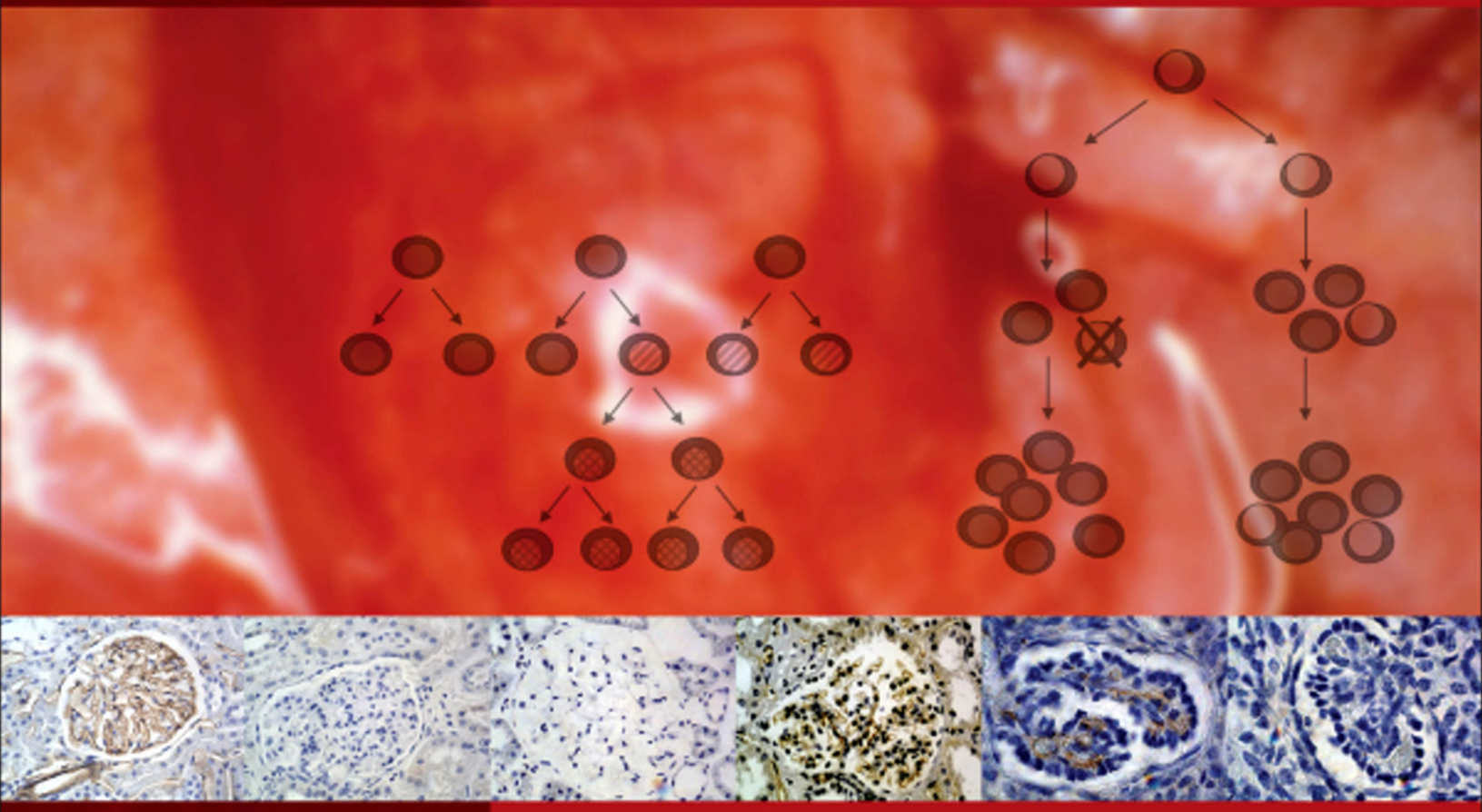
Regenerative Nephrology



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Michael S. Goligorsky

REGENERATIVE NEPHROLOGY

Edited by

MICHAEL S. GOLIGORSKY

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30 Corporate Drive, Suite 400, Burlington, MA 01803, USA
525 B Street, Suite 1800, San Diego, CA 92101-4495, USA

First edition 2011

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British Library Cataloguing-in-Publication Data
A catalogue record for this book is available from the British Library

Library of Congress Cataloging-in-Publication Data
A catalog record for this book is available from the Library of Congress

ISBN: 978-0-12-380928-5

Typeset by TNQ Books and Journals Pvt Ltd.
www.tnq.co.in

Printed and bound in United States of America

10 11 12 13 14 15 10 9 8 7 6 5 4 3 2 1

For Rose and David

Regenerative Nephrology

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Introduction

Somewhere along the tortuous evolutionary path "from fish to philosopher", the ability to regenerate vital organs such as the kidney has been lost, or repressed. This book represents the first attempt to summarize more than a decade of kidney regeneration research. Regenerative nephrology is a subdomain of a broader emerging field of science, regenerative medicine. It is variously defined as "an interdisciplinary field of research and clinical applications focused on the repair, replacement or regeneration of cells, tissues or organs to restore impaired function" [1]; or "regenerative medicine replaces or regenerates human cells, tissue or organs, to restore or establish normal function" [2]; or "the use of cells for the treatment of disease and encompasses both organ repair and the de novo regeneration of an entire organ" [3]. Considering the youth of the field and multitude of remaining hurdles, while providing up-to-date reviews of respective subjects, all contributors to the current book kept an open mind and anticipatory stance, alas, for the lack of a more solid ground. Indeed, the immaturity of this field of knowledge could make one wonder whether the time is ripe for a book on the subject: "Are we there yet?" To me, this situation is reminiscent of Ray Bradbury's finale of "The Martian Chronicles" [4], when a child asks his father of Martians' whereabouts, and the father shows him their own reflection in the water: "'I've always wanted to see a Martian,' said Michael. 'Where are they, Dad? You promised.' 'There they are,' said Dad … and pointed straight down. The Martians stared back up at them for a long, long silent time from the rippling water …". Yes, I believe the field is here to stay, and the chapters collected in this volume are the reflection of it.

During my professional lifetime, a paradigm shift on chronic kidney diseases has occurred. Initially, the prevailing dogma stated that inevitable progression of chronic kidney diseases accelerates as the glomerular filtration rate (GFR) drops below 60 ml/min and becomes inexorably fast when it drops below 25 30 ml/min, with dialysis-free time usually limited to 2 years, even when a low-protein diet was instituted. The process was imagined as an example of classical mechanics when a body is rolling down a sloping, non-resistive surface and gravitational forces are the only driver of the falling object. It has been quite rewarding to witness the collapse of this dogma. The dismantling process started earlier when angiotensin-converting enzyme (ACE) inhibitors, and to a lesser degree statins, phosphate binders and erythropoietin, were introduced into daily clinical practice [5]. Therapy with ACE inhibitors had an impressive success in nephrotic patients in the Ramipril Efficacy in Nephropathy (REIN) extension study and in diabetics (reviewed in Ref. 6). This tide was further enhanced by the observation that, after a prolonged period of normoglycemia in diabetic patients who received a pancreas transplantation, kidney function and glomerular lesions showed improvement [7]. These achievements are masterfully summarized in several reviews [6,8 10]. With infallibility of the dogma being questioned, the ideas of reversibility based on regenerative process began creeping in.

The scientific basis for the new science of regeneration is provided by the plethora of investigations into wound repair. Mythology abounds with the ideas of regeneration. It was the Phoenix that was able to reconstitute itself after it had been consumed by fire; and the deliverer of fire, Prometheus, who was capable of bringing his liver back to the normal size only to provide the persistent eagle with the fresh tissue to sup on the next day. As much as the ancients were sensitive to the delicate ideas of recuperation and restitution, the modern era supplied some mechanistic insights into these processes. An acute, postinjury phase is characterized by the release of soluble mediators increasing vascular permeability, neutrophil ingress and platelet aggregation, while the regenerative phase is marked by the ingrowth of new blood vessels, accumulation of chronic inflammatory cells and fibroblasts in the wound bed (reviewed in Ref. 11). Two outcomes of wound healing are either a complete restoration of structure and function or fibrosis, with the combination of both bridging these two extremes. The simplest distinguishing feature between the two is the chronicity of the injurious agent; however, other stigmata determining full regeneration vis-à-vis fibrosis do exist and are the subject of intense research. Planarians, fish and amphibians, exhibit the ability for the massive regeneration and restitution of lost body parts. This is accomplished by proliferation of stem cells, their migration to the site of injury and formation of a conglomerate of undifferentiated cells,

blastema, which, receiving guidance cues from Wnt and BMP signals, proliferate, differentiate and restore the body plan. In higher organisms, the regenerative potential is much more modest, where the goal is accomplished via compensatory hypertrophy (liver and pancreas), stem cells (liver, pancreas, epithelial, bone, muscle, etc.) and cellular dedifferentiation (in fish: retina, spinal cord, fins; in reptiles: tails) (reviewed in Ref. 12). The end result of wound healing in mammals beyond embryonic age is matrix deposition and scar formation, which in parenchymal organs results in fibrosis. Injury to the kidney is more complex: true kidney regeneration would require formation of new nephrons, but this does not occur in postnatal mammals. Learning how to recapitulate the embryonic conditions, when healing does not result in scarring, and gaining insights into how to achieve restitution of structure functional organization of the lost or damaged tissue, thus preventing scarring and fibrosis, is the goal of regenerative sciences.

Fibrosis is the major process accompanying, and is at least partially responsible for, the functional demise of the kidney, or any other organ in chronic diseases. In the kidney, fibrotic processes may originate from the glomerulus [13], tubulointerstitium [14] and vasculature [15,16], forging the disease progression. In some cases, as in systemic sclerosis, a prototypic example of fibrotic processes with multiorgan involvement, there is clear evidence of vasculopathy preceding fibrosis [17,18]; in others, the sequence of events is more subtle, but the association of fibrosis and vascular dropout is unquestionable [15]. Only in rare cases, employing genetically engineered animals, is the sequence of events reversed. Such are, for instance, transgenic mice overexpressing transforming growth factor-β (TGF-β) receptor type II in fibroblasts [19], caveolin-1-deficient mice [20] and relaxin knockout mice [21], to name a few. Paradoxically, developing hypoxia and induction of proangiogenic factors not only fail to stimulate angiogenesis, but are associated with continuing vascular rarefaction. This phenomenon has been linked to the reduction of endothelial progenitor cells, or their incompetence [22,23]. By all accounts, induction of TGF-β signaling represents a final common pathway for development of fibrosis, while interferon-γ, with or without interleukin-10, serves as a break in this system [24], as recently summarized by Varga and Abraham [18].

One of the intriguing questions is: Why are proregenerative processes not triggered by the encroaching disease? There should exist an element of molecular deception, when a pathological process evades the repair and/or regenerative mechanisms. It is reminiscent of an episode when Odysseus identifies himself to Polyphemus as "Nobody" and, when blinded Polyphemus calls for help and is asked whether he is affected by someone, he responds that "Nobody" attacks him. This stealth mechanism or its opposite, an exaggerated repair response, is counteracted by SOS signaling devices, some of them employing purinergic metabolites [25], others high-mobility group protein-1 (HMGP-1), cytokines and chemokines, to name a few. The molecular mechanisms responsible for either masking the pathological process or inducing exaggerated response to injury, as well as the panoply of SOS signals, remain to be determined. It would be fair to state, therefore, that the imbalance between profibrotic and proregenerative processes, when the former prevails, is a driving force for progression of chronic kidney disease.

It is said that one should avoid cross-disciplinary extrapolation of principles from a particular field of studies to other fields. Do ideas and principles of classical Newtonian mechanics fall into this category and are not to be extrapolated? I believe that the principles of action and counteraction have broader application, and the entire field of regenerative medicine with the discovery of intrinsic mechanisms of repair or counteraction to destructive disease processes is an ample example of this.

Quoting Claude Bernard, "The science of life is a superb and dazzling lighted hall which may be reached only by passing through a long and ghastly kitchen." This collection of chapters should take the reader through this path with the hope that it will be of help for the future entrance to the "lighted hall". Indeed, written by the most prominent scientists, this book should illuminate such a passage. Commencing with the phylogenetic and ontogenetic overviews of kidney regeneration, it proceeds to account for several humoral and immune cell-dependent mediators of regeneration, subjects of intense research which is disclosing novel functions of macrophages and T cells, thus changing our perception of their functional role in inflammation and recovery. The contribution of stem cells to renal repair and trafficking of stem cells from their niches to the sites of repair are discussed in detail in the following two sections of the book. How stem cell repair is modified by disease and in aging and what strategies may improve their regenerative potential is the subject of the following section. The last portion of the book examines the rules for current and future clinical trials, safety of stem cell therapy, tissue engineering, as well as ethical issues related to stem cell therapy and storage of stem cells. Certainly, within the framework of the book it was impossible to embrace all of the exciting developments in the field, but the subjects discussed herein represent the core issues of the main theme of kidney regeneration. If omissions occurred, they are my responsibility. The only excuse I can invoke is the youth of the subject and the lack of precedents attempting to combine the entirety of the

fledgling field of knowledge into a concise and thought-provoking narrative.

In preparing this book, I was tremendously helped by Megan Wickline and Mara Conner from Elsevier, and Patricia Meravy, from New York Medical College, whose assistance, linguistic skills and remarkable insights have been invaluable. I am also indebted to several colleagues, especially Drs M. Little and C. Westenfelder, who provided me with their criticisms of the earlier structural versions of the book. It is now up to the reader to contribute to this process, with my humble assurance that suggestions and critique will be gratefully acknowledged.

Michael S. Goligorsky

February 17, 2010

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CHAPTER

1

Glomerulogenesis and De Novo Nephrogenesis in Medaka Fish: An Evolutionary Approach

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OUTLINE

INTRODUCTION

The kidney plays a pivotal role in controlling humoral conditions, such as electrolytes and osmolarity, as well as blood pressure. Abnormalities of renal function sometimes result in the serious conditions, acute and chronic renal failure, for which dialysis is required. Worldwide, millions of people suffer from end-stage renal failure. Kidney diseases are associated with various systemic diseases including diabetes and autoimmune diseases. The number of kidney disease patients needing dialysis treatment is increasing and is becoming a major issue in the cost of medical care. Therefore, the development of treatments for kidney diseases is an issue that has to be resolved urgently.

Kidney regeneration in humans is one of the major subjects in the development of new therapeutic strategies in renal medicine [1,2]. A well-established characteristic of the adult mammalian kidney is its ability to recover from acute renal failure. This repair response is thought to occur through repopulation of the existing nephron cells [3]. The formation of nephrons is terminated at embryonic or neonate stages, but does not take place in the human adult kidney. It is thought that stem cells for nephrogenesis are not present or are dormant in the human adult kidney. In contrast to the mammalian kidney, the teleost fish kidney is thought to retain the stem cells in adulthood and can reactivate de novo nephrogenesis [4]. Convincing evidence of de novo nephrogenesis in adult fish was provided by

Elger et al., who reported that partial nephrectomy in the skate fish (Elasmobranch *Leucoraja erinacea*) induces proliferation of renal progenitor cells and leads to the formation of a nephrogenic zone [5]. Intriguingly, unilateral nephrectomy of mammalian adult kidneys results in compensatory renal hypertrophy due to cellular hypertrophy but not due to the de novo nephrogenesis [6].

Studies using fish as a model organism have pioneered a new research field in kidney regeneration. However, analysis of the normal development and regeneration process of kidney in fish is required in order to exploit the fish system to develop a regenerative medical approach for human kidney diseases. This chapter introduces the present knowledge on kidney development and regeneration in medaka fish.

MEDAKA AS A MODEL ANIMAL FOR KIDNEY RESEARCH

General Advantages of Using Medaka

Medaka (*Oryzias latipes*) is a small, egg-laying freshwater teleost (Fig. 1.1A) that is widely used as a laboratory animal [10]. Medaka is native to East Asia including Japan, Korea and China. It becomes sexually mature (about 3 cm in body length) within 2 3 months after hatching and spawns daily and year-round under artificial conditions. The transparency of eggs is a distinct advantage for embryological observation and manipulation. In addition, most internal organs including the kidney are visible through the transparent body wall in adult fish if using see-through medaka (STIII, Fig. 1.1B) [8]. The recent publication of the medaka draft genome sequence [11] provides valuable resources on medaka genomic information which facilitate molecular genetics. In comparison to higher vertebrates, the organs in fish are like a minimalist version, using far fewer cells to fulfill the equivalent function in the organism. The kidney of the larva consists of a single pair of nephrons, which facilitates research into kidney development in zebrafish pronephros [12,13]. With these biological characteristics and abundant research resources, medaka is comparable to zebrafish as a model fish. The medaka mesonephros (adult kidney) would be suitable for studies on renal regeneration in fish, as described below, because of its clear histology and small number of nephrons.

Notable Differences Between Humans (Mammals) and Medaka

Mammalian kidneys form from three successive structures, the pronephros, the mesonephros and the metanephros [14], whereas most fish including medaka have only the first two forms [15]. Previous findings suggest that medaka develops more advanced kidneys, which can be referred to as opisthonephros instead of mesonephros: in contrast to some primitive species of teleosts exhibiting segmentally arranged nephrons, medaka mesonephros develops higher order nephron generation with a complex arrangement [15].

Renal cilia in the lumen of the tubules consist of $9+0$ axonemes without dynein arms in mammals, are immotile and are called primary cilia [16]. Recent advances in the understanding of the pathogenesis of polycystic kidney disease (PKD) have proposed the function of immotile primary cilia in the renal tubules: they serve as passive mechanosensors to detect fluid flow rate in the tubule and thereby sense the lumen size [reviewed in Ref. 17]. It is thought that abnormality of their mechanoreceptor functions results in impaired regulation of the renal lumen size and eventually leads to PKD. In contrast to the mammalian kidney, the renal cilia in the fish consist of $9+2$ axonemes with dynein arms, which are features of motile cilia (see Fig. 1.4D). In medaka as well as zebrafish, the maintenance of the renal lumen size requires active beating of the renal cilia, which produce a driving force for intratubular urine flow; loss or reduced motility of the cilia causes PKD [13,18]. Defective ciliary motility causes primary ciliary dyskinesia in humans, as manifested in reversed organ laterality, recurrent respiratory infections and dysmotile sperm flagella, but not PKD. In medaka, however, defects in ciliary motility cause PKD, which has recently been shown in mutants of Kintonun/PF13, which function in preassembly of axonemal dyneins [18], as well as of Dnai2/mii/joi, which constitutes the outer dynein arms of the axomene (authors' own unpublished data and personal communication with Kobayashi). Medaka *kintonun* and *mii/joi* mutants exhibit organ laterality defects and PKD, while human patients having a mutation in these orthologous genes show defective left right polarity formation but not the PKD phenotype [18].

An Example of Medaka Mutant Models for Kidney Disease

A lot of medaka and zebrafish mutants are used as human renal disease models, many of which are related to PKD and are good sources for identifying the genes of human genetic diseases [6,7,13,19,20]. The medaka pc mutant (Fig. 1.1C, D) develops numerous cysts in the kidney and its slowly progressive nature leads to massive enlargement of the kidney in adulthood (Fig. 1.1E) [7]. A recent study revealed that loss of *glis3*

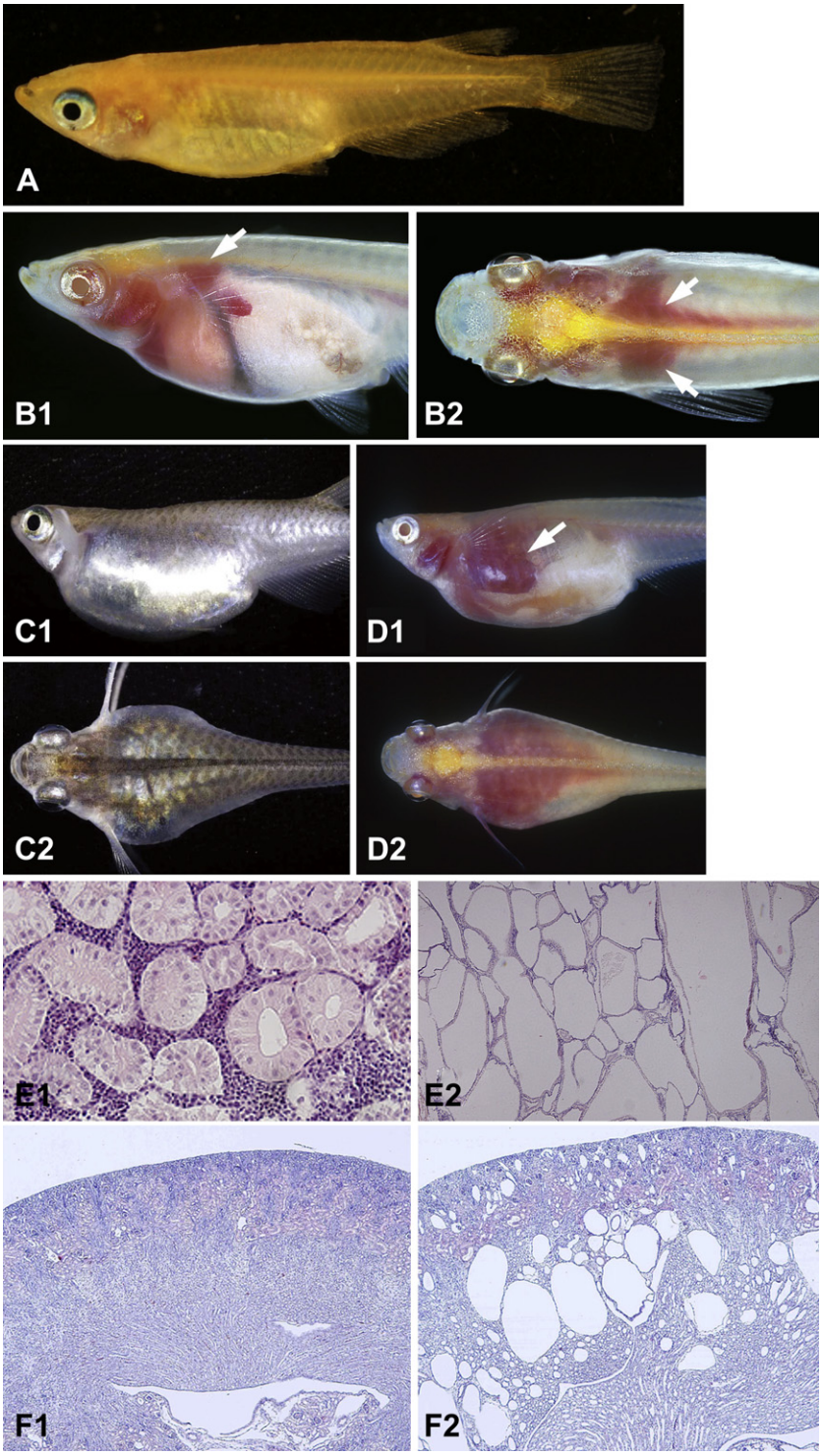

FIGURE 1.1 Medaka as a model organism. (A) Orange–red strain of medaka, a popular strain used in life science research. (B) See through medaka STIII. Reddish kidneys are visible through a transparent body wall (arrows). (C) pc mutant, which manifests a massive abdominal enlargement. (D) pc STII. The kidney expansion can be non invasively observed in a live fish (arrow). (E) Histology of the medaka adult kidney. Severe tubular distension is evident in pc mutant (E2), but not in wild type (E1). (F) Histology of the mouse kidney (postnatal 7 days) deficient for *Glis3*, the orthologue of medaka pc gene. Renal cysts are apparent in *Glis3* (/) kidney (F2), but not in *Glis3* (+/+) kidney (F1). (B1, C1, D1) Lateral views; (B2, C2, D2) dorsal views [7–9]. Please see color plate at the end of the book.

causes renal cyst formation in pc mutant medaka [21]. Further analyses in mice [9] and other reports [22,23] have shown that Glis3/GLIS3, if lost, causes PKD in mammals (Fig. 1.1F). Thus, the PKD phenotype of medaka pc mutant is relevant to human patients with *GLIS3* mutation despite the species difference in the putative function of the renal cilia.

KIDNEY DEVELOPMENT IN MEDAKA

Gross Morphology of Medaka Kidney

Medaka has a pair of kidneys that are located retroperitoneally, extending from the bases of the pectoral fins to the caudal reaches of the abdominal cavity

(Fig. 1.1B). Their large anterior portions, containing most of the nephrons, are much larger than those of zebrafish, whereas the caudal portions are smaller (Fig. 1.2A). Medaka hatchlings have functional pronephros consisting of a single pair of nephrons (Fig. 1.2B, C). The kidney size of the fry increases as mesonephric nephrons develop. The number of nephrons, when represented as corresponding to both mature and immature glomeruli, reaches approximately 200 300 in each kidney within 2 3 months after hatching (Fig. 1.2D). Adult medaka kidneys contain both pronephros and mesonephros, unlike kidneys of some fish species which lose pronephros after mesonephros starts functioning [4,25 27]. The bean-shaped cranial portion is larger than the caudal portion and this proportion persists throughout life.

Histological Anatomy of the Kidney

A medaka hatchling has only pronephros (Fig. 1.2B, C), which consists of a single pair of nephrons having the butterfly-shaped glomus external to the kidney capsule and the tubule connecting the glomus with the duct extending to the urinary bladder (Fig. 1.3A). (Medaka pronephros has glomus but not glomerulus in the sense that the multiple blood vessels go into the single renal corpuscle.)

Mesonephric nephrons distribute throughout the kidney with no particular distinction between medulla and cortex (Fig. 1.3B). The entire kidney is composed of both nephrons and interstitial lymphoid tissue. Three segmental structures of nephron are recognized by hematoxylin & eosin staining of kidney section (Fig. 1.3C, D). The glomerulus is typically a round cell mass covered by a flat epithelium of Bowman's capsule. The proximal segment of the tubule is lined by tall columnar epithelial cells possessing a brush border on the apical surface (Fig. 1.3C, D), which is positive for periodic acid Schiff (PAS) staining (Fig. 1.3E) or *Locus tetragonolobus* lectin staining (Fig. 1.3F). The distal tubular segment has a wide lumen lined by low columnar epithelial cells (Fig. 1.3C).

Under the transmission electron microscope, three important structures are evident in the glomerulus: the capillary blood vessels containing erythrocytes, the capillary endothelium, the glomerular basement membrane (GBM) and the podocytes forming the outer layer of the glomerulus (see Fig. 1.8A, B). The proximal tubule consists of a thick layer of epithelial cells with a brush border on the apical surface and numerous mitochondria on the basal side (Fig. 1.4A). The distal tubule has a scanty brush border of microvilli on the apical side and numerous mitochondria, but no vesicles of the apical endocytotic apparatus (Fig. 1.4B).

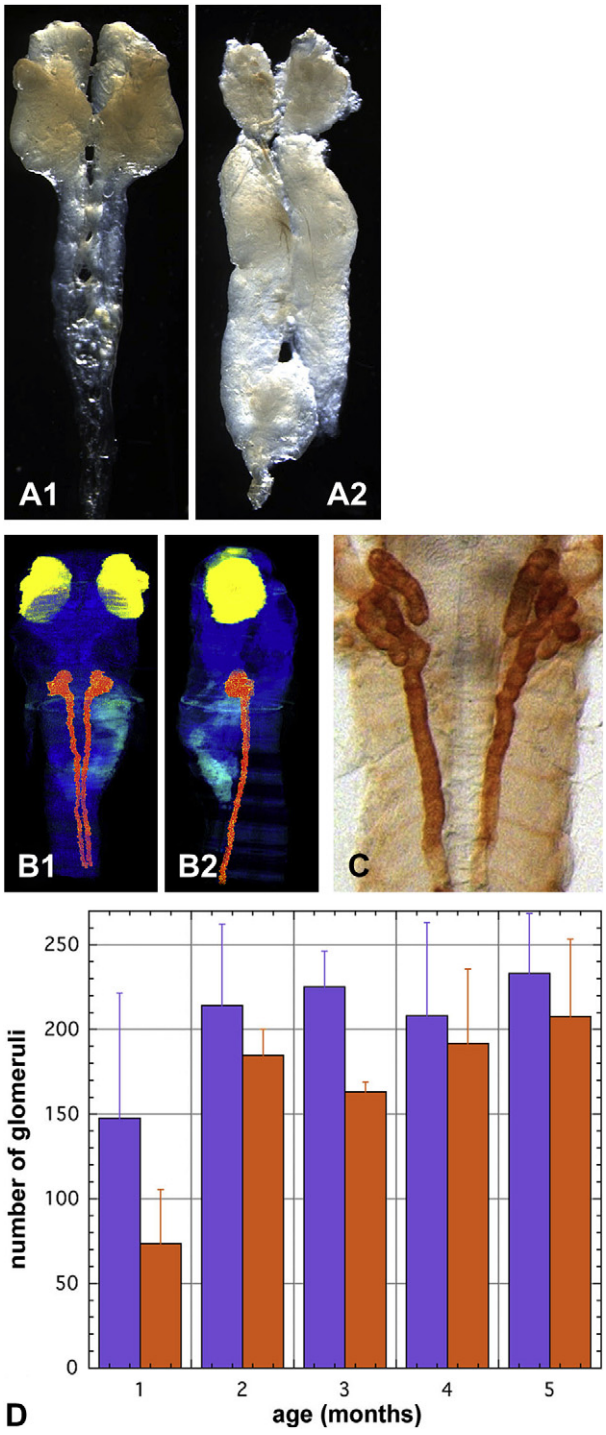

FIGURE 1.2 Gross morphology of medaka kidney. (A) Comparison of adult kidney morphology between medaka (A1) and zebrafish (A2). (B) The pronephros of the hatchling as viewed by 3D imaging. The intrarenal tissues are colored in red. (B1) Dorsal view; (B2) lateral view. The area of pronephric glomus is not colored because it locates externally to the renal tissues. (C) The pronephric tubules and ducts are stained with anti Na^+/K^+ ATPase antibody. (D) The number of pronephric glomeruli in the kidney of the male (blue) and the female (red) during 5 mph. Error bars indicate the standard deviations. The number includes immature developing glomeruli. Anterior to the top (A–C) [15,24]. Please see color plate at the end of the book.

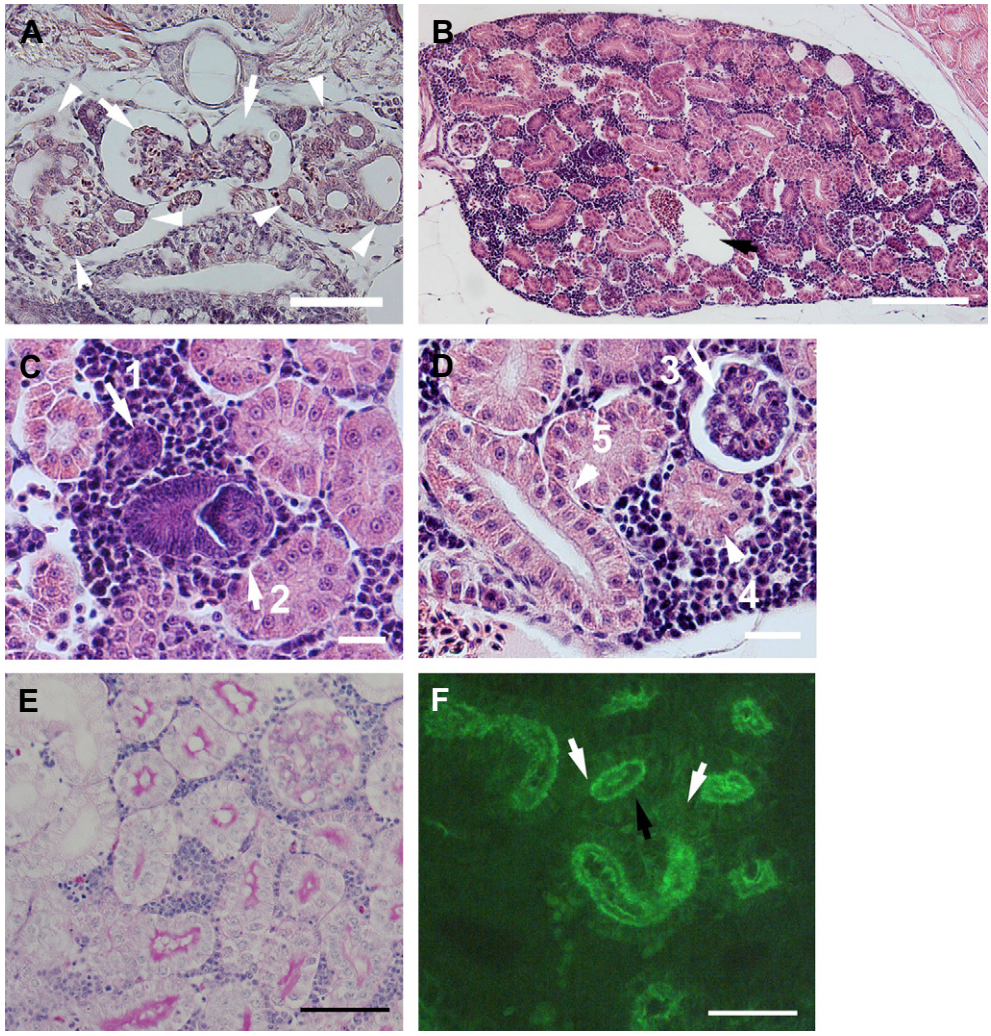

FIGURE 1.3 Histology of medaka kidney. (A) The pronephros of the hatchling. Arrows: the external glomera. Three arrowheads: each of bilateral borders of the renal tissue. (B) Section of the anterior portion of 2 mph medaka. Black arrow: subcardinal vein. (C, D) Magnified image of a section from 2 mph medaka. Arrow 1: mesenchymal condensate; arrow 2: nephrogenic body; arrow 3: mature glomerulus; arrowhead 4: proximal tubule; arrowhead 5: distal tubule. (E) Periodic acid–Schiff (PAS) staining. The proximal tubules but not the distal tubules are positive for PAS stain. (F) *Lotus tetragonolobus* (LTL) staining. The proximal tubules are positively stained by the fluorescent conjugated LTL. Scale bars: 50 μm (A, E, F); 100 μm (B); 20 μm (C, D) [7,15]. Please see color plate at the end of the book.

In both of the tubular segments, monocilia are seen to project into the lumen under the scanning electron microscope (Fig. 1.4C, D). The two segments are distinguishable since the proximal segment has bundles consisting of multiple cilia as well as a dense brush border (Fig. 1.4C). Multiple cilia and monocilia are of the $9+2$ axoneme type on the transmission electron microscope (Fig. 1.4E).

Nephrogenic Stages in Medaka Kidney

In the developing kidney of medaka [10 days posthatching (dph) to 2 months posthatching (mph)], three different stages of nephrogenesis can be distinguished histologically (Fig. 1.3C, D; see also Fig. 1.5B, C, D):

1. Mesenchymal condensation: a cell mass of high cell density strongly stained by hematoxylin, appears in the mesenchyme.
2. Formation of a nephrogenic body: a mesenchymal condensate changes its shape to a tadpole-shaped nephrogenic body; its tail region corresponds to a presumptive tubule. The nephrogenic body may be equivalent to the comma- and S-shaped nephrogenic body in mammalian kidney [29].
3. Maturation of nephron: the glomerulus becomes surrounded by a spacious Bowman's capsule and the tubular segments become eosinophilic possibly because of completion of the mesenchymal epithelial transition.

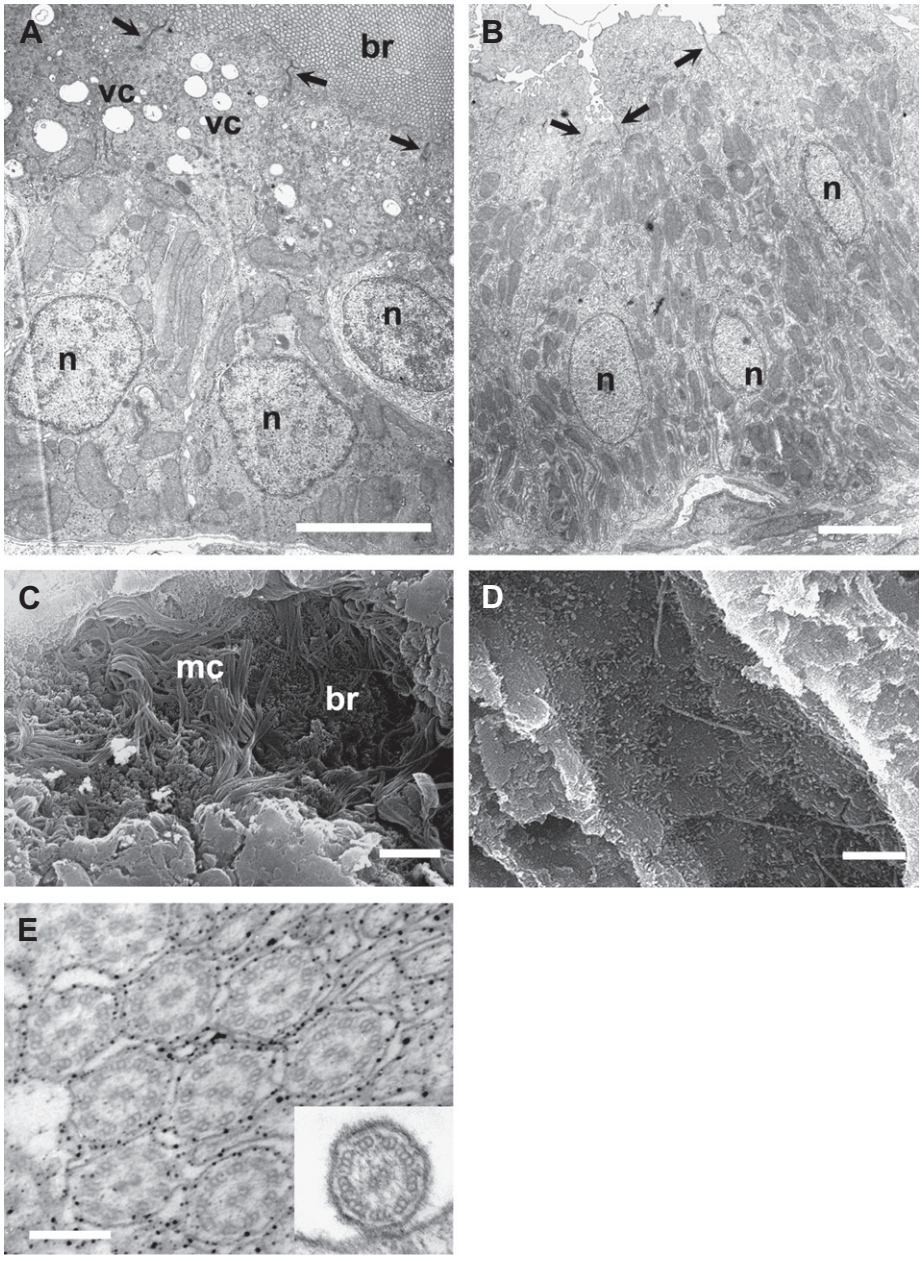

FIGURE 1.4 Electron microscopy. (A) Proximal tubule consisting of tall columnar cells with a dense brush border of microvilli (br), vesicles of apical cystic apparatus (vc), numerous mitochondria, tight junctions (arrow) and basement membranes. (B) Distal tubule consisting of low columnar cells with scanty brush border of microvilli, tight junctions (arrow), numerous mitochondria and basement membrane. (C) Bundles of multiple cilia (mc) and a dense brush border (br) are found in the lumen of the proximal tubule. (D) Monocilia and a scanty brush border are found in the lumen of the distal tubule. (E) Cross sections of multiple cilia and monocilium (insert) of proximal segment. n: nucleus. Scale bars: 4 μm (A, B); 10 μm (C, D); 0.2 μm (E) [28].

Three-dimensional Images of Nephrogenesis in Developing Mesonephros

On the basis of the histological features described above, nephrogenesis in medaka kidney can be viewed in three-dimensional (3D) images reconstructed from serial sections with computer assistance. At the hatching stage, the definitive pronephros, but no sign of developing mesonephric nephrons are observed (Fig. 1.5A). Mesonephric nephrons start to develop at 5 dph as the condensates form in the mesenchyme in the dorsomedial region of the anterior pronephros (Fig. 1.5B, mesenchymal condensation). This condensate acquires a presumptive tubule which has undergone or is undergoing mesenchymal-to-epithelial transition at 7 dph (Fig. 1.5C). The first mesonephric nephron becomes mature within 10 dph, when its glomerulus

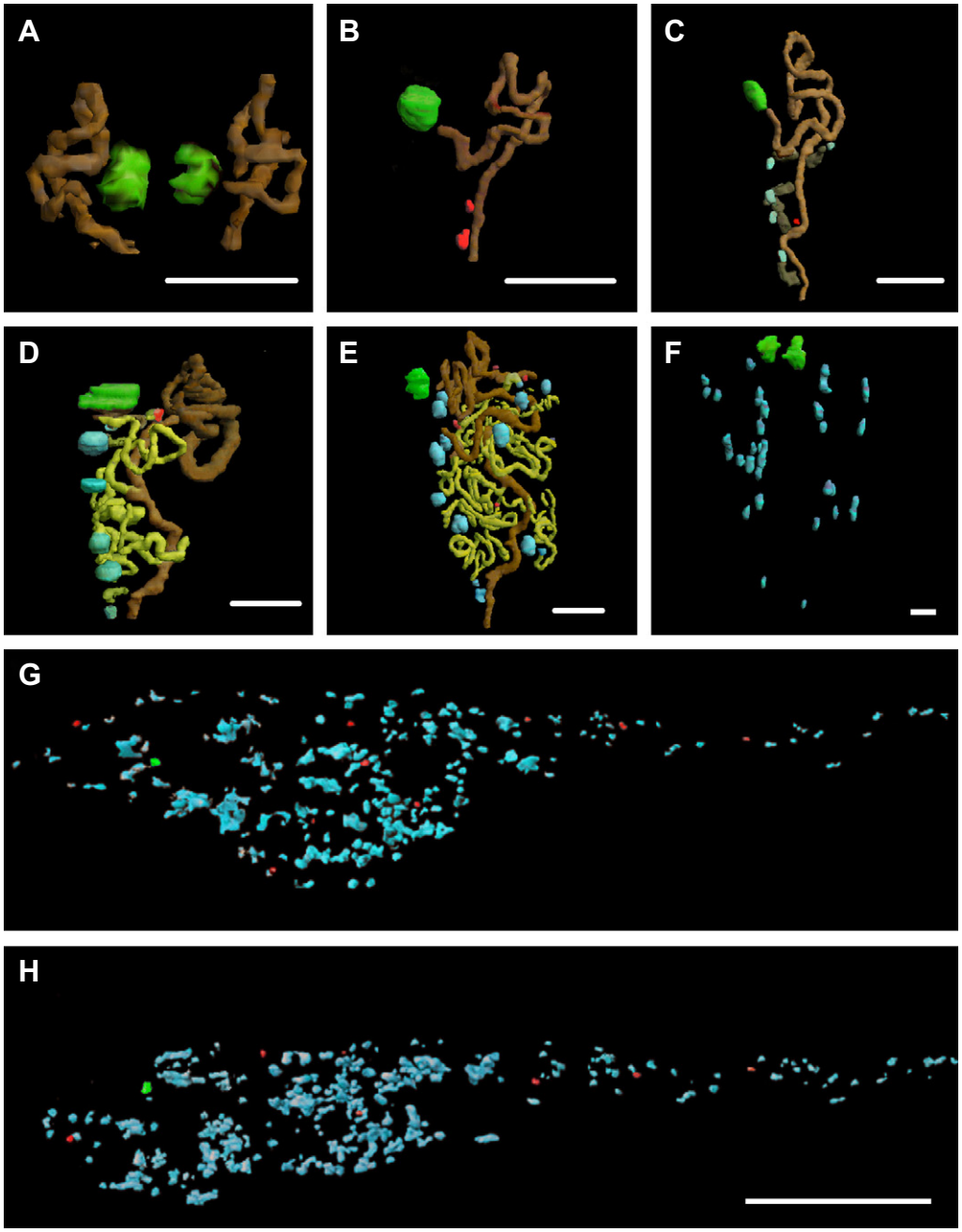

FIGURE 1.5 Three dimensional (3D) images of the nephron. Segments of the nephron are viewed by colorized 3D imaging: pronephric glomera, green; pronephric tubule and duct, brown; mature mesonephric glomeruli, blue; mesenchymal condensates and presumptive glomerular portion of the nephrogenic bodies, red; mesonephric immature tubules with a closed lumen, gray; mesonephric mature tubules with an open lumen, yellow. (A) Hatching stage. Bilateral pronephric nephrons are shown. (B) 5 dph. One or two (two in this case) mesenchymal condensates are observed. (C) 7 dph. Developing nephrons with a tadpole like shape are found. (D) 10 dph. Mature nephrons are found. (E) 15 dph. More than 10 mature nephrons have been formed. (F) 20 dph. Only mature glomeruli which count more than 30 on each side are indicated for bilateral kidneys. (G, H) 4 mph. Glomeruli and mesenchymal condensates are distributed throughout the entire length of the kidney. Anterior to the left. (A–F, H) Ventral views; (G) lateral view; (B–E, G, H) left kidneys. Scale bars: 100 μm [15]. Please see color plate at the end of the book.

has been connected with the pronephric duct by the new tubule with fully differentiated epithelium (Fig. 1.5D). Likewise, mesonephric nephrons continuously develop along the pronephric tubule and duct (Fig. 1.5E, F), rapidly increasing in number until 2 mph. In 4 mph fish, the mature and immature glomeruli are distributed throughout the entire mesonephros (Fig. 1.5G, H). Notably, after the number of nephrons reaches a plateau, approximately 7% of total nephrons are still immature.

wt1 Expression as a Marker of Nephrogenesis

Wilms' tumor suppressor gene 1 (*wt1*), a zinc-finger transcription factor regulating kidney development, is reported to be a good marker of developing pronephric and mesonephric nephrons in zebrafish [30 32]. In medaka, *wt1* expression is detected in the progenitor cells (Fig. 1.6A C) and later the immature glomerulus of pronephric nephrons (Fig. 1.6D G). During mesonephric development, *wt1* expression is first detected in mesenchymal cell mass of the caudomedial end of the pronephric sinus and duct area at 5 dph (Fig. 1.6H, I), which corresponds to the condensate first histologically

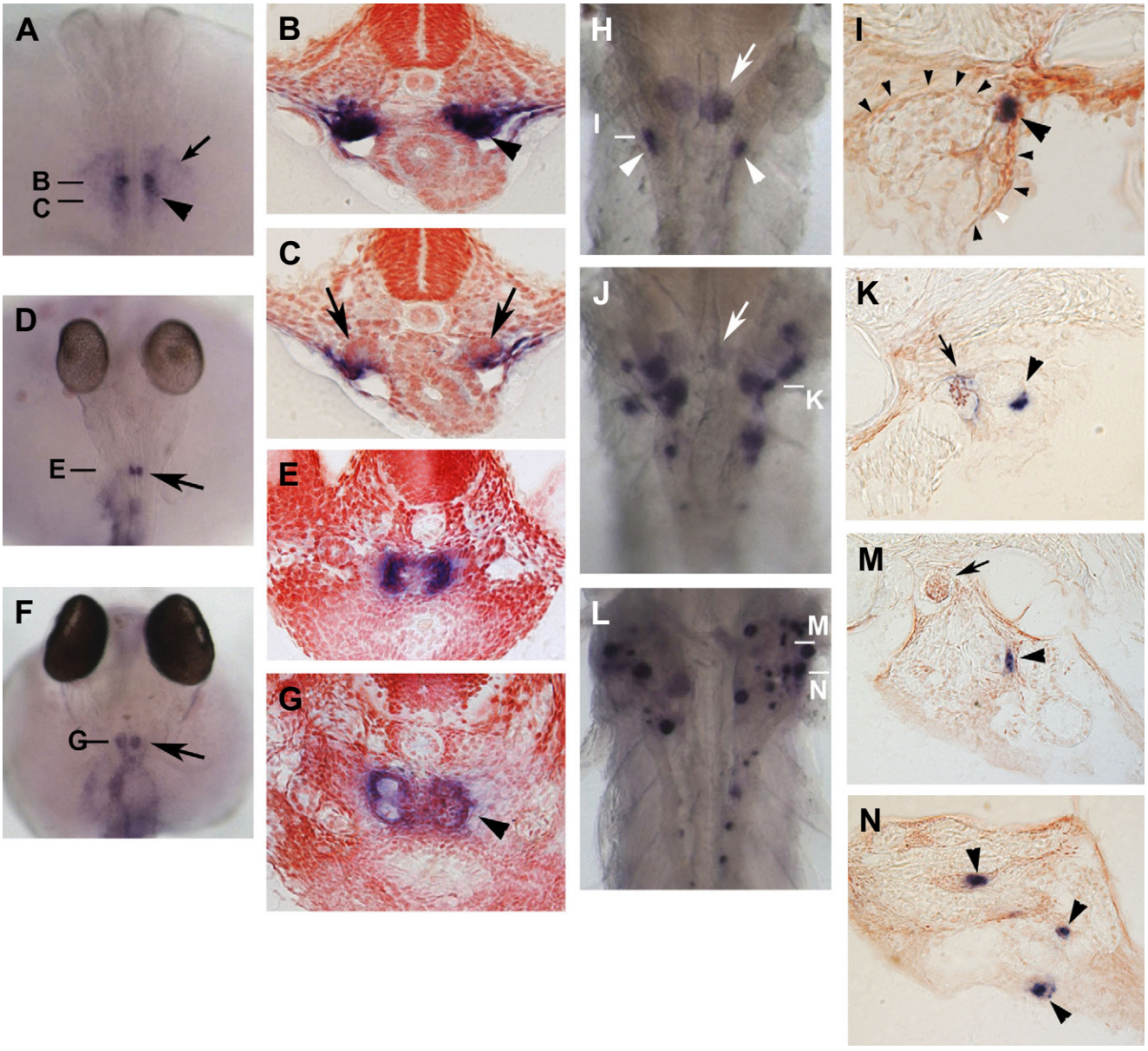

FIGURE 1.6 *wt1* expression in the developing kidney of medaka. (A–C) Stage 25 (18–19 somite stage). *wt1* mRNA is expressed exclusively in the small bilateral spots ventrolaterally to the notochord (arrowhead) and faintly in the surrounding tissues (arrow). (D, E) Stage 31 (gill blood vessel formation stage). The expression domain becomes more confined to the two spots corresponding to the pronephric glomus (arrow). (F, G) Stage 35 (stage at which visceral blood vessels form). The expression becomes limited to the podocytes (arrowhead). (H, I) 5 dph. The first mesonephric nephron progenitor positive for *wt1* appears at the caudomedial end of the pronephric sinus and duct area (white arrowheads in H and black arrowhead in I). White arrow: left pronephric glomus; small black arrowheads: the border of the renal tissue. (J, K) 10 dph. New spots of *wt1* expression appear (black arrowhead). The *wt1* expression of the first mesonephric nephron becomes weaker (black arrow). White arrow: left pronephric glomus. (L–N) 20 dph. The *wt1* expression spots increase (black arrowheads). The first mesonephric nephron has fully developed and lost *wt1* expression (black arrow). Cross sections (B, C, E, G, I, K, M, N) were made at the levels indicated by bars [15]. Please see color plate at the end of the book.

recognized at this particular position in the 5 dph fry (Fig. 1.5B). Subsequently, *wt1*-positive cell masses increase along the pronephric tubule and duct (Fig. 1.6J, L). *wt1* is expressed strongly in the mesenchymal condensate (Fig. 1.6I, K, M, N) and later in the presumptive glomeruli (Fig. 1.6K). Expression of *wt1* disappears when the glomeruli become mature (Fig. 1.6M), indicating that *wt1* is a hallmark of developing nephrons in medaka. The *wt1*-expressing mesonephric nephrons appear in the posterior region in zebrafish (not shown), whereas they are located mainly in the anterior region of the medaka kidney (Fig. 1.6L). The difference in gross morphology of the kidney between medaka and zebrafish may reflect the distinct distribution of mesonephric nephrons.

KIDNEY REGENERATION IN MEDAKA

Nephron Repair in Mammals

Renal recovery from sublethal injury is considered to recruit two different cell sources for repairing the damaged nephrons. One source is surviving tubular epithelial cells, of flattened and squamous shape [33]. They migrate to and cover the denuded basement membrane, and subsequently develop to (repopulate) a cuboidal and columnar epithelium. Another source may be bone marrow stem cells. The plasticity of bone marrow stem cells has been traditionally known as they can give rise to blood cells and connective and adipose tissue cells [1]. Recent studies suggest that bone marrow-derived cells can also contribute to adult renal cells in humans and other animal models [34,35]. When human patients or experimental animals receive a kidney or bone marrow transplant, bone marrow-derived cells repopulate primarily the renal proximal tubule as well as the renal vasculature, interstitium, renal tubules and glomeruli. However, the above two cell sources are incapable of committing de novo nephrogenesis.

Renal Damage with Gentamicin in Medaka

Administration of nephrotoxicants such as gentamicin leads to renal damage in fish. Gentamicin is known to bind specifically to the megalin receptor of nephric tubular epithelial cells of the renal tubular lumen, where its accumulation causes nephric damage, although the molecular mechanism underlying this damage is not clear [36,37]. As has been shown in goldfish and other fish [38 42], in medaka after intraperitoneal injection of gentamicin, the damaged nephrons are found histologically. The optimal sublethal dosage is 75 μg per individual (kidney weight is 2 3 mg in 0.4 0.6 g body weight), which produces severe damage but does not result in instantaneous death. The tubular epithelia become detached from the basement membrane to cause a denuded intact basement membrane (see Fig. 1.8H) and cyst formation at the glomerular and tubular segments 3 days after gentamicin administration (daGa) (Fig. 1.7A, B), both of which are evident with light microscopy. By 7 daGa, the vestiges of the damaged tubules where the basement membrane has become degraded and where the detached epithelia have disappeared from the lumen are apparent (Fig. 1.7C).

When examined under the transmission electron microscope, the damage due to gentamicin administration is visible in the glomerulus as well as in the tubule (Fig. 1.8B F). At 3 daGa, the glomerulus exhibits hypertrophy of the podocytes and capillary endothelia are ectopically positioned, having detached from the GBM (Fig. 1.8B, E). The brush borders on the surface of the tubular epithelial cells have been degraded (Fig. 1.8G, H). The epithelial cells have separated from the basement membrane with cyst-like spaces forming between the two layers. Most of this damage is not detectable by light microscopy (Fig. 1.7B, C).

Renal Repair in Medaka

Both light microscopy and the transmission electron microscopy show that the gentamicin-induced damage becomes less severe at 14 daGa (Fig. 1.7D), although the lining of the podocytes is still slightly disrupted (Fig. 1.8C, F). This may result from recovery of the damaged nephrons through the repair response, which is known to occur in other teleosts such as goldfish, catfish, trout, zebrafish and tilapia [38 42].

Cell proliferation is increased in the gentamicin-administered kidney (Fig. 1.9G), suggesting the repair response in medaka. While PCNA-positive cells are mostly found in the mesenchymal tissues in normal kidney, mature glomeruli and tubules become positive for PCNA at 3 and 14 daGa (Fig. 1.9A, B, C, G). Apoptosis is also detected in response to gentamicin treatment. Whereas TUNEL-positive cells are only detected in the mesenchymal tissues in normal kidney, apoptotic cells appear in the renal tubular epithelia and glomeruli at 3 and 14 daGa (Fig. 1.9D G).

De Novo Nephrogenesis in Medaka

In contrast to mammals, fish are generally capable of regenerating kidney through de novo generation of nephrons. Reimschuessel et al., in pioneering work using goldfish [40], reported increased de novo nephrogenesis in the goldfish kidney exposed to gentamicin.

The de novo nephrogenesis recapitulates the normal development process of pronephros/mesonephros from the mesenchymal tissue. In medaka, reactivation

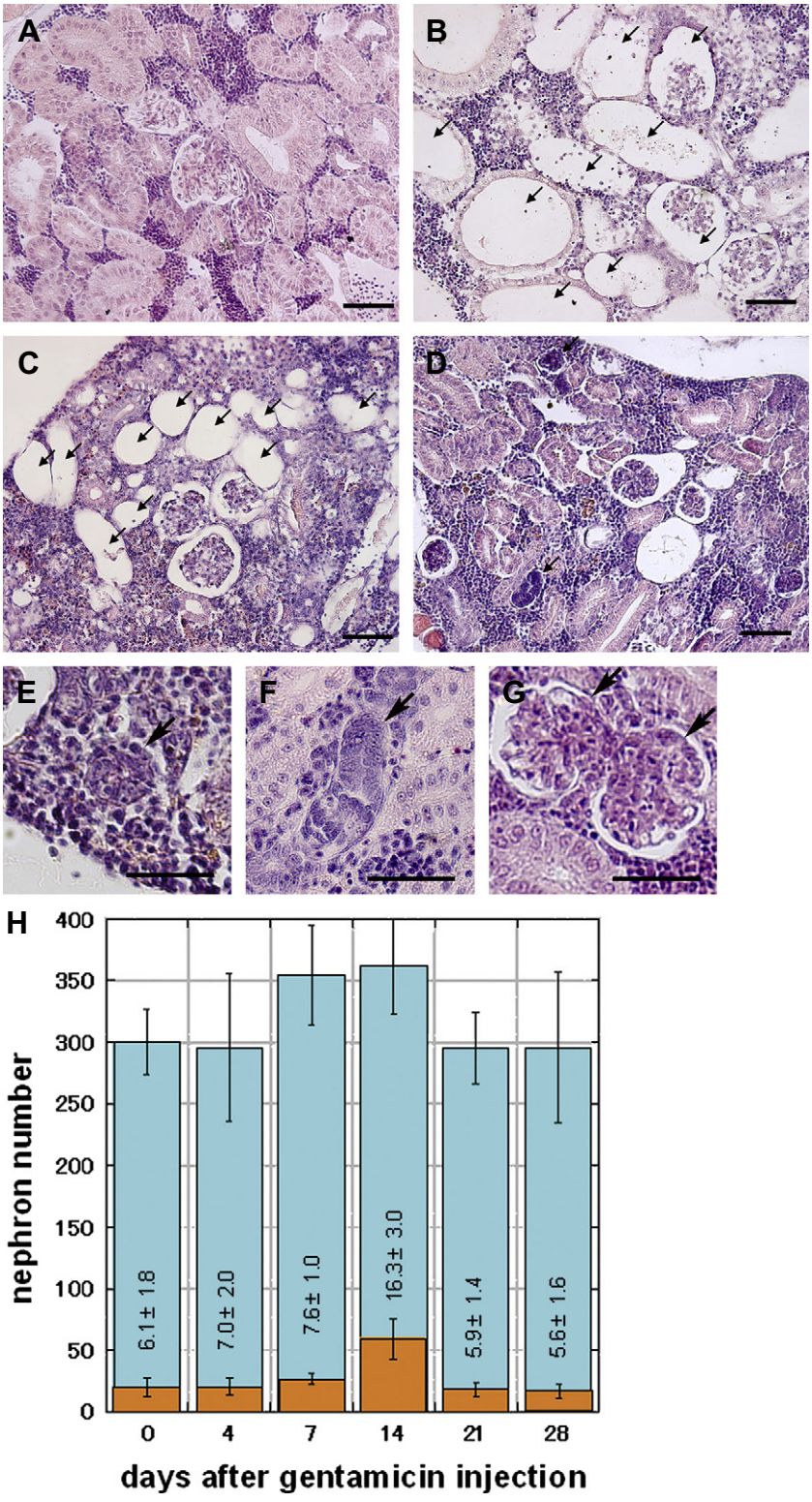

FIGURE 1.7 Histology of kidney regeneration in medaka. (A) Normal kidney (7 days after PBS injection to 3.5 mph medaka) (B–D) Damaged kidneys. Arrows indicate well developed cysts. (B) 3 days after gentamicin injection (daGa) to 3.5 mph medaka. (C) 7 daGa. (D) 14 daGa. (E–G) Magnified images of developing nephrons at 14 daGa. A mesenchymal condensate (E), a nephrogenic body (F) and mature glomeruli (G) are indicated by arrows. (H) Number of developing nephrons and total nephrons (means ± standard deviation). Orange bars: number of developing nephrons; blue bars: total number of nephrons. The ratios of developing nephrons to total nephrons is indicated above orange bars [28]. Please see color plate at the end of the book.

of de novo nephrogenesis seems most apparent at 14 daGa, when a large number of nephrogenic morphologies with small, condensed and basophilic cell clusters appears (Fig. 1.7E, F). These nephrogenic clusters include the mesenchymal condensates and the nephrogenic bodies which are quite similar to the developing nephrons during normal nephrogenesis. It should be noted that developing nephrons are observed in the mesenchymal tissue of normal adult kidney even after the nephron number reaches a near-plateau.

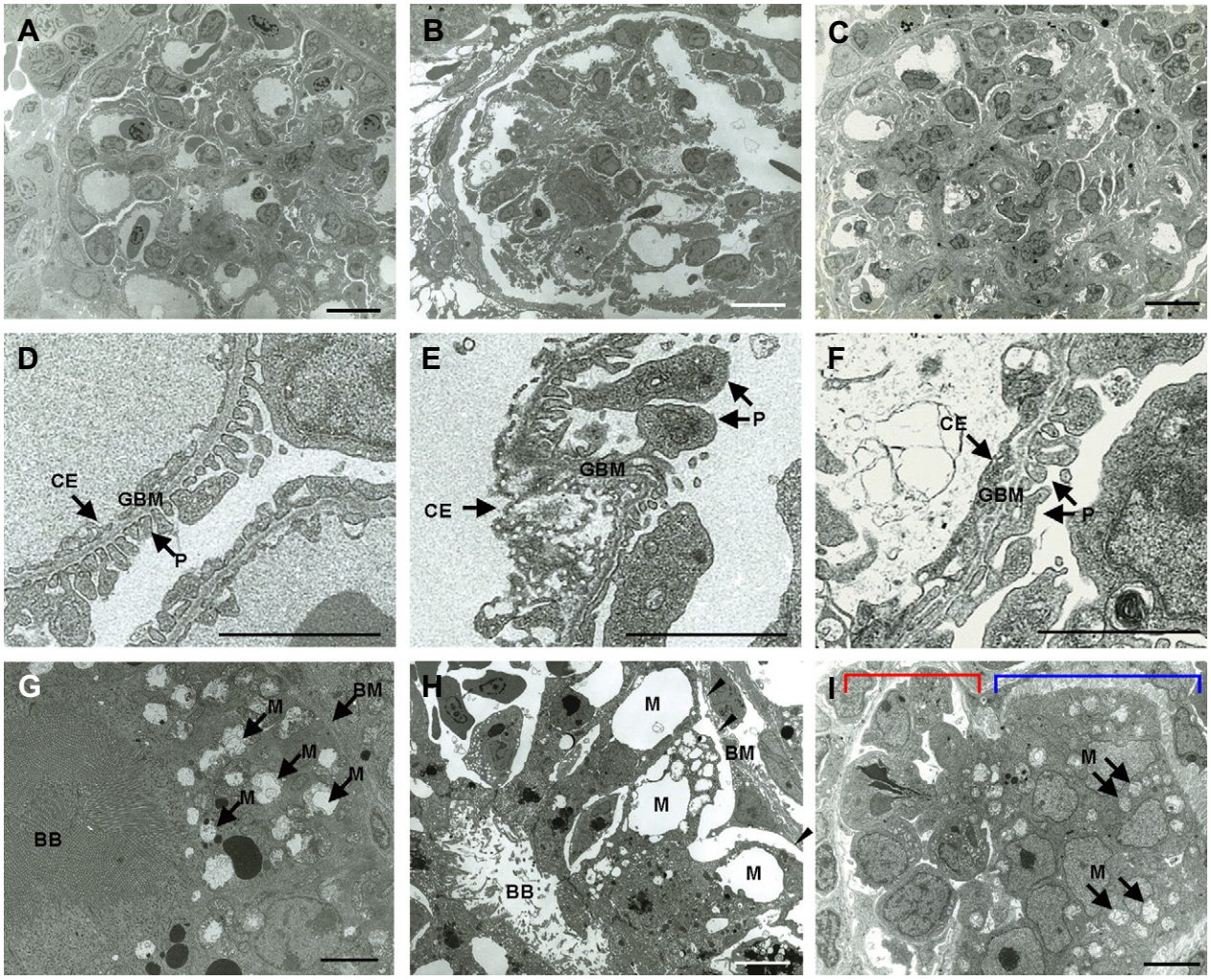

FIGURE 1.8 Transmission electron microscopy of gentamicin administered kidney. (A, D) Normal mature glomerulus. (B, E) Damaged mature glomerulus at 3 daGa. The capillary endothelium is abnormally arranged and detached from the glomerular basement membrane (GBM). The podocytes are hypertrophic. (C, F) Typical mature glomerulus at 14 daGa. The indications of gentamicin induced damage are less severe than at 3 daGa. (G) Normal proximal tubule. The epithelial cell of the proximal tubule has a dense brush border on the apical surface and numerous mitochondria on the basal side. (H) Damaged tubule at 3 daGa. The epithelial cells are detached from the basement membrane (BM). The brush border is severely damaged. (I) Developing nephron (nephrogenic body). At 14 daGa the nephrogenic cell masses become apparent. These clusters appear to consist of two distinct tissue types; one is mitochondria rich, which presumably corresponds to the tubular segment (right blue bar), and the other consists of cells with large nuclei (left red bar). Arrowheads in (H) show the tubular epithelium detached from the BM. Scale bars: 7.7 μm (A–C); 1.4 μm (D–F); 2.9 μm (G); 5.8 μm (H); 3.9 μm (I). CE: capillary endothelium; GBM: glomerular basement membrane; P: podocyte; BB: brush border; BM: tubular basement membrane; M: mitochondria [28].

A significant increase in the number of de novo developing nephrons was consistently found at 14 daGa. In Fig. 1.7(H), nephrons are enumerated in the gentamicin-administered kidney by distinguishing mesenchymal condensates and nephrogenic bodies from mature nephrons. The number of developing nephrons (mesenchymal condensates and nephrogenic bodies) is significantly higher at 14 daGa than at other time-points examined. On transmission electron microscopic analysis, the nephrogenic clusters are frequently found in the images of 14 daGa kidney (Fig. 1.8I).

Although gentamicin administration causes the existing nephrons to degrade significantly until 7 daGa, in Fig. 1.7(H) increases in total nephron number at 7 and 14 daGa are notable. Because these results depend on enumeration of mature and immature nephrons (glomeruli) by histological examination with a light microscope and some portion of glomerular damage was only apparent on examination by transmission electron microscopy, the figure for the number of mature nephrons (glomeruli) in Fig. 1.7(H) includes the number of damaged nephrons that would be repaired or degraded in the following weeks. In addition, there

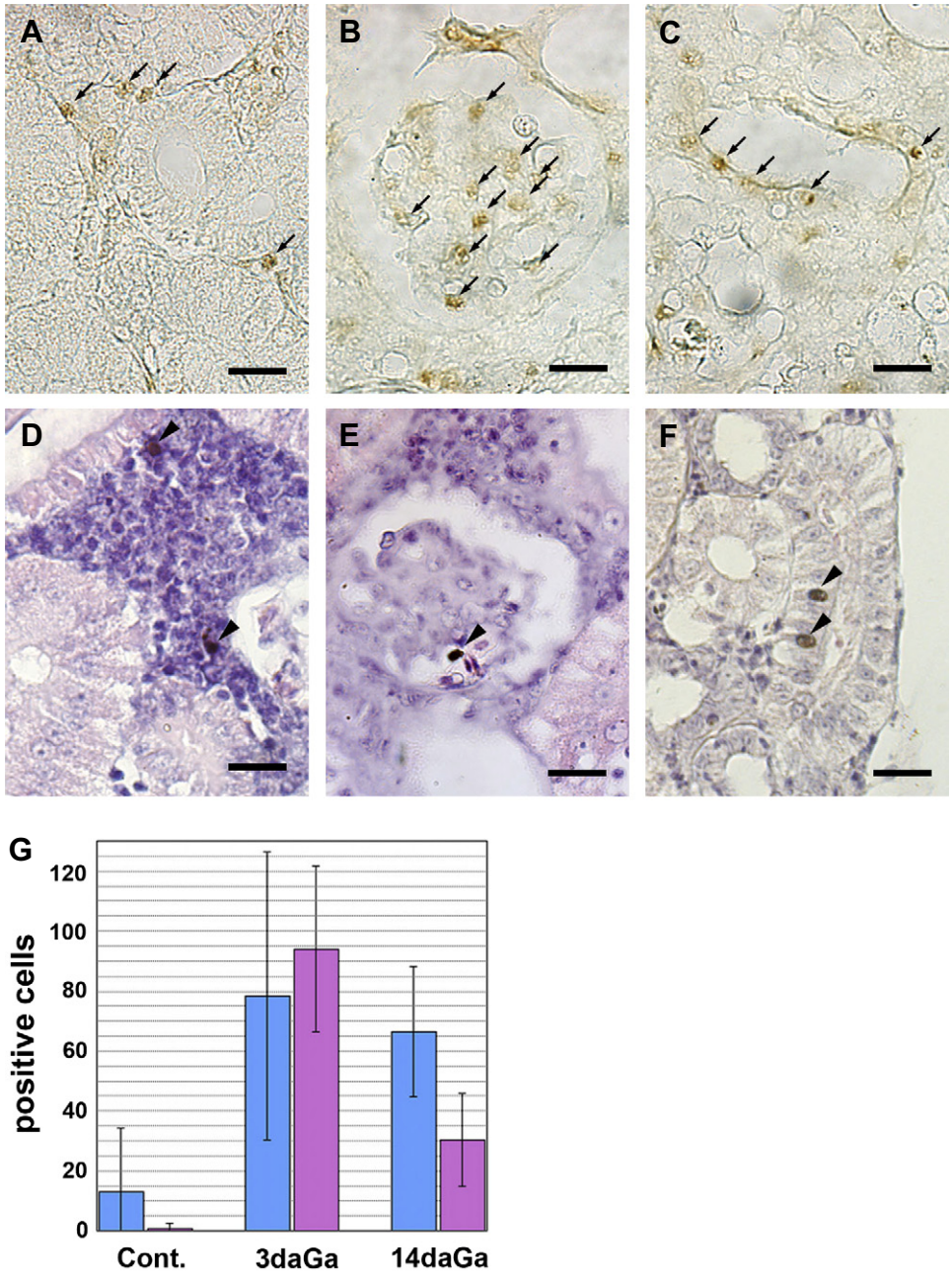

FIGURE 1.9 Detection of cell proliferation and apoptosis in the kidney. (A–C) Proliferating cells detected by PCNA labeling (arrows). (A) PCNA positive mesenchymal cells in the normal kidney. (B, C) The glomerular (B) and the tubular (C) cells positive for PCNA at 3 daGa. (D–F) Apoptosis detected by the TUNEL assay (arrowheads). (D) Apoptotic cells found in the mesenchyme of normal kidney. (E, F) TUNEL positive cells in the mature glomerulus (E) and tubular epithelium (F) at 14 daGa. Arrows indicate positive signals in the area described. Scale bars: 10 μm. (G) Statistics of proliferating and apoptotic cells. Proliferating cells (blue bars) increase significantly at 3 daGa in the area of the nephron (tubular and glomerular segments). Apoptotic cells (red bars) also increase significantly at 3 daGa and at 14 daGa in the nephron area. Bars indicate means ± standard deviation of four individuals. Significant differences are shown by asterisks (*t* test, $*p < 0.005$, $**p < 0.01$) [28]. Please see color plate at the end of the book.

would ordinarily have been a small number of developing nephrons before gentamicin administration, and it is possible that these may not have been affected by treatment with the toxicant for reasons related to their immature stage of development. Thus, the transient increase observed in the total number of nephrons between 7 and 14 daGa may have been due to an increase in the number of developing nephrons combined with the contribution of the remainder of mature damaged nephrons. Since some of these mature damaged nephrons would be degraded during the recovery period, the total number of nephrons at

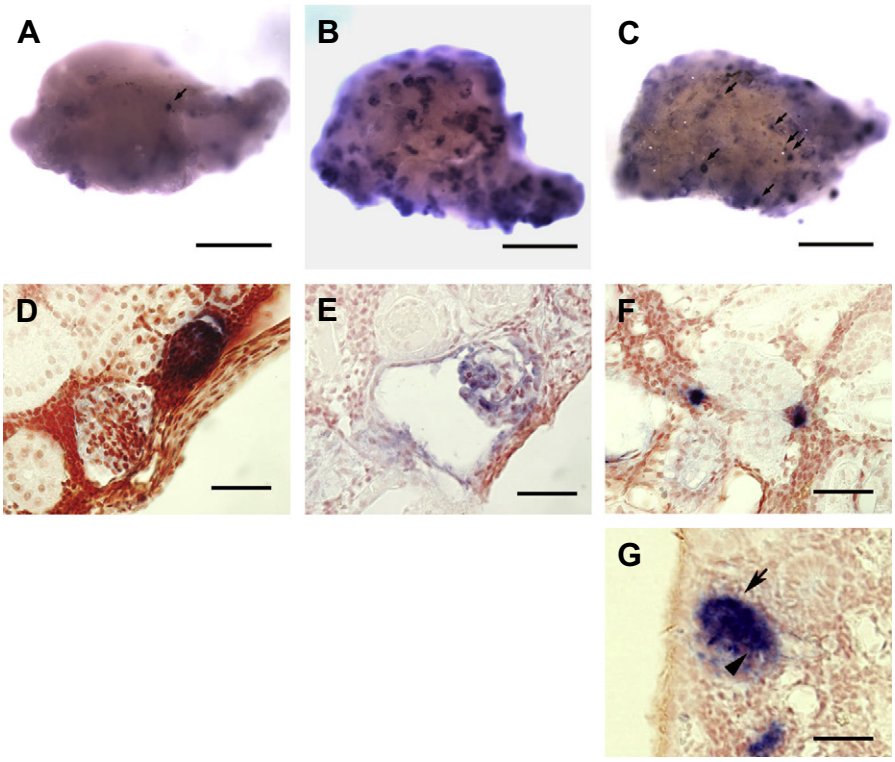

FIGURE 1.10 *wt1* expression in genta micin administered kidneys. (A–C) External views of the whole kidney stained by *wt1* in situ hybridization. Arrows indicate the *wt1* positive signals in the mesenchymal conden sates. (D–G) Plastic sections of *wt1* stained kidney. (A, D) Normal kidney at 3.5 mph. (B, E) 3 daGa. The mature nephrons become positive for *wt1*. (C, F, G) 14 daGa. Small spots of *wt1* positive cell masses corresponding to condensed mesenchyme (F) and nephrogenic bodies (G) appear in the mesenchymal tissues. The arrow indicates a presumptive glomerulus and the arrowhead indicates a presumptive tubule in (G). Scale bars: 0.5 mm (A–C); 20 μm (D–G) [28]. Please see color plate at the end of the book.

21 and 28 daGa would probably have returned to normal levels of around 300.

Recapitulation of *wt1* Expression in Repair and De Novo Nephrogenesis of Medaka Kidney

The appearance of basophilic cell clusters, referred to as mesenchymal condensates and nephrogenic bodies, is a hallmark of de novo nephrogenesis during normal kidney development of fish [4,28], including medaka (Fig. 1.3D, see also Fig. 1.7E, F). Likewise, in normal nephron development, an increase in the number of *wt1*-positive cell mass is also a hallmark for the de novo nephrogenesis (Fig. 1.6). The *wt1*-positive cells appear as a small spot in the mesenchymal condensate and nephrogenic body in the early stages of nephron development. The *wt1* expression becomes limited to the podocytes of the mature glomerulus, and subsequently disappears from the nephron.

Whole-mount in situ hybridization of an adult kidney showed that only a limited number of *wt1*-positive cell masses exist as small spots in the mesenchyme (Fig. 1.10A, D). These spots are only rarely seen in the podocytes of the normal adult kidney. At 3 daGa, *wt1*-positive cells are found in the podocytes of the mature glomeruli (Fig. 1.10B, D). This reactivation of *wt1* expression in the podocytes may reflect the repair response of the damaged nephron, which is detected by transmission electron microscopy (Fig. 1.8B, C). In addition to the podocytes, at 14 daGa small spots in the mesenchyme become positive for *wt1* expression (Fig. 1.10C). These mesenchymal clusters of *wt1*-positive cells appear to include both the mesenchymal condensates (Fig. 1.10F) and the nephrogenic bodies (Fig. 1.10G), suggesting that *wt1* expression in the latter phase reflects de novo nephrogenesis induced by gentamicin administration.

PERSPECTIVES

Stem Cells?

All the regenerative phenomena described above suggest that medaka adult kidney is a useful model for kidney regeneration studies in an evolutionary aspect. The initial response to the renal injury appears to be the repair of damaged nephrons, which may be equivalent to the response of mammalian kidneys [43,44]. Following the repair response, the injured medaka kidney exhibits a second regeneration phase, that is, de novo nephrogenesis. Recapitulation of normal nephron development manifests as the histological appearance of mesenchymal condensates and nephrogenic bodies and de novo expression of *wt1* in these cells. The ability of adult kidney to develop new nephrons suggests that medaka retains renal stem cells throughout life. This nature of medaka kidney may be related to the fact that fish (e.g. medaka) kidneys display slight but continuous growth of mesonephros in

adulthood [15], whereas mammalian kidneys do not generate new nephrons after a specific time-point of development (in rats up to 3 days after birth) [45].

De novo nephrogenesis occurs throughout the renal mesenchymal tissues, implying that there is no specific area where nephrogenic stem cells reside latent in the adult medaka kidney. Renal regeneration through de novo nephrogenesis has previously been reported in other fish [4,5,39 41,46]. Particularly in skates, partial nephrectomy induces renal growth through de novo nephrogenesis. Skates appear to possess a nephrogenic zone containing stem cell-like mesenchymal cells in the ventrolateral portion of the adult kidney. It would be interesting to compare more directly the nephrogenic areas with skate by examining the response to partial nephrectomy in medaka.

From the results to date, it is still unknown whether the reactivation of de novo nephrogenesis in the mesenchyme is accomplished by "multipotent" (one master) renal stem cells in medaka adult kidney. It is possible that progenitor cells in the mesenchyme differentiate into nephrons in response to injury. Medaka adult kidney may retain a wide range of undifferentiated progenitor cells in a quiescent state, which can be observed as mesenchymal condensates and nephrogenic bodies, or just be latent before the formation of nephrogenic clusters. Nevertheless, proliferating cells are often detected in the mesenchymal tissue but not in other tissues of the normal adult kidney, implying that the mesenchymal progenitors possess a capacity for self-renewal.

wt1 Transgenic Fish

Four different stages of nephrogenesis can be defined based on histology using hematoxylin & eosin staining combined with in situ hybridization for *wt1* expression. First, *wt1* is expressed in newly condensed cell masses of the mesenchyme. Second, *wt1* expression also marks nephrogenic bodies, which are more developed than the mesenchymal condensates. Third, when the nephrogenic bodies approach structural maturity, *wt1* is only effective for marking podocytes. Finally, when the nephrons become completely mature, they are no longer positive for *wt1*. In the regenerative process of medaka adult kidney, *wt1* expression appears in a broad range of nephron segments including the glomerulus (Fig. 1.10) in early stages (repair response in the damaged nephron), and later it becomes reactivated in the mesenchyme, where de novo nephrogenesis would occur. Therefore, *wt1* would be a good benchmark for assessing the regeneration of medaka kidney, to trace the repopulation of damaged tissue and de novo nephrogenesis.

In zebrafish, having *wt1a* and *wt1b* paralogues, the regulatory elements that drive *wt1* expression in the kidney have been identified [47]. Bollig et al. established stable transgenic lines with the upstream genomic regions of *wt1a* or *wt1b* recapitulating endogenous expression of the *wt1* paralogues and marking with green fluorescent protein (GFP) reporter developing nephrons in pronephros and mesonephros [47]. In medaka, if possible in a similar way (the enhancer sequence driving glomerular expression of *wt1* does not seem to be conserved in medaka), introduction of the transgene into see-through stock (STII) would enable a live image of the regenerative process in the damaged adult kidney to be acquired.

CONCLUSION

Although medaka mesonephros is evolutionarily different from human metanephros, most of its fundamental features are well conserved, as seen in the histological appearance and cellular nature of nephron development. As in mammalian kidneys, renal stem cells, which derive from mesenchymal cell aggregation, change their shape and differentiate to a tadpole-shaped nephrogenic body and finally form a mature nephron. When damaged by nephrotoxicant (gentamicin), medaka kidney (mesonephros) reboosts the process of nephrogenesis even in adulthood. Thus, medaka has an ability to regenerate nephrons through repopulation of the existing cells and de novo nephrogenesis from the renal stem cells, in response to injury. *wt1* expression marks developing immature nephrons in the development and regeneration processes. Transgenic techniques to label the *wt1*-positive cells with GFP could be utilized to isolate the nephron progenitors and further to identify gene cascade(s) to control differentiation of nephrons. Finally, a great advantage of using medaka as a model should be emphasized: that medaka has only 300 nephrons in each of the definitive kidneys.

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CHAPTER

2

Renal Organogenesis: Growing a Replacement Kidney In Situ from Transplanted Renal Primordia

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OUTLINE

INTRODUCTION

Dialysis and kidney transplantation are mature technologies. They were developed during the first half of the twentieth century and came into wide use during the latter decades of the century [1]. Reflecting on the first year of his nephrology training at Washington University in 1958, Peter A.F. Morrin notes, "The common problems of nephrology were essentially the same as they are today, but our knowledge of the underlying pathophysiology and our ability to deal with them were significantly less. Dialysis for acute renal failure was considered an experimental therapy and limited to a few centers with special interests in the area. There was no effective treatment for chronic renal failure" [2].

Dialysis is life-preserving, but replaces only a small fraction of normal kidney function and has a considerable morbidity [1]. Transplantation is limited by the number of human organs available [3]. While it is certain that dialysis and transplantation will continue to be employed during the foreseeable future, it is likely that

one or more alternatives currently under development will eventually supplant their use.

The use of individual cells including stem cells or differentiated cells to regenerate or repair damaged kidney tissue (cell therapies) offers an alternative to whole organ replacement [4,5]. Since cells are non-vascularized, these approaches can circumvent humoral rejection of xenogeneic tissue mediated by preformed antibodies directed against donor endothelial antigens. Furthermore, cellular rejection can be ameliorated if embryonic cells are transplanted [6,7]. However, cell transplantation to replace the function of structurally complex organs such as the kidney has limitations.

Formation of a functional kidney is dependent on the growth and differentiation of its precursor cells within the intermediate mesoderm into a mature organ consisting of many different cell types. Al Awqati and Oliver have estimated that there are at least 26 terminally differentiated cell types in the kidney of a newborn mouse that arise from at least four cell types present in the undifferentiated metanephric blastema when renal development begins. Delineation of 26 terminally differentiated nephron cell types takes into account cell morphology, location and function [8]. In order for glomerular filtration, reabsorption and secretion of fluid and electrolytes to take place in a manner that will sustain life, individual nephrons must be integrated in three dimensions with one another and with a collecting system, the origin of which is yet another separate structure, the ureteric bud [9]. Concomitantly, vascularization must occur in a unique organ-specific manner from endothelial precursors that may originate from both inside and outside the developing renal primordium [10].

While it is conceivable that endocrine functions of the kidney, such as erythropoietin production, could be recapitulated by transplanting one particular type of renal cell, and it is possible that replacement of one or another type of injured renal cell could enhance the function of damaged tubules, it is difficult to imagine how glomerular filtration and reabsorption in kidneys could be reconstituted de novo by infusion of individual cells. Similarly, programming embryonic stem cells (ESCs) or stem cells from other sources to generate an organ as complex as a kidney presents a formidable [4,5] and thus far unanswered challenge.

Early during its development, the renal metanephric primordium is avascular, making it a suitable cell transplant [4]. Renal primordia are programmed to develop into a kidney in a way that ESCs are not. Therefore, one possible approach to replacing complex kidney functions is through renal organogenesis, or the growing of kidneys in situ originating from transplanted renal primordia/anlagen [1]. This chapter will review the development of methodology for kidney organogenesis and then focus on its application for renal replacement. It will describe alternatives to the use of embryonic kidney primordia as starting material to achieve organogenesis. It will outline current challenges in the use of renal organogenesis technology and conclude by listing a body of experimentally derived general principles.

METHODOLOGY FOR ORGANOGENESIS

The methodology for studies directed towards renal organogenesis derives from a literature describing the transplantation of embryonic kidney. Renal primordia have been transplanted successfully to the chorioallantoic membrane of developing birds [11], subcutaneously [12], into the anterior eye chamber [13 15], into the renal cortex of recipients [16,17], beneath the renal capsule [6,13,18 25] and into the abdominal cavity [6,7,21,25 40]. Most studies that employed renal subcapsular transplantation, placement into the anterior chamber of the eye and onto the chorioallantoic membrane were conducted to define the immune response to fetal kidney transplants or to delineate the means by which renal primordia are vascularized. However, information emerged from these studies leading to approaches that use transplantation to grow a replacement kidney.

Perusal of the literature provides four theoretical reasons why the use of developing metanephroi for transplantation might be advantageous relative to developed kidneys. First, if developing renal primordia are obtained at a sufficiently early stage, antigen-presenting cells (APCs) that mediate direct host recognition of alloantigen or xenoantigen are absent. Second, donor antigens such as major histocompatibility complex (MHC) class I and II may not be expressed on renal primordia. Third, the immune response to transplanted fetal renal tissue differs from that to adult tissue. Fourth, one might expect a transplanted primordium to be supplied by host blood vessels and as such be less susceptible to humoral rejection post-transplantation across a discordant xenogeneic barrier.

Antigen Presentation

In order that a host T-cell-mediated response directed towards antigens from transplanted tissue can be mounted, transplant antigens must first be presented to host T cells. Transplant antigens can be presented by APCs originating from within the donor organ (direct presentation) or by host APCs (indirect presentation). One theoretical advantage gained through the use of embryonic tissue for transplantation is that functional APCs may be absent from the transplants because APCs have yet to mature in the embryos or migrate into the tissue [23,27].

The metanephric kidneys originate in the rat on day 12.5 of a 21 day gestation period [6]. Foglia et al. [22] transplanted kidneys from adult rats or metanephroi from outbred Sprague Dawley rat embryos aged embryonic day (E)15 21, beneath the renal capsule of non-immunosuppressed adult Sprague Dawley hosts. Under these conditions adult kidney transplants undergo acute rejection within 7 days. In contrast, growth and survival of embryonic transplants was age dependent in that enlargement and differentiation in situ over 15 30 days was best for metanephroi obtained from E15 embryos and worsened progressively for those obtained on E16 21. Primordia from E15 embryos showed maturation of renal elements when examined 10 days post-transplantation without rejection, whereas those obtained on E20 had a poor architecture and dense lymphocytic infiltrate. In contrast, liver harvested on E15 transplanted beneath the renal capsule underwent little growth and prompt rejection.

Velasco and Hegre [23] transplanted metanephroi or liver tissue from E15, E17, E18 or E19 inbred Fisher rat embryos with rat major histocompatibility complex (RT1) $RT1^{lvl}$, beneath the renal capsule of RT1-incompatible Wistar Furth adult rats ($RT1^{u}$). All embryonic hepatic grafts were rejected within 10 days. In contrast, the degree of rejection of the metanephroi was age dependent, those from E15 embryos showing minimal or moderate rejection and those from older embryos showing more. If liver and metanephroi from E15 embryos were cotransplanted at different sites, metanephroi underwent a more severe rejection than if they were implanted without liver. APCs populate liver well before E15 in rats, but are not present in circulation until days later. It was speculated that the absence of APCs in metanephroi from E15 embryos together with their presence in liver obtained concurrently explains the differential fate of metanephroi transplanted with or without liver. Under the former conditions, but not the latter, direct presentation of donor antigens to host T cells takes place [23].

MHC Expression

In the mouse, metanephroi arise on day 11.5 of a 20 day gestation period [9]. Statter et al. [24] transplanted metanephroi originating from E14 to adult C57Bl/6 mice ($H\text{-}2^{b}$) beneath the renal capsule of adult congenic B10.A hosts ($H\text{-}2^{a}$). Expression of donor and host-specific class I ($H2K^{b}$) and class II (A^{b}_{β}) transcripts in E14 donor tissue was low and increased progressively in renal tissue from older mice. After transplantation, surviving kidney grafts showed enhanced expression of class I and II transcripts. However, neither class I nor II protein could be detected in transplanted renal primordia.

In human embryos, the metanephric kidneys arise during the first trimester [18 21]. Dekel and co-workers [18 21] carried out a series of investigations in which human adult or embryonic kidney tissue was transplanted beneath the kidney capsule of immunodeficient rats [severe combined immunodeficiency (SCID/Lewis and SCID/nude chimeric rats)]. Human adult kidney fragments transplanted beneath the renal capsule of such rats survive for as long as 2 months. The architecture of the transplanted tissue and the normal structure of glomeruli are preserved. Intraperitoneal infusion post-transplantation of allogeneic human peripheral blood mononuclear cells (PBMCs) results in rejection of adult grafts.

Human fetal kidney fragments transplanted beneath the renal capsule of immunodeficient rats display rapid growth and development. Glomeruli and tubular structures are maintained for as long as 4 months post-transplantation. In contrast to the case for transplanted adult human kidney fragments, infusion of allogeneic human PBMCs into hosts results in either minimal human T-cell infiltration or infiltrates that do not result in rejection or interfere with the continued growth of the human fetal renal tissue. Fetal human kidney grafts have reduced expression of tissue human leukocyte antigen (HLA) class I and II relative to the adult grafts, consistent with reduced effectiveness in inducing an alloantigen-primed T-cell response.

Immune Response

Dekel et al. [20] showed that transcript levels for interferon-γ and interleukin-2 in fetal human kidneys grafted under the renal capsule of immunodeficient rats are markedly reduced after transplantation relative to levels in adult human kidney tissue grafted to the same site. Peak levels of these cytokines appear late after PBMC infusion. Concomitant with these findings, interleukin-4 mRNA is upregulated during the early phase post-PBMC infusion, and interleukin-10 mRNA is expressed throughout the post-PBMC infusion interval. In addition, levels of mRNA coding for chemokines [regulated on activation, normal T cell expressed and secreted (RANTES) and macrophage inflammatory protein-1β (MIP-1β)], their receptor, CCR5, and the cytolytic effector molecule, Fas ligand, are suppressed in the fetal grafts relative to levels in adult grafts. Thus, fetal kidney induces the downregulation of Th1 cytokines, chemokines and Fas ligand, and the upregulation of Th2 cytokines in the grafts. The findings suggest that the human immune response of kidney rejection is dependent on whether the target organ is of fetal or adult origin. An allogeneic immune system appears to mount a T-helper-2-biased response when the target organ is fetal, resulting in enhanced survival of

transplanted tissue relative to adult tissue against which a T-helper-1-biased response is mounted.

Subsequently, Dekel et al. showed that developing human kidneys had restricted expression of multiple factors that determine immune recognition. Thirteen out of 57 genes that were significantly upregulated in adult versus fetal human kidney tissue belonged to the HLA class I and II systems. In addition, molecules that mediate trafficking of leukocytes into the graft such as chemokines RANTES and MCP-1, adhesion molecule E-selectin, proinflammatory cytokines such as osteopontin and complement genes had reduced expressions in embryonic relative to adult kidneys. Reduced immunogenicity of embryonic human or pig kidneys transplanted into immunodeficient mice was confirmed by the absence of cellular rejection following infusion of human PBMCs [21].

Vascularization

The major arterial vessels supplying the kidney originate from lateral branches of the abdominal aorta that terminates in a plexus of arteries in close proximity to the renal pelvis, the renal artery rete [41]. It is a matter of controversy whether the renal microvasculature (smaller vessels and glomerular capillaries) arises exclusively via this angiogenic process, or also in part from endothelial cells resident in the developing metanephros. However, it is clear that during its development, the renal primordium is able to attract at least its major arterial vessels from the developing aorta [10]. In that its blood supply originates at least in part from outside of the developing renal primordium, the kidney may be regarded as a chimeric organ. Its ability to attract its own vasculature in situ establishes the renal primordia as cellular transplants, capable of attracting a blood supply from an appropriate vascular bed [4].

Insight into the origin of the renal microvasculature supply is provided by experiments in which developing kidneys are transplanted to ectopic sites. However, the results of these experiments are somewhat contradictory. One explanation for the differences may be that the means of vascularization is site specific. For mouse or chick metanephroi obtained from E11.5 embryos grafted onto the chorioallantoic membrane of the quail, the vasculature is derived entirely from the host [11]. In the case of metanephroi from E11 12 mouse embryos grafted into the anterior chamber of the eye in genetically identical mice, the glomerular endothelium derives from both donor and host [13 15]. For metanephroi from E15 rat embryos transplanted into the abdominal cavity of mice [29], or from E28 pig embryos transplanted into the abdominal cavity of rats [7,25,35] or mice [32], the microvasculature is largely or entirely host. In all cases, large external vessels derive from the host.

Host immune responses directed against antigens located on the endothelium of a transplanted vascularized organ such as a kidney, or mediated by transplant endothelial cells, are reduced in proportion to the extent that an organ can be transplanted in cell form [4,5] and be supplied by host vessels as it develops in situ. Such reduction would be beneficial in the case of allotransplantation. However, for xenotransplantation it could provide a way to ameliorate humoral rejection (hyperacute rejection and acute vascular rejection) that represents an obstacle to the use of non-primate vascularized organs, such as kidneys from adult pigs, for transplantation into humans [42 47].

Humans and pigs are of comparable size and share a similar renal physiology. The maximum concentrating ability (1080 mOsm/l) and glomerular filtration rate (GFR; 126 175 ml/h or 5 ml/min/kg) of porcine kidneys are similar to those of human kidneys (1200 mOsm/l and 120 125 ml/h or 4 ml/min/kg, respectively) [42]. The specific gravity of pig urine is 1.01 1.05 and the pH 5.5 7.7 (mean 6.40), while those of human urine are 1.002 1.028 and 4.6 8.0, respectively [43]. Physiological differences do exist in renal function between humans and pigs. For example, in adult swine, the normal level of proteinuria is 6 20 mg/100 ml [43,44]. However, experimental observations in the pig-to-primate model have shown that plasma urea, sodium, chloride and potassium remain within normal limits in hosts with good long-term renal function [44]. In view of this level of physiological compatibility between swine and non-human primate renal function, and because pigs are plentiful and can be bred to be pathogen free, pigs represent a suitable kidney donor for humans [42,43].

Hyperacute rejection occurs as a result of the binding of preformed or natural xenoreactive antibodies present in the circulation of hosts to cells of the donor species followed by activation of the host's complement system. Approximately 85% of the natural antibodies in humans that bind to pig cells are directed against galactose-α-1,3-galactose (alpha-gal), a sugar expressed on the vascular endothelium of cells in most mammals, but not in humans, great apes and old-world monkeys. The etiology of acute vascular rejection is multifactorial and incompletely understood. Several of the processes implicated as causative reflect a fundamental incompatibility between host proteins/protein systems and the vascular endothelium of the donor. Factors that are thought to contribute include circulating xenoreactive antibodies that trigger adverse reactions in transplant endothelium, the failure of primate natural killer cells to recognize porcine MHC I molecules, and incompatibilities between porcine proteins/receptors and circulating primate/human proteins such as clotting factors [1].

Humoral rejection following the transplantation of pig kidneys into non-human primates can be ameliorated or overcome through the use of genetically altered organs originating from pigs transgenic for the human complement activator, decay accelerating factor (hDAF) [44 46], or the use of organs from transgenics that do not express alpha-gal [47]. Unfortunately, neither the immunosuppressive regimens used for transgenic-pig to primate kidney transplantation nor the outcomes would be acceptable in humans. Transplantation of kidneys from pigs transgenic for hDAF in combination with host immunosuppression and splenectomy enabled survival for up to 78 days in otherwise anephric cynomolgous monkeys. However, there was a high incidence of adverse events such as development of edema, ascites, vomiting, diarrhea or lymphoproliferative disorders. All recipients had to be euthanized because of renal failure, gastrointestinal hemorrhage or pancreatitis [46]. Survival for as long as 83 days was achieved post-transplantation of kidneys from alpha-gal-deficient donors into immunosuppressed thymectomized and splenectomized baboons, but only if vascularized pig thymic tissue was cotransplanted. Baboons died from serum sickness, infection or myocardial infarction [47].

RENAL ORGANOGENESIS

In contrast to xenotransplantation of whole vascularized organs from pig to primates, cell transplants such as pancreatic islets from pigs can be transplanted into humans without triggering hyperacute or acute vascular rejection [5]. As delineated above, the isolated renal metanephric primordium is a cell transplant. It had been speculated that developing nephrons implanted beneath the renal capsule or into tunnels fashioned in the cortices of host kidneys [16,17] may become incorporated into the collecting system of the host, and thereby increase host renal function. Woolf et al. implanted pieces of sectioned renal primordia originating from embryonic day E13 16 mice into tunnels fashioned in the cortex of kidneys of newborn outbred mice. Differentiation and growth of donor nephrons occurred in the host kidney. Glomeruli were vascularized, mature proximal tubules were formed and extension of metanephric tubules into the renal medulla was observed. However, incorporation of donor nephrons into the collecting system of hosts was not demonstrated.

The present author's group performed experiments similar to those of Woolf et al. in which metanephroi from E15 Sprague Dawley rat embryos were implanted beneath the renal capsule of adult Sprague Dawley hosts. Hosts received no immunosuppression. E15 renal primordia contained segments of ureteric bud and condensing metanephric blastema, but no glomeruli [6]. To determine whether subcapsularly transplanted rat renal primordia became integrated into host kidneys, kidneys of host rats were examined 6 weeks after subcapsular transplantation. To clear blood from the organ, kidneys were back-perfused. This results in a blanching of the kidney as blood is replaced by perfusate. Normally, the entire kidney blanches. However, following perfusion of kidneys that contained a transplanted renal primordia, blood remained in the transplanted structure relative to the host kidney (Fig. 2.1A, cortex arrows). This is likely to reflect a reduced perfusion in chimeric blood vessels (derived from transplant and host kidneys) that have been shown to supply subrenal capsularly transplanted primordia relative to perfusion in those supplying only the host kidney. Blood could be traced into the papilla of the host kidney (Fig. 2.1A, medulla, arrows). Histological examination of kidneys showed that glomeruli (g) in the transplanted kidney (Fig. 2.1B) had been poorly perfused relative to glomeruli present in the host kidney (Fig. 2.1C), in that they contained more red blood cells. Also, glomeruli in the transplanted kidneys (g) were smaller than those in the host kidney (G).

Collecting ducts from transplanted renal primordia migrated towards the papilla of host kidneys in parallel with the vasculature [6]. However, like Woolf et al. [16,17], this study was unable to determine that any connection between the collecting systems of donor and host kidneys was made. In addition, the growth of transplanted renal primordia was constrained by their placement beneath the host kidney capsule.

Isotransplantation/Allotransplantation of Renal Primordia

Renal primordia transplanted into a host rat's fold of mesentery undergo differentiation and growth in hosts that is not confined by a tight organ capsule [6]. A renal primordium in a retroperitoneal dissection from an E15 rat embryo is shown in Fig. 2.2(a). The ureteric bud is delineated by an arrowhead. If transplanted to mesentery with its ureteric bud attached (Fig. 2.2, center panel), the renal primordium enlarges and becomes kidney shaped within 3 weeks (Fig. 2.2b). The ureteric bud differentiates into a ureter (Fig. 2.2b, arrowhead). In contrast to transplanted developed kidneys that undergo acute rejection [6], renal primordia transplanted into non-immunosuppressed hosts have a normal kidney ultrastructure postdevelopment in situ and become vascularized via arteries that originate from the superior mesenteric artery of hosts and veins that originate from the host omentum [30]. Figure 2.3 (a) shows a radiocontrast study that demonstrates that the transplanted metanephros (transplant) is supplied by the host's superior mesenteric artery (SMA).

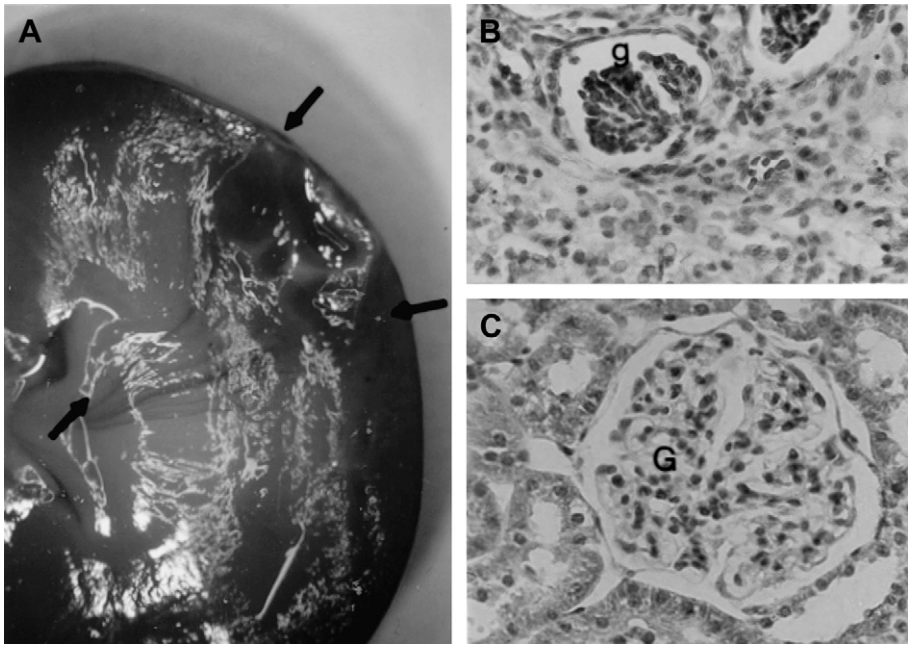

FIGURE 2.1 (A) Photograph of a mid sagittal section obtained following perfusion of a kidney originating from a rat, 6 weeks post transplantation of an E15 renal primordium. Arrows show portions of unperfused transplanted embryonic kidney. (B, C) Photomicrographs of H&E stained kidneys: (B) glomerulus (g) within transplanted embryonic kidney; (C) glomerulus (G) within host kidney. *[Reprinted from Rogers et al., 1998 [6], with permission.]*

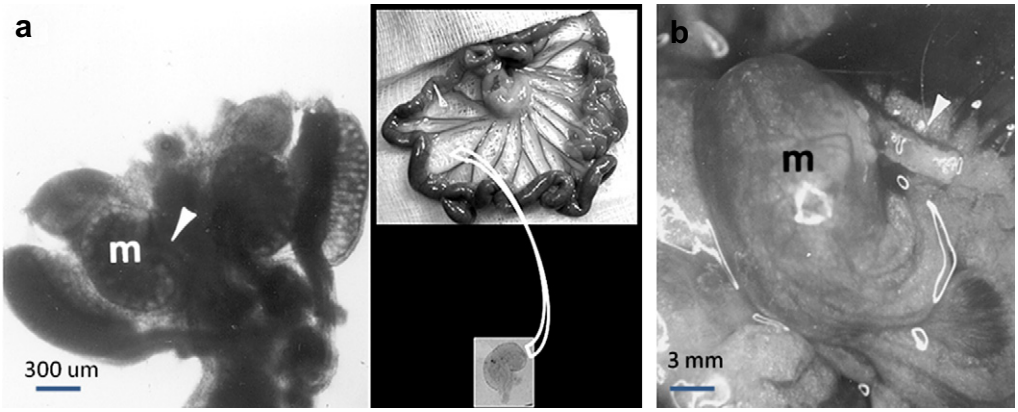

FIGURE 2.2 (a) Photograph of retroperitoneal dissection from an E15 rat embryo showing renal primordium or metanephros (m) and ureteric bud (arrowhead). (b) Photograph of a developed renal primordium or metanephros (m) in the mesentery of an adult host rat 3 weeks post transplantation. Arrowhead shows developed ureter. Magnifications are shown for a and b. The center panel illustrates the transplantation site. *[Reproduced from Hammerman, 2002 [30], with permission.]*

Figure 2.3(b) shows a renal primordium or metanephros (m), 3 weeks after allotransplantation. An artery (a) and vein (v) originating from the host are delineated. A ureteroureterostomy (arrow) between the ureter originating from the transplanted renal primordium (m) and the host ureter is shown in Fig. 2.3(c).

Differentiated structures at 20 weeks postimplantation are illustrated in Fig. 2.4, which shows hematoxylin & eosin (H&E)-stained sections of a developed renal primordium. The cross-sectional diameter of the developed renal primordium shown in Fig. 2.4(a) (~1.2 cm) is about half the diameter of a normal rat kidney [33]. Its ureter (u) is labeled. Figure 2.4(b e) shows a glomerulus (g), proximal tubule (pt), distal tubule (dt) and collecting duct (cd) in the cortex. Electron microscopy of a developed renal primordium [31] reveals normal renal structures (Fig. 2.5). Developed renal primordia transplanted onto the omentum produce urine that is excreted in the normal manner following ureteroureterostomy between transplant and host (Fig. 2.3c), a procedure that can be readily carried out if renal primordia are implanted in close proximity to the host ureter [6].

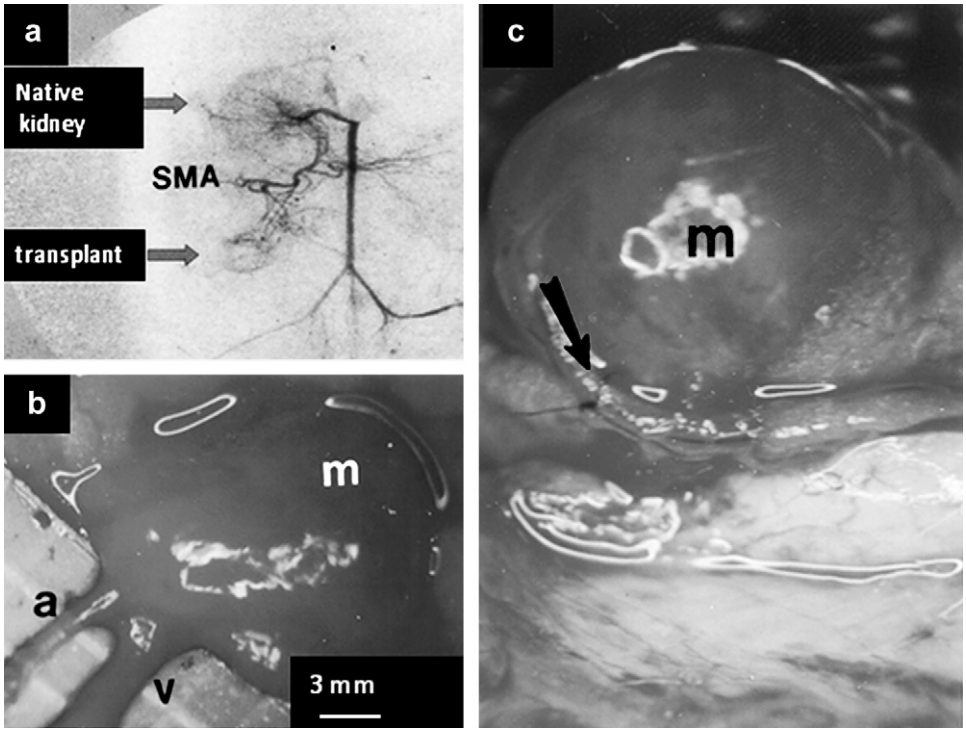

FIGURE 2.3 (a) Radiocontrast image of native kidney and transplant, 6 weeks post transplantation into the peritoneum of a host rat. SMA: superior mesenteric artery. (b) Artery (a) and vein (v) supplying the developed renal primordium or metanephros (m) originating from the host's mesentery. (c) Ureteroureterostomy between transplant and host ureters (arrow). Magnification is shown for b and c (in b). *[Reproduced from Hammerman, 2002 [30] and Rogers and Hammerman, 2004 [33], with permission.]*

Levels of renal function in transplanted renal primordia (GFR) were determined by measuring inulin clearance in otherwise anephric rats. In initial experiments GFRs were very low [6]. However, as shown in Table 2.1, incubation of renal primordia with growth factors before implantation increased GFRs more than 100-fold compared to those in rats with non-growth factor-incubated renal primordia implanted concurrently [30]. GFRs in growth factor-treated renal primordia are about 6% of normal. Renal plasma flow, another parameter of renal function, was measured in transplanted renal primordia by calculating P-aminohippurate (PAH) clearances. The ratio of GFR/PAH clearance (filtration fraction) was 0.6, comparable to filtration fractions measured in rats with reduced renal function [30]. Urine flow rates in transplanted rats are about 12% of the inulin clearance (GFR) measured in growth factor-treated renal primordia (Table 2.1). The urine volume (UV)/GFR of 0.12 demonstrates that transplanted renal primordia can concentrate urine [30].

Hemodialysis provides renal failure patients with GFRs that are about 10% of normal [1]. Therefore, 6% of normal approximates a level of renal function that would be expected to preserve life. In fact, life can be prolonged in otherwise anephric rat hosts by prior transplantation and ureteroureterostomy of one [33] or two [38] renal primordia. Survival as a function of time after removal of all native renal mass (all renal function from the implant) is shown in Fig. 2.6. Control rats (no transplanted renal primordia) lived for 67 ± 2.7 h (range 48 78 h) after removal of all native host renal mass. Rats in the TX group (transplanted primordium, but with the ureteroureterostomy severed such that urine was discharged into the peritoneal cavity) lived for 65 ± 6.0 h (range 55 76 h), no longer than controls. Rats in the TX-EXCR group (transplanted primordium with intact ureteroureterostomy that permitted excretion of urine) lived for 125 ± 12 h (range 108 170 h), significantly longer than control or TX rats [33]. Thus, enhanced survival could not be attributed to a metabolic function of the embryonic renal transplant per se, since it depended on externalization of urine.

Using inbred congenic rats (PVG-RT1^{C} and PVG-RT1^{avl}) it was shown that renal primordia can be transplanted across the RT1 locus into non-immunosuppressed hosts. A state of peripheral immune tolerance secondary to T-cell "ignorance" permits the survival of transplanted renal primordia. The ignorance probably results from the absence of APCs originating from the donor in the embryonic renal tissue, and the consequent absence of direct presentation of transplant antigen to host T cells (presentation by donor dendritic cells to host T cells), as shown previously for subrenal capsular transplants [27].

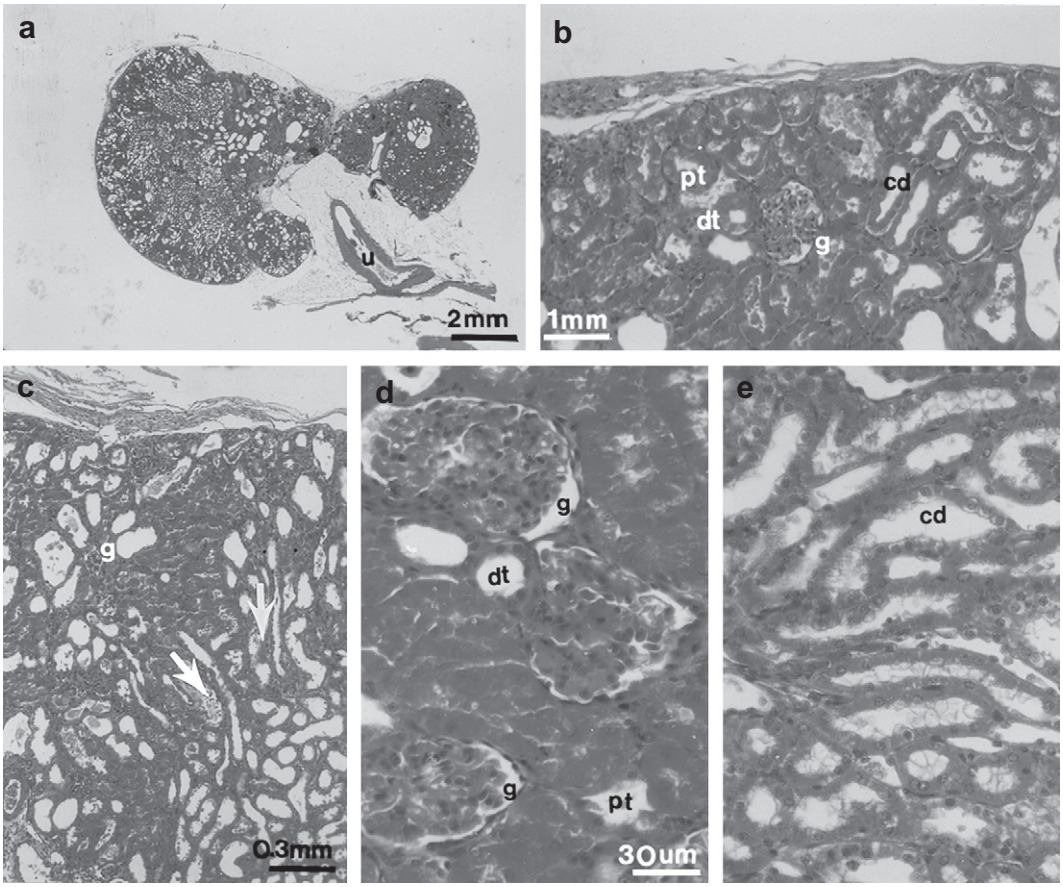

FIGURE 2.4 H&E stained sections of a developed metanephros 20 weeks post transplantation. (a) The ureter (u) is shown. (b) A glomerulus (g), proximal tubule (pt), distal tubule (dt) and collecting duct (cd) in the cortex. (c) A glomerulus (g) and collecting duct (arrow) are labeled. (d) A glomerulus (g), proximal tubule (pt) and distal tubule (dt) are labeled. (e) A collecting duct (cd) is labeled. Magnifications are shown in a–d (for d and e). *[Reproduced from Rogers and Hammerman, 2004 [33], with permission.]*

Preservation Prior to Transplantation

In the case of human renal allotransplantation, there is an unavoidable delay between the time of harvest from donors and the time of implantation into recipients. Before removal from the donor, human renal allografts are flushed with a preservation solution, often University of Wisconsin (UW) solution, and stored subsequently in ice-cold UW solution. Theoretically, renal primordia could be harvested immediately before implantation into humans. However, practically it would be best if primordia could be stored in vitro for a period of time before transplantation. The ability to store primordia would permit distribution to sites for transplantation, distant from the site of harvesting, and would allow time to plan the transplant procedure.

To determine whether renal primordia can be stored in vitro before transplantation, renal primordia from E15 rat embryos were transplanted into the omentum of non-immunosuppressed uninephrectomized (host) rats either directly or suspended in ice-cold UW preservation solution for 3 days before implantation. The size and extent of tissue differentiation preimplantation of E15 renal primordia implanted directly are not distinguishable from the size and differentiation of renal primordia preserved for 3 days. By 4 weeks post-transplantation, renal primordia that had been preserved for 3 days had grown and differentiated such that glomeruli, proximal and distal tubules, and collecting ducts with normal structure had developed. At 12 weeks post-transplantation, GFRs of preserved renal primordia were comparable to those of primordia implanted directly, consistent with the viability of preserved renal primordia [28].

Another approach to preservation was taken by Bottomley et al., who studied the effects of controlled-rate freezing and ice-free vitrification on viability of metanephroi. Renal primordia from E15 Lewis rats were either frozen at a controlled rate using a cryosubstitution solution or cryopreserved in an ice-free state by rapid cooling to 100°C in cryoprotectant, followed by vitrification to 120°C. After cryopreservation, the metanephroi were stored at 135°C for 48 h. After storage the

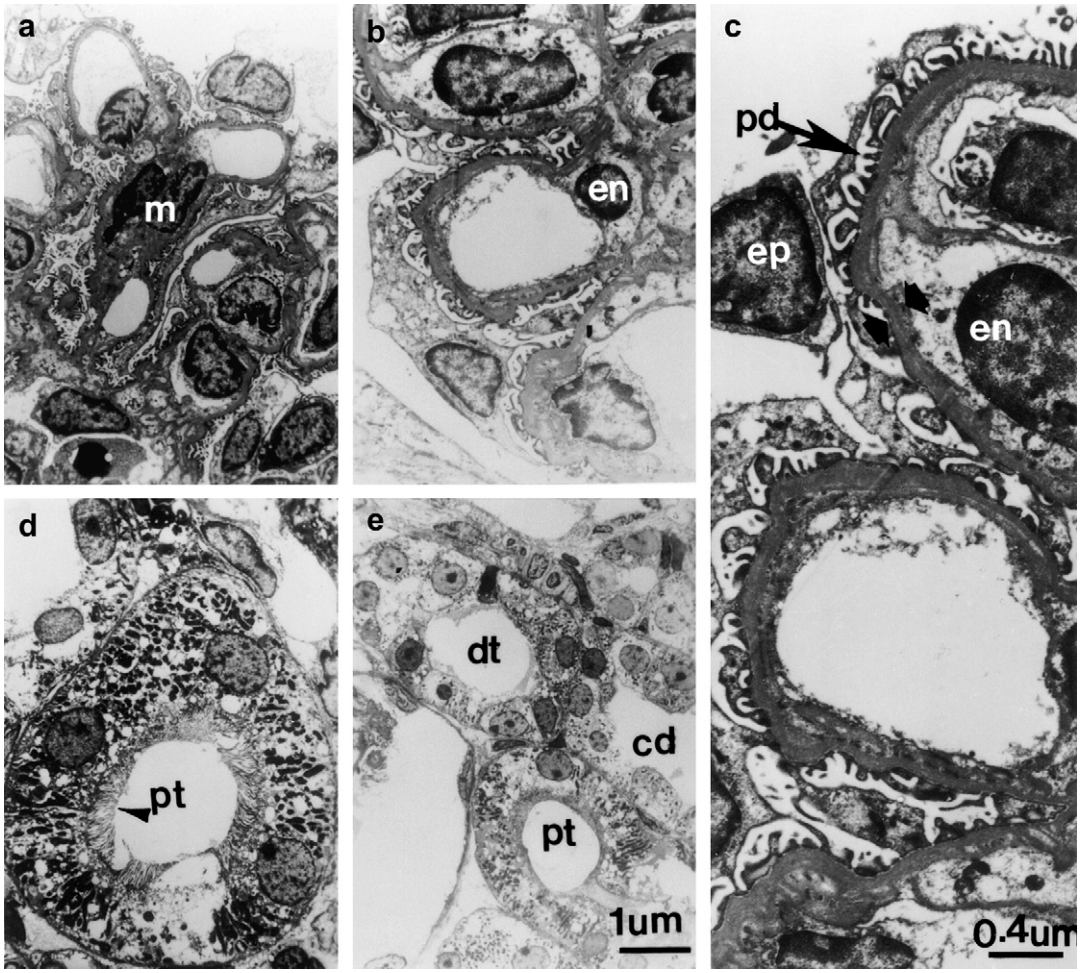

FIGURE 2.5 Electron micrographs of transplanted rat renal primordia. Glomerular capillary loops show labeled: (a) mesangial cell (m); (b) endothelial cell (en); and (c) epithelial cell (ep), endothelial cell (en), podocytes (pd) and a basement membrane (arrows). (d) A proximal tubule (pt) with a brush border membrane (arrowhead); (e) proximal tubule (pt), distal tubule (dt) and collecting duct (cd). Magnifications are shown for c and e. *[Reproduced from Hammerman, 2002 [31], with permission.]*

TABLE 2.1 Urine Volumes and Inulin Clearances

	Vehicle	Growth factors
Urine volume (UV)		
(μl/h)	31 ± 9.1	621 ± 62
Inulin clearance (GFR)		
(μl/min/100 g)	0.24 ± 0.06	27 ± 8.2
(μl/h)	38	5313
UV/GFR	0.8	0.12

Some data are expressed as mean ± SEM.
UV/GFR is calculated using values expressed as μl/h.
Reproduced with permission [30].

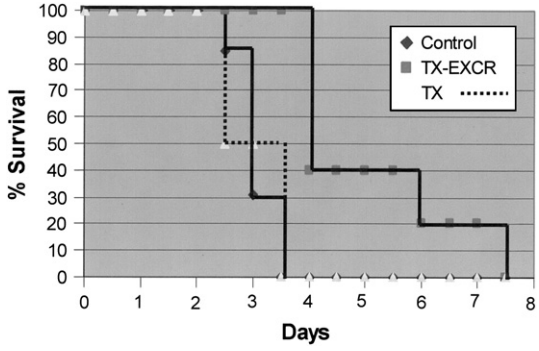

FIGURE 2.6 Survival of rats as a function of time after removal of both kidneys. Control rats (n 13) had no transplanted renal primordia. Rats in the TX group (n 4) had a transplanted renal primordium, but with the ureteroureterostomy severed such that urine was discharged into the peritoneal cavity. Rats in the TX EXCR group (n 5) had a transplanted renal primordium with an intact ureteroureterostomy that permitted excretion of urine. *[Reproduced from Rogers and Hammerman, 2004 [33], with permission.]*

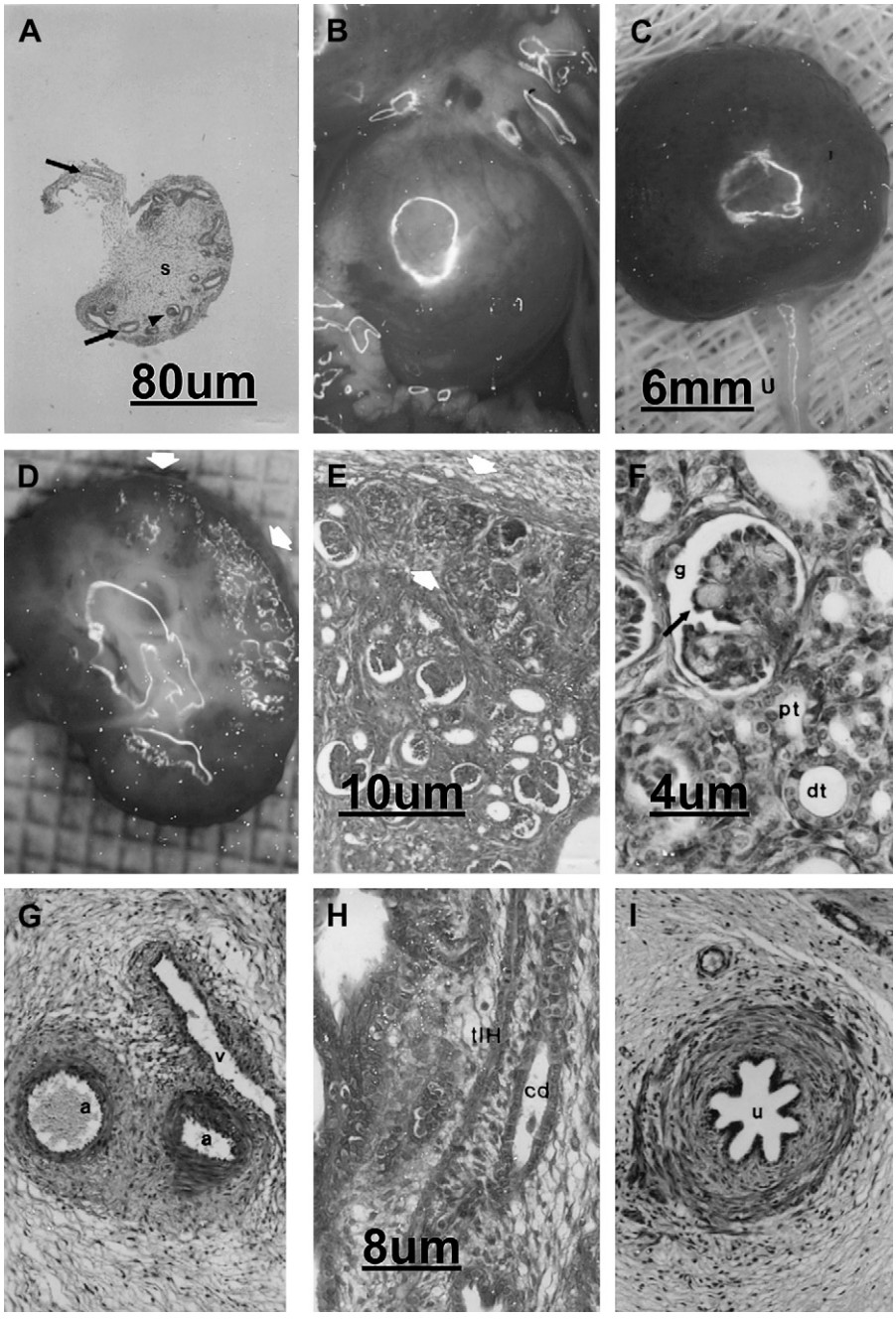

FIGURE 2.7 (A, E–I) Photomicrographs and (B–D) photographs of pig renal primordia. (A) E28 primordium (s: stroma; arrow: ureteric bud; arrowhead: developing nephron). (B–I) Pig renal primordia 7 weeks post transplantation in a rat mesentery: (B) developed primordium in situ; (C) primordium after removal from the mesentery (u: ureter); (D) mid sagittal section of a primordium (arrowheads delineate lobules); (E) cortex with the nephrogenic zone delineated (arrows); (F) cortex with a glomerulus (g), proximal tubule (pt) and distal tubule (dt) labeled (arrow delineates glomerular capillary loop); (G) a large artery (a) and vein (v); (H) medulla with collecting ducts (cd) and thin loop of Henle (tlh) labeled; (I) ureter. Magnifications are shown for A; B–D (in C); E, G and I (in E); for F (in F) and H (in H). *[Reproduced from Rogers et al., 2005 [7], with permission.]*

renal primordia were rewarmed and resuspended in culture media, and their viability was assessed using the AlamarBlue assay and histology. There was no difference in embryonic kidney metabolic activity between either of the cryopreserved groups of metanephroi relative to a control untreated group. Cryosubstitution resulted in ice formation during controlled-rate freezing. In contrast, the amount of ice was significantly

reduced by vitrification. However, vitrified metanephroi showed mitochondrial and nuclear injury at the cellular level [37].

Xenotransplantation of Renal Primordia

Renal primordia were transplanted from an E15 Lewis rat embryo across a concordant xenogeneic barrier into the peritoneum of 10-week-old C57Bl/6J mice. In mice that receive immunosuppression, but not in its absence, the transplanted rat renal primordium undergoes differentiation and growth in situ [29]. To gain insight into the origin of the vasculature (donor versus host) of renal primordia transplanted in the omentum, using a rat-to-mouse model, developing rat renal primordia were stained using mouse specific antibodies directed against the endothelial antigen CD31. The vasculature of the developed rat kidney transplanted into the mouse is largely of mouse origin including glomerular capillary loops. In contrast, the capillary loops in rat renal primordia transplanted into rats do not stain for mouse CD31 [29].

In the pig, metanephroi arise on day 28 of a 120 day gestation period [7,32]. Using a highly disparate model (pig to rodent), E28 pig renal primordia (Fig. 2.7A) consisting of undifferentiated stroma (s) branched ureteric bud (up) and primitive developing nephrons (arrows) were transplanted into the mesentery of Lewis rats [7,35] or C57Bl/6J mice [32]. Five to seven weeks post-transplantation, no trace of the renal primordium could be found in hosts that received no immunosuppression. In contrast, Fig. 2.7(B I) illustrates that by 7 weeks after transplantation in anti-CD45/tacrolimus-treated rats the E28 PRA have undergone differentiation and growth [7]. The developed pig renal primordium is slightly larger in volume (diameter and weight) than a normal rat kidney.

Shown in Fig. 2.8 are glomeruli from rat kidneys and pig kidneys and glomeruli within pig renal primordia transplanted into rats 8 weeks previously, stained with anti-rat endothelial antigen-1 (RECA-1) that is specific for rat endothelium, or anti CD31 that is specific for pig endothelium. The origin of the glomerular vasculature in transplants is rat (host). Non-glomerular renal vasculature is also of host origin [35].

Dekel et al. successfully transplanted renal primordia originating from pig embryos aged E20 21 to E27 28 beneath the renal capsule of immunodeficient mice. Most transplants from the E20 25 donors failed to develop or evolve into growths containing few glomeruli and tubules, but other differentiated derivatives such as blood vessels, cartilage and bone. In contrast, the transplants originating from E27 28 pig embryos all exhibited significant growth and full differentiation into mature glomeruli and tubule. Dekel et al. found mouse CD31 expression in external vessels as well as developing glomeruli and small capillaries of pig renal primordium xenografts, consistent with a host origin for the vasculature of the developed renal primordium cellular transplants. In addition, Dekel et al. transplanted adult pig kidney tissue or E27 28 pig renal primordia beneath the renal capsule or onto the testicular fat of immunocompetent Balb/c mice. Some hosts were treated with CTLA4-Ig. Evaluation of adult or E27 28 embryonic tissues 2 weeks postimplantation into non-CTLA4-Ig-treated hosts showed rejection of tissues. In CTLA4-Ig-treated hosts, most E27 28 renal primordia underwent growth and differentiation. In contrast, all adult kidney grafts had a disturbed morphology, necrotic tissue and a high degree of lymphocyte infiltration. The authors interpreted these data as being consistent with an immune advantage of the developing precursor transplants over developed adult kidney transplants in fully immunocompetent hosts [25].

Dekel and co-workers implanted metanephroi from E70 human embryos intraperitoneally into immunodeficient (SCID) mice. Transplanted kidneys survived for more than 2 months post-transplantation [21]. Hybridization to cDNA arrays of RNA derived from normal human renal primordia at 8, 12, 16 or 20 weeks of gestation demonstrated a subset of 240 genes, the expressions of which changed substantially with time. Clustering analysis of global gene expression in transplants post-transplantation revealed a temporal profile of gene expression similar to that observed in the normal human kidneys during development, consistent with recapitulation of a renal developmental program. Comparison of the expression profiles of developing metanephroi to a Wilms' tumor specimen revealed no similarity, consistent with no threat of malignant transformation after transplantation of human kidney precursors.

ALTERNATIVES TO THE USE OF EMBRYONIC KIDNEY PRIMORDIA TO ACHIEVE ORGANOGENESIS

Use of Embryonic Stem Cells as Starting Material

Human ESCs transplanted into immunodeficient mice differentiate into teratomas containing structures from all three germ layers [48,49]. Differentiated structures include glomerular- and renal tubular-like elements [49], consistent with the potential for ESCs to differentiate into kidney tissue. However, glomerular- and tubular-like structures are not glomeruli and tubules and for reasons delineated in the introduction of this chapter, the structural complexity of kidney makes it unlikely that a functional replacement organ can be generated from ESCs alone.

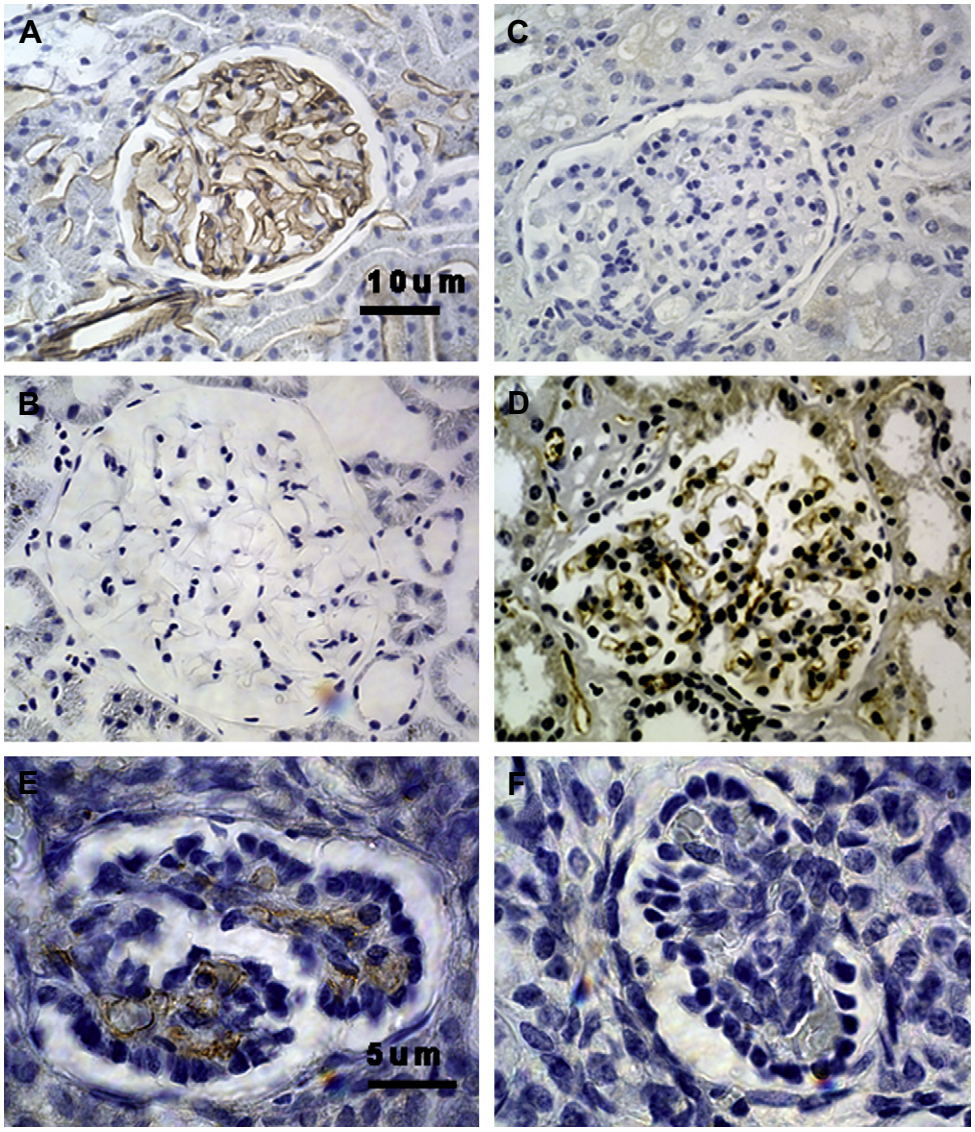

FIGURE 2.8 Photomicrographs of stained sections of: (A, B) rat kidney, and (C, D) pig kidney; and (E, F) a pig renal primordia from an E28 embryo 8 weeks post transplantation into a mouse omentum, stained with RECA 1 (A, B, E) or CD31 (C, D, F). Glomerular capillaries in transplants stain positive for RECA 1 that is specific for rat endothelium (E) and negative for CD31 that is specific for pig endothelium (F). Magnifications are shown for A–D (in A) and E and F (in E). *[Reproduced from Takeda et al., 2006 [35], with permission.]*

A novel step towards use of ESCs in renal engineering was taken by Streenhard et al., who microinjected mouse ESCs that were tagged with a lineage marker into E12 13 mouse metanephroi. ESC derivatives differentiated into lumenized tubules with apical junctional complexes and primary cilia and assembled a basement membrane around their basal aspect following 3 5 days in organ culture. Some of the structures expressed proximal tubule markers. In contrast, there was only rare evidence for integration of ESCs into the glomeruli that form in organ culture [50]. These observations suggest that the developing kidney microenvironment provides cues for entry of ESCs into a nephrogenic program in vitro.

Lanza et al. created bioengineered tissues from cardiac, skeletal and renal cells cloned from adult bovine fibroblasts. They transplanted subcutaneously into an adult bovine host, cultured dispersed kidney cells from its E56 cloned embryo, seeded on collagen coated cylindrical polycarbonate membranes. Cloned and passaged kidney cells expressed renal specific proteins in vitro including synaptopodin, aquaporin-1, aquaporin-2 and Tamm Horsfall protein. After expansion, the cells produced both 1,25(OH)D_3 and erythropoietin [51].

Straw-colored fluid was produced by renal units that had differentiated in situ into glomerulus-like and tubule-like structures and undergone vascularization.

Chemical analysis of collected fluid suggested unidirectional secretion and concentration of urea nitrogen and creatinine. Cells within renal units produced synaptopodin, aquaporins-1 and 2, and Tamm Horsfall protein. No rejection response was detected in hosts to the cloned renal cells [51].

Use of Renal Precursor Cell Components

Advances in understanding the molecular biology of rodent renal development have led to the ability to culture the components of the developing rat kidney (the ureteric bud and metanephric blastema) in isolation from one another. Steer at al. described a method for subculturing and propagating either component in isolation [52]. They propose that ureteric buds can be combined with freshly isolated metanephric blastema to form a large number of rat "neokidneys" derived from a single progenitor in vitro and speculate that neokidneys could be grown from component parts. A colony of neokidneys derived from a single renal component would lead to a large supply of genetically identical renal tissue. In addition, isolated mesenchyme or blastema could be transfected with constructs designed to regulate the growth characteristics of the neokidney or enhance the function of one or more cellular components. Finally, it might be possible to create a chimeric structure using ureteric bud as a scaffold that could be recombined with allo- or xeno-derived non-renal mesenchymal cells. Such cells could be engineered to differentiate into a renal phenotype when exposed to kidney-specific signals from the scaffold [52].

Use of Non-renal Precursor Cells Integrated into Renal Primordia

Yokoo et al. injected human mesenchymal stem cells (hMSCs) labeled with LacZ into E9.5 mouse embryos or E11.5 rat embryos at the site of early renal organogenesis, and subjected the whole embryos to culture. After 48 h of whole culture, metanephroi were dissected from whole embryos and cultured in vitro for 6 days. It was found that hMSC-derived LacZ-labeled cells contribute to renal structures in organ-cultured metanephroi [53]. Subsequently, the investigators implanted LacZ labeled hMSC that had been transfected with glial cell line-derived neurotrophic factor into the nephrogenic site of E11.5 rat embryos. Following 48 h of whole embryo culture, metanephroi containing hMSC were dissected out and transplanted into the mesentery of uninephrectomized rats. No immunosuppression was required. Transplants enlarged over 2 weeks in non-immunosuppressed rats, became vascularized by host vessels and contained hMSC-derived LacZ-positive cells that were morphologically identical to resident renal cells. These findings suggest that self-organs from autologous MSCs can be generated using inherent developmental and angiogenic systems [54].

Fukui et al. assessed whether hMSCs are competent to differentiate into precursors of the collecting duct system by transplanting chicken Pax2-expressing hMSCs into the chicken ureteric bud progenitor region. Transplanted hMSCs migrated caudally with the elongating Wolffian duct and were integrated into the Wolffian duct epithelia. In addition, chicken Pax2-expressing hMSCs started to express human LIM1 after their integration into the Wolffian duct epithelia. These results suggest that chicken Pax2-expressing hMSCs can be competent to differentiate into the Wolffian duct cells by the influence of chicken local signals [55].

Use of Scaffolds

A permissive tissue microenvironment for support of stem cell differentiation and tissue formation must include matrix proteins, and soluble or immobilized growth factors. It must promote cell-to-cell interactions, and account for mechanical forces that shape organ development. Scaffolds serve such functions. Typically, synthetic scaffolds do not have the intricate architecture needed for whole organs and do not provide signals for differentiation [56]. Such functions are better provided by scaffolds made from biological tissues with native cells removed. To ascertain whether native kidney can provide such scaffolding, Ross et al. decellularized rat kidney, seeded the resulting scaffold ex vivo with murine pluripotent ESCs infused antegrade through the renal artery or the ureter, and incubated the mix in growth medium that was either static or perfused through a pulsatile system to simulate the mechanical forces delivered by a native circulatory system. Primitive precursor cells proliferated within glomerular, vascular and tubular matrices. ESCs assumed a flattened endothelial-like appearance in vascular structures, while epithelialization was reflected by expression of cytokeratin in cells lining the vascular basement membranes. Cells not in contact with the basement membrane matrix became apoptotic. These observations suggest that the extracellular matrix can direct regeneration of the kidney [56].

CURRENT CHALLENGES IN THE APPLICATION OF RENAL ORGANOGENESIS TECHNOLOGY

Achieving Adequacy of Blood Flow to Transplants

As reviewed above, the developing metanephros transplant attracts its blood supply from the host.

Optimal glomerular filtration in a native kidney requires a high level of renal blood flow and pressure from the native renal artery which may or may not be achievable through flow and pressure from newly formed renal arteries (Fig. 2.3b). Marshall et al. proposed that the omentum may not provide the ideal site for metanephros transplantation and that sites with increased blood flow would enhance function. To test the hypothesis they transplanted E15 Lewis rat metanephroi into the retroperitoneal fat adjacent to major blood vessels in the peritoneum of unilaterally nephrectomized Lewis rats. Twenty-one days later the transplants were examined and suitable transplants connected to the host urinary system. Approximately 130 days later GFRs of the connected transplants were measured and compared to GFRs of metanephroi transplanted in omentum. GFRs measured in metanephroi transplanted adjacent to major blood vessels were higher than those in metanephroi transplanted to omentum [36].

Achieving Adequate Excretion of Urine: Difficulties with Ureteroureterostomy/ Ureteroneocystectomy

In order for a transplanted metanephric kidney to clear solutes from the blood of the host, urine must be excreted. The issue is not a trivial one. In fact, excretion of urine has been achieved by only two groups of investigators in the setting of isotransplantation or allotransplantation in rats (without a requirement for immune suppression) [6,26,28,33,36 39]. Ureteroureterostomy between the ureter of a developed rat metanephros and the ureter of a rat host is not possible before 17 days post-transplantation because the ureter of the transplant is too friable. If ureteroureterostomy is not performed by 21 days post-transplantation, obstruction results [6]. This is reflected by the formation of urine-filled cysts in transplants [6,25,36 40]. This group has transplanted as many as four metanephroi, each of which undergoes growth and development in the mesentery [27]. They found that only one ureteroureterostomy was possible in the rat because of the small size of transplant and host ureters. However, others have been able to connect ureters from two transplants into the ureter of a host [38].

Immunological reactions following xenotransplantation of metanephroi (e.g. from embryonic pig to rat) and the impact of immune suppression on development of the collecting system of transplants represent potential challenges to achieving adequate excretion of urine after xenotransplantation of renal primordia. Baldan et al. described ureteroneocystectomy as the Achilles' heel in pig-to-small-primate kidney transplantation surgery, citing an incidence of ureteral stenosis as high as 50% in long-term survivors of renal allotransplantation in cynomolgus monkeys [45]. The authors speculate that an immunological reaction to the xenograft (which is far more vigorous in xenotransplantation than in allotransplantation) may at least partially explain the high urological complication rate in their own series and recommend the routine use of ureteral stents when renal xenografts are transplanted into small primates.

Achieving Adequate Growth of Transplanted Renal Primordia

Renal function of developed primordia post-transplantation from embryonic rat-to-rat is low [6,26, 28,33,36 39]. This is probably explained by the fact that rat primordia do not grow to the size of native kidneys after allotransplantation or isotransplantation. However, the kidneys that do develop if ureteroureterostomy is performed in a timely manner and obstruction is avoided (see above) are ultrastructurally normal [31]. Nephron number is approximately 30% of that in native kidneys [34]. In this regard, the phenotype of such kidneys is reminiscent of that observed early in rat models of oligomeganephronic congenital hypoplasia [57,58].

Dilworth et al. transplanted metanephroi from E15 Lewis rat embryos adjacent to the abdominal aorta of uninephrectomized adult female syngeneic Lewis rats. Twenty-one days later, a single metanephros ureter was anastomosed to the host's urinary system and 3 month clearance measurements were performed. Effective renal blood flow (149 ± 33 μl/min/g kidney weight) and GFR (17 ± 9 μl/min/per g kidney weight) were significantly lower in transplanted metanephroi than in control adult kidneys. Renal vascular resistance was higher. Nephron numbers in transplanted metanephroi were significantly greater than those of E21 kidneys, but lower than those of kidneys obtained on postnatal day 1 (P1). Angiotensin II type 2 receptor mRNA expression, a marker of nephrogenesis, was markedly reduced in metanephroi. Aquaporins 1 and 2, the epithelial Na^+ channel and Na K 2Cl cotransporter type 2 mRNA and protein were expressed in transplanted metanephroi. However, the urea transporters A1, 2 and 3 were absent. Vascular markers (α-smooth muscle actin and CD31) were identified in metanephroi and expression did not differ from that of E21 or P1 kidneys. The authors concluded that rat metanephroi continue to develop post-transplantation but only reach a stage of development equivalent to that of a normal rat kidney at birth [39].

The observation that pig primordia transplanted in rats grow to a size larger than native rat kidneys in rat hosts [7] suggests that the hypoplasia may be more characteristic of transplanted rat renal primordia than of pig renal primordia. Hypoplasia can result from excessive cell death in metanephric blastema [57,58]. Rapidly

dividing blastema cells in renal primordia could be placed at risk during the time of relative hypoperfusion that occurs between dissection from donor embryos and revascularization in situ. It may be that pig renal primordia, in which cells divide more slowly over a longer gestation period, are at reduced risk for apoptosis relative to rat primordia during the time of relative hypoperfusion. The finding of kidneys larger than native rat kidneys at 7 weeks postembryonic pig-to-rat xenotransplantation [7] is consistent with this possibility.

CONCLUSION

There is a need for a source alternative to human kidneys to replace renal function in humans with end-stage disease. However, kidney structure is complex, its embryological origin is diverse, and glomerular filtration, reabsorption and secretion depend on precise anatomical relationships between thousands to millions of nephrons and a collecting system [1]. In all probability a solution for end-stage disease will require growing entirely new nephrons or an entirely new kidney from cells already programmed to differentiate into these structures.

In terms of their incorporation into a replacement organ to replace renal function in the long term, endocrine and metabolic functions of kidneys are probably less important than filtration, reabsorption and secretion. Active forms of vitamin D and erythropoietin, routinely administered to patients with end-stage renal failure, can correct endocrine deficiencies [1].

For reasons outlined above, the use of ESCs exclusively to grow a kidney appears to be a technology that will not soon come to fruition. However, the feasibility for use of nuclear-transfer generated cells and tissue as transplants has been demonstrated in a large animal model, the cow [51]. While such a strategy could not be used in humans, as ethical considerations require that preimplantation embryos not be developed in vitro beyond the blastocyst stage, the findings may be applicable to engineered adult native cells or human ESCs.

Approximately a decade has passed since the first demonstration that renal primordia transplanted into a mesenteric site undergo growth and differentiation, attract a vasculature from the host and clear inulin from the host's circulation after ureteroureterostomy [6]. Review of literature from a number of laboratories both antecedent and subsequent to this demonstration [6] supports a body of experimentally derived general principles about renal organogenesis.

- The cellular immune response to transplanted fetal renal tissue differs from that to adult tissue [6,18 23, 27]. If developing renal primordia are obtained at a sufficiently early stage, APCs that mediate direct host recognition of alloantigen or xenoantigen are absent.
- Donor antigens such as MHC class I and II are not expressed on developing renal primordia to the extent that they are expressed on adult kidneys [18,24].
- Unlike developed kidneys, metanephroi are non-vascularized or minimally so. The predominant host origin of the vasculature that does develop in situ under many allogeneic and xenogeneic transplant conditions [25,29,35,53] could serve to ameliorate rejection responses that occur as a result of incompatibilities between hosts and foreign endothelium and currently represent significant obstacles to the use in humans of organs from other species such as pigs.
- Reduction of host renal mass at the time of transplantation enhances the growth of mesenteric transplants [6,40].
- Life-extending renal function can be demonstrated post-transplantation of renal primordia if a means is provided to excrete urine [33,38].
- Apropos of its role as a physiologically compatible kidney donor for humans, the pig can be used as a xenogeneic renal primordia donor. Unlike the case for allotransplantation, xenotransplantation of renal primordia [rat to mouse [29] or pig to rodent [7,25,32,35]] requires that hosts be immunosuppressed.
- The technologies described in this chapter are not mutually exclusive. There are alternatives to the use of embryonic kidney primordia exclusively as starting material to achieve renal organogenesis [48 56].

No matter how it is accomplished, the availability of kidneys via renal organogenesis would result in a paradigm shift in how the world thinks about renal replacement/transplantation: (i) there would be no need to transport kidneys across long distances; (ii) transplantation could be done electively at a convenient time; and (iii) transplantation could be offered to high-risk individuals and could be repeated as needed. Thus, the technology has considerable therapeutic potential.

Acknowledgment

Supported by George M. O'Brien Center DK079333 by grant 1 2008 37 from JDRF.

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CHAPTER

3

Use of Genetic Mouse Models to Study Kidney Regeneration

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OUTLINE

INTRODUCTION

Since the development of the first transgenic mice there has been an explosion of knowledge and experience in the use of genetic manipulation to test gene function in vivo in mice. These technologies have proved to be tractable and powerful tools with significant advances over in vitro studies that were the mainstay of basic sciences. Genetic manipulation in mice has proven to have greater relevance to human disease and greater reproducibility, and has enabled functional studies at a level of sophistication not previously possible. Alongside this explosion of genetic tools in mice, has been the development of an array of mouse models of human conditions including kidney disease and regeneration. Regenerative nephrology, a new field in nephrology, has emerged from these technological advances. This chapter will explain some of the underlying technologies that are in common usage, highlight key areas of technological advance using these methods, and show key areas of pitfalls that may not be readily apparent to a reader.

GENETIC MODIFICATION IN VIVO

Transgenic Expression

Transgenesis is the introduction of foreign DNA from another organism. Since the advent of molecular biological techniques in the 1970s we have been able to use bacteria and subsequently mammalian cells to synthesize proteins. To achieve this the coding sequence for the gene of interest is placed alongside a promoter sequence that is active in the host bacteria or cell in a small circular DNA commonly found in bacteria, called a plasmid. This plasmid is not part of bacterial genomic DNA, but functions in bacteria as a stable source of DNA and is replicated and inherited like genomic DNA during cellular replication. Plasmids can easily be transferred into bacterial cytoplasm. Plasmids can also be persuaded to enter mammalian cell cytoplasm transiently, particularly in transformed cell lines that proliferate well in culture. Bacterial plasmids have been genetically modified to contain key elements of mammalian promoters/enhancers that express at high levels in many types of cells and also contain the 3′ untranslated region (UTR) of genes such as globulin. By placing the coding region or open reading frame of a gene of interest between the promoter/enhancer region and the 3′ UTR of a typical mammalian gene in such a plasmid, and introducing copies of that plasmid into the cytoplasm of mammalian cells it became common practice to transiently generate the protein encoded in that plasmid by the cell that accepts it. Although plasmids do not remain in mammalian cell cytoplasm for more than a few days, linearized plasmid occasionally integrates into the genomic DNA, resulting in stable production of protein by the cell line. Since the establishment of this key advance in molecular biology, many modifications have been made to the development of stable gene expression in mammalian cells. For example, whereas the coding mRNA lacks introns, mammalian genomic DNA contains many introns. Transgenic stable expression of genes in mammalian cells using plasmids may result in progressive silencing of the transgene or removal of the sequence after weeks or months. However designed, introduction of an intronic sequence into the plasmid results in enhanced stable gene expression [1,2]. Manipulation of the standardized 3′ UTR, downstream to the open reading frame, and also the promoter/enhancer regions upstream has resulted in high-level stable expression of many genes in cell lines [3].

Manipulation of the Mouse Embryo

The first transgenic animal was created using mice. With the advent of molecular biological techniques, Ralph Brinster injected DNA in the pronucleus of forming zygotes and observed transgene expression in maturing zygotes [4]. In 1982, several groups reported the first germ line transmitting transgenic mice by DNA injection into the pronucleus of the zygotes, one-cell stage fertilized embryos [5 9]. The team coupled the coding sequence for the enzyme thymidine kinase or the sequence for rat growth hormone, under regulation of the metallothionein promoter.

Around the same time, Martin Evans (UCL) and Matthew Kaufman (Harvard), and independently also Gail Martin, established embryonic stem (ES) cells from mouse blastocysts and grew them in culture in 1981 [10 12] (Fig. 3.1). ES cells can be cultured indefinitely in vitro when cultured in the presence of feeder cells that provide crucial growth factors, including leukemia inhibitory factor (LIF). These cultured ES cells are also susceptible to the introduction of DNA-containing transgenes by transfection techniques such as electroporation, in which cells are transiently damaged by electric shock treatment that punctures holes in the cytoplasm allowing diffusion of extracellular DNA into the cell, before the plasma membrane repairs itself. Incorporation of linearized plasmid into the genomic DNA of ES cells results in transgenic stem cells, which can be placed in a mouse blastocyst and returned to a pseudopregnant mouse uterus where implantation and embryonic and fetal development proceed [13]. It is possible to generate completely ES cell-derived embryos using tetraploid complementation or eight-cell stage embryo injection [14,15].

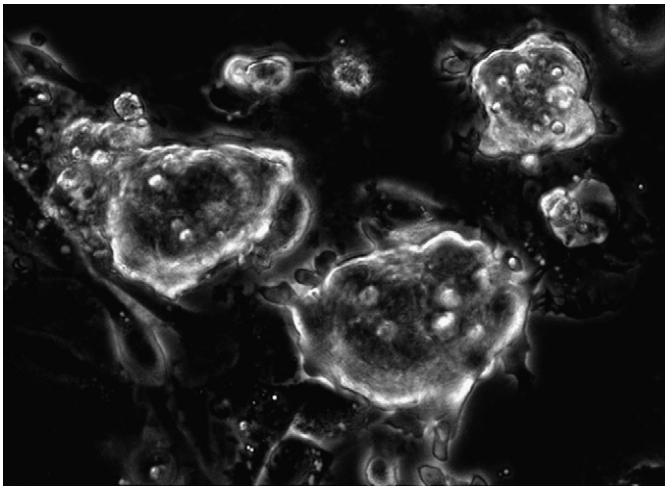

FIGURE 3.1 Photomicrograph of mouse embryonic stem cells in culture growing on feeder cells.

Pitfalls in Transgenic Mice and the Development of the Gene Trap ROSA26 Locus

By the simplified methods described the first transgenic mice evolved. All mice were initially developed predominantly on 129/Sv inbred background since mouse ES cells are traditionally isolated from the 129/Sv strains. This strain restriction has important implications for the interpretation of phenotype (see below) [16]. Incorporation of transgenes into mice is associated with problems. The first is the integration site. The transgene incorporates randomly into the genomic DNA. There may be multiple copies (>50) often in head-to-tail tandem. The local genomic environment of the transgene may have a profound impact on the fidelity of expression. Therefore, despite the use of promoter and enhancer the pattern of gene expression may not truly reflect the expression of gene under the endogenous promoter. The insertion may disrupt or modify endogenous gene expression. Nevertheless, despite these pitfalls, transgenic mice rapidly became established as a tool to study in vivo gene overexpression or de novo expression of foreign or mutated genes, or aberrant expression of genes in cells that would not normally express the gene. In part to circumvent the problems of mosaicism, but also to standardize the insertion of the transgene into the genomic DNA and to standardize ubiquitous expression under the same promoter enhancers, the gene trap system was developed, also known as gene trap ROSA26. Soriano and co-workers were studying random integration of a retrovirus driving the open reading frame for the gene βgeo (known as gene trap) into the mouse genome in ES cells. This virus, reverse orientation splice acceptor (ROSA), integrated at many sites but one clone, numbered 26, resulted in expression of βgeo (βgal, neomycin resistance fusion protein) in all tissues in the mice that resulted from the virally infected ES cells [17,18] (Fig. 3.2). The ROSA26 site was thought to represent a housekeeping gene but although the endogenous promoter at the ROSA26 locus generates transcripts, no proteins are synthesized. The ROSA26 locus has been extremely carefully characterized for ubiquitous activity in the mouse, and following sequencing of the locus a targeting vector that contains 5′ and 3′ regions of the ROSA26 locus was generated that enables insertion of any gene of interest through homologous recombination at this site [17,19]. Insertion of an open reading frame of a gene of interest at this site leads to ubiquitous expression in all cells in all tissues and has become the standard site to express any gene of interest. This system of overexpression under endogenous regulators of gene expression has proven extremely tractable and heralded further advances in technologies that target gene expression [3].

Pitfalls to the ROSA26 Locus

First, expression levels of the gene product in adult mice are significantly lower than in embryonic or fetal mice [20,21], and therefore may not be useful in adult tissues if levels of gene expression above a certain threshold are required. Second, the ROSA26 locus may not be amenable to Cre-mediated recombination at high levels in adult mice. It may be that the ROSA26 locus becomes increasingly inaccessible in certain types of cells following development, possibly owing to chromosomal structure modifications [20].

Coalescence of Ubiquitous Transgenic Gene Expression Under the CAGGS Promoter

The chicken β-actin promoter coupled to the cytomegalovirus (CMV) early intermediate minimal enhancer followed by an intron (CAGGS promoter) can yield high-level transgene expression in the mouse [22]. Single-copy incorporation of this transgenic construct into genomic DNA can lead to widespread stable expression of the gene of interest at high levels in many cell types [23 25]. Despite the use of this system for universal overexpression the transgenic approach remains imperfect. In contrast to ubiquitous expression of genes under the ROSA promoter/enhancers, transgenic expression under short promoter DNA often is not ubiquitous and uniform within tissues and between transgenic lines. This mosaicism of patchy expression levels depending on random insertion of the transgene remains a potential problem for such mouse models using this system [21].

Recent novel strategies combining the strengths of the ROSA26 locus ubiquitous expression and CAGGS promoter/intron system for high-level expression by

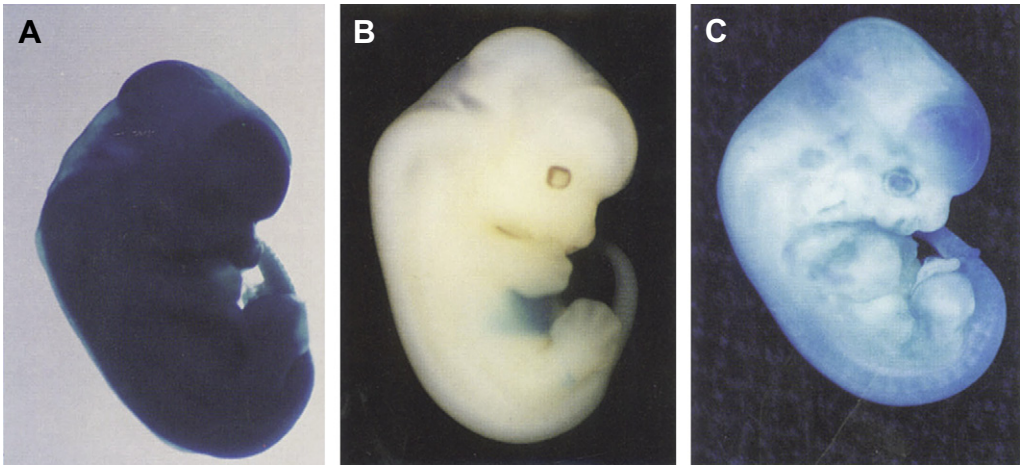

FIGURE 3.2 Gene trap method in embryonic stem cells to detect endogenous promoters with distinct patterns of gene expression. Photo micrographs showing mouse embryos derived from embryonic stem cells that were retrovirally infected and trapped the β gal actosidase–neomycin phosphotransferase βgeo fusion gene using virus with the splice acceptor and construct in reverse orientation to the viral genes (ROSA βgeo) at the ROSA5 locus (A), or in the same orientation to the viral genes (SA βgeo) the SA9 locus (B) or SA3 locus (C). Embryos were fixed and incubated for β gal activity with X gal generating blue color. Note widespread expression of LacZ mice expressing βgeo at the ROSA5 locus. *[Image from Soriano, 1999 [17].]*

inserting CAGGS-driven genes at the ROSA26 locus promise high-level ubiquitous transgenic expression in vivo in a "plug and play" fashion that will enable high-throughput screening of gene function in any given tissue or cell type [26 29].

Diphtheria Toxin Systems for Cellular Ablation In Vivo

Diphtheria toxin (DT) is a protein produced by *Corynaebacteria diphtheriae*. It binds to the heparin-binding epithelial growth factor receptor (EGFR) in human cells enabling its endocytosis and entry to the cytoplasm of subunit A (DTA), where it binds to and inactivates the ribosomal protein eEF-2, disabling the translational machinery which triggers apoptotic cell death rapidly. In humans, whooping cough, a disease of the upper respiratory tract caused by *C. diphtheriae*, frequently results in lethality owing to release of toxin in the upper respiratory tract and circulatory delivery to the myocardium and other vital organs. One milligram of purified DT is lethal to humans. Rodents, including mice, however, have a form of EGFR that does not bind DT and are therefore completely resistant to the toxic effects of extracellular DT [30] (Fig. 3.3). Transgenic expression of the catalytically active subunit DTA intracellularly has been used by molecular biologists in cellular ablation [31 34]. Initially this technique was used to delete stem cells that had not incorporated a transgene faithfully, but transgenic expression of DTA under a cell-specific promoter leads to ablation of any cell that transcribes the transgene. Similarly, transgenic expression of the human heparin-binding EGFR, which is the diphtheria

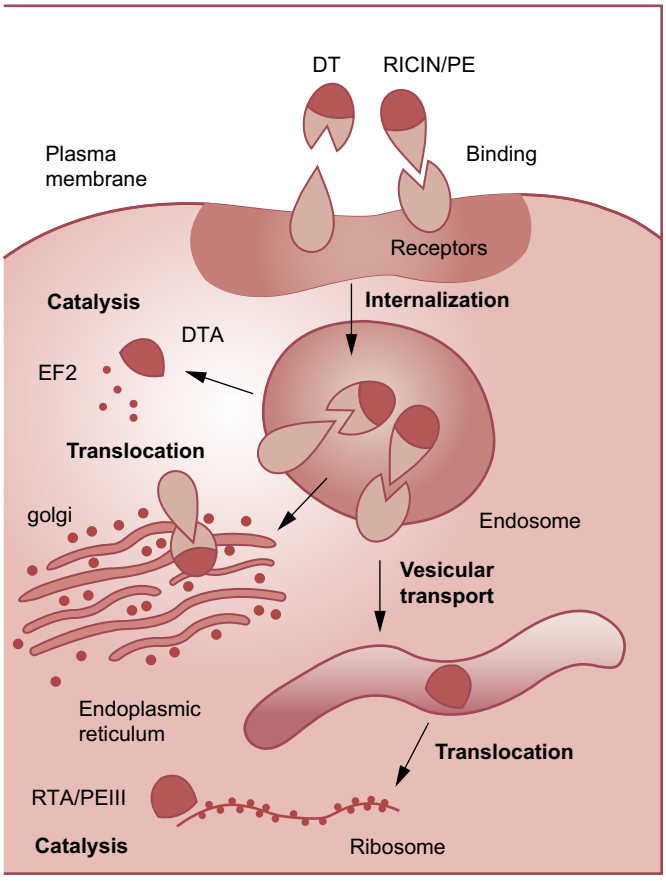

FIGURE 3.3 Mechanism by which diphtheria toxin (DT) enters the cell and disables translational machinery. PE: *Pseudomonas* exotoxin; DTA: diphtheria toxin A; EF2: elongation factor 2; RTA: ricin A chain; PEIII: catalytic domain of *Pseudomonas* exotoxin A.

toxin receptor (DTR), in mouse cells, renders those cells susceptible, like human cells, to extracellular exposure of DT [35]. Transgenic expression of DTR selectively in one of a restricted number of cell types can yield a mouse in which injection of DT into the circulation results in uptake of the DTA subunit only into cells expressing the transgene and rapid loss or ablation of the specific cell-type [30,36 39]. The use of these systems was first reported by Evans in 1989, and Breitman in 1990 [33,40,41]. However, one of the first successful uses of this model system in mice was reported by Lang and Bishop in 1993 [42]. In these studies expression of DTA was transgenically driven by a promoter created by the fusion of a portion of a viral promoter and part of the granulocyte macrophage colony-stimulating factor (GM-CSF). This resulted in restricted expression of the transgene only in macrophages in the peritoneal cavity and the eye, and also inflammatory macrophages and these populations of macrophages were absent. The resultant surviving mice carried a very distinctive phenotype, in the eye. The hyaloid microvasculature of the developing eye, which normally regresses following

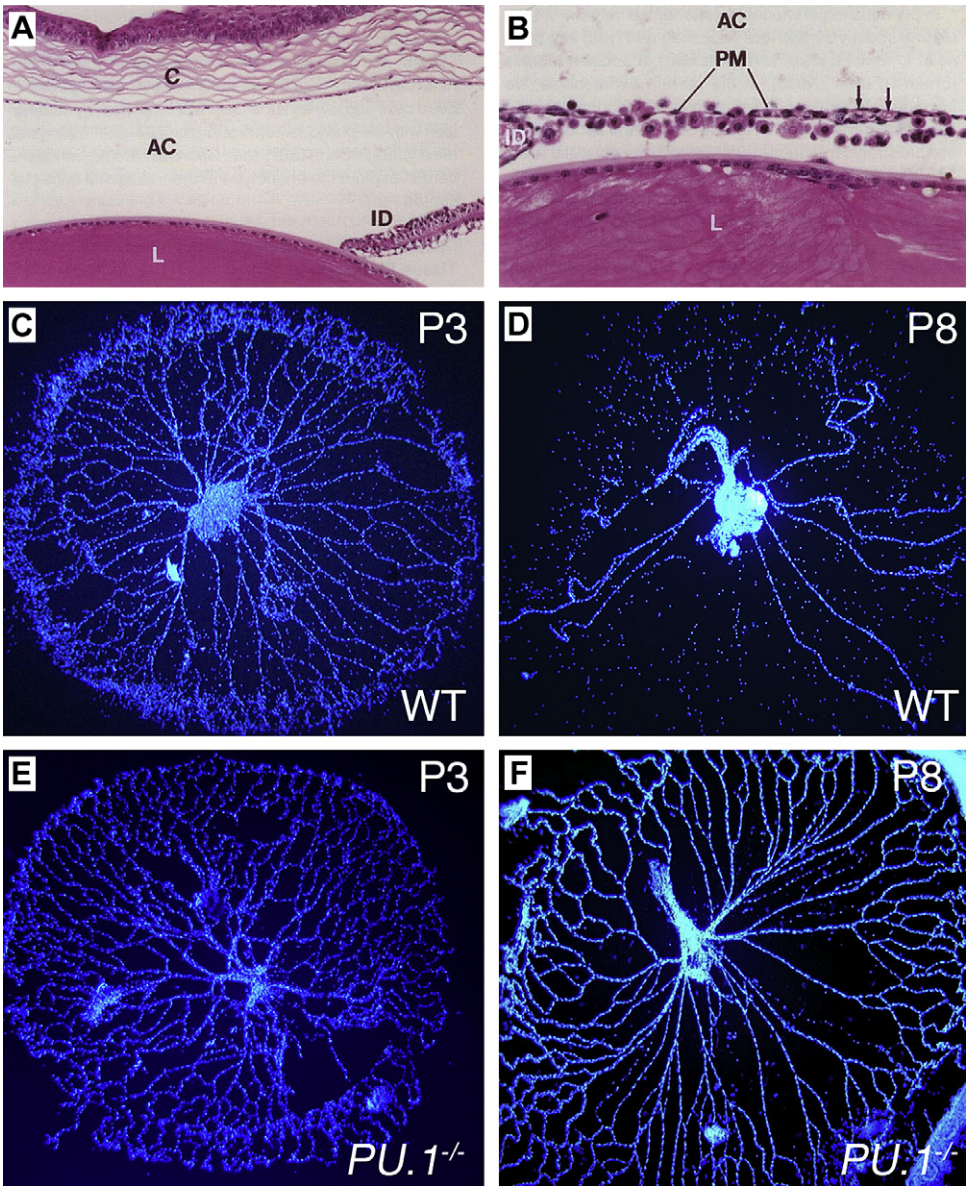

FIGURE 3.4 Effect of diphtheria toxin A (DTA) mediated ablation of hyaloid macrophages in granulocyte−macrophage colony stimulating factor (GM CSF) −DTA mice or macrophage deficiency in PU.1 mutant mice on scheduled regression of hyaloid vasculature in the postnatal eye. (A, B) Photomicrographs of the hyaloid vessels of (A) wild type mice and (B) mice lacking hyaloid macrophages due to expression of the GM CSF DTA transgene. (C, D) Whole mount images of hyaloid vessels in the eye showing scheduled loss of vasculature in the eye between P3 and P8 in wild type mice. (E, F) Scheduled loss of vessels does not occur in PU.1 mice which lack macrophages. *[Images provided by Richard Lang, Cincinnati Children's Hospital.]* Please see color plate at the end of the book.

delivery of newborns, failed to regress in the absence of macrophages in the eye (Fig. 3.4). This novel method was the first genetic system to study macrophage function in vivo by cellular ablation and showed that a major function of macrophages is in tissue remodeling.

Additional ablation systems are available and have been used successfully, including the expression of viral thymidine kinase (Tk) in cells that are then susceptible to the toxic effects of the drug gancyclovir [43 46]. Unlike DT, Tk ablates only dividing cells.

Recombineering and the Advent of Genetic Mutation by Knockin and Knockout Approaches

With the identification of the ROSA locus, which affords universal cellular expression of a gene of choice placed at that locus, it was obvious that inserting a gene of interest under any other endogenous promoter and regulatory sequences would be possible. All that is required is the sequence of the gene, including exons and introns, and the sequences of the promoter upstream and downstream for approximately 2000 bp. However, to modify genomic DNA at will, several key hurdles had to be overcome: first, the capacity to sequence large regions of DNA around the gene of interest; second, the capacity to synthesize a copy of this region of genomic DNA with strategic alterations; and finally, the capacity to "persuade" genomic DNA to be replaced by the constructed DNA sequence (Fig. 3.5). Initially, sequencing of genomic DNA was acquired by tedious sequencing upstream and downstream of the start site of the gene of interest. A length of DNA was then copied from the genomic DNA of the region upstream and downstream of the coding region. One of the exons of the gene was selected and deleted from the copied piece of DNA, usually resulting in a frame shift or premature stop codon in the coding region. This synthesized piece of DNA, which corresponds to the gene except for the absence of one of the exons, when placed in a dividing cell undergoes homologous recombination. The first mouse in which a gene was disrupted in the genomic sequence by this method was reported in 1989 independently by Capecchi, Evans, Smithies and their respective colleagues [47 50]. Genomic DNA undergoes homologous recombination during meiosis, but bacteria and eukaryotes have endogenous systems to repair dsDNA and ssDNA breaks. By relying on endogenous homologous recombination systems in ES cells and a combination of negative and positive selection (Fig. 3.5), spontaneous recombination at the homologous sites will result in insertion of the engineered construct at the specified site in the genomic DNA in a minority of cells that then become resistant to a positive selection.

Genome Modification by Site-specific Recombinases

Site-specific recombinases are widely used to genetically manipulate the mouse genome. These recombinases include Cre, FLP and ΦC31, which recognize specific loxP, FRT and att site sequences, respectively. These recombinases, initially described in bacteriophage viruses and yeast, do not require cofactors to catalyze DNA recombination in eukaryotic cells, which simplifies transgenic designs [51 56]. The site-specific recombination allows removal, insertion and inversion of specific DNA sequences in the genome. These tools have been applied to genetic recombination techniques with high levels of success, enabling tissue-specific gene activation or gene mutation (conditional gene deletion) [57 59].

Inducible Systems and Pitfalls

For temporal gene expression, tetracycline-regulated gene expression system is most commonly used in the mouse [60 63], which was developed by Hermann Bujard in 1992 [64]. In the Tet-Off system, in the absence of doxycycline, tetracycline transcriptional activator (tTA) binds to tetracycline operator (tetO) DNA elements and activates transcription of transgene downstream of CMV minimal promoters adjacent to tetO. After doxycycline administration, doxycycline-bound tTA can no longer bind to tetO, which leads to repression of

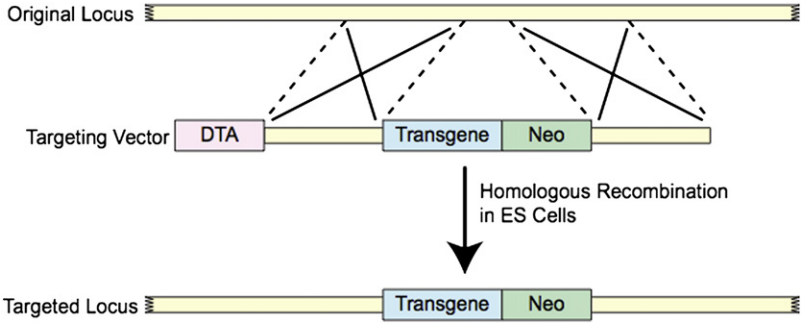

FIGURE 3.5 Method of recombineering. Diagram showing spontaneous recombination of identical segments of genomic DNA occurring in eukaryotic cells that enables the introduction of modified areas of DNA (gene targeting) into the genome. Note the requirement for a positive selection marker, in this case resistance to the antibiotic neomycin (Neo), and a negative selection marker, expression of cytotoxic diphtheria toxin A (DTA) in cells in which the transgenic persists but recombination has not occurred. ES cells: embryonic stem cells.

transgene expression. In contrast, in the Tet-On system, in the absence of doxycycline, the reverse tetracycline transcriptional activator (rtTA) cannot bind DNA. After doxycycline administration, doxycycline-bound rtTA binds to tetO, which activates transgenes downstream of adjacent CMV minimal promoters. The critical consideration for the tetracycline-regulated gene expression system is to place tetO-CMVmin transgenes in neutral loci in the genome so that there is no leaky transgene expression when their transcription is not activated by the tetracycline system and there is a high level of transgene expression when activated by the tetracycline system. It is also noted that the tetracycline system may activate neighboring genes adjacent to tetO elements [65]. GAL4-UAS systems have also been successfully used in the mouse [66], although this system has been most widely used in *Drosophila* fly studies. These bigenic systems, where the promoter and the transgene are separated, have proven useful especially when transgene expression causes lethality and infertility, which prevents maintenance of transgenic lines.

Recombinases fused with a subunit of the estrogen receptor (ER) have become widely used to temporally regulate recombination in the mouse. The nuclear localization subunit of the cytoplasmic ER requires estrogen binding to translocate the receptor into the nucleus where the DNA binding subunit can regulate transcription. The capacity for compartmentalizing proteins to cytosol or nucleus is very attractive for temporal recombination since recombinases need to be in the nucleus to function, so a recombinase fused to the ER (nuclear localization subunit) will remain in the cytosol and be functionally inactive in the absence of estrogens. Unfortunately, endogenous estrogens and other factors have rendered such approaches "leaky" until recently. A mutated form, ER^{T2}, is widely used owing to its complete dependence on the estrogen-mimetic drug tamoxifen for nuclear translocation. In the absence of tamoxifen, ER^{T2} retains fusion proteins in the cytoplasm. Upon tamoxifen administration, tamoxifen binds to ER^{T2}. The tamoxifen-bound ER^{T2} allows translocation of fusion proteins into the nucleus, where recombinases can recombine target sites in DNA.

Fluorescent Proteins, Their Development and Use in Mice

Green fluorescent protein (GFP), an endogenous protein of the jellyfish *Aequorea victoria*, exhibits green fluorescence in response to blue light. Its calcium-dependent fluorescent properties were described by Osamu Shimomura [67,68]. It was originally cloned in 1992 by Douglas Prasher. However, it was first exogenously expressed by three teams in bacteria and worms and found to fluoresce well, outside of jellyfish (Fig. 3.6).

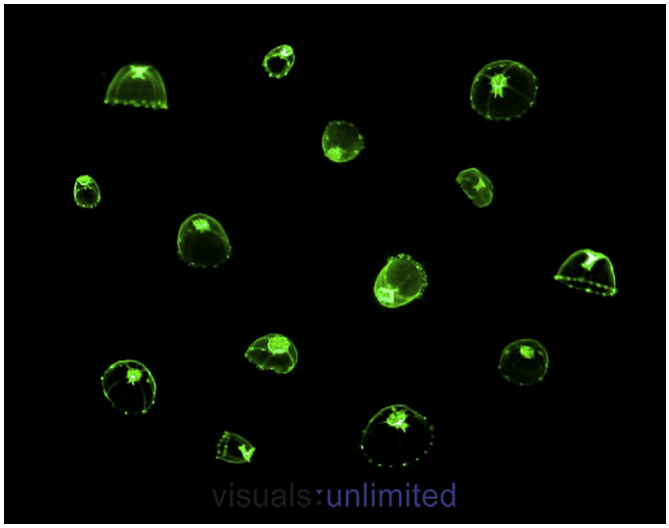

FIGURE 3.6 Fluorescence of the jellyfish *Aequorea victoria*. *(From the worldwide web.)*

This remarkable breakthrough in fluorescent protein technology was awarded the Nobel Prize (2008) and has heralded unprecedented advances in biomedical sciences [67 72]. Following the determination of its crystal structure, the original GFP protein has undergone numerous modifications (beyond the scope of this text) by directed mutagenesis to yield eight different fluorophores: enhanced GFP, blue fluorescent protein (BFP), cyan fluorescent protein (CFP), citrine and yellow fluorescent protein (YFP). Red fluorescent protein (RFP) was cloned from the Red Sea coral *Discosoma* sp. and is also known therefore as DsRed [73]. It fluoresces red with green light. Although it was successfully cloned as a partner to the GFP variants, it was tetrameric, very slow to mature and highly toxic to cells. Through directed mutagenesis requiring 33 substitutions, a monomeric form, mRFP1, was initially described, and this was improved to yield three monomeric proteins that could be linked to other proteins, called RedStar and mCherry and mOrange [74,75]. tdTomato, a dimer, is brighter but less amenable to linking to other proteins. mCherry has perhaps gained most traction as a complementary fluorophore to use in vivo in mice [76]. Fluorescent proteins with a variety of colors have been generated and successfully used in the mouse [77]. These fluorescent proteins also function without cofactors and function at 37°C in mammalian cells, either fused to other proteins via a linker or as unlinked proteins. Monomeric fluorescent proteins, including eGFP and mCherry, are therefore useful to tag proteins of interest. Modified forms are directed to different cellular organelles, plasma membrane or nucleus. Alternatively, these fluorophores have been used in vivo to label recombination events or cell signaling activity or for lineage tracing (Fig. 3.7).

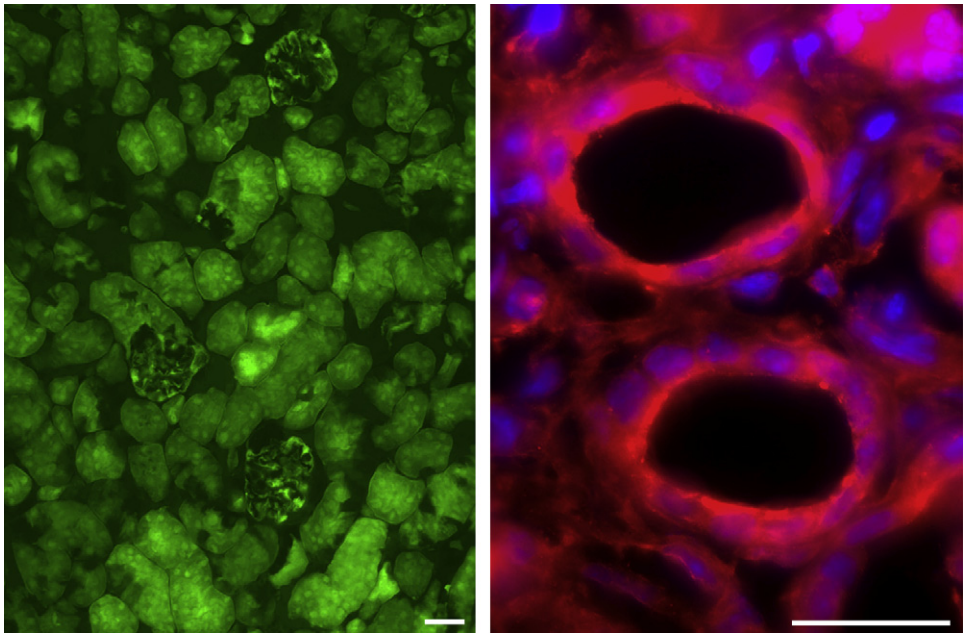

FIGURE 3.7 Fluorescent proteins as fate markers in the kidney. Low power image showing spontaneous GFP fluorescence in a 4 μm section of normal mouse kidney (left) from GFP mouse, and high power image from diseased kidney of dsRED mouse. Note that all cells are positive for the fluorophores. Scale bar 50 μm). Please see color plate at the end of the book.

Pitfalls to In Vivo Genetic Manipulation

Mouse Strain

Thanks to fanciers in Japan and England in the eighteenth and nineteenth centuries, followed by fanciers and biologists, primarily located in Massachusetts, interested in Mendelian inheritance in the later nineteenth and early twentieth century (Lanthrop and Castle), we now have at our disposal many strains of inbred mice. These mice strains have been bred in excess of 150 times in brother × sister matings and are essentially homozygous at all loci. Moreover, each inbred strain is essentially isogenic (genetically identical). Mice are broadly derived from four species/subspecies: *Mus musculus molossinus* (Japan), *Mus musculus domesticus*, *Mus musculus castaneus* and *Mus spretus* [78]. The period before World War I also led to the initiation of inbreeding in rats by Dr Helen King in about 1909 and in mice by Dr C. C. Little in 1909.

The latter project led to the development of the DBA strain of mice, now widely distributed as the two major substrains, DBA/1 and DBA/2, which were separated in 1929 1930. DBA mice are selected for coat color (d dilute, b brown and a agouti). Soon after World War I, inbreeding in mice was started on a much larger scale by Dr L. C. Strong, leading in particular to the development of strains C3H and CBA, and by Little, leading to the C57 family of strains (C57BL, C57BR and C57L). Many of the most popular strains of mice were developed during the next decade, and some are closely related. Evidence from the uniformity of mitochondrion DNA suggests that most of the common inbred mouse strains were probably derived from a single breeding female about 150 200 years ago [79]. The mice were bred for specific purposes: C57BL/6 had increased preference for alcohol and narcotics; FVB mice had large pronuclei and were useful for direct gene transfer into fertilized eggs; and 129 ES cells were selected because of high levels of success in germline transmission. Other mice spontaneously developed tumors.

Phenotypic differences between inbred strains need to be taken into account when designing and interpreting experiments. Strains may vary in characteristics that may not be relevant directly to the phenotype studied but which may nevertheless influence experimental results. The genealogy of mouse strains is both informative and important in experimental design. The genealogy tree is available from ftp://www.informatics.jax.org/pub/datasets/misc/genealogy/genealogy.pdf. For example, with respect to the kidney, different strains of mice have differing susceptibility to kidney diseases, from glomerulonephritis, through diabetic nephropathy to ischemia reperfusion injury (IRI). All studies comparing wild-type mice with mutants must be on the same genetic background. If the mice are on mixed backgrounds, additional controls should be performed for strain combination differences. The extent of this genetic variation was exhibited most clearly in studies of mutation of the protein serum amyloid P/pentraxin-2. Mutant

mice developed by targeted mutation of 129 ES cells grown in C57BL6 embryos developed a spontaneous autoimmune phenotype. However, the strain-matched controls also had a mild autoimmune phenotype. This may be because the segment of the chromosome where pentraxin-2 resides is in the Sle1 susceptibility region of chromosome 1. Selection for 129 Sle1 in C57BL/6 mice predisposes to autoimmunity [16,80].

Mosaicism

Mosaicism is the presence of two heterogeneous genetic types within an individual. Therefore, tissue-specific recombination is mosaicism by definition. The term is often used to describe lower recombination within target tissues where Cre recombinase is expressed. The degree of recombination largely depends on levels of Cre expression and recombination efficiency at target sites. To achieve recombination, a certain level of Cre protein accumulation is required. Also, as described above, different tissues have different accessibility of recombinases to target sites in the genomic DNA, and this may be context, disease or temporally dependent. In addition, owing to the limited time activation, inducible recombinases give lower recombination efficiency. To overcome this type of mosaicism, multiple doses of tamoxifen or tetracycline are required to obtain more complete recombination in target tissues.

Cellular Lineage of Gene Activation

Although the expression of collagen1α1 gene is highly restricted in the adult mouse, the endogenous gene is activated in the blastocyst. This is a serious consideration in designing gene-targeted mice using this locus for analysis. Although expression of a gene such as GFP under the collagen promoter identifies cells and denotes function, activation of a gene permanently or inactivation of a gene permanently under the regulated expression of collagen1α1 would yield no specificity since it is activated in many cells early in embryonic development. With the explosion of in vivo reports of gene function and cell lineage using Cre recombinase to mutate or activate a gene in the genomic DNA, this problem of cell specificity may be rife and extremely opaque to both the investigators and readers. Although Cre may be active only in one cell type in the adult, developmentally it may be active in other cell types and results in genomic recombination (activation or inactivation of the gene or interest) in cell types other than those known to the investigator, and therefore the readers. One such example is the use of the Tie2 promoter to drive Cre. Tie-2 is an endothelial receptor for angiopoietins. Like many other endothelial receptors it is also expressed by leukocytes. In mice, all myeloid cells in the bone marrow transiently express Tie-2 and a subpopulation of myeloid leukocytes continues to express Tie-2. The result is that Cre driven by Tie-2 will recombine genomic DNA in both endothelial cells and myeloid leukocytes [81 84].

DEVELOPMENT OF THE NEPHRON: HOW GENETIC TOOLS HAVE ENABLED UNDERSTANDING OF NEPHRON FORMATION

Nephron Formation During Kidney Organogenesis

The nephron is the basic function unit of the kidney. A kidney contains about 100,000 nephrons in humans and 13,000 in mice. After formation of germ layers during gastrulation, the nephric duct is formed along the intermediate mesoderm of embryos around day 8 in the mouse (Fig. 3.8). At the onset of kidney organogenesis around day 10 in the mouse, the ureteric bud emerges at the posterior end of the nephric duct. Formation of the ureteric bud is induced by signaling from the adjacent metanephric mesenchyme. This tissue interaction largely depends on glial cell line-derived neurotrophic factor (GDNF) and its receptor Ret in the nephric duct. Ectopic expression of GDNF is sufficient to induce ectopic ureteric bud formation at the anterior region of the nephric duct.

Through the reciprocal interactions between the ureteric bud and metanephric mesenchyme, a portion of the metanephric mesenchyme condenses to form the cap mesenchyme. The nephrogenic interstitium (cortical stroma) also arises at this stage. GDNF-Ret signaling continues to be required during kidney organogenesis. The cap mesenchyme and ureteric tip express GDNF and Ret, respectively. The GDNF-mediated tissue interaction between the cap mesenchyme and ureteric tip is essential for ureteric branching, which forms the collecting duct system of the kidney (see Fig. 3.8).

As the ureteric tip branches, a nephron precursor called the pretubular aggregate forms at the newly formed ureteric tip. The pretubular aggregate undergoes mesenchyme-to-epithelium transition (MET) to form an epithelial sphere, the renal vesicle. The renal vesicle undergoes morphological changes to differentiate into the comma-shaped body, S-shaped body and eventually the nephron epithelia. The induction of the nephron also depends on tissue interactions. The ureteric tip-derived Wnt9b induces formation of the pretubular aggregate in mesenchymal tissue. In the mesenchymal tissue, β-catenin is required, suggesting that Wnt9b from the ureteric tip activates the canonical Wnt signaling mediated by β-catenin in the mesenchyme.

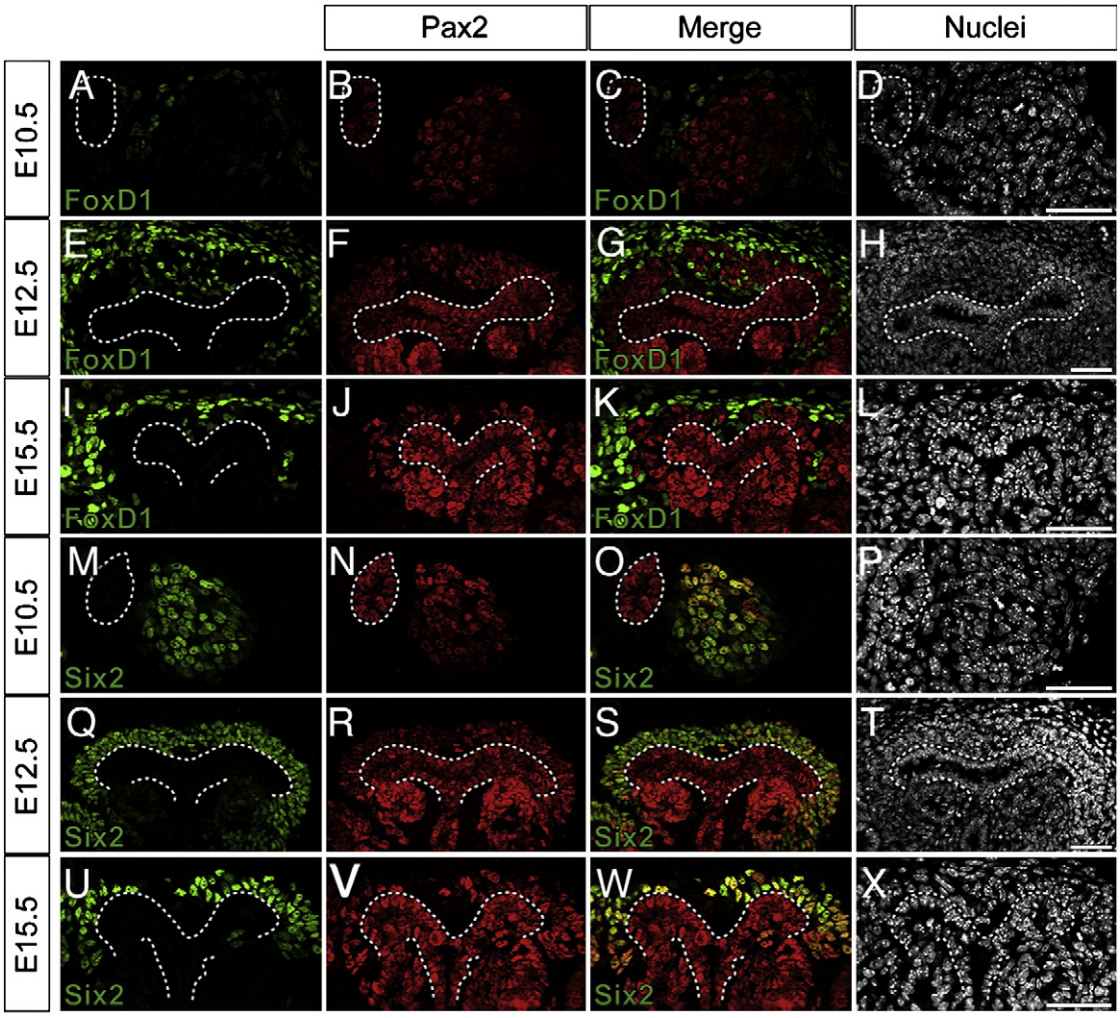

FIGURE 3.8 Mouse kidney progenitor cells of the metanephric mesenchyme from embryonic day 10.5 to 15.5 that give rise to all components of the nephron and the interstitium. Immunofluorescent confocal microscopy of (A–D, M–P) transverse sections of the E10.5 metanephric blastema, (E–H, Q–T) sagittal sections of E12.5 metanephric kidneys, and (I–L, U–X) frontal sections of E15.5 metanephric kidneys. Samples immunostained for Foxd1 (A, E, I), Pax2 (B, F, J, N, R, V) and Six2 (M, Q, U). Nuclei stained for Hoechst 33258 (D, H, L, P, T, X). Merged images (C, G, K, O, S, W). Dashed lines (A–X) indicate nephric duct epithelia. Scale bars 50 μm. *[From Mugford et al., 2008 [85], Figure 1.]* Please see color plate at the end of the book.

