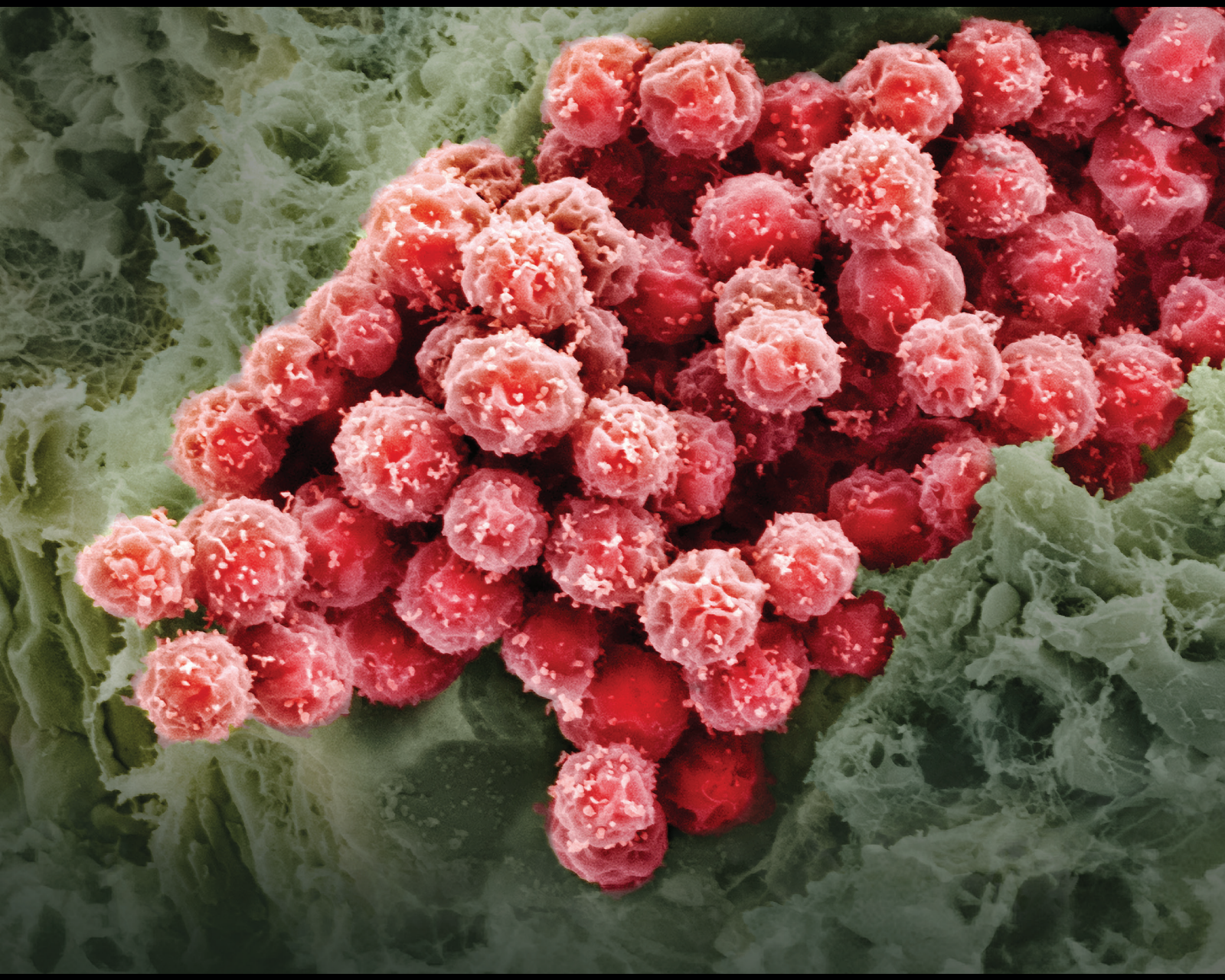
Stem Cells International

# Renal Stem Cells, Tissue Regeneration, and Stem Cell Therapies for Renal Diseases

Guest Editors: Benedetta Bussolati, Akito Maeshima, Janos Peti-Peterdi, Takashi Yokoo, and Laura Lasagni



# Renal Stem Cells, Tissue Regeneration, and Stem Cell Therapies for Renal Diseases

Stem Cells International

# Renal Stem Cells, Tissue Regeneration, and Stem Cell Therapies for Renal Diseases

Guest Editors: Benedetta Bussolati, Akito Maeshima, Janos Peti-Peterdi, Takashi Yokoo, and Laura Lasagni

# Editorial Board

# Contents

Hindawi Publishing Corporation
Stem Cells International
Volume 2015, Article ID 302792, 2 pages
http://dx.doi.org/10.1155/2015/302792

*Editorial*

# Renal Stem Cells, Tissue Regeneration, and Stem Cell Therapies for Renal Diseases

**Benedetta Bussolati,[1] Akito Maeshima,[2] Janos Peti-Peterdi,[3] Takashi Yokoo,[4] and Laura Lasagni[5]**

[1]*Center for Molecular Biotechnology, Department of Molecular Biotechnology and Health Sciences, 10126 Torino, Italy*
[2]*Department of Medicine and Clinical Science, Gunma University Graduate School of Medicine, Maebashi, Gunma 371-8511, Japan*
[3]*Departments of Physiology and Biophysics and Medicine, University of Southern California, Los Angeles, CA 90033, USA*
[4]*Department of Internal Medicine, Jikei University School of Medicine, Tokyo 3-19-18, Japan*
[5]*DENOTHE Center, Department of Clinical and Experimental Medicine, University of Florence, 50139 Florence, Italy*

Correspondence should be addressed to Laura Lasagni; l.lasagni@dfc.unifi.it

Received 1 April 2015; Accepted 1 April 2015



Kidney diseases are a global public health problem, with an incidence that has reached epidemic proportions and continues to climb in the USA and worldwide. This trend is projected to grow in correlation with the global rise in the aged population and the increasing prevalence of conditions that cause renal complications, such as cardiovascular disease, hypertension, and diabetes. Current treatment options for acute and chronic kidney disease include dialysis, which is also associated with substantial morbidity and mortality, and kidney transplantation, which is limited by the supply of compatible organs. Consequently, new methods to alleviate, cure, or prevent renal disease are urgently required to reduce the exponentially growing burden due to acute and chronic kidney disorders and offer alternative therapeutic options to improve patients' survival and quality of life. Several potential regenerative cell-based therapies for the treatment of renal failure are currently under development. The first one is the direct application of stem cells (SCs) to the diseased kidney, which relies on the inherent capabilities of SCs to differentiate, organize, and integrate into the existing tissues to restore function. To this aim different stem cell types have been investigated for their potential to contribute to renal regeneration. In their article "WNT/$\beta$-Catenin Signaling Is Required for Integration of $CD_{24+}$ Renal Progenitor Cells into Glycerol-Damaged Adult Renal Tubules," Z. Zhang et al. described how the endogenous canonical $\beta$-catenin/TCF pathway is reactivated during recovery from AKI and is required for the engraftment of exogenous $CD24^+$ embryonic renal progenitor cells into damaged tubular areas upon injury. These events appear to recapitulate the WNT-dependent inductive process which drives primary nephrogenesis. Review article by F. O. Arcolino et al. entitled "Human Urine as a Noninvasive Source of Kidney Cells" summarizes the recent data regarding human urine-derived cells. Urine contains various types of kidney cells such as podocytes, proximal tubule cells, or renal stem/progenitor cells, which can be easily as well as noninvasively isolated and cultured *in vitro*. Although the characteristics of each cell population are not totally understood, this paper suggests the potential of urine-derived kidney cells as a tool of clinical application for the treatment of various kidney diseases. Stem cell therapy contributes for kidney regeneration not only through direct differentiation and replacement of injured cells but also by secretion of renoprotective or trophic factors. These data are summarized by K. Tsuji and S. Kitamura in their review article "Trophic Factors from Tissue Stem Cells for Renal Regeneration."

Another strategy is based on the prospective design of a therapeutic approach focused on modulation of endogenous kidney regenerative properties by conventional chemical and biological agents able to modulate the activity of resident progenitor cells. Although understanding of regeneration of the nephron may have significance to explore new therapeutic

approaches, the process of regeneration of nephron has yet been poorly understood. The review article "Atlas of Cellular Dynamics during Zebrafish Adult Kidney Regeneration" by K. K. McCampbell et al. provides an essential foundation for future work aimed at elucidating the mechanisms that regulate kidney regeneration following acute kidney injury using adult zebrafish, which maintains the regenerative ability. Akito Maeshima et al. in their review "Diverse Cell Populations Involved in Regeneration of Renal Tubular Epithelium following Acute Kidney Injury" describe recent advances in understanding the regeneration mechanisms of renal tubules, particularly the characteristics of various cell populations contributing to tubular regeneration and highlight the targets for the development of regenerative medicine for treating kidney diseases in humans. Additional information on the renal regeneration capacity could be inferred from studies on kidney organ culture. The review article "Organ *In Vitro* Culture: What Have We Learned about Early Kidney Development?" by A. Rak-Raszewska et al. provides an introduction to the organ culture method and a summary of the progress in the field of kidney developmental biology based on it. Finally, a great advance on the comprehension of the mechanisms of renal regeneration, in particular podocyte regeneration, and on the role of putative population of renal stem/progenitor cells could be obtained from transgenic mouse models, as summarized by D. Lombardi and Laura Lasagni in their review "Transgenic Strategies to Study Podocyte Loss and Regeneration." Finally, a number of different approaches have been applied toward tissue engineering of the kidney as a mean to replace renal function. S. Yamanaka and Takashi Yokoo in their review article "Current Bioengineering Methods for Whole Kidney Regeneration" summarized recent researches involving the use of renal stem cells and renal bioengineering to regenerate functional whole kidneys *de novo*.

The editors hope that the original and review articles integrated in this special issue provide more insights into the advancements and challenges faced by this rapidly expanding field of regenerative medicine and will be helpful and educational for interested readers.

*Benedetta Bussolati*
*Akito Maeshima*
*Janos Peti-Peterdi*
*Takashi Yokoo*
*Laura Lasagni*

Hindawi Publishing Corporation
Stem Cells International
Volume 2015, Article ID 959807, 16 pages
http://dx.doi.org/10.1155/2015/959807

*Review Article*

# Organ *In Vitro* Culture: What Have We Learned about Early Kidney Development?

**Aleksandra Rak-Raszewska,[1] Peter V. Hauser,[2,3] and Seppo Vainio[1]**

[1]*Faculty of Biochemistry and Molecular Medicine, Biocenter Oulu, Oulu University, 90220 Oulu, Finland*
[2]*Renal Regeneration Laboratory, VAGLAHS at Sepulveda, North Hills, CA 91343, USA*
[3]*David Geffen School of Medicine, University of California, Los Angeles, Los Angeles, CA 90095, USA*

Correspondence should be addressed to Aleksandra Rak-Raszewska; aleksandra.rak-raszewska@oulu.fi and Peter V. Hauser; pvhauser@ucla.edu

Received 7 November 2014; Revised 7 January 2015; Accepted 8 January 2015

Academic Editor: Laura Lasagni



When Clifford Grobstein set out to study the inductive interaction between tissues in the developing embryo, he developed a method that remained important for the study of renal development until now. From the late 1950s on, *in vitro* cultivation of the metanephric kidney became a standard method. It provided an artificial environment that served as an open platform to study organogenesis. This review provides an introduction to the technique of organ culture, describes how the Grobstein assay and its variants have been used to study aspects of mesenchymal induction, and describes the search for natural and chemical inducers of the metanephric mesenchyme. The review also focuses on renal development, starting with ectopic budding of the ureteric bud, ureteric bud branching, and the generation of the nephron and presents the search for stem cells and renal progenitor cells that contribute to specific structures and tissues during renal development. It also presents the current use of Grobstein assay and its modifications in regenerative medicine and tissue engineering today. Together, this review highlights the importance of *ex vivo* kidney studies as a way to acquire new knowledge, which in the future can and will be implemented for developmental biology and regenerative medicine applications.

## 1. Introduction

Kidneys develop from a subregion of the embryonic mesodermal tissue, the intermediate mesoderm that generates two key cell types, the epithelial—ureteric bud (UB), and the mesenchymal—metanephric mesenchyme (MM). Reciprocal interactions between UB and MM, via a sequential inductive signalling cascade, regulate the formation of the complex organization of the kidney. The UB gives rise to the collecting system, whereas the MM gives rise to the nephrons, the major functional unit of the kidney. The nephron is composed of the renal corpuscle—the glomerulus, the proximal tubule, the Loop of Henle, and the distal tubule. The latter connects the nephron to the collecting duct system.

To study the cellular mechanisms of organ development, scientists have been culturing organs since the early 1930s, using methods such as *hanging drops* or *watch-glass* cultures [1]. In 1954 Trowell changed the, then common, method of organ culture and introduced a metal grid as a support for a cotton-wool sheet or filter soaked in the culture medium that lifted the organ to grow in the interphase of medium and air [2]. However, the "father of kidney organ culture," Clifford Grobstein, developed the basic method to investigate kidney tubule induction. Although Trowel's technology has been improved during the years, it opened a new dimension to the study of organogenesis (see transformation of the method in the Figure 1) and reflects emerging research trends (see Figure 2). It is also worth noting that the development of kidney organoculture provided an artificial environment that could be easily controlled, enabling exact manipulations of culture conditions, which promoted the field of kidney development tremendously. The aim of this review is not to give detailed descriptions of developmental and molecular processes, which have been reviewed elsewhere, but to

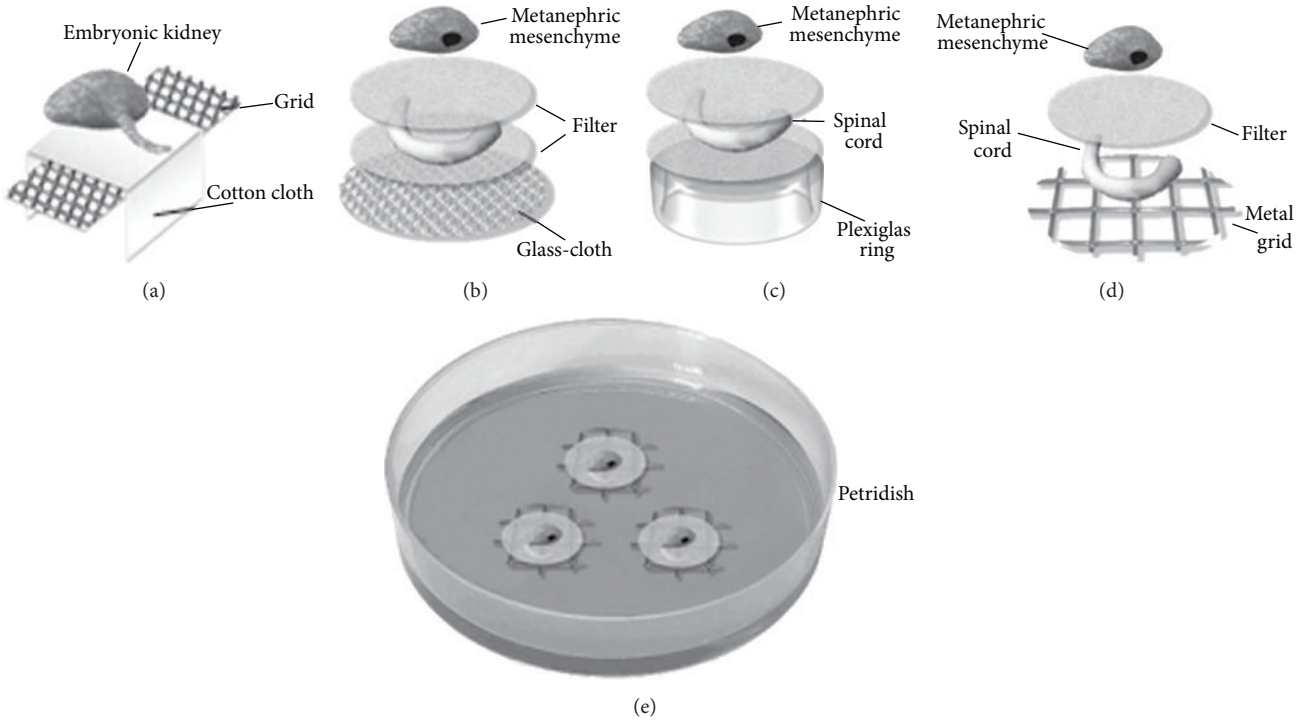



Figure 1: Method setup, from Trowel to Saxen: in 1954 Trowell introduced a new method to culture whole organs. He used a metal grid in support of a cotton sheet or filter that would hold the embryonic kidney; the cotton sheet was soaked with culture medium (see (a)) [2]. Culture medium was added only to the level of the grid to cover the tissue with a thin layer of the medium due to surface tension [2]. This set up became very useful to studying aspects of nutrition and metabolism *in vitro* (a). One year later in 1956, Grobstein slightly modified the method and introduced the "on-the-cloth" (see (b)) and "supported-ring" (c) methods [22]. Both methods used the embryonic spinal cord (eSC) from mouse [43] as inducer. The noninduced mesenchyme was placed on a filter, and a second filter was used to support the eSC. The layout was later called *"sandwich type culture."* The "on-the-cloth" method used glass-cloth as a support for the tissue cultures on the filters (as in (b)) and the "supported-ring" used a Plexiglas ring onto which the filters were cemented (c). In 1962, Saxen combined and simplified these methods (d). He cultured the noninduced mesenchyme and spinal cord separated by a filter [22] on a metal grid [2] to support the tissues on the filter in a simple culture dish (e) [117]. Saxen's modernization has been well taken by others and it is still successfully used nowadays.

provide a brief, yet inclusive, summary of the progress in the field of kidney developmental biology that is based on the *ex vivo/in vitro* kidney culture model.

## 2. Mesenchyme Competence and Induction

At the beginning of the 20th century, the only available technology to study development was explant culture grafting. Embryonic induction, which is the developmental influence of a defined tissue or group of cells over adjacent tissue or cells, has been studied in several embryonic models. For example, Spemann transplanted a small piece of the dorsal blastopore lip into the ventral site of another embryo of the same age and observed that the host embryo developed a second neural plate but located on the ventral site [3]. From this key experiment he concluded that the pattern of development is influenced by the activities of cells in close proximity to each other. Subsequently he called the blastoporal lip the *primary inductor* [3], later renamed to *Spemann's organizer* [4]. Similarly to amphibians, the *primary inductor* was also identified in other vertebrates: reptiles, birds, and mammals, and was named "*primitive streak*" [5, 6]. The primary induction process leads to the development of the three embryonic germ layers. Organogenesis was considered to represent a secondary induction process [7, 8], which mainly occurs between epithelial and mesenchymal tissues. Embryonic induction was later found to be a universal process in the animal kingdom [9]. Many cross-species transplants have also been performed. Based on the tissue conjugation assay, the induction process has been classified as instructive, which describes the dependence of two tissues of each other's signals for appropriate development, or as permissive, when one of the tissues is already committed and the presence of the other tissue merely allows the completion of its differentiation [9, 10]. Similar to other organs, such as teeth, liver, or pancreas, secondary induction is a phenomenon that also controls kidney development.

When Grobstein dissected mouse kidney rudiments at embryonic day (E) 11.0 and separated the uninduced MM from the epithelial structures of the UB, he was able to demonstrate that neither the MM nor the UB developed [11]. However, when the whole kidney rudiment was cultured, "normal" morphogenesis continued, suggesting that the kidney possesses a self-autonomous program that is sufficient to advance organogenesis from E11.5 in mouse. Moreover, from the E11.5 stage, the MM has become committed to

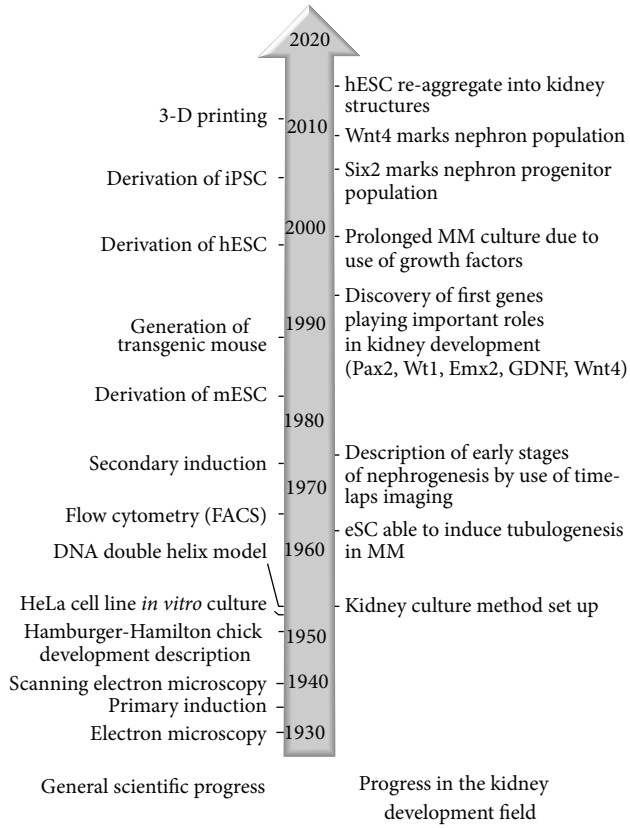



Figure 2: Relation between technology development and scientific progress: advances in biology and medicine are limited by available analytical techniques. Therefore continued progression in the fields like microscopy, immunohistochemistry, or cell biology and especially genetics enabled other biomedical fields, such as developmental nephrology, to flourish; 3D: three dimensional, DNA: deoxyribonucleic acid, ESC: embryonic stem cells, FACS: fluorescently activated cell sorting, MM: metanephric mesenchyme, and eSC: embryonic spinal cord.

develop kidney structures and many embryonic tissues, such as the embryonic spinal cord (eSC), are sufficient to induce it [12] (see also Table 1). This suggests that the induction of nephrogenesis has rather a permissive than instructive character. However, although the renal MM is competent to respond to inductive signals from several embryonic tissues (see Table 1), there is a defined competence window during which the developmental program needs to be activated [13]. Extended preculturing of the MM before exposure to eSC negatively influences induction. The MM remains competent for inductive signals for a limited time after isolation and the response to induction becomes weaker over time [13]. To this day, culturing of MM cells for a prolonged time is not possible, although growth factors, such as Bone Morphogenetic Protein 7 (Bmp7) and Fibroblast Growth Factor 2 (FGF2), can extend MM competence for up to 48 h [14], although even then, the stromal cells seem to expand faster. A culture protocol that preserves MM properties and competence during extended culture, as well as a method to reintroduce competence for induction, would be a breakthrough. It would allow propagating the cells in culture, thereby limiting the number of animals needed for cell isolation. Help in establishing the new culture protocol for competent MM may come from recent knockin and knockout studies. Sine oculis-related homeobox 2 (Six2) is expressed in the induced MM and it has been shown that Six2 descendent cells have the potential to form all parts of the nephron from distal tubule to glomeruli [15]. Six2 is also responsible for the maintenance of nephron progenitors, since Six2 mutants present ectopic and premature nephrogenesis, as well as rapid exhaustion of progenitors [16]. Interestingly, in mice where Forkhead Box D1 (FoxD1) was knocked out in the stromal cell population, the Six2+ cells greatly expanded [17, 18]. It has been suggested that stromal FoxD1+ cells regulate Six2 self-renewal via the Hippo- and BMP-SMAD signalling pathways [17, 18]. More research will be necessary to test if Six2+ MM cells properties can be maintained during *in vitro* culture by factors that allow selective inhibition of these pathways, separately or in combination.

*2.1. Natural MM Inducers.* After eSC has been shown to efficiently induce the MM, it replaced UB as an inductor in subsequent experiments. This initiated a search for the mechanism of kidney induction. eSC, brain and other mesenchymal tissue of different stages of embryonic development have been tested as inducers (see summary in Table 1). The dorsal site of the eSC exhibited stronger inductive effects than the ventral eSC. The eSC regions proximal to the brain (mesencephalon, telencephalon) showed a stronger inductive

Table 1: Natural and chemical MM inducers.

| Natural MM inducers | | | | |
|---|---|---|---|---|
| Organ | Inductive tissue | Age | Strength of signal | References |
| Brain | Whole brain | E11.0 | +++ | Lombard and Grobstein 1969 [19] |
| | | P3 | ++ | |
| | | P7 | + | |
| | | P14 | – | |
| | Dorsal telencephalon | E11.0 | +++ | |
| | | E13.0 | +++ | |
| | | E15.0 | +++ | |
| | Ventral telencephalon | E11.0 | +++ | |
| | | E13.0 | +++ | |
| | | E15.0 | +++ | |
| | Dorsal mesencephalon | E11.0 | +++ | |
| | | E13.0 | +++ | |
| | | E15.0 | +++ | |
| | Ventral mesencephalon | E11.0 | +++ | |
| | | E13.0 | +++ | |
| | | E15.0 | +++ | |
| | Dorsal medulla | E11.0 | ++ | |
| | | E13.0 | ++ | |
| | | E15.0 | + | |
| | Ventral medulla | E11.0 | ++ | |
| | | E13.0 | + | |
| | | E15.0 | + | |
| Bones | Long bones | E14.0 | ++ | Unsworth and Grobstein 1970 [12] |
| Head | Jaw mesenchyme | E11.0 | +++ | Unsworth and Grobstein 1970 [12] |
| | | E13.0 | +++ | |
| | Whole head | E8.0 | +++ | |
| | | E11.0 | +++ | |
| | | E13.0 | +++ | |
| Kidney | Ureteric bud | E11.0 | ++ | Grobstein 1953 [11] |
| | Wolffian duct | E11.0 | ++ | Rosines et al. 2010 [103] |
| Salivary gland | Mesenchyme | E11.0 | + | Unsworth and Grobstein 1970 [12] |
| | Epithelium | E11.0 | ++ | |
| Somites | Posterior somites | E13.0 | – | Unsworth and Grobstein 1970 [12] |
| | Anterior somites | E13.0 | + | |
| Spinal cord | Dorsal SC | E11.0–E19.0 | +++ | Lombard and Grobstein 1969 [19] |
| | | P0 | + | |
| | | P7 | – | |
| | Ventral SC | E11.0–E19.0 | ++ | |
| | | P0 | + | |
| | | P7 | – | |
| Spinal cord from chicken | Dorsal SC | Day 9 | +++ | Lombard and Grobstein 1969 [19] |
| | Ventral SC | Day 9 | – | |

| Chemical MM inducers | | | | |
|---|---|---|---|---|
| Chemical name | Symbol | Role | Strength of signal | References |
| Lithium chloride | LiCl | GSK-inhibitor | ++ | Davies and Garrod 1995 [30], Halt and Vainio 2012 [31] |
| 6-Bromoindirubin-3′-oxime | BIO | GSK-inhibitor | +++ | Brown et al. 2013 [32] Mugford et al. 2009 [81], Kuure et al. 2007 [33] |
| 6-[[2-[[4-(2,4-Dichlorophenyl)-5-(5-methyl-1*H*-imidazol-2-yl)-2-pyrimidinyl]amino]ethyl]amino]-3-pyridinecarbonitrile | CHIR99021 | GSK-inhibitor | ++ | Ye et al. 2012 [35] |

potential than the distal posterior region (medulla). Further, the activity of the inducer seemed to decrease with increased tissue age (up to 7 days of postnatal life) [19]. Various other nonneural tissues also exhibited potential to induce the MM, although with differing outcomes [12] (see Table 1).

Transfilter experiments have been performed using filters of different pore sizes to separate the MM from eSC. It was found that larger filter pores associated with stronger eSC induction, showing more and better-defined tubule development in the MM [13, 20, 21]. In experiments with smaller pores the induction response was slower and weaker, or completely absent [22]. Analysis by electron microscopy revealed that cells developed pseudopodium-like processes that penetrated the filter, thereby generating "bridges" between the MM and eSC [22]. These findings stimulated extensive research to understand whether cell-to-cell contact is essential for induction, or if signalling can occur over long distance. Various chemical compounds with different molecular weights, surface charge, spherical and nonspherical shape, and highly charged molecules were tested and all of them were found to diffuse through the filters faster than the induction factor [23]. Further studies investigated the migration distance of the signalling molecules, different sources of inductive tissues, and the timeframe during which the MM remains competent for induction. Even mathematical models were established to consider the diffusion time through one and two Millipore filters [13, 23]. These experiments led to the rejection of the long distance diffusion as an induction model. Positive confirmation of the cell-to-cell contact requirement for successful induction was established later and was based on advanced tissue preservation methods and electron microscopy [24]. However, with the recent discovery of intracellular vesicles, also called exosomes or microvesicles, a new way of cell-to-cell communication is proposed. Exosomes are small intracellular vesicles (30–100 nm), which carry cellular information, such as various RNAs, proteins, or lipids, and are released by cells [25–27]. Given that they have been detected in blood and urine, they might also have the potential to serve as biomarkers of various diseases [28]. The presence of exosomes in the urine further suggests that they are released also from postnatal kidneys. Nevertheless, their presence and role during embryogenesis are currently unclear. The possibility, however, cannot be ruled out, as Koch and Grobstein in 1963 found, using radioactively labelled eSC, that secreted "molecule" migrated towards the MM on the opposite site of the filter, up to 100 $\mu$m away from its source [29].

*2.2. Small Molecular Chemical MM Inducers.* It has long been known that lithium cations are potent regulators of embryonic development [9], but only years later was lithium studied as a putative inducer of the MM [30, 31]. It appeared that lithium chloride disrupts the Wnt/$\beta$-catenin signalling pathway [32, 33] by inhibiting Glycogen Synthase Kinase-3 (GSK-3) [34] thereby enabling MM induction (see Table 1). Inactivation of GSK-3 by lithium chloride, bromoindirubin-3$'$-oxime (BIO), or 6-[[2-[[4-(2,4-dichlorophenyl)-5-(5-methyl-1H-imidazol-2-yl)-2-pyrimidinyl]amino]ethyl]amino]-3-pyridinecarbonitrile (CHIR99021) prevents apoptosis of the MM and promotes tubulogenesis [32, 33, 35], similar to natural MM inducers, albeit with a more rapid kinetic (A. R.-R. personal observation). Inhibition of GSK-3 leads to cytoplasmic stabilization of $\beta$-catenin, which in turn leads to the activation of target genes by initiation of transcription factors from the TCF/LEF family. Prolonged presence or high concentrations of these molecules are followed by necrosis of the MM [33]. Transient exposure or a low concentration of these compounds is therefore recommended for successful experimental MM induction. Although many small molecules that interfere with the Wnt/$\beta$-catenin pathway have been identified [36], their roles in MM induction have not yet been fully investigated. Nevertheless, two of these small molecules, the inhibitor of Wnt production 2 (IWP2), which acts by repressing Porcupine, and the inhibitor of Wnt response 1 (IWR1) which affects Tankyrases 1 and 2 [36], were shown to completely block the whole kidney development despite presence of the UB [17] reinforcing the importance of Wnt signalling in nephrogenesis [37].

Other factors that are involved in MM induction have been identified during a search for serum-free medium. Animal serum differs from batch to batch in its composition, which may lead to different outcomes of organ culture experiments. Medium that was supplemented with 10% fetal calf serum (FCS) showed strong induction of the MM and tubulogenesis [38]. While serum-free medium alone did not support kidney development, the explanted tissue remained uninduced, without signs of tubulogenesis, even in the presence of spinal cord as an inductor [38]. Supplementation of serum-free medium with 50 g/mL transferrin (TR) was able to support normal induction of kidney development [39]. The effect of TR could not be replaced by epidermal growth factor (EGF), fibroblast growth factor (FGF), or insulin [38]. Thus these are important survival factors for kidney induction preservation.

## 3. Kidney *In Vitro* Culture to Study Renal Development

The progress in the biomedical field (see Figure 2), namely, the technique to generate transgenic knockin [40, 41] and knockout [42] animals, as well as the derivation of mouse and human embryonic stem cell culture [43, 44] in combination with kidney organ culture is very powerful tools. Gene targeting enabled the study of single genes that are responsible for ureteric bud outgrowth, MM induction, and nephron development (see Table 2 and Figure 3).