A Multipotent Self-renewing Nephron Progenitor Population

Recently, genetic studies have identified nephron progenitor cells. *Cited1* and *Six2* are exclusively expressed in the cap mesenchyme of the developing kidney. Using *Cited1-CreER*^{T2} and *Six2-eGFPCreER*^{T2}, it was shown that the cap mesenchyme is a multipotent self-renewing nephron progenitor population (Fig. 3.8) [86]. Fate map analysis using the Cre-loxP system showed that the cap mesenchyme contributes to all cells in the nephron epithelia. Using a genetic label retention assay, evidence has been provided that the cap mesenchyme is maintained by self-renewal. Lastly, clonal analysis indicated that a single cap mesenchyme cell can differentiate into different cell types of the nephron epithelia.

Thus, the presence of the cap mesenchyme allows repetitive formation of the complement of numerous nephrons in the kidney. The Six2+ population of progenitor cells in the mammalian kidney disappears by 5 7 days postpartum, and after this time no new nephrons are formed. Further, Six2-expressing cells have not been identified in adult kidney in health or following injury [86].

Nephron and Interstitium Compartments During Kidney Organogenesis

In kidney disease, nephrons are lost and the fibrotic interstitium expands. Therefore, identification of molecular mechanisms for specification of the nephron and

interstitium, respectively, during kidney organogenesis is crucial to understand and cure kidney disease in adults. A forkhead family transcription factor, Foxd1 (also known as Bf-2), is expressed in the nephrogenic interstitium (stroma) which overlies the cap mesenchyme in the developing kidney (Fig. 3.8). By generating *Foxd1-Cre* mice, it was found that the cortical and medullary interstitium are derived from the nephrogenic interstitium (A. Kobayashi, unpublished data) and these cells mature into pericytes, vascular smooth muscle and mesangial cells of the adult kidney, but not resident macrophages or endothelium [87,88]. These Six2-Cre and Foxd1-Cre alleles are useful tools to genetically manipulate the nephron and surrounding interstitial tissues in the kidney. Fate mapping of these nephron and interstitial tissues revealed cellular regulation during kidney disease and repair (see below). In addition, these Six2-Cre and Foxd1-Cre alleles allow inactivation or activation of certain genes in a specific compartment of the kidney. This will clarify signaling pathways for tissue interactions in the kidney. For example, collecting duct-derived Wnt7b signaling was shown to be mediated by β-catenin in the interstitium during kidney organogenesis [89].

Formation of Other Cell Types in the Kidney

Developmental processes for several tissues in the kidney remain poorly described. These include the vasculature and neurons in the kidney. The vasculature is an important component of the kidney, and increasing studies point to its central role in both nephrogenesis and nephron regeneration after injury [90 95]. However, little is known about how the vasculature develops during kidney organogenesis. One possibility is that it derives from angioblasts originating from blood islands or the aorta gonad mesonephros (AGM) which migrate into the metanephric mesenchyme at about the same time as the ureteric bud invades this condensing mesenchyme. Fate mapping studies of AGM-derived endothelium or blood island-derived endothelium are underway and hopefully will answer this question soon. The other possibility is that a third condensate of peripheral metanephric mesenchyme lying peripheral to Six2+ cap mesenchyme and Foxd1+ interstitial mesenchyme has a distinct vascular lineage. Expression of the transcription factor Osr1 occurs in intermediate mesoderm cells that become Six2+ nephron progenitors and Foxd1+ interstitial progenitors. Osr1+ cells of the condensing metanephric mesenchyme may also become kidney angioblasts [85]. Further studies in this area are required and may prove beneficial for understanding both developmental kidney diseases and regenerative medicine of the adult kidney.

ADULT KIDNEY REGENERATION: HOW GENETIC TOOLS HAVE ENABLED UNDERSTANDING OF NEPHRON REGENERATION

Kidney Repair and Regeneration: A Lack of Model Systems

Unlike many other organs, units of the kidney, nephrons in mammals, are not regenerated. If a nephron becomes sclerotic or absolescent it is not replaced. This fact sets the kidney apart from organs such as liver in which lobules, or functioning units of liver, can be completely regenerated, and muscle, where whole myo-units can be regenerated. Nevertheless, if the nephron and its proximal blood supply remain intact the distal portions of the nephron from proximal tubule onward can be successfully regenerated. Despite widespread loss of epithelial cells in the kidney near complete regeneration can be achieved (Fig. 3.9). Aquatic, non-mammalian vertebrate kidney, which includes many orders of fish, is different. Young kidneys in elasmobranchs and teleosts (fish) can regenerate entire nephrons from precursor cells in a manner highly reminiscent of developmental nephrogenesis in the mammal [96 98]. Perhaps, since there is no mammalian nephrogenesis following injury, many of the model systems used in mice are not true regenerative models. Following mammalian kidney injury there is progressive fibrosis and chronic kidney disease which either stabilizes with hypertrophy of surviving nephrons or progresses toward near complete nephron loss.

Kidney Repair and Regeneration: The Ischemia–Reperfusion Model

This model system, initially developed in rats but now widely adopted in mice, can be singled out as a repairing/regenerative model of the kidney. The model requires clamping of both renal arteries for a defined period while the kidney is maintained at body temperature. Following release of the clamps the organ is immediately returned to the body cavity to maintain temperature. One feature of this model is that there is minimal injury to the glomerulus, therefore all the proximal structures of the nephron and the origin of the nephron blood supply remain following injury. It is perhaps for this reason that repair and regeneration are effective. The model is very temperature sensitive, and sensitive to the strain of the inbred mice used for experimentation. For this reason reproducibility from laboratory to laboratory has been lacking.

The IRI model has yielded significant information about the cellular components of repair by straightforward cell biological studies. Ischemic injury (and

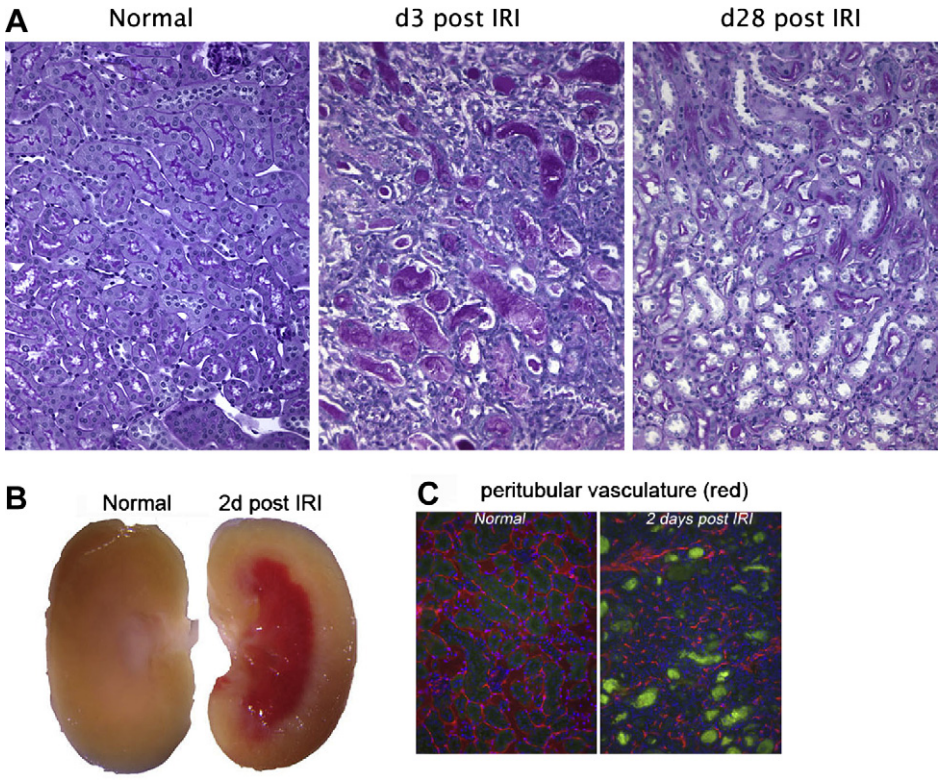

FIGURE 3.9 Injury and repair of the kidney structures. (A) Periodic Schiff stained kidney sections of outer medulla/inner cortex from normal adult kidney, d3 following ischemia–reperfusion injury (IRI) and d28 following IRI regenerated kidney. Note that there is severe injury and disruption to the tubules of the outer medulla but that at d28 there is near complete epithelial regeneration. (B) Whole mouse kidneys showing stasis of blood flow in the medulla 2 days after injury due to disruption of the peritubular vasculature. (C) Immuno fluorescence images of peritubular capillaries of the outer medulla (red) in normal kidney and 2 days following IRI. Note that there is severe disruption of the normal peritubular capillary network. Green shows autofluorescent necrotic debris in tubules. Please see color plate at the end of the book.

many toxic injuries) primarily injures proximal tubule epithelium of the outer medulla (Fig. 3.10). Injury is widely held to be maximal here because of a combination of high aerobic metabolic demand and a sluggish subcortical blood supply which characterizes the nephron. These epithelial cells undergo predominantly necrotic death. Other segments of the nephron are more likely to experience milder injury with apoptotic death [99]. In addition to the obvious epithelial injury there is an underappreciated injury and loss of the peritubular capillary network (Figs 3.9, 3.10). From 2 days to 15 days the lost and damaged portions of the nephron regenerate, as do the peritubular capillaries. However, although there is complete functional recovery from severe (> 90%) functional loss, there is never complete capillary recovery or nephron recovery [100 102]. Moreover, the regeneration of the peritubular capillary plexus remains incomplete [91,95]. In severe examples of this model or a unilateral version of this model, following functional recovery a syndrome of progressive loss of tubular function or even progressive nephron dropout ensues [88,103,104].

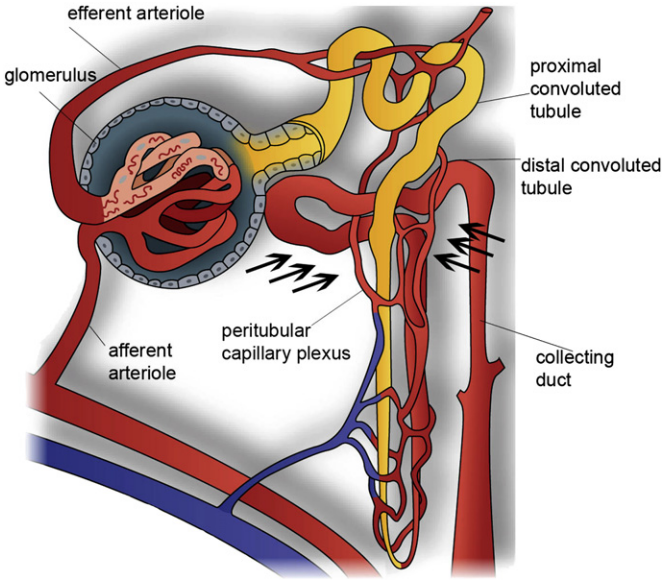

FIGURE 3.10 Diagram of the nephron showing its vasculature. Arrows show the area of maximal injury from reduction in blood flow.

Although there are other toxic injury models, including sepsis triggered by cecal puncture in aged mice, and toxins which trigger death of epithelial cells of the proximal nephron, including gentamicin, carbon tetrachloride or cisplatin, these generally produce either very mild disease or progressive disease. There are reports, however, of these models successfully being used to study regenerative capacity [105]. Understanding why the IRI model predominantly repairs whereas other models characterized by epithelial injury do not may yield key information about mechanisms that promote repair compared with those that result in chronic kidney disease. Recent reports indicate that the obstructive model of kidney disease which results in progressive parenchymal disease is reversible and this reversal results in nephron regeneration [106 108]. The prospect of such a model to study both progression and reversal in a single disease model is tantalizing, but those findings have yet to be reproduced in the literature.

Bone Marrow Chimerism in Mouse Kidney Leukocyte Lineage Tracing: The Origin of Regenerating Cells

To understand the contributions of cells derived from the bone marrow to kidney regeneration, bone marrow transplantation has been employed. It is straightforward to replace bone marrow stem cells completely in rodents by lethal irradiation followed by intravenous infusion of bone marrow stem cells from a donor. Bone marrow transplantation has been used for more than 30 years to trace cells derived from bone marrow stem cells [109]. If the donor stem cells are different from the recipient then cells deriving from bone marrow stem cells can be detected permanently. Permanent genetic or molecular markers of bone marrow stem cells include male stem cells harboring a Y chromosome in female mice, transgenic expression of a marker such as RFP or GFP under a universal promoter, or expression of a marker at the endogenous ROSA26 locus. Other markers include the CD45.1 compared with CD45.2 variant which can be detected readily using specific antibodies. However, CD45 is expressed by leukocytes but may not be expressed by derivatives of hematopoietic stem cells (HSCs) that differentiate into non-hematogenous cells. Three publications supporting the hypothesis that following ischemic injury the proximal tubule cells are regenerated by incorporation of cells derived from bone marrow that differentiate into epithelial cells were published between 2002 and 2003 [110,111]. Two of the studies used LacZ expressed at the ROSA26 locus as the marker of bone marrow cells and one of the studies used the Y chromosome as the fate marker. These studies were published around the time of a range of studies using fate markers of bone marrow HSCs that reported organ regeneration by differentiation of HSCs into myocytes (in muscle), cardiomyocytes (in heart) and a number of other cells in other organs. However, many of the studies were flawed. In subsequent follow-up studies it was not possible to reproduce the findings. In fact, detection of LacZ in the adult kidney is fraught with problems. The bacterial LacZ gene generates β-gal, an enzyme whose activity can be used to generate a blue stain using X-gal solution. LacZ expression can be detected by this blue colorimetric assay. The kidney proximal tubule (the compartment under investigation in these studies) has endogenous mammalian β-galactosidase [112,113]. Its enzyme activity can also trigger blue stain using X-gal solution. Mammalian, endogenous β-gal activity is maximal at low pH owing to its intracellular compartment, and is minimal at pH > 7.4 (Fig. 3.11). By contrast, bacterial β-gal activity is high at neutral or alkaline pH. Therefore, careful attention to tissue pH during staining is a prerequisite for detecting the LacZ fate marker but not endogenous β-gal activity. The early studies showing blue-stained tubules in the kidney of bone marrow chimeras carrying LacZ in HSC-derived cells were not reproduced, probably as a result of this artifact.

Detection of the Y chromosome in male cells has been widely used to detect cell fate in chimeras. It does not rely on expression of a gene product. It should be absolutely unequivocal. Early studies tracing HSC-derived cells in the kidney using the Y chromosome also reported a significant regeneration of epithelium by replacement with HSC-derived cells. These studies were also difficult to reproduce. The method of detection of the Y chromosome uses a fluorescence-tagged or biotin-tagged Y chromosome-specific probe DNA. The Y chromosome detected by this method looks like a small, S-shaped squiggle within the cell nucleus. Unfortunately, the successful application of this probe to tissue sections requires very specific tissue processing. The probe itself has a tendency to aggregation, possibly due to its length or the self-repeating nature of the probe sequence. Although several studies reported detection of the Y chromosome probe in epithelial cells in kidneys following injury, a careful study of kidney epithelium using the Y chromosome probe and confocal microscopy detected significant probe aggregates lying outside the nucleus of epithelial cells. Significantly, these probe aggregates lacked the characteristic S-shape of the Y chromosome but were obvious false-positive results. In each of these studies careful use of positive and negative controls in the initial studies might have averted the artifactual result. Current consensus is that HSC-derived cells do not become epithelial cells after kidney injury.

What about endothelial cells? Does lineage tracing provide any evidence that HSC-derived cells truly can become endothelium and therefore contribute to organ

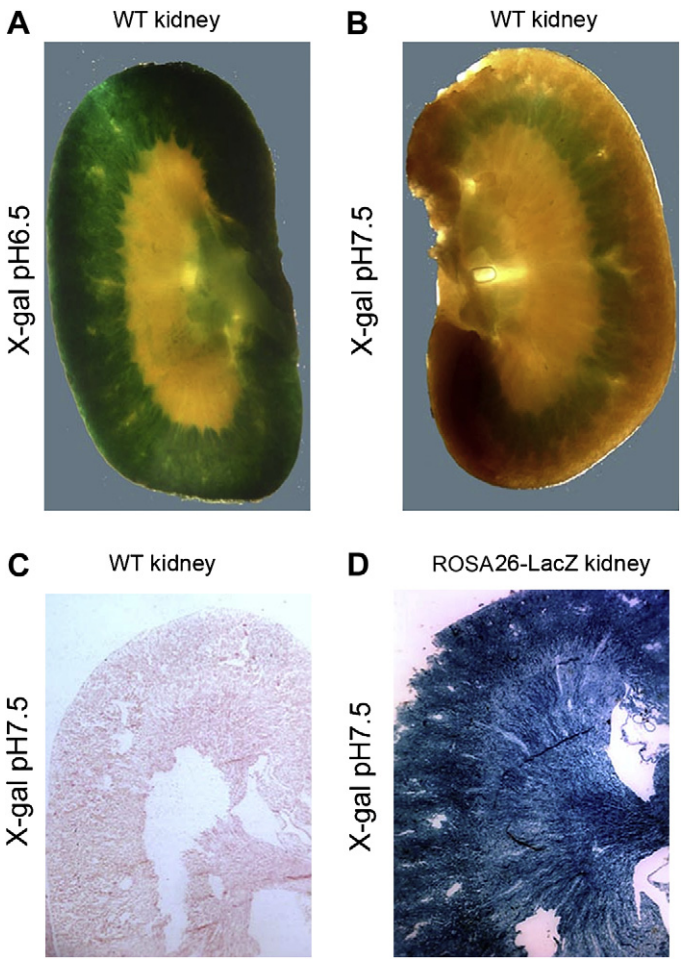

FIGURE 3.11 LacZ detection in the adult kidney. (A, B) Whole mount adult wild type (WT) kidneys incubated with X gal solution for 24 h show blue stain in the cortex when the pH of the solution is 6.5 (A), but at pH 7.5 (B) the blue stain is scarcely detectable. At low pH the X gal detects endogenous β galactosidase in proximal tubules, but at high pH no activity is detected. (C, D) 4 μm kidney sections from wild type and ROSA26 LacZ mice incubated 24 h with X gal solution at pH 7.5. Note that in wild type kidney no LacZ activity is detected but in the reporter mouse expressing bacterial LacZ at the ROSA26 locus, bacterial β gal activity is detected in all cells of the kidney.

regeneration by vasculogenesis? There is significant developmental rationale for the hypothesis that HSCs might differentiate into endothelium. Until recently, it was believed that HSCs and endothelial cells derive from a common cell early in embryogenesis during blood island formation. This hemangioblast cell derived from gonadomesonephric mesenchyme columns and from blood islands has been widely believed to be the common ancestor cell of both the hematopoietic lineage and endothelium [114]. In addition to the developmental rationale, endothelium and hematopoietic lineage cells share many common cellular markers, including CD31, CD34, VEGFR2, Tie-2 and CD146. Very recent studies have cast some doubt on the existence of the hemangioblast common ancestor cell, however. Careful fate tracing studies using the inducible Cre-ER^{T2} protein driven by the VE-cadherin locus, which is expressed by the earliest endothelium at mouse embryonic day (E)8.5, have clearly demonstrated that HSCs derive from endothelial cells rather than the mesenchyme underlying the first embryonic endothelium (Fig. 3.12).

Nevertheless, lineage studies in injured tissues using the *Tie2-GFP* transgenic reporter in vivo indicated that in adult ischemic limb, vessels were regenerated partially from HSC-derived cells, studies that have been reproduced in several tissue beds, including human organ transplantation where the donor endothelium is replaced by recipient endothelium with time [115 117]. Moreover, culture of HSCs in certain conditions in vitro leads to endothelial morphology with tube formation and expression of endothelial markers [118]. These cells have, in some hands, been shown to regenerate endothelium when administered systemically. There are, however, potential problems in the interpretation in some of these lines of investigations. The first set of studies utilized the *Tie-2* promoter to study endothelial cells. However, Tie-2 expression by monocytes and myeloid precursors in addition to endothelial cells indicates that some of the studies that were originally interpreted to show differentiation of myeloid cells into endothelium may have in fact shown a subpopulation of angiogenic myeloid cells promoting repair by local paracrine mechanisms, rather than by differentiation into endothelium. Increasingly, subsets of myeloid lineage cells with proantiogenic functions are being recognized that play roles in vascular

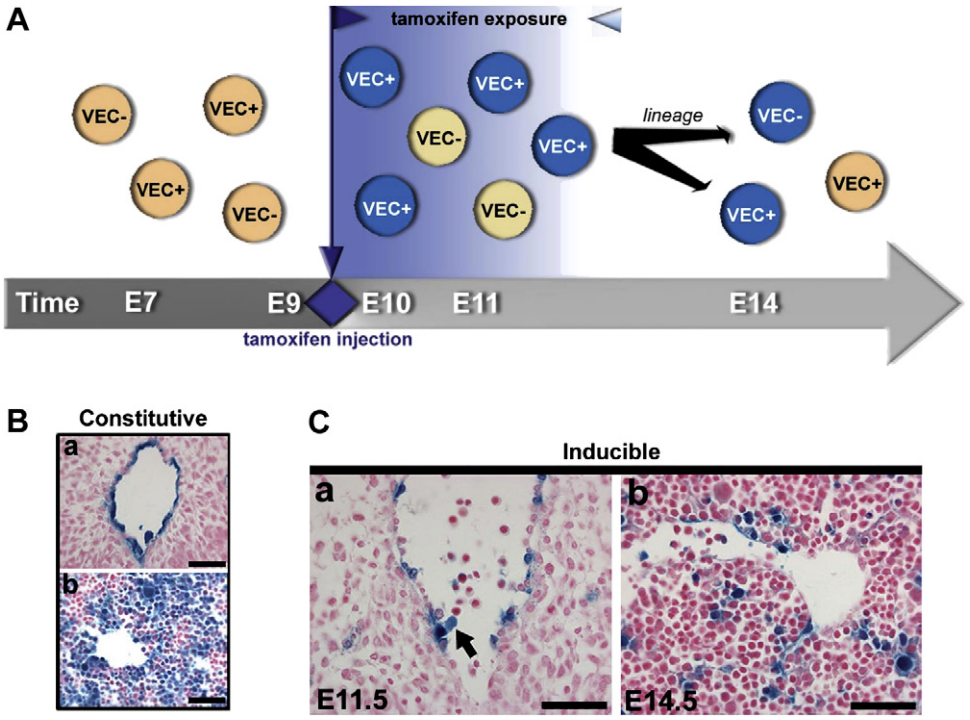

FIGURE 3.12 Fate mapping of endothelial cells arising at mouse embryonic day (E)9.5–10.5 using *VE Cadherin CreER^{T2};R26R* mice. (A) Cartoon showing that cells that expressed VE cadherin (VEC) permanently express LacZ (blue) only if they were exposed to tamoxifen between E9.5 and 10.5. Thereafter, all blue cells derive from the labeled cohort of cells and their fate can be mapped. (B) In *VE Cadherin Cre;R26R* mice where all cells that ever express VEC permanently express LacZ, both endothelium of the aorta (a) and leukocytes in the fetal liver (b) express LacZ. (C) In *VE Cadherin CreER^{T2};R26R* mice pulsed with tamoxifen at E9.5, not only are budding aortic endothelial cells (a) detected that are stained blue but also hematopoietic lineage cells in the liver (b). These mapping studies indicate that the hematopoietic lineage derives from aortic endothelium. *[Adapted from Zovein et al., 2008 [114].]*

homeostasis and repair without differentiating into endothelium [82 84].

There has been considerably less focus in kidney regeneration on the endothelium. In studies from 2005, evidence of vasculogenesis was first supported at a low level by the presence of cells derived from bone marrow in the regenerating peritubular capillary (PTC) walls, coexpressing the endothelial marker CD31. Several subsequent studies have also shown evidence for CD31 expression of HSC-derived cells in PTC walls [100,107,119]. It is quite likely, however, that those findings mistakenly interpreted angiogenic myeloid lineage cells as endothelial cells because of the abundance of shared cell surface markers and the tendency of myeloid cells to take up pericyte-like positions or even form bridging structures, replacing denuded endothelial cells temporarily within the capillary during repair [95,120] (Fig. 3.13). In very recent comprehensive studies lineage tracing of human HSCs recruited to the kidney provided strong evidence for an angiogenic role of progenitor cells and HSCs in vascular repair and regeneration but not in long-term replacement of endothelium [95]. These recent studies implicate PTC regeneration as a prerequisite for nephron tubule regeneration, by paracrine

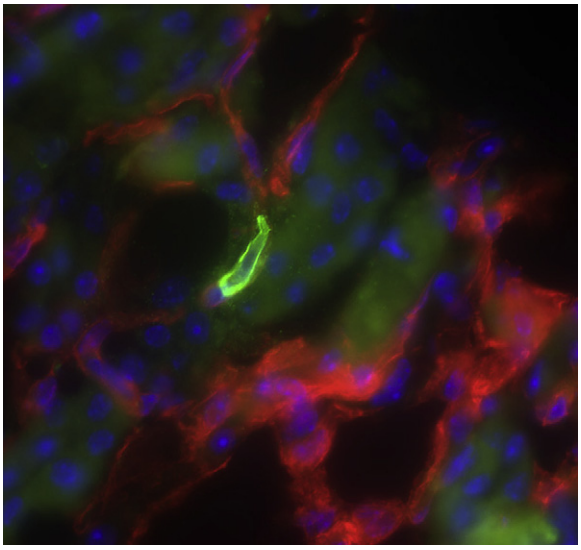

FIGURE 3.13 Human stem cells temporarily replace lost endothelial lost cells in bridging structures in the repairing kidney capillaries. Day 5 post ischemia–reperfusion injury kidney showing mouse endothelium (red) and human stem cell (green). Note that the human stem cell is bridging an area of denuded peritubular capillary. Please see color plate at the end of the book.

signaling events. These studies compare closely to the recently recognized role of angioblasts (endothelial progenitors) in the developing kidney in mesenchyme induction during nephrogenesis by paracrine signaling [90]. It is noteworthy that studies on brain injury in wild-type mice with chimeric bone marrow from Tie2-GFP mice have also identified monocytic bone marrow cells that replace temporarily denuded endothelium in bridging-like structures and express many endothelial markers including Tie-2, but these cells retain leukocyte CD45 expression [120].

Although this remains a contentious area, it is likely that paracrine functions of HSC-derived cells in vascular repair will prove more tractable than endothelial replacement by HSCs in organ regeneration.

Lineage or Fate Tracing in the Mouse Kidney: Origin of Regenerating Epithelial Cells

A major scientific quest of the past decade has been the identification of a mesenchymal cell of the adult kidney that lays dormant but can be reactivated to regenerate the nephron by nephron building, recapitulating a developmental program of nephrogenesis. Several studies have proposed and provided evidence of the existence of such a latent mesenchymal cell in the kidney that retains embryonic multipotency [121 124]. Adult mammalian liver contains oval cells that are dormant interstitial cells with the capacity to become both hepatocytes and cholangiocytes [125 127]. The gut contains epithelial self-renewing progenitor cells at the bases of the crypts, and similarly in the skin, an epithelial self-renewing progenitor is the source of new keratinocytes [128 130]. Furthermore, the brain, despite being a dormant organ, contains cells that have self-renewing multipotency [131]. It makes sense therefore that the adult kidney should contain a cell that has the capacity to renew cells of the nephron. Several studies have pointed to such a cell. The expression of the HSC stem cell marker CD133 by interstitial cells in human kidney biopsies suggested that an interstitial kidney "stem" cell may exist. In addition, several investigators have reported the existence of Oct-4-expressing cells. Oct-4 has also been reported to be a stem cell marker in other contexts [122].

Using genetic tools to ask the question whether an interstitial cell could regenerate the kidney tubules, investigators labeled the whole nephron genetically [132]. As described above, the transcription factor *Six-2* is activated in metanephric mesenchyme fated to become epithelial cells of the nephron. Although Six-2 is expressed in kidney development by mesenchyme fated to become epithelia, it is not detected in postnatal kidney and not activated in injured kidney. Using Cre driven by the Six2 locus, the LacZ reporter under the regulation of the ROSA26 locus (R26R) was activated exclusively in the nephron; alternatively, using the reporter RFP under regulation of the CAGGS promoter, RFP was activated exclusively in the nephron. The activation of the heritable markers LacZ or RFP occurred during kidney development and was permanent in the genomic DNA of those nephron cells. Adult kidneys from these mice were subjected to IRI. In this model there is severe loss of kidney epithelium which then regenerates. If the epithelium regenerates partly from interstitial cells then the heritable marker (LacZ or RFP) will only be present in some of the epithelial cells. If, however, all epithelium is regenerated from epithelial cells that have survived there will be no dilution (Fig. 3.14). In these experiments, all the epithelium was regenerated from surviving epithelial cells (i.e. there was no dilution of epithelial cells). In addition to providing powerful genetic evidence for the absence of an interstitial kidney "stem" cell, the studies corroborated earlier studies that indicated that no cells of bone marrow origin regenerate the kidney tubule.

Oliver et al. detected interstitial cells of the papilla that are low-cell-cycling cells or label-retaining cells, sharing this similarity with other stem cells [124]. Purified cultured cells from the papilla were injected into normal kidney, and were reported to become incorporated into the epithelium of cortex and medulla. Although these and other studies drive the notion that there is an interstitial stem cell in the kidney, the genetic studies from Humphreys et al. provide overwhelming genetic evidence that this is not the case [132]. These genetic findings, however, should not detract from the possibility that it may be possible to generate circumstances in which progenitor cells could be manipulated to become kidney epithelium.

A second hypothesis has been proposed, that there is a population of epithelial self-renewing progenitors within the nephron itself, similar to observations in skin, gut and mammary gland (an epithelial progenitor cell). One thing that strikes the authors is that while skin and gut are constantly turning over (renewal time of gut epithelium is 3 days) and mammary also turns over during lactation, the nephrons are not turning over. The epithelial cells of kidney cortex and medulla show little propensity to divide over several weeks in the adult mouse. The biological requirement for an epithelial stem/progenitor cell as seen in skin and gut is not present in the kidney. Although the studies from Oliver et al. indicate that some low-cycling cells might have been of epithelial origin in the papilla, most epithelium in papilla is collecting duct and therefore derived from ureteric bud, not metanephric mesenchyme. Furthermore, the replacement of cortical epithelial cells by cells of the papilla following injury would require high levels of migration and an expected wave of proliferation in regenerating nephrons from the deepest segments to

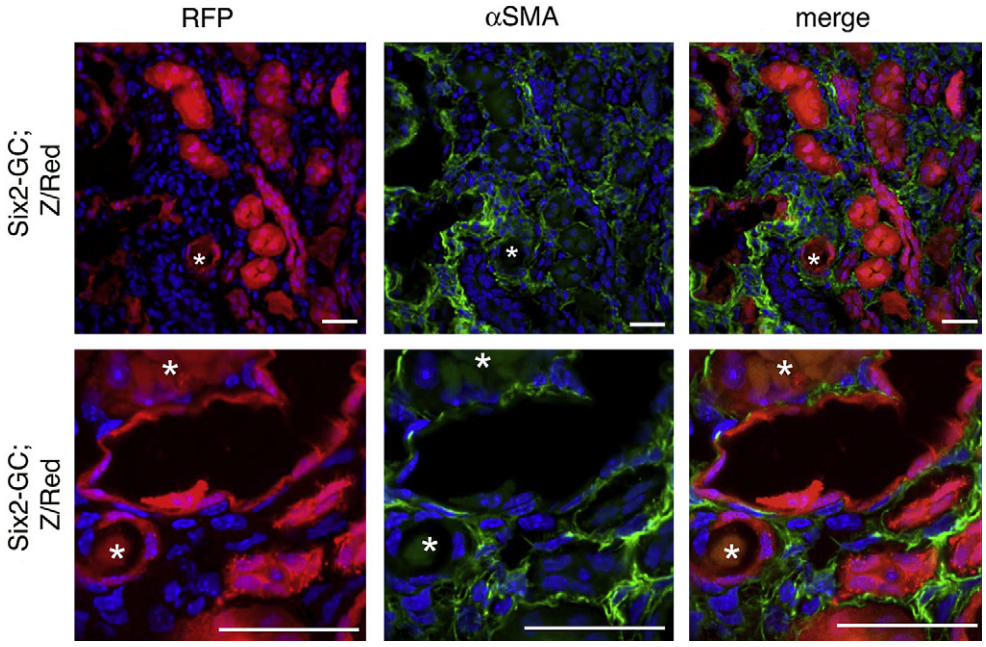

FIGURE 3.14 Fate tracing of epithelial cells of the nephron following ischemia–reperfusion injury. Low power (upper) and high power (lower) confocal images of kidney sections from *Six2 Cre;Z/Red* epithelial fate reporter mice, 15 days following ischemia–reperfusion injury. Kidney sections show native red fluorescent protein (RFP) fluorescence and are colabeled with the myofibroblast marker α smooth muscle actin (α SMA; green). In these mice Six2 is active during nephrogenesis and activates RFP expression in all cells that become tubule epithelium. More than 95% of adult tubule epithelial cells (except for collecting duct) are labeled with RFP permanently regardless of fate. Note that despite widespread loss of kidney epithelium the regenerated epithelium remains 100% positive for the fate marker RFP, indicating that regeneration of the epithelium is by epithelial cells, not cells from outside the epithelium. In addition, note that despite large numbers of α SMA+ myofibroblasts appearing in the interstitium, none of them derives from epithelial cells. * denotes intratubular debris. Please see color plate at the end of the book.

the most superficial segments of the kidney, something that has never been reported. Although no genetic tests have heritably labeled only the kidney proximal tubule, then observed whether dilution of proximal tubule-derived cells from epithelium of other segments of the nephron occurs, several lines of evidence indicate that in the mammalian kidney all injured epithelial cells have similar capacity to regenerate the nephron. Sequential pulse-labeling of regenerating cells with different synthetic nucleotides would be expected to concentrate the different nucleotides in the same cells if epithelial "stem" cells were regenerating the nephron. However, if all cells had an equal propensity to regenerate the nephron there would be a random uptake of sequential nucleotides into cells. In studies that detect the different nucleotides BrdU, CldU, IdU, etc., using different fluorophores, there is no definitive evidence for intraepithelial stem cells in the rodent kidney [132]. Although studies of this nature are by no means definitive, the possibility of a latent progenitor cell within the kidney proximal tubule remains. Latent progenitor cells have been described in the olfactory bulb. In health the olfactory neuroepithelial progenitor cell is not in cell cycle or not turning over, and therefore not replacing cells, but following injury it divides and supplies cells to different compartments of the olfactory bulb [131]. It remains possible that a subpopulation of epithelial cells within the kidney proximal tubule is a progenitor pool, but definitive evidence for this is lacking at this time.

Cellular Ablation in the Mouse Kidney: Role of Myeloid Cells in Organ Repair and Regeneration

Myeloid Cells in Organ Repair and Regeneration

All diseases of the kidney are characterized by the recruitment and local proliferation of monocytes and their tissue derivatives, macrophages and myeloid dendritic cells. Macrophages have well-recognized beneficial functions, including the clearance of cellular and extracellular debris and liberation of growth factors. However, they also have toxic, sterilizing functions that are deleterious to tissues and may contribute to tissue injury. Monocyte-derived cells are characterized as much by their diversity as by their similarities. There are at least three recognized subpopulations of inflammatory macrophages and resident macrophages/dendritic cells that probably have other, distinct functions (Fig. 3.15) [133 135]. Until recently, the function of macrophages in tissue injury has largely been inferred by their presence in injured tissues, the presence of

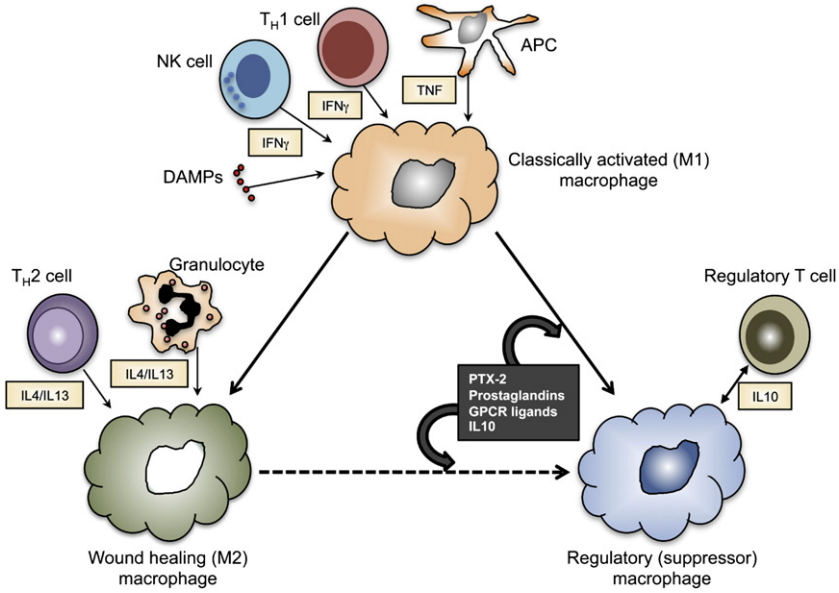

FIGURE 3.15 Schematic showing three different types of inflammatory macrophage, and factors that regulate their activation in sterile inflammation in vivo. Although cell derived and tissue derived factors can regulate the differentiation of recruited monocytes to differentiate into different macrophage subtypes, regulatory or suppressor macrophages may also differentiate from M1 and M2 activated macrophages, triggered by mechanisms that are poorly understood. Pentraxin 2 (PTX 2) opsonized targets, including apoptotic cells, trigger the regulatory macrophage phenotype in vivo that plays a key role in kidney repair and regeneration. NK: natural killer; APC: antigen presenting cell; IFN: interferon; TNF: tumor necrosis factor; DAMP: damage associated molecular pattern; IL: interleukin; GPCR: G protein coupled receptor.

distinguishing markers and the cytokines that they can generate in vitro when activated. A limited number of studies using polyclonal antimacrophage sera suggested deleterious functions for macrophages in glomerular diseases, but these have to be interpreted with caution owing to the lack of specificity of such preparations [136,137]; however, studies using similarly non-specific antisera to ablate macrophages in skin wounds indicated that macrophages promote wound healing [138]. In the 1990s liposomal encapsulated clodronate was developed as a strategy to ablate macrophages in vivo [139,140]. This strategy relies on the selective uptake of liposomes by monocytes and macrophages, delivering toxic levels of the bisphosphonate clodronate. However, liposomes are endocytosed by many cells including neutrophils and endothelial cells, and clodronate has anti-inflammatory effects of its own. Nevertheless, liposomal clodronate is effective and has been widely used to study macrophage function in vivo despite a potential lack of specificity.

To circumvent these problems a genetic approach to macrophage ablation in vivo was developed, relying on the selective susceptibility of human cells but not mouse cells to the toxic effects of DT [30,38]. Humans are greater than 1000 times more susceptible to DT than rodents owing to the cell-surface expression of the human heparin-binding epithelial growth factor receptor which is a receptor for DT (DTR) and transports DT to the cytosol, where it is rapidly lethal. Transgenic expression of this human receptor in mouse cells renders those cells uniquely susceptible to DT (Fig. 3.16). Expression of the DTR under a monocyte macrophage-specific promoter for the integrin CD11b yielded the *CD11b-DTR* mouse. CD11b is a pan-monocyte/macrophage cell-surface marker. Although CD11b is expressed by other cells, including neutrophils, only monocytes, macrophages, dendritic cells and a small population of natural killer T (NKT) cells are susceptible to the effects of DT (Fig. 3.16).

Using this transgenic model, targeted ablation of monocytes and macrophages specifically in models of kidney disease at different time-points has been achieved [30,38,133,141]. In a model of crescentic glomerulonephritis induced by immune-complex formation at the basement membrane of the glomerulus (nephrotoxic nephritis), macrophages promote disease progression (Fig. 3.16), i.e. the overall function of kidney macrophage is deleterious, not reparative. One manifestation of this progression is interstitial fibrosis, another is tubular atrophy. In a second model of immune complex deposition glomerulonephritis, which is analogous to membranoproliferative glomerulonephritis seen in human diseases including cryoglobulinemia or systemic lupus erythematosus, macrophage ablation also ameliorated disease [142]. In both of these models the data suggest that while monocytes and macrophages normally promote safe, non-phlogistic clearance of immune complexes in the glomerulus, the normal safety mechanisms in the innate immune system are overwhelmed, allowing monocyte/macrophage activation and consequent local tissue injury [142,143]; hence the deleterious effects of macrophages. Although within the heterogeneous mix of inflammatory macrophages some may still be performing reparative functions, the net consequence of widespread macrophage ablation in these models of glomerular disease is amelioration of tissue injury.

Another progressive model is the simple model of mechanical injury caused by obstruction of the ureter

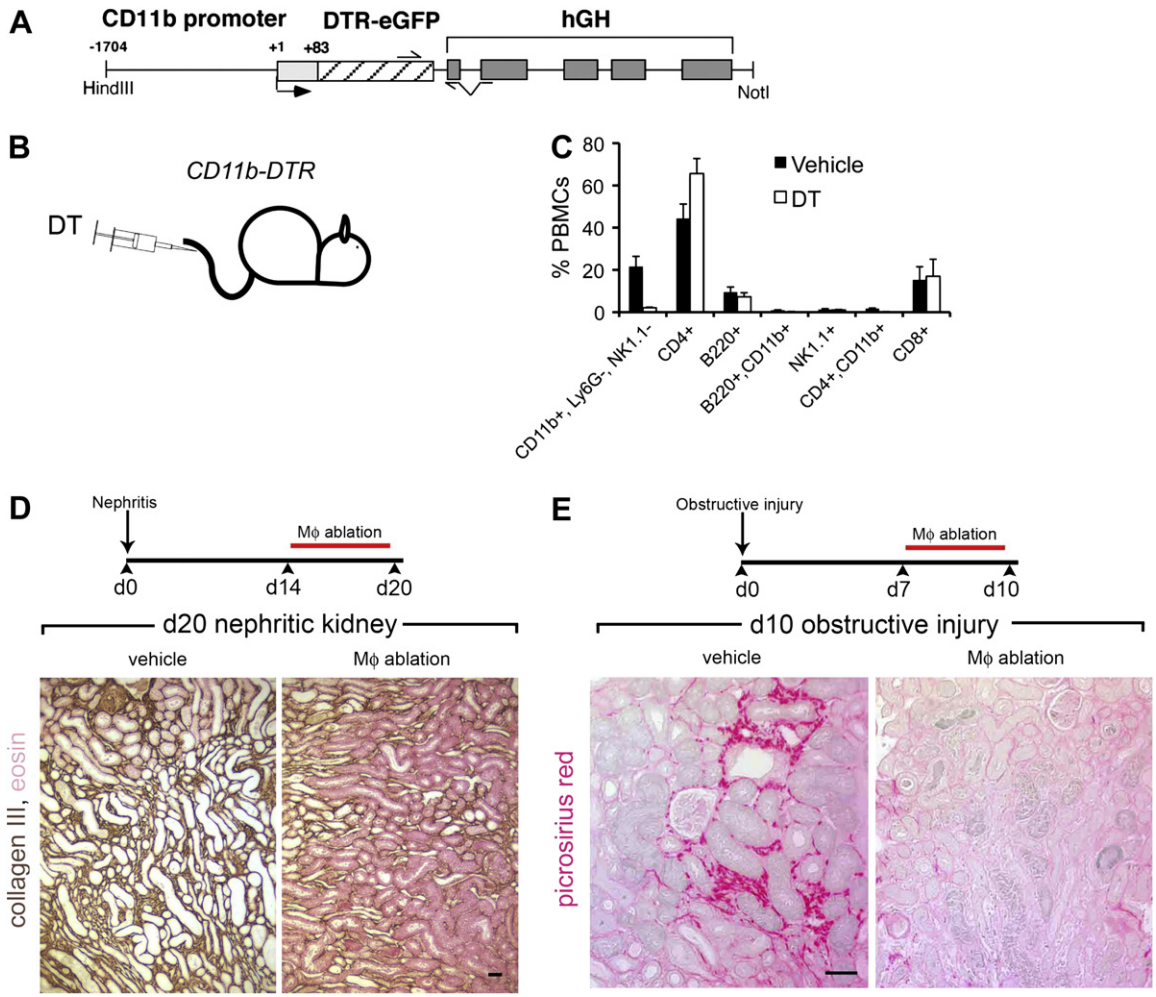

FIGURE 3.16 The diphtheria toxin receptor (DTR) system to study monocyte derived cells in kidney disease. (A) Schematic of the CD11b DTR transgene. Note that the transgene is a fusion of the DTR with enhanced green fluorescent protein (eGFP) but that eGFP is not visible without antibody enhancement. (B) Intravenous (i.v.) or intraperitoneal (i.p.) injection of diphtheria toxin (DT) is required to bring about monocyte and macrophage ablation. (C) Graph showing the percentage of human peripheral blood mononuclear cells (PBMCs) 24 h after vehicle or DT i.v. injection in CD11b DTR mice. Note that DT selectively ablates the $CD11b^{+}$, Ly6G , NK1.1 cells which are monocytes. Neutrophils (not shown) are not affected. (D) Effect of ablation of monocytes/macrophages on tubulointerstitial disease and fibrosis in the nephrotoxic nephritis model of crescentic glomerulonephritis. (E) Effect of ablation of monocytes/macrophages on fibrosis in the ureteral obstructive model of inflammation with fibrosis. Scale bar 50 μm. Please see color plate at the end of the book.

of the kidney, which results in inflammation and fibrosis [133,141] reminiscent of chronic kidney disease (Fig. 3.16). Ablative studies again indicate that macrophages promote fibrosis in response to mechanical injury, suggesting a generalized role for macrophages in fibrosis progression but also suggesting that much of the interstitial disease seen in immunological kidney disease such as NTN may be in response to secondary cellular injury rather than glomerular immune complexes. This finding has been recapitulated by others by preventing recruitment of monocytes from the circulation into the kidney interstitium [144,145].

What about the function of monocytes/macrophages in a reparative model? Macrophage function in the bilateral IRI model, which shares similarities with human acute tubular necrosis, was studied. Surprisingly, macrophage recruitment coincides with repair, not injury, in this model (Fig. 3.17) [146]. This correlation led to the hypothesis that macrophages have the capacity to repair the kidney in the absence of a persistent injury stimulus. In the *CD11b-DTR* mouse model, ablation of macrophages during the repair phase of IRI model (day 3 to 6) indeed prevented normal repair (Fig. 3.17) [146], data recapitulated in independent studies [147]. This failure of normal repair was characterized by (i) retarded functional recovery, (ii) persistent tubule injury, (iii) increased fibrosis, and (iv) impaired regeneration of peritubular capillaries. The extent to which the reparative macrophage represents a subpopulation of macrophages, and the extent to which reparative macrophages

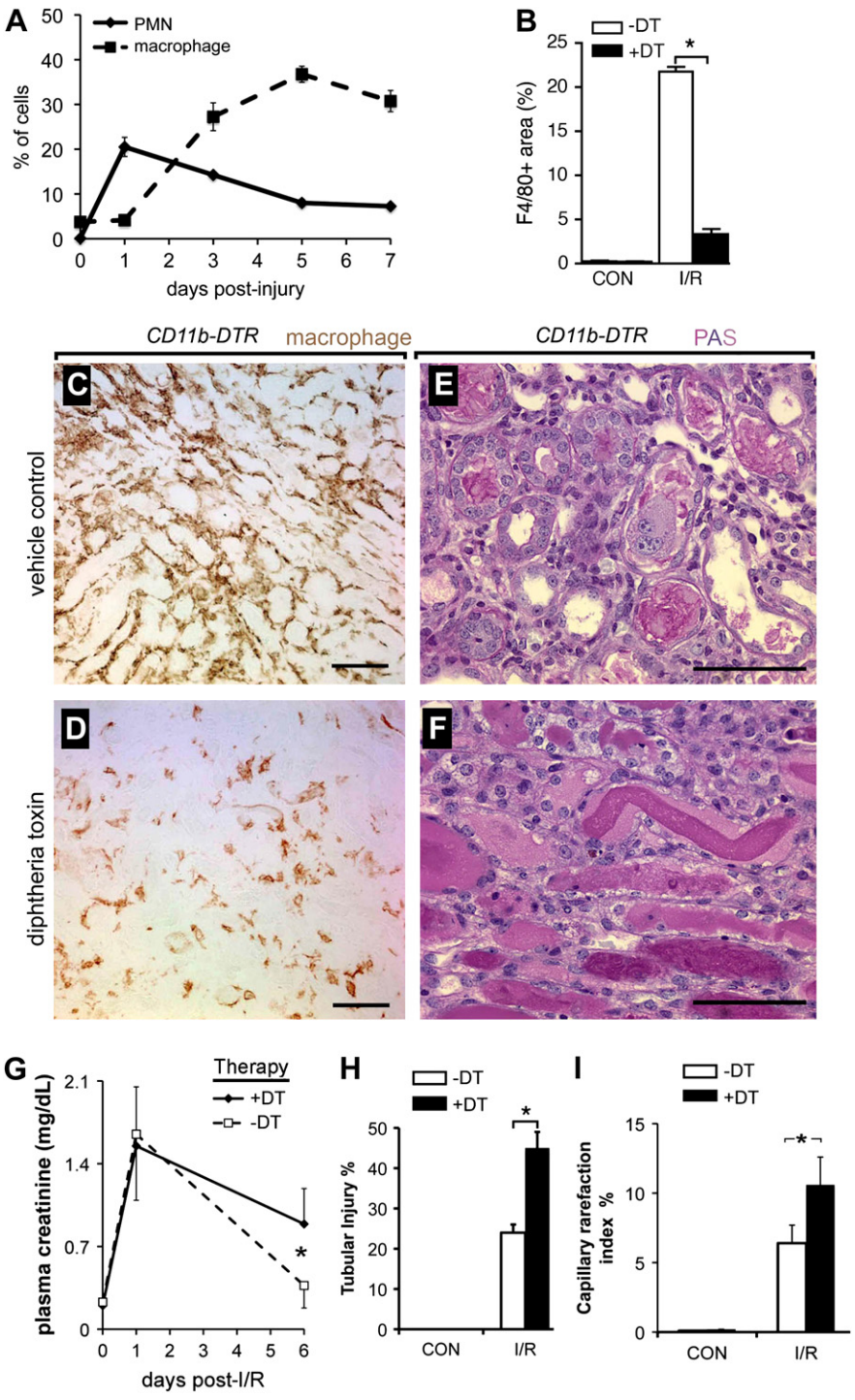

FIGURE 3.17 Macrophages are recruited during repair after ischemia–reperfusion injury (IRI) and ablation of macrophages prevents normal repair and regeneration. (A) Graph showing time course of recruitment of neutrophils and monocytes/macrophages to the kidney after IRI. (B–I) Monocytes/macrophages were ablated by diphtheria toxin (DT) injection or vehicle injection in *CD11b DTR* mice from d3 to d6 post IRI. (B) Quantification of macrophages by immunostaining and morphometry on d6 post IRI. Note marked ablation by DT. (C–E) Macrophage immunostain and periodic acid–Schiff (PAS) stain of kidneys d6 post IRI following ablation or no ablation. Please see color plate at the end of the book.

contribute directly to angiogenesis, prevention of fibrosis and tubule epithelial repair remain unclear. Macrophages have been shown, however, (i) to directly degrade and resorb interstitial matrix, (ii) to liberate angiogenic cytokines and be fundamental in angiogenesis, and (iii) to directly stimulate epithelial regeneration (see below) [148,149]. There are still many holes in our understanding of the molecular mechanisms and hence targetable mechanisms by which monocyte-derived cells promote organ repair.

How is it possible that monocytes/macrophages can promote injury in one disease model and promote repair in another model? Monocyte/macrophage subpopulations have been increasingly described to explain this heterogeneous, sometime dichotomous nature of monocyte-derived cells (Fig. 3.15). It is clear that

macrophages in the repairing kidney express different genes from macrophages in the chronically injured tissue [133,146]. Yet, these effectors derive predominantly from Ly6C^{high} circulating monocytes in both chronic injury states and repairing tissues. Current evidence suggests that monocytes enter the tissue, become activated and differentiate according to whether there is persistent injury or whether the injury stimulus has abated. The factors that regulate such differentiation into reparative versus wound-healing macrophages is currently unknown. Indeed, the mechanisms of activation of monocyte-derived cells in tissue injury are only now starting to be addressed. Elucidation of the mechanisms of macrophage activation in the repairing organ versus the chronically injury organ and elucidation of effector mechanisms will point to novel therapeutic pathways targeted at the monocyte/macrophage (see below). One hypothesis that has evolved from these and other studies is that the homeostatic role of monocyte-derived tissue cells is to clear away, non-phlogistically, damaged cells and debris, and promote tissue homeostasis without becoming activated [150]. Only in exceptional circumstances are the factors that prevent activation overwhelmed. By understanding the factors that overwhelm the system leading to inappropriate activation we may be able to separate deleterious functions of macrophages from beneficial functions.

What are the mechanisms by which inflammatory macrophages can repair a tissue? Current evidence points to three avenues: (i) phagocytosis of tissue debris; (ii) liberation of growth and survival factors for parenchymal cells that promote repair and regeneration; and (iii) angiogenesis.

Following injury of the kidney, tubules fill with necrotic debris and the interstitium becomes expanded. There is damage to vital membranes and destruction of capillaries, and the injury phase is followed by both clearance of damaged tissue and both repair and regeneration of persisting nephron structures and peritubular capillaries. It is clear that the injured kidney will benefit from clearance of debris, repair of membranes and regeneration of the damaged vascular and organ unit structures, all recognized functions of the macrophage.

Bone marrow chimera studies that have separated the contribution resident kidney macrophages from recruited kidney macrophages indicate, first, that local proliferation of endogenous cells accounts for as many as 40% of the total macrophage pool [133], and second, that those resident derived macrophages are hypoactivated and may be functionally discrete, possibly playing discrete roles in angiogenesis. Recruited, monocyte-derived kidney macrophages, in contrast, play roles in injury, fibrogenesis and repair. In support of this, 30% or more of resident kidney macrophages coexpress CD11c, an integrin that may mark myeloid cells with dendritic cell or antigen-presenting cell (APC) function. In fibrogenic models, ablation of CD11c-bearing cells using a transgenic mouse expressing the DTR in CD11c cells only has no impact on fibrogenesis in models of diseases of the kidney, further supporting the notion that the resident pool of monocyte-derived cells (macrophages and dendritic cells) plays discrete roles in kidney disease repair.

Podocyte Regeneration

Although the nephron cannot completely regenerate, the glomerulus is the most proximal structure within the nephron, and is a major target for immune-mediated injury and shear-stress injury from hypertension or hyperfiltration, and is the supplier of blood flow for the remainder of the nephron. The ability of the glomerulus to regenerate will have consequences for the integrity of the remainder of the nephron. The glomerulus may be thought of as a highly specialized vascular bed, since unlike other histologically similar structures such as pancreatic islets, it is not an endocrine tissue; rather, it contains capillary loops that exhibit fenestrated endothelium, unique or highly restricted basement membrane components, and two different types of vascular pericyte cells: the mesangial cell and the podocyte. The latter cell is in a 1:1 ratio with endothelial cells and expresses unique gene products and several gene products that are shared by neurons. In normal glomerulus adult kidney glomerular endothelial cells proliferate more than other cells, perhaps reflecting normal glomerular shear stresses not present in other vascular beds. Increasing evidence has pointed to diseases of the glomerulus, in fact representing diseases of the podocyte selectively. Strikingly, the podocyte has been identified as a cell-type that has limited capacity for proliferation, suggesting that preservation of podocytes is paramount to glomerular survival [151]. However, confusingly, in several diseases, dysregulated, excessive podocyte proliferation is a characteristic [152]. A major question that has emerged is whether the loss of podocytes can result in podocyte regeneration. Several groups have studied podocyte loss using genetic tools. Controlled ablation of podocytes using the DTR system regulated by the podocyte-specific promoter for podocin has yielded some answers [153,154]. Investigators generated a transgenic rat in which podocyte specific ablation studies were performed. Controlled dosing of DT yielded $<20\%$ podocyte ablation at 7 days, 21 40% podocyte ablation and $>40\%$ ablation, with differing outcomes. In the first group, persistent mesangial hyperplasia and expansion occurred and there was expansion of existing podocytes to cover denuded glomerular basement membrane. Glomeruli remained intact and there was only transient proteinuria. In the second group, podocyte ablation resulted in mild persistent

proteinuria, tuft to capsule adhesions and mild segmental glomerulosclerosis. In the third group, high levels of proteinuria ensued and segmental glomerulosclerosis to global glomerulosclerosis developed. These rats also developed tubulointerstitial disease in those with more severe podocyte ablation (Fig. 3.18).

Taking a similar ablative strategy in mice, the nephrin transgenic promoter was used to transgenically express human CD25 in podocytes. A genetically engineered toxin, LMB2, comprising a fusion protein of antihuman CD25 antibodies fused with the *Pseudomonas* exotoxin, selectively kills hCD25-expressing cells. In these transgenic mice, LMB2 ablates podocytes. These authors were also able to determine the degree of podocyte ablation by dose response studies and came to broadly similar conclusions. Strikingly, in these rodent models, regeneration of the podocytes and regeneration of the glomerulus was not apparent. The histological finding was of adaptive remodeling, not podocyte regeneration [155].

In contrast to these models, several studies indicate that podocytes may be regenerated from a parietal cell precursor [156,157], or even that podocytes may be replenished from circulating or bone marrow-derived cells [158]. Podocytes arise from metanephric mesenchyme and activate the transcription factors that determine cell fate for mesenchyme as epithelial and as stromal. Short of a cell fusion process, and given what we now know of the origin of regenerating cells in other compartments of the kidney, the biological likelihood of

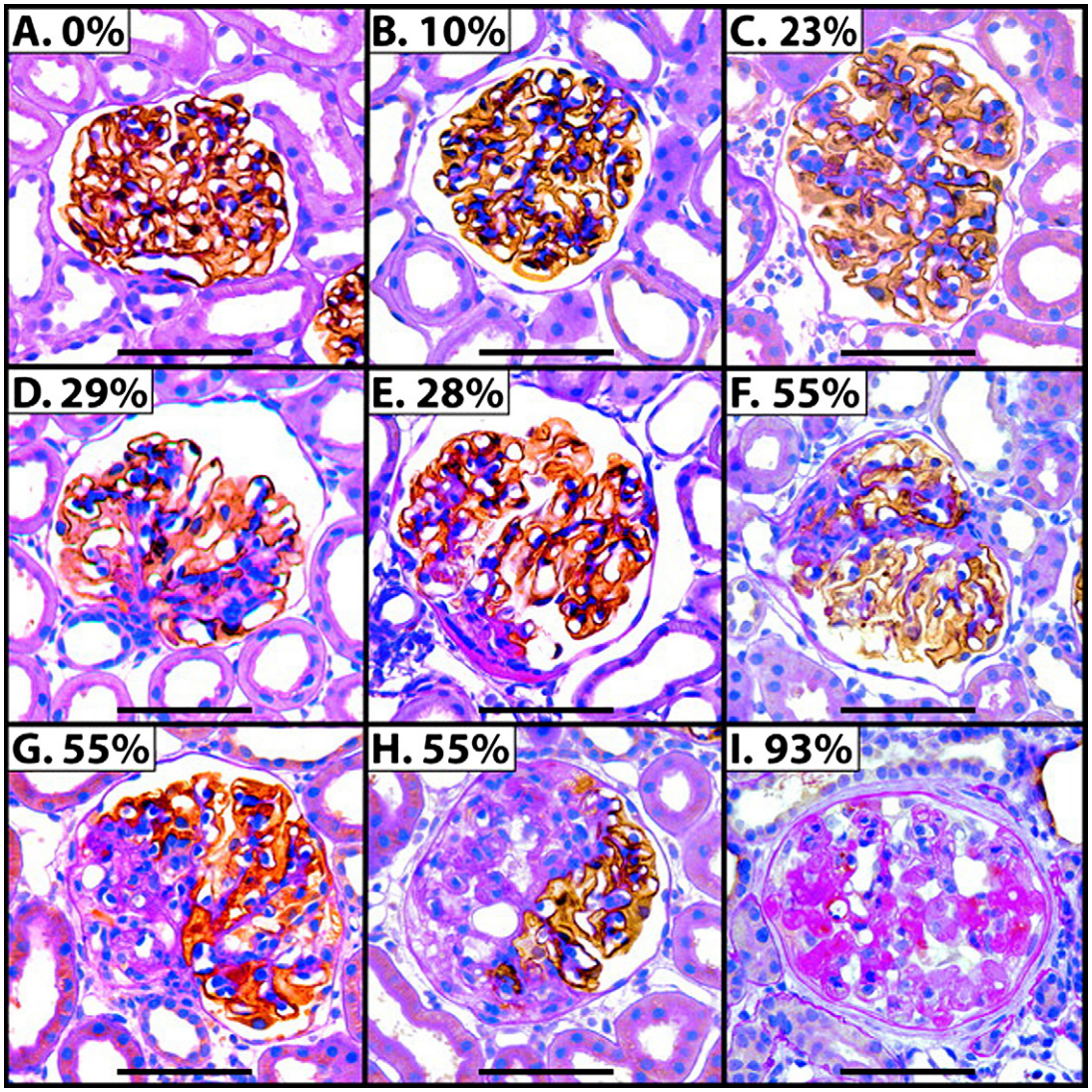

FIGURE 3.18 Effect of selective podocyte ablation on glomerular pathology. Ablation of podocytes by single diphtheria toxin (DT) injection (variable dosing) in *podocin DTR* transgenic rats viewed 28 days later. Podocytes are labeled with Glepp1 (brown) and costained with periodic acid–Schiff (PAS). Note that with 10–30% podocyte ablation there is mesangial hyperplasia with persistent covering of capillary tufts by podocytes but that there are fewer podocytes. Greater than 30% podocyte ablation results in focal segmental glomerulosclerosis or global sclerosis. *[From Wharram et al., 2005 [154], Figure 6.]* Please see color plate at the end of the book.

a marrow-derived podocyte seems low. The podocyte ablation studies in adult rats are uniquely powerful in that a single ablative event is all that is required to trigger all the hallmarks of a range of kidney diseases, and podocyte regeneration is not a feature, despite the presence of parietal precursors and marrow cells. Those studies reporting the existence of podocyte precursors in the parietal epithelium require, in the view of the authors, cautious interpretation. As described in sections above, the specificity of Cre drivers is paramount in interpretation of studies that trace the lineage of cells. Although podocyte-specific genes, loci or promoters have been used to trace podocytes, it is clear that podocyte-specific genes may be activated by injury states in other cells including parietal epithelial cells (PECs) [159,160]. Does the de novo expression of a marker thought to be specific for one cell type (podocyte) in a second cell type (glomerular crescent cell) mean that the second cell derives from the first, or does the de novo gene activation merely reflect a functional alteration in the second cell type? Clearly, the latter explanation predominates. Thus, in tracing the lineage of podocytes using *Cre-LoxP* systems, the appearance of podocyte-derived cells in the injured periglomerular area in models of crescentic glomerulonephritis is likely to reflect activation of podocyte genes in injured PECs in the glomerular crescent, rather than migration and differentiation of podocytes into the crescent. The converse of this study has been lineage tracing of PECs. Studies using conditional Cre activation by doxycycline administration in day 5 pups to genetically label a proportion of PECs with the fate marker *LacZ* report that many podocytes in adult mice derive from parietal epithelial cells [157] (Fig. 3.19). These tantalizing findings use the gamut of genetic tools to label PECs with a fate marker only on day 5 after birth, and then follow the labeled cohort into adulthood. While the studies show very convincing data, two questions arise that relate to the quality control issues raised in the earlier parts of this chapter. First, the investigators used an unusual promoter that is a fusion of a fragment of the human podocalxin promoter (3 kb) and a fragment of the rabbit podocalxin promoter (0.5 kb). Podocalxin is normally expressed in podocytes, not PECs. But with this transgene, expression is instead restricted to PECs, not podocytes. Second, the interpretation of these studies relies on the truly restricted expression of tetracycline-dependent transactivator (rTTA) driven by this promoter, to PECs and not to podocytes (Fig. 3.19). If rTTA is inadvertently activated in podocytes as well as PECs at the time of doxycycline injection that could explain the appearance of the PEC fate marker in podocytes. Third, the studies use rTTA-M2 and docycycline to activate a tetracycline responsive element which drives Cre expression (LC1 transgene), which in turn activates *LacZ* at the ROSA26 locus. If rTTA-M2 activates the tetracycline responsive element (TRE) in the absence of docycycline (even at a low level) the marked expansion of PEC-derived podocytes seen in these studies could be explained by system leakiness rather than cell lineage. Key controls are required to exclude these possibilities. Regardless of some of the technical issues, the studies strongly suggest that at least in early postnatal healthy mouse kidneys, PECs proliferate and differentiate into podocytes. Although the ablative studies in adult rats provide no evidence of PECs supplying new

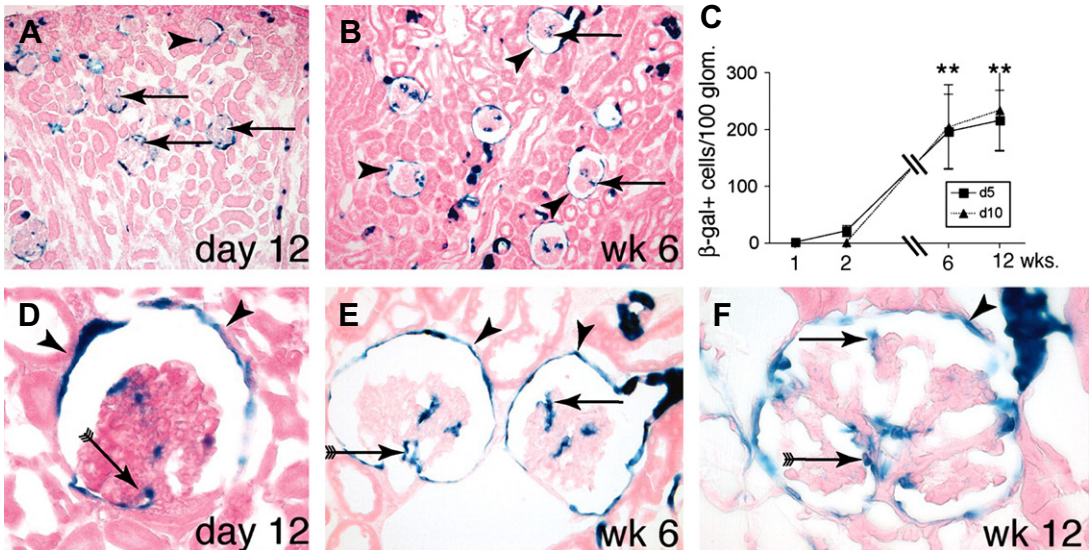

FIGURE 3.19 Lineage tracing of parietal epithelial cells using an inducible Tet inducible Cre LoxP triple transgenic system. pPEC rtTA/LC1/R26R triple transgenic mice were pulsed with tetracycline 5 days postpartum, which resulted in about 75% heritable labeling of PECs at d12 (A) and some other cells in the cortex. By 6 weeks however, there was increasing detection of blue stain podocytes (B, C) which can be seen in images shown in D–F. *[From Appel et al., 2009 [157], Figure 7.]* Please see color plate at the end of the book.

podocytes it remains possible that under stimulatory conditions in adult kidneys PECs can be reactivated to undergo proliferation and differentiation to repopulate the capillary tufts. Further studies are required.

Gene Mutation in the Study of Mouse Kidney Repair and Regeneration

The kidney IRI model exhibits striking necrotic injury to the proximal tubule, particularly in the outer medulla. Hence, much study on regeneration of the kidney after injury has focused on the epithelium. Moreover, histologically the kidney is predominantly epithelium when viewed through the microscope, although numerically endothelial cells may be similar in number to epithelial cells, just much harder to see. Many recent studies of organ regeneration have therefore focused on factors affecting kidney epithelium, and study of the role of stromal cells and endothelium in renal repair remains in its infancy. The following paragraphs describe examples where mouse genetic studies have uncovered and demonstrated convincing in vivo evidence of signaling pathways that regulate repair of the kidney following ischemic injury.

Wnt Signaling Pathway Activation

Mouse genetic studies have recently uncovered the importance of Wingless/Int (Wnt) signaling pathways in kidney regeneration. Wnt paracrine signaling is important in embryogenesis in all organs. Disrupted Wnt signaling results in neoplasia in many tissues [161]. In the kidney, Wnt canonical signaling is of fundamental importance in MET induction, branching morphogenesis and nephron elongation [89,162,163]. The first in vivo indication of the importance of the canonical pathway in the adult kidney was described when the endogenous canonical Wnt inhibitor, APC, was deleted in tubules (from an early developmental stage). This was achieved by expressing Cre transgenically under the control of the Ksp-cadherin promoter and placing *loxP* sites around exon 14 of the APC gene in both alleles. Mice developed polycystic kidneys by 8 weeks of age and many of the cysts developed renal cell carcinomas and organ dysfunction (Fig. 3.20). Dysregulated canonical Wnt signaling therefore predisposes to abnormalities of polarity and abnormalities of proliferation of renal epithelium. Although excessive canonical Wnt signaling results in epithelial cysts and neoplasia, insufficient canonical signaling in adult kidney epithelium has been shown in adults to result in impeded kidney repair following IRI [146]. To study this, receptors for the canonical pathway, Lrp5 and Lrp6 and Fzd4, expressed on epithelial cells were deleted genetically in mice and mice were subjected to kidney ischemic injury. Although injury was similar, the repair phase after injury was different. Evidence of active Wnt canonical signaling in vivo has been demonstrated using genetic techniques. By generating transgenic mice that transcribe the *LacZ* gene in cells when LEF/TCF transcription factors bind to transgenic promoter, comprising a minimal promoter followed by serial consensus sequences for TCF transcription factors, the active Wnt pathway can be detected in cells by *LacZ* transcription (Fig. 3.20). Alternatively, insertion of the LacZ gene at the Axin2 locus will also report canonical Wnt signaling by X-gal blue staining in kidney tissue sections since Axin2 is activated as a negative feedback response to Wnt pathway activation. In this "knockin" mouse and in the transgenic mouse system, evidence has been provided that in the papilla of the normal kidney there is active canonical Wnt signaling, but that following injury to the kidney there is generalized activation of the pathway in many compartments of the kidney, including the epithelium of the kidney medulla and cortex [146].

Following injury of the kidney, there is marked de novo synthesis of Wnt ligands in inflammatory macrophages that are recruited to the kidney and promote repair. An obvious question was whether macrophages might provide Wnt ligands to epithelial cells that promote repair. To test this, the candidate Wnt ligand Wnt7b was deleted in macrophages only using a *Cre-Lox* system (Fig. 3.20). Cre was driven in a myeloid restricted transgene controlled by the *Csf1R* promoter and Exon3 of Wnt7b was flanked by *loxP* sites and was efficiently cleaved, resulting in efficient mutation in monocytes and macrophages of adult mice. In these studies loss of macrophage Wnt7b resulted in impaired proliferative responses in kidney epithelium after injury and an impaired repair phenotype, providing in vivo genetic evidence for macrophages in promoting repair by delivery of Wnt ligands.

Overlap Between Polycystins, Cyst Formation and Epithelial Repair

The fact that dysregulated canonical Wnt signaling can result in cyst generation, failure to elongate the nephron in development and impaired epithelial repair responses following injury suggests that these processes are linked. More than 10 years ago the Pkd1 and subsequently Pkd2 mutation was proven in mice to lead to polycystic kidney disease [165]. Cyst formation may result from dysregulated epithelial proliferation in which cell polarity is disrupted. Heterozygosity, not homozygosity of PKD1 and PKD2 is seen in humans since homozygosity is likely to be embryonically lethal. In mice heterozygous for Pkd1 or Pkd2 cysts develop slowly with aging. A second "hit" has been postulated to be required to trigger cyst formation. In independent studies, mutation of Pkd1 only in collecting duct

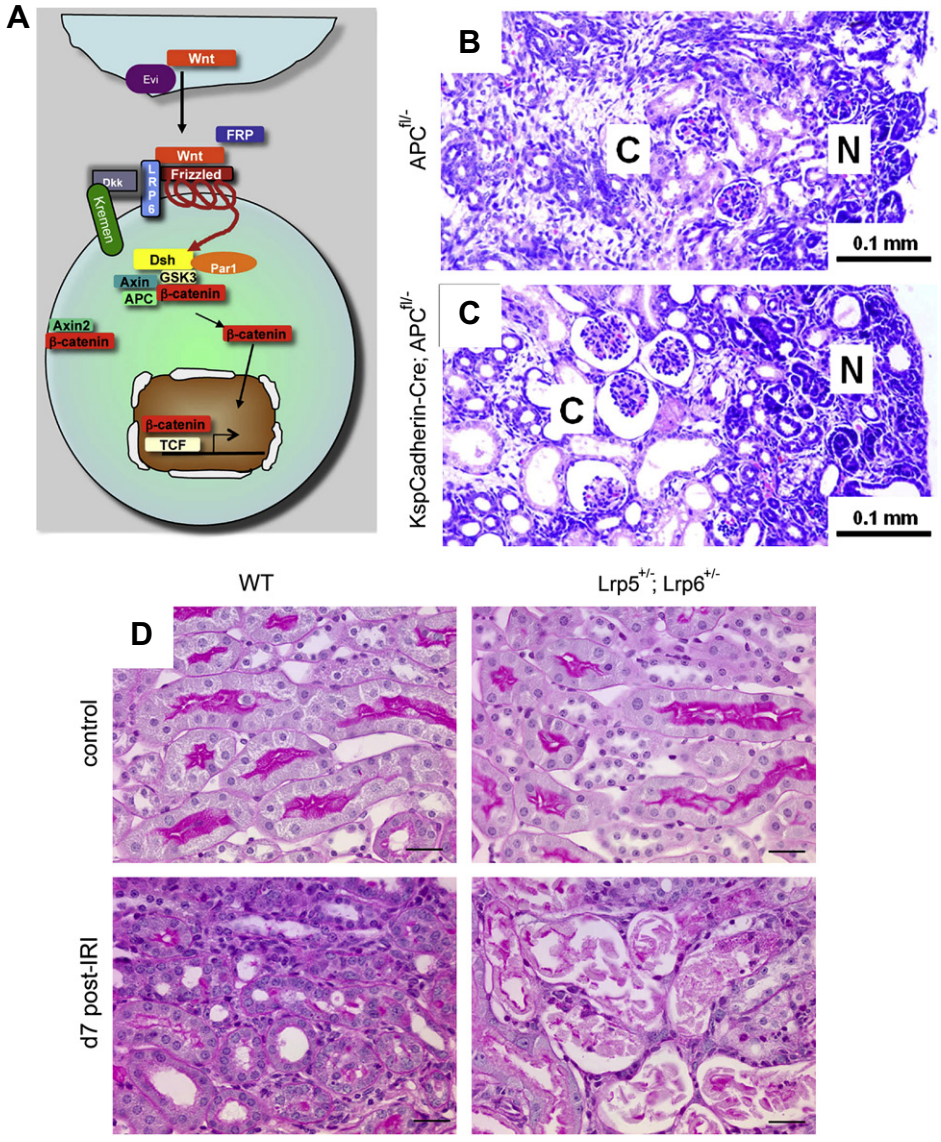

FIGURE 3.20 Canonical Wnt signaling pathway and its role in renal cyst formation and epithelial repair after injury. (A) Cartoon showing a simplified version of the canonical Wnt signaling pathway. (B, C) Newborn kidneys from mice lacking antigen presenting cells in kidney epithelium (C) or controls (B). *[Reproduced from Qian et al., 2005 [164].]* (D) Although adult kidneys of compound heterozygotes at the Lrp5 and Lrp6 loci are normal, epithelial repair after injury is severely impeded at d7 after ischemia–reperfusion injury. Please see color plate at the end of the book.

epithelium and heterozygosity of Pkd2 both show impaired tubule epithelial repair responses in kidneys after ischemic injury [166,167]. In both studies impaired proliferative responses in the affected epithelium were detected, and there was an associated excessive inflammatory response and enhanced fibrogenic response. It seems therefore that abnormal proliferative responses in kidney epithelium after injury hinder repair and that proliferative responses in normal wear and tear of the kidney may expose a genetic predisposition to cyst formation. These genetic studies are telling us that injury and repair and cyst formation are linked and that polycystins and Wnt signaling pathways are also linked, supported by recent identification of a Wnt pathway modulator, Jouberin, that predisposes to adult cyst formation when mutated [105].

Sphingosine-1 Phosphate Receptor Deficiency in Renal Repair

Sphingosine-1 phosphate is generated by many cells via kinases that phosphorylate the membrane lipid sphingosine. It binds to a class of G-protein-coupled receptors (GPCRs) known as receptors S1P receptors. Pharmacological activation of these receptors is beneficial in ischemic injury of the heart and also the kidney, through stimulation of cellular activation pathways including mitogen-activated protein kinase (MAPK) and AKT. The S1P1R has been shown to be crucial in

vascular development. However, it is also expressed by epithelial cells in the kidney. To test whether the beneficial effects of S1P1R stimulation were due to activation of leukocytes, investigators performed IRI studies on RAG1 mice that lack T and B cells. They discovered that S1P receptor stimulation protected the kidney following injury regardless of T and B cells. Next they sought to determine whether S1P1R expression by the proximal tubule was important in directly stimulating epithelial cell survival. To achieve this, mice harboring the homozygous floxed allele for S1P1R were crossed with mice expressing Cre only in epithelial cells of the kidney proximal tubule [phosphoenol pyruvate carboxykinase Cre (PEPCK-Cre)]. In these mice genomic recombination was efficient and there was $>95\%$ deletion of the receptor. Mice were healthy but when kidneys were subjected to ischemic injury there was more severe epithelial injury which did not respond to pharmacological agonists of the receptor [168 171]. These powerful studies highlight not only the importance of the S1P receptor signaling pathway(s) in renal repair but also the efficacy of genetic studies to dissect repair pathways.

LESSONS FROM GENERATION OF THE KIDNEY: CAN WE USE RECENT INSIGHTS AND DEVELOPMENTAL TOOLS TO REGENERATE THE KIDNEY?

Nephron Neogenesis in the Mammalian Kidney: Fiction Only − For Now

The cap mesenchyme cells of mammalian developing kidney are self-renewing multipotent nephron progenitors (Fig. 3.8). These nephron progenitor cells may be able to expand in vitro, and may serve as biomaterials for cell replacement therapy for kidney disease, where nephrons are lost.

De novo nephron formation has not been observed in mammalian kidneys. Powerful genetic studies have failed to identify unequivocally an adult mammalian kidney stem cell, i.e. a cell that resides within the kidney parenchyma that has the capacity to proliferate and differentiate into multiple cell types of the kidney. Yet, a similar kidney progenitor cell is readily identified in adult fish kidney with striking similarities to mammalian Six2+ progenitor cells, and in fish is capable of generating whole new nephrons after injury or resection. Further, in mammalian mammary gland, skin, intestine, liver and brain such a stem/progenitor cell has been convincingly identified and shown in principle to take part in organ regeneration [131,172 175]. Understanding the differences in regulatory mechanisms between these lower vertebrates and mammals may give clues as to how new nephrons can be generated in the mammalian kidney.

Recently, in vivo reprogramming, where defined genetic factors convert one cell type to another in the animal, has been successfully performed [176]. We now understand the gene expression profiles of different cell types at different stages of nephron development [177]. Gene inactivation in the mouse in vivo has identified genetic factors regulating nephron development [178]. It is conceivable that within a few years we will be able to generate nephrons by expressing these nephron genetic factors in existing cells or cells delivered to the adult kidney.

Acknowledgments

This research was funded by the National Institutes of Health (DK73299, DK84077 and DK87389), grants from the Genzyme renal innovation program, Gottschalk Award from the American Society of Nephrology, and grants from Promedior Inc., Baxter Inc., Regulus Pharmaceuticals Inc., and the Peter Tsacoyeanes Pediatric Renal Research Award from the National Kidney Foundation serving New England.

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CHAPTER

4

Endogenous Anti-inflammatory and Proresolving Lipid Mediators in Renal Disease

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OUTLINE

INTRODUCTION

The inflammatory response is, in general, protective and ultimately rids tissues of both the cause and consequences of tissue injury that can accompany host defense [1]. Acute inflammation, defined by its cardinal signs dolor, calor and rubor, may lead to chronic inflammation, scarring and eventual loss of function, if the tissue fails to completely resolve the inflammatory site [1]. The polymorphonuclear neutrophils (PMNs) of the first line of host defense, in this context, must also exit from the inflamed tissues (Fig. 4.1) in order to return to homeostasis and resolve [2]. In recent years it has become widely appreciated that, in addition to the classic diseases associated with inflammation such as psoriasis, periodontal disease and arthritis, uncontrolled inflammation governs the pathogenesis of many other prevalent diseases including cardiovascular and cerebrovascular disease, cancer, obesity and Alzheimer's disease [3 5]. Of interest, another class of arachidonic acid-derived eicosanoids, the lipoxins (LXs) and aspirin-triggered lipoxins (ATLs), were the first mediators recognized to have both endogenous anti-inflammatory and proresolving actions [6 8].

In recent years, the Serhan laboratory has identified novel enzymatic pathways activated during the resolution phase of self-limited inflammatory responses that are initiated from precursors eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), the major n-3 fatty acids, also widely known as the omega-3

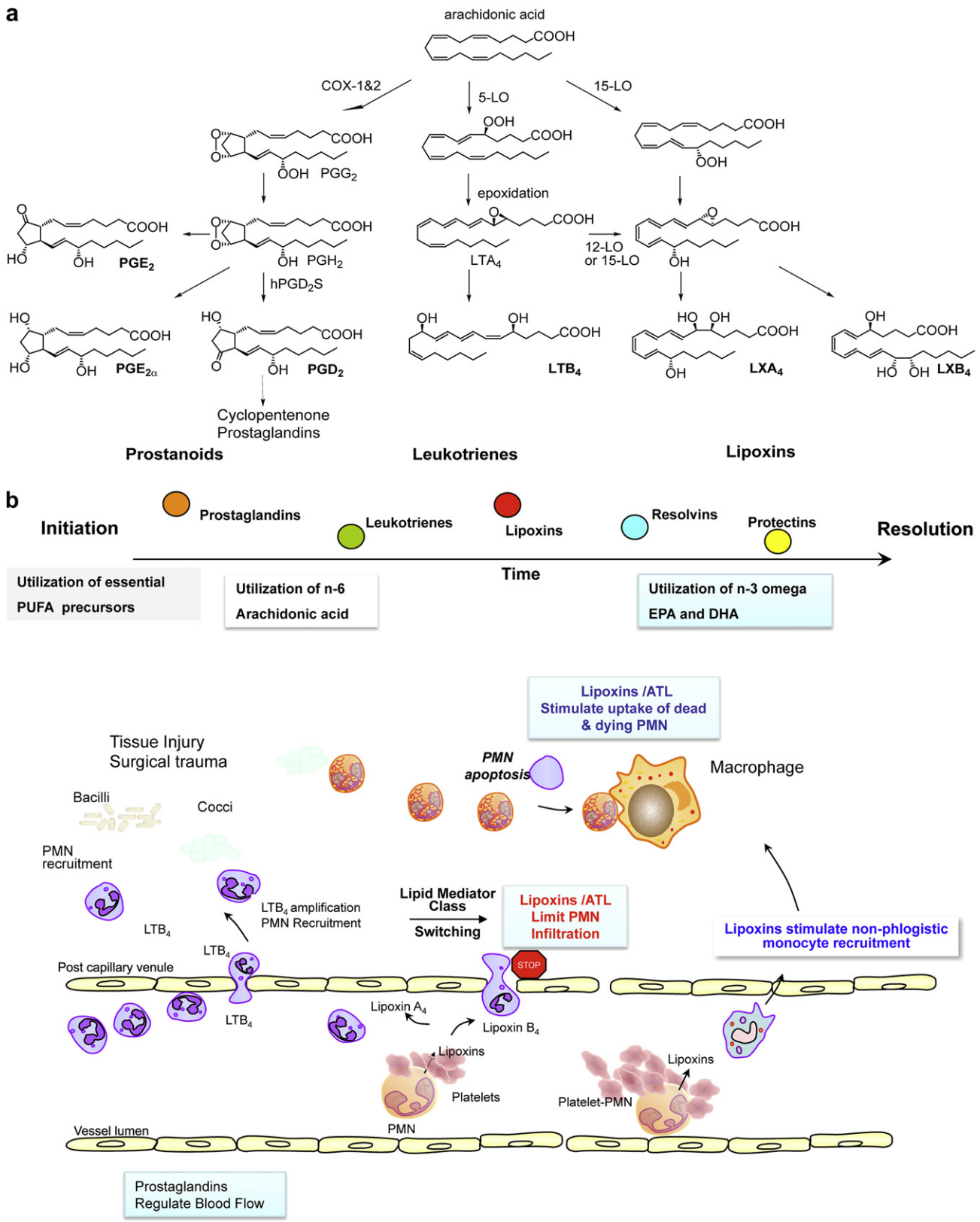

FIGURE 4.1 (a) Eicosanoid mediators in inflammation and resolution. (b) Hypothetical temporal scheme and role of lipid mediators in initiation and resolution of acute inflammation.

polyunsaturated fatty acids (PUFAs) or simply fish oils. These new compounds are biosynthesized in resolving exudates and possess potent actions, controlling the duration and resolution of inflammation [9 11]. The term resolvins, resolution-phase interaction products, was introduced to signify that the new structures are endogenous, locally acting mediators possessing potent anti-inflammatory and immunoregulatory actions [10]. At the cellular level, these include reducing neutrophil infiltration and regulating the cytokine chemokine axis and reactive oxygen species, as well as lowering the magnitude of the inflammatory response [9,10]. The terms protectin and specifically neuroprotectin D1 when generated in the neural tissue [12] were introduced, given the formation and potent anti-inflammatory [11] as well as protective actions demonstrated for the novel and potent DHA-derived 10,17-docasatriene in animal models of stroke [13] and Alzheimer's disease [14]. Both families of mediators, the resolvins and protectins, are potent local-acting agonists of endogenous anti-inflammation and are proresolving mediators [15]. The connection of these new anti-inflammatory mediators [lipoxins (LX), resolvins (Rv) and protectins (PD)] to the control of an acute inflammatory response and its timely resolution are illustrated in Fig. 4.1.

Since the early twentieth century, omega-3 fatty acids (PUFAs) have been known to possess beneficial roles in health and organ function [16]. At high concentrations in vitro, n-3 PUFAs decrease production of proinflammatory prostaglandins, cytokines and reactive oxygen species held to play critical roles in inflammatory diseases [17]. Clinically relevant anti-inflammatory properties were reported with high doses of n-3 fatty acids in both rheumatoid arthritis [18] and periodontal disease [19], whereas the evidence available at this time remains inconclusive for several other conditions [reviewed in Ref. 18]. Of interest, cardiovascular disease was reduced with high-dose n-3 in a multicenter clinical trial [20,21]. Also, blood levels of EPA and DHA were shown to reduce the risk of cardiovascular disease [22]. These findings raised the question of what mechanism(s) underlie the many beneficial actions of n-3 PUFAs.

Because the precursors to both resolvins and protectins are the essential n-3 PUFAs (Figs 4.1 4.2, 4.4), their relation to dietary supplementation by n-3 PUFAs also raises new and interesting questions, given the widely appreciated notion that n-3 supplementation reduces inflammatory diseases. Resolvins and protectins are distinct chemical families that now join the lipoxins [7] as potent agonists of endogenous anti-inflammation and are proresolving chemical mediators of interest in health and particularly renal disease. It is important to note that the biosynthesis of resolvins and protectins is stereochemically controlled via enzymatic reactions that give rise to specific chemical mediators that carry potent bioactions and require precise stereochemistry to activate specific receptors. Resolvins and protectins are, therefore, distinct from the autooxidation products obtained from DHA, EPA or other PUFAs, which may also arise in tissues via interactions with reactive oxygen radicals. These autooxidation products are complex

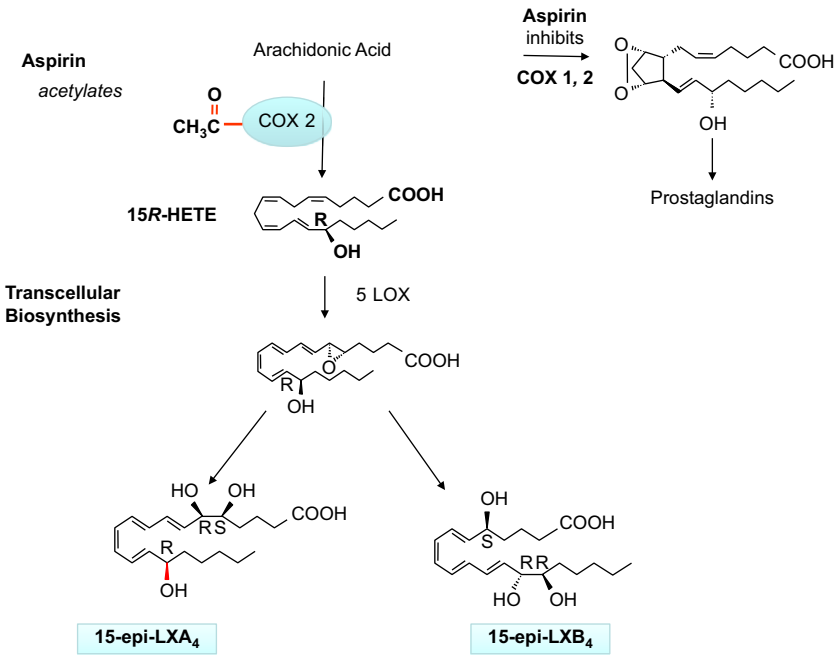

FIGURE 4.2 Transcellular biosynthesis of aspirin triggered lipid mediators: 15 epi lipoxins.

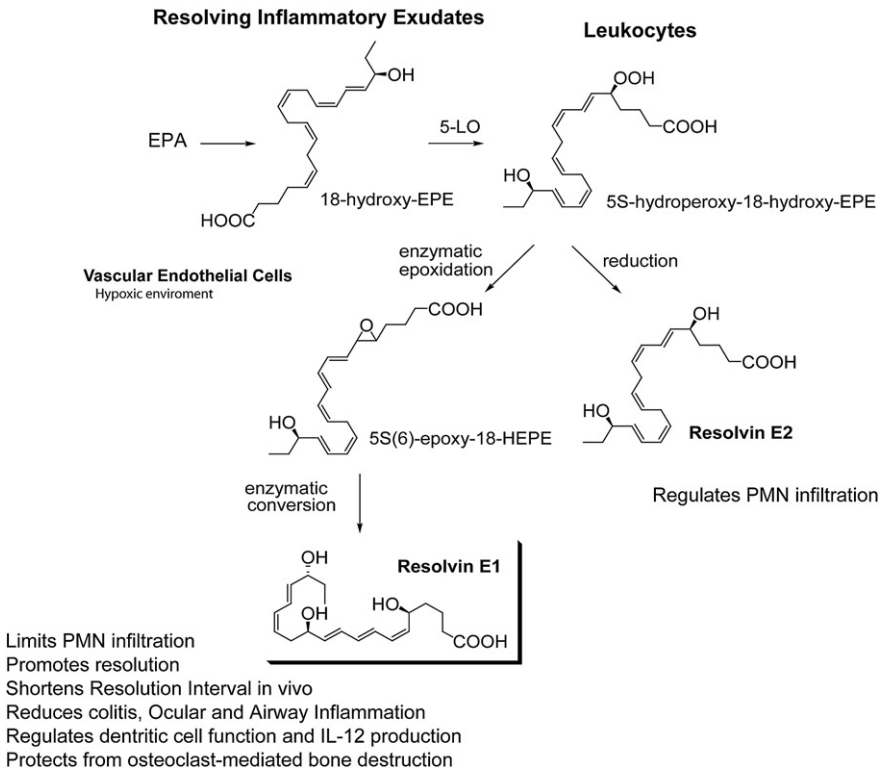

FIGURE 4.3 Biosynthesis of E series resolvins.

mixtures of mono-, dihydroxy- and trihydroxy- products of PUFAs [23] as well as specific isoprostanes and neuroisoprostanes that can serve as biomarkers of oxidative stress [24]. This chapter summarizes recent new findings on lipoxins, resolvin and protectin biosynthesis and their actions, with a focus on their impact in renal tissues.

LIPID MEDIATORS: CHEMICAL AUTACOIDS IN INFLAMMATION AND RESOLUTION

Lipid-derived mediators are well positioned to play important role(s) as signaling molecules in inflammation and tissue regeneration because they are small molecules, local acting, rapidly generated and locally inactivated. Microbial invaders, tissue injury or surgical trauma activate the release and formation of arachidonate-derived eicosanoids. Acute inflammation is characterized by the rapid, time-dependent influx of PMNs into the site, the first line of phagocytic host defense. Proinflammatory prostaglandins and leukotriene B_4 control local blood flow, vascular dilation and permeability changes needed at the site for leukocyte adhesion, diapedesis and recruitment [1,2]. These chemical mediators are enzymatically generated via specific cyclooxygenase (COX) and lipoxygenase (LOX) pathways [25]. As exudates form and pustules are walled off, prostaglandins mediate a number of responses including vasoconstriction, vascular permeability changes, pain, vasodilation and edema. At contained sites of self-limited inflammation, prostaglandin E_2 (PGE_2) and PGD_2 also signal the end or resolution of inflammation by activating the transcriptional regulation of 15-lipoxygenase (LO) in human neutrophils that in turn gives rise to the temporal dissociation of eicosanoids and production of lipoxins [2,26]. Hence, the prostaglandins and leukotrienes are rapidly generated, while the lipoxins are biosynthesized later in the time-course, coinciding with the onset of the resolution phase (Fig. 4.1).

LIPOXINS

Lipoxins were the first family of mediators identified in vivo with anti-inflammatory and proresolving actions (Table 4.1) [6]. Lipoxins are trihydroxytetraene-containing products of arachidonic acid (Fig. 4.1). Their biosynthesis and actions were recently reviewed in detail [for in-depth reviews, see Special Issue on Lipoxins and Aspirin-Triggered Lipoxins [7] and contributions within] and are discussed here in view of their role(s) in anti-inflammation and resolution. Eicosanoid class switching refers to changes in production within the arachidonate-derived family, namely the temporal change in mediator profiles from prostaglandin and leukotriene, to lipoxin.

TABLE 4.1 Mediators in Resolution and Abbreviations

Lipoxins and ATL		Resolvins and protectins
Aspirin triggered lipid mediators		Maresins
PGE_2, PGD_2		Cyclopentenone
		Prostaglandins
Glucocorticoids		Annexin 1
AT RvD1	Aspirin triggered resolvin D1	7*S*,8,17*R* Trihydroxy docosa 4*Z*,9*E*,11*E*,13*Z*,15*E*,19*Z* hexaenoic acid
AT RvD2	Aspirin triggered resolvin D2	7*S*,16,17*R* Trihydroxy docosa 4*Z*,8*E*,10*Z*,12*E*,14*E*,19*Z* hexaenoic acid
AT RvD3	Aspirin triggered resolvin D3	4*S*,11,17*R* Trihydroxy docosa 5,7*E*,9*E*,13*Z*,15*E*,19*Z* hexaenoic acid
AT RvD4	Aspirin triggered resolvin D4	4*S*,5,17*R* Trihydroxy docosa 6*E*,8*E*,10*Z*,13*Z*,15*E*,19*Z* hexaenoic acid
LTB_4	Leukotriene B_4	5*S*,12*R* Dihydroxy eicosa 6*Z*,8*E*,10*E*,14*Z* tetraenoic acid
LXA_4	Lipoxin A_4	5*S*,6*R*,15*S* Trihydroxy eicosa 7*E*,9*E*,11*Z*,13*E* tetraenoic acid
LXB_4	Lipoxin B_4	5*S*,14*R*,15*S* Trihydroxy eicosa 6*E*,8*Z*,10*E*,12*E* tetraenoic acid
PD1/NPD1	Protectin D1/ neuroprotectin D1	10*R*,17*S* Dihydroxy docosa 4*Z*,7*Z*,11*E*,13*E*,15*Z*,19*Z* hexaenoic acid
PGE_2	Prostaglandin E_2	9 *oxo* 11*a*,15*S* Dihydroxy prosta 5*Z*,13*E* dien 1 oic acid
RvE1	Resolvin E1	5*S*,12*R*,18*R* Trihydroxy eicosa 6*Z*,8*E*,10*E*,14*Z*,16*E* pentaenoic acid
RvD1	Resolvin D1	7*S*,8*R*,17*S* Trihydroxy 4*Z*,9*E*,11*E*,13*Z*,15*E*,19*Z* docosahexaenoic acid
RvD2	Resolvin D2	7*S*,16*R*,17*S* Trihydroxy 4*Z*,8*E*,10*Z*,12*E*,14*E*,19*Z* docosahexaenoic acid

ATL: aspirin-triggered 15-epi-lipoxin.

TABLE 4.2 Lipoxins Counter-Regulate Cardinal Signs of Inflammation, Limit Inflammatory Signals and Promote Resolution

Lipoxin and aspirin triggered lipoxin actions	Ref.
Regulate leukocyte traffic	[27]
• Stop PMN and eosinophil infiltration	[28]
• Stimulate non phlogistic monocyte recruitment	[29]
• Stimulate macrophage uptake of apoptotic PMNs	[30]
Redirect chemokine−cytokine axis	[31]
• Block IL 8, IL 1 gene expression	[32]
• Block TNF α actions and release	[33]
• Stimulate TGF β	[34]
Reduce edema	[35]
• Regulate actions of histamine	[36]
Block pain signals	[37]
• LXs/LTs regulate neuronal stem cells, proliferation and differentiation	[38]

PMN: polymorphonuclear neutrophil; IL: interleukin; TNF: tumor necrosis factor; TGF: transforming growth factor; LX: lipoxin; LT: leukotriene.

In this case, PMNs switch from leukotriene B_4 to lipoxin production [2]. Lipoxins, specifically LXA_4 and LXB_4, as well as their aspirin-triggered forms (see below), stop or limit further PMN entry into the exudates as well as counter-regulate the main signs of inflammation (Table 4.2). As new PMNs parachute into exudates, older and apoptotic PMN must be removed from the site in a timely fashion for inflammation to resolve (Fig. 4.1). Once PMNs enter an exudate, they interact with other cells such as neighboring leukocytes, platelets, endothelial, mucosal epithelial and fibroblasts in their immediate vicinity and are able to engage in transcellular biosynthesis to produce LX and eventually new mediators [7,9]. The process of transcellular biosynthesis is defined as the generation of new bioactive mediators that neither cell type can produce on its own. For example, human platelets on their own do not produce LX. When platelets adhere to PMNs, the resulting platelet PMN aggregates become a major intravascular source of LX that in turn halts further PMN diapedesis and recruitment [reviewed in Ref. 39]. Also, when PMNs interact with mucosal epithelial cells in the lung, oral or gastrointestinal mucosa, these PMN biosynthesize LX from precursor 15-hydroxyeicosatetraenoic acid (15-HETE) donated by interactions with mucosal epithelial cells [40 42]. Hence, PMNs switch their phenotype in that they change the profile of lipid mediators that they produce depending on their local environment [2,9]. Exudate PMN switch their lipid mediator phenotype compared to, for example, peripheral blood PMN, which generate predominantly LTB_4 as their main product [2,43]. During the course of inflammation and complete resolution, as discussed below, mediator switching also occurs between families of lipid mediators, namely from eicosanoids to resolvins of the E and D series as well as protectins [9,10,33]. This progression of the exudate is dependent on the availability of substrate, which is supplied to the evolving exudates by local edema [44]. The edema proteins, i.e. albumin, carry circulating n-3 PUFA into the exudate for the transcellular biosynthesis of resolvins and related products.

PRORESOLVING PATHWAYS, ESSENTIAL FATTY ACIDS AND RESOLUTION INDICES

Prostaglandins such as PGE_2 (produced by both COX-1 and COX-2) are generated in the initial phase of inflammation (Fig. 4.1) and have a dual role in stimulating resolution [45]. Signaling pathways leading to prostaglandin E_2 and D_2 actively switch on the transcription of enzymes (15-LOX type 1) required for the generation of lipoxins [2] as well as PUFA-derived resolvins and protectins [33,46]. Selective COX-2 inhibition, by blocking production of PGE_2 and PGD_2, delays the onset of resolution [47]. Hence, COX-2 has a role in both the initiation of acute inflammatory response and the resolution phase. Lipoxins promote resolution by limiting the further recruitment of PMN to sites of inflammation and decreasing reperfusion or reflow tissue injury [reviewed in Ref. 15]. Lipoxins also reduce vascular permeability and promote nonphlogistic (i.e. non-inflammatory) recruitment of monocytes [29] and stimulate clearance of apoptotic neutrophils via macrophages [30] (Table 4.2). Thus, a key process in resolution is the temporal "switch" or transition in the lipid mediator profiles from pro- to anti-inflammatory eicosanoids at sites of inflammation, which has direct implications for the treatment of inflammatory diseases. Drugs that disrupt this switch may have unwanted side-effects in resolution, as do inhibitors of COX and LOX [45,48,49].

COX-2 also plays a key role in the biosynthesis of prostaglandin D_2, which is a precursor to the cyclopentenones (Fig. 4.1). These include prostaglandin J_2, an activator of peroxisome proliferator-activated receptor-γ (PPAR-γ). This property has led to the proposal that cyclopentenone prostaglandins can be anti-inflammatory via regulation of nuclear factor-κB (NF-κB) [reviewed in Refs 45 and 47]. These studies also bring to light the multifunctional role of COX-2 in initiation versus termination of acute inflammation and the induction of proresolving lipid mediators.

Resolution of acute inflammation in murine models involves the appearance in exudates of EPA and DHA, which follow the appearance and accumulation of unesterified arachidonate [33,44]. Precursors are transformed via enzymatic mechanisms to bioactive compounds such as lipoxins, resolvins and protectins that regulate the duration and magnitude of inflammation, namely shorten the period of neutrophil infiltration and initiate clearance of apoptotic PMNs (Table 4.3). LX, Rv and PD1 also increase the expression of CCR5 receptors on T cells and aging PMNs, which enhances the clearance of local chemokines from the inflammatory site [51]. Apoptotic neutrophils are then phagocytosed by macrophages [30], leading to neutrophil clearance and release of anti-inflammatory and reparative cytokines such as transforming growth factor-β_1 (TGF-β_1) [33,70].

A set of resolution indices was introduced as a quantitative means for assessing the major resolution parameters and the impact of specific agents within active resolution [33,49]. These indices permit quantitative assessment of the impact of agents in resolution and the ability to compare their impact in resolution in an unbiased fashion. With these resolution indices defined, specific lipid mediators (e.g. RvE1, LX and PD1) were pinpointed to promote resolution via specific and separate mechanisms. When grossly viewed as the same outcome, namely anti-inflammation, each of these mediators is anti-inflammatory [33]. In addition, it is now clear that anti-inflammation and proresolution are not the same processes [6,49]. These measurable indices are useful tools for evaluating the molecular basis of therapeutic interventions in disease models where inflammation resolution is a component as well as identifying when and where agents may be resolution toxic [6,49].

ASPIRIN IS RESOLUTION FRIENDLY

Hippocrates advocated the use of willow bark in treatment of pain of childbirth and fever. The bark was later found to contain antipyretic substances [71,72]. Salicylates were isolated many years later. In 1899 in Berlin, aspirin was launched on the trademark list in the German Patent Office; Felix Hoffman working at Bayer added the acetyl group to the structure with the goal of enhancing salicylate uptake and actions. Today, aspirin is still one of the most widely used non-steroidal anti-inflammatory drugs. Aspirin's many clinical benefits are still unfolding at the cellular and molecular level and in many clinical studies. Although it is clear that aspirin inhibits prostaglandin and thromboxane formation by acetylating and blocking the catalytic activity of COX-1 and hence is a major mechanism in anti-inflammatory and antithrombotic therapy [73,74], aspirin's well-appreciated ability to limit leukocyte traffic into sites of inflammation, the key mechanism in reducing leukocyte infiltration during inflammation, remained to be established.

Along these lines, aspirin turns on production of the body's own endogenous anti-inflammatory lipid mediators, namely aspirin-triggered lipoxins, as well as resolvins (see below). These novel lipid mediators actively reduce inflammation (Tables 4.1 and 4.2). As depicted in Fig. 4.2, this action of aspirin involves cell cell interactions, for example between COX-2-bearing cells (vascular endothelial cells or epithelial cells) and

TABLE 4.3 Resolvins and Protectins in Experimental Disease Models

Organ system/disease model	Mediator	Actions	Refs
Acute inflammation (murine peritonitis and dermal air pouch inflammation)	Resolvin E1	Reduces PMN infiltration	[9,10,33,50]
	Resolvin E1, protectin D1, ATL/LXA_4, resolvin D1	Upregulates CCR5 expression on late apoptotic human leukocytes	[51]
		Acts as terminator of chemokine signaling during resolution; decreases TNF α formation and actions	
Colitis	Resolvin E1	Decreases PMN recruitment and proinflammatory gene expression	[52,53]
		Improves survival, reduces weight loss	
	Resolvin E1, resolvin D1−4	Genetically engineered *fat 1* mice possess high levels of DHA and EPA, generate resolvins during colitis with less tissue damage	[54]
Periodontitis	Resolvin E1	Reduces PMN infiltration, stops inflammation induced tissue and bone loss	[55−57]
Acute inflammation (murine peritonitis)	Resolvin E2	Stops PMN infiltration	[58]
Thrombogenesis	Resolvin E1	Reduces ADP dependent platelet aggregation	[59]
Ocular	Resolvin E1	Reduces suture induced inflammation	[60]
	Resolvins E1, D1	Reduces hydroxy induced tissue damage	[61]
	NPD1	Reduces choroidal neovascularization	[62]
	PD1	Reduces corneal damage	[63]
Acute inflammation (murine peritonitis)	Resolvin D1	Stops PMN recruitment	[10,64]
Ischemia−reperfusion second organ injury		Reduces lung damage	[44]
Microglial cells		Reduces microglial cell cytokine expression in vitro	[11]
Kidney	Resolvin D1, protectin D1	Protects in renal ischemic injury by limiting PMN infiltration	[65]
		Renoprotective	[66]
		Regulates macrophages	
Murine peritonitis and dermal air pouch inflammation	Resolvins D2, D3, D4	Stops PMN recruitment	[10]
		Reduces peritonitis	
Cecal puncture ligation induced sepsis	Resolvin D2	Enhances microbial clearance	[67]
		Reduces cytokine production	
		Reduces proinflammatory eicosanoids	
		Improves survival	
Acute inflammation (peritonitis)	Protectin D1	Reduces PMN infiltration	[11]
		Upregulates CCR5 expression on late apoptotic human leukocytes; terminator of chemokine signaling during resolution	[51]
		Regulates T cell migration	[68]

(*Continued*)

TABLE 4.3 Resolvins and Protectins in Experimental Disease Models—cont'd

Organ system/disease model	Mediator	Actions	Refs
Liver		Correlates supplements with biosynthesis of PD1 and organ protection in vivo	[46]
		PD1 and 17S HDHA attenuate peroxide induced DNA damage and oxidative stress in hepatocytes and protect from necroinflammatory liver injury in mice	
Lung		PD1 formation is reduced in murine models of asthma	[69]
		PD1 protects from lung damage in vivo	
		PD1 is generated in human asthma, protects from airway inflammation and hyperresponsiveness	
Acute inflammation (peritonitis)	PD1	Reduces PMN infiltration	[33,49]
		Shortens resolution interval (R_i)	
		Downregulates proinflammatory cytokines and chemokines	
		Stimulates anti inflammatory cytokines and chemokines	
Alzheimer's disease	NPD1	Diminished production in human Alzheimer's disease	[14]
		Promotes neural cell survival in vivo	
Stroke	NPD1	Limits ischemic damage	[13]
		Reduces PMN entry into the brain	

ATL/LXA_4: aspirin-triggered 15-epi-lipoxin/lipoxin A_4; NPD1: neuroprotectin D1; PD1: protectin D1; PMN: polymorphonuclear neutrophil; TNF: tumor necrosis factor; DHA: docosahexaenoic acid; EPA: eicosapentaenoic acid; HDHA: hydroxy-docosahexaenoic acid.

leukocytes [9]. By acetylating COX-2, aspirin redirects COX-2's catalytic activity away from generating the intermediate for prostaglandins and thromboxanes towards producing 15*R*-HETE. The COX-2 enzyme with this modification remains catalytically active. This product of vascular endothelial cells and a wide range of other cells are converted to 15-epi-LXA_4 by leukocyte 5-lipoxygenase, termed aspirin-triggered 15-epi-lipoxin A_4 (ATL). ATL shares actions with LXA_4, and appears to be longer acting, resisting rapid dehydrogenation in vivo (Table 4.2). It is important to point out that the role of PMNs is well appreciated in the pathogenesis of rheumatoid arthritis [75], a concept that was recently reaffirmed [76]. Aspirin also increases the hepatic biosynthesis of 15-epi-LX via enhancing p450 production of 15R-HETE in a COX-2-independent pathway in rats [77], which is also likely to contribute to ATL plasma levels in healthy individuals taking low-dose aspirin [78] and in local sites of inflammation [79].

EPA and DHA are each converted via aspirin-acetylated COX-2 to generate bioactive epimers [9 11,13] of resolvins and protectins (see below). Thus, aspirin is unique among anti-inflammatory drugs in that it not only blocks proinflammatory prostaglandins but also jump-starts resolution by enabling the local production of endogenous epimers of resolution-phase mediators that share characteristic features with their counterparts in terms of reducing inflammation and PMN-mediated injury, which are major components in many human diseases.

LXA_4 and ATL display counter-regulatory roles in animal models of disease (Table 4.4), possess local organ-specific functions, and modulate leukotriene formation and their activities. The protective actions of LXA_4 and ATL are ligand-receptor dependent. The principal LXA_4 receptor first described in neutrophils by Fiore et al. [95] is a G-protein-coupled receptor (GPCR) now designated FPR2/ALX, or ALX/FPR2 when the ligand is a lipoxin [96]. ALX/FPR2 binds LXA_4 with high affinity (subnanomolar K_d); it also binds several peptide ligands, and receptor activation can result in either proinflammatory or anti-inflammatory responses [96,97]. Krishnamoorthy et al. recently found that LXA_4 and the resolvin RvD1 (see below) can interact with another GPCR, the "orphan" GPR32 [98]. It is noteworthy that, although LXA_4 and LXB_4

TABLE 4.4 Lipoxin Deficiencies in Human Disease and Lipoxin Treatments in Animal Disease Models

Human disease	Refs	Animal model	Refs
Cardiovascular disease LX/LT	[80]	Rabbit and mouse	[81,82]
Asthma		Mouse Tg hALX	[83]
Aspirin sensitive asthma	[83]		
Cystic fibrosis (classic non resolving)	[84]	Mouse	[84]
Gastrointestinal disease	[85]	Mouse	[86,87]
Renal ischemia–reperfusion injury	–	Mouse	[88]
Glomerulonephritis, poststreptococcal glomerulonephritis	[89]	Gene therapy approach	[90]
Periodontal disease	[35]	Rabbit TgLO	[91]
Rheumatoid arthritis	[92]	Mouse	[93]
Endometriosis	–	Murine endometriosis	[94]

LX: lipoxin; LT: leukotriene.

share many biological activities, LXB_4 does not bind FPR2/ALX and the LXB_4 receptor has not yet been identified. LXA_4 also displays partial antagonism at a subclass of cysteinyl LT receptors (CysLTs), which partly accounts for its counter-regulatory impact on LTD_4 responses [99,100]. Cross-talk between FPR2/ALX and tyrosine kinase growth factor receptors [platelet-derived growth factor (PDGF), epidermal growth factor (EGF), vascular endothelial growth factor-2 (VEGF-2)] also demonstrates a mechanism by which LXA_4 and ATL regulate responses, such as proliferation, angiogenesis and fibrosis [99,101 106]. LXA_4 has been shown to bind the nuclear aryl hydrocarbon receptor (AhR) in dendritic cells, although far higher concentrations of LXA_4 are needed for activation of this receptor compared to the GPCR [107]. Transgenic overexpression of human ALX/FPR2 leads to decreased PMN infiltration with endogenous LXA_4 [108]. Furthermore, ALX/FPR2 is upregulated by glucocorticoids (Table 4.1) [109] and activated by glucocorticoid-induced ligand such as annexin-1 and annexin-derived peptides [110]. Diminished LX and ATL levels are associated with the pathophysiology of several human diseases [15]. LXA_4 and ATL regulate tumor necrosis factor-α (TNF-α)-directed neutrophil actions and stimulate IL-4 in exudates, and thus regulate endogenous mediators in the pathogenesis of inflammatory conditions such as periodontal disease [35]. In many human diseases, LX production appears to be deficient compared to leukotrienes. These include cardiovascular [80], asthma [83], kidney inflammation [111], cystic fibrosis [84], gastrointestinal [85] and periodontal disease [35] (Table 4.4). Designed metabolically stable analogs of LXs and ATLs are useful tools in examining the role(s) and local actions of lipoxins in vivo [112 114]. Administration of LX stable analogs in animal models protects from tissue damage and inflammation [7,115] and enhances resolution [34]. Identification of these anti-inflammatory actions of LXs and ATLs provided strong evidence for the existence of endogenous anti-inflammatory mediators derived from arachidonic acid. Along with reducing PMN influx [116], redirecting chemokines and cytokines [117], and reducing pain [37,118], LX and ATL have the ability to stimulate the removal of apoptotic PMNs by macrophages in vitro [30] and at sites of inflammation in vivo [119]. This proresolving agonist activity is shared by (Table 4.1) annexin 1 [48] and glucocorticoids [120,121], and accelerates the return of the tissue to homeostasis (Tables 4.1 and 4.2).

STATINS AND ANTI-INFLAMMATORY LIPID MEDIATORS

The mechanisms of the well-recognized anti-inflammatory action of statins are of considerable interest. Statins (e.g. atorvastatin) and pioglitazone regulate the production of *S*-nitrosylated COX-2 [122 125]. The *S*-nitrosylated COX-2 produces 15*R*-HETE, which is converted by 5-lipoxygenase to 15-epi-LXA_4. The finding that statins regulate the production of 15-epi-lipoxin A_4 suggests that the anti-inflammatory actions of statins are directly mediated by endogenous production of 15-epi-lipoxin A_4 [123,126]. These widely used drugs aspirin and statins thus have in common a unique ability to trigger the endogenous production of 15-epi-LXA_4. Further studies by Birnbaum et al. show that, when COX-2 is both acetylated and *S*-nitrosylated, the enzyme is inactive, providing potential adverse interactions

among statins, thiazolidinediones and high-dose aspirin. It is likely that this mechanism will also affect the biosynthesis of the aspirin-triggered forms of the resolvins and protectins.

LIPOXINS AND HEME OXYGENASE-1 SYSTEM

Lipoxins exert several direct actions on endothelial cells that are protective and in line with their role in resolution. In this regard, lipoxins stimulate prostacyclin generation by endothelial cells [127] and stimulate nitric oxide (NO) production by vascular endothelial cells [128]. Aspirin acetylation of COX-2 generates 15-epi-lipoxins that in turn stimulate the production of NO by endothelial nitric oxide synthase (eNOS). Aspirin, in either eNOS or inducible nitric oxide synthase (iNOS) knockouts, is not anti-inflammatory in interleukin-1β (IL-1β)-induced murine peritonitis. Both aspirin and 15-epi-LXA_4 had reduced effects on endothelial cell adherence from eNOS and iNOS knockouts compared to wild-type [128]. This suggests that aspirin triggers the production of 15-epi-LXA_4, which increases NO synthesis through both eNOS and iNOS. These findings suggest a tight regulation between the generation of 15-epi-LXA_4 and the production of vascular-derived NO. Also, aspirin-triggered lipoxins and lipoxins block VEGF-stimulated angiogenesis and migration of endothelial cells [101,102]. Aspirin induces heme oxygenase-1 (HO-1) expression in endothelial cells, which is increased by ATLa in a concentration- and time-dependent fashion in human endothelial cells [129]. The induction of HO-1 by LX and ATL appears to regulate, in part, the organ-protective actions observed with lipoxins. In a murine model of sepsis, treatment with ATLa spares lung tissues from inflammatory damage [130].

RESOLVINS ARE NOVEL ENDOGENOUS MEDIATORS: 18*R* E-SERIES AND 17*R* D-SERIES RESOLVINS

In view of the role of LX in resolution and reported beneficial actions of omega-3 in humans, it was of interest to determine whether specialized lipid mediators are involved in the resolution of self-limited inflammation. The mouse air pouch was selected for systematic analysis because acute inflammation and exudate formation are spontaneously resolved in this dorsal skin cavity, which permitted kinetic analysis of chemical mediators and leukocyte traffic. The novel lipid mediators produced from EPA were first isolated from resolving exudates that proved to contain 18*R*-hydroeicosapentaenoic acid (18R-HEPE) as well as several other related bioactive compounds [9]. The first bioactive product isolated from exudates, coined resolvin E1, reduced inflammation (Fig. 4.4) and blocked human PMN transendothelial migration [15]. Structural elucidation was carried out together with both gas chromatography mass spectrometry (GC-MS) and tandem mass spectrometry (MS-MS)-based lipidomic analysis of bioactive fractions obtained following extraction and reverse-phase high-performance liquid chromatography (RP-HPLC). The basic structure of this potent bioactive product in resolving exudates proved to be 5,12,18*R*-trihydroxyeicosapentaenoic acid [9]. Databases were constructed containing known and theoretical fragments produced by MS-MS of putative lipid mediators. These databases were systematically researched in a stepwise fashion using ultraviolet (UV) chromophores and MS-MS spectra, then LC retention times to identify the basic structures of new compounds in the inflammatory exudates. These procedures required constructing algorithms used together with library software for mass spectral analyses [131]. Following assessment of potential bioactions, the complete stereochemistry of the potent bioactive and related isomers was confirmed by total organic synthesis [9,10,50,64,67].

Recombinant COX-2 treated with aspirin generates 18*R*-HEPE as well as 15*R*-HEPE from EPA, which are blocked by selective COX-2 inhibitors [9]. At clinically used doses of either acetaminophen or indomethacin, the oxygenation of EPA to both 18*R*-HEPE and 15*R*-HEPE with isolated recombinant COX-2 continued, although the levels of these products were significantly reduced. These results indicate that the oxygenation of n-3 PUFA to generate novel bioactive mediators can also involve certain of the widely used anti-inflammatory drugs, but not with the selective COX-2 inhibitors [9].

The most likely biosynthetic pathways were recapitulated in vitro with human cell types that biosynthesize these bioactive mediators modeling the resolving murine exudates, which involve cell cell interactions and transcellular biosynthesis (Fig. 4.3). Isolated human vascular endothelial cells in a hypoxic environment treated with aspirin convert EPA to 18*R*-HEPE which is released and then rapidly converted by activated human PMN to a 5[6]-epoxide-containing intermediate. This EPA-derived intermediate is converted to the bioactive resolvin E1 (RvE1). RvE1 possesses a distinct structure consisting of a conjugated triene plus conjugated diene chromophore present within the same molecule (Fig. 4.3). Both biogenic [9] and total organic synthesis was achieved and its complete stereochemical assignment was established [50]. RvE1 proved to be 5*S*,12*R*,18*R*-trihydroxy-6*Z*,8*E*,10*E*,14*Z*,16*E*-eicosapentaenoic acid. The synthetic RvE1 displayed

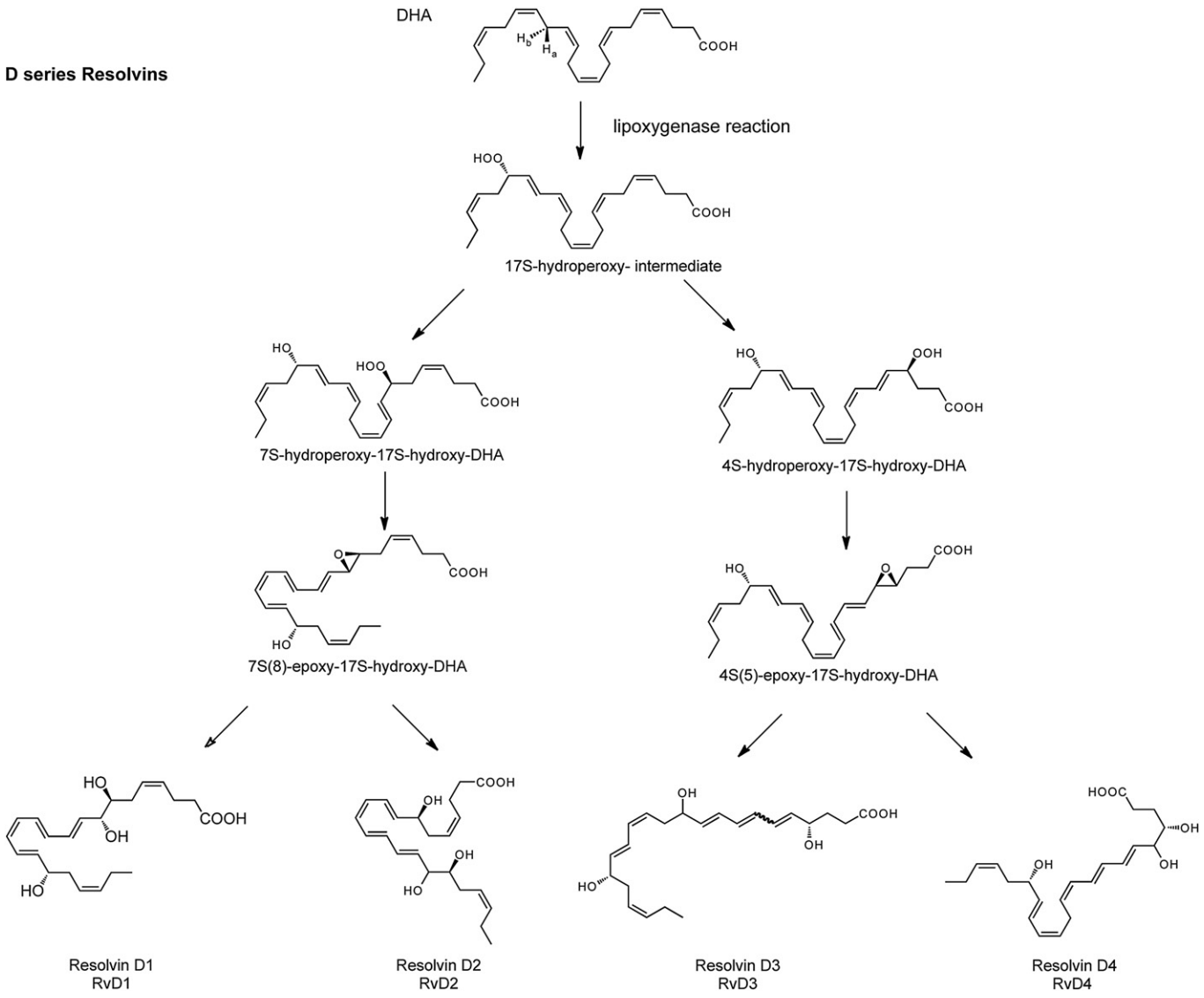

FIGURE 4.4 Biosynthesis of D series resolvins.

potent stereoselective actions in vivo and with isolated cells confirming the original structural assignment (Table 4.3).

To determine the receptors involved in RvE1 actions, a screening library of G-protein-coupled receptors was devised employing counter-regulation of TNF-α, a task that one might expect a resolvin to carry out in vivo [50]. The orphan receptor ChemR23 specifically bound to ^{3}H-labeled RvE1, and attenuated TNF-activated NF-κB signaling [50]. Labeled RvE1 was synthesized using an acetylenic precursor that was tritiated and isolated using RP-HPLC. The main second messenger for RvE1 agonist actions via these GPCRs appears to be activation of intracellular phosphorylation pathways [132]. This intracellular signaling contrasts with proinflammatory mediators that use mobilization of intracellular calcium or cyclic AMP [50,52]. RvE1 also interacts with a second high-affinity GPCR. RvE1 interacts with LTB_4 receptor BLT1 and attenuates LTB_4-induced proinflammatory signals by acting as partial agonist/antagonist on LTB_4 receptor present on human PMNs [133]. ChemR23 is expressed on dendritic cells, monocytes [50] and macrophages [132]. In its active dose range, RvE1 does not activate nuclear receptors [98].

A second bioactive member of the E series resolvins was identified that is produced during RvE1 biosynthesis and shares anti-inflammatory properties with RvE1 [58]. The basic structure of the novel dihydroxyeicosapentaenoid was determined and shown to be 5*S*,18(*R/S*)-dihydroxy-eicosapentaenoic acid, denoted resolvin E2 (RvE2), the reduction product of 5*S*-hydroperoxy, 18-hydroxy-eicosapentaenoic acid (Fig. 4.3). Human 5-lipoxygenase has a pivotal role since it catalyzes the

initial 5-hydroperoxide generation from 18-hydroxy-eicosapentaenoic acid as well as the epoxide required for RvE1 formation. Human neutrophils can biosynthesize RvE2 in greater amounts than RvE1. Resolvin E2 is equipotent to RvE1 when administered intravenously and additive at low doses when given intraperitoneally. These findings suggest that RvE2 acts via different receptors than RvE1 [58].

RESOLVIN AND PROTECTIN BIOSYNTHESIS

Resolving inflammatory exudates from mice given aspirin along with DHA contained novel 17*R*-hydroxy-DHA (17*R*-HDHA) as well as two novel families of bioactive compounds (Figs 4.4 and 4.5). Here too, the potential biosynthetic pathways were reconstructed with human cell types in vitro to establish potential cellular origins and routes for these novel compounds. Hypoxic human microvascular endothelial cells treated with aspirin release 17*R*-HDHA. DHA is a substrate for isolated human recombinant COX-2-producing 13-hydroxy-DHA [10]. With aspirin treatment, COX-2 switches to 17*R*-oxygenation with molecular oxygen to give an epimeric or aspirin-triggered form in exudates and also in blood and brain with both families of resolvins and protectins [10,11]. The aspirin-triggered forms carry a 17*R* alcohol group configuration instead of the carbon 17*S* as when biosynthesized via lipoxygenase mechanisms (Fig. 4.4).

Endogenous DHA is converted to a 17*S* alcohol-containing series of resolvins (RvD1 RvD4; shown in Fig. 4.4), even in the absence of aspirin, as well as docosa-conjugated triene-containing structures via lipoxygenase-initiated mechanisms [11,13]. The complete stereochemistry of protectin D1, which carries a base 10,17-dihydroxydocosatriene structure [10,11], was established and confirmed the original assignment [10,12]. Total organic synthesis of related isomers and matching studies with biologically derived materials showed that endogenous protectin D1, denoted neuroprotectin D1 (NPD1) when produced by neural tissues, was established in isolated human cells and murine cells

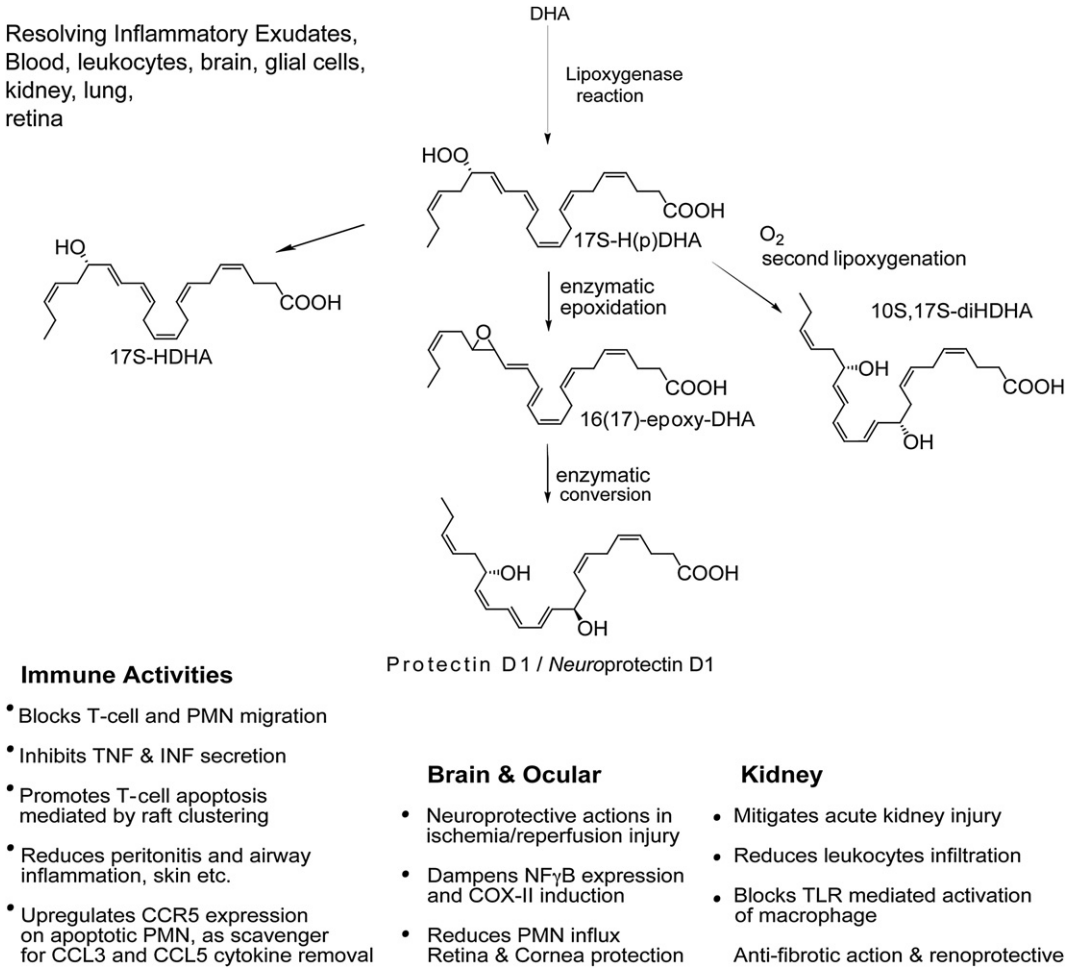

FIGURE 4.5 Biosynthesis of protectin D1/neuroprotectin D1 and related products.

in vivo as 10*R*,17*S*-dihydroxy-docosa-4*Z*,7*Z*,11*E*,13*E*,15-*Z*,19*Z*-hexaenoic acid [12]. The geometry of the double bonds in PD1 and their positions during biosynthesis from the key intermediates in its biosynthesis in situ were formed via an epoxide intermediate generated at the 16[17] position, indicating that biosynthesis of PD1 requires enzymatic steps to generate the potent bioactive molecule (Fig. 4.5). On a molar basis, PD1 proved to be log orders of magnitude more potent than its native precursor DHA [11,12,65].

DHA contained in tissues or taken into the inflammatory site [33,44] is converted to 17*S*-hydroxy-containing protectins and resolvins. The complete stereochemistry via matching studies and total organic synthesis of RvD1 (7*S*,8*R*,17*S*-trihydroxy-4*Z*,9*E*,11*E*,13*Z*,15*E*,19*Z*-docosahexaenoic acid) and the aspirin-triggered form, AT-RvD1 (7*S*,8*R*,17*R*-trihydroxy-4*Z*,9*E*,11*E*,13*Z*,15*E*,19*Z*-docosahexaenoic acid) were achieved [10,64], as well as for RvD2 [67]. These results not only confirmed the potent anti-inflammatory and proresolving actions and original structural elucidation but also now open the possibility to investigate these actions in models of inflammatory diseases.

RESOLVINS AND PROTECTINS IN DISEASE MODELS

In low doses (i.e. only nanogram levels), RvE1 reduces neutrophil transendothelial migration, dermal inflammation [10], peritonitis, dendritic cell migration and IL-12 production (Table 4.3). Synthetic RvE1 blocks PMN infiltration, protects from bone destruction in a rabbit model of periodontal disease [55] and protects against the development of colitis [52]. In several animal models of inflammatory diseases, RvE1 is a potent counter-regulator that protects against leukocyte-mediated tissue injury. Resolvins of the D series also block TNF-α-induced IL-1β transcripts in microglial cells and are potent regulators of PMNs, limiting infiltration into inflamed brain, skin and peritonitis [10,11,13] (Table 4.3). Direct comparisons between the resolvin E versus both of the D series (17*S* and 17*R* epimer aspirin-triggered series) at equal doses demonstrated that the 17*S* series generated by lipoxygenase-initiated mechanisms and the 17*R* series RvDs triggered by aspirin treatment when administered intravenously at 100 ng [~3 μg/kg] in mice display essentially similar actions, reducing PMN infiltration by approximately 50% in peritonitis.

Indomethacin, a widely used anti-inflammatory, was tested for direct comparison at the same doses and gave only approximately 25% reduction in leukocyte infiltration [10,11]. The RvDs (17*S* series) and aspirin-triggered RvDs (17*R* series) are, hence, potent regulators of PMN infiltration in vivo, and the *S* to *R* switch with aspirin treatment in the biosynthesis of the 17-position alcohol in the omega side-chain does not diminish their activity. This implies that the aspirin-triggered 17*R* epimers of protectins and resolvins may each serve as the body's own anti-inflammatory mediators.

The protectins possess the conjugated triene structure and alcohol groups at the carbon 10 and 17 positions, a key feature of this family. PD1 is referred to as neuroprotectin D when generated in the nervous system (Fig. 4.5), and possesses potent actions (Table 4.3). Synthetic PD1 at 10 nM, for example, attenuates human neutrophil transmigration by approximately 50% in vitro, whereas its Δ15-*trans*-isomer is essentially inactive. PD1 is also a potent regulator of PMN in vivo by reducing PMN infiltration (~40% at 1 ng/mouse) in murine peritonitis. PD1 also reduced PMN infiltration when administered after the initiation of inflammation in vivo, and acts in an additive fashion with RvE1 to stop PMN infiltration. PD1 is thus a potent, stereoselective anti-inflammatory molecule in vivo [10 12]. Moreover, these results and those obtained with Bazan and colleagues in neural tissues demonstrate that PD1

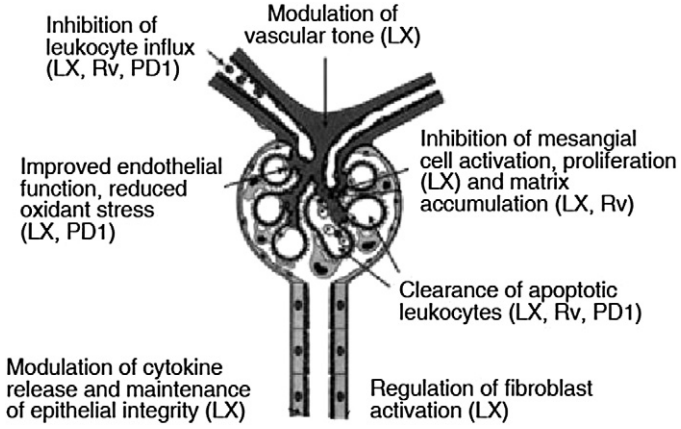

FIGURE 4.6 Lipoxins, resolvins and protectins: targets in renal inflammation, resolution and fibrosis. Multiple sites of action within the glomerulus are depicted including mesangial cells, macrophages and the endothelium. Tubular epithelia and fibroblasts are also targeted.

displays potent immunoregulatory [10 12] and neuroprotective actions [13,14,134], as well as promoting wound-healing capacity [63], being renoprotective [66] and being antifibrotic in kidney [65].

Mouse kidneys produce both D-series resolvins and PD1 in response to bilateral ischemia/reperfusion injury from endogenous sources of substrate. These mediators may play a role in protection against and resolution of acute kidney injury along with protection from eventual organ fibrosis [65]. Fish also biosynthesize both resolvins and protectins [135]. Hence, the resolvins and protectins possess anti-inflammatory actions, are log orders more potent than their precursors EPA and DHA, and are conserved structures in evolution. Their roles in fish, however, remain to be elucidated.

Zymosan-induced peritonitis is a self-resolving exudate and was used to identify anti-inflammatory and proresolving circuits. Administration of ATL analog, RvE1, or NPD1 in this system activated and/or accelerated resolution. The murine air pouch, a widely used model for assessing dermal inflammation, is a cavity lined by fibroblast-like and macrophage-like cells. Intrapouch administration of TNF-α initiates leukocyte infiltration by stimulating the release of chemokines and chemoattractants. In this system, RvE1 is produced in subnanogram amounts. In a TNF-α-induced air pouch dermal inflammation model, administration of 100 ng/mouse of synthetic RvE1 stopped leukocyte infiltration into inflammatory loci by 50 70%. For comparison, topical application of dexamethasone (10 μg/mouse) gave around 60% reduction, and aspirin (1.0 ng/mouse) gave 70% inhibition of leukocyte recruitment. Thus, RvE1 at nanomolar levels is as potent as higher doses of glucocorticoids or aspirin in stopping leukocyte trafficking into an inflammatory site.

In zymosan-induced murine peritonitis, RvE1 (100 ng/mouse) gives 50 60% inhibition of leukocyte infiltration compared to only 25% inhibition of infiltration with indomethacin (100 mg/mouse). Systemic administration of RvE1 dramatically attenuates leukocyte recruitment [50]. RvE2 shares anti-inflammatory actions with RvE1. Intravenous administration of 1 ng of RvE2 resulted in $11.3 \pm 4.8\%$ reduction of PMN infiltration. PMN infiltration was reduced by $17.7 \pm 9.9\%$ and $33.7 \pm 5.0\%$ at 10 and 100 ng, respectively. RvE2 was not significantly different from RvE1 at any of the doses since both significantly reduced PMN infiltration in zymosan-induced peritonitis. When given together, RvE1 and RvE2 had an additive anti-inflammatory impact. When administered intraperitoneally at a 10 ng dose per mouse, RvE2 reduced PMN infiltration by $34.5 \pm 4.5\%$. Unlike RvE2, RvE1 did not stop PMN recruitment when administered intraperitoneally [58].

D-series resolvins are DHA-derived local mediators and exhibit potent anti-inflammatory actions (Table 4.3). In a murine model of peritonitis, both RvD1 and AT-RvD1 showed a dose-dependent decrease in PMN infiltration into the inflammatory site. Maximal inhibition was approximately 35% and occurred at a 10 100 ng dose. RvD1 and AT-RvD1 are equally efficacious in decreasing total leukocyte infiltration, yet AT-RvD1 is statistically more potent than RvD1 at the 10 ng dose. After 4 h of incubation post intravenous administration of compound, the leukocytic infiltration was composed of approximately 70% PMNs and 30% monocytes. Since AT-RVD1 and RvD1 have similar actions on PMNs, the two may have different actions on monocytes [64].

In the setting of acute inflammation, intraperitoneal administration of 1 ng PD1/mouse reduces infiltration of PMNs 2 h after zymosan challenge. Once PD1 was administered, $> 90\%$ of PMN infiltration was blocked. When administered together, RvE1 and PD1 have additive effects. Differential counts on light microscopy revealed that PD1 and its chemical analog 15,16-dehydro-PD1 reduced PMN infiltration and increased nonphlogistic recruitment of monocytes and lymphocytes, all in the context of reducing inflammation and promoting resolution.

The 17*S* series resolvins and PD1 are potent inhibitors of TNF-α-induced leukocyte infiltration in the air pouch model of dermal inflammation via both topical and systemic application. The 17*S* series resolvins reduced PMN recruitment by $82.2 \pm 5.6\%$ when applied topically and by $49.6 \pm 8.2\%$ when given intravenously. Systemic treatment with the 17*S* series resolvins also reduced zymosan A-induced PMN recruitment to the peritoneum by 45.1 0.8%, which was equipotent to the 17*R* series' resolving value. PD1 and synthetic PD1 were also potent systemic inhibitors, reducing PMN trafficking into the exudates by approximately 40%. This was of similar potency to indomethacin, at essentially equal doses [11,12].

In a model of murine zymosan peritonitis, mice were injected intraperitoneally with ATLa, RvE1 or PD1 (300 ng/mouse). These compounds all reduce PMN traffic into cells. PD1 stands apart, displaying different kinetics. RvE1 and PD1 give maximal reduction at 12 h, while the actions of ATLa appear earlier, at around 4 h. Fluorescence-activated cell sorting (FACS) analysis demonstrated that these compounds, at 12 h, did not change the percentage of macrophage populations. As for the resolution indices, ATLa lowered Ψ_{max} without changing R_i or T_{max}. In contrast, RvE1 and PD1 initiated R_i at earlier time intervals. Furthermore, PD1 reduced the duration of R_i. As for their chemokine/cytokine regulation, ATLa reduced proinflammatory cytokines/chemokines [e.g. IL-6, TNF-β and others; see Ref. 33 for a complete list and proteomic analyses], most strikingly at 4 h, while RvE1 and PD1 had maximal

inhibitory actions at around 12 h. It is important to note that in vivo LX generation is at 2 4 h in this system, whereas PD1 levels peak at 12 h, coinciding with the time-points of their bioaction.

Lipoxins and resolvins each control key processes in inflammation in a wide range of experimental animal inflammatory diseases. Lipoxins are produced in gut mucosa and serve to limit persistent inflammation [85]. Patients with ulcerative colitis have low to absent synthesis of LXA_4 and lower mucosal 15-lipoxygenase-2 enzyme levels [85]. The third generation of LX analogs, 3-oxa-LXA_4 analogs, have potent oral efficacy [136] in promoting resolution of 2,4,6-trinitrobenzene sulfonic acid (TNBS)-induced colitis [114]. Mice sensitized to TNBS developed colitis characterized by severe wasting and bloody diarrhea. Treating mice with RvE1 (1 μg/mouse ~0.05 mg/kg) improved mortality. RvE1-treated mice experienced less weight loss and level of shortening of the colonic mucosa. Those treated with vehicle alone showed ulcerations and significant transmural infiltration with PMNs, monocytes and lymphocytes. For comparison, ATLa, which was proven to be protective against dextran sodium sulfate-induced colitis [137], is also protective in colitis. RvE1-treated mice had reduced leukocyte infiltration into the colon, as shown by lower levels of myeloperoxidase present. Serum levels of anti-TNBS immunoglobulin G (IgG) were decreased in the RvE1-treated mice, suggesting a decrease in antigen presentation and B-cell IgG production. Real-time polymerase chain reaction (PCR) showed that mice treated with RvE1 had a significant reduction in TNF-α, IL-12 p40, iNOS and COX-2. No significant effect was seen on expression of interferon-γ (INF-γ), IL-4 and IL-10 in this model. Murine ChemR23 mRNA was shown to be slightly increased in colons obtained from TNBS-treated mice [52,53].

Transgenic *fat-1* mice engineered to express the *Caenorhabditis elegans fat-1* gene that encodes an n-3 fatty acid desaturase are capable of producing n-3 PUFAs from n-6 PUFAs. The *fat-1* mice have a low ratio of n-6/n-3 fatty acids in their systems without the need for dietary interventions. Transgenic *fat-1* mice generate higher tissue levels of endogenous DHA and EPA that are converted to resolvins and protectins. These *fat-1* mice are protected from inflammation and show increased resistance to death from colitis [54]. Dextran sodium sulfate (DSS)-induced colitis is an inflammatory bowel disease model and is characterized by infiltration of inflammatory cells into the lamina propria, lymphoid hyperplasia, epithelial ulceration and focal crypt damage. Induction of DSS colitis resulted in weight loss, bloody stools and changes in general status. The *fat-1* mice showed significantly less weight loss and a delayed progression of diarrhea but no change in fecal bleeding compared to wild type. Using liquid chromatography-UV-MS-MS mediator informatics, RvE1, RvD3 and NPD1/PD1 were identified in colon of *fat-1* transgenics at physiologically active levels. In addition to these, PGE_3 and LTB_5 were also found in *fat-1* mice. The *fat-1* mice also had a decrease in NF-κB protein activity monitored by activated p65 protein and reduced TNF-αmRNA levels. iNOS and IL-1β were reduced in *fat-1* mice, mRNA levels of intestinal trefoil factor 3 (TFF3) were increased in *fat-1* mice, and mRNA levels of Toll-interacting protein (Tollip) were increased in *fat-1* transgenes compared to their wild-type littermates [54].

DHA is stored in synaptic terminals and neuronal plasma membranes. NPD1 is generated in retinal pigment epithelium (RPE) from DHA and protects RPE cells from oxidative stress-induced apoptosis and proinflammatory gene expression. Photoreceptor cell integrity depends on the RPE, and photoreceptor cell degeneration is a feature of retinal degenerative diseases such as retinitis pigmentosa and age-related macular degeneration. RPE cells undergoing oxidative stress generate NPD1 and counteract oxidative stress-induced apoptotic DNA damage in RPE by upregulating anti-apoptotic proteins Bcl-2 and Bcl-x (L), and decreasing proapoptotic Bax and Bad. In addition, NPD1 counteracts leukocyte infiltration and proinflammatory gene expression in brain ischemia reperfusion [138].

In Alzheimer's disease, hippocampal cornu ammonis region 1 has decreased levels of DHA and NPD1. This trend is not seen in the thalamus or occipital lobes of the same brain. AD-hippocampus has decreased expression of key enzymes in NPD1 biosynthesis such as phospholipase A_2 and 15-LOX. NPD1 represses Aβ42-triggered activation of proinflammatory genes and upregulates antiapoptotic genes *Bcl2*, *Bcl-xL* and *Bfl* (A1). Soluble amyloid precursor protein-α stimulates NPD1 biosynthesis from DHA. NPD1 promotes brain cell survival via the induction of antiapoptotic and neuroprotective gene-expression programs that suppress Aβ42-induced neurotoxicity [14].

CORNEAL DAMAGE: EPITHELIAL WOUND HEALING

LXA_4 and NPD1 have roles in wound healing distinct from their PMN-directed actions. Mouse cornea was shown to generate LXA_4 and NPD1. Topical application of LXA_4 or NPD1 (1 μg) increased the rate of re-epithelialization by about 75% and reduced the sequelae of thermal injury. Murine corneal epithelial removal induced neutrophil recruitment into the corneal stroma and increased levels of the proinflammatory chemokine KC (the murine equivalent of IL-8 in humans). Local treatment with LXA_4 or NPD1 increased PMNs in the

cornea yet decreased KC formation by approximately 60%. 12/15-LOX-deficient mice had defective corneal re-epithelialization, PMN recruitment and reduction in endogenous LXA_4 levels [63].

RENAL INFLAMMATION, RESOLUTION AND FIBROSIS

Inflammation is a feature of most renal pathology, and severe acute or chronic renal inflammation may lead to glomerulosclerosis, tubular atrophy, damage to renal vasculature fibrosis and eventual renal failure [139]. The pathogenesis is complex, but irrespective of the initial stimulus, infiltration of neutrophils followed by monocytes and lymphocytes is observed. Resolution of renal inflammation involves removal of apoptotic leukocytes and a change of the cytokine milieu from proinflammatory to anti-inflammatory coupled to restoration of vascular tone [139]. However, the mechanisms by which this occurs and may be subverted in disease are only beginning to be understood. There is growing evidence for a role for anti-inflammatory, proresolution lipid mediators in these processes as in other organs and diseases (see below). As will be discussed below, such mediators target multiple aspects of the inflammatory cascade: inhibiting activation of leukocytes and trafficking from the vasculature, promoting clearance of apoptotic cells, inhibiting the perpetuation of chemotactic signals for additional leukocyte recruitment and contributing to the maintenance of epithelial and mesangial cell integrity resulting in maintenance of kidney functionality and a net antifibrotic phenotype.

Acute glomerulonephritis represents an important cause of acute kidney injury (AKI) that if unresolved may result in chronic kidney disease (CKD). The current mainstay therapy for this disease is immunosuppression. Long-term maintenance treatment is often required with a risk of serious adverse effects. In this regard novel therapeutic strategies that would switch the cellular response from inflammation in favor of resolution mimicking and/or amplifying endogenous homeostatic drives are of great interest [140].

The first compelling experimental evidence for a role for LXs as counter-regulators in renal physiology came from investigations of the glomerular microcirculation, where it was observed that, in contrast to 5-LOX products [LTB_4, C_4 and D_4] generated during the acute inflammatory phase, LXA_4 increased plasma glomerular filtration rate (GFR) and plasma flow and decreased arteriolar resistance subsequent to intrarenal infusion of LXA_4 into the microcirculation. Furthermore, direct antagonism of LTD_4-induced hemodynamic changes by LXA_4 was also reported [141]. In the concanavalin A ferritin model of immune complex glomerulonephritis, treatment of rat neutrophils ex vivo with LXA_4 reduces their subsequent trafficking into inflamed glomeruli. The potential importance of transcellular generation of LXA_4 as a modulator of renal inflammation was demonstrated in a model of nephritis in p-selectin-deficient and wild-type mice, where p-selectin deficiency inhibited platelet endothelial and neutrophil endothelial interaction. The p-selectin mice exhibited exaggerated renal PMN influx and decreased LXA_4 production relative to wild-type mice [142]. Analogously, manipulation of renal 15-LOX expression by intrarenal delivery of 15-LOX cDNA in a rat model of glomerulonephritis was associated with increased LXA_4 production by the kidney and maintenance of renal function relative to non-transfected controls [90].

Modulation by LXs of key inflammatory mediators such as NF-κB, IL-6 and proliferative pathways including Akt and Jak-STAT indicate that LX may not only provide an effective therapy limiting tissue damage arising from inflammation but also modulate the process of renal fibrosis that may follow glomerulonephritis. Acute streptococcal glomerulonephritis typifies a self-resolving condition and it is noteworthy that in a recent investigation maximal LTB_4 levels were observed at the peak of the inflammatory response and then dissipated, whereas LXA_4 levels persisted during resolution and remained elevated. In this setting, increased LXA_4 was shown not only in leukocytes but also in the glomeruli, indicating both a systemic and a local proresolution effect [89].

Mesangial cell proliferation and activation is a key feature of renal inflammation. At a cellular level LXA_4 has been shown to modulate the proliferation of mesangial cells induced by cytokines and growth factors that have been implicated not only in glomerulonephritis but in other disorders such as the development of tubulointerstitial fibrosis [103,106,143]. LXA_4 inhibits LTD_4 binding to human mesangial cells [138]. In addition to acting as a partial antagonist at cysLT receptors, LXA_4 binds to ALX/FPR2 in human mesangial cells [100]. Intriguingly, LXA_4 inhibits PDGF-, EGF- and connective tissue growth factor (CTGF)-stimulated proliferation of mesangial cells independent of cysLT-mediated receptor transactivation [106,144]. Mesangial cell fibronectin and collagen production are critical components of glomerulosclerosis. LXA_4 counteracts PDGF-induced fibrosis related gene expression in mesangial cells and renal epithelia [104], suggesting that LXA_4 is an endogenous regulator of fibrosis and that mimetics may act as potential antifibrotic agents preventing growth factor-induced matrix production and the progression of renal disease. This hypothesis is further enhanced by evidence that LXA_4 modulates TNF-α-induced proliferation and cytokine release as well as CTGF-

mediated release of fractaline, monocyte chemotactic protein-1 (MCP-1) and regulated on activation, normal T cell expressed and secreted (RANTES) [106]. The mechanisms underlying LX inhibition of growth factor (PDGF, EGF)-induced mesangial cell proliferation and gene expression involve the ALX/FPR2 and dephosphorylation of specific phosphotyrosine residues of activated PDGF or EGF receptors [144]. In an antiThy1 model of glomerular nephritis, 15*R/S*-methyl-LXA_4 was shown to be protective against leukocyte influx and mesangial cell proliferation and activation [145]. The downstream consequences of mesangial cell activation include the production of proinflammatory cytokines that may recruit and/or activate local monocytes/macrophages and induce inflammatory responses of tubular epithelia.

Most renal diseases are characterized by macrophage infiltration, historically associated with inflammation and the pathogenesis of fibrosis. However, it is becoming increasingly appreciated that macrophage plasticity is an important determinant of the outcome of inflammation [146]. The classically activated M1 phenotype is associated with defensive inflammatory cytokine production, whereas the M2 phenotype is characterized by reparative responses such as phagocytic clearance of apoptotic leukocytes and production of anti-inflammatory mediators such as IL-10. Growing evidence suggests that there are numerous subtypes of macrophage with defined functions as regards host defense, wound healing and immune regulation [147]. In a murine model of adriamycin-induced nephropathy, it has been shown that manipulating macrophage ex vivo into either an M1 or M2 phenotype [on stimulation with lipopolysaccharide (LPS) or IL-4/IL-13, respectively] dictated renal injury (M1) or resolution and repair (M2) [148]. LXs have been shown to reprogram cytokine-primed macrophages with a classically activated M1 phenotype to an alternatively activated M2 phenotype [119]. Whether distinct macrophage phenotypes are seen in kidney subsequent to LX treatment in experimental models of diseases remains to be investigated. LX-stimulated phagocytosis of apoptotic leukocytes has been well described in vitro and in in vivo models of disease [105]. RvDs and PD1 also modulate macrophage recruitment and activity, inhibiting LPS-induced TNF-α production in bone marrow-derived macrophages in vitro, and furthermore reducing macrophage infiltration in an in vivo model of ischemia reperfusion injury (IRI) [65].

Renal fibrosis, or tissue scarring, is the final outcome of CKD that does not resolve, regardless of etiology, and features include glomerulosclerosis, tubulointerstitial fibrosis, inflammation and loss of parenchyma. Fibrosis in turn cause end-stage renal failure, which requires dialysis and eventually transplantation. Glomerular mesangial cells, interstitial fibroblasts and tubular epithelial cells are the major fibrogenic cell types, and it has been proposed that circulating fibrocytes may also be recruited [149].

In renal fibrosis, myofibroblastic activation of mesangial cells and fibroblasts tends to precede epithelial to mesenchymal transition (EMT). Renal myofibroblasts can be derived from resident fibroblasts, EMT, endothelial to mesenchymal transition (EndMT), bone marrow-derived cells or other mesenchymal cells [150]. Renal fibrosis is characterized by excessive accumulation of extracellular matrix, derived from myofibroblasts, mesangial cells and tubular epithelial cells that have undergone a phenotypic switch. The epithelial/mesenchymal cells, together with inflammatory cells and myofibroblasts, cause basement membrane degradation and migration into the interstitium. To crudely simulate this system, conditioned medium from primary human mesangial cells exposed to PDGF was added to cultured renal epithelia and the epithelia were observed to acquire a more fibroblast phenotype, whereas an attenuation of this response was observed with media from cells exposed to LXA_4 and PDGF [104]. LXs have been implicated to play a role in regulating EMT in human pulmonary epithelial cells [151] and ATL are protective in bleomycin-induced lung fibrosis [152]. As discussed below, evidence for a protective effect of resolvins and protectins in IRI demonstrates attenuation of renal damage, which may reflect modulation of leukocyte recruitment [65] and increased mesangial cell hemoxygenase-1 expression [66,129].

AKI following tubular insult is a common cause of renal dysfunction and mortality, arising as a consequence of sepsis, ischemia or radiocontrast. Therapy for such renal injury is limited, primarily supportive and does not alter the course of the acute renal injury or the increased risk of CKD that follows as a consequence. LXs are potential therapeutics in AKI, as they can influence a variety of relevant pathophysiological functions such as vascular tone, endothelial and epithelial cell injury and cytokine release, and leukocyte recruitment and clearance (see Fig. 4.6). Altered hemodynamic responses during AKI occur as a consequence of many causes, and there is evidence that this endothelial and vasomotor dysfunction may persist [153,154]. LX may improve such endothelial dysfunction, reducing oxidative stress and improving bioavailability of mediators such as NO and HO-1, which play an important role in AKI [155]. As described previously, LXA_4 opposes the vasoconstrictor properties of LTD_4 and attenuates LTD_4-triggered decrease in renal blood flow and this may, in turn, further protect the kidney by reducing activation of the renin angiotensin system and immune-mediated effects of angiotensin II.

The stable aspirin-triggered lipoxin analog, 15-epi-16-(FPhO)-LXA_4-Me, is protective in experimental murine

renal reperfusion injury in vivo [156]. Administration of the ATL analog, before ischemia, resulted in significant functional and morphological protection and attenuated chemokine and cytokine responses. Using a transcriptomic approach to explore the events that underlie this protective effect, it was found that treatment with this analog, before injury, modified the expression of many differentially expressed pathogenic mediators, including cytokines, growth factors, adhesion molecules and proteases [88]. This modulation of transcriptomic responses included many genes expressed by renal parenchymal cells and was not merely reflective of a reduced renal leukocyte recruitment. Of particular interest is the LX induction of suppressor of cytokine signaling-1 (SOCS-1) and SOCS-2 proteins and kidney injury molecule-1 (KIM-1) expression in this setting. SOCS proteins have been identified as inducible feedback inhibitors of cytokine receptors and have been shown to be of crucial importance for the limitation of inflammatory responses, modulating dendritic cell and macrophage function [157], and represent an important target for promoting resolution of inflammation. KIM-1 has recently come to prominence as a more reliable biomarker than serum creatinine in both human and animal models of AKI [158]. KIM-1 may represent not only an AKI prognostic marker but also an important target for therapeutic intervention. KIM-1 additionally confers on epithelial cells the ability to recognize and phagocytose dead cells that are present in the postischemic kidney and contribute to the obstruction of the tubular lumen characteristic of AKI. The importance of clearing apoptotic debris in promoting resolution of inflammation has been outlined earlier, and further exploration of potential LX induction of KIM-1 may have important implications for the development of therapeutics for AKI [159]. In a model of acute antiglomerular basement nephritis, this analog was also shown to be protective, significantly limiting PMN influx [160].

The potential roles of resolvins and protectins in murine renal IRI have been described. Results from studies with Duffield and colleagues established the important principle that resolvins (RvD1) and protectin (PD1) were formed during ischemic injury [65]. Bilateral renal ischemia followed by 24 h reperfusion triggers endogenous biosynthesis and release of the precursor DHA into circulation. Postischemic kidneys also generate PD1, 17*S*-HDHA and to a lesser extent RvD1 and RvD3. In the absence of added DHA, there was no increase in plasma levels of PD1 and RvD1, in contrast to kidney tissue, which demonstrated an increase even in the absence of DHA pretreatment, but there was still an increase in the levels of RvD2, RvD3 and RvD4. Postischemic kidneys showed increased levels of 17*S*-HDHA in both the vehicle group and the DHA-treated group. This suggests that the enzyme 15-LOX is induced in the setting of I/R. Exogenous addition of DHA to mice undergoing renal I/R increased the plasma and kidney tissue levels of RvD6 and RvD2. There was also an increase in the levels of 17*S*-HDHA, RvD1 and RvD3. The administration of DHA did not further increase RvD4 levels in plasma or kidney tissue.

Administration of exogenous RvDs or PD1 before bilateral renal ischemia was associated with reduced leukocytes (PMN and monocyte influx) and an attenuation of the increase in plasma creatinine indicative of renal injury. Treatment with RvD1 or synthetic RvD1 after ischemic injury also demonstrated a protective effect. In contrast, PD1 did not show significant protection after the onset of ischemic injury. Histological examination of tissue 48 h following ischemic injury demonstrated maintenance of tubular integrity in animals pretreated with RvD and PD1. In contrast to what has been described for LX in the maintenance of epithelial integrity, the authors discounted a direct effect of the agents on epithelia, as they were unable to show attenuation of oxidant-induced stress to cultured renal epithelia. However, this does not necessarily restrict the effects to leukocyte recruitment or activation, as endothelial and mesangial cells may also be affected. In these experiments, in addition to significantly reducing monocyte (CD68-positive) recruitment, both RvD and PD1 inhibited LPS-stimulated TNF-α production from rat bone marrow-derived macrophages (RvD was more efficacious than PD). Given the evidence that postinflammatory scarring of the kidney may play a major role in the development of CKD, the authors investigated whether RvD and PD1 altered renal fibrosis by investigating collagen deposition 15 days post-IRI. In animals treated with RvE but not in those treated with PD1, there was a significant decrease in fibrosis [65].

Further evidence for a protective role of PD1 in IRI is provided by Hassan and Gronert [66], who used dietary manipulation to prime endogenous generation of PD1 in murine IRI. In animals fed n-3 PUFAs versus n-6 PUFAs there was a marked attenuation in their resistance to renal IRI (plasma creatinine, PMN influx, inflammatory cytokine production) that was attributed to PD1 biosynthesis and HO-1 upregulation. Systemic treatment with PD1 generated similar effects to those observed with endogenously generated PD1.

PRORESOLVING THERAPEUTICS: AGONISTS OF RESOLUTION?

Additional studies are called for to determine the link between the beneficial roles of n-3 PUFAs relating to improved inflammatory states and the relation to protectins and resolvins. By identifying key players in resolution, promising new anti-inflammatory and

proresolving drugs may be designed [6] without having the notorious side-effects of COX-2 inhibitors [161] or anti-TNF therapies [162 164], and avoiding resolution toxicity [6,47 49]. In the era of COX-2 inhibitors [49,165] deciphering the mechanisms of acute inflammation and its resolution is crucial. An ideal anti-inflammatory and proresolving agent should be able: (i) to dampen the inflammatory response and reduce local tissue damage; (ii) to activate de novo resolution mechanisms; and (iii) not to compromise host defense to infectious disease (i.e. non-immunosuppressive).

CONCLUSION

Inflammation is a central part of renal pathology, and resolution of renal inflammation is vital for the return to normal kidney function following an injury. Evidence to date briefly summarized in this chapter indicates that a new era has emerged in the appreciation of acute inflammation and its progression to resolution, chronic inflammation or fibrosis. The lipoxins, resolvins and protectins each play an active role(s) in controlling and programming resolution of inflammation [9] by serving as agonists on their respective receptors stimulating endogenous anti-inflammatory and proresolving pathways [26]. Lipoxins and resolvins also attenuate nociception through a novel lipid mediator cascade regulating peripheral and spinal sensitization and hyperalgesia [37,166]. Given their potency, stereoselectivity and multiple targets for which they are endogenous regulators, mimicry or amplification of their bioactions may represent attractive targets for novel therapeutics. Hence, the mapping of resolution to uncovering the key players and signaling pathways involving the resolvins and protectins [33] may yield clues for new treatment modalities aiming to reverse and/or prevent the chronic inflammatory state, and possibly new therapeutic alternatives for a wide range of diseases [15].

Acknowledgments

We thank M. Halm Small for manuscript preparation. This study was supported, in part, by National Institutes of Health grant nos GM38765, DK074448 and DE019938 (C.N. Serhan) and the Health Research Board Ireland, Science Foundation Ireland and the EU FP6 Eicosanox Programme (C. Godson).

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CHAPTER

5

Tissue Protection and Regeneration Aided by Erythropoietin and Erythropoietin-derived Peptides

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OUTLINE

INTRODUCTION

In the hematopoietic system, the principal function of erythropoietin (EPO) is the regulation of red blood cell production in order to maintain tissue oxygenation and thus prevent tissue hypoxia. Consequently, following the cloning of the EPO gene [1 3], different recombinant human EPO (rHuEPO) forms (e.g. EPOetin-α and EPOetin-β), and their long-acting analogs darbepoetin-α and the continuous erythropoietin receptor activator (CERA) have been mainly used for treatment of anemia in patients with chronic kidney disease (CKD) and chemotherapy-induced anemia in cancer patients. However, recent experimental studies investigated the ability of exogenous EPO to modulate organ function and cellular responses to diverse types of injury. Thus, in addition to its essential role in the regulation of mammalian erythropoiesis, EPO signaling, activated either by exogenous EPO or by endogenous EPO in an autocrine or paracrine fashion, has emerged as a major tissue-protective survival factor in various non-hematopoietic organs. Indeed, numerous articles have been published on the effects of rHuEPO to prevent ischemia-induced tissue damage in several organs, e.g. brain, heart, liver, blood vessels and kidney (reviewed in [4 10]). In this chapter the focus will be on the effects of EPO and rHuEPO related to the field of nephrology, i.e. the effects on vascular and renal tissue.

TISSUE EXPRESSION OF ERYTHROPOIETIN AND THE ERYTHROPOIETIN RECEPTOR

Characterization of the non-erythropoietic biological effects of EPO and understanding the mechanisms of EPO EPO-receptor signaling activation in non-hematopoietic organs and cell types are critical to the

future development of novel applications for rHuEPO and its derivatives. The primary sites of EPO production reside in the fetal liver and adult kidney where EPO gene expression occurs under the tight control of an oxygen-sensing, hypoxia-inducible factor (HIF)-dependent mechanism [11 14]. In addition, recent studies could identify EPO expression in several extrarenal tissues and cell types, including astrocytes, neurons, the female genital tract, male reproductive organs, mammary glands, placental trophoblasts, bone marrow macrophages and erythroid progenitors [15 22]. The expression of EPO receptors in non-erythroid tissues such as the brain, retina, heart, kidney, smooth muscle cells, myoblasts and vascular endothelium [23 29] has been associated with the discovery of novel biological functions of endogenous EPO signaling in non-hematopoietic tissues. For example, targeted disruption of either EPO or EPO receptor in mice leads to in utero death because of a lack of definitive erythropoiesis in the fetal liver and defects in the onset of the circulatory system, indicating the vital role for EPO and EPO-receptor signaling during vascular development [30].

The EPO molecule is a glycoprotein with a molecular weight of 30.4 kDa, and its tertiary structure is defined by four antiparallel α-helices. Binding of a single molecule to two adjacent EPO receptors on the membrane of target cells leads to homodimerization of the EPO receptor and the triggering of different intracellular signaling cascades. The major mechanism for degradation of EPO in the body occurs in cells expressing the EPO receptor, through receptor-mediated endocytosis of EPO followed by degradation in lysosomes [31]. However, these signaling cascades are highly complex and their relevance for the cellular effects of EPO is not completely understood so far. For example, by using EPO-receptor fusion proteins, it has been shown that distinct conformations of the EPO receptor exist which may activate different intracellular pathways. Thus, which receptor conformation is achieved and which signaling pathway is subsequently activated may depend on the extracellular binding site of EPO [32,33]. Moreover, a physical association between the common β-receptor chain subunit (CD131) and the EPO receptor has been demonstrated by coimmunoprecipitation of the proteins in neuron-like cells. The tissue-protective effects of EPO appear to require the expression of CD131 and a low-affinity, heterodimeric EPO-receptor CD131 receptor, which may exert a different signaling behavior than the "classical" EPO receptor [34,35]. In addition, different classes of EPO receptor with respect to receptor affinity for EPO binding have been described: high (KD90 900 pM) and low (KD20 9000 pM)-affinity EPO receptor [36,37]. The former mediate the well-known hematopoietic effects, whereas the latter seem to be involved in tissue protection by EPO. Collectively, these data indicate that the interaction of EPO with its receptor may be far more complicated than previously believed.

The activated EPO receptor exhibits more than 40 binding sites, and a pivotal molecule that induces intracellular signaling is JAK2 tyrosine kinase. Activation of this kinase leads to tyrosine phosphorylation and dimerization of signal transducers and activators of transcription (STATs). The JAK2 STAT5 signaling pathway not only is responsible for the effects of EPO on red blood cell differentiation, proliferation and survival, but also can mediate protection against programmed cell death (apoptosis) [38,39]. It can therefore be anticipated that the JAK STAT5 pathway plays an important role in the tissue-protective properties of rHuEPO not related to anemia correction. Another important signaling pathway triggered by EPO is phosphatidylinositol 3-kinase (PI3K), which activates Akt (i.e. serine/threonine protein kinase B). EPO-induced activation of the PI3K Akt pathway [40,41] and subsequent inhibition of apoptosis seems to be imperative for tissue protection by rHuEPO, because prevention of Akt phosphorylation abolished the beneficial action of rHuEPO in settings of experimental cardiovascular and neuronal injury. Although all of the above pathways seem to be activated via the classical EPO receptor [42,43] it is currently unknown whether different ligands of the EPO receptor may induce different signaling pathways or whether the single EPO receptor chain associates and forms dimers with other membrane proteins. Theoretically, these novel receptors may be the target of newly designed rHuEPO compounds such as the carbamylated form of the hormone [35].

ERYTHROPOIETIN IN THE VASCULAR SYSTEM

Erythropoietin and the Endothelium

There is evidence that EPO is a major regulator of vascular formation (i.e. angiogenesis) and organ growth in the embryo, and EPO receptors have been found in almost every embryonic tissue [30,44]. Kertesz et al. [30] found both EPO and EPO receptors expressed in the vasculature during embryogenesis, and deletion of either in knockout animals leads to severe angiogenic defects resulting in an embryonic lethal phenotype. These angiogenesis defects can be partially rescued by expressing human EPO during embryogenesis. Furthermore, EPO receptors have been found also on mature endothelial cells, where direct biological effects of EPO have been described, e.g. stabilization of endothelial structures and vascular integrity such as cell cell and cell matrix contacts. Addition of rHuEPO to the culture

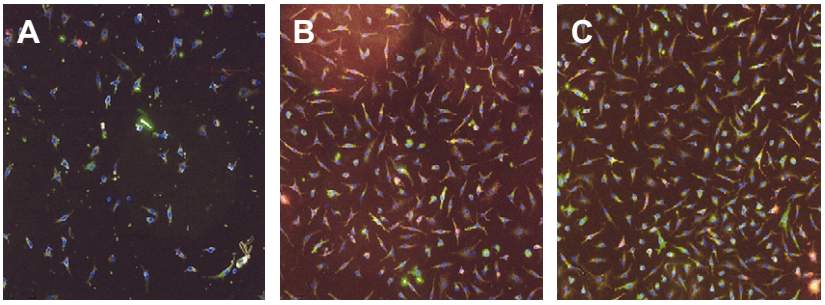

FIGURE 5.1 Endothelial progenitor cells (EPCs) in patients with chronic kidney disease (A) before and (B) after treatment with a standard dose of recombinant human erythropoietin (EPOetin beta). (C) For comparison, EPCs in a healthy control subject. Please see color plate at the end of the book.

medium increases endothelial cell proliferation and protects cells against ischemia and apoptosis [45–47]. The latter effect is thought to be mediated via the PI3K/Akt pathway [47,48]. Since mature endothelial cells obviously do not lose their EPO receptors, anti-apoptotic signaling could persist much longer than in erythrocytes thus rendering endothelial cells more resistant to ischemia-induced cell death. Moreover, mature human endothelial cell lines also respond to rHuEPO by differentiating into primitive vascular structures [49,50]. It is therefore tempting to speculate that EPO preserves its role as a key regulator of vascular protection and even vascular formation in the adult organism.

Recently, activation of endothelial nitric oxide synthase (eNOS) by EPO has been identified as an important mechanism in how EPO affects endothelial cells [51,52]. d'Uscio et al. [52] used a murine model of wire-induced injury of the carotid artery to examine the effect of rHuEPO (1000 IU/kg body weight s.c.) on endothelial repair in wild-type and eNOS-deficient mice. They studied the reactivity and vascular structure of isolated carotid arteries in vitro. The injured arteries exhibited impairment of endothelium-dependent relaxation to acetylcholine, and this was associated with an increase in medial cross-sectional area. In wild-type mice treatment with rHuEPO upregulated expression of eNOS, normalized the vasodilator response to acetylcholine and prevented the injury-induced increase in medial cross-sectional area. These protective effects were not only abolished in eNOS-deficient mice; the authors even observed a significant increase in systolic blood pressure and enhanced medial thickening of injured carotid arteries in these animals. These results show that the vasculoprotective effects of EPO may critically depend on the activation of eNOS, and point to the fact that a functionally active eNOS is crucial for the action of EPO at the vascular level.

Erythropoietin and Endothelial Progenitor Cells

Another aspect of EPO's action on the endothelium is its stimulating effect on endothelial progenitor cells (EPCs). These cells promote endothelial regeneration and orchestrate vascular reparative processes with secretion of proangiogenic cytokines [53]. Findings in patients with CKD together with results from animal and cell culture experiments permit the conclusion that EPO is a potent regulator of EPC proliferation and differentiation (Fig. 5.1), and this effect is mediated, at least in part, via Akt activation [54–56]. In addition, activation of eNOS in EPCs may contribute to EPO's effects in endothelial regeneration, since recent work demonstrated that nitric oxide (NO) production is a key feature of EPCs that is required for endothelial repair [57]. For example, Urao et al. [58] investigated the role of rHuEPO in EPC mobilization and repair of injured endothelium in a wire injury model of the femoral artery in mice. Administration of rHuEPO inhibited neointimal formation and significantly increased the re-endothelialized area. The authors also showed that rHuEPO induced AKT phosphorylation and stimulation of eNOS in EPCs. These results were recently confirmed in a model of postmyocardial infarction heart failure, where rHuEPO-induced neovascularization was also mediated through a combination of EPC recruitment from the bone marrow and improved EPC engrafting [59]. Collectively, these data support the hypothesis that EPO is a key molecule in the process of endothelial (vascular) repair. This is also of importance for physicians caring for patients with CKD, in whom reduced numbers and/or function of EPCs was documented in numerous studies, and possibly contributes to their high cardiovascular morbidity [60]. Moreover, it is plausible that deficient EPC function may hamper vascular and thus tissue repair and regeneration in the kidney as a result of acute or chronic damage such as diabetes or hypertension.

ERYTHROPOIETIN AND THE KIDNEY

Erythropoietin and Acute Kidney Injury

EPO receptors have been found in vascular as well as non-vascular kidney tissue, and it has been shown that rHuEPO activates different survival intracellular pathways in kidney tissue, such as the PI3K/Akt pathway [61]. Moreover, numerous studies have revealed that

administration of rHuEPO protected tissue and whole-organ function in various experimental settings of acute kidney injury (AKI), such as ischemia reperfusion or toxic injury [62 73]. These investigations unequivocally documented that treatment with even a single (high) dose of rHuEPO ameliorates kidney dysfunction by reducing apoptotic cell death in different renal tissue compartments (Table 5.1). In addition, rHuEPO could theoretically increase the local NO bioavailability through stimulation of eNOS and prevent renal vessel injury [51,74]. However, as already discussed, the EPO-mediated increase in vascular NO bioavailability was only seen in intact vessels, whereas administration of rHuEPO provoked vasoconstriction in injured arteries [52].

The encouraging experimental results have certainly prepared the ground for studies exploring the therapeutic potential of rHuEPO in humans with AKI, but before that some safety issues must be resolved. In particular, the dose-dependent effects on the number and activation state of thrombocytes and the stimulation of platelet adherence to (injured) endothelium could mitigate the beneficial effects of rHuEPO on damaged renal (vascular) tissue [75 77]. Recent experimental work revealed that darbepoetin-induced erythropoiesis with increased hematocrit levels is not associated with an increased risk for thrombosis as long as endothelial NO production in intact vessels serves as a compensatory mechanism [78]. In other words, with preserved (local) NO generation, the beneficial vascular actions of EPO prevail over potential adverse effects. Unfortunately, this is almost never the case in the clinical situation, particularly in renal patients in whom NO bioavailability is known to be significantly reduced [79].

It is conceivable, however, that administration of a single high dose or a few repetitive doses of rHuEPO (to avoid the increase in hematocrit levels) to prevent acute ischemic injury may be a promising approach to limit loss of kidney tissue in patients with AKI. In this respect, a small study in patients after coronary bypass grafting has provided encouraging preliminary results [80]. However, data from a prospective multicentre study from France in patients receiving a cadaveric kidney transplant have not revealed a significant effect

TABLE 5.1 In Vivo Studies on Acute Protective Effects of Recombinant Human Erythropoietin (rHuEPO) on the Kidney

Species (ref.)	Experimental model of acute kidney injury	Dose, route and time of application	Mechanism(s) of tissue protection
Rat [61]	Cisplatin nephrotoxicity	100 U/kg rHuEPO i.p. daily for 9 days	Increased regeneration of renal tubular cells
Rat [62]	Ischemia–reperfusion	3000 U/kg rHuEPO i.v. 24 h before ischemia	Heat shock protein 70 activation, reduced caspase activity (reduced apoptosis)
Rat [63]	Hemorrhagic shock	300 U/kg rHuEPO i.v. before resuscitation	Reduced caspase activity (reduced apoptosis)
Rat [64]	Ischemia–reperfusion	200 U/kg rHuEPO i.p. before ischemia, 6 and 24 h after reperfusion, thereafter daily	Reduced downregulation of renal aquaporins (water channels) and sodium transporters
Rat [65]	Ischemia–reperfusion	300 U/kg rHuEPO i.v. 30 min before ischemia, 5 min before reperfusion or 30 min after ischemia	Reduced caspase activity (reduced apoptosis)
Rat [66]	Ischemia–reperfusion	5000 U/kg rHuEPO i.p. 30 min before ischemia	Reduced apoptosis, increased regeneration of renal tubular cells
Mouse [67]	Ischemia–reperfusion	1000 U/kg rHuEPO s.c. for 3 days before ischemia or 5 min before reperfusion	Reduced oxidative stress and lipid peroxidation
Rat [68]	Ischemia–reperfusion	5000 U/kg rHuEPO or 25 μg/kg darbepoetin α i.p. immediately after ischemia or 6 h after ischemia	Reduced apoptosis, increased regeneration of renal tubular cells
Rat [69]	Ischemia–reperfusion	500 U/kg rHuEPO i.p. 20 min prior to ischemia–reperfusion	Reduced apoptosis and inflammation
Rat [70]	Radiocontrast injury + nitric oxide inhibition	3000 U/kg and 600 U/kg rHuEPO i.v. 24 and 2 h before administration of iothalamate + indomethacin	Not reported
Rat [71]	Cisplatin nephrotoxicity	5000 U/kg rHuEPO i.v. before injection of cisplatin	Reduced apoptosis, preserved renal perfusion

i.v.: intravenously; i.p.: intraperitoneally; s.c.: subcutaneously.

of high-dose rHuEPO treatment on the ischemia reperfusion injury after kidney transplantation [81]. In comparison with placebo treatment, administration of EPOetin-β 4 × 30,000 IU i.v. immediately after, 12 h, 7 and 14 days after transplantation did not reduce the incidence of primary kidney graft non-function or need for dialysis. Thus, further studies are needed to explore the potential of rHuEPO to prevent or ameliorate AKI in humans.

Renoprotection by rHuEPO in Chronic Kidney Disease

Even more important than prevention of AKI may be renoprotection by rHuEPO in settings of CKD, that is, for prevention of progression of primary as well as secondary kidney diseases such as diabetic nephropathy. So far, therapeutic efforts in CKD patients have been made only to correct anemia and the putative hypoxic renal tissue damage as a result of anemia [82]. Whereas results of smaller randomized studies suggested that anemia correction with rHuEPO could slow progression [83–85], data from recently published large trials in patients with CKD revealed no beneficial effect on progression [86–88]. However, in later studies, patients with advanced CKD have been studied, and (almost) complete correction of anemia was accomplished within weeks using relatively high doses of rHuEPO; the initial weekly starting dose in the CHOIR study was 10,000 IU of rHuEPO-α [87]. Such an abrupt increase in the hematocrit level in patients with serious vascular problems might have mitigated putative beneficial effects of rHuEPO on the kidney, particularly if one assumes that much lower doses could be adequate for tissue protection.

A hematologically non-effective dose of the long-acting rHuEPO analog darbepoetin (i.e. a dose that did not affect hematocrit levels in treated animals) was used in the established 5/6 nephrectomy remnant kidney model in the rat [89]. This model features progressive injury to the renal microvascular endothelium leading to glomerular sclerosis and ischemia-induced tubulointerstitial damage. It was demonstrated that chronic treatment with darbepoetin conferred renal vascular and tissue protection and preserved renal function in the 5/6 nephrectomized animals. This culminated in significant better survival compared with saline-treated animals (Fig. 5.2). In this experimental setting of CKD, persistent activation of the Akt pathway in endothelial and glomerular epithelial cells was found, along with reduced apoptotic cell death in renal tissue. Further, it was shown that escalating doses of darbepoetin mitigate the protective effects on the remnant kidney tissue and even worsen microvascular renal injury, that is glomerulosclerosis [75]. These

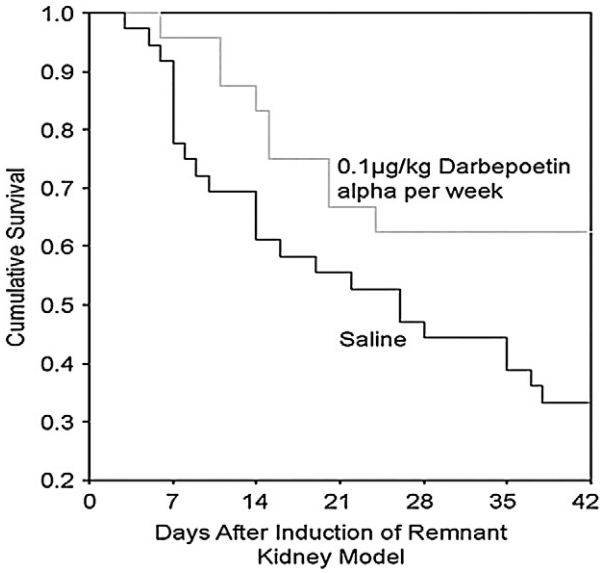

FIGURE 5.2 Kaplan–Meier survival curves of remnant kidney rats treated either with saline or 0.1 µg/kg body weight darbepoetin α. Survival of darbepoetin treated animals (63%) was significantly better ($p < 0.05$) than in the saline treated group (33%). *[Reproduced from Bahlmann et al., 2004 [89].]*

findings were expanded in experimental models of diabetic (db/db mouse), toxic and immunological kidney injury [90–93]. Chronic administration of CERA had beneficial dose-dependent effects on molecular pathways of diabetic kidney damage [90]. However, only the non-hematologically effective (low) dose was also clinically renoprotective, whereas high-dose CERA aggravated albuminuria in this experimental setting despite clear-cut beneficial molecular effects. Moreover, phlebotomy in high-dose CERA-treated mice preserved its tissue-protective effect. These experimental observations could be of considerable clinical relevance, because low-dose rHuEPO treatment may be a safe strategy to avoid potential adverse effects of high-dose therapy, i.e. doses that cause an increase in hematocrit with accompanying changes in rheology and activate thrombocytes. Thus, earlier administration of rather low doses of rHuEPO or analogs may be a feasible way to limit renal tissue damage in patients with CKD.

Similar results were obtained in other experimental models of CKD. For example, Eto et al. [91] examined the mechanisms by which darbepoetin confers renoprotection in a puromycin aminonucleoside-induced model of nephrotic syndrome in the rat. They found that darbepoetin decreased proteinuria in these animals. This protective effect was correlated with the immunohistochemical disappearance of the podocyte injury marker desmin and the immune costimulator molecule B7.1 with the reappearance of nephrin expression in the slit diaphragm. Podocyte foot process retraction and effacement, along with actin filament rearrangement, were all

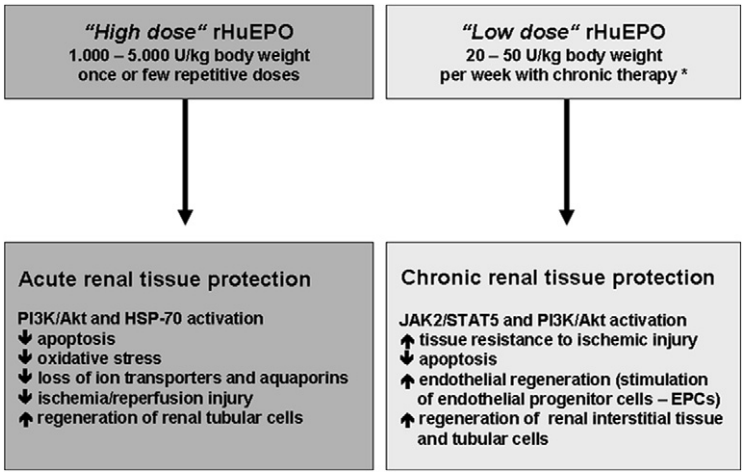

FIGURE 5.3 Established and hypothetical renoprotective mechanisms, and indications for high and low dose therapy with recombinant human erythropoietin (rHuEPO) in clinical settings of acute and chronic kidney injury (alternatively: 0.1–0.3 μg/kg body weight darbepoetin α per week or 0.2–0.6 μg/kg body weight continuous erythropoietin receptor activator once monthly).

reversed by darbepoetin treatment. Furthermore, puromycin treatment of rat podocytes in culture caused actin cytoskeletal reorganization along with deranged nephrin distribution. All these effects in vitro were reversed by darbepoetin. The same authors also investigated the role of rHuEPO in the regulation of heme oxygenase-1, an antioxidative stress protein, in Dahl salt-sensitive rats with low-salt diet, i.e. in a model of chronic tubulointerstitial injury [92]. In this model chronic rHuEPO treatment reduced proteinuria and renal injury, including peritubular capillaries rarefaction. This renoprotection was associated with upregulation of heme oxygenase-1 in kidney tissue. Finally, Logar et al. [93] tested the effects of darbepoetin in preventing podocyte apoptosis in cultured immortalized mouse podocytes treated with low-dose ultraviolet C irradiation to induce apoptosis. Darbepoetin pretreatment significantly reduced podocyte apoptosis, with this effect involving intact JAK2 and Akt signaling pathways. Moreover, in mice with antiglomerular antibody-induced glomerulonephritis chronic darbepoetin treatment significantly reduced podocyte apoptosis, glomerulosclerosis and proteinuria.

Presumably, establishing a minimal effective dose in future studies will allow better cost benefit estimates of rHuEPO treatment in the setting of CKD. Moreover, rHuEPO analogs that maintain tissue-protective effects but are devoid of the action on erythropoiesis (and possibly thrombopoiesis) may represent a valuable alternative. Such molecules, such as the carbamylated form of the hormone, have already been tested in experimental studies, revealing tissue-protective properties comparable to those of classic rHuEPO, but without any effect on hematocrit or on procoagulant activity [94]. The potential of such rHuEPO analogs to prevent loss of renal tissue in patients with CKD awaits further investigation.

CONCLUSION

There is accumulating evidence that the renal therapeutic benefits of rHuEPO go above and beyond ameliorating anemia and accompanying tissue hypoxia. In addition to the well-documented antiapoptotic effect of rHuEPO, stimulation of regenerative cells may play a role in tissue protection/regeneration in CKD patients. It is therefore conceivable that therapy with rHuEPO, whether acute high dose or (early) chronic low dose, may be a feasible way to prevent AKI and/or retard progressive CKD (Fig. 5.3).

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CHAPTER

6

Mast Cells in Kidney Regeneration

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OUTLINE

INTRODUCTION

Chronic kidney disease (CKD) is a growing health problem worldwide as it leads to the development of renal failure with no treatment options except for replacement therapy [1]. CKD can be caused by disorders that affect any of the kidney structures, including glomeruli, renal vessels and the tubulointerstitial compartment. They are either genetically inherited, e.g. autosomal dominant polycystic kidney disease, or acquired. The latter can arise in response to toxic, metabolic (diabetes), hemodynamic (hypertension), postischemic, infectious or autoimmune injury leading to damage of renal tissue. Independent of its origin, the initial injury launches an inflammatory cascade to oppose the injurious insult and initiate tissue repair with eventually limited fibrosis and epithelial regeneration. However, in case of chronic stimulation or defective regulation, the disease will enter into a progressive phase characterized by the development of extensive renal fibrosis with destruction of individual nephrons and blood vessels. Two-thirds of the nephrons will already have been destroyed before CKD

is diagnosed, and from then on the damage progresses to end-stage renal failure regardless of the original cause of injury. The relentless decline in renal function can be slowed by therapies based on converting enzyme inhibitors or angiotensin II (Ang II) receptor type-1 antagonists but they are only partially effective [2]. To date, replacement therapy by either dialysis or transplantation remains the only option for supportive therapy once the ultimate renal failure occurs. Therefore, strategies aiming to shift the balance towards repair and kidney regenerative processes are necessary in order to develop more effective and specific treatments for progressive renal failure and to halt the ever-increasing number of people requiring dialysis and transplantation. Importantly, researchers have now obtained a more subtle view of the inflammatory process, realizing that it represents a highly dynamic entity aiming to fight the injurious insult and to contribute to tissue repair and resolution of the inflammation. Therapeutic strategies must therefore be designed to reinforce these positive regulatory actions while suppressing defective inflammatory mechanisms.

An essential characteristic of progressive renal diseases is the presence of an interstitial infiltrate of leukocytes, which is thought to play a pivotal role in the inherent inflammatory process. Initially thought as being contributory to disease progression, recent data have made it clear that these infiltrating cells also have a variety of positive actions enabling the organism to reduce tissue injury and achieve regeneration. Besides macrophages and T cells, the infiltrate also includes mast cells (MCs), suggesting their functional implication. MCs are of hematopoietic origin. Their precursors, after a short passage in circulation, migrate into tissues where they complete their final maturation under the influence of stem cell factor (SCF) and a variety of other cytokines and growth factors [interleukin (IL)-3, IL-4, IL-9, nerve growth factor (NGF)] [3,4]. They represent a heterogeneous population of cells that can be found in virtually every tissue of the human body, including the kidney [5 7]. While initially studied for their role in allergies, research in the past two decades has revealed that MCs are highly versatile effector cells with multiple roles in innate and adaptive immunity as well as in the inflammatory processes [4,6,7]. Although initially thought to contribute to the pathogenesis of renal diseases, several recent experimental evidences using MC-deficient mice favor a more subtle view. They indicated that MCs can also have beneficial roles in the reparation and tissue remodeling processes that accompany healing. This chapter will review the knowledge that has accumulated on the role of MCs in renal disease, with a particular emphasis on their roles in repair and regeneration.

BASIC BIOLOGY OF MAST CELLS

Origin, Tissue Distribution and Heterogeneity of Mast Cells

MCs were originally described by the German immunologist Paul Ehrlich, who noticed in various connective tissues the presence of cells with metachromatic behavior, i.e. the property to change the color of the applied aniline dyes [6,7]. He named them "Mastzellen", meaning "well-fed", cells owing to the presence of numerous cytoplasmic granules, which he thought to contain phagocytosed material or nutrients (Fig. 6.1). Although many questions remain regarding their differentiation, MCs are of hematopoietic origin, arising from a $CD34^+$ and $CD117^+$ (c-kit) stem cell in the bone marrow [8 10]. After a short passage in blood, committed MC progenitors migrate into tissues where they undergo the final stages of their differentiation under the influence of various cytokines and growth factors. The main survival and developmental factor is SCF, also known as kit-ligand. This is exemplified by the virtual absence of MCs in mice carrying natural mutations in either SCF ($WCB6F_1$-$Kitl^{Sl}/Kitl^{Sl\ d}$ mice) or its receptor c-kit (WBB6F1-$Kit^W/Kit^{W\ v}$ or C57BL/6-$Kit^{W\ sh}/Kit^{W\ sh}$ mice) [7]. However, a series of other cytokines and growth factors may additionally be important in influencing the phenotype and number of MCs, including IL-3, IL-4, IL-9, NGF and transforming growth factor-β_1 (TGF-β_1) [7,11]. Murine bone marrow-derived mast cells (BMMCs) can be cultured in the presence of IL-3 alone, although addition of SCF allows a more mature phenotype to be obtained. By contrast, for growth of human primary MCs from $CD34^+$ stem cells in cord or peripheral blood SCF is absolutely required. MCs are predominant in tissues facing the external environment such as the skin, the airways and the intestine, but can be found in virtually every vascularized tissue. While their numbers may be low in certain types of tissues, e.g. in the kidney, they can increase markedly under chronic inflammatory conditions.

MCs are phylogenetically old cells, which apparently occur in all species containing vascularized tissues [6,7,10]. They are round or elongated cells with a diameter ranging between 10 and 20 μm containing a single nucleus and numerous (up to 1000) secretory granules in their cytoplasm storing histamine (also serotonin in rodents), proteoglycans, cytokines and neutral serine proteases (Fig. 6.1). MC populations are heterogeneous and can be divided into specific subpopulation of cells initially discovered by their differential staining properties with various histochemical dyes, which is mainly based on their differential content in proteoglycans [12]. Thus, rodent MCs were classified into connective tissue type mast cells (CTMCs) and mucosal mast cells (MMCs). By contrast, human MCs were classified

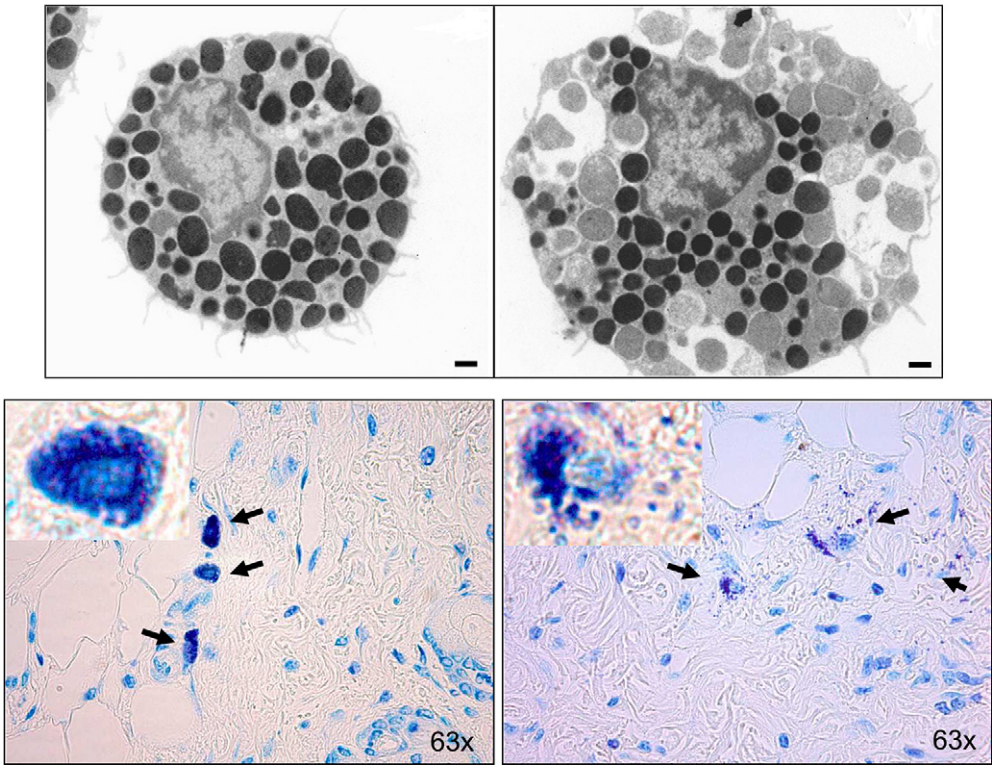

FIGURE 6.1 Resting and stimulated mast cells (MCs). The top panel shows electron micrographs of a resting (left) and stimulated (right) rat peritoneal MC. Scale bar 1μm. (Courtesy of G. Zabucchi and Maria Rosa Soranzo, University of Trieste, Italy.) Resting cells contain numerous cytoplasmic secretory granules that are filled with electron dense material. Stimulated cells have undergone partial exocytosis of granular content by a process implying granule−granule and granule plasma membrane fusion. The bottom panel shows granulated and degranulated mouse connective tissue type MCs in kidney capsules stained with toluidine blue. Note that in degranulated cells, the expelled toluidine blue staining granular matrix can be seen in the tissue surrounding the MC. Inset shows higher magnification (100×) of one of the MCs. Please see color plate at the end of the book.

according to their protease content into tryptase-positive mast cells (MC_T) and tryptase/chymase-positive mast cells (MC_{TC}). However, even within these defined cell populations additional phenotypic differences may exist relating, for example, to protease content. Hence, in human kidney a third subpopulation of chymase-positive MCs has been described (see below) [5].

In addition to the phenotypical heterogeneity the two classes of MCs are further characterized by a functional heterogeneity that can be attributed to their differential mediator content and differential receptor expression. For example, murine MMCs lack tryptase-dependent biological functions. They also have biochemical differences, MMCs being low in histamine and high in activation-elicited cysteinyl leukotriene (LTC) production, while CTMCs have a high histamine content and generating the prostanoid prostaglandin D_2 (PGD_2) in marked preference to LTC. Furthermore, MMCs are also unable to respond to polybasic compounds and complement-activated mechanisms owing to the lack of expression of various G-protein activated receptors including, for example, C3a and C5a receptors for complement fragments. It is likely that additional phenotypic variations occur at the different anatomical sites where they reside, which may depend on the presence of local growth factors and cytokines.

Mast Cell Mediators

Following activation, MCs produce three main classes of mediators (Table 6.1). The first class is contained in secretory granules that are filled with products often ionically bound in preactivated form to the matrix of proteoglycans such as the negatively charged heparin and chondroitin sulfates. They include vasoactive amines such as histamine and serotonin (in rodents only), a number of neutral proteases as well as many lysosomal enzymes such as acid hydrolases [6,8,13]. In rodents, CTMCs express two tryptases (mMCP-6, 7 in mice or rMCP-6, 7 in rats), one β-chymase (mMCP-4 or rMCP-1) and one α-chymase (mMCP-5 or rMCP-5) as well as mast cell-carboxypeptidase A (MC-CPA). Additional phenotypic differences depending on the tissue may exist, as exemplified by the expression of mMCP-9 chymase in MCs of the mouse uterus. MMCs contain two β-chymases (mMCP1 and mMCP-2), but do not

TABLE 6.1 Mediators Secreted by Mast Cells

Type of mediator	Mediator
Preformed in cytoplasmic granules	
Biogenic amines	Histamine, serotonin (in rodents)
Proteoglycans	Heparin and/or chondroitin sulfates
Tryptases	α/β Tryptases, hTMT (human); mMCP 6, 7, 11 (mouse); rMCP 6, 7, 11 (rat)
Chymases	Chymase 1 (human); mMCP 1, 2, 4, 5, 9 (mouse); rMCP 1, 2, 4, 5, 8, 9; hMC CPA (human); mMC CPA (mouse); rMC CPA (rat)
Lysosomal proteases	Cathepsin D, C and E
Lysosomal acid hydrolases	β Hexosaminidase, α glucuronidase
Cytokines	TNF, IL 4, TGF β_1
Matrix metalloproteases	MMP 9
Angiotensinogenase	Renin
Newly synthesized	
Lipid derived	PGD_2, PGE_2, LTB_4, LTC_4, PAF
Cytokines/growth factors	GM CSF, IFN α, IFN β, IFN γ, IL 1α, IL 1β, IL 2, IL3 6, IL9 10, IL 11, IL 12–13, IL 14–15, IL 16, IL 17, IL 18, IL 22, LTβ, M CSF, MIF, SCF, TGF β_1, TNF, EGF, VEGF
Chemokines	CCL 1 (TCA 3), CCL 2 (MCP 1), CCL 3 (MIP 1α), CCL 4 (MIP 1β), CCL 5 (RANTES), CCL 7 (MCP 3), CCL8 (MCP 2), CCL 11 (Eotaxin), CCL 13 MCP 4), CCL 16 (LEC), CCL 17 (TARC), CCL 20 (LARC), CCL 22 (MDC), CXCL 1 (Kc), CXCL2 (MIP 2α), CXCL3 (MIP 2β), CXCL 8 (IL 8), CXCL 10 (IP 10), CXCL 11 (IP 9)
Free radicals	Nitric oxide, superoxide
Blood clotting system	tPA (constitutive), PAI 1 (upon stimulation)

h: human; m: murine; TMT: transmembrane tryptase; MCP: monocyte chemotactic protein; TNF: tumor necrosis factor; IL: interleukin; TGF: transforming growth factor; MMP: matrix metalloproteinase; PG: prostaglandin; LT: leukotriene; PAF: platelet activating factor; GM-CSF: granulocyte macrophage colony-stimulating factor; IFN: interferon; M-CSF: macrophage colony-stimulating factor; MIF: macrophage inhibition factor; SCF: stem cell factor; EGF: epidermal growth factor; VEGF: vascular endothelial growth factor; CCL: chemokine (C C motif) ligand; TCA: T-cell activation; MIP: major intrinsic protein; RANTES: regulated on activation, normal T cell expressed and secreted; LEC: liver-expressed chemokine; TARC: thymus and activation-regulated chemokine; LARC: liver and activation-regulated chemokine; MDC: macrophage-derived chemokine; CXCL: chemokine (C X C motif) ligand; Kc: keratinocyte chemoattractant; IP: interferon-inducible protein; tPA: tissue plasminogen activator; PAI: plasminogen activator inhibitor.
Adapted from Marshall [4], Pejler et al. [13] and Galli et al. [18].

express tryptases or MC-CPA. Similarly, rat MCs do not express tryptases in MMCs, but contain a large variety of chymases (rMCP-2, 3, 4, 8, 9, 10). Human MC_T express αβ-tryptase, while MC_{TC} express both tryptase and the unique α-chymase present in humans. Following release, the change from acid to neutral pH promotes the dissociation of the various substances from the proteoglycan package, albeit at different rates, histamine being released most rapidly. Some products, such as chymase, are released more slowly as it is a highly charged protein, which sticks tightly to the proteoglycan matrix. Its activity partly depends on this association. The proteoglycan content of MC granules also varies in the different MC subtypes. In rodents CTMCs contain heparin, which is not present in MMCs. Conversely, MMCs express chondroitin sulfates E or diB, which are not found in CTMCs [14]. The dominant proteoglycan in human MCs is heparin, which constitutes about 75% of the total, with a mixture of chondroitin sulfates making up the remainder [15]. In humans, the heparin content in MC_T and MC_{TC} is roughly the same. Mice that lack the ability to produce proteoglycans show severe defects in the granule structure of MCs, with reduced content of histamine and impaired storage of certain proteases.

The second class of mediators comprises arachidonic acid metabolites that are de novo generated via either the cyclooxygenase or the lipoxygenase pathway [16]. Cyclooxygenase-dependent products include prostaglandins, in particular, PGD_2. Lipoxygenase-dependent products include LTB_4 and the cysteinyl leukotrienes LTC_4 and its conversion products LTD_4 and LTE_4. All of these lipid compounds have pleiotropic and potent inflammatory actions. For example, PGD_2 represents a potent chemotactic agent for leukocytes (eosinophils, basophils) as well as Th2 and epithelial cell activation via G-protein-coupled DP receptors (DP1 and DP2). It promotes arrest of dendritic cell migration (DP1 receptor). It also elicits bronchoconstriction at about 10-fold lower concentration than histamine via a still unknown receptor and enhances vascular permeability. PGD_2-targeting specific pharmacological inhibitors constitute a promising new strategy for the treatment of asthma [17]. Contrary to the proinflammatory function of PGD_2, its in vivo metabolite 15d-PGJ2 binding to peroxisome proliferator-activated receptor may also participate in the resolution of inflammation by inducing apoptosis, first of infiltrating neutrophils and later of the macrophages recruited to clear these apoptotic cells, thereby supporting the clearance of the inflammatory infiltrate [16]. Cysteinyl leukotrienes via binding to G-protein-coupled CysLT1 and CysLT2 receptors stimulate prolonged constriction of human bronchi and pulmonary smooth muscle cells. They also enhance vascular permeability, promote bronchial

mucus secretion and induce constrictions of arterial, arteriolar and intestinal smooth muscles. LTB_4 acts through G-protein-coupled leukotriene B_4 receptors (BLT1 and BLT2) and may also have some effect on the microvasculature, but was reported to be an important inducer of leukocyte (eosinophil, neutrophil, MC progenitor) chemotaxis, an action that is shared with cysteinyl leukotrienes [16]. In addition, LTB_4 was recently reported to recruit memory $CD8^+$ and $CD4^+$ T lymphocytes to inflammatory sites. Certain types of MCs (CTMCs) also produce the lipid-derived platelet-activating factor (PAF) that acts as a potent activator of platelet aggregation and degranulation. It also increases airway resistance and causes systemic hypotension, suggesting a role for this mediator in anaphylaxis.

The third class of biologically active products secreted by MCs includes a large number of cytokines, chemokines and growth factors [6,7,18]. Some of them are prestored, such as tumor necrosis factor (TNF), TGF-β_1 or IL-4, and ready for secretion and immediate biological action. However, the large majority are newly synthesized and released within a few hours of stimulation. These products participate in a large variety of biological functions. Many of them have proinflammatory functions either by directly inducing responses in tissue resident cells or other inflammatory cells through the interaction with cytokine receptors, and/or by attracting other types of inflammatory cells (chemokines), and/or by promoting the proliferation of inflammatory cells within tissues (growth factors). Some of them, including IL-10 and TGF-β_1, have potent anti-inflammatory activities.

Besides these classical responses, MCs produce also a variety of unclassified products that could be important in inflammatory mechanisms. These include mediators operative in the fibrinolytic pathway such as tissue plasminogen activator (tPA), which is produced by resting cells, while under certain activating conditions (i.e. complement receptors) they can also produce plasminogen activator inhibitor-1 (PAI-1) [19]. MCs also produce nitric oxide and superoxide radicals with known bactericidal, inflammatory and signal-modifying actions.

Together, these mediators are responsible for the numerous biological activities of MCs. Besides the instigation of tissue responses such as vasodilatation and bronchoconstriction initially studied for their importance in allergic processes, these include multiple proinflammatory activities such as the recruitment of other inflammatory cells and induction of host defense mechanisms, adaptive [immunoglobulin E (IgE)-mediated] immunity and immunoregulatory activities such as Ig isotype switching or T-cell polarization as well as tissue remodeling responses. Some of these will be discussed below in more detail, focusing on the mechanisms that may be important in renal inflammation.

Receptors Expressed by Mast Cells

MCs are sentinels that express a large variety of cell surface receptors allowing them to respond to different physiological or pathophysiological situations (Fig. 6.2). Besides the well-studied high-affinity IgE receptor known for its involvement in allergies or adaptive immune responses, other immunoreceptors include Fc receptors (FcR) that bind to IgG subclasses conferring MCs responsiveness to stimulation with immune complexes in host defense, but also in autoimmunity. While mouse MCs express the activating FcγRIII, human MCs express both activating FcγRIIA and under certain conditions FcγRI, the latter being induced by low concentrations of interferon-γ (IFN-γ). Mouse MCs also express the FcγRIIB, while its expression in human MCs is not firmly established [20]. FcγRIIB functions as a general inhibitor of cell activation when coaggregated with another activating immune receptor.

In addition, MCs express various receptors involved in innate immunity [4,6,18]. These include several types of Toll-like receptors (TLRs), able to become activated upon interaction with pathogen-associated molecular products (PAMPs) present in bacteria [i.e. lipopolysaccharide (LPS), flagellin] and viruses (dsDNA, ssRNA and parasites). The initial reports focused on TLR2 and TLR4 but since then other functional TLRs [1,3,6,7,9] have been discovered on MCs [4]. Importantly, MCs also express a variety of receptors or danger-associated molecular products (DAMPs) including the purinergic ATP receptor (P2X7), prostanoid receptors (EP3/4) and TLRs; the latter respond, for example, to high mobility group box 1 protein (HMGB1), a protein released in the surroundings when cells are injured or become necrotic. Stimulation of TLRs can lead to the production of proinflammatory cytokines in the absence of degranulation [4]. MCs have also been shown to express receptors for a number of cytokines, chemokines and growth factors. Many of them, including the chemokine receptor CCR1 or SCF, are known to upregulate the responsiveness to other stimuli [21]. Furthermore, certain of them are able to upregulate the expression of receptors, such as FcγRI by IFN-γ, which can modulate the cellular responsiveness [20]. Some of them, such as receptors for SCF, IL-9 or certain chemokines, may also be a means to attract MCs to a given anatomical site. Thus, it has been shown that IL-9 transgenic mice are particularly enriched in MCs in some tissues, including the kidney [22].

Furthermore, depending on the activating product MCs are able to selectively release a specific set of cytokines/chemokines, suggesting that they have the

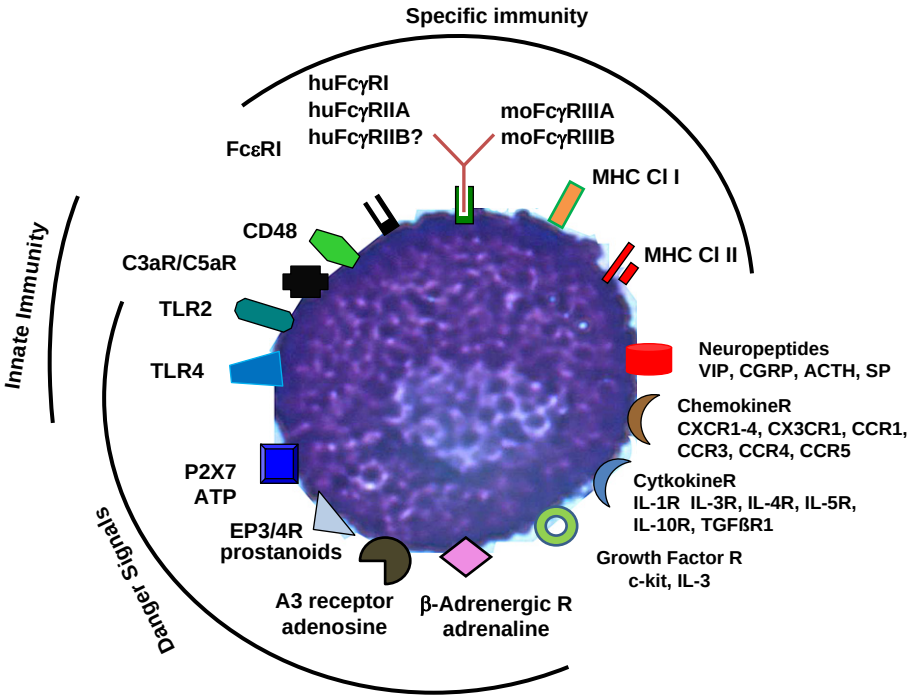

FIGURE 6.2 Examples of surface receptors expressed by mast cells. These can be further classified as receptors involved in specific or adaptive immunity as well as danger signals. hu: human; mo: mouse; FcR: Fc receptor; MHC: major histocompatibility complex; VIP: vasoactive intestinal peptide; CGRP: calcitonin gene related peptide; ACTH: adrenocorticotropic hormone; SP: substance P; CXCR: CXC chemokine receptor; IL: inter leukin; TGF: transforming growth factor; EP3/4R: Prostaglandin E receptor subtype 3 or 4; P2X7: purinergic ATP receptor; TLR: Toll like receptor.

capability of fine tuning the effector response. Several forms of infection or tissue injury also lead to the activation of complement. MCs have long been known to express complement receptors such as CR3 (CD11b CD18), CR4 (CD11c CD18) or receptors recognizing complement split products such as C3aR and C5aR (CD88) [7]. The expression of CR3 and CR4 is variable depending on the type of MC, but can also be upregulated upon activation. C3aR and C5aR are expressed only on certain types of MCs, in general CTMC, indicating functional heterogeneity depending on the anatomical site.

MCs express also a variety of receptors that are activated by polybasic compounds such as compound 48/80, mastoparan and polymers of basic amino acids or a number of related peptides. The responses can be inhibited by pertussis toxin, indicating that they involve G-protein-coupled receptors. As for C3aR and C5aR, CTMCs can be distinguished from MMCs by their property to respond to stimulation with compound 48/80. MCs also respond to stimulation with certain neuropeptides, including substance P (SP), calcitonin gene-related peptide (CGRP), somatotropin release inhibitory factor (SRIF), vasoactive intestinal peptide (VIP) and adrenocorticotropic hormone (ACTH) [23]. This suggests that MCs could represent an important connection between the immune and the central nervous system.

MODEL SYSTEMS TO STUDY MAST CELL FUNCTIONS

A useful model system to study the in vivo function of MCs has been the availability of MC-deficient mice. Several MC-deficient rodents carrying natural mutations such as $Kit^{W}/Kit^{W\ v}$, $Kit^{W\ 41}/Kit^{W\ 41}$ and $Kit^{W\ f}/Kit^{W\ f}$ mice, as well as $Kit^{W\ s}/Kit^{W\ s}$ rats, have been used to investigate MC biology in vivo [7]. The most often used model has been the WBB6F1-$Kit^{W}/Kit^{W\ v}$ mouse; however, C57BL/6-$Kit^{W\ sh}/Kit^{W\ sh}$ mice are nowadays gaining in popularity. Although both types of model are profoundly deficient in MCs, and lack melanocytes and interstitial cells of Cajal, $Kit^{W\ sh}/Kit^{W\ sh}$ mice have fewer abnormalities. In particular, in contrast to W/W^{v} mice they are fertile and not anemic. Thus, they can easily be crossed with mice carrying other abnormalities. As both types of mice exhibit deficiencies not only in the MC compartment, lack of responsiveness in a given experimental model does not necessarily mean that MCs play a role. It is therefore necessary to selectively repair the MC deficiency by the adoptive transfer of genetically compatible in vitro derived BMMCs. They are obtained from bone marrow after culture in IL-3 or culture with a combination of IL-3 and SCF. Sometimes they are also generated from embryonic stem cells (ESCs). Such MC knockin mice can then be used to assess the extent of MC implication.

While the effective restoration of responsiveness may allow reasonable conclusions to be drawn about the implication of MCs, partial or lack of restoration is more difficult to interpret [7]. Notably, it has been found that following adoptive transfer of MCs the anatomical distribution and numbers as well as certain functional properties may not necessarily be completely identical to wild-type mice, which may complicate the interpretation of results [24]. Another MC-deficient strain, WCB6F$_1$-*Kitl*^{Sl}/*Kitl*$^{Sl\ d}$ mice, carrying natural mutations in the ligands of c-kit (Kitl or SCF), has been used less because the absence of biologically active SCF does not enable reconstitution of MCs in these animals [7].

The reconstitution with MCs provides an additional advantage as they allow the reconstitution of mice with MCs deficient in a receptor or particular mediator that are derived from mutant mice or mutant ESCs. Thus, it has been shown in the EAE model of multiple sclerosis that MCs deficient in the activating FcγRIII are unable to restore disease susceptibility, suggesting the implication of pathological IgG immune complexes [25]. Similarly, in the model of autoimmune arthritis induced by the application of arthritogenic antibodies, besides the role of activating FcγR, a crucial role for IL-1β released by MCs in the initiation of the response could be demonstrated [26].

BENEFICIAL ROLE OF MAST CELLS IN INFLAMMATION

While there is no doubt that MCs are implicated in multiple chronic inflammatory diseases, many of these may be the result of the increasing inadequacy of the environment of our modern hygienic society. Indeed, the hygiene hypothesis postulates that owing to the altered challenge with common pathogens encountered during bacterial, viral and parasitic infection, the immune system becomes deviated, leading to inappropriate responsiveness towards otherwise inoffensive antigens such as environmental allergens or towards antigens of the own body, leading to allergy and autoimmune disease [27]. This is supported by epidemiological data showing a tremendous increase in allergic disease in the past five decades. Besides allergies, certain autoimmune diseases such as type 1 diabetes and multiple sclerosis have also increased, probably also promoted by the absence of stimulation with environmental pathogens. Similarly, in renal diseases a constant increase in disease incidence has been observed during the past three decades. In particular, the number of patients developing CKD owing to diabetes and hypertension has risen sharply [28]. A large part of the increase can be attributed to the change in lifestyle as well as to the aging of the population in Western countries. It is possible that inflammatory processes engendered by inappropriately reactive MCs and other inflammatory cells contribute to the severity and accelerated progression.

Indeed, for some of these diseases MCs have been incriminated as major actors of the inherent pathophysiological mechanisms, such as in allergies. Yet, research during the past two decades has also made clear the physiological functions of MCs and their mediators, in particular in host defense against pathogens, where they may have major roles in protection [4,6,23]. They have also revealed a more subtle role of MCs in the inflammatory process that highlight their important function in tissue repair following injury.

Undoubtedly, the first reports of beneficial roles played by MCs were related to their capacity to function in innate and adaptive host defense mechanisms. Because IgE production is markedly upregulated during immune responses to infections with intestinal parasites, with MC numbers being considerably upregulated, this question was investigated in MC-deficient mice. The results clearly showed a more severe disease in the absence of MCs in a parasitic nematode infection model, with higher larval counts and lower worm expulsion in MC-deficient than in wild-type mice [29]. Other parasitic infection models in the skin such as infestations with larval *Haemophysalis longicornis* ticks clearly showed an aggravating role when IgE or MCs were absent in experimental animal models [30]. Besides parasitic infection, MCs play important roles against bacterial infections. In the mid-1990s MCs were shown to exert a powerful protective effect in host defense against sepsis [31,32]. Using the model of bacterial sepsis induced by cecal ligation and puncture, mice-deficient in MCs were found to be highly susceptible to infection and died within a few days, while more than 60% of wild-type mice survived. Further analysis revealed that MC produced TNF, which is rapidly mobilized owing to its presence as a preformed mediator stored in cytoplasmic secretory granules, and is a major protective mediator as neutralizing antibodies abrogated the MC protective effect. However, TNF has also been incriminated in the pathogenesis of sepsis and septic shock. In particular, increasing concentrations and especially persistence of high concentrations of TNF during sepsis have been associated with non-survival. This shows that regulating the inflammatory response is a crucial issue. Given these opposing effects of TNF in the disease process, it is not surprising that strategies aiming to treat sepsis with neutralizing antibodies have essentially failed.

MCs are commonly thought to respond to the presence of toxic products or venoms such as snake or bee venom through the release of tissue-damaging

molecules that can sometimes lead to the induction of anaphylactic shock. However, a recent study has made clear that by responding strongly they might also participate in detoxification. Indeed, MC-released proteases, in particular MC-CPA, were shown to cleave and neutralize the major toxins of snake or bee venoms, or otherwise diminish the toxins' adverse effects [33]. A similar finding also reports the neutralization of endogenously produced endothelin-1 by MC-CPA [34]. These results clearly provide evidence for the physiological function of anaphylactic responses. They call for a rethinking of the associated pathology where systemic distribution of venom may cause anaphylactic shock only in susceptible individuals that are unable to control the systemic activation of MCs.

Several studies have indicated the important contribution of MCs in tissue repair and remodeling processes in response to injury. An important physiological role of MCs could be clearly demonstrated in an in vivo model of healing of skin wounds [35]. Wound closure was significantly impaired in MC-deficient mice. Histomorphometric analyses of MC degranulation at the wound edges revealed distance-dependent MC activation, with MC degranulation being most prominent directly adjacent to the wound. As a consequence, recruitment of other inflammatory cells important for the repair process was promoted, with histamine being an important mediator, as inhibition with the selective histamine H_1-receptor antagonist resulted in significantly delayed skin wound closure.

MCs also participate in hair follicle cycling, a process requiring substantial architectural changes in the skin including proteolysis, angiogenesis and nerve supply rearrangements. Indeed, MC-deficient mice exhibit markedly reduced cycling efficiency [23]. Along the same line, it was found that MCs participate in bone remodeling. MC-deficient mutants when subjected to an induced cycle of bone remodeling showed a delayed onset of the remodeling cycle, a decreased duration and extent of the active formation phase as well as diminished new bone matrix synthesis [36]. This probably involves a paracrine mechanism, which influences osteoclast and osteoblast activity. Indeed, histamine was shown to influence the early rapid phase of osteoclast activation during bone remodeling [37].

MAST CELLS AS REGULATORS OF IMMUNE AND INFLAMMATORY REACTIONS

Immune cell infiltration is generally considered as being causally linked to disease progression and chronicity. Only recently has it become clear that immune cells can also participate in the resolution of inflammation, imposing a highly dynamic view of the inherent processes. For example, the capacity to shift macrophages from a proinflammatory M1 to an anti-inflammatory M2 phenotype, the production of anti-inflammatory cytokines or the activation of regulatory cells, i.e. regulatory T cells, may represent important responsive mechanisms of the organism to resolve injury [38]. Likewise, MCs are able to produce and release a wide range of inflammatory compounds. In general, these are proinflammatory and constitute a necessary response to fight infections or eliminate injury-promoting products. They involve the recruitment, maturation and function of other immune effectors as shown in the model of bacterial sepsis [31,32], the upregulation of adhesion molecules, for example by secretion of TNF [39], the initiation of tissue responses such as bronchial smooth muscle contraction by LTC_4, the degradation of endogenous or exogenous toxins [33,34], and the activation of T-cell responses, for example by performing adjuvant functions [40]. Yet, there is also an increasing awareness that MCs have also many immunomodulatory and even immunosuppressive functions. For example, it is known that by the secretion of Th2-promoting cytokines (IL-4, IL-13) they can promote the production of IgE, interact with T cells for antigen presentation [41] and generally influence the migration, maturation or differentiation and function of other immune effectors by the secretion of numerous immunomodulatory cytokines and chemokines. The first evidence for immunosuppressive functions has come from studies of contact hypersensitivity responses to ultraviolet B (UVB) light in the skin, where it was shown that skin MCs mediate UVB light-induced suppression of contact hypersensitivity induced by a hapten, an effect that is mediated by histamine [42]. More recently, MCs were also shown to suppress contact dermatitis induced by urashiol and chronic irradiation with UVB light and therefore contribute to the resolution of inflammation. It was further shown that IL-10 was importantly contributing to the immunosuppressive function, as reconstitution of MC-deficient mice with MCs from IL-10-deficient mice could not restore the immunosuppressive function, in contrast to the reconstitution with wild-type MCs. Furthermore, MCs were also shown to represent essential intermediates in promoting peripheral tolerance by regulatory T cells in a mouse skin allograft model. This was dependent on IL-9, a cytokine produced by Treg to recruit immunosuppressive MCs [43]. It is not known how MCs contribute to immunosuppression in this model, but it could involve the mutual interaction between these cells to enhance their immunosuppressive functions [44].

INFLAMMATION AND KIDNEY DISEASES: A REGENERATIVE PERSPECTIVE

Kidney diseases are initiated by an injury that can be at the level of either the glomerular, the renal vascular or the tubulointerstitial compartment. The immediate consequence is the initiation of a multistep process with a well-orchestrated succession of events that can be caricatured as four phases that may also happen at least in part in parallel: an inflammatory phase, an anti-inflammatory phase, a repair phase and a regeneration phase (Fig. 6.3). The initial injury is followed by an inflammatory phase predominating in the targeted renal compartment. It is characterized by the release of inflammatory products resulting in increased vascular permeability through activation of endothelial cells followed by leukocyte infiltration including MCs. At the same time kidney-resident cells including mesangial cells, tubular cells and fibroblasts become activated. This inflammatory step is followed by an anti-inflammatory phase that is tightly associated with the initiation of a repair process and even regeneration in some cases. Together these events aim towards the healing of local tissue damage, involving a highly dynamic and complex process that includes, in addition to the infiltrative cells already present from the inflammatory phase, the proliferation of kidney-resident cells and even the putative colonization and differentiation of medullary multipotent stem cells in kidney-resident cells, apoptosis of infiltrative and damaged resident cells, extracellular matrix (ECM) expansion and tissue remodeling, as well as activation of profibrotic cells such as myofibroblasts for local scar formation, which is eventually limited by antifibrotic processes [45]. Ideally, inflammation will resolve without scarring with restoration of tissue structure and function as has, for example, been observed after acute ischemic injury [46]. Resolution of inflammation requires removal of the initiating

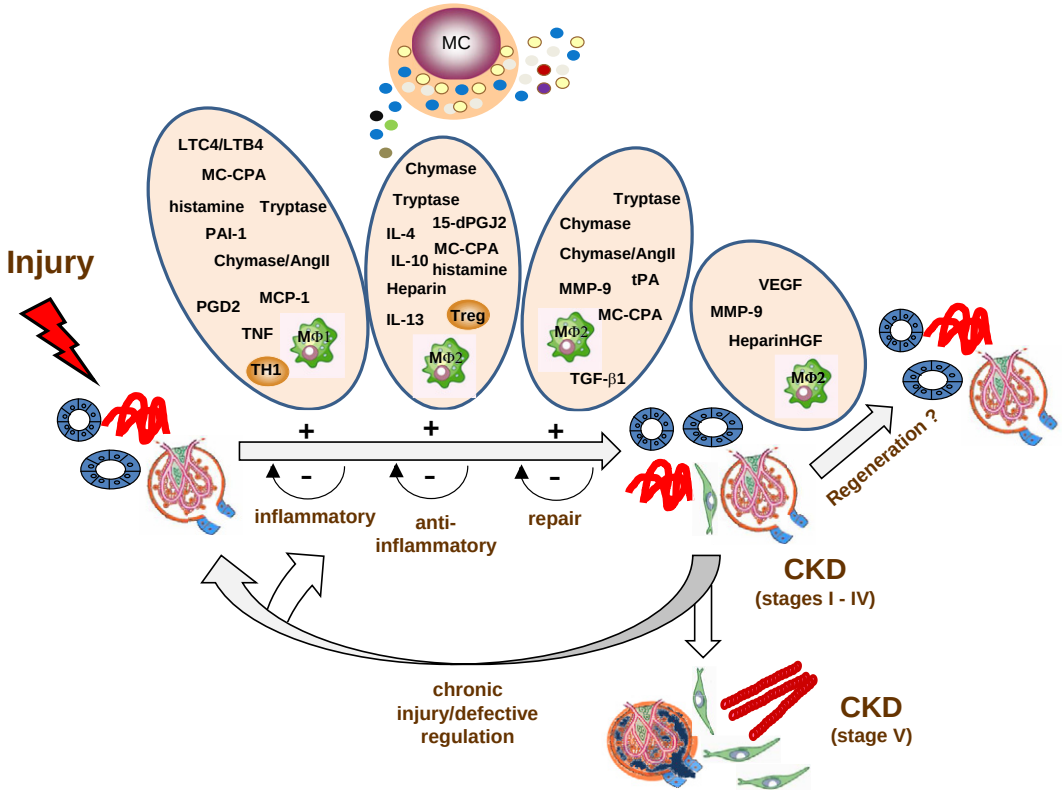

FIGURE 6.3 Mast cell (MC) actions in kidney diseases. A kidney disease is initiated by an injurious event on kidney tissue structures. This engenders an inflammatory response, which can be classified into four successive phases: an inflammatory phase, an anti inflammatory phase, a repair phase and a possible regeneration phase. (Note that these phases may take place also in parallel.) Together these events aim to achieve the healing of local tissue damage. Mast cells act, either systemically or by infiltrating kidney tissue, by secreting a wide range of products that participate in the orchestration of these phases, each phase involving a characteristic set of products. Several of these products can have opposite functions during the various phases, indicating a highly complex process that needs to be controlled dynamically, involving both positive (+) and negative ( ) feedback mechanisms. Any type of chronic injurious event or defective regulation may lead to the installment of an inappropriate inflammatory circuit, shifting the balance from repair and regenerative mechanisms to the progression to the final stages of chronic kidney disease (CKD). LT: leukotriene; MC CPA: mast cell carboxypeptidase A; PAI: plasminogen activator inhibitor; MCP: monocyte chemotactic protein; PG: prostaglandin; TNF: tumor necrosis factor; IL: interleukin; Treg: regulatory T cell; MΦ: macrophage; tPA: tissue plasminogen activator; MMP: matrix metalloproteinase; TGF: transforming growth factor; VEGF: vascular endothelial growth factor; HGF: hepatocyte growth factor.

stimulus, dissipation of mediators and the cessation of cell infiltration. It is accompanied by the clearance of inflammatory cells by migration and apoptosis and by activation of proteases to restore the ECM in its original form. As the inflammatory episode may have led to the loss of renal parenchyma and its replacement by extensive fibrosis, adaptive processes may follow through structural hypertrophy and eventually also regenerative mechanisms. For example, in human renal diseases treatment with Ang II receptor blockers or angiotensin-converting enzyme (ACE) inhibitors can slow down fibrosis development, probably as a result of the disease progression over years leading to irreversible destruction of renal parenchyma. However, it has become clear from animal studies that an early fibrotic process is reversible in several models of progressive renal disease using ACE inhibition or inhibition of Ang II receptors, clearly indicating the regenerative potential of kidneys [2]. Furthermore, cell-tracing studies have demonstrated regeneration and transdifferentiation potential following injury, although the type of cell, intrarenal, hematopoietic lineage marrow cells (HLMCs), mesenchymal stem cells (MSCs) or macrophages, seems to be somewhat a matter of debate and also depends on the experimental model used [38,47 50]. In postischemic kidneys, following reparation, almost normal renal histology and function is restored [51]. In case the inflammation does not resolve owing to the persistence of the initiating insult or inadequate regulation, iterative cycles of repair take place, augmenting the risk of being incomplete, and thereby further increasing the risk of persistent injury with nephron and vessel loss and replacement by fibrotic tissue. Although the human kidney can sustain the destruction of a certain number of nephrons, end-stage renal disease will inevitably develop when more than two-thirds of the renal parenchyma is lost.

Of note is that some participants may be involved during several phases of the process, and even that some of them may have a dual role with sometimes apparent opposite aims. Ang II may exert a main deleterious role with proinflammatory properties during the early inflammatory phase and profibrotic role during the repair phase [2], and MCs contribute to the initial inflammation but may have beneficial effects in the successive phases, as demonstrated by a growing amount of experimental evidence.

MAST CELLS IN KIDNEY DISEASE

Localization of Mast Cells in Normal and Pathological Kidney

MCs can be detected on the basis of their specific staining properties with metachromatic dyes such as toluidine blue or by immunohistochemical staining with antitryptase (recognizing MC_{TC} and MC_T) or antichymase (MC_{TC}). These staining methods are based on granular staining and thus have the disadvantage that they do not necessarily recognize activated MCs that are degranulated. Indeed, hypervacuolated cells with a paucity of metachromatic cytoplasmic granules have been observed in various inflamed kidneys. Other methods based on anti-c-kit or anti-FcγRI alpha chain membrane staining have proven to be inappropriate, as other cells are stained including renal tubular cells for c-kit [52] or macrophages, platelets and eosinophils for anti-FcγRI in humans [6].

Table 6.2 provides some examples from studies in the literature that report the relative increase in MC counts in various renal diseases compared with normal kidney [5]. It can be noticed that all cases of progressive kidney diseases are associated with significantly elevated numbers of MCs in renal tissue. MCs are localized predominantly in the interstitium where they concentrate in areas close to tubules and blood vessels, and are included in mononuclear cell infiltrates associated with fibrotic areas. Although periglomerular localization is also noted, MCs have never been found within glomeruli. Furthermore, staining with tryptase and chymase-specific antibodies showed that both MC_{TC} and MC_T are present, with a predominance of the latter, although the ratio of the various MC subtypes varies with the renal disease. In rejected kidney transplants a third MC type stained with antichymase only has also been found [53]. In most cases, the increase in MC numbers correlated with the concentrations of serum creatinine and with fibrosis and thus poor outcome. It should be mentioned that MCs are also found in significant numbers in the connective tissue of the kidney capsule surrounding the renal

TABLE 6.2 Examples of Mast Cell (MC) Counts in Various Nephropathies

Diagnosis	MC count (cells/mm^2)
Normal kidney	0.5 ± 0.2
Benign nephrosclerosis	1.6 ± 0.4
Membranous nephropathy	6.5 ± 7.7
Lupus nephritis	12.9 ± 15.8
ANCA related nephropathy	18.5 ± 21.1
Membranoproliferative nephropathy	19.8 ± 14.2
Immunoglobulin A nephropathy	21.3 ± 17.7
Diabetic nephropathy	33.0 ± 33.8
Renal allograft rejection	12.7 ± 1.2

ANCA: anti-neutrophil cytoplasmic antibody.
Examples of MC counts were taken from data assembled in Blank et al. [5].

parenchyma. Most intriguingly, the present authors noticed in an experimental model of anti-glomerular basement membrane (GBM) glomerulonephritis that they become activated, as indicated by their degranulated phenotype (unpublished).

Mast Cells in Human Renal Diseases

MCs, by virtue of their mediators and responsiveness to many inflammatory stimuli, could participate in various steps of the inflammatory cascade in kidney diseases through either systemic or local activation. Through mediator secretion they may participate in the removal of injurious insult and in repair processes, or alternatively participate in kidney destruction leading eventually to the final stages of CKD. Although MC numbers are low in normal kidneys, it has become clear that significant numbers of MCs are present in biopsy specimens in various human nephropathies, in particular in advanced stages of disease (Table 6.2). In addition, evidence has been presented showing an increase in SCFs in patients with renal disease. Kitoh et al. [54] showed that serum levels were elevated by about five-fold in patients with end-stage renal failure. This has been interpreted as MCs serving an aggravating role, probably because of their negative connotations in various chronic inflammatory diseases and autoimmune diseases. However, the simple correlation of MC numbers or growth factors with disease severity is clearly insufficient to judge the role of MCs. As indicated above, the described process in kidney diseases appears complex, requiring a finely tuned chain of events composed of proinflammatory components as well as anti-inflammatory and repair components during the resolution phase. Thus, beneficial actions required during the initial phase may not necessarily play the same role during the late phase and could be counterproductive when they are not downregulated. An example is the fibrinolytic system. While during the initial phase of injury formation of fibrin clots is necessary, these clots need to be resolved during the remodeling/repair phase by the action of fibrinolytic enzymes. MCs have been shown to release many products with fibrinolytic action, including tryptase and tPA [19]. Another example is the inactivation of hepatocyte growth factor (HGF) by human MC chymase or murine MCP-4, which during the initial injurious phase may be necessary to limit proliferation of kidney-resident tubular cells, while during the later phase this may prevent epithelial cell regeneration [55].

Multiple studies reported that the increase in the number of MCs is correlated with the degree of tubulointerstitial fibrosis, decline in glomerular filtration rate, leukocyte infiltration and disease progression in a variety of nephropathies, including IgA nephropathy, crescentic glomerulonephritis, diabetic nephropathy and hypertensive nephropathy [5]. MCs were also observed in renal allografts, with high numbers being associated with reduced survival, although this was not the case in all studies [5].

Only a few studies in humans tried to investigate further whether MCs, in addition to being correlated in numbers with disease severity, may also be functionally implicated. In a study with renal allograft patients, retrospective clinical data showed that patients with a history of allergy showed more severe episodes of rejection than non-allergic patients. This indicated that the inappropriate activation of MCs may break tolerance [56]. Few studies have addressed the question of whether infiltrated MCs are actually activated and by which mechanism. Review of the histological analysis in the literature usually shows perfectly granulated toluidine blue-stained or tryptase-positive cells that do not seem to be degranulated, including in the authors' unpublished studies in lupus and IgA nephropathy patients. It is therefore possible that MCs, although correlated with disease severity, may not play a role in the associated pathological process in the long term and only fulfill a role as sentinels without being involved in the chronic development of the disease. Some studies, however, have reported that MCs become activated, as for example in diabetic nephropathy where MCs were reported to be frequently degranulated [57]. These studies further showed that MCs strongly expressed type VIII collagen, suggesting that they may directly contribute to ECM accumulation. Similarly, in cases of severe IgA nephropathy, immunohistological staining with chymase frequently shows the enzyme in the area around MCs, indicating that they have liberated their content, at least partially [58].

Another approach to investigate the functional implication of MCs was by examining MC subtypes. Two studies reported that the relative increase in MC_{TC} compared to MC_T was related to the decline in renal allograft function and fibrosis development, suggesting that pathological function may be associated with the connective tissue type known to release chymase. The possible relevance of the association with fibrosis is highlighted by data showing that human chymase or its murine analog mMCP-4 can function as a major alternative pathway for Ang II generation in addition to ACE [59]. For many years Ang II has been recognized as a major factor involved in the inflammatory process leading to renal fibrosis development. So far, chymase is only known to be produced by MCs. The role of this enzyme in Ang II generation as an alternative for ACE has clearly been demonstrated in renal artery-clamping-induced hypertension [60]. Increased chymase expression was also seen in patients with diabetic nephropathy [61]. In addition to chymase, MCs may present another

possibility of Ang II upregulation, as it has been shown that cardiac MCs and the human MC line HMC-1 can synthesize renin that can be released by degranulation [62]. Another profibrotic action may come from the action of tryptase. This protease, in association with heparin, was shown to promote proliferation of human renal fibroblasts as well as the synthesis of fibronectin and type I collagen, probably by its action on PAR-2 receptors [63].

Taken together, data from several human kidney diseases indicate a correlation of the observed MC infiltrate with disease severity. Although some putative mechanisms of MCs' detrimental role in kidney diseases initiation and progression have been suggested, a clear demonstration of their functional involvement is still missing.

Insights from Animal Models

Antiglomerular Basement Membrane Model

Although MCs are observed in increased numbers of human nephropathies, it is difficult to directly address their participation in disease experimentally. In general, a number of other inflammatory cells are seen to infiltrate the kidney, making interpretation difficult with regard to the participation of one particular cell type. Furthermore, systemic effects independent of the inflammatory cell infiltrate cannot be excluded. The availability of MC-deficient and MC knockin mice circumvents these difficulties and researchers have begun to address the functional role of MCs in various renal disease models.

Several groups have studied the role of MCs using W/W^v mice in an experimental model of Goodpasture disease initially developed by Masugi [64]. It is based on the injection of alloantibodies directed against the GBM [64]. This model involves a heterologous phase of disease development subsequent to the injection of the anti-GBM antibodies derived from a different species. The heterologous phase is characterized by a rapid (within 1 h) and massive, but transient influx of neutrophils that, through FcR- and complement-dependent mechanisms, leads to the initial glomerular injury. The development of an immune response to the injected antibodies by the host promotes the development of the second autologous phase [64] characterized by infiltration of FcR-bearing macrophages and T cells that are effectors of glomerular injury and that further promote disease development towards renal failure. The importance of the T-cell response has been demonstrated in several studies. They suggest a role for CD4 T-cell-driven Th1-type responses with development of delayed-type hypersensitivity-like cellular effectors (CD8 T and macrophages) as well as Th1 Ig isotype production [65]. Recently, defects in regulatory T cells have been implicated as a potential mechanism of pathology [66]. When hosts are preimmunized with normal non-pathological antibodies a few days before injection of the anti-GBM antibody, the appearance of the autologous phase can be accelerated (anti-GBM accelerated model). It should be noted, however, that this model does not exactly reproduce human Goodpasture disease, where anti-GBM are autoantibodies that do not derive from a different species.

When the role of MC in an accelerated model of anti-GBM-induced glomerulonephritis was studied in MC-deficient mice, the results revealed quite different outcomes and conclusions. While two studies reported a beneficial role of MCs [67,68], another study showed that they had an aggravating role [69]. Kanamaru et al. [67] found that following injection of the anti-GBM serum, disease developed rapidly and MC-deficient W/W^v mice exhibited a high mortality, with MC-deficient mice starting to die at day 3 during the early heterologous phase and more than 80% of the mice succumbing within 14 days. Conversely, +/+ mice showed high survival rates. Reconstitution of the MC population in W/W^v mice restored the protection, confirming the essential role of MCs. The striking pathological feature revealed the presence of large subendothelial deposits in W/W^v mice, while they were less prominent in +/+ mice or MC-reconstituted W/W^v mice. These deposits appeared early on in the disease and persisted thereafter. At day 14 a glomerular and interstitial macrophage infiltration in W/W^v mice was recognized, while a T-cell infiltrate was absent in all groups of mice. Similarly, no significant MC infiltration occurred, suggesting at the same time that the beneficial MC action could depend on a systemic activation. Immunohistological data showed that the enhanced deposits were not caused by differences in heterologous and autologous antibodies, but rather contained increased amounts of fibrin and type I collagen in W/W^v mice. This suggests a deficit in remodeling and repair functions in these mice. Furthermore, W/W^v mice were unable to maintain high tPA and urinary-type plasminogen activator (uPA) activity in urine, especially in the later phases of the disease. It was known from experiments performed in fibrinogen-deficient mice that fibrin represents a key factor in glomerulonephritis development and contributes to the rapid progression of the disease [70]. These results suggested that the protective action of MC was due to their capacity to rapidly initiate remodeling and repair. It remained unclear, however, by which mechanism MCs were responsible for this action. Indeed, analysis of the contribution of MC-expressed activating FcR through immune complexes in the protective effects revealed that the contribution of these receptors was minor. Indeed, reconstitution of W/W^v mice with MC derived from mice lacking FcRγ, the common subunit

of activating FcR, showed that they were almost equally as potent as wild-type cells in reconstituting protection. Therefore, other stimuli such as complement fragments, endothelin or other inflammatory mediators that could be produced during the initial inflammatory phase of the disease may play a role [7,64,71]. In the second study, by Hochegger et al. [68], the authors also found an increased mortality after administration of anti-GBM antibodies in W/W^v mice compared to +/+ mice or W/W^v mice reconstituted with bone marrow cells. The rise in mortality appeared, however, relatively late in the disease, between day 14 and day 21. Similarly to our study, W/W^v mice exhibited thick subendothelial deposits in glomeruli as a major pathological feature. The authors also detected increased numbers of macrophages in the interstitium of W/W^v mice and, surprisingly, augmented numbers of interstitial CD4- and CD8-positive T cells at days 14 and 21 that were not observed in our study. In addition, they found considerable infiltration with MCs of the draining lymph nodes while, as in our study, MCs were virtually absent in the kidney. Based on this the authors suggested that MC may be able to induce protection by their ability to modulate the influx of effector T cells and macrophages to the inflammatory sites in the kidney. They speculated that the absence of MCs could disturb the anti-inflammatory counterbalance exerted by regulatory T cells. In the third study, the authors used a non-accelerated model of the disease by injecting the antibodies in two individual doses in the absence of adjuvant, and most of the collected data were scored at day 21 [69]. Under these conditions W/W^v mice were found to have decreased dermal delayed-type hypersensitivity (DTH) responses to the injected antibodies, fewer crescentic glomeruli, and less interstitial infiltrate, glomerular T cells and macrophages compared to +/+ mice or W/W^v mice reconstituted with MC. Examination of renal parameters revealed less serum creatinine and urinary nitric oxide in W/W^v mice than in the control groups, while no difference was found in proteinuria. Immunohistochemical data revealed an augmentation of glomerular adhesion molecule expression. Taken together, these data suggest that MCs, by their ability to promote Th1-dependent effector mechanisms and to enhance glomerular expression of adhesion molecules, aggravate the anti-GBM-induced inflammatory response.

Although the three studies arrived at somewhat different conclusions, they may have actually revealed the delicate balance of the inflammatory reaction as well as the high versatility and sometimes contrasting function of the MC as an effector cell. In our study, owing to the rapid development of glomerulonephritis by the injected anti-GBM alloantibodies, the sentinel action of the MC is revealed, exemplified by its capacity to react to damage with acute effector mechanisms of tissue repair that counteract the injurious process. In the second study, disease develops more slowly, which is likely to be due to the use of an antiserum with less injurious potential. Here, in addition to repair functions the immunoregulatory potential of MCs is revealed to counteract the development of a T-cell-dependent effector mechanism during the autologous phase, which thus dampens disease development. In the third model, owing to an even more slowly developing disease, the non-accelerated model, MCs are contributory in mounting a Th1-directed DTH-mediated immune response that promotes injury. Thus, these studies reveal that MCs are able to exert a continuous spectrum of responses ranging from protection to aggravation in a similar disease model. Besides the specific pathophysiological environment that directs the MC action, their action may also depend on the timing and regulation of the response. One can speculate that, while the immediate action of MCs is destined for repair, more progressive disease may reveal a detrimental action of MC responses as they may become inadequate if not downregulated.

Mast Cells in Lupus Nephritis

Systemic lupus erythematosus (SLE) is an autoimmune disease predominantly affecting young females. It is characterized by the presence of autoantibodies, mostly produced against nuclear cell antigens and almost specifically against double-strand DNA. Circulating immune complexes deposit in various organs and tissues such as skin, joints, the nervous system and kidney. The resulting inflammation leads to various clinical manifestations, which include, besides dermatitis, arthritis and neurological disorders, also glomerulonephritis. The presence or absence of renal lesions is known to be critical for the outcome of SLE since they could lead to renal failure and even to death [72].

The involvement of MCs in at least some SLE manifestations, such as nephritis, has been suspected [73]. In kidney, the number of tubulointerstitial MCs shows an approximately 10-fold increase in SLE nephritis patients compared with healthy individuals and correlates with fibrotic development [74,75]. However, no correlation was found between tubulointerstitial MC number and the clinical outcome of lupus nephritis [76], indicating that although MCs may contribute to the disease in this organ they are not the only factor involved. Here also, the use of MC-deficient mice in an experimental model of pristane-induced lupus nephritis has shed some light on the possible implication of MCs. This model involves the injection with a single dose of the C19 isoprenoid, alkane pristine, inducing a lupus-like disease characterized by the appearance of serum autoantibodies (anti-nRNP/Sm, ribosomal P and Su), deposition of immunoglobulin in glomeruli

and development of glomerulosclerosis in various strains of different genetic background. When pristane was injected into MC-deficient W/W^v and their wild-type littermates, some protective roles of MCs were reported [77], as the absence of MCs led to a diffuse proliferative glomerulonephritis instead of focal segmental glomerulonephritis, and immune deposits in the glomeruli. However, other deposits such as fibrin and collagen have not been examined. A tendency to hypergammaglobulinemia was also noted in some, but not all, MC-deficient W/W^v mice. By contrast, proteinuria was similar in MC-deficient and wild-type mice. As in this work no MC knockin study has been performed, it leaves open the possibility that MC-independent effects could also be at work. Nonetheless, it was relatively interesting to observe that in both anti-GBM and pristane-induced lupus glomerulonephritis a striking pathological feature in the absence of MCs was an enhanced immune deposition in glomeruli, indicating that one of the beneficial effects of MCs is the inhibition of immune deposition in glomeruli and/or the clearance of glomerular immune deposits.

Mast Cells in Renal Fibrosis

Several studies have revealed significant increases in MC numbers in renal interstitium, in peritubular and periglomerular areas. Interstitial increase in MCs was correlated with the progression of fibrosis and decrease of renal function. However, direct evidence that MCs participate in the development of renal fibrosis has not been provided, although several observations in human disease pointed to a fibrogenic potential of kidney-infiltrating MCs. This includes the expression of type VIII collagen mRNA expression by MCs in diabetic nephropathy [78], the expression in MCs of fibroblast-proliferative factor in IgA nephropathy [79] and the presence in MCs of TGF-β_1 in progressive renal disease [80]. It is well accepted that TGF-β_1 and TGF-β_1-initiated Smad signaling pathways are key effector mechanisms for the development of tubulointerstitial fibrosis [81 83] by inducing ECM production and myofibroblast development.

Therefore, it was quite interesting to see that two studies using experimental animal models reported that MCs do not play a major role in the pathogenesis but rather ameliorate renal fibrosis. Miyazawa et al. utilized the puromycin aminonucleoside-nephrosis (PAN) model in MC-deficient W^s/W^s rats [84], leading to the development of diffuse tubulointerstitial fibrosis with ECM expansion and increases in interstitial macrophages and T cells over a period of several weeks [85]. Following administration of puromycin, disease developed in both groups, as shown by the development of proteinuria. At 6 weeks, renal failure developed with a concomitant increase in blood urea nitrogen indicating a significant loss of functional nephrons. No differences were observed in these parameters between the two groups of rats; however, fibrosis development, as quantified by histology and measurement of hydroxyproline levels, was found to be more severe in W^s/W^s rats than in $+/+$ rats. This was associated with increased MC infiltration in the interstitium of $+/+$ rats. Furthermore, profibrotic cytokines IL-4 and TGF-β_1 mRNA levels were higher in the MC-deficient rats. This was probably caused by heparin, a major proteoglycan secreted by MCs, which was shown to inhibit TGF-β_1 production by renal fibroblasts in vitro. A similar protective role was also seen in the unilateral ureteral obstruction (UUO) model when MC-deficient W/W^v mice were examined. The UUO model of tubulointerstitial fibrosis, which is surgically created by ligation of the ureter downstream of one of the two kidneys, reproduces the different stages of obstructive nephropathy leading to tubulointerstitial fibrosis [86]. This includes cellular infiltration, tubular proliferation and apoptosis, epithelial mesenchymal transition (EMT), (myo)fibroblast accumulation, increased ECM deposition and tubular atrophy. These pathological features appear rapidly and are highly reproducible, making this model a choice for the investigation of rapidly evolving renal interstitial fibrosis. Kim et al. [87] applied this model to MC-deficient W/W^v mice and showed significantly increased tubular injury and fibrosis scores with a decrease in cadherin expression and an increase in α-smooth muscle actin compared to wild-type mice 3 weeks after surgical intervention. MC-deficient mice also showed a stronger infiltrate of macrophages and T cells. However, in contrast to the PAN model in rats, MC infiltration has not been investigated, probably because of their absence, suggesting that as in the anti-GBM model they can act through systemic mechanisms. Reconstitution of mice with MCs restored the protective effect, effectively demonstrating the implication of MCs. As already observed in the PAN model, the authors found a significant increase in levels of TGF-β_1, a major mediator of fibrosis development, which could explain the more pronounced fibrotic phenotype. Taken together, these data indicate that MCs are not a major actor involved in the pathophysiology of renal fibrosis, but rather that they may exert a slightly protective effect. The latter could implicate the inhibition of TGF-β_1 production by MC-released heparin.

POSITIVE ACTIONS OF MAST CELL MEDIATORS IN KIDNEY DISEASE

The above studies in humans and mice have highlighted the importance of inflammatory processes in renal disease. The inflammatory response is initially

aimed at leading to recovery and healing. It is characterized by the secretion of soluble factors, the increase in local vascular permeability, activation of kidney-resident cells and leukocyte/lymphocyte infiltration. It functions in a highly orchestrated and timed manner to repair the lesions, promote tissue remodeling and restore tissue homeostasis. However, if the targeted destruction and assisted repair mechanisms are not properly phased, inflammation will lead to persistent tissue damage. After birth, the repair and remodeling process usually leads to the development of scars and limited fibrosis. Under certain circumstances it may also allow replacement of lost cells and regeneration of the renal parenchyma. This is, however, rare, especially in human disease, where chronic injury is often durably installed for some time, making reversal difficult.

The above evidence has made clear that MCs represent an integral part of the inflammatory response in renal diseases. While mostly known for their proinflammatory actions, increasing evidence now exists indicating that they can have beneficial roles by participating in anti-inflammatory responses as well as in the tissue repair and regenerative mechanisms. Various possible mechanisms of action by which MCs can contribute to this repair response are now recounted.

Fibrinolytic System

Fibrin clots build up as an immediate response to tissue injury, but need to be removed during the following tissue remodeling phase. MCs are important cellular actors of the fibrinolytic process, which involves the activation of plasminogen into plasmin, a protease that dissolves fibrin clots. Indeed, MCs are known to produce tPA, as one of the major plasminogen activators [19]. While tPA can be produced constitutively by MCs they also synthesize its natural inhibitor PAI-1; however, only upon stimulation [19]. Recent evidence from an experimental glomerulonephritis model clearly demonstrated that MCs contribute to the dissolution of subendothelial fibrin deposits in vivo [67]. In addition, these experiments have shown that MCs are necessary to maintain high levels of uPA and tPA in the urine at the later phase of glomerulonephritis, suggesting that subendothelial fibrin dissolution is dependent on plasminogen activator production by MCs in the repair phase of glomerulonephritis.

Heparin

Heparin is evolutionarily conserved even in organisms that do not have a coagulation system, suggesting that it participates in other anti-inflammatory activities such as protection from tissue injury by inhibiting integrin-mediated adhesion of inflammatory cells. Heparin can also bind many inflammatory chemokines, cytokines and complement factors, thereby preventing these proinflammatory molecules from interacting with their respective receptors. Consistently, the anti-inflammatory action of heparin has been shown in the course of experimental glomerulonephritis, with beneficial effects of oral heparin-like compounds such as oral pentosan polysulfate in the treatment of glomerulosclerosis [88]. Of note, MCs are the unique producer of heparin in the human body. This highly sulfated glycosaminoglycan, which is released from cytoplasmic granules by degranulation, has potent anticoagulant activity [6,13]. While heparin does not break down existing fibrin clots, it complements the body's natural fibrinolytic system, i.e. the plasminogen-activating system, by preventing reclotting. Similarly, during the repair phase following tissue inflammation and especially in the course of renal diseases, heparin-mediated anti-inflammatory MCs may maintain a non-inflammatory state, allowing an efficient repair action of fibrinolysis through the simultaneous secretion of plasminogen activator by MCs.

Mast Cell Proteases

A variety of proteases such as chymase, tryptase and MC-CPA is uniquely produced by MCs. These enzymes have been considered to be active in the multistep process leading to CKD [13,89]. For example, chymase has been shown to act as a chemoattractant for leukocytes and to be an important local producer of Ang II, independent of ACE. Ang II has been known for many years as a major contributor to renal diseases and inhibition of its action can slow down CKD development and progression. Similarly, tryptase has been reported to activate fibroblasts via PAR-2 receptors, thereby contributing to fibroblast proliferation and synthesis of collagen [13]. Tryptase was also reported to directly activate profibrotic TGF-β, which is produced as a latent inactive complex in the ECM [90]. However, mounting evidence also highlights the protective functions of these proteases, again suggesting that the appropriate balance and timing of their action is a critical parameter. Striking published examples of their beneficial actions constitute the ability to detoxify snake and bee venoms by MC-APA [33]. Importantly for renal disease pathology, MC-CPA is also able to protect mice from an excess of highly toxic endothelin-1, a short peptide with potent vasoconstrictor activities [34]. Endothelin-1 is produced in high amounts in various nephropathies and endothelin receptor antagonism has protective effects by reducing renal vascular fibrosis [2]. Chymases and tryptases have also been reported to cleave and inactivate several types of proinflammatory cytokines including IL-5, IL-6, IL-13 and TNF or the chemokines eotaxin

and RANTES [89]. Similarly, proinflammatory neuropeptides released from nerve endings can be neutralized by the action of chymase and tryptases [89]. Thus, MC proteases may also participate in the downregulation of an inflammatory response. MC proteases also play an important role in the tissue remodeling process. Studies in MCP-4 chymase-deficient mice have revealed that lack of mMCP-4, the functional homolog of human chymase, leads to a slight increase in ECM production [13]. Chymases and tryptases can contribute to matrix destruction by directly cleaving the ECM proteins fibronectin and non-helical collagen. They are also involved in the activation of matrix metalloproteinases (MMPs) (see below).

Matrix Metalloproteinases

Remodeling of the ECM is a key event in the progression of CKD but also in reversal of CKD. Indeed, fibrosis is characterized by the increased deposition of ECM proteins in the repair phase following the inflammatory response through excessive deposition of ECM proteins in the interstitial space as well as in the other renal compartments, glomerular vessels and tubules. Again, when appropriately phased, the regeneration of renal tissue requires the degradation of accumulated ECM proteins, a process largely mediated by MMPs, which in turn are under control of proteases such as plasmin converting the proforms into active forms and of specific tissue inhibitors of metalloproteinases (TIMPs). However, because of their multiple actions MMPs may exert different and even opposite effects during the various phases of renal diseases, with the major challenge being the temporal control of MMP activity during disease development [91]. A direct demonstration of the beneficial potential of MMPs in renal disease was obtained in experimental anti-GBM glomerulonephritis, where MMP9 has a protective action through its profibrinolytic activity [92]. Similarly, in acute kidney injury induced by folic acid, MMP9 deficiency was shown to increase apoptosis and delay recovery of renal function. The effect was attributed to an increased bioavailability of apoptosis-protecting SCFs through increased generation of soluble SCF by MMP9 from a membrane precursor [52]. Whether the generation of soluble SCF also attracts "protective" MCs has not been investigated in this study. While MCs can directly produce certain MMPs, such as MMP2 (gelatinase A) and MMP9, it is thought that their major action may result from their ability to inactivate endogenous inhibitors. Thus, it was shown that MC chymase, when released by degranulation, directly cleaves TIMP-1 [93]. In agreement, mice deficient in mMCP-4, the functional homolog of human chymase, have almost exclusively inactive pro-MMP9 in tissue extract compared to wild-type mice [94]. It is worth noting that the actions of chymase differ from those of β-tryptases, which are not effective activators of MMP9, although their ability to activate prostromelysin/MMP3 provides a potential means of activating MMP9 indirectly via MMP3 [89].

Histamine

Histamine is an important inflammatory mediator mostly produced by MCs, where it is stored in cytoplasmic granules and released by activation. While this product has been recognized as a main inflammatory mediator in allergies, surprisingly little is known as regard to its action in renal disease. Although early studies have reported an association of renal disease with atopy and elevated IgE levels, this was not a consistent observation by various investigators [5]. Surprisingly, however, recent experimental evidence suggested that histamine may have an anti-inflammatory function in the experimental anti-GBM glomerulonephritis model in rats [95]. Indeed, direct administration of histamine led to significant dose-dependent reduction in proteinuria compared to the control antibody-injected group and markedly decreased the number of crescentic glomeruli and macrophage infiltration of the glomeruli. Receptor agonists, such as the H(2)/H(4) agonist Dimaprit, mimicked the effects of histamine on proteinuria and crescent formation and a similar tendency was also observed for Clozapine, an H(4) agonist. An H(1) antagonist, mepyramine, or an H(2) antagonist, ranitidine, did not reverse the protective effect of histamine. Based on this, it was proposed that histamine may alleviate renal injury in anti-GBM glomerulonephritis by its effect on H4 receptors. Indeed, in monocytes activation of H4 receptors was reported to downregulate CCL2 chemokine production [96]. Moreover, histamine was also shown to downmodulate T-cell responses through interaction with H2 receptors [6]. More studies are necessary to substantiate the histamine-mediated anti-inflammatory action in renal disease.

Immunoregulatory Properties of Mast Cells

MCs are able to produce a wide range of cytokines with immunoregulatory properties. As described above in different inflammatory settings, MCs not only promote inflammatory responses, but can also have anti-inflammatory and immunosuppressive functions. In particular, they were necessary for the establishment of peripheral tolerance by regulatory T cells (Treg) in a mouse skin allograft model [43], and a renoprotective effect of Tregs was shown in several models of renal pathology including anti-GBM glomerulonephritis, adriamycin-induced nephropathy and ischemia reperfusion injury [66,97 99]. Transfer of Treg was

shown to be protective, while depletion of Treg was shown to enhance the pathology. However, whether Treg action depends on the presence of MCs, as in the skin, has not been investigated.

MCs also have the ability to downmodulate T-cell responses via the production of soluble mediators. The downregulation of T-cell and macrophage responses via histamine has already been mentioned above. Furthermore, MCs also produce IL-10, with multiple anti-inflammatory actions including the downregulation of T-cell responses, induction of Treg and downregulation of adhesion molecule expression [6]. In renal disease the renoprotective effect of IL-10 in the downregulation of the inflammatory responses in both acute and chronic renal experimental diseases has been demonstrated in knockout mice, as well as after overexpression using viral-derived vectors [100]. MCs also produce the Th2 polarizing cytokines IL-4 and IL-13, which may shift the effector T-cell balance. Both of these cytokines have also been implicated in promoting the generation of alternatively activated macrophages (M2, as compared to classically activated M1). M2 macrophages are thought to suppress immune responses and promote tissue remodeling [38]. Ex vivo programming of macrophages into the M2 phenotype and adoptive transfer clearly ameliorate experimental chronic inflammatory renal disease [101].

Growth Factors

Vascular endothelial growth factor (VEGF) is an important regulator of vascularization in many physiological and pathophysiological processes. However, it is also one of the most potent vascular permeability factors yet described, with a molar potency that exceeds that of histamine 50,000-fold [102]. The importance of VEGF has been demonstrated in various experimental and human renal diseases [102 104]. Indeed, growth and remodeling of glomerular capillaries after injury are necessary in the healing steps of glomerular diseases. Similarly, repair of peritubular capillaries that have been destroyed is important for the renal recovery process. In advanced diabetic nephropathy, the decreased presence of VEGF may be an indicator of an insufficient capillary repair [105]. MCs are the only infiltrating immune cells known to release VEGF from a preformed pool stored in cytoplasmic granules [102]. They may, therefore, represent a cell type able to trigger angiogenesis in a rapid, regulated manner depending on the particular pathophysiological context, which could be important in the fine-tuning of the regeneration response. This may, however, have its downside; for example, during cancer development inappropriately high stimulation of MCs may lead to undesired angiogenic responses [102].

Another important growth factor in renal protection is HGF, which has antifibrotic activities. This growth factor, initially discovered for its implication in liver regeneration, was also shown to have potent antifibrotic activity, a function that can be attributed to its ability to inhibit TGF-β_1-mediated signaling. HGF improved chronic injury in a variety of experimental settings and treatment with a recombinant HGF either at disease onset or after establishment of disease attenuates renal fibrosis [106]. MCs could be important regulators of HGF activity. Indeed, it was shown that MC activation is an important stimulator of HGF, an action that was mediated by heparin [107]. Injection of 1000 U/kg of heparin enhances circulating HGF levels by about 40-fold. In addition, the HGF receptor, Met receptor tyrosine kinase, could be activated much more potently when HGF was complexed to heparin, probably by promoting receptor oligomerization [108]. However, at the same time mMCP-4 chymase was also shown to inactivate HGF by cleavage, suggesting that MCs may also be involved in a feedback loop negatively regulating the activity of a plausible renoprotective HGF in the regeneration phase [55].

Lipid-derived Mediators

MCs generate three arachidonic acid-derived eicosanoids: prostaglandin PGD_2, leukotriene LTB_4 and LTC_4, the parent molecule of the cysteinyl leukotrienes (cysLTs). Although not exclusively produced by MCs, these mediators have powerful proinflammatory functions mediated through activation of specific cognate G-protein-coupled receptors. These products have been described to have potent vasoactive properties and may also mediate leukocyte chemotaxis, placing these mediators to an initial proinflammatory phase. Nevertheless, anti-inflammatory action has been ascribed to 15-deoxy-d(12,14)PGJ2 (15-dPGJ2), an in vivo metabolite of PGD_2. Binding of this metabolite to peroxisome proliferator-activated receptor-γ can induce apoptosis, first of infiltrating neutrophils and later of the macrophages recruited to clear these apoptotic cells, thereby promoting the clearance of the inflammatory infiltrate [16]. Similarly, stimulation of dendritic cells with PGD2 or 15-dPGJ2 inhibited their capacity to generate the Th1-promoting cytokine IL-12 and the Th1-active chemokine CXCL10 in response to the subsequent challenge with antigen or lipopolysaccharide. In a model of renal ischemia reperfusion in rats, 15-dPGJ2 improved renal function (plasma creatinine levels) and reduced the histological signs of renal injury. At the same time, 15-dPGJ2 markedly reduced the expression of inducible nitric oxide synthase (iNOS) and intercellular adhesion molecule-1 during reperfusion and activation

of nuclear factor-κB in renal cells [109]. Thus, certain metabolites of lipid-derived mediators may be useful anti-inflammatory agents and/or regeneration actors that enhance the tolerance of the kidney to renal dysfunction and injury.

CONCLUSION

MCs have now been established as important effectors in kidney disease. While only sparsely present in healthy subjects, they are an integral part of the inflammatory cell infiltrate appearing during disease development. Probably because of the role they play in allergy, they have been regarded as disease-aggravating effector cells, although this was mostly based on correlative data. However, recent experimental evidence imposes a more subtle view with regard to their involvement in renal disease processes, with numerous beneficial roles potentially played by these cells. This rejoins increasing data from the literature recognizing many positive functions of MCs in host defense mechanisms, detoxification and inflammatory diseases [6]. One of their primary functions is to serve as sentinels that participate in the initial inflammatory response by rapidly releasing mediators to counteract an injurious event. These mediators allow orchestration of a response pattern that, when appropriately regulated, funnels smoothly in an anti-inflammatory response and repair phase aiming to restore homeostasis. Besides providing crucial mediators for these various phases, MCs also importantly participate in the regulation of this response by the timed secretion of many immunomodulatory products (Fig. 6.3) that impact on the activity of other immune effectors including T cells and macrophages which participate in the resolution of inflammation. Most importantly, MC-produced mediators can also contribute to the regenerative response, for example, by the provision of growth factors and through the degradation of accumulated ECM proteins during the repair phase. Therefore, it may be postulated that the main role of MCs is to orchestrate remedial strategies to counteract the injurious event and to participate in repair and regenerative responses. However, it should be noted that while beneficial roles of MCs have clearly emerged, chronic stimulation or defective regulatory processes may shift the balance in the opposite direction and MCs may also aggravate the disease, as exemplified by opposite outcomes in related animal models of kidney disease. Therefore, the prime interest of any therapeutic activities is to favor the beneficial actions at the same time as suppressing the negative actions.

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CHAPTER

7

Role of Macrophages in Renal Injury, Repair and Regeneration

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OUTLINE

INTRODUCTION

Macrophages are key mediators in the pathogenesis of most types of primary and secondary human kidney diseases. Many studies in both immune and non-immune-initiated models of kidney disease have shown a clear association between macrophage accumulation and the development of renal injury [1 3]. However, there is emerging evidence that macrophages are pivotal players in the repair of tissue injury. Various strategies have been used to examine the role of macrophages in experimental renal injury including adoptive transfer of macrophages, the systemic depletion of macrophages, blockade of molecules involved in monocyte recruitment into the inflamed kidney and gene modification of macrophages [4 8]. This chapter will review the role of macrophages in renal injury and repair, and after a discussion about the different phenotypes of macrophages, show how experiments using adoptive transfer of different types of macrophages have increased our understanding of the biology of these cells (Table 7.1). Studies using stem cell progenitors of macrophages such as mesenchymal stem cells will not be included in this discussion (this is covered in Chapter 9).

TABLE 7.1 Studies of Adoptive Transfer of Macrophages in Renal Disease

Type of macrophage	Model	Method of delivery	Outcome on renal injury	Ref.
Classically activated macrophages	Adriamycin nephropathy	Intravenous	↑	[9]
IFN γ and/or dexamethasone stimulated macrophages	Nephrotoxic nephritis	Intravenous	↑	[10]
Wound healing (IL 4/IL 13 stimulated) macrophages	Adriamycin nephropathy	Intravenous	↓	[11]
Regulatory (TGF β/IL 10 stimulated) macrophages	Adriamycin nephropathy	Intravenous	↓	[12]
Bone marrow derived IL 4/IL 13 stimulated macrophages	Adriamycin nephropathy	Intravenous	No change	[13]
Wound healing macrophages	Diabetic nephropathy (STZ in eNOS knockout C57BL/6 mice)	Intravenous	↓	[14]
Wound healing macrophages	Diabetic nephropathy (STZ in wild type BALB/c mice)	Intravenous	↓	[15]
Bone marrow derived $CD11b^+$ $CD18^+$ cells transfected with adenovirus containing IL 1 receptor antagonist	Anti GBM glomerulonephritis	Intravenous	↓	[16]
Bone marrow derived macrophages with adenovirus expressing I κB	Nephrotoxic nephritis	Renal artery	↓	[17]
Bone marrow derived macrophages with adenovirus expressing IL 10	Nephrotoxic nephritis	Renal artery	↓	[18]
Alveolar macrophage cell line transfected with adenovirus expressing IL 4	Nephrotoxic nephritis	Renal artery	↓	[19]
Bone marrow derived mononuclear cells expressing endothelial cell markers	Thy1.1 glomerulonephritis	Renal artery	↓	[20]
Bone marrow derived macrophages	Unilateral ureteral obstruction	Intravenous	↓	[21]

IFN: interferon; IL: interleukin; TGF: transforming growth factor; I-κB: adenoviral I-κB; STZ: streptozotocin; GBM: glomerular basement membrane.

INJURY, REPAIR AND REGENERATION OF THE KIDNEY

Kidney disease, particularly in animal models, is characterized by an initial phase of injury, followed by a reparative phase and in some cases regeneration of kidney tissue. The injurious phase is characterized by tissue inflammation, while the reparative phase features the development of fibrosis. The regenerative phase involves proliferation of new cells, derived from either resident cells and/or bone marrow-derived (BMD) cells. This time-course model of renal disease has been elegantly demonstrated by Ricardo and colleagues [22], who studied mice that underwent unilateral ureteral obstruction (UUO) followed by reversal of UUO. Male C57BL/6J mice underwent UUO (using a vascular clamp) for 10 days followed by reversal of UUO (by release of clamp), and were then followed for 1, 2, 4 or 6 weeks. UUO was associated with tubulointerstitial fibrosis (increased collagen and hydroxyproline), tubular cell atrophy and dilatation, and macrophage accumulation. Reversal of UUO was associated with reduced macrophage numbers, decreased hydroxyproline and collagen expression, and restoration of glomerular filtration rate, as well as an increase in the number of viable tubules. This study showed the regenerative capacity of the kidney when the initial injury is removed.

Another study described a model of renal repair whereby mice underwent UUO for different lengths of time before ureteral reimplantation and contralateral nephrectomy. Seven weeks after reimplantation and contralateral nephrectomy, mean blood urea nitrogen levels and proteinuria were increased with longer duration of UUO. In contrast to the previous study, interstitial expansion, fibrosis and macrophage infiltration were similar in kidneys harvested after 10 days of UUO or following 10 weeks of ureter reimplantation,

suggesting that the inflammatory process persisted despite relief of obstruction [23].

The vast majority of studies of macrophages in kidney disease have investigated the role of macrophages in the initial phases of injury and repair. Modulation of macrophage function or number before the initiation of renal injury allows the investigation of the ability of macrophages to abrogate renal injury. Several studies show that the initial degree of macrophage infiltration correlates with subsequent fibrosis, and that blockade of macrophage infiltration (by either depletion or reduction of recruitment) prevents the development of fibrosis [24,25]. Ideally, investigating the capacity of macrophages to facilitate renal repair (rather than reduce injury) requires modulation of macrophage function and/or number after the injurious insult is removed. Therefore, animal models of kidney disease that use a toxic insult are ideally suited as the inciting event does not persist, unlike in immune models such as nephrotoxic nephritis. Models such as Adriamycin nephropathy, or UUO with reversal of UUO, are more suitable for this purpose. Therefore, interventions targeting infiltrating macrophages timed to occur after the toxic insult may be used to address the effect of macrophages on renal repair. Although studies using macrophages specifically for regeneration of kidney tissue are currently limited to BMD macrophages (discussed in this chapter and Chapter 9), this field is a highly active area of research [26].

MACROPHAGE PHENOTYPE

Macrophages are a diverse and dynamic population of cells that has the capacity to perform a wide range of critical functions. Macrophages can secrete a wide range of inflammatory factors, and thus are considered active participants rather than passive bystanders in mediating renal injury. The importance of macrophages in the pathogenesis of various kidney diseases has been investigated in human and animal studies. In human studies, macrophage infiltration is one of the hallmarks of severe and progressive renal disease, and the severity of injury correlates with the intensity of the macrophage infiltrate in patients with glomerulonephritis [1]. Evidence of the pathogenic role of macrophages in various experimental renal diseases has been derived from studies demonstrating that depletion of macrophages or blockade of monocyte recruitment to the kidney reduces renal injury, and that repletion of macrophages restores renal injury. In sites of renal tissue injury, macrophages are activated towards a proinflammatory (M1) phenotype with the high expression of tumor necrosis factor-α (TNF-α), inducible nitric oxide synthase (iNOS) and interferon-γ (IFN-γ). These M1 cells directly damage resident cells through the generation of reactive oxygen species (ROS), nitric oxide (NO), complement factors and proinflammatory cytokines. M1 can also increase the production of extracellular matrix and vasculature through the expression of metalloproteinases and vasoactive peptides. Furthermore, M1 macrophages produce profibrogenic cytokines such as transforming growth factor-β (TGF-β) which promote interstitial fibroblast and myofibroblast proliferation, and their secretion of extracellular matrix (ECM) proteins that accumulate to form a scar.

However, macrophages are a diverse group of cells that also includes subpopulations that have a beneficial rather than harmful role in resolution and repair of tissue injury. Originally, activated macrophages were considered as cells that secreted inflammatory mediators and killed intracellular pathogens [27]. These macrophages were also defined as classically activated macrophages (M1) following stimulation by lipopolysaccharide (LPS) or interferon (IFN-γ) in vitro. Now increasing evidence indicates that macrophages are also able to modulate immune responses through anti-inflammatory cytokines or regulation of T-cell function [28]. For example, decidual macrophages have been shown to possess an immunoinhibitory function at the maternal fetal interface. These cells express indoleamine 2,3-dioxygenase (IDO), which is able to reduce the ability of maternal T cells to mount an immune response against cells of the fetus [29]. In vitro, macrophages incubated with interleukin-4 (IL-4), IL-13 or glucocorticoids can develop a regulatory phenotype (also known as alternatively activated or M2 macrophages) and produce anti-inflammatory cytokines and inhibit T-cell proliferation.

The anti-inflammatory phenotype described above includes a spectrum of macrophages which are heterogeneous in their mode of stimulation and expression of chemokines and surface molecules. Mosser and Edwards [30] recently categorized anti-inflammatory macrophages into wound-healing macrophages (also known as M2a), which are activated by IL-4, and regulatory macrophages (also known as M2c), which are activated by Toll-like receptors (TLRs) in combination with other stimuli. However, macrophages associated with tumors can express characteristics of both wound-healing and regulatory macrophages, while macrophages in adipose tissue in obese individuals can convert from a wound-healing phenotype to a classically activated phenotype in response to adipocyte necrosis. Therefore, the paradigm of macrophage activation is changing. Instead of macrophages being regarded as fixed in phenotype, they are now regarded as responsive to changes in their environment, demonstrating plasticity in their nature. In Mosser and Edwards' model, macrophages may be classified into classically activated

(M1), wound-healing (M2a) or regulatory (M2c). Their phenotype may fall into a blend of any two of the above types (e.g. tumor-associated and obesity-associated macrophages) and may switch from one to another.

A key issue is what happens to macrophage phenotype during the course of kidney disease. Whereas depletion of macrophages early in disease may reduce fibrosis [24], depletion of macrophages later in disease may exacerbate it [21]. In the late stages of kidney disease, the degree of fibrosis correlates inversely with the degree of macrophage infiltration [31], suggesting that the macrophage infiltrate in later disease is antifibrotic rather than profibrotic. Evidence for a change in macrophage phenotype during the course of disease also comes from an animal model of hepatic fibrosis [32] induced by carbon tetrachloride in a transgenic mouse (CD11b-DTR) in which macrophages can be ablated by administration of diphtheria toxin. Conditional macrophage depletion when liver fibrosis was advanced resulted in reduced scarring and fewer myofibroblasts. Macrophage depletion during recovery, by contrast, led to a failure of matrix degradation. These data provide further evidence that functionally distinct subpopulations of macrophages exist in the same tissue and that these macrophages play critical roles in both the injury and recovery phases of inflammatory scarring.

MACROPHAGES IN DIFFERENT DISEASE MODELS

Classically Activated Macrophages in Adriamycin Nephropathy

Although the expression and behavior of classically activated macrophages have been studied extensively, the pathogenic importance of both macrophage number and activation status in kidney disease is not well understood. Significantly, classically activated macrophages can incite and exacerbate inflammation and fibrosis and thus have the potential to impair renal tissue repair.

A study conducted using adoptive transfer of macrophages by the present authors' research group explored the effect of activation status of macrophages in inducing renal injury [9]. This study was carried out using the Adriamycin (doxorubicin) nephrosis (AN) model of kidney disease in severe combined immunodeficient (SCID) mice. AN is a robust experimental model of human focal segmental glomerulosclerosis, characterized by changes in both tubulointerstitial and glomerular compartments. It can be induced in immunocompetent mice [33], and also in SCID mice that are homozygous for an autosomal recessive mutation that leads to an absence of lymphocytes [34]. The use of SCID mice allows the study of the role of macrophages in an in vivo kidney disease model without other potential interactions due to lymphocytes.

In Wang's study [9], SCID mice with AN were treated 6 days after Adriamycin injection with either resting (1×10^6 to 5×10^6) or activated (1×10^3 to 1×10^6) macrophages (M1) (injected intravenously), and the effects on kidney injury were examined after 4 weeks. Transfusion with resting macrophages (M0) in doses between 1 and 5 million cells per mouse did not exacerbate renal injury and fibrosis in comparison with control AN mice. However, transfusion with as few as 10^4 activated macrophages per mouse worsened renal injury and fibrosis. The injury caused by 10^5 and 10^6 activated macrophages was not different from that caused by 10^4 activated macrophages. Similarly, both serum creatinine and urinary protein excretion were significantly worse following transfusion with activated macrophages in doses as low as 1×10^4 (but not 10^3) cells per mouse, but were unaffected by resting macrophages in doses up to 5×10^6 cells per mouse in normal or AN mice. This finding emphasizes the importance of macrophage activation status in causing or exacerbating kidney injury [9]. Similar potency has been shown of IL-4/IL-13-activated macrophages (M2a), which were protective against injury in AN in SCID mice in doses as low as 10^4 per mouse [35].

Activation status of macrophages has also been examined by Nikolic-Paterson's group [10]. Exposure of macrophages to IFN-γ for 18 h (but not 3 h) before adoptive transfer caused a two-fold increase in the degree of proteinuria and glomerular cell proliferation compared with unstimulated cells. This was due to an increase in the number of transferred macrophages within the glomerulus and a significant increase in degree of renal injury per transferred glomerular macrophage [10].

An interesting finding in Wang's study was that transfusion of resting macrophages did not exacerbate kidney injury in comparison to that of untransfused AN mice, even at doses 500 times higher than those at which activated macrophages exacerbated injury. This may be related to the properties of resting macrophages. One study examined the properties and responsiveness to cytokines of macrophages purified from normal and nephritic glomeruli and found that macrophages from normal glomeruli did not generate nitric oxide spontaneously but did so only after treatment with IFN-γ and TNF-α [36]. Another reason may be related to the impaired ability of resting macrophages (M0) to accumulate in sites of (renal) inflammation. However, this result contrasts with that of Nikolic-Paterson's group, who reported that transfusion of resting macrophages did increase proteinuria and mesangial cell proliferation in anti-glomerular basement membrane (GBM) nephritis. The explanation for these contrasting results

may relate to differences in the models, with AN being primarily a model of innate immunity and anti-GBM nephritis of adaptive immunity in which macrophages act as an antigen-presenting cell to T cells. Moreover, under appropriate conditions, resting macrophages may serve as a reservoir for activated macrophages. This has been demonstrated in a two-shot model of Thy1.1 nephritis, in which resting macrophages become activated by interferon only after a second dose of anti-Thy1.1 antibody [37].

The mechanisms underlying the exacerbation of renal injury by activated macrophages have been examined. First, activated macrophages express high levels of major histocompatibility complex (MHC) II and CD86, which are important molecules in the initiation of adaptive immune responses. Second, activated macrophages express inflammatory mediators including nitric oxide and TNF-α, which are known to directly cause renal injury. It is noteworthy that macrophages isolated from AN kidney more than 3 weeks after adoptive transfer still exhibit upregulated inflammatory cytokine expression similar to that of the macrophages immediately after initial activation [9]. Studies from Rees' group demonstrated that macrophages separated from a focus of immunologically mediated inflammation in nephrotoxic nephritis were programmed in vivo, and were unresponsive to activating cytokines such as IL-4, TGF-β and TNF-α. Their studies raise the question of whether macrophage function alters with time during the resolution of inflammation. Thus, transfused macrophages appear to maintain their activation status within the inflammatory focus during the progression of renal injury [36].

To determine the fate of adoptively transferred macrophages, cells were labeled with the fluorescent tracking dye DiI. Fluorescently labeled cells were distributed to kidney, spleen and liver of AN mice 24 h after transfer. Very few of these cells were seen in spleen and liver at day 14 (8 days post-transfusion) and none were detectable at day 28 in AN mice transfused with activated macrophages, whereas in AN mice transfused with resting macrophages, their number in liver and spleen remained constant. In contrast, transfused activated macrophages from a dose of 1×10^4 accumulated progressively in kidneys up to day 28 after Adriamycin. The absolute number of transfused resting macrophages found in kidneys of AN mice, even with transfusion of 5×10^6 cells, was much less than the number of transfused activated macrophages, even with transfusion of as few as 1×10^4 cells. Macrophage recruitment to the kidney is an important step in initiation of inflammation and tissue destruction. Therefore, activated macrophages are more easily recruited into damaged kidney than are resting macrophages. In addition, transfused activated macrophages accumulate progressively at the site of injury [the kidney], rather than in other unaffected organs.

To clarify why activated macrophages accumulated more effectively in sites of injury than resting macrophages, macrophage mobility in response to CCL2 was examined [9]. Activated macrophages demonstrated greater mobility in response to CCL2 than resting macrophages. These data suggest that enhanced responsiveness of activated macrophages to chemotactic stimuli may be a key factor determining their accumulation at sites of injury. Moreover, in that study, activated macrophages expressed higher levels of CCR2, the receptor for CCL2, than resting macrophages. Izikson et al. [38] reported that CCR2 expression is consistent with the ability of monocytes to traffic to sites of inflammation. Furthermore, Xu et al. [39] also showed that inflammatory monocyte recruitment to inflamed tissue was coincident with a reduction in $CCR2^+$ cells in circulation after adoptive transfer of BMD macrophages.

In conclusion, activated macrophages, but not resting macrophages, exacerbate renal injury in murine AN. Activation status of macrophages determines whether and to what extent macrophages cause or exacerbate renal injury. Therapeutic targeting of pathogenic activated macrophages (M1 phenotype) rather than all macrophages is likely to be an effective approach to treating progressive renal disease.

Wound-healing (IL-4/IL-13-stimulated, M2a) Macrophages in Adriamycin Nephropathy

The precedent from other tissues such as lung and skin provides a convincing reason for believing that macrophages play an important role in tissue remodeling and repair. A subset of macrophages that secrete anti-inflammatory cytokines, promote angiogenesis and play a positive role in wound healing and tissue remodeling have been generally referred to as alternatively activated macrophages (M2). M2 cells induced by exposure to IL-4 and IL-13 (M2a) and deactivating cytokines such as IL-10 and TGF-β (M2c) are thought to suppress immune responses and promote tissue remodeling. M2 macrophages have been studied extensively in vitro with respect to their suppressive activity, secretion of anti-inflammatory cytokines and ability to modulate wound healing and angiogenesis. However, whether or not M2 can be potent immune regulators in vivo and used as therapy for kidney disease has not been clear until recently.

The adoptive transfer of both non-activated and LPS/IFN-γ activated macrophages can worsen inflammation in animal models of acute renal injury [10,40,41]. In contrast, inhibition of nuclear factor-κB (NF-κB) by adenoviral I-κB (I-κB) in macrophages leads to an anti-inflammatory phenotype, which has been shown to

ameliorate renal injury in an animal model of glomerulonephritis [17]. In addition, macrophages transfected with anti-inflammatory cytokines have been shown to reduce renal injury in animal models of glomerulonephritis (see below).

Adoptive transfer of cytokine-programmed wound-healing macrophages has the therapeutic potential to reduce renal injury [11]. To determine the effects of wound-healing macrophages in renal disease models independent of endogenous T and B cells, adoptive transfer of macrophages into SCID mice was performed. In this study, macrophages were isolated from spleens of BALB/c mice and stimulated with IL-4 and IL-13 to induce a wound-healing phenotype. These macrophages were then transfused into SCID mice with AN. In these studies, macrophages provided marked protection against both structural and functional damage in AN [11]. Renal injury in AN was marked and characterized by glomerular sclerosis, tubular atrophy and interstitial fibrosis with substantial mononuclear cell infiltration. Renal injury and fibrosis were significantly reduced in mice transfused with wound-healing macrophages compared with control AN mice (Fig. 7.1). Similarly, serum creatinine and urine protein were significantly improved with M2a macrophage transfusion compared with untransfused AN mice. This study provides direct evidence that ex vivo modulation of macrophages by cytokines to an anti-inflammatory phenotype can be an effective strategy for treating experimental chronic inflammatory renal disease. In comparison to other macrophage strategies for ameliorating renal injury in animal models such as genetic modification or chemokine blockade, cytokine manipulation of macrophages to the M2 phenotype provides many of the advantages without the associated risks. The advantages of this method include the simplicity of ex vivo macrophage modulation by cytokines, and the effectiveness of intravenous administration without the need for specialized delivery. Most important is the fact that it avoids limitations of gene therapy which include technical difficulties of gene delivery and risks of infection associated with the viral vector.

The potency of M2a is underscored by the observation that as few as 1×10^4 M2 are required to protect against renal injury [10]. These experiments delineate a lymphocyte-independent mechanism of protection

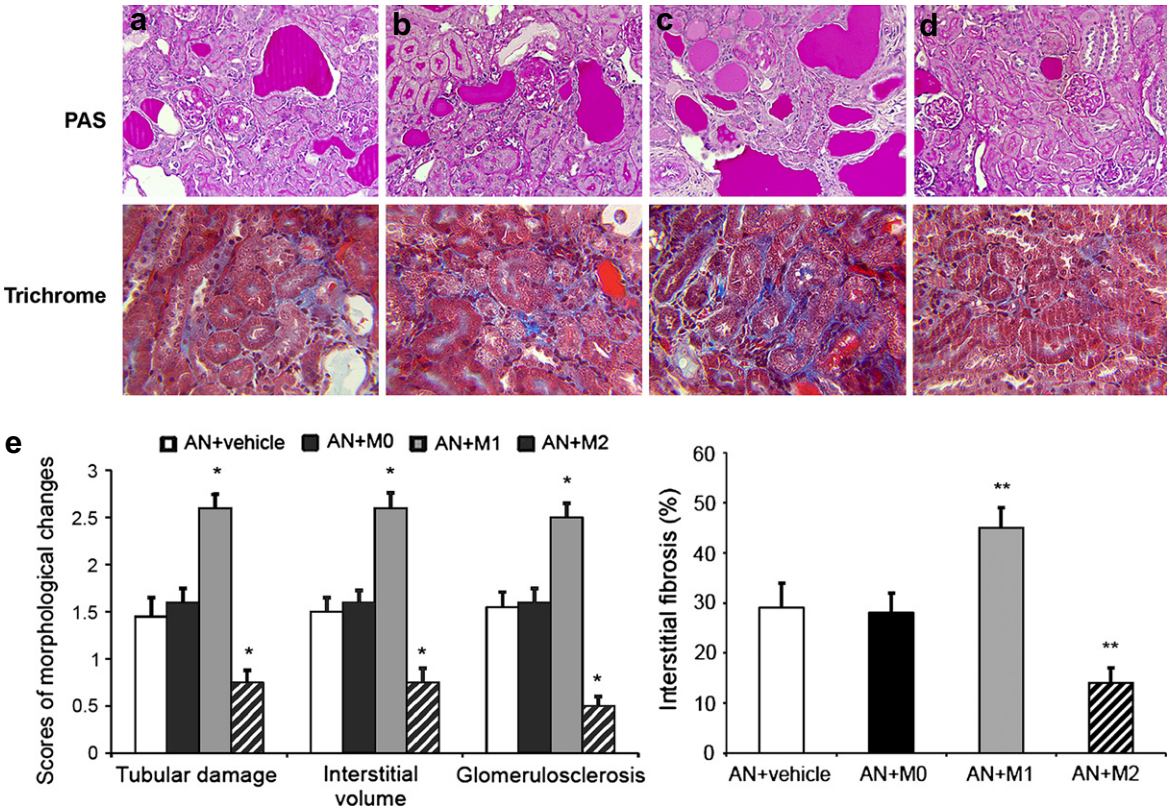

FIGURE 7.1 Effect of M1 and M2a on renal injury and fibrosis. Effect of M0, M1 and M2a macrophage transfusion on histological injury and development of renal fibrosis. (a–d) Representative periodic acid–Schiff and trichrome stained sections of renal cortices at day 28 (original magnification 200×): (a) saline injected Adriamycin nephrosis mice; (b) AN mice transfused with M0 macrophages; (c) AN mice transfused with M1 macrophages; (d) AN mice transfused with M2a macrophages. (e) Kidney injury was assessed semi quantitatively [periodic acid–Schiff (PAS)] and for fibrosis by point counting (trichrome). Each evaluation represents the mean ± standard error of the mean. $*p < 0.05$ vs other three groups (n 7 per group); $**p < 0.05$ vs other two groups. *(Reproduced with permission from Wang et al., 2007 [11].)* Please see color plate at the end of the book.

by M2a. Injected M2a homed to kidneys rather than secondary lymphoid organs and retained their phenotype beyond 4 weeks postinjection, similar to the behavior of adoptively transfused M1 macrophages in AN. The protective effect of M2a was associated with reduced accumulation and downregulated chemokine and inflammatory cytokine expression of the infiltrating host macrophages. This study provides direct evidence that the regulatory effects of M2a are target specific, robust and effective for treating experimental chronic inflammatory renal disease [11].

Another relevant issue is the stability of macrophage phenotype after initial cytokine exposure and transfusion in vivo, an important consideration in the reliability of M2a as a therapeutic approach for kidney diseases. One study has suggested that the microenvironment can reprogram the functional phenotype of macrophages [36]. However, M2a phenotype (expression of CCL17 and mannose receptor), once produced ex vivo, can be maintained for up to 4 weeks in in vitro culture (Fig. 7.2). M2 phenotype and function can also persist in the kidney in vivo, as shown by the following data: (i) M2 macrophages trafficked to inflamed kidney rather than to other unaffected organs, and accumulated progressively in inflamed kidney; (ii) markers of M2 phenotype including CCL17 and mannose receptor were also maintained in transfused IL-4/IL-13-stimulated macrophages for at least 4 weeks after transfusion; and (iii) in inflamed kidney, transfused M2a decreased the number of host macrophages and their expression of inflammatory cytokines including TNFα and CCL2 [11].

In conclusion, adoptive transfer of M2a macrophages in the AN model results in strong protection against renal structural and functional injury. M2a preferentially target the inflamed kidney, and the regulatory effects of M2a are sustained in vitro and in vivo. Thus, macrophage modulation ex vivo to M2a is an effective and reliable strategy for treating experimental chronic kidney disease.

Regulatory (IL-10/TGF-β-stimulated, M2c) Macrophages in Adriamycin Nephropathy

The phenotype of alternatively activated macrophages (M2) is diverse and characterized by anti-inflammatory and regulatory properties. Recently, M2 have been subdivided into M2a, which develop in vitro in response to IL-4 or IL-13, M2b in response to immune complexes and IL-1 or LPS, and M2c in response to IL-10, TGF-β or glucocorticoids [42]. M2c are usually regarded as deactivating macrophages because of their ability to downregulate proinflammatory cytokines and remodel tissue. The primary role of M2c appears to be modulation of the immune response through secretion of regulatory cytokines such as IL-10 and TGF-β.

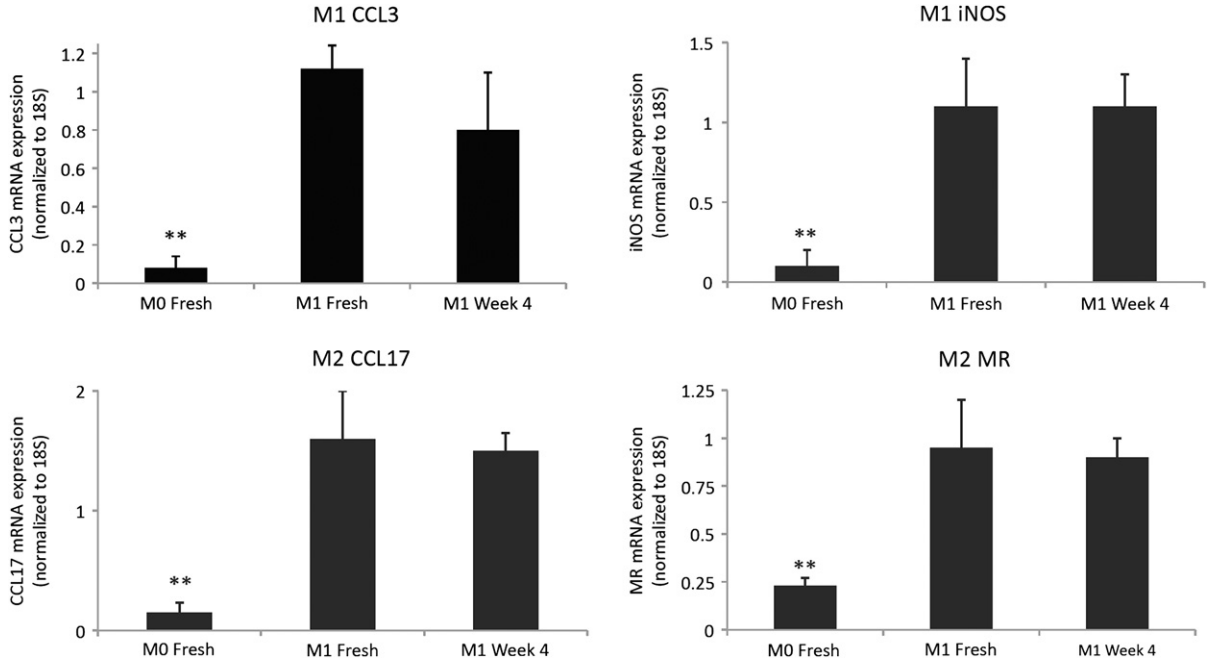

FIGURE 7.2 Persistence of macrophage phenotype after prolonged culture in vitro. Expression of CCL3 and inducible nitric oxide synthase (iNOS) mRNA by M1 macrophages (freshly separated or cultured for 4 weeks) and CCL17 and mannose receptor (MR) mRNA by M2 macrophages (freshly separated or cultured for 4 weeks) was assessed. $^{**}p < 0.01$ control vs other two groups. *(Reproduced with permission from Wang et al., 2007 [11].)*

IL-10 plays a critical role in limiting the duration and intensity of immune and inflammatory reactions. In macrophages, IL-10 inhibits production of proinflammatory cytokines such as TNFα, IL-6 and IL-12, and impairs antigen presentation by monocytes or macrophages via downregulation of MHC II and costimulatory molecules.

TGF-β is a pleiotropic cytokine which mediates a wide variety of effects on cellular differentiation, activation and proliferation. In macrophages and monocytes TGF-β regulates activation, cytokine production, host defense and chemotaxis. TGF-β acts as a negative regulator of CD163-expressing macrophages and inhibits LPS-induced macrophage production of the proinflammatory cytokines TNFα, IL-1α and IL-18 [43,44].

M1, M2a and M2b have been defined by a number of specific biomarkers: iNOS, Cox2 and proinflammatory cytokines for M1; mannose receptor, arginase, CCL17, YM1 and FIZZ1 for M2a; and IL-10 and other TLR-related molecules for M2b. However, there is less certainty about specific biomarkers for M2c. Recently, this group has identified B7-H4 as a specific functional biomarker of M2c. B7-H4, an inhibitory molecule, is a recently discovered member of the B7 family. It has been reported that B7-H4 is expressed in tumor-associated macrophages in human ovarian carcinoma [45]. It has also been shown that regulatory T lymphocytes (Tregs) can convey suppressive activity to macrophages by stimulating B7-H4 expression through IL-10 [46]. A striking finding in this study was that M2c, but not M2a, could convey suppressive activity to T cells via B7-H4, resulting in induction of Tregs. Thus, B7-H4 is potentially a specific biomarker and functional marker of M2c [12].

The effect of transfused M2c was examined in an immunocompetent model of AN. To examine the protective effects of M2c in murine AN, macrophages were separated from spleen and cultured for 48 h with TGF-β and IL-10. M2c cells (1×10^6/mouse) were infused at day 5 after Adriamycin administration. Age-matched normal mice and mice injected with Adriamycin alone were used as normal and disease controls. In vivo, adoptive transfer of M2c significantly reduced tubular atrophy, interstitial expansion and glomerulosclerosis in AN. M2c also reduced proteinuria and increased creatinine clearance significantly. This study demonstrated that M2c were able to diminish inflammatory infiltrates and renal structural and functional injury, as shown for M2a in SCID mice with AN. Moreover, it appeared that M2c were more effective than M2a in reducing renal histological and functional injury [13]. M2c not only deactivated and reduced inflammatory immune cells including $CD4^+$ T cells, $CD8^+$ T cells and renal macrophages, but also reduced tubular damage and glomerulosclerosis. These data demonstrate that macrophages polarized ex vivo to protective (wound-healing and regulatory) phenotypes may be a useful therapeutic strategy for chronic renal inflammatory disease [12].

M2c were found to express high levels of B7-H4, whereas M2a did not. B7-H4 is a member of the B7 family of costimulatory molecules. In the B7 family, the classic costimulatory molecules B7-1 and B7-2 provide critical positive costimulatory signals on interaction with CD28 on resting T cells, whereas B7-H4 is a negative regulator of T-cell responses in vitro that inhibits T-cell proliferation, cell cycle progression and cytokine production. Although mouse B7-H4 ligation of T cells has an inhibitory effect on T-cell activation, the regulatory mechanisms of B7-H4 remain to be defined. A striking finding was that M2c could convey suppressive activity to T cells via B7-H4, resulting in the inhibition of T-cell proliferation. Furthermore, M2c were also able to induce Tregs. This function of M2c was demonstrated both in vitro (partially via B7-H4) and in vivo (in renal draining lymph nodes).

The ability to promote differentiation of Tregs by macrophages has also been reported for lamina propria macrophages [47]. Denning et al. described a population of $CD11b^+F4/80^+CD11c^-$ macrophages in the lamina propria that express several anti-inflammatory molecules, including IL-10, but few if any proinflammatory cytokines. These macrophages can induce *Foxp3*$^+$ Tregs in the lamina propria and inhibit IL-17 production by lamina propria $CD11b^+$ dendritic cells. Recently, Murai et al. [48] showed that IL-10 secreted by a unique subset of macrophages in the lamina propria is responsible for maintaining *Foxp3* expression in Tregs in inflammatory conditions. Lamina propria macrophages produce IL-10, which signals via IL-10 receptor-β on Tregs to maintain *Foxp3* expression and Treg suppressive function. These IL-10-derived signals are essential for the maintenance of *Foxp3* expression in inflamed but not steady-state conditions, perhaps because of the suppressive effects of proinflammatory cytokines on *Foxp3* expression. It is plausible that unique bacterial flora, perhaps through TLR signaling, are responsible for the induction of IL-10 in these macrophages.

This group found that M2c are able to deactivate renal inflammatory (M1) macrophages. This ability of M2c has been shown in coculture with M1, and was further demonstrated in the AN model by their deactivation of endogenous renal macrophages. This novel finding of deactivation of inflammatory macrophages in kidney by M2c may explain their protective effects against renal injury [49].

An interesting question for further study is whether transfused macrophages interact with renal proinflammatory dendritic cells (DCs). Renal dendritic cells (rDCs) were first described as MHC class II-positive

cells with stellate and "mononuclear phagocyte" morphology in the rat renal interstitium, and have been isolated by using their membrane expression of CD11c. Furthermore, in the human kidney, there are two different DC subsets in the tubulointerstitium, $CD68^+$ $BDCA\text{-}1^+$ $DC\text{-}SIGN^+$/ $HLA\text{-}DR^+$ myeloid DCs and $BDCA\text{-}2^+$ $HLA\text{-}DR^+$ lymphoid DCs. Soos et al. [50] demonstrated that CX3CR1 rDCs form an organ-spanning network within the entire interstitial and mesangial space. rDCs have been shown to accumulate in peritubular and periglomerular interstitium in human lupus nephritis [51] and glomerulonephritis [52] and rat crescentic glomerulonephritis [53], indicating that DCs are recruited from the circulation into the site of renal injury. Dong et al. [54] showed that rDCs are a source of proinflammatory mediators in acute urinary obstruction and facilitate accumulation of IL-17 expressing T cells in the kidney. There is also evidence that recruited kidney DCs provide a mechanistic link between glomerular injury and the spread of damage to the tubulointerstitium [55]. Moreover, it has been shown that interstitial macrophages are capable of altering DC function in lung and intestinal disease [55]. Therefore, the interactions between macrophages and DC have potential importance in renal inflammatory disease.

A major consideration in evaluating M2c as a therapeutic approach is the potential for phenotype switch in vivo. Macrophages, unlike T cells which undergo irreversible differentiation upon antigen stimulation, can retain their plasticity and respond to different environmental signals. Numerous studies have examined the stability and longevity of activated macrophages within the host. Several studies have indicated that the phenotype of macrophages can change in vivo over time; for example, it was reported that macrophages in the earliest stages of cancer resembled classically activated macrophages, yet, with tumor growth, macrophages developed a regulatory phenotype [56]. Another example is the observation that macrophages in adipose tissue of non-obese humans have a wound-healing phenotype, yet can switch to a proinflammatory phenotype in obese individuals [30]. It is unclear whether phenotypic deviation can occur in transfused anti-inflammatory macrophages used to treat various diseases. This is very important as transfused macrophages may cause more harm than benefit if they switch from an anti-inflammatory to a proinflammatory phenotype. Importantly, it was previously shown that macrophage phenotype after initial ex vivo cytokine modulation was stable in vitro and little changed in vivo despite microenvironmental changes during the course of disease [11]. However, that study was performed in SCID mice, which are deficient in T and B cells, and the stability of the M2 phenotype could possibly be explained by the lack of cognate immune cells and their secreted proinflammatory cytokines. A more recent study examined the effect of transfused macrophages into an immunocompetent model of AN and found that the phenotype of transfused macrophages did drift, but not towards a distinct M1 or M2 phenotype. The suppressive features of transfused M2c were partially lost, as shown by their reduced expression of anti-inflammatory genes, such as TGF-β, arginase and mannose receptor. The expression of proinflammatory genes by M2c was elevated at weeks 1 and 2, but diminished at week 3. This phenomenon indicates that transfused M2c do not convert to a typical M1 phenotype, and that their phenotype drifts across different stages of disease [49].

Another concern in using M2c as a therapeutic approach is their potential profibrotic effect. M2c express high levels of TGF-β [57], a growth factor linked to renal fibrosis. TGF-β acts on a transmembrane receptor through Smad proteins to regulate key profibrotic genes such as collagen. Macrophage-derived TGF-β may also promote fibrosis by paracrine activation of matrix-producing myofibroblasts and promotion of epithelial mesenchymal transition of tubular epithelial cells (TECs) into myofibroblasts [58]. However, current studies by the present authors in AN mice treated with M2c showed a reduction rather than promotion of renal fibrosis, arguing against this concern. Possible reasons underlying the reduction of fibrosis by M2c include their deactivation of host macrophages and reduction of other profibrotic stimuli. IL-10 and TGF-β secreted by M2c suppress immune responses, dampen inflammation and promote tissue remodeling in early stages of disease, and thereby can further reduce fibrosis. Another possible explanation for the observed reduction in fibrosis is that transfused M2c, after executing their anti-inflammatory effects, decrease their secretion of TGF-β. This group demonstrated that TGF-β expression by transfused M2c in inflamed kidney did reduce progressively from week 1, towards levels seen in resting macrophages. However, IL-10 expression of transfused M2c remained at high levels throughout the observed course of disease. These results suggest that transfused M2c may have a considerable ability to limit inflammation and reduce profibrotic effect as the disease progresses.

In vivo proliferation is another potential concern in the therapeutic use of transfused M2c. It has been reported that fetal macrophages have a high proliferative capacity, but that the proliferative capacity of macrophages declines once permanent hematopoiesis is established [59]. The present authors demonstrated that M2c isolated from the spleen do not proliferate in vitro for up to 3 weeks, nor do transfused M2c isolated from kidneys of AN mice.

A key question is whether M2c exist in various kidney diseases. Several studies have shown some clues to the existence of M2c in vivo. Tumor-associated macrophages (TAMs) are a paradigm for polarized M2 mononuclear phagocytes. In the tumor microenvironment, IL-10, TGF-β and PGE_2 are produced by a variety of tumor cells and by TAM themselves. IL-10 promotes the differentiation of monocytes to mature macrophages and blocks their differentiation to DCs. In addition, IL-10 induces TAM to express M2c-related functions, such as scavenging debris, angiogenesis, tissue remodeling and repair. TAMs express high levels of the mannose receptor (MR) and are poor at presenting antigen. They also have an IL-10^{high}IL-12^{low} cytokine repertoire. In addition, $CD11b^+F4/80^+$ macrophages in the lamina propria express several anti-inflammatory molecules, including IL-10 and TGF-β, which express an M2c-like phenotype. These M2c-like macrophages can induce $Foxp3^+$ regulatory T cells in the lamina propria.

In conclusion, M2c, also known as wound-healing macrophages, are capable of repair of renal injury, and show potential as a means of treating kidney disease.

Macrophages in Unilateral Ureteral Obstruction

UUO is a well-described model that enables study of the factors that contribute to renal fibrosis. The importance of macrophages in UUO was first described by Schreiner [60] in 1988. However, several studies demonstrate an inverse correlation between macrophage number and the degree of fibrosis [31], suggesting that there is a subpopulation of macrophages that is anti-fibrotic. Depletion of macrophages by cyclophosphamide increased fibrosis when administered late (day 14 post-UUO) but not when administered early (day 5 post-UUO). Studies in UUO have shown that BMD macrophages skewed towards an anti-inflammatory phenotype can promote fibrosis via their production of galectin-3 [61].

Data from UUO studies suggest that macrophages tend to be profibrotic/anti-inflammatory or antifibrotic/proinflammatory. In addition to their production of TNF-α and nitric oxide, classically activated macrophages produce matrix metalloproteinases that degrade extracellular matrix and induce TEC (and potentially myofibroblast) apoptosis, thereby reducing TEC production of TGF-β. In contrast, regulatory (IL-10/TGF-β-stimulated) macrophages generate anti-inflammatory cytokines such as TGF-β and galectin-3 which favor myofibroblast activation and extracellular matrix production [31]. As noted previously, macrophages have the capacity to drift between different phenotypes, presumably in response to their microenvironment, and can share properties of any of the recognized phenotypes. Therefore, a challenge for future studies is to find macrophages with an anti-inflammatory antifibrotic phenotype that can be used therapeutically.

Wound-healing (M2a) Macrophages in Diabetic Nephropathy

Macrophages are an important orchestrator of the immune response in diabetic kidney disease. Tubulointerstitial inflammation is a common feature in human diabetic nephropathy, in which macrophages are major components [62]. In addition, both macrophages and lymphocytes ($CD4^+$ and $CD8^+$) are present within the glomeruli of diabetic subjects with kidney disease.

This laboratory has investigated the effect of adoptive transfer of M2a in experimental diabetes. Studies were performed in streptozotocin (STZ)-induced type 1 diabetic wild-type C57BL/6 mice. Kidneys from diabetic mice infused with M2a had less tubular damage, glomerular hypertrophy and interstitial expansion than diabetic mice without M2a. M2a also reduced pancreatic inflammatory cell infiltration and plasma levels of glycosylated hemoglobin A1c. These findings demonstrated that macrophages that have been modified ex vivo by IL-4 and IL-13 to a regulatory phenotype may reduce the severity of both diabetes and diabetic nephropathy. Since glycemic control and islet cell damage were improved in the macrophage-treated group, these cells may reduce renal injury by improving the diabetic milieu [63].

This group also examined the effect of M2a in a murine model of diabetic nephropathy associated with more severe renal injury. Croker and Johnson's laboratory [64] recently described a model of diabetic nephropathy using the endothelial nitric oxide synthase knockout (eNOS −/−) mouse. Streptozotocin-injected eNOS −/− mice developed severe glomerulosclerosis, mesangiolysis and arteriolar hyalinosis associated with hypertension reflecting many of the renal pathological changes in type 1 diabetes. Similar changes were also observed in eNOS knockout mice cross-bred with db/db mice, a model of nephropathy in type 2 diabetes [65]. The present group examined the therapeutic efficacy of IL-4/IL-13-stimulated macrophages (M2a) in a murine model of diabetic nephropathy. Diabetic renal injury was induced by STZ injection in eNOS −/− mice. Diabetic mice treated with M2a had significantly less arteriolar hyalinosis, glomerulosclerosis and tubular damage than control diabetic mice, while functional injury was similar. Glycemic control was similar in both groups. Adoptive transfer of M2a significantly reduced the amount of renal injury due to diabetes. These data suggest that M2a treatment protects against diabetic renal injury by inhibiting arteriolar hyalinosis

and subsequent glomerular damage [Lee and Harris, unpublished and [14]].

Much more work needs to be done before results of these studies can be translated to the clinic. Questions such as the fate of M2a traffic in diabetic kidney disease, the length of time that M2a last in vivo, effectiveness of other M2 phenotypes such as M2c, and their potency remain unanswered. It will also be important to discover whether M2a can ameliorate renal injury in established diabetic nephropathy, a circumstance which more closely resembles that seen in the clinic.

Macrophages in Acute Kidney Injury

Acute kidney injury (AKI) is characterized by infiltration of macrophages, neutrophils, lymphocytes and natural killer (NK) cells soon after the initiation of injury. In the most common model of AKI, ischemia reperfusion injury (IRI), interruption of blood flow to the kidney is followed within minutes by injury to renal parenchymal cells, particularly TECs. TEC injury (necrosis, as opposed to apoptosis) is followed by release of cytokines, complement factors and chemokines that recruit immune cells into the region of damage. After several weeks of IRI recovery phase, fibrosis may occur. The macrophage is the dominant cell type within the cellular infiltrate after IRI, localizes to the outer medulla of the postischemic kidney and persists well into the recovery phase [66]. Depletion studies using clodronate have demonstrated that macrophages promote tissue injury in AKI following the initial phase and fibrosis in the recovery phase [67]. Similarly, inhibition of macrophage recruitment in osteopontin-knockout mice inhibits fibrosis in the recovery phase of IRI [68], suggesting a role of macrophages in the development of fibrosis after the initial phase of injury.

However, it is possible that these depletion studies affect both proinflammatory and regulatory/anti-inflammatory macrophages. Other studies have suggested that macrophages may be involved in the prevention and repair of injury following AKI. Macrophages mediate protection against early renal injury in IRI by the production of heme oxygenase-1, an enzyme that prevents free radical formation [69]. Vinuesa's group investigated the effect of macrophages in the late phase of IRI. Clodronate depletion before induction of IRI was followed by adoptive transfer of RAW 264.7 cells and led to greater regeneration of cells within the kidney compared to non-transfused mice, as shown by positive staining for the proliferation markers, stathmin and proliferating cell nuclear antigen (PCNA), and increased expression of the anti-inflammatory cytokine IL-10 [70]. Further evidence comes from Kelley's group, who showed that colony stimulating factor-1 derived from macrophages and tubular epithelial cells promotes kidney repair in the recovery phase after IRI [71].

Therefore, the role of macrophages in AKI is still not clear. Current data suggest that macrophages may promote renal injury in the immediate postischemic period, but are also involved in repair in the late recovery phase. These data are consistent with the paradigm that macrophages are phenotypically diverse and play opposing roles in tissue injury and repair.

ALTERNATIVE METHODS FOR STIMULATING MACROPHAGES

Adenovirus-transfected Macrophages

Another method for changing the phenotype of macrophages is by adenovirus transfection of gene(s). Yokoo et al. [16] used an adenovirus vector expressing interleukin-1 receptor antagonist (IL-1RA) in BMD CD11b-positive/CD18-positive cells. When administered to mice with anti-GBM disease, these IL-1RA transfected cells tracked to glomeruli, increasing local glomerular expression of IL-1RA, and led to a reduction in albuminuria and glomerular, as well as tubulointerstitial injury.

Inhibition of macrophage NF-κB by I-κB also renders macrophages anti-inflammatory by inhibiting their proinflammatory cytokine production while preserving IL-10 synthesis. Adoptive transfer of BMD macrophages transduced with adenovirus expressing I-κB reduced glomerular infiltration and activation of host macrophages, and reduced functional and histological renal injury in a rat model of nephrotoxic nephritis [17]. The same group then performed a similar experiment, using BMD macrophages transfected with adenovirus expressing IL-10, with comparable results [18]. Macrophages transfected with an IL-4 expressing adenovirus-borne vector also reduce glomerular inflammation in this model [19].

ALTERNATIVE SOURCES OF MACROPHAGES

Bone Marrow-derived Macrophages

BMD macrophages may themselves be capable of modulating renal injury. Nishida and colleagues [21] demonstrated an increase in renal interstitial fibrosis in mice with UUO administered with cyclophosphamide. The profibrotic effect of cyclophosphamide in UUO was associated with a marked reduction in macrophage interstitial infiltration. Adoptive transfer of BMD macrophages (of which over 90% were F4/80 positive) led to

their infiltration within the renal interstitium and amelioration of fibrosis. However, interpretation of these studies requires caution as BMD mononuclear cells may develop into endothelial lineage cells as well. Uchimura and colleagues [20] demonstrated that culture of BMD mononuclear cells for 6 days in culture medium favoring endothelial cell development (Dulbecco's modified Eagle's medium-containing medium supplemented with fibroblast growth factor and vascular endothelial growth factor on fibronectin-coated plates) produced cells expressing markers of endothelial cells, and when injected into the renal arteries of rats with Thy1.1 glomerulonephritis, protected against endothelial injury and mesangial cell activation.

The authors' laboratory has examined the effects of BMD macrophages stimulated ex vivo by IL-4/IL-13 in AN. In vitro, BMD macrophages expressed IL-10, TGF-β, mannose receptor and arginase, as well as suppressed effector macrophages and cytotoxicity of $CD8^+$ T cells. However, BMD macrophages did not reduce tubular cell atrophy, interstitial expansion and glomerulosclerosis, or functional injury in AN. To investigate the failure of BMD macrophages to protect against AN, the transfused macrophages were separated from the kidney and examined. Expression of anti-inflammatory cytokines and regulatory molecules by BMD macrophages was dramatically reduced in inflamed kidney. The loss of suppressive function was linked to their proliferation in inflamed kidney. Macrophages from bone marrow proliferated in the kidney, and the divided cells did not express the regulatory phenotype of M2a. Therefore, BMD macrophages (stimulated ex vivo by IL-4/IL-13) did not protect against renal structural and functional injury in murine AN. This was due to the loss of their regulatory phenotype during proliferation [72].

Therefore, there is conflicting evidence about the effectiveness of BMD macrophages in ameliorating renal injury. Further discussion on the role of BMD cells is included in Chapter 9.

REGENERATIVE POTENTIAL OF MACROPHAGES IN KIDNEY DISEASE

The kidney, and in particular TECs, have a remarkable capacity to regenerate. The ability of macrophages to induce regeneration in kidney tissue has been best examined in studies of their effect on TECs. Kelley's group [71] found that colony-stimulating factor-1 administration led to increased macrophage-dependent and independent TEC proliferation in kidneys exposed to IRI. Moreover, macrophage-derived galectin-3 is an important inhibitor of TEC proliferation in IRI [73]. To determine directly the effect of macrophages on regeneration, Vinuesa et al. [70] depleted macrophages using clodronate and then performed adoptive transfer of a macrophage cell line (RAW 264.7) to mice with IRI. Mice transfused with macrophages 72 h after IRI exhibited greater regeneration as shown by staining for stathmin and PCNA, whereas mice transfused earlier than 72 h post-IRI did not show significant regeneration. This study suggests that when adoptive macrophage transfer is performed in the regenerative phase of renal disease, this not only leads to better tissue preservation, but also contributes to increased regeneration. Both Kelley's and Igarashi's groups independently showed using bone marrow chimeras that proliferating TECs were derived from resident TECs rather than bone marrow [74,75]. Regenerative cells may also be derived from fusion of macrophages with bone marrow cells [76], or with themselves [77], but account for only a minority of regenerated TECs.

CURRENT LIMITATIONS IN THE USE OF MACROPHAGES AS CELLULAR THERAPY IN KIDNEY DISEASE

The manipulation of regulatory cells to treat and prevent chronic kidney disease has great potential; however, major hurdles will need to be overcome before strategies such as adoptive transfer of regulatory cells can be used for treatment in humans.

First, adequate purity and sufficient numbers of macrophages from humans for adoptive transfer have been difficult to achieve thus far. Identifying specific surface markers of regulatory cells (e.g. B7-H4 for M2c) and optimizing culture methods (e.g. use of gene transfection or cytokine stimulation with IL-4/IL-13 in vitro) will help to achieve these aims.

Second, the dose, timing and frequency of use of regulatory cells need to be optimized in human renal disease. Research has focused on the effect of regulatory cells given before or early after onset of renal injury, whereas their effect in advanced disease is not known. Further studies will need to look at their effectiveness in late renal injury. In addition, renal disease has many causes (both immune and non-immune), and so regulatory cell immunotherapy may be useful only for selected patients: the challenge will be to define the responsive group(s). Selecting suitable patients for regulatory cell immunotherapy will involve knowledge on which patients are at highest risk of renal disease, and identify patients with renal disease at an early stage when response to therapy is more likely.

Third, suppressing the immune system with regulatory cells brings with it associated risks, i.e. malignancy and infection, both due to immunosuppression per se, and also transfer of unwanted tumor or infectious agents with the regulatory cells. Optimization of methods of

detection of infectious agents may reduce the risk of transmitting infection.

Fourth, the investigation of the properties of the different phenotypes of macrophages has almost exclusively been done in animal studies and it is too early to extrapolate these data to humans.

Therefore, although it is an exciting prospect that regulatory cells may be used to dampen or even prevent renal disease, greater understanding of this therapeutic strategy, the diseases that may benefit from it and vigorously designed clinical trials are required before these therapies can be applied generally to humans with renal disease.

The past 5 years have seen an exponential growth in our understanding of regulatory immune cells and their role in autoimmune disease, malignancy and transplantation tolerance. Recent data have demonstrated a protective role of regulatory macrophages in dampening glomerular and tubulointerstitial inflammation in a number of models of renal injury. Future research will need to concentrate on how best to harness this ability to protect against renal injury and at the same time avoid systemic immunosuppression. Such studies may pave the way for the clinical use of regulatory cells for both treatment and prevention of renal disease.

CONCLUSION

Macrophages are a heterogeneous group of cells, originally named for their ability to phagocytose, but now understood to be capable of anti-inflammatory as well as proinflammatory function. Wound-healing macrophages (M2a) and regulatory macrophages (M2c) are remarkably potent at reducing renal injury in a number of different renal disease models, and do so by tracking to sites of inflammation within the kidney. These macrophages are capable of direct effects on surrounding tissue, modulation of resident macrophages and induction of regulatory T lymphocytes, thereby reducing the extent of renal inflammation and fibrosis. Macrophages are important across the whole spectrum of kidney disease (non-immune and immune). Understanding the biology of macrophages and their ability to repair renal tissue will enable the future discovery of therapies for kidney disease.

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CHAPTER

8

T-cell Contribution to Injury and Regenerative Processes in Kidney Diseases: Focus on Regulatory T Cells

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OUTLINE

OVERVIEW OF T CELLS IN KIDNEY DISEASES

T cells are key participants in immune responses occurring in many different kidney diseases. The injured kidney not only is the target of the immune system but also actively participates in aggravating or suppressing intrarenal immune responses. Ischemic acute kidney injury (AKI) is often simulated by animal models of ischemia reperfusion injury (IRI). Postischemic kidneys per se contribute to immune responses by recruiting inflammatory cells including T cells and other leukocyte subsets, and generating proinflammatory cytokines and chemokines (Fig. 8.1) [1,2]. Chemokines involved in the recruitment of mononuclear cells, such as CXCR3, stromal cell-derived factor (SDF)-1/CXCL12 and monocyte chemotactic protein-1 (MCP-1)/CCL2, have been directly implicated in the pathogenesis of renal injury after IRI [3 5]. Postischemic kidneys also recruit leukocytes by upregulating the quantity and avidity of adhesion molecules, and a series of steps occur which in turn increases microvascular permeability. Anti-intercellular adhesion molecule-1 (ICAM-1) antibody was shown to protect normal mice from renal IRI, and many studies have demonstrated similar findings in different experimental models [6]. IRI was shown to increase intrarenal vascular permeability and facilitate extravasation of leukocytes by interrupting the integrity of the renal vascular endothelium [7,8]. T cells were suggested to contribute directly to the increased renal vascular permeability after IRI, potentially through T-cell cytokine production, since the production of tumor necrosis factor-α (TNF-α) and interferon-γ (IFN-γ) was increased in CD3 and CD4 T cells from blood and kidney after ischemia and since renal vascular

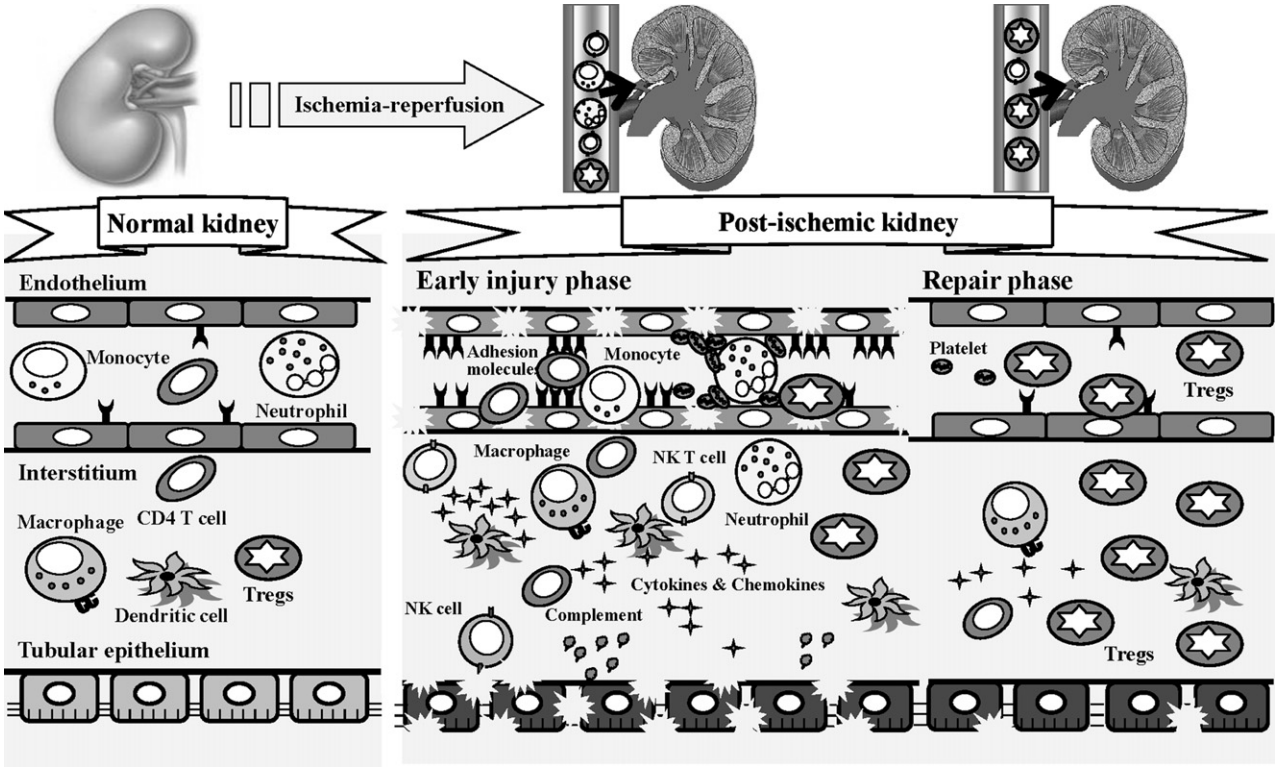

FIGURE 8.1 Regulatory T cells (Tregs) participate in the repair process of postischemic kidneys. Immune response is initiated in the post ischemic kidneys by resident immune cells and rapid influx of immune cells through the disrupted endothelium. T cells, specifically CD4 T cells, contribute to the development of renal injury with major effector cells in the innate immune system such as neutrophils, macrophages and natural killer (NK) cells. The trafficking of Tregs into the postischemic kidney is enhanced with time after ischemia–reperfusion injury and Tregs facilitate the tubular repair process. *[Modified with permission from Jang and Rabb, 2009 [2].]*

permeability measured with extravasation of Evans blue dye was attenuated in CD3 T-cell-deficient mice after IRI [9].

Advances in T-cell biology have fostered better understanding of the role of different T-cell subsets in kidney diseases [10]. Among several subpopulations of T cells, regulatory T cells have been suspected to have a potential role in the regenerative processes in diseased kidneys. It is important to review some background about these fascinating cells. Regulatory T cells were first identified about 30 years ago as "suppressor" T cells capable of suppressing antigen (Ag)-specific responses and transferring tolerance in animal models [11]. The current characteristics of regulatory T cells expressing CD4 and CD25 [the α-chain of interleukin-2 (IL-2) receptor] were first elucidated by Sakaguchi et al. [12]. This study demonstrated the lack of $CD4^+CD25^+$ T cells in neonatally thymectomized mice that developed a fatal autoimmune disease. Transfer of $CD4^+CD25^+$ T cells from normal to thymectomized mice prevented the development of autoimmune disease in thymectomized mice. Nuclear transcription factor Foxp3 (forkhead box P3) was reported as the master gene of $CD4^+CD25^+$ T cells regulating the development and function of these cells [13 15]. Differentiation of most $CD4^+CD25^+$ T cells occurs in thymus after Foxp3 induction in few thymocytes with high affinity to self-Ag major histocompatibility complex (MHC), and these $CD4^+CD25^+$ T cells are called naturally occurring regulatory T cells (nTregs), while their peripheral counterparts are called inducible Tregs (iTregs) [16]. Transfection of Foxp3 in $CD4^+CD25$ T cells was reported to convert them into cells demonstrating suppressor capacity and overexpression of Foxp3 in transgenic mice induced suppressor capacity in $CD4^+CD25$ and CD4 $CD8^+$ T cells [13,17]. However, the importance of Foxp3 as the key factor of Tregs at present is questionable because expression of Foxp3 is induced after TCR stimulation without Treg development and Foxp3 is restricted to Tregs in mice, but not in humans, in which Foxp3 expression occurs in recently activated naïve T cells as well as in memory T cells [18,19]. Although no or low expression of CD127 (IL-7) and the presence of CD27 as a surface marker have been reported as characteristics of Tregs, these markers are yet to be validated [20]. There is another subpopulation of CD4 T cells with suppressor capacity called Tr1 cells. Tr1 cells are known to be generated in periphery from naïve $CD4^+CD25$ T cells on Ag

stimulation with limited costimulation conditions by induction of IL-10 and immature dendritic cells [21,22]. Tr1 cells inhibit naïve and memory T cell responses by secreting suppressor cytokines including IL-10 and transforming growth factor-β (TGF-β), and migrate to inflammation sites, whereas nTregs basically migrate to lymph nodes. Another subset of iTregs is T_H3 cells, which share several characteristics with Tr1 cells and secrete TGF-β [23]. No specific surface marker has been reported regarding Tr1 and T_H3 cells.

Regulatory T cells expressing CD4 and CD25 (Tregs), the best known subset of regulatory cells, were expected to participate in reducing damage and enhance repair in diseased kidneys based on their regulatory effect found in typical immunological diseases such as autoimmune disease and transplantation [12,24]. Tregs have been reported to prevent organ-specific autoimmunity and modulate allogeneic immune responses inducing graft tolerance in transplantation, in both experimental and clinical settings [12,25,26]. Recently, Tregs have been under active investigation in several kidney diseases.

This chapter will discuss the diverse role of T cells in immune responses that occur in several kidney diseases and focus on the regulatory effect of T-cell subsets contributing to the regenerative processes. Kidney diseases in which T cells are implicated in the pathophysiology can be categorized into three different diseases: glomerulonephritis, acute kidney injury (AKI), and transplantation. The role of T cells will be discussed in more detail for each disease.

GLOMERULONEPHRITIS

Glomerulonephritis remains a common cause of chronic kidney disease (CKD) and end-stage renal disease (ESRD). Severe acute or persistent chronic inflammation may result in glomerulosclerosis, tubular atrophy and interstitial fibrosis with deterioration of renal function. The reversal of scarring and the restoration of normal glomerular structure and integrity are required for renal repair following glomerulonephritis. It is notable that severe renal inflammation may be completely resolved spontaneously or by immunosuppressive and conservative treatments in most cases of poststreptococcal glomerulonephritis (PSGN) and in some cases of minimal change disease (MCD). However, other types of glomerulonephritis usually leave different forms of sequelae resulting in renal functional and structural deterioration. Glomerulonephritis models can be classified into two different categories according to a traditional concept of pathophysiology: "immune"-mediated and "non-immune"-mediated glomerulonephritis models.

Immune-mediated Glomerulonephritis

Immune-mediated glomerulonephritis models can be divided further into three different entities: Heymann's nephritis, antiglomerular basement membrane (GBM) disease and lupus nephritis. In Heymann's nephritis (a rat model of membranous nephropathy), T cells infiltrate in glomerulus and interstitium. Monoclonal antibody treatment against T-cell receptor (TCR) α/β, CD4 and CD8 delayed onset of proteinuria, totally prevented proteinuria and markedly reduced proteinuria, respectively [27]. Permanent CD8 T-cell depletion was reported to prevent proteinuria in active Heymann's nephritis, suggesting the essential role of CD8 T cells in the development of glomerular injury in Heymann's nephritis [28].

Tregs were shown to be protective in an animal model of anti-GBM disease. A recent study performing Treg transfer before induction of anti-GBM disease with rabbit antimouse GBM antibody showed markedly reduced functional and structural renal injury in the Treg transfer group [29]. In this report, green fluorescent protein (GFP)-labeled Tregs demonstrated localization of transferred Tregs in the renal-draining lymph nodes and spleen. However, immune complex formation in the glomeruli was not reduced in Treg-treated mice. These findings suggest that Tregs reduce end-organ damage or enhance repair by limiting kidney-specific immune cell activation within regional lymph nodes. There have been studies reporting the existence of Ag (type IV collagen)-specific Tregs in Goodpasture's syndrome patients. Collagen-specific T cells were proinflammatory during the active phase of the disease, but were regulatory when disease became convalescent [30]. The subset of T cells with regulatory function was identified as an IL-10-producing population of CD4 T cells [30,31].

Studies have shown that Tregs are implicated in the pathogenesis of lupus nephritis [32,33]. Foxp3-transduced T cells suppressed the production of autoantibodies in autoimmune-prone CD40L transgenic mice [33]. However, adoptive transfer of Tregs did not suppress the development of lupus nephritis despite inhibiting autoantibody production in lupus-prone mice, implying the existence of a specific population of Tregs involved in lupus nephritis [32]. Late-onset treatment with a CCR1 antagonist that inhibits T-cell infiltration prevented progression of lupus nephritis in a murine lupus model, suggesting a role for T cells in the later phase of lupus nephritis [34].

Non-immune-mediated Glomerulonephritis

Non-immune-mediated glomerulonephritis models include adriamycin nephropathy (AN) and glomerular

compromise in mercuric chloride ($HgCl_2$)-induced nephrotoxicity.

The important pathogenic mechanisms of AN (a rodent model of focal segmental glomerulosclerosis) were shown to include immune responses, unlike its nomenclature. Several studies have reported that T cells are an important mediator of immune response occurring in AN. In contrast to Heymann's nephritis, CD4 T cells were shown to have a protective role against the progression of AN. Depletion of CD4 T cells aggravated glomerular and interstitial injury in AN [35]. Another study reported different data with severe combined immunodeficient (SCID) mice developing AN to a similar extent to wild-type mice, suggesting that T cells are not critical in the development of AN [36]. However, a subsequent study showing that Foxp3-transduced CD4 T cells exhibited a protective effect by reducing glomerulosclerosis, tubular damage and interstitial infiltrates suggested a therapeutic potential of Tregs in this model [37]. Another report supported a beneficial role of Tregs in AN in which SCID mice reconstituted with Tregs expressing high levels of Foxp3 showed reduced glomerulosclerosis and tubular injury with significantly less macrophage infiltration [38]. In this study, in vivo blockage of TGF-β using neutralizing antibodies ameliorated the protective effect of Tregs. These findings suggest a TGF-β-dependent Treg macrophage inhibitory interaction that can explain cognate-independent protection by Treg. Depletion of CD8 T cells protected mice from renal functional and structural deterioration [39]. In contrast, depletion of γδ T cells exacerbated the disease severity in a murine AN model with worse glomerulosclerosis and interstitial inflammation, suggesting a protective role of γδ T cells [40].

In a rat model of toxic renal injury induced by $HgCl_2$, a TGF-β secreting regulatory CD4 T cell line was found to inhibit not only $HgCl_2$-induced antilaminin antibody production, but also increase in serum immunoglobulin E (IgE) concentration and glomerular deposition of immunoglobulin. This implies that TGF-β-producing, autoreactive T cells inhibit both Th1- and Th2-mediated autoimmune diseases [41]. Most studies on T cells in glomerulonephritis have focused on the development or initial injury phase, as reviewed above. Future studies are required to reveal the role of T cells in the regenerative process in glomerulonephritis.

ACUTE KIDNEY INJURY

AKI models can be classified into three different categories according to the cause of the injury: ischemic, sepsis-induced and nephrotoxic AKI.

Ischemic Acute Kidney Injury

Numerous studies have reported the roles of each immune component in the pathogenesis of renal IRI and most studies examining the immune system have been performed in murine models [1,2]. Both innate and adaptive immune systems are now well established as being engaged in the immune response occurring in postischemic kidneys after IRI.

T cells were not expected to participate in the initial renal injury of ischemic AKI based on ideas traditionally about the immunological functions of T cells. The pathophysiological role of T cells in the establishment of initial renal injury following IRI has been elucidated in many studies, both directly and indirectly [42 46]. Previous reports showing that T-cell-targeting medications such as FK506 and mycophenolate mofetil attenuate renal injury after IRI also support the important role of T cells in renal IRI [47,48]. Blockade of the T-cell CD28-B7 costimulatory pathway with CTLA4Ig (a recombinant fusion protein containing a homolog of CD28 fused to an IgG_1 heavy chain) had a protective effect in the early injury phase of cold renal IRI in rats [49]. A subsequent study revealed that CTLA4Ig treatment both on the day of renal IRI and during the first week after IRI reduced proteinuria in a model characterized by progressive proteinuria in uninephrectomized rats that underwent cold IRI, implying that T cells affect the outcome of renal IRI for longer periods as well as the initial period [50].

More direct and detailed mechanisms of T-cell involvement in the initial injury phase of renal IRI were demonstrated in a murine renal IRI model using CD4- and CD8-deficient mice [44]. In this study, CD4 and CD8 double knockout mice were significantly protected from early renal injury and T cells showed a two-fold increase in adherence to renal tubular cells in an in vitro hypoxia reoxygenation setting. A subsequent study by the same investigators identified CD4 T cells as a major pathogenic mediator of renal IRI that acts in the early phase similarly to traditional innate immune components [45]. In this study, another T-cell knockout mouse strain, athymic nu/nu mice, was protected from renal injury after IRI, and adoptive T-cell transfer into these mice restored renal injury, demonstrating that T-cell deficiency did indeed confer renal protection in renal IRI. CD4 knockout mice, but not CD8 knockout mice, were significantly protected from renal injury with lower mortality, and adoptive transfer of CD4 T cells into CD4 knockout mice restored renal injury after IRI. CD28 on T cells and T-cell IFN-γ production were reported as key factors of the effects of CD4 T cells on ischemic AKI in this study. A study on CD4 T-cell subsets in a murine renal IRI model demonstrated that CD4 T cells of the Th1 phenotype are pathogenic

and that the Th2 phenotype can be protective [51]. This study was performed using mice with targeted deletions in the enzyme signal transducers and activators of transcription (STAT)4 and STAT6 that regulate Th1 (IFN-γ producing) and Th2 (IL-4 producing) differentiation and cytokine production, respectively. Renal injury was aggravated both functionally and structurally in STAT6-deficient mice, but STAT4-deficient mice showed mildly improved renal function after IRI. IL-4 was suggested as a protective mediator of the STAT6 pathway since IL-4-deficient mice showed a similar postischemic phenotype to STAT6-deficient mice. Another report showing that inactivation of IL-16 (a T-cell chemoattractant, strongly expressed in distal and proximal straight tubules of the postischemic kidney) by antibody administration and IL-16 deficiency prevented renal injury with less CD4 T-cell infiltration also supported the importance of CD4 T cells in early renal injury after IRI [52]. The kinetics of early trafficking of T cells into postischemic kidney was described in two recent reports [43,53]. Flow-cytometric analyses of mononuclear cells directly isolated from the kidney revealed very early T-cell trafficking into postischemic kidneys at 3 h after IRI and sphingosine-1-phosphate receptor was reported to play an important role in T-cell trafficking.

CD8 T cells have not received much attention in renal IRI, although these cells are a major subpopulation of T cells, along with CD4 T cells. A previous study showed that CD8 knockout mice were not protected from renal IRI, suggesting a limited role of CD8 T cells in renal IRI [45]. However, one recent study using germ-free mice reported aggravated renal injury with increased trafficking of CD8 T cells, but not CD4 T cells, into postischemic kidneys in these mice compared with wild-type controls, implying that CD8 T cells may also contribute to early renal injury but that this contribution may be modified by environmental factors including previous exposure to germs. CD8 T cells isolated from postischemic kidneys were recently reported to produce more IFN-γ than did normal and sham-operated kidneys [54].

The γδ T cells, a minor subset of T cells with T cell receptor (TCR) composed of a γ chain and δ chain, were reported to play a role in early renal injury after IRI since both γδ T-cell-deficient mice and αβ T-cell-deficient mice showed reduced renal injury [55,56]. Greater proportions of $CD3^+CD4^-CD8^-$ double-negative (DN) T cells were found in normal mouse kidneys compared with spleen and peripheral blood and also in postischemic kidneys following thymoglobulin treatment in a murine renal IRI model [54,57]. However, the role of DN T cells in ischemic AKI is yet to be determined. Ag TCR engagement followed by Ag-specific T-cell activation seems to be implicated in the pathogenesis of renal IRI. A recent study using *nu/nu* mice and transgenic DO11.10 mice that have TCRs specifically recognizing chicken OVA peptide demonstrated that diverse TCR repertoire was important for renal IRI in naïve mice without T-cell activation. However, once T cells were activated in an Ag-specific manner through TCR in DO11.10 mice, the restricted TCR repertoire no longer limited the extent of kidney injury [58].

With the use of flow-cytometric analyses of kidney-infiltrating mononuclear cells for the elucidation of dynamic kinetics of T-cell trafficking, it has been shown that T-cell infiltration is increased at 3 h but decreased at 24 h after IRI. In a subsequent study, long-term infiltration of activated and effector-memory T cells into postischemic kidneys was found. In addition, T-cell infiltration, which increased with time, into postischemic kidney was followed up until 11 weeks after IRI and the increased T cells expressed an activation and effector-memory phenotype, implying that T cells may play some role in prolonged renal damage or regeneration processes during the repair phase of renal IRI [59]. The same investigators further investigated the phenotype and the role of infiltrated T cells during the repair phase of renal IRI, and found that infiltration of Tregs increased with time in postischemic kidney during the repair phase [60]. In this study, postischemic kidneys had an increased number of $TCR\text{-}\beta^+CD4^+$ and $TCR\text{-}\beta^+CD8^+$ T cells with enhanced proinflammatory cytokine production, and infiltration of Tregs expressing TCR-β/CD4/CD25/Foxp3 was increased on day 3 and even more on day 10 after IRI. Treg depletion or transfer was performed on day 1 after IRI to avoid affecting initial renal injury and to evaluate the effect of Treg manipulation on the repair process. Treg depletion led to increased proinflammatory cytokine production from kidney-infiltrating T cells and aggravated renal injury. In contrast, Treg transfer reduced proinflammatory cytokine production from T cells in postischemic kidneys and promoted renal regeneration by increasing renal tubular epithelial cell proliferation, measured with Ki-67 (a 360 kDa nuclear protein, expressed by proliferating cells in all phases of the active cell cycle, but absent in resting cells). This study provided the first direct evidence that Tregs are a crucial mediator for renal regeneration in ischemic AKI, even though it was performed in an experimental setting and did not explore the later repair phase when renal fibrosis and tubular atrophy become more apparent. Tregs were also reported to modulate early injury after renal IRI through IL-10-mediated suppression of the innate immune system, and to contribute to the protective effect of ischemic preconditioning in the kidney [61,62]. Treg transfer was performed 2 weeks before renal IRI and significantly attenuated renal injury, with decreased leukocyte accumulation on day 1 after IRI. Adoptive transfer of wild-type Tregs into RAG-1 knockout

mice, which are deficient in mature T cells and B cells, was sufficient to prevent renal IRI, but transfer of IL-10-deficient Tregs did not prevent renal IRI.

Regarding the role of lymphocytes during the repair phase of renal IRI, a recent study identified B cells as another important factor affecting the regeneration process after IRI [63]. Because there are only a few studies that directly deal with regeneration in IRI and since T cells interact with B cells, this report is pertinent to the role of T cells in renal repair. B-cell trafficking kinetics was measured for 4 weeks after renal IRI and the effect of B-cell manipulation was explored. B cells that infiltrated into postischemic kidneys during the repair phase were activated and differentiated into plasma cells that peaked on day 10. Postischemic kidneys of B-cell-deficient mice expressed higher levels of IL-10 and vascular endothelial growth factor, and reduced tubular atrophy with greater tubular proliferation, while adoptive B-cell transfer decreased tubular proliferation and increased tubular atrophy.

Sepsis-induced Acute Kidney Injury

Sepsis-induced AKI (septic AKI) in humans frequently presents as a multiorgan failure accompanied by sepsis. Experimental models of septic AKI are usually induced by administration of lipopolysaccharide (LPS) or cecal ligation and puncture (CLP). There have been few reports regarding the role of T cells in experimental septic AKI models. It would be difficult to investigate this topic because of the difficulty to obtain renal tissue from septic patients with AKI and to simulate a consistent animal model of septic AKI, specifically during the repair phase, given the low likelihood of long-term survival. Therefore, the recent important findings regarding the role of Tregs are reviewed.

Regarding the role of Tregs in sepsis, most studies were performed using blood samples of sepsis patients which revealed an unfavorable role of Tregs, in contrast to experimental data on ischemic AKI. Monneret et al. found a higher percentage of circulating Tregs in the blood samples of septic shock patients [64]. A significant portion of these Tregs expressed CD45RO but not CD69, suggesting that these cells were naturally existing Tregs rather than recently activated by sepsis. Analyses of blood samples obtained between 7 and 10 days after the diagnosis of sepsis showed that the non-survivor group (nine patients) had a higher percentage of Tregs than the survivor group (seven patients). The authors concluded that prolonged existence of Tregs may lead to severe immunoparalysis and result in poor outcome. The same group also demonstrated that the increased proportion of Tregs among CD4 T cells was caused by a selective depletion of $CD4^{+}CD25$ T cells rather than a proliferation of Tregs [65].

Absence of CD127 was reported as an important feature of Tregs implicated in sepsis in humans and mice [66]. Increased $CD4^{+}CD25^{+}CD127$ T cells, which inhibit the proliferative response of peripheral blood mononuclear cells (PBMCs) after simulation with mitogens, were found in septic shock patients, and down-regulation of Foxp3 expression in Tregs using ex vivo transfection with Foxp3 targeting siRNA restored the PBMCs' proliferative response to mitogens in a murine CLP model. Another study analyzing Tregs in sepsis patients and healthy controls also reported that sepsis was associated with an increased percentage of Tregs and elevated plasma levels of soluble CD25 [67]. In this study, four out of 13 patients were reassessed several weeks after hospital discharge and their Tregs showed reduced expression of Foxp3, TGF-β and IL-10 compared with their hospitalization period. Tregs limited the capacity of monocytes to induce Ag-specific response and to secrete proinflammatory cytokines in response to LPS, and inhibited LPS-induced monocyte survival through a Fas/Fas ligand-dependent pathway [68,69]. Coculture study of Tregs and polymorphonuclear neutrophils (PMNs) in a model simulating Gram-negative bacteria infection also demonstrated that the death of PMNs was enhanced by Tregs, and apoptosis of PMNs was more accelerated when Tregs were stimulated with LPS or anti-CD3/CD28 antibodies [70]. Normally, the lifespan of PMNs is prolonged when activated by LPS. The role of human Tregs is well summarized in a recent review paper [71].

Although there is no direct evidence of Treg involvement in septic AKI, a significant effect of Tregs on septic AKI is predicted based on the accumulated data of circulating Tregs in sepsis patients. To elucidate the role of Tregs on septic AKI, establishment of a more suitable animal model with better animal survival is required.

Nephrotoxic Acute Kidney Injury

Nephrotoxic AKI has been induced with several well-known nephrotoxic drugs including cisplatin, gentamicin and cyclosporine. A study using *nu/nu* mice and CD4- or CD8-deficient mice in acute cisplatin nephrotoxicity model revealed that T-cell deficiency protected kidneys both functionally and structurally, with decreasing renal myeloperoxidase activity and proinflammatory cytokine production, suggesting T cells as direct mediators of experimental cisplatin nephrotoxicity [72]. Resveratrol was reported to attenuate cisplatin-induced nephrotoxicity in rats by reducing free radicals and inhibiting the infiltration of inflammatory cells such as T cells and macrophages [73]. Tregs were recently identified as a crucial factor in attenuating early renal injury induced by cisplatin both functionally and structurally [74]. However, there are no

reports regarding the role of T cells in the repair phase of nephrotoxic AKI.

KIDNEY TRANSPLANTATION

Allogeneic kidney transplantation causes a robust immune response in renal allograft. The pathogenic role of T cells in renal allograft rejection and its role as a barrier to tolerance induction have been extensively studied [75]. In this section, the role of Tregs in renal allografts is briefly reviewed.

In solid organ transplantation, two allorecognition pathways have been identified: the direct pathway and the indirect pathway [76]. Tregs have been suggested to interfere with the indirect alloresponse, where recipient Ag-presenting cells present processed donor Ags [77 79]. Direct allorecognition was inhibited with a greater number of Tregs in the absence of indirect allorecognition [77]. In acute rejection, increased mRNA expression of Foxp3 in urine was reported to be associated with better graft survival [80]. Infiltrating Tregs have been found in renal allografts showing acute rejection, implying that Tregs may control effector cells [81]. Treg cell lines obtained from 11 stable renal transplant recipients were able to inhibit both direct and indirect alloproliferation [82]. Treg has been expected to be involved in tolerance induction. The "linked-suppression" phenomenon associated with indirect allorecognition and Tregs was described in inhibition of the alloresponse against other alloantigens mediated by both direct and indirect pathways [83,84]. The decreased number of circulating Tregs in chronic rejection patients compared with stable or clinically tolerant (immunosuppression withdrawn) patients indirectly supports a favorable role of Tregs in the establishment of long-term tolerance in kidney transplantation [85,86]. However, a recent study reported that Foxp3 expression on renal allograft may be a non-specific feature of injured and inflamed sites rather than a feature of allorecognition, and that Foxp3 expression was not associated with a favorable outcome [87]. Modulation of Tregs by immunosuppression is covered in great detail in a recent review [88]. Regarding fibrogenesis in renal allografts, an unfavorable healing process, chemokine receptors involved in T-cell infiltration such as CCR1 and CCR5 [regulated on activation, normal T-cell expressed (RANTES)] were reported to contribute to tubulointerstitial fibrosis in a rat kidney transplantation model [89,90]. Several factors affecting fibrogenesis of renal allografts were summarized in a recent review [91]. Further studies are required to elucidate the precise roles of T-cell subsets, especially Tregs, in tolerance induction and regeneration process after rejection.

CONCLUSION

Distinct T-cell subsets play individual roles during kidney injury and repair depending on the type of kidney disease. Comprehensive understanding about the pathogenic mechanisms of different kidney diseases will be a cornerstone for future studies investigating the contribution of T cells in the regeneration process. Regeneration studies should cover the following three structural sequelae of injured kidneys: glomerulosclerosis, tubular atrophy and tubulointerstitial fibrosis. It is notable that the degree of tubular atrophy was not parallel with the extent of tubulointerstitial fibrosis during the repair phase in a murine renal IRI model [63]. Promoting the regeneration process to restore structural and functional integrity in diseased kidneys is as important as reducing damage during the early injury phase, and more frequently required in most kidney diseases since most patients start receiving diagnostic workup and treatment when they already have established disease. Future studies are required to elucidate the precise roles of T-cell subsets during the repair phase of kidney diseases and to harness these findings to develop novel therapies to enhance regeneration in the injured kidney.

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CHAPTER

9

Mesenchymal Stem Cells

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OUTLINE

INTRODUCTION

Stem cells play fundamental roles in the self-renewal of adult tissues throughout life. Some tissues are characterized by ongoing loss of cells, including the hematopoietic system, intestine and skin, and adult stem cells are responsible for replenishing these cells to maintain tissue homeostasis. Other organs, such as kidney or lung, have a much lower rate of cellular turnover, but are capable of proliferating and repairing after an injury stimulus [1]. While epithelial stem cells can be recruited, proliferate and differentiate to reconstitute some injured tissues, it remains unclear whether the kidney follows this paradigm for epithelial stem cell-based homeostasis and repair after injury [2]. Basal tubule cell turnover in kidney is exceedingly low, and the turnover that can be detected has been proposed to occur by division of terminally differentiated tubular epithelial cells [3]. Soon after injury, by contrast, there is diffuse tubular cell proliferation, potentially reflecting the intrinsic ability of surviving epithelial cells to adapt to the loss of neighboring cells by dedifferentiating and proliferating and ultimately replacing the cells that have died as a result of the insult. Based on the high proliferative capacity of injured kidney, one longstanding model holds that tubular cells themselves are the source of nephron repair [1].

Studies on the role of bone marrow-derived cells (BMDCs) initially challenged this model of dedifferentiation followed by proliferation and redifferentiation of existing tubular cells after injury. Bone marrow contains at least two populations of stem cells in addition to stromal cells. The hematopoietic stem cell (HSC) gives rise to all differentiated blood cell types, and mesenchymal stem cells (MSCs) that give rise to mesenchymal cell types including chondrocytes, osteocytes and adipocytes. While it has long been appreciated that bone marrow-derived inflammatory cells home to injured kidney, recent studies have suggested that BMDCs directly participate in renal injury and repair. MSCs in particular have been reported to protect against experimental renal injury as well as accelerate the repair process in rodent models. As will be reviewed, some reports indicated that the MSCs directly replace dead tubular epithelial, whereas other observations suggest that MSCs regulate the endogenous reparative machinery without transdifferentiating into tubular cells overall, the emerging body of evidence describing MSC modulation of acute kidney injury (AKI) has stimulated a reappraisal of the cellular mechanisms behind renal injury and repair as well as generated considerable excitement at the prospects for novel cell therapies to treat human kidney diseases.

THE MESENCHYMAL STEM CELL

MSCs are an undifferentiated, adult cell type that can be isolated from a variety of tissues but primarily bone marrow stroma. The embryonic lineage of these cells is mesodermal, from mesenchymal cells that give rise to connective tissues such as bone, cartilage and fat as well as blood supply-related organs such as the vasculature and hematopoietic system. The International Society for Cellular Therapy has proposed a set of standards to define human MSCs for scientific and preclinical studies, including adherence to plastic in culture, fibroblastoid appearance, multipotentiality (ability to differentiate into different cell types), expression of typical surface markers such as CD73, CD90 and CD105, and the absence of expression of hematopoietic lineage markers [4]. There is no definitive proof that MSCs are a clonal, self-renewing stem cell, and for this reason many suggest that MSCs refer to "multipotent mesenchymal stromal cells" [5].

MSCs not only reside in bone marrow but have also been isolated from skeletal muscle [6], adipose [7], umbilical cord [8], dental pulp [9], amniotic fluid [10] and other sources. Very recent reports have suggested that MSCs may reside in capillary and microvessel walls throughout the body, sharing markers and characteristics of the vascular pericyte [11]. The finding that purification of pericytes through sorting ($CD146^+$, CD34 , CD45 , CD56) with subsequent in vitro expansion leads to clones of multipotent cells led Caplan to speculate that all MSCs are pericytes [12]. The pericyte phenotype is characterized by expression of CD146, NG2 and platelet-derived growth factor (PDGF)-Rβ and the absence of hematopoietic, endothelial and myogenic markers, as well as multilineage differentiation ability. The attraction of the hypothesis that MSCs are natively associated with blood vessel walls is that it helps to explain why MSCs have been isolated from so many different organs [13]. However, it is not yet clear exactly which subset of perivascular cells contain MSCs and it remains possible that MSCs may derive from other sources.

The functional properties of MSCs make them unique. These multipotent stem cells can differentiate to cells of the mesenchymal lineage such as osteocytes, adipocytes and chondrocytes and potentially other cell types. Directed differentiation can be achieved by culturing MSCs in defined conditions [14]. MSCs are easily cultured, which distinguishes them from embryonic stem cells (which generally require feeder cells and special growth medium) and other adult stem cells. MSCs can be expanded, explaining why they are among the first cells to be used for cellular therapies in humans, since it is not difficult to obtain clinically useful numbers of cells. Finally, MSCs possess immunomodulatory properties that have made them especially attractive for potential use in treating human disease characterized by autoimmunity or inflammation, including graft versus host disease, multiple sclerosis and Crohn's disease [15].

AMNIOTIC FLUID-DERIVED STEM CELLS

The isolation of multipotent stem cells from amniocentesis specimens, termed amniotic fluid-derived stem cells (AFSs), offers a new source of stem cells for use in cellular therapy [10]. AFSs represent about 1% of all amniotic fluid cells and are characterized by expression of the cell surface marker c-kit, as well as other surface antigens also expressed by MSCs such as CD73, CD90 and CD105. Like MSC, they do not form teratomas in vivo, which distinguishes both of these stem cells from embryonic stem cells. AFSs have two important differences from MSCs. First, they are significantly more broadly multipotent than MSCs, and may in fact be pluripotent. Second, AFSs are clonal and therefore are a true stem cell population. Initial studies suggest these cells, like bone marrow-derived MSCs, ameliorate AKI [16]. Whether the properties of AFSs will make them a better candidate for cellular therapies in kidney injury needs to be investigated; however, their accessibility makes them a very attractive candidate for regenerative medicine [17]. The prospect of banking

amniocentesis specimens for future AFS isolation and use in autologous cell therapies, or matching histocompatible donor cells with recipients, represents an important advance in regenerative medicine.

BONE MARROW PLASTICITY AND RENAL REPAIR

The current interest in MSCs for treatment of AKI grew in part from the observations made by Petersen et al. [18] and Theise et al. [19] that BMDCs could develop into hepatocytes. This finding, later reported in humans [20], led to intensive research on the plasticity of BMDCs. Evidence for engraftment of BMDCs was soon reported in other tissues including lung, gastrointestinal tract and skin. Krause et al. demonstrated that a single transplanted HSC could provide hematopoietic reconstitution for a lethally irradiated recipient, and that this single hematopoietic cell could also engraft non-hematopoietic tissues including lung, liver, gastrointestinal tract and skin [21]. These surprising results were followed by studies from Poulsom et al. and Gupta et al., who examined renal biopsies from male patients transplanted with female kidneys. Both groups reported the presence in the allografts of Y-chromosome-positive tubular epithelial cells, varying from less than 1% up to 20% of cells examined [22,23], with similar results found in mouse [24].

Follow-up studies have led to a re-evaluation of the physiological relevance of the initial observations concerning BMDCs transdifferentiating into renal epithelia. It has been proposed that the early results could be due to cell fusion or possible artifactual detection of lineage markers. The inability to repeat some of these findings in other laboratories has also contributed to the debate [25,26]. Not all issues have been resolved, but several conclusions are possible. The method of marking and detecting the bone marrow lineage is critical. Bacterial β-galactosidase transgene activity, in particular, may be problematic owing to high expression of endogenous kidney β-galactosidases and possible leakage of the enzyme by damaged cells, with subsequent uptake by neighboring cells. Green fluorescent lineage markers, such as enhanced green fluorescent protein (GFP), are also subject to misleading artifacts owing to the high intrinsic autofluorescence of the postischemic kidney. This autofluorescence can cause misinterpretation of fluorescent immunostaining. High-resolution marker detection in kidney sections is especially important, with three-dimensional (3D) deconvolution or confocal microscopic techniques required to distinguish between closely apposed and overlying cells and nuclei [27]. Bone marrow-derived leukocytes traffic to the renal interstitium after renal injury, and a superimposed leukocyte nucleus may be mistaken for an epithelial cell nucleus unless such high-resolution imaging is utilized. Cell overlay and intrinsic autofluorescence have also complicated the interpretation of BMDC contribution to myocardial regeneration, emphasizing the challenge of tracking cell fate in vivo, particularly in injured tissues [28,29].

Studies utilizing mice with bone marrow transplants harboring several different lineage markers have led to the conclusion that BMDCs only rarely contribute to the renal epithelial lineage under physiological conditions (at most 1% but probably much less) [30,31]. Using chimeric mice in which GFP-positive bone marrow was transplanted to a GFP-negative recipient, Duffield et al. performed ischemia reperfusion injury (IRI) and tracked the fate of the GFP-positive cells during injury and repair [31]. While they did observe an influx of GFP-positive cells into kidney, these were almost exclusively interstitial cells, 99% of which were leukocytes. This observation is consistent with the important role of interstitial inflammation in the pathogenesis of AKI [32]. Similar results were found using LacZ as a fate marker for bone marrow cells or sex-mismatched transplants utilizing Y-chromosome as a marker for transplanted cells. In the case of Y-chromosome analysis, it is notable that several putative examples of tubular epithelial cells positive for the Y-chromosome were observed by standard fluorescence microscopy, but with high-resolution deconvolution microscopy, these were revealed to be leukocytes overlying epithelial cell but within the interstitium.

Cell fusion is another possible explanation for earlier results and this is emphasized by a study from Grompe and co-workers that convincingly showed a 20 50% fusion of tubular epithelia with BMDCs under long-term and intense genetic pressure [33]. Li et al. employed an elegant fate-mapping technique to quantitate the degree of fusion after IRI [34]. They started with a transgenic mouse that expressed Cre recombinase only in renal epithelial cells: the Ksp Cadherin Cre (Ksp-Cre) driver line. Next, they transplanted bone marrow from donor mice that expressed a reporter gene, EYFP (enhanced yellow fluorescent protein) only after Cre-recombinase-mediated recombination of a LoxP-flanked stop sequence. In resulting mice, any tubular cell that activated expression of EYFP was derived from bone marrow. Next, they performed ischemic injury, and assessed gain of EYFP expression along with ploidy in kidney. After 28 days, they found a substantial number of EYFP-positive immune cells in the renal interstitium, but only very rare EYFP-positive tubular epithelial cells at a frequency of 0.066% of total tubular cells. The authors concluded that cell fusion occurs between BMDCs and epithelia, that injury is required for fusion and that these events are very rare.

MESENCHYMAL STEM CELLS AMELIORATE RENAL INJURY AND ACCELERATE REPAIR

Although endogenous BMDCs do not directly replace renal epithelia during renal repair, several lines of evidence indicate that exogenously administered MSCs do modulate the kidney repair and regenerative response. Intravenous injection of the lineage-negative bone marrow fraction before injury, part of which contains MSCs, blunted the initial rise in blood urea nitrogen (BUN) after IRI [35], whereas whole bone marrow had no protective effect [30]. Injection of purified MSCs almost completely protected against cisplatin-induced rise in BUN, whereas injection of purified HSCs had virtually no protective effect [36]. Similar protection from injected MSCs was found in a glycerol-induced pigment nephropathy model [37] and in a model of IRI [31,38]. Importantly, Westenfelder and colleagues have shown that infused MSCs enhance recovery of rats subjected to IRI even if administered 24 h after the injury, suggesting active participation of these cells in the repair process [38,39]. Taken together, there is good evidence that administered MSCs both protect against AKI in toxic and ischemic rodent models and accelerate the recovery phase [40].

EFFICACY OF MESENCHYMAL STEM CELL THERAPY IN CHRONIC KIDNEY DISEASES

Accumulating evidence suggests that MSC therapy is efficacious not only in the acute injury setting, but also in chronic, progressive kidney diseases. The Kalluri and Cook laboratories have independently used the Col4a3 $^{-/-}$ model of progressive glomerular disease to determine whether administered MSCs might rescue the genetic defect. Both found that transplantation of wild-type MSCs into irradiated Col4a3 $^{-/-}$ recipients led to partial restoration of type IV collagen α3 chain expression in glomerulus, improved glomerular histology, reduction in proteinuria and improved overall kidney histology [41,42]. Transplantation of bone marrow from Col4a3 $^{-/-}$ donors did not ameliorate disease, suggesting that BMDCs homed to the glomerulus, took up residence and secreted wild-type type IV collagen. The precise identity of the bone marrow-derived glomerular cells (podocyte, mesangial cell or other) remains a subject of investigation. Subsequently, Katayama et al. found that irradiation alone ameliorated kidney disease in the same model, suggesting that the BMDCs may have little to do with the observed effects [43,44]. In a follow-up study, LeBleu found that transplantation of wild-type marrow also improved survival in a similar Col4a3 knockout model [45], in contrast with Gross and co-workers, who found reduced fibrosis but no difference in mortality [46]. Most importantly, though, in the LeBleu study, irradiation was not required to rescue the phenotype, confirming that the infused cells were actively participating in glomerular repair. Collectively, these studies demonstrate that the notion of stem cell therapy for Alport's disease, whether MSC or embryonic stem cell, remains a promising one, but that much work is left to do in order to understand the optimal cell source, delivery method and most importantly mechanism of the observed effects.

MSCs may have therapeutic application in other chronic, fibrotic kidney diseases. Using the remnant kidney model, intravenous injection of MSCs in rats every other week resulted in a modest protection of renal function decline at 8 weeks, with reduced fibrosis readouts such as picrosirius red staining, collagen I production and α-smooth muscle actin (α-SMA) expression [47]. Notably reduced were measures of inflammation, suggesting that it was the immunosuppressive effects of MSCs that mediated this antifibrotic effect. However, renal function was equal by 12 weeks in placebo versus MSC-treated groups, suggesting that the anti-inflammatory effects of MSCs may result in only transient protection from progressive renal fibrosis. In a shorter study using the same remnant kidney model, the Noronha group injected MSCs in the subcapsular space, and observed decreased blood pressure, proteinuria and histology scores for fibrosis at 30 days [48]. Longer periods were not investigated. Ezquer et al. examined whether intravenous administration of MSCs might be therapeutic in a diabetic nephropathy model. One month after streptozotocin injection to induce diabetes, these investigators injected C57BL/6 mice with vehicle or two doses of 0.5×10^6 MSCs. Over the next 3 months, vehicle-injected mice developed progressive albuminuria, mesangial expansion and glomerulosclerosis, all of which were substantially improved in mice that received MSCs [49]. Both groups had equivalent hyperglycemia and hypoinsulinemia, however, indicating that the mechanism did not involve an MSC-mediated effect on pancreas regeneration. Moreover, GFP-expressing MSCs persisted in kidneys at 3 months after injection, albeit at low levels, suggesting possible ongoing secretion of renotropic factors.

DO EXOGENOUS MESENCHYMAL STEM CELLS DIRECTLY ENGRAFT INTO INJURED TUBULES?

Morigi et al. and Herrera et al. reported that exogenous MSCs can engraft into injured tubules, and proposed that the ability to transdifferentiate explained

their protective effect [36,37]. Yokoo et al. directly injected exogenous MSCs into developing kidney with subsequent embryo and organ culture and observed MSC incorporation into glomerulus, tubule and interstitium, findings that seem to support the possibility of direct engraftment [50]. In contrast, studies by Lin et al. [30] and the present group [27,31] showed protection from injury by exogenous MSCs but very little or no tubular incorporation. Some of the discordant findings may be explained by different injury models and protocols, as suggested by Broekema et al. [51]; however, the same caveats described previously regarding proof of tubular incorporation of BMDCs also apply to studies of injected MSCs. The nature of the MSC marker, careful 3D microscopic analysis and the possibility of cell fusion all need to be taken into account. It is relevant in this regard that in a follow-up study, Herrera reported much lower tubular incorporation of MSCs (about 2.5%) compared to an earlier report (about 20%) using the same glycerol-induced renal injury model, but a different fluorescence-based cell tracking method of injected MSCs [52].

The bulk of available data indicates that the effects on renal repair of exogenous MSCs are not explained by direct repopulation of the tubule. The timing of renal epithelial cell proliferation, which is dramatically increased within 24 48 h, appears to be too rapid to be explained by transdifferentiation of extrarenal cell types into epithelial cells. In most studies the protective effect of injected MSCs is observed within 24 48 h. When careful lineage analysis has been done, the numbers of MSCs or BMDC-derived epithelial cells appear to be so low (1% or less) that they could not have functionally contributed to repairing the nephron, at least by direct engraftment. Vogetseder et al. have argued that in the uninjured kidney, the small amount of epithelial proliferation present occurs by division of terminally differentiated cells [3,53].

To test definitively whether an endogenous, non-epithelial stem cell population might contribute to the epithelial lineage during mammalian renal repair, a comprehensive fate-mapping study of renal epithelial cells during renal IRI was performed. All mesenchyme-derived renal epithelial cells were genetically labeled using a Cre/Lox strategy. The *Six2* gene is expressed exclusively in metanephric mesenchyme cells fated to become renal epithelia [54], and a transgenic mouse in which the Six2 promoter drives expression of a GFP Cre recombinase fusion protein (the Six2 GC mouse) was crossed against two different reporter mice, the Rosa26-LacZ (R26R) reporter, and the ACTB-Bgeo, -DsRed.MST reporter (Z/Red). Because the *Six2* gene is active very early in nephrogenesis, recombination efficiency was high and 95% labeling of all tubular epithelial cells derived from the metanephric mesenchyme was achieved (collecting duct epithelia are derived from the ureteric bud, and therefore not labeled in this model) [55]. Since interstitial cells and non-renal cells are not labeled, after a cycle of injury and repair, dilution of the fate marker would indicate contribution to the epithelial lineage by unlabeled cells. If repaired tubules still express the fate marker, by contrast, then repairing epithelial cells originated from within the tubule.

Close inspection of kidney sections confirmed that no interstitial cells were labeled, with either LacZ or with red fluorescent protein (RFP). GFPCre fusion protein expression was undetectable after the completion of nephrogenesis (P3 5), whether assessed by epifluorescence or antibody-based detection of GFP (the GFPCre fusion protein retains GFP fluorescence), Six2 mRNA or Six2 protein using a specific antisera. (Since GFPCre expression is regulated by the Six2 promoter, the GFP expression pattern mirrors expression of endogenous Six2 mRNA and protein.) Mice were subjected to renal IRI to trigger the proliferative response. Two days after injury, 47.4% of cells in the outer medulla expressed the proliferation marker Ki67, and these cells coexpressed RFP (Fig. 9.1). Many tubules had flattened epithelial cells characteristic of the dedifferentiated state, and these cells also expressed Ki67, indicating the cells had re-entered the cell cycle, and RFP. When mice were injured and given an injection of bromodeoxyuridine (BrdU) once daily for 7 days, 66.9% of outer

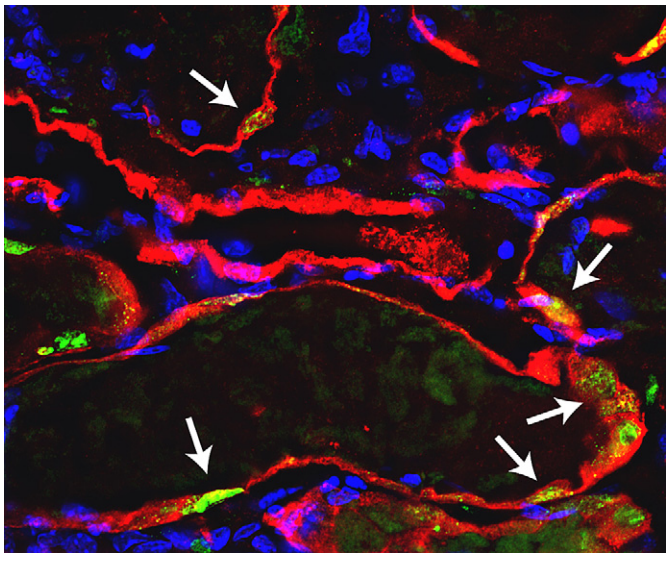

FIGURE 9.1 Kidney repair after ischemia–reperfusion injury (IRI) occurs by proliferation of surviving epithelial cells. Section from a kidney of a mouse with red fluorescent protein (RFP) expression in all epithelial cells 48 h after IRI shows that flattened, dedifferentiated epithelial cells have re entered the cell cycle, as judged by expression of nuclear Ki67 (arrows). Because there was no dilution of the RFP fate marker, mesenchymal stem cells do not directly contribute to epithe lial repair after IRI [55]. Please see color plate at the end of the book.

medulla epithelial cells had incorporated BrdU, compared with 3.5% in uninjured, control kidneys. Despite this robust proliferation, there was no dilution of the fate marker in kidneys that had been allowed to repair for 15 days. Before injury 94.3 ± 3.7% of outer medullary epithelia were RFP positive, and after repair 94.4 ± 2.4% expressed RFP, with similar results calculated for LacZ as the fate marker [55].

If the GFPCre fusion protein were re-expressed in epithelial cells after injury, then a non-labeled interstitial cell that had migrated into the damaged tubule might also be induced to express GFPCre, and thus activate expression of either RFP or LacZ, compromising our ability to detect a dilution of the fate marker. However, re-expression of the transgene, as assessed by sensitive GFP immunofluorescence, was not detected despite very robust GFP fluorescence in the cap mesenchyme of P1 kidneys from Six2-GC mice, a stage where Six2 is still expressed. The researchers were also unable to detect Cre mRNA after injury by qualitative reverse transcription polymerase chain reaction, and endogenous Six2 protein was not detected in adult kidney after injury using a specific anti-Six2 antibody.

Thus, there is now consensus that both endogenous BMDCs and exogenously administered MSCs can give rise to renal epithelial cells only rarely, if at all, and that cell fusion may underlie some of these events. The rarity of transdifferentiation to kidney epithelia indicates that direct tubule repopulation by BMDCs or administered MSCs does not have physiological relevance to renal repair from injury in vivo.

HOMING OF EXOGENOUS MESENCHYMAL STEM CELLS

The mechanisms by which MSCs promote kidney repair remain unclear but an important aspect of the therapeutic effects of MSCs appears to be their ability to home to injured organs. Lange et al. labeled exogenous MSCs with iron-dextran and found these cells located primarily in the renal cortex after IRI in the rat, as assessed by magnetic resonance imaging. These cells remained associated with kidney 3 days after IRI, and histologically they were localized to glomerular capillaries [39]. In a more detailed analysis, fluorescently labeled MSCs were localized by two-photon microscopy to both glomeruli and peritubular capillaries within 10 min of intra-arterial injection into rats subjected to IRI 24 h before [38]. The relative importance of MSCs homing to glomerular versus peritubular capillaries is not known. MSCs have been detected in both compartments in both acute and chronic injury models [56]. Either location may be efficacious, with peritubular MSCs poised to signal to adjacent tubular epithelia and glomerular MSCs potentially able to secrete factors that are filtered into the tubular lumen, where they may bind to and directly regulate damaged epithelial cells. Another unresolved question is whether MSCs bound to the renal microvasculature are capable of migrating into the renal interstitium. Although no direct evidence supports such a possibility at present, it has not yet been examined rigorously.

Recent studies have begun to dissect the signals that regulate MSC homing. Inflammation improves engraftment efficiency of infused MSCs, suggesting the MSC endothelial cell interactions may be important in homing. Indeed, blocking β_1-integrin expressed on MSCs reduced engraftment of MSCs in ischemic myocardium, suggesting that MSC recognition of the endothelial cell VLA-4 adhesion molecule via β_1-integrin was required for engraftment [57]. P-selectin and vascular cell adhesion molecule (VCAM) have additionally been shown to be critical components of the initial steps of interaction between MSCs and endothelium. Using a parallel plate flow chamber, Rüster et al. were able to block MSC rolling and adherence to endothelium by preincubating endothelial cells with either anti-P-selectin or anti-VCAM antibodies, for example [58]. Support for a central role for VLA-4 VCAM-1 interaction in mediating MSC endothelial interactions has also come from studies of the firm adhesion of MSCs to activated endothelium under shear stress conditions [59]. It is clear that molecular interactions guide MSC binding to endothelium and tissue engraftment, rather than trapping in microvasculature, but elucidating the details of this process remains a challenge.

Chemotaxis of MSCs to sites of inflammation is also an area of intensive investigation at present. A number of candidate mediators has been identified, with one attractive candidate being the chemokine stromal cell-derived factor-1 (SDF-1). SDF-1 binds exclusively to its receptor CXCR-4, is expressed in the distal tubule and is upregulated after renal injury [60]. CXCR4 is expressed in MSCs. Its expression is upregulated by hypoxia and the SDF-1/CXCR4 pair is known to regulate HSC migration. Furthermore, hypoxic preincubation of MSCs appears to increase engraftment in vivo [61]. Another promising candidate as a regulator of homing is platelet-derived growth factor (PDGF), which is secreted from the basolateral aspect of human epithelial cells [62]. Cultured MSCs express PDGF receptors and potently migrate in response to exogenous PDGF. This migratory response is enhanced by preincubation of MSCs with tumor necrosis factor (TNF) [63]. Recently, a new candidate for MSC homing has emerged in CD44, which is expressed on MSCs and required for renal localization of injected MSCs after glycerol-induced renal injury. The receptor for CD44, hyaluronic acid, is upregulated in kidney after injury, and

CD44-negative MSCs show reduced migration to injured kidney as well as decreased protection from injury [52]. Elucidating the precise mechanisms controlling MSC migration to injured kidney may have important consequences for human therapy, since effective delivery of these cells to damaged tissue may be critical for therapeutic efficacy [64].

EVIDENCE THAT MESENCHYMAL STEM CELLS REPAIR KIDNEY BY PARACRINE AND ENDOCRINE MECHANISMS

Since MSCs do not directly repopulate the repairing tubule then paracrine and/or endocrine mechanisms must explain their therapeutic effects in AKI. Similar mechanisms are likely to underlie some or all of the therapeutic effects of MSCs in glomerular diseases, although this remains controversial. Given the importance of inflammation in the pathophysiology of AKI [32] it is very important to consider the immunomodulatory properties of MSCs and the role they may play in renoprotection [65,66]. MSCs are immunologically privileged and allogeneic MSCs do not induce a proliferative T-cell response. The mechanisms for this tolerance include low surface expression of both major histocompatibility complex (MHC) class I and II molecules, lack of expression of major costimulatory molecules such as CD40, CD80 and CD86, and direct inhibition of dendritic cell alloantigen-induced differentiation and activation, among others [67]. MSCs also exert anti-inflammatory influences on T cells. Coculture of MSCs with Th1, Th2 or natural killer (NK) cells decreases their secretion of proinflammatory cytokines such as TNF-α and interferon-γ (IFN-γ) and increases their secretion of suppressive and tolerance-promoting cytokines such as interleukin-10 (IL-10); this effect is largely mediated by MSC production of the eicosanoid prostaglandin E_2 (PGE_2) [68]. T cells play important roles in both immune-mediated and ischemic kidney disease, so the ability of MSCs to regulate T-cell function is likely to be relevant for their therapeutic effects in AKI [69]. In support of this notion, Semedo et al. recently measured higher levels of anti-inflammatory cytokines in kidney extracts from MSC-treated animals after IRI [70]. Proinflammatory stimuli such as IFN-γ promoted the immunosuppressive effects of MSCs, including protection from NK cell-mediated cytolysis, enhanced hepatocyte growth factor (HGF) and transforming growth factor-β (TGF-β) secretion and induction of indoleamine 2,3-dioxygenase (IDO), an enzyme that inhibits T-cell proliferation by depleting the essential lymphocyte proliferation cofactor tryptophan [71,72]. Other potentially important immunosuppressive actions of MSCs include suppression of B-lymphocyte proliferation and antibody production, inhibition of dendritic cell activation and potentially the induction of regulatory T cells [67]. These mechanisms are summarized in Fig. 9.2.

MSCs are capable of secreting a broad array of growth factors in addition to cytokines, including granulocyte colony-stimulating factor (G-CSF), stem cell factor (SCF), leukemia inhibitory factor (LIF), macrophage colony-stimulating factor (M-CSF), IL-6 and IL-11 [73]. Secretion of bioactive molecules important for hematopoietic differentiation is not surprising, since MSCs provide the marrow stroma that supports HSC differentiation. Studies have identified other growth factors and chemokines secreted by MSC, such as vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), monocyte-chemoattractant protein-1 (MCP-1), HGF and insulin-like growth factor-1 (IGF-1) [73,74]. Several of these polypeptides enhance epithelial proliferation, modulate inflammation or promote angiogenesis and are therefore good candidates for therapeutic efficacy in renal injury. IGF-1 and HGF, for example, are epithelial mitogens and morphogens that also promote renal blood flow and are known to protect against ischemic injury in murine models [75,76].

The specific paracrine factors secreted by MSCs that may explain their beneficial effects in AKI are now being elucidated. Tögel et al. examined MSC-conditioned

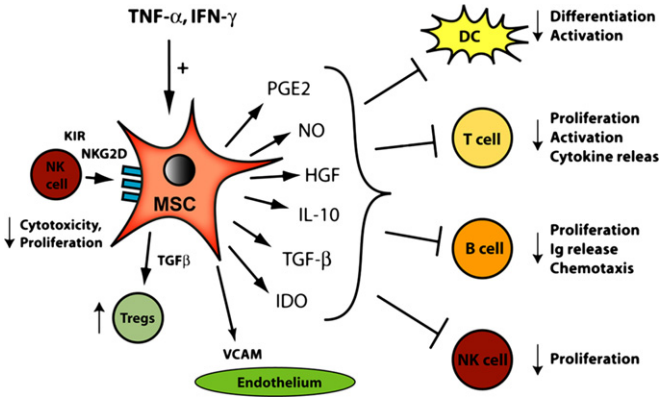

FIGURE 9.2 Mechanisms of immunomodulation by mesenchymal stem cells (MSCs). The immunosuppressive effects of MSCs may be amplified after exposure to proinflammatory stimuli such as tumor necrosis factor α (TNF α) or interferon γ (IFN γ). While natural killer (NK) cells can lyse MSCs via activating lectin NKG2D ligands expressed on MSCs, pre exposure to IFN γ protects MSCs from cytolysis and MSCs inhibit interleukin 2 (IL 2) induced NK cell proliferation. MSCs interact with endothelial cells by cell−cell contact through vascular cell adhesion molecule (VCAM). Soluble factors play very important roles in mediating anti inflammatory effects of MSCs. Prostaglandin E_2 (PGE_2), nitric oxide (NO), hepatocyte growth factor (HGF), IL 10, transforming growth factor β (TGF β) and indoleamine 2,3 dioxygenase (IDO) all exert inhibitory effects on immune effector cells in a paracrine fashion. TGF β derived from MSCs enhances regulatory T cell (Treg) formation, further enhancing anti inflamma tory effects. DC dendritic cell.

medium and found significant levels of VEGF, HGF and IGF-1, and determined that this conditioned medium was capable of enhancing endothelial cell proliferation and differentiation. When MSCs were infused just before IRI, these cells quickly homed to the renal microvascular circulation and there was decreased endogenous cell apoptosis in regions that contained MSCs [77]. These authors propose that important aspect of the MSC-induced renoprotection involves their ability to home to injured microvasculature and inhibit apoptosis. There is strong support for a central role of proangiogenic factors in mediating the protective effects of MSCs. Imberti et al. used an in vitro cisplatin-induced proximal tubule (PT) cell culture model to show that MSCs were able to protect the PT cells from cisplatin-induced cell death. However, MSCs in which IGF-1 was knocked down using siRNA were unable to protect PT cells from damage through increased PT apoptosis and decreased cell proliferation. Importantly, the authors confirmed that MSCs with IGF-1 knockdown failed to protect mice from cisplatin-induced AKI [78]. The Westenfelder laboratory performed a similar study in MSCs with knockdown of VEGF. In this study, rats subject to IRI were protected from AKI when control MSCs were infused, but MSCs with VEGF knockdown failed to protect the rats from AKI [79].