*3.1. MM Influence Ureteric Bud Outgrowth.* Although it is known that the MM develops from the intermediate mesoderm (IM), marked by the expression of odd-skipped related transcription factor 1 (Osr1) [45], a mechanism that leads to the development of the highly specialized MM region at the level of the hind limbs, remains to be revealed. This region, the MM, has been studied extensively over the past decades with the combined use of genetic models and *in vitro* culture. This has led to the identification of genes that

Table 2: Genes important for nephrogenesis.

| Gene abbreviation | Gene full name | Expression | Role in kidney development | References |
|---|---|---|---|---|
| BMP2 | Bone morphogenetic protein 2 | Pretubular aggregate, distal part of early tubules | Inhibiting ureteric bud growth and branching | Godin et al. 1999 [118] |
| BMP4 | Bone morphogenetic Protein 4 | Mesenchymal cells surrounding Wolffian duct and stromal mesenchyme surrounding ureteric bud stalks | Preventing ectopic ureteric bud outgrowth and extra ureteric bud divisions | Miyazaki et al. 2000 [65] |
| BMP7 | Bone morphogenetic Protein 7 | MM | Survival of MM | Dudley et al. 1999 [14] |
| Calb | Calbindin | Ureteric bud epithelial cells and distal part of the nephron | Regulating calcium reabsorption | Davies 1994 [119] |
| Cdh6 | Cadherin 6 | Proximal tubule | Cell polarization, MET, lumen formation | Cheng et al. 2007 [77] |
| CITED1 | Cbp/p300-interactin transactivator 1 | Subpopulation of cells in cap MM | Maintenance of undifferentiated cells within the cap MM | Mugford et al. 2009 [81] |
| E-cadh | E-cadherin | Ureteric bud epithelial and distal tubule cells | Cell polarization, MET, lumen formation | Jia et al. 2011 [119] |
| Emx2 | Empty spiracles protein 2 | Ureteric bud epithelial cells | Regulating ureteric bud functions upon Pax2 induction in the MM | Miyamoto et al. 1997 [51] |
| FGF2 | Fibroblast growth factor 2 | MM | Survival of MM | Dudley et al. 1999 [14] |
| FoxD1 | Forkhead Box D1 | Stromal MM | Differentiation of nephron progenitors | Das et al. 2013 [17], Fetting et al. 2014 [18] |
| GDNF | Glial-cell derived neurotrophic factor | MM | Inducing ureteric bud outgrowth from Wolffian duct, interacting with Ret | Hellmich et al. 1996 [53], Sainio et al. 1997 [54] |
| Osr1 | Odd-skipped related transcription factor 1 | Intermediate mesoderm, MM | Giving rise to MM | Mugford et al. 2008 [45] |
| Pax2 | Paired box gene 2 | Ureteric bud epithelial cells and condensed MM | Expression in the MM ensures high level of GDNF production | Dressler et al. 1990 [46], Rothenpieler and Dressler 1993 [47], Brophy et al. 2001 [48] |
| Ret | Receptor tyrosine-protein kinase | Ureteric bud epithelial cells | Initial ureteric bud outgrowth from Wolffian duct, interacts with GDNF | Shakya et al. 2005 [55] |
| Sall1 | Spalt-like transcription factor 1 | MM | Ensuring high level of GDNF production | Nishinakamura et al. 2001 [52] |
| Six2 | Sine oculis-related homeobox 2 | Subpopulation of cells in cap MM | Maintaining nephron progenitor cells | Kobayashi et al. 2008 [15], Mugford et al. 2008 [45] |
| Wnt4 | Wingless-type MMTV integration site family, member 4 | Cap MM, pretubular aggregate, nephron progenitors | Mesenchymal-to-epithelial transition (MET) | Park et al. 2007 [75] Shan et al. 2010 [76] |
| Wnt9b | Wingless-type MMTV integration site family, member 9B | Ureteric bud stalk epithelial cells | Renewal and differentiation of nephron progenitors and normal ureteric bud branching, MET | Carroll et al. 2005 [73], Park et al. 2007 [75], Karner et al. 2009 [74] |
| Wt1 | Wilms tumor 1 | Cap MM, high levels; stromal MM, low levels; glomerular progenitors | Ensuring high level of GDNF production | Kreidberg et al. 1993 [50] |

contribute to the ureteric bud outgrowth from the Wolffian duct (Figure 3(a)).

The first gene discovered to play a role in kidney organogenesis was paired box gene 2 (Pax2). Its expression has been shown in the UB and in early MM condensates. Loss-of-function studies determined that without Pax2 expression UB branching and nephrogenesis failed [46–48]. The creation of knockout transgenic animals or the construction of loss-of-function models allowed the identification of a number of genes that are involved in embryogenesis. Some

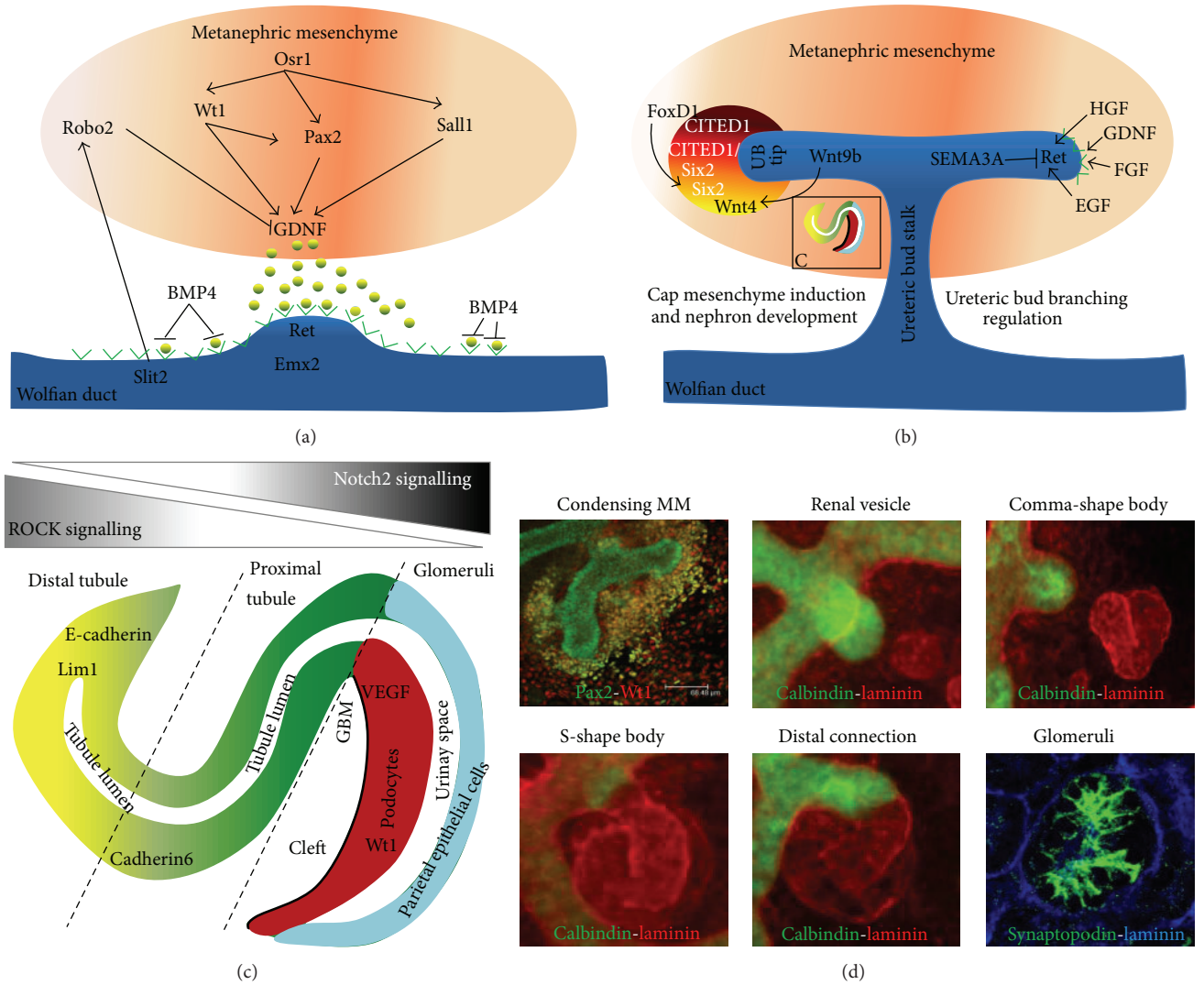



FIGURE 3: Schematic of kidney development: (a) MM influence UB outgrowth from the Wolffian duct. Genes, such as Osr1, Wt1, Pax2, and Sall1, upregulate GDNF production. GDNF is secreted from the MM and binds to Ret receptors and induces budding of the ureteric bud. The ectopic bud outgrowth is prevented by the BMP4 that surrounds Wolffian duct. Slit2 and Robo2 action reduce GDNF production in the anterior part on the MM. (b) Once the UB invades the MM, its branching is regulated by HGF, FGF, GDNF, and EGF inductive action on Ret, while Semaphorin 3A is downregulating the UB branching (left side). At the tip of the UB, the MM condenses and forms heterogenic cell population with expression of CITED1, Six2, and Wnt4. Upon Wnt9b action, Wnt4 induced nephron formation by comma- and S-shape body formation. MET takes place. (c) Distal nephron development depends on ROCK signalling, while proximal nephron—glomeruli development depends on Notch2 signalling. (d) Microphotographs of kidney rudiments developing *in vitro*, presenting all main stages of nephron formation.

affected not only one organ, or led to early embryo death *in utero*, and thus made the analysis of organs at later time points difficult. The use of an *ex vivo* culture system, however, allowed the study of organ development in these mutants. Embryos homozygote for Wilms tumor protein 1 (WT1) knockout [49] die *in utero* between E13 and E15, due to lack of UB outgrowth [50]. Experiments performed *ex vivo* revealed that WT1 double knockout (−/−) MM could be induced with eSC, demonstrating that WT1 is essential for UB budding [50]. Similar results were obtained in Sal-like1 (Sall1) and empty spiracles protein 2 (Emx2) mutants [51, 52]. Kidney rudiments isolated from Sall1 mutant embryos exhibited a failure in UB outgrowth, while MM from Sall1$^{-/-}$ embryos was competent for induction by wild type UB and eSC. It was found that Sall1 knockout mice fail to induce the transcription of the ureter inducing factors such as glial-cell-line-derived neurotropic factor (GDNF), Wt1, Pax2, Wnt4, and BMP7 [52]. The gene Emx2 is expressed in the Wolffian duct and mesonephric tubules, but not in the MM [51]. In combined cultures of mutant UB with wild type MM the induction did not occur and subsequently kidney development was impaired. Emx2 deficient MM, however, was induced when combined with wild type UB. These experiments suggested that kidney agenesis in Emx2$^{-/-}$ homozygous embryonic

mice was caused by a failure to induce UB growth [51]. Without expression of the abovementioned genes, the UB fails to grow out from the Wolffian duct and the cells of the MM remain uninduced and die. Another gene found by *ex vivo* kidney culture to be actively involved in the UB outgrowth was GDNF, a member of the transforming growth factor family $\beta$ (TGF-$\beta$) [53]. GDNF was identified as the main molecule that induces the UB branching from the Wolffian duct [54]. When GDNF soaked beads were placed next to isolated Wolffian ducts in the *ex vivo* culture they induced the formation of ectopic buds. Moreover, the GDNF soaked beads interfered with normal kidney development by inducing additional divisions and irregular branching of the UB [48, 54]. These findings showed for the first time that signals coming from the MM are important for initiation of kidney development, which is marked by the UB outgrowth.

*3.2. Ureteric Bud Development and Branching.* Once the MM produces enough of GDNF, it is secreted from the MM towards the Wolffian duct where it binds to receptor tyrosine-protein kinase (Ret) expressing cells. Observations of $Ret^{-/-}$ mice revealed that the absence of Ret expression is followed by agenesis of the kidney. Moreover, in chimeric mice with a mosaic expression of Ret, where some cells lost Ret expression, the kidneys develop normally, but it was found that the $Ret^{-/-}$ cells rearrange and contribute only to the trunk of the developing UB, and not to the tip, as in wild type mice [55]. After the successful UB outgrowth from the Wolffian duct, the UB invades the MM and after reciprocal molecular cross talk between the MM and the UB, the MM cells become induced and the UB starts to branch.

The use of fluorescently labelled UB, isolated from Hoxb7-GFP transgenic embryos, in combination with *in vitro* organoculture permitted time-lapse imaging and a better visual analysis of the UB branching [56]. The UB exhibits three different consecutive branching patterns: (i) terminal bifid branching, followed by unequal growth of the two new branches and bifid branching of one of them; (ii) terminal bifid branching, followed by trunk elongation and lateral branching within the trunk; (iii) terminal trifid branching, followed by remodelling of the ampulla to yield two distinct branch points [56–58]. The analysis of time-lapse images recorded during UB branching showed that the most common branching type is symmetrical terminal bifurcation (i). Some of the bifid branching events were observed to be asymmetric and the next branching from this segment was rotated 90° from the branch of origin. Trifid and lateral branching occurs with a low rate and appears in later branching generations.

While GDNF has been shown to induce the initial UB outgrowth from the Wolffian duct and to stimulate further branching, another mechanism is necessary to distinguish between the branching zone of the tip and the trunk of a branch. Continued branching of the UB is controlled by a network of Ret [59] and is positively regulated by factors produced by the MM, such as Wnt11, Fibroblast Growth Factor (FGF), Endothelial Growth Factor (EGF) or Hematopoietic Growth Factor (HGF), and Vascular Endothelial Growth Factor (VEGF-A) as in mutants of the abovementioned factors the UB branching is impaired [60–63]. These factors induce kidney development and prevent renal agenesis. However, negative regulators exist that prevent ectopic bud outgrowth and control the UB branching. This group consists of Bone Morphogenic Protein 4 (BMP4), Slit2, roundabout homolog 2 (Robo2), and semaphorins. BMP4 acts along the UB trunk to prevent lateral branching, while Slit2 and Robo2 downregulate the expression of the GDNF in the anterior part of the MM to prevent the ectopic UB outgrowth [48, 64, 65]. Inhibition of the mitogen-activated protein kinase (MAPK) signaling cascade has been demonstrated to reduce UB branching and the length of the branches [58], while inhibition of semaphorin 3A leads to increased branching and kidney expansion [63, 66] (Figure 3(b)).

Although while being a valuable tool, *in vitro* kidney culture, like any other biological model, has limitations. The kidney is a three-dimensional (3D) organ and when grown in two-dimensions (2D) on the culture filter at the air-medium interphase, the morphology of the branching UB in the developing kidney differs from its *in vivo* counterpart. Hence, development of a 3D culture technique, in which the tubular epithelial cells could grow in an environment that better suited then on two-dimensional surface, was necessary. For this, methods have been developed in which the UB have been cultured submerged in extracellular matrix (ECM) compounds, composed of Matrigel and collagen IV, supplemented with a cocktail of growth factors [67].

The UB grown in this manner *in vitro* was able to induce freshly isolated MM to develop functional nephrons [67]. Moreover, UB grown in a 3D ECM culture devoid of contact with MM exhibited a branching pattern similar to that of *in vivo* UB [68]. This culture method further allowed a quantitative morphological analysis of the branching UB structure utilizing fluorescent staining in combination with time-lapse microscopy [69]. They measured the influence of various members of the TGF-$\beta$ superfamily on UB branching morphology and defined the roles of BMP2 and 4, TGF-$\beta$1, Leukaemia Inhibitory Factor (LIF), and activin on UB branching. Based on these findings it was proposed that UB branching is regulated by soluble growth factors and matrix components. Growth factors and matrix components would mediate three signals: (i) stimulus that supports rapid growth and branching, (ii) inhibition that reduces growth and branching (negative feedback loop), and (iii) a signal to stop branching and undergo differentiation [69].

*3.3. Nephrogenesis.* Early observations of the morphological changes after MM induction by eSC focused on tubulogenesis and it is similar to events taking place in the whole kidney culture when the MM is induced by the UB [11, 22]. The first detailed analysis of the events during and after MM induction was performed by time-lapse microscopy on live tissue. The early stages of tubulogenesis were described as (i) undifferentiated MM, competent to receive and respond to induction signals, characterised by very motile cells; (ii) early condensates, formed by MM cells around the UB tips upon induction, characterised by lost motility; (iii) tubule

formation—cells in condensates that undergo mesenchymal-to-epithelial (MET) transition are polarized and form pretubular aggregates with a lumen that elongates and takes S-shape form [70]. The tubules will elongate and give rise to distal and proximal tubules connected by the Loop of Henle. The S-shape body stage is also the start of the glomerular development, with the glomerulus forming at the most proximal site of the S-shaped body. Cells adjacent to the S-shape body basement membrane will become podocytes, and the basement membrane will develop into the thickened glomerular basement membrane (GBM). The thin cell layer on top of the future podocytes is called parietal epithelial cells. They will later give rise to Bowman's capsule. During this process, endothelial and mesangial cells migrate into the developing cleft and give rise to glomerular tuft. Podocytes, GBM, and endothelial cells altogether constitute the renal filtration apparatus [71, 72] (Figures 3(b), 3(c), and 3(d)).

Generation of NIH3T3 cells [24] that express various Wnt genes led to the identification of genes that are essential for nephrogenesis. In classical transfilter experiments it has been found that Wnt family proteins, such as Wnt1, Wnt3a, Wnt4, Wnt7a, and Wnt7b, were able to induce tubulogenesis in the MM [24]. The most interesting of these proteins was Wnt4. Wnt 4 is not only expressed in the mesenchyme, the pretubular aggregate, and the renal vesicle, but is also expressed in the eSC. Wnt4, together with Wnt9b, was later identified as the main factors driving mesenchymal-to-epithelial transition (MET) [73–76]. MET is a process during which the cells in the pretubular aggregates become polarized, and the apical and basolateral sides of the tubes can be distinguished. This process is correlated with lumen formation and tubule development. Wnt9b acts through the canonical Wnt pathway and signals upstream of Wnt4 and Six2 leading to tubulogenesis and therefore promoting the differentiation of nephron progenitors [15]. Wnt9b mutants fail to form pretubular aggregates and fail to undergo tubulogenesis [73]. As tubulogenesis proceeds and nephrons form, they undergo extensive elongation and segmentation (Figure 3(b)). The developing tube becomes polarized and the proximal and distal ends are differentiated. Polarization is controlled by the Notch signalling mechanism [77, 78], whereas Rho-kinase signalling patterns the elongation [79]. Moreover, inhibition of Rho-signalling *in vitro* resulted in UB defects similar to Wnt9b mutant animals [73, 79, 80], demonstrating again that *ex vivo/in vitro* kidney culture is a powerful tool to uncover molecular mechanisms of development. It has been suggested that polarization already starts in the pretubular aggregates, where different levels of E-cadherin and Lhx1 are observed; for example, cells with higher expression of both E-cadherin and Lhx1 are representing the distal part of the future nephron [81]. Although the distal part of the nephron is neurogenic locus notch homolog protein 2 (Notch2) independent, the proximal end requires Notch 2 for normal development. Mice deficient for Notch2 completely lack glomeruli and proximal tubules. Moreover, markers specific for proximal segment—cadherin 6 (cadh6) and *Lotus Tetragonolobus Lectin* (LTL) and early podocytes—WT1 are absent [77] (Figure 3(c)). The development of proximal tubules starts in mouse at around E14 and the proximal tubules start to express brush border antigens [38], whereas the distal end of the tubule develops at E15 and expresses Tamm-Horsfall glycoprotein (TH) [38]. Nevertheless, the extension of the tubes connecting proximal and distal segments, the Loop of Henle, has not been observed under the "sandwich" culture conditions [82].

Only recently, Sebinger et al. highlighted the influence of surface tension of the growth media on UB branching, and further studied the influence of the supporting material on gene expression with the cultured embryonic kidney. Their modified culturing method attempted to maximize UB branching events and increased the survival time of the cultured tissue. The results further suggested the development of Loop of Henle-like structures during *ex vivo* kidney rudiment culture [83].

Developing podocytes express VEGF to attract endothelial cells and develop the vasculature of the glomerulus—the glomerular tuft [84, 85]. Newborn mice in which the endogenous VEGF was blocked by injection with antibodies, or in which VEGF was genetically removed, exhibit glomeruli without capillary tufts and show other vascular defects [84, 85]. However, deletion of VEGF2 specifically from podocytes demonstrates the importance of VEGF paracrine signalling toward endothelial cells via VEGF2 receptor [85], suggesting the role of podocytes in the correct endothelial cell lining of the GBM.

Most information regarding glomerulogenesis has been generated from the genetic models and not in *in vitro* culture, most likely due to the fact that *in vitro* developed glomeruli are avascular. This problem has been solved by implantation of the mouse kidney rudiment on the chorioallantoic membrane (CAM) of a chicken egg, which allows glomerular vascularisation [38, 86, 87]. CAM implantation of kidney rudiments showed that the origin of the endothelial cells and the vasculature of the glomeruli might be of host origin (CAM) when young (E11.5) kidney rudiments were used for interspecies culture (quail, host/mouse, donor) or of mixed origin when older (E12.5) kidney rudiments were used [86, 87]. Interestingly, GBM was deposited by both the quail and the mouse giving rise to completely hybrid structures [87] further suggesting that endothelial cells and podocytes contribute to GBM formation. The developmental processes of the kidney and the interactions of genes during tubulogenesis and glomerulogenesis were in great detail described in reviews by Dressler [88, 89], Vainio et al. [90, 91], and Schell et al. [72].

## 4. Grobstein Assay and Search for Stem/Progenitor Cells

More than 50 years ago, Auerbach and Grobstein disaggregated the MM and allowed it to reaggregate using the eSC as an inductor, which could as well be disaggregated [92]. Although the tissue survived only a few days, early stages of development occurred after reaggregation. This proved that mechanical or chemical tissue disaggregation did not interfere with its inductive abilities, of both sending and receiving signals [92]. The group of Davies deconstructed the Grobstein assay even further and performed experiments

that demonstrated the limits of self-organizational growth of the developing kidney and provided an environment in which the nephrogenic potential of presumptive kidney progenitors could be investigated [93]. In their study, they fully dissociated metanephric kidneys (MM and UB) isolated between E11.5 and E13.5 by dissection and enzymatic treatment. They formed aggregates from the dissociated kidneys by centrifugation and subsequently cultured these aggregates using standard organ culture as shown in Figure 1(e); they called it dissociation–reaggregation or 3D assay. To reduce cellular apoptosis, ROCK inhibitor was added to the culture for up to 24 hours; however, extended exposure to ROCK inhibitor blocked the nephron development. The induced kidney development was limited to the single nephron level, as the developing nephrons were not connected into a tree-like structure with hierarchical organization as in the developing embryonic kidney. Moreover, similar findings were presented with only dissociated–reaggregated MM induced by eSC and demonstrated development of all nephron segments except the descending thin limb of the Loop of Henle. Furthermore, dissociated MM can be successfully manipulated; for example, some genes may be downregulated or overexpressed and then used in the 3D assay to investigate the role of these genes during kidney development [94]. The modification of the Grobstein assay into the dissociation–reaggregation technique provided a method that allowed the introduction of exogenous cells into the embryonic kidney environment in order to test their nephrogenic potential. The dissociation of the embryonic kidney influences the ECM and therefore enabled the movement of exogenous cells and the possible integration into developing kidney structures. The exogenous cells could be of various origins, although stem cells were of highest interest. Depending on their source, stem cells are classified as either embryonic stem cells (ESC) [43] or adult stem cells (ASC) [95]. ESC are pluripotent and are able to differentiate into virtually all cell types, but the use of ESC is restricted in some parts of the world due to ethical implications; adult stem cells on the other hand are multipotent and have only limited differentiation potential to generate certain cell types. Induced pluripotent stem cells (iPS) are pluripotent like embryonic stem cells and generated from somatic cells [96]. Work on mouse ESC (mESC) showed that although they did not inhibit kidney development in the dissociated–reaggregated metanephros, they were only able to integrate into developing ureteric buds, but not nephrons. However, their potential could be enhanced by differentiation towards kidney lineage. Once mESC were differentiated using suspension culture (embryoid bodies assay) to express the mesodermal marker Brachyury (*T*), they were sorted and mixed with dissociated E13.5 embryonic kidney rudiments. Following three days in culture, mESC-derived mesodermal cells showed integration into ureteric bud structures, similarly to undifferentiated mESC, but also into nephrons, including proximal tubules (PT) and glomeruli. Moreover, mESC-derived mesodermal cells, which integrated into proximal tubules, were actually functional, transporting fluorescently labelled anionic molecules from the interstitium to the PT lumen [97].

Experiments with mouse bone marrow-derived mesenchymal stem cells (mBMSC) showed the limited ability of these cells to contribute to renal development [98]. Although mBMSC expressed renal markers such as Osr1, Sall1, Lim1, and GDNF, upon addition to the metanephric kidney in reaggregation experiments, they only localized to the developing renal structures with low frequency and aggregated preferentially to WT1 positive cells. mBMSC further showed detrimental effects on kidney development, depicted by a reduced cell mass of the condensed MM and a fewer number of nephrons. This negative effect could be abolished by stimulation with conditioned medium from neonatal kidney cells (NKC). Treatment with conditioned medium also increased the number of mBMSC integrating into nephrons [98].

Human BMSC are of great interest for the development of new therapies. In studies that investigated the nephrogenic potential of human BMSC (hBMSC) it was found that human and mouse BMSC showed similar characteristics [98]. In the reaggregates, the added hBMSC had a detrimental effect on organoid development and despite stimulation with NKC conditioned medium to rescue kidney development, it did not improve hBMSC integration [98]. Another human cell type tested with great potential for human therapy is human amniotic fluid stem cells (hAFSC). Due to reduced risks of rejection and a lack of ethical concerns, hAFSC would be a great alternative for ESC in tissue engineering and cell therapies. hAFSC have the ability to integrate into renal vesicles and comma- and S-shape bodies, upon microinjection into kidney rudiments [99]. The injected hAFSC showed expression of differentiation markers, such as zona occludens 1 (ZO1), claudin, and GDNF [99]. Moreover, clonal lines of hAFSC have been found to contribute to the formation of renal tissue [100]. Furthermore, in dissociation–reaggregation experiments with hAFSC and mouse kidney rudiments, Siegel et al. demonstrated the crucial role of the mTOR pathway in renal development. Genetic knockdown of mTORC1 or mTORC2 proteins in hAFSC decreased the ability of the cells to integrate into developing renal structures. Promotion of mTOR pathway activity by downregulation of tuberin led to increased hAFSC integration into developing renal structures [100]. Another human cell type tested in the dissociation–reaggregation (3D) assay was human ESC (hESC). The cells showed differentiation towards the renal lineage via stages of normal kidney development, namely, primitive streak, IM, and MM [101]. Differentiated hESC integrated in all developing kidney substructures, whereas undifferentiated hESC disturbed kidney development, corresponding well to the characteristics of mouse ESC in similar experiments [97]. Differentiated hESC not only integrated into all kidney substructures when mixed with mouse renal progenitor cells, but also developed into kidney structures only upon centrifugation, the last step in forming 3D pellets in dissociation–reaggregation assay, independently from any induction [101]. Differentiation protocol of ESC towards the renal lineage might soon be successfully applied to the differentiation of hiPSC, thereby avoiding problems associated with rejection and bypassing ethical concerns.

It seems that *in vitro* organogenesis of the reaggregated tissue is blocked at the step of glomerular development due to missing vascularization. Recently, Xinaris et al. performed experiments showing that the reaggregated cells indeed have the potential to generate vascularized glomeruli, if exposed to the right environment [102]. Similarly to the dissociation–reaggregation experiments described above, they formed aggregates from dissociated mouse metanephric kidneys and cultured them *in vitro* for 5 days. Then, after pretreatment of the aggregates for 4 hours with vascular endothelial growth factor (VEGF), they implanted the aggregates under kidney capsule of unilaterally nephrectomized athymic rats; in addition the recipient rats were also injected with VEGF. After three weeks they recovered the aggregates from the rats and found that glomeruli had developed in the aggregates and that these glomeruli had attracted blood vessels that originated from the mouse. They further found that the tubular structures connected to the glomeruli contained filtrate [102]. These findings might be of great interest for tissue engineering attempts in which human stem cells are added to a guiding rodent kidney cell population.

Although the 3D assay proved to be very useful to test the nephrogenic potential of different cells, the drawback of the technique is that developing UB does not resemble the collecting duct tree developing *in vivo*. These experiments demonstrated that embryonic development could be replicated *in vitro* and indicated the limitations that complicate *in vitro* organogenesis to the current day. The main limitations are the cell material to be used and the need for a controlled interaction of the progenitors with each other.

## 5. Renal Regeneration and Tissue Engineering

Potential approaches of kidney regeneration involve *in situ* repair of damaged tissue using stem cells or *de novo* tissue engineering of functional transplantable tissue. Thus, there is a quest for cells that contribute to or promote regenerative repair or renal development (as described above) or as a source of cells for tissue engineering approaches. Tissue engineering implements the use of cells, bioengineered materials, and suitable biochemical factors with the aim to generate transplantable functional renal tissue. The quest for the optimal cell source is ongoing and the modified Grobstein assay poses as a good platform to test the nephrogenic potential of candidate cells [97, 98, 100–102]. The major obstacle is that in the dissociation, reaggregation assay, the UB does not generate a tree-like hierarchically branched collecting system that is able to drain urine. This drawback could possibly be overcome with use of *in vitro* cultured UB with a suitable stem cell type. Isolated UB has been grown *in vitro* in 3D ECM settings and the cultured UB is capable of inducing freshly isolated MM [67, 103]. Moreover, some UB derived cell lines such as Madin-Darby canine kidney (MDCK) or murine medullary collecting duct 3 (mIMCD-3) cells were able to undergo branching in 3D ECM culture system, however, both required different growth factors for successful UB-like branching. When small aggregates of the UB cell lines were cocultured with freshly isolated MM, they induced tubulogenesis, but branching could not be observed [103]. However, using a micropatterned hydrogel, tubular structures have been generated from dispersed mIMCD-3 cells and from CMUB-1, a mouse ureteric bud-derived cell line. These generated tubular structures exhibited lumen formation and *in vitro* budding towards growth factor soaked beads [104]. On the other hand, investigated MM derived cell lines, BSN, rat inducing metanephric mesenchyme (RIMM-18) and cultured primary MM cells were not competent for signals from freshly isolated UB and tubulogenesis did not occur; these cell lines were also unable to induce UB branching [103].

While engineering of functional renal tissue from UB and MM cell lines, ideally derived from the patient's own cells, faces major challenges due to the complexity of the kidney, similar strategies have been successfully implemented for structurally less complex tissues, such as vaginal and urethral reconstructions [105, 106]. A main hurdle for renal tissue engineering is the correct vascularization of the engineered renal tissue. The modified Grobstein assay could be a useful tool to perform or study *in vitro* vascularization, as it allows the coculture of various cells (e.g., "successful" renal stem cells with endothelial cells) in the embryonic kidney environment. Although the metanephros cultured under *ex vivo* condition develop avascular glomeruli, it might be possible to support the developing glomeruli by vascular system, like the one provided by CAM.

One potential strategy to generate larger tissue structures or whole organs is by 3D printing. However, 3D printing of the kidney is challenged by the need to grow the "printing ink" by culturing all necessary different cell types in high number prior to printing. Further by the development of biomaterial compatible with 3D printing and by the necessary precision of the 3D printer to generate the highly complex architecture of the kidney and its vasculature [107, 108]. But 3D printing has already been implemented for less complex tissue, such as cartilage. An airway splint, printed from biomaterial (polycaprolactone) for a new born with tracheobronchomalacia, has successfully been transplanted [109]. A more complex cartilage that was printed using hydrogel filled with human derived chondrocytes may allow direct cartilage repair in near future [110]. Another approach to engineer implantable functional tissue, or whole organs, is by growing endothelial and/or epithelial or progenitor cells in decellularized adult organs [111–114], which could be obtained from deceased donors. The decellularization process removes all cellular material and leaves the ECM of the organ intact; also some of the growth factors in the ECM remain in place. Removal of the cellular material further removes the human leukocyte antigen (HLA) molecules from the organ thereby minimizing problems associated with graft rejection, as long as the cells that are used to repopulate the organ are derived from the kidney recipient. The main hurdle of this approach is the need for a defined distribution of the different cell types to all compartments of the organ. Researchers assume that the homing mechanism of the ECM will instruct a naive stem cell towards the correct differentiation. However, even if the right cell source that can differentiate into various renal cell types is identified [115, 116], the introduced cells may not follow the existing

matrix layout but populate the organ randomly and generate their own ECM. Cell proliferation would have to be restricted to limit the possibility of overgrowth or tumor formation. In addition, the size of the human kidney is a challenge with regard to the necessary cell number and oxygen supply during culture. However, although many hurdles remain, one study reported the successful decellularization of rat kidneys and their repopulation with epithelial and endothelial cells [114].