Whether the therapeutic effects of MSCs can be entirely ascribed to their ability to home to injured tissues and secrete trophic mediators, i.e. to serve as cellular delivery vehicles for growth factors, is an open question. Some doubt about the relative importance of tissue homing has been raised by a preliminary report in which MSC-conditioned medium itself, when infused in the absence of any cells, also conferred protection from IRI [80]. Given the important immunomodulatory roles of MSCs, it appears unlikely that systemic administration of MSC-produced factors could mimic the pleiotropic protective and prorepair functions of these cells. Support for this latter interpretation is provided by a recent study from Brunswig-Spickenheier et al. [81]. Surprised that porcine MSCs did not provide any protection in a porcine IRI model, the authors investigated whether porcine MSCs possessed functional differences from their rodent or human counterparts. Although porcine MSCs exhibited trilineage differentiation, characteristic antigen profiles and secretion of VEGF and IGF-1, these MSCs failed to inhibit the mixed lymphocyte reaction and expressed large amounts of proinflammatory IL-6. These results certainly highlight the anti-inflammatory properties that are important in mediating kidney protection and repair in rodent and human MSC studies. A summary of the paracrine mechanisms for the therapeutic effects of MSCs in AKI is presented in Fig. 9.3.

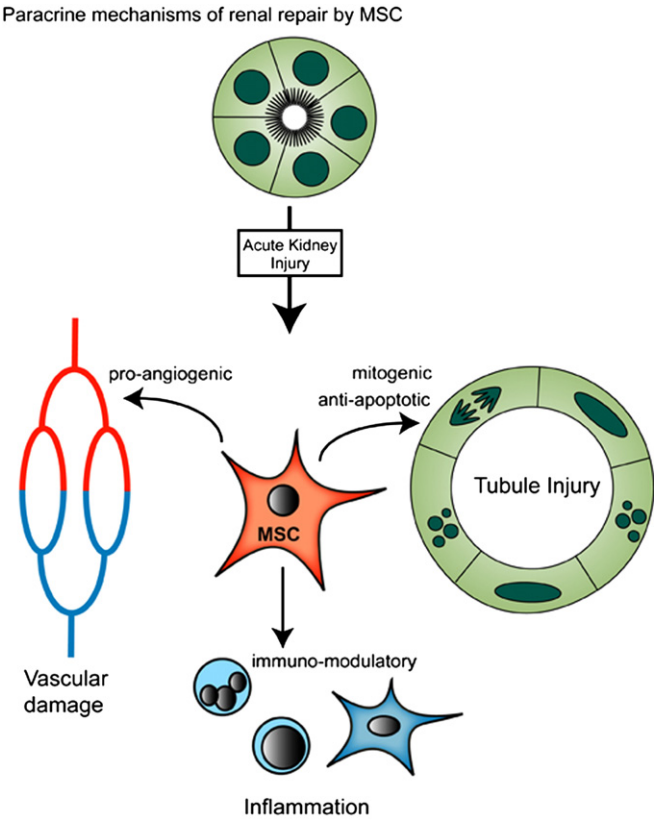

FIGURE 9.3 Paracrine actions of mesenchymal stem cells (MSCs) on injured kidney. After an acute kidney injury, injected MSCs home to sites of kidney injury and may also be recruited from endogenous niches (bone marrow or kidney). MSCs bind to glomerular and/or peritubular capillary endothelium and both protect the kidney from further injury and accelerate repair. Paracrine mediators play impor tant roles in mediating repair, including vascular endothelial growth factor (VEGF), insulin like growth factor (IGF), hepatocyte growth factor (HGF), prostaglandin E_2 (PGE_2) and other soluble factors that exert mitogenic, antiapoptotic, proangiogenic and anti inflammatory effects.

PARACRINE EFFECTS OF MESENCHYMAL STEM CELLS IN OTHER ORGANS

The notion that MSCs generate a paracrine effect on neighboring cells by secretion of soluble factors has gained wide acceptance in other organs. Rose et al. tested the ability of cultured MSCs to transdifferentiate into cardiac myocytes. They derived MSCs from mice in which GFP is driven by a cardiac-specific promoter (α-myosin heavy-chain promoter). These MSCs were cocultured with rat embryonic cardiomyocytes. While 6% of the MSCs became GFP positive, and expressed other cardiac markers such as troponin I and α-actinin, these MSCs never generated action potentials or displayed typical ionic currents of cardiomyocytes [82]. The lack of true functional differentiation of MSCs into cardiomyocytes supports the notion that MSCs exert paracrine effects in cardiac repair. Despite initial reports

that MSCs can transdifferentiate into cardiac myocytes in vivo [83,84], subsequent studies contradicted these findings, suggesting that they were based on staining artifact, fusion or partial expression of cardiac markers without true myocyte functional capacity [reviewed in Ref. 85].

MESENCHYMAL STEM CELL-DERIVED MICROVESICLES AND ACUTE KIDNEY INJURY

A new mechanism by which MSCs protect against AKI is by horizontal transfer of mRNAs contained within shed microvesicles. Bruno et al. demonstrated that microvesicles derived from human MSCs are as effective as MSCs themselves in accelerating recovery from glycerol-induced AKI in severe combined immunodeficiency (SCID) mice in vivo [86]. These microvesicles stimulated epithelial proliferation and inhibited epithelial apoptosis in vitro, and these effects could be abolished by treatment with RNAse. Of particular interest, the microvesicles shed by human MSCs in this study did not simply contain a random assortment of total MSC mRNA, but rather a subset of transcripts, suggesting selective enrichment of reparative RNAs within these vesicles. The authors demonstrated the presence of two human proteins, POLR2E and SUMO-1, encoded by mRNA present in the microvesicles, within nuclei of mouse kidney epithelia, indicating that the transferred mRNAs are translated and suggesting that they exert functional roles within cells. Taken together, these intriguing findings show that microvesicle-mediated transfer of mRNA, which has also been described in endothelial progenitor cells [87] and embryonic stem cells [88], is an additional, potentially important mechanism by which MSCs exert renoprotective effects.

RECRUITMENT OF ENDOGENOUS BONE MARROW MESENCHYMAL STEM CELLS

The observations that exogenously administered MSCs can protect against renal injury naturally leads to the question of whether endogenous MSCs might be recruited to participate in the repair process as well. It has been hypothesized that endogenous bone marrow-derived MSCs may circulate much in the same fashion as HSCs, and some studies have reported that MSC-like cells can be purified from blood, albeit in very low numbers [89]. Whether endogenous MSCs may be recruited from their bone marrow niche and home to sites of injury is an open question. While it is very clear that BMDCs, primarily inflammatory cells, traffic to the interstitium of injured kidney, it is completely unknown whether a subset of these cells is MSCs. In chimeric mice carrying genetic markers expressed in bone marrow, cells are definitively seen in the interstitium of injured kidney [31,90]; however, the presence of MSCs in these populations has not been assessed to date [91]. It is worth noting that the proportion of MSCs compared to non-MSCs in whole bone marrow is very low (0.01% or less), so the great majority of BMDCs in injured kidney represent inflammatory cells such as monocytes, macrophages, neutrophils and lymphocytes.

KIDNEY MESENCHYMAL STEM CELLS

An alternative possibility to homing of bone marrow-derived MSCs is activation of an endogenous kidney MSC population. Organ-specific MSCs are well described and the literature on kidney-derived mesenchymal cell lines is expanding. Gupta et al. reported the isolation of a population of cells from the adult rodent kidney which expressed markers of MSCs (CD90, CD44), expressed Oct4 but not cytokeratin, self-renewed in culture and incorporated into the renal epithelium [92]. The authors called these cells multipotent renal progenitor cells and suggested that these cells were candidate renal stem cells. Bussolati et al. isolated a $CD133^+$ population from the human kidney and found $CD133^+$ cells in the interstitium and within tubular cells. These cells did not express hematopoietic markers but expressed MSC markers (CD29, CD90, CD44 and CD73) [93]. When these cells were injected subcutaneously in Matrigel into SCID mice they developed into tubular structures and expressed proximal and distal tubular markers. When cultured in vitro with VEGF they expressed endothelial markers and when these differentiated cells were injected subcutaneously in Matrigel the cells were reported to form vessels that connected to the endogenous mouse vessels. When injected into mice with glycerol-induced AKI these cells homed to the kidney and reportedly integrated into proximal and distal tubules.

Dekel et al. identified a cell line from renal interstitium based on expression of Sca-1 and the absence of lineage markers [94]. This line was adherent to plastic and exhibited multilineage differentiation potential, and also inhibited the mixed lymphocyte reaction, similar to bone marrow-derived MSCs. Along these lines, Womer and co-workers isolated a $Sca1^+$, $CD24^+$, multipotent renal stromal cell line and showed that it had potent immunomodulatory properties [95]. Specifically, these cells induced differentiation of BMDCs into dendritic cells with low expression of MHC II and decreased CD80 expression, a phenotype associated with self-tolerance. The mechanism for this effect was at least partly through elaboration of IL-6. Both of these studies highlight the important immunomodulatory

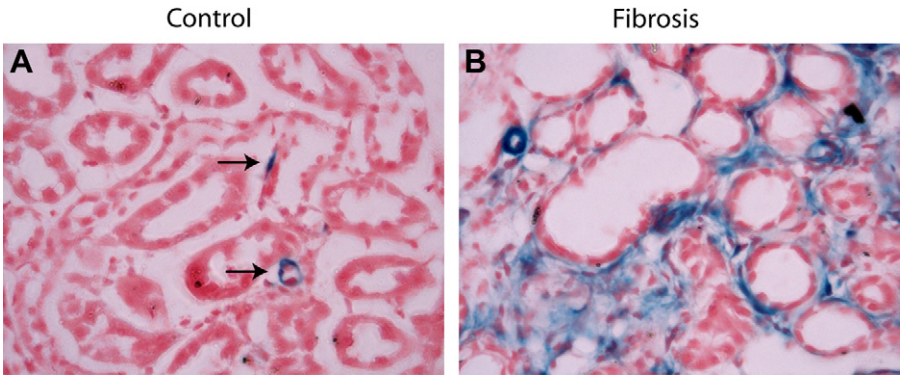

FIGURE 9.4 Lineage tracing perivascular fibroblasts in fibrotic kidney disease. Pericytes/perivascular fibroblasts were genetically labeled by administering Tamoxifen to bigenic mice expressing Tamoxifen regulated CreERt2 recombinase under control of the FoxD1 promoter, as well as a LacZ reporter gene that is activated by Cre recombinase. (A) Labeled cells appear blue in the renal interstitium. (B) After ureteral obstruction, there is dramatic expansion of LacZ positive interstitial cells throughout the fibrotic kidney. These cells gain expression of α smooth muscle actin, indicating that they are myofibroblasts. Whether a subset of pericytes, which are myofibroblast progenitors, might represent kidney specific mesenchymal stem cells requires further investigation [99]. Please see color plate at the end of the book.

roles for cells derived from the kidney which had characteristics of MSCs.

Sagrinati et al. reported the isolation of a $CD133^{+}CD24^{+}$ parietal epithelial cell population from the human adult kidney which could be induced in vitro to express markers of proximal and distal tubular cells, osteogenic cells, adipocytes and neurons [96]. In addition, Plotkin and Goligorsky isolated a multipotent clonal cell line from kidney and showed that these cells could differentiate into erythropoietin-producing fibroblasts under hypoxic culture conditions. These cells migrated to a peritubular and interstitial location, but not a tubular location, after injection in postischemic kidney [97]. In a follow-up study, the Goligorsky group used a Tie2-GFP reporter mouse to derive a clonal mesenchymal cell line from kidney with multilineage differentiation capability. These cells, when transplanted into ischemic kidney, promoted epithelial proliferation and exerted antiapoptotic effects and accelerated recovery, all similar to effects obtained with bone marrow-derived MSCs. Although it is not yet clear whether these groups are working with the same or different cell populations, these observations all support the notion that kidney-specific MSCs exist.

The renal pericyte/perivascular fibroblast has been identified as the progenitor cell for myofibroblasts, the cells responsible for scar formation in chronic kidney disease. In kidney, pericytes are defined by PDGF-Rβ expression, the absence of α-SMA expression, variable expression of NG2 and CD73, and absence of endothelial and hematopoietic markers [98,99]. During development, these stromal cells express the transcription factor FoxD1 [100], and therefore this cell population could be genetically labeled using a FoxD1-Cre driver line crossed to a Rosa26-LacZ reporter line. Bigenic mice expressed LacZ heritably in interstitial cells that were $PDGF\text{-}R\beta^{+}$, α-SMA , CD31 and F4/80 . After fibrotic stimuli, these cells underwent dramatic proliferative expansion and expressed both α-SMA and collagen 1α1 (Fig. 9.4). This is the first study to employ lineage analysis to define the myofibroblast progenitor. Given the close similarities between pericytes and MSCs, including shared expression of surface molecules (PDGF-Rβ, NG2, CD73), a perivascular niche and ability to secrete bioactive trophic molecules [11,97], it will be important to determine whether some subset of the renal pericytes identified by FoxD1 expression in development might represent a kidney-specific MSC. It will also be important to determine whether these lineage-marked pericytes include the mesenchymal cells identified by others [92,96,97,101]. It is intriguing that the 4E clonal cell line isolated from kidney using the Tie2 promoter did exhibit spontaneous myofibroblast differentiation (acquisition of α-SMA expression) in vivo [101]. Tie2, in addition to being expressed in endothelial cells, can be expressed in CD31 pericyte progenitors in vivo [102], and in immortalized pericyte cell lines in vitro [103], raising the possibility that this cell line could have been derived from a pericyte precursor.

CLINICAL TRIALS FOR MESENCHYMAL STEM CELLS IN ACUTE KIDNEY INJURY, GRAFT TOLERANCE AND LUPUS NEPHRITIS

MSCs have had a very good safety record in human studies to date. Unlike pluripotent embryonic stem cells, MSCs do not form teratomas when injected in vivo. In the human trials reported so far, no major adverse effect has been attributed to the injection of this cell type.

Nevertheless, little long-term follow-up information about the consequences of administered MSCs is available. In a recent study, Kunter et al. injected MSCs intrarenally into the rat in a model of glomerulonephritis. While there was a beneficial therapeutic effect on day 60, approximately 20% of the glomeruli of MSC-treated rats contained large adipocytes derived from maldifferentiation of MSCs with pronounced surrounding fibrosis [56]. Ectopic osteogenic maldifferentiation of MSCs has also been observed in a cardiac cryoablation injury model [104]. One possible explanation for the observations of Kunter and colleagues is the high numbers of MSCs injected. These investigators injected 2×10^6 MSCs into renal arteries of rats. By contrast, Westenfelder and others have injected 1×10^5 cells in the suprarenal aorta of mice. Even normalized for kidney size Kunter et al. injected significantly more MSCs and the resulting high local cellular concentration may predispose toward MSC differentiation.

An important advance has been the storage of characterized human MSCs. Because the phenotype of cultured MSCs depends on how they are isolated, the density at which they are plated, the culture medium and other variables, the establishment of MSC banks should accelerate therapeutic application and reduce variability. Indeed, it is not difficult to envision large collections of banked MSCs, similar to cord blood banks for HSCs [105]. With the development of biomarkers for earlier detection of AKI the possibility of one day selecting matched MSCs, thawing them and infusing them within 24 h of the renal insult seems feasible [106].

Indeed, MSC therapies are already being tested in a multitude of human diseases including myocardial infarction, graft versus host disease, Crohn's disease, osteogenesis imperfecta and many others [15]. Perhaps most progress has been made in the field of myocardial infarction, where several small phase I and phase II trials have reported modest improvements in both physiological and anatomic parameters after intracoronary delivery of various BMDC populations, including MSCs [107]. Calls for large randomized trials of cell therapies have been made [108] and the first phase I trial of MSCs in AKI has completed enrollment. This study of cardiac surgical patients enrolled high-risk patients in a dose-escalating phase I clinical trial (www.ClinicalTrials.gov; NCT00733876) designed to test the safety, feasibility and preliminary efficacy of allogeneic MSCs in treatment of AKI. Patients scheduled to undergo on-pump coronary artery bypass grafting or valve surgery and who possess renal risk factors such as preexisting renal disease, diabetes, age >60 years and redo surgery were enrolled. Since allogeneic MSCs need to be expanded in vitro, most surgeries were elective; however, one arm of the study will involve administration of allogeneic MSCs to patients in need of emergent surgery. Preliminary results of the trial have been reported, and there were no adverse effects attributable to MSC therapy. To assess preliminary efficacy, outcomes in test subjects were compared with those of matched historical controls, and this showed a 50% reduction in postoperative length of stay and readmission. At 3 6 months after surgery, subjects who had received MSC therapy had normal serum creatinine, in contrast to historical controls which saw a gradual deterioration of renal function over this time-frame [109]. While the full results of this trial have not yet been reported, these preliminary findings provide validation for the overall concept of MSC therapy for treatment of AKI, although interpretation is subject to the usual concerns with the use of historical controls.

MSCs are being investigated in several other kidney diseases, particularly those in which inflammation plays a prominent role. MSCs for prevention of allograft rejection and induction of tolerance (www.ClinicalTrials.gov; NCT00752479) form the subject of one phase I and II trial designed to explore the safety and effect of syngeneic MSCs in living-related renal allograft recipients with a goal of assessing MSC-mediated graft tolerance. Syngeneic MSCs are expanded ex vivo and administered (2×10^6 MSC/kg body weight) at the time of transplant. A similar trial will involve administration of a second dose of MSCs 2 weeks after allograft surgery (www.ClinicalTrials.gov; NCT00658073). At least two human trials are currently underway investigating the use of MSCs in treatment of lupus nephritis (www.ClinicalTrials.gov; NCT00698191 and NCT00659217).

CONCLUSION

Regenerative medicine has brought together scientists in different fields such as developmental biology, engineering, immunology and medicine with the goal of developing new therapies for the treatment of human disease. The key to realizing the promise of stem cell therapies in kidney disease is to understand better the biology of kidney progenitors in development, and the signaling pathways regulating adult epithelial cell repair and proliferation after an acute insult. Although much remains to be learned about MSCs' underlying biology and long-term safety, MSC therapy holds great promise for the treatment of AKI in humans.

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CHAPTER

10

Endothelial Progenitor Cells and the Kidney

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OUTLINE

INTRODUCTION

As are many other organs, the kidney is capable of self-maintenance under physiological conditions. On a daily basis, epithelial cells are shed in the urine and replaced. Likewise, after tubular necrosis, the tubular epithelium can be fully regenerated. More recently, it has been postulated that even podocyte loss can be repaired from the parietal epithelial niche. Many insults that lead to epithelial damage are associated with endothelial injury and activation.

For example, during ischemia reperfusion injury endothelial cells become physically detached and rarefaction of the capillary bed in the kidney may occur [1]. Recent data suggest that the endothelial pericyte interaction may play an important role in development of renal fibrosis and progression of renal disease. Endothelial activation may drive a regulatory phenotype of pericytes, which is involved in stabilization of the microvascular network, into a myofibroblast phenotype that leads to extracellular matrix deposition [2]. Endothelial integrity and control of endothelial function thus may be important determinants of renal repair processes and, when they fail, of renal disease progression. Careful labeling studies with proliferation markers such as Ki67 have demonstrated, even in healthy subjects, the presence of a very high glomerular and peritubular and endothelial cell turnover. In fact, the proliferation index doubles that of the tubular epithelial cells [3]. This is likely to be a reflection of ongoing microvascular repair. Consequently, endothelial regenerative capacity may be limited owing to subsequent telomere shortening and replicative senescence. This repair mechanism may even be further compromised in renal disease, which is usually characterized by increased redox stress in the vasculature. Such redox stress has been associated with the development of senescence by itself [4].

In recent years, attention has been drawn to circulating cells that could serve as a potential source for endothelial repair and regeneration [5 8]. These cells are often referred to as endothelial progenitor cells (EPCs). However, it turns out that this concept is largely based on different in vitro systems used to characterize these cells. Consequently, phenotypically different circulating cells have been coined EPCs and their in vivo relevance may differ from model to model. This does not distract from the fact that such circulating cells, either as a direct source for endothelial repair or by indirectly

modulating the microenvironment of the vasculature, could be of great importance in endothelial repair and preservation of kidney function. This chapter reviews the various populations that have been proposed as EPCs, the methods used to identify them and their respective in vivo importance.

THE CONCEPT OF ENDOTHELIAL PROGENITOR CELLS

In 1997 Asahara et al. performed a seminal experiment that led to the concept of EPCs. Using magnetic beads coated with an antibody to CD34, they isolated mononuclear blood cells and plated them on fibronectin. After 7 days the investigators found that these cells, when cultured under conditions that favor endothelial cell growth, differentiated into an endothelial-like phenotype. In addition, they demonstrated that labeled $CD34^+$ cells in vivo could home to sites of ischemia where they appear to incorporate into the microcirculation and favor recovery of ischemia [5]. The idea behind these experiments is that the hematopoietic lineages and the endothelial lineages may share a common ancestor in embryology and that in this way this concept of EPCs would recapitulate this phenomenon. Indeed, recent lineage tracing experiments demonstrated in both zebra fish and mice that hematopoietic stem cells originate from a subset of endothelial cells of the aortic floor [9 11].

The concept of repair by EPCs was subsequently expanded by a vast array of studies usually involving a bone marrow chimera model where, in models varying from cancer to ischemic heart disease, incorporation of bone marrow cells could be demonstrated [12,13]. In the renal field incorporation of endothelial cells that apparently were derived from a bone marrow precursor population were found in models of renal disease such as anti-Thy1 glomerular nephritis [14,15] and Adriamycin (doxorubicin) nephrosis [16]. The functional importance of such incorporation was demonstrated by the fact that administering bone marrow cells therapeutically could enhance the recovery of anti-Thy1 glomerular nephritis [15]. Along the same lines, inhibition of apoptosis in Adriamycin nephrosis resulted in increased incorporation of bone marrow cells in the peritubular capillaries, leading to preservation of renal perfusion. This was associated with a reduction in interstitial fibrosis [16].

ENDOTHELIAL PROGENITOR CELLS AS AN INDICATOR OF CARDIOVASCULAR DISEASE

Based on the aforementioned observations, clinicians became interested in measuring EPCs as a potential marker of vascular repair. Unfortunately, a somewhat confusing situation has developed where three different assays have been advanced to assess the number of EPCs. These assays probably reflect a different biology of circulating cells and are hard to relate to the actual in vivo biology of vascular repair.

Early Outgrowth Cells: Myeloid Endothelial Progenitor Cells

When peripheral blood mononuclear cells are isolated by Ficoll gradient centrifugation and cultured on fibronectin-coated tissue culture plates in vascular endothelial growth factor (VEGF)-enriched medium, spindle-shaped cells appear after 3 4 days. These attaching cells incorporate acetylated low-density lipoprotein (LDL), bind various lectins such as *Ulex europaeus* agglutinin and express endothelial markers, such as von Willebrand factor, eNOS or CD31. They display proangiogenic capacities in animal models, notably in ischemic hindlimb [7], and the enumeration of these attaching cells has been associated with the risk of developing coronary heart disease [17]. Even though these cells have been described as EPCs, the phenotypic characterization of the attaching cells is usually not consistent with that of a mature EPC phenotype. For example, markers that are frequently used include the uptake of acetylated LDL and the presence of CD31. Both markers, however, are also present on myeloid cells. Indeed, using lineage tracing methods with endothelial nitric oxide synthase (eNOS) green fluorescent protein (GFP) transgenic mice, it was recently demonstrated that the majority of the endothelial-like cells in this assay are derived from myeloid precursor cells [18]. There are also indications that part of the endothelial markers in the attaching cells could be derived from microparticles taken up from endothelial cells or platelets, thus further defying the concept that the endothelial properties of these cells can be defined by surface markers [19].

Although angiogenic properties have been attributed to these cells when exposed in vitro to VEGF, the circulating myeloid precursors appear to lack such angiogenic properties. Urbich et al. used both freshly sorted human $CD14^+$ cells from Ficoll-isolated human peripheral blood mononuclear cell (PBMCs) and $CD14^+$-derived EPCs (cultured in VEGF-enriched medium) to inject into nude mice that underwent hindlimb ischemia. They showed significant neovascularization after 2 weeks in the mice receiving the $CD14^+$ EPCs compared with the mice that received no cells or fresh $CD14^+$. Moreover, they used CD14 subfractions and cultured under the same conditions as $CD14^+$ to generate EPCs. These CD14 -derived EPCs also showed neovascularization properties [20]. These results strongly suggest that

myeloid cells per se are not capable of inducing angiogenesis, but need a proangiogenic stimulus to acquire this property.

Circulating $CD34^+$ (KDR^+) Endothelial Progenitor Cells

Another method used to identify EPCs is to detect circulating cells according to the expression of their surface markers by flow cytometry.

$CD34^+$ cells can be isolated from human peripheral blood through magnetic beads cell sorting. Dead cells are excluded by propidium iodide, and possible contaminating monocytes are excluded by gating out the $CD14^+$ cells. Cells are subsequently incubated with monoclonal antibodies against the extracellular parts of VEGFR-2 or CD133. Granulocyte colony-stimulating factor (G-CSF) can be used to stimulate the mobilization of $CD34^+$ cells from the bone marrow (from 0.4% to 2.0% of the $CD34^+$ population) to enhance sensitivity of the measurement. Other groups [21] used peripheral blood directly with the monoclonal antibodies, resulting in a count of 0.03% of circulating PBMCs.

During fluorescence-activated cell sorting (FACS) analysis, circulating progenitor cells are characterized as cells located in the lymphocyte gate (based on forward-side scatter) and expressing $CD34^+$ and $CD45^{dim}$. Some research groups also add the presence of the VEGF receptor-2/KDR or the stem cell marker CD133 to characterize the progenitors further. It is customary to express the number of $CD34^+$ cells per 10^3 $CD45^+$ leukocytes.

Numerous studies have shown a correlation between circulating CD34 cells, thus measured, and cardiovascular risk. In addition, most forms of renal disease are associated with reduced numbers of these cells (Table 10.1). It is, however, doubtful whether such reduced numbers also reflect reduced vascular repair. Clearly, $CD34^+$ cells that have been primed in vitro with endothelial cell growth factors exert proangiogenic effects. However, whether naïve $CD34^+$ cells can rescue in vivo ischemia is less clear.

In line with this, clinical studies in cardiology that have used bone marrow mononuclear cells or $CD34^+$ cells have, so far, failed to show an unambiguous effect on repair after ischemic injury. One explanation why circulating $CD34^+$ cells reflect cardiovascular risk in such a robust way could be related to the mobilization of $CD34^+$ cells from the bone marrow appearing to be an eNOS-dependent mechanism. Aicher et al. demonstrated that recruitment of hematopoietic stem cells from the bone marrow is impaired in eNOS knockout mice [32]. Many cardiovascular risk factors impact on the eNOS system. This particularly holds true for uremia, where a long list of factors, including elevated levels of asymmetric dimethyl arginine (ADMA), cytokines, hypertension and impaired glucose tolerance, have all been demonstrated to reduce nitric oxide (NO) availability. Another discussion point with respect to the circulating $CD34^+$ population is whether or not kinase domain receptor (KDR) coexpression would reflect a subtype that is predestined to be involved in endothelial repair. Recent experiments from this laboratory, however, show that the VEGF receptor-2/KDR can be expressed by all $CD34^+$ cells provided they have encountered a platelet-rich injured vessel wall and shear stress [33]. Consequently, KDR expression may be a reflection of vascular injury and probably does not reflect a functional predestination in the bone marrow.

Endothelial Progenitor Cells and Colony-forming Units

Two main methods have been described that define EPCs by their performance in colony-forming assays: the colony-forming unit Hill (CFU-Hill) [34] and the endothelial colony-forming cell (ECFC) assays [35]. Both are based on the number of colonies formed by mononuclear cells plated on a fibronectin-coated tissue culture plate. The CFU-Hill uses the non-adherent cells to form colonies in 4 7 days, and these are named after early outgrowth cells. Following the work of Hill [34], the CFU-Hill has been used in various clinical settings as a marker for disease. In chronic models such as type 1 diabetes [36], chronic obstructive pulmonary disease [37], heart failure [38] and rheumatoid arthritis [39], the number of colonies is found to be decreased. In contrast, acute events such as acute myocardial infarction [40] and unstable angina [41] are associated with an increase in the number of colonies. These discrepancies could be due to chronic versus acute events, but also to the fact that these colonies consist of various types of cells, including T cells and monocytes [42], and do not display postnatal vasculogenesis capacities when implanted into immunodeficient mice [43]. Therefore, the use of CFU-Hill is unlikely to reflect endothelial repair.

ECFCs refer to the long-term culture colonies formed between 7 and 21 days of adherent mononuclear cells obtained from peripheral blood, umbilical cord blood or human umbilical venous endothelial cells. These colonies are made of truly clonal endothelial cells with a proliferative potential and the ability to form blood vessels [42]. Indeed, they express primary endothelium cell surface antigens such as von Willebrand factor, CD31, CD105, CD146, KDR or CD144. Contrary to the CFU-Hill, they do not express myeloid antigens such as CD14, CD115 [macrophage colony-stimulating factor (M-CSF) receptor] or CD45. When replated, they keep forming colonies, whereas CFU-Hill do not. The count

TABLE 10.1 Clinical Studies with EPCs in Chronic Renal Failure

EPC type	Context	Readout	Findings	Improved	Ref.
Myeloid	HD	Number of colonies, migratory capacities	↓ colonies, positive correlation with kT/V	VEGF improves migratory capacities	[22]
	KTx	Number of colonies cultured with patients' serum	More colonies with serum from graft with CrCl > 50 ml/min		[23]
	ESRD	Number of colonies	↓ colonies	8 weeks of rhEPO	[24]
	ESRD	Number of colonies from patients and cultured with patients' serum	↓ colonies in ESRD or with ESRD serum	HD	[25]
	ESRD	Number of colonies and angiogenic properties	↓ colonies, decrease tube formation	Worsened by HD	[26]
	KTx	Number of colonies, migration assay	Improvement of migratory capacities	KTx	[27]
	ESRD	$CD14^+$ EPC count	Non significant increase with decline in GFR		[28]
$CD34^+$	ESRD	Peripheral count of $CD34^+KDR^+$	$CD34^+KDR^+$ increases with eGFR		[29]
	KTx	Peripheral count of $CD34^+$	Decreased in ESRD compared to KTx		[23]
	ESRD	Peripheral count of $CD34^+$	Transient increase under rhEPO	rhEPO	[24]
	ESRD	Peripheral count of $CD34^+$	Decreased circulating cells	HD	[25]
	ESRD	Peripheral count of $CD34^+KDR^+$	$CD34^+KDR^+$ decreased in number	EPO	[26]
	KTx	Peripheral count of $CD34^+$, $CD34^+KDR^+$ and $CD34^+CD133^+$	$CD34^+$ and $CD34^+CD133^+$ decreased in number	KTx	[27]
	ESRD	Peripheral count of $CD34^+$	Decreased in number with age, urea and Pi levels	HD, statins	[28]
	HD	Peripheral count of $CD34^+$	Decreased number compared to healthy control and genomic damage	Just after HD session	[30]
	HD	Peripheral count of $CD34^+$	Decreased number correlates with PTH and phosphate values		[31]

EPC: endothelial progenitor cell; HD: hemodialysis; KTx: kidney transplantation; ESRD: end-stage renal disease; eGFR: estimated glomerular filtration rate; EPO: erythropoietin; Pi: phosphatemia; PTH: parathyroid hormone; VEGF: vascular endothelial growth factor.

of peripheral blood-derived ECFCs is positively correlated with the severity of angiography-proven coronary heart disease [44]. It is speculated that such colonies reflect the capacity of certain resident endothelial cells to be the source of endothelium for angiogenesis [35].

MICROVASCULAR REMODELING BY CIRCULATING CELLS

Clearly, circulating cells participate in neoangiogenesis and repair responses to ischemia. These cells include a variety of hematopoietic cells and probably also a rare circulating true EPC. The latter is probably best reflected in the ECFC assay. It is likely that long-term endothelial preservation requires a source of endothelial cells with high replicative potential. One working hypothesis could be that low-grade maintenance and repair is dependent on these cells. The origin of these ECFCs is unclear. Work from the Yoder group has demonstrated that these cells may reside in the vessel wall [45]. When ischemic insults or more severe microvascular injury occur, the remodeling process is likely to be an integral part of the inflammatory response [46]. While myeloid cells, for example, are thought traditionally of as effector cells of the innate immune system, research in recent years has identified that such cells may also exert anti-inflammatory and repair mechanisms. In several fields of research such myeloid cells have been identified and go by different names such as monocyte type-2, myeloid-derived suppressor cell and Tie2-expressing monocyte [47]. There is controversy over whether such hematopoietic and myeloid cells could actually become true endothelial cells. Using

high-resolution confocal microscopy, Purhonen et al. suggested that such cells may home to the subendothelial space and take up a phenotype similar to that of pericytes [48]. Indeed, it would be very hard to discriminate pericytes from endothelial cells using usual imaging techniques, while several of the so-called endothelial-specific marker genes such as Tie2 also appear to be present on both cells. This does not distract from the potential importance of these cells in maintenance and remodeling of the vascular bed.

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CHAPTER

11

Potential of the Side Population in Regenerative Nephrology

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OUTLINE

WHAT IS THE SIDE POPULATION?

The isolation of a stem or progenitor population can rely on a combination of cell surface markers, enrichment based on location and/or isolation based on functional properties. While the mouse hematopoietic stem cell (HSC) population is often defined on the basis of lack of expression of a series of lineage epitopes, and positive expression of canonical HSC markers such as Sca-1 and c-Kit (CD117), one phenotypically defining property of HSCs is low fluorescence after staining with vital dyes, including Rhodamine 123 and Hoechst 33342.

The term Hoechst "low" side population (SP) was originally described in murine bone marrow preparations where isolation based on dye efflux resulted in 1000 3000-fold enrichment for long-term repopulating HSC activity in bone marrow transplantation assays [1]. This proved as effective as the isolation of HSCs based on sorting for the commonly used combination of lineage cell surface markers [2,3]. As a result, the application of this dynamic measurement alone or in

combination with antibody staining has proven to be a powerful tool in the purification of HSCs from mouse bone marrow.

How and why do these cells efflux dyes? Supravital dyes, including Hoechst 33342, bind to AT-rich regions within the minor groove of DNA in all cells [4]. Hence, the resulting cellular fluorescence intensity is an index of DNA content, and can discriminate between different stages of cell cycle [5]. The efflux of such dyes from a cell results from the activity of membrane efflux pumps, all of which are members of the ATP-binding cassette (ABC) transporter superfamily. ABC transporters are ATP dependent and can move a variety of cargoes into or out of cells, including metal ions, amino acids, sugars, peptides, hydrophobic compounds, inorganic anions, metabolites, xenobiotics and other drugs [6,7]. Some ABC transporters are present on the plasma membrane, while others exist in subcellular membrane compartments such as Golgi and endoplasmic reticulum (ER). ATP transporters are widely expressed throughout the mammalian body, although predominantly in excretory organs including the liver, placenta, kidney, intestine and blood brain barrier [7]. The ABC transporters most commonly associated with the dye-efflux properties of the SP include multidrug resistance 1/P-glycoprotein (Mdr1a/1b mouse/MDR1 human) [8] and breast cancer resistance protein 1 (Bcrp1)/ATP-binding cassette, subfamily G (WHITE), member 2 (ABCG2) [9]. While dye efflux across the plasma membrane may result from other non-ABC transporters within the plasma membrane, ABC transporter-mediated efflux is specifically blocked by calcium channel blockers such as verapamil or reserpine and inclusion of these compounds in the staining media ablates the SP phenotype.

ROLE OF ABC TRANSPORTERS: MARKERS OR MEDIATORS OF STEM CELLS

Given the widespread expression and functional roles of ABC transporters, it may seem unlikely that their presence alone confers a stem cell phenotype. However, enforced expression of at least some of these membrane transporters using retroviral vectors has been shown to have direct functional effects on murine HSCs. Induced expression of ABCG2 in HSCs blocked hematopoietic development [9], whereas overexpression of MDR1 resulted in HSC expansion and myeloproliferative disorder [10]. This suggests that the expression of these transporters may assist in determining cellular differentiation. While the presence of such transporters may contribute to the SP phenotype, they are not exclusive markers that would allow purification based on their presence. In human bone marrow, ABCG2 coexpression with the HSC markers CD34 and CD133 was observed at low or undetectable levels and cells sorted solely on the marker ABCG2 had little colony-forming potential in vitro [11]. This may be due to the presence of multiple ABC transporters on the stem cell population.

A recent study of the SP in the murine myocardium across development suggests that the ABC transporter primarily responsible for this phenotype shifts from Abcg2 during development to Mdr1 in postnatal life [12]. This suggests a distinction between embryonic and adult cardiac SP but does not eliminate the possibility that the two are in the same lineage. The functional significance of Abcg2 in embryonic cardiac SP, however, appears to be in maintaining proliferative capacity and cell survival as loss of this gene results in cell death and reduced division [12]. Similarly, in embryonic stem cells, Abcg2 plays a role in the maintenance of pluripotency [13,14]. In line with this is the observation that during early murine development, all cells of the morula and blastocyst inner cell mass are positive for Abcg2 and Abcb1/P-glycoprotein, whereas this expression is lost as cellular pluripotency gives way to lineage commitment [15].

It has been proposed that efflux of drugs and toxins via these pumps provides protection to the stem cell niche. Indeed, hypoxia can apparently increase the expression of Abcg2 in the cardiac SP. Martin et al. [16] showed this allowed the SP cells to consume hydrogen peroxide after ischemic injury. Increased Abcg2 was elicited in response to hypoxia-inducible factor-2α (HIF-2α) and was accompanied by increased α-glutathione reductase expression, thereby endowing a cell survival benefit in the face of elevated hydrogen peroxide [16]. What appears to occur within the heart is an increase in SP as a percentage of total heart tissue. However, hypoxia may not be causing an increase in SP fraction but simply increasing the levels of expression of Abcg2 on all cells.

EXISTENCE OF SIDE POPULATION FROM OTHER ORGANS AND THEIR ASSOCIATION WITH ORGAN REGENERATION

SP cells have now been isolated from a wide variety of mammalian tissues including skeletal muscle, lung, liver, heart, testis, kidney, skin, brain, fat, uterus and mammary gland (Table 11.1). In some instances, the existence of these populations has been verified in humans. The diversity of tissues harboring SP has largely been reviewed previously [37], although further populations have now been identified, as has evidence of a link with tissue repair and regeneration and/or the presence of stem cell characteristics. The tracheobronchial

TABLE 11.1 Immunophenotype and Gene Expression of Side Population from Various Tissues

Tissue	Lin	CD45	CD34	Sca1	c kit	Thy1	Other	Refs
Bone marrow		+	+/	+	+	+/		[1–3]
Heart				+/			CD44, CD90, CD105, CD106, CD73, Abcg2, Mdr1, CD31	[12,16–18]
Skeletal muscle			+/	+				[19]
Embryonic liver		35–41%	43–46%			11–37%		[20]
Lung	Contains two distinct populations (CD45$^+$ and CD45)							[21,22]
Airway epithelium		+/					Bcrp1, cytokeratin	[23]
Testis				+	+/			[24,25]
Adipose			+				CD31, c met, VEcadherin, Abcg2, Flk1	[26]
Skin			+	+				[27]
Retina						+/		[28]
Ocular surface	Uncharacterized other than Hoescht efflux						Abcg2	[29]
Brain					+			[30]
Pituitary							Sox2	[31]
Uterus					+		CD9 , CD13	[32]
Articular cartilage	Uncharacterized other than Hoescht efflux						Notch 1, Stro1, VCAM +	[33]
Dental pulp	Uncharacterized other than Hoescht efflux						Abcg2, nestin, notch 1, CD31 CD146	[34,35]
Vocal fold	Uncharacterized other than Hoescht efflux						Abcg2	[36]

epithelium of the human lung contains an SP fraction which increases in proportion dramatically in asthmatic lungs [23]. These cells showed stable telomere length and were able to be cultured for 16 passages. These cells were also able to form a differentiated epithelium at an air media interface, suggesting regenerative capacity within the lung. An SP can be isolated from the dental pulp and subfractionation of these cells to obtain the CD31 /CD146 cells yielded a population of cells able to elicit a total regeneration of the pulp and accompanying vasculature in a canine model of pulp amputation [38]. These cells appear to produce proangiogenic factors, suggesting that a tropic activity was responsible for recruitment of endothelial cells rather than transdifferentiation [38].

To test the ability of skeletal muscle-derived SP to enhance regeneration, GFP-positive myoblasts were transplanted into immunocompromised normal mice or dystrophin-deficient mice with or without accompanying CD31 CD45 SP. The presence of the SP fraction increased the number and distribution of GFP-positive myoblasts integrating into the recipient muscle as well as stimulating their proliferation [39]. The SP was shown to produce matrix metalloproteinase-2 (MMP-2), gelatinase and a number of proliferative cytokines, and it was subsequently shown that MMP-2 alone was responsible for enhanced myoblast migration in vivo [39]. MMP-2 activity [40] has also been shown to be important in stem/progenitor cell differentiation. In the tooth this results from the MMP-2-mediated cleavage of dentin matrix protein (DMP1) which then acts directly on the dental pulp progenitors [40]. Another group has shown that the SP itself produces the DMP1, hence these cells induce the production of MMP-2 by adjacent cells and provide the substrate to be activated [34]. Others propose that in the heart the MSCs increase the generation of MMP-2 by the surrounding cardiac fibroblasts [41].

An SP also exists within the stromal compartment of human adult adipose tissue [42]. These cells showed the anticipated mesodermal multipotentiality in vitro but also facilitated scar-free wound healing in vivo [42]. SPs also exist in articular cartilage [33], vocal cord [36] and pituitary gland [31].

DOES DYE EFFLUX EQUAL STEM CELL?

The presence of SP within a variety of solid organs has raised the possibility that low fluorescence following Hoechst staining may represent a common stem cell characteristic. However, other than dye efflux, these populations vary significantly. SP from solid organs all display high expression of Sca-1 and the absence of hematopoietic lineage markers [37]. However, other than these few examples, there is little commonality of epitopes between SP from different organs. Indeed, it would appear that each organ contains a distinct SP in terms of cell surface marker profile.

To be defined as stem cells, SP cells must possess the capacity to differentiate into multiple cell lineages contained in their tissue of residence. For example, murine bone marrow SP represent long-term repopulating HSCs [1,43] able to form all hematopoietic lineages. Arguably only the bone marrow SP fraction has been stringently assessed for this capacity. This may result from the current limitations in suitable assays for tissue-derived stem cells rather than the potential of the SP they contain. As well as being able to generate cell lineages within their tissue of residence, bone marrow SP have the capacity to differentiate into skeletal muscle [19], liver [44], osteogenic [45] and cardiac [46] cells in vivo. Skeletal muscle SP has been reported to give rise to blood derivatives [19] and skin SP to skeletal muscle cells [27]. The hematopoietic capacity of liver SP cells in vitro was less robust [44].

Liver SP cells are able to give rise to a variety of liver-specific cell types, including mature hepatocytes and bile duct epithelium [44]. Welm et al. [47] tested the differentiative potential of mammary gland SP cells after isolation from Rosa26 transgenic mice and transplantation into cleared fat pads of immunocompromised Rag-1 $^{/}$ recipient mice [47]. Donor SP cells were found contributing to both the ductal and alveolar epithelium of the resulting mammary outgrowths [47]. Finally, SP derived from the testis was able to generate the full range of spermatogenic stages when delivered into testes of busulfan-treated mice [24].

In a study of the adipose SP, gene expression at point of isolation identified the expression of a number of stem cell markers (Notch pathway and early vascular precursor genes) as well as the expression of α-smooth muscle actin (α-SMA), CD34, Angpt2, Flk1, CD31, VE-cadherin and c-met [26]. The authors suggest that this points to a pericytic origin for the adipose SP and show that such cells can undergo myogenic and vasculogenic differentiation in vitro and in vivo, as has been reported for other vessel-associated cells. This is reminiscent of the mesenchymal stem cell (MSC) populations of many organs [48]. In most instances, the relationship between MSCs and SP from a variety of organs has not been well investigated. However, a common pericytic location may suggest an overlap in these populations in some instances. In the lung, it has been shown that the CD45 SP contains MSCs in as far as these cells have a capacity to clonically proliferate with stable telomere length, show expression of CD44, CD90, CD105, CD106, CD73 and Sca1, lack classical hematopoietic markers and differentiate into mesenchymal lineages in vitro [49]. Hence, either the line between these various cell definitions is quite blurred or the assays used to distinguish them apart in vitro do not make this distinction.

Other SP have been described as being able to differentiate into endothelium. Adult myocardial SP treated for 4 weeks with vascular endothelial growth factor-A (VEGF-A) in vitro formed vWF-positive endothelial cells [50]. A vasculogenic claim has been made for a subfraction of the dental pulp SP [35], where parallels were drawn between endothelial progenitor cells (EPCs) and the CD31 CD146 fraction of the SP. These cells were also $CD34^{+}$, $Flk1^{+}$ and CD45 and produced VEGF-A, GCSF, GM-CSF and MMP3; however, no evidence was presented to show that these cells themselves became the newly formed vasculature in vivo.

FUSION VERSUS TRANSDIFFERENTIATION: DOES IT MATTER?

Despite the data presented here suggesting multilineage potential for a variety of SP populations, whether this represents transdifferentiation or fusion with another differentiated cell type needs to be assessed carefully. Indeed, what is most critical is whether or not these proposed SP-derived cell types are functional. This has been investigated using the genetic dystrophin-deficient *mdx* mouse strain. Skin SP cells from male donor mice when injected into female *mdx* mice generated dystrophin-expressing Y-chromosome-positive muscle fibers 3 months after transplant [27]. Similarly, in autologous transplantation of muscle SP cells lentivirally transduced with the human dystrophin gene, cells were detected exclusively in the muscles of *mdx5cv* mice after reintroduction via the vasculature [51], again suggesting active homing of these cells to sites of damage [51]. Such studies have substantial implications for autologous cell therapy for genetic muscular disorders and potentially also for other human single gene diseases including cystic fibrosis and Huntington's disease.

Another way to assess claims of SP transdifferentiation potential is to compare this fraction to non-SP fractions within the same organ. Synovial-derived SP cells can respond to BMP7 to produce collagen type II,

suggesting the potential to become cartilage. However, other non-SP cells within the synovium also responded to BMP7 in this way [52], arguing against SP enriching for a stem cell fraction in this organ. Indeed, the ATDC5 chondroprogenitor line contains both SP and non-SP fractions. The SP fraction increases as a percentage of the total in response to hypoxia; however, this fraction does not show any greater chondrocytic potential than the non-SP fraction [53].

While evidence for SP transdifferentiation after in vivo infusion is variable, infusion of SP does result in these cells reaching injured organs, suggesting that homing of SP can occur. Alvarez-Dolado et al. [54] showed that the ensuing engraftment of whole bone marrow from genetically marked reporter mice transplanted into lethally irradiated recipients occurred through fusion of host and donor cells and not through transdifferentiation. Here, multinucleated cells containing donor nuclei were observed in the Purkinje cells of the brain, hepatocytes and cardiac muscle [54]. Subsequently, it was demonstrated that myelomonocytic progeny of a single SP cell engrafted via a fusion event as opposed to direct differentiation into tissue-specific progenitors or mature organ-specific populations [55]. In the case of studies in which donor cells integrated into mdx muscle, the mechanism of apparent integration is harder to establish as myofibers are multinucleated. Revertant muscle function would appear to suggest that, irrespective of whether fusion or transdifferentiation had occurred, functional dystrophin was present. However, even this is complicated by the fact that dystrophin expression in a proportion of myofibers can occur in such mice without transplantation of wild-type cells as a result of exon skipping [56]. This becomes important when considering data using irradiated *Scgd* $^{/}$ mice (mutation that causes loss of δ-sarcoglycan expression, producing progressive cardiomyopathy and muscular dystrophy) transplanted with bone marrow SP cells, where engraftment with donor cells was observed but no expression of δ-sarcoglycan resulted [57].

ORIGIN OF NON-BONE MARROW SIDE POPULATION CELLS

There is accumulating evidence to suggest that SP within the bone marrow or circulation can be recruited to sites of tissue damage, including skeletal muscle [58], heart [59], liver [60,61] and kidney [62,63], during regeneration from certain types of damage. This raises the question of whether SP fractions in specific organs originate from a common pool of cells in the bone marrow and adopt tissue-specific characteristics upon seeding within a specific local environment. There are conflicting data relating to this possibility. In lethally irradiated recipients transplanted with bone marrow SP cells isolated from Rosa26 transgenic mice, 5 months after transplant approximately 40% of host muscle SP cells were LacZ positive, thus being derived from donor cells [64]. Total bone marrow and bone marrow SP cells transplanted into lethally irradiated mice produced lung SP cells that were donor derived and contained both $CD45^{+}$ and CD45 fractions [21]. In lethally irradiated mice transplanted with bone marrow SP cells, donor cells contributed to formation of both $CD45^{+}$ and CD45 hepatic SP populations after DDC treatment [44]. While these confirm an ability for bone marrow SP to home to sites of injury, they do not prove that all organ SP cells are derived from the bone marrow.

The alternative possibility is that organ-specific SP cells arise solely as a consequence of the normal development of that specific organ, but share the SP phenotype as a function of their inherent biological characteristics. This has been established for the testis, where 8 months after transplantation of GFP-positive bone marrow into germ cell-deficient mice (*W54/WV*), the recipient testis SP remained a similar size and contained only negligible GFP-positive cells [25]. In contrast, 50 60% of the $CD45^{+}$ cells in spleen and $>70\%$ of the bone marrow SP were GFP positive, indicating successful engraftment into the hematopoietic compartment, as would be expected [25]. Of note, an analysis of the SP fraction within the liver across embryonic development suggests that this population is likely to have an initial origin in common with the oval cell progenitors of the liver, a population that has long been established as playing a role in liver regeneration [20].

HETEROGENEITY WITHIN DISTINCT SIDE POPULATION FRACTIONS

While any potential stem cell population can be greatly enriched via specific selection protocols, the resultant cell populations remain heterogeneous. This is also the case for SP. While lack of CD45 is regarded as a feature of tissue SP fractions, approximately 75% of murine liver SP cells express CD45. The liver SP cells with the highest efflux capacity are enriched for CD45 cells [44], but both $CD45^{+}$ and CD45 subpopulations express the stem cell markers CD34, c-kit, Sca-1 and Thy-1 [44]. Similarly, while the lung SP represents 0.05 0.07% of the total viable cell population within the lung, 60 70% of these cells are $CD45^{+}$ with the remainder being CD45 [22]. While both lung SP fractions were Sca-1^{+}Lin , the $CD45^{+}$ fraction was $CD34^{+}CD31^{+}$ while the CD45 fraction was CD34 $CD31^{+/}$ [22].

Within the literature, there are also large discrepancies between reported SP abundance within the same tissue. These are likely to reflect variations in

isolation stringency and hence overall heterogeneity. In muscle, the fastest effluxing SP cells are almost exclusively Sca 1$^+$CD45 . However, if the stringency of isolation is dropped by increasing the gate size during sorting to include SP cells closer to the main population (MP), the proportion of Sca-1 CD45 and Sca-1 CD45$^+$ cells significantly increases [65]. This observation has also been made with respect to the bone marrow, where the SP cells shifted from a Sca-1$^+$CD45$^+$ phenotype to a Sca-1 CD45$^+$ as the size of the SP gate increased [65]. It should be noted that the original protocols for isolation of SP cells from bone marrow demonstrated a population that was phenotypically (Lin CD45$^+$Sca-1$^+$) and functionally quite homogeneous in terms of long-term reconstituting HSCs owing to a high level of stringency in the application of the sorting.

Clearly, variation in the level of SP heterogeneity, due to either inherent heterogeneity and/or variations in stringency of isolation between studies, makes the functional assessment of relative enrichment critical. This highlights the need to distinguish between "side population cell" and "stem cell" as the latter may represent only a fraction of the former. An assessment of the stem cell capacity of such cells in vitro and/or in vivo must be employed both to optimize the conditions for isolation of SP cells from individual tissue and to assess the relative enrichment afforded by sorting based on dye efflux. Ultimately, the subfraction of any SP responsible for any observed functional activity may not be able to be definitively identified.

IDENTIFICATION OF SIDE POPULATION CELLS WITHIN THE EMBRYONIC AND ADULT KIDNEY

As for many other organs, the presence of an SP has also been reported in the postnatal kidneys of mouse [66 68], rat [69] and human [70]. An SP fraction was also isolated from embryonic kidney [68]. The kidney as an organ is rich in ABC transporters, given the role of this organ in water and ion flux. Distinct segments of the nephron express distinct ABC transporters depending on their role in excretion and water reclamation. It is not surprising, therefore, that a dye-effluxing population exists within the kidney or that at a proportion of this renal SP is epithelial in nature. However, the accompanying observations regarding the in vitro and in vivo properties of the renal SP and the effect on renal function on the infusion of this population suggest that the presence of a renal SP does not simply reflect an abundance of ABC transporters in the kidney.

While the existence of such a population in kidney was agreed, there were large discrepancies with respect to the abundance of this fraction between different studies. These discrepancies are likely to have resulted from slight variations in tissue dissociation, time, temperature and dye concentration from one experiment or laboratory to the next [65]. For example, the few studies that have examined the murine kidney SP report dramatically different percentages of this population. Asakura and Rudnicki [66] and Hishikawa et al. [67] determined that the SP fraction represented approximately 5% of all total viable cells from the adult mouse kidney. In contrast, Challen et al. [68] report the frequency of the same population to be 0.1%, which is similar to the incidence of SP from adult rat kidney reported by Iwatani et al. [69] of 0.03 0.1% and more in line with the SP frequency more typically associated with other organs. This again highlights the variability in this staining procedure.

SP within the kidney is not confined to mammals. The existence of an SP has been reported within teleosts, including the zebrafish [71] and Gibuna cruian (carp) [72]. Hematopoiesis in these organisms is maintained in the kidney, hence this may reflect the HSC fraction of these organisms and not represent an equivalent to the SP of the mammalian kidney. Indeed, the properties of teleost kidney SP were equivalent to those of HSCs with respect to long-term repopulation capacity [72].

GENE EXPRESSION PROFILE AND IMMUNOPHENOTYPE OF KIDNEY SIDE POPULATION

Several studies have investigated the gene expression profile and immunophenotype of the kidney SP to understand their function and assist in the subfractionation of these heterogeneous populations based on the identification of unique genes encoding cell surface proteins. Using gene expression profiling, Hishikawa et al. [67] identified musculin/MyoR as a novel marker of adult murine kidney SP cells; however, this gene was not expressed in the study of Challen et al. [68]. Challen et al. [68] observed significant differences in gene expression between kidney and bone marrow populations, but a high congruence of expression between adult and embryonic kidney SP (Fig. 11.1A). The genes upregulated in both embryonic and adult kidney SP samples compared to bone marrow included a number of key growth factor receptors, including epidermal growth factor receptor (EGFR) and growth hormone receptor (GHR). The latter is of note given the potential use of exogenous GH therapy in the treatment of end-stage renal disease patients [73,74]. In addition, EGF is known to be a potent regulator of stem cell activity. Kidney SP cells also expressed CD24 and CD133. CD133 has been described as a marker of human

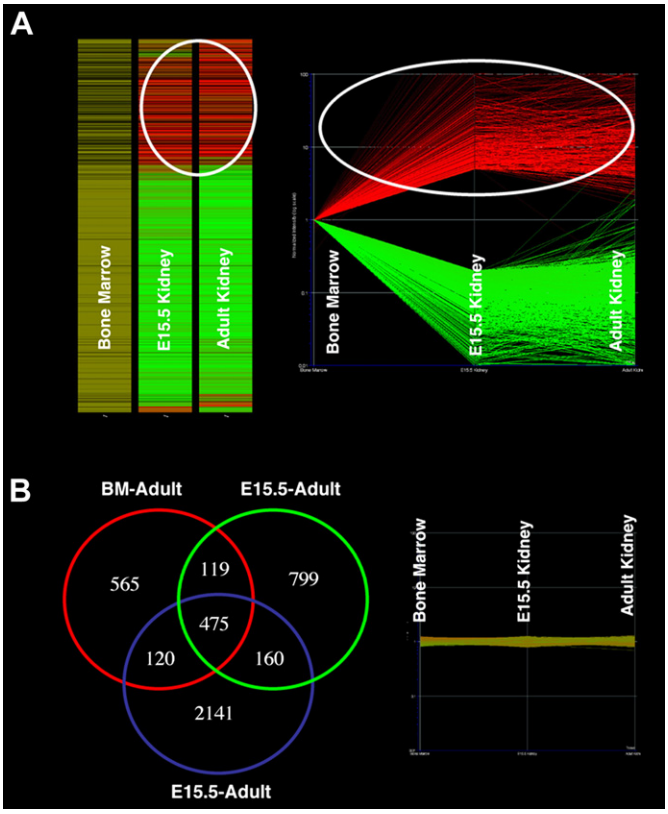

FIGURE 11.1 Affymetrix microarray data analysis. (A) Heat map of Affymetrix gene expression in bone marrow, adult and embryonic kidney shows high congruence between kidney samples. Right hand panel shows relative expression of all genes in kidney side population (SP) compared to bone marrow SP with red representing strongly upregulated and green representing strongly downregulated kidney SP genes. (B) Identification of "common SP genes" or a putative SP molecular signature by determining the genes with less than 25% variance in signal between bone marrow, E15.5 and adult kidney SP samples. The Venn diagram demonstrates a common signature of 475 genes. The right hand panel illustrates the relative expression of these 475 genes across all three samples. Please see color plate at the end of the book.

hematopoietic and neural stem cells and reportedly marks a human adult kidney stem cell population that may contribute to the repair of renal injury [75]. Genes that were upregulated in bone marrow SP cells compared to both kidney SP samples included known hematopoietic and HSC markers (CD34, CD44, CD45, CD53, c-kit, Ly64).

Using immunophenotyping, kidney SP shared some characteristics with bone marrow SP cells, such as high expression of Sca-1 and CD24. However, the kidney SP lacked the canonical HSC marker c-kit (Table 11.2). This again supported the premise that the renal SP is an endogenous population and not a circulating hematopoietic contaminant. In contrast, the only obvious difference in phenotype between the adult and embryonic kidney SP was an increased CD31 antigen in the adult kidney SP fraction.

TABLE 11.2 Immunophenotypes of Bone Marrow, E15.5 Kidney and Adult Kidney Side Population Cells

Marker	Bone marrow	Embryonic kidney	Adult kidney
CD45	+		
CD34	low		
c kit	+		
Sca 1	+	+	+
CD24	+	+	+
CD31	+		low

Gene expression analyses have also been used to try and determine the commonality between kidney and bone marrow SP (Table 11.3, Fig. 11.1B). Such genes are likely to indicate common functional pathways that may reflect similar functions for these populations. Abcg2, a major determinant of the SP phenotype in many tissues, was expressed by all three populations. Abcg2 also represented an SP-specific gene with respect to the non-SP fraction of the kidney, as did CD133, Gas1 and Sca-1. Analysis of the same population using immunophenotyping confirmed that kidney SP cells were CD45 CD34 c-kit Sca-1^{+}CD24^{+}CD31 $^{/low}$.

ORIGIN OF AND CELLULAR HETEROGENEITY WITHIN THE KIDNEY SIDE POPULATION

The expression profile of the renal SP appears to be conserved throughout development, suggesting that this population is established early in metanephrogenesis. Despite the developmental colocation of definitive hematopoiesis and mesonephric development within the aorta goad mesonephric region, the kidney SP shows little HSC activity in vivo [69] and is not likely to represent a direct derivative of hematopoietic progenitors. Indeed, using clustering algorithms to compare the expression profiles of embryonic and adult kidney SP with a variety of HSC profiles isolated using Hoescht efflux and sorting for a Lin c-kit^{+}Sca-1^{+} phenotype (unpublished data in collaboration with Professor Margaret Goodell, Baylor College of Medicine), the kidney SP fractions clustered away from those derived from bone marrow (Fig. 11.2). However, these cells also lack expression of many genes expressed in early metanephric development, indicating that they may not be derived from the metanephric mesenchyme (MM) or ureteric bud (UB) compartments of the developing kidney that give rise to the nephrons and the collecting ducts, respectively. Localization of the SP fraction within the kidney may point to their embryonic origin. This

TABLE 11.3 Cell Surface Transmembrane Proteins Highly Expressed in Bone Marrow, Embryonic Kidney and Adult Kidney Side Population Cells: Note the Expression of the abc Transporter Abcg2 and also the Expression of the Macrophage Markers Csf1r (c-fms) and EMR1 (F4/80)

Probe ID	Gene symbol	Description	UniGene
1437502 x at	Cd24	CD24a antigen	Mm.6417
1437278 a at	Uble1b	Ubiquitin like 1 (sentrin) activating enzyme E1B	Mm.27560
1439462 x at	1110014C03Rik	RIKEN cDNA 1110014C03 gene	Mm.276326
1419872 at	**Csf1r**	**Colony stimulating factor 1 receptor**	**Mm.22574**
1441959 s at	1200003C05Rik	RIKEN cDNA 1200003C05 gene	Mm.278477
1424683 at	1810015C04Rik	RIKEN cDNA 1810015C04 gene	Mm.25311
1433505 a at	Lrrc5	Leucine rich repeat containing 5	Mm.23837
1422906 at	**Abcg2**	**ATP binding cassette, subfamily G (WHITE), member 2**	**Mm.333096**
1416500 at	Sacm1l	SAC1 (suppressor of actin mutations 1, homolog) like	Mm.273671
1448233 at	Prnp	Prion protein	Mm.648
1455820 x at	Scarb1	Scavenger receptor class B, member 1	Mm.282242
1430125 s at	Pqlc1	PQ loop repeat containing 1	Mm.29247
1437378 x at	Scarb1	Scavenger receptor class B, member 1	Mm.282242
1423730 at	C130052I12Rik	RIKEN cDNA C130052I12 gene	Mm.30099
1451652 a at	5033428A16Rik	RIKEN cDNA 5033428A16 gene	Mm.155364
1426397 at	Tgfbr2	Transforming growth factor, beta receptor II	Mm.172346
1416555 at	Ei24	Etoposide induced 2.4 mRNA	Mm.4337
1418444 a at	Mir16	Membrane interacting protein of RGS16	Mm.273142
1431293 a at	1110019C08Rik	RIKEN cDNA 1110019C08 gene	Mm.29482
1425267 a at	3110045G13Rik	RIKEN cDNA 3110045G13 gene	Mm.158962
1424025 at	BC013529	cDNA sequence BC013529	Mm.33716
1424129 at	Mfsd1	Major facilitator superfamily domain containing 1	Mm.271975
1424782 at	2610318G18Rik	RIKEN cDNA 2610318G18 gene	Mm.290488
1416830 at	0610031J06Rik	RIKEN cDNA 0610031J06 gene	Mm.21976
1424462 at	1200009B18Rik	RIKEN cDNA 1200009B18 gene	Mm.59812
1428385 at	March8	Membrane associated ring finger (C3HC4) 8	Mm.27064
1428202 at	1810037C20Rik	RIKEN cDNA 1810037C20 gene	Mm.166485
1460439 at	BC033915	cDNA sequence BC033915	Mm.219459
1447900 x at	Entpd4	Ectonucleoside triphosphate diphosphohydrolase 4	Mm.291443
1452290 at	2310036D22Rik	RIKEN cDNA 2310036D22 gene	Mm.27742
1451161 a at	**Emr1**	**EGF like module, mucin like, hormone receptor like sequence 1**	**Mm.2254**
1448534 at	Ptpns1	Protein tyrosine phosphatase, non receptor type substrate 1	Mm.1682
1424213 at	1200002M06Rik	RIKEN cDNA 1200002M06 gene	Mm.292503
1454390 at	Lrch4	Leucine rich repeats and calponin homology (CH) domain containing 4	Mm.200763
1460478 at	2200002J24Rik	RIKEN cDNA 2200002J24 gene	Mm.45301

(*Continued*)

TABLE 11.3 Cell Surface Transmembrane Proteins Highly Expressed in Bone Marrow, Embryonic Kidney and Adult Kidney Side Population Cells: Note the Expression of the abc Transporter Abcg2 and also the Expression of the Macrophage Markers Csf1r (c-fms) and EMR1 (F4/80)—cont'd

Probe ID	Gene symbol	Description	UniGene
1429276 at	–	*Mus musculus* similar to mKIAA0342 protein (LOC235639), mRNA	Mm.296360
1419288 at	Jam2	Junction adhesion molecule 2	Mm.41758
1419048 at	Pcnx	Pecanex homolog (*Drosophila*)	Mm.86584
1454182 at	5430417C01Rik	RIKEN cDNA 5430417C01 gene	Mm.160094

was attempted by Challen et al. [68] using section in situ hybridization during kidney development for those genes identified as being enriched in the kidney SP versus total kidney. This included genes such as notch1, robo2, CD24a, CD44, Cdh16 and Prgfrb [68]. Many such genes displayed expression in interstitial cells although some were evidence of individual cells within various tubular elements. Both Abcg2 RNA and protein are located in small interstitial cells within the renal stroma [67,68] that coexpress MyoR [67].

As reported for SP in other tissues, SP in the kidney is unlikely to represent a homogeneous population. However, SP from different tissues may contain a subfraction of cells with congruent gene expression and functional properties. To seek such a subpopulation, the commonly expressed transmembrane receptor genes present on bone marrow, embryonic and adult kidney SP were identified [68]. This revealed the presence of a number of characteristic macrophage genes, including colony-stimulating factor-1 receptor (CSF1r/c-fms) and EGF-like module, mucin-like, hormone receptor-like sequence 1 (Emr1/F4/80), suggesting the existence of a macrophage subpopulation within all three SP fractions. Tissue macrophages can be identified in the interstitium of embryonic kidneys as early as E12.5 and persist in this location throughout development and into postnatal life [76]. To investigate this further, renal macrophages were isolated from c-fms-GFP kidneys [77] and subjected to Hoescht staining and SP isolation, revealing that the proportion of macrophages present in the kidney SP fraction (9.3%) was approximately twice that observed in the kidney as a whole (4.1%) [68]. Indeed, a portion of the GFP-positive macrophages was also CD45 F4/80^{+}. This is a clear demonstration of the heterogeneity of the kidney SP. Given the capacity of macrophages to fuse, phagocytose and even produce proregenerative as well as inflammatory cytokines, many of the apparent functional properties of the renal SP may result from this subfraction; however, this has not been formally examined.

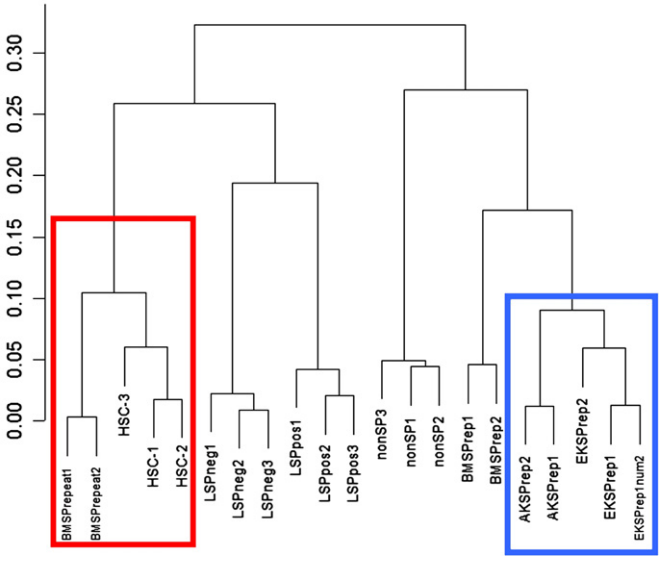

FIGURE 11.2 Microarray data of bone marrow side population (SP) cells generated at the Institute for Molecular Bioscience, University of Queensland (BMSPrepeat1 and 2) and by collaborators at Baylor College of Medicine [hematopoietic stem cell (HSC) 1, 2 and 3] in comparison with embryonic (EKSP) and adult (AKSP) kidney side population fractions. Red indicates that the bone marrow SP preparations clustered with other HSC clusters, whereas the adult and embryonic kidney SP preparations clustered separately from other samples.

RENAL SIDE POPULATION MULTIPOTENTIALITY AND RENAL REGENERATIVE POTENTIAL

It was hoped that a kidney SP would be capable of participating in regeneration after acute and chronic injury. The multipotentiality and renal functional capacity of SP from the adult kidney have been assessed in vitro, ex vivo and in vivo. Kidney SP fractions do display multipotentiality in culture, including osteocytic and adipocytic differentiation potential [67,68]. This suggests a broad mesodermal potential for the kidney SP. Asakura and Rudnicki [66] claimed that 3.3% of kidney SP cells were able to form hematopoietic colonies in vitro. Iwatani et al. also proposed that rat SP cells had hematopoietic potential in vitro, although their

preparations included hematopoietic cells [69]. Of more relevance to kidney, Hishikawa et al. [78] reported that murine kidney SP cultured in the presence of leukemia inhibitory factor (LIF) were able to grow in collagen matrix and produce cadherin-16, a kidney specific cadherin. However, evidence that this represented functional renal tubular epithelium was not presented. An in vivo assessment of the hematopoietic potential of the renal SP showed that approximately 0.03% of bone marrow cells of irradiated recipients expressed GFP, indicating that donor SP cells had engrafted into host bone marrow and produced progeny. Donor cells were also localized in laminin-positive skeletal muscle fibers and albumin-positive hepatocytes. GFP was also detected in the proximal tubules of these transplanted rats [69].

Clearly, the most stringent test of a stem cell lies in its ability to repopulate its organ of origin. This is a challenging task to assess within a solid organ context. Ex vivo incorporation of SP cells into renal structures has also been used to assess the renal potential of this population. Isolated GFP-positive SP from adult kidney were assessed for their capacity to integrate into the developing kidney after microinjection into embryonic kidneys [68]. This resulted in a much higher engraftment of donor cells into developing nephron structures than injection of non-SP cells isolated at the same time. Engraftment into MM- and UB-derived structures was increased approximately 3.5- and 13-fold, respectively, in SP cell injections. However, integration was low in frequency and SP cells were unable to generate entire tubules. A similar result was obtained from injection of kidney SP cells into chick embryos. While donor cells could be identified in chick mesonephroi and metanephroi, these cells did not form entire tubules or glomeruli, nor was there evidence for substantial proliferation. None of these studies establishes whether the apparent integration of SP cells into renal structures represents functional integration or a fusion event.

In the field of regenerative nephrology, the assessment of renal potential in vivo has been approached via the delivery of the candidate population into experimental or genetic models of renal damage [79]. Hishikawa et al. [67] determined that kidney SP cells contribute to renal regeneration in a model of acute renal failure induced by cisplatin injection. It was found that infusion of kidney SP cells significantly suppressed the peak of blood urea nitrogen (BUN) and serum creatinine levels resulting from the induced acute renal failure 7 days after cisplatin injection. Tubular necrosis was also improved by SP cell infusion, pointing to a possible humoral role in the repair process. Of note, this therapeutic effect was not produced by infusion of non-SP kidney cells or bone marrow mononuclear cells, suggesting a specific role for kidney SP cells in renal regeneration following acute insult.

Injection of adult kidney SP cells into an Adriamycin-damage model also appeared to have a positive effect in experimental animals. Injected SP, but not MP, cells were able to be recruited to the damaged organ from the circulation. While the level of recruitment was low (0.05% ~ 1 GFP cell/2000 total kidney cells), no GFP-positive cells were seen in the MP-injected mice. The functional effect of cell delivery was assessed as urinary creatinine/urinary protein levels and total urinary protein content. Urinary creatinine levels of SP-injected mice were closer to control levels than to animals treated with Adriamycin without cell therapy. A similar trend was noted for urine protein content, but neither of these differences was statistically significant owing to the small sample sizes of treatment groups. Indeed, similar trends were seen in those mice injected with MP cells, suggesting that the MP may also confer a benefit. One caveat to these studies is the report suggesting the dose of Adriamycin used may not affect serum creatinine levels [80]. Hence, this may not have been the best model. Despite this, the histological analyses did show a reduction in the occurrence of damaged glomeruli specifically in mice treated with SP cells. To analyze the mechanism of action of this modest improvement in structure and function, this study also examined the localization of the injected SP and MP cells. Neither injected cell population displayed significant migration from the point of injection, proliferation or integration into the glomeruli, the primary site of damage in response to Adriamycin. However, only the SP population showed integration into the recipient organ to any extent, suggesting that any observed beneficial effects were not by way of regeneration of damaged kidney segments, but via a humoral stimulation of proliferation of the surrounding cells, revascularization or ingress of other reparative cell populations.

POTENTIAL MECHANISM OF HUMORAL ACTIVITY

This concept of a humoral mode of action for the SP is in line with what is now accepted for MSCs. As for MSCs, it is assumed that SP cells may produce a variety of secreted growth factors that may regulate the proliferation, mobilization and differentiation of renal epithelial cells in response to acute tubular injury. A number of growth factors that can play such roles has been defined. For example, the expression of insulin-like growth factor-1 (IGF-1), heparin-binding epidermal growth factor-like growth factor (HB-EGF), transforming growth factor-β_1 (TGF-β_1), platelet-derived growth factor B-chain (PDGF-B) and hepatocyte growth factor (HGF) increase in regenerating rat kidneys after damage by toxicants or ischemia [81]. BMP7, a member of the TGF-β superfamily, is important for normal kidney

development and also improves renal repair postinjury [82]. Indeed, administration of recombinant BMP7 to damaged kidneys reduces fibrosis in the glomeruli and interstitial tissue and inhibitors of this protein reduce that antifibrotic action. Colony stimulating factor-1 (CSF1, M-CSF) is also upregulated by the tubular epithelium in response to damage [83], and while this was initially thought to be proinflammatory as a result of the recruitment of macrophages, the latter expressing the CSF-1 receptor, the presence of CSF-1 postinjury improves repair [84].

With respect to which growth factors are produced by the renal SP, SP cells isolated from cisplatin-injured kidneys show elevated expression of VEGF, HGF, BMP7 and LIF [67]. It is of interest, therefore, that Imai et al. [85] have shown that the administration of the histone deacetylase trichostatin A (TSA) also reduces fibrotic kidney damage in the experimental model of nephrotoxic serum nephritis, apparently by increased BMP7 production by the SP. How this is mediated is unclear, although subsequent studies suggest that the induction of histone deacetylase-1 (HDAC-1) and HDAC-2 by the tubular epithelium occurs in response to ischemic injury of the kidney [86]. This subsequently appears to modulate the production of CSF-1, presumably also by the tubular epithelium, and increases the macrophage inflammatory response [87]. These authors suggest that the inhibition of this process would attenuate inflammation. This does not agree with the recent reports of Humphreys and co-workers [84]. However, histone deacetylases such as TSA, suberoylanilide hydroamic acid (SAHA) and valproaic acid (VPA) have already been linked with amelioration of glomerulosclerosis [85,88] and have been recently associated with reprogramming of differentiated cells to a more primitive state as a result of the opening up of chromatin domains [89]. Hence, another alternative is a direct effect of the HDAC inhibitors on the SP sphenotype.

Similarly, Marumo et al. [90] investigated the link between the renin angiotensin system (RAS) and the humoral activity of the renal SP, as inhibition of the RAS also slows the progression of chronic kidney disease, as evidenced by a reduction in collagen and extracellular matrix deposition. Using a model of unilateral ureteric obstruction, Marumo et al. [90] delivered Valsartan, an RAS inhibitor, and observed a concurrent reduction in SP fraction and an increase in the total number of renal $CD45^+$ cells, potentially reflecting an increase in inflammation and/or a phenotypic change in the renal SP.

It is likely that the overall humoral contribution of the SP to renal repair incorporates a number of these observed activities. Indeed, given the heterogeneity of this population, distinct subsets of the SP may play different roles. Indeed, a pro-proliferative activation state of the tissue macrophage population identified within the kidney SP would represent a feasible regenerative humoral activity being mediated by one SP subpopulation.

FUNCTIONAL AND PHENOTYPIC OVERLAP BETWEEN KIDNEY SIDE POPULATION AND OTHER PUTATIVE ENDOGENOUS RENAL STEM CELLS

Over the past 5 years, there have been an increasing number of studies proposing the existence of endogenous renal stem/progenitor cells that may play a role in renal turnover and repair. The SP population is only one such population. Given the accepted and reported heterogeneity of SP isolates, and the potential presence of both epithelial and interstitial elements in the renal SP, it is important to ask what overlap there may be between the SP, isolated based on dye efflux, and other reported renal stem cell populations isolated using other functional or phenotypic isolation protocols (Fig. 11.3). The presence of Sca-1 and absence of hematopoietic lineage markers is a hallmark of a number of other renal progenitor populations, including those described by Gupta et al. [91] and Dekel et al. [92], both of which are thought to reside in the interstitium of the kidney. The population described by Dekel et al. [92] is also $Sca\text{-}1^+Lin^-$ and shows lineage multipotentiality, including myogenic, neural and tubular epithelial potential. These cells also appeared to be able to integrate into the tubular epithelium following ischemic damage, although whether this represented functional integration or fusion and whether these cell were reparative or not is not clear. The presence of other defining SP markers, such as CD24a or MyoR, was not assessed in this study, although these cells were described as $CD44^-$ whereas SP has been reported as $CD44^+$ [68]. Gupta et al. [91] reported the isolation of multipotent renal progenitor cells (MRPCs) after extensive culture from disaggregated adult rat kidneys. These cells expressed the pluripotency marker Oct4. While they were uniformly spindle-shaped and vimentin positive after 4–6 weeks in culture, the analysis of Oct4 expression within the kidney itself suggested that $Oct4^+$ cells were present within the proximal tubules, raising the possibility that these were originally tubular in origin. These cells were also CD90 and Pax2 positive, but negative for Ncam, CD31, CD133, cytokeratin and CD45. The method of isolation used to derive MRPCs was as for the isolation of multipotent adult progenitor cells (MAPCs) from the bone marrow [93]. Isolation required extensive culture and was successful in only 20% of cases. Hence, these cells may have undergone a dedifferentiation event and may not represent a population existing

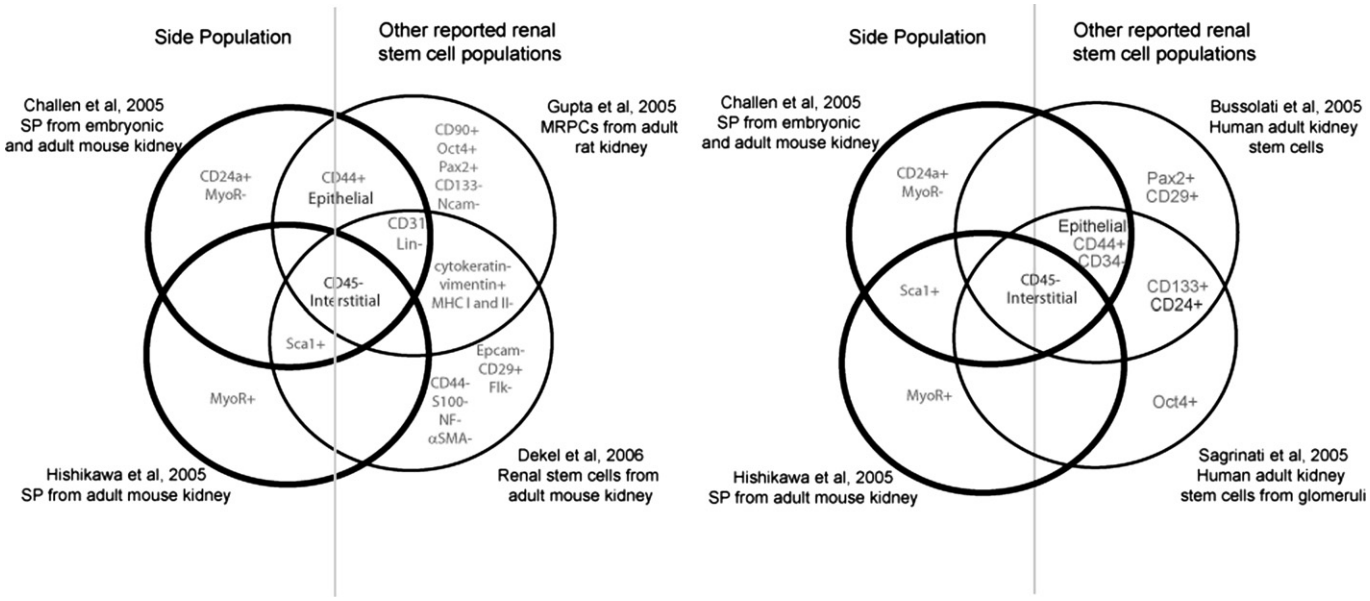

FIGURE 11.3 Comparison of reported rodent kidney side population (SP) immunophenotypes with other potential renal stem cell populations described in rodent (left panel) and human (right panel). MRPCs: multipotent renal progenitor cells. References: Hishikawa et al., 2005 [67], Challen et al., 2005 [68], Bussolati et al., 2005 [75], Gupta et al., 2006 [91], Dekel et al., 2006 [92], Sagrinati et al., 2006 [94].

within the adult kidney in vivo. In contrast, the characterization of kidney SP was performed at the point of isolation, at least in the study of Challen et al. [68]. MRPCs displayed multipotentiality along endothelial, hepatocytic and neural lineages [91]. They also appeared to develop into renal tubules when injected under the kidney capsule. This occurred after culture in LIF, fibroblast growth factor-2 (FGF-2) and TGF-β for 2 weeks. They also appeared to contribute to the renal tubular epithelium when injected intra-arterially into mice subjected to renal ischemia reperfusion injury. However, this did not appear to have any effect on renal function.

It would appear that there is greater similarity between the non-SP cell populations described by Gupta et al. [91] and Dekel et al. [92], in that both of these studies defined populations that were cytokeratin negative, vimentin positive and major histocompatibility complex (MHC) class I and II negative (Fig. 11.3). However, the comparison is flawed as reported population-specific markers identified in one investigation were not always investigated by the opposite study or in analyses of the kidney SP fraction. Without additional measures to increase enrichment of each population so as to determine with which fraction functional activity separates, it remains likely that there is overlap in the cells isolated using each of these approaches.

In studies of potential stem cells derived from human adult kidney, once again populations have been described that are CD45 [75,94] (Fig. 11.3). However, these populations were not assessed for Sca-1 expression and were isolated specifically from within the epithelial fraction of the kidney. Both populations were positive for CD133 and CD24 epitopes (Fig. 11.3); however, the CD24 epitope differs between mouse and human and does not represent the same protein. Conversely, although the presence of an SP within adult human kidney has been reported [70], this was not characterized other than for the presence of reserpine-sensitive dye efflux activity. Hence, a direct comparison cannot be made with other human or rodent endogenous renal populations. If one assumes the equivalence of markers between mouse SP and proposed human renal stem cell populations (Fig. 11.3), there is little overlap between these glomerular and epithelial populations and the SP. Indeed, the $CD24^{+}CD133^{+}$ population in the human kidney has been localized to the urinary pole of the Bowman's space and shown to be able to migrate around the Bowman's space and contribute to podocyte turnover both during development and in response to damage [95,96]. This specialized localization and progenitor capacity could at best only represent a very minor subfraction of the SP.

POTENTIAL REGENERATIVE APPLICATIONS OF KIDNEY SIDE POPULATION

Having determined the existence of an SP within the postnatal kidney and discussed the functional data relating to their capacity to elicit a reparative effect when delivered into animal models of renal damage, the question remains whether this is of relevance to the development of regenerative options for application

within nephrology. There are three possibilities to be explored:

- autologous or allogenic delivery of endogenous renal SP as a cellular therapy
- activation of the endogenous renal SP to assist in repair in situ
- delivery of non-autologous blood/bone marrow-derived SP cells.

In the first option, a donor, who might also be the recipient, would need to undergo renal biopsy to access endogenous SP cells. These cells would then require ex vivo expansion and delivery into the recipient, presumably either directly into the renal parenchyma or via the vasculature. A major challenge to the field of regenerative medicine is delivery of cells, whether this involves delivery into kidney, brain, heart, muscle or any other organ. In animal models such as mice, the delivery of cells via the tail vein results in almost immediate deposition of the majority of cells within the microvasculature of the lung. Injection of cells into the ascending aorta or into the renal artery itself would be more feasible in humans than in some animal models, but would still require the rapid adherence of these cells to the renal vascular beds or an active process of homing. There is some evidence that SP cells can home to sites of injury from the vasculature, and this would be required whether the cells acted via fusion or true transdifferentiation. However, it may not be required if their humoral renoprotective activity is effective when delivered systemically. Bi et al. [97] showed that the delivery of conditioned media from cultured MSC was sufficient to transfer a renoprotective effect, suggesting that systemic cytokines were involved.

Irrespective of potential mechanism, the development of such a treatment would require stringent cell processing facilities and would rely on the ability to expand an SP population without altering their functional phenotype. If this were feasible, the harvesting and repeated delivery of autologous cells might be possible, but would be very expensive. In addition, a patient requiring such treatment would undoubtedly have progressive renal disease and this in itself may render the use of their own SP fraction ineffective. It is not at all clear, given the paucity of animal trials using chronic models of renal damage [79], what is the duration of efficacy and hence the frequency with which such a procedure would be required.

Given the challenges with cell delivery, the second approach would have considerable advantages. This model relies on the hypothesis that appropriate stimulation of the patient's own SP fraction may be sufficient to elicit repair. This will require a great deal more study into the receptor expression and ligand responsiveness of the SP fraction. One may question why an SP fraction is required and whether the delivery of the appropriate cocktail of growth factors would represent a less invasive and hence more feasible treatment of chronic renal disease. Indeed, a fuller understanding of how to manipulate the kidney environment to stimulate repair rather than progression, whether this requires the SP fraction or not, remains a key focus of the experimental nephrology community and it is likely that research in both directions may well converge. One of the disadvantages of this approach, as indeed for the first approach, is the assumption that a patient with chronic renal disease still has an effective and responsive SP fraction. Given the demographics of the chronic kidney disease cohort, this alone will place such patients at a disadvantage.

The final suggestion returns to the concept of using bone marrow-derived SP cells, which are essentially HSCs, to regenerate a chronically damaged kidney. While many studies have examined the ability of cells from the bone marrow to home to and populate a damaged kidney, the data suggest that it is not the HSC fraction but the MSC fraction that possesses this activity. Bone marrow-derived SP cells could be directly delivered into the kidney, but whether this source of SP cells would have the appropriate humoral reparative effect in the kidney is unknown. Such a cell is also unlikely to be able to transdifferentiate into renal tissue. Indeed, there has never been evidence presented to prove that an HSC will spontaneously behave in a pluripotent fashion. The advent of reprogramming technologies means that a pluripotent cell can now be generated from any cell of the body and a cell can be encouraged to swap lineages after overcoming the barriers of its own chromatin packaging and gene regulatory network. Hence, an HSC could be induced to take on another phenotype, but this would seem a highly impractical therapeutic proposal, with the more likely approach involving the direct reprogramming of cells within the kidney to become tubular epithelial progenitors.

CONCLUSION

The adult kidney contains an endogenous SP and this population has been shown to display lineage multipotentiality and a modest capacity to improve renal function when introduced into models of acute renal damage. However, the use of SP cells for renal therapy is currently far from feasible, with the greatest challenge being the identity of the cells themselves and their inherent heterogeneity. In the longer term, if the identity of these cells can be better established, it may be feasible to stimulate this endogenous SP fraction directly to repair the kidney from within. Alternatively, with increased understanding of the humoral activities of

SP cells, it may be possible to mimic this process. Certainly, increasing our understanding of the cells that display this dye-effluxing phenotype within the kidney, together with the information about the nature of the humoral response/s elicited by these and other cell sources during normal responses to acute damage, will continue to move the field towards methods for preventing progression or resolving chronic injury.

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CHAPTER

12

Very Small Embryonic-like Stem Cells and Their Potential Relevance for Kidney Homeostasis

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OUTLINE

INTRODUCTION

Continuous tissue and organ regeneration is one of the important homeostatic mechanisms of the multicellular organism. The senescent, functionally differentiated mature cells in adult organs are continuously being replaced by new ones [1]. This cell turnover is more rapid in the hematopoietic system, intestinal epithelium and epidermis than in the kidney, skeletal muscles, liver and heart. Old or senescent cells in adult organisms are eliminated by various mechanisms involving: apoptosis (e.g. neurons), shedding from the epithelial and epidermal surfaces (e.g. enterocytes, keratinocytes) or active elimination by phagocytosis (e.g. senescent erythrocytes). Thus, to keep a cell mass balance in different tissues and organs, new cells have to be generated continuously to replace eliminated ones [2]. The new cells are supplied by a pool of tissue-committed stem cells

(TCSCs) that reside in specific niches spread throughout the organs and tissues [3]. In the kidney, for example, the presence of the so-called renopoietic system was postulated with TCSCs localized at the urinary pole of Bowman's capsule, from where they can initiate the replacement and regeneration of glomerular, as well as tubular, epithelial cells [4].

In general, when dividing, stem cells have three choices (Fig. 12.1A): they give rise to two daughter stem cells (symmetric division), one stem cell and a differentiated progenitor cell (asymmetric division), or two progenitor cells. This selection of the stem cell fate is a randomly determined process, which follows a probability distribution that can be analyzed statistically, but cannot be determined precisely (stochastic selection).

As mentioned above, while some tissues regenerate quickly, others regenerate at a very slow pace. For example, turnover of the intestinal epithelium takes place every 48 h, the epidermis is exchanged every 14 days, and the half-life of erythrocytes circulating in peripheral blood is 100 150 days. New evidence indicates that even organs such as the kidney, heart or brain also show some degree of cellular turnover, although that occurs very slowly [5]. In the case of the kidney, recent evidence shows that even podocytes, a cell type with limited proliferative capacity under normal conditions, are constantly regenerated from a population of renopoietic cells within Bowman's capsule. To support this, Romagnani and co-workers identified in this region a population of renal $CD133^+CD24^+$ multipotent progenitors that have the capacity for self-renewal and multilineage differentiation [6]. The authors postulated that these cells represent a subset of multipotent embryonic stem/progenitor cells that persist in human kidneys from the early stages of embryonic nephrogenesis.

The novel hypothetical concept presented in this chapter addresses data that support the residence of some very primitive pluripotent stem cells (PSCs) in adult tissues with the ability to differentiate into multiple types of TCSCs. In addition to above-mentioned $CD133^+CD24^+$ multipotent progenitors residing in Bowman's capsule, several investigators have described cells in adult tissues with remarkably broad spectra of differentiation. These cells were described in the literature as multipotent adult progenitor cells (MAPCs), marrow-isolated adult multilineage inducible (MIAMI) cells, multipotent adult stem cells (MASCs) or OmniCytes [7 10]. Recently, the authors' team purified from murine bone marrow (BM) rare $CXCR4^+$ small stem cells that express markers characteristic for embryonic stem cells (ESCs), epiblast stem cells (EpiSCs) and primordial germ cells (PGCs) [11]. Based on the morphology of these cells and unique molecular signature of gene expression, these primitive cells were named very small embryonic-like (VSEL) stem cells.

It cannot be excluded that similar or overlapping populations of primitive stem cells in adult tissues were detected using various experimental strategies and, subsequently, were assigned different names. However, it is an important challenge to understand the biological significance of these primitive stem cells residing in adult tissues. Are these cells merely developmental remnants that are deposited during embryogenesis in the adult tissues, or do they serve as a reserve pool for

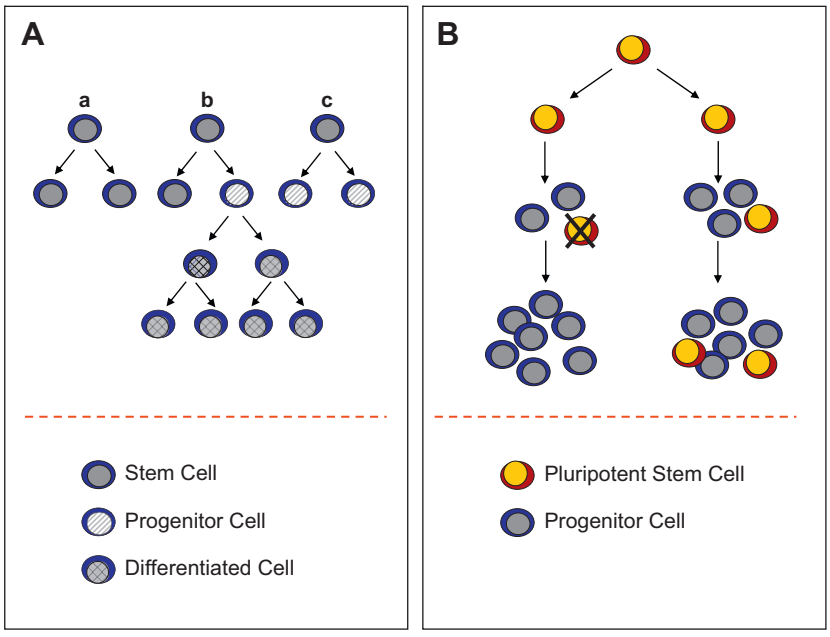

FIGURE 12.1 Stem cell contribution to tissue rejuvenation. (A) Stem cells can give rise to: (a) two daughter stem cells (symmetric division); (b) one stem cell and differentiated progenitor cell (asymmetric division); and (c) two progenitor cells. (B) Pluripotent stem cells that are deposited in adult tissues during embryogenesis/gastrulation may become eliminated after giving rise to tissue committed stem cells (TCSCs) or hypothetically survive among TCSCs and serve as a potential reserve source of TCSCs.

TCSCs (Fig. 12.1B)? If the latter possibility is true, another question emerges: are they continuously supplying and rejuvenating a pool of TCSCs or are these cells a source of TCSCs in emergency situations only, as seen in tissue or organ injury (e.g. kidney damage)? Finally, it is important to explore in the future the relationship between VSELs and TCSCs from the renopoietic system located in Bowman's capsule.

DEVELOPMENTAL OVERVIEW ON STEM CELL COMPARTMENT

It is well known that during embryogenesis the stem cell compartment is heterogeneous and that early stem cells are endowed with a remarkable potential to differentiate into tissues from one, two or even three germ layers [12]. The fertilized oocyte or zygote is unquestionably the most primitive stem cell from which a whole organism develops. The zygote is a totipotent stem cell, the most primitive stem cell which gives rise to both placenta and embryo proper [3]. In a relatively short time, just a few days, the zygote gives rise to the morula and blastocyst. Stem cells in the morula and inner cell mass of the blastocyst are pluripotent. PSCs give rise to TCSCs from all three germ layers (mesoderm, ectoderm and endoderm) that form the embryo proper.

The development of an embryo into an adult organism during embryogenesis is subsequently regulated by the coordinated proliferation, specification and differentiation of TCSCs [3]. As a result of this, one fertilized oocyte gives rise to an adult organism that consists of more than 200 different cell types which total, in the case of humans approximately 10^{14} somatic cells. In adult organisms, various types of TCSCs reside in specific niches (e.g. Bowman's capsule in kidney, bottom of intestinal crypts, hair bulge) and replace senescent cells within various organs [13]. It is also accepted that monopotent TCSCs are endowed with the property of self-renewal and the ability to differentiate into progenitor cells that are committed to particular developmental pathways. Since, as discussed above, somatic cells have a limited half-life, continuous tissue and organ regeneration by TCSCs is one of the important homeostatic mechanisms that maintain the homeostasis of the multicellular organism [13].

DEPOSITION OF DEVELOPMENTALLY EARLY STEM CELLS IN ADULT TISSUES

Reports that monopotent TCSCs are plastic and can change differentiation commitment (trans-dedifferentiate) into stem cells for other tissues are controversial [3]. However, the presence of pluripotent or multipotent stem cells in adult tissues may much better explain some of the positive results demonstrating data on the plasticity of stem cells. Evidence shows that in addition to TCSCs, adult organs harbor a population of more primitive PSCs, that may be envisioned as a reserve stem cells population for TCSCs. As mentioned above, several groups including the present authors have reported on the presence of developmentally primitive stem cell populations with PSC properties that are distributed in adult organs and tissues (e.g. MAPCs, MIAMI cells, MASCs, OmniCytes and VSELs).

These cells reside in adult tissues and could be activated or attracted during stress or tissue injuries to regenerate damaged organs [14 16]. It is well known that damaged organs secrete several factors such as stromal-derived factor-1/CXC chemokine ligand-12 (SDF-1)/(CXCL-12), hepatocyte growth factor/scatter factor (HGF/SF), leukemia inhibitor factor (LIF), and vascular endothelial growth factor (VEGF) which may chemoattract several of the primitive stem cell populations [17]. In support of this, damaged tissues [e.g. kidney during acute renal failure (ARF), infarcted myocardium or stroke area] secrete several of these factors during hypoxia (deprivation of adequate oxygen supply) [14,18,19]. The most important ones are products of genes regulated at the transcriptional level by a transcription factor called hypoxia-inducible factor-1α (HIF-1α). Accordingly, HIF-1α regulates several genes including SDF-1α/CXCL12, HGF/SF and VEGF [20 22]. All these factors (SDF-1, HGF/SF and VEGF) orchestrate accumulation of stem cells in damaged tissues, e.g. myocardium after infarction, SDF-1 interacts with CXCR4 and, as recently reported, with another seven transmembrane-span G-protein-coupled CXC chemokine receptor 7 (CXCR7) as well [23]. It has been demonstrated that SDF-1 CXCR4 and SDF-1 CXCR7 axes play an important role in trafficking of renal progenitors as evidenced, for example, in experimental ARF in mice [24]. To support this notion, in severe combined immunodeficiency (SCID) mice with ARF, SDF-1 is strongly upregulated in cells that surround necrotic areas. Furthermore, intravenously injected renal $CXCR4^{+}CXCR7^{+}$ TCSCs engrafted into injured SDF-1-enriched renal tissue both decreased the severity of ARF and prevented renal fibrosis.

Other important players in the trafficking of stem cells to the damaged tissues are bioactive peptides released during complement cascade (CC) activation. The complement system, which is a part of the body's innate immunity, also becomes activated in injured and hypoxic tissues [25]. It has been reported that the most abundant product of complement cleavage/activation is the small fragment of the third complement component 3 (C3) called C3a, which increases and

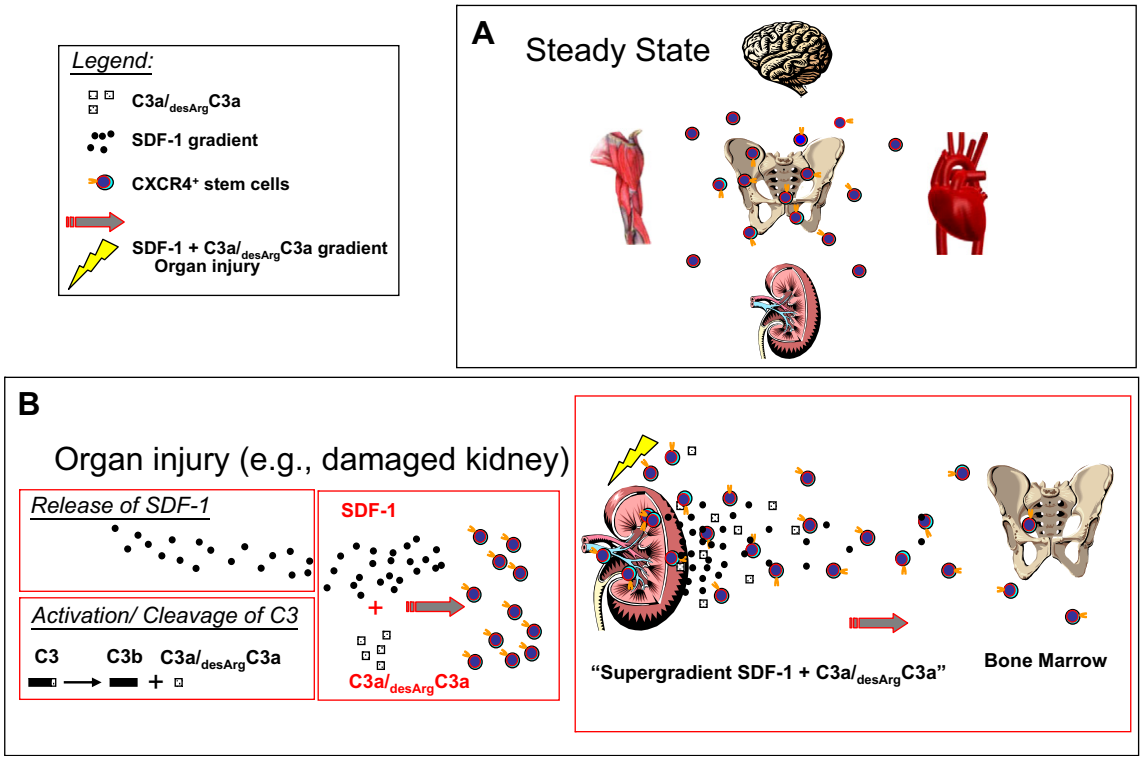

FIGURE 12.2 Very small embryonic like stem cells (VSELs) are mobilized into peripheral blood, e.g. in kidney damage. (A) Under normal steady state conditions, VSELs may circulate in peripheral blood to keep a pool of stem cells in balance in distant niches of the same tissue. (B) The number of these cells increases during stress related to organ/tissue damage. During organ damage (e.g. hypoxic damage of kidney), the level of stromal derived factor 1 (SDF 1) is upregulated in the affected tissues and C3 becomes activated, leading to the accumulation of C3 cleavage fragments (C3a and $_{desArg}$C3a). C3 cleavage fragments enhance/prime the responsiveness of circulating CXCR4^{+} stem cells to an SDF 1 gradient. This leads to more efficient chemoattraction of stem cells for potential regeneration of the damaged tissue by creating a supergradient, as shown in (B) for damaged kidney, for example. In addition to SDF 1, other chemoattractants play important roles here (e.g. hepatocyte growth factor/scatter factor, leukemia inhibitory factor and vascular endothelial growth factor).

sensitizes the responsiveness of stem cells to SDF-1 gradient [26,27]. Recently, other factors were identified from the small cationic peptides family (e.g. cathelicidin, β-II-defensin), which are secreted by granulocytes that accumulate in damaged tissues and, similarly to C3a, increase the responsiveness of TCSCs to the SDF-1 gradient [28]. Thus, in addition to SDF-1/CXCL12 and growth factors, CC cleavage fragments have an important role in the recruitment of stem cells to the damaged tissues, e.g. kidney (Fig. 12.2). However, the interplay of SDF-1 and C3a in kidney regeneration during ARF requires further study. It is also important to assess a potential role of VSELs in this process.

To support this hypothesis, it has been reported that PSCs (e.g. VSELs or OmniCytes) could be mobilized into peripheral blood during several models of organ injury and circulate there in an attempt to enrich and regenerate damaged tissues [14 16]. This physiological mechanism probably plays a more significant role in the regeneration of some small tissue and organ injuries. The regeneration of major tissue or organ damage will require local delivery of a higher number of pluripotent or multipotent stem cells. In general, the regeneration mechanisms are more efficient at a young age, which could be explained by the number of these primitive mobile stem cells (e.g. VSELs) decreasing with age [11,13]. Thus, this aging-related decrease in the number of VSELs could explain both decreasing rejuvenation potential and impaired organ regeneration with advanced age.

ISOLATION OF VERY SMALL EMBRYONIC-LIKE STEM CELLS

Using fluorescence-activated cell sorting (FACS), a homogeneous population of rare small Sca-1^{+}/Lin / CD45 cells has been isolated from BM as well from several other adult tissues including brain, liver, pancreas, kidney, muscles, heart, testes and thymus [11,29]. These VSELs, as determined by real-time quantification reverse transcription polymerase chain reaction (RT-PCR) and by immunohistochemistry, express several markers of PSCs such as SSEA-1, Oct-4, Nanog, and Rex-1, as well as Rif-1 telomerase protein.

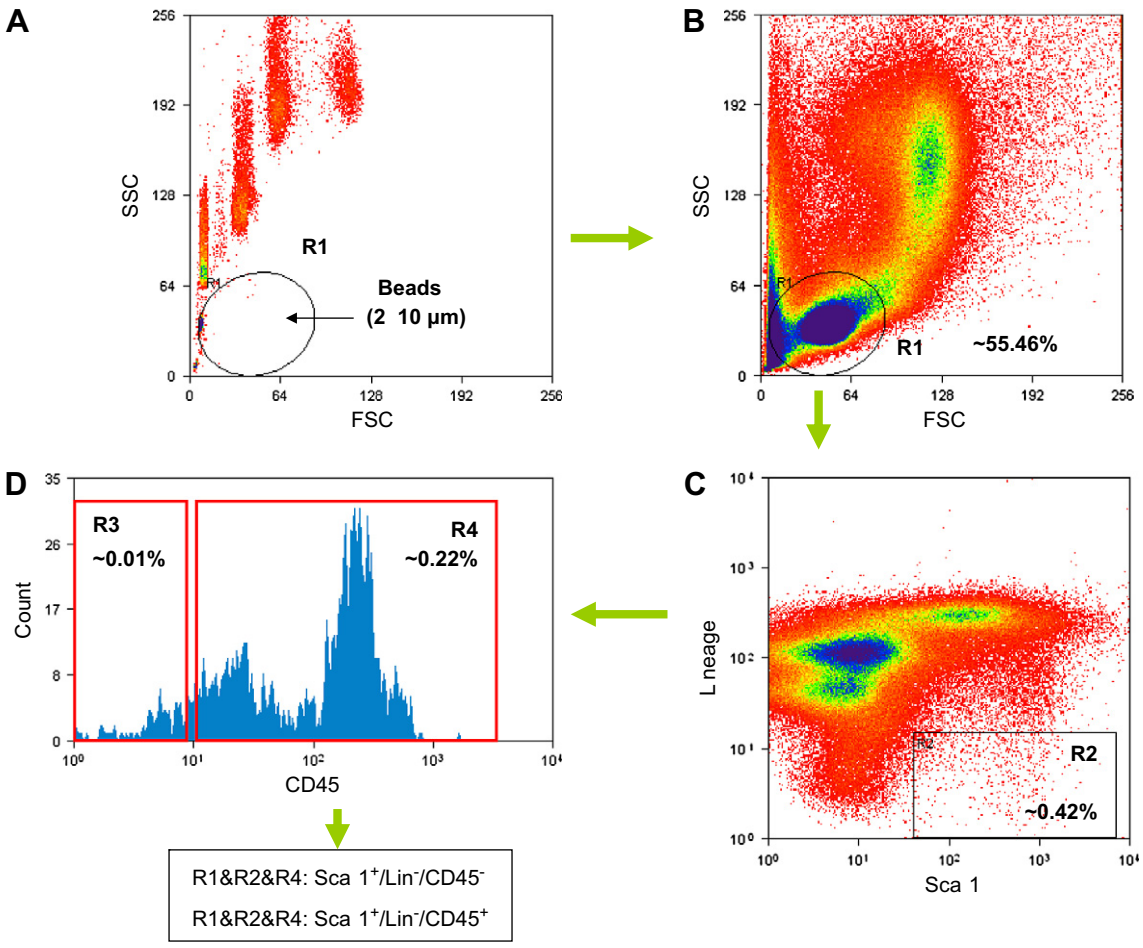

FIGURE 12.3 Sorting strategy for isolation of murine bone marrow derived very small embryonic like stem cells (VSELs) by FACS. Bone marrow derived VSELs were sorted by MoFlo cell sorter (Dako, Glostrup, Denmark) following immunofluorescence staining for Sca 1, CD45 and hematopoietic Lin. (A) Distribution of six predefined, sized bead populations according to their forward and side scatter characteristics (FSCs and SSCs, respectively). Gate R1 includes objects between 2 and 10 µm in size after comparison to bead particles with standard sizes of 1, 2, 4, 6, 10 and 15 µm (Flow Cytometry Size beads, Invitrogen; Molecular Probes, Carlsbad, CA, USA). (B) Bone marrow mononuclear cells visualized on dot plots showing their FSC and SSC signals related to the size and granularity/complexity of the cell, respectively. Small, agranular cells included in region R1 are further visualized based on the expression of Sca 1 and Lin markers (D). Region R2 includes only Sca 1^+/Lin , which are subsequently sorted based on CD45 marker expression into CD45 and $CD45^+$ subpopulations visualized on a histogram (C). Sca 1^+/Lin /CD45 cells (VSELs) are sorted as events enclosed in a logical gate including regions R1, R2 and R3, while Sca 1^+/Lin /$CD45^+$ cells (hematopoietic stem cells) from the gate include regions R1, R2 and R4. Approximate percent contents of each cellular subpopulation are indicated on the plots.

To isolate VSELs from the BM by FACS, a novel size-based approach controlled by size bead markers was used. The overall sorting strategy was to gate in regions containing small events (2 10 µm), shown as region R1 on the dot plot (Fig. 12.3). This region mostly contains cell debris, but also has some rare nucleated cell events. Because most of the sorting protocols exclude events smaller than 6 µm in diameter that contain cell debris, erythrocytes and platelets, these small VSELs are usually excluded from the sorted cell populations. In this sorting strategy for VSELs, the size of the sorted cells is controlled by the beads with predefined sizes (1, 2, 4, 6, 10 and 15 µm in diameter).

The events enclosed in region R1 (Fig. 12.3A, B), which include an average of ~50% of total events, are further analyzed for the expression of Sca-1 and lineage markers (Lin). The Sca-1^+/Lin events shown in region R2 (Fig. 12.3D) consist of 0.38 ± 0.05% of total analyzed BM nucleated cells on average. Cells from region R2 are subsequently sorted according to the expression of CD45 antigen (marker of hematopoietic cells) as Sca-1^+/Lin /CD45 (region R3) and Sca-1^+/Lin /$CD45^+$ (region R4) subpopulations (Fig. 12.3C) that contain VSELs and hematopoietic stem cells (HSCs), respectively. VSELs comprise ~0.03% and HSCs ~0.35% of total BM nucleated cells (Fig. 12.3C). It was found that 95% of Sca-1^+/Lin /CD45 (VSELs) are located within

TABLE 12.1 Characteristics of Human and Murine Bone Marrow-derived Very Small Embryonic-like Stem Cells

Source of cells	Murine BM derived VSELs	Human CB derived VSELs
Size (diameter)	3–5 μm	4–7 μm
Nucleus	Large: contains euchromatin Diploid number of chromosomes	Large: contains euchromatin Diploid number of chromosomes
Cytoplasm	Tiny rim of cytoplasm enriched in mitochondria	Tiny rim of cytoplasm enriched in mitochondria
Surface markers	Sca 1^+, $CXCR4^+$, CD45 , lin MHC I HLA DR , CD90 CD105 CD29	$CD133^+$, $CXCR4^+$, CD45 , lin , MHC I HLA DR , CD90 CD105 CD29
ESC markers	SSEA 1Oct 4, Nanog, Rex 1, High telomerase activity	SSEA 4Oct 4, Nanog, Rex 1High telomerase activity

BM: bone marrow; VSEL: very small embryonic-like stem cells; CB: cord blood; ESC: embryonic stem cell; MHC: major histocompatibility complex; HLA: human leukocyte antigen.

the 2 6 μm size range, while 86% of Sca-1^+/Lin / $CD45^+$ (HSCs) are found in the 6 10 μm size range [30]. Thus, using flow cytometry and the size marker beads, it was confirmed that the majority of Sca-1^+/ Lin /CD45 cells isolated from adult BM are unusually small (< 6 μm). In general, VSELs are larger than peripheral blood platelets and smaller than erythrocytes. Direct transmission electronic microscopy (TEM) analysis revealed that these cells display several features typical for ESCs such as small size, a large nucleus surrounded by a narrow rim of cytoplasm and open-type chromatin (euchromatin) [11].

Despite their small size, VSELs possess diploid DNA. They do not express major histocompatibility complex-I (MHC-I) and HLA-DR antigens and CD90 CD105 CD29 . Thus, on one hand, VSELs do not express histocompatibility antigens (MHC-I and HLA-DR) similarly to ESCs and, on the other, they do not express typical markers for mesenchymal stem cells (MSCs) (CD90 CD105 CD29) (Table 12.1). This further supports their unique phenotype.

Recently, evidence has also mounted to suggest that similar cells are also present in human umbilical cord blood (UCB) and BM, particularly in young patients (Table 12.1). Accordingly, using a novel two-step isolation procedure, i.e. removal of erythrocytes by hypotonic lysis combined with multiparameter sorting, a population of human cells was isolated that is similar to VSELs previously described in murine BM [31]. These CB-isolated (CB) VSELs are very small (4 6 μm), are highly enriched in a population of $CXCR4^+AC133^+CD34^+$lin CD45 UCB mononuclear cells (MNCs), possess large nuclei containing unorganized euchromatin, and express nuclear embryonic transcription factors Oct-4 and Nanog as well as surface SSEA-4 protein. Further studies are needed to determine whether human UCB-VSELs are endowed with PSC properties similarly to their murine BM-derived counterparts. At this point, it was possible to differentiate them in cultures over OP9 cells into early HSCs [32]. The presence of very small cells with ESC markers (Oct-4 and SSEA-4) that are able to grow neurospheres was recently confirmed in UCB by another group of investigators [33,34].

REGENERATION POTENTIAL OF VERY SMALL EMBRYONIC-LIKE STEM CELLS

The data indicate that if plated over a C2C12 murine myoblastic cell line feeder layer, ~5 10% of purified VSELs are able to form spheres that resemble embryoid bodies that could be grown from established ESCs [11]. Cells from these VSEL-derived spheres (VSEL-DSs) are composed of immature cells with large nuclei containing euchromatin and are $CXCR4^+SSEA\text{-}1^+Oct\text{-}4^+$, just like purified VSELs. Similar spheres were also formed by VSELs isolated from murine fetal liver, spleen and thymus. The formation of VSEL-DSs was associated with a young age in mice and no VSEL-DSs were observed in cells isolated from older mice (> 2 years) [11]. This age-dependent content of VSELs in BM may explain why the regeneration process is more efficient in younger individuals. There are also differences in the content of these cells among BM MNCs between long- and short-lived mouse strains. The concentration of these cells is much higher in the BM of long-lived (e.g. C57Bl6) than in short-lived (DBA/2J) mice [11]. It would be interesting to identify the genes responsible for tissue distribution and expansion of these cells, as they could be involved in controlling the life span of mammals.

Because VSELs express several markers of PGCs (fetal-type alkaline phosphatase, Oct-4, SSEA-1, CXCR4, Mvh, Stella, Fragilis, Nobox, Hdac6), they could be closely related to a population of epiblast/germ-line-derived PGCs [3]. VSELs are also highly mobile and respond robustly to an SDF-1 gradient, adhere to fibronectin and fibrinogen, and may interact with BM-derived stromal fibroblasts [11]. Confocal microscopy and time-lapse studies revealed that these cells attach rapidly to, migrate

beneath or reside in invaginations formed by cell membranes of marrow-derived fibroblasts (emperipolesis). Because fibroblasts secrete SDF-1 and other chemoattractants, they may create a homing environment for small $CXCR4^+$ VSELs. This robust interaction of VSELs with BM-derived fibroblasts has an important implication, namely that isolated BM stromal cells may be contaminated by these tiny cells from the beginning.

It is anticipated that VSELs could become an important source of PSCs for regeneration. Thus, researchers working with animal models must determine whether these cells could be efficiently employed in the clinic or whether they are merely developmental remnants found in the BM that cannot be harnessed effectively for regeneration. Preliminary data indicate that they play a role in regeneration of myocardium in experimental model of murine myocardial infarction if injected directly into the infarcted area. Furthermore, VSEL isolated freshly from the BM do not possess immediate hematopoietic activity (they neither grow hematopoietic colonies nor radioprotect lethally irradiated recipients); however, if plated over a supportive OP9 cell line, these CD45 VSELs give rise to colonies of $CD45^+CD41^+$Gr-1 Ter119 cells whose phenotype resembles that of the earliest hematopoietic cells that are derived in vitro from established embryonic cell lines [32]. This hematopoietic differentiation of VSELs was accompanied by upregulation of mRNA for several genes regulating hematopoiesis (e.g. *PU-1*, *c-myb*, *LMO2*, *Ikaros*). More importantly, the $CD45^+$CD41 Gr-1 Ter119 cells expanded from VSEL isolated from GFP^+ mice, when transplanted into wild-type animals, protected them from lethal irradiation and differentiated in vivo into all major hematopoietic lineages (e.g. Gr-1^+, $B220^+$ and $CD3^+$ cells) [32]. Thus, it is proposed that VSELs are a population of BM-residing PSCs that may give rise to long-term engrafting HSCs.

DEVELOPMENTAL ORIGIN OF VERY SMALL EMBRYONIC-LIKE STEM CELLS

From a developmental point of view, cells that are "immortal" in mammals are those that belong to the germ line (Fig. 12.4). Accordingly, the germ line passes genomic and mitochondrial DNA to the next generations and creates mortal soma, which help the germ line to fulfill its reproductive mission [3]. The most primitive cell in the germ line is the zygote, mentioned above, which is a result of the fusion of two gametes (oocyte and sperm) during the process of fertilization. Germline potential is subsequently maintained in blastomeres of the morula and in the cells of the inner cell mass (ICM) of the blastocyst. At the level of the blastula, however, a part of the cells that surrounds the ICM of the blastocyst buds out from the germ-line lineage and differentiates toward trophoblasts, which give rise to the placenta. After implantation of the blastocyst in the uterus, a germ-line potential is maintained in the epiblast.

At 7.25 days postconception (dpc) in mice, a proximal portion of the epiblast PSCs is specified into a population of germ-line stem cells, PGCs, that will migrate to the genital ridges, where they subsequently differentiate into oocytes or sperm during gametogenesis [35]. Shortly after PGC specification, the remaining epiblast PSCs, which are envisioned to be related to the germ-line lineage, become specified to multipotent or unipotent stem cells for developing tissues and organs. These primitive epiblast/germ-line-derived PSCs, as hypothesized, are not completely eliminated from the developing organism by the differentiation process. The authors believe that some of them survive into adulthood as VSELs among TCSCs (Figs 12.1 and 12.4).

GERM LINE AS ORIGIN AND SCAFFOLD SYSTEM FOR TISSUE-COMMITTED STEM CELLS IN THE ADULT BODY

A recent study by this group provides more data on the unique molecular signature of VSELs (Table 12.2) and provides more evidence that these cells are somehow related to the population of migratory PGCs that become specified early in development in the proximal epiblast.

PGCs are the most important population of stem cells. As precursors of gametes, they are directly responsible for passing genetic information on to the next generation. However, if these developmentally early cells are isolated from the developing embryo after 11 dpc (during the time when they migrate to the genital ridges) and cultured ex vivo, they surprisingly will undergo rapid terminal differentiation or apoptosis [36]. They do not complement blastocyst development, are not able to provide fully functional nuclei during nuclear transfer in the process of clonote formation, and do not grow teratomas. Therefore, these cells lack the currently approved criteria of pluripotentiality. This also indicates that PGCs must be protected somehow from uncontrolled expansion by certain important regulatory mechanisms.

One explanation for this obvious lack of pluripotentiality is that PGCs, in contrast to other somatic cells, undergo the reprogramming and erasure of the somatic imprints, an epigenetic mark, and as a result of this some of the genes are differentially methylated on either maternal or paternal chromosomes [35]. The parent-of-origin specific DNA methylation pattern regulates the monoallelic transcription of imprinted

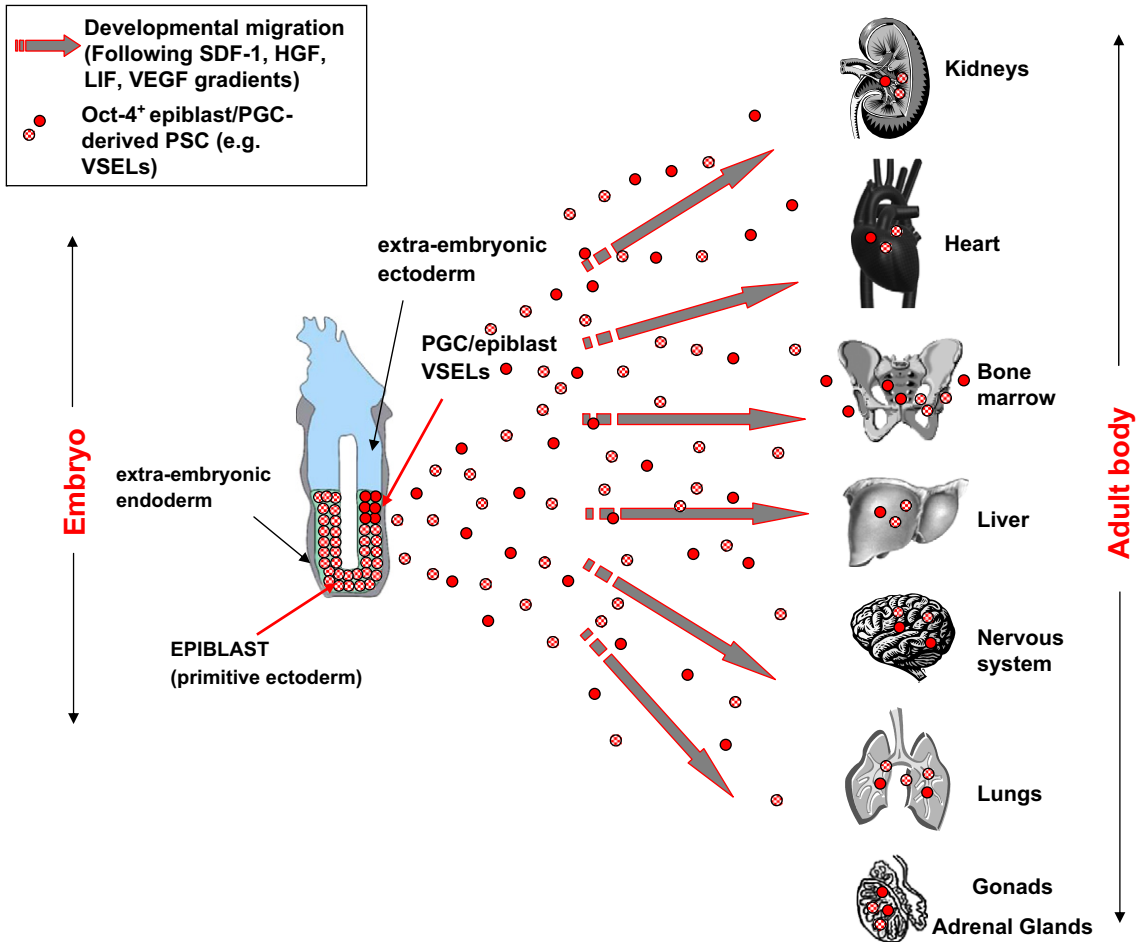

FIGURE 12.4 Epiblast/germ line derived very small embryonic like stem cells (VSELs) as origin and scaffold of stem cell compartment in an adult organism. From the developmental and evolutionary point of view, the germ line carries the genome (nuclear and mitochondrial DNA) from one generation to the next. Somatic cell lines bud out from the germ line during ontogenesis to form soma that will help germ line derived gametes fulfill this mission. The germ potential is established in the fertilized oocyte (zygote) and subsequently retained in the morula, ICM of the blastocyst, epiblast, primordial germ cells (PGCs) and mature gonocytes (oocytes and sperm). The first cells that bud out from the germ lineage are trophoectodermal cells that will give rise to the placenta. Subsequently, during gastrulation, the epiblast is a source of pluripotent stem cells (PSCs) for all three germ layers (mesoderm, ectoderm and endoderm) and PGCs. It is hypothesized that at this stage some epiblast/germ line derived stem cells could be deposited as Oct 4^+ VSELs in peripheral tissues and organs (red circles).

genes such as *H19*, insulin-like growth factor 2 (*Igf2*), Igf2 receptor (*Igf2R*), ras protein-specific guanine nucleotide-releasing factor 1 (*Rasgrf1*) and small nuclear ribonucleoprotein polypeptide N (*SNRPN*) [37]. As a result of the imprints, for example, Igf2 is expressed from the paternal chromosome while H19 is expressed from the maternal chromosome.

The process of erasure of the somatic imprints occurs very early during gastrulation when PGCs begin to migrate to the genital ridges [35]. Erasure is one of the basic mechanisms that prevents their uncontrolled proliferation and parthenogenesis, and prevents potential teratoma formation by these cells. However, if PGCs are plated over murine fetal fibroblasts in the presence of selected growth factors, i.e. LIF, basic fibroblast growth factor (bFGF) and kit ligand, they may undergo epigenetic changes forced by in vitro culture conditions that regain partially the somatic imprints, which endows them with "immortality" [38]. Thus, this change of PGCs' fate is connected with a proper recovery of somatic imprinted genes. This immortalized population of PGCs, known as embryonic germ cells (EGCs), is in many aspects the equivalent to ESCs. For example, similarly to ESCs, EGCs contribute to all three germ layers including the germ lineage after injection into a blastocyst (blastocyst complementation assay), provide functional nuclei for the clonote after nuclear transfer and form teratomas after injection into living mice. Thus, it is evident that proper somatic imprints are vital for cells from the germ line to retain full pluripotentiality. Based on this, the authors postulated and recently confirmed that erasure of the somatic imprints for some

TABLE 12.2 Molecular Signature of Bone Marrow-derived Very Small Embryonic-like Stem Cells

	ESCs	EpiSCs	PGCs	VSELs
PLURIPOTENCY				
Oct4	+++	+++	+++	++
Nanog	+++	++	+	+
Klf4	+++	+++		+++
c Myc	+++	+++		++
Sox2	+++	+++	+	++
Stat3	+++	+++		+
EPIBLAST				
Rex1	+++	+	+	+
Gbx2	+	+++	+	+++
Fgf5	+	+++	++	+++
Nodal	+	+++		+++
GERM LINE				
Fragilis	+++	++	++	+
Stella	+++		+++	+++
Blimp1	+		+++	+++
Prdm14	+		+++	++
Dppa2	+		++	+++
Dppa4	+		++	+++
Mvh	+		+++	+++
DNA METHYLATION				
Dnmt1	+++	+++	+	+++
Dnmt3a	+++			++
Dnmt3b	+++	+	cyto	++
Dnmt3L	+++	+		+++

The level of gene expression is presented as: : no expression; +: low level; ++: medium level; +++: high level of expression; cyto: cytoplasmic localization.
ESC: embryonic stem cell; EpiSC: epiblasy stem cell; PGC: primordial germ cell; VSEL: very small embryonic-like stem cell.

developmentally crucial imprinted genes is involved in controlling the quiescent status of VSELs [39].

MOLECULAR MECHANISMS GOVERNING QUIESCENCE OF VERY SMALL EMBRYONIC-LIKE STEM CELLS: INVOLVEMENT OF IMPRINTED GENES

Oct-4$^+$ VSELs isolated from murine BM do not proliferate in vitro if cultured alone and the quiescence of these cells is epigenetically regulated by DNA methylation of genomic imprinting, which is an epigenetic program that ensures the parent-of-specific monoallelic transcription of imprinted genes.

TABLE 12.3 DNA Methylation Status of Crucial Imprinted Genes in Murine Very Small Embryonic-like Stem Cells

	VSEL	HSC	MSC	ESC
Igf2 H19	↓	N	N	↑
Rasgrf1	↓	N	N	N
Igf2R	↑	N	N	↑
KCNQ1	↑	N	N	↓
Peg1/Mest	↑	N	N	N
SNRPN	N	N	N	N

VSEL: very small embryonic-like stem cell; HSC: hematopoietic stem cell; MSC: mesenchymal stem cell; ESC: embryonic stem cell; ↑: hypermethylation; ↓: hypomethylation; N: normal status.
Changes at mRNA level: VSEL: increase in mRNA for *H19*, *p57^{KIP2}*, *Igf2R*; decrease in mRNA for *Igf2*, *Rasgrf1*.

The imprinted genes play a crucial role in embryogenesis, fetal growth, totipotential status of the zygote and pluripotency of developmentally early stem cells [37]. Importantly, the significance of a proper monoallelic imprint of the *Igf2-H19* gene was demonstrated as being crucial in generating viable parthenogenic mice using two haploid sets of maternal genomes [40]. The expression of imprinted genes is regulated by DNA methylation on differentially methylated regions (DMRs), which are CpG-rich *cis*-elements in their loci. VSELs freshly isolated from murine BM erase the paternally methylated imprints (e.g. Igf2-H19, Rasgrf1 loci); however, they hypermethylate the maternally methylated ones (e.g. Igf2R, Kcnq1-p57^{KIP2}, Peg1 loci) (Table 12.3).

Because paternally expressed imprinted genes (*Igf2*, *Rasgrf1*) enhance the embryo growth but maternally expressed genes (*H19*, *p57KIP2*, *Igf2R*) inhibit cell proliferation [37], the unique genomic imprinting pattern observed on VSELs demonstrates growth-repressive imprints in these cells. As supported, VSELs highly express growth-repressive imprinted genes (*H19*, *p57^{KIP2}*, *Igf2R*) and downregulate growth-promoting ones (*Igf2*, *Rasgrf1*), which explains the quiescent status of these cells [39]. Importantly, the quiescent pattern of genomic imprinting was progressively recovered during the formation of VSEL-DSs, in which these cells proliferate and differentiate. These results suggest that epigenetic reprogramming of genomic imprinting should maintain the quiescence of the most primitive pluripotent adult stem cells (e.g. Oct-4$^+$ VSELs) deposited in the adult body and protect them from premature aging and tumor formation.

As a result of the unique DNA methylation pattern of imprinted genes, VSELs express lower amounts of

Igf2, but highly express the non-signaling receptor Igf2R, which functions at the cell surface as a decoy receptor for Igf2 and prevents its binding to tyrosine kinase receptor, i.e. Igf1R [41]. Furthermore, another paternally imprinted gene, *Rasgrf1*, which is one of the Ras-specific guanine nucleotide exchange factors (GEFs) and activates the Ras protein, is expressed at very low levels in VSELs. This gene could be also linked to insulin/Igf1 signaling [42]. Therefore, these results suggest that the unique expression of imprinted genes in VSELs could negatively regulate the insulin/Igf1 signaling in these PSCs and maintain their quiescence. Therefore, it is postulated that potential modulation of mechanisms controlling genomic imprinting in VSELs will be crucial for developing more powerful strategies to unleash the regenerative potential of these cells for efficient employment in the clinical setting.

MOLECULAR SIGNATURE OF mRNA FROM ADULT BONE MARROW-DERIVED VERY SMALL EMBRYONIC-LIKE STEM CELLS SUPPORTS THEIR DEVELOPMENTAL EPIBLAST/GERM-LINE ORIGIN

As mentioned above, the authors' recent study provides more data on unique molecular signature of VSELs (Table 12.2) and supports a concept that these cells indeed are related to the population of migratory PGCs that become specified early in development in the proximal epiblast.

Although purified adult BM-VSELs share several markers characteristic for EpiSCs as well as migratory PGCs, there are some discrepancies between migratory PGCs and VSELs. First, migratory PGCs demethylate both paternally and maternally DMRs, although each imprinted locus shows a different sensitivity to demethylation [43]. In contrast, VSELs deposited into adult BM show a different imprint pattern depending on the parental origin for DMRs [39]. This could be related to differences in the expression level and subcellular location of de novo DNA methyltransferase 3a and 3b (*Dnmt3a* and *Dnmt3b*) and its associated protein (*Dnmt3L*). VSELs, in contrast to migratory PGCs, highly express these de novo Dnmts in the nucleus [39,43]. Furthermore, VSELs express the same genes (*Klf4*, *c-Myc*, *Stat3*, *Snai1*, *Ecat1*), which are highly expressed in ESCs but not in PGCs [44]. Although VSELs are similar to migratory PGCs, they still show some differences in gene expression, which could be explained by the different modulatory effects of the microenvironments in niches where they reside (genital ridge for PGCs and BM for VSELs).

Accumulating evidence also indicates that PGCs could somehow be related to HSCs, another population of highly migratory stem cells. To support this hypothesis: (i) a tight spatiotemporal overlap exists between the migration route of PGCs and the developmental origin of HSCs (first in extraembryonic tissues in yolk sac blood islands and then in the AGM region) [45,46]; (ii) PGCs were described as being able to grow HSC colonies [47]; and (iii) BM-VSELs may differentiate into HSCs in cocultures with OP9 cells [32]. Thus, this part of the developing embryo, in addition to PGCs, could also be the potential origin of other related, highly migratory stem cells (e.g. VSELs, HSCs?).

POTENTIAL ROLE OF VERY SMALL EMBRYONIC-LIKE STEM CELLS IN PATHOLOGY: POTENTIAL CANCER STEM CELLS?

In parallel, evidence has accumulated that stem cells may play an undesirable role in the development of some pathologies. It may be hypothesized that if VSELs are mobilized at the wrong time and home to the wrong place (e.g. into areas of chronic inflammation) they may exert unwanted effects. For example, BM-derived stem/progenitor cells have been implicated in the pathogenesis of lung fibrosis, ocular pterygia and diabetic neuropathy. The potential involvement of VSELs in these processes requires further study.

It has also been recently postulated by several investigators that several types of cancer (e.g. brain tumors, melanomas, and prostate, colon and lung cancers) arise from accumulation of mutations and maturation arrest of normal stem/progenitor cells rather than by the dedifferentiation of already differentiated cells [48 52]. This hypothesis is based on the logical assumption that self-renewing stem cells residing in organs and tissues, and not mature differentiated somatic cells, may acquire and accumulate mutations during a lifetime. These mutations are subsequently maintained in stem cell compartments, and self-renewing stem cells may be subjected to additional mutations and epigenetic changes to the point where the genome is destabilized and uncontrolled neoplastic proliferation is initiated.

However, the overall concept that developmentally primitive cells exist in adult tissues that could turn into tumors is not so novel. In the nineteenth century, several investigators proposed that cancer may develop in populations of cells that are left in a dormant state in developing organs during embryogenesis. This "embryonic rest hypothesis of cancer's origin" was initially postulated by Virchow, Cohnheim and Beard, who suggested that adult tissues may contain embryonic remnants that normally lie dormant, but that can be

activated to become cancerous. In agreement with those theories, in 1910 Wright proposed a germinal cell origin of Wilms' tumor (nephroblastoma).

Oct-4$^+$ VSELs that express several epiblast/germ-line markers may somehow unify and fully support all cancer stem cell origin theories [53]. First, it may be assumed that if the genomic imprinting in VSELs is not erased, these rare cells may retain postdevelopmental in vivo pluripotency and grow teratomas and teratocarcinomas. Second, if PGCs that are developmentally closely related to VSELs migrate astray from their major migratory route to the genital ridges and ultimately settle down in various organs, they may give rise to germinal tumors [54]. Such tumors are found not only in the gonads and retroperitoneal space, but also in the mediastinum of the thorax and brain. Third, if VSELs acquire some critical epigenetic changes that lead to perturbation of some developmentally critical genes such as, for example, Igf2-H19, Kcnq1 or Rasgrf1, they may develop into several types of pediatric sarcomas (e.g. Wilms' tumor, rhabdomyosarcoma, neuroblastoma or Ewing's sarcoma). In support of this, there exists some correlation between the number of these Oct-4$^+$ cells that persist in postnatal tissues and the coincidence of these types of tumors in pediatric patients [55]. Furthermore, patients with Beckwith Wiedemann syndrome, who very often develop Wilms' tumor, display perturbation in the expression of imprinted genes. Accordingly, such patients are diagnosed with a loss of imprint on the H19-Igf2 locus that leads to overexpression of pro-proliferative Igf2 and downregulation of growth-inhibitory H19 [56]. Similarly, perturbation of imprint in the Kcnq1 locus may lead to downregulation of $p57^{KIP2}$ and important inhibitors of cyclin-activated kinases, that negatively control cell cycle and cell proliferation [56]. Fourth, it is possible that if VSELs are mobilized at the wrong time into peripheral blood and deposited in areas of chronic inflammation instead of playing a role in regeneration, they may contribute to the development of other malignancies (e.g. stomach cancer or lung cancer).

Furthermore, it has been proposed that cancer is a chromosomal rather than a genetic disease. Accordingly, carcinogenesis could be initiated by aneuploidies induced by cancerogenes, which misbalance gene expression and lead to the selection of aneuploid clones of transformed cells [57]. In this context, VSELs as highly fusogenic cells could be potential fusion partners for somatic cells. In this scenario, VSELs would provide several transcripts that are characteristic for early developmental cells (e.g. Oct-4, Nanog, Klf-4). In contrast, somatic cells as partners in fusion would supply chromosomes that show proper genomic imprinting. The formation of such heterokaryons could be a first step in the selection of aneuploid immortal cells.

Finally, it is postulated that mobile VSELs could be chemoattracted by the expanding hypoxic microenvironment of tumor tissue and potentially be involved in tumor expansion and growth by providing vessels and stroma. In this case, because the expanding tumor secretes several chemoattractants for VSELs (e.g. SDF-1, HGF/SF, VEGF), these cells could wrongly recognize the expanding tumor as regenerating tissue. Thus, after being mobilized into the peripheral blood, VSELs could be incorporated into the tumor microenvironment to provide vessels and stroma. In support of this, the kidney was reported to secrete SDF-1, which chemoattracts from BM and other tissue-residing CXCR4$^+$ primitive stem cells [18]. Further studies are needed to compare the similarities and differences of these primitive CXCR4$^+$ cells to VSELs identified by this team.

APPLICATIONS TO OTHER AREAS OF HEALTH AND DISEASE

VSELs isolated from adult tissues are an alternative and not ethically controversial source of stem cells for regenerative medicine. However, several questions need to be answered regarding this timely issue, especially in view of the current and widely performed clinical trials with BM-derived stem cells in cardiology and neurology, before VSELs can find their potential application in regenerative medicine. It is also anticipated that stem cells will be used in nephrology to regenerate kidney damaged as a result of ARF.

First, there is the obvious problem of isolating a sufficient number of VSELs from the BM, UCB or mobilized peripheral blood (mPB). The number of these cells among BM MNCs is very low. For example, VSELs represent approximately 1 cell in 10^5 BM MNCs. Furthermore, these cells are enriched in the BM of young mammals and their number decreases with age. It is also likely that if VSELs are released from the BM, even if they are able to home to the areas of tissue or organ injury, they may function only in the regeneration of minor tissue injuries. Heart infarction or stroke, on the other hand, may involve severe tissue damage beyond the effective repair capacity of these rare cells. Second, the allocation of these cells to the damaged areas depends on homing signals that may be inefficient in the presence of proteolytic enzymes released from leukocytes and macrophages associated with damaged tissue. For example, matrix metalloproteinases (MMPs) released from inflammatory cells may degrade SDF-1 locally and perturb homing of CXCR4$^+$ stem cells. Thus, VSELs may potentially circulate as a homeless population of stem cells in peripheral blood and return to the BM or home to other organs. Third, to reveal their full regenerative potential, these cells have to be fully functional and thus require the

appropriate activation signals from unidentified factors. Finally, efficient ex vivo culture conditions have to be developed that will allow for efficient expansion of VSELs without supportive feeder layer cells (e.g. C2C12, OP9 cells).

Nevertheless, the data strongly indicate that VSELs could potentially provide a therapeutic alternative to the controversial use of human ESCs and strategies based on therapeutic cloning. The controlled modulation of somatic imprint status in VSELs as hypothesized here, a proper de novo methylation of somatic imprinted genes on maternal and paternal chromosomes, could increase the regenerative power of these cells. The coming years will bring important answers to these challenges.

Acknowledgments

This work is supported by NIH grants R01 CA106281 01 and R01 DK074720 and the Stella and Henry Hoenig Endowment to MZR, and NIH grant P20RR018733 from the National Center for Research Resources to MK.

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CHAPTER

13

Induced Pluripotent Stem Cells

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OUTLINE

INTRODUCTION

An increasing number of patients with end-stage renal failure are undergoing dialysis therapy worldwide. It causes both medical and medicoeconomic problems. Renal transplantation has proven a successful therapy for most patients with end-stage renal failure, as the therapy results in a significant improvement in the patient's quality of life, prolongs survival and is considered cost-effective [1]. However, the annual increase in the number of new patients with end-stage renal disease who need a renal transplant, and the widening gap between the demand for and the supply of donor kidneys have led to a progressive shortage of donor organs for transplant. This has become a serious issue and is worsened by the problem of limited graft survival due to immune rejection [1].

Among the strategies to overcome these problems is kidney regeneration using stem cells. Stem cells may be divided into two large categories: organ-specific or somatic stem cells and pluripotent stem cells. In contrast to organ-specific stem cells that generally have a limited potential for growth and differentiation, pluripotent stem cells, such as embryonic stem cells

(ESCs) [2 4] and induced pluripotent stem (iPS) cells [5 7], have a virtually unlimited replicative capacity on culture dishes and are theoretically able to give rise to any cell type in the body. Stem cells have increasingly been used as a model system for understanding developmental mechanisms. In addition, in vitro culture and differentiation of stem cells offer unique opportunities for disease modeling, drug discovery, toxicology and cell replacement therapy [8]. The generation of specific functional cell types from ESCs has been demonstrated, including neural cells (several kinds of neuron and glia), vascular endothelia and smooth muscle, cardiomyocytes, hematopoietic cells, pancreatic insulin-producing cells and hepatocyte-like cells [8]. However, the protocol for in vitro differentiation of pluripotent stem cells into renal lineage cells has not been fully established.

Other approaches to regenerate kidney have also been investigated using organ-specific local stem cells within the kidney and bone marrow-derived hematopoietic stem cells [9]. Kidney regeneration using mesenchymal stem cells localized in bone marrow has also been examined [10]. However, the approaches are still being developed and the role of these stem cells in kidney regeneration remains to be well defined.

Therapeutic approaches using human ESCs face two major problems. One is the ethical issue derived from the use of human fertilized eggs, and the other is immune rejection in any cell or tissue transplantation due to histocompatibility antigenic differences between ESCs and patients. These problems have been overcome by a breakthrough experiment by Takahashi and Yamanaka. They identified four factors normally found in ESCs, Oct3/4, Sox2, c-Myc and Klf4, that were sufficient to reprogram both mouse and human somatic cells to closely resemble mouse and human ESCs [5 7]. They named these iPS cells. Since iPS cells can be generated from somatic cells of patients, clinical approaches using iPS cells are not associated with the two above problems (use of human fertilized egg and immune rejection). In the next natural step after iPS cell creation, significant progress has been made in redifferentiating iPS cells into somatic cells. As is the case with ESCs, iPS cells have been redifferentiated into several somatic tissues, including active motor neurons [11], insulin-secreting islet-like clusters [12], hepatocyte-like cells [13,14] and a number of cardiovascular cells (arterial endothelium, venous endothelium, lymphatic endothelium, cardiomyocytes), but not kidney [15,16].

This chapter first summarizes the mechanisms of kidney development and the research on the directed differentiation of ESCs into renal lineages based on the knowledge of kidney development. In vitro generation of kidney using the undifferentiated cell mass in amphibian eggs, similar to mammalian pluripotent stem cells in that the cell mass can differentiate into various organs in vitro, is also described as a reference to kidney regeneration in mammals. Recent advances in the iPS cell research and technology are then reviewed, and finally the future direction of iPS cells in the field of regenerative nephrology is described.

MECHANISMS OF KIDNEY DEVELOPMENT

Vertebrates develop successively three kidneys: pronephros, mesonephros and metanephros. The three kidneys consist of a basic functional unit, the nephron, although the number of nephrons differs among kidneys [17]. The kidneys are derived from a portion of the early embryonic germ layer, the intermediate mesoderm, that is located between the lateral and paraxial mesoderms [18]. A lineage tracing experiment has demonstrated that intermediate mesodermal cells expressing *Odd-skipped related* (*Osr*)*-1*, an essential transcriptional factor for intermediate mesoderm and kidney development, give rise to all components consisting of mammalian adult kidney metanephros [18]. Metanephros is formed by the reciprocal interaction between two tissues derived from the intermediate mesoderm: the metanephric mesenchyme and the ureteric bud [17]. The ureteric bud induces the metanephric mesenchyme to differentiate into the epithelia of glomeruli and renal tubules. By creating a novel culture system which uses the coculture with the cell line expressing a renal-epithelializing factor *Wnt4*, it was demonstrated that mouse metanephric mesenchyme contains multipotent progenitor cells that can give rise to several kinds of epithelial cells found in adult kidney, such as glomerular podocytes and epithelia of proximal and distal renal tubules and the loop of Henle [19]. The progenitor cells are contained only in the cell population strongly expressing *Sall1*, a zinc finger transcriptional factor that is essential for kidney development. It was also shown that the progenitor cell population can reconstitute a three-dimensional kidney structure in vitro, which contains glomerulus- and tubule-like components in an organ culture setting (Fig. 13.1A C). The presence of progenitor cells was also demonstrated in vivo using a lineage tracing experiment for *Six2*, which is also an essential transcriptional factor for the formation of kidney [20].

The progenitors in the metanephric mesenchyme differentiate into epithelia after Wnt stimulation, but a cell fate decision is required for further differentiation towards glomerular podocytes, proximal or distal renal tubules, or the loop of Henle. The molecular mechanisms of the cell fate decision are largely unknown,

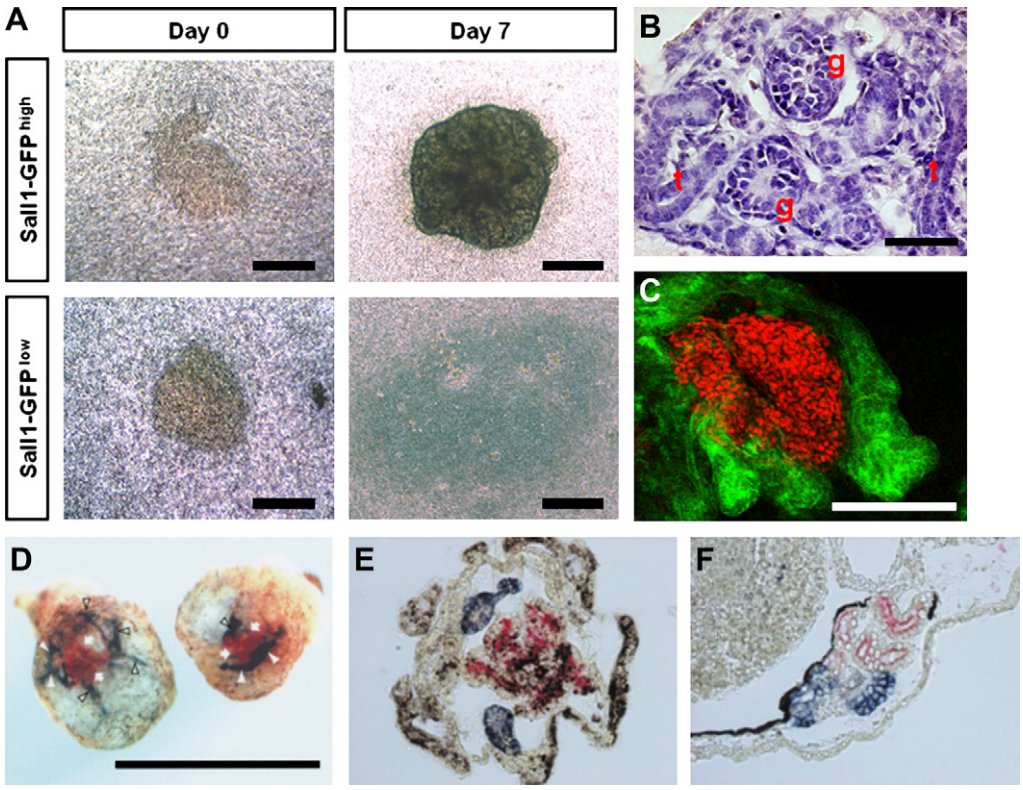

FIGURE 13.1 In vitro generated kidneys. (A–C) Metanephric kidney formed in vitro from multipotent progenitor cells in embryonic mouse kidney. (A) Metanephric cells strongly expressing *Sall1* (Sall1 GFP^{high} cells; upper panels) that contain multipotent progenitors differentiate into kidney structure in an organ culture setting, while those weakly expressing *Sall1* (Sall1 GFP^{low} cells; lower) disappear. (B) Hematoxylin & eosin staining of sections of kidney structure formed in vitro from Sall1 GFP^{high} cells. Tubule (t) and glomerulus like structures (g) are seen. (C) Double staining with WT1 (red, podocyte marker) and *Lotus tetragonolobus* lectin (LTL, green, proximal tubule marker) of kidney formed from Sall1 GFP^{high} cells. (D–F) Pronephric kidney formed in vitro from an undifferentiated cell mass in amphibian eggs (animal cap). Double immunostaining with pronephric tubule specific antibody 3G8 (red) and pronephric duct specific antibody 4A6 (blue) of kidney structure formed in animal cap treated with activin A and retinoic acid (D, E) and a stage 40 *Xenopus* larva (F). (D) Whole mount staining. (E, F) Section staining. Scale bars: (A) 500 μm, (B, C) 25 μm, (D) 1 mm. Please see color plate at the end of the book.

although it was demonstrated that *Notch2* is required for the differentiation of proximal nephron structures, such as podocytes and proximal renal tubules, as *Notch2* deletion leads to the impaired formation of these proximal structures [21]. There may be many other molecules involved in the lineage commitment, and they remain to be elucidated. Metanephric mesenchyme may contain at least two other precursor populations in addition to the epithelial progenitors: (i) vascular precursor cells that can give rise to vascular and glomerular endothelial cells, vascular smooth muscle cells (pericytes) and mesangial cells, and (ii) stromal precursors eventually differentiating into interstitial cells within the adult kidney. The ureteric bud is known to elaborate the lower urinary tract system, from collecting ducts through renal pelvis and ureters to a part of the urinary bladder [17]. Most of the mechanisms of the lineage commitment, by which the intermediate mesoderm gives rise to the ureteric bud, stromal and vascular cells, are unknown. Further investigations are required to elucidate the mechanisms, which will eventually help us to completely understand the commitment of multiple cell lineages within a kidney and reproduce it in vitro from pluripotent stem cells.

ANIMAL CAP IN FERTILIZED EGGS OF AMPHIBIANS

Amphibians have been used as experimental animals for research into developmental biology mainly because of the ease with which they can be handled and observed. In amphibian eggs, the ectodermal cell mass of mid-blastula embryos, called the animal cap, is similar to ESCs and iPS cells in mammals in that it possesses a potential of multipotent differentiation. The animal cap can be easily excised from fertilized eggs and cultured as explants in vitro in simple saline solution. After treatment with differentiation-inducing factors, the animal cap can differentiate into various tissues and organs in vitro [22]. The pronephros is a simple excretory organ of *Xenopus* larvae with only

one nephron, consisting of the glomus, a filtering unit equivalent to the glomerulus in metanephros, tubule and duct. When animal caps were treated with a combination of activin A, a protein inducer belonging to transforming growth factor-β (TGF-β) superfamily, and retinoic acid, differentiation into pronephric tubules was observed [23]. In the same differentiation system, the marker gene expression for pronephric glomus was also detected [24]. It was demonstrated that the pronephric duct, a third component of the pronephric kidney, can be generated in the explants, in addition to the pronephric tubule and glomus, and that the pronephros formed in vitro is similar to that in *Xenopus* embryos both histologically and in gene expression [25] (Fig. 13.1D F). The pronephric tubule and duct also show similar ultrastructure to those of *Xenopus* embryos by electron microscopy.

The pronephros formed in vitro from the animal cap shows a temporarily similar gene expression pattern to that in *Xenopus* embryos. Using the in vitro system to generate pronephric tissue, the molecular mechanisms underlying pronephros development have been investigated and some molecules involved in the formation of the pronephros were identified [22]. Although the pronephros produced in vitro from the animal cap cannot be directly translated to clinical applications, this system should facilitate the study of kidney regeneration and may promote a shift from tissue engineering to clinical applications. Indeed, this induction method established for the animal cap using activin A and retinoic acid has been applied in a differentiation experiment from mouse ESCs into renal lineages [26].

EMBRYONIC STEM CELLS

ESCs are pluripotent stem cells derived from the inner cell mass of fertilized eggs in mammals. The first derivation of mouse ESCs by Evans and Martin was described in 1981 [2,3] and human ESCs was established by Thomson about 20 years later [4]. Both mouse and human ESCs have a virtually unlimited replicative capacity and are theoretically able to give rise to any cell type in the body. Since their derivation, mouse ESCs have mainly been used as a research tool to generate experimental mouse models by combination with gene targeting techniques. However, following successes in human ESC derivation, the regenerative medical strategy has been considered closer to clinical applications, which aim at accomplishing the functional recovery of dysfunctional organs by transplanting organ-specific cell types generated from ESCs in vitro. The in vitro differentiation of ESCs into specific cell types of various organs has since been more vigorously investigated to develop cell transplantation therapies.

In vivo injection of ESCs into immunocompromised mice can produce teratoma, which are tumors containing cells of all three embryonic germ layers (ectoderm, mesoderm and endoderm). Glomerulus- and tubule-like structures can be formed in teratomas derived from mouse [27] and human ESCs [4], which demonstrates that mouse and human ESCs do have a potential to differentiate into kidney lineage cells. Furthermore, fundamental research aiming to establish methods to efficiently induce mouse ESCs into renal progenitors and fully differentiated renal cell types have already been carried out [26,28 31]. Mouse ESCs treated with the combination of hepatocyte growth factor (HGF) and activin A in addition to the transfection of a renal-epithelializing factor Wnt4 [28], the combination of activin A, bone morphogenetic protein (BMP)-7 and retinoic acid [26], activin A alone [29], BMP-4 alone [30], or the combination of four chemical compounds [31] produced cells expressing markers for intermediate mesoderm, developing kidney and fully differentiated renal cells. In some reports, the induced cells formed tubule-like structures or were incorporated into developing mouse kidneys [26,28,29]. Few reports have described the directed differentiation of human ESCs into a renal lineage. It was shown that human ESCs differentiated in vitro into cells expressing WT1 and rennin, marker genes for glomeruli, following treatment with growth factors [32]. Combinational treatment with activin A, retinoic acid and BMP-4 or BMP-7 also induces marker gene expression for intermediate mesoderm and developing kidney from human ESCs [33].

In summary, these data suggest that ESCs are a potential source for kidney regeneration, although the efficiency of generation and the induction rate of renal lineage cells from ESCs are unknown in many cases. It remains to be determined whether renal lineage cells produced from ESCs have excretory or endocrinological functions as in the kidney. It is also unknown whether tubule-like structures or cells integrated into the kidney are metanephric or of earlier kidneys (pronephros and mesonephros). The research on kidney regeneration using ESCs is in its infancy and further studies are required to develop this research field.

CREATION OF INDUCED PLURIPOTENT STEM CELLS

Development involves two distinct cellular processes: division and differentiation. Division is the means by which one cell gives rise to two daughter cells, and is indispensable for the growth of an organism and the renewal of fully developed tissues. Differentiation refers to the process by which a cell specializes to perform a particular biological function in an adult.

Differentiation usually occurs through a combination of cell cell interactions, exposure to diffusible factors and other positional cues that ultimately alter gene expression, conferring a specific cellular identity and function.

One of the more remarkable observations made in the past century was that differentiation is not a unidirectional process. Instead, it can be turned back much like the hands of a clock. This rewinding of the developmental clock is termed nuclear reprogramming and is often defined as the process whereby an adult somatic nucleus has a developmental potential restored to it [34]. Nuclear reprogramming has been accomplished in three ways: (i) somatic cell nuclear transfer (SCNT) or cloning, (ii) cell fusion, and (iii) factor-based reprogramming to produce iPS cells.

SCNT, a procedure in which the nucleus of an adult cell is injected into an unfertilized egg whose chromosomes have been removed, has demonstrated that the genome of adult cells can be reset to an embryonic state [35,36]. Using this strategy researchers have generated cloned embryos that possess the potential to develop into an adult animal or become an ESC line that is genetically identical to that of the donor nucleus [37,38]. These experiments established that no irreversible changes are made to the genome during development and further showed that animal oocytes harbored factors that could accomplish nuclear reprogramming.

In a related series of experiments, several researchers have shown that when somatic cells are fused with ESCs, the resulting tetraploid hybrid cells silence the expression of somatic genes and establish a program of transcription indistinguishable from ESCs, indicating that ESCs contain the necessary reprogramming activities to accomplish this transformation [39,40]. Thus, the cytoplasm of the enucleated oocyte and the ESC is able to re-establish the pluripotent state via a mechanism dependent on global epigenetic and transcriptional changes.

The mechanism by which this transformation occurred and the mediators of nuclear reprogramming were largely undefined until Takahashi and Yamanaka identified four factors normally found in ESCs that could reprogram mouse somatic cells (see Introduction).

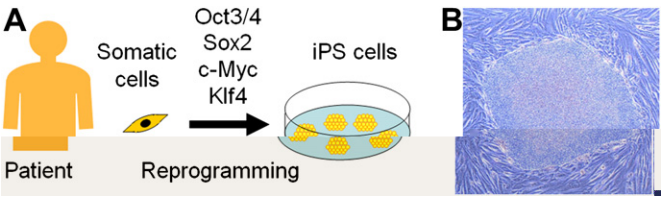

FIGURE 13.2 (A) Induced pluripotent stem (iPS) cells can be established by introducing genes encoding four transcription factors (Oct3/4, Sox2, Klf4 and c Myc) into fibroblasts. (B) An iPS cell colony generated from adult human dermal fibroblasts by introducing the four factors using retroviral vectors. Please see color plate at the end of the book.

They called these iPS cells [5] and these stem cells were also derived from human somatic cells 1 year later (Fig. 13.2A, B) [6,7]. Since this first report, the technique has been rapidly confirmed, improved and subsequently applied to successfully reprogram somatic cells. This technology has since given birth to an entire research field that has progressed at a phenomenal pace.

CELLS AND ANIMALS TO USE FOR THE GENERATION OF INDUCED PLURIPOTENT STEM CELLS

The rate at which iPS cell lines are being created is rapidly increasing. While most iPS cells were created from fibroblasts in the beginning, cell lines have also been created from adult liver and stomach cells [41], adult neural stem cells [42], keratinocytes [43], hematopoietic cells [44 46], fetal cells harvested during both amniotic fluid and chorionic villus sampling, and several other somatic cell types [44]. It appears that the reprogramming is possible in almost any cell type, although the efficiency of the reprogramming process varies among cell types. Finding the most suitable somatic cell types to accomplish reprogramming remains a main challenge for iPS cell research.

The derivation of iPS cell lines from species other than human or mouse cells expands the research potential of iPS cell in additional animal models. The generation of iPS cells was reported from adult rhesus monkey fibroblasts [47]. Two separate groups also created iPS cells from adult rat cells [48,49].

In addition, iPS cells have been derived from somatic pig cells by two other groups. One group used tetracycline-inducible human Oct4/Sox2/Klf4/c-Myc/Nanog/Lin28 delivered via lentiviruses to transduce primary pig ear fibroblasts and primary bone marrow cells into iPS cells [50]. The other group transduced porcine fetal fibroblasts into iPS cells using human Oct4/Sox2/Klf4/c-Myc delivered with lentiviruses [51]. It has been reported that iPS cells were generated from canine cells by the combination of retroviral transduction and chemical inhibitors [52]. It is possible that culture conditions for the derivation or maintenance of iPS cells from species other than human or mouse have not been fully established. However, the availability of model animal iPS cells offers a new and potentially powerful model for therapeutic applications.

MECHANISTIC UNDERSTANDING OF REPROGRAMMING PROCESSES

One of the major questions in iPS cell research seeks to define the underlying mechanism by which nuclear

reprogramming is accomplished. This includes identifying the molecular players, the key cellular and molecular events and the likely ways in which this process might fail. The goal is to make the iPS induction process safer and more efficient, and one day to manipulate the underlying cellular state of any cell, thereby generating specific cell types at will. Indeed, it was demonstrated that in vivo expression of the combination of three defined factors, Ngn3 (also known as Neurog3), Pdx1 and Mafa, which are key developmental regulators of the pancreas, can reprogram differentiated exocrine cells in adult mice into cells that closely resemble beta-cells [53]. It has also been shown that the combination of three factors, Ascl1, Brn2 (also known as Pou3f2) and Myt1l, rapidly and efficiently converted mouse embryonic and postnatal fibroblasts into functional neurons in vitro [54]. The neurons generated were named induced neuronal (iN) cells.

The nature of the factors required for the reprogramming of somatic cells into iPS cells has been elucidated. In particular, Oct4 has emerged as a central molecule in iPS cell reprogramming; for example, neural stem cells can be reprogrammed to iPS cells with Oct4 alone, which was not unexpected as they express high levels of endogenous Sox2 [42]. Until recently, no cell line had been reprogrammed without Oct4. However, it has been shown that the orphan nuclear receptor Nr5a2 (also known as Lrh-1) can replace Oct4 in the derivation of iPS cells from mouse somatic cells in part through activating Nanog expression [55]. While Yamanaka's original four-factor combination remains the most widely used, several other combinations have been shown to generate iPS cells. One of the earliest alternative combinations was developed by the Thomson group. They successfully used Oct4/Sox2/Nanog/Lin28 to create iPS cell lines [7]. Recently, it has been shown that mouse embryonic fibroblasts (MEFs) were able to reprogram using Esrrb, which is an orphan nuclear receptor, in combination with Oct4, Sox2 and c-Myc [56].

Several reports have shown that some of the reprogramming factors can be replaced with chemicals. In these cases, chemicals either directly activate the expression of reprogramming factors or in some way compensate for their activity. A Harvard group added valproic acid (VPA), known to be a histone deacetylase (HDAC) inhibitor and a widely used antiepileptic drug, to newborn human skin (fibroblast) cells in culture and was able to create iPS cells with only two reprogramming factors, Oct4 and Sox2 [57], eliminating the need for two potent cancer-promoting genes, *c-Myc* and *Klf4*. Another group used a combination of the small molecules BIX-01294 and BayK8644 to generate iPS cells from MEFs that were transfected with only Oct4 and Klf4 [58]. It has also been demonstrated that a small-molecule inhibitor of TGF-β signaling can replace Sox2 by inducing Nanog expression [59]. The promise of these approaches is to generate iPS cells using chemicals alone. However, great care should be taken over the toxicity of chemicals, including their carcinogenicity.

IMPROVING METHODS FOR INDUCED PLURIPOTENT STEM CELL INDUCTION

The original method to generate iPS cells uses genome-integrating viral vector, retroviral or lentiviral vectors [5 7], and it causes the problem of potential carcinogenesis. To overcome this issue, several groups have recently developed alternative iPS cell production methods. The iPS cells produced in each new method appear to be very similar to those produced in the traditional method. Each new method has its own advantages and disadvantages compared with the original one, and each provides insight into how scientists may be able to develop iPS cells that are safe for use in clinical trials.

Adenoviruses were used to deliver reprogramming factors into adult mouse liver cells [60]. Newborn mouse fibroblasts were also transduced; however, these were transgenic and required doxycycline induction of Oct4 expression for iPS generation. In addition, three human iPS cell lines were established from fibroblasts using adenoviruses [61]. The adenoviral approach is advantageous because it avoids integrating exogenous genes into the genome, avoiding the potential of insertional mutagenesis. The virus needs to be present for only a short time to accomplish reprogramming. However, the technique is inefficient compared with iPS cell transduction with retroviruses, it still uses cancer-promoting genes, and the adenovirus may still integrate into the host DNA at low frequencies. Recently, a Japanese group succeeded in the generation of human iPS cells by introducing reprogramming factors with Sendai viral vectors [62]. Sendai virus is an RNA virus and carries no risk of altering the host genome. They also showed that Sendai virus-derived transgenes were decreased during cell division. This approach may provide a solution for insertional mutagenesis.

The Yamanaka group reported success in generating murine iPS cells without using any viruses [63]. They successfully reprogrammed mouse cells by transfection with two plasmid constructs carrying the reprogramming factors; the first plasmid expressed c-Myc while a second, polycistronic plasmid expressed Oct3/4, Klf4 and Sox2. In a related approach, another group used one polycistronic construct expressing all four factors to achieve nucleofection in MEFs and induced iPS cell formation [64]. These methods avoid viruses entirely

but still require cancer-promoting genes to accomplish reprogramming. As with the adenoviral strategies, plasmid-based approaches are much less efficient than retroviral methods and begin with embryonic skin cells, which may be more amenable to reprogramming than adult skin cells. Moreover, transfected plasmids have been shown to integrate into the host genome and therefore pose a risk of insertional mutagenesis [65].

Three separate research groups addressed the low efficiency of non-retroviral approaches to iPS cell induction by using the piggyBac transposon system to deliver the Oct4/Sox2/Klf4/c-Myc reprogramming factors to MEFs [66 68]. piggyBac is unusual among transposon systems because upon re-excision of the exogenous genes, no footprint mutations are left in the host cell genome.

Fibroblasts from Parkinson's disease patients have been reprogrammed using floxed doxycycline-inducible lentiviral vectors that can be excised using Cre-recombinase [69]. While this strategy yielded human iPS cells with global transcriptomes that more closely resembled those of human ESCs, a genomic footprint (the loxP site) was left behind, so the mutagenicity of the retroviral approaches remains.

One report described the use of the episomal vector oriP/EBNA1 to generate iPS cells from human foreskin fibroblasts [70]. This vector is duplicated as an extrachromosomal episome once per cell cycle and is stable as long as drug selection is used. In the absence of drug selection, the episomal vector is lost at a rate of 5% per iPS cell generation. After a few generations, iPS cells that do not carry the vector can be isolated. The major disadvantage to this approach is its low efficiency.

It was reported that iPS cells were successfully generated using recombinant proteins [71]. The protein reprogramming factors were delivered into MEFs by conjugating the proteins to polyarginine, a short peptide that mediates protein transduction. A parallel approach was shown to work in human fibroblasts by fusing the Oct4/Sox2/Klf4/c-Myc factors to cell-penetrating peptide sequences [72]. The major advantage of these protein-based strategies is that exogenous genes are not introduced. However, the strategy is again rather inefficient. Improved and efficient methods to derive iPS cells need to be developed without the integration of transgenes.

INCREASING EFFICIENCY OF INDUCED PLURIPOTENT STEM CELL INDUCTION

The efficiency of reprogramming adult fibroblasts remains low ($< 0.1\%$). Whether this frequency reflects the need for the precise timing, balance and absolute levels of expression of the reprogramming genes, or selection for rare genetic/epigenetic changes either initially present in the somatic cell population or acquired during prolonged culture remains unsolved. Although considerable advances have been made in identifying the complex networks involved, it is not yet understood how these factors maintain pluripotency, how growth factors control and stabilize these networks, or how these cells respond so precisely to differentiation cues. Certain small molecules seem to improve the efficiency of the iPS cell generation process, including VPA [73], 5-aza-cytidine [74] and BIX01294 [75]. More chemicals that improve the efficiency of iPS cell reprogramming are expected to be identified. It has also been shown that mouse ESC-specific microRNAs (miRNAs), miR-291-3p, miR-294 and miR-295, can replace c-Myc and enhance the efficiency of three factor (Oct4/Sox2/Klf4)-induced reprogramming from MEFs [76]. It may be possible that other small RNAs can replace additional factors, and further study of the targets of these miRNA may offer insights into the mechanisms of reprogramming. Ultimately, the goal is to develop a cocktail of reprogramming factors that efficiently and reliably transduces somatic cells to iPS cells.

Attention has focused on p53 as a key player in the efficiency of iPS cell transduction. In 2008, it was demonstrated that adding p53 siRNA (small interfering RNA) to the Oct4/Sox2/Klf4/c-Myc reprogramming factors increased the rate of iPS cell colony formation by up to 100-fold [77]. However, many of the resulting iPS cells were only partially reprogrammed, and none yielded teratomas in vivo.

The central role of p53 in controlling iPS cell transduction has been better defined in several newly published papers. The Yamanaka group showed that in homozygous p53 knockout MEFs, 10% of the cells could be transduced to iPS cells with three reprogramming factors (Oct4/Sox2/Klf4) [78]. They further showed that terminally differentiated, p53 null T cells could be turned into iPS cells. Another group arrived at similar conclusions when they transfected cells with p53 shRNA (small hairpin RNA), and when they transduced $p53^{+/}$ and $p53^{/}$ MEFs into iPS cells [79]. They further showed that reducing p21 and Bax levels, two factors downstream of p53, also increased the efficiency of iPS cell transduction. Two other groups focused on the Ink4/Arf locus, which is responsible for inhibiting Mdm2, which in turn is the main destabilizing enzyme of p53 [80,81]. One group found that downregulating tumor suppressors contained in the Ink4/Arf locus increased the efficiency of iPS cell transduction. The other group showed that cells with low endogenous Ink4a/Arf locus products are more readily reprogrammed into iPS cells, and that genetic ablation of p53 converts non-reprogrammable somatic cells into cells that could be transduced to iPS cells. Lastly, it was also shown that p53 is responsible for preventing

iPS cell transduction of G3 Terc $^{-/-}$ MEFs, which are cells with short telomeres [82]. Taken together, these data show that the molecular network surrounding p53 strongly inhibits iPS cell transduction and the disruption of this network increases the efficiency of iPS cell generation many-fold.

While the precise molecular mechanisms behind p53 inhibition of iPS cell reprogramming is unknown, a newly published study suggests that the very process of reprogramming upregulates tumor suppressor expression. It was found that the Oct4/Sox2/Klf4/c-Myc reprogramming factors induced DNA damage and chromatin remodeling, thereby resulting in the development of senescence characteristics, including p16, p21 and p53 expression [83]. Vitamin C improves mouse and human iPS cell generation by reducing p53 levels and alleviating cell senescence while maintaining an intact DNA repair machinery [84].

COMPLETE PLURIPOTENCY

Previously, iPS cells had not been shown to contribute fully to all cell types in adult organisms. In a significant breakthrough, three separate groups have reported that in mice, MEF-derived iPS cells could be injected into tetraploid blastocysts and result in the live birth of mice entirely derived from iPS cells [85–87]. Different iPS cell lines were varyingly effective at producing viable offspring, with some cell lines showing early termination of fetal development. The success of this approach seems related to the age of the somatic cells from which the iPS cells were derived. With these reports, the debate over the equivalence of ESCs and iPS cells with regard to pluripotency has been resolved, at least in mice.

DISEASE MODELING WITH PATIENT-DERIVED INDUCED PLURIPOTENT STEM CELLS

Currently, there is one major question being addressed by iPS cell research. Researchers are seeking to produce iPS cell lines that capture the genotypes of disease. These cell lines would offer an unprecedented opportunity to understand pathobiology and pathophysiology, identify abnormalities in the development or function of differentiated cells affected by disease, develop therapies that render these cells resistant to disease and provide sources of material for cell replacement therapy. Ultimately, the goal is to develop new therapies where treatment is either non-existent or insufficient.

Numerous groups have reported the generation of disease-specific iPS cell lines. A Harvard group has generated iPS cells from patient fibroblasts of a familial form of amyotrophic lateral sclerosis (ALS) [88]. Another Harvard group has produced iPS cells from patients with 10 different genetic diseases, including Parkinson's disease, type 1 diabetes, Duchenne and Becker's muscular dystrophy, adenosine deaminase deficiency-related severe combined immunodeficiency (ADA-SCID), Shwachman Bodian Diamond syndrome, Gaucher's disease type III, Huntington's disease, Down's syndrome and the carrier state of Lesch Nyhan syndrome [89]. Another two groups have added to the list of disease-specific iPS cells by creating human cell-based models of spinal muscular atrophy (SMA) [90] and Parkinson's disease [69].

iPS cells were also generated from skin fibroblasts of a patient with homozygous beta-thalassemia and subsequently differentiated into hemoglobin-producing hematopoietic cells [44]. In theory, these cells could be treated with gene therapy to yield autologous hematopoietic cells that function normally. This goal was advanced by another group when they derived iPS cells from dermal fibroblasts harvested from Fanconi anemia patients, corrected the genetic defect using lentiviral vectors encoding for FANCA and FANCD2, and subsequently derived hematopoietic progenitor cells that were phenotypically disease free [91].

In a publication demonstrating the broad utility of disease-specific iPS cells, the generation of iPS cells was reported from patients with familial dysautonomia (FD). The iPS cells were redifferentiated into cells of all three germ layers and tissue-specific missplicing of the protein responsible for FD was demonstrated [92]. During redifferentiation, novel insights were also gained into the pathogenesis of FD: a possible mechanism for the tissue specificity of FD was demonstrated, and defects in cell differentiation and migration were uncovered. Lastly, the in vitro model was successfully used to screen candidate drugs. These approaches using disease-specific iPS cells can be also applied to genetic disorders in the nephrology field.

TOXICOLOGY SCREENING USING INDUCED PLURIPOTENT STEM CELLS AND EMBRYONIC STEM CELLS

Other clinical applications using ESC or iPS cell technology include the use of stem cell-derived cells or tissues for in vitro toxicology screening. For example, it has been shown that human ESC or iPS cell-derived cardiomyocytes can be used to examine toxic effects of drug compounds on cardiomyocytes, such as drug-induced QT interval prolongation which can lead to sudden cardiac death and is a major safety concern for the drug industry [93]. Similar approaches may include the use of ESC or iPS cell-derived hepatocytes and renal

cells in testing the hepatotoxicity and nephrotoxicity of drug compounds, which are common and major problems in clinical practice.

FUTURE DIRECTIONS IN INDUCED PLURIPOTENT STEM CELL RESEARCH AND TECHNOLOGY

In the rapidly developing field of iPS cell research, improved and more efficient iPS cell derivation protocols are expected in the near future (Fig. 13.3). Key will be the development of methods that do not rely on the integration of transgenes but are still highly efficient. As shown by the recent tetraploid complementation studies, the age at which somatic cells are harvested plays a key role in the pluripotency of the derived iPS cells. As such, it will be important to identify somatic cell types that are easily harvested and harbor the fewest mutations. It may be advisable to collect cord blood from newborns as they have been shown to be candidates for reprogramming and would at that time harbor very few mutations. In addition, cord blood cells possess the immunological immaturity of newborn cells and several hundred thousand immunotyped cord blood units are readily available through a worldwide network of cord blood banks. Two separate groups have already reported the successful derivation of human iPS cells from human cord blood cells [94,95].

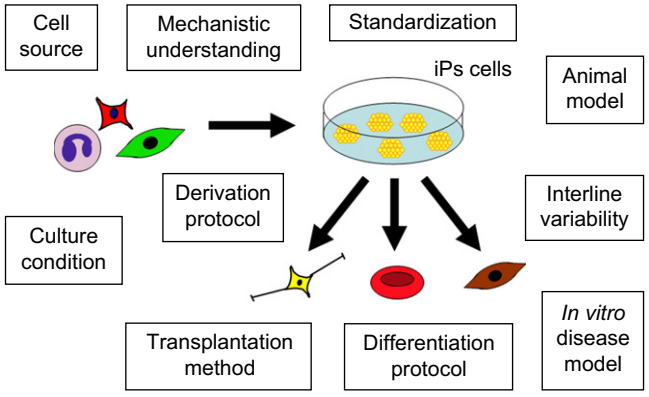

FIGURE 13.3 Questions and future directions in induced pluri potent stem (iPS) cell research and technology. Suitable somatic cell sources, safer and more efficient derivation protocols, and both deri vation and maintenance culture conditions need to be established for iPS cells in the near future. Elucidation of the mechanisms of iPS cell reprogramming would help to overcome the problems of low frequency and inefficient iPS cell transduction. The criteria for iPS characterization and standardization need to be developed, which would make it possible to solve the problem of interline variability of iPS cell lines. Protocols to efficiently differentiate iPS cells into various organ lineages including kidney remain to be established. The creation of animal disease models using iPS cells from species other than mouse and disease modeling using patient derived iPS cells would contribute to curing patients with intractable disorders. Strategies for the transplantation of iPS derived specific cell types into diseased organs also need to be developed for the embodiment of cell replacement therapies.

We are only beginning to understand the mechanism and kinetics of iPS cell reprogramming. Elucidation of these would overcome the current problems of low-frequency and inefficient iPS cell transduction. An integrative genomic analysis of the reprogramming process demonstrated that the repression of lineage-specific transcription factors and DNA demethylation are critical and inefficient steps [74]. The question of quality standards for iPS cells has begun to be addressed [96]. A minimum set of criteria for iPS characterization includes: (i) pluripotent stem cell morphology and unlimited self-renewal; (ii) expression of pluripotency markers and downregulation of differentiation markers; (iii) reprogramming factor independence; and (iv) "proof of functional differentiation through the highest stringency test acceptable".

The promise of iPS cell technology includes applications in both patient care and advanced cellular research. Currently, incomplete silencing of viral transgenes and even continued dependence on exogenous factors to maintain pluripotency are barriers to fulfilling the promise of iPS cells.

Significant differences exist in the differentiation potential of different human ESC lines, even though the observable differences in the pluripotent state are marginal [97]. These results also suggest that pluripotent stem cells derived by other means, including iPS cells and ESCs produced by SCNT, may show a similar variability. Thus, although the demonstration of complete iPS cell pluripotency via the tetraploid complementation studies was significant, it remains necessary to develop and standardize differentiation protocols that assess the differentiation potential of iPS cell lines, which would address the problem of interline variability.

Although the transcriptional and genomic characterization of iPS cells is fairly established, no proteomic characterization has been performed. The recent discovery of microRNAs presents another area of potential research and characterization; a Stanford group performed the first "miRNA-ome" analysis of human iPS cells compared with human ESCs and fibroblasts [98].

CUES TO REGENERATE KIDNEY LINEAGE CELLS FROM INDUCED PLURIPOTENT STEM CELLS AND EMBRYONIC STEM CELLS

To date, few reports have described the generation of renal lineage cells from iPS cells. However, the phenotype of iPS cells is indistinguishable from ESCs and accumulated experiences and knowledge using ESCs to

generate mesodermal and renal lineage cells are potentially applicable to iPS cell differentiation experiments.

By mimicking signals used in embryonic development, to the extent that they are known, a stepwise protocol was explored to differentiate ESCs or iPS cells into some specific cell types in adult organs, such as pancreas and liver [12 14,99]. For the generation of insulin-producing cells in pancreas, human ESCs were first differentiated into definitive endoderm, then foregut endoderm, followed by pancreatic progenitors, then endocrine precursors, and eventually insulin-expressing endocrine cells [99]. These stepwise approaches may be used in generating renal lineage cells from ESCs or iPS cells, and should involve directing the pluripotent stem cells first to form intermediate mesoderm, then the renal progenitors, followed by the eventual formation of functional renal cells found in adult kidney.

There are significant differences in the differentiation potential among different human ESC lines, and some cell lines have a propensity to differentiate into certain lineages or cell types [97]. These findings suggest that iPS cell lines may show similar differences in differentiation potential, and underscore the importance of using suitable stem cell lines for renal lineage differentiation.

Directed differentiation of pluripotent stem cells into specific organ lineages is usually carried out through the combination of cell cell interactions (coculture with primary cells or immortalized cell lines), exposure to diffusible factors (growth factor, cytokine, chemical) and other positional cues (extracellular matrix) that ultimately alter gene expression, conferring a specific cellular identity and function. Direct genetic manipulations on pluripotent stem cells using the overexpression of cDNA, siRNA or shRNA have also been performed to generate specific cell types. It has recently been demonstrated that high-throughput screening of chemical compounds can be used to identify a small molecule that has a potential to induce the differentiation of pluripotent stem cells into specific tissue lineages [100]. In the report, a chemical compound, (−)-indolactam V, known to activate protein kinase C (PKC) signaling, was shown to induce the differentiation of a substantial number of pancreatic progenitor cells from human ESCs. It was also shown that the activated PKC signal is involved in pancreatic specification in human ESC differentiation culture [100]. Similarly, chemical screens in an unbiased high-throughput method can be applied for the identification of chemical compounds to induce mesodermal and renal lineage differentiation from pluripotent stem cells and the elucidation of mechanisms or pathways involved in renal lineage commitment. By taking the cues described into account, continued investigations are needed to establish methods to efficiently differentiate pluripotent stem cells into renal lineage.

CONCLUSION

In a short period, iPS cells have proven to be a major new frontier in biological research. iPS cells have been created in human and several animal models including mice, rats, pigs and primates. They have been generated from numerous somatic cell types, and disease-specific iPS cells have been created from dozens of diseases. iPS cells have also been shown to develop autonomously into full-term mice via tetraploid complementation. Over the coming months and years, iPS cell generation efficiency will be improved.

Research towards the kidney regeneration strategy using iPS cells is in its infancy. Continued efforts to elucidate the developmental mechanisms of mesoderm formation, specification into kidney lineages and nephron specification should be made. These investigations are necessary for the establishment of differentiation protocols from iPS cells into renal lineages. Further investigations using pluripotent stem cells as a model for kidney development will facilitate our understanding of kidney precursor specification, which eventually will lead to the development of new cell-based therapies for the treatment of renal diseases, in addition to other clinical applications such as drug discovery, disease modeling and toxicology (Fig. 13.4). iPS cell-based disease modeling and drug discovery will yield new discoveries, culminating in the development of low-cost, patient-specific cell-based therapies. Because of the annual increase worldwide in the number of patients with end-stage renal failure who have to undergo dialysis therapy and need a renal transplantation, regenerative medicine strategies using iPS cells need to be developed.

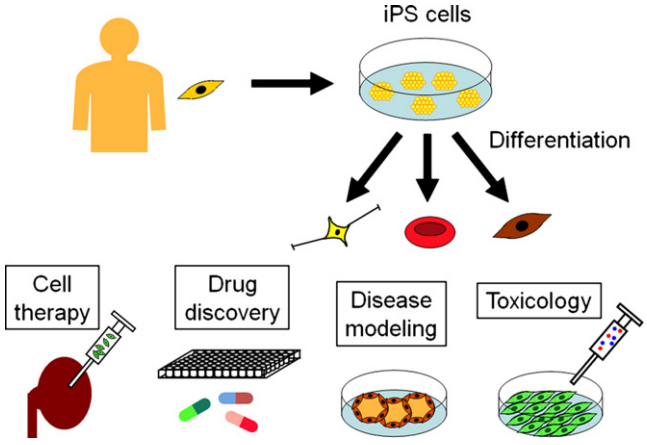

FIGURE 13.4 Possible applications of induced pluripotent stem (iPS) cell technology. iPS cells generated from somatic cells of patients with intractable diseases can be differentiated into the affected cell types and used to develop cell therapy and to study disease mecha nisms (disease modeling). The cells can also be used as tools in drug discovery and toxicology.

Acknowledgment

The authors would like to thank members of CiRA, Kyoto University, for scientific support and helpful discussions.

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CHAPTER

14

Methods of Isolation and Culture of Adult Stem Cells

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OUTLINE

INTRODUCTION

The advances in the methods of isolation and in vitro or ex vivo manipulation of stem cells that have been taking place in the past few decades have changed the perspectives in the field of regenerative medicine. For one thing, these methods have allowed for the understanding that almost all, if not all, tissues contain primitive cells that are able to give rise to mature, tissue-specific cells. In addition, the feasibility of generation of specialized cell types in vitro creates the perspective that cells lost owing to pathological processes or accidents can be replaced by cells produced from these primitive cells.

The natural path for tissue reconstruction would be, therefore, isolating and manipulating stem cells to obtain mature cells. The relative numbers of stem cells in different tissues are, however, intrinsically low, which calls for their expansion in vitro before differentiation so that an adequate number of mature cells is yielded. Consequently, different methodologies focus on the in vitro expansion of stem cells.

Expansion of stem cells in vitro is not always easy. Perhaps unnoticed is the fact that primitive cells are slowly cycling under physiological conditions in vivo, as demonstrated by experiments that focused on label-retaining cells [1 5] or cell sorting based on metabolic activity [6]. In humans, the lifespan of most cells is limited by progressive erosion of the extremities of chromosomes, which occurs at each cell division cycle owing to the lack of expression of the enzyme telomerase by somatic cells [7]. Therefore, if stem cells underwent as

many divisions as required to maintain some tissues that exhibit a high turnover rate such as the blood or skin, replicative senescence would be achieved very quickly and cell replacement would soon cease. This does not happen, only because stem cells do not replicate frequently; instead, they occasionally give rise to less immature cells that also possess some self-renewal capacity which, in turn, give rise to other cells in a more advanced stage of maturation. It is, therefore, the hierarchical structure in which stem cells are intrinsically embedded rather than stem cells themselves that is responsible for the maintenance of the different tissues. However, the existence of such a hierarchy adds complexity to stem cell research because it is sometimes difficult to tell exactly which cells are stem cells. One of the simplest definitions of a stem cell is a cell that is able to self-renew and also give rise to at least one type descendant at a more advanced differentiation state. In that case, many instances of the cellular hierarchy of a given tissue would represent stem cells. As an alternative to this and other stem cell concepts, stem cells could be defined as cells that reside at the top of the cellular hierarchy of the tissues to which they belong.

Research on hematopoiesis has greatly contributed to the current paradigms that concern stem cells. Early studies have shown that the hematopoietic compartment of mice can be ablated by irradiation and rescued by transplantation of bone marrow cells. Later, it was found that some cell populations within bone marrow are able to reconstitute hematopoiesis. However, depending on the cell population used, restored hematopoiesis lasts for shorter or longer periods [see Ref. 8 for a review]. Therefore, it is the bone marrow cell populations able to restore long-term hematopoiesis that contain the cells at the top of the hematopoietic hierarchy, which correspond to hematopoietic stem cells (HSCs). Experiments such as these have contributed to the definition of stem cells by showing that stem cells can not only self-renew and give rise to more differentiated cells in vivo, but also sustain their downstream cell hierarchy for a lifetime.

Although many studies have established that stem cells are present in almost all, if not all tissues, it is not always easy to recognize these cells. Perhaps the most important advance in the identification of stem cells was the development of monoclonal antibody technology, which allowed reproducible detection of molecules present on different types of live cells [9]. Consequently, cell populations could be defined based on the expression of a set of molecules on their surfaces. The conjugation of monoclonal antibodies with fluorescent fluorochromes and the subsequent introduction of fluorescence-activated cell sorting (FACS) systems [10] allowed for the isolation of specific cell populations based on the presence or absence of specific molecules. In spite of this, to date, most methodologies used for the isolation of stem cells fail to provide the whole range of cells that represent the stem cell compartment of a given tissue. When different combinations of surface molecules in addition to the ability to exclude the Hoechst 33342 dye were used to purify HSCs by FACS, it was found that none of the combinations could encompass the entire HSC compartment [11]. A possible explanation for such a finding is that stem cell compartments are heterogeneous.

In contrast to a prospective approach based on the expression of surface molecules, enrichment of stem cells can also be achieved by other means. Mesenchymal stem cells (MSCs), for example, can be enriched by adherence to a plastic surface and propagation in culture [12]. In contrast to HSCs, which tend to differentiate or not proliferate when cultured [13], MSCs show a marked ability to proliferate under simple culture conditions (see Methods for the Isolation and Culture of Mesenchymal Stem Cells, below). This characteristic is, however, incongruent with the fact that the cellular turnover rate in most mesenchymal tissues is far lower than in others such as blood or skin. The low turnover rate in mesenchymal tissues hinders the use of functional assays to identify which cell is at the top of the hierarchy in their corresponding tissue.

To date, cultured cell populations are operationally defined as MSCs based on their ability to differentiate along osteogenic, adipogenic and chondrogenic pathways in vitro [14] and to form bone when implanted in vivo [15]. One of the oldest criticisms of cell culture, namely, that the conditions are artificial and may therefore skew results, affects MSC research as well. Some mature mesenchymal cells can dedifferentiate in culture and exhibit characteristics of in vitro-defined MSCs [16,17], and this undermines the use of markers expressed by in vitro MSCs to seek for MSCs in vivo. Notwithstanding, several studies have found that perivascular cells give rise to mature cells in vivo under physiological conditions or injury situations (reviewed in ref. [18]). However, a hierarchy for perivascular cells has not been defined yet. It is possible that some of these perivascular cells are bona fide stem cells, whereas others represent progenitor cells. For this reason, the study of the characteristics of subpopulations of perivascular cells shows promise to answer the question as to which cells correspond to MSCs in vivo. The evidence indicates that cells that surround endothelial cells, referred to as pericytes herein, are the cells that most likely fulfill this role. Indeed, $CD146^{+}$ cells from the bone marrow (adventitial reticular cells) have been shown to transfer the hematopoietic supportive environment in vivo [19], and fresh $CD146^{+}/CD34^{-}$ cells from various tissues have been shown to form muscle fibers

when directly injected into cardiotoxin-injured mice [20]. However, although CD146 is a marker for pericytes, it is also a marker for endothelial cells [21]. Further, these studies do not exclude the possibility that the $CD146^{+}$ cells used represent progenitor cells rather than stem cells.

In an attempt to put studies such as those mentioned above in perspective, the authors have proposed a model for the role of perivascular cells under physiological conditions [22] and later extended it to include the behavior of perivascular cells during tissue injury [18]. In these models, pericytes correspond to stem cells. Under physiological conditions, these cells would physically support blood vessels, secrete some factors important for tissue maintenance and give rise to progenitors only at a very low rate. Under injury conditions, however, some of the pericytes in or at the surroundings of the insulted site would become activated, proliferate and secrete various soluble factors that would contribute to resolution of the lesion. The behavior of pericytes facing tissue injury can be seen, thus, as a process rather than a single action. This behavior can be compared to that of B cells: under physiological conditions, B cells remain as B cells until exposed to a specific context, when they become activated, proliferate and become effector cells called plasma cells.

Primary MSC cultures contain various cell types and mimic some aspects of tissue injury as cells are deprived of specific signals provided by their native environment. It is possible that these conditions allow for pericyte activation as well as changes in other cell types present; consequently, in vitro MSCs can be viewed as cell populations that contain effector cells for tissue injury. Accordingly, the secretion of paracrine factors by in vitro MSCs is a remarkable characteristic of these cells and accounts for most of their therapeutic effects [23,24]. These unique aspects of MSCs show importance when defining which cells to isolate for use in a therapeutic approach. If the most important cells present in MSC cultures comprise the progeny of pericytes, direct isolation of these cells could improve the efficiency of a therapeutic protocol. In contrast, isolated pericytes that are not exposed to the conditions that lead to the establishment of MSC cultures may not exhibit the full range of trophic and immunomodulatory properties characteristic of in vitro MSCs.

Whereas adhesion to plastic is a simple selection technique that can be used to obtain cell populations with promising therapeutic properties, other isolation procedures may provide more specialized cells. The combination of FACS with the ability of some cells to exclude specific dyes, for example, also provides a means of enrichment for some cell types. This was first demonstrated for the enrichment of HSCs. It was found that bone marrow cells that exclude the Hoechst 33342 dye are enriched for the presence of HSCs by a 1000-fold factor [25]. A FACS strategy based on poor Hoechst uptake has also been found to be effective for the enrichment of slowly cycling kidney cells with tubulogenic capacity [26]. Likewise, the expression of some enzymes associated with a primitive phenotype can be detected by incubation of the cells with substrates that become fluorescent after modification by the enzyme and used as a criterion for isolation by FACS [27].

Another method that has been used for the enrichment of stem cell-containing populations consists of centrifugation of a stem cell-containing tissue in density gradients, which provides a layer of mononuclear cells devoid of erythrocytes. Although this method is mentioned last in this introductory text, it was one of the first to be used for the enrichment of stem cell-containing populations. More specifically, it has been widely used for obtaining bone marrow mononuclear cells (BMMNCs), which correspond to a highly heterogeneous population that contains at least two different types of stem cell (HSCs and MSCs). BMMNCs obtained by centrifugation in density gradients have been widely used in many different experimental protocols. The main advantages of using BMMNCs include minimal manipulation and presence of different cell types that may be beneficial for therapeutic purposes. The disadvantages of BMMNCs include a low frequency of stem and progenitor cells and the presence of immune cells that can cause complications. Consequently, the use of BMMNCs in experimental therapeutic protocols has declined, and the use of more defined cell populations is usually preferred.

In view of the above, it is clear that each type of stem cell has its own particularities when it comes to history, biology, conceptual problems, isolation and in vitro or ex vivo manipulation. The success of a given experimental therapeutic protocol is dependent, therefore, on the cell population used and the enrichment method utilized to obtain such a population. To date, bone marrow transplantation and umbilical cord blood (UCB) transplantation for hematological diseases (e.g. leukemias, anemias) are the only clinically established applications of stem cells. All other types of stem cell therapy are experimental, and should follow appropriate ethical guidelines. Even for non-hematological diseases, however, BMMNCs or mobilized peripheral blood stem cells collected by leukapheresis are the most usually applied stem cell populations. Purified HSCs are also used, but much less frequently. The methods used to isolate and cultivate some of these cell types will be discussed. Given the growing number of studies that investigate the use of MSCs for cell therapy of many diseases, including kidney regeneration, special attention will be given to methods involved in the processes used for the isolation, enrichment and culture of this cell type.

METHODS FOR THE ISOLATION AND CULTURE OF BONE MARROW STEM CELLS

Based on the premise that all tissues have their own stem cell compartment, it should be possible to isolate stem cells from any of them. However, as mentioned earlier, our poor knowledge of morphological or molecular stem cell markers hinders their prospective identification in most cases. The stem cell fraction of many tissues may be enriched by different methods, and further cultivation selects cells that are operationally described as stem cells by their properties of proliferation and differentiation into mature cells. It should always be taken into consideration, however, that the in vitro cultivation conditions do not reproduce the in vivo niche, so that we end up with cells that are certainly different from their physiological counterparts. Nevertheless, their "stemness" level is high enough for basic, preclinical or clinical studies to be conducted with consistent results and conclusions [28].

For most tissues, the isolation of stem cells aims at allowing the investigation of basic biological characteristics. Adult stem cell populations usually employed in clinical trials include mononuclear cells obtained from the bone marrow, cord blood or peripheral blood after mobilization, as well as MSCs isolated from the bone marrow or adipose tissue. The isolation and cultivation of bone marrow-derived stem cells will be presented below.

Isolation of the Bone Marrow or Cord Blood Mononuclear Fraction

The mononuclear fraction of bone marrow includes lymphocytes, monocytes, several types of blast cells, and at least three populations of stem/progenitor cells: HSCs, MSCs and endothelial progenitor cells (EPCs). EPCs were first described as occurring in circulating blood [29], and their origin in bone marrow was later proposed [30]. However, some studies indicate that bone marrow-derived EPCs do not significantly contribute to neovascularization/angiogenesis, which could be attributed to poor knowledge of the hierarchy of the EPC compartment [31]. The application of EPCs in kidney disease has been reviewed [32].

It has been suggested that the bone marrow also contains a plethora of versatile tissue-committed, non-hematopoietic stem cells [33]. It is accepted today that the main therapeutic activity of bone marrow cells for non-hematological diseases is due to MSCs [34]. The frequency of MSCs is much more limited in the UCB [35], so that the mononuclear fraction of cord blood is more appropriate for the treatment of hematological diseases than other pathologies.

The isolation of mononuclear cells from human blood was first reported by Dr Arne Bøyum in 1968 [36], and has been used successfully ever since. The method is based on the lower buoyant density of mononuclear cells compared to erythrocytes and polymorphonuclear leukocytes (granulocytes). Most mononuclear cells have densities below 1.077 g/ml, so that they can be isolated by centrifugation on an isoosmotic medium with this density. The two types of most widely used reagents are Lymphoprep™ (AXIS-SHIELD PoC AS) and Ficoll-Paque™ (GE Healthcare), which have recently been shown to yield BMMNC fractions equivalent in terms of the composition and quantity of cell types [37].

For clinical trials, bone marrow is usually collected by aspiration from the anterosuperior iliac crest, performed approximately 3 h before the reinfusion and with the patient under sedation. The volume aspirated is around 80 ml, in a medium containing anticoagulant preservative. When the cells are to be used for research purposes, the procedure may be different. Although some institutions have access to bone marrow samples donated for research, in most cases alternatives to whole bone marrow must be implemented, including the use of bone marrow remnants in bags and nylon meshes used for collecting bone marrow for transplantation [38]. When bone marrow is collected for research purposes, it is usually obtained by a single puncture of the iliac crest using a 20 ml syringe. The quality of the sample depends on some factors. The site where the bone marrow is aspirated from can influence the amount of cells obtained as more or less medullar blood or fat can be present.

Human UCB is collected after clamping of the cord during vaginal deliveries. The blood is collected into sterile blood bags containing anticoagulant. The volume of blood collected varies usually between 70 and 120 ml. In either type of sample, the anticoagulant used is an important factor, which is sometimes overlooked. Heparin is a very popular (and effective) anticoagulant. However, heparin has soluble factor-binding activities that can influence the quality of the cells obtained. Hence, the amount of heparin should be just enough to prevent coagulation. Alternatives to heparin include sodium citrate and ethylene-diaminetetraacetic acid (EDTA), which are Ca^{2+} chelating agents and tend not to interfere with the quality of the cells when used at the right concentrations.

Bone marrow or cord blood is then diluted 1:1 with 2 mM EDTA in phosphate-buffered saline (PBS-EDTA), and light-density mononuclear cells are separated by centrifugation on Ficoll-Hypaque 1.077, at $400 \times g$ for 30 min. If the method, which is conventionally used for the isolation of mononuclear cells from peripheral blood, results in contamination with red cells, an enrichment step with 3% gelatin in 0.9% saline may be used

before centrifugation on Ficoll-Hypaque. For that, the sample is mixed 1:1 with gelatin solution and incubated at room temperature for 2 h. The mononuclear-enriched supernatant is collected and centrifuged (10 min, 400 × *g*) and the pellet is centrifuged on Ficoll-Hypaque.

Cells are then counted using a hemocytometer or Neubauer chamber. Although intact cells can be discerned from damaged cells by counting under a phase-contrast microscope, viable cells can be more accurately identified using a stain such as trypan blue. To assess cell viability, an aliquot of the parent cell suspension is mixed with an equal volume of 0.4% trypan blue in PBS and placed in the hemocytometer. Cells permeable to trypan blue are stained blue and correspond to non-viable cells. These are scored along with the viable cells and cell viability is calculated by dividing the number of viable cells by the total number of cells; red blood cells (RBCs) are recognized by eye and not included.

For clinical studies, the mononuclear fraction is collected, washed in a heparinized saline solution containing 5% autologous serum and filtered for removal of cellular adjunctive. The cells are counted, suspended in saline/5% autologous serum in the concentration determined and administered to the patient by local or systemic injection. A small fraction should be separated for sterility and viability tests, immunophenotyping by flow cytometry to determine the cell subpopulations present, and for functional analyses.

Isolation and Cultivation of Hematopoietic Stem Cells

Human HSCs are characterized as CD34- or CD133-positive, CD38- and lineage-negative cells. For clinical applications, even in cases when the treatment is referred to as hematopoietic stem-cell transplantation, the cells which are used are actually populations enriched, but not purified, for HSCs [39]. A usual method of enrichment for HSCs which avoids the need for bone marrow collection is mobilization with differentiation/growth factors such as the granulocyte colony-stimulating factor (G-CSF), followed by blood collection by leukapheresis [40]. More recently, combinations of growth factors and other molecules, such as plerixafor, have been developed for more efficient concentration of HSCs in the peripheral blood [41].

With much less frequency, patients receive purified HSCs [42], which may be isolated from bone marrow, peripheral blood after mobilization or UCB [43]. In these cases, the cells are selected by reaction with anti-CD34 or anti-CD133 antibodies followed by two alternative methods of isolation of positive cells. The first one is FACS, as described below for the isolation of pericytes. The second method, which preserves more of the biological characteristics of the cells, is magnetic cell sorting (MACS). In this case, the cells are collected, washed and incubated with an antibody conjugated to magnetic microbeads at 4°C. The cells are then washed, resuspended in the appropriate buffer and loaded on a magnetic separation column which is placed in a magnetic field. Cells negative for the marker are then eluted from the column, which preferentially retains the positive cells. After removal from the magnetic field, the positive cells are eluted. To increase purity of the sorted cells, the eluted cells may be subjected to an additional purification cycle.

All blood cells are derived, through a series of maturational cell divisions regulated by the hematopoietic microenvironment, from a small common pool of HSCs. HSCs are of interest not only because of their developmental capacity but also because of their therapeutic role in hematological disorders and gene therapy. They share the main characteristics presented by adult stem cells: (i) they constitute a very small compartment, estimated between less than 0.05% and up to 0.5% of cells in the bone marrow; (ii) they are normally quiescent or slowly cycling, as shown for instance by their resistance to treatment with 5-fluorouracil or 4-hydroperoxycyclophosphamide. Estimates of periodicity of mitosis vary widely, and the direct examination of the cell cycle of long-term cells indicates that at any moment only 4% of them are in the S/G2/M phases. Besides bone marrow, HSCs can be found in UCB and in peripheral blood, particularly after mobilization treatments.

When stem cells divide, they may return to the G0 phase of the cell cycle, generating more stem cells, or generate large numbers of committed progenitors with a progressively restricted differentiation potential. Homeotic genes have a fundamental role in the regulation of these and other cellular processes [44]. Intermediate steps, characterized by the progressive loss of the self-renewal capacity and the commitment to a specific cell lineage, result in the transition from stem cells to mature hematopoietic cells.

Despite much effort, the in vitro cultivation of HSCs is still poorly developed [45]. Many different combinations of growth/differentiation factors [46], or functional parameters [47], are under investigation to define the conditions allowing expansion of this cell population with maintenance of stemness. However, as different assays detect and analyze hematopoietic cell types specifically stimulated by the experimental conditions used, and the correspondence among the assays is not always easily established, different names have been given to the cell types observed, as detailed below.

Till and McCulloch established, in 1961, the first quantitative assay for cells with a radioprotective effect. Although these cells, which were denominated spleen colony-forming units (CFU-S), do not represent the

more primitive stem cell pool, the assay is useful for the investigation of early events in hematopoiesis. In vivo assays involving the engraftment of human hematopoietic cells into immunodeficient mice have shown the existence of pluripotent cells, either by limiting dilution analysis or by clonal integration of a retroviral marker gene [48,49]. The expression "marrow repopulating ability", derived from in vivo studies, refers to primitive HSCs capable of repopulating the bone marrow of lethally irradiated mice [50].

Two different types of cells with marrow repopulating ability have been distinguished in the mouse. Initial engraftment (short-term repopulation) is a characteristic of CFU-S. Long-term engraftment is attributed to a different cell type and is possible only if the animals also receive short-term repopulating cells. A cell type which is more primitive than CFU-S (pre-CFU-S) is considered to be responsible for long-term marrow repopulating ability [reviewed in Ref. 51].

The development of in vitro cultivation systems for the study of hematopoiesis allowed the identification and quantification of several different types of hematopoietic precursor cells. The colony formation assay [52] allows the identification and quantification of early progenitor cells, capable of forming colonies when cultured in semisolid medium. In this assay, cells are grown in vitro in methylcellulose or other highly viscous media, containing also different combinations of growth and differentiation factors. These semisolid media reduce cell movement and allow individual cells to develop into cell clones that are identified as single clusters (< 50 cells) or colonies (> 50 cells) of differentiated cells after a culture period of 7 14 days. These colonies are the progeny of single cells called colony-forming cells (CFCs) or colony-forming units (CFUs), and the composition of the colonies determines which CFU is being assessed. These clonogenic assays allow the identification, for instance, of CFU-blasts which give rise to colonies composed of cells with blast-like morphology, of CFU-GEMM which originate multilineage colonies (granulocytes, erythrocytes, monocytes and megakaryocytes), CFU-meg which give rise to megakaryocyte colonies, and so on [53]. Other types of stem/precursor cells identified by this culture system include the high proliferative potential colony-forming cells (HPP-CFC), defined by the ability to form very large colonies (> 5 mm) containing approximately 50,000 cells, and the low proliferative potential colony-forming cells (LPP-CFC), that give rise to colonies smaller than 1 mm. These primitive HSC pools are considered to comprise cell types such as BFU-E (erythrocyte blast-forming unit) and CFU-GM (precursor of granulocytes and monocytes) [50].

Another culture system currently used for the study of hematopoietic progenitors is the delta assay [54]. In this system, cells are grown first in liquid culture for 1 week and then replated onto semisolid medium. Colonies observed after this period indicate the number of hematopoietic progenitors more primitive than those normally obtained after 14 days of growth in semisolid medium.

The development of hematopoietic cells in these culture systems depends on the continuous presence of colony-stimulating factors (CSFs). The investigation of results obtained with addition of different types of stimulating molecules has been the key to the discovery and characterization of many of the hematopoietic growth factors [55]. Colony-forming assays, therefore, allow the study of the role of growth factors or cytokines in the determination of the lineage along which CFCs differentiate.

Expansion of HSCs is not possible with the use of standard semisolid systems; consequently, alternative culture assays have been developed for the sustained production or self-renewal of clonogenic cells. In the long-term culture (LTC) system originally described by Dexter for murine cells [56] and later adapted for human cells [57], cells are cultivated at a lower temperature in a culture medium containing high concentrations of horse serum and hydrocortisone. This supportive microenvironment allows the self-renewal of stem cells over a period of several months. The long-term culture of bone marrow cells employs primary adherent layers of stromal cells which include MSCs as important components. These stromal cells provide a complex functional extracellular matrix which allows direct cell cell contacts between different cell types and secrete cytokines and low molecular weight substances which are required for the controlled differentiation and proliferation of hematopoietic progenitor cells. This system can maintain primitive stem cells, induce early differentiation of a fraction of the primitive progenitors and prevent their terminal differentiation.

A second type of widely used culture system for hematopoietic cells is the Whitlock Witte long-term bone marrow culture [58], which was initially developed for murine bone marrow. In this lymphoid culture system, a "poor" culture medium containing 5% fetal calf serum and no cortisone permits the growth of freshly isolated bone marrow cells that develop into a confluent adherent stromal cell layer within 2 3 weeks. Whitlock Witte cultures can reconstitute the B-lymphocyte compartment in immunocompromised mice, but do not maintain primitive multilineage hematopoietic precursors such as CFU-S [50]. Pluripotent HSCs and early precursors can also be identified by the LTC assays as cobblestone area-forming cells (CAFCs) [59] and long-term culture-initiating cells (LTC-ICs). Limiting dilution analysis is used for the quantification of these cells [60].

The transduction of CFU cells and LTC-ICs using retroviral vectors indicates that the in vitro progenitor assays currently available measure functionally different, and presumably less quiescent, populations than the long-term repopulating stem cells [61]. However, none of these culture systems is able to maintain hematopoiesis indefinitely, showing that the in vivo niche of HSCs has not yet been adequately reproduced in vitro. It is not probable, therefore, that clinical application of isolated, cultivated HSCs will be available in the near future.

ISOLATION AND CULTURE OF RENAL PROGENITOR CELLS

Among the methodologies described for the isolation of kidney progenitor cells with tubulogenic capacity, two [26,62] have been selected for discussion in this chapter. In the first, slowly cycling cells have been isolated from the kidneys of rats; in the second, $CD133^+$ cells have been isolated by MACS.

In the work by Maeshima et al. [26], 7-week-old rats were implanted with an osmotic pump that released 5-bromo-2-deoxyuridine (BrdU) and the pump was removed after 1 week. Two weeks after removal of the pump, the rats were euthanized; the kidneys were removed and sequentially digested with collagenase and trypsin. The cells were cultured in Dulbecco's modified Eagle's medium (DMEM)-F12 containing 10% fetal bovine serum (FBS) for 48 h and incubated with 10 μg/ml Hoechst 33342 for 1 h at 37°C. The $Hoechst^{low}$ cells isolated by FACS were found to give rise to various cell types when cultured under specific conditions or implanted into cultured E15 metanephroi. Although the methodology used to isolate cells is similar to that used to obtain side population cells, the rationale is different: incorporation of BrdU to DNA renders it less susceptible to incorporation of Hoechst 33342; hence, $BrdU^+$ cells become apparent as $Hoechst^{low}$ cells [63]. Incubation of $BrdU^+$ cells with 50 μM verapamil, an inhibitor of the drug efflux protein responsible for the exclusion of Hoechst 33342, indicated that the $Hoechst^{low}$ cells isolated are not side population cells.

Bussolati et al. [62] isolated cells with progenitor characteristics from the normal cortical region of human kidneys obtained after surgery. $CD133^+$ cells were isolated by MACS and expanded under conditions described for human multipotent adult progenitor cells [64]: culture on fibronectin-coated dishes in a medium consisting of 60% DMEM and 40% MCDB-201 with 2% FBS, insulin transferrin selenium (ITS), linoleic acid bovine serum albumin (LA-BSA), 1×10^{-9} M dexamethasone, 1×10^{-4} M ascorbic acid-2 phosphate, 10 ng/ml hPDGF-BB and 10 ng/ml epidermal growth factor. These cells were able to exhibit epithelial and endothelial characteristics in vitro and in vivo. The cultured cells derived from $CD133^+$ kidney cells localized to proximal and distal tubules when injected into mice subjected to acute tubular injury by intramuscular glycerol injection; some of the engrafted cells seemed to have differentiated into epithelial cells.

METHODS FOR THE ISOLATION AND CULTURE OF MESENCHYMAL STEM CELLS

Isolation and Culture of Mesenchymal Stem Cells from Human Bone Marrow

Bone marrow mesenchymal stem cells (BMMSCs) are produced by cultivation of the mononuclear fraction, isolated as described earlier. The rationale is that the selection of proliferative, adherent cells present in the BMMNC fraction results in the enrichment for MSCs, which are further characterized by immunophenotyping and differentiation assays.

BMMNCs are counted and plated in culture-treated, polystyrene culture dishes or flasks at a density of around 175,000 cells/cm^2. In practical terms, this corresponds to 1×10^7 cells per 100 mm dish in 7 ml of complete culture medium. Accurate cell counting is important to minimize variation between experiments. Since centrifugation in density gradients does not eliminate all RBCs from the BMMNCs, acetic acid can be added to an aliquot of the cell suspension to deplete the former before counting. This is done by mixing an aliquot of the parent solution with an equal volume of a 4% acetic acid solution.

MSCs are usually cultured in FBS-containing medium. The most commonly used basal media are DMEM and an alpha modification of Eagle's minimum essential medium (αMEM). FBS concentrations range from 10 to 20% (v/v). Media especially formulated for MSCs are also available from a growing number of companies. Alternatives to FBS include serum replacement solutions, available from some companies, human AB serum and platelet lysate [65]. Owing to the increasing interest in the clinical use of MSCs, the latter has been gaining attention as medium supplement because it can eliminate the need for xenogeneic components. For small laboratories conducting basic research experiments, however, the use of platelet lysate can be more difficult as it is not currently commercially available. Hence, FBS is still an important item in MSC research. Since FBS batches can vary considerably depending on the supplier and other factors, it is advisable to test some batches from different suppliers. Some companies provide FBS batches which have been

prescreened to provide optimal growth of MSCs. If the use of prescreened FBS is not an option, FBS samples from different suppliers can be tested by means of fibroblastic colony formation and differentiation assays. Although the use of heat-inactivated serum is very popular, FBS inactivation seems to offer no benefit to MSC growth; instead, it may decrease performance [66]. Such a decrease in performance may be attributed in part to the fact that complement proteins, the target of heat inactivation, are important for MSC migration and, possibly, proliferation [67].

After bone marrow processing and plating, cells are maintained in an incubator with a water-saturated atmosphere which consists of a mixture of environmental air with 5% CO_2. DMEM has been formulated for use in combination with an atmosphere that contains 10% CO_2; in spite of this, most laboratories that use DMEM as the basal medium maintain MSCs in 5% CO_2. Three days after the establishment of the culture, the used medium is aspirated and replenished with fresh complete medium. In this process, most non-adherent cells are discarded. At this point in time, it is usually not possible to visualize discrete fibroblastic colonies. The cells are cultured for about 2 more weeks with two medium changes a week. During this time, MSCs become noticeable as discrete fibroblastic colonies that increase in size until they cover most of the bottom of the dish, i.e. are subconfluent. At this point, cells are enzymatically harvested and replated in new culture vessels to increase cell numbers. First, the old medium is aspirated and the plate is rinsed with a Ca^{2+}- and Mg^{2+}-free saline solution such as Tyrode's balanced salt solution, Hank's balanced salt solution or PBS to remove traces of FBS and divalent cations. A Ca^{2+}- and Mg^{2+}-free solution containing 0.25% trypsin and 0.5 mM EDTA is added to the cells, and the dish is incubated at 37°C for 5 10 min to speed up the process. The volume of trypsin EDTA solution added usually ranges from 1 to 2 ml/25 cm^2. Although it is convenient to use the cell incubator to perform trypsinization, it is recommended that the cells be incubated in the absence of added CO_2 because the buffering systems of saline solutions are developed to maintain a physiological pH at normal atmospheric CO_2 concentrations. The high CO_2 concentration in the incubator rapidly acidifies the trypsin EDTA solution, which drastically diminishes trypsin activity.

After trypsinization, serum or a serum-containing solution or medium is used to inactivate trypsin. In some laboratories, newborn calf serum is added directly to the cell suspension at a ratio of 1 ml per 2 ml of trypsin EDTA solution. In other laboratories, culture medium containing 5 10% FBS is used for this purpose. Cells are then mechanically dispersed by pipetting, transferred to centrifuge tubes and centrifuged. Seven minutes of centrifugation at 250 × *g* are effective to pellet the cells. The temperature during centrifugation can be set to around 22°C or room temperature. After centrifugation, the cells are resuspended in complete culture medium, counted and replated at 2500 cells/cm^2.

The process of harvesting the MSCs from one dish and replating in another one is referred to as "passage". Primary cultures are defined as "passage 0" or "P0". Cells derived from the first passage are "first passage cells". The term "passage" can also comprehend the period required for cells to become subconfluent or confluent: at the end of the first passage, i.e. when first passage cells become confluent or nearly confluent, cells are trypsinized and again seeded in fresh dishes at 2500 cells/cm^2. Although MSCs cultured under appropriate conditions can be in vitro expanded for several passages, cells are often used for the experiments at the end of the second or third passage. At this point, it is advisable to freeze cells for future experiments. Freezing can be done by resuspending 5×10^5 MSC aliquots in 1 ml of freezing medium (90% FBS + 10% DMSO), transferring to a freezing vial, placing the vial in a 80°C freezer overnight and transferring it to liquid N_2 the next day. The freezing process can be enhanced by the use of devices or systems that control the freezing rate. The number of vials to be frozen depends on the projected future use.

Isolation and Culture of Mesenchymal Stem Cells from Murine Bone Marrow

The mouse is the entry-level species for a range of preclinical models including those involving MSCs. The isolation of murine bone marrow-derived MSCs (mBMMSCs) is in line with the development of therapeutic strategies in which human bone marrow is to be used. The establishment of mBMMSC cultures is not as easy as it is for their human counterparts. Unlike hBMMSC cultures, mBMMSC initial cultures exhibit heavy contamination by hematopoietic cells and are not highly proliferative. Serial passaging can be used to favor the enrichment for mBMMSCs [68], but it requires some persistence as the hematopoietic contaminants are not easily depleted by this process.

To establish mBMMSC cultures, a mouse is euthanized by cervical dislocation and briefly embedded in 70% ethanol. Under aseptic conditions, the femora and tibiae are removed and freed from connective tissue. A 5 ml syringe coupled with a 21 G needle is filled up with complete culture medium (DMEM containing 3.7 g/l sodium bicarbonate, 10 mM HEPES and 10% FBS). Bone marrow is obtained from the femur by inserting the needle through the distal extremity of the bone, cutting the proximal end and flushing the bone marrow out into a sterile container by applying pressure to the plunger. The same syringe is used to mechanically disaggregate bone marrow by passing it through the

needle with some aspiration/expulsion cycles. The same procedure is used for the tibia, except that the needle is inserted through the proximal end of the bone and the distal end is cut to allow for expulsion of the bone marrow. Cells are counted as described above for the isolation of hBMMSCs.

To initiate primary cultures, a cell suspension at a concentration of 5×10^6 cells/ml is prepared in complete culture medium. This suspension is plated in a well of a six-well dish at 3.5 ml/well. The dish is placed in an incubator with humidified atmosphere containing 5% CO_2 at 37°C. Three days after plating, the non-adherent cells are removed by changing the medium. The cells are further cultured with two medium changes per week until they become confluent. When confluence is achieved, the cells are rinsed with a Ca^{2+}- and Mg^{2+}-free saline solution and incubated for 10 min with a solution containing 0.25% trypsin and 0.5 mM EDTA at 37°C. After incubation with trypsin EDTA, the detached cells are resuspended in complete culture medium and centrifuged at 250 400 $\times$ *g* for 10 min at room temperature. For primary cultures, it is natural that many cells remain attached to the well after trypsinization. After centrifugation, the supernatant is discarded and the cell pellet is resuspended in 10.5 ml of complete medium. This cell suspension is distributed along three wells of a fresh six-well dish (3.5 ml/well). Cells are cultured with two medium changes per week until they are at least nearly confluent. When confluence is achieved, cells are trypsinized; the contents of each well are split into two new wells and further cultured. This process is repeated until a population of highly proliferative, flat cells becomes predominant. A considerable amount of time is required for the cells of the initial passages to become confluent (weeks). As the cultures are passaged and consequently enriched for cells with MSC characteristics, the interval between seeding and confluence becomes shorter, and the split ratios should be increased accordingly so that cells are passaged twice a week. It is recommended that flow cytometric analysis of some MSC and hematopoietic markers be checked before using the cells in experiments.

MSCs can be obtained from other murine tissues as well. The establishment of MSC cultures from most murine tissues other than bone marrow is usually easier, possibly because some cell types present in murine bone marrow inhibit the growth of MSCs. Isolation of MSCs from other murine organs requires enzymatic disaggregation with type I collagenase. Details regarding collagenase digestion are provided in the section below and can be applied to all murine tissues other than bone marrow. The culture procedures for culturing mMSCs from other organs are basically the same as described for mBMMSCs. Usually, mMSC cultures derived from organs other than bone marrow can be established more quickly; however, differences related to the tissue of origin are evident between these populations. Consequently, mMSCs from different organs should not be considered equivalent.

Isolation and Culture of Mesenchymal Stem Cells from Human Adipose Tissue

The main difference between the isolation of human adipose tissue-derived mesenchymal stem cells (hATMSCs) and hBMMSCs is that adipose tissue requires enzymatic disaggregation to provide a cell suspension. This enzymatic disaggregation is performed mainly by type I collagenase, which is actually a group of different molecules that contain collagenolytic and tryptic activities.

Adipose tissue samples can be obtained as large fragments from lipectomies or small fragments from liposuction procedures, the latter form being the most common. Liposuction material contains, in addition to small fragments of adipose tissue, oil and a mixture of blood and saline solution. The fat fragments remain afloat and are transferred with a 25 ml pipette to 50 ml centrifuge tubes to which 25 ml of Hank's balanced salt solution (or other saline solution) have been previously added. The tubes are then capped and the contents mixed by vigorous shaking. These tubes are centrifuged at 400 $\times$ *g* for 10 min at room temperature. It is important to refrain from using low temperatures during manipulation of these samples to avoid the formation of a compact gelatinous mass by the adipose tissue fragments. At the end of centrifugation, four phases can be recognized: the topmost, which consists of oil; the adipose tissue fragments under the oil; a pellet consisting of blood cells at the bottom of the tube; and a column of saline solution between the pellet and the adipose fragments. The oily layer is removed with a Pasteur pipette and the buoyant tissue fragments are transferred to a second set of tubes loaded with 25 ml of Hank's balanced salt solution. After mixing and centrifuging, the oil at the top of the phases is removed with a Pasteur pipette and the adipose tissue fragments are transferred to fresh 50 ml centrifuge tubes to estimate the volume.

When the volume of adipose tissue fragments is known, an equal volume of type I collagenase solution is prepared by dissolving collagenase in the digestion solution of choice and filtering it through a 0.22 μm pore membrane. Collagenase activity requires Ca^{2+}, and for this reason the digestion solution must contain this ion. PBS with added Ca^{2+} (4 μM) can be used as a digestion solution. Alternatively, collagenase can be dissolved in culture media such as DMEM, RPMI 1640 or αMEM. To achieve maximal quality of the cells after digestion, FBS can be added to the digestion solution at concentrations ranging from 10 to 20%.

Two other factors, collagenase concentration and incubation time, influence the quality of the resulting

cells. Short incubation times with high collagenase concentrations may be effective to achieve a good level of cell dissociation, but collagenase can be toxic to cells at high concentrations. This toxicity can be counteracted by addition of FBS, which can inactivate tryptic but not collagenolytic activity. The high cost of collagenase also points to the use of a minimum amount required to obtain a cell suspension with low levels of aggregated cells. In the authors' experience, a final collagenase concentration as low as 100 units per ml of PBS with an incubation time of 40 min at 37°C is effective at dissociating the stromal vascular fraction from mature adipocytes. However, the resulting material consists mainly of intact microvessels. If these microvessels are plated under MSC conditions, MSC cultures will be successfully established. However, standardization of the number of cells plated will be compromised because the cells cannot be precisely counted before plating.

Collagenase digestion is carried out at 37°C with intermittent gentle agitation. Considering that the cells in the adipose tissue fragments are deprived of most survival signals they had in the native adipose tissue, at this temperature the duration of digestion should be limited to a minimum to preserve the quality of the cells. Again, addition of FBS to the digestion solution counteracts the negative effects of extending incubation time. It is important to emphasize that the use of FBS is contraindicated if the stromal vascular cells are intended to be used in humans. In that case, other additives such as human albumin or autologous plasma can help.

After enzymatic disaggregation, the digested samples are transferred to 50 ml centrifuge tubes and centrifuged at $250 \times g$ for 7 min. The supernatants, which include oil, floating adipocytes and digestion solution, are discarded. The cell pellets are resuspended in RPMI 1640 medium containing 20% FBS (RPMI/20) and centrifuged again. These cell pellets usually contain relatively high amounts of RBCs. Although cells at this point can be counted as described above for isolation of hBMMSCs, it is convenient to deplete RBCs by incubation of the cells in 160 mM NH_4Cl for 10 min at room temperature. After RBC lysis, cells are centrifuged again and resuspended in the culture medium of choice. Cells are counted and plated at 1×10^6 cells per 100 mm dish in 7 ml of medium (around 17,500 cells/cm^2). From this point on, cells are cultured as described above for hBMMSCs.

ISOLATION AND CULTURE OF HUMAN ADIPOSE TISSUE-DERIVED PERICYTES

As mentioned earlier in this chapter, pericytes are the best candidates for the role of MSCs in vivo. Consequently, these cells may constitute important therapeutic agents in the future.

Pericytes can be isolated by FACS after staining with antibodies that recognize pericyte markers. However, the use of pericyte markers alone does not ensure that the population obtained is devoid of other cell types. Pericytes maintain a strict physical association with endothelial cells, and there is no guarantee that the disaggregation process completely separates these two cell types from each other. A pericyte bound to an endothelial cell can be detected by the cell sorter as a single event. If a single pericyte marker is used and the positive cells are selected, these may contain endothelial cells among other contaminant cell types. Furthermore, although some molecules are known to be preferentially expressed on pericytes, these may also be expressed by other cells too. In some recent works [19, 20], CD146 has been used as a marker to isolate pericytes; however, this molecule is also expressed by endothelial cells. A second marker is, thus, necessary to discriminate and negatively select endothelial cells. CD34 has been used to this end.

After defining the sorting strategy, it is necessary to define the conditions required to obtain a cell suspension that meets the minimal quality criteria for FACS. Adipose tissue is an interesting source of cells because it can be obtained in large amounts and the cells that result from collagenase digestion correspond to a vascular stromal fraction which is enriched for pericytes. The conditions used for the digestion of adipose tissue from liposuction material are crucial for the success of pericyte isolation by FACS. The enzymatic disaggregation conditions described above for the isolation of hATMSCs have been optimized to provide cells that are suitable for FACS. In fact, the isolation and culture of pericytes is performed in parallel with the establishment of hATMSCs from the same sample so that both populations can be compared.

After enzymatic disaggregation, cells are washed once and counted. An aliquot containing 100,000 cells is reserved to provide a control tube for FACS. A fraction of the parent cell suspension is reserved for the establishment of hATMSC cultures. The remaining cells are subjected to immunostaining for FACS. Cells are centrifuged and resuspended in RPMI/20. This medium is also used for all washes during immunostaining. The immunostaining procedure is as follows: a primary antibody [mouse immunoglobulin G (IgG)] that recognizes a pericyte marker is added to the cells at a dose of 1 μg per 1×10^6 cells in a volume of 100 μl and incubation for 20 min at room temperature. Cells are resuspended in 5 ml RPMI/20 and centrifuged. The supernatant is discarded and the cells are resuspended in 100 μl RPMI/20. An antimouse IgG antibody conjugated with a fluorochrome (e.g. FITC) is added at a dose of 1 μg per 1×10^6 cells and the tube is incubated for 20 min at room temperature, protected from light. Cells are

resuspended in 5 ml RPMI/20, centrifuged, and the supernatant is discarded. The cells are resuspended in 100 μl RPMI/20 and an antibody (sheep IgG) that recognizes an endothelial cell marker is added at a dose of 1 μg per 1×10^6 cells. The tube is incubated at room temperature for 20 min, protected from light. Then, 5 ml of RPMI/20 is added to the tube and the cells are centrifuged. The supernatant is discarded and the cells are resuspended in 100 μl of RPMI/20. An antisheep IgG antibody conjugated with a different fluorochrome (e.g. PE) is added at a dose of 1 μg per 1×10^6 cells, and the tube is incubated for 20 min at room temperature, protected from light. Then, 5 ml of RPMI/20 is added to the cells, and the tube is centrifuged again. After discarding the supernatant, cells are resuspended in RPMI/20 at a concentration of 1×10^7 cells/ml. The control tube is immunostained in a similar fashion except that the primary antibodies used, which must match host species and isotype, are unspecific. It is not possible to perform FACS without an appropriately stained control tube.

After immunostaining, the cells are taken to the flow cytometry core and the control tube is run in the cell sorter to determine baseline fluorescence for each marker. Data are acquired and stored for future reference. Quadrants are drawn to help discernment between single-positive cells for each marker as well as double-positive cells. The cells prepared for FACS are then run through the cell sorter. If cell aggregation is visible, cell clumps can be removed with the use of a cell strainer. Cells that are positive for the pericyte marker and negative for the endothelial cell marker used are selected for sorting. Data are again acquired and stored for future reference. Cell sorting can be a lengthy process if large amounts of cells are processed at a time and the frequency of target cells in the sample is low. Usually, about 20 min are spent for 1×10^7 input cells in addition to the time required to prepare the cell sorter.

Some cell sorters offer the convenience of dispensing the sorted cells directly in culture dishes. In that case, six-well dishes containing 2 ml of culture medium per well are a good choice. It is recommended that at least 30,000 cells be plated per well. Cells subjected to FACS are exposed to harsh conditions and are usually more prone to the negative effects of antifungal agents such as amphotericin B, and hence it is advisable to add these to the medium in some of the wells but not all. Conditions used for the culture of pericytes include addition of a substrate to the dishes to facilitate cell attachment and also to provide other signals. Collagen [69] and 0.2% gelatin [20] have been reported to be suitable substrates for pericytes. Hence, if cells are collected directly in six-well dishes, these must be previously coated with the substrate of choice. Media used for the culture of pericytes include DMEM containing 5% FBS and 5 ng/ml bFGF [69], Endothelial Cell Growth Medium 2 and DMEM containing 20% FBS [20].

After the establishment of primary cultures, pericytes can be further expanded by subculturing the cells with trypsin EDTA when cultures approach confluence. If low-serum culture conditions are used, trypsin concentration should be low (0.05%), while EDTA concentration can be maintained at 0.5 mM. Seeding densities can be as low as 1000 2000 cells/cm^2. After brief expansion in culture, it is recommended that a fraction of the cells be frozen before characterizing the cells.

Acknowledgments

The financial support from Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) and Fundação de Amparo à Pesquisa do Estado do Rio Grande do Sul (FAPERGS) is gratefully acknowl edged. L. da S. M. holds a postdoctoral fellowship from FAPESP.

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CHAPTER

15

Stem Cell Niche in the Kidney

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OUTLINE

INTRODUCTION

Stem cells are one of the fundamental underpinnings of tissue biology [1,2]. They allow adult tissues to be replenished by fresh cells throughout life. Additional stem cells lie dormant, but can be activated at particular life-cycle stages, or following injury. These potent agents are controlled within restricted tissue microenvironments known as "niches" [1,2]. Accurately identifying stem cells in vivo remains the biggest obstacle to progress in understanding stem cell biology. Normal stem cells within tissues can rarely be identified by means of histological methods [1,2]. Some properties that have been widely assumed to mark stem cells, such as preferential bromodeoxyuridine (BrdU) label retention (caused by an expected tendency of stem cells to divide more slowly than many of their progeny) have frequently proven to be unreliable where definitive independent markers are available [3 6]. However, three functional properties characterize stem cells obtained from different tissues: (i) the ability to self-renew, which is a prerequisite for sustaining the stem cell pool; (ii) the ability to generate at the single cell level differentiated progeny cells, in general of multiple lineages; and (iii) the ability to functionally reconstitute a given tissue in vivo [1,2]. The typical adult stem cell, present in many tissues, is considered multipotent as it can only give rise to differentiated cell types from the tissue of origin, in contrast to embryonic stem cells, which can give rise to cells from the three somatic germ layers (ectoderm, mesoderm and endoderm), as well as to germ cells, and are thus pluripotent [1,2].

STEM CELLS IN THE ADULT KIDNEY

The kidney is one of the few organs that undergo mesenchymal epithelial transition during their development [7]. Structures present in the adult kidney arise from reciprocal interactions between two discrete embryonic appendages: the ureteric bud and metanephric mesenchyme [7]. The adult kidney contains more than 24 mature cell types arranged in distinct vascular, interstitial, glomerular and tubular compartments [7]. This unique organogenesis and structural complexity of the adult kidney has been the cause of many challenges to the identification and characterization of kidney stem cells [7,8]. In addition, the kidney has been regarded as an organ incapable of regeneration, since no new nephrons appear after 36 weeks of

Regenerative Nephrology DOI: 10.1016/B978-0-12-380928-5.10015-6

gestation in humans as a result of the exhaustion of progenitor mesenchyme [7,8]. This does not rule out the existence of a renal stem cell able to elicit repair through replacement of postnatal renal cell types as opposed to formation of new nephrons [7–9]. Indeed, the mammalian kidney shares with the majority of organs the ability to repopulate and at least partially repair structures that have sustained some degree of injury [7–9]. Tubular integrity can be rescued after acute damage, and even severe glomerular disorders sometimes may undergo regression and remission, suggesting that glomerular injury is also reparable [10,11]. For all these reasons, the existence of renal stem cells has been a matter of long debate.

Attempts to identify adult kidney stem cells were made on the basis of the broad principles of stem cell biology, such as prolonged cell-cycling time (label-retaining cells), ability to extrude Hoechst dye (side population cells), by restrictive cell culture conditions, or by using markers expressed by other stem cells or developing kidney [12–14]. Some of these studies suggested the existence of putative interstitial renal stem cells [12–14]. In particular, Oliver et al. [13] identified a population of BrdU-label-retaining cells within the interstitium of renal papilla in the rat kidney. However, as already reported, the use of BrdU labeling does not seem to be a specific method for identification of stem cells [4,5]. In addition, this hypothesis has recently

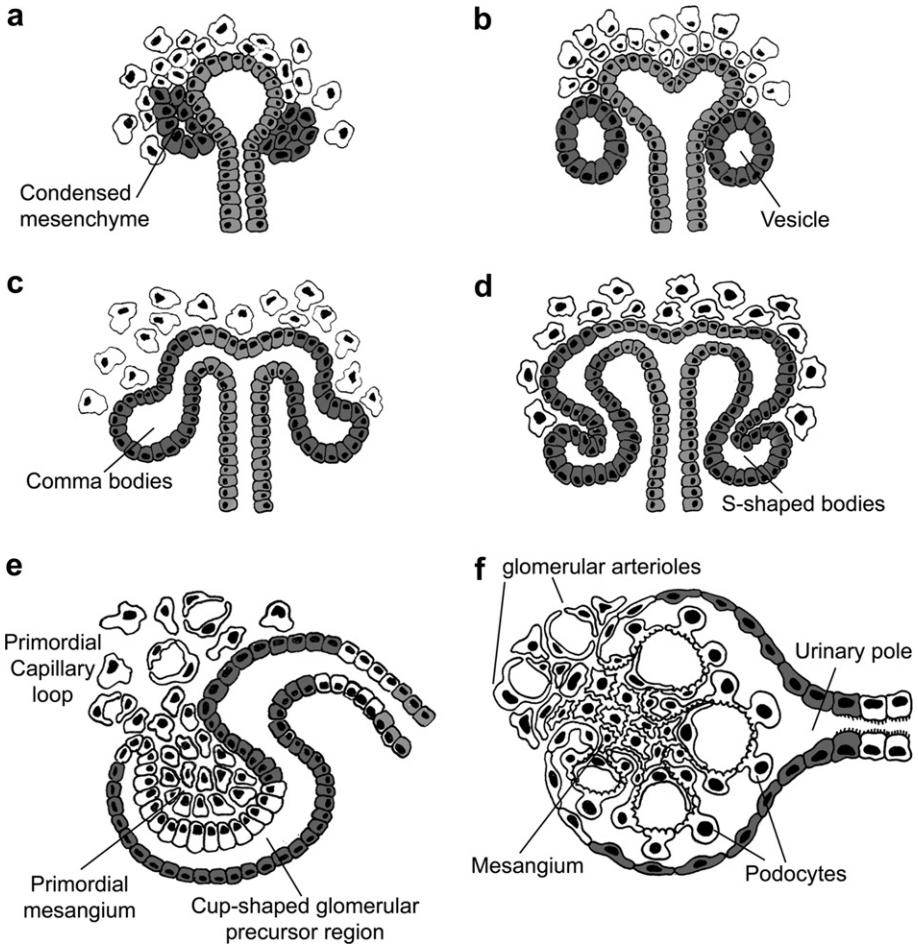

FIGURE 15.1 Series of schematics depicting the morphological events and the localization of $CD24^{+}CD133^{+}$ renal stem cells during the different phases of nephron development. Mesenchymal cells near the tips of the branching ureteric bud are induced and differentiate through a series of forms: (a) aggregate, (b) renal vesicle, (c) comma shaped bodies, and (d) S shaped bodies. Also shown is (e) the developing vasculature within the glomerular cleft of an S shaped body and (f) the glomerulus in a more mature nephron. Cells of the ureteric bud are stained in green (light gray in the figure). $CD24^{+}CD133^{+}$ renal stem cells are stained in red (dark gray in the figure). (a) $CD24^{+}CD133^{+}$ renal stem cells localize in the condensed mesenchyme but not in the uninduced mesenchyme or in the ureteric bud. (b) $CD24^{+}CD133^{+}$ renal progenitors localize in a primary vesicle but not in the uninduced mesenchyme or in the ureteric bud. (c) $CD24^{+}CD133^{+}$ renal progenitors localize in the comma bodies but not in the uninduced mesenchyme or in the ureteric bud. (d) $CD24^{+}CD133^{+}$ renal stem cells localize in the S shaped body, in the proximal loop, as well as in the distal loop, but not in the uninduced mesenchyme or in the ureteric bud. (e) $CD24^{+}CD133^{+}$ renal stem cells localize in an S shaped body after colonization by primordial capillaries and mesangium. (f) In maturing glomeruli, $CD24^{+}CD133^{+}$ renal stem cells selectively persist as a subset of cells of Bowman's capsule localized opposite to the vascular pole. *[From Romagnani, 2009 [9]. © AlphaMed Press and Wiley Blackwell.]*

been questioned by a study by Humphreys et al., who have developed a method for distinguishing the source of kidney tubular regeneration based on studies in transgenic rodents for the homeodomain transcriptional regulator Six2 [15]. In this model, the Six2 promoter drives a fusion protein of green fluorescent protein (GFP) and Cre recombinase, which is expressed transiently in renal epithelial precursors during the developmental period of active nephrogenesis [15]. GFPCre expression is not present in the adult, and this expression is not observed after injury. When Six2-GFPCre mice are crossed with a floxed STOP reporter strain, Cre-dependent removal of the stop sequence in progeny leads to constitutive and heritable expression of a marker gene so that all mesenchyme-derived renal epithelial cells, from Bowman's capsule to the junction of the connecting segment and collecting duct, are heritably labeled [15]. In contrast, the entire interstitial compartment is unlabeled [15]. Thus, the maintenance of labeled tubules postinjury supports a model of epithelial tubule repair due to surviving tubular epithelial cells or stem/progenitor cells localized within the labeled nephron. In addition, in an update of their previous work, Oliver et al. [16] demonstrated that following ischemic injury, label-retaining cells of the papilla migrate in the interstitium and to a lesser extent in collecting ducts, but only occasionally within the cortex. Finally, more recent results suggest that label-retaining cells of the papilla represent a population of cells that exits the cell cycle during embryonic development because it differentiates early [17]. Taken together, these findings indicate that papillary interstitial cells or other types of interstitial stem/progenitor cells do not directly contribute to regeneration of glomerular or tubular epithelial cells, while their contribution to collecting ducts or interstitial cells regeneration remains to be established [12 17]. These observations also indicate that repair of injured renal epithelium is predominantly accomplished by intrinsic, surviving tubular epithelial cells or a subset of stem/progenitor cells localized within the nephron [16,17].

However, converging data definitively demonstrated the existence of a population of stem/progenitor cells within the Bowman's capsule of adult human kidney [9,18,19]. These renal stem cells were identified through the assessment of the presence of both CD24, a surface molecule that has been used to identify different types of human stem cells [20], and CD133, a marker of several types of adult tissue stem cells [21]. The results showed that both markers were coexpressed by a subset of parietal epithelial cells selectively localized at the urinary pole of Bowman's capsule [18]. Once isolated, $CD24^{+}CD133^{+}$ renal stem cells were found to lack lineage-specific markers, to express transcription factors that are characteristic of multipotent stem cells, and to exhibit self-renewal, high clonogenic efficiency and multidifferentiation potential [18]. When injected intravenously in severe combined immunodeficiency (SCID) mice that had acute kidney injury, $CD24^{+}CD133^{+}$ renal stem cells regenerated tubular structures from different portions of the nephron and also reduced the morphological and functional kidney damage [18]. The identification of $CD24^{+}CD133^{+}$ renal stem cells has been confirmed by results obtained in transgenic mice, which suggest that endogenous cells of the nephron are responsible for repair of injured tubular epithelium [15], and leads to the hypothesis that, from the urinary pole of Bowman's capsule, $CD24^{+}CD133^{+}$ renal stem cells may initiate the replacement and regeneration of glomerular, as well as tubular epithelial cells in adult human kidney [18] (Fig. 15.1).

ONTOGENESIS OF THE RENAL STEM CELL NICHE

Regenerative biology draws on the understanding of normal developmental processes. It is generally believed that adult stem/progenitor cells directly derive from the organ-specific embryonic progenitor that is involved in organogenesis during fetal life [22,23]. Nephrons, the basic functional units of the kidney, are generated repetitively during kidney organogenesis from a mesenchymal stem cell population (Fig. 15.1). Development of the mature mammalian kidney results from reciprocal signaling between the branching ureteric bud tips and the undifferentiated metanephric mesenchyme, which leads to the aggregation and condensation of renal epithelial stem cells to form the renal vesicle, which then undergoes transformation in S-shaped body [7]. At this stage, the proximal end of S-shaped body becomes invaded by blood vessels, differentiates into podocytes and parietal epithelial cells, and then generates glomeruli. Simultaneously, the middle and the distal segments of the S-shaped body begin to express proteins that are characteristic of tubular epithelia [7].

The existence of renal stem cells in the metanephric mesenchyme is supported by the observation that some metanephric mesenchyme-derived cells display multidifferentiation potential [7,24,25]. In embryonic kidneys, coexpression of CD133 and CD24 characterized a subset of cells in the cap mesenchyme, renal vesicles and S-shaped bodies that displayed self-renewal and multidifferentiation potential (Fig. 15.1). Thus, to track down embryonic renal stem cells, the expression of CD133 and CD24 during kidney development was evaluated. During nephron development, coexpression of CD133 and CD24 remained selectively localized to cells of the urinary pole of Bowman's capsule [26]. When injected into mice with acute renal failure, $CD24^{+}CD133^{+}$ renal

embryonic stem cells regenerated cells of different portions of the nephron, reduced tissue necrosis and fibrosis, and significantly improved renal function [26]. In agreement with their nature, $CD24^+CD133^+$ renal embryonic stem cells also expressed high levels of the stem cell-related transcription factor BmI-1, which is a critical regulator of self-renewal in different types of stem cells [27,28]. The existence of a putative metanephric mesenchyme cell with stem cell properties had already been suggested by studies performed by the group of Reisner, who demonstrated that functioning renal tissue can be reconstituted by metanephric mesenchyme derived from kidneys of 8 weeks of gestation [29]. Indeed, fetal kidney tissue obtained from 10 to 14 weeks of gestation maintained the property to generate de novo functional nephrons, but generated a smaller number of mature glomeruli and tubuli than kidneys of 8 weeks of gestation [29]. Accordingly, $CD24^+CD133^+$ renal embryonic stem cells are enriched in the kidneys of 8 9 weeks of gestation, substantially decrease during 10 14 weeks of gestation, and represent $< 2\%$ of whole renal cells in adults. In both fetal and adult kidney, $CD24^+CD133^+$ stem cells persist as parietal epithelial cells localized at the urinary pole of Bowman's capsule, supporting the concept that $CD24^+CD133^+$ renal embryonic stem cells directly derive from renal embryonic stem cells and the urinary pole of Bowman's capsule represents a stem cell niche, which is a specific site in adult tissues where stem cells reside [29,30]. In agreement with this hypothesis, blastocyst-derived mouse embryonic stem cells once differentiated towards renal tubular cells selectively migrated to the tubuloglomerular junction following their injection into developing kidneys [31].

More recently, studies performed in transgenic rodents for the homeodomain transcriptional regulator Six2 have confirmed the existence in the cap mesenchyme of a multipotent nephron progenitor population [32]. Indeed, *Six2*-expressing cells give rise to all cell types of the main body of the nephron during all stages of nephrogenesis [32]. Pulse labeling of *Six2*-expressing nephron progenitors at the onset of kidney development suggests that the *Six2*-expressing population is maintained by self-renewal and is multipotent, generating the multiple domains of the whole cortical nephron [32]. Notably, podocytes, proximal and distal tubule structures were all descendants of a $Six2^+$ cell, further confirming that a single multipotent progenitor is the source of both the glomerular and tubular epithelial cells that constitute the adult nephron [32].

THE ADULT RENAL STEM CELL NICHE

Stem cells are functionally defined by their ability to self-renew and to differentiate into the cell lineages of their tissue of origin [1,2]. The ability to self-renew is maintained through a process called symmetric division, which consists of the generation of another cell which maintains the functional and phenotypic properties of a stem cell [1,2]. However, once activated, epithelial stem cells can generate a proliferating progeny, which is often referred to as transiently amplifying cells or committed progenitors, through a process called asymmetric division [1,2]. In their normal environment, committed progenitors retain the capacity to divide and then differentiate along a particular cell lineage to make the tissue. In the kidney, $CD24^+CD133^+$ stem/progenitor cells are localized at the urinary pole of Bowman's capsule and exhibit self-renewal properties as well as the potential to differentiate in tubular cells or podocytes [18,19]. Additional studies recently performed in adult mice have confirmed that parietal epithelial cells of the glomeruli represent a population of stem/progenitor cells with renal regenerative capacity [33]. Of note, in vitro induction of an epithelial mesenchymal transition induced the regression of adult renal progenitors of Bowman's capsule to the phenotype of the embryonic renal progenitor [33]. Accordingly, following their injection under contralateral kidney capsule of unilaterally nephrectomized mice, these renal progenitors generated novel renal tissue including neoglomerular and tubular structures at 3 weeks postengraftment [33].

Thus, the discovery of a population of renal stem cells localized at the urinary pole of Bowman's capsule suggests its possible involvement in regeneration of adult tubular, as well as of glomerular, cells. However, the role of these renal stem cells in tubular and glomerular injury may be different. Indeed, following acute tubular injury, the kidney undergoes a regenerative response leading in most cases to recovery of renal function [34]. The cell source for regenerating cells is poorly understood but several studies suggest that the tubular epithelium can be self-renewing after acute kidney injury and that differentiated tubular cells proliferate and migrate to replace the neighboring dead cells [34 36] (Fig. 15.2). Indeed, the tubular epithelium is resting in G1 rather than G0 and, as such, is primed to enter the cell cycle if injured [35,36] (Fig. 15.2). Accordingly, in most adult epithelial tissues, proliferation and migration of epithelial cells ensure replacement of adjacent injured cells, as previously reported for the skin [1,38]. However, during chronic injury or in cases of extended damage, resident stem cell compartments are also activated and critically participate in regeneration [1,38].

The discovery of $CD24^+CD133^+$ renal stem cells and their localization at the urinary pole of Bowman's capsule suggests that these cells may participate in tubular regeneration, progressively migrating and differentiating in

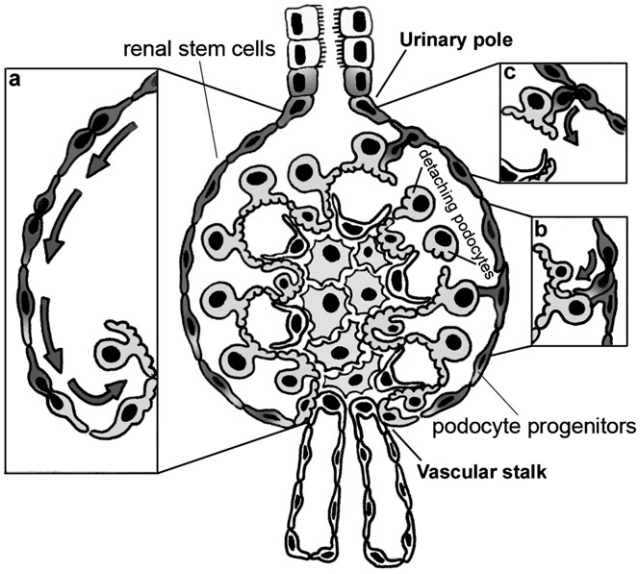

FIGURE 15.2 Renal stem cells regenerate podocytes. Renal stem cells are localized at the urinary pole. A transitional cell population (podocyte progenitors) displays features of either glomerular epithelial stem cells or podocytes and localizes between the urinary pole and the vascular stalk. Cells that express only podocyte markers and the phenotypic features of differentiated podocytes localize at the vascular stalk of the glomerulus or within the glomerular tuft. Proposed mechanisms of podocyte regeneration are depicted in more detail in (a–c). (a) Glomerular epithelial stem cells can generate novel podocytes by progressively migrating, proliferating and differentiating towards the vascular stalk. This occurs as the kidney grows, during childhood and adolescence, and may also occur following an injury which allows a slow generation of novel podocytes, such as following uninephrectomy. (b, c) In glomerular disorders characterized by severe podocyte death/detachment, glomerular epithelial stem cells generate cell bridges between Bowman's capsule and the glomerular tuft, which may allow a quick replacement of lost podocytes. (b) Bridging parietal epithelial cells may acquire podocyte markers following injury, and directly replace the lost podocytes. The directions of migration, proliferation and differentiation of glomerular epithelial stem cells to regenerate lost podocytes are indicated by the arrows. (c) Cell bridges may provide a slide for the migration, proliferation and differentiation of an adjacent progenitor and a quick replacement of lost podocytes. *[From Lasagni and Romagnani, 2010 [37]. © American Society of Nephrology.]*

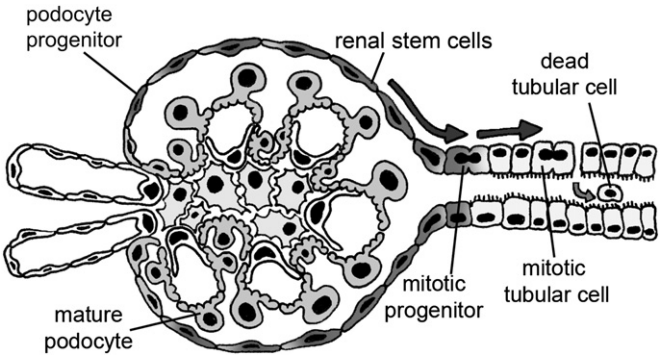

FIGURE 15.3 Proposed mechanisms of tubular cell regeneration. Renal stem cells are localized at the urinary pole and are in close contiguity with tubular renal cells at the tubuloglomerular junction. A transitional cell population displays mixed features of renal progenitors and proximal tubular cells and localizes at the tubuloglomerular junction. When tubular cells are injured, the adjacent differentiated tubular cells divide to replace the lost cells. Repeated cycles of proliferation can exhaust the regenerating capacity of tubular epithelial cells. However, renal progenitors can maintain the bulk of proliferating cells through generation of novel tubular progenitors at the tubuloglomerular junction. The directions of differentiation and proliferation are indicated by the arrows (dark gray). The light gray arrow points to the dead tubular cell. *[From Lazzeri et al., 2010 [39]. © Lippincott Williams & Wilkins.]*

proximal tubular cells at the tubuloglomerular junction (Fig. 15.3). Several previous studies demonstrated that the proximal tubule arises at a variety of angles from Bowman's capsule and that the tubuloglomerular junction has an area of intermediate appearance, with prominent microvilli on parietal cells in humans, mammals and fish [40,41] (Fig. 15.3). These findings suggest that parietal epithelium may be able to change to tubular tissue and that this may particularly occur as the kidney grows [40,41]. Accordingly, $CD24^{+}CD133^{+}$ renal stem cells isolated from adult human kidneys generated novel tubular structures and reduced the morphological and functional kidney damage once injected in mice affected by acute renal failure, suggesting that these cells can participate in tubular regeneration in adult human kidneys [17,18]. Thus, renal stem/progenitor cells may contribute to tubular epithelium repair by generating novel tubular-committed progenitors when proliferation of neighboring unwounded tubular cells is exhausted, as may occur during chronic tubular injury (Fig. 15.3). In addition, transdifferentiation of parietal epithelial cells of Bowman's capsule in cells with a proximal tubule phenotype has been reported during many types of chronic tubular injury, in association with interstitial fibrosis and tubular atrophy, or during aging [42–47]. Taken together, these results suggest that injured tubular cells are mostly regenerated through proliferation of neighboring differentiated tubular cells, but that renal progenitors may participate in tubular regeneration during severe or chronic tubular injury. However, further studies are needed to verify this possibility.

By contrast, evidence is already available to demonstrate that renal stem cells are essential for glomerular regeneration. The glomerular tuft comprises three resident cell types: mesangial cells, endothelial cells and podocytes [48]. Some studies have shown that primary injury to each of these cell types may be associated with glomerular disease [48]. However, injury to endothelial and mesangial cells can be repaired by proliferation of adjacent cells [48]. In addition, several lines of evidence suggest that bone marrow-derived stem cells physiologically contribute to mesangial and endothelial cell regeneration [49–52]. By contrast, podocytes cannot divide, and podocyte depletion is a common

determining factor that can result in glomerulosclerosis [53,54]. Recently, the first evidence was provided that podocytes can be regenerated by the $CD24^{+}CD133^{+}$ renal stem cells of Bowman's capsule [55]. In addition, it was demonstrated that $CD24^{+}CD133^{+}$ renal stem cells are arranged in a precise sequence within Bowman's capsule of adult human kidneys [55] (Fig. 15.2). A subset of more undifferentiated cells expressing renal progenitor markers in the absence of podocyte markers is localized at the urinary pole of Bowman's capsule and acts as a bipotent progenitor for both tubular cells and podocytes, and exhibits self-renewal potential [55] (Fig. 15.2), as shown by clonal analyses demonstrating that this subset of parietal epithelial cells represents multipotent epithelial stem cells and not simply a mixture of unipotent progenitors [55]. This was accomplished by first culturing the progeny of cells derived from single $CD24^{+}CD133^{+}$ cells and then by their transplantation into SCID mice affected by focal segmental glomerulosclerosis (FSGS) [55]. A transition population expressing both renal stem cells and podocyte markers is localized between the urinary and the vascular pole of Bowman's capsule, exhibits a committed differentiative potential only towards the podocyte lineage and lacks self-renewal properties (Fig. 15.2) [55]. Finally, differentiated cells, which do not express renal stem cell markers, but display high levels of podocyte specific markers, localize at the vascular pole of Bowman's capsule (Fig. 15.2) [55].

These findings obtained in human kidneys were confirmed in a parallel study performed in murine kidney by Appel et al. [56], who also demonstrated that transitional cells with morphological and immunohistochemical features of both parietal epithelial cells and podocytes could be detected in proximity of the glomerular vascular stalk. More importantly, using genetic tagging of parietal epithelial cells in rodents, the same authors unequivocally demonstrated that podocytes are recruited from parietal epithelial cells, which proliferate and differentiate from the urinary to the vascular stalk, then generating novel podocytes (Fig. 15.2) [56]. This occurs as the kidney grows, during childhood and adolescence [56], and may also take place following an injury which allows a slow, regulated generation of novel podocytes, such as uninephrectomy.

The discovery that renal stem cells of Bowman's capsule represent a potential source for podocyte generation provides the basis for a novel concept that injured podocytes can be replaced. However, in glomerular disorders characterized by acute or severe podocyte loss, regeneration requires other pathways that should allow faster replacement of injured podocytes. Indeed, the possibility that parietal epithelial cells can also migrate from Bowman's capsule to the capillary tuft in different areas than the vascular pole was previously suggested by some studies, which proposed that areas of adhesion as well as bridges between Bowman's capsule and the glomerular tuft may represent such sites of migration (Fig. 15.2) [57,58]. A recent study using genetic tagging of parietal epithelial cells demonstrated that bridges between Bowman's capsule and the glomerular tuft in experimental models of glomerular disorders are exclusively generated by parietal epithelial cells [59]. Taken together, the results of these studies suggest that, following podocyte injury, parietal epithelial cells generate cell bridges between Bowman's capsule and the glomerular tuft. This may provide a slide for the migration and differentiation of an adjacent progenitor and a quick replacement of lost podocytes (Fig. 15.2), or alternatively bridging parietal epithelial cells may acquire podocyte markers following injury, as recently suggested [60], and directly replace the lost podocytes (Fig. 15.2). In summary, a large body of evidence indicates that Bowman's capsule of adult kidneys contains a population of renal stem cells that can replace lost podocytes through multiple mechanisms and allow glomerular regeneration.

However, as in other adult organs, a response to injury can shift the balance from regeneration to repair depending on a complex interaction between the stem cell compartment and the surrounding environment [2,38,61]. In normal conditions, the response of stem cell to injury is strictly regulated. As a consequence of resident stem cell depletion and/or uncontrolled growth, healing problems can manifest in every injured tissue and induce previously functional tissue to become a patch of cells and disorganized extracellular matrix that is referred to as a scar [2,38,61]. Therefore, at least in proteinuric glomerular disorders, glomerulosclerosis could be envisioned like a scar induced by insufficient or exaggerated responses of renal stem cells to podocyte injury. Taken together, these results support the concept that the outcome of glomerular disorders may depend on a strictly regulated balance between the degree of podocyte injury and the amount of regeneration provided by renal stem cells.

THE RENAL NICHE IN AGING

Several studies suggest that, as already reported for other types of adult tissue stem cells [62,63], the regenerative capacity of renal stem cells may be limited [39,62 64]. Previous observations suggest that repair of podocyte injury is possible when less than 20% of podocytes are lost, while 20 40% podocyte loss results in a scarring response, until at greater than 60% podocyte loss glomerulosclerosis cannot be avoided. These results suggest that the amount of podocyte injury greatly influences the possibility of regeneration provided by renal

stem cells, and that glomerulosclerosis may occur in those glomerular disorders where the amount of podocyte injury exceeds the possibility of regeneration (Fig. 15.4). In addition, renal stem cells display a different regenerative potential in distinct ages of life [56], and exhibit the highest regenerative potential until adolescence [56], which explains why glomerular disorders have a better prognosis during childhood than in adult life, while FSGS is more frequent in older stages of life (Fig. 15.4). This also provides a further possible explanation for the progressive increase in prevalence of glomerulosclerosis with aging, which may be related to an exhaustion of the self-renewal potential of renal stem cells. Indeed, a reduced self-renewal potential with aging has been described in other adult stem cells [62,63,65 70].

Accumulated DNA damage and loss of DNA repair has been suggested to be one of the mechanisms underlying age-dependent stem cell decline [62]. However, the most important modulator of the stem cells' regenerative potential is represented by the surrounding environment [65 67]. It is well known that after birth the stem cell niche regulates the balance between self-renewal and differentiation by maintaining a specialized microenvironment [65 69]. Previous studies demonstrated that factors present in the young niche environment could restore the proliferative and regenerative capacity of aged stem cells in the niches of adult tissues [67]. Accordingly, very recent data suggest that the regenerative potential of renal stem cells can be enhanced or inhibited through the use of different culture conditions [33]. More importantly, injection of renal stem cells under the contralateral kidney capsule of unilaterally nephrectomized mice generated novel renal tissue including neoglomerular and tubular structures, a finding that was not observed following injection under the capsule of normal kidneys [33]. This suggests that the regenerative potential of renal stem cells is strictly dependent on the surrounding environment and that the process of kidney growth generates favorable conditions for regeneration [33]. Although a recent study has reported a detailed characterization of renal stem cells [70], little information is available about which cells support their growth and differentiation and which factors maintain their function and number. Therefore, additional studies are required to clarify this relationship and how it changes during progressive glomerulosclerosis or during aging.

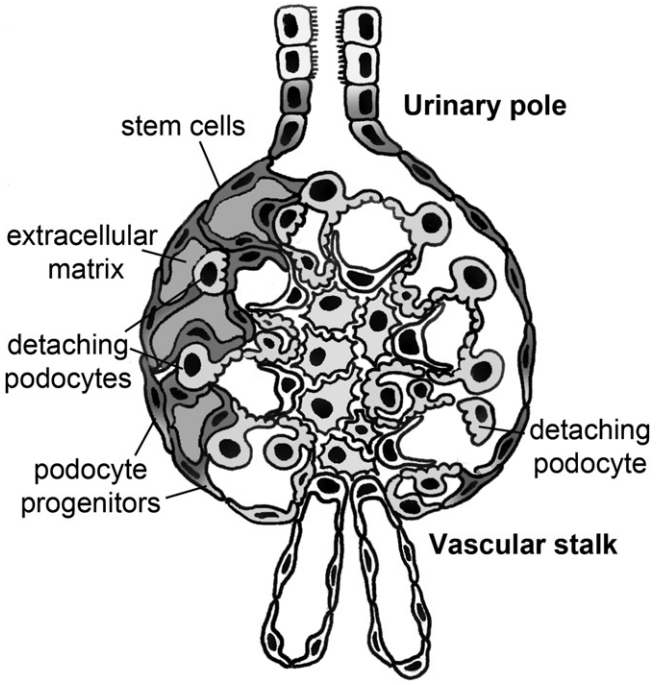

FIGURE 15.4 Limits and defaults of glomerular epithelial stem cells' regenerative potential. Age, genetic alterations and environ mental factors can limit the regenerative response of glomerular epithelial stem cells, thus impairing podocyte replacement when the amount of injury is extended. If regeneration is impaired, podocyte loss requires filling of the injury by extracellular matrix, which can be produced by glomerular epithelial stem cells when exposed to TGF β, which is highly secreted by podocytes exposed to proteinuria. *[From Lasagni and Romagnani, 2010 [37]. © American Society of Nephrology.]*

THE RENAL NICHE IN GLOMERULAR DISORDERS

It has been widely recognized that a disruption in the strictly regulated balance of stem cell self-renewal and differentiation not only impairs regenerative mechanisms but can even generate disorders [71 73]. For example, myeloproliferative disease arises as a result of aberrant proliferation of hematopoietic stem cells [71,72], while in aplastic anemia hematopoietic stem cells are reduced in number and there is fatty replacement in bone marrow, resulting in pancytopenia [73]. These stem cell-related disorders are generated by intrinsic genetic alterations and/or by alterations in the surrounding environment [71 73].

In the glomerulus, the response to podocyte injury may cause aberrant epithelial cell proliferation, hypercellular lesions formation and Bowman's space obliteration, as seen in collapsing glomerulopathy and in crescentic glomerulonephritis [74 78]. Until now, theories explaining the origin of aberrant epithelial cells in collapsing glomerulopathy and crescentic glomerulonephritis have been controversial [74 84]. One possibility is that these cells are exclusively of parietal epithelial origin [74 79], while another is that some dedifferentiated podocytes acquire markers of parietal epithelial cells [80 84].

Following the identification within Bowman's capsule of a population of renal stem cells that can

generate novel podocytes, the authors explored the possibility that hyperplastic epithelial cells in crescentic glomerulonephritis and collapsing glomerulopathy may result from an aberrant proliferative response of renal stem cells to podocyte injury. This would easily explain the presence in these lesions of cells with an intermediate phenotype between parietal epithelial cells and podocytes [74 84]. Accordingly, it was recently demonstrated that the majority of cells present in the hyperplastic lesions of patients with collapsing glomerulopathy or crescentic glomerulonephritis exhibits the renal stem cell markers CD133 and CD24, with or without coexpression of podocyte markers [85]. Therefore, it is suggested that the glomerular hyperplastic lesions are generated by renal stem cells of Bowman's capsule at different stages of their differentiation towards mature podocytes (Fig. 15.5A) [85].

Support for this hypothesis came from lineage tracing experiments performed in transgenic mice with genetically labeled parietal epithelial cells in the nephrotoxic nephritis model of inflammatory crescentic glomerulonephritis, as well as in the Thy-1.1 transgenic mouse model of collapsing glomerulopathy [85]. In both models, genetically labeled parietal epithelial cells constituted the majority of cells that composed early extracapillary proliferative lesions and almost the totality of proliferating cells. Le Hir et al. [86] suggested that the development of the crescent is initiated by cell bridges that are formed between the tuft and Bowman's capsule [86]. Since lineage tracing experiments demonstrated that bridging between Bowman's capsule and the tuft is generated by parietal epithelial cells [59], it is suggested that following massive podocyte injury, renal stem cells generate cell bridges with the glomerular tuft in several areas of the glomerulus, providing a slide for a quick replacement of lost podocytes (Fig. 15.5A). However, numerous areas of podocyte injury and renal stem cell proliferation heavily distort glomerular structural integrity, thus altering the polarity of renal stem cell division. A polarized proliferation is a critical determinant of a correct stem cell differentiation [65 69], and this may explain why a disruption in renal stem cell polarity initiates their abnormal proliferation and the development of hyperplastic glomerular lesions, thus impairing repair (Fig. 15.5A). Both crescentic glomerulonephritis and collapsing glomerulopathy are characterized by the death of numerous podocytes during a short time interval and by an aberrant proliferation of renal stem cells, suggesting that they may not be pathogenically distinct but rather represent two faces of the same disorder [87,88].

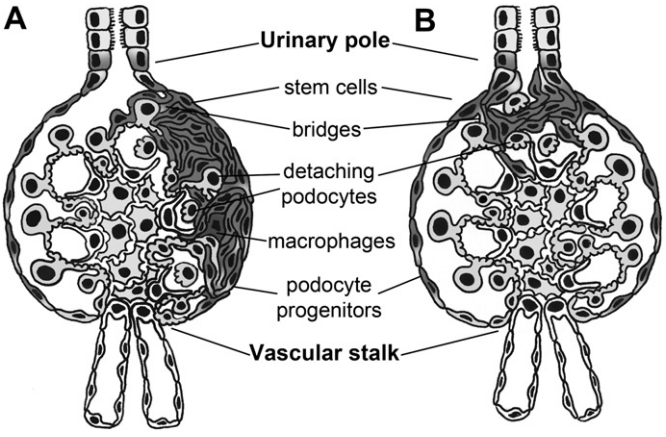

FIGURE 15.5 Dysregulated glomerular epithelial stem cells can generate disorders. Aberrant proliferation of glomerular epithelial stem cells can generate hyperplastic lesions. (A) Following massive podocyte injury, glomerular epithelial stem cells generate cell bridges with the glomerular tuft in several areas of the glomerulus to quickly replace lost podocytes. However, numerous areas of podocyte injury distort glomerular structural integrity, thus altering the polarity of glomerular epithelial stem cells' division and initiating their abnormal proliferation and the development of extracapillary hyperplastic lesions, as well as crescents. Macrophages can also be included within the lesions. Similar processes may occur in crescentic glomerulone phritis and collapsing glomerulopathy. (B) Replacement of podocytes in physiological conditions follows a gradient, with novel podocytes that are progressively added at the vascular stalk. Thus, the tip podocytes represent the "oldest" podocytes of the glomerular tuft, which suggests that they may be more susceptible to injury related to heavy proteinuria. Glomerular epithelial stem cells may proliferate and migrate from the urinary pole of Bowman's capsule towards the tuft in an attempt to replace the podocytes that died in response to heavy proteinuria, and generate the tip lesion. *[From Lasagni and Romagnani, 2010 [37]. © American Society of Nephrology.]*

Although epithelial cell proliferation is most characteristic of and most prominent in crescentic glomerulonephritis and collapsing glomerulopathy, some epithelial cell proliferation is also observed in histopathological lesions typically found in other podocytopathies, such as the tip lesion and FSGS [77]. It was recently demonstrated that also in the tip lesion and in FSGS characterized by mild levels of hyperplasia, renal stem cells are the main constituents of the proliferative lesions [85], which raises the question of how distinct etiopathogenetic factors can initiate abnormal regenerative processes. It has been previously demonstrated that FSGS is induced as a consequence of at least 40 60% podocyte loss. However, in this glomerular disorder, in front of a massive podocyte injury, hyperplastic glomerular lesions generated by renal stem cells are usually mild [76]. This suggests that FSGS may be the consequence of an insufficient proliferation of glomerular epithelial stem cells, which impairs the correct replacement of injured podocytes and requires filling of the injury by extracellular matrix (Fig. 15.4) [85]. When placed in presence of transforming growth factor-β (TGF-β), which is highly secreted by podocytes exposed to proteinuria [88], renal stem cells can also produce and deposit high amounts of extracellular matrix [85]. In addition, the regenerative potential of

renal stem cells is reduced in aging patients, where FSGS is more frequent (Fig. 15.4) [76]. Other reasons underlying the different response of renal stem cells to a massive podocyte injury in FSGS in comparison with collapsing glomerulopathy or crescentic glomerulonephritis are currently unknown, but may be related to the type of injury and/or to the different genetic background of patients. Accordingly, a recent study demonstrated that podocyte damage leads to glomerular injury with a complete histological pattern of collapsing glomerulopathy related to high parietal epithelial cell proliferation in mice with knockout for the cell cycle inhibitor p21 [89], but to segmental lesions and mild intraglomerular proliferation in wild-type mice [89].

Finally, renal stem cells are also the main constituents of the tip lesion [85]. The tip lesion has been described in several proteinuric conditions, including podocytopathies, membranous nephropathy [90], postinfectious glomerulonephritis [91] and diabetic nephropathy [92]. Since replacement of podocytes in physiological conditions follows a gradient, with novel podocytes that are progressively added at the vascular stalk, the tip podocytes represent the "oldest" podocytes of the glomerular tuft. This should make the tip podocytes more susceptible to injury, thus suggesting that they may be the first to die in response to heavy proteinuria (Fig. 15.5B). On this basis, Haas and Yousefzadeh [93] have already argued that the tip lesion is a response to prolonged heavy proteinuria. Consistently, experimental evidence in vitro and in models of disease has documented that the exposure of podocytes to excessive amounts of plasma proteins promotes podocyte dysfunction and injury followed by tuft adhesion and sclerosis [89,94]. Thus, renal stem cells may proliferate and migrate from the urinary pole of Bowman's capsule towards the tuft in an attempt to replace the podocytes that died in response to heavy proteinuria, and generate the tip lesion (Fig. 15.5B).

CONCLUSION

Remission of disease and regression of renal lesions are widely observed in experimental animals and even in humans [10,57]. However, the repair of injured renal tissue in mammals is not sustained by the generation of new nephrons and frequently leads to a non-functioning mass of fibrotic tissue. A large body of evidence has recently shown that the parietal epithelium of Bowman's capsule represents a reservoir of renal progenitors in adult kidney deputed to replacement and regeneration of podocytes, as well as of proximal tubular cells [18,19,33,37,39,55,56,64,70,80,85,95]. This finding provides a new point of view for the understanding of renal physiology and pathophysiology. Indeed, the first main outcome of the discovery of renal stem cells is that they encourage regeneration and promote functional repair of glomerular injury, and even prevention and treatment of glomerulosclerosis may be possible. In addition, the discovery that renal progenitors of Bowman's capsule can not only regenerate podocytes but also cause hyperplastic glomerular lesions in crescentic glomerulonephritis, collapsing glomerulopathy, FSGS or the tip lesion, suggests that glomerular disorders may at least in part result from abnormal or inefficient regenerative responses to podocyte injury.

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CHAPTER

16

Bioartificial Stem Cell Niches: Engineering a Regenerative Microenvironment

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OUTLINE

INTRODUCTION

With the rapidly increasing demand for stem cells, the problems related to their storage and preservation, maintenance and expansion become ever more acute. The requirements for stem cell banking would place the need for preserving quiescence and stemness at the top of the list, whereas the requirements for transplantation of stem and progenitor cells would give preference to the possibility of increasing the mass of stem cells without losing their properties. Inevitably, the constellation of different requirements should orbit around the physiological ways of preserving stem cells, i.e. stem cell niches. The niches are defined as the umbrella microenvironment supporting cell attachment and quiescence by sheltering cells from proliferation and differentiation signals, enhancing cell survival, regulating stem cell division and renewal, and coordinating the population of resident stem cells to meet the actual requirements of an organ [1]. The way in which these

diverse functions are accomplished by the niches may vary from one type of stem cells and their niche to another, yet the general outline is preserved. It includes specific components of the extracellular matrix (ECM) that create a scaffold and a boundary to the niche, cell cell and cell matrix adhesion molecules, locally stored growth and/or quiescence factors, and a set of instructions for proliferation and/or migration that can be triggered on demand. The vicinity of vascular beds, often covered with sinusoidal endothelial cells, provides a portal for entry into the circulation.

DESIGN CRITERIA FOR CREATING A BIOARTIFICIAL NICHE

The design criteria for matrices for encapsulation of cells for cell therapy include not only technological criteria from chemistry, biology and engineering, but also real-world marketing, regulatory and financial constraints [2]. A biocompatible material suitable for a three-dimensional (3D) bioartificial niche should be easy to use, have user-determined composition and compliance, and permit seamless transitions from in vitro to preclinical to clinical use. Replacing the complex native ECM environment with a minimalist semi-synthetic ECM allows cells to remodel the provisional matrix into a tissue-specific architecture [3]. These semi-synthetic extracellular matrices (sECMs) are "living" biopolymers based on chemically modified hyaluronan (HA), a ubiquitous ECM glycosaminoglycan that is evolutionarily conserved from the simplest prokaryotes to complex tissues of eukaryotes. The sECMs allow inclusion of the appropriate biological cues needed to simulate the complexity of the ECM of a given tissue and offer a manufacturable, highly reproducible, flexible, FDA-approvable and affordable vehicle for cell expansion and differentiation in 3D.

For a biomaterial to create a bioartificial niche, it must recapitulate the principal functions of the natural ECM in orchestrating cell proliferation, migration, differentiation, angiogenesis and invasion [4,5]. A bioartificial niche should orchestrate the integration of biochemical and mechanical cues in the cellular microenvironment [6,7] and allow versatility in engineering a regenerative microenvironment [8 10]. Specifically, the performance criteria should include: (i) experimental control of composition and compliance; (ii) control of bioresorption in vitro and in vivo; (iii) flexibility of physical form to allow for both cell encapsulation and biofabrication; (iv) batch-to-batch consistency; (v) ease of use at physiological temperature and pH; (vi) transparency for ease of visualization; and (vii) the ability to translate preclinical results into a clinical product. In addition, the pharmaceutical industry requires a bioartificial niche for drug evaluation purposes that is compatible with high-throughput screening (HTS) platforms [11]. One solution is a modular platform of sECMs that enables the user to add soluble factors, attachment peptides, matricellular proteins, cross-linkers and macromomers in the combinations required for a given cell type and microenvironment [12].

CHEMISTRY OF BIOARTIFICIAL NICHES

The cornerstone idea behind artificial stem cell niches investigated thus far is in the generation of scaffolds with the properties of the ECM [13]. In this regard, chemically engineered synthetic hydrogels (as opposed to natural substances like fibrin or collagen) have been extensively studied based on their mechanical and structural properties that emulate the ECM. The governing design principles include controlled degradation, local delivery of macromolecular regulators (growth factors, cytokines and adhesion molecules), and tolerance and integration into the native neighboring tissues.

A growing number of fibrous scaffolds and hydrogels is in development for the expansion of primary cells and progenitor cells in 3D [6,9,14,15]. A hydrogel is a cross-linked network (of either natural or synthetic polymers) that is hydrophilic and swells substantially in water. Hydrogels can be as much as 95% water by volume when fully swelled [16]. Hydrogels suffer from a lack of mechanical strength owing to the large amount of swelling observed in aqueous environments. Often, hydrogels are formed from a combination of different polymer chains where one serves to provide mechanical support for the gel, while the other adds favorable properties such as binding sites for cells or encapsulated biomolecules (such as growth factors or other proteins) [6]. Hydrogels are sometimes formed as interpenetrating networks where two different polymers are separately cross-linked in the same reaction vessel [16].

Among the naturally derived biomaterials is an in situ cross-linkable sECM that is based on chemically modified HA [3,17]. HA plays a critical role in morphogenesis [18], and chemically modified HA derivatives have generated considerable interest as biomaterials for tissue engineering [19,20]. As specified in the design criteria above, this versatile sECM offers user-controllable composition and compliance, to which a variety of growth factors or matricellular proteins may be added [12,21]. The elastic moduli of hydrogels can be tailored to match the cell type to achieve optimal regeneration of new tissue [22 24].

Among synthetic hydrogels, poly(ethylene glycol) diacrylate (PEGDA), alginate, poly(ε-caprolactone) (PCL), oligo(poly-ethylene glycol) fumarate (OPF), poly(*N*-isopropylacrylamide) (PNIPAAm), poly(hydroxyethyl methacrylate) [p(HEMA)] and others (Fig. 16.1), the

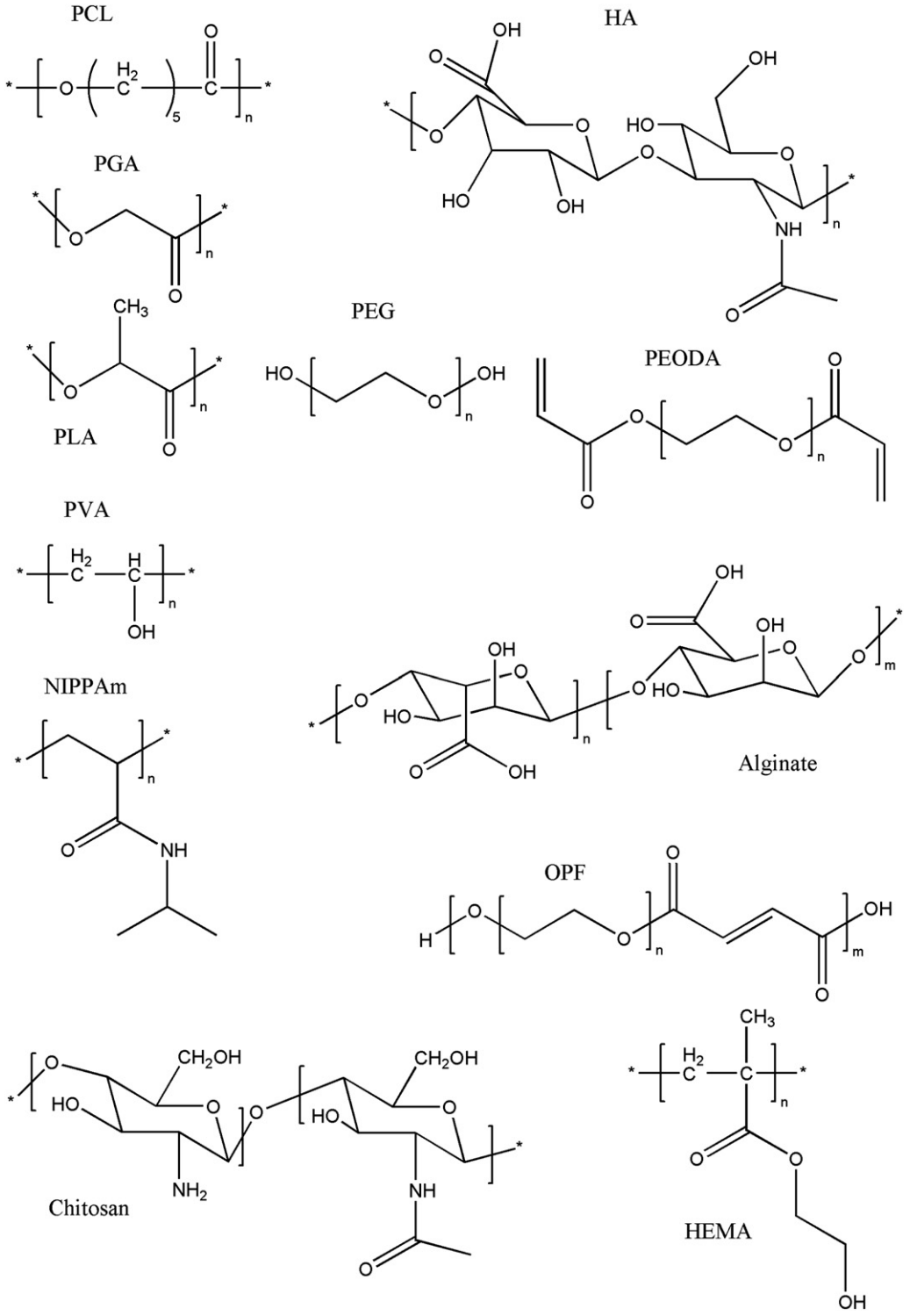

FIGURE 16.1 Chemical structures of some common hydrogel materials. PCL: poly(ε caprolactone); PGA: poly(glycolic acid); PLA: poly (lactic acid); PVA: poly(vinyl alcohol); NIPPAm: *N* isopropylacrylamide; HA: hyaluronic acid; PEG: poly(ethylene glycol); PEODA: poly (ethylene oxide) diacrylate, also called PEGDA: poly(ethylene glycol) diacrylate; OPF: oligo(poly ethylene glycol) fumarate; HEMA: hydrox yethyl methacrylate.

use of hyaluronic acid-based (HA) hydrogels has received significant attention. HA is a non-sulfated linear polysaccharide of (1-β-4) D-glucuronic acid and (1-β-3) *N*-acetyl-D-glucoasamine, and is a major constituent of the ECM during early embryogenesis, as well as in adult connective, epithelial and neuronal tissue [25]. HA hydrogels are capable of storing, propagating and differentiating stem cells [26]. Cross-linking HA hydrogels allows for the manipulation of their mechanical properties such as rigidity and elasticity [23], whereas the application of hyaluronidase permits rapid dissolution of the hydrogels. These cross-linked 3D scaffolds can be coated with various adhesion molecules and have been used to preserve pluripotent stem cells for 3 weeks [27]. Different purposes dictating different adhesion molecules and motifs, incorporation or coating of scaffolds with RGD, YIGSR or IKVAV peptides or their combinations have been reported [13]. Similarly, inductive growth factors such as transforming growth factor-β (TGF-β), hepatocyte growth factor (HGF), or basic fibroblast growth factor (bFGF) incorporation into hydrogels has been shown to improve regeneration of muscle and bone in vivo [28,29]. The size of pores, which is a function of the degree of cross-linking, affects stem cell behavior: pores of 30 60 μm allow for more rapid cell growth than pores of 60 130 μm [30]. The ability to incorporate stem cells, adhesion molecules and factors responsible for the maintenance and regulation of quiescence or proliferation of these cells provides this bioartificial stem cell carrier/niche with substantial flexibility to enable regulation of stem cell fate. Real-time control over the properties of scaffolds can be achieved by using switchable coatings sensitive to physical parameters such as temperature, light, pH and electromagnetic field [31] to regulate protein-surface and cell-surface interactions.

FABRICATION TECHNIQUES

Three techniques for the fabrication of hydrogels are typically used. These are chemical cross-linking, photo-cross-linking and sol-gel synthesis. In addition to these major methods, some hydrogels are formed through cryogelation, spinning, bioprinting, centrifugal casting or a microfabrication process.

Chemical Cross-linking

The most common form of gelling polymers to form hydrogels is through the addition of a bifunctional chemical compound that will react with functional groups on the polymer backbones to cross-link them. This usually involves reactions with carboxyl, amine or thiol groups that branch off the main chain of the polymer. Sometimes heat or agitation is required for the cross-linking to occur. Depending on the concentration of cross-linker and gel precursors in the solution the gelling can take anything from minutes to hours to complete [32]. Some systems require alternate forms of cross-linking, such as alginate, which is cross-linked by the addition of a divalent cation, most commonly Ca^{2+} [33 37]. Chemical cross-links are also most common because the chemicals are typically non-toxic, which is necessary for both in vitro and in vivo applications, and allow for a much greater control over the location of the linkages.

Photo-cross-linking

Photo-cross-linking is typically achieved by the addition of an ultraviolet (UV)-sensitive photoinitiator, although some initiators are also susceptible to visible light [32]. The initiator begins cross-linking the hydrogel through a radical polymerization of double bonds in the hydrogel precursors. This cross-linking technique requires the addition of double bonds to the gel backbone in order to function properly. The advantages of photo-cross-linking over chemical cross-linking are that the reaction usually completes on a shorter timescale, seconds to minutes, and tends to release almost no heat, whereas chemical cross-linking may require heating to proceed [32]. However, radical polymerization tends to be more difficult to control. As a more energetic reactive species, radicals can react at unpredictable places and do not necessarily target the same atom every time. This leads to a more random hydrogel that can have different properties at different locations throughout the gel.

Methacrylated HA (HA-MA) was recently employed in research on cutaneous and corneal wound healing [38], embryonic stem cell expansion [26], and drug and growth factor delivery [39]. In addition, photo-cross-linked HA-MA provided a 3D microenvironment suitable for mesenchymal stem cells (MSCs) to differentiate into a chondrogenic phenotype [40].

Sol-gel Synthesis

Sol-gel synthesis uses a simple phase transition to convert the hydrogel precursors to a stable gel form. This effect is usually pH [41 46] or temperature [42 47] dependent and can be very useful for drug delivery applications where an injected hydrogel solution mixed with a drug molecule transitions at or below body temperature to form a gel [46,47]. The drug molecule then diffuses slowly out of the gel in a time-controlled manner based on the degree of physical cross-linking. Cells can also be encapsulated in this manner, and remain viable [47].

Cryogelation

This synthesis technique, like sol-gel synthesis, also creates a physically cross-linked hydrogel. When the gel precursors are at room temperature, they form a solution, although a potentially viscous one. When frozen, the ice crystals that form tend to aggregate the polymer chains together. Upon thawing, the chains form noncovalent bonds resulting in a hydrogel [48 50]. This technique can be used to store molecules or even cells for extended periods. However, the polymers for which this synthesis technique is possible tend to be synthetic and thus do not contain many adhesive sites for cells. This problem is typically overcome by the addition of some other polymer such as collagen or chitosan that contains adhesion sites for the cells [48 50].

Spinning

Spinning is a technique where a hydrogel precursor solution is extruded through a needle to form microscaled or nanoscaled fibers. This is typically achieved using electrospinning without a cross-linking agent. In electrospinning, a large voltage is applied to the needle as the solution is pushed through with a syringe pump. As the charged solution exits the needle, a "taylor cone" is formed where the electrostatic repulsion is high enough that a very fine stream of solution erupts from the needle. During flight, the solvent dries out and the charge moves to the surface of the forming fiber. This causes the fiber to whip around owing to the electrostatic repulsion felt over small bends (Fig. 16.2D). The fiber is finally collected on a grounded plate [51 55], or for aligned fibers, separated collecting plates [53,56] or a spinning mandrel or disc [52,53,57 59]. In addition to electrospinning, hydrodynamic spinning is a potential fabrication technique [60]. In hydrodynamic spinning, a similar extrusion process is used; however, the addition of a cross-linker is required. The diameter of the fibers formed is determined by the flow rate through the apparatus, and hollow fibers can be formed from the combination of three concentric streams [60].

Bioprinting

The technique of bioprinting consists of two components. First, cell aggregates, cellularized sECM hydrogels or cell-seeded microspheres comprise the "bioink." The cell-free polymers that provide the substratum for the bioink are the "biopaper." Bioprinting consists of stepwise assembly of bioink and biopaper components into an organ-appropriate 3D architecture using a three-axis printer [61,62]. For example, vascular networks can be printed by a "scaffold-free" process involving automated deposition of sausage-like cell aggregates and agarose

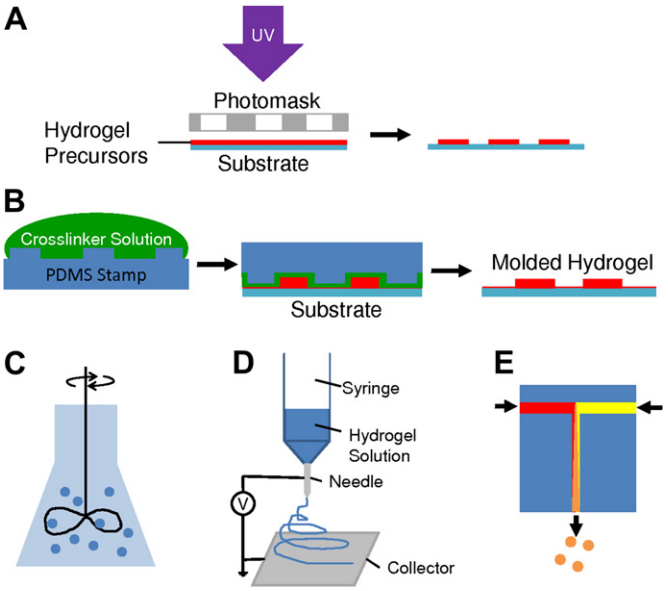

FIGURE 16.2 Schematic diagrams of various microfabrication techniques. (A) In photolithography, a thin layer of hydrogel precursor is applied to a substrate, typically glass, before being exposed to ultraviolet (UV) light through a photomask. This causes cross linking in regions exposed to UV light. The remaining hydrogel precursor solution can then be washed away. (B) In micromolding, the poly(dimethyl siloxane) (PDMS) stamp is "inked" with a chemical cross linker solution before being pressed into a thin layer of hydrogel precursor on a substrate. The PDMS stamp is removed after a short while, leaving behind a cross linked and molded hydrogel. (C) In emulsification, a rod agitates a solution of hydrogel precursors dis solved in an organic solvent that is mixed with an aqueous phase which contains a cross linker. This agitation results in small droplets of organic hydrogel precursor in the aqueous cross linker solution. The cross linker diffuses into the droplets which form small beads upon gelation. (D) An electrospinning set up shows the hydrogel solution being extruded through a syringe and collecting on a groun ded plate. The "taylor cone" forms at the opening of the needle as electric charges built up in the hydrogel force it into a thin stream which will dry to a final thickness of nanometers to micrometers. (E) Microfluidics shows two precursor solutions being pumped into a T junction which then mix to form cross linked hydrogel microspheres.

tubes [63]. In each case, computer-assisted design is used to guide the deposition of cells in geometries that recapitulate the structure of the target tissue or organ [64]. After printing, the engineered construct is allowed to mature and gain functionality in a bioreactor or in vivo environment [63,65].

Centrifugal Casting

Living polymers, such as the sECMs described below, offer the unique opportunity of forming a wide variety of tubular structures in the body, including potentially kidney nephrons, by centrifugal casting [66]. This technique was first validated by sandwiching a layer of fluorescently labeled endothelial cells between two layers of an in situ cross-linkable sECM by axial centrifugation

at approximately 11 × g [67]. After centrifugally coating the inside of a Dacron vascular prosthesis with the sECM, a cell suspension was applied and centrifugally cast as the gel cross-linked during centrifugation. The heavier cells were entrapped between the two layers, remained viable and remodeled the cross-linked HA gelatin sECM. Cells in the sECM suspension can also be applied to laser-machined micropores of a sheet of small intestinal submucosa (SIS), which affords a robust construct that retains the cell-seeded hydrogel and permits rapid biofabrication of a tubular tissue in a bioreactor-free fashion [68].

Microfabrication

Complex patterns can also be generated in hydrogels using various microfabrication techniques, typically by reshaping a preformed hydrogel. Microspheres of hydrogels can be formed through the use of emulsification polymerization [69]. Here a mechanical agitation of the hydrogel precursors in an organic phase causes microspheres to form which can then be gelled through a variety of cross-linking methods (Fig. 16.2C). Size can be controlled through the addition of surfactant molecules and the degree of agitation [69].

Photolithography is another useful technique for the fabrication of complex patterns [69]. A thin layer of hydrogel precursor is applied to a substrate. Then a photomask with the desired pattern is placed over the hydrogel and the entire assembly is exposed to UV light, which causes gelation of the pattern (Fig. 16.2A). The remaining hydrogel can then be washed away. Photolithography is capable of producing complex features on a submicrometer to millimeter scale. However, photolithography is essentially a 2D approach and multiple steps may be required to achieve a 3D structure.

Microfluidics can also be used to create hydrogels [69]. Here, small channels are formed in a polymer such as poly(dimethyl siloxane) (PDMS) by molding it from a master. The device is then sealed by placing the PDMS mold onto a piece of glass, or a stronger seal can be formed by air plasma treatment of the PDMS before inversion on glass [70]. Hydrogel precursors can be pumped in through multiple inlets and by controlling the flow rate and design of the channels, various morphologies can be generated (Fig. 16.2E). Typically, rods and spheres are formed but hybrid particles can also be created where each side has a different composition [69]. In addition, microfluidics can be combined with photolithography to capture cells in a particular location by cross-linking the surrounding hydrogel [69].

Finally, micromolding is a simple technique for the fabrication of hydrogels with micrometer-scale features [69]. Micromolding is based on a technique called soft lithography, where a PDMS stamp is molded from a prefabricated silicon wafer (Fig. 16.2B). Hydrogel precursors are molded with the PDMS stamp and subsequently gelled by the addition of a cross-linking agent through the mold, by irradiation for photopolymerization or by a sol-gel transition. Micromolding is a fast and efficient technique for generating 3D structures and can support features down to the nanoscale.

COMMON HYDROGEL MATERIALS

The proper materials selection is important when designing a hydrogel for a specific application. Depending on the use, a hydrogel needs to exhibit different properties in vivo. There are three general classes of biocompatibility for both natural and synthetic hydrogels. There are biomimetic hydrogels that emulate a naturally occurring component of the target implant site, biodegradable hydrogels that slowly dissolve in vivo to release drug molecules or cells, and bioinert hydrogels that have very little interaction with the biological tissue. Each type of interaction can be relevant for a particular application.

Biomimetic Hydrogels

These materials tend to have very similar properties to the implant site, whether they are natural or synthetic, and are usually able to integrate seamlessly with the actual tissue. Collagen/gelatin and HA are two natural compounds that are major components of the ECM. Other ECM proteins such as fibrin, fibronectin and laminin have also been used to fabricate hydrogels [6,71]. Synthetic hydrogels can be combined with other naturally occurring polymers to create biomimetic implants, such as those used for bone repair [6,16,72], which must be stronger than a natural material on its own.

Biodegradable Hydrogels

Most natural hydrogel materials are biodegradable by enzymes that are naturally produced by the body. Among synthetic hydrogels, those made from polymers of amino acids, such as poly(lactic acid) (PLA) and poly (glycolic acid) (PGA), are easily biodegradable. Others, such as PCL and OPF, are polyesters that can be slowly degraded through hydrolysis. Biodegradable hydrogels can be tuned to degrade at different rates by varying cross-linker concentration [6,73] and through the addition of other monomers to form block copolymers that may degrade at a faster or slower rate [6].

Bioinert Hydrogels

The last class of hydrogels is bioinert hydrogels. These elicit essentially no immune or inflammatory responses

when implanted. Poly(ethylene glycol) (PEG) and poly (vinyl alcohol) (PVA) are two examples of synthetic bioinert hydrogels (Fig. 16.1). PEG and PVA are very hydrophilic and offer very few attachment sites for cells. These materials can be used to help isolate encapsulated cells from an immune response [6] or serve as long-term repositories for drug delivery [6]. They are also very stable in water because the backbone of PVA is all carbons, and that of PEG is polyether. PEG is commonly used as a surface treatment for other implants because of its ability to be conjugated to other functional groups through terminal alcohol domains.

EXAMPLES OF HYDROGEL APPLICATIONS

Example of Bioprinting

The injection or implantation of masses of cells in vivo results in mostly homogeneous tissues that fail to recapitulate the complexities of mature, functional organs. Bioprinting may provide a solution, although many technological hurdles exist in order to print a functional organ. Paramount among these challenges are the need to re-create the complex cellular organization within the neotissues of an engineered organ, and the need to create a vascular network within the construct that can be functionally connected to the recipient [74].

In an effort to produce a biomaterial suitable for use in biopapers and bioinks, a methacrylated gelatin derivative (GE-MA) was combined with HA-MA to produce a biocompatible hydrogel [75]. Using a two-step photocross-linking strategy, an extrudable gel-like fluid was formed by partial cross-linking of the mixture. This GE-MA/HA-MA gel was then extruded from a syringe into a defined pattern of cell-free and fibroblast-loaded hydrogels. A second irradiation made the structure more rigid, and the printing irradiation sequence was iterated to build a tubular construct. Cells remodeled the printed ECM and secreted collagen.

Example of a Biomimetic Hydrogel

Native HA has several shortcomings for use in regenerative medicine, including its relatively short residence time in vivo, its poor mechanical strength, the inability of most cells to attach to HA and the inability to form stable structures. A large number of chemical modifications of HA [20,76] has been developed to make materials more "fabricatable" for regenerative and reparative medicine [19]. Fabricatable HA derivatives can be divided into two major categories [77]. In monolithic derivatives, the modification gives a chemical form that cannot form new chemical bonds in the presence of cells or tissues, and can only be altered by fabrication, e.g. spinning, weaving, printing or machining. In living derivatives, the modification permits the formation of new covalent bonds in vivo or ex vivo, enabling fabrication of a new physical form, often a hydrogel, during cell encapsulation or injection of a cell suspension.

The development of living HA derivatives was motivated by the need to create an in situ cross-linkable equivalent of the ECM. HA was selected as the basic building block for this covalently cross-linked sECM [3,78]. To create a modular, clinically versatile and readily manufactured sECM, a thiol-introduction chemistry [79] was developed based on the modification of the carboxylate groups of glycosaminoglycans (GAGs), proteins and other synthetic polymers by hydrazide formation. These mild aqueous conditions do not alter other chemical groups required for biological function of the GAG. Moreover, when the hydrazide reagent contains a disulfide bond [79,80], thiol-modified HA, sulfated GAGs and even proteins such as gelatin [81,82] could be prepared and cross-linked into hydrogels under cytocompatible physiological conditions. For example, thiol-modified macromonomers spontaneously, but slowly, cross-linked in air only to a hydrogel; this gel could be dried to give a thin film or lyophilized to produce a porous sponge [83]. Alternatively, cross-linking with difunctional electrophiles can be accomplished, in the presence or absence of cells, to give injectable and biocompatible hydrogels [78,81].

Different physical properties and rates of biodegradation can be obtained by controlling several parameters for the biocompatible HA hydrogels [84]: (i) molecular weight of starting HA employed; (ii) percentage 3, 3′-dithiobis (propanoic hydrazide) (DTPH) modification; (iii) concentration of HA-DTPH; (iv) molecular weight of PEGDA; and (v) ratio of thiols to acrylates. The authors have also investigated polyfunctional acrylates, acrylamides and additional thiol-reactive groups, e.g. PEG divinyl sulfone, PEG bis-haloacetates and PEG bis-haloamides [85,86].

The importance of clinically useful modular sECMs for reparative medicine and for drug delivery in three dimensions [17] was recently reviewed [2]. By incorporating attachment factors to an HA-based hydrogel sECM was produced that supported cell attachment, growth and proliferation in 3D [87]. This was achieved both with lyophilized macroporous sponges and with injectable materials [81]. The gelatin component could be replaced by either an RGD peptide [88] or selected domains of fibronectin [89]. As discussed herein, these materials have also been used for centrifugal casting of endothelial cells [67]. To enhance cell growth and simultaneously enhance the rate of neovascularization, an HA-based sECM containing co-cross-linked thiol-modified heparin (HP-DTPH) was found to give a half-life for bFGF release of over 1 month in vitro [90]. By varying

the GAG-DTPH composition, the presence or absence of gelatin [91], the release of vascular endothelial growth factor (VEGF), angiopoetin-1, platelet-derived growth factor (PDGF), TGF-β_1 and keratinocyte growth factor (KGF) could be modulated. Furthermore, dual release [92] of VEGF with KGF, PDGF, bFGF or angiopoetin in vivo led to enhanced vascularization and vascular maturation in vivo [11,93 95].

Growth factor delivery is also effective in preclinical models. The delivery of bFGF from a chondroitin sulfate film containing HP-DTPH to control release resulted in a highly significant improvement in healing of full-thickness cutaneous wounds in diabetic mice [96]. In addition, even particulate growth factor mixtures can be delivered in an sECM. In a rat femoral defect model, sECMs were used to deliver human demineralized bone matrix (DBM) to enhance bone repair [97]. While untreated controls showed little new bone formation after 8 weeks, the sECM sponge containing DBM showed a three-fold increase in new bone growth in the defect at 4 weeks postinjury.

Example of Cellular Delivery

An sECM hydrogel using a thiol-modified HA was also examined for repair of an osteochondral defect in a rabbit patellar groove [98]. In untreated control defects, extensive scar tissue accumulated after 12 weeks. However, simply filling the defect with the HA-gelatin based sECM hydrogel resulted in regeneration of both new osteal and chondral tissues. More robust remodeling was accomplished by the addition of autologous MSCs to the sECM hydrogel, suggesting that localized cell delivery and retention can add an important dimension to the regenerative process. Nonetheless, the fundamental role of the sECM is to enhance the natural biological repair processes mediated by endogenous cells.

RECRUITMENT AND DELIVERY OF STEM AND PROGENITOR CELLS TO AND FROM ARTIFICIAL NICHES

The ability to attract and home circulating stem cells is a potentially important feature of bioartificial niches that can be used to store in vivo autologous stem cells. An example of such usage has been described by Bakshi et al. [99], who implanted hydrogel scaffolds soaked in brain-derived neurotrophic factor in the injured spinal cord. The studies showed significant angiogenesis and axonal in-growth in these scaffolds. Implantation of 3D collagen scaffolds containing immobilized heparin and stromal cell-derived factor-α (SDF-1α) resulted in the accumulation of $CD11b^+$ and $CD11c^+$ myeloid cells and fibroblasts recruited early after implantation [100]. In studies of HA-hydrogel, the present authors used Sca-1/GFP^+ mice with implanted scaffold precoated with SDF-1 to detect its impregnation with GFP-expressing cells (Ratliff et al., unpublished observations). Similarly, when injected into the kidney pole, these scaffolds also became impregnated with stem cells. The described approach, namely mobilization of stem cells of interest and their capture, possibly even a selective capture, using variably treated hydrogels, may be an important adjunct to collection and storage of autologous stem cells.

Selective engagement of integrins may have important instructive consequences for stem cell differentiation. When MSCs are forced to express $\alpha_5\beta_1$-integrin through manipulations of the central cello-binding domains of fibronectin, osteogenic differentiation of cells was facilitated [101]. In the case of endothelial progenitor cells (EPCs), Sales et al. [102] compared precoating elastomeric poly(glycerol sebacate) scaffolds with laminin, fibronectin, fibrin, collagen types I/III or elastin and demonstrated that fibronectin coating promoted proliferation, reduced apoptosis and instructed EPCs to bias the endothelial over smooth muscle cell phenotype.

Scaffolds seeded with stem cells can be used to propagate certain functions to a host that lacks them in vivo. For instance, transplantation of scaffolds seeded with the embryonic islet-like cells to streptozotocin-diabetic mice [103] results in improved glycemic control.

Recruitment of endogenous progenitor cells is a powerful approach of creating biological function in a regenerative microenvironment. In a recent study of the restoration of brain tissue in a mouse model of stroke, Carmichael and co-workers explored the use of the sECM HyStem-HP, consisting of cross-linked HA and heparin, for delivery and retention of neural progenitor cells (NPCs) into the stroke cavity [104]. The experimental model used both brain-derived and embryonic stem cell-derived NPCs, and compared the survival of cells implanted in the sECM matrix with cells delivered in buffer only. Glial scar formation by activated astrocytes and recruitment of endogenous neurons into the stroke cavity were also examined. HyStem-HP promoted survival of implanted cells in the stroke cavity, reduced inflammatory infiltration, diminished cell stress and enhanced recruitment of native neurons. Stem cell transplantation into an infarct cavity in a clinically appropriate sECM hydrogel may provide a translational therapy for stroke recovery.

Recruitment of MSCs may also be possible to accelerate treatment in an alternative to the traditional concept of cell therapy. The usual concept envisions harvesting stem cells from patients, propagating and differentiating these progenitors in vitro, and subsequently reinjecting the manipulated autologous cells into the patient. An alternative paradigm that could be easier, safer and more practical would involve attracting

endogenous stem cells and precursor cells to the defect site for de novo tissue regeneration. As a proof of concept, Heprasil, an sECM containing covalently linked gelatin, heparin and HA, was loaded with hepatocyte growth factor (HGF), a pleiotropic cytokine of mesenchymal origin that exerts strong chemoattractive effect on MSCs. Indeed, slow release of HGF from Heprasil induced migration of human bone marrow MSCs into the scaffold in vitro, providing support for the use of an HGF-Heprasil sECM for MSC recruitment in regenerative medicine [105].

EXTRACELLULAR SCAFFOLD AS A MOLD FOR KIDNEY REGENERATION

The idea of emulating ECMs for re-creating niche environments has been brought to the extreme by using an actual matrix cleared of cells and preserving the 3D architecture of an organ. Ott et al. [106] used sodium dodecyl sulfate (SDS) perfusion to decellularize rat heart, only to repopulate it afterwards with neonatal cardiocytes and observe contractions. With electrical stimulation, such reconstituted hearts could generate modest pump function. Ross et al. [107] used sequential detergent enzymatic perfusion of rat kidneys with Triton X-100, DNase and repeated Triton X-100, followed by SDS to achieve decellularization. Scanning electron microscopy showed excellent preservation of the cell-free ECM. After seeding the scaffolds with pluripotent murine embryonic stem cells, preparations were perfused and proliferation and differentiation of cells were observed. Unfortunately, no functional studies of such reconstructed kidneys were presented.

HYDROGEL ENCAPSULATION OF ENDOTHELIAL PROGENITOR CELLS PROTECTS CELLS FROM CYTOTOXINS AND IMPROVES THEIR VIABILITY

This group recently compared the viability of EPCs encapsulated in HA-hydrogel versus EPC cultured on the surface and then exposed to a powerful toxin, doxorubicin (Adriamycin). Encapsulated EPCs showed superior viability [108]. Moreover, the ability of encapsulated EPCs to participate in neovascularization was tested in a mouse model of femoral artery ligation. The effects of encapsulated cells were compared with the intravenous infusion of the equivalent number of EPCs. As shown in Fig. 16.3, cell therapy accelerated restoration of blood flow to the ischemic hind limb; however, HA-hydrogel-encapsulated EPC, released through the gradual enzymatic degradation of the hydrogel, showed a much steeper restoration of blood

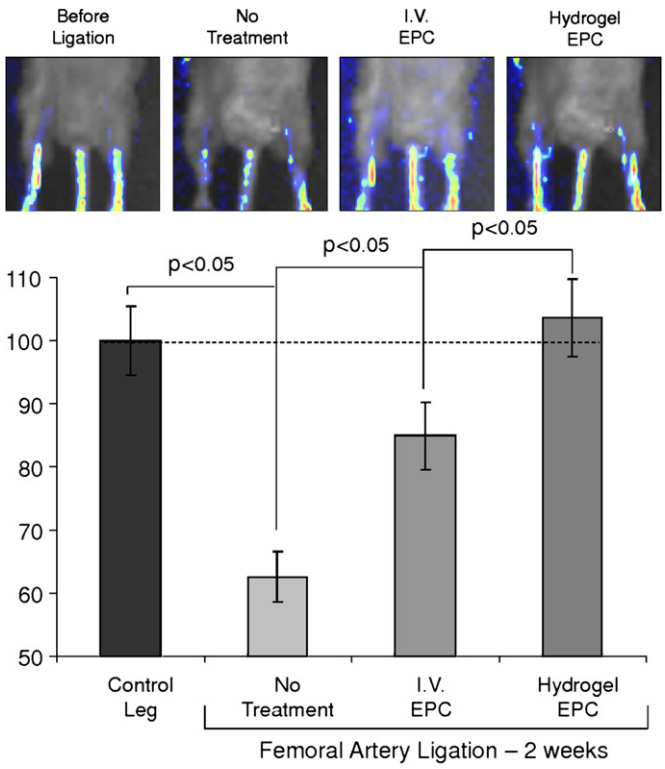

FIGURE 16.3 Femoral artery ligation is performed in mice that are then treated with endothelial progenitor cells (EPCs) through either tail vein injection or hydrogel implantation. (A) Images of laser Doppler perfusion on the site of ligation after 2 weeks in mice given different treatments. (B) Laser Doppler flowmetry data (percentage perfusion) in the ligated vs the control hindlimb after 2 weeks. Please see color plate at the end of the book.

flow than the equivalent amount of EPCs infused intravenously. These data argue favorably for the improved therapeutic efficacy of EPCs encapsulated in hydrogels.

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CHAPTER

17

Imaging of Transplanted and Native Stem Cells

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OUTLINE

INTRODUCTION

Utilization of stem cells for therapeutic approaches is becoming an attractive alternative to conventional treatments, especially for diseases refractory to other treatments. The main objective of cell-based therapies is to repopulate the damaged tissue with functional cells, or to use the cells to administer therapeutic agents to the target tissue. Use of stem cells in animal models has been shown to regenerate renal tubular [1 3], mesangial [4] and endothelial cells in kidney diseases such as acute kidney ischemia or glomerulopathy. Endothelial progenitor cells (EPCs) have been used in the stenotic kidney of a swine model of chronic renal artery stenosis and it was demonstrated that EPCs integrated into renal vascular and tubular structures and improved renal function [5]. Stem cells have also been shown to regenerate different organ and systems, such as pancreas [6,7], joints [8 10], musculoskeletal system [11] and components of the cardiovascular system [12 14], and have also been used as adjuvant treatment for malignancies [15]. In addition to EPCs, a wide range of cell types, including mesenchymal stem cells (MSCs) [8,11,16 18], embryonic stem cells

(ESCs) [19 21], bone marrow-derived hematopoietic stem cells (HSCs) [22,23] and neural stem cells [24,25], have been applied in different systems.

A significant body of knowledge has been acquired with regard to the biology of stem cells and the potential benefit of their use in tissue and organ regeneration by ex vivo imaging (histology). However, such techniques cannot address questions regarding the biology of stem cells in living subjects, such as temporal and longitudinal patterns of cell location (due to migration), viability and functional status (interactions with tissue, differentiation, etc.) after delivery. Thus, the need for an imaging technique to track the biology and behavior of stem cells before and after transplantation becomes of paramount importance for the advancement of the field of cell-based regenerative medicine.

The capacity to study cell biology and cell survival has been limited in part owing to limitations in imaging technology. Traditional ex vivo histology/immunohistochemistry assays are useful for ex vivo cell biology study, but for stem cell tracking they are limited to biopsy specimens or to information obtained at the time of euthanasia of the animals. A non-invasive approach would permit a longitudinal study (in the same subject) of the biology of stem cells, at the same time minimizing the interference of biological variables (because the same subject can be used for multiple studies) [26,27]. Furthermore, a non-invasive approach has the potential to disturb the microenvironment minimally, allowing a more physiological study. Moreover, such strategies have the potential to be translated for clinical use, and some non-invasive imaging has indeed been adapted to image stem cells in clinical trials.

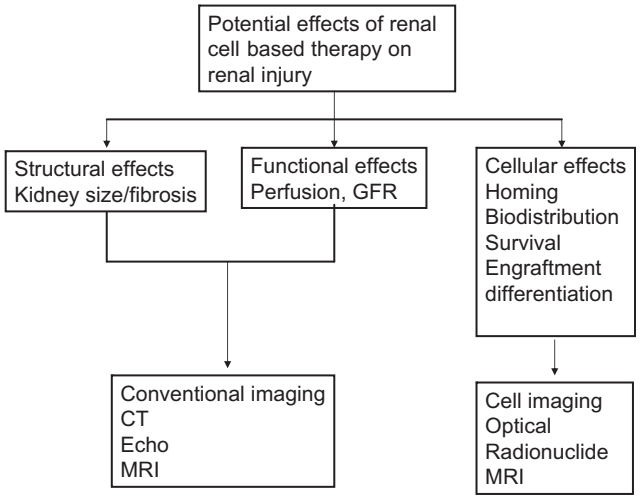

FIGURE 17.1 Schematic outlining the potential application of imaging in cell based therapy of renal diseases. GFR: glomerular filtration rate; CT: computed tomography; MRI: magnetic resonance imaging.

In addition to histological technique, current available mainstream non-invasive image modalities for stem cell tracking include optical imaging (OI), magnetic resonance imaging (MRI) [28], radionuclide positron emission tomography (PET) and single-photon emission computed tomography (SPECT). These techniques not only provide structural and functional information after cell therapy, but may also be capable of tracking stem cells in vivo longitudinally, and following their migration and transdifferentiation. Figure 17.1 shows the potential role of imaging in cell-based therapy of renal diseases. This chapter will discuss using these modalities for stem cell imaging and their application in kidney diseases.

METHODOLOGIES FOR LABELING STEM CELLS

To render stem cells visible under any imaging modality, proper labeling of cells is important. There are two major strategies for the labeling of stem cells for imaging: direct labeling and indirect labeling (reporter genes). In a direct labeling strategy, labeling agents are introduced into the cells by simple incubation with the tracer before transplantation, and stem cells are transplanted and then followed in vivo. In an indirect labeling system, protein-encoding genes can be transiently or stably introduced in the cell, thus becoming reporters of gene expression and enabling cell-tracking following transduction. Stem cells incorporated with reporter gene ex vivo are then transplanted into living subjects. These stem cells express a special protein (e.g. enzyme, cell surface receptor) that interacts with an exogenously given substrate or light excitation, resulting in signal which can be detected non-invasively in vivo or by histology.

IMAGING MODALITIES

Histology

Histology is a conventional tool to track stem cells mainly using microscopy and, because of its invasiveness, especially in animal research. Stem cells need to be labeled with tracers before transplantation, which enable them to be distinguished from host cells.

Direct Labeling

The most effective tracer of cell morphology is a fluorescent probe, which has the capacity for localized introduction into a cell or organelle, as well as long-term retention within that structure, and is biologically inert and non-toxic. Fluorescent tracers can be used to

investigate the movements of labeled cells in culture, tissues or intact organisms. There are four major different types of fluorescent probes for stem cell tracking.

- *Membrane tracers*: DiI, DiO, DiD, DiR, DiA and their analogs. Di dyes can be introduced into membranes by direct application of dye onto the cell by loading from culture media [29]. Lateral diffusion of the dye within the membrane eventually stains the entire cell. These probes are widely used for stem cell tracking and long-term assays of cell cell association [30]. DiI dye has been applied in EPC labeling and tracking in the kidney. EPCs were incubated with CM-DiI for 30 min, then transplanted into the stenotic kidney of pigs with unilateral renal artery stenosis. Red fluorescence EPCs could be visualized in kidney sections as late as 4 weeks after transplantation (Fig. 17.2). Furthermore, combining Di dye with other immunohistological staining, it was possible to track stem cell change in phenotype (transdifferentiation). For example, a histological section from a kidney injected with Di dye-labeled EPCs (fluorescent red) was stained with an antibody against the endothelial marker CD31 (labeled fluorescent green). The cells showing both red and green fluorescence (i.e. yellow) were mainly confined to perivascular capillary structures, indicating the ability of EPCs to transdifferentiate into mature vascular endothelial cells (Fig. 17.2).
- *Fluorescent microspheres*. Those microspheres contain approximately 10^2 to 10^{10} fluorescent dyes per bead and are the most intensely fluorescent tracers available [31]. Furthermore, they are often biologically inert and physically durable, which makes fluorescent beads particularly useful as long-term markers for transplantation studies. Submicrometer microspheres can be taken up by phagocytosis. This feature enabled EPCs to be labeled by incubation with microspheres in the culture medium, and then EPCs (yellow fluorescence) were tracked in the kidney 4 weeks after injection (Fig. 17.2).
- *Cell-permeant cytoplasmic labels* (thiol-reactive CellTracker probes) yield fluorescent products that are retained in many live cells through several generations and are not transferred to adjacent cells,

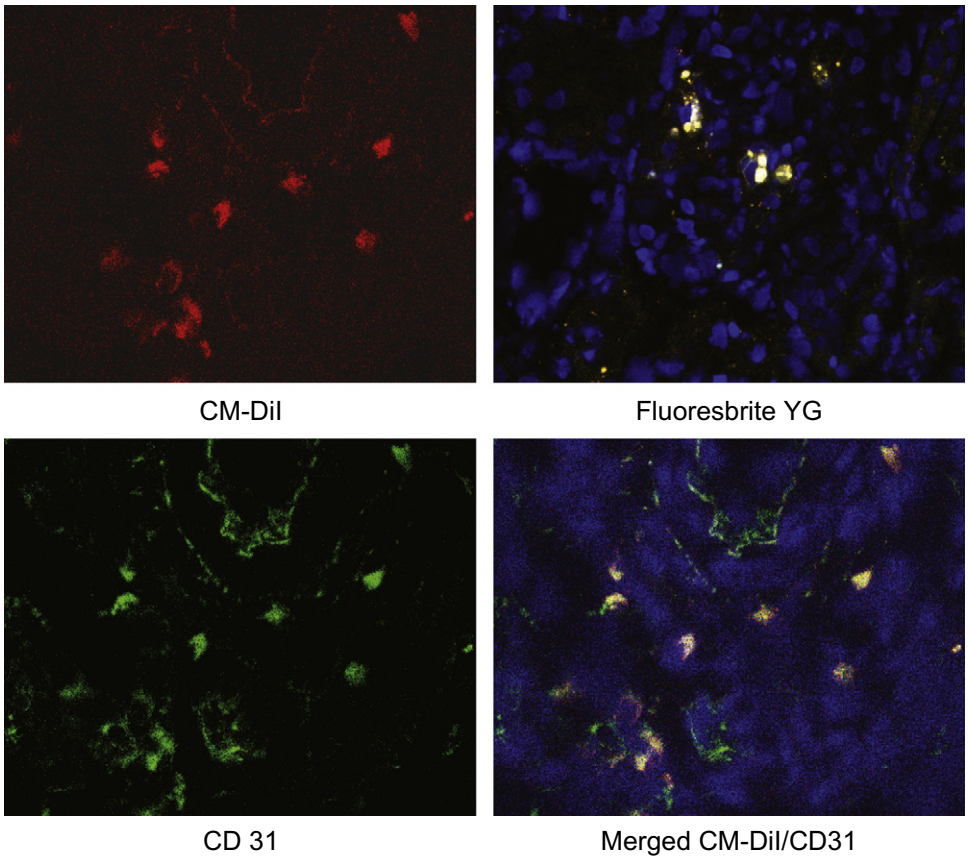

FIGURE 17.2 Top: Representative images of endothelial progenitor cells (EPCs) labeled with fluorescence dye (CM DiI, red, left) and yellow–green (YG) microspheres (right) in the kidney 4 weeks after transplantation. Bottom: Representative merged images of CM DiI labeled EPCs and the same kidney immunostained for the endothelial marker CD31, indicating transdifferentiation of EPCs to vascular endothelial cells. Please see color plate at the end of the book.

except possibly by transport through gap junctions. These probes constitute excellent long-term tracers for transplanted cells or tissues [32].

- *Fluorescent dextran conjugates*. Dextran conjugates are ideal cell lineage tracers because they are relatively inert, exhibit low toxicity and are retained in cells for long periods [33]. However, they are membrane-impermeant probes, and usually need to be loaded into cells by invasive techniques such as microinjection, whole-cell patch clamping or scrape loading. This may limit the application of dextran conjugates in stem cell tracking.

Histological techniques are also used to track iron-labeled stem cells in the kidney in conjunction with MRI studies. Prussia blue staining is specific for iron, and comparing MRI images with histological sections is useful to confirm and determine the accuracy of MRI tracking.

Indirect Labeling

For histology, cells can be labeled with a fluorescent reporter gene, and then when the tissue is excised, the labeled cells that remained in the tissue can be easily identified using histological methods [34]. Similar to in vivo conditions, different reporter genes emit light in different spectra permitting detection of corresponding fluorophores in the sample. These applications play a very important role in the study of stem cell biology in cell cultures and ex vivo. Furthermore, owing to their light emission and detection characteristics, they are commonly used as a basic strategy when designing novel reporter gene constructs. A good example for reporter genes is green fluorescent protein (GFP) [3,35]. Stem cells isolated from GFP transgenic animals can be allogenically transplanted into the host body; those cells consistently express GFP and can be visualized using fluorescence microscopy.

In summary, although histology is a traditional ex vivo imaging technique, which can only provide information obtained by invasive techniques, it is still a basic and powerful tool that allows accurate cell tracking, and provides cell fusion information and cell tissue interaction, especially in animal studies.

Optical Imaging

The principle of OI is similar to that underlying the acquisition of a photograph. It uses a light that excites a fluorophore and a detector, such as a highly sensitive charge-coupled device (CCD), which captures the probe's emission once resolved by an appropriate filter. The ability to image at single cell level, lack of radiation and low cost make this modality a basic imaging technique for stem cell tracking, especially in small animals.

Direct Labeling

This method employs an incident of light that excites a fluorophore and emitted light is captured by a CCD camera. So far, no single ideal optical contrast agent is available. In general, all OI contrast agents need to be biocompatible, possess a tolerable toxicity profile, be small in size and exude a bright signal.

- *Fluorescent dyes*. These fluorochromes are continuously being designed (hundreds are commercially available) and include DiI, DiO, DiD and CM-DiI. They have proven effective for in vitro cell labeling (as discussed in the last section) and in vivo cell tracking with OI.
- *Targeted probes*. Colloidal quantum dots (QDs) have a narrow emission and continuous broad absorption spectrum (i.e. broad excitation spectrum), which allows fluorescent excitation by any wavelength below the emission maximum.

Indirect Labeling

One of the main advantages of fluorescent reporter genes is that different fluorophores (e.g. red or green fluorescent proteins) can be used to label different cell populations, permitting their concomitant imaging [36].

- *Bioluminescence*. Bioluminescent imaging typically involves the luciferase (luc) gene and based on luc-mediated oxidation of D-luciferin (with emission spectra: 400 620 nm). A chemical process following intravenous administration of luciferin generates light emission. Photon generation takes place exclusively at the site of luciferase expression; therefore, the target-to-background signal ratio is extremely high, thus allowing for detection at lower wavelengths with a maximal penetration depth of 3 cm.
- *Fluorescent proteins* [37]. The first representative of this class of proteins was GFP, which has been commonly used for cell tracking with OI in small animal models.

Perhaps the most frequent application of fluorescent reporter genes in cell imaging is for ex vivo analysis, where they can be used for cell sorting. For example, not all stem cells transduced with a bioluminescent or PET reporter gene incorporate the gene of interest into their genome. A common method of sorting and selecting the cells that did incorporate the gene of interest takes advantage of fluorescent reporter gene cell markers (e.g. Gfp, Rfp) [34].

Application of Optical Imaging in the Kidney

Few studies have applied OI to track stem cells in the kidney. Tögel et al. [38] tested bioluminescence to track MSCs in a mice acute kidney injury (AKI) model using the OI technique. Bone marrow-derived MSCs were infected with luciferase/neomycin phosphotransferase.

The transinfection had no impact on MSC biology, as tested by a multidifferentiation assay. Transinfected MSCs (1×10^5) were injected into mice through the jugular vein or carotid artery. Immediately or 24 h after injection, animals were imaged using the Xenogen IVIS 100 system. MSCs showed distinct accumulation in the kidney only in AKI mice, mainly after the cells had been administered by intra-arterial injection. Importantly, intravenous injection of MSCs resulted in substantial accumulation in the lung (Fig. 17.3), so that fewer cells reached the kidney. This study demonstrated that bioluminescence is sensitive for in vivo MSC tracking, and is suitable for non-invasive kidney localization of injected MSCs in small animals.

Limitations

The fluorescence direct imaging and reporter gene approaches have limited tissue penetration (around 2 mm), restricting the use of these techniques to superficial tissues in small animals (e.g. mice) [26]. Furthermore, most common fluorescence imaging devices do not have tomographic capabilities, which further limits the identification of deeper organs. Several studies are underway to attempt to provide tomographic fluorescence imaging [39 41].

There are also some other drawbacks to the use of fluorescent reporter genes. Similar to other imaging modalities, the detected signal constitutes only a small fraction of the emitted signal. Significant efforts are underway to use multiple cameras, which may enable detection of a higher fraction of the emitted signal [42]. Novel developments, such as time-domain imaging [43 45], incorporate the time domain in the analysis, and have the potential to provide depth information of the fluorescent signal. However, such strategies are under development and not ready to be routinely applied by mainstream imagers. In addition, fluorescence techniques are associated with background autofluorescence, which necessitates a stronger signal to overcome the noise in the data. The presence of

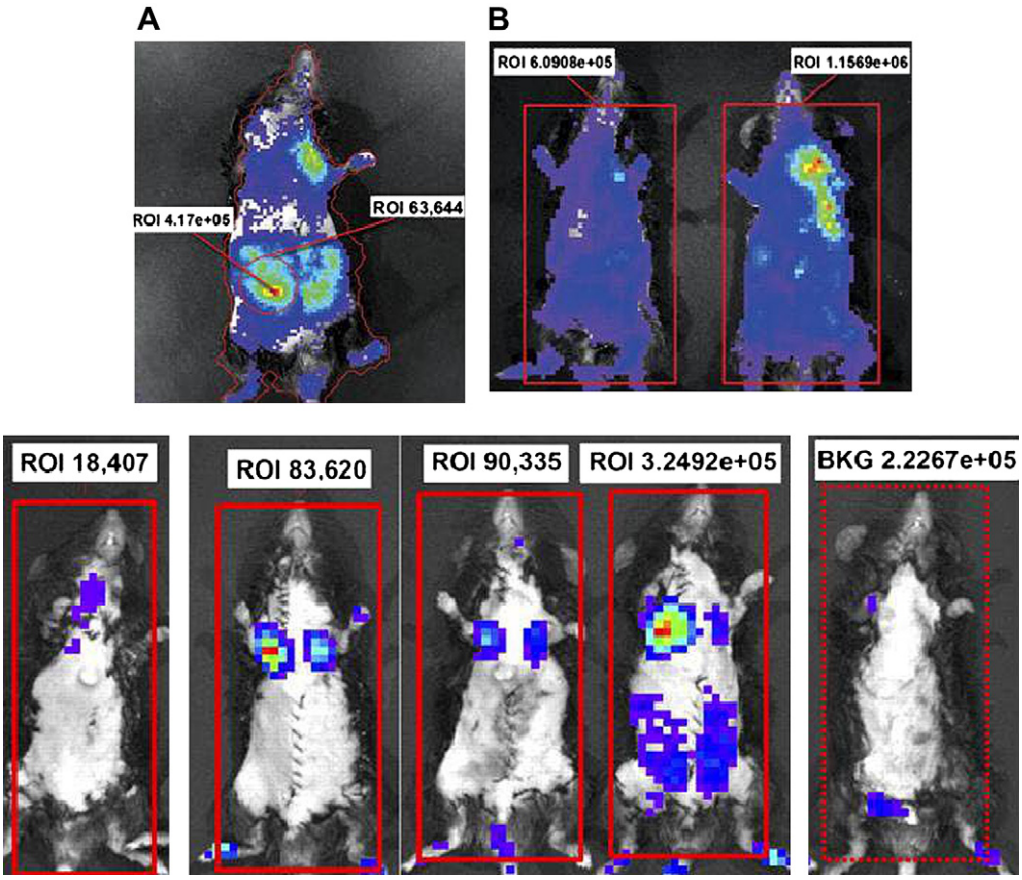

FIGURE 17.3 Optical imaging of mesenchymal stem cells (MSCs) in the kidney. (A) Immediately after intra arterial infusion, animals with acute kidney injury (AKI) showed distinct accumulation of cells in kidneys (as shown by green/red areas). (B) Normal animals show diffuse whole body distribution with eventual accumulation in the lungs in some animals. Bottom: Intravenous injection in a normal animal (first on the left) showed accumulation of MSCs in the lungs. In the middle panels three different AKI animals showed accumulation of cells in the lungs immediately after injection, and only one animal with AKI showed retroperitoneal uptake indicating cell localization in the kidneys. *[Adapted from Tögel et al., 2008 [38] with permission.]* Please see color plate at the end of the book.

autofluorescence could be a significant disadvantage, especially when the emitted signal is not high, leading to a low signal-to-noise ratio.

Magnetic Resonance Imaging

MRI provides excellent soft tissue contrast with high resolution. Thanks to a large number of available MRI techniques to generate contrast in the images, a number of anatomical, physiological and metabolic variables can be measured almost simultaneously and under physiological conditions. Partly because of this versatility, MRI has evolved into one of the most powerful imaging tools in radiology and biomedical sciences. Furthermore, the high resolution of MRI enables its use for the visualization of single cells against a homogeneous background. The availability of responsive and targeted contrast agents extends applications of MRI from visualization of cell location to characterization of molecular and cellular signaling events and their functional status, such as enzyme or receptor expression.

Direct Labeling

To visualize cells against the background of host cells and to increase the likelihood and sensitivity of their detection with MRI, cells need to be labeled with specific MRI contrast agents. Four types of agents are currently available for MRI cell imaging, and selection of a specific agent depends on the purpose of imaging.

- *Iron oxide-based contrast agents.* Superparamagnetic iron oxide particles (SPIOs) are the most commonly used strategy in MRI cell labeling. These highly magnetic particles induce changes in T2 relaxivity, which makes them detectable in vivo [46 48]. In a direct labeling strategy, stem cells are prelabeled with SPIOs, before transplantation and imaging, so that the signal originating from the SPIOs is considered to reflect number of cells retained in the tissue.
- *Lanthanide chelates and Mn^{2+}-based contrast agents.* Paramagnetic compounds such as Mn^{2+} ions or lanthanide chelates usually affect T2 less than SPIOs, but are commonly used for their increase in T1 relaxation, resulting in a hyperintense (bright) contrast in T1-weighted images. Manganese-based contrast agents such as manganese chloride ($MnCl_2$) or manganese oxide (MnO_2) nanoparticles have also been used for cell labeling. However, their toxicity prevents their widespread application.
- *Contrast agents based on heteronuclear MRI.* The majority of MRI contrast agents used for clinical and experimental applications is based on modulating the contrast in ^{1}H-based MRI [49]. Although relatively sensitive, this presents a major challenge for discriminating labeled cells from the background signal and motivates the search for a less abundant contrast molecule. Most heteronuclear approaches to MRI-based cell imaging have focused on ^{19}F-MRI and spectroscopy. One advantage of ^{19}F-MRI and spectroscopy is the ability to quantify the signal as long as the signal-to-noise ratio is sufficiently high. Other potential applications of heteronuclear MRI and spectroscopy include ^{13}C and ^{31}P.
- *Responsive contrast agents.* Contrast agents that change contrast owing to their changed magnetic properties (e.g. relaxation, chemical shift) in response to dynamic changes in physiological, enzymatic and other metabolic properties are frequently referred to as responsive contrast agents [50]. Contrast agents that are (chemically) modified by cellular changes have initially been developed for reporting on the physiological status and metabolic activity of cells. Most of these contrast agents are lanthanide chelates with one or more potential coordination sites for water that can be blocked in the inactive state of the contrast agent. Using these agents enables detection of the interaction between transplanted cells and host organs. A potential limitation is the remaining influence of the "inactive" contrast agent on T1 relaxation owing to the secondary coordination sphere of the water, which further decreases its sensitivity.

Special Cell-labeling Approaches for Magnetic Resonance Imaging

Useful approaches for stable labeling of cells with MRI contrast agents include incorporation of the contrast agent in vitro before transplantation and engraftment into the host, systemic application and subsequent specific internalization of the contrast agent by the targeted cells, receptor-specific binding or internalization of targeted contrast agent to targeted cells, or the generation and accumulation of contrast by genetically modified cells.

- *In vivo cell labeling.* In vivo cell labeling applications include localized injection of iron oxide particles, which allows monitoring of the migration of endogenous stem cells. In a recent study [51], iron oxide particles were injected in the area of stem cell generation, and labeled cells that translocated along the rostral migratory stream to the target area, assumed to represent stem cells.
- *Targeted contrast agents for in vivo cell labeling.* To achieve more specific labeling of particular cell types by systemic administration of a contrast agent, targeted agents are the most promising approach. Targeted contrast agents are chemically modified so

that they accumulate specifically in certain tissue types, usually owing to the presence of particular cell types. Active accumulation occurs by specific binding or uptake due to biomarkers bound to the contrast agents, such as antibodies or small peptides. Antibodies bound to iron oxide particles or lanthanide chelates accumulate at sites of high expression of the targeted receptor. The most common approach is to use Tat peptides to derivatize either coated nanoparticles or lanthanide chelates. Biological applications of targeted contrast agents include inflammation, angiogenesis, apoptosis, tumors and atherosclerosis. These approaches are often hampered by insufficient accumulation of the contrast agent owing to either low cell densities or low receptor expression on the cell surface.

Indirect Labeling: Magnetic Resonance Reporter Genes

MRI-based reporter genes strategies are based on the production of intracellular metalloproteins [52]. As previously described, iron is a paramagnetic substance that induces changes in relaxivity (i.e. T2* effect) that can be detected using specific imaging sequences, and the goal of these strategies is to accumulate large quantities of iron intracellularly for its detection. Two major metalloproteins have been used in MRI reporter gene techniques: ferritin and tyrosinase. Ferritin is a metalloprotein that functions as an iron depot and can contain up to 4000 iron atoms. For example, cells transduced with the ferritin reporter gene were delivered to the brain of mice [53]. After several days, enough iron signal was "collected" inside the transplanted cells (due to the overexpression of ferritin) to enable the non-invasive monitoring of transplanted cells using MRI. In a recent study [54], the investigators used a replication-defective adenovirus vector to deliver the ferritin transgenes. Following focal inoculation of the vector into the mouse brain, they monitored the reporter activity using in vivo time-lapse MRI. They observed robust contrast in virus-transduced neurons and glia for several weeks (Fig. 17.4). This technology is adaptable to monitor transgene expression in vivo in many tissue types and has numerous biomedical applications, such as visualizing preclinical therapeutic gene delivery.

Tyrosinase has also been used as an MRI reporter gene [55]. Tyrosinase participates in the production of melanin, which has high affinity for iron, which increases relaxivity. Tyrosinase has been transfected to fibroblasts and embryonic kidney cells as well as breast cancer cells, and resulted in increased signal. However, iron toxicity limited this approach. Melanin production also produces deleterious reactive oxygen species and thus exhibits significant toxic effects.

Magnetic Resonance Stem Cell Imaging in the Kidney

Most studies applying MRI for tracking stem cells were performed in the rat AKI models using direct labeling. These studies followed SPIO-labeled MSCs immediately [56], 3 days [57] or up to 8 days after transplantation [58]. SPIO labeling caused a strong R2* signal intensity loss in renal cortex, with a label half-life longer than 11 days [46]. Intra-arterially injected SPIO-labeled MSCs could also be detected in liver, spleen and bone marrow. Thus, qualitative and quantitative in vivo cell tracking and monitoring of organ distribution of intra-arterially injected MSC(SPIO) in AKI is feasible with MRI [48]. Intravenously injected MSCs were found to home to focal areas of glomerular damage and can also be detected using ex vivo MRI [59]. Ittrich et al. [48] evaluated SPIO-labeled MSC in a rat AKI model. MSC were incubated for 24 h with the commercially available SPIO Ferucarbotan® without addition of transfection agent, and then injected into the kidney through a catheter placed in the aorta. Animals were scanned with a 3 T MRI scanner. Images clearly showed MSC accumulation in renal cortex up to 14 days after transplantation (Fig. 17.5). R2* relaxometry method was used to quantify the renal retention of MSC and cell distribution to different organs. Lange et al. [57] also used MRI to monitor the physical presence of MSC in the injured kidney in a renal ischemia reperfusion rat model. The iron used to label bone marrow-derived MSC in this study was carboxy-dextran-coated iron oxide nanoparticles. MSCs (1.5×10^6) were injected into the thoracic aorta, and MRI scanning was performed 72 h later. In MRI images, iron-labeled MSCs showed a pattern of signal loss of outer cortex, which remained consistent until day 3. These were confirmed by histological analysis using Prussia blue staining, which showed predominant MSC accumulation in glomerular capillaries. In another study, by Bos et al. [46], MSC were prelabeled with SPIO Endorem® and the transfection agent Superfect®. MSC (2.5×10^6) were injected into the kidney through the renal artery, and rats were imaged at 4 and 8 days after injection using a conventional 1.5 T MRI scanner. T2*-weighted postinjection MRI images showed a signal intensity loss in the renal cortex, which was restricted in the kidney injected with MSCs. Furthermore, quantitative evaluation revealed a 100% increase in R2* value immediately after MSC injection, which returned to baseline at day 8.

Limitations

MRI-based cell tracking offers high resolution and anatomical details for soft tissue imaging as well as the ability to generate contrast based on a variety of physiological parameters. However, several limitations need to be considered.

Many contrast agents are administered in coated or chelated form to reduce toxicity. Commercially available and clinically tested contrast agents are often used in concentrations much higher than clinically applied. Immediate toxic effects can easily be tested by growth and viability studies in cell suspensions. Iron in high concentrations is known to interact with a number of other metabolic pathways and may cause oxidative stress. The transfection agents used for SPIO internalization may also affect cell viability.

Once cells have been labeled with contrast agents, the local intracellular concentrations are subject to changes. The most obvious change is due to dilution in the process of cell proliferation. This would require an overload with contrast agents for highly proliferative cells to visualize them over prolonged periods. In addition to loss of cell detectability, release of contrast agents in vivo and uptake by host cells may result in misinterpretation of MRI-based cell tracking.

Although MRI visualizes cells in vivo, quantification of cell numbers is challenging. Limitations for the in vivo quantification of loaded cells can be overcome if the signal generated by the contrast agent can be observed directly, as in ^{19}F-MRI.

There are also a few limitations for the use of metalloproteins as MRI reporter genes [52]. The signal is dependent on the accumulation of iron inside the cells and the duration of their retention. In addition, when cells divide the signal is diluted and can be lost until the cells accumulate enough iron again to generate a sufficient signal. Furthermore, the relaxation depends on the iron loading conditions: R2 relaxivity is high at low

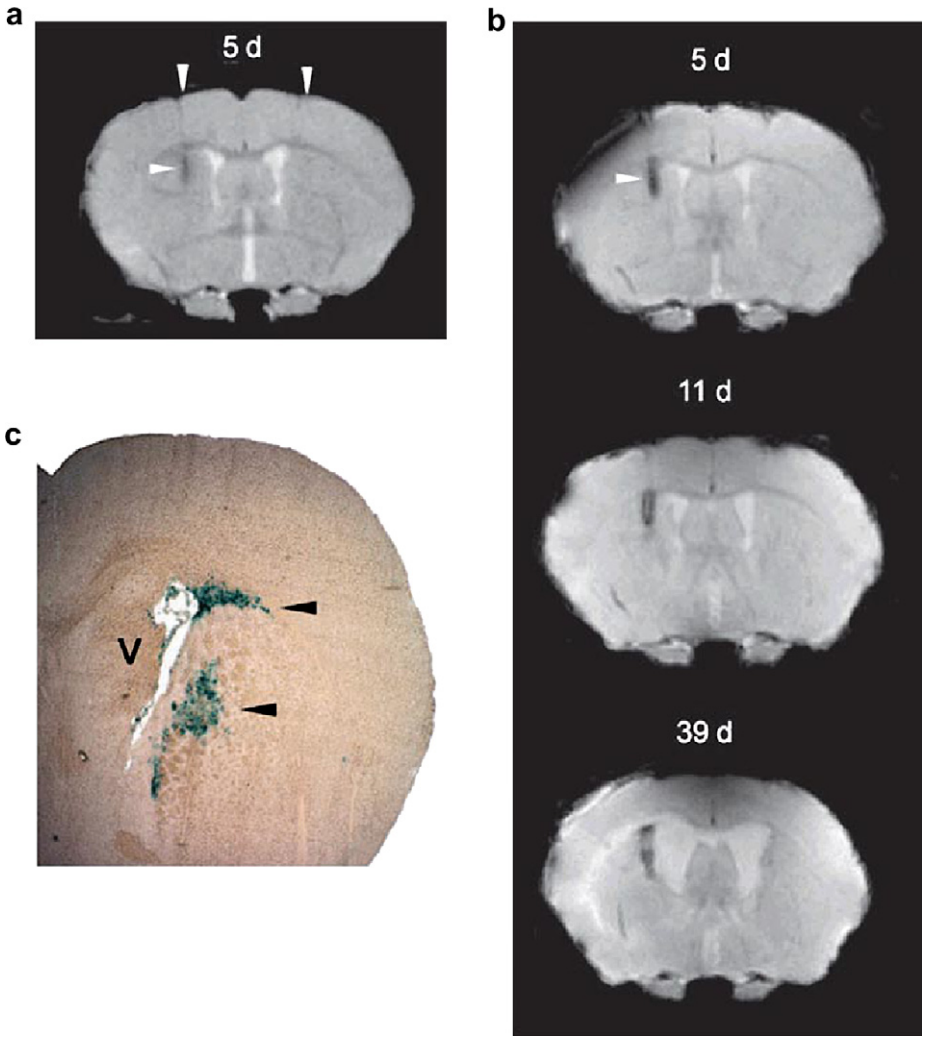

FIGURE 17.4 In vivo longitudinal monitoring of magnetic resonance imaging (MRI) reporter gene expression in the mouse brain. (a) T2 weighted image 5 days after injection, showing the inoculated site (left arrow). (b) Time lapse T2* weighted images obtained 5, 11 and 39 days after injection. (c) X gal stained image showing histological pattern similar to MRI. *[Adapted from Genove et al., 2005 [54] with permission.]* Please see color plate at the end of the book.

iron doses and decreases at intermediate iron loading conditions, and when iron conditions are high, T2 relaxation remains constant, which may hamper quantification of the detected signal. Similar to SPIOs, when cells die the accumulated iron continues to be detectable (until cells are dissolved or eliminated by phagocytosis) and thus the signal is neither representative nor a linear indicator of cell viability.

Radionuclide Labeling (PET/SPECT)

The strategy for direct labeling of cells with radioisotopes resembles that used with SPIO-based techniques, which is to introduce a labeling agent to the cell before transplantation. Because of the short half-life of isotopes, these strategies may be useful for the initial localization of transplanted cells, but will not be sufficient to monitor stem cells for extended periods. One of the major advantages of SPECT and PET imaging is their high sensitivity (nanomolar and femtomolar, respectively), which permits the detection of relatively low signals [60 62]. Furthermore, these imaging techniques may be clinically applicable.

Radioisotopes

Radioisotopes used for this purpose have different physical half-lives (e.g. ^{99m}Tc: 6 h; ^{18}F: 109 min; ^{111}In: 2.8 days), which dictate the length of time for which they can be followed after labeling. For example, ^{111}In-labeled cells are used clinically to track the homing of inflammatory cells and localize inflammatory processes [63,64]. This methodology is now being applied to labeling of stem cells using different isotopes [e.g. ^{111}In for SPECT, ^{18}F-fluoro-deoxyglucose (^{18}F-FDG) for PET] [65 68]. ^{18}F-FDG, with a physical half-life of 109 min, may allow tracking of cells for a few hours after transplantation [69,70]. An isotope with a longer half-life (e.g. ^{111}In) may allow cells to be followed for up to a few days. ^{64}Cu, with a relatively long physical half-life, has also been used to monitor C6 rat glioma cells using PET [71].

Direct Labeling PET/SPECT Imaging

SPECT and PET have relatively lower spatial resolution than MRI, which may impede signal localization. The recent development of integrated PET-CT and SPECT-CT has overcome much of its limitation in spatial resolution. Kraitchman et al. [66] combined SPECT, CT

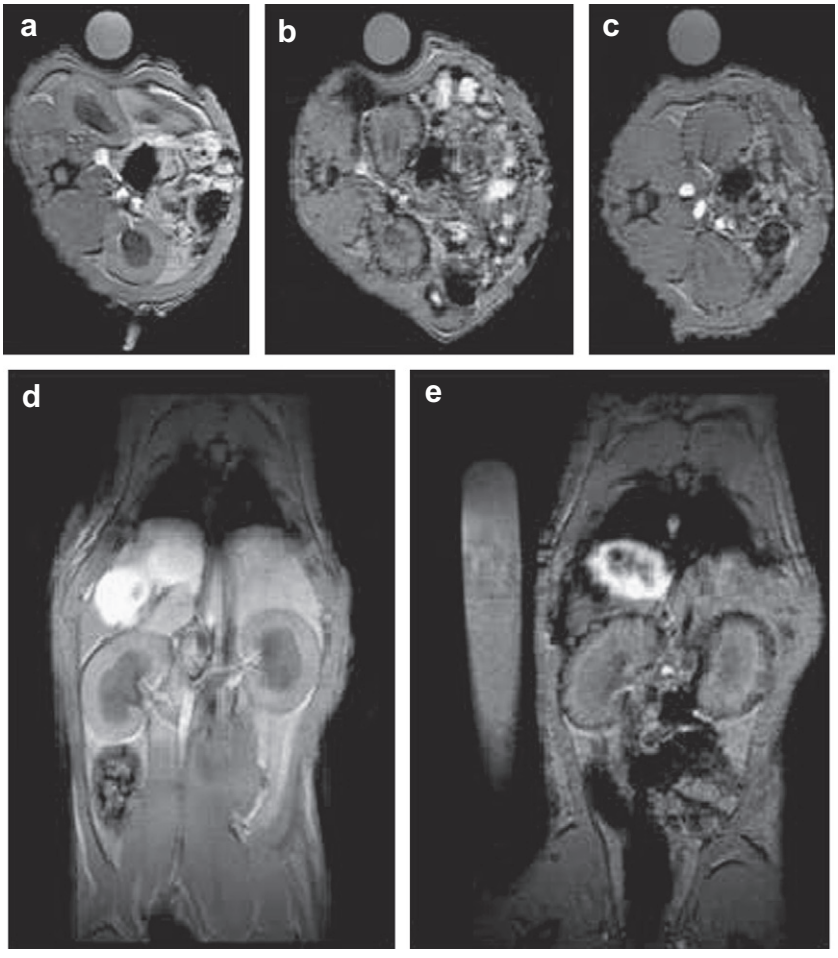

FIGURE 17.5 Accumulation of superparamagnetic iron oxide particles (SPIO) labeled mesenchymal stem cells (MSCs) in the renal cortex of rats. (a, d) Baseline. T2* weighted gradient echo in vivo magnetic resonance image showing a significant signal loss in the cortex. (b, e) Immediately, and (c) 14 days after MSC injection. *[Adapted from Ittrich et al., 2007 [48] with permission.]*

and MRI to track MSCs in a canine myocardial infarction model. MSCs were labeled with both a radioisotope (^{111}In) and SPIO (Feridex-PLL), and then injected intravenously. SPECT-CT was performed immediately and 2, 4 and 8 days after injection. Redistribution of the labeled MSCs after intravenous injection from initial localization in the lungs to non-target organs such as the liver, kidney and spleen was observed within 24 48 h after injection. Focal and diffuse uptake of MSCs in the infarcted myocardium was already visible in SPECT-CT images in the first 24 h after injection, persisted until 7 days after injection and was validated by tissue counts of radioactivity (Fig. 17.6). In contrast, MRI was unable to demonstrate targeted cardiac localization of MSCs, in part because of its lower sensitivity. One of the main advantages of PET/SPECT imaging is that it can provide tomographic and volumetric information, thereby improving localization and quantification of the detected signal. In addition, while lower than the sensitivity of optical imaging (10^{-15} mol/l), the sensitivity of PET is in the femtomolar range (10^{-12} mol/l), which is higher than SPIO-MR (10^{-9} mol/l).

Indirect Labeling PET/SPECT Reporter Genes

The principles of PET/SPECT reporter gene-based cell imaging are similar to other systems using this approach. Of the main three reporter gene systems available for PET or SPECT cell imaging, the most commonly used is based on production of a herpes simplex virus type 1 thymidine kinase enzyme (HSV1-TK) that phosphorylates an exogenous substrate, such as ^{18}F-9-[4-fluoro-3-(hydroxymethyl)butyl]guanine (^{18}F-FHBG) and leads to its cellular retention. Because the enzyme can phosphorylate many molecules of the radioactive substrate, the signal in the tissue is amplified and easily detectable. However, this method requires the substrate to cross the cell membrane. Alternatively, a cell-membrane receptor-based approach often relies on a mutant version of the

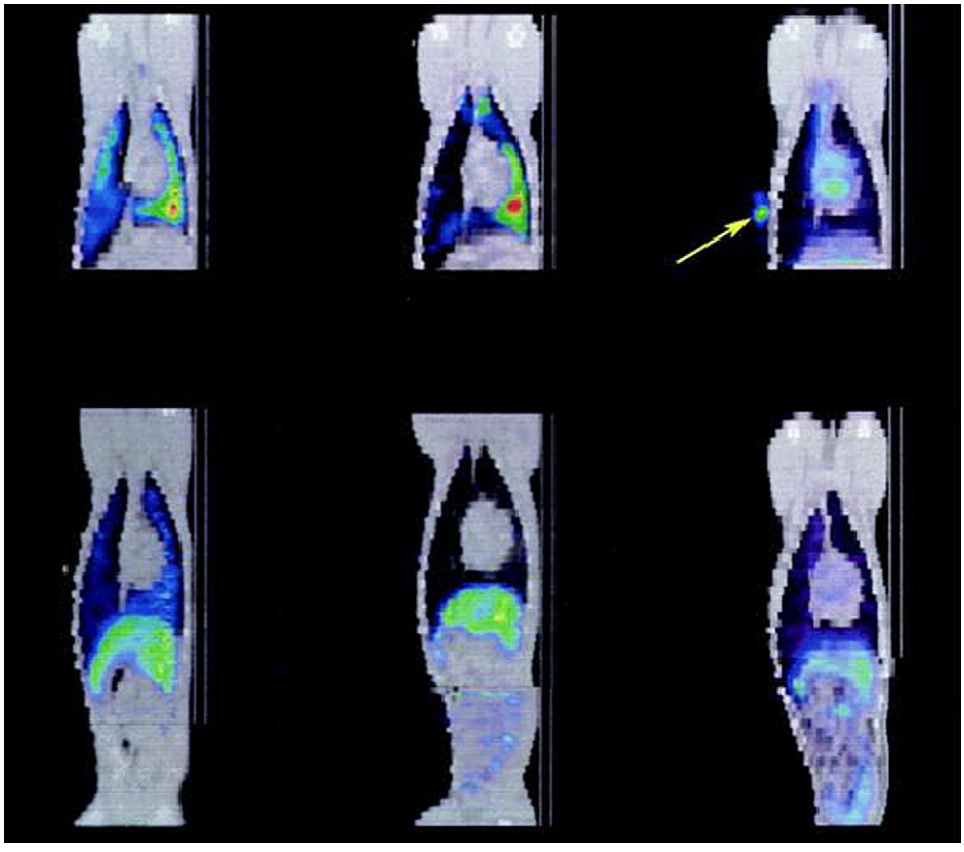

FIGURE 17.6 Coronal fused single photon emission computed tomography (SPECT, color) and computed tomography (CT, gray scale) images of a dog with (top left) and without (top middle) myocardial infarction (MI) during the first hours after intravenous injection of ^{111}In oxine labeled mesenchymal stem cells (MSCs), showing predominant lung uptake with increased uptake to the dependent left lung (green–yellow color towards right). In a dog with MI that received ^{111}In oxine without MSCs (top right), the tracer behaves primarily as a blood pool agent, with uptake visible in left and right ventricles of the heart. A reference marker (arrow) containing ^{111}In oxine was placed on the chest wall on the dog that did not receive MSCs (top right). Redistribution of ^{111}In oxine labeled MSCs to predominantly the liver occurs at 24 h after intravenous injection in both a representative infarcted (bottom left) and non infarcted (bottom middle) dog. In an infarcted dog injected intravenously with ^{111}In oxine only (i.e. no MSCs), a similar pattern of redistribution to the liver is observed at 24 h after injection (bottom right). *[Adapted from Kraitchman et al., 2005 [66] with permission.]* Please see color plate at the end of the book.

dopamine receptor (D_2R) [72]. An important advantage of this strategy is elimination of the need for the substrate to cross the cell membrane to interact with the reporter protein. In addition, as it is an endogenous protein, there is less risk of an immune reaction. However, since each receptor interacts with only one substrate molecule, the amplitude of the signal that it generates may limit this approach. These reporter gene PET methods have been shown to be useful for visualization of transplanted cells and monitoring their fate in the heart [34,73] and pancreas [74,75]. Lastly, the sodium-iodine symporter (NIS) [76 78], which is used by the thyroid gland to transport iodine into the cells in exchange for sodium, has been sequenced and cloned, and used as a reporter gene in the thyroid and other organs. This system has been used not only for PET (with ^{124}I as the substrate), but also for imaging with SPECT (using ^{123}I or ^{99}Tc-perthechnetate as the substrate).

This indirect labeling technique has not been applied in stem cells studies in the kidney. The most common use of reporter gene imaging for cell monitoring involves delivery of cells carrying the reporter gene, and subsequent administration of a substrate to interact with it. However, limited access to and interaction of the cells with the administered substrate immediately after transplantation, for example, due to reduced blood supply, may limit the detection of short-term engraftment of transplanted cells. One option to circumvent this limitation is by exposure of the cells to the substrate (tracer) before transplantation, which facilitates the interaction between the cells and substrate, and increases the signal for monitoring cell homing to the tissue of interest. Long-term monitoring of transplanted stem cells can still be achieved following the standard protocol since the vascular network, and consequently the interaction between transplanted cells and substrate, seem to improve over time.

Limitations

These imaging modalities are limited by several practical considerations. While PET is very versatile for detection of different metabolic and anatomical processes, the production of reporter probes for PET is complex, and requires expertise in chemistry and tight quality control. In addition, depending on the half-life of the radioisotope, it requires proximity to a cyclotron, thereby limiting the widespread utility of this method. Furthermore, because PET detects only photons of 511 keV, it cannot discern different signals. In contrast, SPECT can detect simultaneous signals (e.g. ^{201}Tl and ^{99}Tc), its tracer labeling is less complex and more practical, but its limited spatial resolution compared to PET may restrict its use to spatial localization of a few cells, and SPECT is less versatile for tracer production. Therefore, if an available SPECT probe happens to be suitable to address the research questions, its production and availability make the research more practical and feasible.

Other Imaging Modalities

Multidetect computed tomography (MDCT) has excellent resolution, which provides detailed anatomical descriptions of various structures. The lack of specific tracers for stem cell labeling that can be detected in vitro but do not alter stem cell function restricts its use on its own in cell tracking strategies. This technique is often combined with other modalities for better cell imaging and evaluation of cell-based therapy [79]. Furthermore, MDCT has been used to characterize functional effects of EPCs 4 weeks after transplantation [5].

Ultrasound is another commonly available imaging tool in clinical practice for evaluation of organ structure and function. Kuliszewski et al. [80] reported recently that microbubbles targeted to a genetically engineered cell surface marker on EPCs exhibit specific binding to EPCs in vitro. These targeted microbubbles bind to EPCs engrafted in vivo within Matrigel plugs and can be detected by their enhancement on contrast-enhanced ultrasound imaging. The need for genetic modulation of cells and lack of suitability for long-term tracking restrict its use for clinical stem cell studies.

Multimodality Approach

Clearly, each imaging modality is associated with strengths and weakness for tracking stem cells. One attractive approach to address this limitation is to use a combination of modalities together offering tomographic capability (e.g. PET or MRI), long half-life, good anatomical detail and tracking of cell viability. For example, Cao et al. [81] used a versatile triple-fusion (fluc-mrfp-ttk) reporter gene transduced into embryonic stem cells to be detected by the mrfp for single cell fluorescence image, fluc for bioluminescence imaging (OI) and ttk for PET (Fig. 17.7). This strategy allowed characterization and monitoring of the kinetics of the engrafted cell survival, proliferation and migration.

The multimodality approach allows complementary analysis of cell localization and viability (e.g. PET plus MRI). So far, no such approaches have been reported in the kidney. However, several studies have been conducted in stem cell tracking in myocardium [82 84] and in spinal cord injury [85].

Imaging of Native Stem Cells

As discussed earlier, SPIO can be injected stereotactically into the tissue area of interest, and the iron label then incorporates into cells in the area of its primary deposition. When specific cells migrate out of the labeled

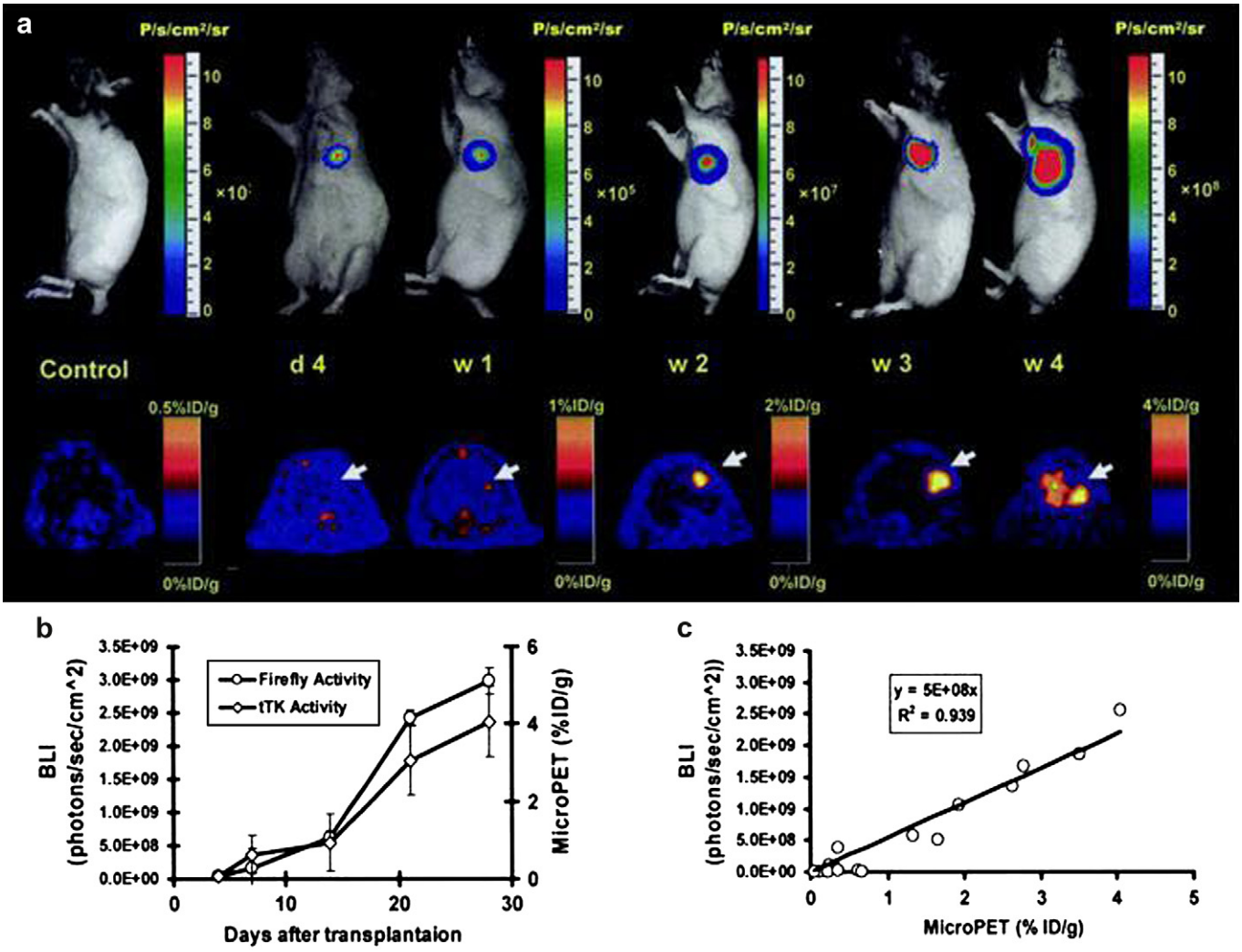

FIGURE 17.7 Molecular imaging of transplanted cells with bioluminescence and positron emission tomography (PET). (a) Animals were imaged for 4 weeks to assess longitudinal cell survival. A representative study animal injected with triple fusion cells showed significant bioluminescence (top) and PET (bottom) signals at day 4, week 1, week 2, week 3 and week 4. In contrast, control animals showed only background activities. (b) Quantification of imaging signals showed a drastic increase in fluc and ttk activities from week 2 to week 4. Extracardiac signals were observed during subsequent weeks. (c) Quantification of cell signals showed a robust in vivo correlation between bioluminescence (BLI) and PET imaging (r^2 0.92). *[Adapted from Cao et al., 2006 [81] with permission.]* Please see color plate at the end of the book.

region, trails of newly generated T2*-weighted contrast may be detectable. Shapiro et al. [51] successfully applied this method to label progenitor cells and track their migration from the subventricular zone to the olfactory bulb along the rostral migratory stream. Immunohistochemistry confirmed the presence of the particles inside neural progenitor cells with increasing degrees of maturity up to the olfactory bulb. This approach takes advantage of the migratory mobility of the stem cells and may allow tracking of native stem cells in vivo.

A recent report used proton nuclear magnetic resonance spectroscopy to detect neural progenitor cells with a peak spectrum at 1.28 ppm in mouse brain [86]. This signal is suggested to associate with progenitor cells, and thus has potential to be applied in native stem cell imaging. However, the reliability and specificity of the spectral analysis remain to be determined.

CONCLUSION

Over the past decade, major strides have been made towards improvement of stem cell imaging techniques for better understanding of cell biology and tracking the fate of stem cells after transplantation. This chapter has outlined some of the most important imaging techniques used for the detection and monitoring of progenitor and stem cells. It focused on imaging technologies

including histology, OI, MRI and radionuclide imaging, which enable short-term or long-term monitoring of stem cell biology in vivo or in vitro. So far, no single technique can answer all of the research questions and address all of the needs. Indeed, in the future, the use of multimodality imaging tools may be found to be the most useful and instructive method in this exciting and rapidly evolving field. Applying these techniques to clinical use is still challenging, but is likely to be resolved over the next few years.

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CHAPTER

18

Stem Cell Injury and Premature Senescence

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OUTLINE

INTRODUCTION

The preceding chapters have outlined the plethora of examples of stem cell participation in regenerative processes. An obvious question that arises with thus accrued knowledge is: why do chronic kidney diseases tend to progress? Is it a matter of a simple imbalance between the intensity of destructive processes of a particular disease and the regenerative processes linked to stem cells? Or do regenerative processes become compromised and, if this is the case, what are the mechanisms and how this ineptitude could be overcome? These straightforward enquiries will guide us next to the thick of the stem cell cycle, from quiescence and maintenance of stemness to mobilization, engraftment and differentiation into a mature cell.

The idea that stem cells may become dysfunctional in the course of a disease derives from the demonstration of their defective regenerative capacity, compromised clonogenicity, migration and capillary formation. Similar to all somatic cells, stem cells are subjected to various exogenous and endogenous stressors that could impair their competence; however, such impairment of stem cells has much more ominous consequences owing to their extended lifespan. There are plenty of examples of stem cell incompetence developing in the course of a disease. For instance, patients with systemic lupus erythematosus manifest reduced levels of circulating hematopoietic stem cells (HSCs) and endothelial progenitor cells (EPCs) even during remission [1]. Mesenchymal stem cells (MSCs) are functionally abnormal in immune thrombotic microangiopathy [2]. Aging is the most common cause of stem cell dysfunction [3]. Recent studies showed that bone marrow-derived cells (BMDCs) and bone marrow-derived EPCs from db/db mice are functionally incompetent,

Regenerative Nephrology DOI: 10.1016/B978-0-12-380928-5.10018-1

whereas adoptive transfer of BMDCs from syngenic non-diabetic dbm mice significantly improved vasculopathy and insulin sensitivity, and partially improved renal function in db/db recipients [4]. Hyperglycemia has been reported to reduce survival and impair the function of circulating EPCs [5]. In addition, EPC dysfunction has been documented in type 1 and 2 diabetes, coronary artery disease (CAD), atherosclerosis, vasculitis with kidney involvement and end-stage renal disease [6 11]. There is emerging evidence that senescence may serve as an important mechanism mediating EPC dysfunction. Decreased numbers and an increased proportion of senescent EPCs has been reported in patients with pre-eclampsia or hypertension [5,12]. Angiotensin II can induce EPC senescence through the induction of oxidative stress and its influence on telomerase activity [13]. Oxidized low-density lipoprotein (OxLDL) induces EPC senescence and dysfunction [14]. Collectively, these data are supportive of the "stem cell aging" hypothesis of organismal aging [15]. There is emerging evidence that senescence may serve as an important mechanism mediating EPC dysfunction. Dzau et al. [16] summarized clinical situations associated with abnormalities in the number and function of EPCs (Table 18.1).

TABLE 18.1 Diseases Characterized by Alterations in EPC Levels and Function

Disease	Number	Function
CARDIAC		
CAD	Reduced	Reduced
CHF	Reduced	Reduced
Unstable angina	Reduced	ND
Myocardial infarction	Increased	Reduced
VASCULAR		
Atherosclerosis	Reduced	Reduced
Acute injury/inflammation	Increased	ND
Peripheral limb ischemia	Increased	ND
Transplant arteriopathy	Reduced	ND
In stent restenosis	Reduced	ND
Hypertension	Reduced	Reduced
Hyperlipidemia	Reduced	Reduced
Diabetes	Reduced	Reduced
CHRONIC KIDNEY DISEASE		
Hemodialysis	Reduced	Reduced

CAD: coronary artery disease; CHF: congestive heart failure; ND: not determined.
From Dzau et al. (2005) [16].

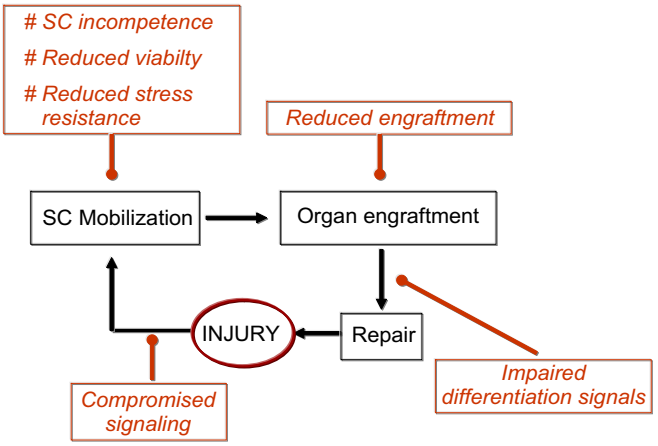

FIGURE 18.1 Potential sites of stem cell (SC) incompetence resulting in disruption of regenerative processes.

The precise definition of stem cell incompetence is lacking. Based on the accepted functions of stem cells, i.e. self-renewal and asymmetric (less proven in mammals) proliferative capacity, mobilization, recruitment and engraftment, cell-dependent or paracrine signaling, viability and resistance to the existing stressors [17], it can be extrapolated that stem cell incompetence is characterized by the dysfunction of at least one of these properties, as depicted in Fig. 18.1, which illustrates the cycle of injury to regeneration involving all these functions of stem cells and sites where these functions can be perturbed.

From the outset, it should be recognized that an element of uncertainty creeps into all methods of detection of stem cell populations during this train of events. Considering the stem cell cycle, its identifiable feature is that along the path of self-replication, differentiation and maturation an individual cell undergoes perpetual metamorphosis (Fig. 18.2) marked by the changing landscape of the defining markers. Unless there is a one-to-one exchange of a maturing cell with a differentiating

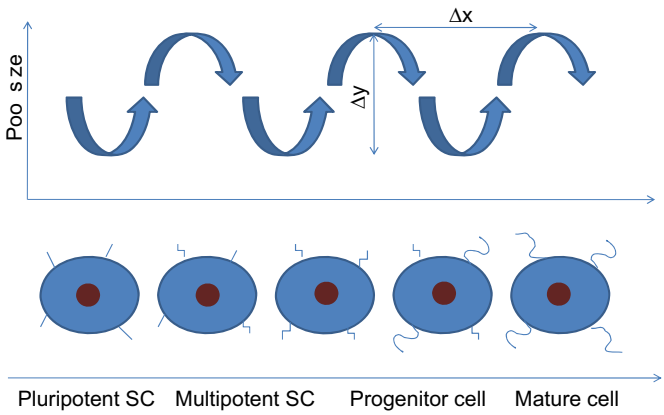

FIGURE 18.2 Changing pool size and epitope markers during stem cell (SC) maturation create problems with their detection.

one, which is quite unlikely, the size of the pool of cells characterized by each set of markers should have an oscillatory profile. Thus, each measurement of the population of such cells inevitably represents a snapshot, while the broader picture of oscillations remains hidden from view. Until accelerating and inhibitory feedback loops along the stem cell cycle are better refined and integrated into the whole picture, it will be difficult to tell whether any particular measurement was performed at the acme or nadir point of the curve. Accepting this uncertainty may help us to understand some discrepancies in the experimental results.

Hence, examples of stem cell dysfunction abound and some general or cell-specific mechanisms can be discerned, as outlined below.

MOLECULAR MECHANISMS OF STEM CELL EXHAUSTION

Reactive Oxygen Species

In general, most adult stem and progenitor cells show superior resistance to acute challenge with oxidants owing to their higher levels of glutathione and superoxide dismutases [18] compared with their mature progeny. Chronic challenge with oxidants, however, dissipates this advantage. Ito et al. [19] demonstrated that chronic states of oxidative stress induced either by an inhibitor of glutathione synthesis or by deletion of Atm (ataxia telangiectasia mutated) gene, acting through the activation of p38 mitogen-activated protein (MAP) kinase, shorten the lifespan of HSCs through increase in proliferation, resulting in stem cell exhaustion and bone marrow failure, expressed as defective bone marrow reconstitution. Fanconi anemia is an example of reactive oxygen species (ROS)-induced impairment of hematopoiesis [20]. Mitochondrial dysfunction and increased generation of ROS may be involved in the developing stem cell incompetence [21].

Another example of ROS-induced stem cell incompetence is represented by Adriamycin (doxorubicin) toxicity. Adriamycin toxicity has been ascribed in part to mitochondrial dysfunction due to the excessive production of ROS [22,23]. Potential downstream signaling of ROS may explain why Adriamycin-associated nephropathy (AAN) has been attributed to activation of p38 MAP kinase and transforming growth factor-β_1 (TGF-β_1)/Smad signaling [24]. It is instructive that both the kidney-resident side population cells, as well as the side population-excluded main population of cells, adoptively transferred to mice with AAN decreased proteinuria [25]. Deleterious effects of Adriamycin on glomerular endothelial cells have been linked to the development of proteinuria [26]. Vascular dysfunction manifesting in blunted responses to acetylcholine was also demonstrated in in vitro studies of isolated thoracic aorta obtained from Adriamycin-injected rats [27], arguing for the development of endothelial dysfunction under this condition. The viability of EPCs after Adriamycin administration was examined in vitro [28]. EPCs obtained from AAN mice showed impaired clonogenicity. Cultured EPCs treated with various doses of Adriamycin exhibited apoptosis, necrosis and signs of stress-induced premature senescence. Previous studies on Adriamycin nephropathy showed that bone marrow-derived cells engrafted the kidneys, and transdifferentiated into endothelial cells and myofibroblasts, which coexpressed endothelial markers suggestive of endothelial mesenchymal transformation [29]. In the case of Adriamycin-associated nephropathy, EPCs also develop signs of impaired viability, premature senescence and functional incompetence, as judged by the higher frequency of apoptosis, staining for senescence-associated β-galactosidase and reduced clonogenic potential. The finding that exogenous intact EPCs improve structural and functional consequences of Adriamycin administration [28] not only further supports the role of these cells in kidney regeneration, but also argues in favor of EPC competence as the necessary prerequisite for accomplishing regenerative functions. Other examples of ROS-induced stem cell incompetence have been recently reviewed [30] and are presented below in the context of various initiating factors.

Proinflammatory Cytokines

Cytokines modulate various aspects of stem cell behavior. In MSCs pretreated with dexamethasone and committed to osteogenic differentiation, interleukin-6 (IL-6) and its soluble receptor (sIL-6R) enhance osteoblastic differentiation [31]. This effect depends on the expression of Gp130 and requires phosphorylation of Stat-3. Indeed, IL-6 is a robust marker of inflammation and endothelial dysfunction and its levels correlate with atherosclerotic burden in carotid arteries of patients with chronic kidney disease (CKD) [32]. Whether IL-6 can participate in the development of vascular calcification by misdirecting vascular wall MSC differentiation remains to be examined. IL-6 and tumor necrosis factor-α (TNF-α) inhibit differentiation and maturation of a broad range of stem cells, including neuronal, whereas IL-4 promotes these processes [33]. Macrophage inhibitory protein-1α (MIP-1α) inhibits proliferation of multipotent stem cells [34], although these data could not be confirmed (M. Ratajczak, personal communication). TGF-β_1 can also provide inhibitory instructions for stem cell proliferation [35], although this issue is in need of re-evaluation. Members of the TGF-β family, bone morphogenetic proteins (BMPs), have emerged as negative regulators of proliferation

in stem cells of intestinal crypts and hair follicles [36]. In combination with leukemia inhibitory factor (LIF), BMPs, acting via Smad pathway on Id genes, suppress differentiation of embryonic stem cells and maintain their self-renewal [37].

The chemokine RANTES (regulated on activation, normal T cell expressed and secreted) promotes homing of bone marrow-derived endothelial progenitors to inflamed glomeruli, although in the particular case of a rat model of anti-Thy-1.1 glomerulonephritis induced by mesangiolysis, manipulation of EPC homing does not appear to affect the outcome [38]. IL-10 secretion by monocytes/macrophages activated by LPS is enhanced by the reprogramming executed by bone marrow-derived MSCs in a cyclooxygenase- and Toll-like receptor-4 (TLR-4)-dependent manner, thus resulting in better survival of endotoxemic mice [39].

Delta-like-1/fetal antigen-1 (dlk1), a member of the epidermal growth factor-like homeotic protein family known as a modulator of MSC and HSC differentiation, is overexpressed during liver and muscle regeneration and at early stages of liver fibrosis, and has been found to stimulate IL-1α, IL-1β, IL-6, CXCL1 (growth-related oncogene-α/keratinocyte), CXCL2 (macrophage inflammatory protein-2/GRO-β) CXCL3 (GRO-γ), CXCL6 (granulocyte chemotactic protein-2), CXCL8 (IL-8) and CCL20 (MIP-3α) in human MSCs, where it inhibits adipocytic and osteoblastic differentiation [40].

Proatherogenic Risk Factors

It has been demonstrated that atherosclerotic risk factors inversely correlate with the number of circulating EPCs [6]. Hyperglycemia, type 1 and 2 diabetes and hypertension are well-established causes of EPC dysfunction [5]. OxLDL inhibits vascular endothelial growth factor (VEGF)-induced differentiation of EPCs via deactivation (dephosphorylation on Ser^{473}) of the serine/threonine kinase Akt pathway [41]. Several potential mechanisms mediating this effect of oxLDL exist: activation of protein phosphatase-2A or lipid phosphatases, such as phosphatase and tensin homolog deleted on chromosome 10 (PTEN) or inositol-5 phosphatase-2 (SHIP-2) inactivating phospholipid products of PI-3 kinase. Many effects of proatherogenic factors on stem cells are mediated via ROS. Much less information is available on the role of proatherogenic factors in modulating HSCs and MSCs.

mTOR–Wnt Pathway

The canonical Wnt/β-catenin pathway is one of the key regulators of stem cell self-renewal [42]. Consisting of 19 secretory cysteine-rich glycoproteins, Wnt signal via Frizzled receptors and members of low-density lipoprotein receptors LRP5 and LRP6 [43]. Activation of this pathway plays a role in embryonic development, tissue maintenance and regeneration, with persistent activation inducing various tumors or cell senescence [44,45]. Wnt3A and Wnt5A are essential in modulating self-renewal of HSCs [43]. An inhibitor of Wnt signaling, Dickkopf-1, is necessary for HSC re-entry into the cell cycle [46]. Recent findings in epidermal stem cells indicate that prolonged Wnt signaling results in a mammalian target of rapamycin (mTOR)-mediated exhaustion of stem cells and their premature senescence [47] and mTOR inhibitor, rapamycin, protects stem cells against developing senescence. This mechanism of developing senescence is considered as epithelial protection against uncontrolled proliferation and tumorigenesis. Another inhibitor of mTOR, the regulated in development and DNA damage-1 (REDD-1), is a hypoxia-induced protein that may have relevance to diverse pathological conditions [48]. Other potential targets of Wnt signaling include increased expression of *HoxB4* and *Notch1* genes [49]. Extensively studied in embryogenesis, the role of Notch signaling is much less examined in adult stem cells. There is, however, evidence that it can restore regenerative capacity of skeletal muscle of old mice and its inhibition impairs it [50].

Adrenergic and GABA Receptor Stimulation

Hematopoietic Lin Sca^{+} progenitor cells (HPCs) express adrenergic receptors. Catecholamine stimulation of HPCs results in enhanced formation of ROS, activation of the p38/MAPK pathway and decreased capacity to form granulocyte/macrophage colonies, which is reversible upon antioxidant or p38 inhibition treatment [51]. Adrenergic stimulation also leads to transcriptional activation of p16, invoking the possibility of cell cycle arrest and premature senescence of HPCs. This mechanism, prevalent in aging, congestive heart failure and a variety of other conditions, may have important implications for defective regenerative and immune defense processes.

Activation of γ-aminobutyric acid-A ($GABA_A$) receptors negatively controls embryonic and neuronal stem cell proliferation independent of differentiation, apoptosis or damage to DNA [52]. The effects of GABA are transduced via S-phase checkpoint kinases and the histone H2AX. With the broadening specter of GABA receptor expression, its role in adult stem cells remains to be discovered.

Micro-RNAs as Potent Modifiers of Stem Cell Proliferation and Differentiation

The past decade has witnessed the emergence of micro-RNAs (miRNAs) as key regulators of stem cell

proliferation and differentiation in embryonic development and in adult life [53]. It is estimated that humans harbor about 1000 different miRNAs, 19 25 nucleotide-long non-coding RNA transcripts, and 700 of them had been confirmed at the time of writing (sequences deposited in http://microrna.sanger.ac.uk). The complexities of their synthesis have been partially decoded. RNA polymerases II and III generate long primary miRNA transcripts, which undergo cleavage by nuclear RNase Drosha and DiGeorge syndrome critical region gene 8 (DGCR8) protein to produce an ≈70 nucleotide-long precursor, (pre)-miRNA, that is exported to the cytoplasm and employs another RNase, Dicer, to generate the mature miRNA. Mature miRNA in turn forms a complex with Argonaute proteins, a catalytic portion of the RNA-induced silencing complex (RISC), binds to the 3′ untranslated region (3′UTR) of target RNAs, and either accelerates their degradation (perfect complementation) or induces translational suppression (imperfect complementation). The predominant miRNAs expressed in embryonic stem cells are limited to four loci: miR-290 295 (most abundant), miR-15 16, miR-17 92, and miR-21 clusters. In *Dicer1* or *DGCR8* null mice, ESCs lack expression of the pluripotent marker Oct4, fail to proliferate, and embryonic lethality occurs on E7.5 [54]. In contrast, conditional *Dicer1* mutant mice are viable and although they express Oct4, cells fail to differentiate towards endodermal and mesodermal lineages upon stimulation [55], suggesting that one of the main functions of miRNAs is to permit differentiation by silencing pluripotency factors.

One of the silencing mechanisms is attributed to miR-134, which is induced by retinoic acid and targets two positive regulators of Oct4, Nanog and liver receptor homolog-1 (LRH-1) [56]. In adult HSCs, miR-181 appears to be responsible for differentiation towards B-lymphoid lineage without affecting T-cell lineage, whereas overexpression of miR-223 and miR-142 is responsible for upregulation of the T-lymphoid lineage [57].

Cell cycle progression represents another target of miRNAs. Along these lines, the miR-290 cluster represses cyclin E-cdk2 inhibitors [58] and the miR-302 cluster targets cyclins D1 and D2 [59], thus promoting rapid G1-S checkpoint transition characteristic of ESCs. A similar function is executed by miR-92b, which represses the cyclin-dependent kinase inhibitor p57 [60]. In contrast, miR-24, which is upregulated during physiological terminal differentiation of HSCs, muscle and neuronal cells, inhibits cell cycle progression by repressing E2F2 [61], an example of its role in differentiation to the mature phenotype.

Regulation of miRNA biogenesis by cytokines is exemplified by TGF-β/BMP signaling that induces miR-21 expression with the subsequent downregulation of the programmed cell death 4 (PDCD4) [62]. miR-221 and miR-222 inhibit normal erythopoiesis and modulate angiogenesis by targeting c-Kit receptor for stem cell factor [63,64].

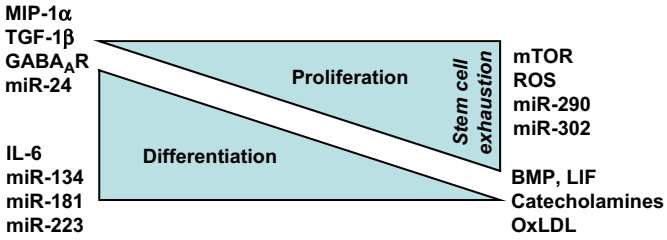

FIGURE 18.3 Modulators of differentiation and proliferation of stem cells. See text for the details. MIP: macrophage inhibitory protein; TGF: transforming growth factor; miR: micro RNA; IL: interleukin; mTOR: mammalian target of rapamycin; ROS: reactive oxygen species; BMP: bone morphogenetic protein; LIF: leukemia inhibitory factor; OxLDL: oxidized low density lipoprotein.