While the attempts to create a bioartificial kidney are very promising, many obstacles remain that need to be overcome to develop a potential treatment for kidney patients. Current experiments are mostly performed on rodents and if successful will have to be translated to nonhuman primates before entering human trials and clinical application.

## 6. Summary

The culturing of whole organs has been an ambitious goal since the early 1950s; during the last decade this radical idea has come into reach. One method that has been central to the advancement of the field is the Grobstein assay. As a relatively simple and cheap method, it provided a platform that could be changed to fit the needs of novel ideas. In that way, the modifications to the Grobstein assay somewhat reflect the prevailing concepts and scientific trends over the decades since its introduction.

It helped to explore the basic mechanisms of renal development. In the early stages, questions of mesenchymal induction and competence have been explored. Tissues, such as the embryonic spinal cord or the salivary gland that can act as natural inducers and chemical inducers, such as lithium and CHIR99021, of the metanephric mesenchyme have been defined. The method was essential to delineate genes that control and influence renal development from the outbranching of the Wolffian duct, such as Ret and GDNF, to the hierarchical branching of the ureteric bud, like Wnt9b, the induction of the MM and it's maintenance, such as FGF2 and Bmp7, and the formation of the functional nephron, for example, Cdh6. The Grobstein assay also allowed testing of the nephrogenic potential of different stem cell types. Stem cells such as human and mouse BMSC and human and mouse ESC and hAFSC have been tested and their differentiation and integration potential has been described. The findings of kidney organ culture lead the way to regenerative medicine, which ultimately aims to reconstruct or engineer transplantable functional renal tissue. The approaches that are currently pursued are repopulation of a decellularized organ, 3D printing, and reformation of the metanephros from dissociated renal progenitor cells. All approaches are challenged by the structural complexity of the kidney and by the quest for the optimal cell source that is used to regenerate the kidney. However, successful bioengineering of structurally simpler tissues is leading the way to overcome the challenges in generating bioartificial kidneys in the future.

## Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

## Authors' Contribution

Aleksandra Rak-Raszewska and Peter V. Hauser contributed equally.

## References

[1] E. Borghese, "The development in vitro of the submandibular and sublingual glands of *Mus musculus*," *Journal of Anatomy*, vol. 84, part 3, pp. 287–302, 1950.

[2] O. A. Trowell, "A modified technique for organ culture in vitro," *Experimental Cell Research*, vol. 6, no. 1, pp. 246–248, 1954.

[3] H. Spemann, *Embryonic Development and Induction*, Yale University Press, New Haven, Conn, USA, 1938.

[4] L. Saxen and S. Toivonnen, *Primary Embryonic Induction*, Logos Press, 1962.

[5] V. Hamburger and H. L. Hamilton, "A series of normal stages in the development of the chick embryo," *Journal of Morphology*, vol. 88, no. 1, pp. 49–92, 1951.

[6] P. P. L. Tam and R. R. Behringer, "Mouse gastrulation: the formation of a mammalian body plan," *Mechanisms of Development*, vol. 68, no. 1-2, pp. 3–25, 1997.

[7] L. Saxen, E. Lehtonen, M. Karkinen Jaaskelainen, M. Karkinen-Jääskeläinen, S. Nordling, and J. Wartiovaara, "Are morphogenetic tissue interactions mediated by transmissible signal substances or through cell contacts?" *Nature*, vol. 259, no. 5545, pp. 662–663, 1976.

[8] S. Vainio, I. Karavanova, A. Jowett, and I. Thesleff, "Identification of BMP-4 as a signal mediating secondary induction between epithelial and mesenchymal tissues during early tooth development," *Cell*, vol. 75, no. 1, pp. 45–58, 1993.

[9] J. B. Gurdon, "Embryonic induction—molecular prospects," *Development*, vol. 99, no. 3, pp. 285–306, 1987.

[10] L. Saxen and H. Sariola, "Early organogenesis of the kidney," *Pediatric Nephrology*, vol. 1, no. 3, pp. 385–392, 1987.

[11] C. Grobstein, "Inductive epithelio-mesenchymal interaction in cultured organ rudiments of the mouse," *Science*, vol. 118, no. 3054, pp. 52–55, 1953.

[12] B. Unsworth and C. Grobstein, "Induction of kidney tubules in mouse metanephrogenic mesenchyme by various embryonic mesenchymal tissues," *Developmental Biology*, vol. 21, no. 4, pp. 547–556, 1970.

[13] S. Nordling, H. Miettinen, J. Wartiovaara, and L. Saxén, "Transmission and spread of embryonic induction. I. Temporal relationships in transfilter induction of kidney tubules in vitro," *Journal of Embryology and Experimental Morphology*, vol. 26, no. 2, pp. 231–252, 1971.

[14] A. T. Dudley, R. E. Godin, and E. J. Robertson, "Interaction between FGF and BMP signaling pathways regulates development of metanephric mesenchyme," *Genes & Development*, vol. 13, no. 12, pp. 1601–1613, 1999.

[15] A. Kobayashi, M. T. Valerius, J. W. Mugford et al., "Six2 defines and regulates a multipotent self-renewing nephron progenitor population throughout mammalian kidney development," *Cell Stem Cell*, vol. 3, no. 2, pp. 169–181, 2008.

[16] M. Self, O. V. Lagutin, B. Bowling et al., "Six2 is required for suppression of nephrogenesis and progenitor renewal in the developing kidney," *The EMBO Journal*, vol. 25, no. 21, pp. 5214–5228, 2006.

[17] A. Das, S. Tanigawa, C. M. Karner et al., "Stromal-epithelial crosstalk regulates kidney progenitor cell differentiation," *Nature Cell Biology*, vol. 15, no. 9, pp. 1035–1044, 2013.

[18] J. L. Fetting, J. A. Guay, M. J. Karolak et al., "FOXD1 promotes nephron progenitor differentiation by repressing decorin in the embryonic kidney," *Development*, vol. 141, no. 1, pp. 17–27, 2014.

[19] M. N. Lombard and C. Grobstein, "Activity in various embryonic and postembryonic sources for induction of kidney tubules," *Developmental Biology*, vol. 19, no. 1, pp. 41–51, 1969.

[20] C. Grobstein, "Some transmission characteristics of the tubule-inducing influence on mouse metanephrogenic mesenchyme," *Experimental Cell Research*, vol. 13, no. 3, pp. 575–587, 1957.

[21] E. Lehtonen, J. Wartiovaara, S. Nordling, and L. Saxen, "Demonstration of cytoplasmic processes in Millipore filters permitting kidney tubule induction," *Journal of Embryology and Experimental Morphology*, vol. 33, no. 1, pp. 187–203, 1975.

[22] C. Grobstein, "Trans-filter induction of tubules in mouse metanephrogenic mesenchyme," *Experimental Cell Ressearch*, vol. 10, no. 2, pp. 424–440, 1956.

[23] J. Wartiovaara, S. Nordling, E. Lehtonen, and L. Saxén, "Trans-filter induction of kidney tubules: correlation with cytoplasmic penetration into Nucleopore filters," *Journal of Embryology and Experimental Morphology*, vol. 31, no. 3, pp. 667–682, 1974.

[24] A. Kispert, S. Vainio, and A. P. McMahon, "Wnt-4 is a mesenchymal signal for epithelial transformation of metanephric mesenchyme in the developing kidney," *Development*, vol. 125, no. 21, pp. 4225–4234, 1998.

[25] C. Tetta, E. Ghigo, L. Silengo, M. C. Deregibus, and G. Camussi, "Extracellular vesicles as an emerging mechanism of cell-to-cell communication," *Endocrine*, vol. 44, no. 1, pp. 11–19, 2013.

[26] D. Sun, X. Zhuang, S. Zhang et al., "Exosomes are endogenous nanoparticles that can deliver biological information between cells," *Advanced Drug Delivery Reviews*, vol. 65, no. 3, pp. 342–347, 2013.

[27] G. Raposo and W. Stoorvogel, "Extracellular vesicles: exosomes, microvesicles, and friends," *Journal of Cell Biology*, vol. 200, no. 4, pp. 373–383, 2013.

[28] D. Y. P. Fang, H. W. King, J. Y. Z. Li, and J. M. Gleadle, "Exosomes and the kidney: blaming the messenger," *Nephrology*, vol. 18, no. 1, pp. 1–10, 2013.

[29] W. E. Koch and C. Grobstein, "Transmission of radioisotopically labeled materials during embryonic induction in vitro," *Developmental Biology*, vol. 7, pp. 303–323, 1963.

[30] J. A. Davies and D. R. Garrod, "Induction of early stages of kidney tubule differentiation by lithium ions," *Developmental Biology*, vol. 167, no. 1, pp. 50–60, 1995.

[31] K. Halt and S. Vainio, "In vitro induction of nephrogenesis in mouse metanephric mesenchyme with lithium introduction and ureteric bud recombination," *Methods in Molecular Biology*, vol. 886, pp. 23–30, 2012.

[32] A. C. Brown, S. D. Muthukrishnan, J. A. Guay et al., "Role for compartmentalization in nephron progenitor differentiation," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 110, no. 12, pp. 4640–4645, 2013.

[33] S. Kuure, A. Popsueva, M. Jakobson, K. Sainio, and H. Sariola, "Glycogen synthase kinase-3 inactivation and stabilization of $\beta$-catenin induce nephron differentiation in isolated mouse and rat kidney mesenchymes," *Journal of the American Society of Nephrology*, vol. 18, no. 4, pp. 1130–1139, 2007.

[34] R. S. Jope, "Lithium and GSK-3: one inhibitor, two inhibitory actions, multiple outcomes," *Trends in Pharmacological Sciences*, vol. 24, no. 9, pp. 441–443, 2003.

[35] S. Ye, L. Tan, R. Yang et al., "Pleiotropy of glycogen synthase kinase-3 inhibition by CHIR99021 promotes self-renewal of embryonic stem cells from refractory mouse strains," *PLoS ONE*, vol. 7, no. 4, Article ID e35892, 2012.

[36] A. Voronkov and S. Krauss, "Wnt/beta-catenin signaling and small molecule inhibitors," *Current Pharmaceutical Design*, vol. 19, no. 4, pp. 634–664, 2013.

[37] K. Stark, S. Vainio, G. Vassileva, and A. P. McMahon, "Epithelial transformation metanephric mesenchyme in the developing kidney regulated by Wnt-4," *Nature*, vol. 372, no. 6507, pp. 679–683, 1994.

[38] P. Ekblom, A. Miettinen, I. Virtanen, T. Wahlström, A. Dawnay, and L. Saxén, "*In vitro* segregation of the metanephric nephron," *Developmental Biology*, vol. 84, no. 1, pp. 88–95, 1981.

[39] I. Thesleff and P. Ekblom, "Role of transferrin in branching morphogenesis, growth and differentiation of the embryonic kidney," *Journal of Embryology and Experimental Morphology*, vol. 82, pp. 147–161, 1984.

[40] R. Jaenisch, "Transgenic animals," *Science*, vol. 240, no. 4858, pp. 1468–1474, 1988.

[41] R. Jaenisch and B. Mintz, "Simian virus 40 DNA sequences in DNA of healthy adult mice derived from preimplantation blastocysts injected with viral DNA," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 71, no. 4, pp. 1250–1254, 1974.

[42] B. H. Koller, L. J. Hagemann, T. J. R. Doetschman Hagaman et al., "Germ-line transmission of a planned alteration made in a hypoxanthine phosphoribosyltransferase gene by homologous recombination in embryonic stem cells," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 86, no. 22, pp. 8927–8931, 1989.

[43] E. D. Avner, W. E. Sweeney Jr., N. P. Piesco, and D. Ellis, "Growth factor requirements of organogenesis in serum-free metanephric organ culture," *In Vitro Cellular & Developmental Biology*, vol. 21, no. 5, pp. 297–304, 1985.

[44] M. J. Evans and M. H. Kaufman, "Establishment in culture of pluripotential cells from mouse embryos," *Nature*, vol. 292, no. 5819, pp. 154–156, 1981.

[45] J. W. Mugford, P. Sipilä, J. A. McMahon, and A. P. McMahon, "Osr1 expression demarcates a multi-potent population of intermediate mesoderm that undergoes progressive restriction to an Osr1-dependent nephron progenitor compartment within the mammalian kidney," *Developmental Biology*, vol. 324, no. 1, pp. 88–98, 2008.

[46] G. R. Dressler, U. Deutsch, K. Chowdhury, H. O. Nornes, and P. Gruss, "Pax2, a new murine paired-box-containing gene and its expression in the developing excretory system," *Development*, vol. 109, no. 4, pp. 787–795, 1990.

[47] U. W. Rothenpieler and G. R. Dressler, "*Pax-2* is required for mesenchyme-to-epithelium conversion during kidney development," *Development*, vol. 119, no. 3, pp. 711–720, 1993.

[48] P. D. Brophy, L. Ostrom, K. M. Lang, and G. R. Dressler, "Regulation of ureteric bud outgrowth by Pax2-dependent activation of the glial derived neurotrophic factor gene," *Development*, vol. 128, no. 23, pp. 4747–4756, 2001.

[49] K. Pritchard-Jones, J. Renshaw, and L. King-Underwood, "The Wilms tumour (WT1) gene is mutated in a secondary leukaemia in a WAGR patient," *Human Molecular Genetics*, vol. 3, no. 9, pp. 1633–1637, 1994.

[50] J. A. Kreidberg, H. Sariola, J. M. Loring et al., "WT-1 is required for early kidney development," *Cell*, vol. 74, no. 4, pp. 679–691, 1993.

[51] N. Miyamoto, M. Yoshida, S. Kuratani, I. Matsuo, and S. Aizawa, "Defects of urogenital development in mice lacking Emx2," *Development*, vol. 124, no. 9, pp. 1653–1664, 1997.

[52] R. Nishinakamura, Y. Matsumoto, K. Nakao et al., "Murine homolog of SALL1 is essential for ureteric bud invasion in kidney development," *Development*, vol. 128, no. 16, pp. 3105–3115, 2001.

[53] H. L. Hellmich, L. Kos, E. S. Cho, K. A. Mahon, and A. Zimmer, "Embryonic expression of glial cell-line derived neurotrophic factor (GDNF) suggests multiple developmental roles in neural differentiation and epithelial-mesenchymal interactions," *Mechanisms of Development*, vol. 54, no. 1, pp. 95–105, 1996.

[54] K. Sainio, P. Suvanto, J. Davies et al., "Glial-cell-line-derived neurotrophic factor is required for bud initiation from ureteric epithelium," *Development*, vol. 124, no. 20, pp. 4077–4087, 1997.

[55] R. Shakya, T. Watanabe, and F. Costantini, "The role of GDNF/Ret signaling in ureteric bud cell fate and branching morphogenesis," *Developmental Cell*, vol. 8, no. 1, pp. 65–74, 2005.

[56] S. Srinivas, M. R. Goldberg, T. Watanabe, V. D'Agati, Q. Al-Awqati, and F. Costantini, "Expression of green fluorescent protein in the ureteric bud of transgenic mice: a new tool for the analysis of ureteric bud morphogenesis," *Developmental Genetics*, vol. 24, no. 3-4, pp. 241–251, 1999.

[57] Q. Al-awqati and M. R. Goldberg, "Architectural patterns in branching morphogenesis in the kidney," *Kidney International*, vol. 54, no. 6, pp. 1832–1842, 1998.

[58] T. Watanabe and F. Costantini, "Real-time analysis of ureteric bud branching morphogenesis in vitro," *Developmental Biology*, vol. 271, no. 1, pp. 98–108, 2004.

[59] B. C. Lu, C. Cebrian, X. Chi et al., "Etv4 and Etv5 are required downstream of GDNF and Ret for kidney branching morphogenesis," *Nature Genetics*, vol. 41, no. 12, pp. 1295–1302, 2009.

[60] F. Costantini, "Renal branching morphogenesis: concepts, questions, and recent advances," *Differentiation*, vol. 74, no. 7, pp. 402–421, 2006.

[61] J. A. Davies, "Do different branching epithelia use a conserved developmental mechanism?" *BioEssays*, vol. 24, no. 10, pp. 937–948, 2002.

[62] S. Ishibe, A. Karihaloo, H. Ma et al., "Met and the epidermal growth factor receptor act cooperatively to regulate final nephron number and maintain collecting duct morphology," *Development*, vol. 136, no. 2, pp. 337–345, 2009.

[63] J. E. Cain, S. Hartwig, J. F. Bertram, and N. D. Rosenblum, "Bone morphogenetic protein signaling in the developing kidney: present and future," *Differentiation*, vol. 76, no. 8, pp. 831–842, 2008.

[64] U. Grieshammer, L. Ma, A. S. Plump, F. Wang, M. Tessier-Lavigne, and G. R. Martin, "SLIT2-mediated ROBO2 signaling restricts kidney induction to a single site," *Developmental Cell*, vol. 6, no. 5, pp. 709–717, 2004.

[65] Y. Miyazaki, K. Oshima, A. Fogo, B. L. M. Hogan, and I. Ichikawa, "Bone morphogenetic protein 4 regulates the budding site and elongation of the mouse ureter," *Journal of Clinical Investigation*, vol. 105, no. 7, pp. 863–873, 2000.

[66] A. Tufro, J. Teichman, C. Woda, and G. Villegas, "*Semaphorin3a* inhibits ureteric bud branching morphogenesis," *Mechanisms of Development*, vol. 125, no. 5-6, pp. 558–568, 2008.

[67] E. Rosines, R. V. Sampogna, K. Johkura et al., "Staged *in vitro* reconstitution and implantation of engineered rat kidney tissue," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 104, no. 52, pp. 20938–20943, 2007.

[68] T. N. Meyer, C. Schwesinger, K. T. Bush et al., "Spatiotemporal regulation of morphogenetic molecules during in vitro branching of the isolated ureteric bud: toward a model of branching through budding in the developing kidney," *Developmental Biology*, vol. 275, no. 1, pp. 44–67, 2004.

[69] K. T. Bush, H. Sakurai, D. L. Steer et al., "TGF-$\beta$ superfamily members modulate growth, branching, shaping, and patterning of the ureteric bud," *Developmental Biology*, vol. 266, no. 2, pp. 285–298, 2004.

[70] L. Saxen and J. Wartiovaara, "Cell contact and cell adhesion during tissue organization," *International Journal of Cancer*, vol. 1, no. 3, pp. 271–290, 1966.

[71] S. E. Quaggin and J. A. Kreidberg, "Development of the renal glomerulus: good neighbors and good fences," *Development*, vol. 135, no. 4, pp. 609–620, 2008.

[72] C. Schell, N. Wanner, and TB. Huber, "Glomerular development—shaping the multi-cellular filtration unit," *Seminars in Cell & Developmental Biology*, vol. 36C, pp. 39–49, 2014.

[73] T. J. Carroll, J. S. Park, S. Hayashi, A. Majumdar, and A. P. McMahon, "Wnt9b plays a central role in the regulation of mesenchymal to epithelial transitions underlying organogenesis of the mammalian urogenital system," *Developmental Cell*, vol. 9, no. 2, pp. 283–292, 2005.

[74] C. M. Karner, R. Chirumamilla, S. Aoki, P. Igarashi, J. B. Wallingford, and T. J. Carroll, "Wnt9b signaling regulates planar cell polarity and kidney tubule morphogenesis," *Nature Genetics*, vol. 41, no. 7, pp. 793–799, 2009.

[75] J. S. Park, M. T. Valerius, and A. P. McMahon, "Wnt/$\beta$-catenin signaling regulates nephron induction during mouse kidney development," *Development*, vol. 134, no. 13, pp. 2533–2539, 2007.

[76] J. Shan, T. Jokela, I. Skovorodkin, and S. Vainio, "Mapping of the fate of cell lineages generated from cells that express the Wnt4 gene by time-lapse during kidney development," *Differentiation*, vol. 79, no. 1, pp. 57–64, 2010.

[77] H.-T. Cheng, M. Kim, M. T. Valerius et al., "Notch2, but not Notch1, is required for proximal fate acquisition in the mammalian nephron," *Development*, vol. 134, no. 4, pp. 801–811, 2007.

[78] K. Surendran, S. Boyle, H. Barak et al., "The contribution of Notch1 to nephron segmentation in the developing kidney is revealed in a sensitized Notch2 background and can be augmented by reducing Mint dosage," *Developmental Biology*, vol. 337, no. 2, pp. 386–395, 2010.

[79] N. O. Lindstrom, P. Hohenstein, and J. A. Davies, "Nephrons require Rho-kinase for proximal-distal polarity development," *Scientific Reports*, vol. 3, article 2692, 2013.

[80] L. Michael, D. E. Sweeney, and J. A. Davies, "A role for microfilament-based contraction in branching morphogenesis of the ureteric bud," *Kidney International*, vol. 68, no. 5, pp. 2010–2018, 2005.

[81] J. W. Mugford, J. Yu, A. Kobayashi, and A. P. McMahon, "High-resolution gene expression analysis of the developing mouse kidney defines novel cellular compartments within the nephron progenitor population," *Developmental Biology*, vol. 333, no. 2, pp. 312–323, 2009.

[82] F. Morel, "The loop of Henle, a turning-point in the history of kidney physiology," *Nephrology Dialysis Transplantation*, vol. 14, no. 10, pp. 2510–2515, 1999.

[83] D. D. R. Sebinger, M. Unbekandt, V. V. Ganeva, A. Ofenbauer, C. Werner, and J. A. Davies, "A novel, low-volume method for

organ culture of embryonic kidneys that allows development of cortico-medullary anatomical organization," *PLoS ONE*, vol. 5, no. 5, Article ID e10550, 2010.

[84] Y. Kitamoto, H. Tokunaga, and K. Tomita, "Vascular endothelial growth factor is an essential molecule for mouse kidney development: glomerulogenesis and nephrogenesis," *Journal of Clinical Investigation*, vol. 99, no. 10, pp. 2351–2357, 1997.

[85] K. Sison, V. Eremina, H. Baelde et al., "Glomerular structure and function require paracrine, not autocrine, VEGF-VEGFR-2 signaling," *Journal of the American Society of Nephrology*, vol. 21, no. 10, pp. 1691–1701, 2010.

[86] H. Sariola, P. Ekblom, E. Lehtonen, and L. Saxen, "Differentiation and vascularization of the metanephric kidney grafted on the chorioallantoic membrane," *Developmental Biology*, vol. 96, no. 2, pp. 427–435, 1983.

[87] H. Sariola, "Incomplete fusion of the epithelial and endothelial basement membranes in interspecies hybrid glomeruli," *Cell Differentiation*, vol. 14, no. 3, pp. 189–195, 1984.

[88] G. R. Dressler, "Tubulogenesis in the developing mammalian kidney," *Trends in Cell Biology*, vol. 12, no. 8, pp. 390–395, 2002.

[89] G. R. Dressler, "The cellular basis of kidney development," *Annual Review of Cell and Developmental Biology*, vol. 22, pp. 509–529, 2006.

[90] K. Halt and S. Vainio, "Coordination of kidney organogenesis by Wnt signaling," *Pediatric Nephrology*, vol. 29, no. 4, pp. 737–744, 2014.

[91] I. Pietilä and S. J. Vainio, "Kidney development: an overview," *Nephron—Experimental Nephrology*, vol. 126, no. 2, pp. 40–44, 2014.

[92] R. Auerbach and C. Grobstein, "Inductive interaction of embryonic tissues after dissociation and reaggregation," *Experimental Cell Research*, vol. 15, no. 2, pp. 384–397, 1958.

[93] M. Unbekandt and J. A. Davies, "Dissociation of embryonic kidneys followed by reaggregation allows the formation of renal tissues," *Kidney International*, vol. 77, no. 5, pp. 407–416, 2010.

[94] S. Juntilla, U. Saarela, K. Halt et al., "Functional genetic targeting of embryonic kidney progenitor cells *ex vivo*," *Journal of the American Society of Nephrology*, 2014.

[95] M. Herrera and M. Mirotsou, "Stem cells: potential and challenges for kidney repair," *The American Journal of Physiology—Renal Physiology*, vol. 306, no. 1, pp. F12–F23, 2014.

[96] K. Takahashi and S. Yamanaka, "Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors," *Cell*, vol. 126, no. 4, pp. 663–676, 2006.

[97] A. Rak-Raszewska, B. Wilm, D. Edgar, S. Kenny, A. S. Woolf, and P. Murray, "Development of embryonic stem cells in recombinant kidneys," *Organogenesis*, vol. 8, no. 4, pp. 125–136, 2012.

[98] M. Kuzma-Kuzniarska, A. Rak-Raszewska, S. Kenny et al., "Integration potential of mouse and human bone marrow-derived mesenchymal stem cells," *Differentiation*, vol. 83, no. 3, pp. 128–137, 2012.

[99] L. Perin, S. Giuliani, D. Jin et al., "Renal differentiation of amniotic fluid stem cells," *Cell Proliferation*, vol. 40, no. 6, pp. 936–948, 2007.

[100] N. Siegel, M. Rosner, M. Unbekandt et al., "Contribution of human amniotic fluid stem cells to renal tissue formation depends on mTOR," *Human Molecular Genetics*, vol. 19, no. 17, pp. 3320–3331, 2010.

[101] M. Takasato, P. X. Er, M. Becroft et al., "Directing human embryonic stem cell differentiation towards a renal lineage generates a self-organizing kidney," *Nature Cell Biology*, vol. 16, no. 1, pp. 118–126, 2014.

[102] C. Xinaris, V. Benedetti, P. Rizzo et al., "In vivo maturation of functional renal organoids formed from embryonic cell suspensions," *Journal of the American Society of Nephrology*, vol. 23, no. 11, pp. 1857–1868, 2012.

[103] E. Rosines, K. Johkura, X. Zhang et al., "Constructing kidney-like tissues from cells based on programs for organ development: toward a method of in vitro tissue engineering of the kidney," *Tissue Engineering Part A*, vol. 16, no. 8, pp. 2441–2455, 2010.

[104] P. V. Hauser, M. Nishikawa, H. Kimura, T. Fujii, and N. Yanagawa, "Controlled tubulogenesis from dispersed ureteric bud-derived cells using a micropatterned gel," *Journal of Tissue Engineering and Regenerative Medicine*, 2014.

[105] T. Cui, R. Terlecki, and A. Atala, "Tissue engineering in urethral reconstruction," *Archivos Espanoles de Urologia*, vol. 67, no. 1, pp. 29–34, 2014.

[106] A. M. Raya-Rivera, D. Esquiliano, R. Fierro-Pastrana et al., "Tissue-engineered autologous vaginal organs in patients: a pilot cohort study," *The Lancet*, vol. 384, no. 9940, pp. 329–336, 2014.

[107] S. V. Murphy and A. Atala, " 3D bioprinting of tissues and organs," *Nature Biotechnology*, vol. 32, no. 8, pp. 773–785, 2014.

[108] A. Skardal and A. Atala, "Biomaterials for integration with 3-D bioprinting," *Annals of Biomedical Engineering*, 2014.

[109] D. A. Zopf, S. J. Hollister, M. E. Nelson, R. G. Ohye, and G. E. Green, "Bioresorbable airway splint created with a three-dimensional printer," *The New England Journal of Medicine*, vol. 368, no. 21, pp. 2043–2045, 2013.

[110] X. Cui, K. Breitenkamp, M. Lotz, and D. D'Lima, "Synergistic action of fibroblast growth factor-2 and transforming growth factor-beta1 enhances bioprinted human neocartilage formation," *Biotechnology and Bioengineering*, vol. 109, no. 9, pp. 2357–2368, 2012.

[111] C. Booth, T. Soker, P. Baptista et al., "Liver bioengineering: current status and future perspectives," *World Journal of Gastroenterology*, vol. 18, no. 47, pp. 6926–6934, 2012.

[112] S. H. Mirmalek-Sani, G. Orlando, J. P. McQuilling et al., "Porcine pancreas extracellular matrix as a platform for endocrine pancreas bioengineering," *Biomaterials*, vol. 34, no. 22, pp. 5488–5495, 2013.

[113] H. C. Ott, T. S. Matthiesen, S. K. Goh et al., "Perfusion-decellularized matrix: using nature's platform to engineer a bioartificial heart," *Nature Medicine*, vol. 14, no. 2, pp. 213–221, 2008.

[114] J. J. Song, J. P. Guyette, S. E. Gilpin, G. Gonzalez, J. P. Vacanti, and H. C. Ott, "Regeneration and experimental orthotopic transplantation of a bioengineered kidney," *Nature Medicine*, vol. 19, no. 5, pp. 646–651, 2013.

[115] A. C. O'Neill and S. D. Ricardo, "Human kidney cell reprogramming: applications for disease modeling and personalized medicine," *Journal of the American Society of Nephrology*, vol. 24, no. 9, pp. 1347–1356, 2013.

[116] B. Song, J. C. Niclis, M. A. Alikhan et al., "Generation of induced pluripotent stem cells from human kidney mesangial cells," *Journal of the American Society of Nephrology*, vol. 22, no. 7, pp. 1213–1220, 2011.

[117] L. Saxen, *Organogenesis of the kidney*, Cambridge University Press, Cambridge, UK, 1987.

[118] R. E. Godin, E. J. Robertson, and A. T. Dudley, "Role of BMP family members during kidney development," *International Journal of Developmental Biology*, vol. 43, no. 5, pp. 405–411, 1999.

[119] L. Jia, F. Liu, S. H. Hansen, M. B. A. Ter Beest, and M. M. P. Zegers, "Distinct roles of cadherin-6 and E-cadherin in tubulogenesis and lumen formation," *Molecular Biology of the Cell*, vol. 22, no. 12, pp. 2031–2041, 2011.

Hindawi Publishing Corporation
Stem Cells International
Volume 2015, Article ID 547636, 19 pages
http://dx.doi.org/10.1155/2015/547636

*Research Article*

# Atlas of Cellular Dynamics during Zebrafish Adult Kidney Regeneration

**Kristen K. McCampbell, Kristin N. Springer, and Rebecca A. Wingert**

*Department of Biological Sciences and Center for Zebrafish Research, University of Notre Dame, Notre Dame, IN 46556, USA*

Correspondence should be addressed to Rebecca A. Wingert; rwingert@nd.edu

Received 7 November 2014; Accepted 7 January 2015

Academic Editor: Takashi Yokoo



The zebrafish is a useful animal model to study the signaling pathways that orchestrate kidney regeneration, as its renal nephrons are simple, yet they maintain the biological complexity inherent to that of higher vertebrate organisms including mammals. Recent studies have suggested that administration of the aminoglycoside antibiotic gentamicin in zebrafish mimics human acute kidney injury (AKI) through the induction of nephron damage, but the timing and details of critical phenotypic events associated with the regeneration process, particularly in existing nephrons, have not been characterized. Here, we mapped the temporal progression of cellular and molecular changes that occur during renal epithelial regeneration of the proximal tubule in the adult zebrafish using a platform of histological and expression analysis techniques. This work establishes the timing of renal cell death after gentamicin injury, identifies proliferative compartments within the kidney, and documents gene expression changes associated with the regenerative response of proliferating cells. These data provide an important descriptive atlas that documents the series of events that ensue after damage in the zebrafish kidney, thus availing a valuable resource for the scientific community that can facilitate the implementation of zebrafish research to delineate the mechanisms that control renal regeneration.

## 1. Introduction

The vertebrate kidney is comprised of functional units known as nephrons, which are epithelial tubules that cleanse the bloodstream of metabolic waste through vascular filtration and subsequent urine production [1]. Vertebrates form up to three kidney structures that are comprised of nephrons during development, termed the pronephros, mesonephros, and metanephros [1, 2]. A conserved trait of nephrons among these kidney forms across diverse terrestrial and aquatic vertebrate species is that they display a fundamentally similar regional organization along their length, containing a renal corpuscle that serves to filter blood, proximal and distal tubule segments that are specialized to perform discrete tasks in solute reabsorption and secretion, and a collecting duct that transports urine out of the organ and modifies salt and water levels [3, 4].

Acute kidney injury (AKI) is a devastating and often lethal condition in which nephron epithelial cells are destroyed by damage from ischemia or toxin exposure, typically affecting proximal tubule segments [5]. While there is compelling evidence from work in various fish and mammalian models that vertebrate nephron epithelial tubule cells can be robustly regenerated after some forms of AKI damage [6, 7], there is still a poor understanding of the mechanisms that mediate this regeneration response, and there are ongoing controversies regarding the cell(s) of origin that enable kidney regeneration in different species [2, 8, 9].

The zebrafish, *Danio rerio*, has emerged as a genetically tractable vertebrate model to study renal biology and associated medical conditions such as AKI, both in the embryonic and in adult settings [10]. The zebrafish embryo kidney, which is functional pronephros, consists of a pair of segmented nephrons that share a blood filter and each contains two proximal and two distal tubule segments [11, 12]. This structure forms by 1 day post fertilization (dpf) and blood filtration commences at approximately 2 dpf [13, 14], thus proffering a rapid and anatomically simple system

for research on nephron patterning [15–18], identification of essential genes [11–13, 19], tubulogenesis [20, 21], and physiology and disease modeling [22–26]. In comparison, the adult zebrafish kidney, or mesonephros, is a single, relatively flat organ attached to the dorsal body wall that consists of characteristic bilaterally symmetric regions referred to as the head (or anterior), trunk (or medial), and tail (or posterior) (Figure 1(a)) [27]. The mesonephros begins to form between about 12 and 14 dpf, with the progressive addition of nephrons to the existing pronephric pair [28]. Over the lifespan of the zebrafish, the mesonephros continues to accumulate nephrons—a phenomenon linked to their continual adult growth (measured in tip to tail length of the animal) and the associated increasing excretory demands [28]. In the typical zebrafish adult at around 6 months of age, this mesonephric kidney is estimated to possess approximately 450 nephrons, with the most densely populated sites of nephrons in the head and trunk [28]. The adult nephrons have similar segments as found in the pronephros but are grouped in branched arrangements (Figure 1(a)) [29], which like other fishes do not show a regular orientation of nephrons as seen in the cortex and medulla of the mammalian metanephric kidney [6]. Zebrafish mesonephric nephrons commonly have shared distal tubule segments (Figure 1(a)) and drain into a pair of major collecting ducts that span the length of the organ [10, 29]. The stroma, consisting of the cells interspersed between mesonephric nephrons, is the site of adult hematopoiesis and also contains an intriguing populace of mesenchymal renal progenitors [10, 28, 29]. These renal progenitors provide a continual source of new nephrons that are produced in a process termed nephron neogenesis or neonephrogenesis, which occurs during the aforementioned processes of mesonephros development and when the mesonephros grows in response to naturally increasing biomass of the aging fish [10, 28, 29].

Overall, the complexity of the zebrafish mesonephric kidney provides a useful adult setting for renal biology studies and shows promise for identifying genetic components of the renal regeneration response that can complement research in traditional mammalian AKI models such as the mouse and rat [10]. To date, kidney regeneration paradigms in adult zebrafish have included nephrotoxin administration, specifically of the aminoglycoside antibiotic gentamicin to induce widespread nephron tubule damage [10, 28, 29], as well as the creation of several transgenic strains that were engineered to induce ablation of particular epithelial cell types in the nephron blood filter [30, 31]. Among the former, previous studies have demonstrated that two major events transpire following gentamicin-induced AKI in the adult zebrafish kidney organ: (1) neonephrogenesis, or the production of new nephrons due to the activation of the aforementioned stromal renal progenitors, which is detectable by histology based on the appearance of basophilic nephron units [10, 28, 29], and (2) partial functional restoration in existing nephrons around 4 days post injury (dpi), suggestive that the damaged nephron tubule epithelium recovers rather rapidly from chemical insult [29]. Despite these observations, the precise temporal sequence of molecular events that transpire within zebrafish nephrons during AKI, including cell death and proliferation, has received relatively little scrutiny. Some alterations in gene expression have been annotated in prior work, such as the transient abrogation of transcripts encoding *slc20a1a* [29], a sodium-dependent phosphate transporter that is normally localized to the proximal tubule epithelium, but further observations have been quite limited.

A significant impediment toward the pursuit of characterizing kidney regeneration aspects in zebrafish has been the paucity of histological and other molecular labeling methodologies tailored for use in this model organism. More recently, we have adapted a number of techniques to illuminate renal structures that can now be utilized [32]. For example, we demonstrated that the various proximal tubule segments could be distinguished by unique combinations of lectin staining, dextran uptake, and alkaline phosphatase (AP) reactivity (Figure 1(a)) [32]. Further, distal tubules are distinguished by labeling with a different lectin (Figure 1(a)) [32].

Here, to obtain a more detailed understanding of the regeneration events associated with zebrafish renal injury and identify cellular attributes enabling the demarcation of tubule segments, we performed extensive histological and immunofluorescence studies to annotate the sequence of tissue changes that result following gentamicin nephrotoxicity. For this work, we used and/or modified several traditional histology protocols for use with zebrafish renal tissues and also implemented a number of our recently developed nephron labeling methodologies [32]. With these tools, we now demonstrate for the first time that following gentamicin-induced AKI, nephron proximal tubule epithelial regeneration proceeds over approximately one week. We have now documented the detailed succession of cell death and proliferation in proximal tubules during this interval. Through additional structural and functional assays, we show that proximal tubules throughout the post-AKI zebrafish kidney regain absorptive capacity between 14 and 21 dpi. Further, we show that neonephrogenesis occurs in a partially overlapping time frame as nephron epithelial regeneration, beginning around 5 dpi and progressing over the subsequent two weeks, and show that regenerating populations in both existing and new nephrons express the renal transcription factor Pax2. Taken together, these descriptive and functional studies provide an essential foundation for future work aimed at elucidating the mechanisms that regulate kidney regeneration following AKI in the adult zebrafish.

## 2. Results

The aminoglycoside antibiotic gentamicin is an established nephrotoxin that has been used to model AKI by inducing renal tubule damage in the adult zebrafish [10, 28, 29], as well as other fish species, such as goldfish and medaka [6, 33]. In the latter studies, the histology of nephron phenotypes after gentamicin administration was documented, including assessment of cell proliferation by such measures as detection of proliferating cell nuclear antigen (PCNA) labeling and the incorporation of 5-bromo, 2′deoxyuridine (BrdU) [6, 33]. Previous studies in zebrafish have documented the appearance of gentamicin-damaged nephrons at 1 dpi and the incorporation of BrdU in immature nephrons [29], but

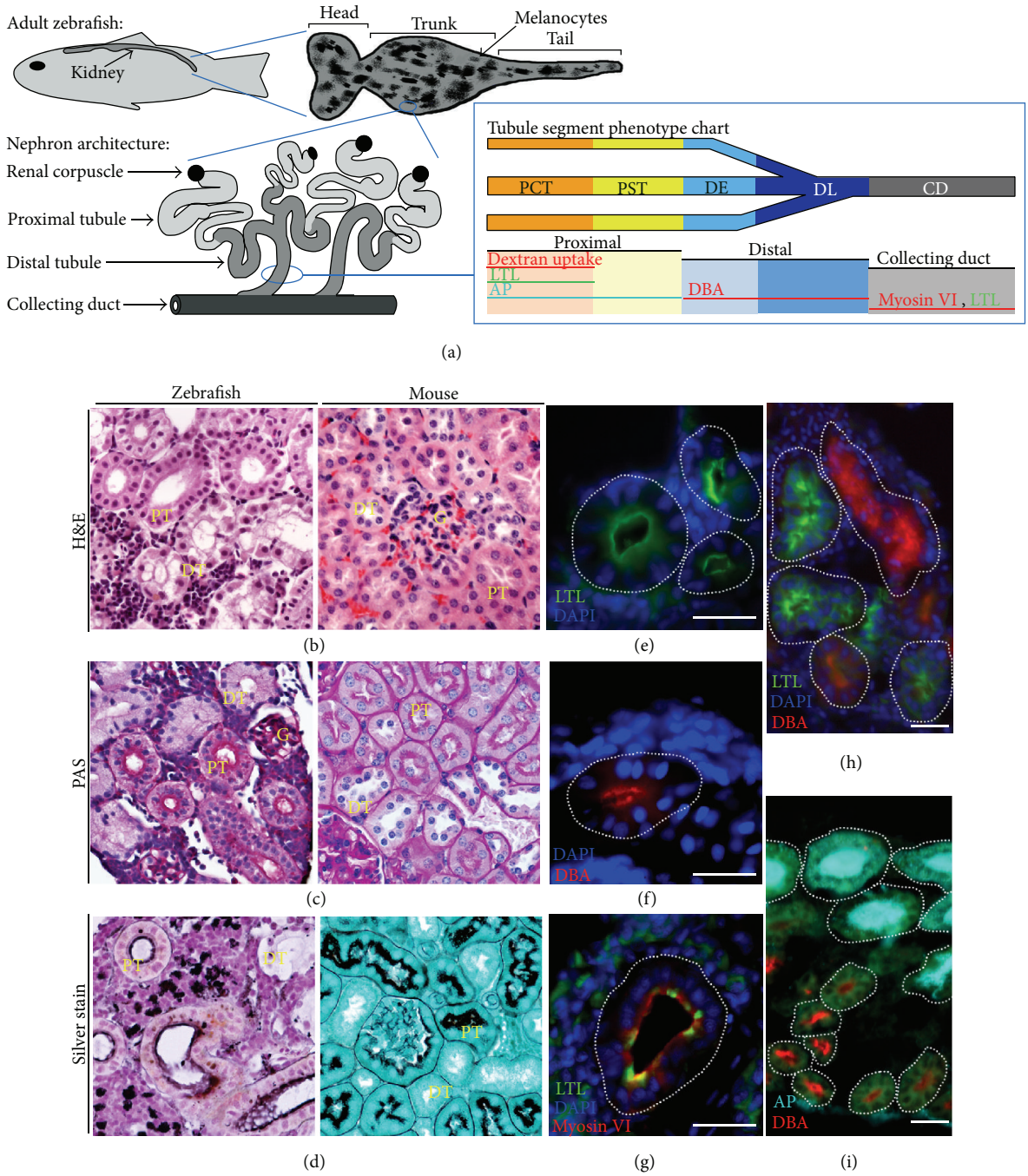



Figure 1: Anatomy of the zebrafish kidney and the identification of histological stains to distinguish renal structures. (a) The adult zebrafish kidney is comprised of arborized arrangements of nephrons that share common distal late tubule segments and drain into major collecting ducts (schematic adapted from [32]). ((b)–(d)) Histological staining in the zebrafish kidney revealed similarities to that of mammalian organ structure. (b) H&E and (c) PAS staining of wild-type zebrafish and mouse kidney tissue emphasized the brush border and elongated cells characteristic of the proximal tubule (PT, yellow label) compared to the pale pink hue of the distal tubule (DT, yellow label). Blood filters or glomeruli (G, yellow label) were as indicated. (d) Silver staining highlighted the brush border in a deep brown hue and additionally stained hyaline droplets located in the PT, while DT structures lacked the brown labeling. Zebrafish tissue sections 60x, mouse tissue sections 40x. ((e)–(i)) Fluorescent labeling of zebrafish tubules and collecting duct, with perimeters of each respective structure outlined in white dots, and nuclei ((e)–(h)) labeled with DAPI. Scale bars, 25 $\mu$m. (e) LTL (green) stained the PT, while (f) DBA (red) stained the DT, and these tubule populations are mutually exclusive (h). (g) LTL also stained the collecting ducts, which were distinguished by a myosin VI antibody. (i) In transgenic *Tg:enpep:egfp* zebrafish, PT structures labeled with ELF-97 to detect alkaline phosphatase were mutually exclusive to DBA-stained distal tubules, and tubules were identified by labeling with anti-eGFP (representative panel reprinted with permission from [32]).

the sequence of cellular alterations in gentamicin-damaged nephrons over time has not been determined. To examine and document such cellular changes, we first adapted histological methods that would enable characterization of renal structures (Figure 1) and then utilized these and other approaches to explore the spatial and temporal sequence of proximal tubule cell death and proliferation (Figures 2–8).

*2.1. Histological Stains Distinguish Renal Structures.* The adult zebrafish kidney is comprised of pinwheel-like arrangements of nephrons in which several nephrons connect to individual branched distal tubules that drain into the collecting ducts (Figure 1(a)) [10, 29, 32]. Hematoxylin and eosin (H&E) staining is a basic method that distinguishes the proximal tubule from the distal tubule based partly on the presence of a brush border: the proximal tubule possesses a brush border, whereas distal tubules do not [34]. The brush border, found on the luminal side of the proximal tubule epithelial cells, is lined with densely packed microvilli, forming a surface that greatly increases the surface area of the cells, facilitating their reabsorption functions. When paraffin sections of the kidney from healthy adult zebrafish were stained with H&E, the brush border was prominent as a thick pink stained band at the apical side of dark-pink stained proximal tubule cells, which showed characteristic elongated shapes, and formed a dilated lumen (Figure 1(b)). In comparison, the cells of the distal tubule had a narrow lumen and stained with a much lighter pink hue, allowing clear distinction of segment identity (Figure 1(b)). H&E staining in the healthy adult murine kidney revealed a comparable staining result (Figure 1(b)).

Periodic acid-Schiff (PAS) is a staining method used to detect polysaccharides in tissues, and the reagents in this stain have an affinity for the brush border of the mammalian proximal tubule [35]. Adult zebrafish kidney paraffin sections were stained with PAS, which revealed that their nephrons possessed numerous characteristics conserved with the murine kidney (Figure 1(c)). Namely, the zebrafish proximal tubules were discernible as their brush borders stained a very deep shade of magenta and the cell cytoplasm was dark pink (Figure 1(c)). The basement membranes of the glomerular capillary loops and tubular epithelium were also stained with a similar deep shade of magenta, while epithelial cells of the distal tubule were stained pale pink in color (Figure 1(c)).

Methenamine silver stains are able to detect proteins and have been documented as a marker for basement membranes in mammals [36, 37]. Applying this stain to tissue paraffin sections of the zebrafish kidney, the basement membranes of tubules and glomeruli were visualized by a dark brown hue, and the brush borders of the proximal tubules were also stained dark brown (Figure 1(d)). In addition, the silver stain revealed the presence of hyaline droplets in the proximal tubules, which has also been documented in mammals [38].

*2.2. Tubule and Duct Compartments Can Be Distinguished Further with Labeling Methods Using Lectins, Myosin VI Expression, and Alkaline Phosphatase Reactivity with ELF-97.* Lectins are sugar-binding proteins of nonimmune origin that are expressed throughout nature [39]. Specifically in the kidney organ, *Lotus tetragonolobus* lectin (LTL) marks the proximal tubules, and the targets of *Dolichos biflorus* agglutinin (DBA) include the distal tubules and collecting ducts [39]. Previous studies requiring the ability to distinguish proximal versus distal tubules and quantify such structures in mice have utilized LTL and DBA staining with great success [39–42]. Similarly, for the identification of tubular segments in a medaka fish model of polycystic kidney disease, LTL was used as a proximal tubule marker, and DBA was used as a distal tubule marker [43]. Tissue cryosections of zebrafish adult kidneys were subjected to staining with LTL and DBA (Figures 1(e)–1(i)), as well as whole mount staining (data not shown). The binding specificity of the lectins was conserved in zebrafish, with LTL and DBA labeling distinct tubules (Figures 1(e) and 1(f), resp.), and these labels were mutually exclusive both in cryosection (Figure 1(h)) and in whole mount preparations (data not shown) [32]. Interestingly, the major collecting ducts in the zebrafish kidney, which are a pair of large drainage ducts that extend the entire length of the organ [28], were distinguished via colabeling of LTL and an antibody to detect myosin VI (Figure 1(g)). These were uniquely identified as each zebrafish kidney contained only two such structures, one on each symmetric side of the organ (data not shown).

As proximal tubules possess a brush border, a fluorescence-based method known as ELF- (enzyme labeled fluorescence-) 97 was used to determine if this activity could be localized in adult zebrafish kidneys. The brush borders of epithelial cells in the intestine are known to express high levels of endogenous alkaline phosphatase activity [44]. One previous study reported ELF-97 reactivity in the adult zebrafish kidney, suggesting that ELF-97 staining may be a viable way to label proximal tubule cells [45]. Tissue cryosections of adult zebrafish kidneys stained with the ELF-97 phosphatase were counterstained with the distal tubule marker DBA. The ELF-97 signals were localized to the proximal tubules, which have very prominent brush borders of microvilli that project into the tubule lumens (Figure 1(i), data not shown) [32]. In contrast, tubules that were identified by DBA completely lacked any ELF-97 precipitate (Figure 1(i), data not shown), and these observations were confirmed in whole mount kidney preparations as well (data not shown) [32]. These observations are in agreement with the knowledge that vertebrate nephron distal segments do not possess a brush border and would therefore not be stained with ELF-97 [4, 46].

*2.3. Histological Characterization of Gentamicin Injury Time Course.* Next, we utilized these various histological tools to analyze the phenotype of zebrafish nephrons following AKI. An intraperitoneal injection of gentamicin was administered to induce AKI in adult zebrafish, based on a previously established dosage [28, 29, 47]. The injected fish were sacrificed at several time points and their kidneys fixed, sectioned, and then stained with H&E (Figure 2(a)). At 1 dpi, sufficient nephron damage was induced that resulted in a denuded basement membrane and an immense amount of intraluminal cellular debris. The proximal tubular epithelium had become vacuolated and massive disorganization was

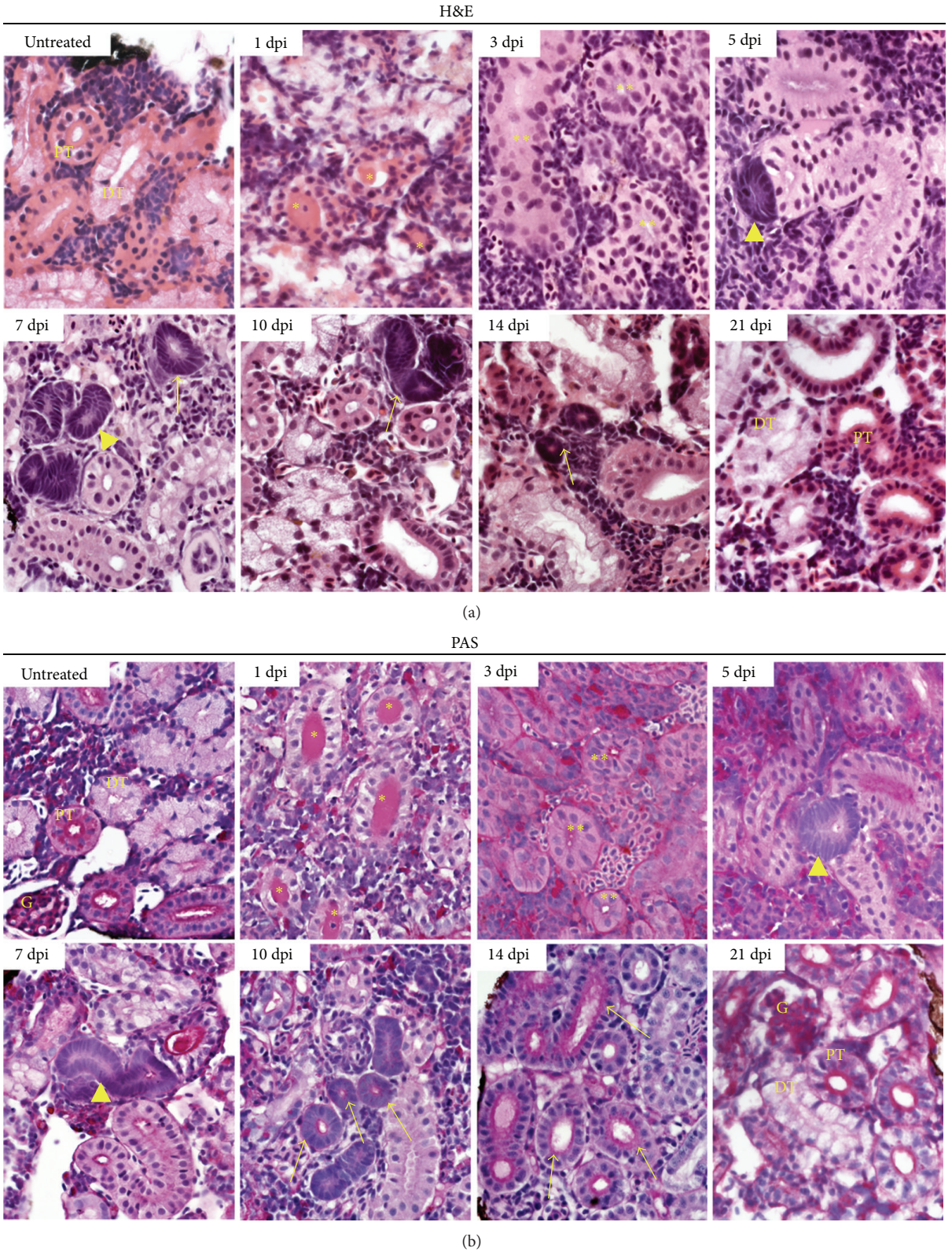



Figure 2: Histological staining reveals the process of regeneration after gentamicin-induced injury in adult zebrafish. (a) H&E and (b) PAS staining demonstrated extensive nephron damage and tubule destruction, followed by regenerative events that were completed by 21 dpi. The formation of basophilic cellular aggregates (darkest purple cellular staining) was indicative of new nephron formation. Both time courses were completed over a three-week period. 60x. DT: distal tubule; G: glomerulus; PT: proximal tubule. Yellow labels: asterisk (∗) indicates luminal debris; double asterisk (∗∗) indicates restoration of cells to tubules; arrowhead indicates neonephrogenic cluster; arrow indicates neonephron with visible lumen.

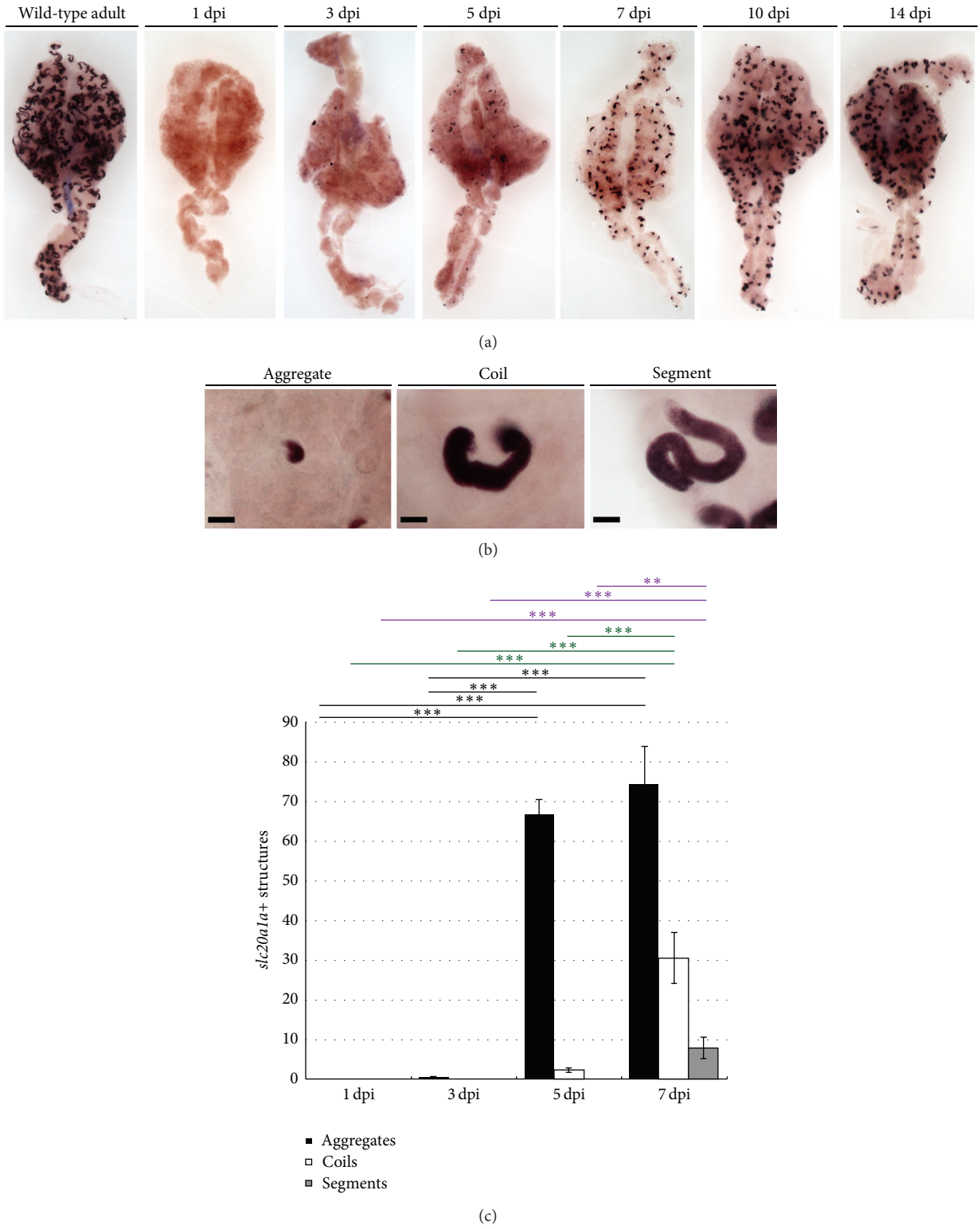



FIGURE 3: Dynamics of *slc20a1a* expression over the course of zebrafish adult kidney injury and regeneration. (a) Whole mount *in situ* hybridization of adult kidneys over a two-week injury time course revealed the initial absence of *slc20a1a* transcripts, followed by an increase in transcript levels and domains to near-normal levels by 14 dpi. Throughout the time course, new nephrons first appeared as small cellular aggregates that form coils and eventually elongated into PCT segments that are indistinguishable from preexisting, regenerated PCT tubules. 3x magnification. (b) Representative images of the progressive stages of nephron formation. Scale bar, 25 $\mu$m. (c) Quantification shows that *slc20a1a* positive structures during regeneration. Labels: $**$ indicates $P < 0.01$; $***$ indicates $P < 0.001$; black indicates aggregate; green indicates coil; purple indicates segment.

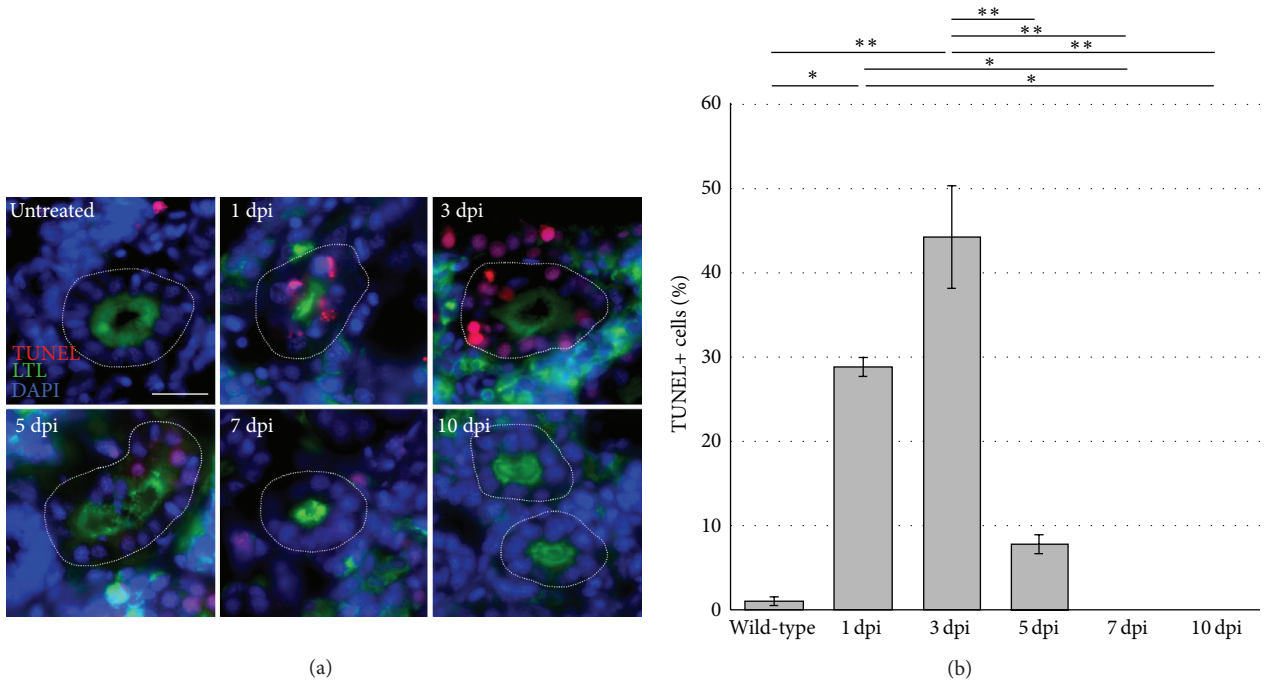



Figure 4: Detection of proximal tubule cell death after gentamicin injury. (a) Kidney tissue was assayed for TUNEL-positive cells in uninjured kidneys and at 1, 3, 5, 7, and 10 dpi. The peak of TUNEL reactivity in cells was identified within LTL-positive proximal tubules at 1, 3, and 5 days after kidney injury. By 7 and 10 days, the level of TUNEL-positive cells decreased, returning to basal levels previously established in untreated kidneys. Proximal tubules were labeled by LTL. (b) Quantification of TUNEL labeling in nephron proximal segments. Scale bar, 25 $\mu$m. Labels: $*$ indicates $P < 0.05$; $**$ indicates $P < 0.01$.

evident. Cellular disruption was still apparent at 3 dpi, but within the tubular spaces, cells with a mesenchymal structure were congregated. At 5 dpi, cells within the tubules were more organized, appearing as a visually intact single layer of cells with a hint of a luminal opening. In addition, a small number of basophilic, dark purple cellular clusters had emerged, which is a trait of neonephrogenesis in fish [6]. By 7 dpi, numerous basophilic cellular aggregates had formed, some containing a lumen, and a majority of cellular debris was cleared. Tubular lumens continued to form in the aggregates at 10 dpi and lumens that were evident at 7 dpi had widened. In addition, tubule cells displayed shades of pink similar to that of proximal and distal tubules. By 14 dpi, many of the aggregates possessed obvious brush borders, indicative of a proximal segment, and the kidney tissue overall was analogous to that of an uninjured adult fish. Kidney tissue staining at 21 dpi revealed a similar wild-type appearance with nonexistent cellular aggregates.

A second histological analysis was completed to stain zebrafish kidneys with PAS (Figure 2(b)). PAS was utilized because the stain emphasizes the brush borders of the proximal tubule more effectively than H&E, and thus we hypothesized that this staining method could provide additional insight into the establishment of epithelial character in regenerated proximal tubules. At 1 dpi, the appearance of PAS-positive intraluminal cellular debris was readily observed in distal tubules, as observed in H&E stained samples. Interestingly, hyaline casts have previously been documented as being PAS-positive in the injured kidney of other vertebrate species [48]. Thus, this suggests that the luminal cellular debris was the result of cell death and subsequent sloughing within the renal tubules in areas located upstream of the distal segments, presumably localized to the proximal regions. Kidney tissue at 3, 5, and 7 dpi corresponded closely to that of previously described tissue in the H&E time course. Interestingly, dark magenta linings were noted in many tubules that had only subtle lumens, suggesting they were putative newly regenerated proximal tubules. At 10 dpi, numerous aggregates that have formed possessed a PAS-positive brush border, and by 14 dpi, the kidney tissue was indistinguishable from wild-type tissue. Again, 21 dpi tissue staining revealed an absence of cellular aggregates, suggesting that the regeneration process of neonephrogenesis had been completed. Interestingly, the location of basophilic cellular aggregates that appeared throughout both histological time courses was closely juxtaposed to a nephron tubule (Figure 2). Particularly, at later time points (e.g., 7–10 dpi), each aggregate was associated with a plumbing event into a preexisting proximal tubule based on PAS reactivity, though these events were detectable as early as the 5 dpi time point as well (Figure 2, data not shown). Further, the aggregates themselves displayed the characteristic of PAS reactivity, with dark magenta apical staining (Figure 2, data not shown). This staining character of the basophilic aggregates suggests the largely intriguing notion that new nephrons possess a proximal nature.