So far, the role of miRNAs in developing stem cell incompetence has been underinvestigated. Expression profiling of miRNAs in senescing mouse embryo fibroblasts revealed that miR-290 is commonly upregulated [65] and it correlates with other senescence-associated genes such as *p19Arf*, *p16* and *p21*. This pathway is also involved in stress-induced premature senescence triggered by nocodazole treatment. The subject is ripe for investigation of miRNAs in various forms of stem cell dysfunction.

In conclusion, these molecular mechanisms (Fig. 18.3), solely or in combination, contribute to stem cell incompetence which manifests itself in the diverse dysfunctional states, as discussed below.

IMPAIRMENT OF STEM CELL FUNCTION

Mismatched Niche Capacity

The ability of stem cell niches to adjust their capacity to harbor stem cells in accord with physiological requirements and the loss of this capacity under diverse pathological conditions have been recently noticed. Zhang et al. [66] and Calvi et al. [67] have disclosed complex relations within the bone marrow osteoblastic HSC niche intricately regulated by parathyroid hormone and bone morphogenetic protein leading to the increased niche capacity. The questions related to the capacity of niches have come into focus in investigations of cancer cells, which may create their own niche and/or hijack the pre-existing ones [68]. Moreover, the first evidence linking stem cell niches with disease initiation and progression has arrived from studies of primary myelofibrosis, which is associated with an imbalance between endosteal and vascular niches resulting in clonal cell proliferation [69].

In an attempt to visualize potential sites of stem cell residence, MSC used as a bait were used presented to ex vivo control and postischemic kidney sections, and stem cell adhesion was examined as a surrogate marker of niche capacity [145]. Specificity of MSC adhesion was confirmed by demonstrating that (i) 3T3 cells displayed 10-fold lower adhesion, and (ii) MSC adhesion was CXC chemokine receptor-4 (CXCR4)/stromal cell-derived factor-1 (SDF-1) dependent. MSC adhesion was asymmetrical, with postischemic sections exhibiting greater than two-fold higher adhesion than controls, and showed a preference for perivascular areas. Pretreatment of kidney sections with cRGD resulted in increased MSC adhesion (through displacement of resident cells), whereas blockade of CXCR4 (with AMD3100) or inhibition of $\alpha_4\beta_1$(VLA4) integrin or its counterpart, vascular cell adhesion molecule-1 (VCAM-1), reduced adhesion. The difference between adhered cells under cRGD and control conditions reflected prior occupancy of the binding sites with endogenous cells. AMD3100-inhibitable fraction of adhesion reflected CXCR4-dependent adhesion, whereas maximal adhesion was interpreted as the MSC-lodging capacity of the kidney. The data demonstrated that (i) CXCR4/SDF-1-dependent adhesion increases in ischemia, (ii) preoccupancy of MSC binding sites is decreased, and (iii) the capacity of MSC binding sites is expanded in postischemic kidneys. A similar technique was used to study MSC adhesion to myocardium obtained from control and Adriamycin-treated mice, which revealed that Adriamycin cardiomyopathy is associated with increased niche capacity and preoccupancy, with only a minor CXCR4/SDF-1 dependence of adhesion (unpublished observations). This field remains in its infancy and requires further exploration.

Impaired Mobilization

Impaired stem cell mobilization can occur owing to the inadequacy of mobilizing signals, their shielding or impaired detachment from their niches. Hematopoietic stem and progenitor cells, akin to lymphocytes, exhibit constitutive trafficking routes from the bone marrow to various extramedullary organs, where they reside for several days and then return to the bloodstream via lymphatics [70]. Sphingosine-1 phosphate receptors govern the egress of stem cells from the tissues to lymphatic capillaries. This sentinel cycle allows for replenishment of organ-resident stem cells and for their extramedullary proliferation upon local activation by TLR agonists. Along this route, sphingosine-1 phosphate and receptors and TLRs can be potentially targeted by disease processes, resulting in either impaired drainage or excessive local proliferation.

A classical mobilization agent, granulocyte colony-stimulating factor (G-CSF), executes dislodgment of stem cells from the bone marrow niche by activating neutrophil elastase, which in turn cleaves SDF-1 [71]. A closely related granulocyte/macrophage CSF mobilizes 5 100-fold higher numbers of HSCs, MSCs and EPCs compared with baseline levels [72]. Caveolin-1 deficiency blocks efficient dimerization of CXCR4, thus facilitating stem cell mobilization, but negatively affecting stem cell engraftment [73], whereas its overexpression may result in enhanced SDF-1/CXCR4 binding in the bone marrow and defective mobilization.

Mobilization of EPCs from their respective niches can be accomplished by mechanical injury and ischemic stress through generation of hypoxia-inducible factor-1 (HIF-1)-regulated release of vascular endothelial growth factor, erythropoietin and SDF-1, as well as by placental growth factor, G-CSF and granulocyte macrophage colony-stimulating factors [74 79]. Some proinflammatory cytokines, such as IL-8, are potent stimulators of EPC and HSC mobilization, thus linking this response to stressors with proinflammatory conditions [79]. An additional mechanism for EPC mobilization has been attributed to apoptotic endothelial microparticles [80], a feedback mechanism of a well-timed surge in stem cells when injury occurs. Microparticles (microvesicles) shed from the live cells have been implicated in the bidirectional transfer of genetic information between stem cells and injured somatic cells [81]. In relation to the kidney, microvesicles derived from injured tubular epithelia may induce phenotypical changes in MSCs through expression of some tubular cell markers [82].

Several investigators have addressed the question of whether stem cells can be efficiently delivered to areas of tissue ischemia to preserve or restore end-organ function by participating in vasculogenesis. In a model of myocardial infarction in bone marrow-transplanted mice, histological analysis showed donor-derived endothelial cells in areas of neovascularization at the border zone of the infarct [83]. These experimental data have been strongly supported by clinical observations. Adams et al. [84] found increased levels of circulating EPCs in patients with CAD after exercise-induced myocardial ischemia. Lambiase et al. [85] showed an inverse correlation between the density of coronary collaterals and numbers of peripheral EPCs in CAD. Such an inverse correlation between the number and function of circulating EPCs and the severity of various chronic cardiovascular and renal diseases is a rather general rule [86].

Pharmacological mobilization of EPCs can be achieved using statins, VEGF, erythropoietin, angiotensin-converting enzyme (ACE) inhibitors and estrogens, to

name a few [87 91]. These agents exert their effects at least in part via activation of endothelial nitric oxide synthase (eNOS), making nitric oxide (NO) production an obligatory part of the response to mobilizing agents. Mice deficient in eNOS showed reduced mobilization [92]. Considering the fact that eNOS is usually uncoupled (the state of dissociation of C-terminal reductase domain from the N-terminal oxygenase domain resulting in the reduced generation of NO, enhanced generation of the superoxide anion and peroxynitrite) in many diseases, and in view of the fact that mobilization of EPCs is dependent on functional eNOS, it is not surprising that this function may become impaired.

Acute ischemia is one of the potent signals to mobilize EPCs; this has been unequivocally documented in humans and in experimental animals with myocardial ischemia, ischemic stroke and renal ischemia [84,93 96]. Despite the apparent universality of this response to ischemic insult, the precise molecular mechanisms responsible for it remain uncertain.

What are stress signaling (SOS) molecules, produced by and discharged from the ischemic tissue, which are capable of downstream mobilization and recruitment of stem and endothelial progenitor cells? Uric acid and high-mobility group protein (HMGP) are prototypical alarm signals activating the innate immune system, which exhibit a short-lived surge after ischemia reperfusion injury. Previous studies demonstrated that exogenous uric acid leads to a rapid mobilization of EPCs and HSCs and protection of the kidney against ischemic injury [96]. Kuo et al. [97] demonstrated that monosodium urate (MSU) in vitro and in vivo resulted in exocytosis of Weibel Palade bodies with the release of IL-8, von Willebrand factor (vWF) and angiopoietin-2 into the culture medium and circulation, respectively. In TLR-4-deficient mice, acute elevation of uric acid level by injection of MSU did not result in the release of vWF and angiopoietin-2 to the circulation, suggesting that uric acid affected exocytosis of Weibel Palade bodies via this receptor. The release of IL-8 in response to elevated uric acid level required both TLR-2 and TLR-4. These findings outline a novel paradigm linking postischemic repair and inflammation via the release of the constituents of Weibel Palade bodies and further broaden the spectrum of alarm signaling to establish constituents of Weibel Palade bodies as potential second messengers not only for proinflammatory responses but also for mobilization of stem cells. The important feature of this response is that it is only a brief transient surge of uric acid that has this signaling function, whereas chronic elevation of uric acid not only lacks this type of alarm signaling, but inhibits ischemia-induced mobilization [96].

Interactions between HSCs and stromal cells are mediated in part through $\alpha_4\beta_1$ (VLA4)/VCAM-1 interaction [98]. Accordingly, inducible ablation of $\alpha_4\beta_1$ (VLA4) or conditional ablation of VCAM-1 is associated with the enhancement of G-CSF-induced mobilization of HSCs [99 101]. An additional mechanism of leptin-induced upregulation of $\alpha V\beta_5$ and α_4 integrins may explain enhanced adhesion to neointimal lesions [102], but the same phenomenon would appear to inhibit EPC mobilization, thus requiring further analysis. The issue of adhesion molecules participating in stem cell adhesion to respective niches has been comprehensively reviewed elsewhere [103]. There remain multiple unanswered questions related to the efficacy of stem cell mobilization by intrinsic or pharmacological means in CKD, which need to be addressed in future studies.

Impaired Engraftment

The process of engraftment is just as complex as is a similar process of leukocyte egress; it includes tethering, adhesion, transmigration and final lodging of stem cells from the circulatory bed to the tissues. Adhesion and transmigration are initiated in part by integrins. The pathways involved in homing of bone marrow-derived stem cells have been identified as $\alpha_4\beta_1$-integrin and thymosin β_4 [104].

Many ligands participate in the recruitment of stem cells to specific tissues. Homing of HSCs, EPCs and a subpopulation of MSCs which expresses CXCR4 is governed mainly by the α-chemokine SDF-1 [105,106]. Both CXCR4 and SDF-1 are expressed in the normal kidney and their expression is already enhanced 24 h after ischemia [107]. Impaired engraftment can occur owing to reduced CXCR4/SDF-1 expression or function.

There are many factors affecting binding of SDF-1 to its cognate G-protein-coupled seven-transmembrane domain receptor CXCR4 [105]. SDF-1/CXCR4 binding activates several pathways, such as FAK/Paxillin, PI-3K/Akt, MEK, Jak/STAT, nuclear factor-κB (NF-κB), matrix metalloproteinases (MMPs) and phosphatases Ship 1, 2 and CD45, thus regulating cell motility and chemotaxis, adhesion, survival and secretion. Upon receptor activation, CXCR4 undergoes oligomerization/dimerization, a process that takes place in lipid-rich domains and requires caveolin-1 expression [73]. Other potential ligands engaged in homing of stem cells, albeit significantly less explored, include chemokines MCP-3 (homes MSCs) and GRO-1 (homes bone marrow-derived EPCs, especially in tumors).

The knowledge of where to home may be also affected by impaired niche capacity, as discussed above.

Impaired Signaling and Transformation

Impaired signaling and transformation of stem cells can occur as a result of either perturbed paracrine secretion or differentiation into a mature cell. The capacity of multipotent stem cells to differentiate towards mature cells of diverse lineages has a stochastic and an instructive component. Usually, it is most probably the latter that is in error under pathological conditions. Mesenchymal stem cells undergo osteogenic differentiation when stimulated by epidermal growth factor (EGF), but not by the closely related platelet-derived growth factor (PDGF). Proteome-based comparison of tyrosine phosphorylated proteins in MSCs showed that more than 90% overlapped upon stimulation with either agonist, whereas the phosphatidylinositol protein kinase B pathway was activated exclusively by PDGF [108], suggesting that this kinase represents a checkpoint for MSC differentiation. Blocking this pathway converts the effect typical of PDGF to that resembling EGF. It remains to be seen whether this mechanism may participate in the development of ectopic calcification.

The ability of circulating progenitors to differentiate towards endothelial or smooth muscle cell lineages has been furthered by studies of a parabiotic model in which a wild-type mouse and a transgenic mouse expressing green fluorescent protein (GFP) are conjoined subcutaneously via anastamosing circulations. Tanaka et al. [109] demonstrated that mechanical injury to femoral arteries of wild-type mice resulted within 4 weeks in a chimerism of cells comprising developing neointima: 15% and 31% of parabiotic partner-derived GFP-positive cells were detected in the intimal and medial layers, respectively, with some cells expressing α-smooth muscle actin (α-SMA), others CD31. Studies using adoptive transfer of MSCs to mice with acute renal ischemia showed that in this in vivo model the majority of transplanted cells differentiated towards α-SMA-expressing cells and a smaller portion towards CD31-expressing cells, whereas in vitro the proportions were reversed [110].

A dramatic example of the inappropriate TGF-β signaling resulting in hypercellular lesions of different podocytopathies has been described by Smeets et al. [111], demonstrating that the population of $CD133^{+}CD24^{+}$ renal progenitors proliferated and deposited extracellular matrix, thus contributing to the development of the so-called tip lesion and crescent formation.

Mechanical stress may be another important instructive signal for stem cell differentiation decision. When bone marrow MSCs were subjected to flow- or pressure-induced stress, as opposed to static culture conditions, a 12-fold higher proportion of the cells acquired smooth muscle/myofibroblastic characteristics [112]. These observations may have far-reaching significance for fibrotic processes where distorted or enhanced mechanical forces exerted by fibrogenic transformation of the parenchyma could bias the differentiation decisions.

Impaired Self-protection

The loss of stem cell viability and resistance to ongoing stress may occur in various disease states associated with oxidative stress. Ito et al. [19] convincingly demonstrated that chronic states of oxidative stress induced either by an inhibitor of glutathione synthesis or by deletion of Atm (ataxia telangiectasia mutated) gene, shorten the lifespan of HSCs through an increase in proliferation, stem cell exhaustion and bone marrow failure, expressed as defective bone marrow reconstitution. This is an example of ROS inducing an accelerated type of replicative senescence. This author's group demonstrated that oxidative stress in type 2 diabetic db/db mice is associated with an increased proportion of apoptotic EPCs under basal conditions and reduced viability under conditions of stress [4]. Similar findings were described in type 2 diabetic patients: EPCs exhibited impaired proliferation, adhesion and incorporation into vascular structures [113]. Evidence was provided that pharmacological restoration of stem cell competence, both ex vivo and in vivo using a seleno-organic antioxidant and peroxynitrite scavenger, is possible [4]. Holmen et al. [114] described a most intriguing phenomenon whereby circulating inflammatory endothelial cells expressing two inflammation-associated markers, vascular adhesion protein-1 and major histocompatibility complex (MHC) class I-related chain A, in addition to high levels of inducible NOS and neutrophil-activating chemokines, contribute to the impairment of proliferation, migration and clonogenicity of EPCs in patients with Wegener granulomatosis. This mechanism may have much broader pathological implications.

To draw a preliminary conclusion, stem/progenitor cells are differentially affected in different models of CKD. For instance, in Adriamycin nephropathy the prevailing defect could be traced to the inability of stem/progenitor cell homing to the kidney owing to the cytotoxic effect of this anthracycline antibiotic; in diabetic nephropathy the defect may be explained predominantly by oxidative stress and reduced viability of stem/progenitor cells; in unilateral ureteral obstruction, pathology of stem/progenitor cell predilection towards myofibroblastic differentiation may be palpably responsible for the ensuing tubulointerstitial fibrosis and scarring. These hypothetical pathogenic mechanisms require in-depth investigation, as the delineation of the causes of stem/progenitor cell incompetence is a prerequisite to designing effective therapies.

IN VIVO AND EX VIVO THERAPY OF STEM AND ENDOTHELIAL PROGENITOR CELLS

The success of several therapeutic interventions has been linked in part to their effect on stem cells. These therapies have been comprehensively reviewed [115]. The effect of peroxisome proliferation-activated receptor-γ (PPAR-γ) agonists has been recognized [116]. Aged mice treated with growth hormone or insulin-like growth factor-1 (IGF-1) showed improvement in EPC number and function, the effect mediated via an increase in telomerase activity and expression and activity of eNOS due to phosphorylation by phosphoinositide-3-kinase [117]. Similar improvements in vasculopathy and insulin resistance were documented in db/db mice treated with ebselen, which is a peroxynitrite scavenger, an antioxidant and a glutathione peroxidase mimetic seleno-organic compound [4].

However, the most daring approaches to improve stem cell function involve ex vivo therapy of isolated cells. An example of this approach is a striking improvement in vascular and renal function of db/db mice by ex vivo treatment of bone marrow-derived cells with ebselen before their retransplantation to db/db mice [4]. Attempts at genetic engineering for therapy of EPCs ex vivo involved overexpression of eNOS or heme oxygenase-1 [118], which resulted in inhibition of neointimal hyperplasia. Overexpression of telomerase reverse transcriptase resulted in the improved neovascularization of ischemic limb [119]. Pretreatment of bone marrow-derived cells or EPCs with eNOS enhancers improved the regenerative capacity of these cells infused intravenously in models of hindlimb ischemia and acute myocardial infarction [120]. Pharmacological inhibition of MAP-kinase p38 improves the functional properties of circulating stem cells [121]. In addition, preconditioning studies are underway to use nonspecific caspase inhibitors, antioxidants and gene therapy to overexpress HIF-1, Bcl-2, Akt and heat shock proteins to improve stem cell functions [122]. These, as yet tentative, mechanistic attempts at correcting stem cell incompetence and improving their survival and homing await future refinement and verification.

POTENTIAL MECHANISMS OF STEM CELL-INDUCED ORGAN REPAIR

The participation of EPCs in reparative processes has been the subject of several excellent reviews [123 125]; therefore, this issue will be discussed only briefly.

Direct incorporation of stem cells substituting for the damaged cells has been initially advocated, but by and large refuted. Towards this end, incorporation into a presumably defective intimal layer has been demonstrated by Asahara et al. [126]. EPC transplantation into diabetic mice resulted in vascular engraftment and restoration of blood flow in hindlimb ischemia [127]. Cross-grafting aortic segments between Balb/c and Tie-2/LacZ mice demonstrated chimerism of endothelial cells in the intimal layer, thus arguing in favor of EPC incorporation into the vessel [128]. This process may be facilitated and directed in part by platelet adhesion to the site of vascular injury, resulting in adhesion and maturation of circulating EPCs to endothelial cells [129]. Usually, the direct EPC engraftment varies within the 1 25% range [130]. There is growing debate over whether bone marrow-derived cells or EPCs actually repair vascular endothelium or injured cardiomyocytes [131,132] with the emphasis shifting towards progenitors within the vascular wall. Recent studies on MSC adhesion to the surface of the kidney serving as a surrogate marker of stem cell niches revealed that perivascular adhesion is a preferred site (Ratliff et al., unpublished observations). In addition to direct stem cell substitution for the injured mature cells, various pathways of indirect communication between these partners are emerging, as summarized in Fig. 18.4 and described below.

Paracrine effects of stem cells. Proteomic screening of the medium conditioned by the embryonic fibroblasts revealed that IGF-2 is the most prevalent among the growth factors supporting survival and self-renewal of pluripotent human stem cells [136]. Basic fibroblast growth factor (bFGF) stimulates production of IGF-2 and a host of TGF family members in this paracrine interaction. Conditioned medium from MSCs

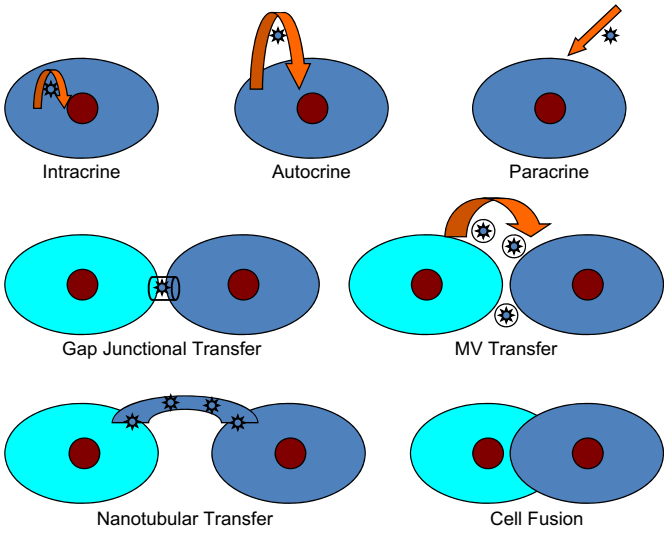

FIGURE 18.4 Modes of information transfer between stem cells and injured somatic cells. In addition to autocrine and paracrine mechanisms, stem cells employ gap junctional communication, microvesicular (MV) and nanotubular information transfer, and cell fusion [133–135]. *[Modified from Re and Cook, 2009 [133].]*

overexpressing Akt-1 has been reported to decrease the size of myocardial infarction and apoptosis in mice [137]; however, the secreted molecules responsible for this effect have not been reported.

Cultured peripheral or bone marrow-derived cells give rise to at least two populations of EPCs: early, VEGFR2$^+$ and VE-cadherin$^+$ cells coexpressing myeloid CD14 and pan-leukocytic CD45 (4 7 days) markers, and late outgrowth cells emerging after 2 3 weeks from CD14-non-myeloid population and expressing CD34, VEGFR2 and AC133 [123 125]. Both populations are capable of inducing neovascularization, but the mode of action differs. While early outgrowth EPCs have limited capacity for population doubling and induce transient angiogenesis, late outgrowth EPCs expand to more than 100 population doublings. Cell therapy with both populations results in enhanced engraftment and neovascularization in hindlimb ischemia [123 125]. Early outgrowth EPCs exert their angiogenic effect mainly by secretory products, whereas late outgrowth cells do so by direct engraftment. The secretome of EPCs has been studied using a combination of proteomic techniques which revealed angiogenic factors such as thymidine phosphorylase as a major agonist, MMP-9, IL-8, pre-B-cell enhancing factor and macrophage migration inhibitory factor, among others [138].

Cell cell fusion occurs on a one-to-one basis. Two types of fusion exist: homotypic cell fusion between similar cells resulting in multinucleated cells, and heterotypic cell fusion between cells of different lineages [139]. Both processes occur under physiological conditions and in chronic inflammatory processes, as for instance, in cell fusion of myelolymphoid cells with non-hematopoietic cells. Cell fusion events have been elegantly documented between bone marrow-derived and tubular epithelial cells after ischemic injury [140]. Notably, this mechanism of repair may be partially responsible for reprogramming or transdifferentiation, which hitherto was attributed to stem cell plasticity, and may represent a rescue mechanism whereby genetic material is rejuvenated.

Tunneling nanotube (TNT) formation between cultured cells has been described [141] and proven to be a viable mechanism of organellar exchange between the partners (Fig. 18.4). This mechanism has been shown to account for mitochondrial transfer between adult stem cells and somatic cells and rescue their respiration [142]. This mechanism, although difficult to demonstrate in vivo and therefore studied in cultured cells, is believed to play a significant role in intercellular communication. Yasuda et al. [28] presented human umbilical vein endothelial cells (HUVECs) with EPCs, each cell type labeled with differentially emitting fluorophores, and observed that exchange occurs under basal conditions. EPC-to-HUVEC flux increased three-fold after exposure of HUVECs to a cytotoxic dose of Adriamycin. The TNT mechanism of organellar exchange may provide the means for a single EPC to exchange organelles with multiple HUVECs.

Each of these mechanisms may be targeted by disease processes resulting in aberrant regeneration, even when the other functions of EPCs remain intact.

USE OF ARTIFICIAL NICHES TO STORE AND DELIVER ENDOTHELIAL PROGENITOR CELLS

One of the stumbling blocks of stem cell therapy consists of a low percentage of transplanted cells surviving in the circulation or after engraftment. This problem may be circumvented by delivering cell therapy in synthetic scaffolds. The cornerstone idea behind artificial stem cell niches investigated thus far is the generation of scaffolds with properties of the extracellular matrix [143]. The creation of artificial stem cell niches represents an attempt to mimic the natural niche by providing cells with a low-oxygen environment within these avascular scaffolds, but ensuring their ability to preserve the phenotype, quiescence, recruitability and protection from noxious stimuli. Hyaluronic acid hydrogels are capable of storing, propagating and differentiating stem cells [144]. Studies of EPCs encapsulated in an artificial stem cell niche manufactured from polymeric hyaluronic acid have been initiated, and demonstrated that it is a dynamic storage compartment that not only preserves cells and improves the resistance of EPCs to cytotoxic and genotoxic insults, but also allows for the recruitment of stem/progenitor cells on demand, and improves their engraftment of affected organs and their regenerative potential (see Chapter 16 for details).

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CHAPTER

19

Regeneration and Aging: Regulation by Sirtuins and the NAD^+ Salvage Pathway

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OUTLINE

INTRODUCTION

It is well accepted that preventing the insults that can lead to renal damage is preferable to managing renal disease once it has manifest. This is a feasible strategy in some instances, avoidance of high blood pressure being a prime example. However, as with many conditions, preventing renal damage often is not an option because the pathology has advanced beyond the point at which such a strategy is relevant. In this context, the notion of tissue regeneration is emerging an attractive potential therapy.

Although considered as a therapeutic strategy, regeneration is also an innate property of adult organs, albeit often of limited capacity. Indeed, strategies that harness the innate regenerative capacity of organs may prove to be an effective mode of regenerative medicine. However, the innate response to severe or sustained kidney damage is primarily fibrosis, not regeneration. This reality highlights a challenge to the field of regenerative nephrology, as the barriers to innate renal regeneration will also be barriers to therapeutic regenerative strategies.

Regenerative Nephrology DOI: 10.1016/B978-0-12-380928-5.10019-3

In this chapter, one of the major barriers to renal regeneration, namely cell and tissue aging, is discussed. Aging of the vasculature, a process that can profoundly limit functional repair and regeneration, will be emphasized. Molecular pathways that regulate cellular aging will be reviewed, focusing on the production and consumption pathways of the oxidized form of nicotinamide adenine dinucleotide (NAD^+), including sirtuin-1 (SIRT1)-dependent cascades, cascades that are emerging as key determinants of regeneration versus aging.

AGING OF THE KIDNEY

Aging of Parenchymal and Stromal Compartments

The histological hallmarks of renal aging include glomerulosclerosis, tubular atrophy, interstitial fibrosis and arterial thickening [1]. Thus, the aging process is not confined to the working parenchymal cells, but also involves the interstitial components of the kidney, including the vasculature (Fig. 19.1). This is important because, although regenerating renal parenchyma may be the primary goal of regenerative therapy, its success will depend on the status of the extracellular matrix, stromal cells and the vasculature.

The capacity of the kidney parenchyma to regenerate is evident by the proliferative response of renal epithelial cells. Normally, the proportion of tubular cells proliferating at any given time is less than 1%. However, a burst of proliferation can occur in response to acute damage, a response that serves to regenerate the tubules [2]. Importantly, this proliferative activity declines with age [3]. Regenerative proliferation may also come from progenitor cell in niches within the renal papilla, Bowman's capsule and proximal tubule [3,4], cells which will also be subjected to aging.

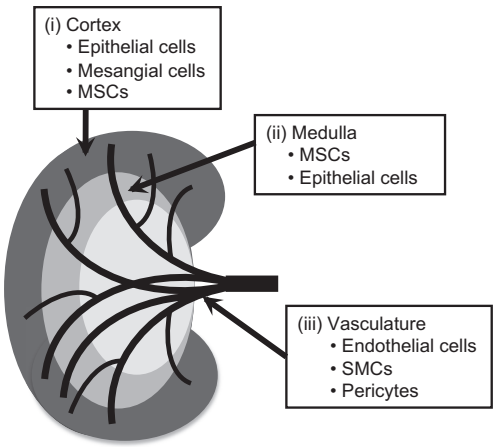

FIGURE 19.1 Aging of the kidney. The aging process in the kidney involves impairment of (i) the function and proliferative potential of renal epithelial cells, (ii) the repair, regenerative and cytokine control functions of mesenchymal stem cells (MSCs) in the interstitium, and (iii) the function, repair and regenerative capacities of endothelial cells and smooth muscle cells (SMCs) in the vascula ture. Aging of other potential progenitor cells, including pericyte or mesangial like cells, also warrants consideration.

In association with this, several age-related changes in the extracellular matrix (ECM) may themselves impede the ability of parenchymal cells to replicate. These changes include increased collagen cross-linking, collagen glycation, and oxidation, nitration and racemization of various ECM or ECM-regulatory proteins [5]. Studies on collagen glycation have been particularly informative in highlighting how the ECM can promote cellular aging. Endothelial cells cultured on glycated collagen display features of premature senescence, including senescence-associated β-galactosidase activity and upregulation of p53 and $p14^{ARF}$. Similar aging attributes are found in vivo in diabetic rats and a functional linkage with reduced nitric oxide (NO) availability has been found [6].

Aging of cellular elements of the interstitium can also be expected to impact the capacity of the kidney for regeneration. Mesenchymal stem cells (MSCs) can regulate regeneration in at least two ways. The classic stem cell paradigm may apply whereby MSCs divide occasionally to produce daughter cells that will produce committed lineages of mature differentiated cells. In this regard, kidney-derived mesenchymal cells have been found to differentiate towards endothelial and SMC lineages to support new blood vessel formation [7] and juxtaglomerular cells [8], among other phenotypes. In addition, there is growing evidence that MSCs can operate outside this paradigm and contribute to remodeling as a nidus of paracrine signaling [9]. Aging of MSCs will almost certainly compromise either paradigm. In general, there is a less permissive environment for mesenchymal cell remodeling with aging due to senescence of MSCs, telomere shortening and increased burden of reactive oxygen species (ROS) [10,11].

Aging of the Renal Vasculature

The aging vasculature can impact on renal function and its potential for regeneration in several ways. First, aged renal vasculature can lead to impaired autoregulation of the afferent and efferent arterioles. The resulting flow dysregulation can lead to hyperperfusion glomerular injury [12,13] and expansion of the mesangial matrix. The capacity to regenerate glomeruli will thus depend on the extent to which this aspect of vascular function can be preserved.

Another form of age-related vascular dysfunction is arterial or arteriolar occlusive disease leading to

ischemic injury. The resulting canonical damage cascade can drive both glomerular and tubulointerstitial pathology. Importantly, aging-related renal ischemia probably proceeds even in the absence of overt arterial occlusive disease. Studies in the rat kidney have identified aging-related increases in expression of key hypoxia-induced genes, including hypoxia-inducible factor-1α (HIF-1α) and vascular endothelial growth factor (VEGF) [14].

A third relevant feature of the aging vasculature is increased stiffness [15]. Arterial stiffness will increase pulse wave velocity and transmit uncompensated force to the distal microvasculature. This can have important consequences for renal function and it has been determined that albuminuria correlates with large artery stiffness [14,16,17].

Finally, emerging evidence suggests that vascular pericytes have putative roles as progenitor populations [18,19]. Although this has yet to be demonstrated for pericytes of the renal vasculature, pericytes from diverse vascular beds have been found to have broad multipotency, suggesting another means by which the aging vasculature could compromise organ regeneration. Collectively, therefore, aging of the vasculature can have diverse adverse consequences for kidney function and restorative capacity.

CELLULAR BASIS OF TISSUE AGING

Cellular Senescence

The cellular basis of tissue aging is the subject of a number of excellent reviews [20,21] and will not be reviewed in detail. However, one important paradigm of tissue aging is that of cellular senescence. Although best studied in culture, cell senescence is strongly implicated in age-related pathologies as well as the recognized decline in tissue regenerative potential with age [22].

Cellular senescence was first described by Hayflick and Moorfield, who observed that human fibroblasts replicating in culture eventually, and irreversibly, arrested their growth [23,24]. Growth arrest during senescence appears to be a programmed event and normally occurs in the G1 phase of the cell cycle. Importantly, however, senescence is not a mode of death. Provided ambient conditions are satisfactory, senescent cells remain viable and can maintain metabolic activity indefinitely [25]. Indeed, studies have revealed that senescent cells can be resistant to apoptosis [26] and show reduced autophagy [27]. However, senescent cells are not believed to be neutral with respect to tissue function. Their inability to replicate means that they cannot participate in reparative or homeostatic processes. Senescence of stem cells will similarly abrogate stem cell-based regeneration. In addition, senescent cells have been shown to be proinflammatory [28], further compromising tissue health.

Telomere-dependent and Telomere-independent Cell Senescence

One widely discussed hypothesis for explaining replicative senescence relates to telomeres. Telomeres are DNA protein complexes at the ends of the chromosomes that protect the chromosomes from degradation, recombination and fusion [29]. DNA replication at these chromosomal ends sites is semi-conservative, meaning that the duplication process is not complete. To overcome this, telomere length can be maintained by telomerase, a ribonucleoprotein that adds telomeric DNA to the chromosome, using the RNA of this complex as a template. In somatic cells, telomerase activity is too low for this telomere regeneration strategy to be effective. The result is that telomeres successively shorten with each cell division.

Critical telomere shortening produces telomere dysfunction, which is thought to trigger senescence. A primary pathway by which senescence is triggered is through the p53 tumor suppressor pathway. Telomere dysfunction can lead to ATM-dependent stabilization of p53 with resultant transactivation of p21. p21 expression, in turn, will inhibit cyclin-dependent kinase, one consequence of which will be hypophosphorylation of the retinoblastoma protein (Rb). Hypophosphorylated Rb remains bound to the E2F transcription factor and thereby imposes growth arrest. In the kidney, the role of telomerase has recently been elucidated using a mouse model. The regenerative capacity of acutely injured kidneys was found to be impaired in telomerase-deficient mice [30]. These mice displayed both increased senescence and increased apoptosis. Although this differs from the classic notion that senescent cells are resistant to apoptosis, it also highlights the fact that there are multiple roles for the telomerase enzyme [31].

Senescence can also proceed in the absence of cell division and telomere shortening. In this case, irreversible growth arrest is triggered by one or more stresses and the term stress-induced premature senescence (SIPS) is applied. This form of senescence may be particularly relevant to the in vivo situation where cell proliferation rates may be low. Although cells subjected to SIPS do not display telomere attrition, they share many attributes with those subjected to replicative senescence and are thus deleterious to tissue function. One of the most physiologically relevant inducers of SIPS is oxidative stress. The accumulation of ROS is known to induce cumulative damage to mitochondrial DNA and membrane proteins during aging. Relevant

signaling targets include promoters of genes encoding the tumor suppressors $p16^{INK4a}$ and p^{14ARF} [32].

MOLECULAR MECHANISMS UNDERLYING CELLULAR AGING

Lessons from Lower Organisms

Studies of lower organisms have proven to be a fruitful means of discovering pathways that may underlie human aging and senescence. One of the major advances was the discovery that the lifespan of yeast, worms, flies and rodents can be extended by caloric restriction [33]. Although several pathways are likely to underlie this response, the process in general falls under the category of "what doesn't kill you makes you stronger". Thus, caloric restriction is believed to activate adaptive stress responses that evolved to help organisms to survive periods of starvation [34].

One of the molecular cascades that has been linked to this response involves a class of protein deacetylases known as sirtuins. Sirtuins remove the acetyl group from a number of target proteins through a unique two-step reaction [35]. Although this may seem unnecessarily complex, this two-step deacetylation reaction requires the hydrolysis of NAD^+, a requirement that ensures that the activity of sirtuins is regulated by NAD^+ availability. The importance of this level of regulation will be discussed below.

The best studied sirtuin in lower organisms is silent information regulator-2, or Sir2. In the absence of Sir2, yeast (*Saccharomyces cerevisiae*), worms (*Caenorhabditis elegans*) and flies (*Drosophila melanogaster*) no longer show extended longevity in response to nutrient restriction [36 39]. Likewise, extra copies or increased expression of the *Sir2* gene extends the lifespan of yeast, worms and flies [40 43]. The relevant mechanisms by which Sir2 confers lifespan extension in these species include silencing of telomeres and inhibiting the activity of cell death proteins such as p53 and Ku70, through NAD^+-dependent deacetylation [43 46]. The extent to which these specific pathways translate to sirtuin function in humans is uncertain. However, as discussed below, mammalian sirtuins have diverse actions that can positively affect cellular health in several ways.

NAD^+-consuming Reactions and Mammalian Cell Health

NAD^+ (the oxidized form of nicotinamide adenine dinucleotide) is classically known as a cofactor for the hydride transfer enzymes of intermediary metabolism [47]. These well-known reactions entail the recycling of NAD^+ [or nicotinamide adenine dinucleotide phosphate (NADP)] between oxidized and reduced forms. However, in recent years, entirely unexpected roles for NAD^+ have been discovered. These new, but vital, roles are not based on redox events, but instead are characterized by NAD^+ consumption. This is because NAD^+ is not a cofactor for these reactions, but rather is a substrate that is enzymatically degraded.

Three classes of mammalian NAD^+-consuming enzymes are currently recognized: (i) ADPribose transferases and poly(ADPribose) polymerases (PARPs), (ii) cADPribose synthases, and (iii) sirtuins (Fig. 19.2). The wide range of reactions mediated by these enzymes includes DNA repair, chromatin silencing, transcriptional regulation, metabolic switching, calcium mobilization and

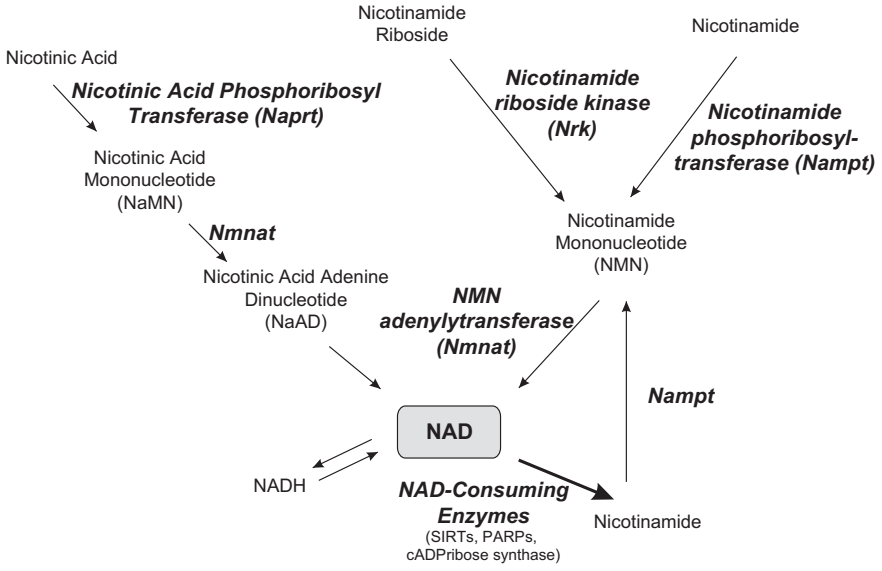

FIGURE 19.2 Biosynthesis and utilization of NAD^+. NAD^+ can be synthesized from several precursors including nicotinic acid, nicotinamide riboside and nicotinamide. The rate limiting enzymes are Naprt, Nrk and Nampt, respectively. NAD^+ can also be degraded during critical reactions catalyzed by SIRTs, PARPS and cAD Pribose synthases. These reactions liberate nico tinamide, which is salvaged by Nampt to regenerate NAD^+. Nmnat is common to all pathways that use these three precursors, although isoforms are differentially located within the cell.

lifespan regulation [47 50]. Although all three classes perform indispensable functions within cells, only the sirtuins (particularly the founding member and mammalian homolog of Sir2, SIRT1) have been consistently implicated in mammalian cell survival and replicative longevity.

Sirtuins and Cellular Metabolism

There are seven known mammalian sirtuins, or SIRTs. SIRT1, 2, 3, 5 and 7 have deacetylase activity, consistent with Sir2 in lower organisms. SIRT4 and SIRT6 have ADP-ribosyltransferase activity which, like SIRT-mediated deacetylase activity, requires NAD^+.

The best studied mammalian SIRT is SIRT1, an enzyme that impinges on an array of intracellular processes. One of the primary roles of SIRT1 is as a master regulator of metabolism, by virtue of its activity in fat, muscle, liver and pancreas. SIRT1 activity mobilizes fatty acids in white adipose tissue and prevents preadipocyte differentiation. These "fat-losing" processes proceed via SIRT1-mediated repression of peroxisome proliferator activated receptor-γ (PPAR-γ) interactions with its cofactors, nuclear receptor corepressor (NCoR) and silencing mediator of retinoid and thyroid hormone receptor (SMRT) [51]. In skeletal muscle, SIRT1 deacetylates and activates PPAR-γ coactivator-1α (PGC-1α), which serves to increase fatty acid utilization and aerobic capacity [52,53]. A similar activation of PGC-1α in the liver results in increased gluconeogenesis [54]. Control of hepatic gluconeogenesis by SIRT1 is also conferred by deacetylation, and associated nuclear trapping, of the forkhead box transcription factor, FoxO1 [55].

Importantly, the SIRT1-mediated reduction in adipose tissue and enhanced skeletal muscle aerobic capacity together serve to increase insulin sensitivity [52]. SIRT1 can also directly increase the action of insulin in these tissues by repressing protein tyrosine phosphatase 1B expression [56], a negative regulator of insulin signaling. In addition, SIRT1 enhances glucose-stimulated insulin secretion by the pancreas by repressing expression of uncoupling protein-2 [57] and protects β-cells from glucose toxicity during hyperglycemia, through deacetylation of FoxO1 [58].

Although the salutatory effects of SIRT1 on the metabolic milieu have yet to be thoroughly evaluated in the context of renal disease, the importance of glucose control to renal performance, and of glucose and lipid metabolism to renovascular health, highlight the central role that SIRT1-mediated metabolic cascades may play in the kidney. Recent data have shown that the SIRT1 activator, resveratrol, ameliorates glucose-mediated dysfunction of glomerular epithelial cells [59]. Thus, the data to date suggest that SIRT1 is critical to establishing a favorable metabolic milieu for tissue repair and regeneration.

Sirtuins and Endothelial Cells

Endothelial cell senescence is a feature of aging and vascular disease. In addition to growth arrest, senescent endothelial cells are characterized by decreased NO and prostacyclin production, increased plasminogen activator inhibitor-1 (PAI-1) expression and enhanced monocyte adhesion properties [60], phenomena that contribute substantially to the pathogenesis of vascular disease.

All seven SIRTs are expressed in human vascular endothelial cells [61]. As with the metabolic cascades noted above, however, most studies of sirtuins and endothelial cells have focused on the role of SIRT1. There is an association between SIRT1 activity and NO metabolism. In vitro, SIRT1 has been found to deacetylate lysines 496 and 506 of endothelial nitric oxide synthase (eNOS), thereby stimulating its activity and the endothelial cell production of NO [62]. Corresponding studies in mice subjected to caloric restriction confirmed enhanced deacetylation of eNOS in response to SIRT1 activation in vivo [62]. Moreover, mice given low doses of red wine, the predominant source of the activator of SIRT1 resveratrol, show coordinate increases in SIRT1 and eNOS expression [63].

SIRT1 also plays a role in regulating endothelial cell longevity and resistance to senescence. Inhibition of SIRT1 expression and activity in human umbilical vein endothelial cells has been shown to induce premature senescence, characterized by increased PAI-1 expression and decreased expression and activity of eNOS [64]. Consistent with this, overexpression of SIRT1 [64,65], activation of SIRT1 with resveratrol [65] or induction of SIRT1 expression by the PDE3 inhibitor cilostazol [66] prevented oxidative stress-induced premature senescence and the associated inflammatory phenotype in endothelial cells. Cilostazol appears to induce SIRT1 expression through an eNOS-dependent pathway [66], a mechanism previously identified in adipose tissue of mice subjected to caloric restriction [67].

SIRT1 also has angiogenic actions. Potente et al. demonstrated that NAD^+-dependent deacetylation of FoxO1 by SIRT1 inhibits the antiangiogenic activity of FoxO1 in endothelial cells, and that neovascularization after acute hindlimb ischemic injury was blunted in endothelial cell-specific SIRT1-deficient mice [61]. Moreover, studies of putative circulating human endothelial progenitor cells (EPCs) exposed to high glucose suggest that the resulting oxidative stress diminishes SIRT1 expression and activity, with concomitant increases in FoxO1 acetylation [68]. Thus, SIRT1 may promote vascular regeneration by deacetylation pathways, in both mature and progenitor endothelial phenotypes, outcomes that would be expected to favor the restoration of optimal kidney function.

Sirtuins and Vascular Smooth Muscle Cells

Vascular smooth muscle cells (SMCs) are central to the regulation of blood flow as well as the repair of damaged vasculature. Deteriorated function of vascular SMCs with aging can have profound effects on tissue homeostasis and regenerative potential. As one important example, the efficiency with which SMCs stabilize atherosclerotic lesions can determine whether the lesion will rupture, a potentially fatal event. Vascular SMC senescence has recently been identified as a feature of atherosclerotic lesions [69]. Senescent SMCs may be particularly dangerous because the resulting proinflammatory and non-reparative state could incite lesion disruption and acute vascular occlusion. Strategies to prevent the premature senescence of SMCs could thus be a promising approach for reducing vascular insufficiency of regenerating organs, including the kidney, if molecular targets can be identified.

A role for SIRT1 in regulating SMC senescence is emerging, although it appears to be context dependent. As noted, SIRT1 has been reported to inhibit senescence in endothelial cells [64] and a similar response has been observed for human cancer cells and fibroblasts [44,70]. However, in other cells SIRT1 has also been reported to promote senescence [71,72]. In still other studies SIRT1 was found to have no effect on senescence at all [73]. One study found that overexpression of SIRT1 in SMCs led to a modest reduction in the accumulation of senescent cells and a modest extension in SMC replicative longevity [74]. However, SMC lifespan was markedly extended when SIRT1 was overexpressed together with nicotinamide phosphoribosyltransferase (Nampt), the rate-limiting enzyme for the NAD^+ salvage pathway (Fig. 19.3, and discussed further below) [74]. Furthermore, it was found that SIRT1 activity declined, concomitant with the capacity to regenerate NAD^+, as SMCs underwent replicative aging. Thus, understanding the role of SIRT1 in human cell aging, and the potential benefits of activating SIRT1 for tissue regeneration, must take into account the prevailing metabolic conditions in which the enzyme operates.

The role of SIRT1 may also extend beyond regulating vascular SMC aging. SIRT1 appears to contribute to the control of blood pressure through actions on SMCs and the renin angiotensin system [68]. SIRT1 was found to inhibit expression of the angiotensin II (Ang II) type 1

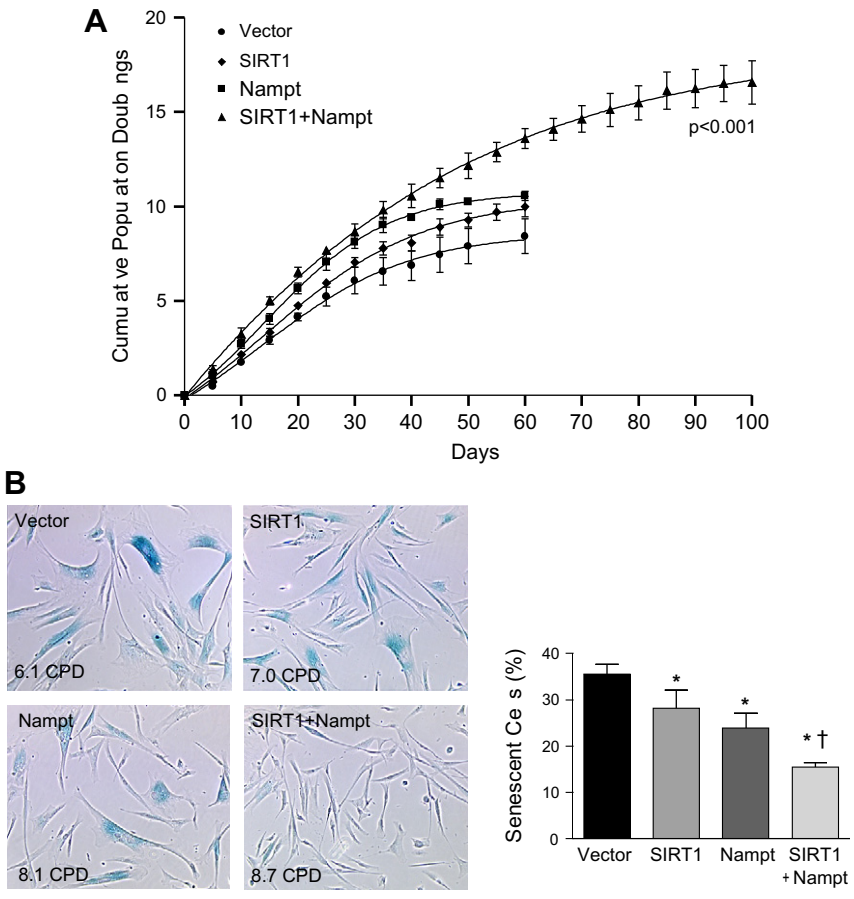

FIGURE 19.3 Marked extension of human smooth muscle cell (SMC) lifespan by SIRT1 and Nampt. (A) Population doubling of HITC6 SMCs transduced with retrovirus containing control vectors, or cDNA encoding SIRT1, Nampt, or both SIRT1 and Nampt. The pronounced increase in replicative lifespan in SIRT1−Nampt SMCs corre sponds to a substantial decrease in SMC senescence (B), as depicted by the blue staining of senescence associated β galactosidase activity ($*p < 0.05$ vs vector, $\dagger p < 0.05$ vs SIRT1 and Nampt). *[Reprinted from Ho et al., 2009 [73] with permission from Elsevier.]*

receptor (AT1R) and administration of resveratrol to mice diminished aortic AT1R expression and blunted Ang II-induced hypertension [68]. Combined with the ability of SIRT1 to activate eNOS and enhance endothelium-dependent vasodilation [68], these observations suggest that SIRT1 plays a significant role in controlling hypertension at the level of the vessel wall.

NAD+ BIOSYNTHESIS AND CELL HEALTH

NAD+-producing Enzymes

The discovery of NAD+-consuming reactions, and the roles of SIRT1 in mammalian cell survival and longevity, has led to a major rethinking of the significance of pathways that generate NAD+. Whereas dietary maintenance may meet baseline NAD+ requirements, pathways that locally supply or regenerate NAD+ are emerging as critical determinants of cell function, particularly during tissue stress or disease [75]. Currently, there are 16 enzymes known to contribute to NAD+ production in humans. However, the enzymes that generate NAD+ from nicotinic acid, nicotinamide and nicotinamide riboside appear to be critical to active and remodeling tissue (Fig. 19.2). This is because these enzymes can generate NAD+ by salvaging these precursors from within the cell [75,76].

Nampt is the enzyme that catalyzes the salvage reaction whereby nicotinamide is recycled to produce nicotinamide mononucleotide, which can then be converted to NAD+ [77 79]. Nampt has been found to impact insulin secretion [80], inflammation [81,82] and lymphocyte development [83]. Nicotinamide mononucleotide adenylyltransferase (Nmnat) is a distal NAD+-generating enzyme that has recently been found to prevent neurodegeneration and axonal degeneration [84,85]. Nicotinamide riboside kinase (Nrk) catalyzes a newly discovered mammalian NAD+ supply pathway that extends the lifespan of lower eukaryotes [75,76,86]. The local generation of NAD+ is thus vital for longevity and regeneration in various systems. However, the distribution of NAD+ biosynthetic enzymes is variable among tissues. The specific complement of pathways and biosynthetic enzymes in normal and diseased organs may thus be a determinant of the aging versus regenerative profile of the tissue [75,87 89].

Nampt is an NAD+-regenerating Enzyme

Nampt was identified as being substantially upregulated as SMCs entered a program of differentiation and enhanced resistance to stress [77]. When first discovered, Nampt was thought to be a cytokine and was termed pre-B-cell colony-enhancing factor (PBEF) [90]. However, the present authors and two other groups subsequently revealed its role instead as an enzyme in the NAD+ salvage pathway [77,91,92]. There are also several reports, and many review articles, discussing Nampt as an adipokine termed visfatin [93,94]. However, the index report in *Science* describing an adipokine role for Nampt has now been retracted [95]. In addition, the often contradictory findings relating circulating Nampt (visfatin) to various metabolic conditions are confounded by the uncertain validity of Nampt enzyme-linked immunosorbent assay (ELISA) kits that rapidly appeared on the market after the initial report in *Science* [93]. There is evidence, however, that an extracellular form of Nampt might have important actions [80,96], including regulating glucose uptake in tissues that do not synthesize significant amounts of Nampt, such as the brain. The extent to which the release of Nampt from cells is regulated is uncertain. Regardless, locally produced Nampt is likely to be important to several organs and Nampt has been found to be expressed in endothelial cells, SMCs and kidney-derived mesangial cells [77,79,97,98].

From a biochemical and functional standpoint, there are interesting parallels between Nampt and one of the enzymes found to mediate lifespan in yeast. The first step in regenerating NAD+ from nicotinamide in *S. cerevisiae* is deamidation of nicotinamide by the *PNC1* gene product, to form nicotinic acid. PNC1 is induced by cell stress and nutrient restriction. Increasing the gene dose of *PNC1* extended the lifespan of this organism, by depleting nicotinamide and activating Sir2 [99]. However, a gene homologous to *PNC1* in humans does not appear to exist. Rather than an initial deamidation step, NAD+ salvage in humans requires the conversion of nicotinamide to nicotinamide mononucleotide, the reaction catalyzed by Nampt. Nampt was substantially upregulated when SMCs were subjected to the stress of complete serum withdrawal [77]. Yang et al. found similar upregulation of Nampt following serum withdrawal from fibrosarcoma HT1080 cells and from primary cardiomyocytes [100], supporting the concept that Nampt is a stress-responsive gene. Mammalian Nampt may thus be a functional equivalent of yeast Pnc1 [91,99,100].

Nampt in Vascular Cell Aging and Regenerative Capacity

Deletion of Nampt in the germ line leads to embryonic death by day 10.5 [80]. Nampt has been found to be essential for lymphocyte development in vivo and for maintaining cell viability during genotoxic stress [83]. Overexpression of Nampt in SMCs stimulated

protein deacetylase activity and enabled the cells to shift to an apoptosis-resistant cell that could migrate to endothelial channels [77]. Investment of endothelial channels by SMCs is an important step in postnatal vascular regeneration that is not effectively achieved with angiogenic growth factors.

A programmed diminution in the capacity to regenerate NAD^+ from nicotinamide is a critical precursor of human SMC senescence. Moreover, by overexpressing Nampt cellular lifespan was lengthened, a phenomenon observed in SMCs, endothelial cells and fibroblasts derived from a patient with Hutchinson Gilford progeria syndrome [78,79]. These investigations further established that this antiaging phenomenon was mediated by enhanced SIRT1 deacetylase activity which, in turn, holds p53 levels below those that induce senescence [78]. These findings implicate a Nampt SIRT1 p53 axis as a fundamental determinant of human cell aging.

The lifespan-extending effects of Nampt on endothelial cells were particularly noteworthy as Nampt enabled these cells to overcome the stress of high glucose [79]. Furthermore, Nampt overexpression completely abrogated the induction of polyploidy in endothelial cells, a feature of endothelial senescence that itself may contribute to endothelial cell dysfunction [98]. This protective action of Nampt was mediated by increased SIRT1 activity, which reduced cellular ROS and the associated oxidative stress stimulus for the induction of polyploidy. Collectively, these findings launched the concept that variations in NAD^+ supply systems may critically impact vascular regeneration, a concept supported by others [96]. Given the central role of the vasculature in the progression of kidney disease, reliable NAD^+ production may also be crucial to the successful restoration of kidney function by way of its support of vascular regeneration.

CONCLUSION

Recent discoveries regarding the cellular and molecular basis of aging have provided fascinating insights into the mechanistic basis of aging-related disease. Moreover, these developments have implications for potential regenerative strategies to reverse the effects of aging- and disease-related damage in organs such as the kidney. Regeneration and aging can be considered as opposing, and possibly mutually exclusive, processes. Strategies to suppress replicative or stress-induced aging, if they can be realized, would thus hold considerable promise as a means of optimizing a range of regenerative efforts. Methods of enhancing NAD^+ supply, NAD^+ regeneration and sirtuin activity are exciting possibilities in this regard.

Acknowledgments

Supported by grants from the Heart and Stroke Foundation of Ontario (T7081) and Canadian Institutes of Health Research (FRN 11715). JGP holds a Career Investigator Award from the Heart and Stroke Foundation of Ontario.

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CHAPTER

20

Bone Marrow Mesenchymal Stem Cells in Organ Repair and Strategies to Optimize their Efficacy

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OUTLINE

INTRODUCTION

Bone marrow-derived mesenchymal stem cells (BMMSCs) are currently considered one of the cell types with great clinical potential for cell-based therapy [1]. When transplanted systemically, mesenchymal stem cells (MSCs) are able to migrate to sites of injury and exert their therapeutic action via paracrine release of trophic factors instead of a direct repopulation of the damaged tissue. Experimental studies have reported the use of BMMSCs as preferred stem cells for therapy of acutely damaged heart [2], lung [3] and liver [4]. Some experimental studies using BMMSCs have been translated into clinical trials, with results that are sometimes modest and controversial [5]. Even in the kidney, stem cell therapy has been approached to support the limited renal capacity to recover following acute ischemic or toxic injury. Acute kidney injury (AKI) is a potentially devastating disorder with an extremely serious outcome. Several pharmacological therapies for AKI have been attempted, with disappointing results [6]. BMMSC therapy has been identified as a powerful

treatment to regenerate damaged renal tissue in experimental models of AKI [7–9]. To maximize treatment efficacy and translate these results to patients with highly compromised renal function, we need to understand how to enhance the efficiency of MSCs in terms of migration, engraftment, production of cytokines and growth factors, and differentiation in the pathological environment of kidney failure. These issues will be addressed in this chapter.

DO ADULT BONE MARROW-DERIVED STEM CELLS PARTICIPATE IN KIDNEY REPAIR?

Pioneering preclinical studies have reported the existence of a bone marrow–kidney axis with bone marrow cells contributing to tubular epithelial cells [10] physiological processes of cell turnover or regeneration and to mesangial cells, podocytes and endothelial cells [11,12]. In mice, Poulsom et al. showed the presence of Y-chromosome-positive cells in kidneys obtained from female recipients of male bone marrow [10], demonstrating the existence of traffic of these cells into the kidney. Moreover, the percentage of Y-chromosome-positive tubular epithelial cells (*Lens culinaris* lectins positive), which ranged from 0.6 to 6.8% [10], suggested a contribution of bone marrow cells to the renal epithelium. Although the presence of male tubular cells after acute renal injury was a rare event, several reports showed that extrarenal cells could directly participate in the regeneration of tubules. In acute tubular necrosis, male patients receiving a kidney transplant from female donors showed Y-chromosome-positive cells in tubular epithelium and in glomeruli, where they expressed vimentin, suggesting a podocyte phenotype [10]. Similarly, Gupta et al., studying the origin of tubular cells in male patients with resolving acute tubular necrosis who had received a kidney transplant from a female donor, documented 1% of the tubular cells positive for the Y-chromosome and negative for the hematopoietic marker CD45 in the kidneys after injury [13]. Another study in gender-mismatched transplantation, with male patients receiving a female renal allograft, demonstrated that stable tubular epithelial chimerism (from 2.4 to 6.6%) occurred regularly in renal allografts, in 72% of the patients [14]. However, this study does not clarify whether the Y-chromosome-positive cells found in the transplanted kidney came directly from the bone marrow, represented circulating stem cells or derived from other organs. After acute tubular injury induced by folic acid treatment in mice, bone marrow-derived cells recruited to the kidney proliferated and generated tubular cells and glomerular mesangial cells [15]. Little evidence for cell fusion between indigenous renal tubular cells and bone marrow-derived cells was found [15]. Others have obtained conflicting results [16]. Recently, enhanced green fluorescent protein (eGFP)-labeled bone marrow-derived stem cells (BMSCs) were transplanted in sublethally irradiated syngeneic recipient mice. Four weeks after transplantation, ischemia reperfusion injury was induced. On day 7 postdamage eGFP-positive BMSCs were clearly detectable in ischemic kidney tissue and contributed towards 10% of regenerated proximal tubular cells [17]. None of these reports discriminated whether cells from bone marrow which home to the kidney were hematopoietic stem cells (HSCs) or MSCs.

Studies on bone marrow-derived HSC mobilization with a cocktail of cytokines showed conflicting results with potentially negative effects on kidney regeneration [18], whereas others exhibited renoprotection in an AKI model induced by folic acid [15]. In addition, transplantation studies with GFP-positive hematopoietic lineage cells or male cloned cultured BMMSCs into irradiated female mice have shown that both populations can engraft the bone marrow; however, only hematopoietic cells could transdifferentiate into renal tubular epithelial cells, playing a role not only in normal turnover but also in the repair of $HgCl_2$-induced tubular injury [19]. In different animal models bone marrow-derived cells were able to differentiate into podocytes, mesangial and endothelial cells [20–22]. However, the contribution of extrarenal cells to kidney repair remains controversial, with studies indicating that repair of injured nephrons is mainly accomplished by intrinsic, surviving tubular epithelial cells [23]. In chimeric mice expressing GFP or β-gal in bone marrow-derived cells, subjected to ischemic injury, some interstitial cells (mainly leukocytes) but not tubular cells expressed GFP, whereas β-gal-positive signals were attributed only to endogenous cells [24], excluding a direct contribution of bone marrow-derived cells to renal repair [24,25].

BONE MARROW MESENCHYMAL STEM CELL THERAPY CONTRIBUTES TO RENAL REPAIR

Mesenchymal Stem Cells

MSCs are currently considered one of the cell types with good clinical potential for cell-based therapy because of their relative ease of expansion, immunoprivileged and immunosuppressive character, great tropism for damaged organs and the spectrum of growth factors which regulate crucial cellular biological function at the place of engraftment [26–30]. MSCs, also defined as stromal cells, represent a heterogeneous cell population of multipotent stem cells that mostly resides

in the bone marrow. It has been estimated that MSCs represent 0.01 0.0001% of the nucleated cells of adult human bone marrow. These cells can differentiate into the mesodermal cell lineage as adipocytes, osteocytes and chondrocytes, but they also have endodermic and neuroectodermic potential [27,29]. However, the evidence that BMMSCs can transdifferentiate into functional tissue-specific cell types both in vitro and in vivo to promote tissue repair is still controversial. To understand more about BMMSC biology, the key questions to address are: where are they anatomically located, and what is their natural function? In the bone marrow of the trabecular bone, mesenchymal/stromal progenitor cells at different stages of maturation are functional components of the HSC niche. In the endosteal niche, stromal progenitor cells work together with osteoblasts in maintaining HSCs in a quiescent state, while in the vascular niche perivascular stromal cells and sinusoidal endothelial cells regulate the proliferation, differentiation and recruitment of HSC to the systemic circulation [27] via local release of specific cytokines. In vitro data document that human BMMSCs are able to release several factors [31 33], among them stem cell factor (SCF), leukemia inhibitory factor (LIF), stromal cell-derived factor-1 (SDF-1), oncostatin M (OSM), bone morphogenetic protein-4 (BMP-4), fms-like tyrosine kinase-3 (Flt-3), transforming growth factor-β (TGF-β) (Table 20.1) [28,30,34], supporting self-renewal rather than differentiation of immature HSCs in the bone marrow. Moreover several interleukins (Table 20.1) [28,35], act on committed hematopoietic cells toward blood cell lineages. Recent studies indicate a new niche for MSCs, showing the presence of perivascular cells coexpressing markers of pericytes and MSCs in multiple organs [36] including the kidney [37]. These findings may suggest a functional role for MSCs in the control of microvascular stability.

TABLE 20.1 Expression of Cytokines and Growth Factors by Human Bone Marrow Mesenchymal Stem Cells

Cytokines and growth factors	Ref.
IL 6, IL 7, IL 8, IL 11, IL 12, IL 14, IL 15	[35]
IL 1, IL 10, IL 27	[28]
LIF, M CSF, Flt 3, SCF	[35]
HGF, PGE_2	[28]
TGF β, MMP 2, TSP 1	[34]
PDGF, Ang 1, PA, MMP 9	[33]
HO 1	[27]
VEGF, MCP 1, IGFBP 2, IGFBP 4	[32]
EGF, IGF 1, bFGF, SDF 1, FGF 2	[31]

IL: interleukin; LIF: leukemia inhibitory factor; M-CSF: macrophage colony-stimulating factor; Flt: fms-like tyrosine kinase; SCF: stem cell factor; HGF: hepatocyte growth factor; PGE: prostaglandin E; TGF: transforming growth factor; MMP: matrix metalloproteinase; TSP: thrombospondin; PDGF: platelet-derived growth factor; Ang: angiopoetin-1; PA: plasminogen activation; HO: heme oxygenase; VEGF: vascular endothelial growth factor; MCP: monocyte chemotactic protein; IGFBP: insulin-like growth factor binding protein; EGF: epidermal growth factor; IGF: insulin-like growth factor; bFGF: basic fibroblast growth factor; SDF: stromal cell-derived factor; FGF: fibroblast growth factor.

The use of these cells for clinical protocols are of interest because BMMSCs can be easily isolated from a small aspirate of bone marrow, rapidly expanded in vitro, maintaining their genetic stability [26], and collected or dispatched from the laboratory to the bedside. Moreover, cultured BMMSCs have been successfully infused in humans for safety and early clinical testing for the treatment of genetic disorders [38], and in ischemic cardiomyopathies [39], hematological diseases [40], Crohn's disease and spinal cord injury [41]. There is a general consensus that cultured human BMMSCs express variable levels of CD105 (endoglin), CD73 (ecto-5′-nucleotidase), CD44, CD90 (Thy1), CD71 (transferrin receptor), the ganglioside GD2 and CD27 (low-affinity nerve growth factor receptor), whereas they do not express hematopoietic markers CD45, CD34 and CD14. Owing to the low constitutive expression of major histocompatibility complex (MHC) class II [42,43] and the absence of costimulatory molecules such as CD80, CD86 and CD40/CD40L, BMMSCs escape the immune system and therefore can be injected into allogeneic host without rejection [42,44], in the absence of conditioning regimens. In vivo data obtained in humans [43], baboons [45] and rodents [46] showed that BMMSCs inhibit T-cell responses to cellular and to non-specific mitogenic stimuli, targeting both naïve and memory $CD4^+$ and $CD8^+$ T cells [43]. There is also evidence that BMMSC-induced T-cell suppression occurs independently of MHC matching with either stimulatory cells or responder lymphocytes in the mixed lymphocyte reaction (MLR) [45]. BMMSC-mediated immunomodulation is promoted by close cellular contact but also by several soluble factors, including hepatocyte growth factor-1 (HGF-1), TGF-β, indoleamine 2,3-dioxygenase (IDO) and prostaglandin E_2 (PGE_2) (Table 20.1) [27,28,34]. More recently, nitric oxide and insulin-like growth factor (IGF) binding protein [47,48] have also been shown to play a key role. Immunosuppressive effects of BMMSCs are increased by inflammatory cytokines such as interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α) [47]. Scanty and conflicting data are available on the immunogenicity and immunomodulatory effects of BMMSCs in vivo. Allogeneic BMMSC infusion in rats with AKI significantly ameliorated renal dysfunction, although the effect on animal survival was very modest [49]. Intramyocardial injection of BMMSCs from allogeneic donors in pigs 3 days after infarction resulted in long-term engraftment, decreased scar formation and near-normalization

of cardiac function [50]. A recent study demonstrated long-term allogeneic BMMSC survival, engraftment and restoration of cardiac function following transplantation into chronically scarred myocardium [51]. Based on their immunosuppressive properties, allogeneic BMMSCs were used as a promising therapeutic approach to enhance skin engraftment in immunocompetent baboons [45] and reduced graft-versus-host disease in mice [52] and humans [40]. In addition, pretransplant infusion of BMMSCs prolonged the survival of semi-allogenic heart transplant through the generation of regulatory T cells in unconditioned recipient mice [53]. At variance, conflicting results showed that BMMSC injection failed to prevent graft rejection [46] and slightly prolonged graft survival [54] in fully MHC-mismatched vascularized heart transplant in rodents.

The great potential of BMMSCs for cell therapy may rest on their ability to migrate to the site of acute tissue damage or inflammation injury, promoting tissue repair. This peculiar property of BMMSCs has been demonstrated in the setting of bone fracture [55], cerebral ischemia [56] and infarcted heart [57], as well as in the damaged kidney [8,9,58], where BMMSCs can promote tissue regeneration through the local delivery of mitogenic and vasculotropic factors [59,60], as described below.

Functional Effect of Bone Marrow Mesenchymal Stem Cells in Acute Kidney Injury

A number of reports highlights that MSCs of bone marrow origin have a role in regeneration after acute renal failure. The renoprotective effect of BMMSCs was first documented by this group in the experimental model of AKI induced by the nephrotoxic drug cisplatin. Male BMMSCs, transplanted in female mice 1 day after cisplatin injection, restored renal function and tubular structures [8]. The engraftment of BMMSCs into the damaged kidney was investigated by tracking Y-chromosome-positive cells, which were detected, although to a very low extent, in tubuli displaying binding sites for *L. culinaris* lectins. This study also showed that BMMSCs significantly accelerated proliferation of tubular cells to a remarkable extent, as proven by the presence of Ki-67 signal in the cells. The effect of human BMMSCs was also investigated in immunodeficient non-obese diabetic/severe combined immunodeficiency (NOD/SCID) mice with cisplatin-induced AKI. Renal functional recovery, associated with a therapeutic effect on survival, was observed in these animals accompanied by an increase in cell proliferation, and reduction of apoptosis and tubular lesions [9]. These cells were found predominantly in tubulointerstitial areas (Fig. 20.1), with the rest equally distributed in tubuli and glomeruli [9]. Human BMMSC treatment also induced protection against AKI-related peritubular capillary changes consisting of endothelial cell abnormalities, inflammatory cell infiltration, and low endothelial cell and lumen volume density as assessed by morphometric analysis. The present group has reported even more pronounced positive results by using human MSCs obtained from cord blood, with improvement of renal function and significant prolongation of survival [61]. This renoprotective effect appeared to be mediated by a complex action of human cord blood MSCs on tubular cells involving lowering oxidative

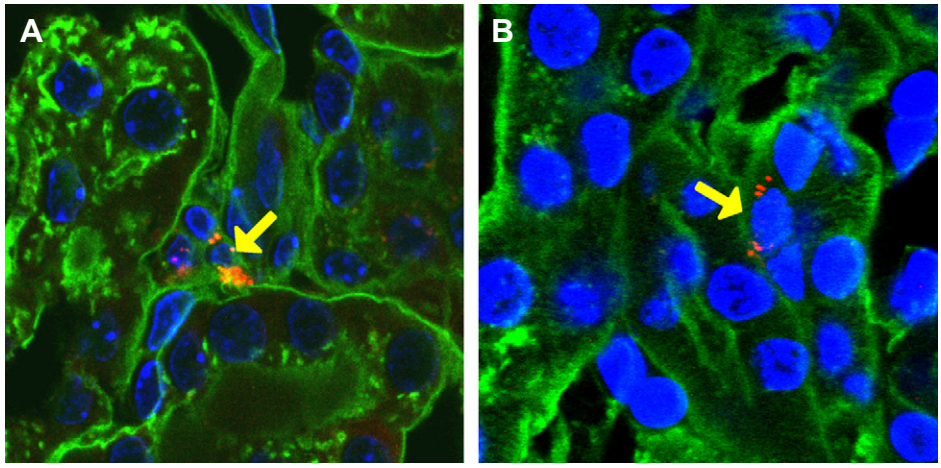

FIGURE 20.1 Human bone marrow mesenchymal stem cells (BMMSCs) engraft the damaged kidney in immunodeficient NOD SCID mice with cisplatin induced acute kidney injury. Representative images, taken 3 days after intravenous cell injection, show PKH 26 labeled BMMSCs (red) localized in the interstitium (A, arrow) or tubuli (B, arrow) of renal tissues of cisplatin mice stained with fluorescein isothiocyanate (FITC) labeled wheatgerm agglutinin (WGA, green) and 4′,6′ diamidino 2 phenylindole dihydrochloride hydrate (DAPI) nuclear staining (blue). Original magnification 630×. Please see color plate at the end of the book.

stress, apoptosis and inflammation counterbalanced by the activation of prosurvival and mitogenic signaling [61]. In another mouse model of glycerol-induced AKI, mouse BMMSCs differentiated into tubular epithelial cells (intravenously injected GFP-positive BMMSCs were positive to cytokeratins), and promoted epithelium proliferation and renal function [7]. In rats, administered BMMSCs had highly renoprotective capacity and enhanced the recovery from ischemia reperfusion AKI [62]. Here, iron-dextran-labeled BMMSCs were located in the kidney cortex after injection, as demonstrated by magnetic resonance imaging [63]. In the same rat model, Tögel et al. studied intracarotid administration of fluorescence-labeled BMMSCs and, despite no differentiation occurring, they found kidney protection [63]. Another study by the same authors reported that both autologous and allogeneic BMMSCs were safe and effective in an ischemia reperfusion model of AKI in rats, and reduced late renal fibrosis by ameliorating renal function in surviving animals [49].

The effectiveness of BMMSCs in the treatment of acute kidney damage has been here clearly highlighted; however, it is important to know the extent of cell recruitment and engraftment in the target organ, in order to manage patient treatments and for the success of the therapy. Cell delivery methods and the intrinsic capacity of the cells to migrate into the kidney could affect the results and make a difference. Several studies have made use of cell-tracking methodologies to quantify administered stem cells in target tissues, but mainly provided no evidence that BMMSCs enter the renal parenchyma in significant numbers [24,63,64]. Tögel et al., using fluorescently labeled MSCs injected in ischemic rats, showed that fluorescent cells were detected only exceptionally at 24 h in scarce numbers in the kidney and in the renal microvasculature for only 1 2 days after infusion [63]. In an ischemia reperfusion model in rats, iron-dextran-labeled BMMSCs intravenously injected through the carotid artery were found on day 3 at a frequency of 30,000 cells per kidney [62]. Recent data showed that in cisplatin-induced AKI, injected murine BMMSCs predominantly localized in peritubular areas and to a lesser extent in the tubular epithelium, with scattered BMMSCs found in glomeruli [60]. In the same AKI model, 84% of human BMMSCs engrafted peritubular areas, whereas only 8% were found in tubules and 8% in glomeruli (Fig. 20.1) [9]. In these animals, PKH-26-labeled human BMMSCs localized in renal tissues at a frequency of 3.4 ± 0.76 per 10^5 renal cells [9]. In agreement with these findings are data in a model of glycerol-induced AKI showing that injected murine BMMSCs were recruited largely in peritubular areas, but still resulting in kidney recovery [58]. Nevertheless, eGFP-fluorescent BMMSCs injected into mice subjected to ischemia reperfusion were not found in the kidney parenchyma at 24 h, 48 h, 7 days or 15 days after ischemic injury [24].

Paracrine Effect of Bone Marrow Mesenchymal Stem Cells

The very low engraftment of infused BMMSCs in the damaged renal tissue and the preferential localization of stem cells to peritubular interstitial areas [9,63] would exclude a direct repopulation of the tubules by BMMSCs being responsible for the renoprotective effects. The evidence supports paracrine action of BMMSCs once engrafted in the damaged kidney, via the local release of soluble factors with regenerative potential for renal cells (Fig. 20.2). Relevant to the former interpretation are data showing that in the experimental model of ischemia reperfusion injury in rats, the protective effect on renal function and structure by BMMSCs was due to their intrinsic ability to produce high levels of growth factors such as HGF, vascular endothelial growth factor (VEGF) and IGF-1 [59,63]. Kidneys of rats, 24 h after BMMSC infusion, showed a significant reduction in the expression of proinflammatory genes coupled with an increased gene expression of basic fibroblast growth factor (bFGF), TGF-β, and of the antiapoptotic and anti-inflammatory B-cell lymphoma-2 (Bcl-2) and interleukin-10 (IL-10) compared with kidneys of untreated animals. More recently, the administration of conditioned medium derived from murine BMMSCs to cisplatin mice with AKI ameliorated renal dysfunction, enhanced tubular regeneration and limited cell apoptosis [64]. That IGF-1, a mitogenic and prosurvival factor, could represent a powerful regenerative agent in MSC-mediated renal repair rests on data showing that administration of IGF-1 gene-silenced BMMSCs in mice with cisplatin-induced AKI limited their protective effect on renal function and tubular injury [60]. Similarly, VEGF knocked down by small interfering RNA reduced the effectiveness of BMMSCs and decreased animal survival [49]. It has also been shown that suppression of HGF production impairs the ability of administered adipose-derived MSCs to promote revascularization in a mouse ischemia model [65]. Altogether, these data support the paradigm that growth factors delivered locally by BMMSCs and by resident renal cells could act in concert to mitigate renal injury and hasten repair (Fig. 20.2). The combined actions of several factors that decrease inflammation, oxidative stress and apoptosis at the same time would result in a greater renoprotective action than any single growth factor treatment, which could manifest systemic side-effects [66]. Stem cell release, at the site of tubular injury, of HGF, IGF-1

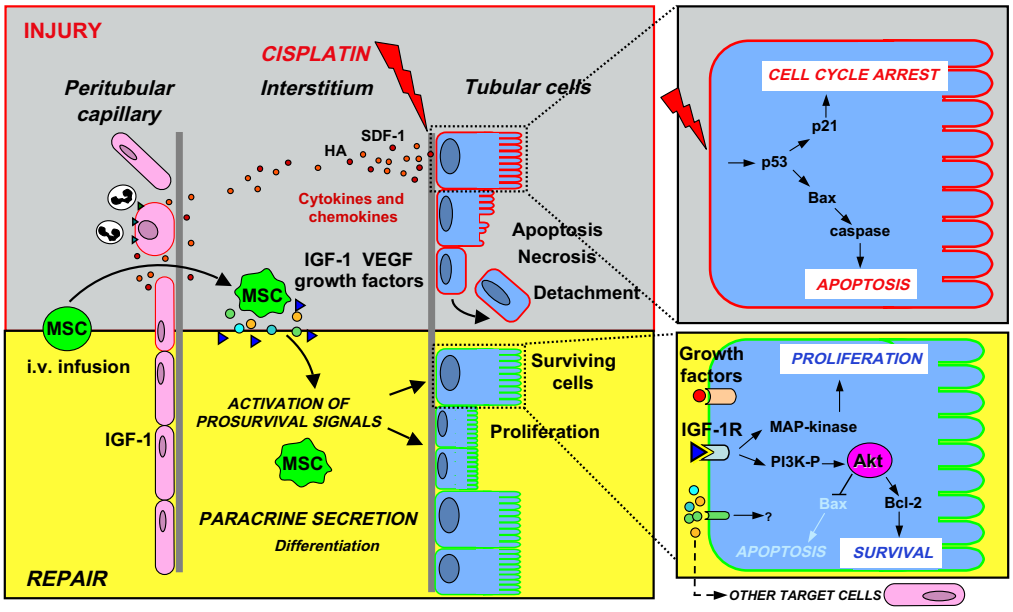

FIGURE 20.2 Proposed mechanism of bone marrow mesenchymal stem cell (BMMSC) mediated renal tubular repair after acute injury. The effect of BMMSCs, which systemically injected have the capability to home to renal damaged tissues, on tubular functional and structural repair passes through induction of cell proliferation and inhibition of apoptosis, both mediated by BMMSC derived growth factors including insulin like growth factor 1 (IGF 1) and vascular endothelial growth factor 1 (VEGF 1). These and the other growth factors supplied by BMMSCs may be also responsible for the overall improvement in the microvascular environment, acting directly or eventually via stimulation of still unknown target cells. HA: hyaluronic acid; SDF: stromal cell derived factor; MAP kinase: mitogen activated protein kinase.

and VEGF which possess mitogenic and chemotactic activity, could be instrumental to the recruitment of circulating or resident stem cell/progenitor cells [67] which contribute to the reparative processes of the kidney. Of note, HGF, IGF-1 and FGF-2 have been shown to induce resident progenitor cell activation and migration within the infarcted border zone of the heart [68].

Together, these data have set the basis for initiation of clinical trials of AKI. However, several critical points need to be addressed, including the long-term risk of maldifferentiation into unwanted cells [69] and the potential contribution of stem cells to malignancy [1].

STRATEGIES TO ENHANCE STEM CELL EFFECTS

At present, converging pieces of evidence suggest that BMMSC-mediated renal regeneration passes through a paracrine secretion of mitogenic and vasculotropic factors. Therefore, it is evident that potentiating renal migration, engraftment, survival and paracrine effects of administered stem cells, using preconditioning or genetic modification strategies, could contribute towards optimizing the final beneficial effect of the therapy. This is particularly true when we approach pathologies such as chronic renal failure, featuring severely compromised renal function and histology.

Signals, Receptors and Trafficking of Bone Marrow Mesenchymal Stem Cells

Migration of systemically administered BMMSCs, endogenous stem cells or resident tissue stem/progenitor cells is a prerequisite to exert any type of effect at sites of injury. Recruitment of BMMSCs occurs through a complicated multistep process (Fig. 20.3). As a first step, BMMSCs sensing the chemotactic factors from the remote injured tissue are released from their storage niche into circulation in a process known as mobilization. Then, stem cells migrate to the site of injury, where they interact with resident renal cells.

Knowledge about the nature of factors secreted from the injured tissue to mobilize BMMSCs from the bone marrow and guide them to the target tissue is relatively limited. However, it is believed that growth factors, cytokines and chemokines, which are upregulated under injury conditions and released into the circulation, can stimulate BMMSCs to downregulate the adhesion molecules that hold them in their niche. Many of these secreted mediators serve as chemotactic signals that guide BMMSCs to the appropriate microenvironment.

Chemokines and their receptors play a critical role in regulating the mobilization, trafficking and homing of BMMSCs. Chemokines are a family of chemotactic cytokines that were first identified on the basis of their ability to stimulate the migration of diverse cell types, particularly those of lymphoid origin [70,71]. They are

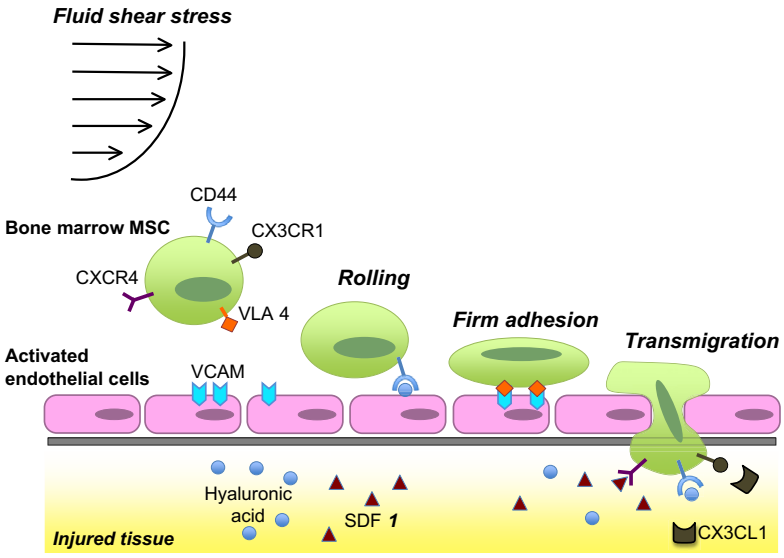

FIGURE 20.3 Multistep process of stem cell homing, adhesion and engraftment in damaged tissues. Chemokines released at the sites of the injury recruit bone marrow mesenchymal stem cells (BMMSCs) that interact with microvascular endothelial cell adhesive proteins through a multistep process including rolling and then stable adhesion. Following this, adherent stem cells transmigrate through the endothelial layer and reach the target injured area where they can exert their paracrine action. VLA: very late antigen; VCAM: vascular cell adhesion molecule; SDF: stromal cell derived factor.

distinguished from other cytokines by being the only members of the cytokine family that bind to the superfamily of G-protein-coupled seven-transmembrane domain receptors. There are approximately 50 human chemokines, which segregate into four categories based on the position of the cysteine residues within the primary amino acid sequence: CC chemokines, CXC (C X3 C motif) chemokines, the CX3C family, and lymphotactin as the sole member of its family [71 73]. Cells are able to produce and release chemokines in a cell- and stimulus-specific manner and their expression is upregulated in response to a range of diseases [74]. Consequently, expression of the appropriate chemokine receptors on BMMSCs is crucial for migration and engraftment in the damaged tissue. BMMSCs express a unique set of chemokine receptors [31,75 80]: seven CC chemokine receptors, five CXC chemokine receptors and CX3CR1 (Table 20.2). In cell biological assays, all of the tested chemokines, including CXCL12 (C X C chemokine ligand 12), CXCL13, CXCL16, CCL19 (C C chemokine ligand 19), CCL5 and CCL25, stimulated chemotaxis of BMMSCs, and CXCL12 induced cytoskeleton F-actin polymerization [75]. In a recent report, Ponte et al. demonstrated expression of CCR2, CCR3, CCR4 and CXCR4 in human BMMSCs and found that TNF increases CCR2, CCR3 and CCR4 expression [31].

Chemokines, by interacting with their specific receptors, are able to induce integrin upregulation and to activate various downstream signaling events. Integrins are known to mediate the firm adhesion of BMMSCs to endothelial cells, playing an important role in their transendothelial migration and subsequent engraftment [81,82].

Engraftment of BMMSCs to the sites of target tissues involves a cascade of events, including rolling of stem cells in the blood vessels and stable adhesion onto the endothelial cell surface lining the capillaries (Fig. 20.3). Then, transendothelial migration, extravasation from the blood vessels and migration of BMMSCs through

TABLE 20.2 Chemokine Receptors and Adhesion Molecules Expressed by Human Bone Marrow Mesenchymal Stem Cells

	Ref.
CHEMOKINE RECEPTORS	
CCR1, CCR7, CCR9, CXCR4, CXCR5, CXCR6	[75]
CCR1, CCR4, CCR7, CCR10, CXCR5	[76]
CCR1, CCR7, CXCR4, CXCR6, CX3CR1	[77]
CCR6, CXCR4	[78]
CXCR4	[79]
CCR2, CCR3, CCR4, CXCR4	[31]
CXCR1, CXCR2, CCR2	[80]
ADHESION MOLECULES	
ALCAM	[85]
ICAM 1, ICAM 2, vitronectin R β chain	[86]
ICAM 3	[87]
L selectin	[88]
LFA 3, VLA α_1, VLA α_2, VLA α_3, VLA α_5, VLA $\alpha_6\beta_4$ integrin	[89]
NCAM	[90]
HCAM, VCAM, hyaluronate receptor, VLA β chain	[91]

CCR: C C motif chemokine receptor; CXCR: C X C motif chemokine receptor; ALCAM: activated leukocyte cell adhesion molecule; ICAM: intercellular adhesion molecule; LFA: lymphocyte function-associated antigen; VLA: very late antigen; NCAM: neural cell adhesion molecule; HCAM: homing-associated cell adhesion molecule; VCAM: vascular cell adhesion molecule.

the extracellular matrix occur into the target injured area (Fig. 20.3). A wealth of data supports the notion that BMMSCs may utilize adhesion molecules for homing to sites of damaged tissues, similar to the adhesion molecules engaged by leukocytes for recruitment to sites of inflammation [83,84]. Indeed, many of the molecules known to be involved in the tethering, rolling, adhesion and transmigration of leukocytes from the bloodstream into tissues are known to be expressed on BMMSCs. These molecules include integrins, selectins, cellular adhesion molecules (CAMs) and chemokine receptors (Table 20.2).

Adhesion molecules mediating BMMSC endothelial cell interaction include various integrins and CAMs, such as very late antigen (VLA), $\alpha_{1\ 6}$, VLA-β, β_4, vitronectin Rα, vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-2/3 (ICAM-1/2/3), activated leukocyte cell adhesion molecule (ALCAM), homing-associated cell adhesion molecule (HCAM) and neural cell adhesion molecule (NCAM) (Table 20.2) [85 91]. These adhesion molecules and their counterligands are expressed on either BMMSCs or endothelial cells. Approximately half of human BMMSCs express the integrin VLA-4. In vitro studies demonstrated that, under conditions of shear stress, binding of human BMMSCs to endothelial cells was mediated by VLA-4 [78]. Treating endothelial cells with a neutralizing antibody to its counterpart adhesion molecule VCAM-1 induced a decrease in human BMMSC adherence, thus indicating the involvement of the VLA-4 VCAM-1 axis of stem cell endothelial cell interaction. Likewise, three-dimensional systems revealed that BMMSC transmigration through the endothelium requires the interaction of VCAM-1 and VLA-4, and triggers the clustering of β_1 integrins and secretion of matrix metalloproteinases (MMP-2) [82]. Furthermore, it has been demonstrated that BMMSCs adhere to cardiac microvascular endothelium and that adhesive properties of both cell types can be increased by the proinflammatory cytokines TNF-α and IL-1β under shear stress conditions [81]. Consistently, in vivo, activation by BMMSCs with TNF-α before injection significantly enhanced cardiac homing of BMMSCs. This response was completely blocked by pretreating either cardiac microvascular endothelium or BMMSCs with anti-VCAM-1 antibodies, but not by anti-ICAM-1 antibodies, suggesting that cytokine-induced adhesion is, at least partly, mediated by the VCAM-1 VLA-4 axis.

Chemokine-mediated BMMSC migration has been documented in various experimental models. After myocardial infarction, the levels of SDF-1 protein have been observed to rise significantly in the left ventricle of mice, especially in the cardiomyocytes and blood vessels within the infarct zone, but not in remote areas of the myocardium [92]. When BMMSCs were intravenously administered into mice 48 h after induction of myocardial infarction they migrated to ischemic cardiac tissue. The importance of SDF-1 and its receptor CXCR4 in BMMSC migration was confirmed by the administration of a specific CXCR4 receptor antagonist, AMD3100, which significantly inhibited BMMSC recruitment to the infarcted site. Furthermore, when the myocardium was transduced with an adenoviral vector containing SDF-1, which led to an increase in SDF-1 expression, BMMSC detection in the heart was significantly increased [92]. In support of these findings, SDF-1 levels in humans have also been observed to rise in patients after myocardial infarction [93].

In the kidney, the SDF-1 CXCR4 axis regulates vascular development [94] and mediates kidney regeneration in ischemic AKI [95]. More precisely, it has been shown that SDF-1 expression in the mouse kidney increased after ischemia reperfusion-induced AKI and decreased in the bone marrow, thereby reversing the normal gradient between bone marrow and the periphery. This phenomenon causes mobilization of bone marrow-derived cells into the circulation and their subsequent homing into the injured kidney. Correspondingly, Mazzinghi and colleagues have demonstrated in mice that SDF-1 is strongly upregulated in resident cells surrounding necrotic areas after AKI [96]. Blocking of CXCR4 or CXCR7 receptors on renal stem/progenitor cell surfaces reduced their engraftment and abrogated their beneficial effect on injured kidney when infused in mice with AKI [96]. In addition to their role in mediating cell migration, chemokines may play important autocrine and paracrine roles. SDF-1 promotes the growth, survival and development of BMMSCs, which may act in an autocrine manner via CXCR4 [97]. Recently, a novel function of CD44, a homing molecule for BMMSCs, was demonstrated by Sackstein et al. [98]. They found that a distinct glycoform of CD44-mediated trafficking of human BMMSCs to bone in NOD/SCID mice through interactions with E-selectin, which is constitutively present on marrow vasculature [98]. Moreover, the adhesion protein CD44 and its main ligand hyaluronic acid (HA) are thought to be involved in several processes mainly including cytoskeleton rearrangement and cell migration. Consistently, in vitro studies have shown that HA promoted a dose-dependent migration of BMMSCs which was abrogated by an anti-CD44 blocking antibody [58]. In the same study, stem cells injected into mice with AKI migrated to the injured kidney where HA expression was increased. Renal localization of the BMMSCs was blocked by preincubation with the CD44 blocking antibody or by soluble HA. Stem cells derived from CD44 knockout mice did not localize to the injured kidney and did not accelerate morphological or functional recovery. Reconstitution by transfection of CD44 knockout stem cells with cDNA

encoding wild-type CD44 restored in vitro migration and in vivo localization of the cells to injured kidneys, suggesting that CD44 and HA interactions recruit exogenous BMMSCs to injured renal tissue and enhance renal regeneration [58].

A fundamental role for another chemokine, CCL2, in mediating BMMSC migration to ischemic brain tissue was also documented. The injured brain tissue extract induced the migration of BMMSCs in vitro, which was significantly diminished by the neutralizing CCL2 antibody [56]. CCL3 and CXCL8 have also been proposed as important mediators of BMMSC migration to damaged cerebral tissue [99].

Strategies to Increase Bone Marrow Mesenchymal Stem Cell Migration

BMMSCs express a heterogeneous repertoire of chemokine receptors (Table 20.2) that are instrumental to the process of homing to different tissues in response to a wide spectrum of stimuli. Since BMMSC-specific surface receptors and adhesive molecules contribute to mobilization, migration and stem cell interactions with resident cells, the modulation of these trafficking signals may allow a more precise targeting of stem cells to the damaged areas. A potential strategy to enhance the migratory capacity and engraftment efficiency of BMMSCs may be ex vivo preconditioning with compounds recruited from the tissue injury milieu or the developmental machinery. In this direction, several studies have examined different factors for their properties to modulate migration. It has been demonstrated that preconditioning of rat BMMSCs with IGF-1 increased the expression levels of the chemokine receptor CXCR4 and enhanced the migratory response of BMMSCs to SDF-1 [100]. An IGF-1-induced increase in BMMSC migration in response to SDF-1 was attenuated by phosphoinositide-3 kinase (PI3K) inhibitor (LY294002 and wortmannin), suggesting that IGF-1 exerts its effect via PI3/Akt signaling. Glial cell line-derived neurotrophic growth factor (GDNF), a potent regulator of kidney organogenesis and prosurvival factor for neurons [101], increased the in vitro motility of cultured BMMSCs and had a cytoprotective effect against apoptotic death [102].

Epidermal growth factor (EGF) is secreted from platelets and macrophages in wounded tissues [103] and exhibits various actions on many cell types, including migration and proliferation [104]. It has been shown that adipose-derived MSCs, expressing EGF receptor, proliferated and migrated in vitro in response to EGF without changing the undifferentiated state [105]. Another fundamental regulator of organ formation during embryogenesis and tissue homeostasis in adults is HGF, a pleiotropic cytokine of mesenchymal origin exhibiting mitogenic, morphogenic, motogenic and differentiating properties [106]. HGF is involved in kidney [107] and liver regeneration [108] and displays in vivo cytoprotective activity in different cell types of injured organs [108]. Short-term exposure of BMMSCs to HGF induced the activation of its cognate Met receptor and the downstream effectors extracellular signal-regulated protein kinase-1/2 (ERK-1/2), p38 mitogen-activated protein kinase (p38MAPK) and PI3K/Akt, while long-term exposure to HGF resulted in cytoskeletal rearrangement and cell migration [109]. The effects of HGF on BMMSC migration were abrogated by the PI3K inhibitor wortmannin, suggesting that HGF-induced migration is PI3K/Akt dependent. bFGF participates in kidney development as a morphogen and is re-expressed in renal tissue during the recovery phase of AKI [110]. bFGF was released from endothelial cells and macrophages during hypoxia or vascular injury and promoted endothelial proliferation and migration [111,112]. In vitro, bFGF increased the migratory activity of BMMSCs through activation of the Akt/protein kinase B (PKB) pathway [113]. In the same study, erythropoietin, IL-6, SDF1-β and VEGF were also analyzed for their promigratory properties. All tested factors were able to increase BMMSC migration, but to a lower extent than bFGF. Preliminary data from the authors' laboratory have shown that incubation of BMMSCs with TNF-α, IGF-1 or GDNF increases the in vitro migration of stem cells and induces cytoskeletal rearrangements. In addition, it emerged from in vivo experiments that preconditioning of BMMSCs with IGF-1 before injection increases the engraftment of PKH26-labeled BMMSCs into the injured kidney and totally restores renal function in cisplatin-induced AKI (unpublished data by C. Xinaris).

Like growth factors, some cytokines appear to achieve similar effects on MSC migratory behavior. It has been reported that preincubation of BMMSCs with TNF-α, a potent proinflammatory cytokine, enhances migration towards chemokines in vitro and simultaneously induces the expression of the chemokine receptors CCR2, CCR3 and CCR4 [31]. Another study has shown that short stimulation (24 h) of BMMSCs with a cocktail of cytokines, including Flt-3 ligand, SCF, IL-6, HGF and IL-3, resulted in upregulation of both cell surface and intracellular CXCR4, increasing in vitro stem cell migration in response to SDF-1 and homing to the bone marrow of irradiated NOD/SCID mice [114]. Moreover, transplantation of cytokine-treated BMMSCs resulted in faster hematological recovery and higher levels of donor chimerism in bone marrow through CXCR4 than in non-treated cells. Finally, preconditioning with SDF-1 suppressed BMMSC apoptosis, enhanced their survival, engraftment and vascular

density, and improved myocardial function via SDF/CXCR4 signaling [115].

Besides strategies using growth factors or cytokines, hypoxic preconditioning of BMMSCs before their delivery was also tested. Short exposure of BMMSCs to 1% oxygen increased the expression of chemokine receptors CX3CR1 and CXCR4, as well as the in vitro migratory capacity in response to fractalkine (CX3CL1) and SDF-1, respectively, in a dose-dependent manner [116]. Hypoxic conditions also rapidly induced BMMSC migration and three-dimensional capillary-like structure formation in Matrigel [117]. In addition, MMP-2 mRNA expression and protein secretion were downregulated in BMMSCs, while those of membrane type-1 (MT-1)-MMP were strongly induced by hypoxia.

Genetic modification of stem cells before transplantation represents a novel approach to increase the migratory capacity and homing of BMMSCs. Recently, several studies have focused on engineering cells to express key cell surface receptors and homing molecules involved in stem cell recruitment and migration. Adenoviral overexpression of IGF-1 in BMMSCs induced massive stem cell mobilization via SDF-1 signaling and culminated in extensive angiomyogenesis in the infarcted heart, thus acting in an autocrine and paracrine fashion to activate survival signaling in MSCs and the host myocytes [118]. IGF-1 also promoted multiple growth factor expression in BMMSCs, including SDF-1 and VEGF, which stimulated bone marrow and endothelial progenitor cell mobilization to the ischemic myocardium [118]. The accelerated mobilization of stem cells resulted in extensive neovascularization and myogenesis in the infarcted heart. Furthermore, engineering of BMMSCs to overexpress CXCR4, the SDF-1 receptor, similarly led to greater homing of MSCs and improved left ventricular function compared with control MSCs when the cells were delivered within 24 h after myocardial infarction [119].

As discussed above, overexpression of the receptors or homing factors can improve the migratory potential and engraftment of MSCs in various types of tissue. Other strategies for achieving positive effects have been studied. Upregulation of endothelial nitric oxide synthase (eNOS) expression has also been shown to improve the migratory capacity of BMSCs [120]. Stem cells from patients with coronary artery disease had reduced migratory capacity compared with BMSCs from healthy subjects [121]. Pretreatment of BMSCs from patients with the eNOS transcription enhancer AVE9488 (AVE) resulted in restoration of SDF-1-mediated stem cell migration and significantly increased neovascularization after infusion of cells into a hindlimb ischemia model [120].

Strategies to Enhance Bone Marrow Mesenchymal Stem Cell Survival and Efficacy

As described above, BMMSCs represent an ideal population for the treatment of ischemic diseases because of their tropism for damaged tissues and regenerative properties. However, a major limitation of cell therapy is the high sensitivity of transplanted stem cells to the hypoxic and inflammatory environment of ischemic tissues. Several strategies leading to enhanced stem cell survival and paracrine activity have been investigated by genetic modification [122 126] or by using preconditioning agents [127] before stem cell infusion. In particular, early data proved the hypothesis that BMMSCs that had been retrovirally transduced with the prosurvival gene *Akt*, when infused into rats with ischemic heart damage, were more resistant to apoptosis after hypoxia reoxygenation and more efficiently restored cardiac repair and function by preventing the process of cardiac remodeling [122]. Similarly, a significant increase in cardiomyocyte survival, vascular density and cardiac function was observed in animals with acute myocardial infarction transplanted with BMMSCs genetically modified to overexpress the antioxidant and antiapoptotic genes heme oxygenase-1 (*HO-1*) [123] or *Bcl-2* [124]. In both studies, the number of genetically modified BMMSCs that survived within the damaged tissue and their efficiency in tissue repair were significantly increased. On the basis of recent evidence that SDF-1 has growth and survival benefits in CXCR4-expressing BMMSCs [97], SDF-1-overexpressing BMMSCs were also tested in acute myocardial infarction [126]. The results showed that these cells were more resistant to hypoxic conditions and markedly preserved cardiomyocytes within the infarcted zone [126]. Gene-modified BMMSCs have also shown additive or enhanced therapeutic effects in preclinical models of AKI [125]. BMMSCs that had been genetically modified with the serine protease kallikrein by adenovirus transduction were more resistant to oxidative stress-induced apoptosis and secreted higher levels of VEGF in culture medium than control BMMSCs. In vivo, infusion of kallikrein-modified BMMSCs enhanced protection against acute ischemic kidney injury by inhibiting apoptosis and inflammation [125]. Other strategies to improve the ability of grafted stem cells to survive and produce paracrine factors can be achieved by ex vivo pretreatment with melatonin, known for its antioxidant and stimulating activity on cytokine production [127]. In a rat model of AKI, melatonin preconditioning allowed the long-term survival of BMMSCs within the kidney associated with angiogenesis, proliferation and recovery of renal function. In vitro, melatonin increased the resistance of BMMSCs to hydrogen peroxide-induced apoptosis by promoting

overexpression of the antioxidant enzyme catalase and superoxidase dismutase [127]. Of note, GDNF, a member of the transforming growth factor family, was cytoprotective against oxidative stress-induced apoptotic death of cultured kidney-derived MSCs [102].

Together, these findings suggest that the use of preconditioning agents or genetic modification of BMMSCs could represent an innovative and safe strategy for increasing the efficacy of stem cell therapy for damaged organs.

CONCLUSION

This overview indicates that BMMSCs are potent tools for many clinical applications in the evolving field of regenerative medicine. Although early preclinical and clinical data demonstrate the safety and effectiveness of MSC therapy mainly in acute injury, before BMMSCs are used therapeutically in patients there are many questions to be answered. How should the cells be delivered for localized therapy? How many cells should be injected for a given application? When should the cells be injected? Additional information is also required regarding the therapeutic efficacy of administered cells and the mechanisms of engraftment, homing and in vivo differentiation. There is also a need to carry out appropriately designed experimental studies to verify the long-term safety of these therapies and to examine the risks of maldifferentiation or malignancy. The effective use of stem cell therapy also demands the development of methods for large-scale culture, storage and delivery to the patient.

Understanding the mechanisms involved in stem cell-based tissue repair will expand the current knowledge on the key factors that govern stem cell migration, engraftment, function and survival which, if appropriately exploited, could enhance the therapeutic effect of stem cells. Emerging strategies, such as pretransplant preconditioning of BMMSCs with growth factors or cytokines, or exposure to hypoxia, known to influence cell survival, proliferation and migration, are currently being tested to improve the regenerative potential of stem cells. Moreover, genetic modification of stem cells has been broadly investigated in vitro and less so in preclinical models, with the goal of enhancing their homing, survival and efficacy. Genetically engineered BMMSCs are also being studied as a vehicle for the in vivo delivery of anti-inflammatory molecules, mitogenic and prosurvival factors, therapeutic genes or drugs. The treatment of severely compromised organs, such as in chronic renal diseases, may take advantage of the global enhancement of stem cell action. However, although there are encouraging preclinical results, more studies need to be conducted to overcome critical issues and allow a more informed transfer to the clinic.

Acknowledgments

The authors are indebted to Antonella Piccinelli, Cinzia Rota and Valentina Benedetti for their helpful collaboration in preparing the figures, and Manuela Passera for valuable assistance in organizing the references.

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CHAPTER

21

Treatment of Acute Kidney Injury with Allogeneic Mesenchymal Stem Cells: Preclinical and Initial Clinical Data

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Regenerative Nephrology DOI: 10.1016/B978-0-12-380928-5.10021-1

ACUTE KIDNEY INJURY: CLINICAL AND EXPERIMENTAL ISSUES

Clinical Acute Kidney Injury

In 1951 Homer Smith introduced the term "acute renal failure" in his textbook *The Kidney: Structure and Function in Health and Disease*, describing the abrupt decrease in renal function, caused by various renal insults, resulting in nitrogenous waste product accumulation. Currently, acute renal failure (ARF) due to acute kidney injury (AKI) is defined as the abrupt partial or complete loss of kidney functions that results in disturbed volume, electrolyte and acid base balance, and uremic, multisystem complications due to retention of toxic waste products, a heightened inflammatory state and generation of reactive oxygen species (ROS), together resulting in life-threatening multiorgan complications with high mortality rates, particularly in elderly patients and those with significant other comorbidities [1 4].

Because of widely divergent definitions of ARF, in 2004 the Acute Dialysis Quality Initiative (ADQI) (www.ccm.upmc.edu/adqi/ADQI2/ADQI2g1.pdf) published a consensus definition for this syndrome, the Risk Injury Failure Loss Endstage renal disease (RIFLE) classification [5]. The definition is based on the change in serum creatinine and/or urine output and combines severity grades based on these variables as well as on clinical outcomes, together combined into the RIFLE criteria (risk, injury and failure, with the outcome classes loss and end-stage kidney disease). The term acute kidney injury (AKI) was introduced to define the syndrome broadly, identifying both early and mild forms as well as severe forms, requiring renal replacement therapy. The ADQI group subsequently published a simplified version of the RIFLE classification, known as the AKI Network classification [6]. The categories risk, injury, and failure were replaced by stages 1, 2 and 3, respectively. An absolute increase in creatinine of at least 0.3 mg/dl was added to stage 1; patients needing renal replacement therapy are automatically classified as stage 3. The RIFLE outcome categories loss and endstage renal disease were deleted.

Clinical settings that result most commonly in AKI include ischemia reperfusion insults of the kidney due to major surgery, shock, sepsis, trauma and nephrotoxins such radiocontrast medium, aminoglycosides, cisplatin and others [7]. Comorbidities that greatly enhance the susceptibility of patients to develop severe AKI include underlying renal disease [8], a recently challenged risk factor [9], older age, diabetes mellitus, nephrotic syndrome, congestive heart failure, chronic obstructive pulmonary disease and prolonged cardiopulmonary bypass times in patients undergoing coronary artery bypass graft (CABG) or valve surgery [10 12].

Severity and extension of injury are related to the nature and duration of the causative agent or insult. Pathological data were initially limited to autopsy findings, based on which AKI has long been termed acute tubular cell necrosis (ATN). The subsequent utilization of renal biopsies demonstrated that in patients with significant AKI only very limited tubular cell necrosis is seen, and only discrete cell loss, affecting mainly the proximal tubule and occasionally the medullary thick ascending limb (mTAL) and rarely the collecting duct. This pattern has recently been described as "physiologic and pathologic dissociation" [13].

The morphological manifestations of AKI develop in several overlapping phases. Initially, ultrastructural changes manifest as cytoskeletal alterations leading to changes in the polarization of proximal tubular cells and loss of their brush border [14]. Cell detachment is a consequence of these cytoskeletal alterations and eventually some cells undergo apoptosis or may become necrotic if an insult is severe and rapid. This is followed by repair and regeneration. Cell loss and detachment make the tubular epithelium appear flattened and expose areas of denuded basement membrane. Detached tubular cells are seen in the tubular lumen and can be cultured from the urine. Debris as well as dead and viable cells cause tubular obstruction downstream. Vascular changes in AKI affect mainly the peritubular capillaries and result in vascular congestion mainly in the corticomedullary region. The acute vascular insult results in leaks and subsequent rarefaction of peritubular capillaries with distension of surviving capillaries [15].

Data on the renal pathology of human AKI remain limited because the lack of systematic studies describing early, intermediate and late changes. In contrast, the renal pathology in animal models of AKI has been extensively described. Yet, the morphological changes in animal kidneys cannot be directly compared to the human situation since the majority of animal studies are conducted in otherwise healthy animals, i.e. animals that lack comorbidities that are regularly present in patients with significant AKI. It holds, therefore, that effective therapies can only be developed if cellular, molecular, pathophysiological and morphological manifestations of AKI are fully understood and thus comprehensively targeted.

The incidence of AKI is becoming more common in the hospital, mainly caused by an aging population subjected to high-risk procedures while being affected by high rates of cardiovascular, diabetic, hepatic and pulmonary comorbidities, rendering these patients particularly susceptible to AKI [4]. AKI is also becoming an increasingly important cause of end-stage renal disease (ESRD) since a significant percentage of

patients with AKI progresses to ESRD within 2 years after diagnosis (United States Renal Disease Survey, 2006) [1,2].

It is well recognized that, despite the provision of intensive, continuous renal replacement and all other needed supportive therapies, morbidity and mortality, exceeding 50%, and associated treatment costs in patients with dialysis requiring degrees of AKI have remained disturbingly high [16]. This dismal situation, together with the late development of ESRD post-AKI and high cost burdens, continue to make the development of effective interventions a therapeutic urgency. Despite the fact that extensive preclinical studies with novel agents showed and continue to show renoprotective efficacy in experimental AKI, the data from a subsequent clinical trial that evaluated insulin-like growth factor-1 (IGF-1) were either inconclusive or failed to demonstrate efficacy [17,18]. The use of the highly original Renal Assist Device in a group of patients with severe AKI was promising, as its use improved both renal outcomes and patient survival [19]. Currently, however, this complex system is not available in the clinic.

In conclusion, the frequency of AKI in hospitalized patients and its impact on the outcome of underlying comorbidities, as well as the high mortality and therapeutic costs associated with AKI, urgently warrant the development of effective therapeutic interventions. The reality, however, is that there is no specific pathophysiology-based treatment approach, and care remains limited to supportive measures such as fluid, electrolyte and blood pressure management and, as needed, provision of various renal replacement modalities.

Treatment Resistance of Clinical Acute Kidney Injury

There are numerous reasons why significant clinical AKI has essentially remained and/or appears to be unresponsive to renal replacement therapy (RRT) and resistant to numerous novel interventions that have been tested in clinical trials [19]. All utilized modalities of hemodialysis provide only partial replacement of renal functions and, in addition, are invasive and thereby affected by their associated complications.

It is well documented that more elderly and severely ill patients with significant comorbidities are being subjected to high-risk surgical procedures, resulting in an increased incidence of AKI. Recent studies have confirmed that different dialysis modalities and higher doses of dialysis in patients with AKI do not improve outcomes [20]. The ideal treatment for AKI targets its complex pathophysiology directly by interfering with all major cellular and molecular processes causing AKI, and thereby improving adverse multisystem complications. At the time of writing, essentially all novel therapeutic approaches in AKI lack a solid, broad-based pathophysiological focus.

Experimental Models of Acute Kidney Injury

While it is well recognized that major comorbidities increase the susceptibility of patients to developing severe AKI, practically all animal studies that show therapeutic benefit in AKI have ignored this critical fact by having been conducted in otherwise healthy animals. This approach is in frank contrast to AKI in the hospital setting, where patients with comorbidities such as diabetes or other multisystem diseases are at highest risk for severe AKI. These limitations of commonly used models are in part responsible for the slow and sometimes unsuccessful translation of experimental therapies into clinical practice. The present authors recently began to address this issue, showing that AKI induced in rats with underlying chronic kidney disease (CKD) (5/6th nephrectomy), i.e. acute on chronic renal failure, can be readily treated with mesenchymal stem cells (MSCs) (unpublished data).

For the purpose of simplification, clinical and experimental AKI have been divided into categories based on ischemic and toxic insults. For each of these, a number of animal models is available in which their respective pathophysiology and experimental therapies can be studied. These models are widely used; however, they are associated with significant limitations and their pathophysiology only reflects certain aspects of human injury [21].

Ischemic models (primarily warm ischemia with reperfusion) most commonly use clamping of the renal arteries with complete cessation of blood flow for varying times, causing different degrees of injury. The principal determinant of injury in this setting is tissue hypoxia due to blood flow cessation with changes in outer medullary microcirculation, subsequent induction of inflammation and tubular cell injury, mainly affecting the S3 segment of the proximal tubule. Reperfusion induces formation of ROS with resulting cellular injury. While hypoxemia and hypotension are commonly seen clinically, complete cessation of blood flow with massive cellular injury and frank cortical necrosis, as seen in animal models, is rare.

Nephrotoxic models include exposure to cisplatin, heavy metals, aminoglycoside antibiotics and glycerol-induced rhabdomyolysis with heme pigment release. These agents cause multifactorial tissue damage, including apoptosis and hypoxia, mainly in tubular cells, but some are not nephrotoxic in humans or toxicity can be prevented, e.g. by vigorous hydration before and after the administration of cisplatinum [22].

Several models have been developed that claim to be similar in their pathophysiology to the decline in renal

function that is associated with sepsis. These sepsis models include endotoxin administration, bacterial infusion, cecal ligature and perforation, and intraperitoneal infusion of bacteria. While sepsis-associated AKI is an important independent contributor to morbidity and mortality, these models only satisfy some aspects of the complicated pathophysiology of AKI in critically ill patients [23 25]. The cecal ligation model is primarily a model of cytokine storm and to some extent neglects the fluid imbalance and vascular aspects of human sepsis. The response to endotoxin is very variable across species and rather inconsistent in rodents. There is also considerable variability in morbidity and mortality of animals among laboratories using these models.

Design of Clinical Trials

The design of a successful clinical trial requires that a suitable clinical setting is chosen in which the efficacy of a novel intervention can be reliably tested. For example, lack of a proper study design probably contributed to the apparent failure of a trial with IGF-1 in AKI patients [17,18]. In these studies, the enrolled subjects represented an inhomogeneous cohort of patients with ARF in whom a tested intervention was given at widely different time-points after the initial insult, and the study drugs were harmful because they caused hypotension.

These examples strongly argue for study designs that test novel therapies in a clinical setting where the time-point of a potential renal insult and the subsequent intervention are clearly defined [26]. Mindful of this, the present authors chose to test the safety and preliminary efficacy of allogeneic MSC therapy in patients undergoing on-pump CABG and/or valve surgery, the potential renal insult, and precisely defined the postoperative time-point at which allogeneic MSCs, i.e. the therapy, were given (see below). In addition, realistic endpoints were chosen, changes in novel biomarkers for AKI were assessed and the RIFLE criteria were used to define AKI (see below). Based on the same rationale, a clinical trial will be conducted in recipients of cadaveric kidney transplants who, being at high risk of delayed graft function (DGF), will be treated with allogeneic MSCs. In this setting, both the renal insult (cold ischemia time, surgery) and time of cell administration are well defined, facilitating the optimal assessment of safety and preliminary efficacy of such an intervention.

Novel Diagnostic Biomarkers for Acute Kidney Injury

Under most steady-state conditions serum creatinine levels are a reasonable indicator of glomerular filtration rate (GFR) but are not specific for an acute renal injury per se. There can be marked injury with relative preservation in GFR and vice versa. An ideal marker for AKI would identify risk, provide prognostic information for the possible development of AKI, specifically diagnose both renal injury and loss of function, and indicate successful recovery with resolution of the causative derangements. In recent years several novel biomarkers for kidney injury have been identified. The most promising include kidney injury molecule-1 (KIM-1), interleukin-18 (IL-18), neutrophil gelatinase-associated lipocalin (NGAL) and cystatin C [27,28]. Since a rise in serum creatinine in patients with AKI may not be the result of an actual renal injury, and even if caused by an actual injury, it is often delayed by 24 48 h, its levels may be affected by its generation rate or by changes in extracellular volume. From this it follows that sole reliance on serum creatinine levels is inadequate for the proper management of this complication. In addition, a decrease in urine output, which can also be the result of prerenal or postrenal complications and thus not represent actual renal injury, may further prevent the prompt institution of potentially effective therapies. The introduction, therefore, of such novel diagnostic biomarkers for AKI is essential for its early and specific diagnosis, permitting the administration of therapies within a few hours after a given insult. This approach is expected to significantly improve overall outcomes in patients with AKI. However, whether this is seen with the currently available treatment options remains to be shown.

Pathophysiology-based Therapy

The authors believe that single pharmacological agents that focus only temporarily on some aspects of the highly complex pathogenesis and pathophysiology of AKI (summarized in Fig. 21.1) are inferior to stem cell-based interventions. The reason for this may lie in the fact that infused MSCs, as described below, home to the site of injury, adhere to the microvascular endothelium in a regulated fashion, as has been shown in the heart [29], interpret and respond to cues from the damaged microenvironment by changing their own gene expression profile and that of responsive renal cells to a cytoprotective, anti-inflammatory and repair-stimulating pattern. After effectively carrying out these actions, which requires generally less than 72 h in the kidney, MSCs detach in a regulated fashion, leave the kidney and undergo anoikis/apoptosis in the circulation. As further discussed in detail below, these complex organ protective and regeneration-stimulating effects of MSCs are mediated by paracrine and endocrine mechanisms, and not by their differentiation into and replacement of lost target cells or significant fusion with target cells.

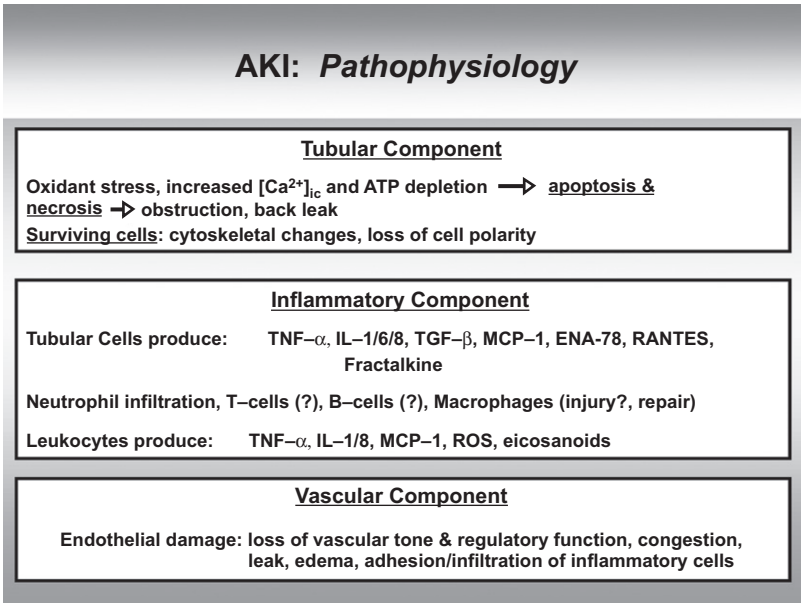

FIGURE 21.1 Major components of the pathophysiology of acute kidney injury (AKI). TNF: tumor necrosis factor; IL: interleukin; TGF: transforming growth factor; MCP: monocyte chemotactic protein; ENA: epithelial neutrophil activating protein; RANTES: regulated on activation, normal T cell expressed and secreted; ROS: reactive oxygen species.

Injury to the kidney or other solid organs may result in the transient expression or export of the biology of the stem cell niche, i.e. the creation of a facultative niche, in which small numbers of administered MSCs function as they do in the bone marrow or in a pericyte location, i.e. they respond to insults at these sites by carrying out anti-inflammatory, cytoprotective and repair-supporting programs. These nursing effects of MSCs are well recognized in clinical bone marrow transplantation, where these cells are known to facilitate hematopoietic stem cell (HSC) engraftment and afford their protection through identical paracrine actions [30,31].

TREATMENT OF ACUTE KIDNEY INJURY WITH STEM CELLS: PRECLINICAL STUDIES

Pathophysiology and Repair of Acute Kidney Injury

Ischemia reperfusion and toxic insults to the kidney result in a rapid succession of pathophysiological events or stages of AKI that lead to loss of function (Fig. 21.1). Molitoris and Sutton recently described these successive pathophysiological stages as an initiation phase, injury phase, vascular extension phase and recovery phase [32]. These phases are characterized by decreases in renal blood flow, decline in GFR, vascular congestion, tubular and vascular damage, epithelial cell depolarization, transepithelial and vascular leaks, cell loss due to apoptosis and cell necrosis, tubular obstruction and accumulation of inflammatory cells in the microvasculature. At the molecular level, the generation of ROS and upregulation of proinflammatory cytokines and other maladaptive responses are recognized as major mediators of the evolving renal injury [33 35].

The kidney, particularly in younger individuals or those with less severe degrees of kidney injury, is readily capable of repairing itself, achieving within days return of normal or adequate kidney function. Organ repair, as is now well established, is primarily carried out by intrinsic renal cells [36 38]. At the gene level, re-expression of developmental programs that are active in organogenesis participate in the overall repair process [39]. It appears likely that additional contributions to the repair process are carried out by circulating stem and progenitor cells, such as MSCs, HSCs and endothelial progenitor cells (EPCs) [40,41]. In addition, a contribution of kidney intrinsic stem cells to the repair process has been suggested by Oliver et al. [42,43]. These investigators identified cells with stem cell characteristics in the rodent papilla, a location that may represent bona fide stem cell niches. Post-AKI, these cells appear to migrate towards the cortical areas of tissue damage, where they may support the repair process. MSCs that are thought to reside in a pericyte location within the kidney [44] may similarly participate in local repair, a possibility that awaits investigation.

It is apparent, however, that the collective capacity of these kidney intrinsic and extrinsic organ-protective and repair-stimulating mechanisms is overwhelmed when the insult is too severe. This is the reason why several investigations have tested whether the therapeutic boosting of stem or progenitor cell delivery to the injured kidney would improve outcomes. The efficacy

of these cell-based interventions was reproducibly demonstrated in cisplatin-, glycerol- and ischemia reperfusion injury (IRI)-induced models of rodent AKI (see below).

Hematopoietic Stem Cell Mobilization and Administration

The initial rationale for the subsequent administration of multipotent, bone marrow-derived stem cells was based on the hypothesis that the acutely injured kidney would generate signals for stem cell mobilization, from the bone marrow, and their homing to the kidney. There, they would adhere to endothelial cells at sites of injury and contribute to repair by differentiation into and replacement of lost renal cells. This response, it was hypothesized further, may represent another recapitulation of a developmental program, according to which stem cell delivery, via the primitive circulation of the embryo, is boosted during organogenesis. Data were generated that were partly in support of this generally held paradigm. Bilateral or unilateral IRI AKI in mice resulted in the prompt mobilization, from the bone marrow, of $CD34^+$ cells (HSCs, endothelial progenitor and other cells) into the circulation and recruitment to the injured kidney only. The mobilization and homing signal that was strikingly upregulated in the injured kidney was stromal cell-derived factor-1 (SDF-1) or CXC chemokine receptor-12 (CXCR12), a chemokine that has a central function in hematopoietic bone marrow niches, where it is produced by reticular and mesenchymal precursor cells, i.e. MSCs [30]. It facilitates recruitment and engraftment of intravenously administered HSCs to the bone marrow niches of a bone marrow transplant recipient. Both HSCs and MSCs express CXCR4, the cognate receptor for SDF-1, albeit only in low numbers in MSCs [45 48]. Physiologically, and even after bone marrow ablation, the highest SDF-1 levels are found in the bone marrow. However, this gradient was reversed following AKI, resulting in higher renal than bone marrow levels of SDF-1. Both in vivo and in vitro, inhibition of CXCR4 activation reversed the recruitment of $CD34^+CXCR4^+$ cells to the injured kidney or their migration across a transwell, identifying SDF-1 as the principal mobilization and homing signal in this experimental setting. Similar roles of the SDF-1 CXCR4 axis have been identified in the injured brain, liver, heart and other organs [49,50]. Of note was the fact that the renal expression of CXCR4 was also upregulated following AKI, a response whose ultimate significance remains to be elucidated [50].

Since AKI resulted in a significant increase in $CD34^+$ cell numbers both in the circulation and in the kidney, it was hypothesized that these multipotent cells must have the capacity to contribute to organ protection and repair. Accordingly, it was tested whether G-CSF and cytoxan-induced augmentation of circulating $CD34^+$ cell numbers at the time of AKI induction would exert a robust kidney protective effect, i.e. in analogy to the beneficial effects that stem cell mobilization had in a mouse myocardial infarct model [51]. In the latter, mobilized HSCs were reported to differentiate and engraft in the myocardium, resulting in overall better outcomes. Unexpectedly, outcomes (survival, renal function, histopathology) in animals that were pretreated with an HSC mobilization protocol were extremely poor compared with vehicle-treated controls (Fig. 21.2). This was the result of concurrent and marked increases in peripheral neutrophil numbers, which abrogated any potential beneficial effects of mobilized HSCs by causing frank renal cortical necrosis. Animals rendered neutropenic before the induction of AKI had significantly improved outcomes, confirming that the adverse actions of stem cell mobilization were exerted by the renal delivery of high numbers of neutrophils. However, several studies with HSCs alone or following bone marrow transplantation and mobilization in experimental AKI suggested their therapeutic potency, mediated by their engraftment following differentiation [52,53], while administration of HSCs to animals with AKI by other investigators were without benefit [54]. From this, it follows that stem cell/progenitor cell mobilization protocols that are associated with a simultaneous rise in circulating leukocytes must be avoided. A recent elegant study by Park et al. [55] demonstrated that spontaneous mobilization and recruitment to the kidney of HSCs, MSCs and EPCs play an important role in the postobstructive repair of the kidney that is injured by unilateral ureteral obstruction (UUO). Additional evidence for the role of the SDF-1 CXCR4 axis in MSC recruitment to the obstructed kidney was also provided.

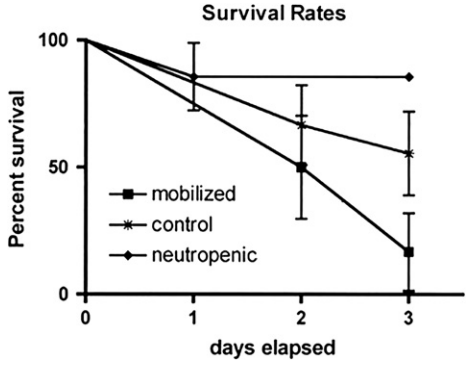

FIGURE 21.2 Stem cell mobilization before induction of acute kidney injury (AKI) in mice worsens (mobilized), while neutropenia (neutropenic) improves animal survival compared with controls with AKI (control) (Kaplan–Meyer analysis). *[Reproduced from Tögel et al., 2004 [41], with permission from Kidney International.]*

In analogy of what has been shown for HSCs, the SDF-1 CXCR4 axis is also involved in the recruitment of CXCR4-expressing MSCs that are administered to animals with AKI [56 58]. This also applies, at least in theory, to the homing of EPCs given to rodents with AKI. Herrera et al. identified CD44, hyaloadherin, the receptor for hyaluronic acid that is expressed on MSCs, as an important mediator of MSC recruitment by the kidney that is injured by glycerol-induced rhabdomyolysis [59]. In this model, interstitial hyaluronic acid is robustly upregulated, facilitating intrarenal binding of administered MSCs and subsequent improvement of kidney function.

Administration of Endothelial Precursor Cells

Patschan et al. demonstrated that interventions that use EPC administration were highly effective in improving renal function by repairing microvascular injury and hemodynamics in animals with AKI [60]. Details of this work are described in Chapter 18. In analogy to these observations, MSCs elicit a similar vasculoprotective effect early after AKI in rats [61] and such treatment of AKI protected microvascular density later after the renal insult [62].

Administration of Multipotent Marrow Stromal Cells

Background

This second type of adult stem cell in the bone marrow cannot be readily mobilized into the circulation, in part owing to its extravascular localization both in the bone marrow niches and in their pericyte location on blood vessels [30,49,63,64]. Bone marrow niches are low oxygen pressure compartments in which MSCs and HSCs are localized both adjacent to the endostium (endosteal or osteoblastic niche) and next to sinusoids (perivascular niche) [30]. At these sites, MSCs secrete, together with reticular cells and megakaryocytes, soluble factors in a paracrine fashion, which importantly include SDF-1. These collectively regulate the maintenance and survival of HSCs in their respective niches [30]. Unlike relatively large MSCs, comparably small HCSs can readily enter the peripheral circulation via characteristic fenestrations in the walls of sinusoidal vessels.

In 1970, Friedenstein et al. first described fibroblast-like cells growing out in culture of bone explantants and bone marrow suspensions [65], cells that were subsequently named MSCs [64]. Friedenstein recognized the bone-forming potential of these cells and distinguished this new cell population from cells undergoing hematopoietic differentiation. These cells were initially named colony-forming units of fibroblasts (CFU-F) that could be readily obtained from the bone marrow, spleen and thymus of mice. Being clonogenic, these cells could additionally be induced to differentiate into osteocyte precursors, chondrocytes, adipocytes and bone marrow stroma cells in vitro. Subsequent studies reported that MSCs can also differentiate into cells from unrelated germ-line lineages, but some of these reports have been questioned and the full nature of the differentiation potential or plasticity of MSCs is a matter of debate that will have to await full elucidation of their in vitro and in vivo biology [49].

In an effort to standardize the nomenclature, a consortium of experts suggested the name "multipotent mesenchymal stromal cells" to refer to this population of fibroblast-like cells that are plastic adherent and can be differentiated into mesenchymal lineages [66]. The defining property of MSCs is their ability to differentiate into various mesenchymal lineages, including bone, cartilage and adipose tissue, as well as tendon, muscle and marrow stroma [67]. In the laboratory, trilineage differentiation into bone, adipose tissue and cartilage is taken as a major defining criterion for MSCs.

The research community is still debating whether these cells have properties of true stem cells and can therefore be called stem cells. This depends largely on the criteria by which a stem cell is defined, and whether that includes the necessity to differentiate into cell types of unrelated germ-line layers. Potential artifacts, induced during in vitro culture and by in vivo tracking methods, and their fusion with target cells, have raised questions as to their true differentiation into cells of non-mesodermal germ layers. Further complicating the issue of their stemness is the fact that true stem cells must be tightly interacting with niche cells to exist as such within a niche system [31]. Since these aspects of the MSC biology remain incompletely understood, the question as to their definitive stemness (self-renewal, differentiation into a mesodermal cell type) remains unsettled, a point that may or may not be important.

The nature of resident MSCs in vivo is only poorly described, and many of their in vivo characteristics are unknown. It has been shown that MSCs can be derived from many tissues, including the bone marrow, adipose tissue, vasculature, cord blood and umbilical cord matrix [68]. MSCs in the microvasculature are found in a pericyte location, from which, it has been suggested, they exert their organ-protective and vasculoprotective activity when a local injury occurs [69]. They locally elicit potent anti-inflammatory effects that cooperate with their antiapoptotic and repair-inducing trophic actions. Despite defined functional differences between tissues of origin, MSCs express a number of common surface markers (CD29, CD44, CD49a f, CD51, CD73, CD90, CD166, CD271, Stro-1) and lack expression of

markers typical of hematopoietic lineages including CD11b, CD14 and CD45 [49,63].

A number of bone marrow-derived pluripotent cells that have the ability to differentiate into cells of mesodermal, endodermal and neuroectodermal lineages have been described.

The yield of MSCs from various tissues is very low. Their frequency has been estimated to be in the order of 0.001 0.01% of total nucleated cells, a percentage that is also dependent on harvesting methods and separation processes [49]. Adipose tissue contains the highest numbers of MSC at approximately 400×10^6/ml [64]. MSCs can be expanded in vitro to hundreds of millions of cells from a 10 20 ml bone marrow aspirate. Most clinical studies have used fetal calf serum to expand MSCs in vitro, adding a potential biohazard to the culture system and potentially alloimmunizing the recipient [70]. Effective culture expansion in animal serum-free media, using platelet lysate or other additives, is now well established [71]. Because of their ability to proliferate extensively in vitro, MSCs seem ideal candidates for large-scale industrial expansion under good manufacturing practice (GMP) conditions. Development of culture systems that include closed bioreactors with larger overall surface areas for effective cell expansion is currently ongoing, and is expected to improve the economy and efficiency of the cell culture process, and thus provide for more efficient large-scale production in commercial applications [72].

MSCs do not induce significant alloreactivity in vivo because they express low levels of major histocompatibility complex (MHC) class I antigens and do not express MHC class II as well as costimulatory molecules including CD40, CD80 and CD86 on their surface [73]. These features protect MSCs from natural killer (NK) cell-mediated lysis and make them attractive for large-scale applications in clinical practice [74]. Figure 21.3 summarizes the currently identified immune-modulating activities that are exerted by MSCs [75]. Additional details are provided in Chapter 9.

Treatment with allogeneic human MSCs in the clinical setting did not induce MSC antibody production or T-cell priming, while antibodies against xenogenic tissue culture medium components such as fetal bovine serum were found [70]. Because of this immune privilege, MSCs have become the premier cell type for clinical applications, representing an off-the-shelf allogeneic cell product.

Allogeneic MSCs have already been successfully used clinically in a number of different diseases. Horwitz et al. showed that allogeneic MSCs derived from the same donor who provided the initial bone marrow transplant for pediatric patients with osteogenesis imperfecta were able to engraft in the majority of recipients, resulting in improved growth velocity and bone mineralization. However, their overall contribution to bone cells was very low (in the range of 1%) and declined over time [76]. Lazarus et al. reported that the combined administration of hematopoietic stem cells and MSCs from the same marrow donor in patients undergoing a bone marrow transplant for hematological malignancies was safe, feasible and resulted in improved bone marrow engraftment [77]. It was reported that steroid-refractory acute graft-versus-host disease was resolved in six out of eight transplant recipients after MSC treatment [78].

To date, MSCs, also termed multipotent marrow stromal cells, have been the mainstay of non-hematological cell therapy in the clinical setting. This is facilitated by

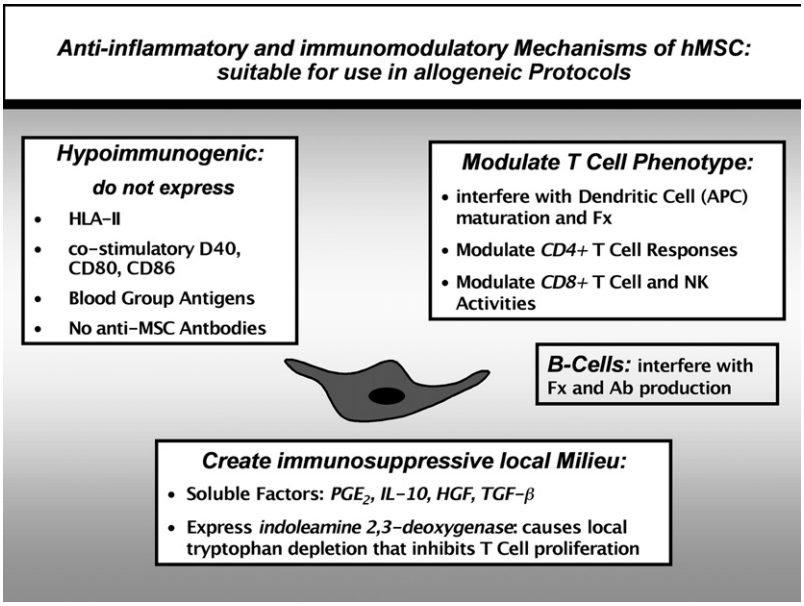

FIGURE 21.3 Anti inflammatory and immunomod ulatory mechanisms expressed by human mesenchymal stem cells (hMSCs). HLA: human leukocyte antigen; APC: antigen presenting cell; Fx: function; NK: natural killer; Ab: antibody; PGE: prostaglandin; IL: interleukin; HGF: hepatocyte growth factor; TGF: transforming growth factor.

the fact that they can be safely administered in an allogeneic fashion, and their use in most conditions has been safe so far. Intravenous infusions of MSCs has been shown to improve repair of multiple organs such as bone [79], ischemic brain [80], heart [81] and pancreas [82], and found to favorably modulate the immune system (see above). Importantly, these beneficial effects were shown to be independent of long-term engraftment but instead were mediated by local effects of these cells after transient vascular adhesion or engraftment at the site of injury. Figure 21.4 summarizes the spectrum of trophic actions that have been identified in endogenous and administered MSCs. Importantly, in humans, no long-term adverse effects have been reported so far.

Administration of Multipotent Marrow Stromal Cells in Experimental Acute Kidney Injury

In recognition of the biology of MSCs in the bone marrow niches and as pericyte-like cells in practically all blood vessels, as discussed above and in Chapter 9, together with their effective use in a number of experimental and clinical disorders, their therapeutic potential and mechanisms of action were tested in rats with IRI AKI.

As stated above, the ideal treatment of AKI would address its complex pathogenesis and pathophysiology directly, comprehensively and throughout its successive phases, thereby interfering with harmful processes that lead to the full expression of this syndrome, while initiating and carrying out regenerative programs that will result in return of function (Fig. 21.1). Cell therapy appears to possess the unique capacity to address virtually all major aspects of the pathophysiology of AKI, thereby providing a fundamentally novel therapeutic approach that appears vastly superior to single-agent pharmacotherapy. Cell therapy has, in principle, the potential to replace cells that are lost through injury, to enhance the survival of sublethally injured cells that are about to undergo apoptosis, to stimulate regeneration by boosting mitogenesis, to decrease inflammation at the site of injury, to modify cytokine secretion patterns of damaged cells, to enhance recovery of damaged endothelial cells and thereby improve microcirculation, and to activate tissue-resident stem cells at the site of injury [49].

Since the pathophysiology of AKI involves mainly tubular and vascular damage, an optimally effective agent to prevent or attenuate AKI should have vascular, hemodynamic and tubular effects. Cells are capable of interpreting and reacting to microenvironmental changes, such as hypoxia, oxidant stress or altered cytokine and growth factor levels, in real time and can thereby specifically react to pathological derangements at sites of injury. Such actions have the capacity to change the microenvironment at the site of injury from one that is deleterious to one that results in a favorable outcome.

Despite the substantial theoretical advantages of cell therapy in clinical applications, the main safety concern with cell therapy is their long-term engraftment with potential adverse effects such as fibrosis, scar formation, ectopic differentiation and tumorigenesis, issues that are further addressed in Chapter 23. Exogenously administered MSCs have been shown to protect kidney function and improve kidney repair in a number of animal models of AKI, and it was shown in humans that bone marrow cells contribute to renal cell types including tubular cells [54,83,84].

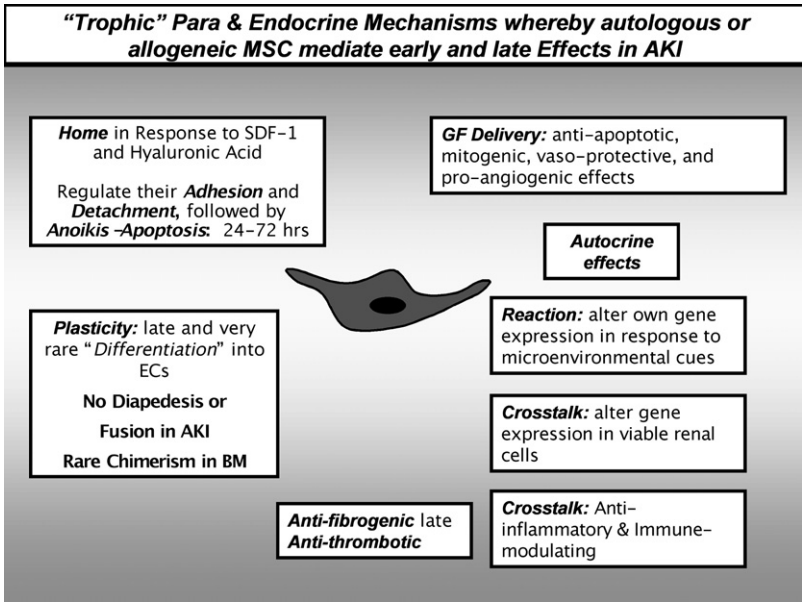

FIGURE 21.4 Trophic paracrine and endocrine mechanisms expressed by mesenchymal stem cells (MSCs). AKI: acute kidney injury; SDF: stromal cell derived factor; BM: bone marrow; GF: growth factor.

Treatment of Cisplatinum and Glycerol-induced Acute Kidney Injury with Mesenchymal Stem Cells

Injection of MSCs protected against cisplatin-induced AKI [54], while infusion of purified HSCs had no protective effect in the same setting. Fluorescence in situ hybridization (FISH) for Y-chromosomes in a model in which male MSCs were injected into female hosts showed that donor-derived MSCs contributed and integrated into the tubular epithelium. The exact quantity of the contribution was not determined, but proved to be very small in later studies. Herrera et al. provided data showing a relatively large contribution of 20% of donor derived MSCs to tubular epithelium in a glycerol model of AKI [84]. Other studies from different groups did not observe significant engraftment of donor-derived cells and thus argue against long-term engraftment or significant fusion with target cells, and significant restoration of epithelia by donor-derived cells [36,37,85,86].

The process of MSC differentiation and incorporation into tubular epithelia is rare and inconsistent, and occurs after renal function is essentially normalized, and therefore cannot explain the improvement in outcome compared to controls. Subsequent studies using more rigorous methodologies showed that mechanisms independent of differentiation are responsible for the overall improvement of AKI in animal models [38,86].

Most of the diverging data regarding renal engraftment of differentiated MSCs can be explained by differences in the models and, most importantly, by the cell labeling and tracking methods that were used [87]. Methodically more rigorous studies, cited above, established the absence of significant and long-term engraftment of MSCs that have assumed an epithelial cell phenotype, and thus further argue for paracrine mediator mechanisms [88]. Timing of cell delivery relative to injury and recovery from injury, seen as early as within 24 48 h, does not correlate with the very rare and late engraftment of differentiated MSCs. This discrepancy represents another powerful argument in favor of an overall paracrine mode of action of these cells. Rigorous lineage analysis provided conclusive evidence that most epithelial regeneration is undertaken by endogenous tubular cells.

Based on these observations in the kidney and other organs it is currently generally accepted that paracrine mechanisms underlie the organ-protective activity of administered MSCs in most tested injury models. These mechanisms involve immunomodulation (Fig. 21.3), secretion of growth factors and antiapoptotic actions (Fig. 21.4). Immunomodulation involves all of the MSC properties described above, including T-cell modulation, effects on NK cells, and suppression of B-lymphocyte proliferation and antibody production [57]. MSCs secrete a broad range of growth factors that have been shown to be renoprotective and data have been published that link some of these factors, namely vascular endothelial growth factor (VEGF) and IGF-1, directly to renoprotection [61,62,89]. Data showing remote endocrine effects of MSCs injected intraperitoneally provide further evidence of the paracrine/endocrine hypothesis of action [90]. Recent data show that MSC-derived microvesicles enhanced proliferation in vitro and conferred resistance of tubular epithelial cells, via horizontal transfer of mRNA, to apoptosis in vivo [91].

Homing mechanisms of administered cells are largely unknown but a number of studies provide evidence of the involvement of cytokines such as SDF-1 in this process [50]. Hypoxic preincubation of MSCs appears to increase engraftment in vivo and the mechanism involves upregulation of the SDF-1 CXCR4 axis [92]. Another potential homing receptor is CD44, which is expressed on MSC and binds to upregulated hyaluronic acid in the interstitial space of the kidney in glycerol-induced AKI [59]. The migration response of MSCs is induced by growth factors and chemokines, the latter augmented by preincubation of MSC with tumor necrosis factor-α (TNF-α) [45]. Understanding the processes involved in homing and migration of stem cells after injury is vital in designing effective therapies that optimize the targeting efficiency of therapeutic cells to injured organs.

Treatment of Ischemia Reperfusion Acute Kidney Injury in Rats with Autologous and Allogeneic Mesenchymal Stem Cells

RENAL FUNCTION AND HISTOPATHOLOGY

As others in the field had primarily focused on toxic models of AKI (see above), the present authors chose to investigate the therapeutic utility of syngeneic and later allogeneic MSCs in the rat with IRI AKI. It was established that the immediate or delayed administration, at 24 h after reflow, of syngeneic as well as allogeneic MSCs promptly protected renal function and hastened subsequent recovery of function, associated with decreased injury and apoptotic scores, and increased proliferative indices (Fig. 21.5) [86]. Instead of using an intravenous, intraperitoneal or intraparenchymal route of administration, as used by others, these cells were injected into the suprarenal aorta. This was done to avoid the induction of potential respiratory distress in animals (and in future study subjects), an adverse effect that has been shown to result from trapping of these large cells in the microcirculation of the lungs [93]. In addition, the reasoning was that with this administration route a smaller cell dose for the effective treatment of AKI may be sufficient. Extensive dose response studies in rats with IRI AKI were

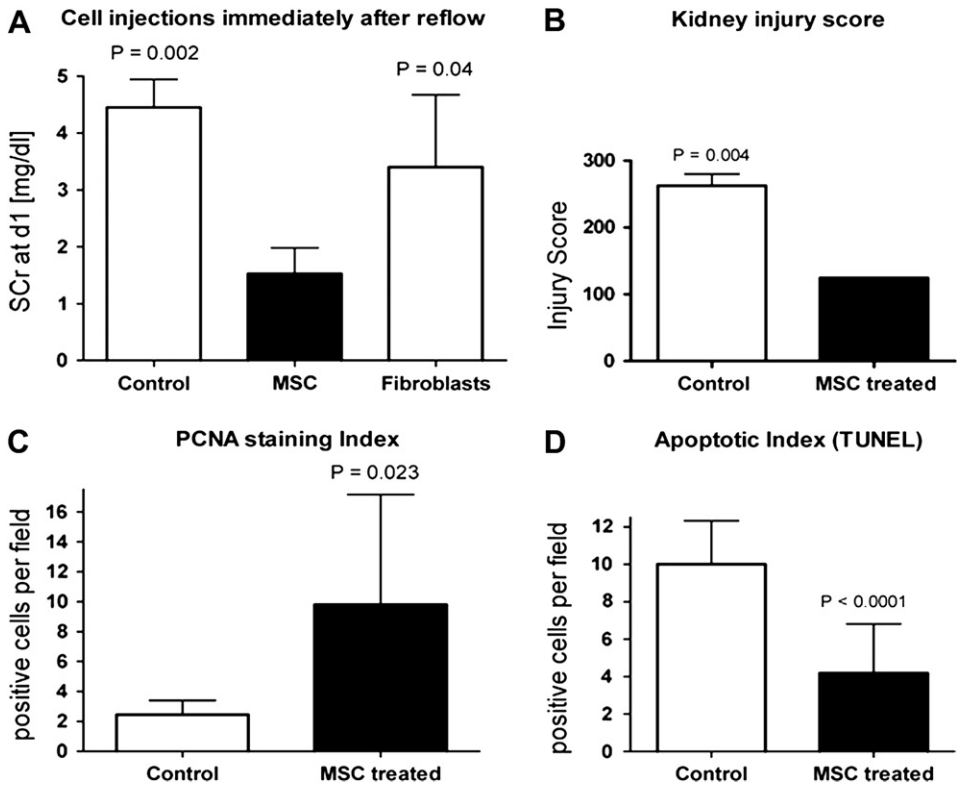

FIGURE 21.5 Treatment of severe acute kidney injury (AKI) in rats with human mesenchymal stem cells (MSCs) significantly (A) improves renal function, (B) improves renal injury scores, (C) stimulates mitogenesis and (D) inhibits apoptosis, while syngeneic fibroblasts are not kidney protective (A). SCr: serum creatinine; PCNA: proliferating cell nuclear antigen; TUNEL: terminal deoxynucleotidyl transferase dUTP nick end labeling. *[Reproduced from Tögel et al., 2005 [86], with permission from the American Journal of Physiology.]*

performed, which found that this therapy is safe and effective when either syngeneic or allogeneic MSCs are infused [93]. Infusion of identical doses of syngeneic fibroblasts to animals with AKI failed to improve outcomes, demonstrating that the organ-protective effects are specific to MSCs.

CELL TRACKING

Renal function was significantly protected as early as 24 h after reflow and MSC administration, a time-frame that would make it difficult for these cells to differentiate into target cells and to replace lost cells by engraftment. Since these observations were in conflict with the then generally held paradigm that organ repair by multipotent stem cells is carried out in this fashion, systematic MSC tracking studies were carried out in vivo, using a number of different techniques. The in vivo location of tagged MSCs in the kidney of rats with IRI AKI was monitored by two photon laser confocal microscopy (O'Brien Center, Division of Nephrology, University of Indiana, Indianapolis, USA). Administered MSCs, appropriately labeled, were primarily detected in glomeruli and less frequently in peritubular capillaries [86]. Their numbers within superficial glomeruli and peritubular capillaries fell rapidly within 1 2 days of follow-up. These in vivo data were confirmed in histological studies.

Next, the location of iron sucrose-loaded MSCs was monitored by magnetic resonance imaging (MRI). These cells were administered to rats with IRI AKI after ensuring that iron tagging of these cells did not reduce their viability, surface marker expression and ability to undergo trilineage differentiation [94]. Signal extinction on MRI was most prominent in the renal cortex of rats with IRI AKI, indicating the presence of iron-loaded MSCs in that location. The intensity of signal extinction in the kidney declined within 2 3 days after injury, akin to the pattern observed by confocal microscopy. The iron-tagged MSCs in this study protected renal function as effectively as normal cells. These MRI findings were confirmed histologically, showing that Prussian blue-stained MSCs were predominantly found in a glomerular location, and only small numbers were transiently detected in the postglomerular circulation and in the bone marrow.

A third in vivo cell tracking approach used bioluminescence imaging (BLI) of mouse MSCs that were transfected with the firefly luciferase (FFL) gene. Mice with AKI were infused with FFL-transfected MSCs, FFL substrate was administered intraperitoneally and the

whole-body distribution of resulting BLI signals was repeatedly assessed using a highly sensitive CCD camera [88]. Intravenous administration of FFL MSCs, as expected, resulted in significant pulmonary but also kidney signals, reflecting the presence of MSCs. Kidney signals declined again within 1 2 days, as observed with the other in vivo methods.

From these studies, it was concluded that administered MSCs are primarily and transiently detected in glomeruli and less frequently in the postglomerular circulation of rats with IRI AKI. On average, within 24 h of administration and AKI, one to three MSCs were present in glomeruli, and approximately one in three examined glomeruli contained no MSCs at this time-point. Since each rat kidney contains approximately 30,000 nephrons, only about 60,000 of the total number of infused MSCs per kidney are detectable at 24 h postinjury. However, having this number of cells transiently present in the kidney is sufficient to improve drastically the course of IRI-induced AKI in rats.

Genomic and non-genomic methods were used to corroborate the above findings. Accordingly, female rats with IRI AKI were treated with male MSCs, and kidney tissues were examined using FISH of cryosections for Y-chromosome-positive cells, and reverse transcription polymerase chain reaction (RT-PCR) for Sry expression. In parallel studies, bone marrow-derived MSCs from human placental alkaline phosphatase (hPAP) transgenic Fischer 344 rats (a generous gift from Dr Sandgren, University of Wisconsin, Madison, WI, USA) were administered to Sprague Dawley rats with IRI AKI. This transgene can be easily tracked by immunohistochemistry and RT-PCR. Neither of these genomic methods detected any cells at 24 h postinfusion. In addition, the renal delivery of carboxy-fluorescein diacetate (CFDA)-labeled MSCs that were infused into the suprarenal aorta of rats with IRI AKI was evaluated by immunohistochemistry. Venous blood contained no CFDA-positive cells at 2, 24 and 72 h post-AKI after their infusion, while kidneys, liver, lungs, bone marrow and spleen contained labeled cells at 2 h. With this method, no tagged cells were detectable in the kidneys at 24 and 72 h postinjury.

Together, these tracking data demonstrate that administered MSCs are present in the injured kidney for about 24 h after infusion. None of the renal tissues examined with these techniques showed evidence of early engraftment of administered cells. Their possible differentiation and subsequent engraftment were re-examined 5 days post-AKI [61]. At this time-point the very rare but definitive presence in or engraftment of CFDA-positive cells into the endothelium of peritubular capillaries was detected; these cells were likely to have derived from CFDA-labeled MSCs. Since renal function by this time post-AKI had almost normalized following MSC infusion, the contribution of this phenomenon to early renal repair is unlikely or of unknown immediate benefit.

In summary, the functional, histological and cell tracking data demonstrate that syngeneic and autologous MSCs, infused into the suprarenal aorta, protect renal function post-IRI AKI in rats and accelerate the repair process, a response not obtained with fibroblasts. At the tissue level, MSCs were transiently located in glomerular capillaries, reduced cellular apoptosis and inflammation, and stimulated mitogenesis, responses already apparent by 24 h postinjury. The significance of the late and very rare engraftment of MSCs that had differentiated into renal endothelial cells is uncertain.

In conclusion, these initial data strongly suggest that the mode of action of MSCs in IRI AKI is primarily mediated by differentiation-independent, paracrine antiapoptotic, mitogenic and anti-inflammatory mechanisms. The nature of these mechanisms was further investigated.

Mediator Mechanism of Mesenchymal Stem Cells in Ischemia–Reperfusion Acute Kidney Injury

Homing

Homing of mobilized or administered $CXCR4^+$ to the kidney is significantly mediated by injury-induced upregulation of SDF-1 [50]. Transwell studies showed that MSC migration across this barrier is specifically stimulated by SDF-1 when added to the bottom well or when derived from ATP-depleted proximal tubular cells. This chemokine is central to the recruitment to and engraftment of $CD34^+$ cells in the bone marrow following a bone marrow transplant [31]. Anchoring or retention of HSCs in the bone marrow niches is also mediated by the SDF-1 CXCR4 axis, while disruption of this retention by activated leukocytes or blockers of CXCR4 results in mobilization of HSCs and other cells into the periphery [95].

Adhesion

Adhesion, like homing, of CXCR4-expressing MSCs to endothelial cells, such as those in the renal or cardiac microcirculation, is significantly mediated by upregulated SDF-1 in the injured organ. Others showed that CD44-expressing MSCs are recruited to intrarenal sites where hyaluronic acid levels are upregulated by injury, a process that appears essential for the renoprotective activity of these cells in glycerol-induced AKI [59]. In addition, adhesion of MSCs to vascular endothelial cells is mediated by very late antigen-4 (VLA-4; $\alpha_4\beta_1$ integrin) expressed on MSCs and vascular cell adhesion molecule-1 (VCAM-1) expressed on endothelial cells

[29,96], paralleling in part the molecular mechanisms of leukocytes. The expression levels of these adhesion molecules on both endothelial cells and MSCs are upregulated by TNF-α and IL-1β, thereby increasing MSC adhesion intensity to resist higher shear stresses. Importantly, cell cell contact between MSCs and T cells, following antigen activation, was shown to be mediated by intercellular adhesion molecule-1 (ICAM-1) and VCAM-1 on MSCs, an interaction that appears critical for their immunosuppressive actions, and expression levels of these adhesion molecules are upregulated by interferon-γ (INF-γ) and proinflammatory TNF-α and IL-1β.

Detachment and Fate of Mesenchymal Stem Cells

Detachment of MSCs from the activated renovascular endothelium is likely to result from the downregulation of the involved adhesion molecules (see above) and SDF-1 levels, mediated by the anti-inflammatory and trophic actions of MSCs. As shown below, treatment of IRI AKI in rats with MSCs drastically decreases renal expression levels of INF-γ, inducible nitric oxide synthase (iNOS) and proinflammatory TNF-α and IL-1β, while upregulating anti-inflammatory IL-10 [86]. Renal SDF-1 expression levels decline in concert with the effective treatment of experimental AKI with MSCs (unpublished observations). Furthermore, it appears likely that MSCs, following detachment from intrarenal adhesion sites, home back to the bone marrow and/or undergo apoptosis in the circulation, the latter due to the fact that optimal survival of these cells depends on their adhesion to a stromal matrix or basement membrane. At 3 months postinfusion of genomically tagged, allogeneic MSCs to rats with IRI AKI, message signatures of these cells was only rarely detected in the bone marrow, while brain, liver, spleen, lungs, muscle, blood vessels and liver showed no such low-level evidence of chimerism [93]. Although it is likely that additional mechanisms for these processes will be identified, the molecular adhesion and detachment mechanisms of MSCs in the kidney proposed here, and their fate after detachment are illustrated in Fig. 21.6.

Hemodynamic Actions

The authors tested whether MSC or syngeneic endothelial cell administration immediately following reflow post-IRI improves renal blood flow and/or renocortical blood flow, potential mechanisms whereby these cells could protect renal function. However, the early decrease in total renal and cortical blood flow, and the rise in renovascular resistance, as measured with perivascular flow probes, a laser Doppler system and a carotid blood pressure probe, were not improved by either MSCs or endothelial cells [61]. These data demonstrate that the tested cells do not afford early functional protection by improving renal hemodynamics, whereas Patschan et al. demonstrated beneficial effects of EPCs in this setting [60]. It should also be emphasized at this point that the direct injection of large numbers of MSCs into the renal artery can result in abrupt deterioration of renal blood flow, resulting in infarcts and loss of function. In addition, in an anti-Thy-1 model of glomerulonephritis, this route of administration was shown to lead to late and significant ectopic maldifferentiation of MSCs into glomerular adipocytes [97], as discussed in detail in Chapter 23.

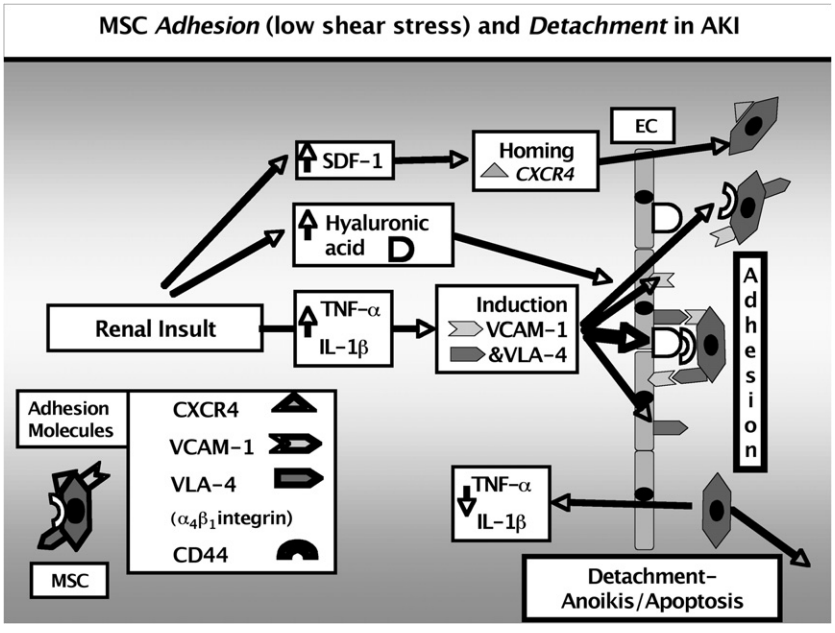

FIGURE 21.6 Intrarenal adhesion, detachment and fate of human mesenchymal stem cells (MSCs) during the course of acute kidney injury (AKI). Engaged molecular adhesion mechanisms are illus trated. With recovery, MSCs detach and return to the circulation, where the majority undergoes anoikis/apoptosis. SDF: stromal cell derived factor; TNF: tumor necrosis factor; IL: interleukin; VCAM: vascular cell adhesion molecule; VLA: very late antigen; EC: endothelial cell.

Anti-inflammatory Actions

It is well established that a central pathogenic mechanism in AKI is inflammation, mediated by inflammatory cells and upregulated proinflammatory cytokine levels (see above). Therefore, this investigation examined whether the histologically apparent decrease in inflammatory cells within the MSC-treated kidney was paralleled by changes in the expression levels of proinflammatory and anti-inflammatory cytokines and other factors. Figure 21.7(A C) shows the comparative gene expression ratios in kidneys from MSC- and vehicle-treated rats with severe AKI. Functional renoprotection obtained with MSCs was paralleled by the striking suppression of proinflammatory TNF-α and IL-1β, INF-γ, and upregulation of anti-inflammatory IL-10 [86]. In addition, renal iNOS expression was absent following MSC administration. Cytokine protein array data partially paralleled the PCR expression patterns.

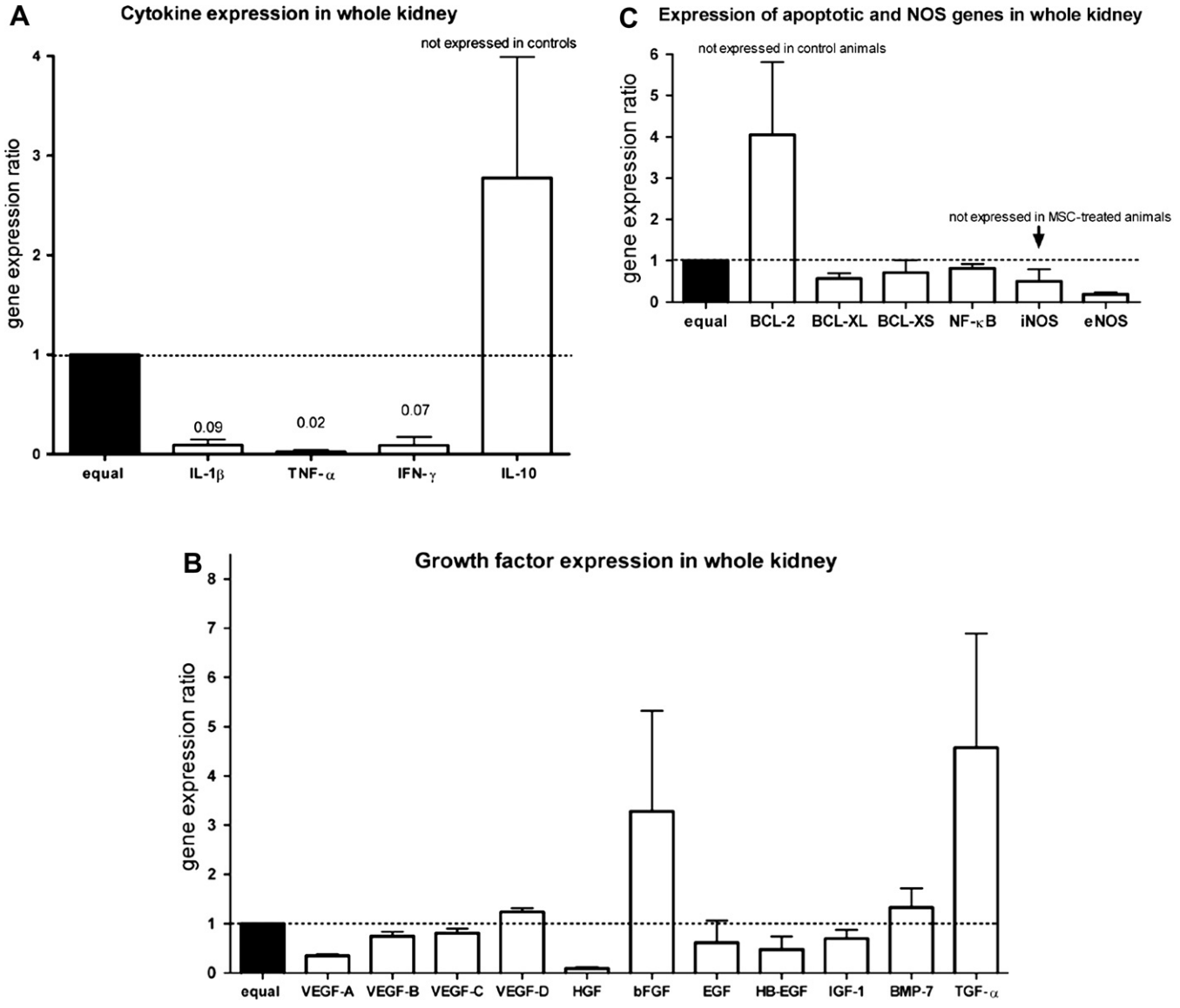

FIGURE 21.7 Mesenchymal stem cell (MSC) infusion to rats with acute kidney injury (AKI) caused in renal cells (A) significant suppression of proinflammatory interleukin 1β (IL 1β), tumor necrosis factor α (TNF α) and interferon γ (IFN γ) expression, while that of anti inflammatory IL 10 was upregulated; (B) upregulation of basic fibroblast growth factor (bFGF) and transforming growth factor β (TGF β) (VEGF: vascular endothelial growth factor; HGF: hepatocyte growth factor; EGF: epidermal growth factor; HB EGF: heparin binding epidermal growth factor; IGF: insulin like growth factor; BMP: bone morphogenetic protein); (C) upregulation of antiapoptotic B cell lymphoma 2 (BCL 2) and down regulation of inducible nitric oxide synthase (iNOS) expression (NF κB: nuclear factor κB; eNOS: endothelial nitric oxide synthase). Data show comparative gene expression ratios in AKI kidneys following treatment with MSCs or vehicle. A value above or below 1 indicates that gene expression in MSC treated animals has increased or decreased, respectively, compared with vehicle treated controls. *[Reproduced from Tögel et al., 2005 [86], with permission from the American Journal of Physiology.]*

In additional studies, it was demonstrated that media, conditioned by human or rat MSCs, as well as human and rat MSCs per se, dose-dependently inhibited T-cell proliferation when tested in an allogeneic mixed lymphocyte reaction (MLR) protocol [98].

Very unexpectedly, it was discovered that MSC administration in pigs with IRI AKI, a large animal model thought to be representative of human AKI, was completely ineffective yet safe [99]. In vitro, porcine MSCs (pMSCs) exhibited comparable trilineage differentiation, surface antigen profiles, and VEGF-A and IGF-1 secretion compared to human and rodent MSCs. However, in striking contrast to hMSCs, pMSCs failed to inhibit T-cell proliferation in the MLR, while inducing robust production of IL-6. Together these data indicate that pMSCs lack the immune-modulating activity of rodent and human MSCs, which may explain why pMSCs are ineffective in the pig model of AKI. It may be concluded that these data directly demonstrate that effective targeting of the inflammatory component of AKI is essential to the successful treatment of AKI. The complex and already clinically tested anti-inflammatory and immune-modulating actions of MSCs are summarized in Fig. 21.3.

Trophic Actions

The renoprotective activity of intrinsically produced or administered growth factors such as IGF-1, hepatocyte growth factor (HGF), epidermal growth factor (EGF), basic fibroblast growth factor (bFGF), heparin-binding fibroblast growth factor (HB-FGF), bone morphogenetic protein-7 (BMP-7), erythropoietin, VEGF and others is well established [49,63,64]. These act on their cognate receptors in the injured kidney, some of which are upregulated, and thereby elicit beneficial antiapoptotic, mitogenic, angiogenic and limited anti-inflammatory responses. Several of these growth factors are secreted by MSCs, which makes it theoretically possible to use MSCs as vehicles to deliver this mix of factors into an injured organ. MSCs express the receptors for a number of growth factors that they secrete, providing the basis for autocrine actions of currently known and unknown significance. There is evidence that MSCs interpret and respond to physiological and potentially harmful cues from the environment, e.g. in the bone marrow stem cell niches, by altering their own gene expression profile. A process termed licensing has been suggested to be required for the full expression and activation of the pleiotropic effects of MSCs [56]. As in the bone marrow stem cell niches, this cross-talk between MSCs and adjacent cells can instruct responsive targets to favorably alter their gene expression profile.

Mindful of these mechanistic possibilities, the growth factor expression profiles of kidney-protective MSCs and ineffective fibroblasts were compared. In MSCs, VEGF, HGF and IGF-1 expression exceeded that seen in fibroblasts, while fibroblasts showed higher EGF, HB-FGF, BMP-7 and bFGF expression levels [86]. Media conditioned by MSCs secreted HGF, IGF-1 and VEGF-A, and exerted significant antiapoptotic and proliferative effects in endothelial cells, particularly when MSCs were cultured in hypoxic media, while fibroblast-conditioned media lacked these activities. These data suggest that MSCs possess vasculotropic effects, which were subsequently investigated (see below).

Since functional protection of injured kidneys by MSC administration was associated with significantly reduced tissue injury scores and inflammatory cell numbers and, importantly, with markedly lower apoptotic and higher mitogenic indices, renal tissues were examined for changes in comparative gene expression profiles that would match these beneficial responses. Compared with vehicle-treated animals with AKI, MSC administration caused a significant increase in the expression of antiapoptotic B-cell lymphoma-2 (Bcl-2) in renal cells, as well as bFGF and transforming growth factor-β (TGF-β) (Fig. 21.7A C). In addition, it was directly demonstrated in injured kidneys that the frequency of apoptotic cells in areas where MSCs were attached to glomerular and peritubular endothelial cells was significantly lower than in areas where no MSCs were present [61]. Together, these MSC-induced responses correspond with the organ-protective antiapoptotic and regeneration-supporting tropic actions elicited by MSCs, which are summarized in Fig. 21.4.

Role of Vascular Endothelial Growth Factor Expression by Mesenchymal Stem Cells

The trophic actions of MSCs are thought to be primarily mediated by the dynamic release of various growth factors from MSCs that are present in the injured kidney (see above). Imberti et al. demonstrated that IGF-1 secretion by MSCs is critical to their organ-protective effects in AKI [89]. Since VEGF-A is also secreted by MSCs and has been clearly shown to beneficially affect the course of AKI, a recent study tested whether knockdown of VEGF-A in MSCs, using siRNA technology, diminished their protective actions in IRI AKI in rats [62]. Media, conditioned by VEGF knockdown MSCs or by wild-type MSCs, had a significantly reduced ability to stimulate proximal tubular cell proliferation in vitro. Compared to treatment with wild-type MSCs, functional protection and animal survival were markedly reduced in rats infused with 34 VEGF knockdown MSCs, identifying VEGF release by MSCs as another important mediator of their trophic actions in AKI.

Vascular Actions

What has been termed the extension phase of AKI is characterized by dysregulation and damage of the intrarenal microvasculature, causing congestion, leaks and secondary worsening of the parenchymal injury [32]. The authors investigated whether the delivery of MSCs to the injured kidney exerted beneficial effects on vascular function and thus organ repair. As discussed above, neither MSCs nor endothelial cells improved total or cortical renal blood flow early after IRI, and MSC-conditioned media inhibited endothelial cell apoptosis and stimulated proliferation in vitro, the latter being desirable vascular effects. The vasculotropic potential of MSCs was further examined and compared to that of aortic endothelial cells. Both cell types showed similar gene expression profiles for angiogenic factors, such as VEGF, bFGF, HGF and TGF-β, while IGF-1 was exclusively expressed in MSCs. Only confluent MSCs express VEGF receptors flt-1 and flk-4, explaining their response to VEGF which, when tested, resulted in their assumption of an endothelial cell-like phenotype, demonstrated by robust induction of DiI-low-density lipoprotein uptake. After short culture of MSCs on Matrigel, followed by standard culture, MSCs led to de novo expression of CD31 and von Willebrand factor (vWF), both endothelial cell-specific gene products. The in vitro angiogenic potential of MSCs and aortic endothelial cells alone was tested by growth on Matrigel, and similar capillary tube formation was observed. However, when both cell types were cocultured on Matrigel, endothelial cells formed tubes that were decorated by MSCs, which assumed a pericyte-like phenotype in this tube assay (Fig. 21.8).

Subcutaneous injection of MSCs in a Matrigel plug resulted, within 7 days, in the formation of vascular structures in rats. As discussed above, 5 days after the administration of CFDA-tagged MSCs, very rare CFDA-positive endothelial cells were detected in the wall of peritubular capillaries, suggesting potential differentiation and engraftment. Taken together, these data demonstrate that MSCs possess the capacity to protect and repair damaged intrarenal vessels through paracrine and potentially angiogenic effects, thereby contributing to the recovery of function post-AKI.

Fusion and Diapedesis

Fusion of administered MSCs with target cells in the kidney is another mechanism whereby their protective and regenerative activities could be mediated. The current authors detected no evidence for this phenomenon, whereas Li et al. [85] discovered low-frequency cell fusion. Active outmigration of MSCs from the renal

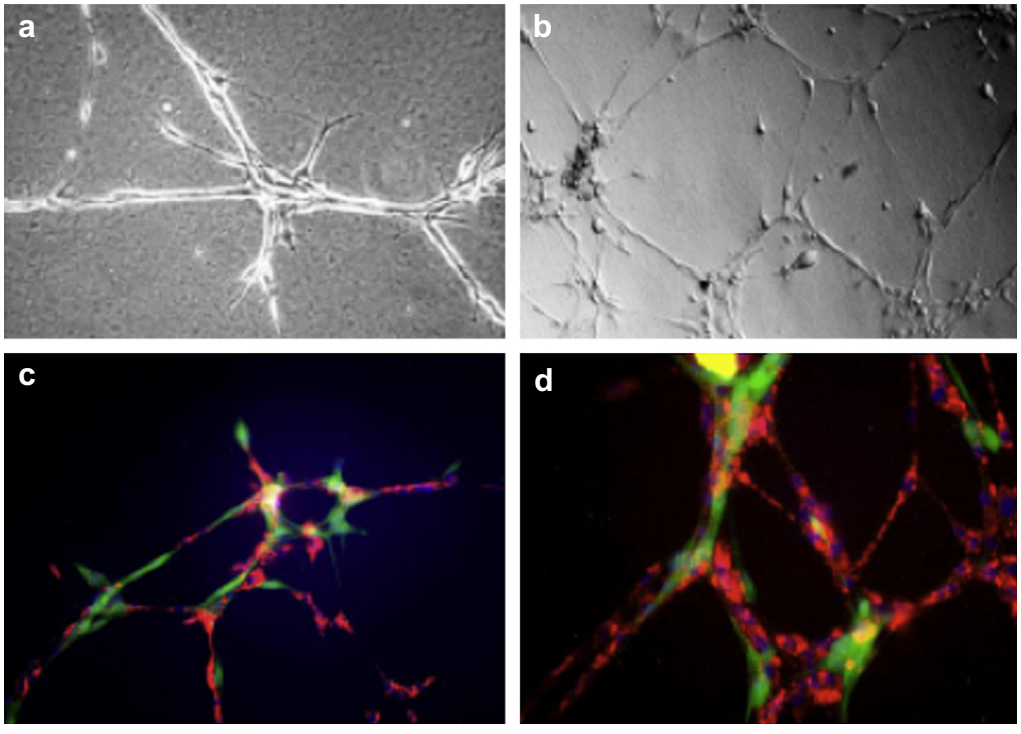

FIGURE 21.8 Both (a) mesenchymal stem cell (MSCs) and (b) endothelial cells (ECs) undergo capillary tube formation when seeded on Matrigel. (c, d) When both cell types are cocultured on Matrigel coated slides, EC cells form tubes (green; PKH26 labeled) that are decorated by MSCs (red; CFDA labeled), suggesting that the latter act like pericytes. Nuclei are stained blue with Hoechst 33342 dye. *[Reproduced from Tögel et al., 2007 [61], with permission from the American Journal of Physiology.]* Please see color plate at the end of the book.

vasculature, via diapedesis, was not observed at any point after AKI.

Late Consequences of Ischemia–Reperfusion Acute Kidney Injury Therapy with Allogeneic Mesenchymal Stem Cells

It is now well documented that even mild forms of clinical AKI can initiate the subsequent development of CKD and ESRD [1,2], and independently contribute to increased mortality late after cardiac surgery [48]. Basile [100] and other investigators have clearly demonstrated that AKI results in reduced renal VEGF expression, and its administration ameliorates secondary damage following recovery from renal IRI. These issues were addressed experimentally by comparing late outcomes in rats with severe IRI AKI that were initially treated with either allogeneic MSCs or vehicle [93]. Renal function post-IRI improved significantly in MSC-treated animals and mortality was reduced. At 3 months, the GFR was remeasured in all surviving animals. Vehicle-treated animals showed a significant reduction in GFR, while renal function in MSC-treated rats was slightly above baseline. On trichrome stains of the kidney, reduction in fibrosis scores in MSC-treated animals was associated with significant downregulation of profibrotic plasminogen activator inhibitor-1 (PAI-1), tissue inhibitor of metalloproteinase-1 (TIMP-1) and TGF-β expression. In parallel experiments, the renal microvessel density was assessed at 4 weeks post-AKI in MSC- and in vehicle-treated rats, and the results were compared with those obtained from animals treated with VEGF knockdown MSCs. Animals treated with wild-type MSCs had significantly higher renal microvesicle density and better renal function than vehicle-treated and VEGF knockdown MSC-treated animals, respectively. These data provide further evidence of the early and later therapeutic significance of VEGF expression by MSCs that are used to treat AKI.

In summary, the understanding of the mechanisms whereby MSCs improve experimental AKI and its late consequences remains incomplete in many areas. In Figs 21.3, 21.4, 21.6 and 21.9, identified and suggested modes of action are summarized. Based on current knowledge, although still in evolution, a working model could be advanced that demonstrates that allogeneic MSCs therapy is able to target all principal pathophysiological mechanisms that result in AKI and secondary CKD (Fig. 21.10). Specifically, infused MSCs express CXCR4 and CD44, which mediate their recruitment to the injured kidney, where SDF-1 and hyaluronic acid levels are increased. MSCs are detected for less than 3 days post-IRI AKI in glomeruli, and less commonly in peritubular capillaries. Low shear stress adhesion at these sites is carried out by SDF-1 receptors, CXCR4, expressed on MSCs that interact with SDF-1, produced by stressed endothelial cells and other cells. Similar interactions between CD44, expressed on MSCs, and hyaluronic acid have been shown. Upregulated proinflammatory TNF-α, IL-1β and INF-γ at sites of injury induce increased expression of ICAM-1, VCAM-1 and VLA-4 ($\alpha_4\beta_1$ integrin) on both endothelial cells and MSCs, facilitating a more robust adhesion. The cell cell contact that is carried out in this fashion is critical to the immune-modulating, anti-inflammatory actions of MSCs. Through the release of growth factors and cytokines, MSCs inhibit apoptosis and stimulate mitogenesis of renal cells, protecting function and augmenting the repair process that is undertaken by

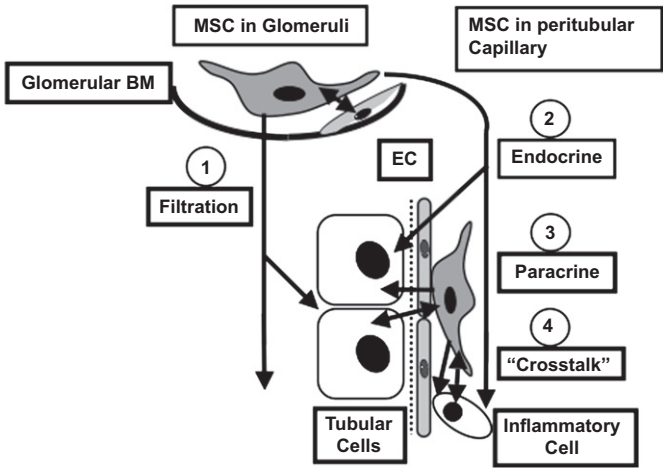

FIGURE 21.9 Proposed intrarenal actions of administered mesenchymal stem cells (MSCs) in acute kidney injury. Infusion of MSCs into the suprarenal aorta results in their delivery into the renal circulation, homing to sites of renal injury and temporary adhesion to predominantly glomerular and also postglomerular capillaries. Secretion of growth factors and cytokines by MSCs from their intraglomerular location delivers these to both the luminal and basolateral aspects of injured proximal tubules by filtration (1) across the glomerular basement membrane (BM) and release into post glomerular capillaries (2). This pattern of growth factor delivery is serendipitous, since receptor expression for some growth factors is upregulated in viable tubular cells and receptor distribution in sublethally injured, depolarized proximal tubular cells is both luminal and basolateral. In addition, adherent MSCs act in a paracrine fashion (3) both in glomeruli and in the postglomerular circulation, targeting glomerular and microvascular endothelial cells (EC; gray), as well as inflammatory cells. Cross talk (4) between MSCs and adjacent renal and inflammatory cells induces changes in gene expression profiles that are beneficial in MSCs, renal and inflammatory cells. It is thought that low shear stress adhesion of MSCs to endothelial cells is medi ated by integrin (very late antigen 4) and vascular cell adhesion molecule 1 receptors that are upregulated in parallel on both endo thelial cells and MSCs by elevated tumor necrosis factor α and interleukin 1β, and the stromal cell derived factor 1–CXCR4 axis. MSC mediated downregulation of these proinflammatory cytokines causes downmodulation, in turn, of these adhesion molecules and detachment of MSCs, followed by their anoikis/apoptosis in the circulation.

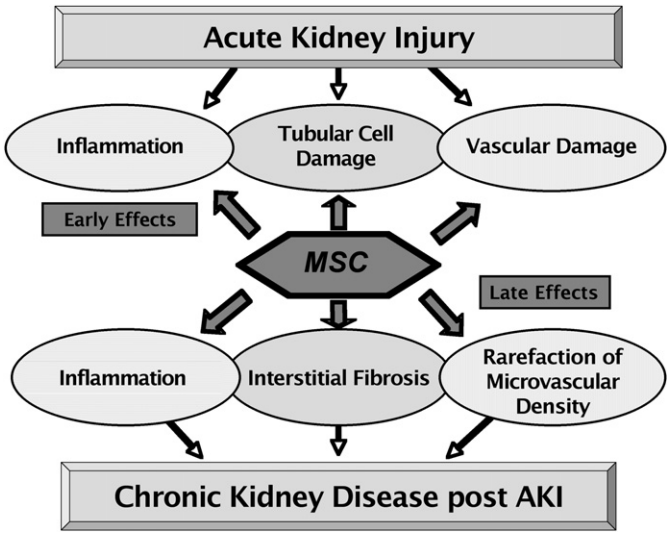

FIGURE 21.10 Administered mesenchymal stem cells (MSCs) target the major pathological processes in acute kidney injury (AKI) through their early paracrine actions, manifested by effective functional protection and stimulated repair. Improvement of early outcomes achieved by infused MSCs translates into the prevention of AKI induced chronic kidney disease, a highly beneficial late effect of this therapy.

kidney-intrinsic cells. MSCs interpret cues present in the microenvironment at sites of injury, respond to these by adjustments in their own gene expression repertoire, known as licensing, and instruct renal cells through paracrine signals to adjust their gene expression profiles favorably. Together, these trophic actions of MSCs collaborate with their anti-inflammatory actions, resulting in the protection and repair of renal epithelial and microvascular endothelial cells. Attached MSCs, in turn, cause downregulation of proinflammatory TNF-α, IL-1β and INF-γ, and upregulation of anti-inflammatory IL-10, and subsequent downregulation of adhesion molecules. This, together with return of renal blood flow and increased shear stress, results in the detachment and disappearance of MSCs from the kidney. Rarely, small numbers of circulating MSCs are recruited to the bone marrow, while the majority undergo apoptosis unless they are able to attach at other sites, a process that appears essential for their survival.

The administration of MSCs for the treatment of AKI improves outcomes early but significantly prevents late deterioration in renal function by inhibition of renal fibrosis and maintained microvascular density. When MSCs are administered into the suprarenal aorta, they do not induce physical complications such as renal infarcts or respiratory distress, and do not undergo ectopic maldifferentiation. Unless MSCs are extensively passaged, oncogenic transformation and karyotypic instability does not occur. MSCs are unique cells that lack human leukocyte antigen-2 (HLA-2) expression, together with absent expression of costimulatory cofactors CD40, CD80 and CD86, and they possess broad immune-modulating activity, elicited in various T-cell groups, in B cells and dendritic cells, and by upregulation of regulatory T cells (Tregs), together mediated by the release of various soluble factors, and probably by cell cell contacts. These characteristics, together with their very short residence time in the kidney, their very rare engraftment in the kidney and their rapid disappearance from the kidney and entire organism, make their use in allogeneic protocols safe, as has been amply demonstrated in various clinical trials. Mindful of these very favorable characteristics, the safety and preliminary efficacy of allogeneic MSCs were tested in a Food and Drug Administration (FDA)-approved phase I trial, described below.

PHASE I CLINICAL TRIAL

PREVENTION AND TREATMENT OF POSTOPERATIVE ACUTE KIDNEY INJURY WITH ALLOGENEIC MESENCHYMAL STEM CELLS IN PATIENTS WHO REQUIRE ON-PUMP CARDIAC SURGERY

Rationale

The well-documented incidence of significant AKI in high-risk patients undergoing on-pump cardiac surgery, associated morbidity, mortality and costs, together with the lack of an effective therapy in this setting, was the primary motivation for the conduct of this trial. Because of the large body of data showing the effectiveness and safety of administered allogeneic MSCs in experimental animal models of AKI, and the demonstrated safety and efficacy of these cells in several completed and ongoing clinical trials (see www.clinicaltrials.gov), a dose-escalating phase I clinical trial (www.clinicaltrials.gov; nct00733876) was designed to test the safety, feasibility and preliminary efficacy of MSCs in cardiac surgery patients at high risk of AKI. To obtain interpretable data from a small number of study subjects, this clinical setting was chosen, as it allows the clear assessment of preoperative risk factors for AKI, allows reliable collection of renal function and biomarker data preoperatively, and clearly identifies the time-point at which the potential renal injury occurs (on-pump cardiac surgery) and the time when allogeneic MSCs are infused (the intervention). Study endpoints were defined and changes in renal function were assessed using RIFLE criteria [5].

Study Design

Time-course

Close monthly or more frequent follow-up visits for 6 months following surgery and MSC administration were used to assess overall safety. Annual follow-ups at postoperative years 1, 2 and 3 were chosen to determine whether any late adverse events, such as ectopic maldifferentiation or oncogenic transformation of previously administered, allogeneic MSCs, would become manifest.

Study Objectives

The primary objective was the demonstration of early (from time of surgery until 6 months after surgery) and late safety (up to 3 years) of suprarenal MSC administration by absent therapy-specific adverse events or severe adverse events.

The secondary objective was assessment of preliminary efficacy compared to outcomes of well-matched, historical case controls from the same institution, and by comparison of matched data available on www.STS.com. Specific outcomes were assessed by the following endpoints: (i) postoperative development of AKI, as assessed by the RIFLE criteria, and need for dialysis (temporary or permanent); (ii) length of hospital stay; (iii) readmission rates within 30 days of surgery; (iv) 30 day postoperative mortality; and (v) deterioration of renal function at 6 months and later after surgery and cell infusion.

Patient Selection

After Institutional Review Board approval for the study had been obtained at the two participating study sites, 16 study subjects were enrolled, 11 at Intermountain Medical Center, and five at St Mark's Hospital, Salt Lake City, Utah, USA. These needed on-pump cardiac surgery (CABG and/or valve) and were identified as being at high risk for postoperative AKI owing to underlying renal disease (CKD stages 1 4), diabetes mellitus, age >65 years, congestive heart failure (CHF), chronic obstructive pulmonary disease (COPD), hypertension and cardiopulmonary bypass time >2 h. Major exclusion criteria were unstable cardiac function, active infections, a history of cancer within the last 5 years, age <18 years, pregnancy, prisoner, or recent or current participation in another clinical trial.

Preoperative Data Collection

Medical history and physical examination, medications, detailed demographic data, renal function (creatinine clearance, urinalysis, estimated GFR, RIFLE classification), complete blood count, renal ultrasound and postvoid residual in male patients, echocardiogram and coronary angiogram data, as available, were recorded. Urinary and serum samples were obtained and frozen for later assay of creatinine, and levels of the biomarkers neutrophil gelatinase-associated lipocalin (NGAL) and IL-18 (these assays were generously performed by Drs Prasad Devarajan and Michael Bennett, Cincinnati Children's Hospital, Cincinnati, OH, USA).

Allogeneic Mesenchymal Cell Stem Therapy

After completion of surgery and after the study subject had come off-pump and was hemodynamically stable, a predetermined dose of allogeneic MSCs was infused via a pediatric catheter into the suprarenal aorta. The first five patients received the lowest MSC dose/kg body weight. After Data Safety Monitoring Board (DSMB) review of the outcomes of the first cohort of study subjects found no safety issues, permission was given to enroll the next five study subjects, who were treated with an intermediate dose of cells. After DSMB review of their outcomes did not raise safety concerns, the final group of study subjects was enrolled and received the highest dose of allogeneic MSCs.

Postoperative Data Collection

Clinical and laboratory data, including biomarker levels, were collected frequently after surgery and up to the day of discharge.

Observations

Demographic Data of Study Subjects

Table 21.1 summarizes the demographic profiles, risk factors for postoperative AKI and type of surgery in all study subjects. Using these data, closely matched historical case controls were identified for each study subject, and respective outcomes were compared.

Safety

To date, none of the study participants has missed follow-up appointments or withdrawn from the study. The majority of study subjects has, at the time of writing, been followed for 12 18 months after undergoing cardiac surgery and MSC administration. In the opinion of the principal investigators (JD, DA), the members of the DSMB and investigator sponsor, no adverse or serious adverse events that are or may be attributable to the suprarenal administration of allogeneic MSCs, via a femoral catheter, have been observed, indicating that at early and intermediate follow-up times, no safety concerns have arisen.