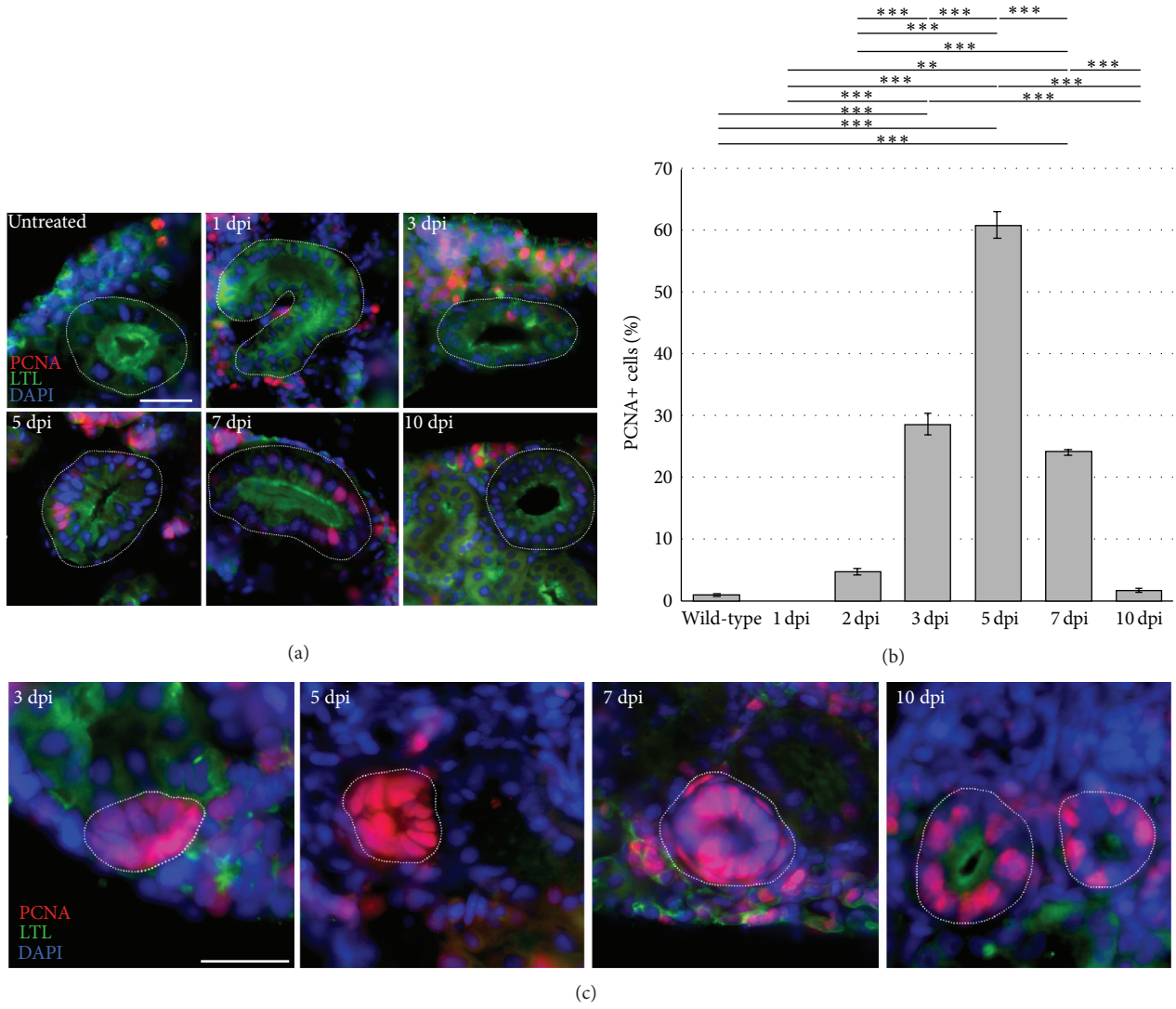



Figure 5: Cell proliferation in the regenerating proximal tubule epithelium and in neonephrogenic clusters. Kidney tissue was assayed for PCNA-positive nuclei (red), and proximal tubules were identified by LTL labeling (green). Nuclei were stained with DAPI (blue). (a) Most epithelial proliferation occurred at 3, 5, and 7 days after kidney injury. Scale bar, 25 $\mu$m. (b) Quantification of PCNA expression in nephron proximal tubular segments. Labels: $**$ indicates $P < 0.01$; $***$ indicates $P < 0.001$. (c) Epithelial proliferation marked by PCNA (red) occurred at high levels in cellular aggregates at 3 and 5 dpi. At 7 dpi, aggregates that have formed lumens continued to express intense levels of PCNA. At 10 dpi, PCNA was still abundant in tubules that have become LTL-positive (green).

*2.4. slc20a1a Expression Time Course in the Injured Kidney.* The solute transporter *slc20a1a*, which marks the proximal convoluted tubule (PCT) segment in the adult nephron (Figure 1(a)) as well as the embryo, has been used as a marker of proximal tubule segments after nephrotoxin injury in the zebrafish [29]. To correlate spatiotemporal alterations of this gene with our histology time courses, gentamicin-injected zebrafish kidneys were examined using *in situ* hybridization with *slc20a1a* (Figure 3). At 1 dpi, the gentamicin-induced nephron damage was so catastrophic that *slc20a1a* transcript expression was completely abrogated (Figure 3(a)), consistent with prior observations [29]. The appearance of a small number of *slc20a1a*+ cellular aggregates at 3 dpi (0.58 ± 0.28) was followed by substantial increases in this number at 5 dpi (66.66 ± 3.9) and 7 dpi (74.5 ± 9.4) (Figures 3(a) and 3(b)). Small coiled structures first appeared at 5 dpi (2.3 ± 0.55) and were numerous throughout the kidney by 7 dpi (30.6 ± 6.5) (Figures 3(a) and 3(b)). A high number of aggregates also persisted between 7 and 10 dpi; however, the quantity declined as more coils appeared (Figure 3(a), data not shown). Further, the emergence of segment structures, which closely phenocopied uninjured PCT segments, appeared at 7 dpi, with an incidence of 7.9 ± 2.6 structures per kidney (Figures 3(a) and 3(b)). At 14 dpi, *slc20a1a* expression was analogous to that of wild-type adult kidneys, with the majority of structures stained resembling segments (Figure 3(a)). To further analyze these observations, we performed ANOVA statistical analyses to compare the number of aggregates, coils, and segments, which revealed that there was a significant increase in aggregates between 3 and 7 dpi, accompanied

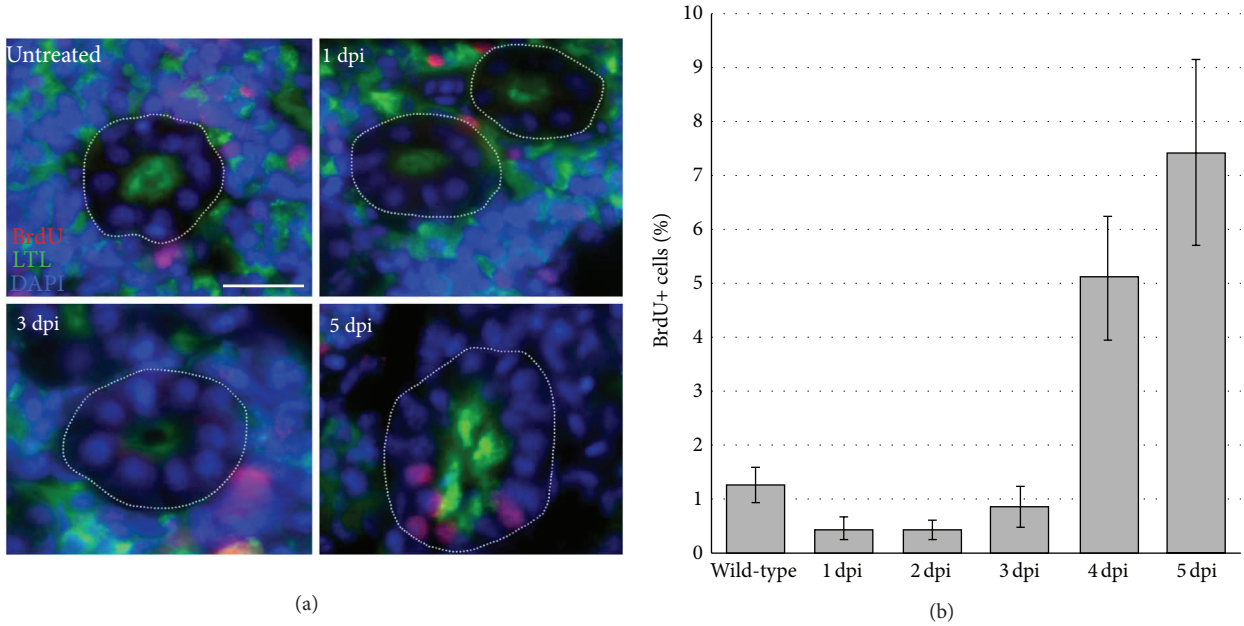



FIGURE 6: Regenerating proximal tubules show BrdU incorporation after gentamicin injury. (a) Kidney tissue was assayed for BrdU-positive nuclei (red), and proximal tubules were identified by LTL labeling (green). Nuclei were stained with DAPI (blue). (b) Quantification showed that BrdU incorporation occurs progressively at 3, 4, and 5 dpi.

by the emergence of coils and segments at 5 and 7 dpi, which was also significant (Figure 3(c)). This suggests that throughout the time period following nephrotoxicant injury, new nephrons first appear as small cellular aggregates that eventually coil and elongate into healthy, normal functioning nephrons, in keeping with the qualitative observations of basophilic aggregates and coils in histological data as well as previous observations of nephron formation [28, 29]. The populace of coils and segments likely also includes the population of existing nephrons that regenerate the damaged proximal tubule epithelium, most likely at time points from 5 dpi onward. However, additional studies are needed to distinguish new nephrons that express *slc20a1a* from existing nephrons that show tubular regeneration.

*2.5. Cellular Death and Proliferation during Kidney Regeneration.* The TUNEL method is a useful and specific label for nuclear DNA fragmentation [49], which is a signature of cellular apoptosis. Previous AKI studies with gentamicin in zebrafish have not examined the timing and location of nephron tubule cell death, though this agent is well known to destroy renal proximal tubule cells. To confirm this directly and to assess whether cell death occurred in other tubule segments, we implemented a combination of TUNEL and LTL labeling to localize when and where cell death occurred in proximal tubules compared to other segments following gentamicin exposure. While uninjured kidneys showed very low levels of TUNEL-positive cells (0.95% ± 0.43%), TUNEL reactivity escalated dramatically in the nuclei of tubular cells, specifically within LTL-positive proximal tubules at 1 and 3 dpi (Figures 4(a) and 4(b)). At 1 dpi, 28.78% ± 1.12% of cells in LTL-positive proximal tubules were TUNEL-positive, which climbed to an incidence of 44.11% ± 6.03% of proximal tubule cells at 3 dpi (Figures 4(a) and 4(b)). By 5 dpi, only 7.82% ± 1.05% of proximal tubule cells were TUNEL-positive (Figures 4(a) and 4(b)). By 7 and 10 days after injection, the level of TUNEL-positive cells in proximal tubules decreased even further, returning to approximately basal levels that had been established in kidneys that were untreated (Figures 4(a) and 4(b)). The rapid elevation in TUNEL reactivity from 1 to 3 dpi followed by rapid decline over 3 to 10 dpi was statistically significant (Figure 4(b)). Renal tubules that were LTL-negative were not found to be TUNEL-positive (data not shown). Similar trends were observed in independent studies in which TUNEL staining was performed in conjunction with ELF-97 to label the proximal tubule (data not shown). Overall, these data show that cell death was occurring largely in the proximal tubule and that it transpires in a wave that peaks at approximately 3 dpi.

Next, we evaluated the dynamics of cell proliferation following gentamicin exposure using PCNA labeling (Figure 5). PCNA is found in varying concentrations within the cell during the cell cycle and is in maximum quantities during the S phase [50]. As with the cell death analysis, the use of LTL labeling in conjunction with the use of an antibody to detect PCNA allowed for examination of cell proliferation in proximal nephron tubules compared to the rest of the renal tubules and ducts. At basal levels in untreated kidneys, 0.97% ± 0.21% of cells were positive in the LTL-positive proximal tubules (Figures 5(a) and 5(b)). At 1 dpi, no cells were found to express PCNA (Figures 5(a) and 5(b)). However, by 3 dpi, 28.6% ± 1.69% of LTL-positive tubular cells were positive for PCNA (Figures 5(a) and 5(b)). The percentage of PCNA positive cells in proximal tubules peaked at 5 days after injury, reaching 60.9% ± 2.16% of all proximal tubule cells. This incidence declined to 24.1% ± 0.51% at 7 dpi and then further

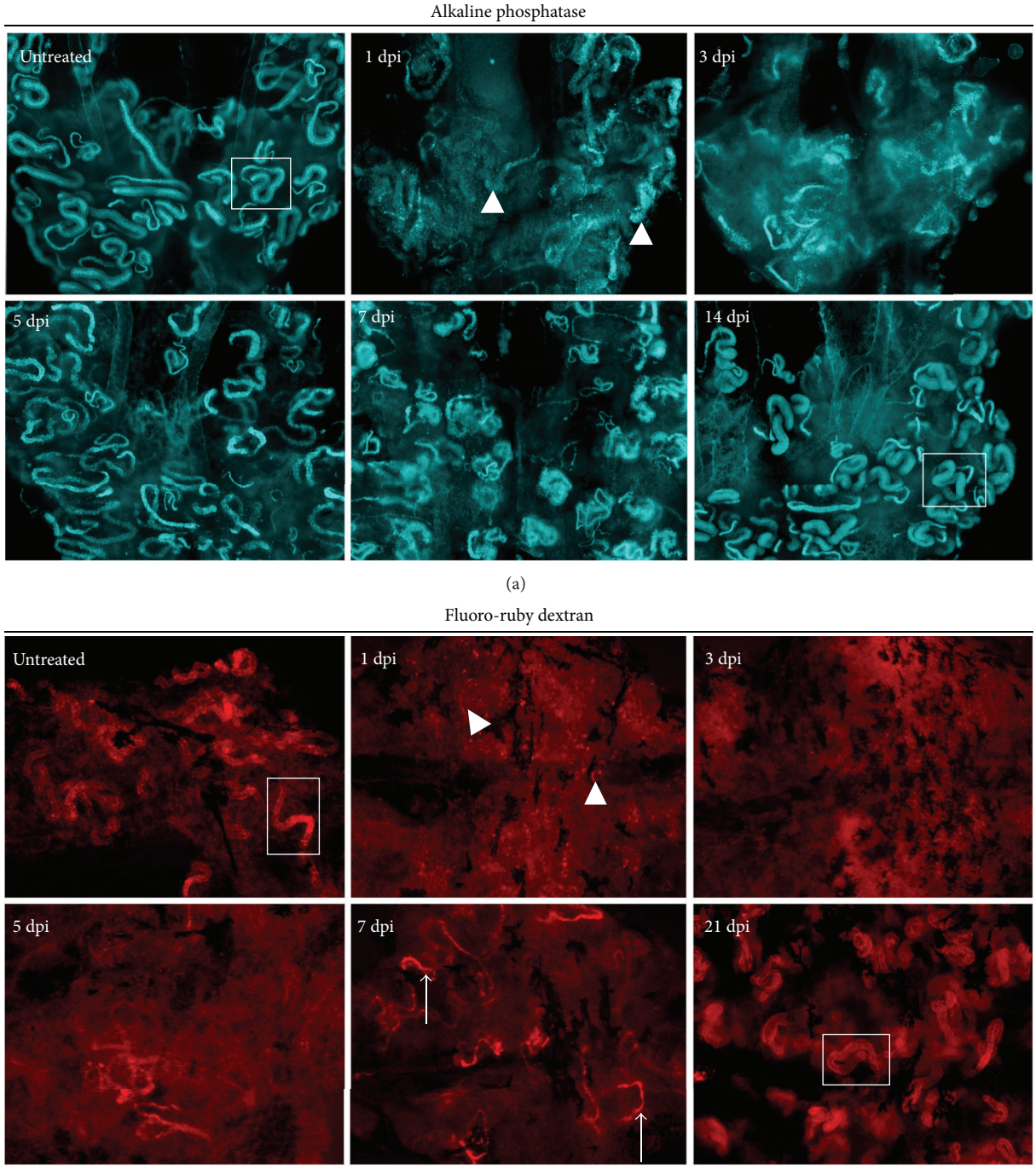



Figure 7: Changes in ELF-97 staining and proximal tubule functionality after gentamicin injury. Whole mount labeling of (a) alkaline phosphatase reactivity and (b) uptake of fluoro-ruby dextran in the zebrafish kidney during recovery from AKI. (a) Untreated kidneys contained discrete PCT-PST structures (white box) that were destroyed at 1 dpi (white arrowheads). Restoration of discrete PCT-PST structures was completed by 14 dpi. (b) Nephron reabsorption was visualized in injured kidneys, in which the PCT was demarcated by uptake of fluoro-ruby dextran, while at 1 dpi, such structures were absent (white arrowheads). Partial uptake was sporadically observed at 5–7 dpi but was not consistent throughout the kidney organ until 21 dpi when PCT segments were distinctly visualized (white box).

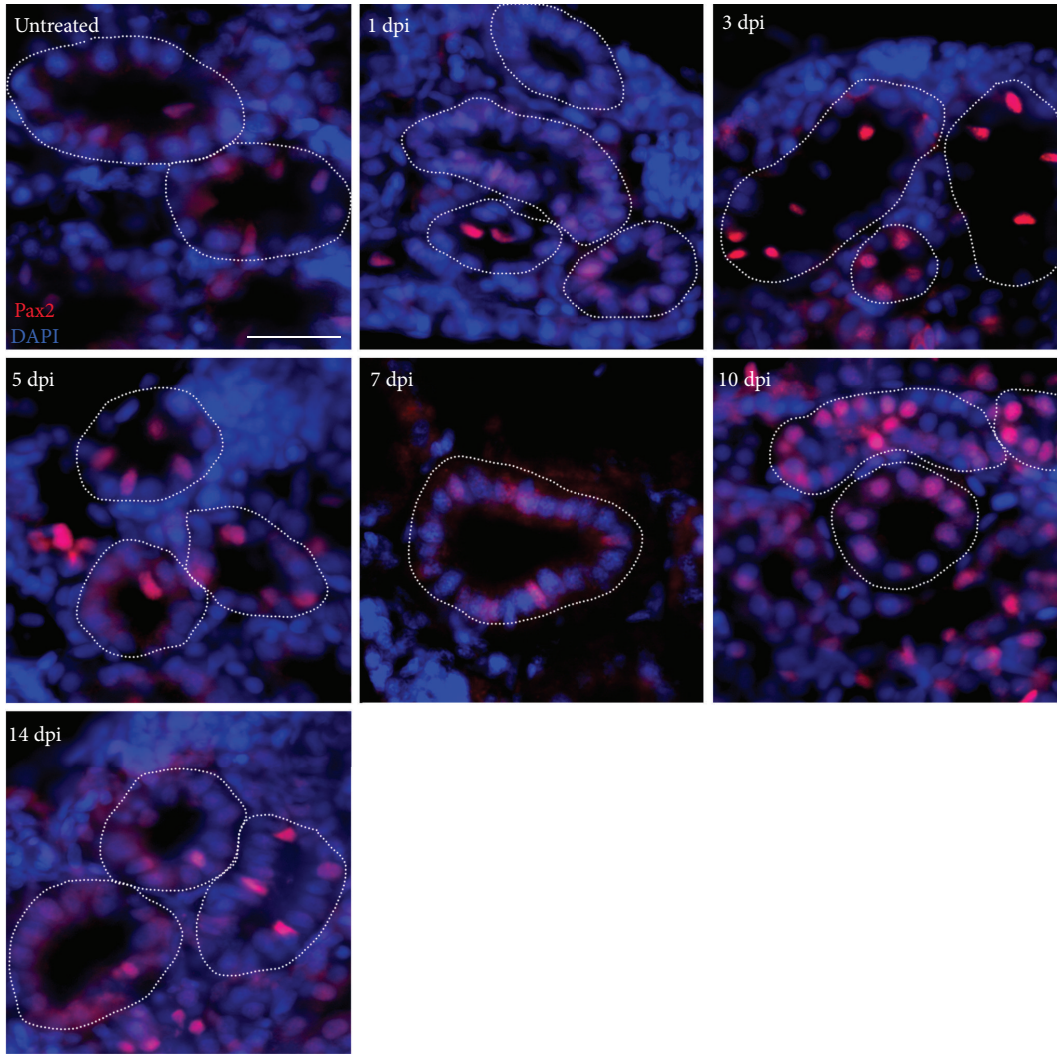



Figure 8: The Pax2 transcription factor marks cells in the regenerating proximal tubule and neonephrogenic clusters. (a) Expression of Pax2 in the regenerating kidney tubule. Pax2-positive nuclei (red) were identified in kidney tubule cryosections at baseline and over a two-week span after injury. Elevated Pax2 expression was detected at 5, 7, and 10 dpi. Nuclei were stained with DAPI (blue). Scale bar, 25 $\mu$m. (b) Intense Pax2 immunoreactivity demarcated neonephrogenic units. High levels of Pax2 expression (red) were present in coil-like structures at 5 dpi. At 10 dpi, Pax2 continued to stain profound numbers of cells in the coil-like neonephron structures. Nuclei are stained with DAPI (blue). Scale bar, 25 $\mu$m.

to 1.74% ± 0.39% by 10 dpi. PCNA staining was also completed in combination with the proximal tubule marker ELF-97, which showed similar dynamics in the regenerating nephron epithelium, with the proliferation at 3, 5, and 7 dpi (data not shown).

Interestingly, high levels of PCNA were observed in neonephrogenic kidney structures during this time course as well (Figure 5(c)). Beginning at 3 dpi, when aggregates are forming, intense PCNA expression was found in the entire structure colocalizing with DAPI-stained nuclei (Figure 5(c)). By 5 and 7 dpi, when aggregates have formed lumens and transform into coil-like neonephrons, high levels of PCNA were still present (Figure 5(c)). At 10 dpi, PCNA continued to be abundant in these neonephrogenic structures that now had a brush border that stained positive for LTL (Figure 5(c)). Based on these observations, it appears as if the intensely stained PCNA-positive neonephron structures have become proximal tubules. However, genetic fate mapping is needed to definitively track the progression of these structures and label them as having a proximal fate.

As another measure to evaluate cell proliferation, we performed BrdU pulse-chase experiments in zebrafish following gentamicin-induced AKI (Figure 6). For this examination, an intraperitoneal injection of BrdU was administered to uninjured zebrafish and gentamicin-injected zebrafish 24 hours prior to each time point for analysis over a 5-day course, and kidneys were then analyzed by immunofluorescence to detect BrdU-positive cells in proximal tubules labeled with LTL (Figure 6). In uninjured kidneys, we found that 1.25% ± 0.32% of proximal tubule cells had incorporated the BrdU label (Figures 6(a) and 6(b)). After gentamicin injection, at 1, 2, and 3 dpi, there was a low incidence of BrdU incorporation (0.44% ± 0.19%; 0.42% ± 0.18%; 0.83% ± 0.37%, resp.) (Figures 6(a) and 6(b)). At 4 and 5 dpi, however, the incidence of BrdU-positive cells in LTL-stained proximal tubules increased to 5.09% ± 1.16% and 7.42% ± 1.73%, respectively (Figures 6(a) and 6(b)). While ANOVA statistical analysis failed to show that the increase in BrdU incorporation was significant, the overall trend of elevated BrdU incorporation over time in the regenerating proximal tubules is consistent with the observations of PCNA incorporation over time (Figure 5). Taken together, these data show that proliferation in regenerating proximal tubules occurs within the first week following injury.

*2.6. Restoration of Additional Proximal Tubule Structural Characteristics and Absorptive Functions following Gentamicin-Induced AKI.* To further visualize the regeneration of proximal tubule structures following gentamicin-induced injury and to assess the restoration of proximal tubule physiological function, we utilized alkaline phosphatase staining and dextran uptake assays, respectively [32]. In whole mount kidney preparations, these assays enable a three-dimensional assessment of nephrons throughout the organ (Figure 7) [32]. Labeling of alkaline phosphatase activity with ELF-97 specifically enables the visualization of the entire proximal tubule, both the PCT and PST segments, which are connected and have distinguishing diameters, with the PCT being distinctively wide compared to the narrow diameter of the attached PST (Figure 7(a)) [32]. At 1 and 3 dpi, alkaline phosphatase reactivity was diminished and dispersed throughout the kidney, with few PCT structures evident (Figure 7(a)). At 5 dpi and 7 dpi, tubules were more distinctly labeled with alkaline phosphatase, but wide PCT-like tubules were rarely observed (Figure 7(a)). By 14 dpi, there was a clear restoration of PCT-PST structures labeled with alkaline phosphatase reactivity (Figure 7(a)).

In parallel, we examined renal uptake of fluorescently labeled dextran moieties, an assay that determines whether the PCT epithelial cells are capable of endocytosis [32]. Uninjured kidney tubules evinced PCT-specific uptake of fluoro-ruby dextran or fluorescein dextran, while this property was abrogated following gentamicin injury at 1 and 3 dpi (Figure 7(b); data not shown). Between 5 dpi and 19 dpi, PCT uptake of fluoro-ruby or fluorescein dextran was sporadic, with labeling detected in only a few nephron tubules, and not until the 21 dpi time point was PCT uptake consistent across nephrons of the entire organ (Figure 7(b), data not shown). This suggests that regeneration of PCT functionality requires up to three weeks, even though at two weeks the nephron tubules appear structurally intact by alkaline phosphatase labeling (Figure 7(a)) and other histological methods (Figure 2).

*2.7. Pax2 Expression Marks Regenerating Tubular Epithelial Cells and Neonephrogenic Structures.* The process of nephrogenesis is controlled by specific genes that can either enhance or inhibit cell survival and direct subsequent proliferation and differentiation events [51, 52]. One such gene is the *Pax2* transcription factor. During development when renal cells undergo a mesenchymal to epithelial transition into condensed cellular aggregates and differentiate into nephron tubules, they express an increased level of Pax2; later in nephrogenesis, the transcription factor is downregulated [53–55]. In order to restore organ or tissue function in adult animals after undergoing physical damage or injury, it has been suggested that the regeneration process may recapitulate specific developmental processes [56, 57]. In keeping with this notion, previous research has demonstrated that Pax2 is reexpressed in nephron tubular cells following AKI in the adult mouse [58] as well as in zebrafish embryonic nephrons subsequent to gentamicin-induced AKI [59].

In order to test the hypothesis that developmental genes are reexpressed during regenerative events and explore whether zebrafish adult nephrons similarly show tubular Pax2 expression following AKI, an antibody to Pax2 was used for immunolabeling during the adult zebrafish injury and repair time course (Figure 8). Analysis of Pax2 expression in the injured kidney revealed that this developmental transcription factor was expressed in the epithelial tubules at a low level in the uninjured nephron tubule and was more strongly expressed in tubular cells at multiple time points over a two-week time span after injury (Figure 8(a)). Notably, a higher expression level of Pax2 was present between 5 and 7 dpi (Figure 8(a)). A low level was still detectable in the repaired tubules by 14 dpi, comparable to the untreated kidney (Figure 8).

Further, similar to PCNA expression patterns in neonephrogenic structures, Pax2 was expressed at high levels in the cells of coiled bodies and other neonephrogenesis structures at 5 and 10 dpi, respectively (Figure 8(b)). The tubules containing Pax2-positive cells were distinguished from the neonephrogenic structures based on lumen diameter. Tubules that were undergoing repair possessed a larger lumen, representative of a tubule that was previously established and functioning in the kidney. In contrast, lumens of the neonephrons were very small and appeared to expand in time beyond that which is documented in this time course. Taken together, these data demonstrate that expression of Pax2 accompanies regeneration of the proximal tubule epithelium as well as neonephrogenesis in the zebrafish kidney.

## 3. Discussion

To date, three modes of kidney regeneration have been characterized following exposure to nephrotoxins or mechanical injuries: (1) tubular epithelium regeneration, in which existing nephrons are repopulated after cells have been destroyed, (2) compensatory renal hypertrophy, in which remaining kidney structures enlarge, typically observed following unilateral nephrectomy, and (3) nephron neogenesis from renal mesenchymal progenitor/stem cells [60]. Vertebrate species vary with regard to whether they can perform several or all three of these feats [6, 60]. For example, humans and other mammals are incapable of developing new nephrons following either gestation or the neonatal period, a feature known to be associated with renal stem cell exhaustion during metanephros ontogeny [52, 61–67]. In contrast, fish and amphibians have versatile regenerative traits throughout juvenile stages as well as adulthood that include the formation of entirely new nephrons [10]. While various fish species, including goldfish, medaka, skate, trout, tilapia, and toadfish, can undergo kidney regeneration [33, 68–71], the zebrafish provides an advantage to discover the pathways and signaling events involved in kidney regeneration due to their genetic tractability. Before undertaking traditional genetic or chemical screens using zebrafish to identify the cast of components in renal regeneration, however, it is vital to have a thorough understanding of the progression of tissue changes that transpires following toxicant exposure.

Here, we have further characterized the spatiotemporal sequence of cellular and gene expression changes associated with regeneration of the zebrafish nephron tubular epithelium and also annotated a number of features associated with neonephrogenesis. In sum, our work has revealed that the injured nephron tubule epithelium is regenerated within one week of damage, involving partially overlapping waves of cell death and proliferation that is accompanied by Pax2 expression (Figure 9). Functionality of the regenerated nephrons is subsequently restored between 2 and 3 weeks following damage. In agreement with prior studies, we found that neonephrogenesis commences by approximately 5 dpi, with nephron clusters forming new nephrons over the subsequent week, and show for the first time that the new nephrons possess the proximal tubule feature of PAS reactivity (Figure 9). While this suggests that new nephrons have proximal character, genetic fate mapping studies are needed to ascertain what functional segment(s) the new nephrons come to possess. Given the highly branched nature of the nephron arrangements in the zebrafish mesonephros, it is an attractive hypothesis that new nephrons commonly plumb into preexisting proximal segments, thus adding to the filtration and bulk reabsorption functions of the kidney while utilizing the existing distal and collecting duct systems for fine-tuning of salt balance in the urinary stream.

*3.1. Toolkit for Cellular and Molecular Renal Studies in Zebrafish.* These studies provide a new and important descriptive atlas of the cellular changes that transpire during zebrafish adult kidney regeneration. Moreover, in the pursuit of these studies we have refined a number of histological methods for their application in the adult zebrafish kidney. Together, this set of information and methodologies provide a resource for further studies in this promising regeneration model. Three histological stains that have proven to be valuable include H&E stain, PAS stain, and silver stain. The H&E stain distinguishes the proximal tubules from the distal tubules based essentially on the presence of a brush border (a marked feature of proximal tubules). The reagents in the PAS stain have a high affinity for the brush borders of proximal tubules and allow a more effective characterization of the varying structures within the kidney tissue. The silver stain also stains the brush borders a discernable dark brown color, allowing distinction from distal tubules. Notably, the silver stain also labels hyaline droplets formed from protein reabsorption, which are located specifically in the proximal tubule. The use of lectin stains to distinguish tubule compartments based on sugar-binding proteins also plays a vital role in this novel toolkit. LTL is a robust proximal tubule marker, labeling the brush borders of the tubule; DBA is a marker for the distal tubules. Finally, the ELF-97 staining method, which detects high levels of endogenous alkaline phosphatase activity in brush borders, is a consistent technique to differentiate the proximal tubules from the distal tubules. A major limitation of working with these and other markers noted above involves incompatibilities with kidney tissue fixation requirements. We have found the most success in zebrafish renal histology procedures by fixing the organ in two different ways: fixation in paraformaldehyde (with or without antigen retrieval prior to immunolabeling) or ethanolic formaldehyde. These methodologies should prove to be useful to further study renal regeneration in the adult kidney, as well as established and emerging models of embryonic nephron injury and regeneration [22, 59, 72–75]. However, not all markers work with both fixatives in combination with immunofluorescent antibodies, and a current limitation in the zebrafish system is the paucity of commercially available antibodies. Future work will mostly likely benefit by examinations of gene expression by additional *in situ* hybridization studies in whole mount or sections [32]. These methods enable the spatial and temporal localization of gene transcripts, which is feasible for any gene(s) of interest because the zebrafish genome has been sequenced and because appropriate reagents are commercially available for gene expression studies.

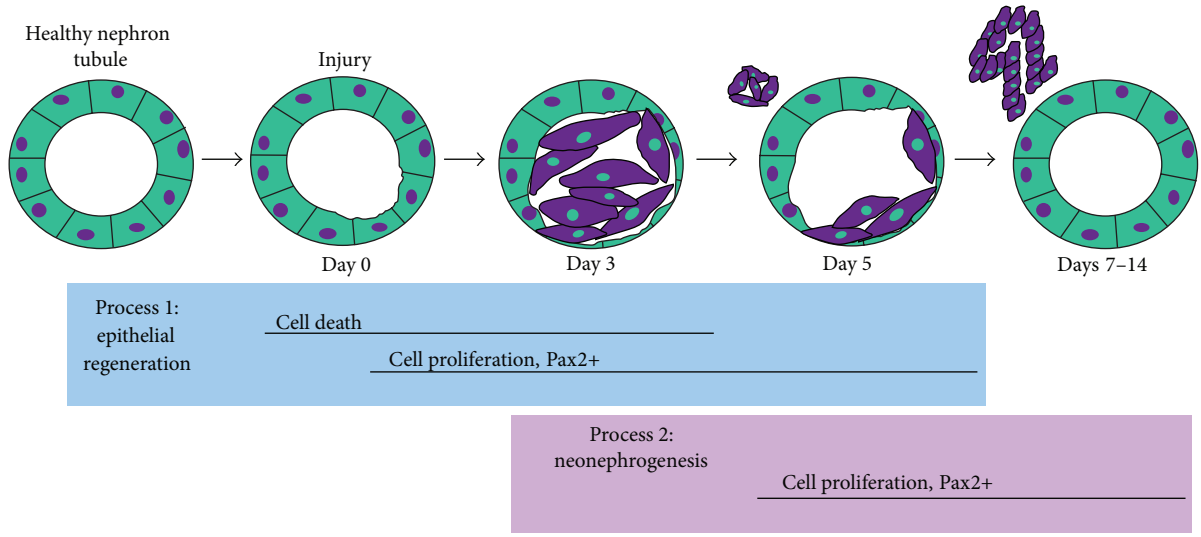



FIGURE 9: Summary of major cellular events in zebrafish kidney regeneration. After injury to proximal tubules of nephrons within the adult zebrafish kidney, successive and overlapping waves of cell death and cell proliferation occur. Cell proliferation is accompanied by Pax2 expression. Neonephrogenesis entails abundant Pax2 expression and cell proliferation in neonephron structures during the regeneration process.

*3.2. Stem Cells and Their Roles in Zebrafish Adult Kidney Regeneration.* Studies in various mammalian species have demonstrated that intratubular proliferation occurs in the healthy nephron tubule [76–78]. Similarly in the present work, we have documented a low level of proliferation in the uninjured zebrafish proximal tubule based on PCNA reactivity and BrdU incorporation in LTL+ nephron cells. Fate mapping studies in the mouse have clearly demonstrated that intratubular nephron populations replenish the injured nephron [79, 80]. However, the nature of these regenerating cells in the damaged mammalian kidney tubules remains a topic of intense debate. At present, two hypotheses exist that describe the cellular attributes of this intratubular cell source. One scenario involves the dedifferentiation of epithelial cells that will migrate and proliferate to repair injured tubules. The second scenario posits that stem/progenitor cells located within the tubule undergo division and amplification in response to tissue damage. There is experimental evidence among mammalian models that supports both models—fate mapping studies in the mouse support dedifferentiation [8], while there is also opposing evidence from human kidney research that unique subpopulations of renal cells with features suggestive of stem cell character are located among the nephron epithelial cells, and that these cells fuel tubular regeneration [9]. Whether there are differences across mammalian species or whether additional studies will eventually reconcile these conflicting data remains a fascinating area of current nephrology research.

As such, an important aspect of future renal regeneration research with regard to the zebrafish model will be to clarify the origin of reparative epithelia in existing nephrons through transgenic genetic fate mapping. Such lineage analysis will be vital for evaluating the origins of the cells in repaired tubules. Further, since renal cells can be isolated by flow cytometry and then operationally tested through transplantation procedures [81], *in vivo* experiments to test the replicative and differentiation potential of putative intratubular stem/progenitor cells in zebrafish, if they are identified, are likely to become feasible.

*3.3. Complexities and Benefits in Comparing Zebrafish and Mammals.* Ultimately, the degree to which the zebrafish kidney is "unique" from higher vertebrates, including mammals, is an important biological question to understand. For example, the zebrafish adult kidney stroma is the site of hematopoiesis—thus the microenvironment in the zebrafish kidney is arguably quite distinct from mammalian counterparts in which blood production ensues elsewhere. Research that uncovers these or other differences and how they impact renal regeneration capacities may provide vital insights into methods that could be used to stimulate similar reparative responses to treat kidney injury and disease or assist in designing reprogramming strategies [82–84]. The data presented here provide a valuable foundation for researchers that aim to embark on such genetic and cellular studies to ascertain the identities of the molecules and signaling pathways that activate and regulate renal regeneration in zebrafish.

## 4. Experimental Procedures

*4.1. Zebrafish Strain and Maintenance.* Adult zebrafish of the Tübingen wild-type strain were raised and maintained at 28.5°C on a 14-hour light: 10-hour dark cycle at an average luminance of 200 lux [85] in the Center for Zebrafish Research at the Notre Dame Freimann Life Science Center. All protocols were approved by the IACUC of the University of Notre Dame, animal protocols 13-021 and 16-025.

*4.2. Gentamicin Injections and Kidney Dissections.* For gentamicin injections, zebrafish were anesthetized with a diluted working solution of 0.02% Tricaine, made using a 0.2% Tricaine pH 7.0 stock for approximately 2-3 minutes and transferred to an injection mold. Fish received an intraperitoneal injection of 2.5 mg/mL gentamicin and were returned to a clean system tank. At various time points, adults were euthanized with an overdose of Tricaine and fixed with either 4% paraformaldehyde/1X PBS/0.1% dimethyl sulfoxide (DMSO) or 9 : 1 ethanolic formaldehyde (100% ethanol : 37% formaldehyde). The kidneys of adult fish were dissected as previously described [27, 32]. Briefly, fish were euthanized with 0.2% Tricaine pH 7.0 for approximately 5 minutes. Dissection needles were used to pin open the body walls by attaching them to a dissection tray that contained 4% paraformaldehyde/1X PBS/0.1% DMSO. The samples were fixed overnight at 4°C. The following day, the fixative was removed from the tray and fine forceps were used to detach the kidney from the dorsal wall.

*4.3. Whole Mount In Situ Hybridization.* Whole mount *in situ* hybridization (WISH) on adult kidneys was performed as previously described [27, 32]. Briefly, kidneys were fixed in 4% paraformaldehyde/1X PBS/0.1% DMSO and pigmentation in the organ was removed by hydrogen peroxide treatment. Kidneys underwent permeabilization and hybridization steps in a humidified chamber at 70°C overnight. Samples were then incubated in blocking buffer at room temperature (10% bovine serum albumin and 5% fetal calf serum) and following extensive washes, digoxigenin-labeled probes were detected with alkaline phosphatase conjugated to an antidigoxigenin antibody. NBT/BCIP (Sigma-Aldrich) served as the enzymatic substrate for the purple color reaction. Color reactions were stopped with 4% paraformaldehyde/1X PBS.

*4.4. Tissue Cryosections.* As described previously [32], adult fish were fixed in either 4% paraformaldehyde/1X PBS/0.1% DMSO or 9 : 1 ethanolic : formaldehyde overnight at 4°C, and the kidneys were dissected out the next day. Samples were washed with 5% sucrose/1X PBS, cryoprotected in 30% sucrose/1X PBS overnight at 4°C and then subsequently washed in 1 : 1 tissue freezing medium (TFM, Triangle Biomedical Sciences): 30% sucrose/1X PBS overnight at 4°C. The following day, samples were embedded in 100% TFM. Serial sections of approximately 12–14 $\mu$m thickness were transversely cut through the entire kidney. Frozen cryosections were mounted onto glass microscope slides (TruBond 380 Microscope Slides, Tru Scientific) and allowed to air-dry for 1 hour at 50°C. Slides were stored at −80°C until use.

*4.5. Histology Analysis.* For all histological stains, adult fish were euthanized at various time points by an overdose of Tricaine and fixed overnight in 4% paraformaldehyde/1X PBS/0.1% DMSO. Kidneys were dissected out, washed in 70% ethanol at 4°C, and then were paraffin-embedded and serially sectioned on a microtome. After slides were deparaffinized and rehydrated, sections were stained with hematoxylin and eosin, periodic acid-Schiff, or methenamine silver (Notre Dame Integrated Imaging Facility–Histology Core). Mouse kidney sections (a generous gift from the Notre Dame Histology Core) were treated with the same histological protocol.

*4.6. BrdU Incorporation.* Cell proliferation was assayed through BrdU incorporation. Adult zebrafish were anesthetized in 0.02% Tricaine pH 7.0 and intraperitoneally injected with 5 mM BrdU (Molecular Probes) 24 hours before sacrifice. Cells that incorporated BrdU were visualized by immunofluorescence analysis.

*4.7. Immunofluorescence.* Slides were thawed for 30 minutes at 50°C and then rehydrated in 1X PBS/0.05% Tween-20. Cryosections were incubated at room temperature in blocking solution 1X PBS/0.05% Tween-20/10% fetal calf serum/1% DMSO for 2 hours and then placed at 4°C for overnight primary antibody incubation. Primary antibodies were diluted in block and included mouse anti-Green Fluorescent Protein monoclonal antibody (1 : 500; Sigma-Aldrich), rabbit anti-Pax2 polyclonal antibody (1 : 50; Covance), rabbit anti-myosin VI antibody (1 : 50; Sigma-Aldrich), mouse anti-BrdU (1 : 50; Molecular Probes), and mouse anti-proliferating cell nuclear antigen (PCNA) polyclonal antibody (1 : 1000; Sigma-Aldrich). Following primary antibody incubation, cryosections were washed in 1X PBS/0.05% Tween-20 and incubated in secondary antibody solution for 2 hours at room temperature. Secondary antibodies were diluted 1 : 500 in 1X PBS/0.05% Tween-20 and included Alexa Fluor 488 and 568 goat anti-mouse IgG and 594 goat anti-rabbit IgG (Molecular Probes). Nuclei were labeled with DAPI (Molecular Probes) for 5 minutes. Cryosections were washed with 1X PBS and mounted with Vectashield Mounting Medium (Vector Laboratories). Antigen retrieval was performed by incubating slides between 95°C and 100°C for 40 minutes in preheated 10 mM sodium citrate buffer for Pax2 and PCNA labeling or by incubating cryosections with 2 M HCl at 37°C for 30 minutes for BrdU labeling. Sections were then washed and immunolabeled as described above.

*4.8. Identification of Sectioned Tubule Segments.* Tubular segments of the kidney were identified by utilizing the following markers: fluorescein *Lotus tetragonolobus* lectin (LTL, Vector Laboratories) diluted 1 : 100 in 1X PBS for 2 hours to label the proximal tubule; enzyme labeled fluorescence-(ELF-) 97 (Molecular Probes) diluted 1 : 20 in detection buffer (included in kit) for 1 hour to label the proximal tubule; rhodamine *Dolichos biflorus* agglutinin (DBA, Vector Laboratories) diluted 1 : 100 in 1X PBS for 2 hours to label the distal tubule [32]. If colabeling with an antibody, LTL and/or DBA stains were applied directly after the secondary antibody incubation. DAPI immunolabeling followed LTL and/or DBA as described above. For ELF-97 colabeling [32], substrate solution was applied directly after the secondary antibody solution. The reaction was stopped with wash buffer 1X PBS/25 mM EDTA/5 mM levamisol pH 8.0, incubating the cryosections with fresh buffer 3 times for 15 minutes each, and then visualized in ELF-97 mounting medium.

*4.9. Whole Mount Kidney Morphology Assays*

*4.9.1. Dextran Labeling.* Whole mount labeling of proximal convoluted tubule segments in the kidney was followed as previously described [32]. In short, adult zebrafish were anesthetized and injected intraperitoneally with 50 mg/mL fluoro-ruby dextran (Invitrogen) and returned to a clean system tank. The next day, the fish were sacrificed and the kidney was dissected out for fluorescent tubule visualization.

*4.9.2. ELF-97 Labeling.* Whole mount labeling of pan-proximal segments was performed as previously described [32]. Briefly, kidneys were subjected to fixation, dissection, permeabilization, and pigmentation removal. The kidneys were blocked and then incubated in ELF-97 substrate solution for 30 minutes. Once the reaction was stopped, multiple washes were performed and the fluorescent proximal segments were visualized.

*4.9.3. LTL and DBA Labeling.* Whole mount labeling of pan-distal segments in the kidney was conducted as previously described [32]. In brief, kidneys were fixed, dissected, and permeabilized and pigment was removed. After blocking, kidneys were incubated in the respective staining solution. Once the staining solution was removed with several washes, the fluorescent signal(s) could be visualized.

*4.10. TUNEL Assay.* Apoptotic cells were identified with the TUNEL assay, using the ApoAlert DNA Fragmentation Assay Kit (Clontech Laboratories) and the TACS 2 TdT Replenisher Kit (Trevigen). Adult zebrafish were fixed in 9 : 1 ethanolic formaldehyde and their kidneys were dissected, embedded, and cryosectioned as previously described. Cryosections were thawed at 50°C for 1 hour, permeabilized with 1X PBS for 20 minutes, 0.1% sodium citrate buffer/0.1% Triton X-100 for 2 minutes, and again with 1X PBS for 5 minutes at room temperature. Equilibration buffer was applied directly to the cryosections for 10 minutes, followed by the addition of biotinylated DNTPs and TdT enzyme (both at a concentration of 1 : 50 in equilibration buffer) for 2 hours at 37°C. The labeling reaction was terminated by incubating cryosections in 2X SSC stop buffer for 15 minutes. Positive nuclei were visualized by applying Alexa Fluor 568 Streptavidin diluted in 1X PBS (1 : 200, Molecular Probes) for 1 hour.

*4.11. Statistical Analysis.* Statistical significance among experimental groups was analyzed using the one-way ANOVA followed by Tukey's HSD multiple comparisons test using R version 3.0.3. Data shown are mean ± SEM. Significance was accepted at $P < 0.05$ or greater.

## Key Findings

(i) A suite of histological stains was characterized to provide tools to identify distinguishing features of zebrafish adult kidney anatomy, including nephron proximal tubule traits.

(ii) Cell death and proliferation in the injured proximal tubule are dynamic and transpire in successive waves of activity the first week following injury, while functional restoration occurs over the subsequent weeks.

(iii) Spatiotemporal immunofluorescence studies revealed that Pax2 is expressed both in the epithelial population in regenerating nephrons and in neonephrogenic clusters that are associated with the production of de novo nephrons after AKI.

## Abbreviations

AKI: Acute kidney injury
AP: Alkaline phosphatase
BrdU: 5-Bromo, 2′deoxyuridine
CD: Collecting duct
dpi: Days after injury
DAPI: 4′,6-Diamidino-2-phenylindole
DBA: *Dolichos biflorus* agglutinin
DE: Distal early
DL: Distal late
DT: Distal tubule
ELF-97: Enzyme labeled fluorescence-97
G: Glomerulus
H&E: Hematoxylin and eosin
LTL: *Lotus tetragonolobus* lectin
N: Neck
PAS: Periodic acid-Schiff
Pax2: Paired box gene 2
PCNA: Proliferating cell nuclear antigen
PCT: Proximal convoluted tubule
PST: Proximal straight tubule
PT: Proximal tubule
T: Tubule
TUNEL: Terminal deoxynucleotidyl transferase dUTP nick end labeling
WISH: Whole mount *in situ* hybridization.

## Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

## Acknowledgments

The authors thank Dr. Clay Connor for kindly providing his zebrafish histology and immunofluorescence expertise during the refinement of methodologies that were employed in the study. They also thank Shanna Newton, Elvin Morales, and Charles Renner for performing exploratory studies related to this project. They thank the entire staff of the Department of Biological Sciences for ongoing support and the Center for Zebrafish Research at Notre Dame for their dedication and care of our zebrafish aquarium. They especially thank the Notre Dame Integrated Imaging Facility and Histology Core for reagents and other research supports. Finally, they thank the members of our research lab for their comments, discussions, and insights about this work. The

research is supported by National Institutes of Health (NIH) Grant nos. DP2OD008470, K01DK083512, and R01DK100237 and March of Dimes Basil O'Connor Starter Scholar Grant Award no. 5-FY12-75. Support was also provided from startup funding to RAW from the University of Notre Dame, College of Science and Department of Biological Sciences, as well as a Gift to the University of Notre Dame from Elizabeth and Michael Gallagher to support stem cell research. Kristin Springer was supported by summer fellowship funding from the Glynn Family Honors Program and the College of Science at the University of Notre Dame.

## References

[1] L. Saxen, *Organogenesis of the Kidney*, Cambridge University Press, Cambridge, UK, 1987.

[2] K. K. Mccampbell and R. A. Wingert, "Renal stem cells: fact or science fiction?" *Biochemical Journal*, vol. 444, no. 2, pp. 153–168, 2012.

[3] P. Romagnani, "Toward the identification of a 'renopoietic system'?" *Stem Cells*, vol. 27, no. 9, pp. 2247–2253, 2009.

[4] R. A. Wingert and A. J. Davidson, "The zebrafish pronephros: a model to study nephron segmentation," *Kidney International*, vol. 73, no. 10, pp. 1120–1127, 2008.

[5] J. V. Bonventre and L. Yang, "Cellular pathophysiology of ischemic acute kidney injury," *Journal of Clinical Investigation*, vol. 121, no. 11, pp. 4210–4221, 2011.

[6] R. Reimschuessel, "A fish model of renal regeneration and development," *ILAR Journal*, vol. 42, no. 4, pp. 285–291, 2001.

[7] A. Benigni, M. Morigi, and G. Remuzzi, "Kidney regeneration," *The Lancet*, vol. 375, no. 9722, pp. 1310–1317, 2010.

[8] B. D. Humphreys, "Kidney injury, stem cells and regeneration," *Current Opinion in Nephrology and Hypertension*, vol. 23, pp. 25–31, 2013.

[9] P. Romagnani, L. Lasagni, and G. Remuzzi, "Renal progenitors: an evolutionary conserved strategy for kidney regeneration," *Nature Reviews Nephrology*, vol. 9, no. 3, pp. 137–146, 2013.

[10] K. K. McCampbell and R. A. Wingert, "New tides: using zebrafish to study renal regeneration," *Translational Research*, vol. 163, no. 2, pp. 109–122, 2014.

[11] R. A. Wingert, R. Selleck, J. Yu et al., "The cdx genes and retinoic acid control the positioning and segmentation of the zebrafish pronephros," *PLoS Genetics*, vol. 3, no. 10, pp. 1922–1938, 2007.

[12] R. A. Wingert and A. J. Davidson, "Zebrafish nephrogenesis involves dynamic spatiotemporal expression changes in renal progenitors and essential signals from retinoic acid and irx3b," *Developmental Dynamics*, vol. 240, no. 8, pp. 2011–2027, 2011.

[13] I. A. Drummond, A. Majumdar, H. Hentschel et al., "Early development of the zebrafish pronephros and analysis of mutations affecting pronephric function," *Development*, vol. 125, no. 23, pp. 4655–4667, 1998.

[14] G. F. Gerlach and R. A. Wingert, "Kidney organogenesis in the zebrafish: insights into vertebrate nephrogenesis and regeneration," *Wiley Interdisciplinary Reviews—Developmental Biology*, vol. 2, no. 5, pp. 559–585, 2013.

[15] R. W. Naylor, A. Przepiorski, Q. Ren, J. Yu, and A. J. Davidson, "HNF1$\beta$ is essential for nephron segmentation during nephrogenesis," *Journal of the American Society of Nephrology*, vol. 24, no. 1, pp. 77–87, 2013.

[16] Y. Li, C. N. Cheng, V. A. Verdun, and R. A. Wingert, "Zebrafish nephrogenesis is regulated by interactions between retinoic acid, mecom, and notch signaling," *Developmental Biology*, vol. 386, no. 1, pp. 111–122, 2014.

[17] C. N. Cheng, Y. Li, A. Marra, V. Verdun, and R. A. Wingert, "Flat mount preparation for observation and analysis of zebrafish embryo specimens stained by whole mount *in situ* hybridization," *Journal of Visualized Experiments*, vol. 89, Article ID e51604, 2014.

[18] C. N. Cheng and R. A. Wingert, "Nephron proximal tubule patterning and corpuscles of Stannius formation are regulated by the *sim1a* transcription factor and retinoic acid in zebrafish," *Developmental Biology*, vol. 399, no. 1, pp. 100–116, 2015.

[19] P. T. Kroeger Jr., S. J. Poureetezadi, R. McKee et al., "Production of haploid zebrafish embryos by *in vitro* fertilization," *Journal of Visualized Experiments*, no. 89, Article ID e51708, 2014.

[20] G. F. Gerlach and R. A. Wingert, "Zebrafish pronephros tubulogenesis and epithelial identity maintenance are reliant on the polarity proteins *Prkc iota* and *zeta*," *Developmental Biology*, vol. 396, no. 2, pp. 183–200, 2014.

[21] R. McKee, G. F. Gerlach, J. Jou, C. N. Cheng, and R. A. Wingert, "Temporal and spatial expression of tight junction genes during zebrafish pronephros development," *Gene Expression Patterns*, vol. 16, no. 2, pp. 104–113, 2014.

[22] D. M. Hentschel, K. M. Park, L. Cilenti, A. S. Zervos, I. Drummond, and J. V. Bonventre, "Acute renal failure in zebrafish: a novel system to study a complex disease," *The American Journal of Physiology—Renal Physiology*, vol. 288, pp. F923–F929, 2005.

[23] C. C. Cosentino, B. L. Roman, I. A. Drummond, and N. A. Hukriede, "Intravenous microinjections of zebrafish larvae to study acute kidney injury," *Journal of Visualized Experiments*, no. 42, Article ID e2079, 2010.

[24] S. A. Rider, C. S. Tucker, J. del-Pozo et al., "Techniques for the *in vivo* assessment of cardio-renal function in zebrafish (Danio rerio) larvae," *Journal of Physiology*, vol. 590, no. 8, pp. 1803–1809, 2012.

[25] L. Ebarasi, A. Oddsson, K. Hultenby, C. Betsholtz, and K. Tryggvason, "Zebrafish: a model system for the study of vertebrate renal development, function, and pathophysiology," *Current Opinion in Nephrology and Hypertension*, vol. 20, no. 4, pp. 416–424, 2011.

[26] L. M. Swanhart, C. C. Cosentino, C. Q. Diep, A. J. Davidson, M. de Caestecker, and N. A. Hukriede, "Zebrafish kidney development: basic science to translational research," *Birth Defects Research C—Embryo Today: Reviews*, vol. 93, no. 2, pp. 141–156, 2011.

[27] G. F. Gerlach, L. N. Schrader, and R. A. Wingert, "Dissection of the adult zebrafish kidney," *Journal of Visualized Experiments*, no. 54, Article ID e2839, 2011.

[28] W. Zhou, R. C. Boucher, F. Bollig, C. Englert, and F. Hildebrandt, "Characterization of mesonephric development and regeneration using transgenic zebrafish," *The American Journal of Physiology—Renal Physiology*, vol. 299, no. 5, pp. F1040–F1047, 2010.

[29] C. Q. Diep, D. Ma, R. C. Deo et al., "Identification of adult nephron progenitors capable of kidney regeneration in zebrafish," *Nature*, vol. 470, no. 7332, pp. 95–100, 2011.

[30] W. Zhou and F. Hildebrandt, "Inducible podocyte injury and proteinuria in transgenic zebrafish," *Journal of the American Society of Nephrology*, vol. 23, no. 6, pp. 1039–1047, 2012.

[31] J. Huang, M. McKee, H. D. Huang, A. Xiang, A. J. Davidson, and H. A. J. Lu, "A zebrafish model of conditional targeted podocyte

ablation and regeneration," *Kidney International*, vol. 83, no. 6, pp. 1193–1200, 2013.

[32] K. K. McCampbell, K. N. Springer, and R. A. Wingert, "Analysis of nephron composition and function in the adult zebrafish kidney," *Journal of Visualized Experiments*, no. 90, Article ID e51644, 2014.

[33] N. Watanabe, M. Kato, N. Suzuki et al., "Kidney regeneration through nephron neogenesis in medaka," *Development Growth and Differentiation*, vol. 51, no. 2, pp. 135–143, 2009.

[34] P. Vinay, A. Gougoux, and G. Lemieux, "Isolation of a pure suspension of rat proximal tubules," *The American Journal of Physiology*, vol. 241, no. 4, pp. F403–F411, 1981.

[35] J. B. Longley and E. R. Fisher, "A histochemical basis for changes in renal tubular function in young mice," *Quarterly Journal of Microscopical Science*, vol. 97, part 2, pp. 187–195, 1956.

[36] C. M. Tsai and C. E. Frasch, "A sensitive silver stain for detecting lipopolysaccharides in polyacrylamide gels," *Analytical Biochemistry*, vol. 119, no. 1, pp. 115–119, 1982.

[37] K. Amann and C. S. Haas, "What you should know about the work-up of a renal biopsy," *Nephrology Dialysis Transplantation*, vol. 21, no. 5, pp. 1157–1161, 2006.

[38] D. B. Jones, "Nephrotic glomerulonephritis," *The American Journal of Pathology*, vol. 33, no. 2, pp. 313–329, 1957.

[39] L. Watson, J. Vassallo, G. G. Cantor et al., "Lectin histochemistry: an alternative to immunohistochemistry for identifying specific structures in rat renal papillary necrosis," *HistoLogic*, vol. 41, pp. 28–31, 2008.

[40] F. Cachat, B. Lange-Sperandio, A. Y. Chang et al., "Ureteral obstruction in neonatal mice elicits segment-specific tubular cell responses leading to nephron loss," *Kidney International*, vol. 63, no. 2, pp. 564–575, 2003.

[41] H.-T. Cheng, M. Kim, M. T. Valerius et al., "Notch2, but not Notch1, is required for proximal fate acquisition in the mammalian nephron," *Development*, vol. 134, no. 4, pp. 801–811, 2007.

[42] R. G. B. Bonegio, L. H. Beck, R. K. Kahlon, W. Lu, and D. J. Salant, "The fate of Notch-deficient nephrogenic progenitor cells during metanephric kidney development," *Kidney International*, vol. 79, no. 10, pp. 1099–1112, 2011.

[43] E. Mochizuki, K. Fukuta, T. Tada et al., "Fish mesonephric model of polycystic kidney disease in medaka (*Oryzias latipes*) pc mutant," *Kidney International*, vol. 68, no. 1, pp. 23–34, 2005.

[44] H. Harris, "The human alkaline phosphatases: what we know and what we don't know," *Clinica Chimica Acta*, vol. 186, no. 2, pp. 133–150, 1990.

[45] W. G. Cox and V. L. Singer, "A high-resolution, fluorescence-based method for localization of endogenous alkaline phosphatase activity," *Journal of Histochemistry and Cytochemistry*, vol. 47, no. 11, pp. 1443–1455, 1999.

[46] R. F. Reilly, R. E. Bulger, and W. Kriz, "Structural-functional relationships in the kidney," in *Diseases of the Kidney and Urinary Tract*, Lippincott Williams & Wilkins, Philadelphia, Pa, USA, 8th edition, 2007.

[47] I. A. Drummond and A. J. Davidson, "Zebrafish kidney development," *Methods in Cell Biology*, vol. 100, pp. 233–260, 2010.

[48] C. Ronco, R. Bellomo, and J. Kellum, *Critical Care Nephrology*, Elsevier Saunders, Philadelphia, Pa, USA, 2nd edition, 2009.

[49] Y. Gavrieli, Y. Sherman, and S. A. Ben-Sasson, "Identification of programmed cell death in situ via specific labeling of nuclear DNA fragmentation," *Journal of Cell Biology*, vol. 119, no. 3, pp. 493–501, 1992.

[50] K. M. Connolly and M. S. Bogdanffy, "Evaluation of proliferating cell nuclear antigen (PCNA) as an endogenous marker of cell proliferation in rat liver: a dual-stain comparison with 5-bromo-2′-deoxyuridine," *Journal of Histochemistry and Cytochemistry*, vol. 41, no. 1, pp. 1–6, 1993.

[51] A. S. Woolf, "Multiple causes of human kidney malformations," *Archives of Disease in Childhood*, vol. 77, no. 6, pp. 471–473, 1997.

[52] M. H. Little and A. P. McMahon, "Mammalian kidney development: principles, progress, and projections," *Cold Spring Harbor Perspectives in Biology*, vol. 4, no. 5, Article ID a008300, 2012.

[53] G. R. Dressler, U. Deutsch, K. Chowdhury, H. O. Nornes, and P. Gruss, "*Pax2*, a new murine paired-box-containing gene and its expression in the developing excretory system," *Development*, vol. 109, no. 4, pp. 787–795, 1990.

[54] P. J. D. Winyard, R. A. Risdon, V. R. Sams, G. R. Dressler, and A. S. Woolf, "The PAX2 transcription factor is expressed in cystic and hyperproliferative dysplastic epithelia in human kidney malformations," *Journal of Clinical Investigation*, vol. 98, no. 2, pp. 451–459, 1996.

[55] R. Attar, F. Quinn, P. J. D. Winyard et al., "Short-term urinary flow impairment deregulates PAX2 and PCNA expression and cell survival in fetal sheep kidneys," *The American Journal of Pathology*, vol. 152, no. 5, pp. 1225–1235, 1998.

[56] R. Bacallao and L. G. Fine, "Molecular events in the organization of renal tubular epithelium: from nephrogenesis to regeneration," *The American Journal of Physiology—Renal Fluid and Electrolyte Physiology*, vol. 257, no. 6, pp. F913–F924, 1989.

[57] A. Wallin, G. Zhang, T. W. Jones, S. Jaken, and J. L. Stevens, "Mechanism of the nephrogenic repair response: studies on proliferation and vimentin expression after 35S-1,2-dichlorovinyl-L-cysteine nephrotoxicity *in vivo* and in cultured proximal tubule epithelial cells," *Laboratory Investigation*, vol. 66, no. 4, pp. 474–484, 1992.

[58] M. Imgrund, E. Gröne, H.-J. Gröne et al., "Re-expression of the developmental gene *Pax-2* during experimental acute tubular necrosis in mice," *Kidney International*, vol. 56, no. 4, pp. 1423–1431, 1999.

[59] C. C. Cosentino, N. I. Skrypnyk, L. L. Brilli et al., "Histone deacetylase inhibitor enhances recovery after AKI," *Journal of the American Society of Nephrology*, vol. 24, no. 6, pp. 943–953, 2013.

[60] Y. Li and R. A. Wingert, "Regenerative medicine for the kidney: stem cell prospects & challenges," *Clinical and Translational Medicine*, vol. 2, no. 1, article 11, 2013.

[61] D. Herzlinger, C. Koseki, T. Mikawa, and Q. Al-Awqati, "Metanephric mesenchyme contains multipotent stem cells whose fate is restricted after induction," *Development*, vol. 114, no. 3, pp. 565–572, 1992.

[62] J. Qiao, D. Cohen, and D. Herzlinger, "The metanephric blastema differentiates into collecting system and nephron epithelia in vitro," *Development*, vol. 121, no. 10, pp. 3207–3214, 1995.

[63] M. Self, O. V. Lagutin, B. Bowling et al., "Six2 is required for suppression of nephrogenesis and progenitor renewal in the developing kidney," *EMBO Journal*, vol. 25, no. 21, pp. 5214–5228, 2006.