Preliminary Efficacy

LENGTH OF STAY AND 30-DAY READMISSION RATES

In comparison to case controls, the length of hospital stay in the entire group of study subjects was reduced

TABLE 21.1 Demographic Data of Study Subjects

Patient no.	Gender	Age (years)	AKI risk factors	Surgical procedures
001	M	59	DM 3, hypertension, pump time 116 min	CABG × 3
002	F	79	Hypertension, CHF, aortic stenosis, pump time 88 min	Aortic valve replacement
003	M	74	CKD 2, mitral regurgitation, CHF, hypertension, pump time 123 min	Mitral valve repair, CABG × 1
004	F	66	DM 2, aortic aneurysm, pump time 155 min	Aortic root replacement, aortic graft
005	F	70	CKD 2, CAD, CHF, hypertension, pump time 135 min	CABG × 4
006	M	65	DM 2, hypertension, CAD, pump time 127 min	CABG × 5
007	M	78	CKD 2, DM 2, CAD, hypertension, pump time 59 min	CABG × 2
008	M	70	CKD 2, DM 2, CAD, hypertension, pump time 171 min	Aortic valve replacement, CABG × 4
009	M	67	CKD 2, DM 2, aortic stenosis, murmur, pump time 98 min	Aortic valve replacement
010	F	62	CKD 2, DM 2, CHF, COPD, hypertension, pump time 100 min	Aortic valve replacement
011	M	66	CKD 2, DM 2, CAD, hypertension, pump time 179 min	CABG × 5
012	M	75	CKD 2, aortic stenosis, hypertension, pump time 127 min	Aortic root replacement
013	M	75	CKD 2, DM 2, pump time 136 min	CABG × 4
014	M	86	CKD 3, CHF, hypertension, pump time 120 min	Mitral valve replacement
015	M	81	CKD 2, CHF, pump time 207 min	CABG × 3
016	F	82	CKD 2, DM 2, CAD, hypertension, pump time 245 min	Aortic valve replacement, ascending aorta replacement, CABG × 1

M: male; F: female; DM: diabetes mellitus; CHF: congestive heart failure; CKD: chronic kidney disease; CAD: coronary artery disease; CABG: coronary artery bypass graft.

from 11 to 7 days. Similarly, in comparison to case controls, the 30 day postoperative readmission percentages in all MSC-treated subjects were reduced from 18.5% to 6%. Notably, none of the study subjects with CKD required rehospitalization within 30 days of surgery.

RENAL OUTCOMES

Preoperatively, mild to moderate underlying CKD was documented in 12 of the 16 study subjects. Using the RIFLE criteria, acute changes in postoperative renal function and at discharge were assessed in the entire group and in patients with pre-existing CKD. Obtained data were then compared to historical case controls that closely matched the demographic profiles, risk factors and type of cardiac surgery of study subjects. Figure 21.11(a) shows these data for the entire study group, and Fig. 21.11(b) the results for the group with underlying CKD. As shown, none of the MSC-treated individuals and none of the 12 subjects with underlying CKD developed AKI at the time of discharge, and renal function, assessed in this fashion, actually improved at the time of discharge. In contrast, 15% of historical case controls and 19% of historical case controls with CKD met the RIFLE criterion for failure. These outcomes between study subjects and case controls were significantly different. Furthermore, none of the study subjects required renal replacement therapy at any time after surgery, while approximately 6% of historic controls were temporarily dialyzed and a mortality of 6% was documented.

Figure 21.12(a) depicts serum creatinine levels from all study subjects over time, and Fig. 21.12(b) shows the corresponding data from those with pre-existing CKD. In comparison to matched historical case controls, serum creatinine levels and thus renal function in all study subjects, including in those with CKD, have remained stable to this date, while renal function in case controls progressively and significantly declined over the same period of follow-up.

Using changes in serum and urinary NGAL and IL-18 levels as surrogate biomarkers for AKI, none of these met the criteria for AKI in the entire group of study subjects (unpublished observations).

Summary and Conclusion

This small phase I trial has demonstrated that the administration of allogeneic MSCs to a cohort of cardiac surgery patients who are at high risk for postoperative AKI is safe, feasible and, up to the present time of follow-up, well tolerated. Although underpowered, the

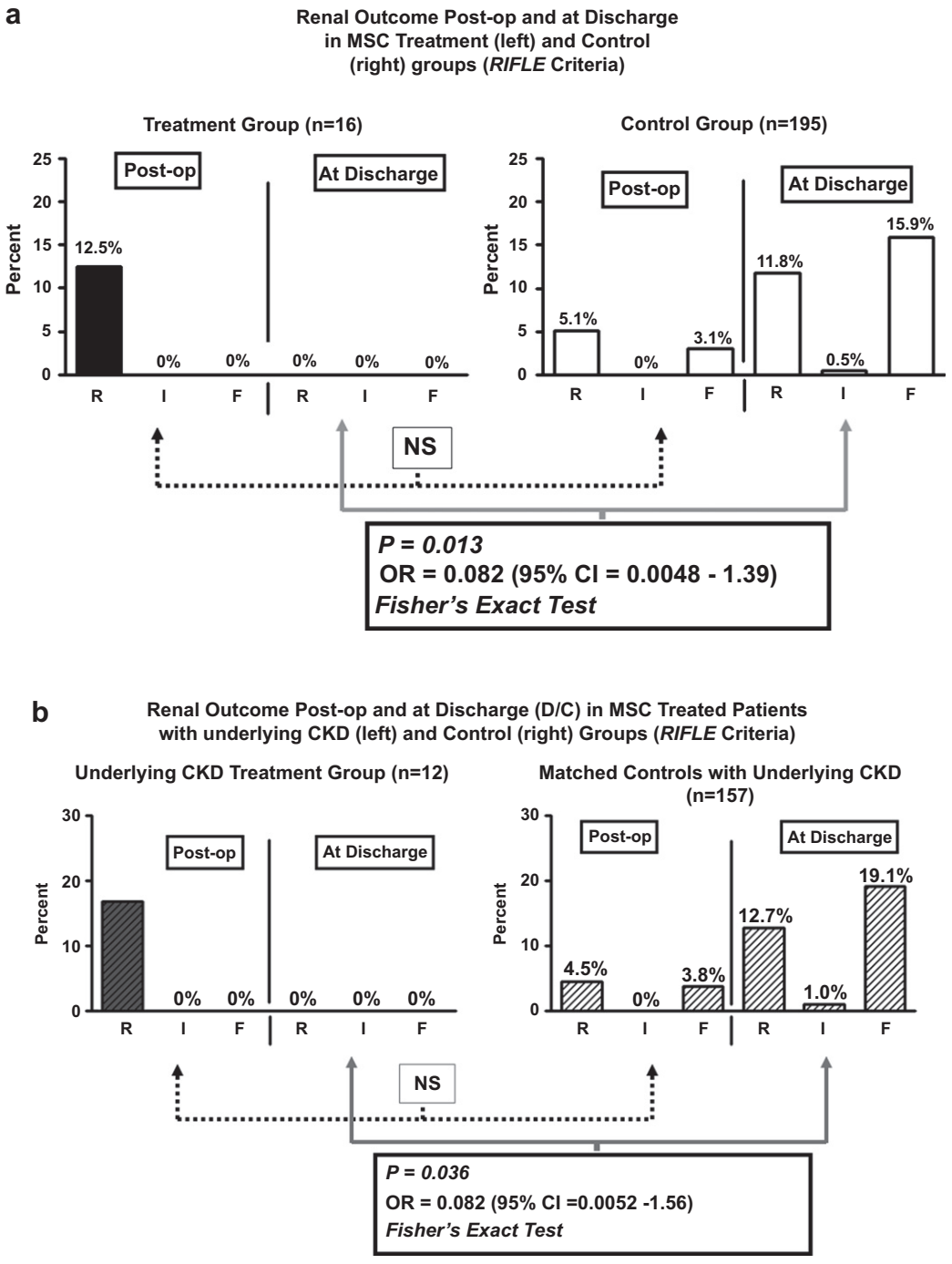

FIGURE 21.11 Assessment of renal outcomes in study subjects and historical case controls by Risk–Injury–Failure–Loss–Endstage (RIFLE) criteria. (a) Postoperative profiles were not different between treatment (left panel) and control groups (right panel). At discharge, none of the mesenchymal stem cell (MSC) treated individuals met the RIFLE criteria for risk, injury or failure, while a significant increase in these was noted in controls. (b) Similar early outcomes were observed when study subjects with underlying chronic kidney disease (CKD) were compared with controls with CKD, except that the incidence of failure at the time of discharge was higher. Identical results for all data were obtained when outcomes in only four historical case controls for each study subject were analyzed (not shown). NS: not significant; OR: odds ratio; CI: confidence interval.

preliminary efficacy of this intervention was examined by comparing outcomes in study subjects to those of recent, well-matched historical case controls from the same institutions and surgeons. The postoperative infusion of allogeneic MSCs improved renal function early and prevented a postoperative deterioration in renal function that was noted in case controls, requiring dialysis in some. The tested surrogate biomarkers for AKI, NGAL and IL-18 levels in blood and urine, did not rise above baseline, further indicating that MSC infusion

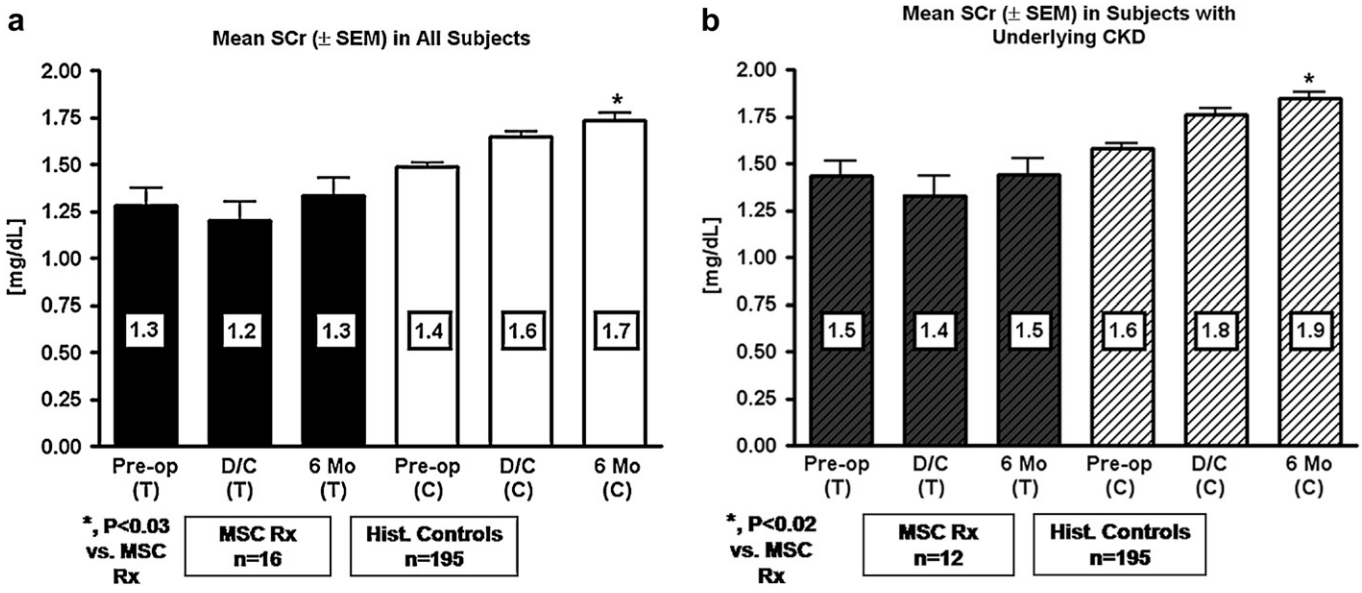

FIGURE 21.12 Assessment of early and late renal outcomes in study subjects and historical case controls. (a) Serial measurements of serum creatinine (SCr) levels in mesenchymal stem cell (MSC) treated subjects and case controls show that at 6 months postsurgery, renal function in the entire group of study subjects remained at baseline, while SCr levels in case controls rose significantly, although only by 0.3 mg/dl above preoperative levels. (b) A similar pattern was observed when SCr levels were compared between MSC treated subjects and case controls with underlying chronic kidney disease (CKD), again demonstrating that early administration of MSCs appears to reduce the likelihood of CKD development after acute kidney injury (AKI).

did not cause a renal insult. In comparison to case controls, length of hospital stay and 30 day postoperative readmission rates in all MSC-treated subjects were reduced by about 40%. Notably, none of the study subjects with CKD required rehospitalization. At 6 months and up to 16 months of follow-up, renal function, as reflected by unchanged serum creatinine levels, remained at baseline in MSC-treated subjects, while there was a gradual deterioration in renal function in case controls, which was more pronounced in those with underlying CKD.

In conclusion, the early and late results of this phase I trial largely parallel those obtained in preclinical studies, thereby validating the relevance of the experimental AKI models that were used (see above). Untested but suggested is the likelihood that these encouraging results are mediated by the same paracrine mechanisms that MSCs very temporarily but effectively exert in experimental AKI, i.e. a combination of anti-inflammatory and tropic actions that simultaneously target all major pathophysiological components of this serious complication. The hypoimmunogenic and immune-modulating characteristics of MSCs, and their very brief residence time in the kidney and entire organism, critically contribute to the safety of this cell-based therapy. Although the preliminary efficacy data are promising, they are of insufficient power. Accordingly, an adequately powered phase II trial will be conducted, in which the efficacy of this MSC therapy is tested, using a multicenter, double-blind, placebo-controlled and randomized design in a similar cohort of cardiac surgery patients, patients who are at high risk for postoperative AKI. If the definitive efficacy of allogeneic MSC therapy for this indication is demonstrated, their utility in other settings of AKI will be tested, with the expectation that this novel intervention can eventually be introduced into general clinical practice to improve the still poor outcome of patients with various vforms and degrees of this complication.

Caveat

To date, both autologous and allogeneic MSCs have been administered to several hundred patients, and have so far been proven extraordinarily safe. Importantly, no major adverse events that preclude their future use have been documented in the allogeneic setting. Despite these promising observations, the generation of a complete safety profile for this form of cell therapy requires the collection of sufficient long-term follow-up data. This process is incomplete but ongoing. Concerns about ectopic maldifferentiation of MSCs have been raised in studies in which these cells were directly infused into renal arteries of rodents with experimental glomerulonephritis [97]. Similarly, the ectopic differentiation potential of MSCs into mesenchymal lineages was observed when high numbers of these cells differentiated into bone after they were injected into injured

rodent hearts [101]. Long-term culture of MSCs, particularly when carried out under conditions known to cause cell stress, may result in karyotypic instability and potential immortalization and transformation, indicating that their use may be associated with this type of potential risk [102]. Their broad immune-modulating actions, while beneficial in certain settings, may potentially facilitate opportunistic infections and the expansion of malignancies [103].

Acknowledgments

The authors' research was supported in part by funds from the Merit Review program of the Veterans Administration, Washington, DC, the National Institutes of Health, the American Heart Association, the National Kidney Foundation, the Western Institute for Biomedical Research and Allocure, Inc. The human MSCs that were administered to study subjects in the phase I clinical trial were prepared in the cGMP Cell Therapy Facility of the University of Utah. The outstanding skills of the PIs (Dr John Doty, Intermountain Medical Center, Murray, Utah; Dr David Affleck, St Mark's Hospital, Salt Lake City, Utah), Co PIs (Drs Benjamin Horne, Brent Muhlestein, Intermountain Medical Center; Drs Shreekanth Karwande, Gilbert Schorlemmer, St Mark's Hospital) and study nurses (Jean Flores, Intermountain Medical Center; Analee Creer, St Mark's Hospital) are gratefully acknowledged. The contribu tions of the members of the Data Safety and Monitoring Board for this trial, Drs C. Kablitz, George R. Reiss and Srinivasan Beddhu are also greatly appreciated. Finally, the excellent bench work of Zhuma Hu, Dr Ping Zhang and Dr Anna Gooch was invaluable for the successful conduct of the preclinical studies.

Competing interests

F. Tögel declares that he has no competing interests. C. Westenfelder is a consultant to Allocure, Inc.

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CHAPTER

22

Clinical Trials in Renal Regenerative Medicine

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OUTLINE

INTRODUCTION

Kidney disease is a growing global public health issue. Chronic kidney disease (CKD) has a prevalence of over 10% in the general population and this number is increasing by almost 3% per year [1,2]. Advanced CKD is associated with a more than three-fold increased risk for cardiovascular events and an almost six times increased mortality [3]. Acute kidney injury (AKI) is also an increasing clinical problem, with an incidence of 15 patients per 1000 patient years (Medicare database). Mortality rates of AKI are up to 80% and more than 10% of the survivors eventually develop end-stage renal disease (ESRD). More than 350 new cases of ESRD per 1,000,000 population are seen every year, according to the United States Renal Data System (USRDS) 2009 annual data report. Thus far, therapeutic strategies for kidney disease have focused on the reduction of tissue damage and supportive care.

However, renal disease and renal disease progression can be envisioned as the result of the kidney injury in combination with an insufficient regenerative response. Both glomeruli and tubuli are known to have regenerative capacity in humans [4 6]. In the past decade, the recognition that growth factors and progenitor cells are involved in the regeneration of tissues has heightened interest in enhancing tissue regeneration (Table 22.1). Preclinical trials have shown that therapeutic strategies can enhance renal regeneration. Therapeutic regeneration may be an interesting therapeutic strategy in renal disease.

Clinical trials investigate the relation between a certain input variable (e.g. a therapeutic intervention or prognostic test) and a certain output variable (e.g. organ function, disease outcome, patient survival) in a well-defined patient population, and aim to estimate phenomena in a population, by making measurements on samples from the target population [7]. Clinical trials form the cornerstone for the introduction of new prognostic and therapeutic procedures in the clinic. The number of clinical trials in nephrology is quite low compared with other internal medicine disciplines [8]. There appears to be a barrier in the translation of basic science to human studies, which may relate to regulatory burden, high research costs and lack of funding. However, despite similar hurdles, strategies to enhance regeneration in cardiovascular medicine and hepatology have been translated into clinical trials with positive results. These results, combined with promising preclinical studies on therapeutic renal regeneration, suggest that clinical introduction of therapeutic renal regeneration is at hand. This chapter will discuss regenerative strategies in other organ systems, (pre)clinical studies

TABLE 22.1 Therapeutic Approaches Used in Clinical Trials on Regenerative Medicine

Therapy	Subtype	Administration
Growth factor therapy	Recombinant human	Subcutaneous
	Gene therapy	Systemic intravenous
	Plasmid	Local intra arterial
	Adenoviral	Direct tissue injection
Cell therapy	Unselected BMDCs	Systemic mobilization
	Selected BMDCs	Systemic intravenous
	MSCs	Local intra arterial
	CD133	Direct tissue injection
	CD34	
	Cultured MNCs	
	Mature differentiated cells	

BMDCs: bone marrow-derived cells; cultured MNCs: progenitor cells derived from cultured peripheral blood-derived mononuclear cells.

on renal regenerative therapies and the translation of successful preclinical strategies into clinical trials on therapeutic renal regeneration.

The first section of this chapter will review strategies and outcome parameters applied in prognostic and therapeutic clinical trials on regeneration in other disciplines. The focus will be on cardiac neovascularization because of the crucial role of the vasculature in renal function, maintenance and repair, and on hepatic regeneration because kidney and liver are both highly vascularized parenchymatous organs. In the second section the available clinical data on renal regeneration will be discussed. The third section of this chapter will discuss translation of animal experiments from cage to clinic. As the majority of preclinical studies so far have investigated therapeutically enhanced regeneration in models of acute tubular damage, the primary focus will be on the translation of therapeutic strategies for acute tubular damage.

CLINICAL TRIALS OF REGENERATIVE MEDICINE IN OTHER DISCIPLINES

Therapeutic Neovascularization in Cardiovascular Medicine

Blood vessels are of crucial importance for renal function, maintenance of renal integrity and renal repair [9]. Cardiovascular studies aiming at the generation of new blood vessels (neovascularization) may have great relevance for regenerative nephrology. During neovascularization, proliferating mature endothelial cells are one source of new endothelial cells. Growth factors play an important role in the regulation of endothelial cell proliferation. In addition, circulating endothelial progenitor cells stimulate neovascularization, both by structural participation in the vasculature and by paracrine effects (Table 22.1) [10]. Both prognostic studies to assess regenerative capacity and therapeutic trials to enhance regeneration, using growth factors or stem cells, have been performed in patients with cardiovascular disease (CVD). The different diagnostic and therapeutic strategies, as well as the outcome parameters used in these studies, will be discussed here.

Prognostic clinical trials have shown that parameters of regeneration relate to clinical outcome in CVD. Elevated plasma levels of hepatocyte growth factor (HGF) were associated with improved collateralization and a favorable prognosis in patients with myocardial ischemia [11,12]. A decreased number of circulating endothelial progenitor cells (EPCs), suggesting low regenerative capacity, correlated with increased cardiovascular mortality in patients with coronary artery disease [13,14]. However, others report data that seem conflicting. Serum growth factor levels in patients with angina correlated with the development of myocardial infarction and several studies have shown positive correlations between concentrations of circulating growth factors and myocardial damage and mortality [15 23]. The increase in circulating growth factors or progenitor cells depends on both the amount of tissue injury and the quality of the regenerative response and therefore may reflect not only the process of regeneration but also the amount of tissue damage.

Therapeutic trials of growth factor administration to enhance neovascularization in patients with cardiac ischemia showed variable results [24,25]. Growth factors regulate the proliferation and migration of endothelial (progenitor) cells, and form a promising strategy to enhance neovascularization. Therapeutic use of recombinant growth factors allows for the precise control of the administered dose. Recombinant growth factors can be administered systemically (e.g. intravenous injection) or locally (e.g. intracoronary or intramyocardial administration). Local delivery can specifically increase the local concentration while reducing possible systemic adverse events, but requires invasive procedures such as catheterization or the surgical implantation of minipumps.

In the Vascular endothelial growth factor in Ischemia for Vascular Angiogenesis (VIVA) trial recombinant human vascular endothelial growth factor (rhVEGF) in patients with stable angina was shown to improve clinical parameters [26]. Fibroblast growth factor (FGF) has been shown to increase the formation of capillaries, reduce myocardial ischemia, increase left ventricular ejection fraction and lower the rate of angina recurrence

[27 30]. However, these results were not reproduced in the large, multicenter, randomized, double-blind, placebo-controlled FGF-2 Initiating RevaScularization Trial (FIRST) [31]. The disadvantage of recombinant protein therapy is the short half-life in vivo due to rapid degradation by circulating proteases [32 34] and hence the need for a relatively high dose and/or repeated administration of the recombinant protein to induce a therapeutic effect. This may increase the occurrence and intensity of adverse events. Therefore, administration of plasmids has been used to deliver proangiogenic factors locally and continuously. All clinical studies that used gene therapy to enhance neovascularization used local administration by either intracoronary infusion or intramyocardial injection. Plasmids have a good safety profile because they are not integrated into the host genome and plasmid levels become undetectable within weeks of administration because of breakdown by nucleases. Plasmids of several isoforms of FGF and VEGF enhanced myocardial perfusion and myocardial wall motion, and reduced angina in patients with chronic myocardial ischemia [35 42]. However, results were not very consistent: some found increased perfusion without functional improvement, while others found functional improvement without increased perfusion. In addition, although some studies demonstrated increased plasma growth factor levels after transfection and improved cardiac function, others demonstrated improved cardiac function without a significant increase in plasma growth factors [38 40]. Moreover, the GENASIS trial, designed to enhance exercise tolerance using VEGF-C plasmid in "no-option" coronary artery disease patients, was stopped prematurely because of the high likelihood of lack of effect [43]. The limited effect of plasmids may be the flipside of their good safety profile: low transfection efficacy and rapid breakdown.

Viral vectors can be used to obtain a more efficient transfection and sustained gene expression. However, the use of viral vectors can be complicated by an inflammatory response [39,44]. In addition, the integration into the host genome raises important safety concerns, specifically the possibility of insertional mutagenesis with subsequent malignant transformation [45,46]. In the REVASC study, "no-option" patients with coronary artery disease were treated with replication-deficient adenovirus containing VEGF [47]. This study showed improvement in exercise-induced ischemia and decreased angina symptoms but no increase in myocardial perfusion. The Angiogenic GENe Therapy (AGENT) trials used replication-deficient adenovirus containing the human FGF gene to enhance neovascularization in patients with angina [48 51]. Although the safety profile was good and initial results were promising, the AGENT-3 and 4 trials were stopped prematurely because the interim analysis indicated that a significant difference in the primary efficacy endpoint was unlikely. In these trials adenoviral transfection did not increase the plasma levels of FGF. In summary, gene therapy, using either plasmids or replication-deficient adenoviruses, has not been shown to give consistently positive clinical results.

Progenitor cells have also been used in clinical trials to enhance neovascularization in patients with ischemic heart disease. Recent meta-analyses demonstrate that intracoronary and intramyocardial infusion of bone marrow-derived cells (BMDCs) in patients after myocardial infarction appears to be safe and shows a significant, consistent, clinically relevant but moderate improvement in left ventricular ejection fraction, left ventricular end-systolic volume and myocardial lesion area [52 55]. Initially, it was speculated that BMDCs would enhance regeneration by reducing apoptosis of cardiomyocytes and stimulating proliferation of myocardial (progenitor) cells. However, more recent data suggest that the beneficial actions of BMDCs on myocardial function are mainly due to enhanced neovascularization and paracrine effects [10]. BMDC infusion 5 7 days after myocardial infarction appeared to be superior to early administration, suggesting that postinfarction inflammation may attenuate BMDC-enhanced regeneration.

Infusion of subpopulations of BMDCs or cultured progenitor cell populations has also been used to enhance regeneration in patients with myocardial infarction. In the Transplantation of Progenitor Cells and Regeneration Enhancement in Acute Myocardial Infarction (TOPCARE-AMI) trial [56 59] both intracoronary infusion of cultured autologous peripheral blood-derived progenitor cells and bone marrow mononuclear cells improved coronary blood flow reserve, coronary conductance capacity, myocardial viability, regional wall motion, end-systolic left ventricular volume and left ventricular ejection fraction. The $CD34^{+}$ subpopulation of BMDCs reduced angina frequency and increased exercise time, whereas the $CD133^{+}$ subpopulation enhanced myocardial perfusion and left ventricular function [60 62]. Another interesting subpopulation is the mesenchymal stromal cell, also known as the mesenchymal stem cell (MSC). MSCs have been shown to have therapeutic properties in several preclinical studies [63]. They may offer advantages over BMDCs. MSCs are immunologically privileged cells that are not rejected after allogeneic transplantation [64]. However, it should be noted that under certain conditions MSCs are subject to immune rejection [65,66]. MSCs home to areas of injury and can be infused intravenously [67 71]. Preclinical studies have shown that MSCs are able to enhance neovascularization [72,73]. Hare et al. infused in vitro expanded allogeneic MSCs intravenously in patients after myocardial infarction and showed a reduction in ventricular arrhythmias and global symptom score without significant adverse events [64]. Furthermore, ejection fraction was increased

and reverse remodeling was seen using magnetic resonance imaging (MRI). The mechanism by which MSCs enhanced cardiac function was not evaluated. Because of the clinical effect combined with the possibility of allogenic use and a good safety profile, MSCs are a promising new therapeutic strategy to enhance regeneration. Infusion of (subpopulations of) BMDCs appears to enhance myocardial perfusion and, subsequently, myocardial function in patients after myocardial infarction.

The outcomes of clinical trials on cardiac neovascularization were evaluated at different levels (Table 22.2). Several authors investigated whether infused BMDCs reach the infarcted region in human myocardium, by isotopically labeling BMDCs and tracing them using positron emission tomography (PET). They demonstrated that some of the injected BMDCs home to infarcted areas of the myocardium [74 76]. This property also makes them ideal vehicles for the delivery of genes, e.g. for paracrine stimulation of regeneration [77,78]. Homing differed between the various subpopulations of BMDCs. The direct effect of the intervention on tissue vascularization was assessed by quantitative coronary angiography, including coronary flow reserve. The effect on myocardial perfusion was assessed by contrast-enhanced MRI and myocardial perfusion scintigraphy [single-photon emission computed tomography (SPECT)]. In addition,

TABLE 22.2 Outcome Evaluation in Current Therapeutic Clinical Trials on Cardiac Neovascularization and Hepatic Regeneration (Shaded) and Possible Strategies to Evaluate Outcome of Therapeutic Clinical Trials on Renal Regeneration (Unshaded)

	Intervention	Intervention evaluation	Cell/tissue outcome	Clinical outcome

Heart	• Growth factor therapy
	• Cell therapy
		• Growth factor concentration
		• Progenitor cell mobilization
		• Progenitor cell tracking
			• Capillary density
			  • Angiography
			• Cardiac perfusion
			  • SPECT
			  • MRI
			  • Coronary blood flow
			  • Reserve
			  • Conductance
			• Cardiac function
			  • Contractility
			  • Nuclear
			  • Ultrasound
			  • MRI
			  • Ventriculogram
			• Heart failure
			  • Natriuretic peptides
				• Angina score
				• Exercise tolerance
				• Reinfarction
				• Clinical heart failure
				• Quality of life
				• Survival
				• Adverse effects

Liver	• Portal vein embolization
	• Growth factor therapy
	• Cell therapy
		• Growth factor concentration
		• Progenitor cell mobilization
			• Histology
			  • Progenitor cell activation
			  • Hepatic cell proliferation
			• Liver function
			  • Bilirubin
			  • Albumin
			  • Transaminases
			  • Total protein
			  • Prothrombin time
			• Liver volume
			  • CT
				• Survival
				• Adverse effects

Kidney	• Growth factor therapy
	• Cell therapy
		• Growth factor concentration
		• Growth factor binding protein concentration
		• Growth factor receptor expression
		• Progenitor cell tracking
			• Histology
			  • Damage score
			  • Tubular cell proliferation
			  • Sclerosis/fibrosis score
			  • Progenitor cell
			  • Number
			  • Differentiation
			• Renal function
			  • See Table 22.4
			• Renal volume
			  • CT
			  • Ultrasound
				• Hospitalization
				• Need for renal replacement therapy
				• Survival
				• Adverse effects

Outcome includes assessment of intervention efficacy (intervention evaluation), and assessment of outcome at cell, tissue and organ level, as well as clinical outcome.
SPECT: single-photon emission computed tomography; MRI magnetic resonance imagaing; CT: computed tomography.

the functional consequences of the intervention were evaluated by parameters such as left ventricular ejection fraction, end systolic volume and end diastolic volume, with the aid of echocardiography, left ventricular angiography and MRI, respectively. Heart failure was further quantified using N-terminal pro-brain natriuretic peptide (NT-proBNP) and N-terminal pro-atrial natriuretic peptide (NT-proANP) serum levels [79]. Besides these functional parameters, clinical parameters such as exercise duration, angina score, recurrence of myocardial infarction, any revascularization procedure, death, clinical heart failure and quality of life were evaluated. Finally, possible side-effects of treatment were evaluated such as neointima accumulation, edema formation, inflammatory response, restenosis, elevated liver enzyme concentrations, increased tumor/teratoma formation, neovascularization in non-target organs, vascular malformations, increased atherogenesis or plaque destabilization and arrhythmias [39,50,64,80 83].

Hepatic Regeneration

The liver shares several characteristics with the kidney. It is a highly vascularized parenchymatous organ that under normal conditions has a relatively low cell turnover, but is also able to recover from acute damage by an enormous capacity to proliferate. Under physiological conditions, as few as one out of 2000 3000 hepatocytes divides to maintain the physiological liver mass. However, liver damage or loss of liver mass can stimulate the regenerative capacity until the tissue mass has been restored by the proliferation of mature parenchymal liver cells [84]. In rodents, up to 75% of surgically removed liver mass can be regenerated within 1 week [85]. This model reflects what happens in human liver after partial hepatectomy (e.g. in case of tumor resection). Compensatory hyperplasia is also seen after liver transplantation when the recipient is larger than the donor [86].

Hepatic cell renewal is thought to be derived from three different sources. Mature hepatocytes and cholangiocytes are the earliest and most important source of tissue repair [87]. In times of overwhelming cell loss, with longstanding iterative injury, or when hepatocyte replication is impeded, regeneration occurs via a second, poorly defined, cell compartment. This compartment seems to arise from a less differentiated cell population within the terminal branches of the intralobular biliary tree. Finally, bone marrow-derived hepatocytes and cholangiocytes have been reported in human bone marrow transplant recipients, although the relevance of this source for hepatocyte renewal is debated [88 90]. BMDCs may enhance hepatic regeneration by other mechanisms than transdifferentiation. BMDCs have been shown to enhance liver regeneration by fusing with mature, differentiated liver cells [91,92]. Furthermore, BMDCs can stimulate mature hepatocytes by paracrine mechanisms or by stimulation of neovascularization, which subsequently stimulates liver regeneration [93,94].

No trials have been performed that investigated the relation between regenerative parameters and liver disease prognosis. However, assessment of regeneration in liver biopsies may be used to evaluate the severity of disease [95]. Human hepatic progenitor cells can be identified immunohistochemically in liver biopsies using markers such as OV6, CK7 and CK19. Livers of patients with massive necrosis showed increased numbers of hepatic progenitor cells over time. In addition, these cells migrated from their ductal progenitor cell niche into the liver lobule. The location and number of hepatic progenitor cells correlate to the severity of hepatic disease [96,97]. Here, regenerative parameters may be viewed as an indirect parameter of organ damage, similar to the relation between serum levels of growth factors and CVD risk, because increased damage can also initiate increased regeneration.

Therapeutic trials to enhance liver regeneration, in patients with advanced stages of cirrhosis and in patients with a partial hepatectomy because of a hepatic malignancy, used several techniques [98 106]. First, portal vein embolization or ligation has been shown to cause hypertrophy of the contralateral hepatic lobe [104 107]. Makuuchi et al. first reported a beneficial effect of this novel approach for routinely inducing contralateral hypertrophy in patients with cholestatic liver disease, chronic hepatitis or cirrhosis [108,109]. However, this strategy will not be applicable for the kidney because a reduction in nephron mass causes detrimental hemodynamic and structural changes in the remaining nephrons [110,111]. A second strategy to enhance liver regeneration in patients with cirrhosis or patients with partial hepatectomy is mobilization of progenitor cells from the bone marrow using granulocyte colony-stimulating factor (G-CSF). Mobilization of $CD34^+$ bone marrow-derived stem cells using G-CSF in patients with alcoholic steatohepatitis increased hepatic progenitor cell proliferation in liver biopsies, increased serum levels of regenerative growth factors (e.g. HGF) and improved liver function as measured by the Model for End-Stage Liver Disease (MELD) score [103]. Third, peripheral infusion of undifferentiated BMDCs, obtained by bone marrow aspiration, has been shown to increase the number of proliferating cells in hepatic biopsies and subsequently the amount of viable hepatic tissue in patients with liver cirrhosis [98]. Similar results have been shown for portal infusion of BMDC subpopulations. Autologous $CD133^+$ BMDCs infused in patients after partial hepatectomy enhanced liver volume assessed by computer tomography (CT) [100,101]. As a fourth strategy, a combination of G-CSF-induced mobilization, $CD34^+$ cell isolation using

apheresis, in vitro expansion and local infusion of $CD34^+$ cells was used in patients with (alcoholic) liver disease. This strategy decreased serum bilirubin and transaminases, and improved ascites and Child Pugh score, in uncontrolled trials [99,102].

Finally, intraperitoneal infusion of allogeneic fetal hepatocytes was used to treat fulminant hepatic failure [112]. Ethical considerations may hamper the routine clinical use of such a strategy. Taken together, mobilization as well as local and systemic infusion of selected and unselected BMDCs can enhance liver mass and improve liver function in patients with cirrhosis and alcoholic steatohepatitis.

The outcome of these therapeutic strategies for regeneration of hepatic tissue was quantified at different levels (Table 22.2). Progenitor cell mobilization from the bone marrow into the circulation using colony-stimulating factors was quantified by fluorescence-activated cell sorting (FACS) analysis of peripheral blood. The direct effect of therapeutic intervention at a cellular level has been evaluated by quantifying proliferation of hepatic (progenitor) cells in liver biopsies. Liver function was assessed using serum parameters such as albumin, prothrombin time, transaminases and bilirubin. Liver volume was serially measured using CT, and patient survival was evaluated. Finally, possible adverse events were assessed. Bone marrow infusion was complicated by a short period of fever, and leukapheresis by a reversible thrombocytopenia. In addition, infection rate, bleeding complications, alterations in liver perfusion and the appearance of focal liver lesions were assessed in liver regeneration trials; however, no increased incidence of these adverse effects was found in the intervention groups.

CLINICAL TRIALS ON RENAL REGENERATION

Thus far, no clinical trials have specifically addressed therapeutic renal regeneration. However, some trials that used growth factors in patients with AKI or CKD have reported possible regenerative effects of these factors. In addition, prognostic studies have evaluated the relation between several parameters and renal disease prognosis. Although most of these parameters reflect inflammation or tubular damage, some can be considered parameters of regeneration.

Prognostic clinical trials in nephrology have mainly used markers of tubular inflammation and damage in blood and urine to predict renal prognosis (Table 22.3). Increases in protein biomarkers such as urinary kidney injury molecule-1 (KIM-1), neutrophil gelatinase-associated lipocalin (NGAL) or interleukin-18 (IL-18) have been shown to correlate with a worse renal prognosis [113,114]. A relation between tissue and urinary mRNA levels of genes encoding for molecules involved in the pathogenesis of renal disorders was found to be related to the outcome of various renal diseases 1 12 months after biopsy [115 119]. One recent prognostic clinical trial demonstrated a relation between increased VEGF mRNA level and improved clinical outcome in patients with lupus nephritis class III or IV. Levels of VEGF mRNA, assessed in renal biopsies, correlated negatively with crescent formation, histological activity index and endocapillary proliferation. Furthermore, renal VEGF mRNA levels accurately predicted deterioration of renal function in the next 12 months, defined as doubling of serum creatinine or the development of ESRD [120]. To the authors' knowledge, this is the only prognostic clinical trial that correlates the level of a regenerative parameter with improved clinical outcome. Other studies reported that higher plasma and urine levels of VEGF and HGF were associated with worse renal survival. This discrepancy is similar to that observed in cardiovascular studies and again may be explained by the fact that levels of growth factors are determined by both the extent of tissue injury and the quality of the regenerative response and therefore reflect both injury and regeneration.

TABLE 22.3 Biomarkers of Renal Damage

Serum	Urine
Neutrophil gelatinase associated lipocalin (NGAL)	Neutrophil gelatinase associated lipocalin (NGAL)
Cystatin C	Cystatin C
Pro atrial natriuretic peptide (pro ANP)	γ Glutamyltransferase (GGT)
Neutrophil CD 11b	IL 18
Interleukin 6 (IL 6)	Glutathione *S* transferase (GST)
IL 8	γ GST
IL 10	π GST
	A GST
	Alkaline phosphatase (AP)
	Lactate dehydrogenase (LDH)
	β_2 Microglobulin
	N Acetyl β D glucosamidase (NAG)
	α_1 Microglobulin
	Kidney injury molecule 1 (KIM 1)
	Sodium–hydrogen exchanger isoform 3 (NHE 3)
	Matrix metalloproteinase 9 (MMP 9)
	Liver fatty acid binding protein (FABP)

Although no therapeutic clinical trials have primarily aimed to enhance renal regeneration, insulin-like growth factor-1 (IGF-1) administration was found to improve renal function in clinical trials that may, at least in part, be through regenerative mechanisms. IGF-1 in the circulation is bound to binding proteins (IGFBPs), of which seven subtypes have been identified. These carrier proteins are responsible for the bioavailability of the hormone, but mechanisms are ill-defined [121]. IGF-1 was used to enhance glomerular filtration rate (GFR) and renal plasma flow (RPF) [122,123]. However, IGF-1 is also abundantly expressed during tubular regeneration and may act as a renotropic agent, which can induce hypertrophy of tubules and glomeruli [123 127]. Furthermore, IGF-1 reduces protein breakdown and exerts a generalized anabolic action [128 130]. In animal models of ischemia reperfusion, continuous subcutaneous administration of IGF-1, for 4 days starting 30 min after reperfusion, not only reduced serum creatinine and blood urea nitrogen and enhanced inulin clearance post-ischemia reperfusion, but also reduced histological damage and mortality [131]. The latter suggests that the beneficial effects of IGF-1 are mediated not only by temporary hemodynamic effects but also by structural changes in the kidney. In 1993, the first uncontrolled clinical trial, in four patients with CKD and a creatinine clearance between 25 and 60 ml/min, showed that administration of recombinant human (rh) IGF-1 (s.c. twice daily for 4 days) increased inulin and para-aminohippurate (PAH) clearance in all patients during treatment [132]. After cessation of rhIGF-1, PAH clearance remained elevated in two out of four patients whereas inulin clearance returned to baseline values in all patients. In addition, IGF-1 treatment caused a temporary increase in renal volume, which returned to baseline after cessation of therapy. The acute and partially reversible effect on renal function suggests that IGF-1 stimulates the functional reserve of the damaged kidney. The persistently elevated PAH clearance in two patients may suggest some structural improvement in the kidney.

In a subsequent uncontrolled trial in nine patients with more severe renal failure (inulin clearance below 21 ml/min/1.73 m^2) treatment with rhIGF-1 (s.c. twice daily for 4 27 days) caused an increase in GFR and RPF and a decrease in blood urea nitrogen and plasma phosphate [133]. No difference in renal volume was observed by CT. However, the effects of IGF-1 did not persist over time, possibly owing to increased removal of IGF-1 by the increase in GFR, downregulation of IGF-1 receptors, and reduced availability of the administered IGF-1 due to altered IGF-binding proteins during treatment. Furthermore, side-effects were reported: jaw pain, nasal congestion, Bell's palsy, pericarditis and gingival hyperplasia [121].

Franklin et al. were the first to perform a randomized, placebo-controlled, double-blind trial aimed at preventing decline in renal function after major vascular surgery by administration of IGF-1 (twice daily for 3 days) starting immediately after surgery [134]. They showed that 3 days after surgery the IGF-1-treated group had a significantly higher creatinine clearance than the control group. However, there was no improvement in secondary endpoints: length of intensive care unit stay, length of hospital stay, length of intubation and creatinine at discharge. No side-effects were reported. In a subsequent double-blind, randomized, placebo-controlled clinical trial in 72 patients with AKI due to surgery, trauma, hypotension or sepsis, rhIGF-1 (s.c. twice daily for 14 days) caused no improvement in clinical recovery or renal function, but even a tendency towards delayed recovery of GFR and urine flow rate, despite a significant increase in serum IGF-1 concentrations [135]. These results were in contrast to the promising results of the previous animal experiments and clinical trials. The authors speculated that the role of IGF might differ between rodents and humans as well as between different disease state or etiology [136]. Furthermore, the first dose of IGF-1 was given as late as 6 days after onset of AKI and only continued for 2 weeks. Finally, the bioavailability of IGF-1 might have been affected by the IGFBPs.

The variable results on the potential role of IGF to enhance renal function illustrate that promising strategies in animal models do not guarantee successful clinical results. Furthermore, these studies underline the fact that evaluation of bioavailability of the therapy (e.g. by measuring growth factor binding proteins) is necessary to evaluate whether the therapy reaches its target. In addition, these trials stress the difference between improvement of renal function by hemodynamic alterations and structural renal regeneration. The acute improvement in renal function probably represents the functional reserve of the kidney, whereas a persistent increase in renal function after cessation of growth factor therapy may reflect structural improvement, and therefore renal regeneration. The latter, however, should be confirmed by renal biopsy, because a lowered serum creatinine as a result of hyperfiltration may be harmful in the long term [110,111]. Finally, timing of the initiation of therapy may influence its efficacy, as was also demonstrated in cardiovascular trials.

FUTURE PERSPECTIVES IN CLINICAL REGENERATIVE NEPHROLOGY

Prognostic Clinical Trials

Prognostic assessment of the outcome of renal disease is important to guide clinical decisions. To adequately

predict renal outcome, which can be envisioned as the balance between renal damage and renal repair, both detrimental processes and (potential) regenerative capacity should be quantified. Nowadays, prognostic information about renal diseases is obtained by combining the damage inflicted and the intensity of the current disease process [137 139]. Quantification of potential regenerative capacity will improve prognostic evaluation, which is crucial to guide clinical decisions, especially when toxic therapies are considered, for example in patients with glomerulonephritis or with rejection after kidney transplantation.

Several new biomarkers have been introduced to evaluate the severity of renal damage, as reviewed by Coca et al. (Table 22.3) [113,140 143]. Considerably fewer data are available on evaluation of renal regenerative capacity [120]. The regenerative capacity of the kidney may be assessed using blood or urine samples and renal biopsies. Trials in cardiovascular medicine have demonstrated a relation between circulating growth factors, such as HGF, and angiogenic capacity [11]. Growth factors such as IGF-1, HGF and epidermal growth factor (EGF) have been shown to be upregulated in renal regeneration and to enhance renal recovery in animal models [144 147]. These regenerative growth factors can be related to outcome parameters. Renal (progenitor) cells can also be evaluated to assess regenerative capacity. Proliferating tubular cells are the most important source of new tubular cells after AKI [148]. Progenitor cells, both local and circulating, are thought to enhance this process, predominantly by paracrine stimulation [149]. The number of progenitor cells may correlate with regenerative capacity. In addition, functional parameters such as migration and proliferative capacity of both progenitor and mature tubular cells may correlate with renal prognosis, comparable to the relation between EPC function and cardiovascular prognosis [150]. Furthermore, regenerative potential might be evaluated at the protein and mRNA level in renal biopsies or urine. Similar to previous studies that correlated increased tissue and urinary mRNA levels of genes encoding for molecules involved in the pathogenesis of renal disorders with worse outcome, an increase in mRNA levels of regenerative factors, such as HGF, may be an independent predictor of improved disease outcome [115 119]. Assessment of mRNA and protein levels of factors involved in renal regeneration such as HGF, IGF-1 and EGF in renal biopsy, urine samples or blood may be able to provide prognostic information.

Clinical trials correlate a certain prognostic or therapeutic intervention with an outcome parameter, which can cover the complete spectrum from histology to mortality. In animal studies the damage to the kidney is often standardized, and the intervention group is compared with a control group with the same amount of damage. In human trials the research population as well as the cause and degree of kidney injury will be more heterogeneous, necessitating not only the inclusion of a large number of patients but also multifactorial analysis of the correlation between parameters of renal damage, disease progression, regeneration and outcome. In prognostic clinical trials, regenerative parameters should therefore be considered in the context of initial renal damage and possibly a continuing and damaging process.

The continuing process of injury to the kidney can be evaluated using specific disease parameters such as antineutrophil cytoplasmic antibodies (ANCAs) in the case of Wegener's disease or anti-double stranded DNA antibodies in the case of systemic lupus erythematosus. More specific parameters of renal damage can also be used (Table 22.3). Immunohistochemistry is an important source of information on both the disease and regenerative process in animal experiments. In clinical trials protocol biopsies yield small amount of tissue and are performed only at a limited number of timepoints. Using standardized scoring systems, histological improvement and quantification of (progenitor) cell proliferation can be used as a parameter of damage, disease and possibly regeneration [137 139]. However, with only a few small sequential biopsies, the risk of sample bias is great. Another outcome parameter could be kidney volume assessed by ultrasound or CT. Furthermore, commonly used renal function tests can be used, such as creatinine clearance and proteinuria (see also Table 22.4) [151]. Finally, clinical outcome parameters, such as quality of life, hospitalization, need for renal replacement therapy and mortality, should be used. Information about regenerative potential will cover an important blind spot in disease prognostication in renal medicine.

Intervention Trials

Clinical trials will have to determine in which patient population a new therapeutic intervention will be investigated. Diabetic nephropathy is the leading cause of CKD that ultimately progresses to ESRD. The number of patients with diabetic nephropathy is steadily increasing worldwide. Diabetic nephropathy has been shown to be a reversible glomerular disease [4,6]. Therefore, patients with diabetic nephropathy form a relevant population for therapeutic enhanced regeneration. However, the number of preclinical data on therapeutically enhanced regeneration in models of diabetes-induced renal failure is limited (Table 22.5). In contrast, numerous preclinical studies have investigated therapeutically enhanced regeneration in acute tubular damage models, both toxic and ischemic. Although generally considered a reversible renal disease, AKI is

TABLE 22.4 Clinical Renal Function Tests

Parameter	Test
GFR	Inulin clearance
	^{125}I Iothalamate clearance
	^{51}Cr Ethylenediaminetetra acetic acid (EDTA) clearance
	^{99m}Tc Mercaptoacetyltriglycine (MAG_3) clearance
	Iothalamate sodium clearance
	Iohexol clearance
	Diatrizoate meglumine clearance
	Creatinine clearance
	Urea clearance
	Cystatin C clearance
RPF	P aminohippurate (PAH) clearance
	^{131}I Hippuran clearance
	^{99m}Tc Mercaptoacetyltriglycine (MAG_3) clearance
Renal concentrating ability	Water deprivation test
Renal diluting capacity	Water loading test
Urinary acidification	Urinary anion gap
	Urine osmolal gap
	Alkali loading test
	Urine P_{CO_2} Blood P_{CO_2}
	Sodium sulfate infusion test
	Response to loop diuretic
Tubular function in AKI	Fractional excretion of sodium
	Fractional excretion of urea
	Fractional excretion of uric acid
	Urinary β_2 microglobulin
	Urinary amylase
	Urinary lysozyme
	Urinary retinol binding protein
Glomerular damage	Non selective proteinuria

GFR: glomerular filtration rate; RPF: renal plasma flow; AKI: acute kidney injury.

associated with a 28-fold increase in the risk of developing stage 4 or 5 CKD [205]. Moreover, AKI is independently associated with a two-fold increased risk of death. Based on the extensive preclinical experience on therapeutically enhanced regeneration in models of acute tubular damage, clinical trials to enhance renal regeneration in patients with acute tubular damage will soon be initiated. A phase I clinical trial using allogeneic MSC infusion in patients with AKI after on-pump coronary artery bypass grafting has been initiated by Westenfelder et al. (see www.clinicaltrials.gov) [206].

In the translation of renal regenerative strategies from cage to clinic it is important to take into account differences between species, differences between renal injury models and the clinical context of renal disease. The well-known ischemia reperfusion model in rodents differs significantly from the acute tubular necrosis seen in human AKI [207]. Renal failure in animal models is induced as an isolated defect, whereas additional organ systems are often affected in the clinical setting. Furthermore, in contrast to many clinical situations, in the animal model renal blood flow is suddenly and completely interrupted, and also suddenly and completely reversed. This discrepancy causes differences in histological damage, which is milder in the proximal tubular and more severe in the distal parts of the nephron in rodent AKI than in human AKI [207,208]. AKI following temporary interruption of renal perfusion during elective cardiac or vascular surgery is more comparable to the preclinical experimental situation: acute interruption and resumption of renal perfusion in otherwise healthy individuals. These patients therefore form a relevant study population for translating therapeutic renal regeneration into clinical trials.

Interventions to enhance renal regeneration can be separated into growth factor-based therapy and cell therapy. In animal experiments on AKI, promising results of recombinant growth factor treatment have been shown. Single and repeated injections of EGF, glial cell line-derived neurotrophic growth factor (GDNF) and HGF attenuate renal damage after ischemia reperfusion, even when administered up to 24 h after ischemic damage was inflicted [131,145,147,152,153,209 212]. These growth factors reduced peak serum levels of creatinine and urea nitrogen and enhanced their reversal. Furthermore, they increased RPF, GFR and tubular cell proliferation, and improved histological damage. Decreased levels of *N*-acetyl-β-D-glucosaminidase suggested reduced tubular damage, whereas reduced fractional sodium excretion and enhanced tubular phosphorus reabsorption suggested improved tubular function. Clinical parameters such as body weight and mortality also improved. Others used prolonged administration of IGF-1 by a subcutaneous pump to enhance renal recovery after ischemia reperfusion [131]. Of note, the same growth factors improve tubular recovery after toxic AKI [144,146,213,214]. All these growth factors have already been used in humans [215 217]. As discussed above, most recombinant growth factors have a relatively short half-life, necessitating repetitive administration to ensure adequate tissue concentrations.

TABLE 22.5 Frequently Used Animal Models of Renal Damage with the Primary Location of the Damage and the Human Analog

	Model	Primary damage	Human analog
Acute tubular damage	IRI	Tubular damage	Ischemic ATN [77,152–167]
	Glycerol	Tubular damage	Rhabdomyolysis [71,168–171]
	Cisplatin	Tubular damage	Toxic ATN [149,172–179]
	Folic acid	Tubular damage	Toxic ATN [178,179]
	Gentamycin	Tubular damage	Toxic ATN
	$HgCl_2$	Tubular damage	Toxic ATN [152,180–182]
Chronic tubular damage	Unilateral ureteric obstruction	Tubular damage	Mechanical ATN [183–185]
Acute glomerular damage	Anti Thy1.1 glomerulonephritis	Glomerular damage	Mesangioproliferative/ microangiopathy [186,187]
Chronic glomerular damage	STZ induced DM	Glomerular damage	Diabetic nephropathy [188–191]
	Adriamycin	Focal segmental glomerulosclerosis	Chronic progressive renal fibrosis [192,193]
	5/6 Nephrectomy	Chronic renal failure	End stage renal failure [194–196]
	Anti Thy1.1 glomerulonephritis combined with habu/snake venom	Glomerulosclerosis	Chronic progressive glomerulosclerosis [197]
	Renal artery stenosis	Glomerulosclerosis	Hypertensive nephropathy [198]
Genetic glomerular damage	Collagen 1α2 deficiency	Dysfunctional basement membrane	Alport syndrome [199]
	Collagen 4α3 deficiency	Dysfunctional basement membrane	Alport syndrome [200–202]
	HIgA GN	Immune complex glomerulonephritis	IgA nephropathy [203,204]

IRI: ischemia reperfusion injury; STZ: streptozotocin; DM: diabetes mellitus; ATN: acute tubular necrosis; HIgA GN: high serum immunoglobulin A (IgA) mouse model for IgA nephropathy.

However, because recovery from AKI occurs within weeks, repetitive administration appears feasible. Although growth factor therapy using transfection has already been successful in the preclinical setting, clinical safety and proof of principle studies will probably use recombinant proteins [218]. The results of clinical trials on growth factor-enhanced regeneration using gene therapy have so far been disappointing (see Therapeutic Neovascularization in Cardiovascular Medicine, above). Local delivery can be achieved by a catheter in the renal artery or by an implanted pump. The latter may be particularly useful after aortic surgery, when the renal artery is already exposed during the operation. Whether continuous local administration of EGF or HGF and early initiation of therapy may enhance renal regeneration after AKI in humans is a very interesting research question [134,219]. Gupta et al. demonstrated that activation of the notch pathway using notch ligand Delta-like-4 (DLL4) enhances regeneration in an experimental ischemia reperfusion model [154]. The notch pathway is an evolutionarily conserved pathway that is responsible for tissue morphogenesis during development and regeneration of several organs including the kidney [154]. This pathway is currently also under investigation as a potential target for therapeutic regeneration in cardiovascular medicine [220].

A limited number of studies has investigated the capacity of growth factors to enhance renal function in patients with CKD [121]. Although some functional improvement has been demonstrated in these clinical studies, renal regeneration has not unequivocally been demonstrated. However, successful therapeutic regeneration has been demonstrated in several preclinical models of CKD including streptozotocin-induced diabetes. CKD is characterized by the triad of glomerulosclerosis, interstitial fibrosis and tubular atrophy [221]. Transforming growth factor-β (TGF-β) has been identified as a main mediator leading to progression of CKD [222]. TGF-β activity is counterbalanced by factors such as HGF and bone morphogenetic protein-7 (BMP-7) [223,224]. Studies involving enhanced regeneration using BMP-7 are limited [225]. However, HGF administration attenuated renal damage and antibody-mediated HGF inhibition enhanced renal damage in

a number of CKD models, including diabetic nephropathy [188,226,227]. HGF has been shown to reduce TGF-β-induced fibrosis and enhance tubular and endothelial regeneration [228]. In rodent models of streptozotocin-induced diabetes rhHGF and HGF plasmids were shown to attenuate proteinuria, glomerular damage, interstitial α-smooth muscle actin (α-SMA) expression, myofibroblast activation, apoptosis and TGF-β expression [189,190], as well as a rise in blood urea nitrogen and creatinine. Moreover, reversal of mesangial sclerosis was demonstrated, suggesting structural regeneration. Note that HGF therapy did not improve control of diabetes. In contrast, one study showed that HGF treatment in an obese diabetic mouse model decreased GFR and increased proteinuria, without changes in histology [229]. This study used a very low dose of HGF. HGF may be an interesting new strategy to treat diabetic nephropathy. Note that many forms of CKD finally evolve in a common final pathway to ESRD. Targeting these common events may inhibit or even reverse CKD independently of the underlying mechanism [221,230].

Cell therapy using different stem or progenitor cell populations has been shown to enhance renal recovery in animal models. For clinical purposes in the near future, BMDCs (or subpopulations of BMDCs) appear most promising, because of ethical issues related to fetal and embryonic cells, and the risk of tumor and teratoma formation with the use of embryonic stem cells and induced pluripotent cells [231,232]. Although local progenitor cells have been identified, the number of studies demonstrating therapeutically enhanced regeneration using these cells is limited. Different local progenitor cells were isolated from healthy donor kidneys and enhanced renal regeneration in a reversible renal damage model in genetically identical animals. Problems in translating these experiments combined with the limited preclinical data will delay the clinical introduction of local renal progenitor cells as a therapeutic strategy [155,156,192]. The potential therapeutic role of BMDCs in renal recovery after acute tubular damage was first shown by Kale et al. They demonstrated that complete ablation of the bone marrow worsens the course of ischemia reperfusion-induced AKI and that infusion of lineage-depleted BMDCs can restore the repair process [233]. Although it was primarily thought that BMDCs differentiated into several renal cell types and participated structurally in tissue repair, later studies demonstrated that the numerical contribution of BMDCs to renal repair was relatively low (0.06 8%) [157,178]. Many of the cells identified as bone marrow-derived renal cells appeared to be the results of cell fusion [234].

Both hematopoietic stem cells (HSCs) and MSCs, which provide stromal support for the HSCs, have been studied for their effects on renal regeneration in animal models of acute tubular damage. Morigi et al. demonstrated that MSCs but not HSCs provide protection against acute tubular injury [235]. In several animal models it has been demonstrated that MSCs enhance renal regeneration after acute toxic or ischemic tubular damage [158,159,168]. Moreover, human MSCs have been shown to stimulate renal recovery after cisplatin-induced acute tubular injury in immunodeficient mice [172]. MSCs do not enter the renal parenchyma in significant numbers, nor do they contribute structurally to tubular cell repopulation. The therapeutic effect of MSCs in AKI is primarily mediated by stabilization of the renal vasculature and paracrine factors [149,156,160,172,206,236,237]. Bi et al. demonstrated that medium conditioned by MSCs induced migration and proliferation of renal epithelial cells [149]. In addition, intraperitoneal injection of conditioned medium twice daily reduced renal injury after cisplatin-induced acute tubular damage. MSCs lack the expression of various major histocompatibility complex and costimulatory cell-surface markers and secrete anti-inflammatory cytokines, supporting their potential use as an allogeneic graft without the need for immunosuppression. In addition, MSCs have been shown to home to the injured kidney using platelet-derived growth factor (PDGF) stromal-cell derived factor-1 (SDF-1) receptor interaction and CD44 hyaluronic acid interaction [68 71]. Several clinical safety and efficacy trials are underway using MSCs in the field of nephrology to treat refractory lupus erythematodes, renal allograft rejection and chronic allograft nephropathy (www.clinicaltrials.gov). Moreover, Westenfelder et al. are currently executing a clinical trial to investigate MSC-enhanced therapeutic renal regeneration in patients with AKI following cardiac surgery. MSC-conditioned medium will also be an interesting clinical strategy. Further identification of regeneration-stimulating factors in conditioned medium may overcome the need for MSC isolation and culture. Proposed factors are IGF-1, HGF, EGF and VEGF [149,160,173,238 240]. However, Bruno et al. demonstrated that the effect of MSCs on renal recovery after acute tubular injury is also mediated by mRNA transfer from MSCs to target cells using microvesicles [241]. Therefore, growth factors alone may not be able to fully mimic the effect of MSCs and MSC-conditioned medium. In addition, infusion of MSCs offers the advantage of continuous local production of paracrine factors including microvesicles. The current study by Westenfelder et al. will clarify whether MSCs enhance renal recovery after acute tubular damage. Future clinical trials will have to evaluate whether autologous MSCs can be replaced by donor MSCs or even conditioned medium. Note that MSCs not only enhance regeneration, but have anti-inflammatory capacities,

and can therefore reduce the inflicted damage [160]. This stresses the importance of assessing both destructive and regenerative processes.

Cell therapy has also been used to enhance regeneration in CKD. EPCs were shown in one study to restore renal function in experimental chronic renovascular disease, by enhancing neovascularization and reducing remodeling and fibrosis [198]. MSCs have also been used to enhance renal regeneration in animal models of CKD, such as 5/6 nephrectomy and a progressive model of anti-Thy-1.1-induced glomerulonephritis. In these animal studies MSCs were shown to reduce proteinuria and improve renal function [186,187,194]. In streptozotocin-induced type 1 diabetes in mice, single infusion of MSCs not only improved glucose tolerance and prevented the development of diabetic glomerulopathy, but also reversed proteinuria [191]. Whether the reversal of proteinuria was an indirect effect of improved glucose regulation or the result of MSC-enhanced renal regeneration is unclear. MSCs were found in the kidney up to 2 months after MSC infusion. According to www.clinicaltrials.gov, several clinical trials are currently investigating MSC infusion in patients with type 1 diabetes. It will be interesting to see whether MSC infusion can enhance renal function independently of glucose regulation. In addition, MSC tracing (see below) will further elucidate the mechanisms of MSC-enhanced renal regeneration in diabetic patients.

Outcome parameters of clinical trials on therapeutic renal regeneration include parameters of initial damage, parameters of disease progression and regenerative parameters (see Prognostic Clinical Trials, above). Because HGF and MSCs not only enhance regeneration but also reduce fibrosis and inflammation, both processes have to be monitored during clinical trials to assess their specific effect on regeneration. In addition, direct effects of the intervention, including adverse effects, should be evaluated. During growth factor therapy, changes in growth factor binding proteins and expression of growth factor receptors should be evaluated because they can influence the efficacy of therapy [133]. In kidney biopsies the presence of growth factor receptors should be assessed during treatment, preferably over time. In clinical trials, where cells are administered to enhance renal regeneration, tracking of the cells could provide important information about homing, transdifferentiation and participation in tissue regeneration. Infused cells can be traced using different techniques. First, the presence of the infused cells in the kidney can be evaluated immunohistochemically, using chemical or genetic markers. The latter can be intrinsic characteristics of the allogeneic donor cells [e.g. human leukocyte antigen (HLA) markers, Y-chromosome] or can be added to the cells by transfection. Double staining for both a tracing marker and a differentiation marker will elucidate whether infused cells structurally contribute to newly formed tissue, or whether only paracrine mechanisms are involved. Another technique to trace injected cells is in vivo imaging [reviewed in Ref. 242]. Stem cells can be traced in vivo using direct radionuclide labeling. This technique has been used in cardiovascular regeneration trials and has a high sensitivity [74 76,242,243], but a disadvantage is the possible adverse effect of radiation on both labeled cells and native cells of the patient. Newer techniques include labeling agents for MRI, which has a higher spatial resolution than PET. However, this technique has a low sensitivity and there are concerns about the effect of labeling on cell proliferation and differentiation. In addition, neither MRI labeling nor direct radionuclide labeling can evaluate the viability of the labeled cells, in contrast to indirect radionuclide labeling. For indirect radionuclide labeling a reporter gene is transferred to cells before infusion into the patient. Later, cells can be detected by an intravenously administered, radiolabeled probe, which is specific for the reporter gene. This probe will accumulate solely in viable transduced cells. This technique has not yet been applied clinically.

Besides efficacy, the safety of the intervention should be considered. There is concern that growth factor therapy may exacerbate pre-existing sclerotic, fibrotic, cancerous or atherogenic conditions, although this concern has not been borne out in human trials thus far. Nonetheless, patients with malignancies are currently excluded from clinical trials with growth factor-enhanced regeneration [244]. HGF treatment in clinical trials until now has not shown any adverse effects [245 247]. A potential concern with long-term use of rhIGF-1 is the induction of glomerulosclerosis [219]. However, this has not been observed in trials, and prolonged growth hormone treatment in children with chronic renal failure appears not to be associated with a more rapid loss of renal function. Adverse effects of BMDC infusion have been demonstrated in animal models. Systemic administration of BMDCs can be hampered by non-specific homing to other organs and mechanical entrapment of cells in capillary beds, especially in the lung. This may even affect pulmonary hemodynamics [248,249]. In addition, concerns have been raised regarding MSC-derived tumor formation, ectopic tissue formation and stimulation of existing tumors to metastasize by MSCs [250 253]. Finally, BMDCs can contribute to renal interstitial fibrosis and are able to maldifferentiate into adipocytes in the glomerulus [161,187,193]. Future trials using growth factors or BMDCs should include long-term follow-up of both beneficial and adverse events.

Taken together, increased insight into molecular and cellular mechanisms of renal damage and repair has provided us with tools to monitor and even influence these processes. We are on the verge of the clinical introduction of therapeutic renal regeneration. With the

extensive preclinical data on AKI, taking therapeutically enhanced regeneration to a clinical trial is an important translational step with great potential impact. Patients undergoing elective cardiovascular surgery, with increased risk of renal failure, will not only provide the opportunity to obtain proper informed consent, but also allow for preoperative isolation and preparation of autologous BMDCs that can be used therapeutically. In addition, the sudden and complete occlusion and reperfusion of the kidney in relatively healthy individuals resembles the situation in preclinical experiments in which regeneration was successfully stimulated. Therapeutic regeneration to treat CKD, in particular diabetic nephropathy, also forms an interesting subject for clinical investigation, not least because of its increasing relevance to public health. In both AKI and CKD, HGF and MSC therapy appear very promising strategies for therapeutic regeneration. Both HGF and MSCs have been shown not only to enhance regeneration, but also to attenuate fibrosis and inflammation. Outcome should be evaluated in the context of both the initial damage, before initiation of therapy, and possible persistent disease activity during therapeutic regeneration. The near future will reveal whether regenerative medicine will be a turning point in the treatment of AKI and progressive CKD.

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CHAPTER

23

Potential Risks of Stem Cell Therapies

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OUTLINE

INTRODUCTION

No medical treatment can ever be considered completely safe. For example, much excitement greeted the prospects of gene therapy, but the field suffered a severe setback when several patients from the first clinical trials developed leukemia [1]. Malignancies, mostly related to applications of embryonic stem cells (ESCs), have evolved to be a major concern in stem cell therapies as well. Other concerns relate to the validity of experimental results. For example, stem cell markers are often prone to technical misinterpretation or may be affected by heterogeneity of the cell preparations. Consequently, this chapter will address both the medical safety aspects of stem cell therapies and the scientific "safety" of stem cell-related data (Fig. 23.1).

Currently, nephrological stem cell studies are almost exclusively performed in animal disease models. These

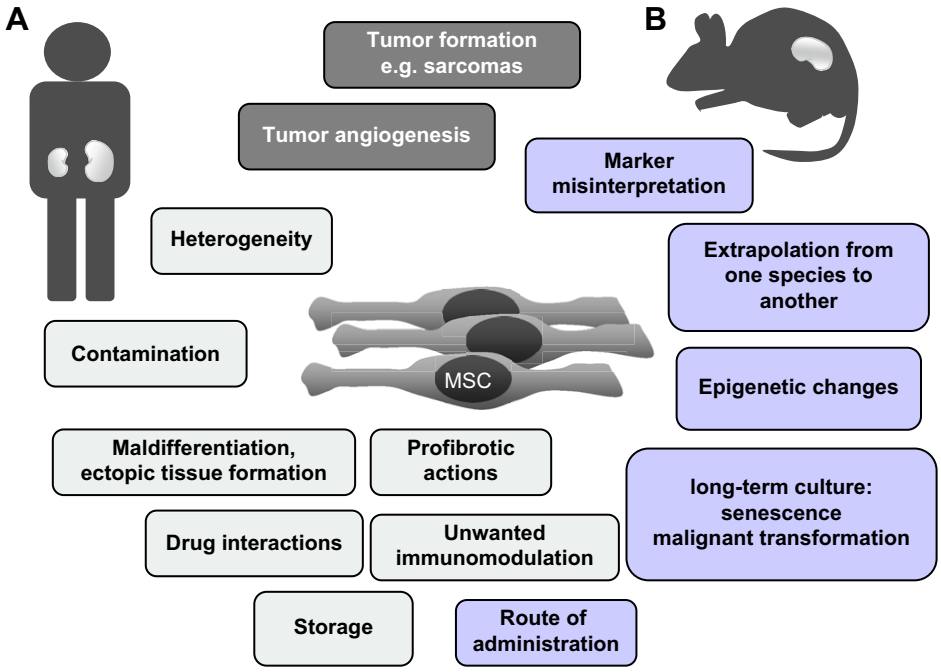

FIGURE 23.1 Factors that affect the safety of stem cell therapies: (A) with respect to clinical studies, and (B) with respect to the validity of research results. MSC: mesenchymal stem cell.

mostly use rodents, sometimes crossing species borders by transplanting human stem cells into immunodeficient [e.g. severe combined immunodeficiency (SCID)] mice. Only a handful of clinical phase I studies on stem cells in renal diseases is on its way. The cells that usually come to mind when thinking about stem cell therapies are ESCs. But up to March 2010, no clinical studies using ESCs or induced pluripotent stem (iPS) cells in renal diseases were registered on the National Institutes of Health (NIH) site (www.clinicaltrials.gov). Rather, ongoing clinical trials in nephrology mostly investigate tolerance induction by hematopoietic stem cells (HSCs) or mesenchymal stem cells (MSCs) after kidney transplantation. In addition, very few studies involving MSCs target other renal diseases, such as a Chinese study on refractory systemic lupus erythematosus and a single-center study on acute kidney injury (AKI) [2]. Consequently, this chapter will focus mainly on safety issues regarding MSCs. However, with the rapid developments in the field, including the first Food and Drug Administration (FDA) approval of an ESC study on spinal cord injury, safety aspects concerning stem cells other than MSCs may gain importance in the near future and will consequently be mentioned throughout this chapter.

Characteristics of Mesenchymal Stem Cells

MSC stands for mesenchymal stem cell or, more precisely, multipotent mesenchymal stromal cell, following the recommendations of a recent position paper by the International Society for Cellular Therapy (ISCT). MSCs have to fulfill certain consensus criteria regarding shape and surface markers, adherence, self-renewing capacity and in vitro differentiation potential, described in detail in an ISCT publication [3].

Although researchers still have to deal with the lack of indisputable criteria defining MSCs, making it necessary to constantly standardize the widely differing protocols for isolation, expansion and characterization, the emerging (and considerable) therapeutic potential of MSCs renders them strong candidates for human therapies. Recent studies have established animal serum-free culture conditions for human MSCs [4,5], a prerequisite for clinical use with respect to biological safety, and successfully established isolation and expansion of large numbers of MSCs derived by washings of bone marrow collection bags [6]. If not mentioned otherwise, "MSCs" in this chapter will always refer to bone marrow-derived MSCs.

The preference for MSCs in nephrological research can be understood when comparing MSCs to ESCs. The apparently less powerful and versatile MSCs are favorable in many practical aspects: they can be cultured easily and without significant ethical concerns from adult bone marrow aspirates (and several other organs, e.g. adipose tissue and umbilical cord blood) and expanded under simple, inexpensive conditions in vitro. Their phenotypic stability has been considered superior to that of ESCs and, initially, no teratoma

formation or other malignant transformation had been observed. However, recently, Tolar et al. [7] described cytogenetic aberrations in mouse MSCs after several passages in vitro and (unwanted) sarcoma formation of transduced MSCs in recipient mice in vivo. Since then, several alarming observations on MSCs of different species have been published and will be discussed later (Potential Clinical Problems of Stem Cell Therapies, below).

Another appealing aspect of MSCs is that they can be obtained as syngeneic material from a patient before a calculated medical risk, e.g. major surgery, and later be readministered in case of organ failure (e.g. AKI). This idea led to the first phase I trial of allogeneic, intra-aortic MSC administration in AKI, mentioned above.

Allogeneic transplantation of MSCs seems feasible given a growing number of studies investigating and mostly confirming their tolerance-inducing effects by modulation of the immune response and their ability to escape T-cell recognition. These and other favorable properties of MSCs are currently being evaluated in several preclinical and clinical studies [reviewed in Ref. 8].

Older studies on MSCs focused mainly on their ability to adopt non-mesenchymal phenotypes, e.g. neural precursors and cardiomyocytes. However, methods employed to verify differentiation of MSCs into other phenotypes in vivo are technically problematic and prone to misinterpretation (see Caveats of Stem Cell Markers and Tracing Methods and Fig. 23.3, below). In more recent studies, beneficial effects of MSCs were largely related to paracrine effects, e.g. secretion of cytokines and growth factors, rather than to differentiation into new phenotypes [9 13]. This is well in line with knowledge on the feeder function of MSCs for embryonic and hematopoietic stem cells.

The therapeutic potential of MSC-conditioned media opens up the prospect of cell-free stem cell therapy, which is potentially less risky than living cell injection.

Sometimes risks are easier to identify when recapitulating the natural processes. What is the physiological (and thus safe) contribution of stem cells to kidney regeneration? Bone marrow-derived stem cells in general may contribute to cell turnover and repair in the kidney [14]. In an attempt to visualize this contribution, mice transgenic for enhanced green fluorescent protein (eGFP) were used as bone marrow donors and differentiation of bone marrow cells into glomerular cells expressing desmin was described in wild-type recipients [15]. In rats with anti-Thy1.1 nephritis, differentiation of invading bone marrow cells into endothelial or mesangial cells was also reported [16,17]. These data were interpreted to reflect a regular contribution of bone marrow to glomerular cell turnover, which is modified during disease and initiated the first studies which supplemented exogenous MSCs in renal disease models. Nevertheless, transplantation of MSCs is not a naturally occurring process. First, the cells are isolated from their niche, disrupting the regular microenvironment with its signals and interactions. Second, the cells are subjected to an artificial cell culture milieu. Third, the cells are forced mostly without preconditioning into a completely new environment, e.g. the myocardium or the glomerulus. It should not come as a surprise that the sum of these processes can sometimes lead to unexpected MSC behavior, as discussed later in this chapter (see Non-malignant Maldifferentiation and Unwanted Actions of Stem Cells and Fig. 23.6A,B).

The potential of MSCs for renal repair has been shown in several rodent models of AKI, where the course of glycerol, cisplatin or ischemia reperfusion -induced acute renal failure (ARF) was improved by MSC injection shortly after disease induction [9, 18 20]. Follow-up in these studies was usually short and no unwanted effects were noted. More recently, the first long-term studies in renal disease were published. Beneficial effects of bone marrow-derived cells [21,22] or MSCs [11] were noted in a model of chronic progressive renal fibrosis, i.e. in mice that are genetically deficient of the collagen α3(IV) chain (Alport mice). More studies investigating effects of MSCs in chronic fibrosis are coming up and will allow us to gain a more complete picture of possible long-term wanted and unwanted effects of these cells (see Potential Clinical Problems of Stem Cell Therapies, below).

Characteristics of Induced Pluripotent Stem Cells

A possible and powerful future alternative to MSCs is iPS cells. These cells were introduced in 2007 when they were created by two research teams by inserting genes encoding transcription factors into somatic human skin cells [23,24]. Since then, various reprogramming methods for generation of iPS cells from a variety of tissue sources have been described. Nevertheless, iPS cells can lead to teratoma formation and considerable variations in the safety of iPS cell lines exist [25]. In particular, reactivation of c-Myc was associated with tumor growth in iPS cell offspring and chimeric mice derived from c-Myc-negative iPS cells were much less prone to tumor formation [26,27].

There are no published studies using iPS cells in renal disorders yet, but initial data show that murine iPS cells can differentiate into cells expressing renal lineage markers in vitro. In particular, activin may enhance the differentiation of iPS cells to tubular cells [28].

TECHNICAL CAVEATS IN STEM CELL PREPARATION AND ADMINISTRATION

Standardization of Stem Cell Methodology and Quality Control

A recent publication [29] described a "simplified culture and polymerase chain reaction identification assay for quality control performance testing of stem cell media products". The authors wanted to offer a quick and sensitive assay/tool for validation and quality control for, in particular, MSC in research, and clinics and found it in a combination of culture assay, gene microarray and polymerase chain reaction (PCR). When this reportedly inexpensive low-technology assay indeed does live up to the expectations, it will certainly contribute to stem cell safety by facilitating to discriminate undifferentiated stem cells from early stage differentiating cells.

Apart from the ISCT position papers on MSCs mentioned above [3,30], a position paper on MSCs in solid organ transplantation with special focus on safety concerns has been published by MISOT, the Mesenchymal stem cell In Solid Organ Transplantation group [31] and another paper on human stem cell research has been published by the World Federation of Neurology [32].

Not only in MSCs, but also in other progenitor cells small alterations in the culture conditions can induce a shift in cell phenotype. As an example, in cultured human fetal kidney epithelial progenitor cells, expression of E-cadherin was calcium dependent [33]. Effects of needle diameter and flow rate on viability, phenotype and multilineage differentiation capacity of MSCs were tested in the past, and no significant interference was found [34].

Heterogeneity of Stem Cell Preparations

Heterogeneity in a stem cell preparation can strongly influence both its therapeutic potential and its safety. Moreover, heterogeneity may be mistaken for true plasticity or transdifferentiation across tissue lineage boundaries. Bone marrow, apart from hematopoietic stem cells, contains subpopulations of tissue-committed stem cells and primitive pluripotent stem cells which are released from the marrow to the blood upon injury [35]. Thus, bone marrow per se is a heterogeneous stem cell/progenitor cell preparation. Even if aiming at distinct bone marrow subgroups, current isolation protocols do not guarantee homogeneity. As an example, MSC preparations are notoriously heterogeneous [36,37] (Fig. 23.2). These cells can be isolated from a multitude of tissue types but cannot easily be defined by phenotypic or

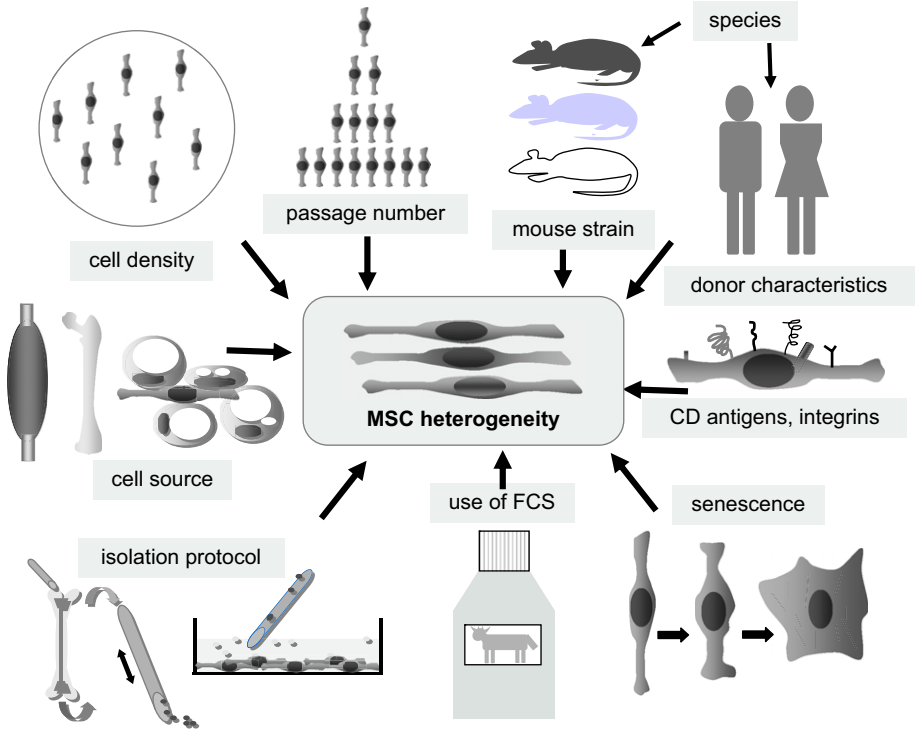

FIGURE 23.2 Factors influencing heterogeneity of mesenchymal stem cell (MSC) preparations. Determinants include the source of the MSCs (e.g. muscle, bone marrow, adipose tissue), donor characteristics (in both humans and rodents, e.g. gender and strain), isolation protocols, initial plating density, use of fetal calf serum (FCS), number of passages and cellular senescence.

functional characteristics [3]. Even in a seemingly homogeneous MSC preparation, cells may be rapid dividers, slow dividers or non-dividers. In human MSCs vascular cell adhesion molecule-1 (VCAM-1) positivity and fibromodulin (FMOD) positivity predicted low progenitor activity and limited differentiation capacity, whereas small, spindle-shaped cells divided very slowly and were therefore discussed to be very early, quiescent progenitors [38]. The existence of distinct clonal subpopulations in cultures of bone marrow MSCs has been supported by several studies. Recently, proteomic profiling of clonal subpopulations has been performed and demonstrated differential expression of 11 out of 83 proteins within the clones. These proteins were related to cytoskeleton and cellular structure, calcium binding and intermediate filaments [39].

The cell source is another confounder to heterogeneity and, for example, MSCs from different human materials such as placenta, cord blood and bone marrow express different cytokine profiles [40].

Apart from the cell source, cell density, culture conditions (including the use or non-use of fetal calf serum) and the number of passages all affect the differential expansion of specific cell subsets and may affect the therapeutic potential of the final cell preparation. For example, a study attempting to standardize MSC protocols found it comparatively simple to repeatedly obtain homogeneous MSC preparations, but culture conditions then had a major impact on their proteome and transcriptome [37].

To complicate matters further, the nomenclature for MSCs differs widely. Attempts to clarify both nomenclature regarding MSCs and definition of minimal consensus criteria for their functional and phenotypic properties have been made by the ISCT, as mentioned above [3,30].

In addition to heterogeneity resulting from differences in technical aspects, there is intraspecies and interspecies variability. For example, in rodents cross-strain transplantation data suggest that MSCs from different strains possess different immunogenic potential and induce variable immunological responses [41]. Another ISCT publication recently compared bone marrow-derived MSC lines from four frequently used rat strains (Fisher and Lewis, both inbred, and Wistar and SPRD, both outbred) [42]. They analyzed cell surface markers, population doubling times throughout 10 passages, and their differentiation capacity and proliferation rates in cocultures with spleen cells. Findings in these four rat strains were much more uniform than in different mouse strains, where key factors such as differentiation potential and cell surface epitopes can differ widely [43]. A publication from 2009 investigated human first passage MSCs from different people with regard to their proteomic profiles and found seemingly identical proteomic patterns and functionally similar properties of the cells [44].

Finally, it is often desirable to verify rodent data in large-animal models, e.g. pigs, before embarking on human studies. In this respect it is of note that a recent study could not confirm the beneficial effects of MSCs observed in rodents with renal ischemia reperfusion injury in an analogous porcine model [45]. In that study, porcine MSC preparations failed to exhibit the same immune-modulating abilities as human or rat MSCs and induced the secretion of proinflammatory interleukin-6 (IL-6) in the recipient. Similarly, immunosuppressive effects varied broadly when comparing human, monkey and mouse MSCs [46]. In yet another large-animal study, autologous MSC injection in sheep with ischemic kidney injury caused no benefit, although the injected cells could be localized to the kidneys [47]. All the above caveats are likely amplified in xenograft models, e.g. transplantation of human adipose-tissue derived MSCs in immunocompetent C57BL/6 mice with AKI [48].

In summary, there is an urgent need to develop reliable reagents, widely accepted guidelines and standards for MSC preparations together with a definition of precise molecular markers to define subtypes of MSCs.

Caveats of Stem Cell Markers and Tracing Methods

In their seminal work from 2002, Terada et al. [49] shattered the common belief in almost unlimited plasticity, i.e. transdifferentiation of stem and progenitor cells, by demonstrating spontaneous cell fusion as a mechanism by which bone marrow cells (BMCs) can adopt the (unexpected) phenotype of differentiated cells from other lineages (Fig. 23.3A). Transdifferentiation so far had mostly been deduced from donor-specific genes such as Y-chromosomes in differentiated cells within a female cell recipient. The authors cocultured mouse BMCs expressing GFP and a puromycin resistance gene with mouse ESCs in the presence of IL-3. Later, ESCs were removed from the culture by addition of puromycin. The remaining GFP-positive clones resembled ESCs in many features and were first assumed to represent BMC-derived ESCs. Only genetic analyses revealed quite unexpectedly that coculture of BMCs with ESCs did not induce ESC formation from BMCs. Instead, the surprisingly tetraploid and hexaploid DNA content of the GFP-positive cell clones and consecutive PCR amplification of multiple microsatellites that were polymorphic between genomes of ESCs and BMCs suggested that the BMC-ESC clones were created simply by fusion of these two cell types. These findings were consecutively reproduced by the authors in four repetitions of the experiment and showed dependency on IL-3 [49]. Which exact bone marrow cell type might

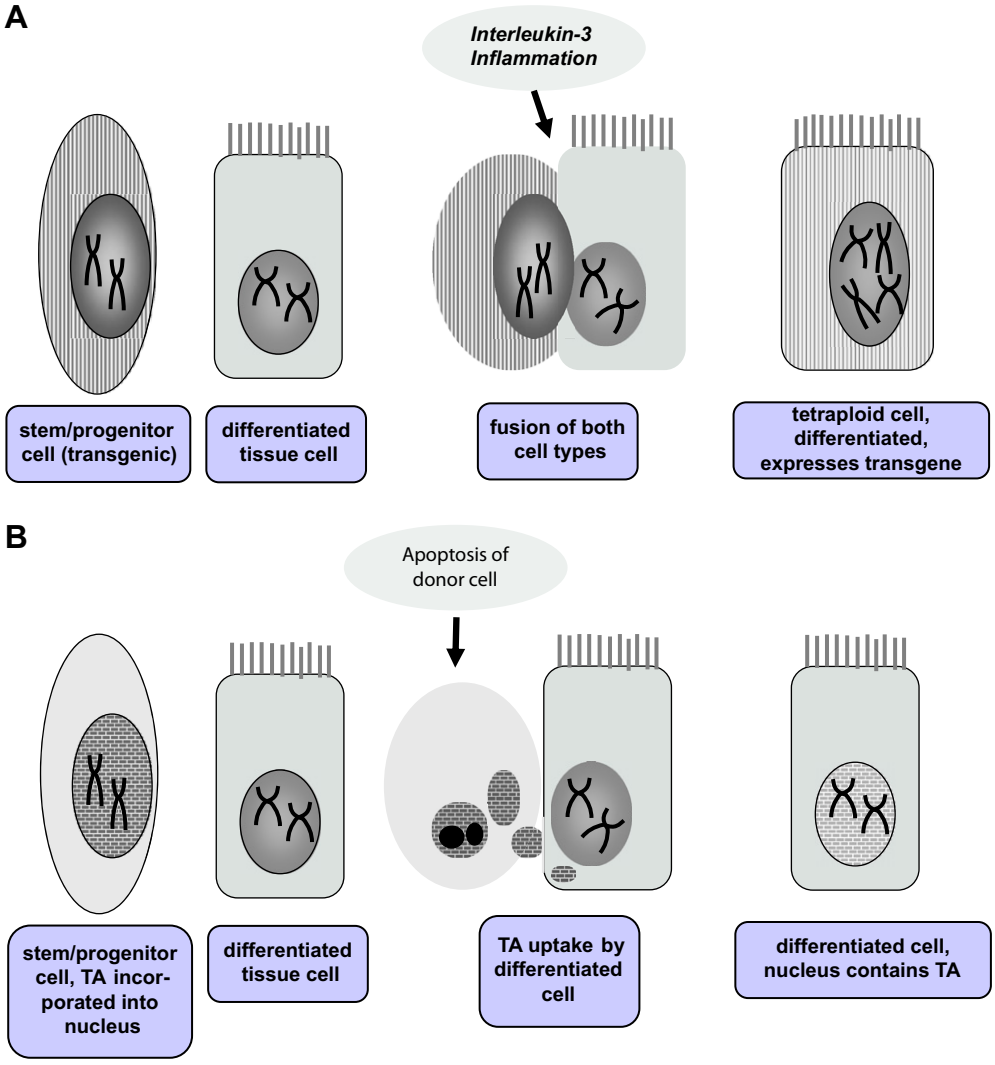

FIGURE 23.3 Caveats of stem cell tracing. (A) Fusion events between donor stem/progenitor cells and fully differentiated resident cells of the recipient can mimic differentiation of the donor cells. External factors, e.g. interleukin 3 and inflammatory conditions, have been discussed to promote the fusion events. (B) Transplanted stem/progenitor cells that were labeled with thymidine analogs (TA) release the dye upon apoptosis/necrosis. Neighboring differentiated recipient cells can take up the TAs and incorporate them into the nucleus upon cell division. Consecutive staining for TAs can falsely be interpreted as differentiation of the TA labeled stem/progenitor cell.

have been responsible for the fusion events could not be clarified and the authors noted that fusion was a very rare event. In an equally important work, Ying et al. showed similar mechanisms in cocultures of GFP-positive, puromycin resistance gene-positive progenitor cells of the central nervous system with ESCs [50]. Again, tetraploid cell clones were formed exhibiting the genome of both cell types.

Fusion phenomena have since widely been accepted as a major caveat in interpreting events that seemingly visualize transdifferentiation of a stem cell into a differentiated cell. It has become common knowledge that the presence of not only donor cell markers but also recipient cell markers in an apparently transdifferentiated cell needs to be evaluated. Since 2002, observations that had led to assumptions of plasticity and transdifferentiation have been revisited and analyzed more closely. Since then, paracrine effects of injected stem cells have widely superseded the belief in plasticity as the main mediator of their beneficial actions [51]. It should be mentioned that spontaneous fusion between mammalian cells had already been described as early as 1961 [52].

The first report on fusion events between renal and bone marrow cells after unilateral ischemia reperfusion injury using two transgenic, gender-mismatched mouse strains as cell donors and recipients was published in 2007 [53] and confirmed the above findings. Notably, fusion was observed in only seven out of 10,000 tubular cells, whereas 180 out of 10,000 tubular cells were

estimated to be donor derived. The authors demonstrated that injury was a prerequisite for fusion and no such event occurred in healthy recipients of bone marrow transplants.

Another concern regarding transdifferentiation events came in 2006 [54]. Thymidine analogs such as bromodeoxyuridine (BrdU), which have been used for cell tracking by incorporating into DNA during S phase, can be transferred from labeled dead donor cells to (dividing) neighboring recipient cells, thus mimicking transdifferentiation (Fig. 23.3B). This observation was made when 3 12 weeks after transplantation of thymidine analog-labeled, GFP-positive live stem cells into the central nervous system, few local GFP-positive cells but many thymidine analog-labeled neurons and glia cells were found. A control group using thymidine analog-labeled dead progenitor cells caused the same strong thymidine analog staining in the surrounding tissue, as did thymidine analog-labeled fibroblasts, whereas supernatants of labeled cells did not. This was the first conclusive report on false labeling of neural cells and it certainly applies to stem cell transplantation in other organs as well. It is well documented that the majority of injected stem cells dies quickly, thus offering large quantities of thymidine analogs to be processed by endogenous DNAses and to be incorporated into dividing recipient cells.

Another method to identify putative stem cells is the efflux of certain dyes, most prominently Hoechst 33342 as described in the so-called side population cells and other hematopoietic progenitors [55]. This method has been used to identify putative stem cells in the adult human kidney [56] but, overall, cells sorted for dye efflux are quite heterogeneous [57] with all the consequences arising from heterogeneity as described above.

Even old-fashioned markers such as β-galactosidase (β-gal) expression in transgenic stem cells came under scrutiny in 2005 when Duffield and Bonventre discussed whether prior detection of apparent transdifferentiation of β-gal-expressing stem cells into tubular cells might in reality mirror increased intrinsic β-gal activity in the injured tubules [12]. Another traditional method of cell tracking, the technically demanding Y-chromosome detection by fluorescence in situ hybridization (FISH) in gender-mismatched stem cell transplantation in ARF was discussed by the same authors to be prone to misinterpretation through artifacts caused by superimposition of a tubular cell and an infiltrating bone marrow cell [12].

Another principle for stem cell detection is quiescence. One of the first descriptions of intrarenal stem cells was published by Oliver et al. in 2004 [58], who described slow-cycling, BrdU-retaining cells in the renal papilla of adult mice that re-entered the cell cycle in AKI, thus giving the impression that they were recruited for repair. Later, others have shown that even fully differentiated epithelial cells can retain BrdU, merely because they are quiescent, which renders BrdU retention alone an unreliable stem cell marker [59].

Another new marker predominantly used to characterize human glomerular progenitor cells is CD133 [60]. Kidney-derived $CD133^{+}$ cells were later found to be able to contribute to tumor angiogenesis [61]. Nevertheless, is has to be kept in mind that CD133 is one of the most widely accepted markers for cancer stem cells (CSCs) and has been described previously in renal malignancies and Wilms' tumor [62], demonstrating the need to isolate putative progenitor cells based not exclusively on expression of one marker, but rather on a combination of several markers (e.g. $CD24^{+}$) [63,64].

Contamination of Stem Cell Preparations

Risk factors for microbial contamination of stem cells include the source/donor as well as the methods of cell collection, expansion and, maybe, manipulation [65] (Fig. 23.4). MSCs are susceptible to human herpes viruses and transmission from the donor to the severely immunocompromised recipient has been a concern. Nevertheless, Sundin et al. did not find human herpes simplex virus (HSV), cytomegalovirus (CMV), varicella zoster virus (VZV) or Epstein Barr virus (EBV) in MSCs from herpes virus-seropositive donors [66]. In 2008, a retrospective analysis of 20 MSC preparations from healthy human donors uncovered one preparation with persistence of parvovirus B19 DNA. The persistence of this virus is facilitated by the expression of B19 receptor and a coreceptor on human MSCs. These cells can transmit B19 to bone marrow (hematopoietic stem) cells in vitro, underscoring the importance of monitoring B19 transmission of MSC products [67]. In another investigation of MSC susceptibility to specific viruses, all tested human MSCs were found to express the human herpes virus-6 virus receptor CD46, but none of the cell samples was found to harbor the virus [68].

In the limited and restricted ESC lines, much effort was invested in securing the absence of infection. The International Stem Cell Initiative 2007 published its comparative findings on 59 human ESC lines worldwide and found no indications of (significant) contamination with mycoplasma, cytopathic viruses or bacteria [69].

The recent development of protocols to replace fetal bovine serum (FBS) in clinical-scale expansion of MSCs is certainly a step in the right direction to prevent bovine prion, viral and zoonose contamination of the cell preparation and product. Human platelet lysates from buffy coats still hold certain risks, but are undoubtedly superior to FBS [5].

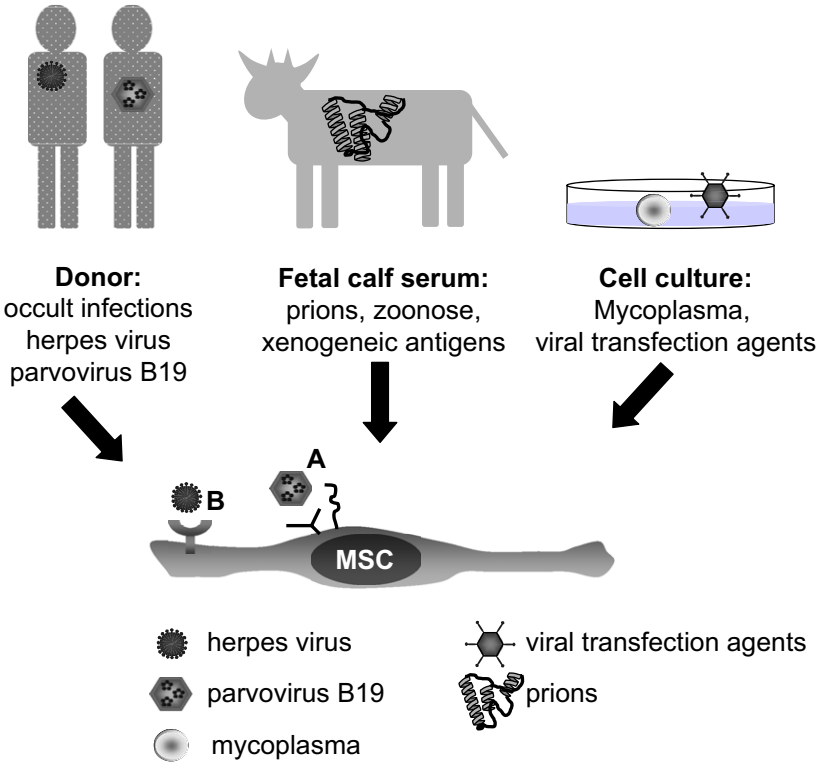

FIGURE 23.4 Potential sources of contamination in stem cells and stem cell products. Several sources have to be taken into consideration: occult infections of the donor, especially human herpes virus 6 and parvovirus B19, as mesenchymal stem cells (MSCs) express both B19 receptor and coreceptor (A) and human herpes virus 6 receptor CD46 (B).

Storage of Mesenchymal Stem Cells

Therapeutic use of MSCs is hard to imagine without cryopreservation. One study found a viability of approximately 90% in cryopreserved human MSCs from several donors and no negative influence of cryoconservation for up to 3 years on osteogenic potential [70]. Chin et al. tested the feasibility and safety of off-site MSC culture for therapeutic use in ischemic cardiomyopathy. MSCs isolated from bone marrow aspirates were submitted to freeze-controlled cryopreservation with dimethyl sulfoxide (DMSO) and rapidly thawed immediately before intramyocardial injection in three patients. Again, cell viability was around 90% and patients were followed up for 1 year, remaining well [71]. Others confirmed the resistance of MSC towards freezing and storage during ex vivo culturing [72], suggesting that cells derived from the same bone marrow aspiration might be used in the same patient at different time-points [73]. Thus, cryopreservation of MSCs can probably be considered a safe and convenient storage method for both therapeutic and research applications, at least with respect to viability and osteogenic potential. However, with respect to immunomodulation, a recent study suggests that human MSCs should be stored for no longer than 6 months (and pooled from different donors) for optimal results [74]. Nevertheless, even freezing for over 30 months allowed MSCs to suppress lymphocyte proliferation [74]. In contrast to these data, liquid storage of human MSCs in saline or medium at +4°C or room temperature rapidly reduced their viability [75].

Strategies and Problems of Stem Cell Delivery

Different routes of stem cell delivery have been tested in various animal and disease models; not all have been efficient. In broad categories, investigators have delivered stem cells either systemically (usually intravenously) or locally.

For intravenous stem cell therapy, the capillary network of the lung imposes a major obstacle to site-directed delivery of stem cells (pulmonary first pass effect). For example, in rats the majority of labeled, intravenously injected cells, be they MSCs, multipotent adult progenitor cells (MAPCs), bone marrow-derived mononuclear cells (BMMCs) or neural stem cells, are trapped in the lung and only a few cells reach the arterial system, rendering any calculation of therapeutic dose questionable [76]. MSCs performed worst in this respect and cell size is likely to be the key determinant [77]. This is a major confounder in xenotransplantation experiments, when human stem cells are injected into immunodeficient mice. The issue of cell size also stresses the importance of using cells from early passages, as aging (stem)

cells increase in size (see above), and the importance of optimized trypsinization and separation of adherent stem cells so that single-cell suspensions are obtained before injection. MSCs also express adhesion molecules such as intercellular adhesion molecule-1 3 (ICAM-1 3) and VCAM-1 [78] and blocking them may decrease the first pass effect. Vasodilators may also be used to decrease the pulmonary first pass effect [76].

Following the primary entrapment in the lung, there may be an unpredictable time-curve of MSC rebound [79], resulting in a gradual redistribution of MSCs within 48 h to other organs, e.g. liver, spleen, kidney and bone marrow [80,81]. There are also safety concerns that stem cells injected intravenously can damage the lung tissue itself, resulting in cystic structures with adjoining fibrosis [79]. Finally, an (unwanted) influence of lung tissue on the MSC phenotype cannot be ruled out.

In other experimental and clinical situations, stem cells were applied locally. In general, strategies for local stem cell delivery are naturally more prone to side-effects such as bleeding, embolization, infection and even overdosing.

In the present authors' hands, intravenous injection of MSCs in a rat model of acute glomerulonephritis did not exert any benefit, whereas intra-arterial delivery accelerated glomerular healing [82]. In particular, in kidney transplantation local delivery of MSCs is feasible, for example via preflushing of the organ with an MSC solution before engraftment. However, in AKI models benefits from stem cells did not necessitate local delivery.

Another factor in stem cell delivery that may decide over success or failure is timing, especially in studies where immunomodulation by MSCs is needed. MSCs need to be licensed, i.e. they must spend time in an appropriate microenvironment with certain cytokines to allow them to acquire their immunosuppressive phenotype. Supernatants of cultured MSCs alone did not exert immunosuppressive effects, whereas supernatants from MSCs cocultured with activated T cells did [2]. These findings are supported by an in vivo study in mouse autoimmune encephalitis, where administration of MSCs before, but not after disease induction could abrogate the disease [83].

Measures aimed at enhancing homing of exogenous or endogenous stem cells could also be useful. Stem cell migration and engraftment have been studied in various publications, especially in myocardial infarction [84]. The basic requirements, e.g. adhesion to endothelial cells, extravasation of MSCs and the molecules involved, have been described by Ruster et al. [85].

A systematic analysis of the route of administration in regenerative cell therapy in the kidney is still pending, but such an effort was accomplished in 2010 for intravenous cell delivery in neurological disorders. That meta-analysis of 60 preclinical studies encouragingly revealed large treatment effects. Use of established stem cell lines, genetically modified cells and overall cell dose proved to be especially important [86].

Potential Drug Effects on Stem Cells

When asking whether stem cell preparations are safe for the recipient, one may also ask whether recipients are safe for the donated stem cells. It comes as no surprise that high-dose chemotherapy for treatment of hematological malignancies induces HSC damage, and it has been shown recently that the damage extends to MSCs as well [87, 88]. Autologous MSC cotransplantation in hematopoietic stem cell rescue could consequently be of lower value than expected. Chemotherapy targets rapidly dividing cells, and thus slow-cycling stem cells may remain largely unaffected in the short run. Nevertheless, even seemingly innocent drugs such as vitamin D_3 or triiodothyronine can dose-dependently induce stem cells to adopt a certain phenotype that may not have been wanted in the first place [89,90].

The steroid dexamethasone was shown to act antiproliferatively on MSCs in vitro [91]. Other drugs with unwanted MSC side-effects are the thiazolidinediones, i.e. insulin sensitizers. Unexpectedly, they can cause anemia, possibly by inducing adipogenesis in MSCs and thus disturbing the normal bone marrow environment [92]. Another example is the mammalian target of rapamycin (mTOR) inhibitor rapamycin, which modulates the MSC differentiation potential and inhibits the development of an osteogenic phenotype [93], whereas it was reported to promote the osteoblastic differentiation of human ESCs [94]. Recombinant vascular endothelial growth factor (VEGF) protein inhibits the MSC expression of bone morphogenetic protein-2 (BMP-2), resulting in an inhibition of osteogenic differentiation [95].

Even common drugs such as acetyl salicylic acid can induce apoptosis via the Wnt/β-catenin pathway in MSCs [96,97]. Non-steroidal anti-inflammatory drugs (NSAIDs; e.g. diclofenac or parecoxib) did not inhibit MSC proliferation or their direct osteogenic differentiation potential, but blocked chondrogenesis. They may thereby ultimately contribute to bone loss [98]. These observations may be of particular relevance for orthopedic MSC-based studies, since orthopedic patients frequently are placed on NSAIDs in parallel.

Exogenous growth factors and hormones may modify stem cell functions as well. A prominent example is erythropoetin (EPO). MSCs express EPO receptors and respond to EPO with increased proliferation in vitro [99]. As will be shown later in this chapter, MSCs can

promote tumor growth via enhanced angiogenesis, which applies to EPO as well.

Apart from drugs, stem cells can be affected by physical challenges such as irradiation. Ionizing radiation in mice induced marked (and long-term) phenotypic and functional changes in hematopoietic progenitor cells [100]. MSCs exhibit reduced osteogenic potential after irradiation [101] and this may explain why irradiation of soft tissue (which is known to contain MSCs) can suppress ectopic calcification in rats [102].

Sick People May Have Sick Stem Cells: How to Choose the Right Donor

Stem cells can be affected by systemic diseases or show signs of a disease on their own. For example, MSCs from osteopenic rats showed less osteogenic differentiation in vitro [103] and MSCs from patients with thrombocytopenic purpura showed an impaired proliferative capacity and a lower inhibitory potential on activated T-cell proliferation compared to healthy controls [104]. However, others did not find significant differences in bone marrow progenitors from patients with or without hematological diseases, and the clonogenic potential of MSCs and HSCs was not altered [141].

Of particular relevance for nephrology are observations that renal failure has a pronounced effect on various stem cell functions. In patients with uremia, the frequency of functionally active peripheral blood endothelial progenitor cells is markedly reduced [105]. Renal transplantation could normalize the phenotype of these cells [106]. Similarly, c-kit$^+$CD34$^+$ bone marrow cells from patients with advanced age, anemia and renal failure exhibited impaired functions, in particular a reduced proangiogenic potency, compared with controls. After xenotransplantation into mice with ischemic limbs, the animals that received c-kit$^+$CD34$^+$ bone marrow cells from such patients showed worse blood reflow recovery than those from controls or patients with diabetes or hypertension [107]. Other variables in addition to advanced age, anemia and renal function that led to reduced proangiogenic activity in this model included high serum triglycerides, C-reactive protein and IL-6 levels [108].

Similar to uremia, in patients with systemic sclerosis autologous MSCs were found to have a significantly reduced angiogenic potential [109]. However, altered MSC behavior in immunological diseases is not universal and MSCs of patients with active Crohn's disease maintained their immunomodulatory capacity, thus rendering them potentially useful for syngeneic cell therapy [110].

Gender may also affect stem cell functions. In a study on bone healing, female rats yielded significantly fewer MSCs in bone marrow isolations and the reduced number was associated with delayed bone formation, although functional characteristics of male and female MSCs were similar [111]. Furthermore, in patients with leukemia who had received a stem cell transplant, 10 years later, secondary tumors developed significantly more often when the bone marrow donor had been female compared to male (4.6% vs 1.8%). These results indicate that stem cells from women may be functionally different, but the data are too preliminary to draw firm conclusions.

Another issue governing stem cell frequencies and functions is critical illness and/or exposure of blood to artificial surfaces. One study recovered mesenchymal, hematopoietic and epithelial progenitor cells from patients on extracorporeal membrane oxygenation [112]. MSCs with trilineage differentiation capacity were present in 18 out of 58 blood samples, but not in normal controls. Thus, potentially a stress reaction of the bone marrow to severe shock, inflammation and/or the foreign surface led to altered stem cell frequency in the peripheral blood. The latter is supported by data showing that mechanical stimulation or shear stress can alter the phenotype of MSCs towards an endothelial differentiation [113].

In summary, testing of stem cells for functional abnormalities in patients with non-hematological, non-malignant diseases has just begun. Currently, healthy young male donors without medication seem the most reliable choice.

Safety Issues Regarding Culture of Embryonic Stem Cells

Human ESCs are typically grown on mouse feeder layers in the presence of animal-derived serum replacements. This can lead to the incorporation and expression of immunogenic non-human sialic acid on the stem cells [114]. Sera from most humans contain antibodies against this sialic acid (Neu5Gc), which were demonstrated to damage ESCs in vitro and would probably lyse the cells in vivo as well. For human use, stem cell culture conditions eliminating all animal material therefore seem mandatory. The first stem cell lines maintained exclusively under cell- and serum-free conditions have been published [115].

Concerning the comparability among various ESC lines, several differences in the expression of lineage markers were detected and reported by the International Stem Cell Initiative 2007 [69]. Several imprinted genes showed generally similar allele-specific expression patterns, but some gene-dependent variation was observed. Notably, some female lines expressed readily detectable levels of X-inactive specific transcripts (XIST), important for the inactivation of the X-chromosome, whereas others did not.

POTENTIAL CLINICAL PROBLEMS OF STEM CELL THERAPIES: TUMOR FORMATION, ECTOPIC TISSUE AND OTHER UNWANTED EFFECTS

Risks of Long-term Mesenchymal Stem Cell Culture

The lack of specific single MSC markers and their low frequency in the bone marrow usually necessitate their expansion in vitro before use in vivo. The longer MSCs are cultured, the higher the risk of unwanted changes in phenotype or loss of differentiation capacity, e.g. replicative senescence.

Potential for Malignant Transformation

While the potential of ESCs for malignant transformation is well established, it has been noted only since 2005 that adult stem cells can do the same after about 8 months in culture [116]. This may in part relate to the observation that (at least mouse) MSCs with an increasing number of population doublings develop chromosomal instabilities [117] (Table 23.1). However, in cultured rat MSCs, a markedly aneuploid karyotype and progressive chromosomal instability has been detected in all passages analyzed [128].

In MSCs of children in vitro analyses for properties leading to oncogenic transformation (i.e. alterations in p53, p16, RB, H-RAS, hTERT, kariotype) remained negative and no tumor formation was shown after transplantation into SCID mice [130]. In contrast, human MSCs cultured for 5 106 weeks underwent spontaneous malignant transformation in 11 out of 24 cultures and formed lung tumors when transferred into immunodeficient mice [129]. Apparently, MSC transformation depends on several factors, among these culture conditions, the origin of the cells and the time of culture. A central role for p53 could be shown in controlling proliferation, differentiation and spontaneous transformation of MSCs. Knockouts for p53 exhibited signs of genomic instability and changes in expression of c-myc [127].

Further links between stem cells and cancer will be discussed in a later section (Stem Cells and Malignancies).

Functional Changes Resulting from Aging and Senescence of Cultured Mesenchymal Stem Cells

Donor age reduces the proliferative capacity of MSCs. Mostly independent of donor age, another process called replicative senescence can lead to changes in cultured cells that may resemble aging. Nevertheless, senescence and aging must be distinguished and MSCs from an old donor may not equal senescent MSCs from a young donor. Aging is "the sum of primary restrictions in regenerative mechanisms of multicellular organisms" [131], limiting cell replenishment and lifespan, whereas senescence relates more to functional changes inducing an irreversible arrest of cell division but with the (functionally deranged) cell staying alive, as opposed to apoptosis [132]. Thus, senescene represses genes that support cell cycle progression and upregulate its inhibitors, like p53/p21 and p16/RB [133]. This fits the hypothesis that senescence may represent a fixed program where genes involved in the proliferation machinery are downregulated, and this has indeed been shown in specimens from several donors, suggesting a common molecular program [134]. Ultimately this might have been caused by accumulation of cellular defects, e.g. oxidative stress, telomere loss and DNA damage. Whatever the cause, senescence-associated changes in phenotype, global gene expression and miRNA expression profiles are continuously acquired and increase from the onset of in vitro culture, even before reaching senescent passage numbers (shown to start within passage 7 12 in human MSCs) [134]. This emphasizes the importance of short-term culture.

Others reported human MSCs to undergo replicative senescence after in vitro culturing for several months as evidenced by prolonged cell population doubling times, cell enlargement, increase in podia, accumulation of actin, loss of differentiation capacity and DNA methylation pattern changes [135]. Senescent cells also release metalloproteinases, inflammatory cytokines and growth factors that can alter the tissue environment and may contribute to malignant transformation [131,136]. In a study investigating age-related changes in MSCs of Wistar rats aged 3, 7, 12 or 56 weeks, MSCs from 56-week-old donors not only showed reduced proliferation and fibroblast colony-forming units, but also accumulated oxidized proteins and lipids and had a decreased activity of antioxidative enzymes [137]. Oxidative damage thus may affect the functionality of MSC preparations from old donors just as much as decreased proliferation. Other groups reported a decrease in colony-forming unit potential of MSCs from aged donors [summarized in Ref. 131] and those cells too exhibited a decreased growth rate [138]. Most groups also report age-related decreases in osteogenic differentiation and it has been assumed that this is accompanied by an increase in adipogenic potential [139], culminating in senile osteoporosis. For example, secretion of osteoblast-stimulating transforming growth factor-β (TGF-β) was found to be reduced in MSCs from aged mice and, together with reduced BMP-2/4, may contribute to their adipogenic switch [140]. However, there is also a report on an early loss of adipogenic differentiation potential during MSC aging [141].

Senescence-associated changes do not exclusively affect MSCs but also affect human hematopoietic progenitor cells (HPCs). However, the overlap of

TABLE 23.1 Examples of Protumorigenic Stem Cell Actions

Cell type	Recipient	Findings	Ref.
Murine MSCs	Mouse	Immunosuppressive effects of MSCs favor tumor growth when cotransplanting MSCs and a melanoma cell line.	[118]
Human ESCs	Rat	Teratocarcinoma formation by xenogenic ESCs in experimental stroke	[119]
Mouse BMDCs	Mouse	*Helicobacter* infection of the stomach recruits BMDCs that later form epithelial cancers	[120]
Mouse vascular progenitors from BM	Nude mice	Recruitment by vascularized tumors (U 87 glioma cell line derived)	[121]
Adipose tissue derived MSCs from young children	–	Spontaneous malignant transformation in long term culture	[116]
hTERT immortalized human MSCs	–	Give rise to CSCs on long term culture	[122]
Murine MSCs	Mouse	Accumulated chromosomal instability leading to malignant transformation/sarcoma formation	[117]
Human MSCs	Mouse	MSCs within tumor stroma of a weakly metastatic human breast cancer cell line promote metastasis	[123]
Murine MSCs, non virally labeled	Mouse	MSCs cause sarcoma formation	[7]
Murine MSCs expressing PAX FKHR	Mouse	In combination with secondary mutations giving rise to alveolar rhabdomyosarcoma	[124]
MSCs	Mice with autoimmune diabetes	MSC transplantation causes malignant lesions	[125]
Human neural stem cells	Patient with ataxia teleangiectasia	Intracerebral tumor formation by donor cells	[126]
MSCs		Malignant transformation regulated by p53	[127]
Rat MSCs	–	Malignant transformation long term culturing	[128]
Human MSCs	Immunoincompetent mice	Long term culture causes frequent malignant transformation, MSCs form lung tumors upon transplantation into mice	[129]
Human $CD133^+$ renal progenitor cells	SCID mice	$CD133^+$ progenitors cotransplanted with renal carcinoma cells contributed to tumor vascularization in SCID mice	[61]

MSC: mesenchymal stem cell; BMDC: bone marrow-derived cell; BM: bone marrow; hTERT: human telomerase reverse transcriptase; SCID: severe combined immunodeficiency; ESC: embryonic stem cell; CSC: cancer stem cell.

differentially regulated genes between MSCs and HPCs was only limited [142], suggesting that senescence-induced changes in expression profile vary among the affected cell types.

Other Changes Associated with Long-term Culture

It has been shown that MSCs from low passages secrete more VEGF than those from later passages. The latter were also less protective in a mouse myocardial infarction model [143]. Another functional impairment of MSCs after repeated passaging in culture is a loss of homing capacity [144]. Finally, ex vivo expanded MSCs lost part of their therapeutic potential in ischemic stroke when cultured for extended periods [145]. This may relate to their paracrine activity, since tissue levels of trophic factors in a rat stroke model were higher after injection of human MSCs from passage 2 than after injection of those from passage [6].

Stem Cells and Malignancies

Nephrology has been spared reports about stem cell-induced malignancies or fatalities in humans so far. In rodents, it has been known for a while that injections of pluripotent embryonic cells or embryonic stem cell-derived precursor cells into the central nervous system can lead to malignant transformation of these cells [146], mostly forming teratomas or teratocarcinomas [119]. Nevertheless, several stem cell types have been used in preclinical studies for neurological diseases. A randomized phase II clinical trial investigated the stereotactic transplantation of cultured human neuronal

cells (5 10 million), derived from the human precursor cell line NT2/D1, into the brains of patients with motor deficits after strokes [147]. The maximum follow-up was 2 years with some benefit in daily living and only rare major adverse effects such as strokes. Nevertheless, hopes for stem-cell based therapies encountered a major setback in February 2009, when Amariglio et al. [126] described a donor-derived brain tumor after neural stem cell transplantation in an Israeli boy with ataxia teleangiectasia. The multifocal glioneuronal neoplasm was first diagnosed 4 years after human fetal stem cells had been injected intracerebellarly and intrathecally in a Russian clinic. Microsatellite and human leukocyte antigen (HLA) analysis could track the tumor to at least two different donors [126], and indeed the boy had received repeated injections.

Another example of a possible causative role of stem cell transplantation in the subsequent development of cancer is the presence of second solid cancers after allogeneic stem cell transplantation [148]. Patients who had received graft donations from women were at a higher risk for second solid cancers when compared not only to the general population but also to recipients of male grafts.

Apart from these clinical reports, most concerns about a putative role of stem cells in direct or indirect formation of malignancies stem from experimental data. In addition, the striking similarities between stem cells and CSCs have led to much discussion.

Phenotypic Similarity and Local Proximity between Mesenchymal Stem Cells and Cancer Stem Cells

The existence of CSCs was first hypothesized more than a century ago by Rudolf Virchow and S. Paget [149]. It took until 1964 for the first cells with CSC characteristics to be isolated from germ cell-derived teratomacarcinomas [150]. The prospective identification and purification of CSCs were successfully accomplished in patients with leukemia in 1997 [151] and extended to solid tumors such as breast cancer [152].

CSCs have for a long time been considered a very rare population within a tumor and at the same time the only tumor cells with the capacity for unlimited self-renewal [153]. Their existence could explain why most cancers return although they had been dramatically reduced in size by conventional anticancer drugs until no longer detectable. Explanations for the persistence of CSCs after therapy include their dormant state or low cycling frequency and expression of both antiapoptotic and multidrug resistance proteins [149].

Very recently it was found that not only very rare cells, but actually 27% of human melanoma cells could seed a tumor in severely immunocompromised mice [154], and the respective cells did not all express the standard stem cell markers such as CD133. These findings have challenged the hitherto existent understanding of CSCs as rare intratumor cells with unique tumor-forming potential and it remains unclear whether CSCs are a real entity, whether they represent "stem cells gone bad" or whether mature but abnormal cells somehow acquired stem cell-like characteristics. Indeed, stem cells and CSCs share many features, such as self-renewal (asymmetric cell division ensuring that the stem cell numbers remain constant). One of the possible mechanisms leading to formation of a malignant phenotype from a "regular" stem cell is loss of polarity control. Asymmetric division occurs perpendicular to the cell's assumed polarity, resulting in both a stem cell and a more committed daughter cell [155]. In theory, if non-asymmetric stem cell division generates only committed daughter cells, depletion of the original stem cell pool ultimately occurs. If non-asymmetric stem cell division generates only (dysfunctional) replicating stem cells, rapid tumor growth may occur [156].

Another theoretical model for interactions between stem cells and CSCs is the "seed and soil" model, as first proclaimed by S. Paget in 1889 [157]. According to that theory, stem/progenitor cells are recruited to the circulation upon tumor signaling and home to organs, where they prepare a premetastatic niche (the "soil"), giving circulating tumor cells the successful extravasation, survival and growth in these locations (the "seed"). In 2007, MSCs were shown to act in accordance with this theory in a mouse model of breast cancer [123,158] (Fig. 23.5). Not surprisingly, their potent proangiogenic potential, mediated by secretion of angiogenic factors such as VEGF [159,160], can promote growth of vascularized tumors [121]. Another mode by which MSCs accelerate tumor growth is in the allogeneic setting through their immunosuppressive effects [118,161,162]. MSCs are likely to have chemotactic properties and their tropism for the tumor microenvironment has been acknowledged for a while, possibly because damaged tissues, wounds and tumor stroma secrete many of the same inflammatory molecules [163,164]. Irradiation of (tumor) tissue can significantly enhance the homing of MSCs by amplifying the local inflammatory signaling [165,166]. This is eligible when modified MSCs carrying therapeutic genes are used but otherwise destructive because it would facilitate survival of remaining CSCs within the irradiated area when supported by homing MSCs. Examples of successful studies using tumor tropism of genetically modified MSCs exist, e.g. Loebinger et al. employed tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-expressing MSCs injections to eliminate metastases in a lung cancer model [167]. Oncologists are currently evaluating the safety of such approaches for modulation of carcinogenesis in MSC-mediated gene therapies [162], aiming at delivering anticancer agents within the tumor.

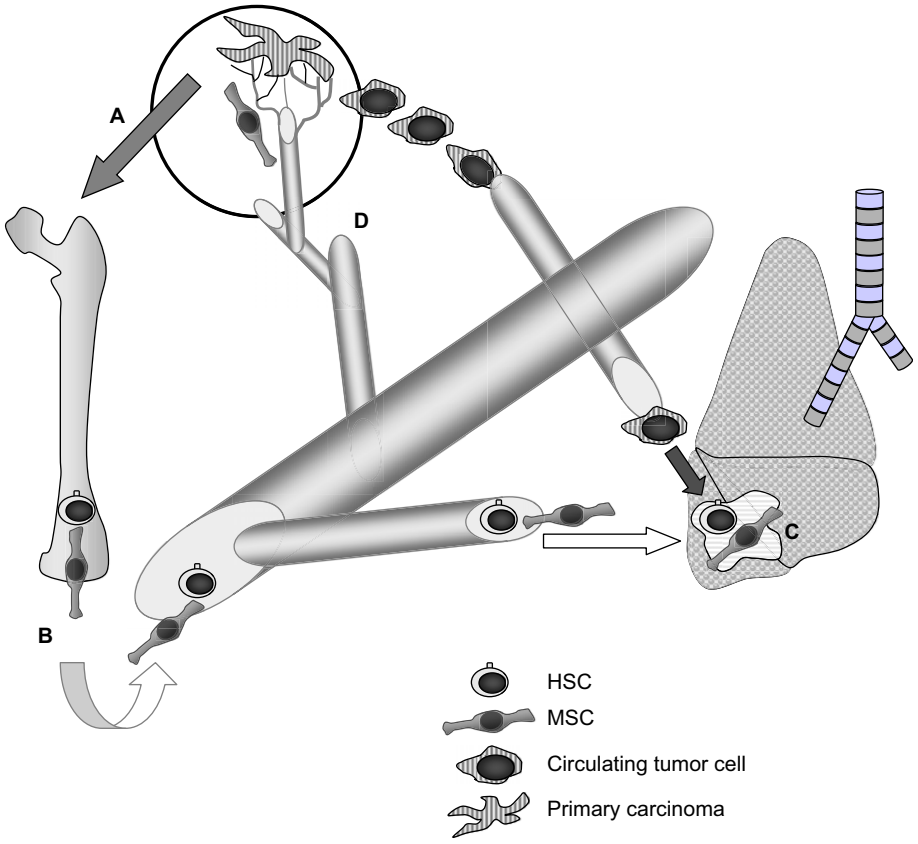

FIGURE 23.5 Potential contributions of mesenchymal stem cells (MSCs) and hematopoietic stem cells (HSCs) to tumor dissemination [158]. (A) The primary carcinoma sends signals that lead to (B) recruitment of progenitor cells to the circulation. (C) HSCs and MSCs form pre metastatic niches that facilitate engraftment of circulating tumor cells (seed and soil). (D) MSCs home to tumor stroma and contribute to angiogenesis.

The changes that MSCs undergo when infiltrating tumor stroma were further elucidated by Spaeth et al. in 2009. In three different adenocarcinoma xenograft models, they found evidence for an MSC transition into tumor-associated fibroblasts (TAFs), cells that are essential for many aspects of tumor growth and progression by providing structural and functional support [168]. This support includes contributions to microvascularization, stromal networks and tumor-stimulating paracrine factors (e.g. IL-6) by TAFs. The transition of MSCs to TAFs again confirms notions on profibrotic actions of MSCs and their general similarity to fibroblasts (see Mesenchymal Stem Cells and Fibrosis, below).

Do all MSCs migrate to tumor sites? Probably not. Again, heterogeneity of MSC preparations seems to be a safety concern. Ho et al. showed in 2009 that subgroups of MSCs show markedly enhanced migratory potential to malignant gliomas [169], and these cells show high expression of matrix metalloproteinase-1. Are there any clinical data confirming these experimental findings? In a very recent study by Worthley et al., 18 female patients from the Australian Bone Marrow Transplant Registry were identified who developed solid organ tumors after receiving male allogeneic stem cell transplants. With extensive histological testing, no donor-derived malignant cells were found. In two of these patients, suffering from rectal adenoma and gastric cancer, donor-derived TAFs were identified, supporting the above-mentioned hypotheses [170].

Not only MSCs, but other proangiogenic progenitor cells that are recruited into solid tumors and may promote vasculogenesis have been identified. These are mostly $VEGFR1^{+}$ hematopoietic precursor cells and $VEGFR2^{+}$ circulating endothelial precursor/progenitor cells (CEPs) [171]. In bone marrow transplantation experiments, the recruitment of these cells could be demonstrated to be essential for tumor growth.

Besides homing to tumor sites, transition into TAFs and enhancement of tumor angiogenesis, other unwanted effects of MSCs on diverse aspects of tumor progression have been observed. Some relate to the well-known supportive role of MSCs for hematopoiesis, since the secreted growth factors and cytokines can both stimulate and negatively regulate hematopoiesis at various steps. In addition, MSCs express adhesion

molecules that are involved in homing of circulating cells to the bone marrow [162]. Possible examples of a negative impact of these properties are as follows. MSCs from myeloma patients have been shown to support the growth of myeloma stem cells significantly better than MSCs from healthy control patients [172]. MSCs have been reported to induce critical prosurvival pathways in leukemic cells upon direct cell contact [173]. Cross-talk between MSCs and cells from yet another hematological malignancy, chronic lymphatic leukemia (CLL), resulted in CLL cell protection from both spontaneous and drug-induced apoptosis [174]. In another report, MSCs facilitated early entry of breast cancer cells into the bone marrow, possibly by regulation of the stromal cell-derived factor-1α (SDF-1α) and CXC chemokine receptor-4 (CXCR4) axis [175].

Another observation visualizing the proximity between stem cells and malignancies is the fact that in many tissues, CSCs express CD133 and CD44. The same markers can be found in "benign" CEPs and MSCs, respectively [149]. CD44 is found in many human cancers and correlates with outcome in several of these [149]. This may be related to the quantity of CSCs within these tumors or to the number of intratumor MSCs, shown above to promote tumor growth [123]. Besides MSCs, CEPs and HSCs belong to the helpful allies in tumor metastasis, forming the premetastatic niche [176]. Other bone marrow cells have been shown to contribute cancerous microvascular endothelial cells to tumor vessels [177]. These examples point out how well-intended administration of bone marrow preparations may unwillingly nourish occult tumors. Will stem cells from other locations behave differently? Bruno et al. published findings in 2006 in which $CD133^+$ resident renal progenitor cells were isolated from 30 human renal carcinomas. These cells could not induce tumor formation when transplanted alone into immunodeficient mice, but supported the growth of cotransplanted renal carcinoma cells [61] and these results were reproduced with $CD133^+$ renal progenitor cells that were derived from healthy kidneys.

Another concern is that since similarities between CSCs and non-malignant stem cells are widespread, the preparation and enrichment of potential stem cells from tissues and organs may be at risk of unwillingly expanding malignant cells in vitro before transplantation, unless the isolation methods include a step to identify (and discard) these hidden CSCs. An example to demonstrate the similarities between CSCs and stem cells is the large number of genes coexpressed in MSCs, multipotent mesenchymal precursors from paraxial mesoderm and Wilms' tumor cell lines with WT1 mutations [178]. Cell lines from five Wilms' tumors with WT1 mutations also showed stem cell-like features in their differentiation potential (adipogenic, chondrogenic, osteogenic, myogenic) [178] and could therefore possibly mimic MSCs in vitro.

Mesenchymal Stem Cells Can Give Rise to Malignant Lesions

Can MSCs themselves form the primary malignant lesion or is their role in cancer merely supportive? As described above, irrespective of culture conditions rat MSCs exhibited a markedly aneuploid karyotype and progressive chromosomal instability in all analyzed passages [128] (Table 23.1). The genome of cultured human MSCs has been reported to be much more stable [179]. One possible explanation is the early onset of senescence halting cell replication. However, in both humans and rodents spontaneous immortalization and malignant transformation of MSCs after culture expansion have been reported [128]. Aneuploidization has been named among other changes associated with osteosarcoma formation from cultured MSCs [180].

In vivo, alarming observations have recently been made in experiments with MSCs in non-obese diabetic (NOD) mice with autoimmune type 1 diabetes. When injected with autologous NOD MSCs, the mice developed soft tissue and visceral tumors. This did not happen when MSCs from healthy strains where injected. NOD mice are inbred and may be more prone to tumor formation, but the findings nevertheless raise concerns [125]. The first clinical trials using MSCs in type 1 diabetes are already underway.

In another study, where irradiated mice had received injections of cultured, labeled MSCs, sarcomas developed in the lungs of all animals and in the extremities of two out of 17 [7]. Several animals died prematurely. The authors found all of the used MSC cultures to be cytogenetically abnormal after several passages in vitro. Cells from the tumors exhibited the same abnormalities. In another model, mouse MSCs expressing the PAX-FKHR fusion gene (which is hypothesized to determine the alveolar rhabdomyosarcoma progenitor to the skeletal muscle lineage) have recently been identified as being at risk of giving rise to alveolar rhabdomyosarcomas when PAX-FKHR is coupled with secondary mutations, leading to activation of critical cell signaling pathways [124]. For a selection of studies linking (M)SCs to malignancies, see Table 23.1.

What Can be Done to Avoid Unintended Support of Tumors by Transplanted Mesenchymal Stem Cells?

Avoiding the whole-cell injection approach has been one suggestion. There are several publications of interest when considering administration of stem cell products rather than viable cell injection. One was published by Bruno et al. in 2009. Following the hypothesis that MSC-mediated effects in ARF models are largely paracrine, the authors isolated microvesicles from human

MSC cultures and tested these both in vitro and in vivo using an ARF model in SCID mice [181]. Microvesicles have been discussed as a means of cell-to-cell communication and stem cells in general have been discovered to release these exosomic particles [182, 183]. The injected microvesicles, mean size 135 nm and containing several important adhesion molecules, protected the mice from acute tubular injury, most probably because of their mRNA content [181]. In a technically more simple approach, Bi et al. in 2007 suggested that besides intravenous and intraperitoneal injection of MSCs, even culture supernatants or conditioned media of these cells could significantly ameliorate ARF in cisplatin-injected mice [184]. The effects of these conditioned media may be mediated by growth factors, microvesicles, exosomes and/or micro-RNAs (miRNAs). The latter have been identified as a regulatory element in MSCs and could potentially be used to modulate stem cell differentiation as well as specific cellular pathways [185], not only in the stem cells themselves, but also in other cells in their proximity [186].

Trophic Paracrine and Direct Cell-to-Cell Effects of Mesenchymal Stem Cells: Risks and Benefits

As shown above, MSCs exert strong proangiogenic actions. However, surprisingly MSCs can also exhibit antiangiogenic effects. Thus, in a Matrigel angiogenesis assay MSCs inhibited endothelial cell-derived capillary growth [187]. In an in vivo tumor model, high numbers of MSCs injected directly into the tumor induced apoptosis, ultimately abrogating tumor growth. This effect was mediated by the production of MSC-derived reactive oxygen species (ROS) and direct cell-to-cell interactions (see below). MSCs have been shown to transfer mitochondria to other cells as well [188] and can thereby communicate ROS generating signals [187]. These latter observations raise concerns that high numbers of MSCs in a single location could exert cytotoxic effects not only in tumors but also in inflamed tissue, especially when they aggregate.

Strong tropism of injected MSCs to several solid tumors or inflammatory sites in mice was demonstrated by in vivo imaging [164, 189, 190]. Thus, administered MSCs with a proangiogenic profile could unwillingly promote growth of an (occult) solid tumor. Many of the tumor-infiltrating MSCs later differentiated into osteoblasts (lung metastasis) or adipocytes (subcutaneous tumors) [189], ultimately limiting their unwanted paracrine (stem cell) effects. Vice versa, this may be used therapeutically and MSCs manipulated to secrete cytotoxic factors, for instance, have been used to target various tumors, with promising results, for example in colon carcinoma [191], and this can be enhanced further by prior irradiation of the tumor [192].

Apart from paracrine actions, stem cells also affect tissues by direct cell-to-cell interactions. One of the primary functions of MSCs is support for hematopoietic progenitor cells. Their ex vivo expansion, migratory potential and stemness can be preserved in culture by direct cell-to-cell contact with an MSC feeder layer [193]. MSCs can express connexin-43, a specific gap junction protein, and are therefore capable of forming gap junction channels with neighboring cell types, leading to functional coupling between MSCs and, for example, endothelial cells [194]. MSCs may thereby mediate dose-dependent cytotoxicity and interfere with angiogenesis. This effect was abolished by administration of peptides that antagonized connexin-43-dependent gap junction formation [187].

Another mechanism by which stem cells can influence signaling pathways of neighboring cells is the secretion of microparticles that can be enriched for pre-miRNAs, strong mediators of intercellular communication [186].

Non-malignant Maldifferentiation and Unwanted Actions of Stem Cells

Given that bone, adipose and stromal tissues are all present in normal bone marrow and the maintenance of this microenvironment regulates the lymphohematopoietic system, the commitment of MSCs towards one of these phenotypes may be stronger than has been assumed so far.

In the 1950s reports were already emerging of ectopic bone marrow transplantations leading to formation of new bone in inappropriate locations, e.g. the anterior chamber of the eye [195]. In particular, one bone marrow transplantation experiment is of interest as it showed surprising ossicle formation in sponges carrying differently isolated bone marrow preparations that had been transplanted under the renal capsules of mice [196]. Outside transplantation, the formation of ectopic bone or heterotopic ossification as well as fat tumors is well recognized, for example in benign symmetric lipomatosis (Launois Bensaude syndrome). Maldifferentiation of resident or invading MSCs may be a causal factor involved in this. Other clinical phenomena possibly related to MSC maldifferentiation have been described. Extensive bone metaplasia including osseous matrix, osteoblasts and osteocytes was observed in a renal transplant nephrectomy of a young girl after several rejection episodes [197]. Renal osseous metaplasia has also been described in the context of extramedullary hematopoiesis [198, 199]. Finally, MSC subtypes forming osteogenic structures in inadequate, scaffold-free, subcutaneous tissue without supplementation of osteogenic factors were demonstrated in mice [200].

The authors have recently provided the first evidence for adipogenic maldifferentiation of injected MSCs in the kidney [82]. This study followed an initial report that rat MSCs injected into a renal artery could accelerate recovery from AKI in rat anti-Thy1.1 glomerulonephritis, probably via paracrine effects, such as the release of VEGF and TGF-β_1, rather than by differentiation into resident glomerular cell types [10]. Next, the long-term effects of early MSC administration in chronic anti-Thy1.1 nephritis induced by prior uninephrectomy were investigated. Again, rat MSCs localized to most glomeruli, ameliorated AKI and reduced glomerular tuft adhesions [82]. About 2 months later, MSC-treated rats in comparison to rats receiving medium alone exhibited reduced proteinuria, better renal function, better preservation of glomeruli and reduced tubulointerstitial fibrosis. However, about 20% of the glomeruli of MSC rats contained single or clusters of large adipocytes with pronounced surrounding fibrosis, both of which severely distorted the normal glomerular morphology. Several lines of evidence demonstrated that these adipocytes apparently derived from the injected MSCs (Fig. 23.6A, B). Thus, in this glomerulonephritis model the early beneficial effect of MSCs in preserving damaged glomeruli and maintaining renal function was offset by a long-term partial maldifferentiation of intraglomerular MSCs into adipocytes accompanied by glomerular sclerosis, reflecting the dichotomy of MSC action.

This novel observation of orthodox MSC differentiation in an unorthodox location raised considerable concerns about the safety of MSC-based cell therapies. Notably, others working in the field have started to include long-term observations in their studies. As an example, no unwanted differentiation of MSCs has been observed in animals with AKI at 3 months after systemic MSC injection [19]. However, in that study, the MSCs did not localize to the kidney but rather migrated to the bone marrow.

When searching PubMed, only one clinical case with similar glomerular findings, i.e. glomerular adipocyte formation, could be found: a young woman with tuberous sclerosis underwent nephrectomy for multiple tumors of the kidney and histopathological findings included intraglomerular microlesions of angiomyolipomas [202]. Moreover, our findings of intraglomerular adipogenic maldifferentiation of MSCs resemble the recent case of a patient with lupus nephritis who had received HSC injections to the renal region and developed several abdominal masses of unusual composition and unknown dignity several months later [242].

The findings about maldifferentiation that resemble the authors' own experiences most closely have been made in a murine cardiac cryodamage model. Direct injection of MSCs into the damaged myocardium carried the highest risk for formation of osteogenic, probably MSC-derived lesions [203]. Intravenous injection of MSCs or injection of unfractionated bone marrow in the same model appeared to be free of adverse events. The above data also resemble findings obtained in the lung where murine MSCs were trapped, and formed "cysts" with adjacent collagen depositions resulting in severe lung damage [79]. Morphologically, in routine histology these cysts might have easily equaled

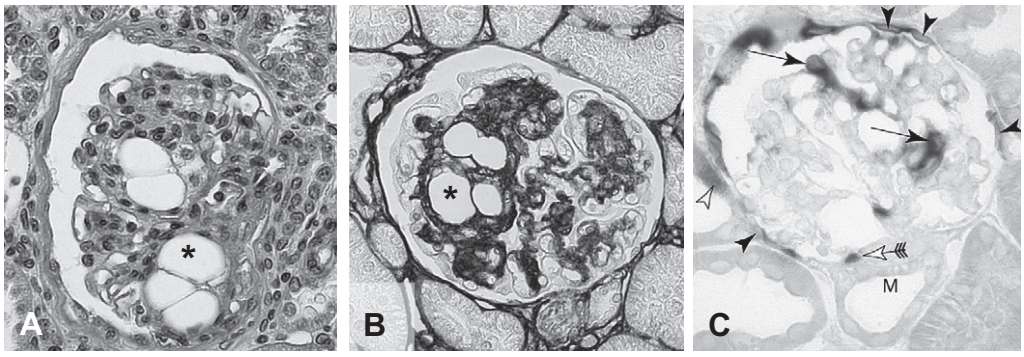

FIGURE 23.6 (A, B) Evidence for intraglomerular maldifferentiation of mesenchymal stem cells (MSCs) in Lewis rats with anti Thy1.1 nephritis on day 60 after disease induction, day 58 after injection of 2×10^6 cells into the renal artery. (A) Periodic acid–Schiff (PAS) staining exhibited "vacuolar" changes (*) that were positive for triglycerides in oil red O staining (not shown). (B) Staining for collagen type IV shows an intense fibrotic area around the "vacuoles". Magnifications: 400×. [Adapted from Kunter et al., 2007 [82].] (C) Endogenous progenitor cells: recruitment of podocytes from parietal cells in juvenile mice. Cre recombination was induced in triple transgenic PEC rtTA/LC1/R26R newborn mice by administration of doxycyclin for 3 days. At 6 weeks, the glomeruli were double stained with an enzymatic X gal (dark gray)/eosin staining to visualize genetically tagged cells. As expected, parietal cells lining the inner side of Bowman's capsule were genetically tagged (black arrowheads). Close to the vascular pole, genetically tagged transitional cells could be identified (arrow with tails; M: macula densa). On the capillary convolute, at least two genetically tagged podocytes can be seen (arrows), which have been recruited from parietal cells in juvenile mice. Within the tubules, solitary cells are also marked sporadically by the PEC rtTA transgenic mouse (empty arrowhead). Magnification: 600× [201].

adipocytes, keeping in mind that tissue embedding usually completely washes out the lipids.

Adverse effects of bone marrow cells on coronary artery stenosis have also been reported [204]. In the first study, granulocyte colony-stimulating factor (G-CSF) was administered for 5 days, followed by bare metal stent implantation and intracoronary peripheral blood stem cell infusion. In-stent restenosis was found at an unexpectedly high rate in G-CSF-treated patients and further enrollment stopped [205]. In the second study [206], 21 patients with recent myocardial infarction and bare metal stenting received intracoronary injections of $CD133^+$-enriched bone marrow cells. The authors found multiple indications for adverse effects of the cell-treated group, leading to reduced coronary blood flow in both stents and distal non-stented lumina. These effects might be partially caused by proinflammatory actions of G-CSF or the mobilized bone marrow cells.

Another unwanted effect of MSCs in patients with high cardiovascular risk may relate to their capacity to produce renin: murine and human MSCs express the nuclear hormone receptor liver X receptor-α (LXR-α). Upon activation of this receptor by its ligands (e.g. 22-hydroxycholesterol) or cyclic AMP, expression of renin was stimulated. Upon overexpression of LXR-α, they even differentiated into renin-producing juxtaglomerular-like cells [207].

Mesenchymal Stem Cells and Fibrosis

Several publications have dealt with the apparent similarities of MSCs with fibroblasts [208]. Beside their phenotypic similarities, making specific cell identification difficult, they show extensive overlap when comparing gene and miRNA expression profiles [209], and adult human fibroblasts are equivalent to MSCs concerning their immunoregulatory function [210].

MSCs produce various matrix proteins, including nine different collagen subtypes and fibronectin [78], and the profibrotic potential of MSCs has been used to enhance wound healing in diabetic mice [211,212]. Apart from producing extracellular matrix, MSCs contribute to wound healing by releasing various mediators including epidermal growth factor (EGF), VEGF, TGF-β_1 and SDF-1 [78].

Organ fibrosis is wound healing gone wrong. Renal fibrosis results from glomerular proteinuria, chronic hypoxia with loss of peritubular capillaries, misdirected glomerular ultrafiltrate, tubular protein leakage and/or direct toxic insults, e.g. drugs, oxidative stress. This offers a wide potential for MSC actions. However, these are difficult to predict and this group provided the first evidence of both a profibrotic and antifibrotic role of MSCs in renal disease [10,82]. Others have shown that bone marrow-derived mesangial cell progenitors from ROP Os/+ mice, a model of spontaneous glomerulosclerosis, can transmit glomerulosclerosis when transplanted into congenic +/+ mice [213]. In another profibrotic example, bone marrow-derived cells differentiated into renal tubulointerstitial myofibroblasts after ischemia reperfusion injury [214].

Profibrotic MSC actions also affect non-renal organs. In mouse models of liver damage, profibrotic actions including myofibroblast transformation of human MSCs have been described [215,216]. These findings were independent of donor age. In a rat model of chronic heart allograft rejection [217], MSCs migrated to sites of chronic rejection and differentiated towards a fibroblast phenotype. In murine lung fibroblasts proliferation and matrix production were induced by MSC-conditioned media, possibly mediated via TGF-β_1 and Wnt isoforms [218]. Thus, there appear to be both direct and indirect ways by which MSCs can boost fibrotic processes.

MSCs can also exert antifibrotic effects. In contradiction of the findings by Salazar et al. [218], conditioned media from MSCs did not induce, but rather attenuated fibroblast proliferation and collagen synthesis in vitro [219]. In vivo, MSCs have been shown recently to attenuate renal fibrosis in 5/6 nephrectomized rats, possibly by immunomodulation [220], and there are reports on antifibrotic actions in animal models of cardiac disease [221,222]. The latter may involve stimulatory effects of MSCs on matrix metalloproteinase secretion by cardiac fibroblasts, enhancing their ability to degrade matrix, but MSC-conditioned media also decreased the viability of cardiac fibroblasts in vitro [222].

Unwanted Immunological Effects of Transplanted Stem Cells

MSCs have been reported to be tolerance inducing. They are believed to be non-immunogenic, do not stimulate alloreactivity and escape cytotoxic T cell and natural killer cell lysis activity [67,223,224]. They are thus believed to be suitable for allogeneic or even xenogeneic transplantation [225]. In a novel report from 2009, MSCs after interferon-γ (IFN-γ) stimulation had the ability to cross-present exogenous antigens, inducing an effective $CD8^+$ T-cell immune response. This novel concept of MSCs as antigen-presenting cells led to hypotheses on their therapeutic value in cancer and infections [226].

The potential for adverse effects on the immune system is illustrated by a study that aimed to improve the course of murine collagen-induced arthritis by injecting $Flk\text{-}1^+$ MSCs. Unexpectedly, the MSCs did not ameliorate the arthritis but rather led to increased secretion of proinflammatory cytokines including IL-6 and IL-17. In vitro coculture indicated that these cells promoted lipopolysaccharide-primed splenocyte proliferation [227]; therefore, MSC injections could contribute

unintentionally to chronic autoimmune disease by proinflammatory actions.

Another concern comes from observations in a patient who received MSC infusions for treatment of severe gastrointestinal graft-versus-host disease (GVHD) and initially responded well to the therapy. However, he developed CMV-associated gastroenteritis, possibly due to MSC-induced immunosuppression [228]. In another MSC-treated patient, EBV post-transplant lymphoproliferative disorder developed; again, MSC-induced immunosuppression was discussed being as causative or at least confounding [228]. When considering possible applications of MSCs and their chondrogenic and anti-inflammatory potential in rheumatoid arthritis, the risk of unwanted side-effects of the immunosuppressive effects of these cells needs to be kept in mind [229].

Heterogeneity of stem cell preparations is a potential problem with respect to immunological actions. Among the most widely established surface markers for human and rodent MSCs is CD90 (Thy1) [3]. Decreased expression of CD90 on human MSCs is associated with reduced immunosuppressive potency [230]. This is a relevant finding, as most clinical applications of MSCs so far have been in protocols aiming at amelioration of GVHD after bone marrow transplantation, and reduced immunosuppressive potency of MSCs would thus risk enhanced GVHD [8]. There is indeed one GVHD study in which cotransplanted donor-derived allogeneic MSCs unexpectedly promoted donor graft rejection in a non-myeloablative setting [231], whatever the cause.

A DIFFERENT CONCEPT: STIMULATION OF INTRARENAL RESIDENT STEM CELL POPULATIONS

A conceptually different approach to administering bone marrow- or peripheral blood-derived stem cells is the administration or, even better, stimulation of intrarenal stem cells. The current understanding is that the kidney possesses a much greater capacity for self-repair than believed before [12,13]. Proximal tubule cells have been shown to be able to repair tubular damage rapidly without recruiting external stem cells [232].

In 2005, Bussolati et al. reported the isolation of renal progenitor cells from human glomeruli [60]. These $CD24^{+}CD133^{+}$ cells from Bowman's capsule ameliorated AKI in SCID mice with rhabdomyolysis. Although discussed to be embryonic renal multipotent progenitors, these cells did not show tumorigenic potential [64]. When combining $CD24^{+}CD133^{+}$ with podocalyxin (PDX) positivity or negativity and location at the urinary versus the vascular pole, the respective progenitor cells differed significantly in their regenerative potential following podocyte damage [233]. Bowman's capsule was recently identified as a progenitor cell niche, from which podocytes are derived [201] (Fig. 23.6C). Understanding the regulation of this process may open new therapeutic approaches to treat progressive podocyte loss, a key process in the pathogenesis of glomerulosclerosis. However, in both rodents and humans such intrarenal progenitors also contribute to hyperplastic lesions in podocytopathies and in particular to crescentic glomerulonephritis [234,235]. Thus, it is clear that any therapeutic manipulation of intrarenal progenitors may have adverse consequences as well.

THE HUMAN RISK FACTOR: STEM CELL CURES

Patient advocacy groups start to turn to stem cell experts with the intention to discuss the financial and emotional risks of "overseas experimental cures" for an array of diseases ranging from Alzheimer's and diabetes to autism and spinal injury. The marketing of overseas stem cell therapies via the internet has been around for some years. Often clinics use the testimonials of patients as a marketing technique, hiding the fact that much of what they offer is not supported by clinical trials and is therefore untested. If patients go abroad for poorly documented therapies, it is unclear who will monitor them subsequently. However, it is also clear that a lot of psychological and legal pressure is generated by patients, their advocates and the public.

In response to increasing concerns surrounding unregulated and unproven stem cell therapies offered to patients overseas, several non-profit organizations have developed handbooks designed to help individuals to understand what stem cells are, which stem cell treatments are considered safe and effective by specialists, which are considered experimental, and which are unproven and the safety of the treatment is unknown. [For more information, see Ref. 236.]

The non-profit organization International Society for Stem Cell Research (ISSCR) has published a similar handbook [237]. The ISSCR warns patients especially of therapies that claim success on the basis of individual patient testimonial, where one cell type is reported to treat several diseases and where the source of the cell is not clearly documented, claims that there are no risks, and substantial open or hidden costs.

CONCLUSION

As has been shown in this chapter, challenges and risks for stem cell-based therapies are multifaceted. Risks that derive from ex vivo expansion in the presence of xenogenic compounds can hardly be calculated. In addition,

most adult stem cells experience a long replicative history with both intracellular and extracellular influences in vitro before use. It may be assumed that common consensus in the stem cell field exists on the need for a robust regulatory and ethical framework for good manufacturing practice (GMP). All stem cell products need to be profoundly characterized, reproducible and, as far as by all means possible, biologically predictable.

Have any patients with side-effects possibly associated with MSC therapies been reported? In 2007, Le Blanc and Ringden [238] summarized the experiences with MSC injections in humans published to that date. They listed 14 clinical studies (e.g. hematological diseases, malignancies, osteogenesis imperfecta) with a total number of 142 patients treated. While many studies reported encouraging benefits of MSC (co)transplantation, none of the described outcomes included adverse events or MSC-associated toxicity. In many patients, the transplanted MSCs were difficult to detect later on. Reinders et al. estimated the total number of patients who have been treated with MSCs at more than 1000 [2]. Naturally, little is known about long-term effects and side-effects yet.

It should be borne in mind that stem cells and stem cell products are novel therapies whose manufacturing process as well as the adequate methods to confirm their safety, lack of contamination, absence of malignant transformation and predictable function still need further adjustments. Several reviews have dealt with optimization of stem cell culture conditions, a comprehensive field of ongoing interest that cannot be discussed here owing to space limitations [239,240].

Xenotransplantation studies (e.g. human donor, immunocompromised rodent recipient) cannot fully mimic the situation in patients, especially in respect of immune responses. Animal models of renal diseases are limited in their capacity to reflect the human situation. Furthermore, compared with rodent cell donors, human cell donors tend to be much more diverse in terms of age, gender, comedication and potential diseases. They should be screened for infectious and, where appropriate, genetic diseases. In the cell culture, animal components should be avoided as far as possible. Replacements include human platelet lysates or even chemically defined components. Adherence to GMP procedures is strongly recommended in any case.

Before initiating clinical studies, preclinical studies in rodent and/or large-animal models should be performed and long-term observations for maldifferentiation, ectopic tissue formation and malignant transformation completed. For these observations, the stem cells should be securely trackable. Researchers should be alert to any unanticipated phenomena (especially in modified or manipulated cells) and look for the unexpected, otherwise, for example, maldifferentiation can easily be underdiagnosed. If the unforeseen should happen in a preclinical model, these findings need to be communicated to clinical researchers without delay.

Animal studies should further help to optimize cell dosage, administration route and factors protecting the cells from post-transplantation apoptosis and enhancing their homing.

For regulatory aspects concerning clinical stem cell research, the authors strongly recommend reading the *Guidelines for the clinical translation of stem cells*, published by the Task Force for the Clinical Translation of Stem Cells of the ISSCR, a multidisciplinary group of researchers, clinicians, ethicists, regulatory officials from 13 countries [241]. The guidelines provide excellent information and recommendations on topics as cell processing, manufacture, preclinical and clinical studies, as well as details on informed consent of patients and issues of social justice.

Note in Proof

D. Thirabanjasak and co-authors in june 2010 reported the case of a female patient with severe lupus nephritis who underwent percutaneous injections of autologous peripheral stem cells (probably HSCs) into the regions of both kidneys in a private clinic [242]. The stem cell injections could not improve renal function and the patient had to start hemodialysis. Six months after the treatment, she presented with hematuria and flank pain and was diagnosed with renal masses, liver masses and even a mass in the right adrenal gland (MRI). 11 months after treatment, she underwent nephrectomy. The left kidney exhibited several solid masses showing angiomyeloproliferative and myeloproliferative components. The biological/malignant potential of the lesions remained unclear. No histology of the liver and right adrenal gland was obtained and the patient later died of dialysis-related complications. The authors convincingly discussed the lesions in all organs to be most likely derived from the stem cell injections. Disappointingly, no further information could be obtained from the private clinic to correlate the findings with the number and location of the injections.

Acknowledgments

This work has received funding from the European Community's Seventh Framework Programme [FP7/2007–2013] under grant agreement no HEALTH F5 2008 223007 STAR TREK and from the German Research Foundation (Deutsche Forschungsgemeinschaft), grant SFB TRR57.

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CHAPTER

24

Tissue Engineering in Urology

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OUTLINE

INTRODUCTION

Patients suffering from diseased or injured genitourinary organs are often treated with reconstructive surgery or transplants, but there is a severe shortage of donor tissue and organs. The aging population grows, and the need for organs grows with it. Physicians and scientists have begun to look to the fields of regenerative medicine and tissue engineering to provide new options for these patients. These fields apply the principles of cell transplantation, material science and bioengineering to construct biological substitutes that can significantly improve the quality of life of the urological patient by eliminating the need for intensive grafting procedures or transplant surgery.

Tissue engineering, one of the major components of regenerative medicine, follows the principles of cell transplantation, materials science and engineering to develop biological substitutes that can restore and maintain normal organ function. Tissue engineering strategies generally fall into two categories: the use of acellular matrices designed to direct the body's natural ability to use its own cells to regenerate damaged tissue, and the use of matrices seeded with cells in the laboratory to produce novel tissues and organs. Acellular tissue matrices are usually prepared by manufacturing artificial scaffolds, or by removing cellular components from donor tissues via mechanical and chemical manipulation to produce collagen-rich matrices ([1 4]; and see Chapter 16). These matrices slowly degrade after implantation and are replaced by the extracellular matrix (ECM) proteins secreted by the ingrowing cells. Cells themselves can also be used for therapy via injection, either with carriers such as hydrogels or alone.

The most common way to use cells in tissue engineering is to obtain a small piece of donor tissue and dissociate it into individual cells in the laboratory. These cells are either implanted directly into the host or

expanded in culture and attached to a support matrix. The cell matrix construct is then reimplanted into the host. The source of the donor tissue can be heterologous (such as bovine), allogeneic (same species, different individual) or autologous. Ideally, autologous cells are used, because in this case both structural and functional tissue replacement will usually occur with minimal complications. To accomplish this, a biopsy of tissue is obtained from a host, the cells are dissociated and expanded in culture, and the expanded cells are implanted back into the same host [2,5 12]. The use of autologous cells, although it may cause an inflammatory response, avoids rejection and thus, the deleterious side-effects of lifelong immunosuppression can be avoided.

However, for many patients with extensive end-stage organ failure, a tissue biopsy may not yield enough normal cells for expansion and transplantation. In other instances, primary autologous cells cannot be expanded from a particular organ, such as the pancreas. In these situations, stem cells are envisioned as an alternative source of cells from which the desired tissue can be derived. Stem cells can be derived from discarded human embryos (human embryonic stem cells), from fetal tissue or from adult sources (bone marrow, fat, skin). However, there are ethical issues involved in the use of embryonic stem cells (ESCs) and most human applications are currently banned in the USA. Despite this, the field of stem cell biology is advancing rapidly, and cutting-edge techniques such as therapeutic cloning and somatic cell reprogramming circumvent some of the ethical questions and offer a potentially limitless source of these cells for tissue engineering applications.

This chapter will review the major components of most tissue engineering techniques, and will describe how these techniques are being applied to the reconstruction and regeneration of the genitourinary system.

BASIC COMPONENTS OF TISSUE ENGINEERING

Cells

Native Cells

In the past, one of the limitations of applying cell-based regenerative medicine techniques to organ replacement was the inherent difficulty of growing certain cell types in large quantities. Even when some organs, such as the liver, have a high regenerative capacity in vivo, cell growth and expansion in vitro can be difficult. By studying the privileged sites for committed precursor cells in these organs, as well as by exploring the conditions that promote differentiation and/or self-renewal of these cells, it has been possible to overcome some of the obstacles that limit cell expansion in vitro. One example is the urothelial cell. Urothelial cells could be grown in the laboratory setting in the past, but only with limited success. Several protocols were developed over the past two decades that identify the undifferentiated cells in a mixed culture of cells isolated from the urinary tract, and keep them undifferentiated during their growth phase [11,13 16]. Using these methods of cell culture, it is now possible to expand a urothelial culture that initially covered a surface area of 1 cm^2 to one covering a surface area of 4202 m^2 (the equivalent of one football field) within 8 weeks [11]. These studies indicated that it should be possible to collect autologous bladder cells from human patients, expand them in culture and return them to the donor in sufficient quantities for reconstructive purposes [11,14 19]. Major advances in cell culture techniques have been made within the past decade, and these techniques make the use of autologous cells possible for clinical application.

Embryonic Stem Cells

In 1981, pluripotent cells were found in the inner cell mass of the human embryo, and the term "human embryonic stem cell" (hESC) was coined [20]. These cells are able to differentiate into all cells of the human body, excluding placental cells (only cells from the morula are totipotent; that is, able to develop into all cells of the human body). These cells have great therapeutic potential, but their use is limited by both biological and ethical factors.

The political controversy surrounding stem cells began in 1998 with the creation of hESCs derived from discarded embryos. hESCs were isolated from the inner cell mass of a blastocyst (an embryo 5 days postfertilization) using an immunosurgical technique. Given that some cells cannot be expanded ex vivo, ESCs could be the ideal resource for tissue engineering because of their fundamental properties: the ability to self-renew indefinitely and the ability to differentiate into cells from all three embryonic germ layers. Skin and neurons have been formed, indicating ectodermal differentiation [21 23,24]. Blood, cardiac cells, cartilage, endothelial cells and muscle have been formed, indicating mesodermal differentiation [25 27]. Pancreatic cells have been formed, indicating endodermal differentiation [28]. In addition, as further evidence of their pluripotency, ESCs can form embryoid bodies, which are cell aggregations that contain all three embryonic germ layers while in culture, and can form teratomas in vivo [29]. These cells have demonstrated longevity in culture and can maintain their undifferentiated state for at least 80 passages when grown using current published protocols [30 31].

However, in addition to the ethical dilemma surrounding the use of ESCs, their clinical application is limited because they represent an allogenic resource and thus have the potential to evoke an immune

response. New stem cell technologies (such as somatic cell nuclear transfer and reprogramming) promise to overcome this limitation.

Therapeutic Cloning (Somatic Cell Nuclear Transfer)

Somatic cell nuclear transfer (SCNT), or therapeutic cloning, entails the removal of an oocyte nucleus in culture, followed by its replacement with a nucleus derived from a somatic cell obtained from a patient. Activation with chemicals or electricity stimulates cell division up to the blastocyst stage.

At this point, it is extremely important to differentiate between the two types of cloning that exist: reproductive cloning and therapeutic cloning. Both involve the insertion of donor DNA into an enucleated oocyte to generate an embryo that has identical genetic material to its DNA source. However, the similarities end there. In reproductive cloning, the embryo is then implanted into the uterus of a pseudopregnant female to produce an infant that is a clone of the donor. A world-famous example of this type of cloning resulted in the birth of a sheep named Dolly in 1997 [32]. However, there are many ethical concerns surrounding such practices, and as a result, reproductive cloning has been banned in most countries (see also Chapter 25).

While therapeutic cloning also produces an embryo that is genetically identical to the donor, this process is used to generate blastocysts that are explanted and grown in culture, rather than in utero. ESC lines can then be derived from these blastocysts, which are only allowed to grow up to a 100-cell stage. At this time the inner cell mass is isolated and cultured, resulting in ESCs that are genetically identical to the patient. This process is detailed in Fig. 24.1. It has been shown that nuclear transferred ESCs derived from fibroblasts, lymphocytes and olfactory neurons are pluripotent and can generate live pups after injection into blastocysts. This shows that cells generated by SCNT have the same developmental potential as blastocysts that are fertilized and produced naturally [33 36]. In addition, the ESCs generated by SCNT are perfectly matched to the patient's immune system and no immunosuppressants would be required to prevent rejection should these cells be used in tissue engineering applications.

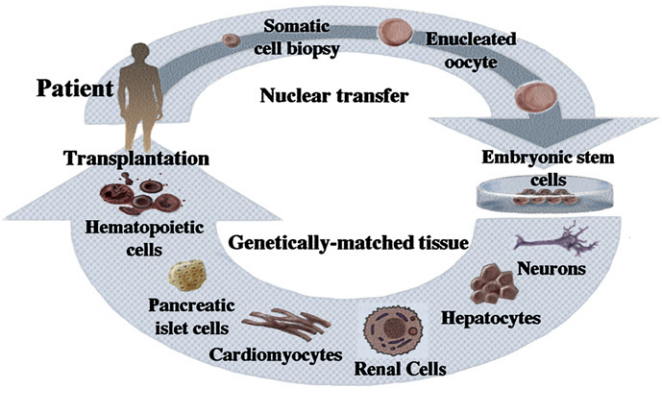

FIGURE 24.1 Strategies for therapeutic cloning in regenerative medicine.

Although ESCs derived from SCNT contain the nuclear genome of the donor cells, mitochondrial DNA (mtDNA) contained in the oocyte could lead to immunogenicity after transplantation. To assess the histocompatibility of tissue generated using SCNT, Lanza et al. microinjected the nucleus of a bovine skin fibroblast into an enucleated oocyte [37]. Although the blastocyst was implanted (reproductive cloning), the purpose was to generate renal, cardiac and skeletal muscle cells, which were then harvested, expanded in vitro and seeded onto biodegradable scaffolds. These scaffolds were then implanted into the donor steer from which the cells were cloned to determine whether cells were histocompatible. Analysis revealed that cloned renal cells showed no evidence of T-cell response, suggesting that rejection will not necessarily occur in the presence of oocyte-derived mtDNA. This finding represents a step forward in overcoming the histocompatibility problem of stem cell therapy.

Although promising, SCNT has certain limitations that require further improvement before its clinical application, in addition to the ethical considerations regarding the potential of the resulting embryos to develop into cloned embryos if implanted into a uterus. In addition, this technique has not been shown to work in humans. The initial failures and fraudulent reports of nuclear transfer in humans reduced enthusiasm for human applications [38 40], although it was recently reported that non-human primate embryonic stem cell lines were generated by SCNT of nuclei from adult skin fibroblasts [41,42].

Before SCNT-derived ESCs can be used as clinical therapy, careful assessment of quality of the lines must be determined. For example, some cell lines generated by SCNT have contained chromosomal translocations and it is not known whether these abnormalities originated from aneuploid embryos or if they occurred during ESC isolation and culture. In addition, the low efficiency of SCNT (0.7%) and the inadequate supply of human oocytes further hinder the therapeutic potential of this technique. Still, these studies renew the hope that embryonic stem cell lines could one day be generated from human cells to produce patient-specific stem cells with the potential to cure many human diseases that are currently untreatable.

Reprogrammed Somatic Cells

Recently, exciting reports of the successful transformation of adult cells into pluripotent stem cells through

a type of genetic reprogramming have been published. Reprogramming is a technique that involves dedifferentiation of adult somatic cells to produce patient-specific pluripotent stem cells, eliminating the need to create embryos (see also Chapter 13). Cells generated by reprogramming would be genetically identical to the somatic cells (and thus, the patient who donated these cells) and would not be rejected. Yamanaka was the first to discover that mouse embryonic fibroblasts (MEFs) and adult mouse fibroblasts could be reprogrammed into an induced pluripotent state [43]. These induced pluripotent stem (iPS) cells possessed the immortal growth characteristics of self-renewing ESCs, expressed genes specific for ESCs, and generated embryoid bodies in vitro and teratomas in vivo. When iPS cells were injected into mouse blastocysts, they contributed to a variety of cell types. However, although iPS cells selected in this way were pluripotent, they were not identical to ESCs. Unlike ESCs, chimeras made from iPS cells did not result in full-term pregnancies. Gene expression profiles of the iPS cells showed that they possessed a distinct gene expression signature that was different from that of ESCs. In addition, the epigenetic state of the iPS cells was somewhere between that found in somatic cells and that found in ESCs, suggesting that the reprogramming was incomplete.

These results were improved significantly by Wernig et al. in 2007 [44]. In this study, DNA methylation, gene expression profiles and the chromatin state of the reprogrammed cells were similar to those of ESCs. Teratomas induced by these cells contained differentiated cell types representing all three embryonic germ layers. Most importantly, the reprogrammed cells from this experiment were able to form viable chimeras and contribute to the germ line like ESCs, suggesting that these iPS cells were completely reprogrammed.

It has recently been shown that reprogramming of human cells is possible [45,46]. Yamanaka and coworkers generated human iPS cells that are similar to hESCs in terms of morphology, proliferation, gene expression, surface markers and teratoma formation [45]. Thompson's group showed that retroviral transduction of the stem cell markers *OCT4*, *SOX2*, *NANOG* and *LIN28* could generate pluripotent stem cells [46]. However, in both studies, the human iPS cells were similar but not identical to hESCs. Although reprogramming is an exciting phenomenon, our limited understanding of the mechanism underlying it currently limits the clinical applicability of the technique, but the future potential of reprogramming is quite exciting.

Placental and Amniotic Fluid Stem Cells

Recently, it has been shown that pluripotent cells may be derived from the amniotic fluid and placenta. Both amniotic fluid and placenta are known to contain multiple partially differentiated cell types derived from the developing fetus. Stem cell populations have been isolated from these sources. Called amniotic fluid and placental stem cells (AFPSCs), they express embryonic and adult stem cell markers [47]. The undifferentiated stem cells expand extensively without a feeder cell layer and double every 36 h. Unlike hESCs, AFPSCs do not form tumors in vivo. Lines maintained for over 250 population doublings retained long telomeres and a normal complement of chromosomes. AFPSCs are broadly multipotent, and human lines can be induced to differentiate into cell types representing each embryonic germ layer, including cells of adipogenic, osteogenic, myogenic, endothelial, neuronal and hepatic lineages. Examples of differentiated cells derived from AFPSCs and displaying specialized functions include neuronal lineage secreting the neurotransmitter L-glutamate or expressing G-protein-gated inwardly rectifying potassium (GIRK) channels, hepatic lineage cells producing urea and osteogenic lineage cells forming tissue engineered bone. In this respect, they meet a commonly accepted criterion for pluripotent stem cells, without implying that they can generate every adult tissue. The cells could be obtained either from amniocentesis or chorionic villous sampling in the developing fetus, or from the placenta at the time of birth. They could be preserved for self-use and used without rejection, or they could be banked. A bank of 100,000 specimens could potentially supply 99% of the US population with a perfect genetic match for transplantation. Such a bank may be easier to create than with other cell sources, since there are approximately 4.5 million births per year in the USA [47].

Biomaterials

In the most common tissue engineering procedures, isolated cells are seeded onto a scaffold composed of an appropriate biomaterial. These biomaterials replicate the biological and mechanical function of the native ECM found in tissues in the body by serving as an artificial ECM. Biomaterials provide a three-dimensional space for the cells to develop into new tissues with appropriate structure and function (see also Chapter 16). They can also allow delivery of appropriate bioactive factors (e.g. cell adhesion peptides, growth factors) to the developing tissue [48] to help regulate cellular function. As the majority of mammalian cell types is anchorage dependent and will die if no cell adhesion substrate is available, biomaterials provide this substrate that can deliver cells to specific sites in the body with high loading efficiency. Biomaterials can also provide mechanical support against in vivo forces so that the predefined three-dimensional structure of the engineered implant is maintained during tissue development.

The ideal biomaterial should be biodegradable and bioresorbable, and support the replacement of normal tissue without inducing inflammation. Incompatible materials are destined for an inflammatory or foreign-body response that eventually leads to rejection and/or necrosis. Degradation products, if produced, should be removed from the body via metabolic pathways at an adequate rate so that the concentration of these degradation products in the tissues remains at a tolerable level [49]. The biomaterial should also provide an environment in which appropriate regulation of cell behavior (adhesion, proliferation, migration and differentiation) can occur. Cell behavior in the newly formed tissue has been shown to be regulated by multiple interactions of the cells with their microenvironment, including interactions with cell-adhesion ligands [50] and with soluble growth factors. Since biomaterials provide temporary mechanical support while the cells undergo spatial reorganization into tissue, the properly chosen biomaterial should allow the engineered tissue to maintain sufficient mechanical integrity to support itself in early development, while in late development, it should begin to degrade so that it does not hinder further tissue growth [48].

In general, three classes of biomaterials have been used for engineering tissues: naturally derived materials (e.g. collagen and alginate), acellular tissue matrices (e.g. bladder submucosa and small intestinal submucosa) and synthetic polymers [e.g. polyglycolic acid (PGA), polylactic acid (PLA), and poly(lactic-co-glycolic acid) (PLGA)]. These classes of biomaterials have been tested with respect to their biocompatibility [51 52]. Naturally derived materials and acellular tissue matrices have the potential advantage of biological recognition. However, synthetic polymers can be produced reproducibly on a large scale with controlled properties such as strength, degradation rate and microstructure, which would aid in the preparation of easily used, off-the-shelf scaffold material.

Naturally Derived Materials

Collagen is the most abundant and ubiquitous structural protein in the body, and may be readily purified from both animal and human tissues with an enzyme treatment and salt/acid extraction [53]. Collagen implants, under normal conditions, degrade through a process involving phagocytosis of collagen fibrils in fibroblasts [54]. This is followed by sequential attack by lysosomal enzymes including cathepsins B1 and D. Under inflammatory conditions, the implants can be rapidly degraded, largely by matrix metalloproteins (MMPs) and collagenases [54]. However, the in vivo resorption rate of a collagen implant can be regulated by controlling the density of the implant and the extent of intermolecular cross-linking: the lower the density, the greater the space between collagen fibers and the larger the pores for cell infiltration, leading to a higher rate of implant degradation. Collagen contains cell adhesion domain sequences (e.g. RGD) that may assist in retaining the phenotype and activity of many types of cells, including fibroblasts [55] and chondrocytes [56].

Alginate, a polysaccharide isolated from seaweed, has been used as an injectable cell delivery vehicle [57] and a cell immobilization matrix [58] owing to its gentle gelling properties in the presence of divalent ions such as calcium. Alginate is relatively biocompatible and is approved by the Food and Drug Administration (FDA) for human use as wound dressing material. Alginate is a family of copolymers of D-mannuronate and L-guluronate. The physical and mechanical properties of alginate gel are strongly correlated with the proportion and length of polygluronic block in the alginate chains [57].

Acellular Tissue Matrices

Acellular tissue matrices are collagen-rich matrices prepared by removing cellular components from tissues. The matrices are often prepared by mechanical and chemical manipulation of a segment of tissue [1 4]. These matrices slowly degrade upon implantation, and are replaced and remodeled by ECM proteins synthesized and secreted by transplanted cells or on in-growing cells.

Synthetic Polymers

Polyesters of naturally occurring α-hydroxy acids, including PGA, PLA and PLGA, are widely used in tissue engineering. These polymers are FDA approved for a variety of applications, including sutures [59]. The ester bonds in these polymers are hydrolytically labile, and they degrade by non-enzymatic hydrolysis. The degradation products of PGA, PLA and PLGA are non-toxic natural metabolites and are eventually eliminated from the body in the form of carbon dioxide and water [59]. The degradation rate of these polymers can be tailored to the application by altering crystallinity, initial molecular weight and the copolymer ratio of lactic to glycolic acid. In general, the optimal degradation time ranges from several weeks to several years. Since these polymers are thermoplastics, they can be easily formed into a three-dimensional scaffold with a desired microstructure, gross shape and dimension by various techniques, including molding, extrusion, solvent casting [60], phase separation techniques and gas foaming techniques [61]. Many applications in tissue engineering often require a scaffold with high porosity and ratio of surface area to volume. Other biodegradable synthetic polymers, including poly(anhydrides) and poly(orthoesters), can also be used to fabricate scaffolds for tissue engineering with controlled properties [62].

TISSUE ENGINEERING OF SPECIFIC GENITOURINARY ORGANS

Investigators around the world, including the author's laboratory, have been working towards the development of several cell types, tissues and organs for clinical application. The following subsections will describe this research in detail.

Urethra

Various biomaterials without cells, such as PGA and acellular collagen-based matrices derived from decellularized small intestine and bladder, have been used in animal models for the regeneration of urethral tissue [4,63 67]. Some of these biomaterials, like acellular collagen matrices derived from bladder submucosa, have also been seeded with autologous cells for urethral reconstruction. The author's laboratory has been able to replace tubularized urethral segments with cell-seeded collagen matrices [68,69].

Acellular collagen matrices derived from bladder submucosa by this laboratory have been used experimentally and clinically. In animal studies, segments of the urethra were resected and replaced with acellular matrix grafts in an onlay fashion. Histological examination showed complete epithelialization and progressive vessel and muscle infiltration, and the animals were able to void through the neourethras [4]. These results were confirmed in a clinical study of patients with hypospadias and urethral stricture disease [70]. Decellularized cadaveric bladder submucosa was used as an onlay matrix for urethral repair in these patients. Patent, functional neourethras were noted in these patients with up to a 7-year follow-up (Fig. 24.2). The use of an off-the-shelf matrix appears to be beneficial for patients with abnormal urethral conditions and obviates the need for obtaining autologous grafts, thus decreasing operative time and eliminating donor site morbidity.

Unfortunately, the above techniques are not applicable for tubularized urethral repairs. The collagen matrices are able to replace urethral segments only when used in an onlay fashion. However, if a tubularized repair is needed, the collagen matrices should be seeded with autologous cells to avoid the risk of stricture formation and poor tissue development [68]. Therefore, tubularized collagen matrices seeded with autologous cells can be used successfully for total penile urethra replacement.

Bladder

Currently, gastrointestinal segments are commonly used as tissues for bladder replacement or repair. However, gastrointestinal tissues are designed to absorb specific solutes, whereas bladder tissue is designed for the excretion of solutes. Owing to the problems encountered with the use of gastrointestinal segments, numerous investigators have attempted alternative materials and tissues for bladder replacement or repair.

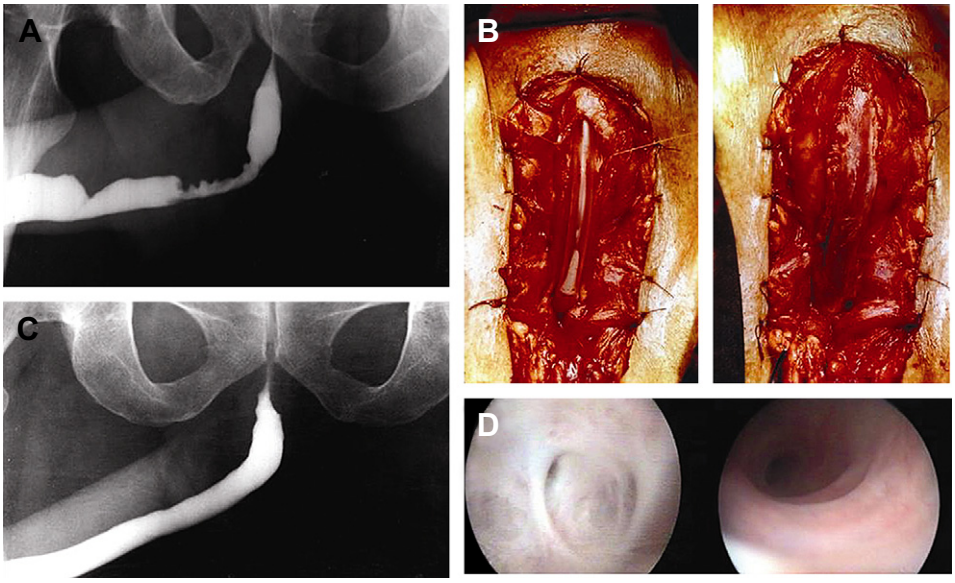

FIGURE 24.2 Urethral repair using a collagen matrix. (A) Representative case of a patient with a bulbar stricture. (B) During surgery, strictured tissue is excised, preserving the urethral plate on the left side, and the matrix is anastamosed to the urethral plate in an onlay fashion on the right. The boxes in both photographs indicate the area of interest, including the urethra, which appears white in the left photograph. In the left photo graph, the arrow indicates the area of stricture in the urethra. On the right, the arrow indicates the repaired stricture. Note that the engineered tissue now obscures the native white urethral tissue in an onlay fashion in the right photograph. (C) Urethrogram 6 months after repair. (D) Cystoscopic view of urethra before surgery on the left side, and 4 months after repair on the right side. Please see color plate at the end of the book.

The success of cell transplantation strategies for bladder reconstruction depends on the ability to use donor tissue efficiently and to provide the right conditions for long-term survival, differentiation and growth. Urothelial and muscle cells can be expanded in vitro, seeded onto polymer scaffolds, and allowed to attach and form sheets of cells [71]. These principles were applied in the creation of tissue engineered bladders in an animal model that required a subtotal cystectomy with subsequent replacement with a tissue engineered organ in beagle dogs [12]. Urothelial and muscle cells were separately expanded from an autologous bladder biopsy and seeded onto a bladder-shaped biodegradable polymer scaffold. The results from this study showed that it is possible to tissue engineer bladders that are anatomically and functionally normal. Clinical trials for the application of this technology are currently being conducted.

A clinical experience involving engineered bladder tissue for cystoplasty reconstruction was conducted starting in 1999. A small pilot study of seven patients was reported, using a collagen scaffold seeded with cells either with or without omentum coverage, or a combined PGA collagen scaffold seeded with cells and omental coverage (Fig. 24.3). The patients reconstructed with the engineered bladder tissue created with the PGA collagen cell-seeded scaffolds showed increased compliance, decreased end-filling pressures, increased capacities and longer dry periods [72] (Fig. 24.4). Although the experience is promising in terms of showing that engineered tissues can be implanted safely, it is just a start in terms of accomplishing the goal of engineering fully functional bladders. Further experimental and clinical work is being conducted.

Kidney

The kidney is a complex organ with multiple cell types, derived from combining anlagen, and a complex functional anatomy that renders it one of the most difficult to reconstruct [5,73]. While the metanephros is responsible for the development of the proximal section of the nephrons, the ureteric bud forms the collecting ducts and distal structures. The large vessels of the kidney are induced from extrarenal tissues. Divergent embryological origin converges to produce at least 26 distinct functional cells in the kidney [74]. Previous efforts in tissue engineering of the kidney have been directed toward the development of extracorporeal renal support systems made of biological and synthetic components [75 83] and these ex vivo renal replacement devices are known to be life-sustaining. However, there would be obvious benefits for patients with end-stage kidney disease if these devices could be implanted long term without the need for an extracorporeal perfusion circuit or immunosuppressive drugs.

The possibility was explored of seeding an implantable biomaterial with a heterogeneous population of renal cells to evaluate function and viability. Atala et al. [84] plated donor rabbit kidney cells, including distal tubules, glomeruli and proximal tubules, in vitro and after expansion, these were seeded onto biodegradable polyglycolic acid scaffolds and implanted subcutaneously into athymic mice. The implants consisted of individual cell types and a mixture of all three. When examined histologically, progressive formation and organization of the nephron segments within the polymer fibers were noted. In addition, bromodeoxyuridine incorporation into the renal cell DNA was confirmed [84]. It was unclear whether the tubular structures found on the scaffolds occurred de novo from the implanted cells or if they merely represented fragments of donor tubules which had survived the original dissociation and culture process intact. To investigate this question further, mouse renal cells were harvested and expanded in culture. Subsequently, a single cell suspension was created from the isolated cells, and these were seeded on biodegradable polymers and implanted into immune-competent synergic hosts. In this experiment, renal epithelial cells reconstituted tubular structures in vivo. The analyses of the retrieved implants indicated that the renal epithelial

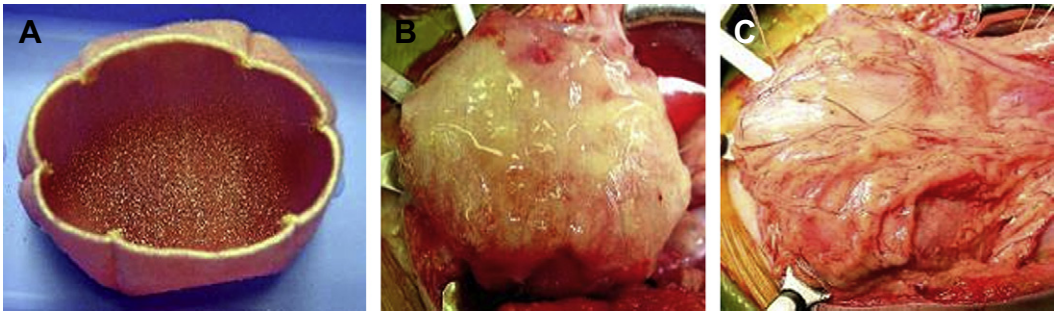

FIGURE 24.3 Construction of an engineered bladder. (A) Scaffold material seeded with cells for use in bladder repair. (B) The seeded scaffold is anastamosed to native bladder with running 4 0 polyglycolic sutures. (C) Implant covered with fibrin glue and omentum. Please see color plate at the end of the book.

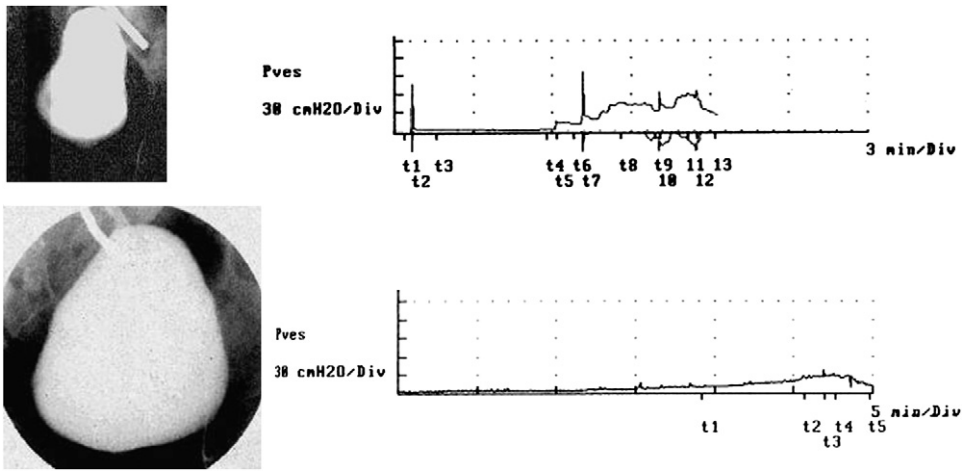

FIGURE 24.4 Cystograms and urodynamic studies of a patient before and after implantation of the tissue engineered bladder. (A) Preop erative results indicate an irregular bladder in the cystogram and abnormal bladder pressures as the bladder is filled via urodynamic study. (B) Postoperatively, findings are significantly improved.

cells first organized into a structure with a solid center. Next, canalization into a hollow tube could be seen at 2 weeks. Histological examination with nephron-specific lectins revealed successful reconstitution of proximal tubules, distal tubules, loops of Henle, collecting tubules and collecting ducts. These results clearly showed that single cell suspensions grown in vitro are capable of reconstituting tubule structures. The tubules contained homogeneous cell types within each tubule [85].

Further investigations by this group have demonstrated that renal tubular development from digested renal units is possible in the murine model as well. Joraku et al. [86] developed an in vitro method for cultivation of renal cells which allowed for development of tubular structures. This technique involves digestion of the entire murine kidney followed by cultivation of the resulting cells in specific renal media. Immunohistochemistry revealed cells expressing proximal and distal tubule markers as well as markers for glomerular and endothelial cells. When these cells were allowed to grow on rat-tail type 1 collagen, cells from the thick ascending loop of Henle stained positive for Tamm Horsfall protein in an architectural pattern reminiscent of natural tubules [86].

Yoo et al. [87] evaluated whether murine renal cells grown in vitro could produce functional results in vivo. They harvested the cells, expanded them in culture and seeded them onto a tubular device constructed from polycarbonate. At one end of the tubular device was a silastic catheter which terminated into a reservoir. The device was subcutaneously implanted into athymic mice. The implanted device demonstrated extensive vascularization in addition to glomerular formation and highly organized tubular architecture. Immunohistochemistry for alkaline phosphatase showed positivity in the proximal tubule-like structures. Osteopontin, which is secreted by the proximal and distal tubular cells and the cells of the thin loop of Henle, was found on immunocytochemical staining of the tubular sections. In addition, the ECM of the newly formed tubules stained uniformly positive for fibronectin. Importantly, fluid collected in the reservoir of the device. This fluid was yellow and the uric acid concentration was 66 mg/dl (compared with 2 mg/dl in plasma). The creatinine concentration of the fluid (27.91 ± 7.56 mg/dl) was 8.2 times higher than that found in the serum (4.49 ± 0.08 mg/dl) [87]. These studies demonstrate that single cells can form multicellular structures and become organized into functional renal units, and are capable of unidirectional excretion of solutes through production of a urine-like fluid.

Next, the principles of both tissue engineering and therapeutic cloning were applied in an effort to produce genetically identical renal tissue in a large animal model, the cow (*Bos taurus*) [88] (Fig. 24.5). Bovine skin fibroblasts from adult Holstein steers were obtained by ear notch, and single donor cells were isolated and microinjected into the perivitelline space of donor enucleated oocytes (nuclear transfer). The resulting blastocysts were implanted into progestin-synchronized recipients to allow for further in vivo growth. After 12 weeks, cloned renal cells were harvested, expanded in vitro, then seeded onto biodegradable scaffolds consisting of polycarbonate membranes and three collagen-coated cylindrical silastic catheters (Fig. 24.5A). The ends of the three membranes of each scaffold were connected to catheters that terminated into a collecting reservoir. This created a renal neo-organ with a mechanism for collecting the excreted urinary fluid (Fig. 24.5B). These devices were transplanted subcutaneously into the same steer from which the genetic material originated, and then retrieved 12 weeks after implantation.

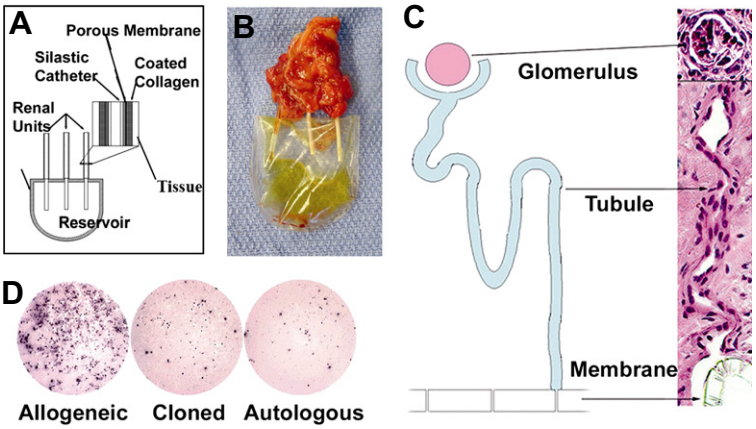

FIGURE 24.5 Combining therapeutic cloning and tissue engineering to produce kidney tissue. (A) Illustration of the tissue engineered renal unit. (B) Renal unit seeded with cloned cells, 3 months after implantation, showing the accu mulation of urine like fluid. (C) Clear unidirectional conti nuity between the mature glomeruli, their tubules and silastic catheter. (D) Enzyme linked immunosorbent spot (ELISPOT) analyses of the frequencies of T cells that secrete interferon γ (IFN γ) after stimulation with allogeneic renal cells, cloned renal cells or nuclear donor fibroblasts. Cloned renal cells produce fewer IFN γ spots than the allogeneic cells, indicating that the rejection response to cloned cells is diminished. The presented wells are single representatives of duplicate wells. Please see color plate at the end of the book.

Chemical analysis of the collected urine-like fluid, including urea nitrogen and creatinine levels, electrolyte levels, specific gravity and glucose concentration, revealed that the implanted renal cells possessed filtration, reabsorption and secretory capabilities. Histological examination of the retrieved implants revealed extensive vascularization and self-organization of the cells into glomeruli and tubule-like structures. A clear continuity between the glomeruli, the tubules and the silastic catheter was noted (Fig. 24.5C). Immunohistochemical analysis with renal-specific antibodies revealed the presence of renal proteins, reverse transcription polymerase chain reaction (RT-PCR) analysis confirmed the transcription of renal specific RNA in the cloned specimens, and Western blot analysis confirmed the presence of elevated renal-specific protein levels.

Since previous studies have shown that bovine clones harbor oocyte mtDNA [89 91], the donor egg's mtDNA was thought to be a potential source of immunological incompatibility. Differences in mtDNA-encoded proteins expressed by cloned cells could stimulate a T-cell response specific for mtDNA-encoded minor histocompatibility antigens when the cloned cells are implanted back into the original nuclear donor [92]. Maternally transmitted minor histocompatibility antigens in mice have been shown to stimulate both skin allograft rejection in vivo and cytotoxic T-lymphocyte expansion in vitro [92] that could prevent the use of these cloned constructs in patients with chronic rejection of major histocompatibility matched human renal transplants [93,94]. A possible T-cell response to the cloned renal devices was tested for using delayed-type hypersensitivity testing in vivo and enzyme-linked immunosorbent spot (ELISPOT) analysis of interferon-γ-secreting T cells in vitro. Both analyses revealed no evidence of a T-cell response, indicating that rejection of cloned renal cells will not necessarily occur in the presence of oocyte-derived mtDNA (Fig. 24.5D). This finding may represent a step forward in overcoming the histocompatibility problem of stem cell therapy [94].

These studies demonstrated that cells derived from nuclear transfer can be successfully harvested, expanded in culture and transplanted in vivo with the use of biodegradable scaffolds on which the single suspended cells can organize into tissue structures that are genetically identical to that of the host. These studies were the first demonstration of the use of therapeutic cloning for regeneration of tissues in vivo.

Investigations into the possibility of using other cell types to produce renal tissue have also been promising. Perin et al. were able to induce AFPSCs to differentiate into renal tissues. They labeled human AFPSCs with a green fluorescent protein and microinjected the cells into developing murine embryonic kidneys. The labeled cells assisted in forming both C- and S-shaped bodies, and the cells expressed RNA for early markers of renal development [95]. This study demonstrates that AFPSCs can differentiate into renal lineages when cultured in vitro with renal precursors. While promising, further in vivo studies and functional assays are required before AFPSCs can be used in clinical applications.