[64] S. Boyle, A. Misfeldt, K. J. Chandler et al., "Fate mapping using Cited1-CreERT2 mice demonstrates that the cap mesenchyme contains self-renewing progenitor cells and gives rise exclusively to nephronic epithelia," *Developmental Biology*, vol. 313, no. 1, pp. 234–245, 2008.

[65] A. Kobayashi, M. T. Valerius, J. W. Mugford et al., "Six2 defines and regulates a multipotent self-renewing nephron progenitor population throughout mammalian kidney development," *Cell Stem Cell*, vol. 3, no. 2, pp. 169–181, 2008.

[66] F. Costantini and R. Kopan, "Patterning a complex organ: branching morphogenesis and nephron segmentation in kidney development," *Developmental Cell*, vol. 18, no. 5, pp. 698–712, 2010.

[67] C. Hendry, B. Rumballe, K. Moritz, and M. H. Little, "Defining and redefining the nephron progenitor population," *Pediatric Nephrology*, vol. 26, no. 9, pp. 1395–1406, 2011.

[68] R. Reimschuessel and D. Williams, "Development of new nephrons in adult kidneys following gentamicin-induced nephrotoxicity," *Renal Failure*, vol. 17, no. 2, pp. 101–106, 1995.

[69] C. J. Salice, J. S. Rokous, A. S. Kane, and R. Reimschuessel, "New nephron development in goldfish (*Carassius auratus*) kidneys following repeated gentamicin-induced nephrotoxicosis," *Comparative Medicine*, vol. 51, no. 1, pp. 56–59, 2001.

[70] J. Augusto, B. Smith, S. Smith, J. Robertson, and R. Reimschuessel, "Gentamicin-induced nephrotoxicity and nephroneogenesis in *Oreochromis nilotica*, a tilapian fish," *Disease of Aquatic Organisms*, vol. 26, no. 1, pp. 49–58, 1996.

[71] M. Elger, H. Hentschel, J. Litteral et al., "Nephrogenesis is induced by partial nephrectomy in the elasmobranch *Leucoraja erinacea*," *Journal of the American Society of Nephrology*, vol. 14, no. 6, pp. 1506–1518, 2003.

[72] C. S. Johnson, N. F. Holzemer, and R. A. Wingert, "Laser ablation of the zebrafish pronephros to study renal epithelial regeneration," *Journal of Visualized Experiments*, no. 54, Article ID e2845, 2011.

[73] E. D. de Groh, L. M. Swanhart, C. C. Cosentino et al., "Inhibition of histone deacetylase expands the renal progenitor cell population," *Journal of the American Society of Nephrology*, vol. 21, no. 5, pp. 794–802, 2010.

[74] S. J. Poureetezadi and R. A. Wingert, "Congenital and acute kidney disease: translational research insights from zebrafish chemical genetics," *General Medicine*, vol. 1, no. 3, article 112, 2013.

[75] A. Palmyre, J. Lee, G. Ryklin et al., "Collective epithelial migration drives kidney repair after acute injury," *PLoS ONE*, vol. 9, no. 7, Article ID e101304, 2014.

[76] T. Nadasdy, Z. Laszik, K. E. Blick, L. D. Johnson, and F. G. Silva, "Proliferative activity of intrinsic cell populations in the normal human kidney," *Journal of the American Society of Nephrology*, vol. 4, no. 12, pp. 2032–2039, 1994.

[77] A. Vogetseder, A. Karadeniz, B. Kaissling, and M. L. Hir, "Tubular cell proliferation in the healthy rat kidney," *Histochemistry and Cell Biology*, vol. 124, no. 2, pp. 97–104, 2005.

[78] A. Vogetseder, T. Palan, D. Bacic, B. Kaissling, and M. Le Hir, "Proximal tubular epithelial cells are generated by division of differentiated cells in the healthy kidney," *The American Journal of Physiology—Cell Physiology*, vol. 292, no. 2, pp. C807–C813, 2007.

[79] F. Lin, A. Moran, and P. Igarashi, "Intrarenal cells, not bone marrow-derived cells, are the major source for regeneration in postischemic kidney," *Journal of Clinical Investigation*, vol. 115, no. 7, pp. 1756–1764, 2005.

[80] B. D. Humphreys, M. T. Valerius, A. Kobayashi et al., "Intrinsic epithelial cells repair the kidney after injury," *Cell Stem Cell*, vol. 2, no. 3, pp. 284–291, 2008.

[81] C. Q. Diep and A. J. Davidson, "Transplantation of cells directly into the kidney of adult zebrafish," *Journal of Visualized Experiments*, no. 51, article 2725, 2011.

[82] C. Sagrinati, E. Ronconi, E. Lazzeri, L. Lasagni, and P. Romagnani, "Stem-cell approaches for kidney repair: choosing the right cells," *Trends in Molecular Medicine*, vol. 14, no. 7, pp. 277–285, 2008.

[83] C. Hopkins, J. Li, F. Rae, and M. H. Little, "Stem cell options for kidney disease," *Journal of Pathology*, vol. 217, no. 2, pp. 265–281, 2009.

[84] E. E. Morales and R. A. Wingert, "Renal stem cell reprogramming: prospects in regenerative medicine," *World Journal of Stem Cells*, vol. 6, no. 4, pp. 458–466, 2014.

[85] M. Westerfield, "Recipes," in *The Zebrafish Book*, chapter 10, University of Oregon Press, Eugene, Ore, USA, 4th edition, 2011.

Hindawi Publishing Corporation
Stem Cells International
Volume 2015, Article ID 964849, 8 pages
http://dx.doi.org/10.1155/2015/964849

*Review Article*

# Diverse Cell Populations Involved in Regeneration of Renal Tubular Epithelium following Acute Kidney Injury

**Akito Maeshima, Shunsuke Takahashi, Masao Nakasatomi, and Yoshihisa Nojima**

*Department of Medicine and Clinical Science, Gunma University Graduate School of Medicine, 3-39-15 Showa, Maebashi 371-8511, Japan*

Correspondence should be addressed to Akito Maeshima; amaesima@gunma-u.ac.jp

Received 5 November 2014; Accepted 6 December 2014

Academic Editor: Benedetta Bussolati



Renal tubular epithelium has the capacity to regenerate, repair, and reepithelialize in response to a variety of insults. Previous studies with several kidney injury models demonstrated that various growth factors, transcription factors, and extracellular matrices are involved in this process. Surviving tubular cells actively proliferate, migrate, and differentiate in the kidney regeneration process after injury, and some cells express putative stem cell markers or possess stem cell properties. Using fate mapping techniques, bone marrow-derived cells and endothelial progenitor cells have been shown to transdifferentiate into tubular components in vivo or ex vivo. Similarly, it has been demonstrated that, during tubular cell regeneration, several inflammatory cell populations migrate, assemble around tubular cells, and interact with tubular cells during the repair of tubular epithelium. In this review, we describe recent advances in understanding the regeneration mechanisms of renal tubules, particularly the characteristics of various cell populations contributing to tubular regeneration, and highlight the targets for the development of regenerative medicine for treating kidney diseases in humans.

## 1. Introduction

Renal tubules express several types of transporter that are involved in renal reabsorption and secretion, as well as ion channels for the maintenance of body fluid balance. These cells comprise polarized mature epithelial cells with the capacity to regenerate following acute kidney injury. After the insult occurs, surviving tubular cells rapidly lose epithelial cell properties and acquire a more mesenchymal phenotype. The dedifferentiated cells migrate into the regions where cell necrosis, apoptosis, or detachment has resulted in denudation of the tubular basement membrane. They proliferate and eventually differentiate into mature epithelial cells with polarized lumen, completing the repair process [1].

The process of restoration and maturation of damaged epithelium after renal injury has many parallels with the developmental process during kidney organogenesis. Soluble factors involved in kidney development have been identified by gene targeting techniques, in vitro tubulogenesis models, and organ culture systems, and most of these also have been demonstrated to regulate kidney recovery as potential renotrophic factors [2]. These factors have been shown to be epithelial cell mitogens in vitro and to induce tubular cell proliferation after injury when exogenously administered. With recent fate mapping techniques that facilitate cell lineage tracing, various cell populations or cell-cell interactions have been revealed to be intricately involved in tubular regeneration after acute kidney injury (Figure 1).

In this review, we highlight recent advances regarding the regeneration mechanisms of renal tubules after injury, particularly focusing on possible cell populations and their interactions, which contribute to the repair process of renal tubules after injury.

## 2. Regeneration Process of Renal Tubules after Injury

Renal tubular epithelium has a huge capacity for regeneration after injury. During the repair process, surviving tubular cells actively proliferate and differentiate into mature tubular cells to reconstruct their functional structures. Sophisticated lineage tracing studies have demonstrated that it is unlikely that extrarenal cells enter the tubule and differentiate into

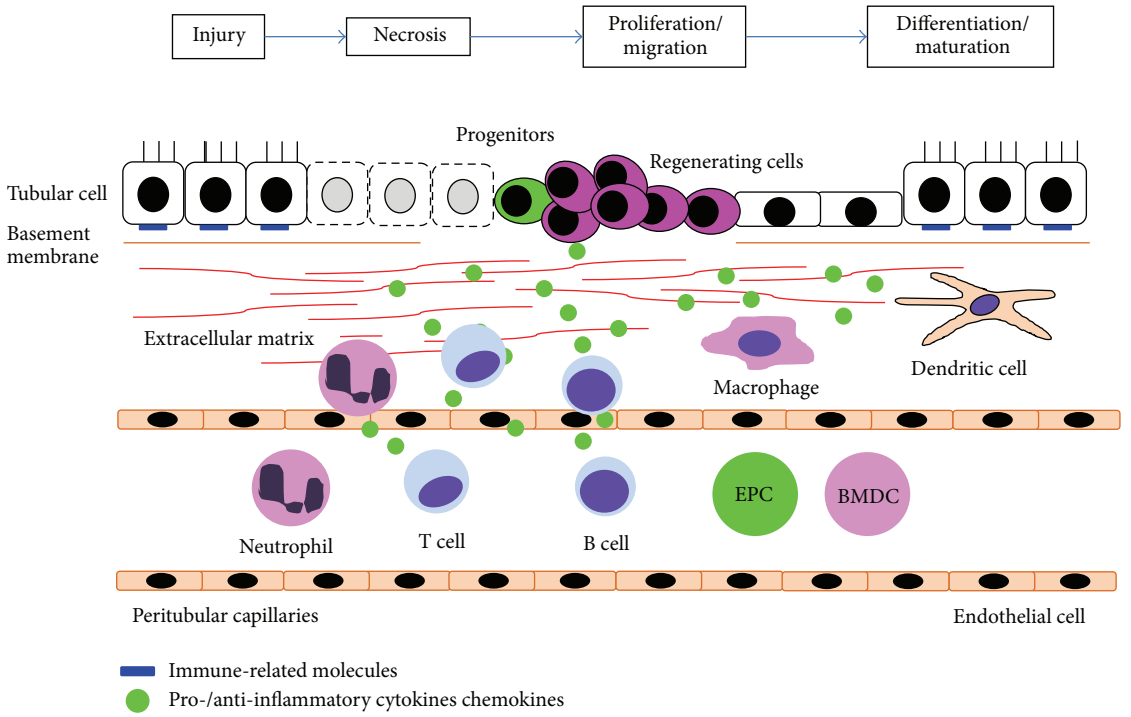



Figure 1: Diverse cell populations involved in tubular regeneration after injury.

epithelial cells in mice. It is more likely that tubule recovery is controlled by a number of intratubular cells with a substantial regenerative capacity [3, 4].

*2.1. Potential Progenitor Cells Engaged in Tubular Repair.* Despite the structural complexity of the adult kidney, attempts to identify cell populations contributing to the regenerative process have been based on the broad principles of stem cell biology. To conserve growth potential and prevent genetic injury during mitosis, stem cells cycle slowly and are recruited only as demanded by tissue turnover. To identify slow-cycling stem cells, a pulse label of 5-bromo-2-deoxyuridine (BrdU), followed by a chase period, is commonly used, allowing the detection of slow-cycling label-retaining cells (LRCs). LRCs have been identified in renal tubules of normal rat kidneys, and regenerating cells during tubular repair are essentially derived from LRCs [5–7]. The number of these LRCs declines with age, resulting in reduced regenerative capacity after injury in the aging kidney [8]. Other groups also found LRCs in tubules [9, 10], papilla [11], and renal capsules [12]. A previous study demonstrated that there is a unique cell population in rat kidneys that self-renews for more than 200 population doublings, without evidence of senescence. These cells were able to differentiate into renal tubules when injected intra-arterially after renal ischemia [13]. Another report revealed that a promising cell line derived from the S3 segment of the proximal tubules could be maintained for long periods without transformation and that the cells were partly replaced in injured tubules when engrafted to the kidney after renal ischemia [14]. These results suggest the presence of a tubular progenitor cell population with high proliferation capacity. Fate mapping studies applying targeting strategies with progenitor-specific labeling and clonal analysis tools will enable us to examine which cell population predominantly contributes to tubular regeneration after kidney injury.

In human, a resident population with progenitor characteristics was identified along the nephron [15]. Stem cell marker CD133-positive cells were localized in the Bowman's capsule of the glomeruli, in the proximal tubules as well as in the inner medullary papilla region [16, 17]. The embryonic renal markers and mesenchymal stem cell markers were found to be expressed in these cells. The number of cortical CD133-positive tubular cells increased in patients with acute renal injury [18]. These cells might be able to obtain progenitor property after damage. It has been also demonstrated that CD133/CD24 double-positive renal stem/progenitor cells secreted chemoattractants, such as MCP-1 or IL-8, that can induce recruitment or mobilization of hematopoietic or mesenchymal stem cells [19, 20]. Pharmacological manipulation of renal progenitors is one of the promising strategies to enhance intrinsic capacity of tubular regeneration after injury.

*2.2. Immune-Related Molecules Expressed by Tubular Cells.* Tubular epithelial cells play an important role in the inflammatory process after acute kidney injury. Recent studies have highlighted the importance of several tubular cell surface proteins in mediating the interaction between tubular cells and inflammation during tubular repair.

Renal tubular epithelium constitutively expresses toll-like receptors (TLRs), a family of pattern recognition receptors that detect motifs of pathogens and host material released during injury. TLRs mediate signal transduction pathways

that control the expression of proinflammatory cytokines and chemokines. TLR4 is upregulated in tubular epithelial cells after renal ischemia, while TLR4 deficiency decreases the renal ischemic injury-induced production of proinflammatory cytokines and chemokines and inhibits macrophage and neutrophil accumulation [21]. Lack of TLR2 expression in kidney parenchymal cells also inhibits renal ischemic injury. Production of proinflammatory cytokine is reduced in TLR2-deficient mouse kidneys when compared with wild-type kidneys [22]. In vitro data have demonstrated that stimulation with lipopolysaccharide upregulates the expression of TLR2, TLR3, and TLR4 and secretion of C-C chemokines, in cultured mouse tubular cells, thus suggesting the involvement of tubular TLRs in mediating interstitial leukocyte infiltration and tubular injury [23].

The complement system has been shown to mediate renal ischemia-reperfusion injury. Normally, proximal tubular epithelial cells express Crry, a complement inhibitor, on the basolateral membrane. After renal ischemia, Crry production decreases, thus allowing the deposition of C3 on the tubular epithelium and stimulation of proinflammatory CXC chemokine production. These chemokines attract neutrophils and macrophages to the injured kidney [24]. In support of a protective role for proximal tubular Crry expression, Crry-deficient mice are more susceptible to renal ischemic injury [25]. Activation of C5a receptor (C5aR) is known to induce the recruitment of neutrophils and macrophages and activates these to produce cytokines, chemokines, and adhesion molecules. After renal ischemia, C5aR expression was upregulated in tubular epithelial cells. Blockade of the C5aR pathway with a specific C5aR antagonist abolished upregulation of CXC chemokines and significantly reduced renal damage [26].

Suppressor of cytokine signaling 3 (SOCS-3), a key intracellular negative regulator of several signaling pathways, has been found to be strongly expressed in renal proximal tubules after acute kidney injury. Conditional proximal tubular knockout mice showed accelerated tubule recovery after renal injury, which is associated with increased numbers of infiltrating anti-inflammatory M2 macrophages during the repair phase. These results suggest that upregulated expression of SOCS-3 in damaged proximal tubules inhibits the regeneration process and modulates macrophage phenotype [27].

## 3. Recruitment of Inflammatory Cells during Tubular Regeneration

Repeated or persistent injury results in cell death and limited tubule regeneration. Recruitment of leukocytes is recognized as a major mediator of tubular cell injury. The initial stages of inflammation are characterized by migration of leukocytes to the activated vascular endothelium, followed by transmigration into the interstitium. During the injury phase, tubular epithelial cells express proinflammatory cytokines and chemokines, which recruit neutrophils, M1 macrophages, and various proinflammatory lymphocyte subsets [28].

*3.1. Neutrophils.* Neutrophils rapidly respond to injury, and adherence of neutrophils to the vascular endothelium is a crucial process in the initiation of damage to ischemic tissues [21]. Neutrophil accumulation is one of the hallmarks of renal ischemic injury, and depletion of neutrophils inhibits acute kidney injury [29]. However, blockade of neutrophil function or neutrophil depletion provides only partial protection against renal injury, indicating that other leukocytes, such as macrophages, B cells, and T cells, also mediate acute kidney injury [30].

*3.2. Lymphocytes.* Lymphocytes are also important for tubular repair after ischemic injury. B cells migrate to the ischemic kidney and differentiate into plasma cells, which limit tubule regeneration after acute kidney injury. Consistent with these data, B cell-deficient mice are protected from renal ischemic injury [31, 32]. However, the adoptive transfer of purified B cells back into these mice does not repair kidney injury after ischemia. On the other hand, when compared to B cell-deficient mice without serum transfer, the transfer of serum from wild-type mice results in higher serum creatinine levels after renal ischemia, thus suggesting that the absence of a circulating factor is responsible for the protection observed in B cell-deficient mice. Other investigators have reported no protection from renal ischemic injury in RAG-1-deficient mice lacking both B cells and T cells [33].

The role of T cells in the pathogenesis of renal ischemic injury has been analyzed in different mouse models. T cells have a major role in vascular permeability, potentially through production of cytokines during ischemic injury. Gene microarray analysis has shown that production of TNF and interferon-gamma increased in CD3-positive and CD4-positive T cells from the blood and kidney after renal ischemia. In mice lacking T cells expressing CD3, CD4, and CD8, the increase in renal vascular permeability was attenuated after ischemic injury [34, 35]. In mice lacking CD4-positive and CD8-positive T cells, serum creatinine levels and renal histology decreased significantly after renal ischemia. Reconstitution of mice with CD4-positive T cells alone, but not CD8-positive T cells alone, restored kidney function after renal injury [36]. In addition, RAG-1-deficient mice lacking both B and T cells are also protected from renal ischemic injury and adoptive transfer of CD4-positive T cells from wild-type mice reconstitutes injury. Importantly, transfer of CD4-positive T cells from IFN-gamma-deficient mice failed to repair injury in this model [37], thus suggesting that IFN-gamma-producing CD4-positive T cells mediate the early phases after renal ischemic injury. Exposure of renal tubular epithelial cells to 2 h of hypoxia followed by 1 h of reoxygenation increased T cell adhesion by more than twofold. Phorbol ester treatment, which activates integrins, increased T cell adhesion. These data suggest that T lymphocytes mediate renal ischemic injury. Adhesion of infiltrating T cells to renal tubular cells is a critical step underlying postischemic tubular dysfunction [38].

Among several lymphocyte subsets with anti-inflammatory properties, regulatory T cells ($T_{REG}$ cells) have been shown to promote tubular repair, possibly through the modulation of proinflammatory cytokine production of other

T-cell subsets after acute kidney injury. Increased $T_{REG}$ cell trafficking into the kidneys was observed after ischemic injury. Infusion of $T_{REG}$ cells after initial injury reduced interferon-gamma production by T cell receptor (TCR) $\beta$+CD4+ T cells, improved renal repair, and reduced cytokine generation. In contrast, partial depletion of $T_{REG}$ cells with an anti-CD25 antibody potentiated ischemia-induced kidney damage and resulted in more neutrophils, macrophages, and innate cytokine transcription in the injured kidney [39, 40]. Mice deficient in $T_{REG}$ cells had a greater accumulation of inflammatory leukocytes after renal ischemia than mice containing $T_{REG}$ cells [41].

Natural killer T (NKT) cells, a unique subset of T lymphocytes with surface receptors and functional properties shared with conventional T cells and natural killer (NK) cells, have also been shown to be involved in tubular regeneration after injury. Invariant NKT (iNKT) cells possess a conserved invariant TCR together with the NK cell marker NK1.1. Renal ischemic injury leads to an increase in activated CD4+CD69+ cells, and the number of IFN-gamma-producing iNKT cells in the kidney increases significantly after renal ischemia. Blockade of NKT cell activation with an anti-CD1d monoclonal antibody, NKT cell depletion with an anti-NK1.1 monoclonal antibody in wild-type mice, or use of iNKT cell-deficient mice inhibited the accumulation of IFN-gamma-producing neutrophils after renal injury and reduced acute kidney injury [42]. These studies suggest that neutrophil activation and infiltration is regulated by iNKT cells.

*3.3. Macrophages.* Macrophages are derived from monocytes in the blood and are named for their role in phagocytosis. In addition, macrophages produce proinflammatory cytokines that can stimulate the activity of other leukocytes. Macrophages are involved in all phases of tissue injury, including regeneration [43]. Several macrophage-derived cytokines not only have immunosuppressive effects but also enhance the repair process. Macrophages produce Notch ligands, which stimulate the proliferation of tubular epithelial cells [44]. Blockade of Notch signaling with a gamma-secretase inhibitor suppresses Notch-2-driven induction of survivin and its autocrine capacity to regenerate tubules in a model of ischemic kidney injury [45]. IL-6 produced by interstitial macrophages in renal outer medulla mediates renal ischemic injury [46].

Macrophages infiltrate the injured kidney shortly after renal ischemia and this infiltration is mediated by CX3CR1 signaling pathways [47]. Depletion of kidney and spleen macrophages, using liposomal clodronate, prior to renal ischemic injury prevented acute kidney injury, and adoptive transfer of macrophages reconstituted acute kidney injury [48]. It was recently reported that neutrophil accumulation is controlled by vascular-resident CD169-positive macrophages after renal ischemia [49].

It is thought that proinflammatory stimuli preserve M1 macrophage activity that induces tubular atrophy. Sustained secretion of TNF partially accounts for these antiregenerative effects, which potentially triggers tubular cell injury. Therefore, tubular regeneration can be observed only when M1 macrophages are deactivated [50, 51]. In contrast, macrophage colony stimulating factor-1 signaling leads to proliferation of M2 macrophages with proregenerative properties, which support the resolution of inflammation and acceleration of tubular regeneration during the recovery phase of acute kidney injury [51]. During tubular recovery after injury, M2 macrophages secrete Wnt7b, which interacts with Wnt receptors on the surviving tubular epithelial cells and accelerates tubule recovery via Wnt-$\beta$-catenin signaling [52].

*3.4. Dendritic Cells.* Dendritic cells, an important link between innate and adaptive immunity, are abundant in the normal mouse kidney. Upon stimulation, dendritic cells develop into a mature cell type characterized by high levels of class II major histocompatibility complex (MCH class II) and costimulatory molecules and low phagocytic capacity. Mature dendritic cells are specialized in T cell activation. However, dendritic cells are also important in the innate immune response by releasing proinflammatory factors, interacting with NKT cells via CD40-CD40L and presenting glycolipids via the CD1d molecule to activate iNKT cells.

Dendritic cells play a role in acute kidney injury. After acute kidney injury, renal dendritic cells produce the proinflammatory cytokines TNF, IL-6, C-C motif chemokine 2, and C-C motif chemokine 5, and depletion of dendritic cells before ischemia substantially reduces the levels of TNF produced by the kidney [53]. In a separate study, dendritic cells were shown to accumulate in the renal draining lymph nodes after renal ischemic injury and induce T cell proliferation in an antigen-specific manner, thus suggesting that renal dendritic cells are involved in the adaptive immune response to renal ischemic injury [54].

The activation of intrarenal dendritic cells by factors released from dying tubular cells also promotes tubule regeneration. Necrotic tubular cells release a number of intracellular molecules that act as immunostimulatory DAMPs, specifically activate TLR4 on intrarenal dendritic cells, and induce secretion of IL-22 [55]. Dendritic-cell-derived IL-22 activates the corresponding receptor and subsequent STAT3 and Erk signaling in surviving tubular cells, leading to the enhancement of tubular regeneration and recovery [55].

## 4. Role of Peritubular Capillary Endothelium in Tubular Recovery

The integrity of vascular endothelium is determined by the balance between endothelial turnover and repair. In the kidney, peritubular capillary endothelium is reported to act as a source of factors required for tubular recovery after injury [56]. However, a considerable decrease in the density of peritubular capillary is observed following acute ischemic injury indicating that, unlike renal epithelial tubular cells, the renal vascular system lacks comparable regenerative potential [57, 58]. After ischemic insult, damaged endothelial cells slough into the circulation, and replacement occurs via the induced proliferation of neighboring endothelial cells and/or by the recruitment of EPCs (endothelial progenitor cells) from

the circulation. Growing evidence suggests that the bone marrow is a rich source of immature EPCs. Circulating EPCs can be recruited into vascular beds to maintain normal physiologic homeostasis/repair. Similarly, it has been reported that renal ischemia mobilizes EPCs and induces the accumulation of EPCs in the renal medulla, and transplantation of EPC-enriched cells from the medullary parenchyma affords partial renoprotection after renal ischemia, thus suggesting a role for recruited EPCs in functional rescue [59, 60]. Paracrine mechanisms of therapeutic effect of EPC are also reported. Microvesicles derived from EPC protect the kidney from ischemic acute injury by delivering their RNA content, the microRNAs cargo which converts survived resident renal cells into a more regenerative state [61].

Peritubular capillary endothelium also plays an important early role in the inflammatory response to kidney damage by promoting the accumulation of leukocytes. After renal ischemic injury, capillary endothelium is activated, leading to an increase in vascular permeability [62], which promotes recruitment of leukocytes into the kidney. In addition to changes in the integrity of the endothelial cell layer of the renal vasculature, renal ischemic injury upregulates the expression of adhesion molecules that facilitate leukocyte-endothelial cell interactions. The expression of intracellular adhesion molecule 1- (ICAM-) 1 increases in the kidney after renal ischemic injury. Mice lacking ICAM-1 are protected from renal ischemic injury [29]. Leukocyte adhesion to endothelial cells leads to inflammation and extension of cellular injury. In addition, renal endothelial cells upregulate the expression of CX3CL1 (fractalkine), a ligand for the CX3CR1 receptor expressed on macrophages that mediates macrophage recruitment in the inflamed kidney, and pre-treatment with a neutralizing CX3CR1 monoclonal antibody reduces the severity of acute kidney injury [47].

## 5. Recruitment of Bone Marrow-Derived Cells (BMDCs) during Tubular Regeneration

Similar to the results observed in other organs, BMDCs appear in the kidney in response to renal injury. BMDCs significantly contribute to the regeneration of the renal tubular epithelium, differentiate into renal tubules [63–65], or promote proliferation of both endothelial and epithelial cells after injury [66]. Based on these data, cell therapy with BMDCs has been extensively examined and reported to be effective. In light of their ease of accessibility, BMDCs are strong candidates as a cell source in stem cell therapy. Stem cell factor and granulocyte colony-stimulating factor (G-CSF) induce hematopoietic stem cells (HSCs) homing to the injured kidney, leading to significant enhancement of the functional recovery of the kidney [67, 68]. In contrast to the reports above, boosting of peripheral stem cell numbers was found to be associated with increased severity of renal failure and mortality. High numbers of activated granulocytes appeared to obscure the potential renoprotective effects of HSC [69]. There are several reports against the potential of BMDCs to transdifferentiate into tubular cells after injury. Transgenic mice that express GFP in BMDCs [70], in mature renal tubular epithelial cells [71], or in all mesenchyme-derived renal epithelial cells [72] revealed that, while BMDC recruitment occurs, tubular recovery after renal ischemia is predominantly elicited via proliferation of endogenous renal tubular cells.

Mesenchymal stem cells (MSCs) derived from bone marrow also have been reported to enhance the intrinsic tubular recovery in several acute kidney injury models in a paracrine manner. Treatment with MSCs promoted proximal tubular cell proliferation, reduced apoptosis, and preserved microvascular integrity, leading to the amelioration of renal tissue oxygenation [73–76]. It is considered that MSCs interact with resident cells in endocrine and paracrine manners through the release of growth factors, cytokines, or extracellular vesicles [77, 78]. MSC-derived conditioned medium, which contains renotrophic factors and anti-inflammatory factors, was shown to be able to enhance kidney repair after injury [77, 79, 80]. BMDCs including MSCs are now considered to contribute to the regenerative process by producing protective and regenerative factors, rather than by differentiating to directly replace damaged cells.

## 6. Conclusions

Recently, many new concepts in the regeneration process of renal tubules after acute kidney injury have emerged. Growing evidence suggests that the immune system supports the regeneration process of the kidney after injury. Using gene targeting techniques or fate mapping analysis, the involvement of diverse cell populations including tubular cells, inflammatory cells, resident renal endothelial cells, endothelial progenitor cells, and bone marrow-derived cells has been clarified. Critical roles for neutrophils, lymphocytes, and macrophages have been established in mouse models of acute kidney injury. In addition, several studies have reported that complement, TLRs, and numerous cytokines and chemokines are clearly involved in amplifying the immune response to kidney injury. However, the complex interplay between tubular cells and neighboring cells in renal ischemic injury is not yet fully understood. Termination of renal inflammation will enable tubular regeneration by inhibiting tubular cell necrosis or by driving a phenotype switch from proinflammatory to anti-inflammatory immune cells. In this context, specific and selective anti-inflammatory drugs will be required to suppress systemic and local causes of renal inflammation to promote regeneration in patients with kidney diseases. Specific activation of surviving tubular epithelial cells with regenerative capacity also represents an attractive approach to enable sufficient reepithelialization and nephron survival after renal injury. These new concepts will provide important clues for identifying new targets for the development of clinically relevant treatment strategies for acute kidney injury.

## Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

## References


[1] J. V. Bonventre, "Dedifferentiation and proliferation of surviving epithelial cells in acute renal failure," *Journal of the American Society of Nephrology*, vol. 14, supplement 1, pp. S55–S61, 2003.

[2] A. Maeshima, M. Nakasatomi, and Y. Nojima, "Regenerative medicine for the kidney: renotropic factors, renal stem/progenitor cells, and stem cell therapy," *BioMed Research International*, vol. 2014, Article ID 595493, 10 pages, 2014.

[3] B. D. Humphreys, M. T. Valerius, A. Kobayashi et al., "Intrinsic epithelial cells repair the kidney after injury," *Cell Stem Cell*, vol. 2, no. 3, pp. 284–291, 2008.

[4] T. Kusaba, M. Lalli, R. Kramann, A. Kobayashi, and B. D. Humphreys, "Differentiated kidney epithelial cells repair injured proximal tubule," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 111, no. 4, pp. 1527–1532, 2014.

[5] A. Maeshima, S. Yamashita, and Y. Nojima, "Identification of renal progenitor-like tubular cells that participate in the regeneration processes of the kidney," *Journal of the American Society of Nephrology*, vol. 14, no. 12, pp. 3138–3146, 2003.

[6] S. Yamashita, A. Maeshima, and Y. Nojima, "Involvement of renal progenitor tubular cells in epithelial-to-mesenchymal transition in fibrotic rat kidneys," *Journal of the American Society of Nephrology*, vol. 16, no. 7, pp. 2044–2051, 2005.

[7] A. Maeshima, H. Sakurai, and S. K. Nigam, "Adult kidney tubular cell population showing phenotypic plasticity, tubulogenic capacity, and integration capability into developing kidney," *Journal of the American Society of Nephrology*, vol. 17, no. 1, pp. 188–198, 2006.

[8] M. Miya, A. Maeshima, K. Mishima et al., "Age-related decline in label-retaining tubular cells: Implication for reduced regenerative capacity after injury in the aging kidney," *The American Journal of Physiology—Renal Physiology*, vol. 302, no. 6, pp. F694–F702, 2012.