Genital Tissues

Reconstructive surgery is required for a wide variety of pathological penile conditions, such as penile carcinoma, trauma, severe erectile dysfunction, and congenital conditions such as ambiguous genitalia, hypospadias and epispadias. One of the major limitations of phallic reconstructive surgery is the scarcity of sufficient autologous tissue.

The major components of the phallus are corporal smooth muscle and endothelial cells. The creation of autologous functional and structural corporal tissue de novo would be beneficial. Autologous cavernosal smooth muscle and endothelial cells were harvested, expanded and seeded on acellular collagen matrices and implanted in a rabbit model [96,97]. Histological examination confirmed the appropriate organization of penile tissue phenotypes, and structural and functional

studies, including cavernosography, cavernosometry and mating studies, demonstrated that it is possible to engineer autologous functional penile tissue. The author's laboratory is currently working on increasing the size of the engineered constructs.

Congenital malformations of the uterus may have profound implications clinically. Patients with cloacal exstrophy and intersex disorders may not have sufficient uterine tissue present for future reproduction. The possibility of engineering functional uterine tissue was investigated using autologous cells [98]. Autologous rabbit uterine smooth muscle and epithelial cells were harvested, then grown and expanded in culture. These cells were seeded onto preconfigured uterine-shaped biodegradable polymer scaffolds, which were then used for subtotal uterine tissue replacement in the corresponding autologous animals. Upon retrieval 6 months after implantation, histological, immunocytochemical and Western blot analyses confirmed the presence of normal uterine tissue components. Biomechanical analyses and organ bath studies showed that the functional characteristics of these tissues were similar to those of normal uterine tissue. Breeding studies using these engineered uteri are currently being performed.

Similarly, several pathological conditions, including congenital malformations and malignancy, can adversely affect normal vaginal development or anatomy. Vaginal reconstruction has traditionally been challenging owing to the paucity of available native tissue. The feasibility of engineering vaginal tissue in vivo was investigated [99]. Vaginal epithelial and smooth muscle cells of female rabbits were harvested, grown and expanded in culture. These cells were seeded onto biodegradable polymer scaffolds, and the cell-seeded constructs were then implanted into nude mice for up to 6 weeks. Immunocytochemical, histological and Western blot analyses confirmed the presence of vaginal tissue phenotypes. Electrical field stimulation studies in the tissue-engineered constructs showed similar functional properties to those of normal vaginal tissue. When these constructs were used for autologous total vaginal replacement, patent vaginal structures were noted in the tissue-engineered specimens, while the non-cell-seeded structures were noted to be stenotic [99].

CONCLUSION

Efforts in regenerative medicine are currently underway experimentally for virtually every type of tissue and organ in the human body. As regenerative medicine incorporates the fields of tissue engineering, cell biology, nuclear transfer and materials science, personnel who have mastered the techniques of cell harvest, culture, expansion and transplantation, as well as polymer design, are essential for the successful application of these technologies to extend human life. Various tissues are at different stages of development, with some already being used clinically, a few in preclinical trials and some in the discovery stage. Recent progress suggests that engineered tissues may have an expanded clinical applicability in the future and may represent a viable therapeutic option for those who would benefit from the life-extending benefits of tissue replacement or repair.

Acknowledgment

The author wishes to thank Jennifer L. Olson PhD for editorial assis tance with this manuscript.

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CHAPTER

25

Ethics in Regenerative Medicine

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OUTLINE

INTRODUCTION

Regenerative medicine entails a broad range of methodologies that utilize cells or tissues as therapeutic tools to rebuild or repair damaged or diseased structures and restore their function. The source of therapeutic cells can be autologous, allogenic or xenogenic. If allogenic, then these cells can be harvested without harm or risk of harm or cooperation with harm to another human being or harvested with corresponding harm. Ethical issues abound in experimental medicine but those that arise uniquely in regenerative medicine reflect this latter circumstance, wherein one human life is used as an object to satisfy the needs or desires of another.

The developmental potential of embryonic cells and fetal cells and tissues has attracted the compelling interest of investigators (Fig. 25.1). The interest of the political establishment and the media are similarly engaged in part because the ethics of regenerative medicine are so frequently intertwined with the ethics of abortion. The ethics of human embryonic stem cells (hESCs) and of abortion both hinge on the respect, or the absence of respect, accorded to the human organism at a prenatal stage of development. In hESC research, which historically required destruction of a live embryo to make a new cell line [1,2], the biological fact that a new human life appears at fertilization is ever at risk of subordination to a utilitarian calculus. In this calculus, utility to another determines which life will or will not be accorded respect and rights consistent with human dignity and, if so, then to what extent. The ferocity of the abortion debate, together with the contradictions anticipated if the respect mandated to a human embryo by an investigator were to exceed that accorded to a human fetus by its erstwhile mother, helps to drive the stem cell debate.

Another driver of the debate is the issue of scientific freedom of inquiry. Researchers militate for unrestricted access to tools for basic investigation and therapeutic intervention and the perspectives of this lobby are largely sacrosanct. However, the public frequently funds or is asked to fund such research and the broader society is divided on the question of medical progress if achieved at the cost of destroying healthy human embryos. Even more germane

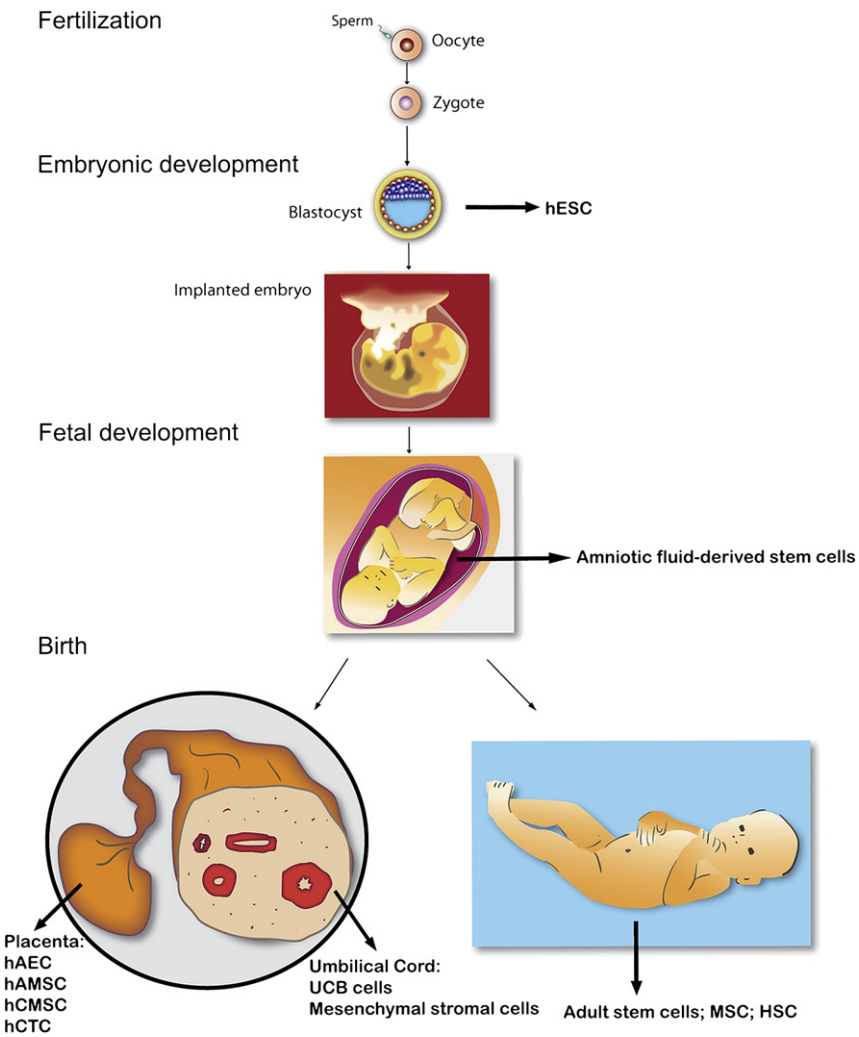

FIGURE 25.1 Human development from fertilization to birth with indicated time points for isolation of various types of stem cells. The placenta and an enlarged transverse view of the umbilical cord are shown in the circle. hAEC: human amniotic epithelial cells; hAMSC: human amniotic mesenchymal stromal cells; hCMSC: human chorionic mesenchymal stromal cells; hCTC: human chorionic trophoblastic cells; hESC: human embryonic stem cells; UBC: umbilical cord blood; MSC: mesenchymal stem cells; HSC: hematopoietic stem cells.

than its role as the source of research funding, the public is the presumptive recipient of the therapies that are the rationale for much of the publicly funded research. Some have suggested that the monolithic prerogatives of investigators must yield to those of the divided, and consequently more circumspect, body politic.

The opposition of many patients to aspects of regenerative medicine as currently practiced is not only to a therapy that requires the destruction of an embryo or fetus for its immediate implementation, but also to a therapy that entailed such destruction, perhaps years before, in the creation of the therapeutic cell lines [3,4]. The issue of "cooperation" with morally suspect practices complicates the use of organs from condemned prisoners, tissues from electively aborted fetuses [5] and human embryonic stem cell lines derived through the destruction of healthy human embryos (Table 25.1) [1,6,7].

TABLE 25.1 Moral Consideration of Cells and Tissues as a Function of Origin and Circumstances of Harvesting

	Common ground	Requiring cooperation	Generally objectionable
Embryonic	Dead embryo created for reproductive purposes that died despite best efforts	Surplus embryo from in vitro fertilization for reproductive purposes	Embryo created for destructive harvesting
Fetal	Spontaneous abortion	Therapeutic abortion	Abortion after conception for the purpose of abortion and harvesting
Adult	Deceased adult	Prisoner after legal execution	Execution of political prisoner for the purpose of harvesting

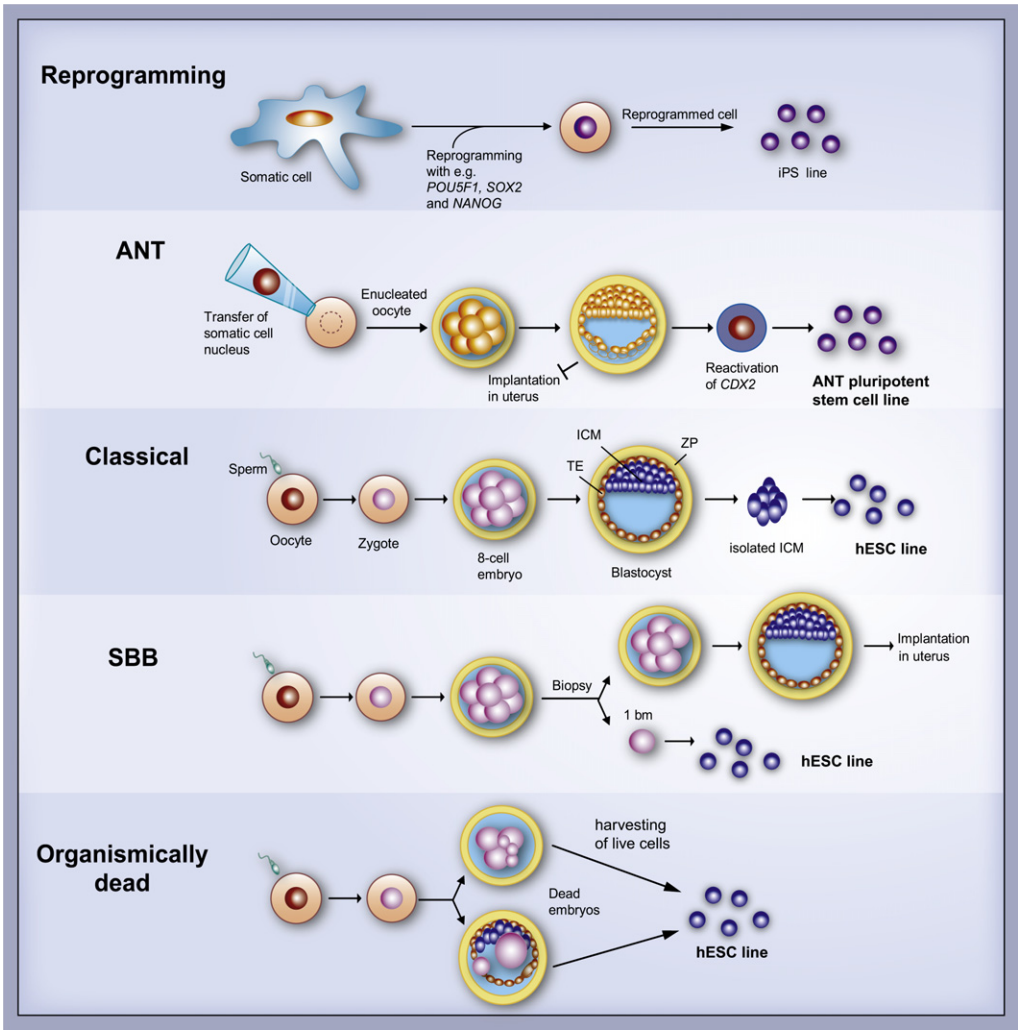

FIGURE 25.2 Classical and alternative strategies for derivation of pluripotent stem cell lines without the destruction of viable human embryos. ANT: altered nuclear transfer; SBB: single blastomere biopsy; iPS: induced pluripotent stem cells; ICM: inner cell mass; TE: trophectoderm; ZP: zona pelucida; hESC: human embryonic stem cell; bm: blastomere.

The division of opinion on the destructive use of prenatal human beings cannot be solved by splitting the difference. The contest, framed in its most positive terms, pits the imperative to respect human dignity and treat human beings as subjects and never as objects against the duty to devise and implement methods to relieve human suffering. This division is not economic and thus cannot be solved by splitting the difference. The difficulties with persuasion and the unseemliness of coercion have argued in favor of a search for a common ground. Alternative approaches that spare human life at the earliest stage of development and yet achieve the objectives of scientific inquiry have sparked considerable interest (Fig. 25.2). These alternative approaches were reviewed in a 2005 White Paper from the US President's Council on Bioethics. Considerable progress has been made in this area and in the following sections the current status of the alternative embryo-sparing approaches is reviewed.

ALTERNATIVE METHODS

Adult Stem Cells

Adult stem cells (ASCs) represent ethically acceptable source of stem cells and are eligible for federal funding in the USA and elsewhere (Fig. 25.2). ASCs reside within different tissues as tissue-specific progenitors that are employed during tissue self-renewal and/or differentiation. When transplanted into the host, ASCs exhibit lower tumorigenic potential than hESCs. ASCs play an important role in the lifelong maintenance of tissue homeostasis [8,9]. Any homeostatic disturbance can

disrupt a dynamic balance between self-renewal and differentiation which can lead to excessive differentiation, resulting in the exhaustion of the stem cell pool or overproduction resulting in tumorigenesis [8 12].

Bone marrow represents a typical adult stem cell source, containing different cell populations that have the potential to migrate and transdifferentiate into cells of diverse phenotypes [13,14]. The plasticity of bone marrow-derived stem cells has been broadly studied. Two major subsets of bone marrow cells include hematopoietic stem cells (HSCs) and mesenchymal stem cells (MSCs). HSCs are commonly identified by the expression of $CD34^{+}$ cell surface antigen; HSCs have been studied extensively and used clinically for bone marrow transplantation in a variety of hematological disorders [1,13,14].

MSCs are a population of cells present in adult tissues including the bone marrow and adipose tissues [15]. They have been shown to differentiate into multiple tissues including neuronal, adipose, muscle, liver, lungs, spleen and gut. MSCs derived from adipose tissue are particularly attractive because they are easily accessed and not in short supply.

In summary, ASCs represent an ethically acceptable source of stem cells, albeit currently of limited therapeutic use except for HSCs. The major reasons are the challenges involved in isolating scarce ASCs, their restricted differentiation potential, and difficult and often unsuccessful *ex vivo* expansion [1]. If these drawbacks were successfully resolved, ASCs could become an important source of tissue-specific stem cells for regenerative therapies.

Multipotent Stem Cells from Extraembryonic Tissues

Umbilical Cord as Stem Cell Source

The umbilical cord is another non-controversial source of stem cells (Fig. 25.1). Stem cells are isolated from two compartments: umbilical cord blood (UCB) and umbilical cord matrix, i.e. Wharton's jelly [16 19]. UCB contains stem cells which have been successfully used for transplants in the treatment of leukemia [14]. They are useful clinically, owing to their naïve immune status [17,18]. UCB stem cells give rise to cardiac, neural, hepatic and dermal tissues in vitro, in addition to hematopoietic lineage [18,20,21]. The isolation of UCB stem cells is non-invasive; they are easily collected and banked. However, the main drawback is the limited number of cells available from a single unit, estimated to represent only 5% of the optimal dose required for adult hematopoietic transplantation [17].

Besides UCB stem cells, mesenchymal stromal cells can be isolated from the Wharton's jelly of the umbilical cord [16,22 24]. Mesenchymal stromal cells can be expanded more efficiently *ex vivo* than UCB stem cells and were shown to differentiate into bone, cartilage, muscle, fat and neural tissue under suitable conditions [24 27]. Thus, they are intensively studied for clinical and tissue engineering applications.

Currently, numerous facilities exist that offer long-term storage of UCB stem cells in the hope of future applications of these cells for broad therapeutic purposes [20]. However, at this time the only realistic therapeutic application is for the treatment of leukemias and lymphomas. Physicians should have this information in mind when advising parents wishing to store the cord blood or the umbilical cord of their yet to be born child.

Placenta, Amnion and Amniotic Fluid as Stem Cell Source

The placenta and amnion are additional extraembryonic sources of multipotent stem cells. Fetal placenta has four distinct regions: amniotic epithelial, amniotic mesenchymal, chorionic mesenchymal and chorionic trophoblastic [28]. From these regions, the following cell populations are isolated: human amniotic epithelial cells (hAECs), human amniotic mesenchymal stromal cells (hAMSCs), human chorionic mesenchymal stromal cells (hCMSCs) and human chorionic trophoblastic cells (hCTCs) [28,29]. Placenta is readily available upon birth and represents an ethically non-controversial source of stem cells (Fig. 25.1). However, various placenta-derived cells display variable differentiation potential and self-renewal has not been clearly demonstrated [28,29]. Therefore, further investigations are required to determine the full potential of these various placental stem cells in order to predict their eventual applicability in therapy.

Amniotic fluid-derived stem cells are isolated from the amniotic fluid, which is routinely isolated during amniocentesis for prenatal diagnosis (Fig. 25.1) [15,30 33]. The origin of amniotic fluid-derived stem cells is still under investigation, although current theories suggest that their source is either urinary and pulmonary secretions from the fetus or potentially ultrafiltrate from the plasma of the mother entering through the placenta. Thus, it is not certain whether these cells are an intermediate between adult and embryonic stem cells. Nevertheless, these cells express some markers of pluripotency (such as *POU5F1*), display high proliferative capacity, differentiate into multiple lineages and do not form tumors in vivo [15,30 33]. Amniotic fluid-derived stem cells are phenotypically most similar to hAMSCs. The major drawback is their isolation during amniocentesis with the well-known risk of miscarriage due to the procedure. While this risk is accepted when amniocentesis is indicated for the purpose of prenatal genetic diagnosis it would not be acceptable exclusively for the purpose of stem cell isolation [15,31].

While all of these extraembryonic tissues represent non-controversial sources of stem cells that could be valuable for specific therapeutic purposes, it is beyond reasonable speculation whether they could replace or substitute for hESCs and their derivatives *in vivo* given that the full potential of therapeutic application of hESCs is unknown at this time [1].

Reprogramming of Somatic Cells: Induced Pluripotent Stem Cells

Somatic cells can be reprogrammed into induced pluripotent stem (iPS) cells (Fig. 25.2) by the addition of several genes, as initially reported by Yamanaka's and Thomson's laboratories [1,34,35]. Yamanaka and colleagues generated human iPS cells through retrovirus-mediated transfection of the genes *POU5F1*, *SOX2*, *KLF4* and *c-MYC*, while Thomson and colleagues generated iPS cells without the oncogene *c-MYC*, inducing the pluripotent state by retroviral transfection of *POU5F1*, *SOX2* and *NANOG* [1,33,35]. It was demonstrated that iPS cells are similar to hESCs by morphology, proliferative capacity and pluripotency. Since these initial reports on iPS cell derivation, a variety of different methods has been reported for reprogramming (see Chapter 13) [36 39].

Reprogramming is an exciting approach as it does not require the use of human oocytes and/or embryos. Induced pluripotent stem cells opened new possibilities particularly for interrogating human disease. Derivation of patient-specific iPS cells will recapitulate disease pathology and this type of research will give important insight into the pathogenesis of many degenerative diseases. Also, iPS cells are an extensive source of histocompatible pluripotent stem cells, which makes them particularly appealing for clinical use. However, there are several important concerns to be resolved: the low efficiency of reprogramming, tumor formation, and developmental equivalence between iPS cells and hESCs. Two recent reports show that reprogrammed cells fail to differentiate or do not differentiate as efficiently as hESCs [40,41]. These findings clearly demonstrate that iPS cells are distinct population of pluripotent stem cells and indicate the imperfections in the reprogramming process. Further research is needed to diagnose and overcome these deficiencies of iPS cells prior to their use in regenerative medicine.

Altered Nuclear Transfer

Altered nuclear transfer (ANT) is a theoretical method for derivation of pluripotent stem cells that would avoid embryo destruction [1,6,42,43]. It is based on somatic cell nuclear transfer (SCNT), with a modified nucleus that would lead to an embryo-like structure incapable of implantation (Fig. 25.2). It has been shown in mice that embryos with a mutation in the *Cdx2* gene die at the blastocyst stage since trophectoderm fails to form, while the ability to derive hESCs from the inner cell mass was unchanged [44,45]. It remains unknown whether CDX2 is the optimal target in human development and whether such embryos could be used to derive stem cells [46]. It is also unclear whether these implantation-disabled structures could be considered as biological artifacts and not as defective embryos [1,46]. Finally, the requirement for a large supply of human oocytes, if ANT should prove successful, implicates further ethical challenges [1].

Single Blastomere Biopsy

Lanza and co-workers developed single blastomere biopsy (SBB) for the purpose of deriving hESCs in a manner that would avoid embryo destruction [47 50]. This approach uses a technique that was originally developed for preimplantation genetic diagnosis (PGD) [1,51 53], the difference being the derivation of hESCs from a single removed blastomere rather than genetic testing [49,50]. A biopsied embryo can continue developing and reach the blastocyst stage and beyond, which was demonstrated with more than a decade of experience with PGD [1,53]. This method has been used successfully to derive hESC lines [1,47 50]. Although this approach bypasses the ethical issue of embryo destruction it brings the risk associated with embryo biopsy [54] which is accepted as the part of PGD procedure, but it would be considered unjustified in the absence of a clinical indication [1]. In addition, US regulations forbid research on an embryo that imposes greater than minimal risk, unless the research is for the direct benefit of the fetus [55]. To date, none of the hESC lines derived by SBB has been approved for National Institutes of Health (NIH) funding [56].

Organismically Dead Embryos

Embryos engendered by *in vitro* fertilization (IVF) procedures frequently fail to develop properly and are classified as non-viable [57 59]. Many non-viable embryos have undergone an irreversible arrest of normal development. Aneuploidy is widespread but mosaicism is common with normal cells distributed among the abnormal. Even a few- celled embryo is an organism with cell-cell communication essential for normal growth and development. Normal cells deprived of integration fail to develop and die. Irreversibly arrested, non-viable embryos satisfy the universal concept of death for multicellular life: an irreversible loss of integrated organismic function. We proposed that hESCs could be derived from cells harvested from

human embryos that have died, despite best efforts, during the course of IVF for reproductive purposes [1]. This approach, wherein live cells are harvested from dead embryos is analagous to the harvesting of essential organs from deceased donors and the established ethics of essential organ donation could be applied to the derivation of new hESC lines [1,60 62]. The use of brain death as a criterion for diagnosing the death of a developed human [62,63] informs the use of irreversible arrest of normal development in diagnosing the death of a developing human. The utility of a crisp diagnosis of death in the developed human avoids the inappropriate application of scarce medical resources and is the foundation of essential organ transplantation. Similar benefits would accrue to the diagnosis of death in the developing human.

The natural history studies that provided the foundation for diagnostic criteria for brain death inspired similar studies of non-viable embryos. Observation of non-viable embryos that arrest and then decompose could provide a stage of development and a duration of arrest that define a time beyond which, having failed to develop, no development is possible. In a retrospective study of non-viable embryos, we determined that many of the non-viable embryos rejected for clinical use were organismically dead: the failure of normal cell division for 48 h was irreversible and, despite the possible presence of individual living cells, indicated an irreversible loss of integrated organismic function [1,62]. A follow-up prospective study examined the progression of non-viable embryos in extended culture [60]. The analysis showed that developmental arrest observed in some human embryos by embryonic day 6 (ED6) following IVF cannot be reversed by extended culture in conditions suitable for preimplantation embryos, as no morphological changes indicative of normal developmental progression were seen and no unequivocal instances of further cell divisions were observed [60]. These observations are in line with standard IVF practice; such embryos are not transferred or cryopreserved because they are known not to produce live offspring [60,64 71]. Although morphological categorization of embryos is of limited value in predicting cell number, the higher cell number associated with cavitation may predict greater potential for the success of hESC derivation [60]. Also, the majority of irreversibly arrested embryos contain a high proportion of vital cells regardless of the stage of arrest, indicating that harvesting cells and deriving hESCs from such non-viable embryos should be feasible [60].

To date, 14 hESC lines have been successfully derived from non-viable embryos that were irreversibly arrested, according to these criteria, thus demonstrating the feasibility of this alternative approach [72 74]. The first cell line (hES-NCL9) was derived from 132 arrested embryos [73]. In a different study, 11 hESC lines were derived from 413 poor-quality embryos rejected for clinical use [72]. Recently, our group has derived two hESC lines: CU1 and CU2 from 159 ED6 irreversibly arrested, non-viable human embryos [74]. Pluripotency and differentiation potential were demonstrated *in vitro* and/or *in vivo* for all 14 hESC lines derived [72 74]. While it is common for arrested embryos to be aneuploid [58,59,75 77], analysis revealed normal or apparently normal karyotype in all 14 hESC lines derived to date [72 74].

Derivation of hESC lines from organismically dead embryos has the likelihood of being accepted by the strongest opponents of hESC research [1]. This approach is unique because it defines a common ground in the stem cell debate. Eleven hESC lines derived by Daley and colleagues have been included in the NIH stem cell registry and are available for research with NIH funding [56]. Despite the low efficiency of isolation of hESCs from organismically dead embryos, large-scale derivation is feasible because in the US alone nearly half a million such embryos are generated yearly as a by-product of IVF for reproductive purposes [1,60]. Available data suggest that hESCs derived from organismically dead embryos are of equal quality to lines derived by the classical, inner cell mass (ICM) derivation approach, but further characterization of these lines is needed [1].

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CHAPTER

26

Stem Cell Banking

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OUTLINE

INTRODUCTION

Stem cell-based strategies have become a tool for a diverse range of medical applications such as organ and tissue repair, a complement to organ transplantation (mainly by modulating the immune response) and an alternative to organ transplantation [1 4].

Hematopoietic stem cells (HSCs) derived from bone marrow were the first adult stem cells used for therapy [5]. Later, peripheral blood [6] and cord blood [7] were consolidated as alternative sources of HSCs for transplantation. The experience gained in hematopoietic progenitor banking for transplantation provides a model for general stem cell banking. Many cord blood banks, both private and public, have been established in recent years and play an important role throughout the world [8]. To date, stem cells from different sources have been used mainly for research purposes. Potential medical applications of stem cells include inflammatory, neurodegenerative, musculoskeletal and metabolic diseases, and diseases of the heart and blood vessels [9,10].

The variety of cell lineages with different functions in the kidney makes it difficult to find the stem cell [11]. However, most of the cells that reveal stemness conditions (ability to generate differentiated progeny of multiple cell types, ability to repopulate tissues in vivo and self-renewal) from different human tissues (e.g. bone marrow, adipose tissue, dental pulp and cord blood) show similar cell structure and, therefore, similar requirements for storage processing [12,13].

The possibility of re-creating in the laboratory a pluripotent state in stromal fibroblastic cells and other differentiated somatic cell types [e.g. induced pluripotent stem (iPS) cells] introduces the idea of personalized stem cell therapy and may lead to the development of substances for regenerative medicine [14 16]. Nevertheless, much still needs to be learned regarding the behavior of iPS cells before they or their products can be used in the clinic [17].

Future clinical applications will require a large number of cells and therefore the development of stem cells banks will be necessary. Cell banking comprises the preservation of a cell stock in the originally obtained state. These banks must ensure the availability, quality and safety of the stored cell products, especially when the stored cells are mainly for clinical use. Critical issues involving stem cell banking are summarized in Table 26.1.

TABLE 26.1 Critical Issues in Stem Cell Banking

Collection area	Stem cell bank	Transplant center
Donor selection	Reception	Selection
Stem cell collection	Processing	Transplantation
	Cryopreservation and storage	Follow up
	Biological controls	
	Shipment to transplant center	
	Follow up	

BASIC PRINCIPLES OF CELL BANKING

Stem cell banking for therapeutic use requires several standards to be maintained:

- *Quality*: development of processing methods to generate cells in compliance with current good manufacturing practices (GMP) to ensure the quality of the products. Commission Directive 2003/94/EC of October 2003 regulates the principles and guidelines of GMP with respect to medicinal products for human use and investigational products in Europe [18].
- *Safety*: applying strict measures to avoid the risk of disease transmission. Cellular therapy products are derived from human sources and, therefore, carry the risk of transmitting infectious agents.
- *Efficiency*: providing products that maintain the biological properties that are useful for human health (clinical perspective) and performing all the activities related to stem cell banking at a lower cost without reducing the quality.
- *Traceability*: allowing the ability to track specific information at every step in the process chain. This must be achieved confidentially.
- *Transparency*: offering accurate information to users and institutional organisms according to current concepts in medicine and regulations.
- *Benchmarking*: establishing indicators to compare the model with other ones and optimizing the services by implementing a continuing program of quality improvement.

To meet these issues and ensure reliable quality, the bank needs a quality assurance system of management. This system must comply with current professional standards and statutory legislation. Basic principles of GMP guidelines involve quality assurance, personnel, premises and equipment, documentation, production, quality control, complaints and product recalls, and self-inspection and quality audits [18]. Moreover, the design of stem cell bank facilities must focus on guaranteeing the quality and safety of the cell products.

The personnel working in the bank must be familiar with the quality requirements, carrying out appropriate training programs, and must know their responsibilities (management, medical advisory, technical work, quality assurance). The standard operating procedures and associated documents of the cell bank must be included in a manual and be periodically reviewed to ensure their suitability.

The implementation of such a quality assurance system allows relationships to be established among cell banks for clinical use based on a common principle of applying standards that are as high as necessary, which is important to encourage cooperation at the international level.

By concentrating resources and expert knowledge, centralization is an efficient policy for cell and tissue banks at regional and national levels. Centralization is especially important for public non-profit banks. The international network of umbilical cord blood banks, intended to achieve the fast and efficient location of products according to human leukocyte antigen (HLA) compatibility criteria, is a good example of the implementation of common standards for international cooperation in stem cell banking. However, there continues to be a major effort to standardize banking and regulate all the steps to provide the highest quality for patient use. Organizations such as the American Association of Blood Banks, the American Red Cross, the American Society of Blood and Marrow Transplantation, the European Blood and Marrow Transplantation Society, Eurocord, the Foundation for the Accreditation of Hematopoietic Cell therapy, the International Society for Hematotherapy and Graft Engineering, the Joint Accreditation Committee of ISHAGE-Europe and EBMT, Netcord and the National Bone Marrow Donor program make sure that the quality and standards in cord blood banking are established and met [19].

DONATION

Proper informed consent has to be obtained for the intended use (autologous and/or allogeneic administration) as well as for retrieval, processing, testing, storage and data management. In addition, the possibility of alternative use in research must be included when cells are not used for therapeutic purposes. Other possibilities could be a change from the autologous to allogeneic condition, once autologous use has been completely discarded and when donor selection criteria are in compliance. In any case, the donor must be informed and advised in a comprehensive manner according to the scientific and clinical evidence.

Donor selection criteria are based on an assessment of medical and behavioral history. Potential donors must

answer a questionnaire specially designed to identify specific risk factors for hepatitis B and C, human immunodeficiency virus (HIV), syphilis and other infectious diseases. This is the first step in donor screening and the most important one in order to avoid the risk of transmissible disease. The exact questions to be asked, the information to be obtained and the source of that information have to be regularly reviewed and updated by specialized professionals belonging to accredited scientific societies, international health institutions and government institutions (American Association of Tissue Banks, American Association of Blood Banks, International Society for Cell Therapy, European Association of Tissue Banks, Food and Drug Administration, European Medicines Agency, etc.). The kind of stem cells to be collected should also be covered in the questionnaire [20].

The second step taken to minimize the risk of infection is testing donor blood samples for specific infectious markers (hepatitis B and C, HIV, syphilis, etc.) [21]. The decision on the diseases to be screened depends mainly on the origin of the donor and also on the geographical scope of the bank's services (different countries have distinct requirements). Epidemiological institutions offer current information on this issue. Samples need to be stored for additional testing, especially in the case of products that can be stored for long periods. Donor samples must be tested in laboratories accredited by international societies.

It is important to take into account that cell manipulation proceedings usually start before the donor's test results are known. As biological products of human origin, tissues, cells and associated samples are considered to be risk materials for disease transmission, and protective measures must be implemented during manipulation. In addition, the risk of contamination in the opposite direction (from the operator and the environment to the biological products) has to be avoided. This concern will be present in all of the following procedures.

COLLECTION

Cells and tissues are usually collected under operating theater conditions. An aseptic environment is fundamental to minimizing the risk of contamination. If cells became contaminated at this step, this condition will be maintained in the following phases of processing, because of the difficulty in applying sterilization procedures which may irreversibly damage cells [22]. For instance, microbiological contamination can lead to 1 15% of cord blood being discarded [23,24].

Cells are collected as a suspension (e.g. hematopoietic progenitors) or included in tissue fragments (e.g. adipose tissue). In either case, specific environmental conditions (container, temperature, transport solution, etc.) must be established to maintain cell viability and function according to cell bank requirements. If hematopoietic progenitors are the stem cell source (e.g. bone marrow or peripheral blood), coagulation must be avoided, for example by collecting the cells in acid citrate dextrose (ACD) or citrate phosphate dextrose (CPD) solutions. Umbilical cord blood is usually collected in a bag containing approximately 23 ml of anticoagulant solution CPD-A [25] and maintained at 4°C until processing. For tissue fragments, a basal nutrient medium [e.g. Dulbecco's modified Eagle's medium (DMEM) or Medium 199] can be used as the transport solution, with the addition of antibiotics to avoid the growth of microorganisms. If processing of the tissue fragment is to be delayed, then donor serum (5 10%) can be added to the medium.

In addition to cells or tissues, associated biological samples must be included in the package for the bank in a suitable condition for the intended use, and documentation (informed consent, detailed description of products, data related to the collection process, etc.) must be included and must be legible. External labeling must include the center of origin and person responsible, advice on handling, center of destination and person responsible, etc.

PROCESSING

Upon arrival, the package contents (cells, tissue fragments, samples, documentation, etc.) are checked to assess their suitability. The origin of every individual product must be identified unequivocally to ensure traceability.

The areas where the cells are exposed to the environment during processing are considered critical because of the high risk of contamination, so they must be exhaustively controlled. The specifications on the air quality in the different areas of the bank must be defined and an environmental monitoring system used, according to this classification, which includes a particulate count (Table 26.2) and microbiological cultures (Table 26.3) [18].

To maintain the aseptic chain, it is important to use flow cabinets with grade A air quality and at least a grade C background. Depending on the air flow direction two types of cabinet can be distinguished: horizontal, where the air flow blows from the side facing the operator, parallel to the work surface, and is not recirculated (Fig. 26.1); and vertical, where the air blows down from the top on to the work surface and is drawn through the work surface and either recirculated or vented (Fig. 26.1). The first type permits more

freedom of movement and the second, which includes a front panel, offers more protection for products and technicians.

To achieve the highest efficiency from stored products, it is very important to prepare them according to optimal processing and storage protocols.

TABLE 26.2 Classification in Function of Airborne Particulates

	At rest		In operation	
	Maximum permitted number of particles/m^3 equal to or above			
Grade	**0.5 μm**	**5 μm**	**0.5 μm**	**5 μm**
A	3,500	0	3,500	0
B	3,500	0	3,500	2,000
C	350,000	2,000	350,000	20,000
D	3,500,000	20,000	Not defined	Not defined

Adapted from the European Commission Guide to Good Manufacturing Practice. Annex 1: Manufacture of Sterile Medicinal Products [18].

TABLE 26.3 Classification in Function of Recommended Limits for Microbiological Monitoring of Clean Areas During Operation

Grade	Air sample (cfu/m^3)	Settle plates (diam. 90 mm) (cfu/4 h)	Contact plates (diam. 55 mm) (cfu/plate)	Glove print, 5 fingers (cfu/glove)
A	< 1	< 1	< 1	< 1
B	10	5	5	5
C	100	50	25	–
D	200	100	50	–

cfu: colony-forming unit.
Adapted from the European Commission Guide to Good Manufacturing Practice. Annex 1: Manufacture of Sterile Medicinal Products [18].

Cell Suspension

Organs and tissues contain different cell lineages with different functions and capabilities. Whether stem cells are present in all tissues is still a matter of debate. For stem cell banking, the cells intended for therapeutic purposes must be selectively isolated and concentrated.

Cells can be isolated from tissue fragments by two techniques. In the first, explant culture, tissue is chopped finely and the pieces are seeded on to the culture surface. It is recommended that a high concentration (30 50%) of serum is used in the nutrient medium, so that surface tension holds the small pieces in place until adhesion. Then, after a few days, outgrowth of cells is achieved by migration from the tissue to the culture surface (Fig. 26.2A). Later, when the culture reaches subconfluence, cells can be detached and suspended in isotonic solution. The main advantage of this technique is that tissue is minimally manipulated, but cell yields are lower than those obtained with enzymatic digestion, because some cells in the tissue fragment do not contact the culture surface.

The second isolation technique is enzymatic digestion. Tissue disaggregation is achieved by digestion of the extracellular matrix. Trypsin and collagenase are by far the most commonly used enzymes for this objective. This technique yields a higher number of cells, quantitatively and qualitatively (including more cell lineages of the whole tissue), than explant culture (Fig. 26.2B). However, prolonged exposure to enzymes may result in cell damage. This technique can also be used for whole organ processing [26].

The cell suspension is the starting point for further processing. In the case of blood, cells are already suspended in plasma, a liquid medium. The next step is the specific selection of cell types with potential for use in cell therapy.

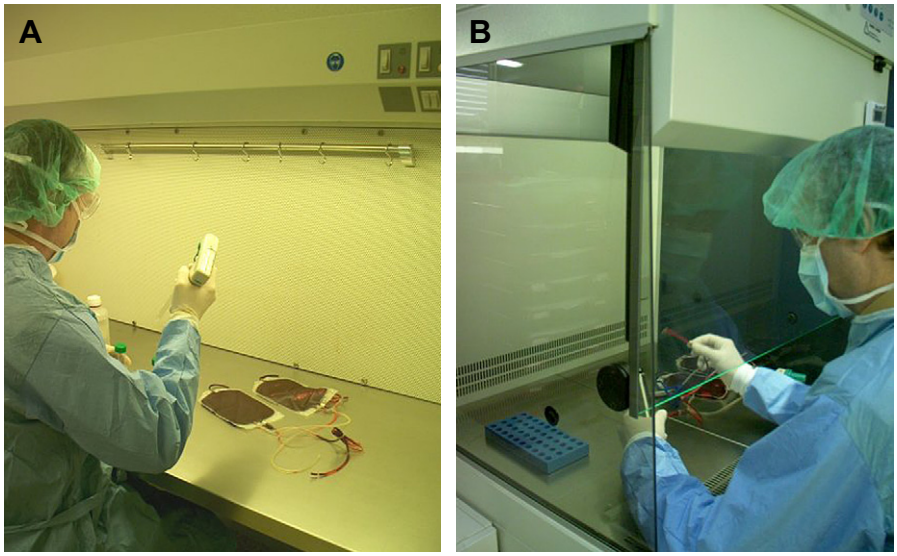

FIGURE 26.1 (A) Horizontal laminar flow cabinet; (B) vertical laminar flow cabinet.

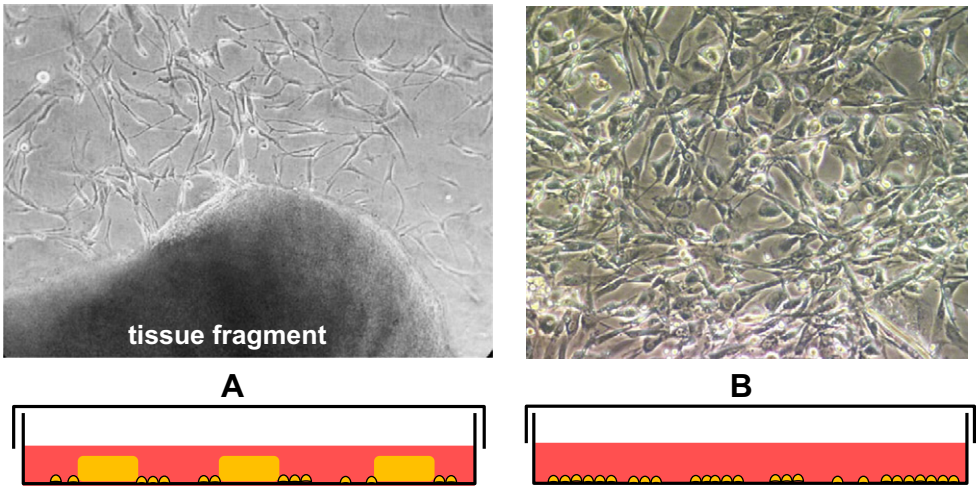

FIGURE 26.2 (A) Cell culture as explants. The cells migrating out of the explant and proliferating are known as the outgrowth. This method may be preferable when the sensitivity of cells to the enzyme used for digestion could affect cell survival. (B) Cell culture after enzymatic digestion of tissue. This method generates larger cultures more rapidly than explant culture.

Cell Selection

The presence of a high number of red blood cells in the sample is a problem because these cells can interfere with stem cell processing. Different methods are used to reduce or eliminate erythrocytes. For example, the hematocrit in umbilical cord blood can be reduced by adding hydroxyethyl starch, which induces erythrocyte aggregation and therefore facilitates sedimentation by gravity. Two different automatic devices have been developed specifically for this purpose: Sepax and AXP. Sepax consists of a centrifuge and a pneumatic system with vacuum or pressure capability to fill or empty the separation chamber and lines, using sterile, disposable processing kits with different configurations for dedicated protocols [27]. The AXP AutoXpress™ platform is an automated fully closed system, specifically designed to reduce cord blood to a precise volume. This system has been developed with integrated sampling capability and a cryoprotectant line incorporating a sterile filter, potentially providing a truly closed system [28]. To eliminate residual erythrocytes entirely, for example from enzymatically digested adipose tissue, a specific lysis buffer (e.g. containing ammonium chloride) can be used. However, some authors have reported potential detrimental effects on stem cells with this treatment.

Further cleaning of the cell suspension can be achieved using a sieve to retain residual extracellular matrix (e.g. fibers) or other tissue residues. In addition, with adequate pore size, this device can be used for cell selection purposes, as a function of cell size.

Then, cells can be concentrated as a pellet in the bottom of a tube or a bag through centrifugation or stratification in layers. The position in the layer depends on cell size and density (Fig. 26.3). It is possible to create

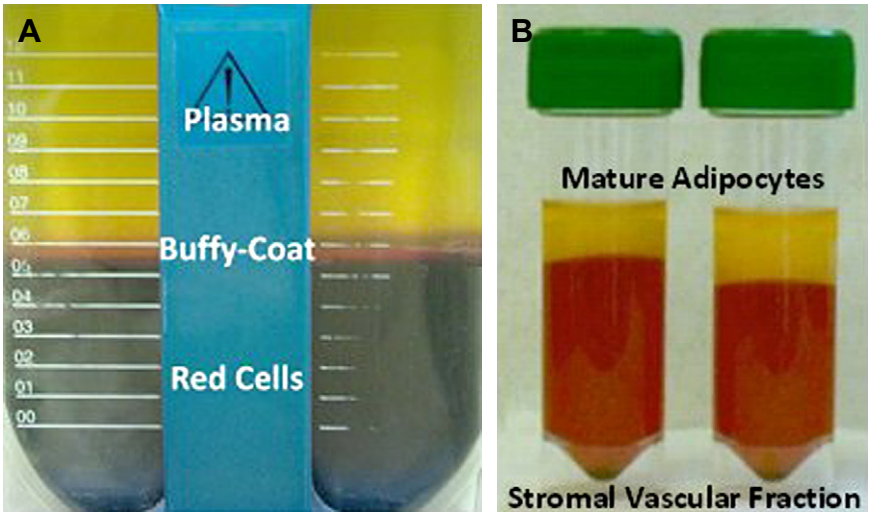

FIGURE 26.3 (A) Bag containing blood after centrifugation to separate hemoderivatives (plasma on the top, platelets and leukocytes in the intermediate buffy coat layer, and red cells on the bottom). (B) Adipose tissue enzymatically digested. After centrifugation the supernatant contains the mature adipocytes, which float owing to their high lipid content, and the pellet contains the stromal vascular fraction containing blood cells, fibroblasts, pericytes, endothelial cells and preadipocytes.

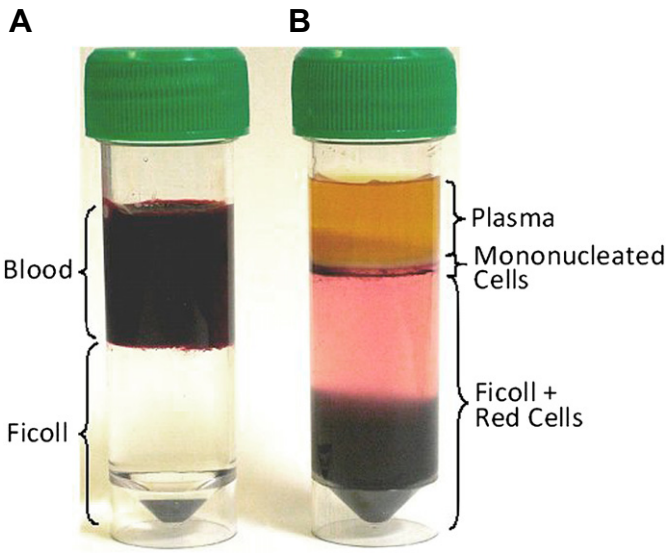

FIGURE 26.4 (A) The cell suspension (blood in the example) containing the target cells is top loaded over the Ficoll gradient. (B) Following centrifugation, various clearly defined layers are obtained.

a density gradient using solutions with monitoring density (e.g. Ficoll) (Fig. 26.4) [29].

Cell separation by centrifugal elutriation can be performed in a closed system with automated devices designed for this objective (Fig. 26.5), resulting in improved cell yield and high reproducibility [27].

For finer cell selection, microbeads with paramagnetic properties bearing antibodies against specific cell surface antigens can be used to capture cell populations based on their immunophenotype (Fig. 26.6). Positive selection uses antigens addressed against cells of interest [30] and negative selection uses antigens directed against cells that are not required [31]. In another immunology-based method, using fluorescent particles instead of paramagnetic beads, in combination with flow cytometry, a mixture of cells with different immunophenotypes can be sorted and each type of cell collected in a different container. This is called fluorescence-activated cell sorting (FACS) and is based on the specific light scattering and fluorescent characteristics of each cell (Fig. 26.7).

The interaction with the substrate in cell culture conditions is another property that can be used for cell selection. In selective adhesion, different cell types have different affinities for the culture substrate and will attach at different rates after seeding. In selective detachment, once cells have adhered to the culture surface and grown, they can be detached using specific solutions (e.g. trypsin/EDTA). Different cells types show different sensitivity to these products.

Once the cells have been isolated and concentrated, they can be stored or, if necessary, expanded in culture, maintaining their phenotype and therapeutic potential by using a suitable nutrient medium (basal medium supplemented with 10 20% serum and antibiotics), under suitable environmental conditions (usually 37°C, 95% humidity and 5% CO_2). As mentioned already, culture conditions are also used for cell selection. Supplementation of the medium with bovine-derived serum, as usually used in research work, is not recommended without further processing [32]. The proposed criteria for current GMP require that animal-derived supplements should be avoided or replaced with synthetic supplements [18]. Autologous human serum is a useful supplement for basal culture media; it is disposable in transfusion centers with high safety requirements and suitable for most human cell types intended for cell-based therapies. In addition, these

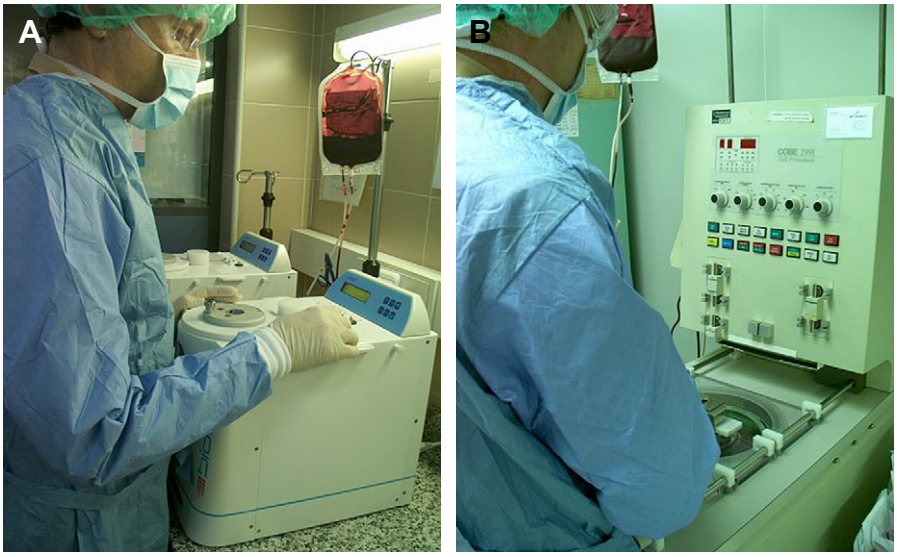

FIGURE 26.5 The use of (A) semi automated (Sepax) or (B) fully automated (Cobe) devices for centrifugal elutriation reduces cell manipulation. The risk of con tamination is thus minimized, which is very important in the case of cells intended for clinical use.

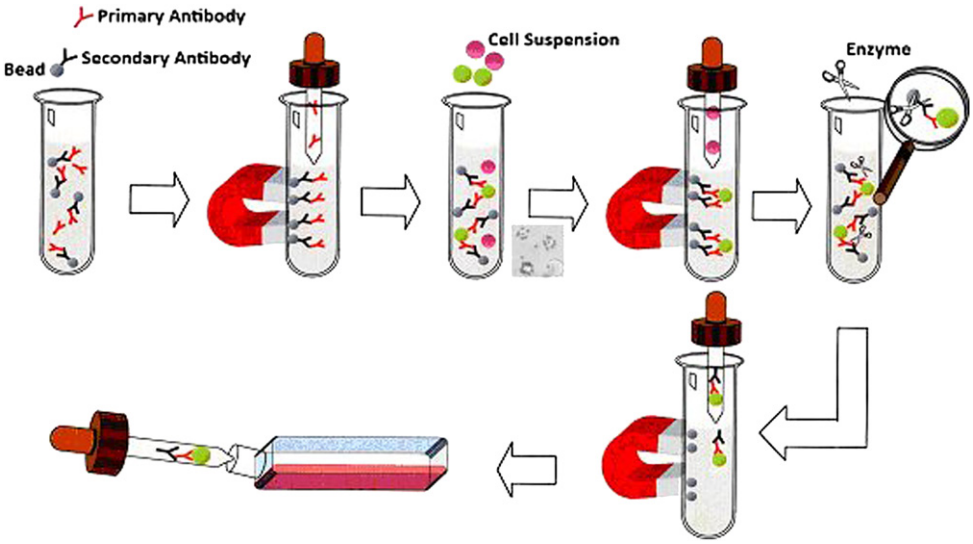

FIGURE 26.6 Cell selection with paramagnetic beads. The primary antibody recognizes the specific cell surface antigen of the target cell. The secondary antibody is directed against the primary antibody. With the use of a magnet, first the excess of non bound material is rinsed and then, after incubation with the cell suspension, the cells with the specific surface antigen are selected. Finally, the bead is enzymatically released and the cells can be stored or seeded in culture.

centers provide platelet lysate with a high content of different growth factors and cytokines, another hemo-derivative suitable for cell growth [33].

Processing in the bank is a critical step with respect to contamination risk, especially when cells or tissues are openly manipulated. Microbiological monitoring to assess the maintenance of the aseptic chain must be implemented.

To provide a cell profile of the product to be stored, additional testing can be performed, for example phenotypic cell characterization by flow cytometry (e.g. $CD34^+$ and $CD45^+$ cells to identify hematopoietic progenitor cells), and HLA typing for compatibility studies and cell viability.

FIGURE 26.7 Fluorescence activated cell sorting (FACS) is a technology developed for counting and examining cell populations. Each cell is individually assessed according to physical characteristics (e.g. size, cytoplasmic complexity) or immunological phenotype (expression of specific antigens on the cell membrane).

Cryopreservation

Freezing is the method used most for successful long-term preservation of cells and tissues. To maintain cell viability, it is necessary to suspend cells in a cryoprotective solution and design freezing and thawing protocols. Two main obstacles have to be overcome: cell damage associated with ice crystal growth and osmotic stress [34 36]. A cell density between 10^6 and 10^7 cells/ml in the cryoprotective solution is recommended.

Dimethyl sulfoxide (DMSO) and glycerol are the products most often used as cryoprotectants [37,38]. Both are permeating substances, but DMSO permeates more rapidly than glycerol. However, cryoprotectants can produce detrimental effects at high concentrations [34,39]. DMSO is a cryoprotector widely used for stem cells derived from bone marrow, cord blood, dental pulp, placenta and adipose-derived adult stem cells [40]. Cryopreservation of stem cells from adipose tissue using 10% DMSO has been shown to have no effect on the cell characteristics (phenotype, proliferation and osteogenic differentiation [41]. Cryoprotectant concentrations from 3% to 10% are usually used. HSCs can be successfully cryopreserved using DMSO concentrations between 5 and 10% [42].

Moreover, a stepwise protocol to add cryoprotectant sequentially, increasing gradually until reaching the final concentration, is advisable. During cooling, the

FIGURE 26.8 Controlled rate freezer devices include a freezing chamber, a module with the software for the design and storage of cooling programs, and a container for nitrogen supply, in addition to exhaustive control, data recording and high reproducibility of processing.

liquid surrounding the cells is gradually frozen (because extracellular ice develops first), then solutes are released to the liquid fraction and it becomes hyperosmotic. As a consequence, cells lose water to equilibrate. By reducing water inside the cell, the risk of formation of ice crystals, which can cause mechanical damage to cell structures, will be reduced. Nevertheless, excessive hyperosmotic exposure can cause irreversible damage in cell membranes. Cells have critical limits to shrinkage and swelling that must not be exceeded [34,39].

The presence of these substances lowers the freezing point, so that cell volume reduction during cooling is postponed to a lower temperature, and reduces the amount of ice crystals formed. The term supercooling defines a state in which unfrozen water is present at a temperature below its nominal freezing point. At this stage, the induction of ice crystal nucleation can be achieved by mechanical vibration or by rapidly lowering the temperature. This is known as seeding and decreases heterogeneity in the nucleation process.

The freezing rate is a significant factor in viability assurance after storage and thawing. Controlled-rate freezers (Fig. 26.8) use accurate programs for cell freezing [42]. The main advantage of this apparatus is that it reaches a temperature close to that of nitrogen vapor. Cooling rates between 1 and 3°C/min are suitable for a high number of human cell lineages.

Nevertheless, modest variations in cooling rates can be tolerated by cell suspensions, in which case other freezing technologies can be used, but with a reduction in cell survival compared with controlled-rate freezing. The most common method to achieve freezing uses passive cooling devices, where the material is placed in a precooled polystyrene box or a precooled polycarbonate container with ethanol or isopropanol (Fig. 26.9) at −80°C. Once the specimen is frozen, it is incubated in nitrogen vapor before being submerged in the liquid phase. Previous qualification and validation are required of the complete procedure, standardizing conditions (e.g. box size, wall thickness and time to reach temperatures at each stage) and providing tools to avoid disturbances in temperature out of range (e.g. temperature monitoring system with acoustic and visual alarms). Non-controlled freezing has been shown to be effective for HSCs from bone marrow [43], peripheral blood [44,45] and cord blood [46,47].

Vitrification has been proposed as an alternative to overcome problems observed with other methods of cryopreservation. It requires an ultrarapid cooling rate and the presence of solutes at high concentration; then the liquid solution becomes increasingly viscous until reaching a glassy solid state, avoiding crystallization. Nevertheless, thawing can involve problems related to the risk of recrystallization if the warming rate is not

FIGURE 26.9 Passive cooling devices can be used as an alternative for freezing samples near the storage temperature.

FIGURE 26.10 Cryopreservation bag containing hematopoietic progenitor cells, in an aluminum case.

FIGURE 26.11 Storage area containing nitrogen tanks.

rapid enough. At present, only a very small volume and thin samples can be successfully processed by vitrification. This methodology is very useful, for example, for cryopreservation of an oocyte, which is suspended in a very small droplet (no more than 1 μl), giving excellent results regarding survival and clinical outcome. Nevertheless, some authors have encouraged research into the vitrification of complex systems, proposing the kidney as the most challenging organ to vitrify and rewarm successfully [48].

Inclusion of the freezing bag containing the cell suspension in an aluminum case (Fig. 26.10) has several advantages. It protects the bag against breakage, facilitates organization in storage containers, maintains a homogeneous thickness in the liquid content, improves heat exchange during freezing and thawing, and avoids temperature gradients in the sample.

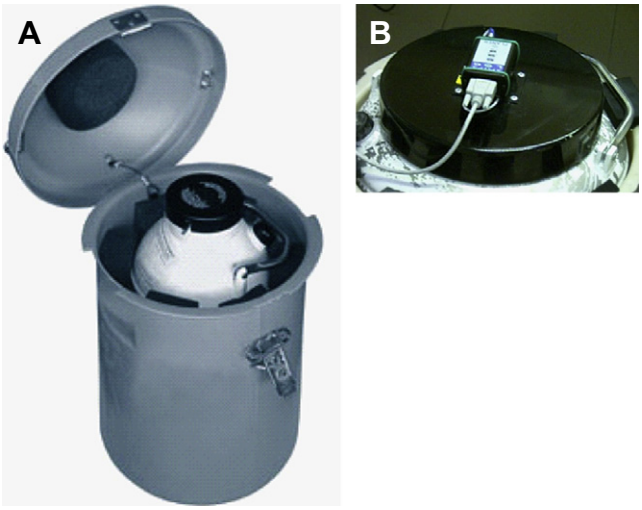

FIGURE 26.12 (A) Dry shipper: vapor phase nitrogen tank mostly used for the transportation of cryopreserved samples. (B) Temperature monitoring system for dry shipper.

STORAGE

Once the product has been successfully frozen, it has to be stored. Labeling must be adequate to maintain integrity and legibility during storage, identifying the product unequivocally and ensuring traceability.

Long-term cryopreservation is critical for stem cell banking: the lower the temperature the longer the storage. The liquid nitrogen (196°C) phase appears to be useful in achieving this aim (Fig. 26.11). In addition providing to an ultralow temperature, this liquid phase maintains a constant temperature, avoids undesirable oscillations and is easily monitored. The highly efficient recovery of functional hematopoietic progenitor and stem cells from cord blood cryopreserved for 15 years has been demonstrated [49].

Because cells are stored before screening test results are available, the frozen product must remain in quarantine while awaiting the final decision on its release or rejection. The adoption of measures to avoid the risk of either cross-contamination or environmental contamination is particularly relevant not only during quarantine but also during storage. Several authors have shown that liquid nitrogen should be considered as a contamination risk element in cell and tissue banking [50 53].

To avoid the presence of liquid media that could be used as a vehicle for contaminating microorganisms, storage tanks based on the technology used in dry shipping have been developed (Fig. 26.12A). The dry shipper was designed for delivery of biological samples classified as non-hazardous at ultralow temperatures. These

dewars have an insert containing a hydrophobic absorbent material. The absorbent repels moisture and humidity but absorbs liquid nitrogen, allowing maintenance of a temperature in the range of the nitrogen vapor phase. Although the presence of microorganisms seems to be unavoidable with time as a consequence of successive tank openings during stem cell banking in a nonaseptic environment, the absence of liquid could decrease the risk of cross-contamination during storage [54,55].

Selection of Cells for Transplantation and Delivery

The transplant program is fully dependent on the bank for the quality of stem cells. Stored stem cells can be used for autologous and allogeneic purposes. In the first case, selection is made mainly on the basis of cell quantity. In the second case, HLA matching is mandatory for some types of cells. The selection of HSCs for transplantation requires combining both cell dose and HLA matching. Possible HLA-matched cord blood units for patients are found from computerized registries [56].

Cell products can be shipped on long journeys in the frozen state, using dry shippers. Dry shippers allow the cells to remain frozen and can maintain temperatures below −150°C for 14 days. Upon arrival, temperature is controlled (Fig. 26.12B). According to international standards, the temperature must be continuously controlled during transport.

Labeling and additional documentation must be included in the package, bearing the following information: name of the cell bank, address and contact references (personnel, telephone number), product description, identification code, origin, processing, HLA typing (if necessary), cryoprotective solution composition, required storage temperature, advice on handling and thawing instructions.

Thawing and Viability Assessment

The thawing procedure requires as much attention as freezing to ensure cell survival. There are two stages to consider: proper thawing and removal of the cryoprotectant.

In general, rapid rates of warming are used, to avoid the growth of ice crystals in the frozen sample. This can be accomplished by submerging the frozen cell container in a waterbath at 37°C. However, the liquid medium represents a potential risk of environmental microorganism contamination, in spite of the use of antiseptic substances diluted in the water. Microbiological monitoring must be implemented at this stage. An alternative to water is an electric dry-warming device that uses warmed gel pads and can be located inside the clean room where devices containing water are not indicated (i.e. are restricted) [57].

Especially for products stored in the liquid phase of nitrogen, the use of a two-step thawing process is suggested: first, slow thawing (e.g. incubation at room temperature until reaching approximately −100°C) to prevent thermal stresses created by rapid warming of the frozen material at ultralow temperature [35]; and second, rapid thawing at 37°C.

Once the ice has dispersed, elution of the cryoprotectant solution with basal culture medium takes place in a single-step or a stepwise (especially when a high cryoprotectant concentration is used) dilution process. Stepwise elution reduces the risk of membrane injury by minimizing critical variations in cell volume due to instantaneous exposure of the cryoprotectant-containing cells to an isotonic medium. Moreover, human serum or sucrose can be added to the basal culture medium to minimize dilution shock [58].

A small sample of the thawed cell suspension can serve as a processing control, to assay the membrane integrity (i.e. viability) and potency. Cell membranes are implied directly in the events related to cooling and warming rates. Trypan blue is a vital stain used for routine viability assessment. Cells with damaged membrane are stained blue, while cells with intact membrane remain uncolored (Fig. 26.13). This method requires only conventional microscopy. Another possibility is the use of acridine orange ethidium bromide stain. In this case viable cells appear green and nonviable cells red, but it is necessary to use a microscope with fluorescent light [59] (Fig. 26.13). These methods have the disadvantages that only a small number of cells can be analyzed and the method is observer dependent. Determination of viability using flow cytometry and fluorescent dye is more accurate and reliable [60]. To measure the potency of the sample, the in vitro growth of colony-forming units (CFUs) and cell differentiation are assessed [61].

Information System

Information is a strategic factors in stem cell banking management. Any information system must be updated regularly, must assure quality, and must be transparent, coherent and available. A three-module set (general, scientific/technical and educational) is proposed as a base on which to develop a system for information in a cell bank, and to provide functionality to users. *General* module includes data management and record keeping from bank activities (donation, processing, transplantation, biovigilance, etc.), and reports provided to institutional organizations and users of the bank's services; in addition, indicators need to be established to monitor the efficiency of cell banking. *Scientific and*

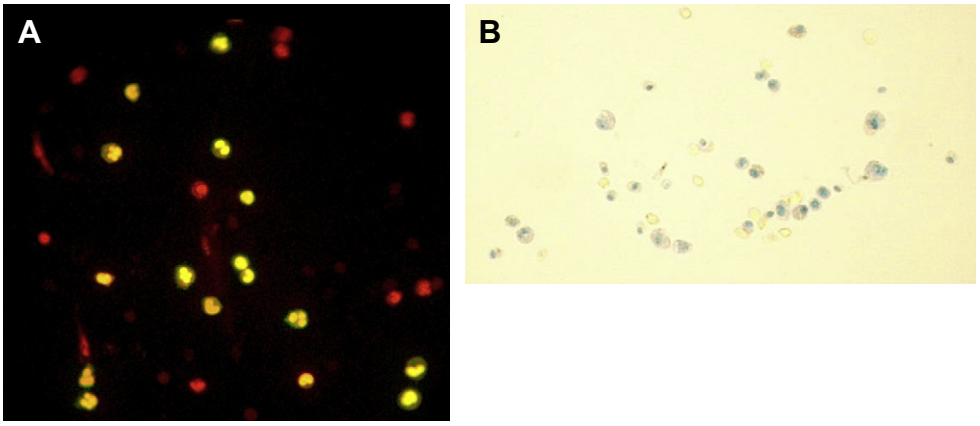

FIGURE 26.13 (A) Acridine orange–ethidium bromide stain. Green cells: viable cells; orange–red cells: non viable cells. (B) Trypan blue stain. Non colored cells: viable cells; blue cells: non viable cells. Please see color plate at the end of the book.

technical module to facilitate the access to databases for the design of strategies based on updated evidences to decision making and risk evaluation. With this aim, available knowledge of the precise and concise operations and procedures within a cell bank is fundamental. *Educational* module, as a tool for transmitting the particular knowledge in a cell bank, not only to health professionals but also to the public, as cell banks exist owing to people's altruism.

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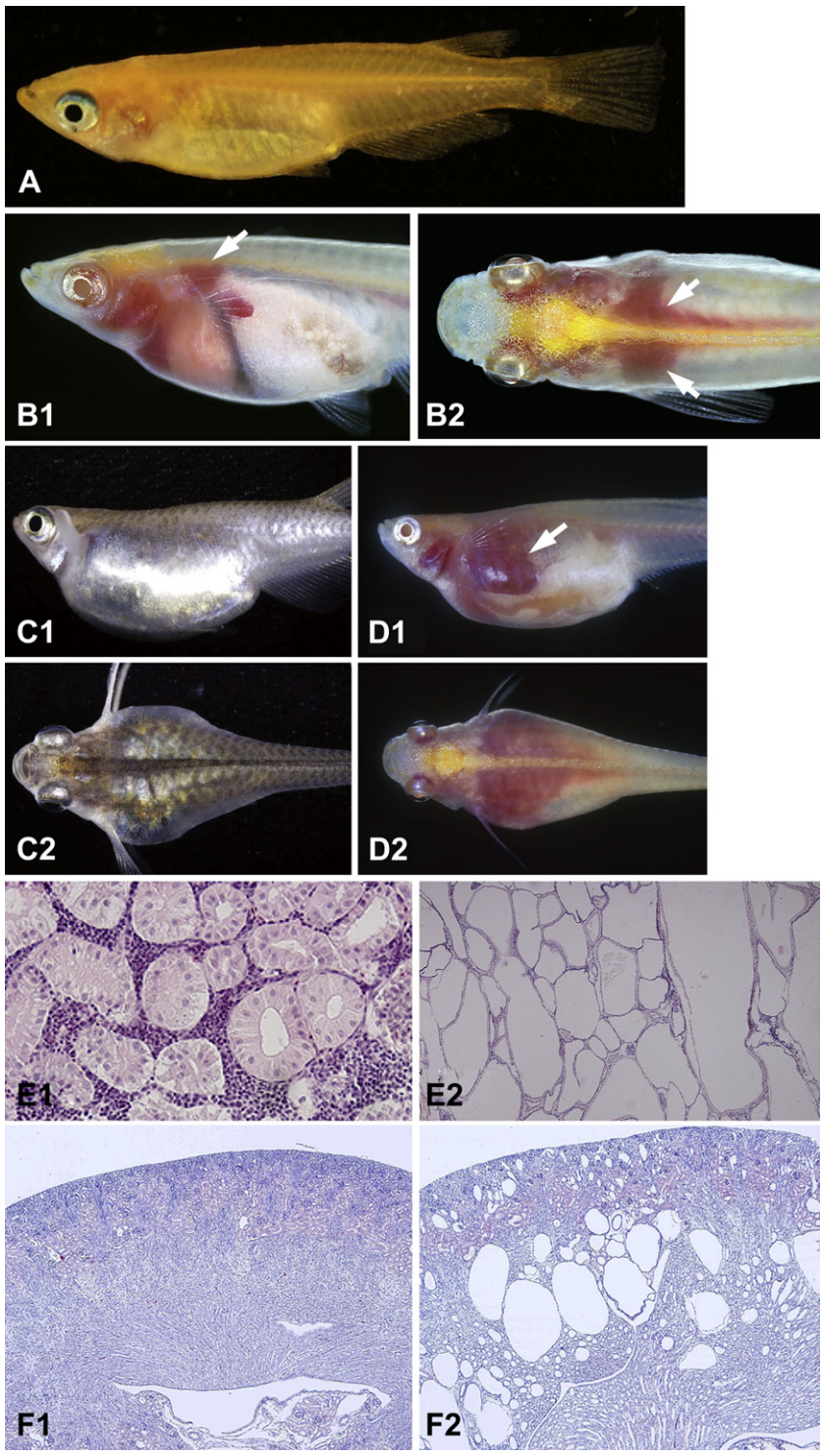

FIGURE 1.1 Medaka as a model organism. (A) Orange–red strain of medaka, a popular strain used in life science research. (B) See through medaka STIII. Reddish kidneys are visible through a transparent body wall (arrows). (C) pc mutant, which manifests a massive abdominal enlargement. (D) pc STII. The kidney expansion can be non invasively observed in a live fish (arrow). (E) Histology of the medaka adult kidney. Severe tubular distension is evident in pc mutant (E2), but not in wild type (E1). (F) Histology of the mouse kidney (postnatal 7 days) deficient for *Glis3*, the orthologue of medaka pc gene. Renal cysts are apparent in *Glis3* (/) kidney (F2), but not in *Glis3* (+/+) kidney (F1). (B1, C1, D1) Lateral views; (B2, C2, D2) dorsal views [7–9]. Please see black and white figure on p. 5.

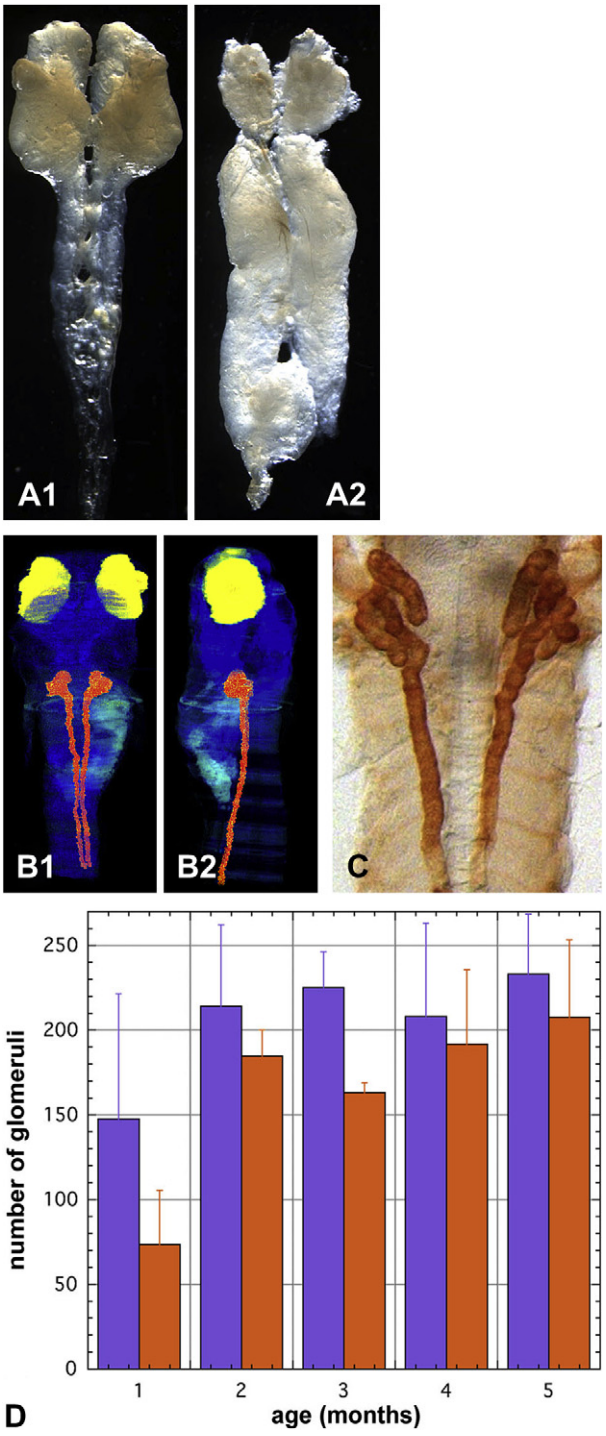

FIGURE 1.2 Gross morphology of medaka kidney. (A) Comparison of adult kidney morphology between medaka (A1) and zebrafish (A2). (B) The pronephros of the hatchling as viewed by 3D imaging. The intrarenal tissues are colored in red. (B1) Dorsal view; (B2) lateral view. The area of pronephric glomus is not colored because it locates externally to the renal tissues. (C) The pronephric tubules and ducts are stained with anti Na^+/K^+ ATPase antibody. (D) The number of pronephric glomeruli in the kidney of the male (blue) and the female (red) during 5 mph. Error bars indicate the standard deviations. The number includes immature developing glomeruli. Anterior to the top (A–C) [15,24]. Please see black and white figure on p. 6.

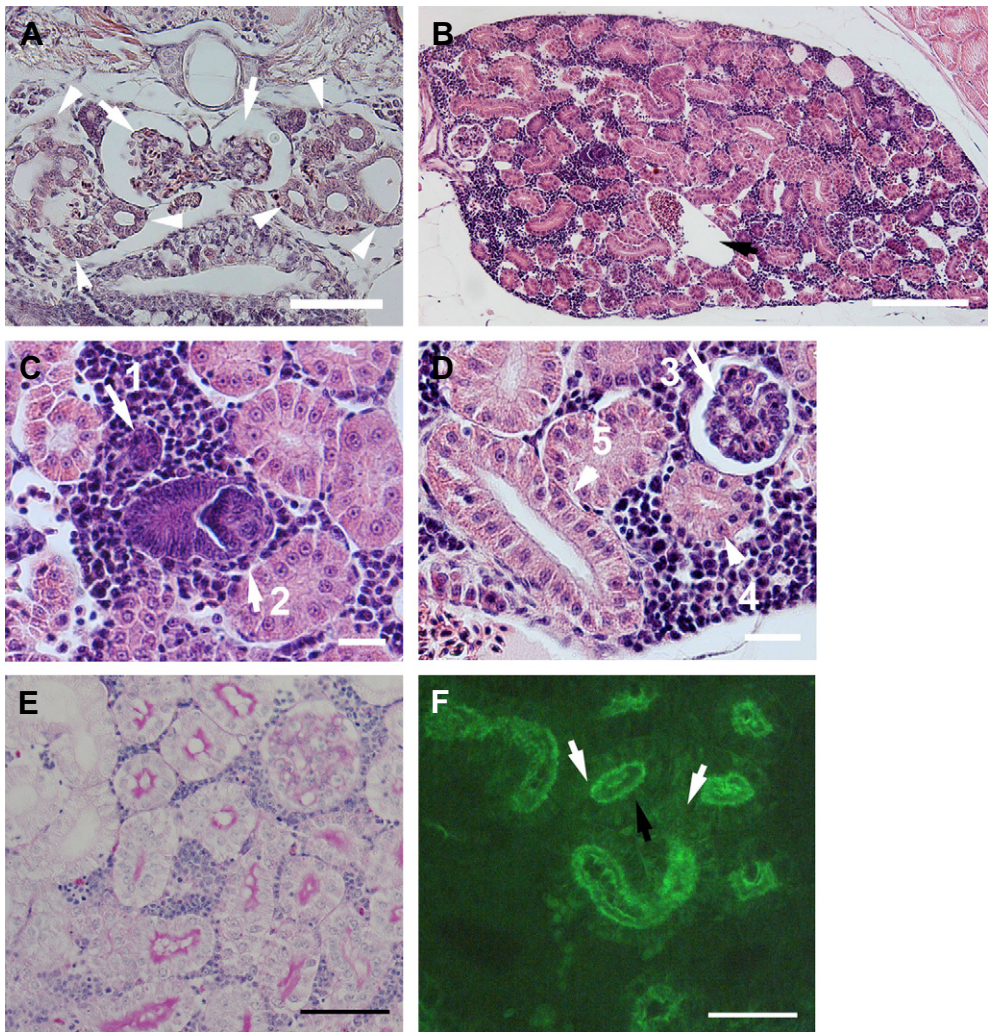

FIGURE 1.3 Histology of medaka kidney. (A) The pronephros of the hatchling. Arrows: the external glomera. Three arrowheads: each of bilateral borders of the renal tissue. (B) Section of the anterior portion of 2 mph medaka. Black arrow: subcardinal vein. (C, D) Magnified image of a section from 2 mph medaka. Arrow 1: mesenchymal condensate; arrow 2: nephrogenic body; arrow 3: mature glomerulus; arrowhead 4: proximal tubule; arrowhead 5: distal tubule. (E) Periodic acid−Schiff (PAS) staining. The proximal tubules but not the distal tubules are positive for PAS stain. (F) *Lotus tetragonolobus* (LTL) staining. The proximal tubules are positively stained by the fluorescent conjugated LTL. Scale bars: 50 μm (A, E, F); 100 μm (B); 20 μm (C, D) [7,15]. Please see black and white figure on p. 7.

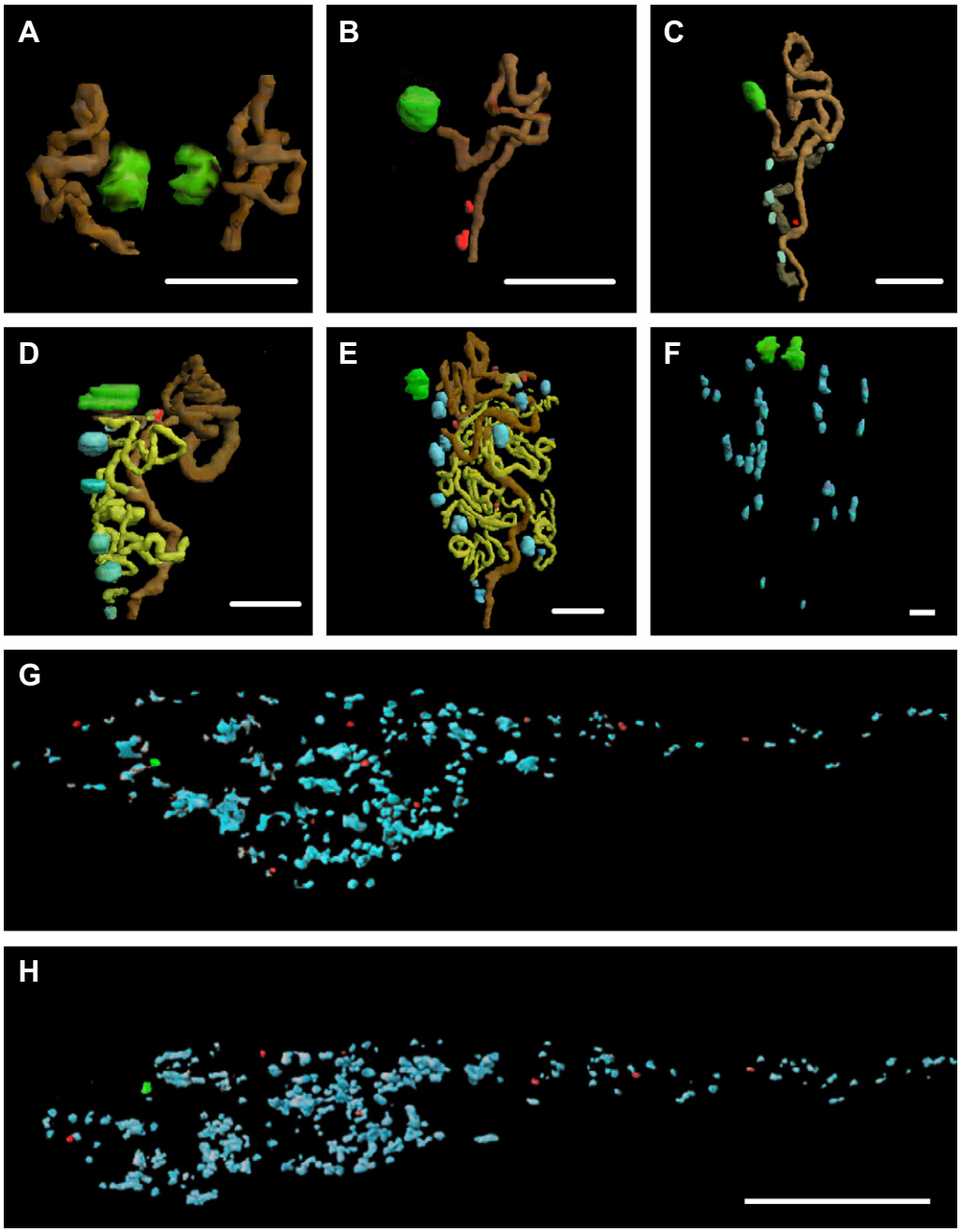

FIGURE 1.5 Three dimensional (3D) images of the nephron. Segments of the nephron are viewed by colorized 3D imaging: pronephric glomera, green; pronephric tubule and duct, brown; mature mesonephric glomeruli, blue; mesenchymal condensates and presumptive glomerular portion of the nephrogenic bodies, red; mesonephric immature tubules with a closed lumen, gray; mesonephric mature tubules with an open lumen, yellow. (A) Hatching stage. Bilateral pronephric nephrons are shown. (B) 5 dph. One or two (two in this case) mesenchymal condensates are observed. (C) 7 dph. Developing nephrons with a tadpole like shape are found. (D) 10 dph. Mature nephrons are found. (E) 15 dph. More than 10 mature nephrons have been formed. (F) 20 dph. Only mature glomeruli which count more than 30 on each side are indicated for bilateral kidneys. (G, H) 4 mph. Glomeruli and mesenchymal condensates are distributed throughout the entire length of the kidney. Anterior to the left. (A–F, H) Ventral views; (G) lateral view; (B–E, G, H) left kidneys. Scale bars: 100 μm [15]. Please see black and white figure on p. 9.

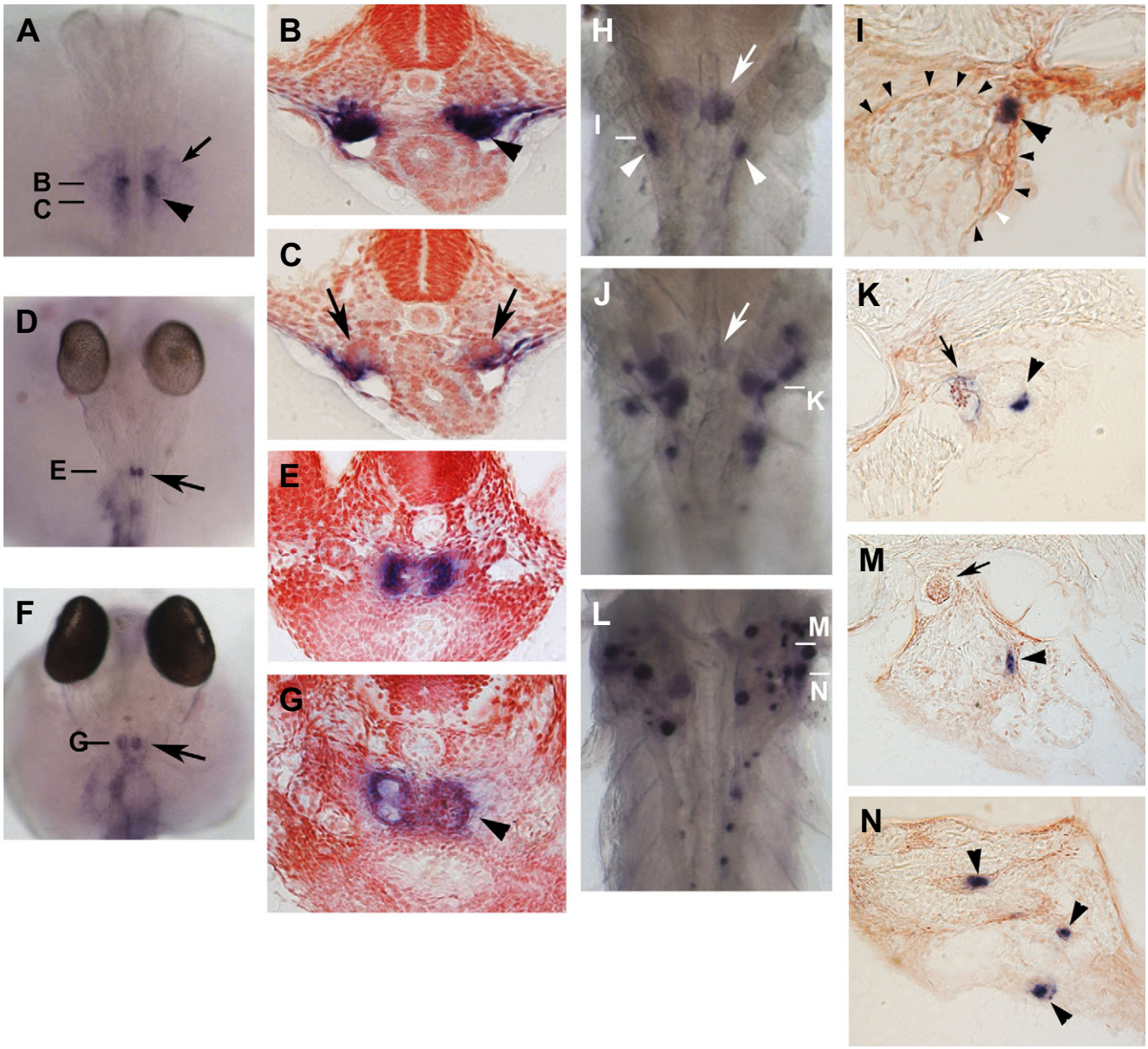

FIGURE 1.6 *wt1* expression in the developing kidney of medaka. (A–C) Stage 25 (18–19 somite stage). *wt1* mRNA is expressed exclusively in the small bilateral spots ventrolaterally to the notochord (arrowhead) and faintly in the surrounding tissues (arrow). (D, E) Stage 31 (gill blood vessel formation stage). The expression domain becomes more confined to the two spots corresponding to the pronephric glomus (arrow). (F, G) Stage 35 (stage at which visceral blood vessels form). The expression becomes limited to the podocytes (arrowhead). (H, I) 5 dph. The first mesonephric nephron progenitor positive for *wt1* appears at the caudomedial end of the pronephric sinus and duct area (white arrowheads in H and black arrowhead in I). White arrow: left pronephric glomus; small black arrowheads: the border of the renal tissue. (J, K) 10 dph. New spots of *wt1* expression appear (black arrowhead). The *wt1* expression of the first mesonephric nephron becomes weaker (black arrow). White arrow: left pronephric glomus. (L–N) 20 dph. The *wt1* expression spots increase (black arrowheads). The first mesonephric nephron has fully developed and lost *wt1* expression (black arrow). Cross sections (B, C, E, G, I, K, M, N) were made at the levels indicated by bars [15]. Please see black and white figure on p. 10.

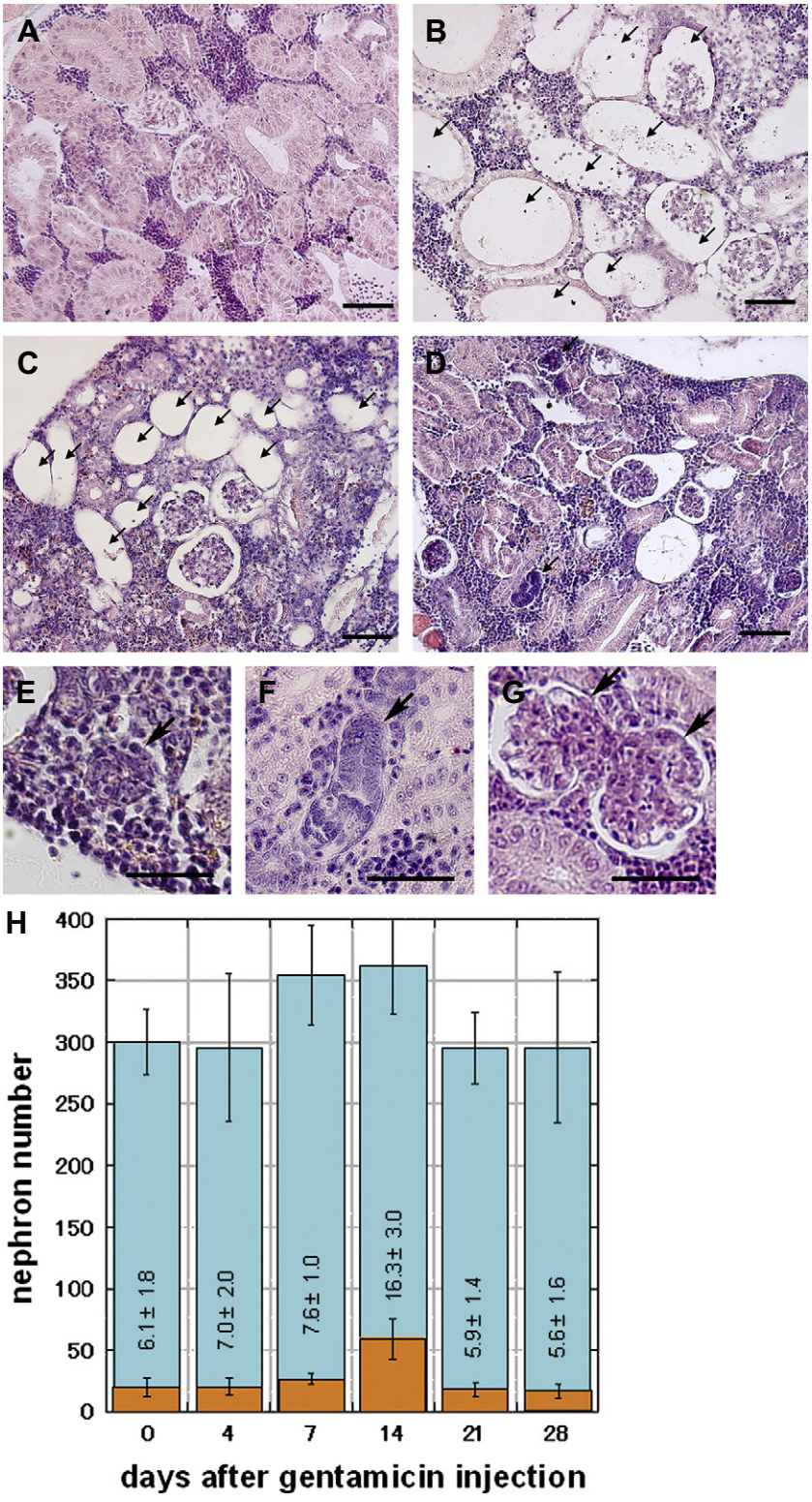

FIGURE 1.7 Histology of kidney regeneration in medaka. (A) Normal kidney (7 days after PBS injection to 3.5 mph medaka) (B–D) Damaged kidneys. Arrows indicate well developed cysts. (B) 3 days after gentamicin injection (daGa) to 3.5 mph medaka. (C) 7 daGa. (D) 14 daGa. (E–G) Magnified images of developing nephrons at 14 daGa. A mesenchymal condensate (E), a nephrogenic body (F) and mature glomeruli (G) are indicated by arrows. (H) Number of developing nephrons and total nephrons (means ± standard deviation). Orange bars: number of developing nephrons; blue bars: total number of nephrons. The ratios of developing nephrons to total nephrons is indicated above orange bars [28]. Please see black and white figure on p. 12.

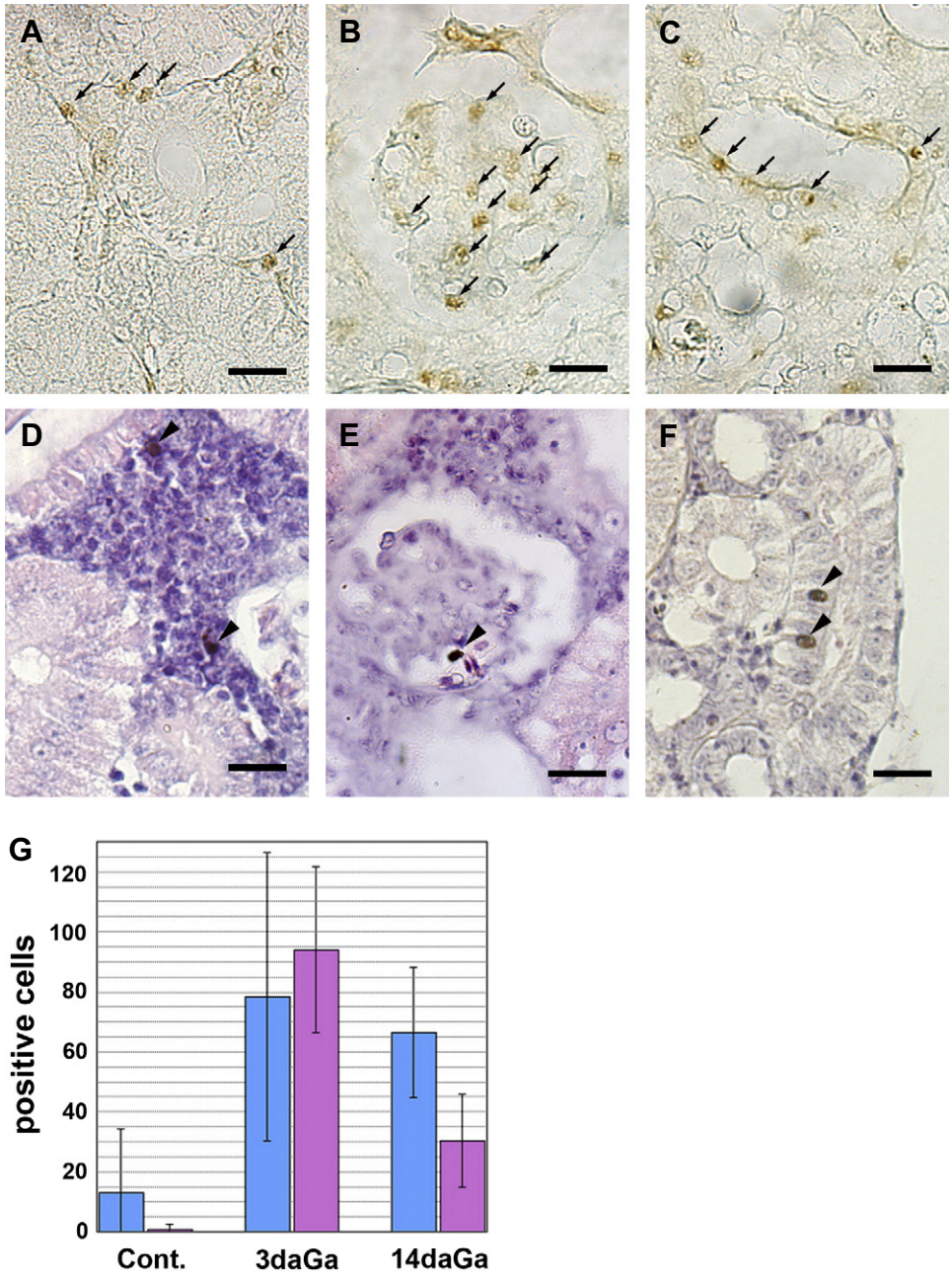

FIGURE 1.9 Detection of cell proliferation and apoptosis in the kidney. (A–C) Proliferating cells detected by PCNA labeling (arrows). (A) PCNA positive mesenchymal cells in the normal kidney. (B, C) The glomerular (B) and the tubular (C) cells positive for PCNA at 3 daGa. (D–F) Apoptosis detected by the TUNEL assay (arrowheads). (D) Apoptotic cells found in the mesenchyme of normal kidney. (E, F) TUNEL positive cells in the mature glomerulus (E) and tubular epithelium (F) at 14 daGa. Arrows indicate positive signals in the area described. Scale bars: 10 μm. (G) Statistics of proliferating and apoptotic cells. Proliferating cells (blue bars) increase significantly at 3 daGa in the area of the nephron (tubular and glomerular segments). Apoptotic cells (red bars) also increase significantly at 3 daGa and at 14 daGa in the nephron area. Bars indicate means ± standard deviation of four individuals. Significant differences are shown by asterisks (*t* test, $*p < 0.005$, $**p < 0.01$) [28]. Please see black and white figure on p. 14.

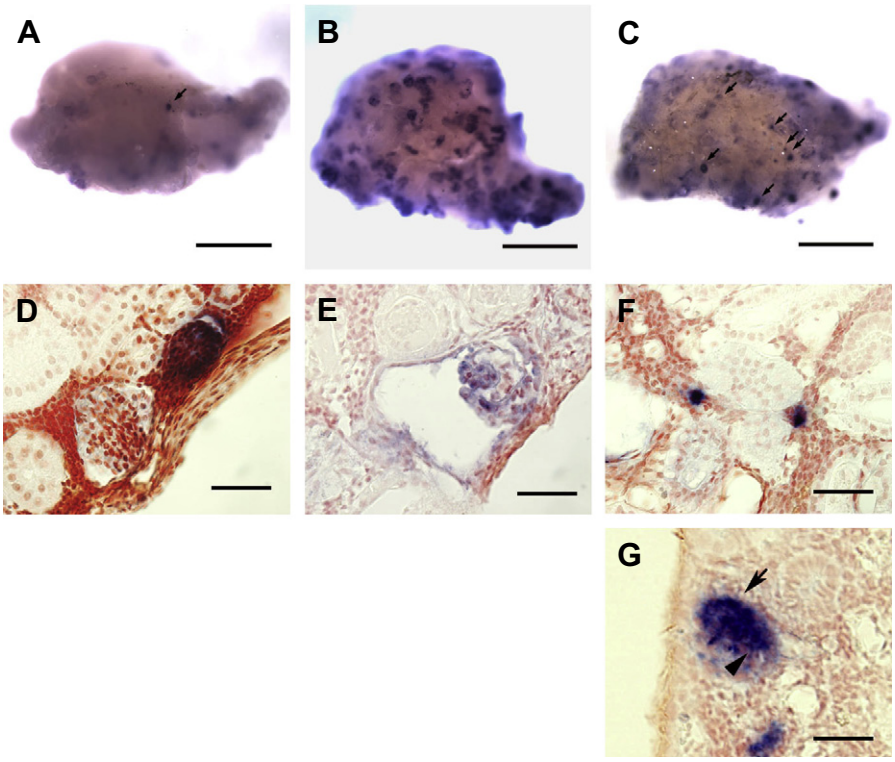

FIGURE 1.10 *wt1* expression in genta micin administered kidneys. (A–C) External views of the whole kidney stained by *wt1* in situ hybridization. Arrows indicate the *wt1* positive signals in the mesenchymal conden sates. (D–G) Plastic sections of *wt1* stained kidney. (A, D) Normal kidney at 3.5 mph. (B, E) 3 daGa. The mature nephrons become positive for *wt1*. (C, F, G) 14 daGa. Small spots of *wt1* positive cell masses corresponding to condensed mesenchyme (F) and nephrogenic bodies (G) appear in the mesenchymal tissues. The arrow indicates a presumptive glomerulus and the arrowhead indicates a presumptive tubule in (G). Scale bars: 0.5 mm (A–C); 20 μm (D–G) [28]. Please see black and white figure on p. 15.

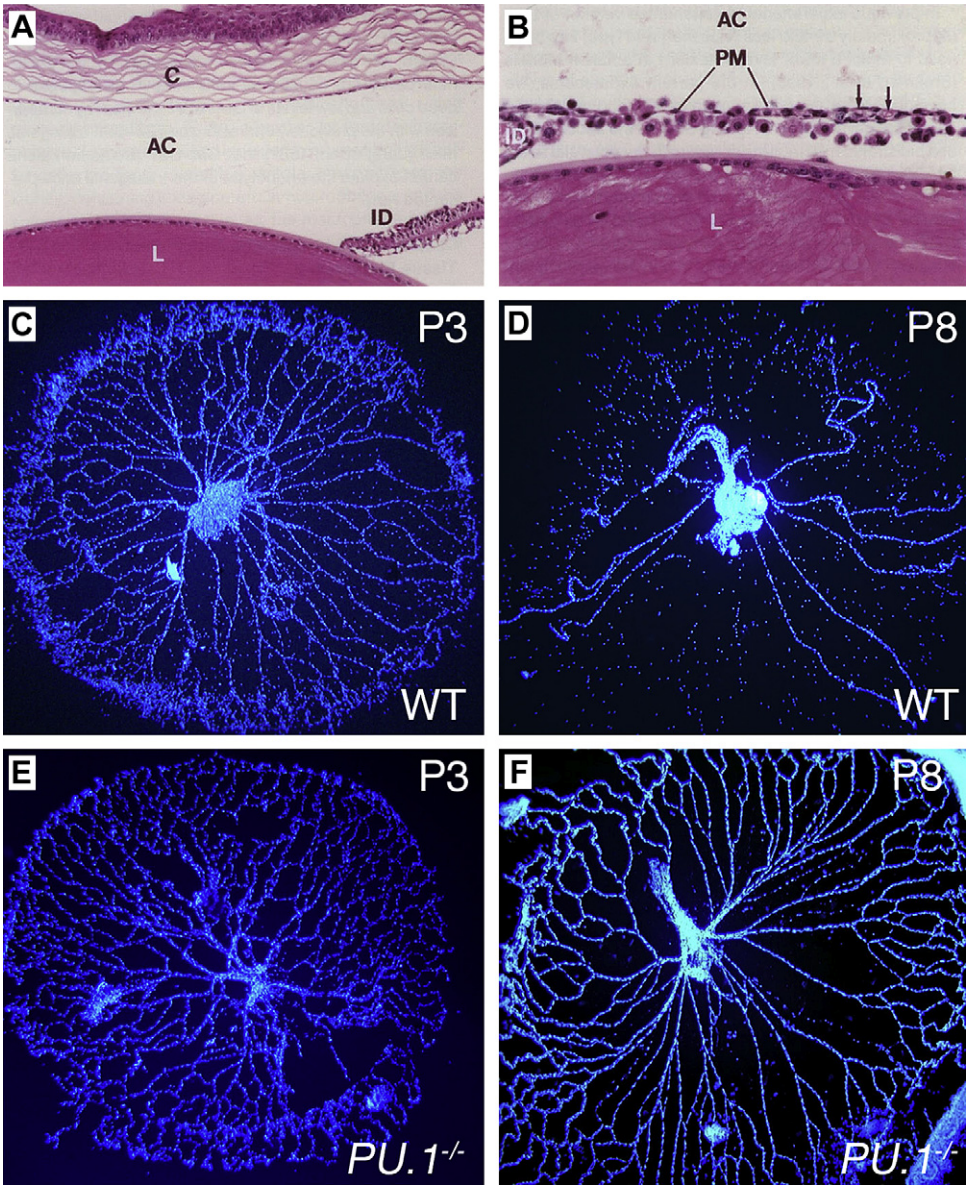

FIGURE 3.4 Effect of diphtheria toxin A (DTA) mediated ablation of hyaloid macrophages in granulocyte−macrophage colony stimulating factor (GM CSF) −DTA mice or macrophage deficiency in PU.1 mutant mice on scheduled regression of hyaloid vasculature in the postnatal eye. (A, B) Photomicrographs of the hyaloid vessels of (A) wild type mice and (B) mice lacking hyaloid macrophages due to expression of the GM CSF DTA transgene. (C, D) Whole mount images of hyaloid vessels in the eye showing scheduled loss of vasculature in the eye between P3 and P8 in wild type mice. (E, F) Scheduled loss of vessels does not occur in PU.1 mice which lack macrophages. *(Images provided by Richard Lang, Cincinnati Children's Hospital.)* Please see black and white figure on p. 41.

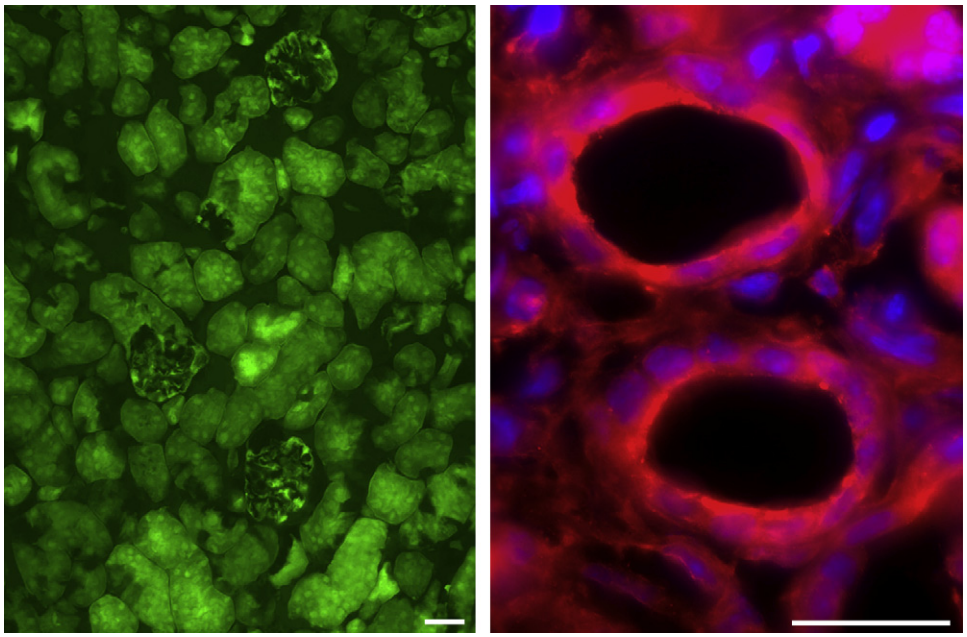

FIGURE 3.7 Fluorescent proteins as fate markers in the kidney. Low power image showing spontaneous GFP fluorescence in a 4 μm section of normal mouse kidney (left) from GFP mouse, and high power image from diseased kidney of dsRED mouse. Note that all cells are positive for the fluorophores. Scale bar 50 μm). Please see black and white figure on p. 44.

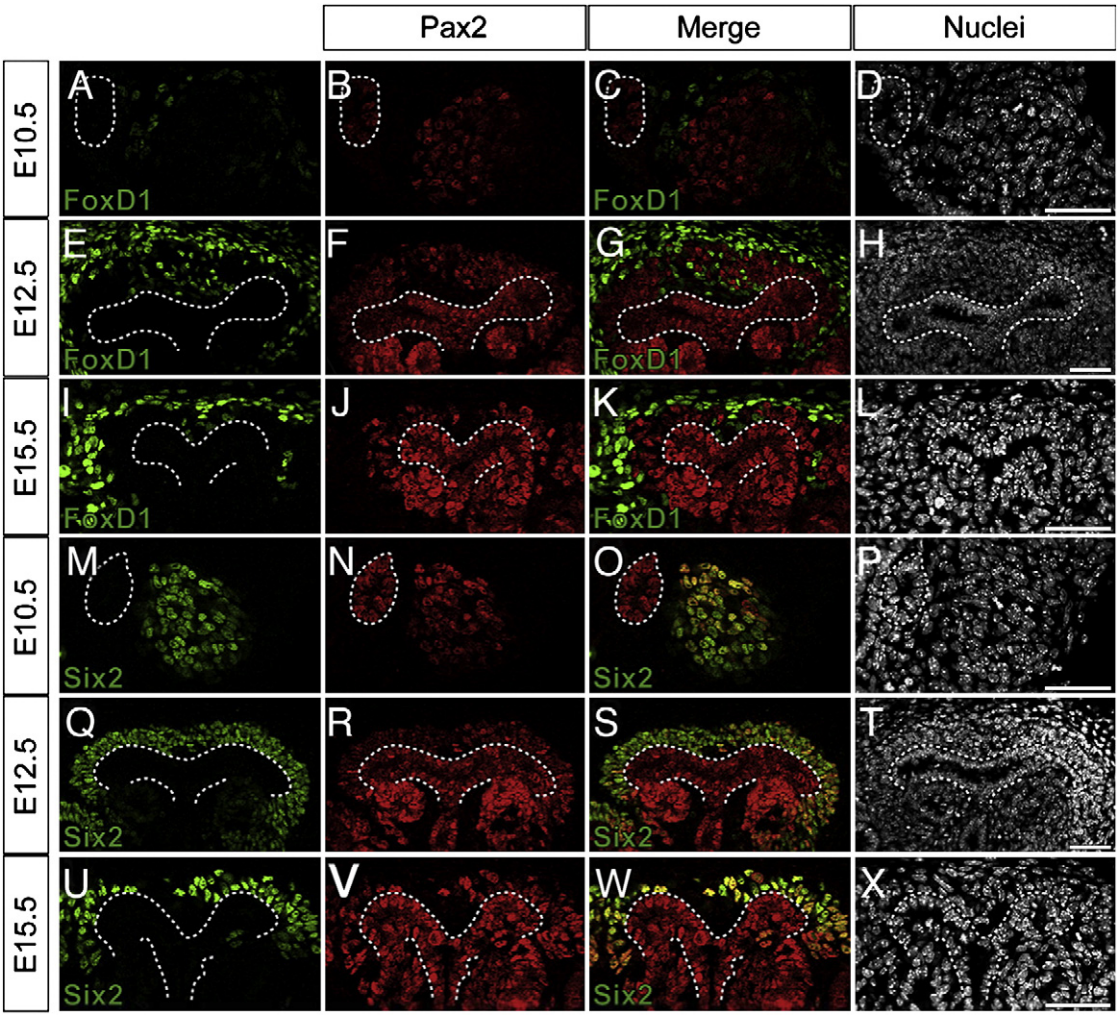

FIGURE 3.8 Mouse kidney progenitor cells of the metanephric mesenchyme from embryonic day 10.5 to 15.5 that give rise to all components of the nephron and the interstitium. Immunofluorescent confocal microscopy of (A–D, M–P) transverse sections of the E10.5 metanephric blastema, (E–H, Q–T) sagittal sections of E12.5 metanephric kidneys, and (I–L, U–X) frontal sections of E15.5 metanephric kidneys. Samples immunostained for Foxd1 (A, E, I), Pax2 (B, F, J, N, R, V) and Six2 (M, Q, U). Nuclei stained for Hoechst 33258 (D, H, L, P, T, X). Merged images (C, G, K, O, S, W). Dashed lines (A–X) indicate nephric duct epithelia. Scale bars 50 μm. *[From Mugford et al., 2008 [85], Figure 1.]* Please see black and white figure on p. 46.

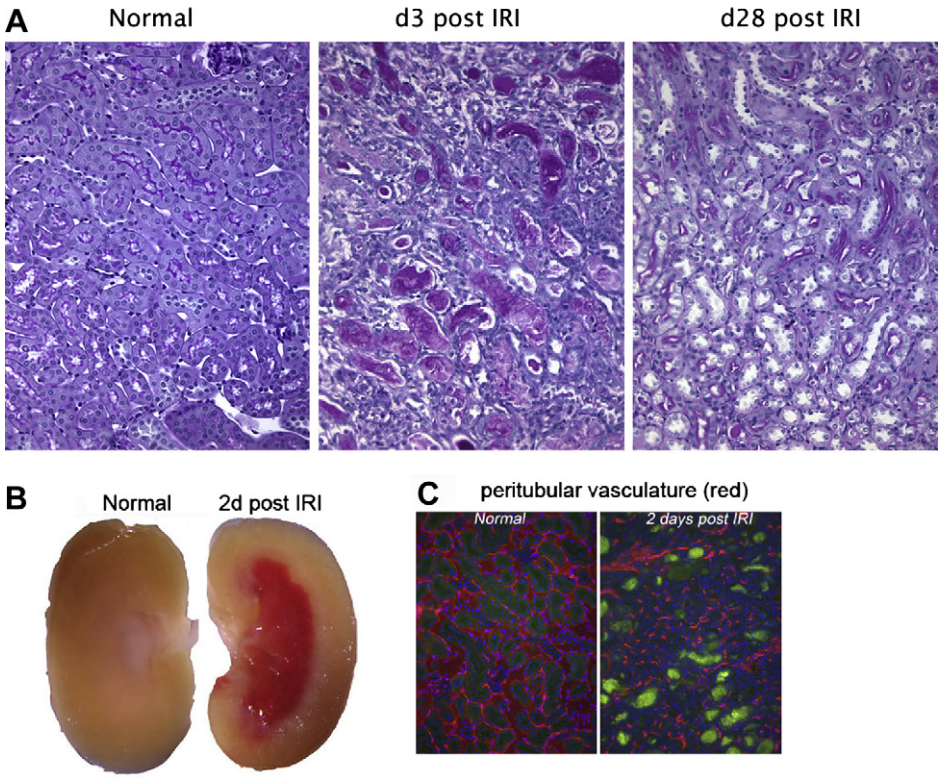

FIGURE 3.9 Injury and repair of the kidney structures. (A) Periodic Schiff stained kidney sections of outer medulla/inner cortex from normal adult kidney, d3 following ischemia–reperfusion injury (IRI) and d28 following IRI regenerated kidney. Note that there is severe injury and disruption to the tubules of the outer medulla but that at d28 there is near complete epithelial regeneration. (B) Whole mouse kidneys showing stasis of blood flow in the medulla 2 days after injury due to disruption of the peritubular vasculature. (C) Immuno fluorescence images of peritubular capillaries of the outer medulla (red) in normal kidney and 2 days following IRI. Note that there is severe disruption of the normal peritubular capillary network. Green shows autofluorescent necrotic debris in tubules. Please see black and white figure on p. 48.

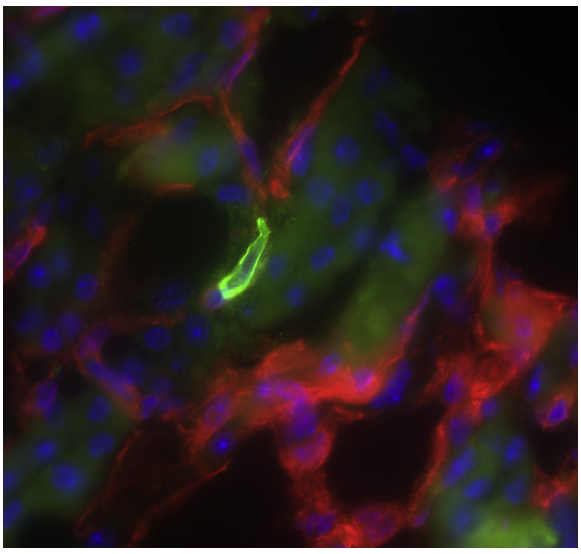

FIGURE 3.13 Human stem cells temporarily replace lost endothelial lost cells in bridging structures in the repairing kidney capillaries. Day 5 post ischemia–reperfusion injury kidney showing mouse endothelium (red) and human stem cell (green). Note that the human stem cell is bridging an area of denuded peritubular capillary. Please see black and white figure on p. 51.

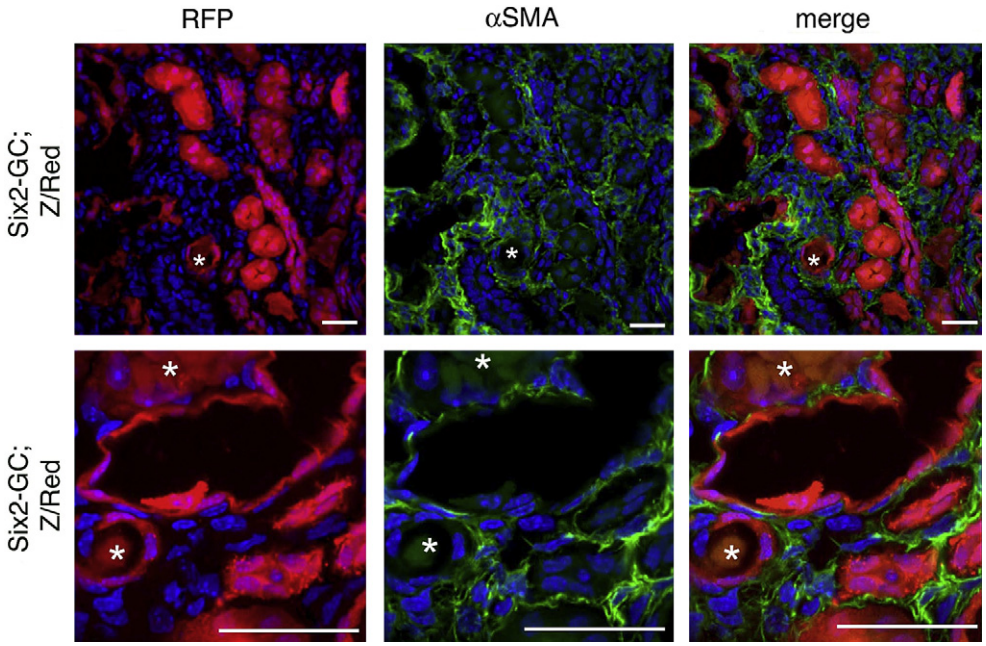

FIGURE 3.14 Fate tracing of epithelial cells of the nephron following ischemia−reperfusion injury. Low power (upper) and high power (lower) confocal images of kidney sections from *Six2 Cre;Z/Red* epithelial fate reporter mice, 15 days following ischemia−reperfusion injury. Kidney sections show native red fluorescent protein (RFP) fluorescence and are colabeled with the myofibroblast marker α smooth muscle actin (α SMA; green). In these mice Six2 is active during nephrogenesis and activates RFP expression in all cells that become tubule epithelium. More than 95% of adult tubule epithelial cells (except for collecting duct) are labeled with RFP permanently regardless of fate. Note that despite widespread loss of kidney epithelium the regenerated epithelium remains 100% positive for the fate marker RFP, indicating that regeneration of the epithelium is by epithelial cells, not cells from outside the epithelium. In addition, note that despite large numbers of α SMA+ myofibroblasts appearing in the interstitium, none of them derives from epithelial cells. * denotes intratubular debris. Please see black and white figure on p. 53.

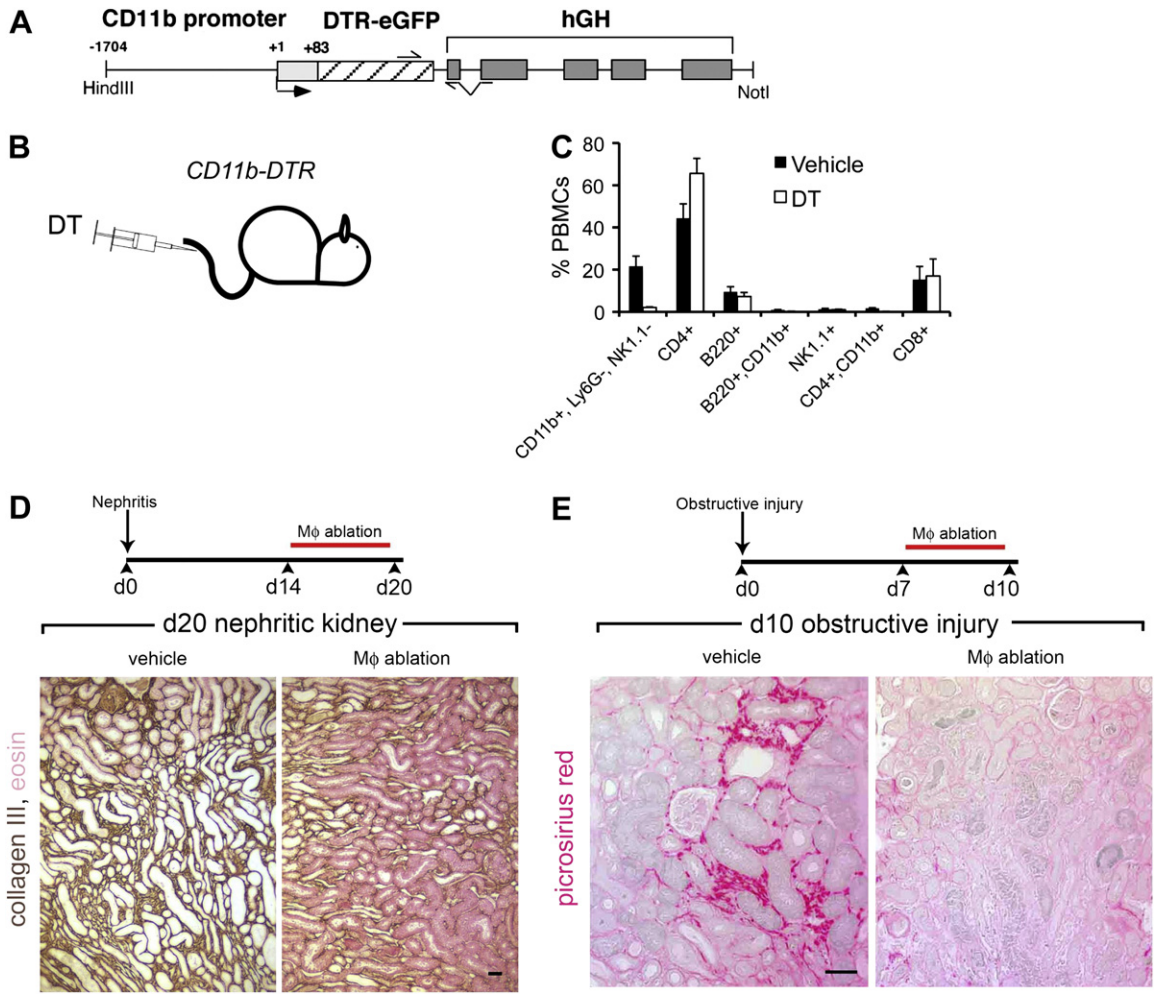

FIGURE 3.16 The diphtheria toxin receptor (DTR) system to study monocyte derived cells in kidney disease. (A) Schematic of the CD11b DTR transgene. Note that the transgene is a fusion of the DTR with enhanced green fluorescent protein (eGFP) but that eGFP is not visible without antibody enhancement. (B) Intravenous (i.v.) or intraperitoneal (i.p.) injection of diphtheria toxin (DT) is required to bring about monocyte and macrophage ablation. (C) Graph showing the percentage of human peripheral blood mononuclear cells (PBMCs) 24 h after vehicle or DT i.v. injection in CD11b DTR mice. Note that DT selectively ablates the $CD11b^{+}$, Ly6G , NK1.1 cells which are monocytes. Neutrophils (not shown) are not affected. (D) Effect of ablation of monocytes/macrophages on tubulointerstitial disease and fibrosis in the nephrotoxic nephritis model of crescentic glomerulonephritis. (E) Effect of ablation of monocytes/macrophages on fibrosis in the ureteral obstructive model of inflammation with fibrosis. Scale bar 50 μm. Please see black and white figure on p. 55.

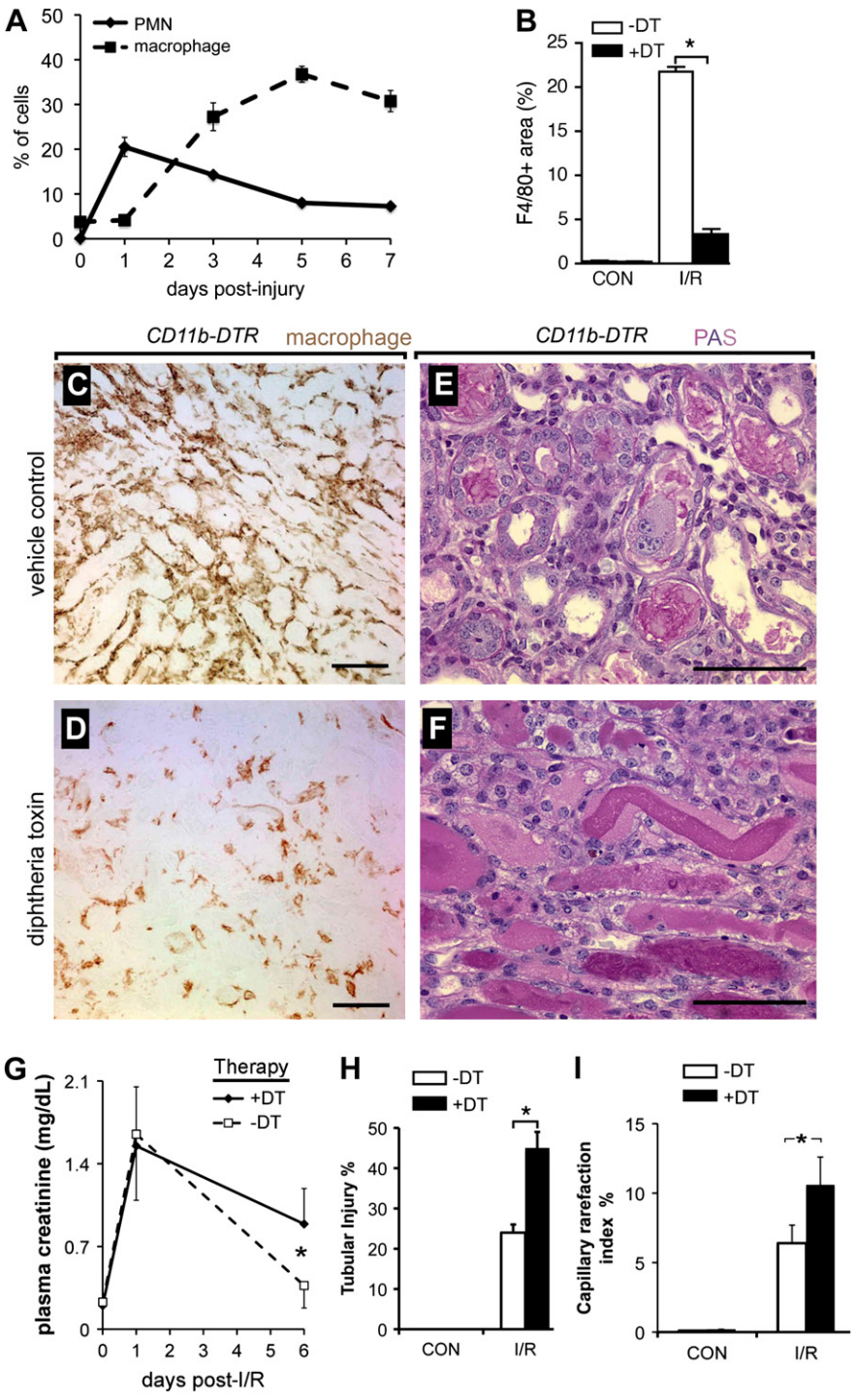

FIGURE 3.17 Macrophages are recruited during repair after ischemia–reperfusion injury (IRI) and ablation of macrophages prevents normal repair and regeneration. (A) Graph showing time course of recruitment of neutrophils and monocytes/macrophages to the kidney after IRI. (B–I) Monocytes/macrophages were ablated by diphtheria toxin (DT) injection or vehicle injection in *CD11b DTR* mice from d3 to d6 post IRI. (B) Quantification of macrophages by immunostaining and morphometry on d6 post IRI. Note marked ablation by DT. (C–E) Macrophage immunostain and periodic acid–Schiff (PAS) stain of kidneys d6 post IRI following ablation or no ablation. Please see black and white figure on p. 56.

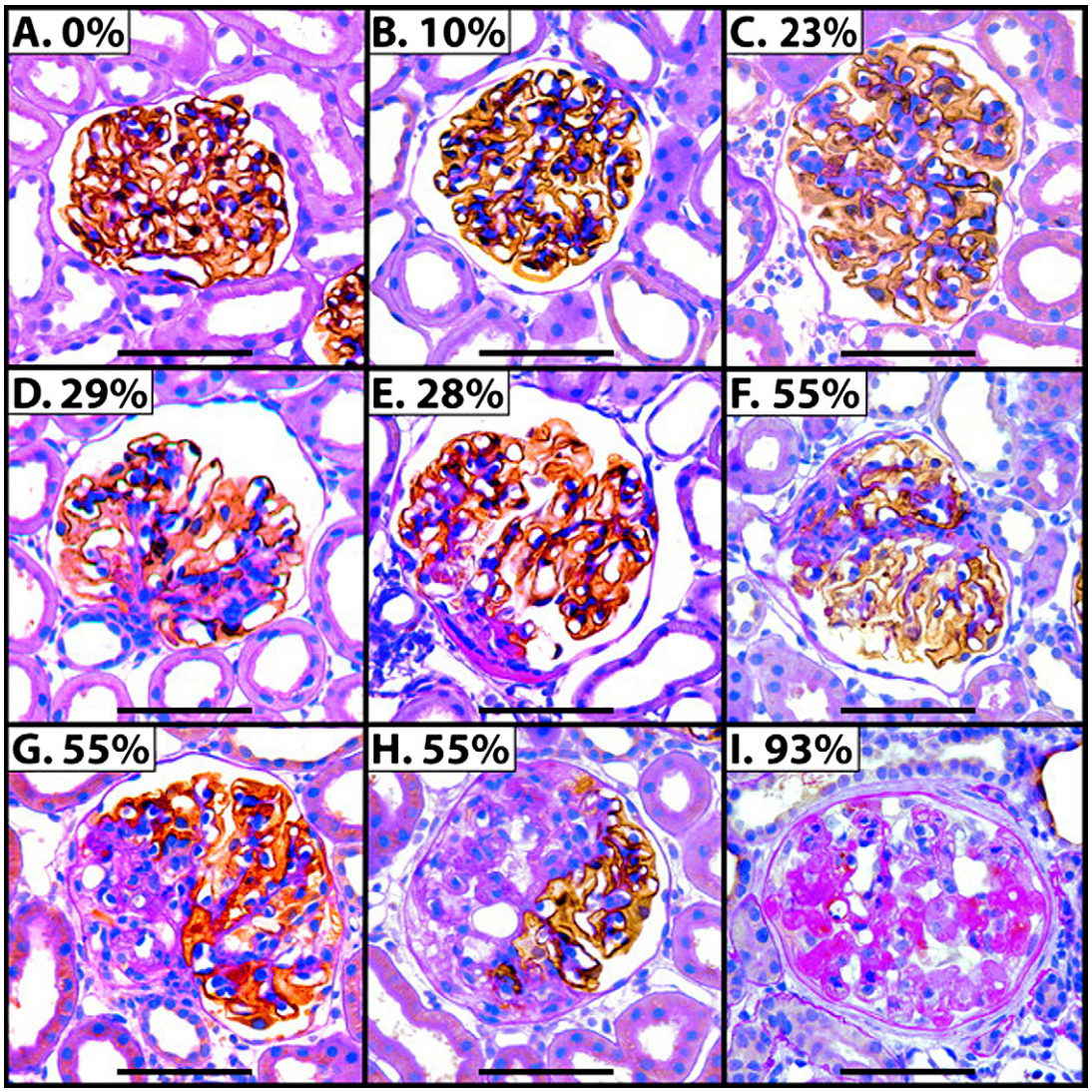

FIGURE 3.18 Effect of selective podocyte ablation on glomerular pathology. Ablation of podocytes by single diphtheria toxin (DT) injection (variable dosing) in *podocin DTR* transgenic rats viewed 28 days later. Podocytes are labeled with Glepp1 (brown) and costained with periodic acid−Schiff (PAS). Note that with 10−30% podocyte ablation there is mesangial hyperplasia with persistent covering of capillary tufts by podocytes but that there are fewer podocytes. Greater than 30% podocyte ablation results in focal segmental glomerulosclerosis or global sclerosis. *[From Wharram et al., 2005 [154], Figure 6.].* Please see black and white figure on p. 58.

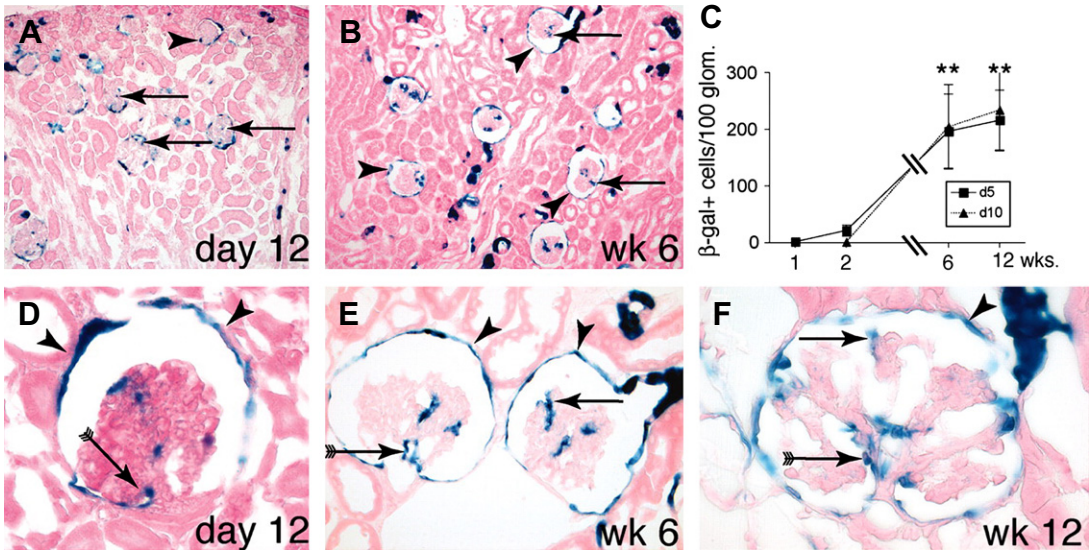

FIGURE 3.19 Lineage tracing of parietal epithelial cells using an inducible Tet inducible Cre LoxP triple transgenic system. pPEC rtTA/LC1/R26R triple transgenic mice were pulsed with tetracycline 5 days postpartum, which resulted in about 75% heritable labeling of PECs at d12 (A) and some other cells in the cortex. By 6 weeks however, there was increasing detection of blue stain podocytes (B, C) which can be seen in images shown in D–F. *[From Appel et al., 2009 [157], Figure 7.]* Please see black and white figure on p. 59.

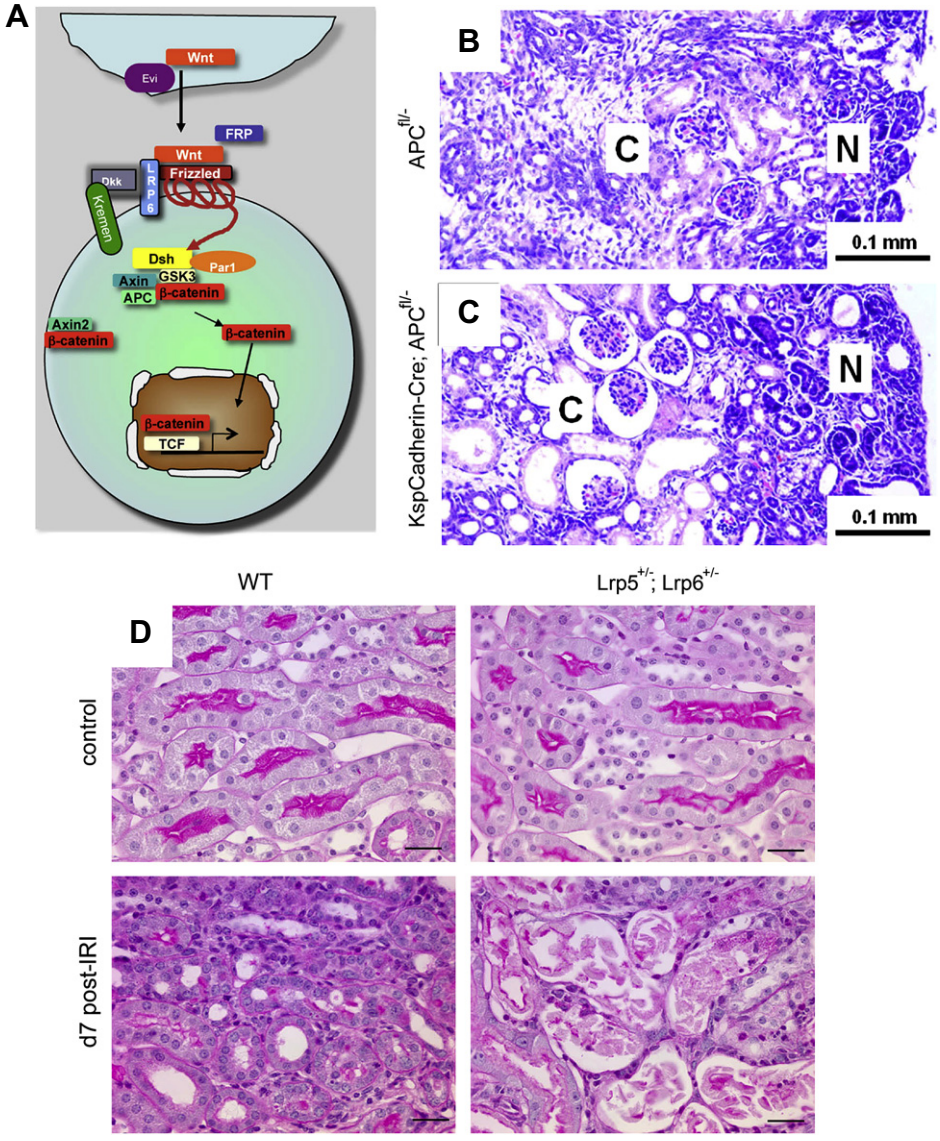

FIGURE 3.20 Canonical Wnt signaling pathway and its role in renal cyst formation and epithelial repair after injury. (A) Cartoon showing a simplified version of the canonical Wnt signaling pathway. (B, C) Newborn kidneys from mice lacking antigen presenting cells in kidney epithelium (C) or controls (B). *[Reproduced from Qian et al., 2005 [164].]* (D) Although adult kidneys of compound heterozygotes at the Lrp5 and Lrp6 loci are normal, epithelial repair after injury is severely impeded at d7 after ischemia−reperfusion injury. Please see black and white figure on p. 61.

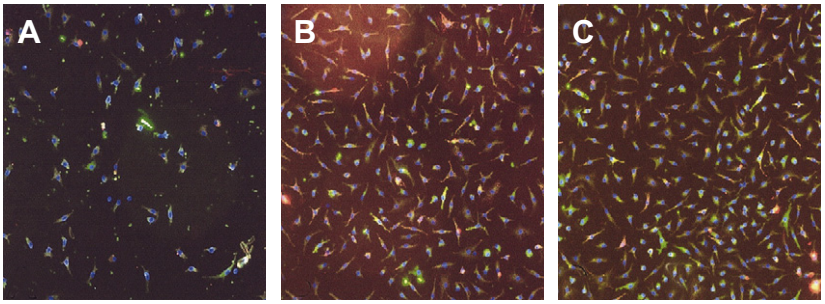

FIGURE 5.1 Endothelial progenitor cells (EPCs) in patients with chronic kidney disease (A) before and (B) after treatment with a standard dose of recombi nant human erythropoietin (EPOetin beta). (C) For comparison, EPCs in a healthy control subject. Please see black and white figure on p. 95.

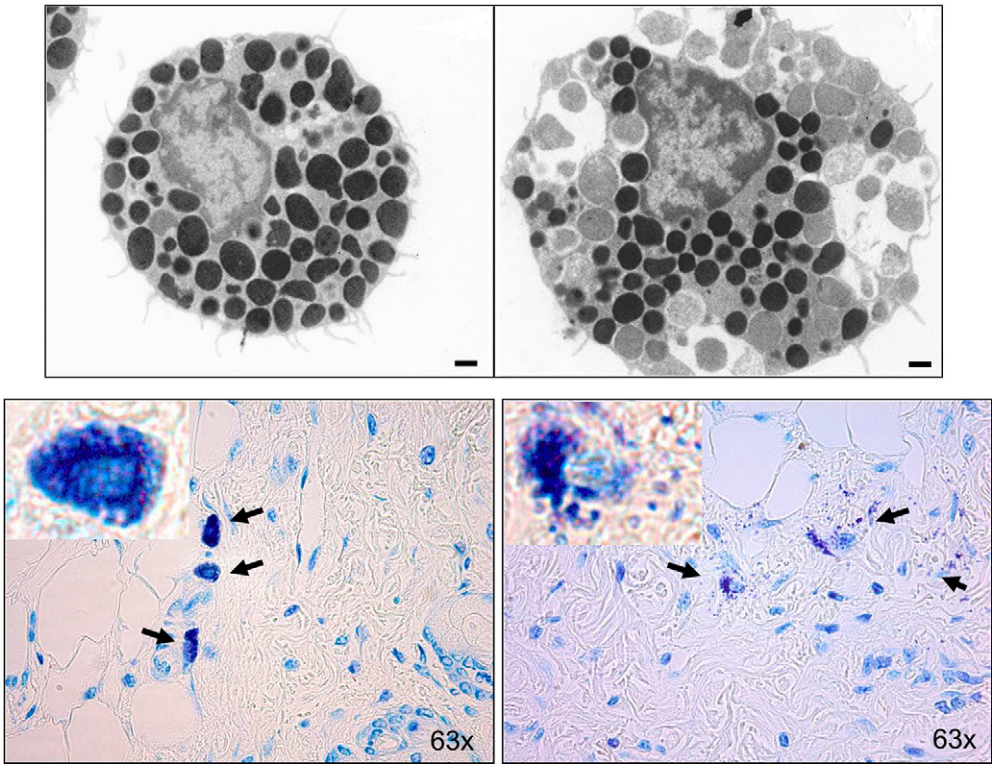

FIGURE 6.1 Resting and stimulated mast cells (MCs). The top panel shows electron micrographs of a resting (left) and stimulated (right) rat peritoneal MC. Scale bar 1μm. (Courtesy of G. Zabucchi and Maria Rosa Soranzo, University of Trieste, Italy.) Resting cells contain numerous cytoplasmic secretory granules that are filled with electron dense material. Stimulated cells have undergone partial exocytosis of granular content by a process implying granule−granule and granule plasma membrane fusion. The bottom panel shows granulated and degranulated mouse connective tissue type MCs in kidney capsules stained with toluidine blue. Note that in degranulated cells, the expelled toluidine blue staining granular matrix can be seen in the tissue surrounding the MC. Inset shows higher magnification (100×) of one of the MCs. Please see black and white figure on p. 105.

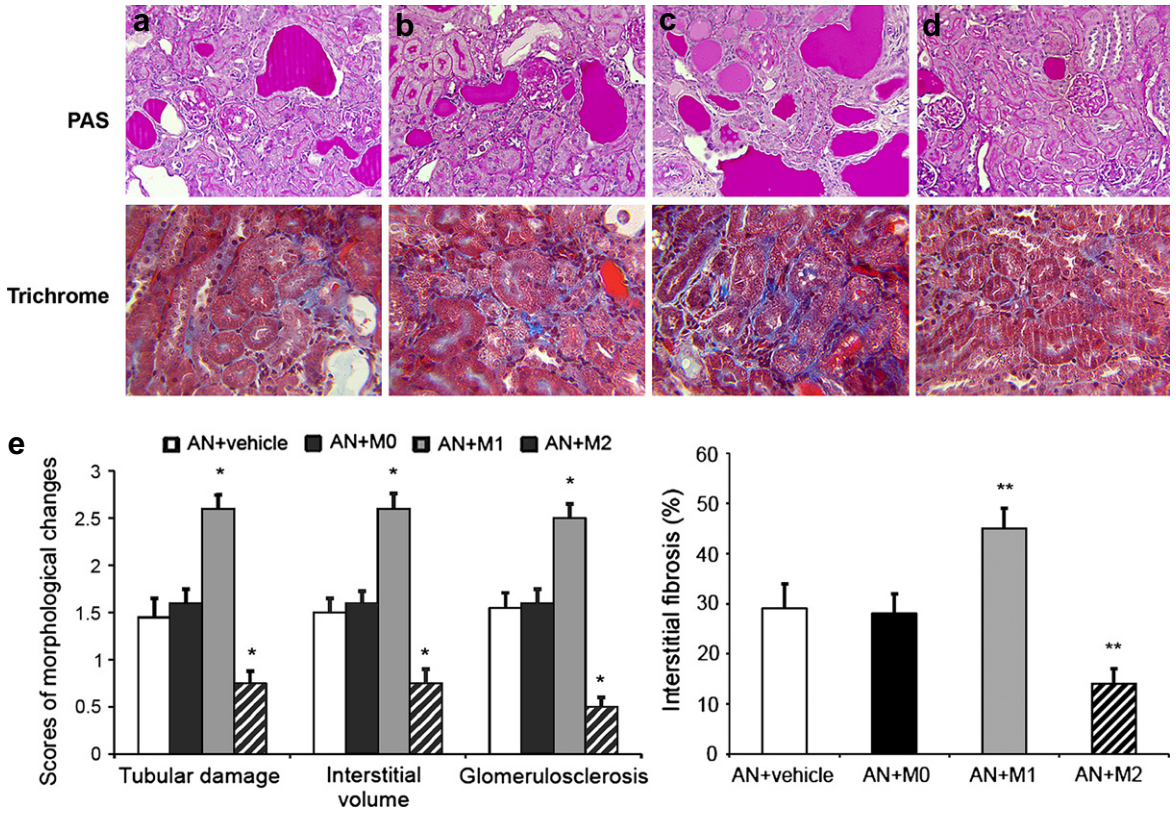

FIGURE 7.1 Effect of M1 and M2a on renal injury and fibrosis. Effect of M0, M1 and M2a macrophage transfusion on histological injury and development of renal fibrosis. (a–d) Representative periodic acid–Schiff and trichrome stained sections of renal cortices at day 28 (original magnification 200×): (a) saline injected Adriamycin nephrosis mice; (b) AN mice transfused with M0 macrophages; (c) AN mice transfused with M1 macrophages; (d) AN mice transfused with M2a macrophages. (e) Kidney injury was assessed semi quantitatively [periodic acid–Schiff (PAS)] and for fibrosis by point counting (trichrome). Each evaluation represents the mean ± standard error of the mean. $*p < 0.05$ vs other three groups (n 7 per group); $**p < 0.05$ vs other two groups. *(Reproduced with permission from Wang et al., 2007 [11].]* Please see black and white figure on p. 130.

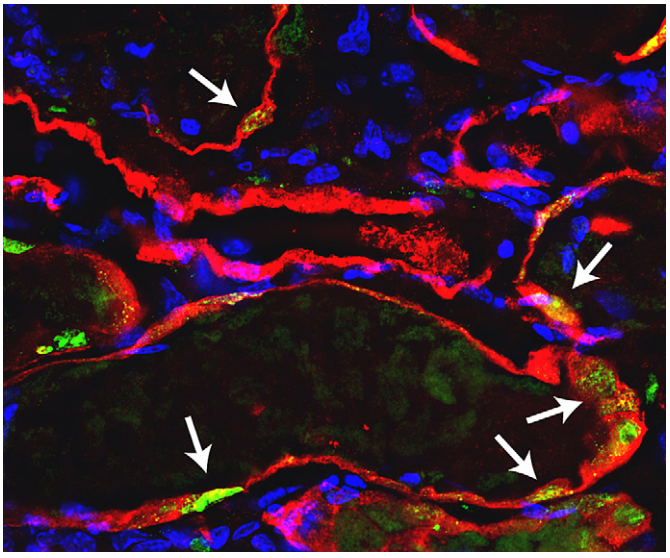

FIGURE 9.1 Kidney repair after ischemia–reperfusion injury (IRI) occurs by proliferation of surviving epithelial cells. Section from a kidney of a mouse with red fluorescent protein (RFP) expression in all epithelial cells 48 h after IRI shows that flattened, dedifferentiated epithelial cells have re entered the cell cycle, as judged by expression of nuclear Ki67 (arrows). Because there was no dilution of the RFP fate marker, mesenchymal stem cells do not directly contribute to epithelial repair after IRI [55]. Please see black and white figure on p. 157.

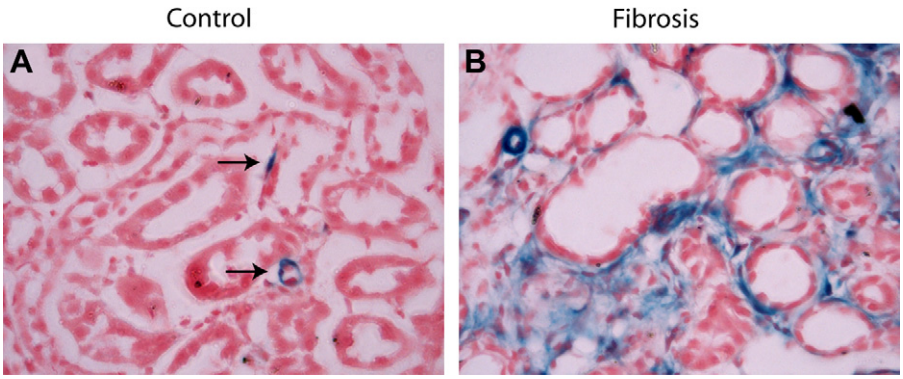

FIGURE 9.4 Lineage tracing perivascular fibroblasts in fibrotic kidney disease. Pericytes/perivascular fibroblasts were genetically labeled by administering Tamoxifen to bigenic mice expressing Tamoxifen regulated CreERt2 recombinase under control of the FoxD1 promoter, as well as a LacZ reporter gene that is activated by Cre recombinase. (A) Labeled cells appear blue in the renal interstitium. (B) After ureteral obstruction, there is dramatic expansion of LacZ positive interstitial cells throughout the fibrotic kidney. These cells gain expression of α smooth muscle actin, indicating that they are myofibroblasts. Whether a subset of pericytes, which are myofibroblast progenitors, might represent kidney specific mesenchymal stem cells requires further investigation [99]. Please see black and white figure on p. 162.

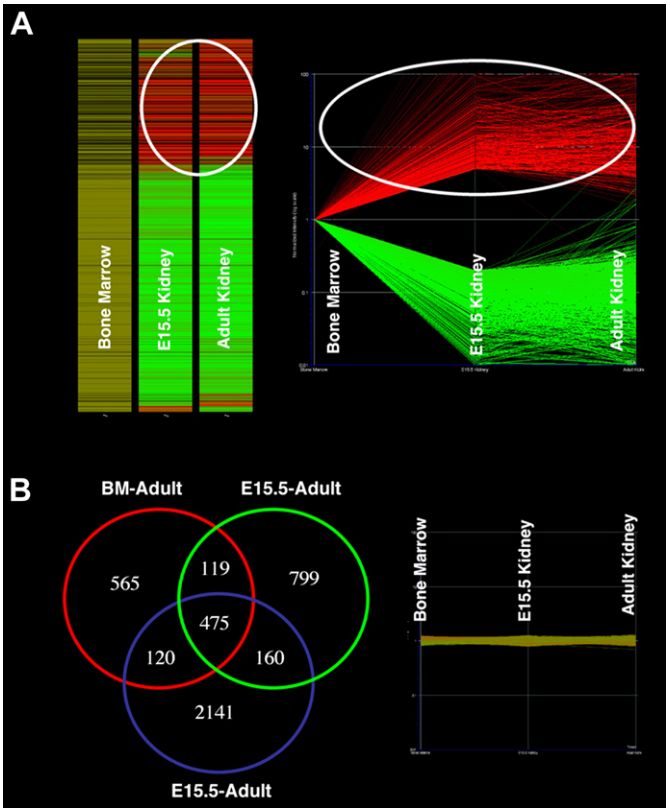

FIGURE 11.1 Affymetrix microarray data analysis. (A) Heat map of Affymetrix gene expression in bone marrow, adult and embryonic kidney shows high congruence between kidney samples. Right hand panel shows relative expression of all genes in kidney side population (SP) compared to bone marrow SP with red representing strongly upregulated and green representing strongly downregulated kidney SP genes. (B) Identification of "common SP genes" or a putative SP molecular signature by determining the genes with less than 25% variance in signal between bone marrow, E15.5 and adult kidney SP samples. The Venn diagram demonstrates a common signature of 475 genes. The right hand panel illustrates the relative expression of these 475 genes across all three samples. Please see black and white figure on p. 179.

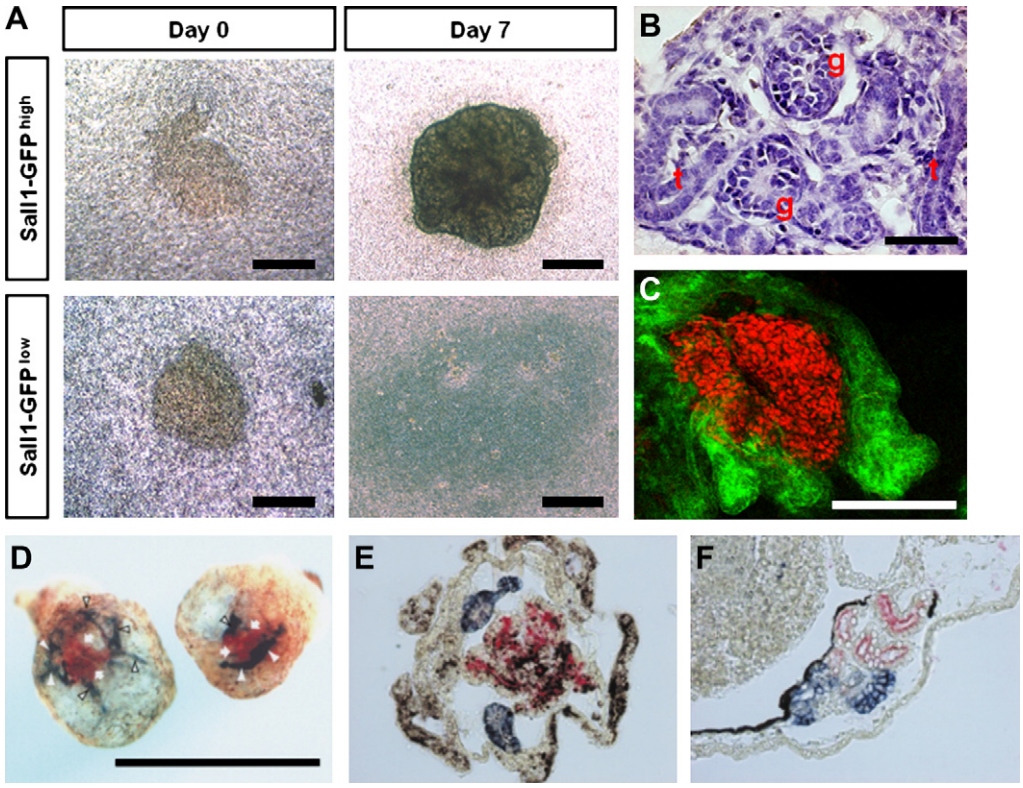

FIGURE 13.1 In vitro generated kidneys. (A–C) Metanephric kidney formed in vitro from multipotent progenitor cells in embryonic mouse kidney. (A) Metanephric cells strongly expressing *Sall1* (Sall1 GFP^{high} cells; upper panels) that contain multipotent progenitors differentiate into kidney structure in an organ culture setting, while those weakly expressing *Sall1* (Sall1 GFP^{low} cells; lower) disappear. (B) Hematoxylin & eosin staining of sections of kidney structure formed in vitro from Sall1 GFP^{high} cells. Tubule (t) and glomerulus like structures (g) are seen. (C) Double staining with WT1 (red, podocyte marker) and *Lotus tetragonolobus* lectin (LTL, green, proximal tubule marker) of kidney formed from Sall1 GFP^{high} cells. (D–F) Pronephric kidney formed in vitro from an undifferentiated cell mass in amphibian eggs (animal cap). Double immunostaining with pronephric tubule specific antibody 3G8 (red) and pronephric duct specific antibody 4A6 (blue) of kidney structure formed in animal cap treated with activin A and retinoic acid (D, E) and a stage 40 *Xenopus* larva (F). (D) Whole mount staining. (E, F) Section staining. Scale bars: (A) 500 μm, (B, C) 25 μm, (D) 1 mm. Please see black and white figure on p. 205.

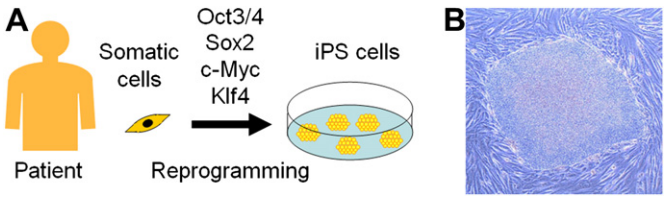

FIGURE 13.2 (A) Induced pluripotent stem (iPS) cells can be established by introducing genes encoding four transcription factors (Oct3/4, Sox2, Klf4 and c Myc) into fibroblasts. (B) An iPS cell colony generated from adult human dermal fibroblasts by introducing the four factors using retroviral vectors. Please see black and white figure on p. 207.

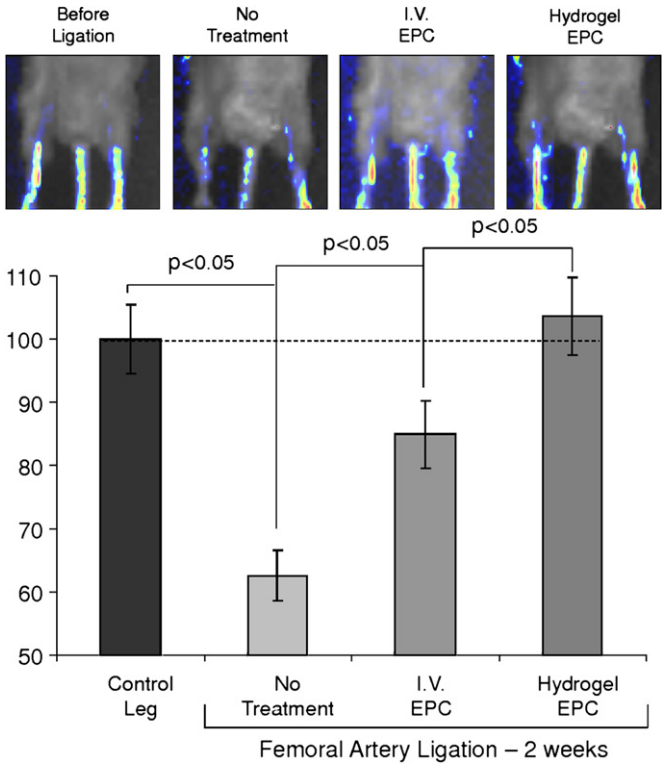

FIGURE 16.3 Femoral artery ligation is performed in mice that are then treated with endothelial progenitor cells (EPCs) through either tail vein injection or hydrogel implantation. (A) Images of laser Doppler perfusion on the site of ligation after 2 weeks in mice given different treatments. (B) Laser Doppler flowmetry data (percentage perfusion) in the ligated vs the control hindlimb after 2 weeks. Please see black and white figure on p. 253.

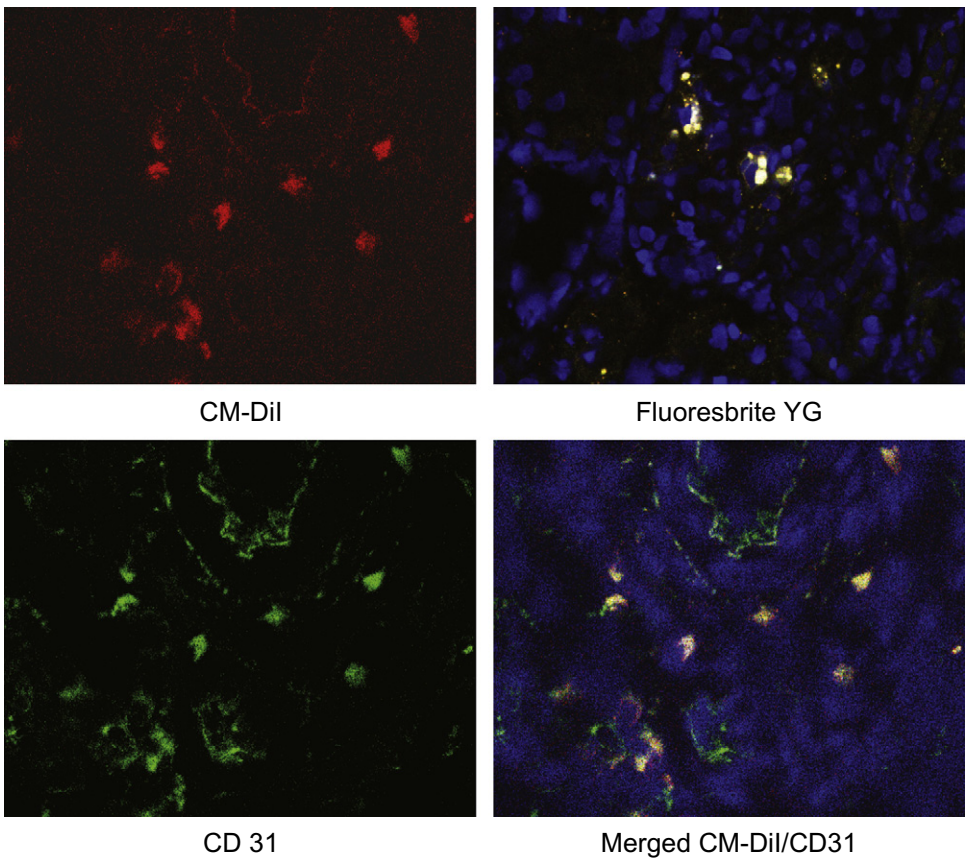

FIGURE 17.2 Top: Representative images of endothelial progenitor cells (EPCs) labeled with fluorescence dye (CM DiI, red, left) and yellow−green (YG) microspheres (right) in the kidney 4 weeks after transplantation. Bottom: Representative merged images of CM DiI labeled EPCs and the same kidney immunostained for the endothelial marker CD31, indicating transdifferentiation of EPCs to vascular endothelial cells. Please see black and white figure on p. 259.

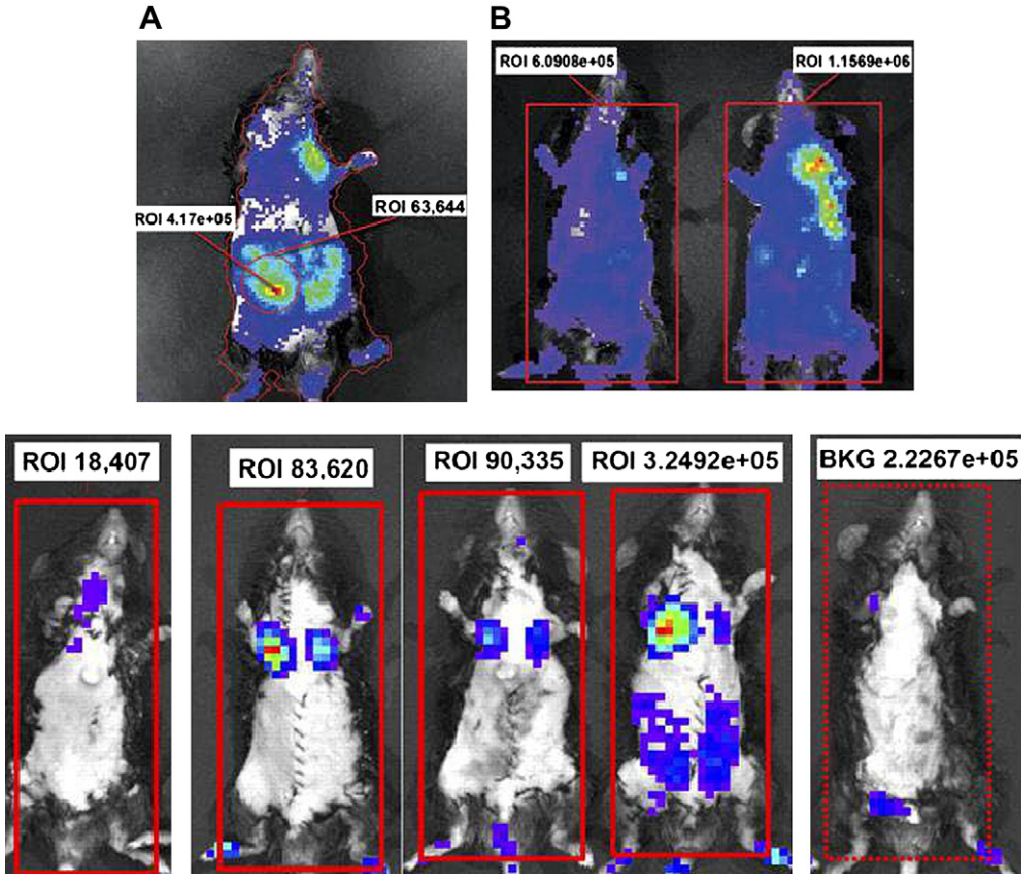

FIGURE 17.3 Optical imaging of mesenchymal stem cells (MSCs) in the kidney. (A) Immediately after intra arterial infusion, animals with acute kidney injury (AKI) showed distinct accumulation of cells in kidneys (as shown by green/red areas). (B) Normal animals show diffuse whole body distribution with eventual accumulation in the lungs in some animals. Bottom: Intravenous injection in a normal animal (first on the left) showed accumulation of MSCs in the lungs. In the middle panels three different AKI animals showed accumulation of cells in the lungs immediately after injection, and only one animal with AKI showed retroperitoneal uptake indicating cell localization in the kidneys. *[Adapted from Tögel et al., 2008 [38] with permission.]* Please see black and white figure on p. 261.

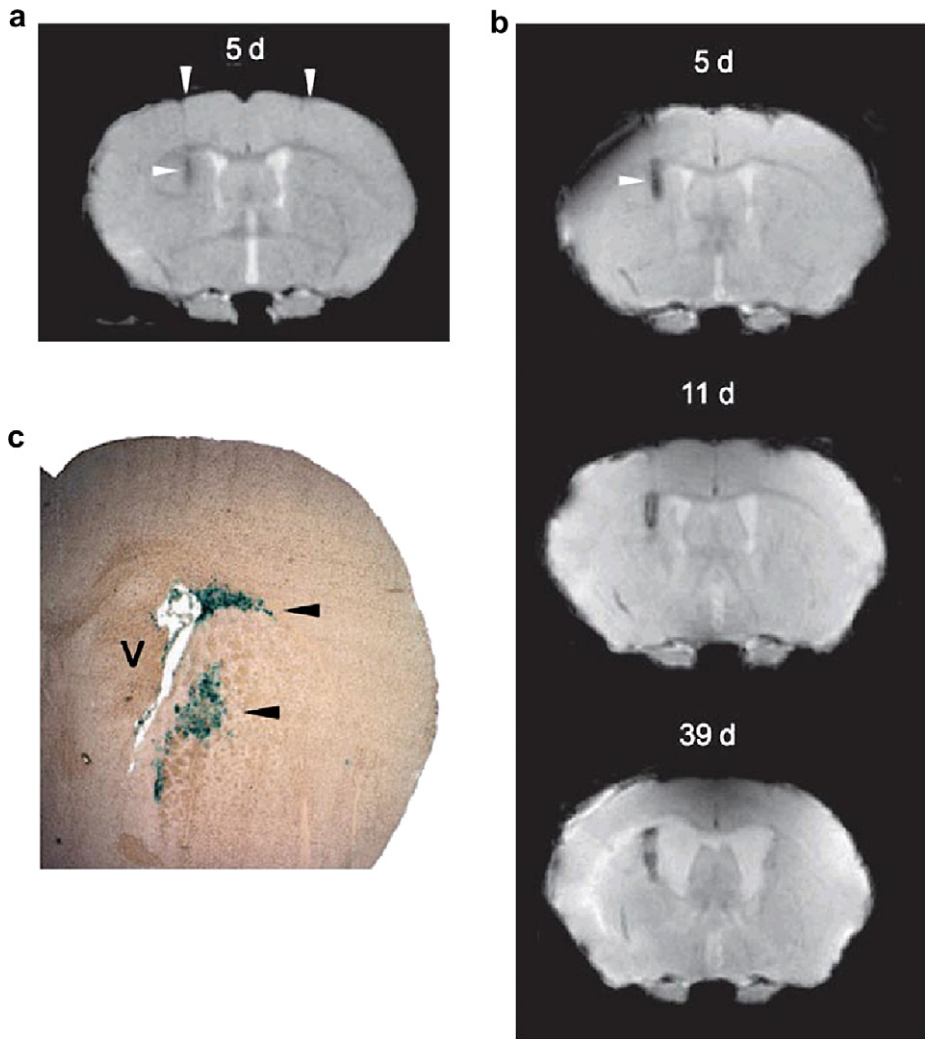

FIGURE 17.4 In vivo longitudinal monitoring of magnetic resonance imaging (MRI) reporter gene expression in the mouse brain. (a) T2 weighted image 5 days after injection, showing the inoculated site (left arrow). (b) Time lapse T2* weighted images obtained 5, 11 and 39 days after injection. (c) X gal stained image showing histological pattern similar to MRI. *[Adapted from Genove et al., 2005 [54] with permission.]* Please see black and white figure on p. 263.

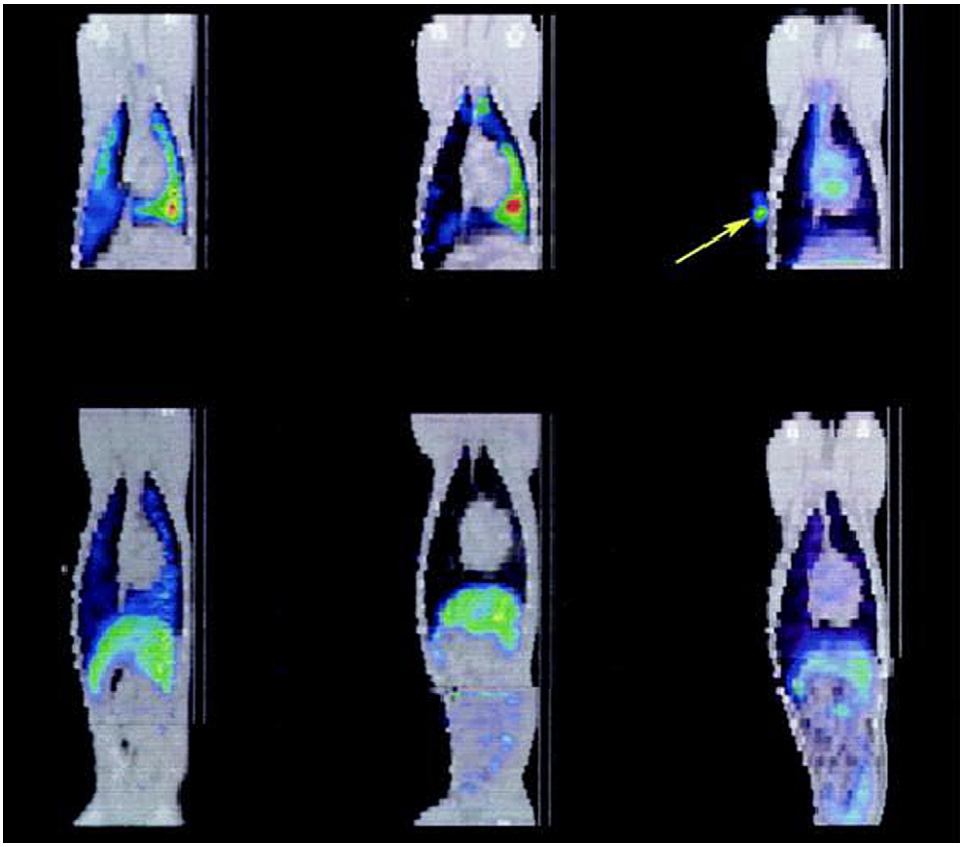

FIGURE 17.6 Coronal fused single photon emission computed tomography (SPECT, color) and computed tomography (CT, gray scale) images of a dog with (top left) and without (top middle) myocardial infarction (MI) during the first hours after intravenous injection of ^{111}In oxine labeled mesenchymal stem cells (MSCs), showing predominant lung uptake with increased uptake to the dependent left lung (green−yellow color towards right). In a dog with MI that received ^{111}In oxine without MSCs (top right), the tracer behaves primarily as a blood pool agent, with uptake visible in left and right ventricles of the heart. A reference marker (arrow) containing ^{111}In oxine was placed on the chest wall on the dog that did not receive MSCs (top right). Redistribution of ^{111}In oxine labeled MSCs to predominantly the liver occurs at 24 h after intravenous injection in both a representative infarcted (bottom left) and non infarcted (bottom middle) dog. In an infarcted dog injected intravenously with ^{111}In oxine only (i.e. no MSCs), a similar pattern of redistribution to the liver is observed at 24 h after injection (bottom right). *[Adapted from Kraitchman et al., 2005 [66] with permission.]* Please see black and white figure on p. 266.

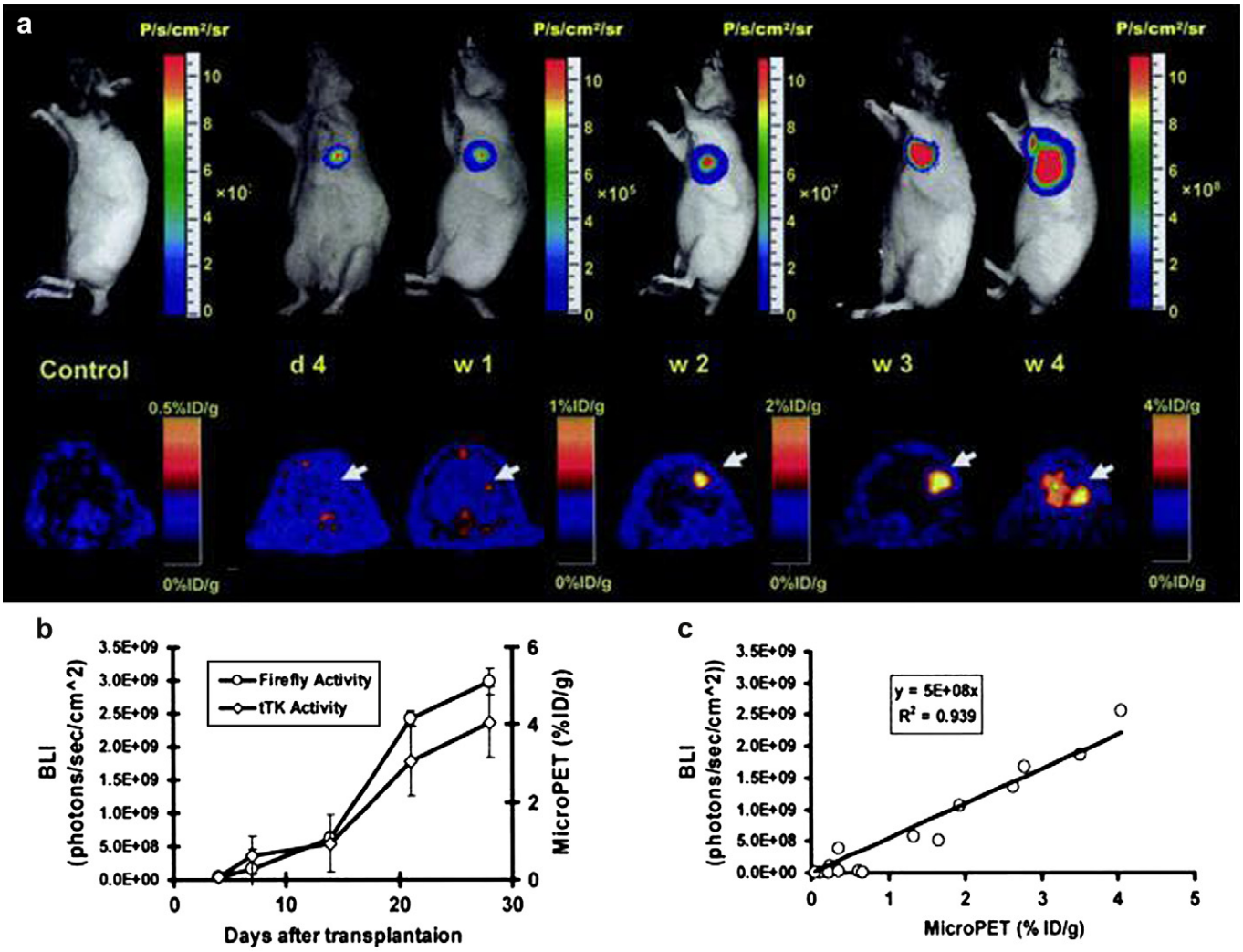

FIGURE 17.7 Molecular imaging of transplanted cells with bioluminescence and positron emission tomography (PET). (a) Animals were imaged for 4 weeks to assess longitudinal cell survival. A representative study animal injected with triple fusion cells showed significant bioluminescence (top) and PET (bottom) signals at day 4, week 1, week 2, week 3 and week 4. In contrast, control animals showed only background activities. (b) Quantification of imaging signals showed a drastic increase in fluc and ttk activities from week 2 to week 4. Extracardiac signals were observed during subsequent weeks. (c) Quantification of cell signals showed a robust in vivo correlation between bioluminescence (BLI) and PET imaging (r^2 0.92). *[Adapted from Cao et al., 2006 [81] with permission.]* Please see black and white figure on p. 268.

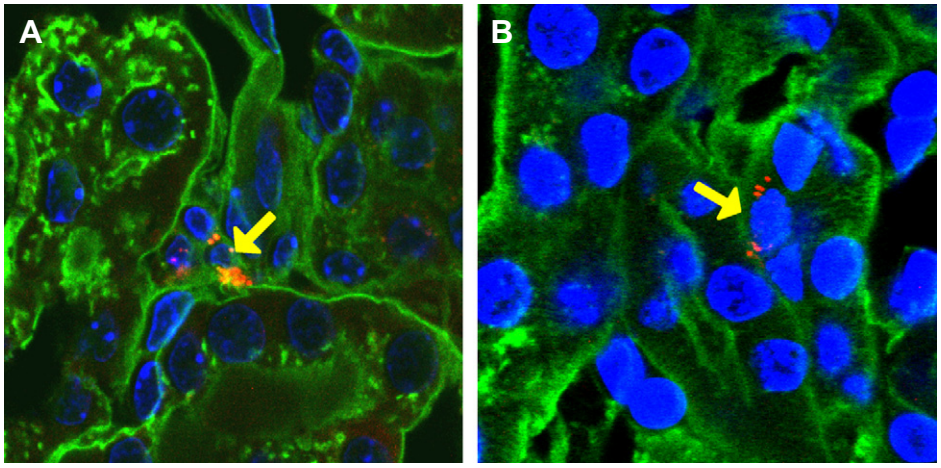

FIGURE 20.1 Human bone marrow mesenchymal stem cells (BMMSCs) engraft the damaged kidney in immunodeficient NOD SCID mice with cisplatin induced acute kidney injury. Representative images, taken 3 days after intravenous cell injection, show PKH 26 labeled BMMSCs (red) localized in the interstitium (A, arrow) or tubuli (B, arrow) of renal tissues of cisplatin mice stained with fluorescein isothiocyanate (FITC) labeled wheatgerm agglutinin (WGA, green) and 4′,6′ diamidino 2 phenylindole dihydrochloride hydrate (DAPI) nuclear staining (blue). Original magnification 630×. Please see black and white figure on p. 302.

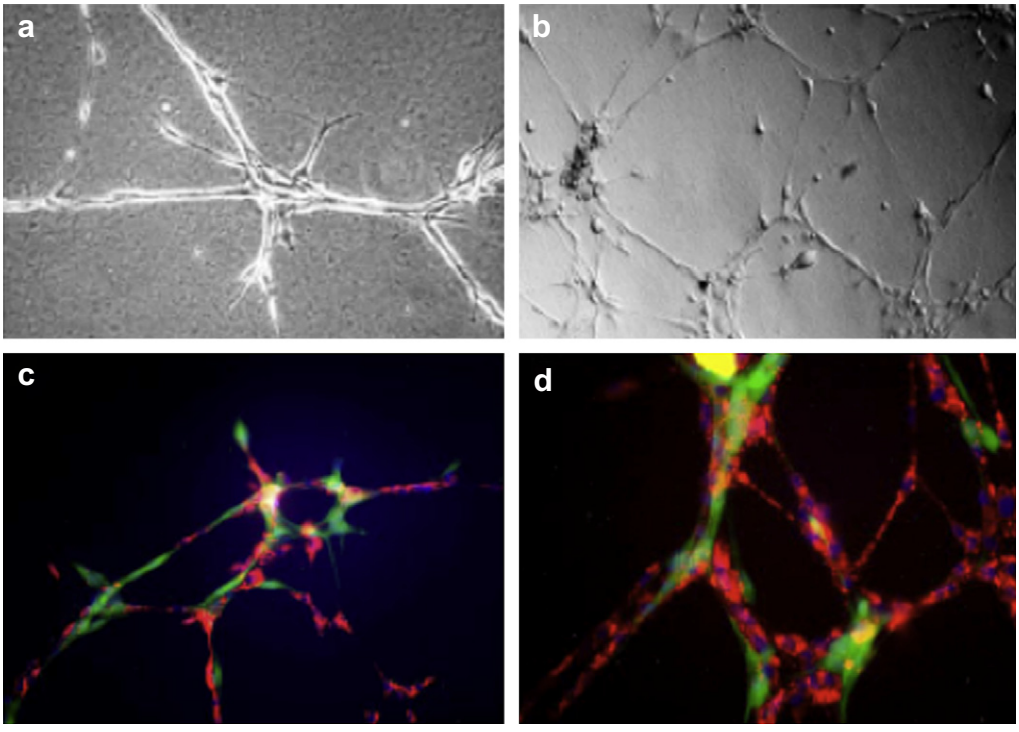

FIGURE 21.8 Both (a) mesenchymal stem cell (MSCs) and (b) endothelial cells (ECs) undergo capillary tube formation when seeded on Matrigel. (c, d) When both cell types are cocultured on Matrigel coated slides, EC cells form tubes (green; PKH26 labeled) that are decorated by MSCs (red; CFDA labeled), suggesting that the latter act like pericytes. Nuclei are stained blue with Hoechst 33342 dye. *[Reproduced from Tögel et al., 2007 [61], with permission from the American Journal of Physiology.]* Please see black and white figure on p. 330.

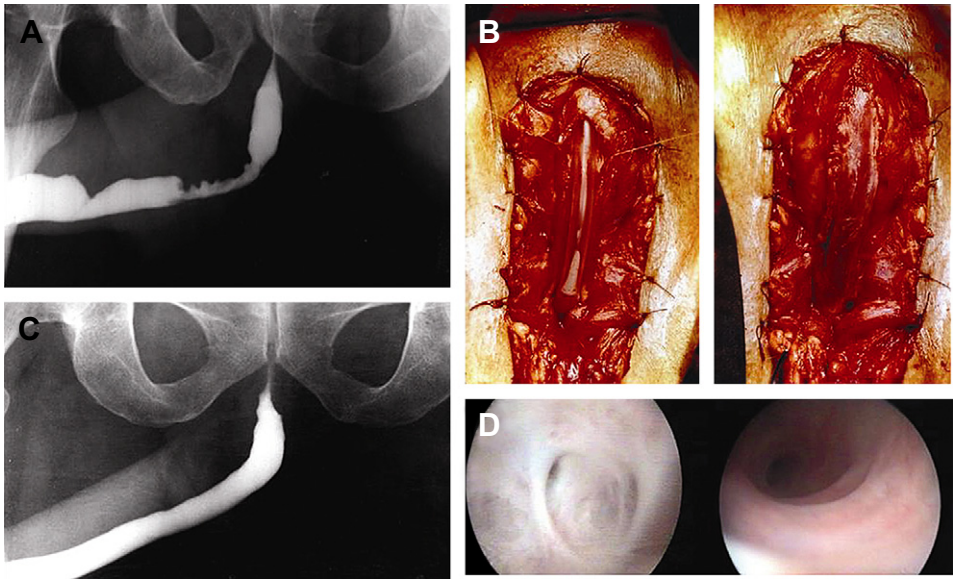

FIGURE 24.2 Urethral repair using a collagen matrix. (A) Representative case of a patient with a bulbar stricture. (B) During surgery, strictured tissue is excised, preserving the urethral plate on the left side, and the matrix is anastamosed to the urethral plate in an onlay fashion on the right. The boxes in both photographs indicate the area of interest, including the urethra, which appears white in the left photograph. In the left photo graph, the arrow indicates the area of stricture in the urethra. On the right, the arrow indicates the repaired stricture. Note that the engineered tissue now obscures the native white urethral tissue in an onlay fashion in the right photograph. (C) Urethrogram 6 months after repair. (D) Cystoscopic view of urethra before surgery on the left side, and 4 months after repair on the right side. Please see black and white figure on p. 394.

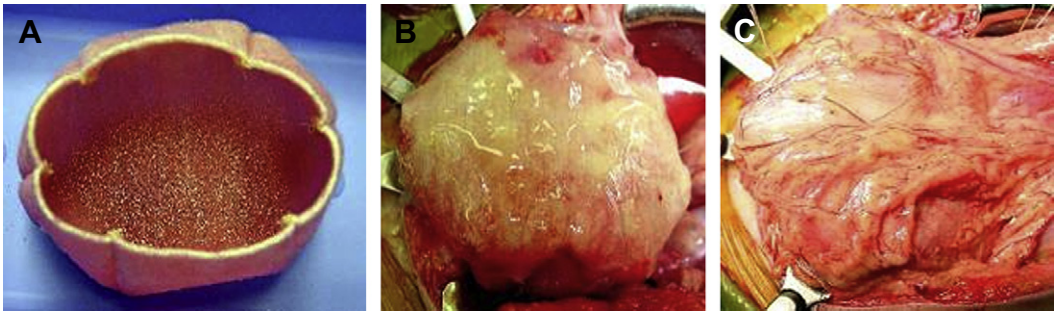

FIGURE 24.3 Construction of an engineered bladder. (A) Scaffold material seeded with cells for use in bladder repair. (B) The seeded scaffold is anastamosed to native bladder with running 4 0 polyglycolic sutures. (C) Implant covered with fibrin glue and omentum. Please see black and white figure on p. 395.

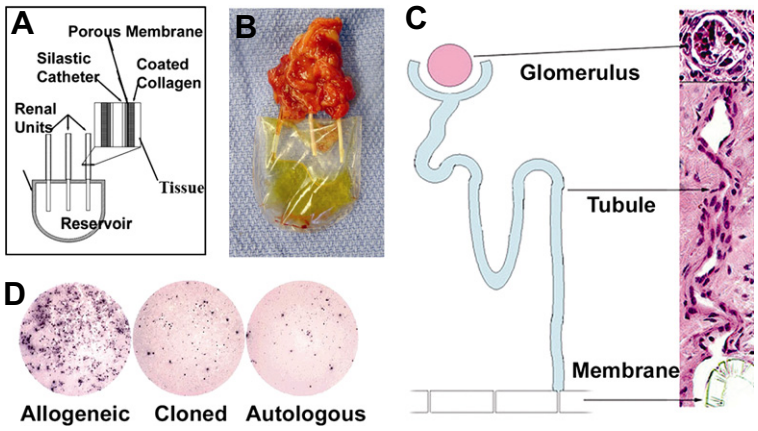

FIGURE 24.5 Combining therapeutic cloning and tissue engineering to produce kidney tissue. (A) Illustration of the tissue engineered renal unit. (B) Renal unit seeded with cloned cells, 3 months after implantation, showing the accu mulation of urine like fluid. (C) Clear unidirectional conti nuity between the mature glomeruli, their tubules and silastic catheter. (D) Enzyme linked immunosorbent spot (ELISPOT) analyses of the frequencies of T cells that secrete interferon γ (IFN γ) after stimulation with allogeneic renal cells, cloned renal cells or nuclear donor fibroblasts. Cloned renal cells produce fewer IFN γ spots than the allogeneic cells, indicating that the rejection response to cloned cells is diminished. The presented wells are single representatives of duplicate wells. Please see black and white figure on p. 397.

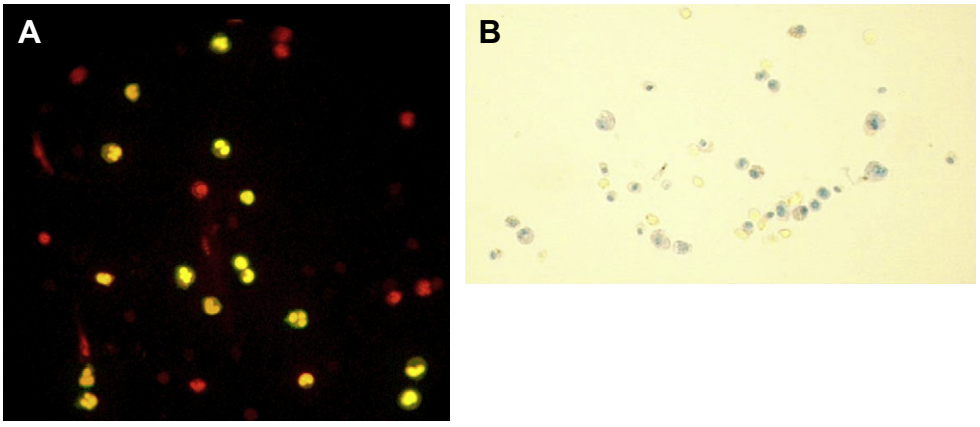

FIGURE 26.13 (A) Acridine orange–ethidium bromide stain. Green cells: viable cells; orange–red cells: non viable cells. (B) Trypan blue stain. Non colored cells: viable cells; blue cells: non viable cells. Please see black and white figure on p. 419.