[9] A. Vogetseder, A. Karadeniz, B. Kaissling, and M. Le Hir, "Tubular cell proliferation in the healthy rat kidney," *Histochemistry and Cell Biology*, vol. 124, no. 2, pp. 97–104, 2005.

[10] Y. Fujigaki, T. Goto, M. Sakakima et al., "Kinetics and characterization of initially regenerating proximal tubules in S3 segment in response to various degrees of acute tubular injury," *Nephrology Dialysis Transplantation*, vol. 21, no. 1, pp. 41–50, 2006.

[11] J. A. Oliver, O. Maarouf, F. H. Cheema, T. P. Martens, and Q. Al-Awqati, "The renal papilla is a niche for adult kidney stem cells," *Journal of Clinical Investigation*, vol. 114, no. 6, pp. 795–804, 2004.

[12] H.-C. Park, K. Yasuda, M.-C. Kuo et al., "Renal capsule as a stem cell niche," *American Journal of Physiology: Renal Physiology*, vol. 298, no. 5, pp. F1254–F1262, 2010.

[13] S. Gupta, C. Verfaillie, D. Chmielewski et al., "Isolation and characterization of kidney-derived stem cells," *Journal of the American Society of Nephrology*, vol. 17, no. 11, pp. 3028–3040, 2006.

[14] S. Kitamura, Y. Yamasaki, M. Kinomura et al., "Establishment and characterization of renal progenitor like cells from S3 segment of nephron in rat adult kidney," *FASEB Journal*, vol. 19, no. 13, pp. 1789–1797, 2005.

[15] B. Bussolati, S. Bruno, C. Grange et al., "Isolation of renal progenitor cells from adult human kidney," *The American Journal of Pathology*, vol. 166, no. 2, pp. 545–555, 2005.

[16] C. Sagrinati, G. S. Netti, B. Mazzinghi et al., "Isolation and characterization of multipotent progenitor cells from the Bowman's capsule of adult human kidneys," *Journal of the American Society of Nephrology*, vol. 17, no. 9, pp. 2443–2456, 2006.

[17] B. Bussolati, A. Moggio, F. Collino et al., "Hypoxia modulates the undifferentiated phenotype of human renal inner medullary CD133$^+$ progenitors through Oct4/miR-145 balance," *American Journal of Physiology—Renal Physiology*, vol. 302, no. 1, pp. F116–F128, 2012.

[18] B. Smeets, P. Boor, H. Dijkman et al., "Proximal tubular cells contain a phenotypically distinct, scattered cell population involved in tubular regeneration," *Journal of Pathology*, vol. 229, no. 5, pp. 645–659, 2013.

[19] F. Sallustio, L. de Benedictis, G. Castellano et al., "TLR2 plays a role in the activation of human resident renal stem/progenitor cells," *The FASEB Journal*, vol. 24, no. 2, pp. 514–525, 2010.

[20] F. Sallustio, V. Costantino, S. N. Cox et al., "Human renal stem/progenitor cells repair tubular epithelial cell injury through TLR2-driven inhibin-A and microvesicle-shuttled decorin," *Kidney International*, vol. 83, no. 3, pp. 392–403, 2013.

[21] H. Wu, G. Chen, K. R. Wyburn et al., "TLR4 activation mediates kidney ischemia/reperfusion injury," *Journal of Clinical Investigation*, vol. 117, no. 10, pp. 2847–2859, 2007.

[22] J. C. Leemans, G. Stokman, N. Claessen et al., "Renal-associated TLR2 mediates ischemia/reperfusion injury in the kidney," *The Journal of Clinical Investigation*, vol. 115, no. 10, pp. 2894–2903, 2005.

[23] N. Tsuboi, Y. Yoshikai, S. Matsuo et al., "Roles of toll-like receptors in C-C chemokine production by renal tubular epithelial cells," *The Journal of Immunology*, vol. 169, no. 4, pp. 2026–2033, 2002.

[24] J. M. Thurman, A. M. Lenderink, P. A. Royer et al., "C3a is required for the production of CXC chemokines by tubular epithelial cells after renal ishemia/reperfusion," *The Journal of Immunology*, vol. 178, no. 3, pp. 1819–1828, 2007.

[25] J. M. Thurman, D. Ljubanović, P. A. Royer et al., "Altered renal tubular expression of the complement inhibitor Crry permits complement activation after ischemia/reperfusion," *The Journal of Clinical Investigation*, vol. 116, no. 2, pp. 357–368, 2006.

[26] B. De Vries, J. Köhl, W. K. G. Leclercq et al., "Complement factor C5a mediates renal ischemia-reperfusion injury independent from neutrophils," *The Journal of Immunology*, vol. 170, no. 7, pp. 3883–3889, 2003.

[27] N. Susnik, I. Sörensen-Zender, S. Rong et al., "Ablation of proximal tubular suppressor of cytokine signaling 3 enhances tubular cell cycling and modifies macrophage phenotype during acute kidney injury," *Kidney International*, vol. 85, no. 6, pp. 1357–1368, 2014.

[28] L. Li, L. Huang, S.-S. J. Sung et al., "The chemokine receptors CCR2 and CX3CR1 mediate monocyte/macrophage trafficking in kidney ischemia-reperfusion injury," *Kidney International*, vol. 74, no. 12, pp. 1526–1537, 2008.

[29] K. J. Kelly, W. W. Williams Jr., R. B. Colvin et al., "Intercellular adhesion molecule-1-deficient mice are protected against ischemic renal injury," *The Journal of Clinical Investigation*, vol. 97, no. 4, pp. 1056–1063, 1996.

[30] M. J. Burne-Taney and H. Rabb, "The role of adhesion molecules and T cells in ischemic renal injury," *Current Opinion in Nephrology and Hypertension*, vol. 12, no. 1, pp. 85–90, 2003.

[31] M. J. Burne-Taney, D. B. Ascon, F. Daniels, L. Racusen, W. Baldwin, and H. Rabb, "B cell deficiency confers protection

from renal ischemia reperfusion injury," *Journal of Immunology*, vol. 171, no. 6, pp. 3210–3215, 2003.

[32] H. R. Jang, M. T. Gandolfo, G. J. Ko, S. R. Satpute, L. Racusen, and H. Rabb, "B cells limit repair after ischemic acute kidney injury," *Journal of the American Society of Nephrology*, vol. 21, no. 4, pp. 654–665, 2010.

[33] P. Park, M. Haas, P. N. Cunningham, L. Bao, J. J. Alexander, and R. J. Quigg, "Injury in renal ischemia-reperfusion is independent from immunoglobulins and T lymphocytes," *American Journal of Physiology: Renal Physiology*, vol. 282, no. 2, pp. F352–F357, 2002.

[34] M. Liu, C.-C. Chien, D. N. Grigoryev, M. T. Gandolfo, R. B. Colvin, and H. Rabb, "Effect of T cells on vascular permeability in early ischemic acute kidney injury in mice," *Microvascular Research*, vol. 77, no. 3, pp. 340–347, 2009.

[35] V. Savransky, R. R. Molls, M. Burne-Taney, C.-C. Chien, L. Racusen, and H. Rabb, "Role of the T-cell receptor in kidney ischemia-reperfusion injury," *Kidney International*, vol. 69, no. 2, pp. 233–238, 2006.

[36] M. J. Burne, F. Daniels, A. El Ghandour et al., "Identification of the $CD4^+$ T cell as a major pathogenic factor in ischemic acute renal failure," *The Journal of Clinical Investigation*, vol. 108, no. 9, pp. 1283–1290, 2001.

[37] Y.-J. Day, L. Huang, H. Ye, L. Li, J. Linden, and M. D. Okusa, "Renal ischemia-reperfusion injury and adenosine 2A receptor-mediated tissue protection: the role of $CD4^+$ T cells and IFN-$\gamma$," *The Journal of Immunology*, vol. 176, no. 5, pp. 3108–3114, 2006.

[38] H. Rabb, F. Daniels, M. O'Donnell et al., "Pathophysiological role of T lymphocytes in renal ischemia-reperfusion injury in mice," *American Journal of Physiology: Renal Physiology*, vol. 279, no. 3, pp. F525–F531, 2000.

[39] M. T. Gandolfo, H. R. Jang, S. M. Bagnasco et al., "$Foxp3^+$ regulatory T cells participate in repair of ischemic acute kidney injury," *Kidney International*, vol. 76, no. 7, pp. 717–729, 2009.

[40] G. R. Kinsey, L. Huang, A. L. Vergis, L. Li, and M. D. Okusa, "Regulatory T cells contribute to the protective effect of ischemic preconditioning in the kidney," *Kidney International*, vol. 77, no. 9, pp. 771–780, 2010.

[41] G. R. Kinsey, R. Sharma, L. Huang et al., "Regulatory T cells suppress innate immunity in kidney ischemia-reperfusion injury," *Journal of the American Society of Nephrology*, vol. 20, no. 8, pp. 1744–1753, 2009.

[42] L. Li, L. Huang, S.-S. J. Sung et al., "NKT cell activation mediates neutrophil IFN-$\gamma$ production and renal ischemia-reperfusion injury," *The Journal of Immunology*, vol. 178, no. 9, pp. 5899–5911, 2007.

[43] M. Lech and H.-J. Anders, "Macrophages and fibrosis: how resident and infiltrating mononuclear phagocytes orchestrate all phases of tissue injury and repair," *Biochimica et Biophysica Acta: Molecular Basis of Disease*, vol. 1832, no. 7, pp. 989–997, 2013.

[44] B. Bielesz, Y. Sirin, H. Si et al., "Epithelial Notch signaling regulates interstitial fibrosis development in the kidneys of mice and humans," *Journal of Clinical Investigation*, vol. 120, no. 11, pp. 4040–4054, 2010.

[45] J. Chen, J.-K. Chen, E. M. Conway, and R. C. Harris, "Survivin mediates renal proximal tubule recovery from AKI," *Journal of the American Society of Nephrology*, vol. 24, no. 12, pp. 2023–2033, 2013.

[46] M. L. Kielar, R. John, M. Bennett et al., "Maladaptive role of IL-6 in ischemic acute renal failure," *Journal of the American Society of Nephrology*, vol. 16, no. 11, pp. 3315–3325, 2005.

[47] D.-J. Oh, B. Dursun, Z. He et al., "Fractalkine receptor (CX3CR1) inhibition is protective against ischemic acute renal failure in mice," *American Journal of Physiology—Renal Physiology*, vol. 294, no. 1, pp. F264–F271, 2008.

[48] Y.-J. Day, L. Huang, H. Ye, J. Linden, and M. D. Okusa, "Renal ischemia-reperfusion injury and adenosine 2A receptor-mediated tissue protection: role of macrophages," *American Journal of Physiology: Renal Physiology*, vol. 288, no. 4, pp. F722–F731, 2005.

[49] K. Karasawa, K. Asano, S. Moriyama et al., "Vascular-resident CD169-positive monocytes and macrophages control neutrophil accumulation in the kidney with ischemia-reperfusion injury," *Journal of the American Society of Nephrology*. In press.

[50] S. Lee, S. Huen, H. Nishio et al., "Distinct macrophage phenotypes contribute to kidney injury and repair," *Journal of the American Society of Nephrology*, vol. 22, no. 2, pp. 317–326, 2011.

[51] M.-Z. Zhang, B. Yao, S. Yang et al., "CSF-1 signaling mediates recovery from acute kidney injury," *The Journal of Clinical Investigation*, vol. 122, no. 12, pp. 4519–4532, 2012.

[52] S.-L. Lin, B. Li, S. Rao et al., "Macrophage Wnt7b is critical for kidney repair and regeneration," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 107, no. 9, pp. 4194–4199, 2010.

[53] X. Dong, S. Swaminathan, L. A. Bachman, A. J. Croatt, K. A. Nath, and M. D. Griffin, "Resident dendritic cells are the predominant TNF-secreting cell in early renal ischemia-reperfusion injury," *Kidney International*, vol. 71, no. 7, pp. 619–628, 2007.

[54] X. Dong, S. Swaminathan, L. A. Bachman, A. J. Croatt, K. A. Nath, and M. D. Griffin, "Antigen presentation by dendritic cells in renal lymph nodes is linked to systemic and local injury to the kidney," *Kidney International*, vol. 68, no. 3, pp. 1096–1108, 2005.

[55] O. P. Kulkarni, I. Hartter, S. R. Mulay et al., "Toll-like receptor 4-induced IL-22 accelerates kidney regeneration," *Journal of the American Society of Nephrology*, vol. 25, no. 5, pp. 978–989, 2014.

[56] M. Miya, A. Maeshima, K. Mishima et al., "Enhancement of in vitro human tubulogenesis by endothelial cell-derived factors: implications for in vivo tubular regeneration after injury," *The American Journal of Physiology—Renal Physiology*, vol. 301, no. 2, pp. F387–F395, 2011.

[57] D. P. Basile, "The endothelial cell in ischemic acute kidney injury: implications for acute and chronic function," *Kidney International*, vol. 72, no. 2, pp. 151–156, 2007.

[58] M. Hörbelt, S.-Y. Lee, H. E. Mang et al., "Acute and chronic microvascular alterations in a mouse model of ischemic acute kidney injury," *American Journal of Physiology—Renal Physiology*, vol. 293, no. 3, pp. F688–F695, 2007.

[59] D. Patschan, K. Krupincza, S. Patschan, Z. Zhang, C. Hamby, and M. S. Goligorsky, "Dynamics of mobilization and homing of endothelial progenitor cells after acute renal ischemia: modulation by ischemic preconditioning," *American Journal of Physiology: Renal Physiology*, vol. 291, no. 1, pp. F176–F185, 2006.

[60] F. Becherucci, B. Mazzinghi, E. Ronconi et al., "The role of endothelial progenitor cells in acute kidney injury," *Blood Purification*, vol. 27, no. 3, pp. 261–270, 2009.

[61] V. Cantaluppi, S. Gatti, D. Medica et al., "Microvesicles derived from endothelial progenitor cells protect the kidney from ischemia-reperfusion injury by microRNA-dependent reprogramming of resident renal cells," *Kidney International*, vol. 82, no. 4, pp. 412–427, 2012.

[62] T. A. Sutton, H. E. Mang, S. B. Campos, R. M. Sandoval, M. C. Yoder, and B. A. Molitoris, "Injury of the renal microvascular

endothelium alters barrier function after ischemia," *American Journal of Physiology: Renal Physiology*, vol. 285, no. 2, pp. F191–F198, 2003.

[63] R. Poulsom, S. J. Forbes, K. Hodivala-Dilke et al., "Bone marrow contributes to renal parenchymal turnover and regeneration," *The Journal of Pathology*, vol. 195, no. 2, pp. 229–235, 2001.

[64] S. Kale, A. Karihaloo, P. R. Clark, M. Kashgarian, D. S. Krause, and L. G. Cantley, "Bone marrow stem cells contribute to repair of the ischemically injured renal tubule," *The Journal of Clinical Investigation*, vol. 112, no. 1, pp. 42–49, 2003.

[65] F. Lin, K. Cordes, L. Li et al., "Hematopoietic stem cells contribute to the regeneration of renal tubules after renal ischemia-reperfusion injury in mice," *Journal of the American Society of Nephrology*, vol. 14, no. 5, pp. 1188–1199, 2003.

[66] B. Li, A. Cohen, T. E. Hudson, D. Motlagh, D. L. Amrani, and J. S. Duffield, "Mobilized human hematopoietic stem/progenitor cells promote kidney repair after ischemia/reperfusion injury," *Circulation*, vol. 121, no. 20, pp. 2211–2220, 2010.

[67] G. Stokman, J. C. Leemans, N. Claessen, J. J. Weening, and S. Florquin, "Hematopoietic stem cell mobilization therapy accelerates recovery of renal function independent of stem cell contribution," *Journal of the American Society of Nephrology*, vol. 16, no. 6, pp. 1684–1692, 2005.

[68] M. Iwasaki, Y. Adachi, K. Minamino et al., "Mobilization of bone marrow cells by G-CSF rescues mice from cisplatin-induced renal failure, and M-CSF enhances the effects of G-CSF," *Journal of the American Society of Nephrology*, vol. 16, no. 3, pp. 658–666, 2005.

[69] F. Tögel, J. Isaac, and C. Westenfelder, "Hematopoietic stem cell mobilization-associated granulocytosis severely worsens acute renal failure," *Journal of the American Society of Nephrology*, vol. 15, no. 5, pp. 1261–1267, 2004.

[70] J. S. Duffield, K. M. Park, L.-L. Hsiao et al., "Restoration of tubular epithelial cells during repair of the postischemic kidney occurs independently of bone marrow-derived stem cells," *Journal of Clinical Investigation*, vol. 115, no. 7, pp. 1743–1755, 2005.

[71] F. Lin, A. Moran, and P. Igarashi, "Intrarenal cells, not bone marrow-derived cells, are the major source for regeneration in postischemic kidney," *The Journal of Clinical Investigation*, vol. 115, no. 7, pp. 1756–1764, 2005.

[72] B. D. Humphreys, M. T. Valerius, A. Kobayashi et al., "Intrinsic epithelial cells repair the kidney after injury," *Cell Stem Cell*, vol. 2, no. 3, pp. 284–291, 2008.

[73] M. B. Herrera, B. Bussolati, S. Bruno, V. Fonsato, G. M. Romanazzi, and G. Camussi, "Mesenchymal stem cells contribute to the renal repair of acute tubular epithelial injury," *International Journal of Molecular Medicine*, vol. 14, no. 6, pp. 1035–1041, 2004.

[74] M. Morigi, B. Imberti, C. Zoja et al., "Mesenchymal stem cells are renotropic, helping to repair the kidney and improve function in acute renal failure," *Journal of the American Society of Nephrology*, vol. 15, no. 7, pp. 1794–1804, 2004.

[75] M. Morigi, M. Introna, B. Imberti et al., "Human bone marrow mesenchymal stem cells accelerate recovery of acute renal injury and prolong survival in mice," *Stem Cells*, vol. 26, no. 8, pp. 2075–2082, 2008.

[76] C. Lange, F. Tögel, H. Ittrich et al., "Administered mesenchymal stem cells enhance recovery from ischemia/reperfusion-induced acute renal failure in rats," *Kidney International*, vol. 68, no. 4, pp. 1613–1617, 2005.

[77] B. Imberti, M. Morigi, S. Tomasoni et al., "Insulin-like growth factor-1 sustains stem cell-mediated renal repair," *Journal of the American Society of Nephrology*, vol. 18, no. 11, pp. 2921–2928, 2007.

[78] B. Bi, R. Schmitt, M. Israilova, H. Nishio, and L. G. Cantley, "Stromal cells protect against acute tubular injury via an endocrine effect," *Journal of the American Society of Nephrology*, vol. 18, no. 9, pp. 2486–2496, 2007.

[79] N. Eliopoulos, J. Zhao, M. Bouchentouf et al., "Human marrow-derived mesenchymal stromal cells decrease cisplatin renotoxicity in vitro and in vivo and enhance survival of mice post-intraperitoneal injection," *American Journal of Physiology: Renal Physiology*, vol. 299, no. 6, pp. F1288–F1298, 2010.

[80] F. Tögel, P. Zhang, Z. Hu, and C. Westenfelder, "VEGF is a mediator of the renoprotective effects of multipotent marrow stromal cells in acute kidney injury," *Journal of Cellular and Molecular Medicine*, vol. 13, no. 8 B, pp. 2109–2114, 2009.

Hindawi Publishing Corporation
Stem Cells International
Volume 2015, Article ID 724047, 10 pages
http://dx.doi.org/10.1155/2015/724047

*Review Article*

# Current Bioengineering Methods for Whole Kidney Regeneration

**Shuichiro Yamanaka and Takashi Yokoo**

*Division of Nephrology and Hypertension, Department of Internal Medicine, Jikei University School of Medicine, Tokyo 105-8461, Japan*

Correspondence should be addressed to Takashi Yokoo; tyokoo14@gmail.com

Received 3 October 2014; Revised 13 January 2015; Accepted 13 January 2015

Academic Editor: Laura Lasagni



Kidney regeneration is likely to provide an inexhaustible source of tissues and organs for immunosuppression-free transplantation. It is currently garnering considerable attention and might replace kidney dialysis as the ultimate therapeutic strategy for renal failure. However, anatomical complications make kidney regeneration difficult. Here, we review recent advances in the field of kidney regeneration, including (i) the directed differentiation of induced pluripotent stem cells/embryonic stem cells into kidney cells; (ii) blastocyst decomplementation; (iii) use of a decellularized cadaveric scaffold; (iv) embryonic organ transplantation; and (v) use of a nephrogenic niche for growing xenoembryos for *de novo* kidney regeneration from stem cells. All these approaches represent potentially promising therapeutic strategies for the treatment of patients with chronic kidney disease. Although many obstacles to kidney regeneration remain, we hope that innovative strategies and reliable research will ultimately allow the restoration of renal function in patients with end-stage kidney disease.

## 1. Introduction

Transplantation represents the ideal method of restoring organ function in patients with organ failure. However, the lack of suitable available donor organs is a serious problem worldwide [1]. Regenerative medicine has the potential to provide the ultimate treatment for various diseases by using autologous cells to reconstruct new organs and replace failing organs, and it has thus garnered considerable attention in recent years.

There are currently no published clinical reports regarding the regeneration of functional organs for the treatment for terminal organ failure. Kidney regeneration is particularly difficult because of the anatomical complexity of the organ [2], and reconstruction of the kidney's cubic structure is hard. The kidneys function to maintain homeostasis and are responsible for blood filtration and urine production, as well as the control of endocrine functions via erythropoietin (Epo) and vitamin D. To date, regeneration of all the constituent cells of the kidney has not been achieved. However, it is possible that kidney regeneration may be achieved by gradual advances in stem cell research and cellular engineering (Figure 1).

The regeneration of an entire kidney may be difficult, but functional recovery of as little as 10% of kidney filtration function might allow for the withdrawal of dialysis in patients with end-stage kidney disease (ESKD) [3], thus greatly increasing quality of life. The regeneration of a functional kidney represents a huge practical challenge. However, research in the field of regeneration is continuing to make progress, with the aim of overcoming ESKD through kidney regeneration. In this review, we consider current advances in kidney regeneration, including induced pluripotent stem cell (iPSC) technologies and renal bioengineering, and we discuss some of the associated limitations and challenges.

## 2. Directed Differentiation of Induced Pluripotent Stem Cells and Embryonic Stem Cells into Kidney Cells

Previous studies have suggested that pluripotent stem cells (PSCs) have the potential to differentiate into any cell type in the body and self-assemble into heterogeneous tissues or organs [4–6]. PSCs have indeed been shown to generate mature cells *in vitro*. PSCs therefore represent an important

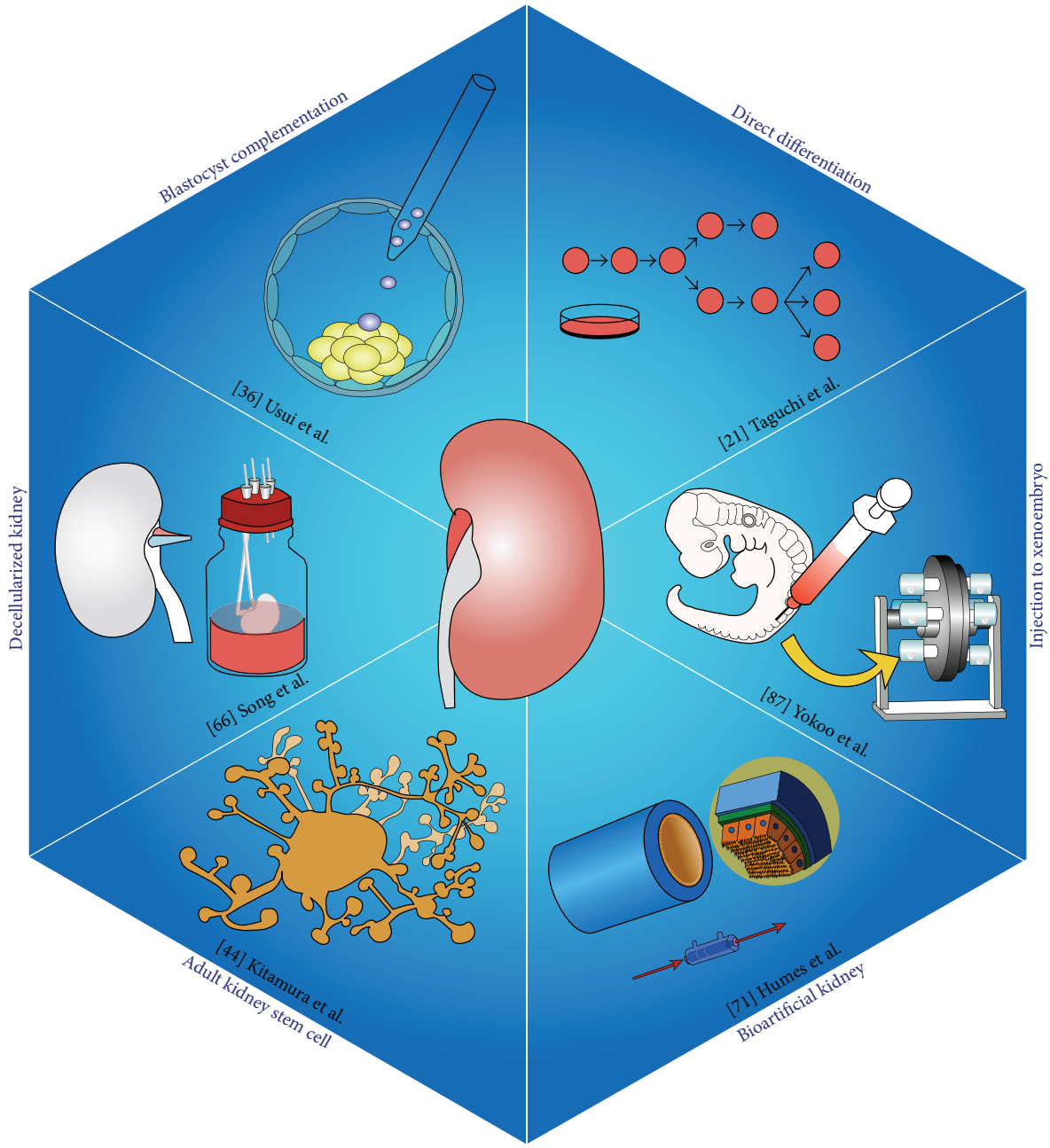



Figure 1: Schematic representation of the main strategies for kidney regeneration.

cell type for bioengineering strategies aimed at kidney regeneration. PSCs include both embryonic stem cells (ESCs) and iPSCs. ESCs are derived from embryos and grown in primary culture [4], while iPSCs are produced from terminally differentiated cells using transfection factors such as c-myc, Oct4, Klf4, and Sox-2 [5, 6]. PSCs have been differentiated successfully into various types of cells and tissues, including intestine [7], hepatic [8, 9], neural [10, 11], hematologic [12], pancreatic [13, 14], and cardiac lineages [15]. This approach, whereby iPSCs or ESCs are differentiated into kidney or other progenitors, is termed "directed differentiation" and is accomplished by the sequential application of chemicals or growth factors.

Recent progress in stem cell research has generated human nephron progenitor cells, including intermediate mesoderm (IM) and metanephric mesenchyme (MM) cells [16–21]. An understanding of the processes and molecular mechanisms of kidney development is important for the production of appropriate cells to generate nephrons. The kidney is derived from IM and arises from the ureteric bud and MM following precisely timed interactions between multiple signals [22]. Different studies have used different growth-factor protocols in human PSCs (hPSCs); however, the Wnt agonist CHIR99021 is commonly used to promote mesoderm differentiation [16].

High-throughput chemical screening and low-molecular-weight chemical compounds have recently been used in directed differentiation for kidney formation. An efficient method has been developed for inducing the differentiation of IM cells from hiPSCs/hESCs using a combination of activin A and CHIR99021 to generate mesoderm, followed by combined treatment with bone morphogenetic protein 7 (BMP7) and CHIR99021 [17, 18]. This protocol induced hiPSCs to develop into odd-skipped-related 1 (OSR1)+ IM cells with an efficiency greater than 90% [18]. Furthermore, high-throughput screening of approximately 1,821 chemical compounds identified two retinoic acid receptor agonists, AM580 and TTNPB, that efficiently induced the differentiation of human iPSCs (hiPSCs)/ESCs into IM cells [23]. The high-throughput screening system used a hiPSC reporter line, in which the green fluorescent protein (GFP) coding sequence was knocked into the OSR1 gene locus, allowing small molecules that increased the induction rate of OSR1+ cells to be identified quantitatively by flow cytometry. GFP expression was examined using an LSR Fortessa cell analyzer equipped with a high-throughput sampler (BD Biosciences, San Jose, CA, USA). They proposed that small chemical compounds were less expensive and more consistent than growth factors and might therefore be more suitable for generating IM cells [18, 23]. This study demonstrated the feasibility of monitoring the nephrogenic-differentiation capacity of hiPSCs and provided a new strategy for investigating the efficiency and specificity of methods of achieving renal differentiation of hiPSCs.

Many studies have attempted to generate MM progenitor cells by direct differentiation from PSCs. Xia et al. tried to differentiate PSCs into ureteric bud (UB) lineage cells by stepwise treatment of iPSCs/hESCs with a combination of fibroblast growth factor 2 (FGF2) and BMP4 for 2 days, followed by combined treatment with activin A and BMP2 for another 2 days. After the 4 days, IM-like cells expressing PAX2, OSR1, WT1, and LHX1 were produced. The UB markers HOXB7, RET, and GFRA1 were upregulated in these cells after an additional 2 days of differentiation, implying that PSCs could be driven towards UB progenitor-like cells. Additionally, *in vitro* differentiation of PSCs generated cells with a UB-committed IM fate with the potential to assemble spontaneously into complex, chimeric three-dimensional (3D) structures upon coculture with murine embryonic kidney cells [19]. However, the differentiation efficiency was poor, though the reasons for this were unclear.

The strategy of direct differentiation from PSCs has made significant advances in the past few years [20, 21]. Takasato et al. [20] and Taguchi et al. [21] noted that nephron progenitor cells were derived from PSCs. The developmental origin of the kidney is well known. Takasato et al. [20] induced a primitive streak from hESCs using activin A (or CHIR99021) and BMP4. The protocol up to this point was similar in both groups [20, 21], but the subsequent protocols used to induce IM differentiation from posterior primitive streak differed. The differentiated hESCs formed renal vesicles combined with dissociated embryonic mouse kidney cells in the study by Takasato et al., involving the integration of human cells into mouse renal structures [20]. In contrast, Taguchi et al. demonstrated more reasonable protocols for inducing renal structures such as nephrons and proximal tubules [21]. They proposed the concept of "posteriorization" for inducing nephron progenitors from PSCs in a mouse and human model [21]. Briefly, nephron progenitor cells of the MM could be derived from posteriorly located IM. Brachyury, encoded by the T gene, is a representative marker of the primitive streak and posterior nascent mesoderm [24]. Posteriorly located T+ mesoderm is a putative part of the axial progenitor cells recently identified as the source of the caudal body trunk [25]. They also assumed that there were differences in the developmental processes between the posteriorly located MM and anteriorly located mesodermal tissues such as the heart, which have been successfully induced from PSCs by way of the T+ state with an initial short period of differentiation [26]. The competence of the IM differed both temporally (E8.5 versus E9.5) and spatially (anteroposteriorly), and OSR1+ or PAX2+ cells were considered to represent a homogeneous population [21]. This study indicated that a precursor of the UB was located anteriorly in IM cells lacking T at E8.5 and was already segregated from that of the MM localized in the T+ posterior nascent mesoderm. Surprisingly, the T+ posterior nascent mesoderm was negative for OSR1 but nevertheless contained nephron progenitors [21]. Lineage-tracing experiments demonstrated that the MM precursor could be traced back to the OSR1+ posterior IM at E9.5 and T+/OSR1+ posterior mesoderm at E8.5 [21]. The same study also established an efficient protocol for inducing metanephric nephron progenitors from the posterior nascent mesoderm using a combination of high-CHIR99021 (10 $\mu$M) and BMP4 for posteriorization, followed by combined treatment with mid-CHIR99021 (3 $\mu$M) and BMP4 with the addition of retinoic acid and activin A. A final step involved the addition of low-CHIR99021 (1 $\mu$M) and FGF9 for 1 day each. They finally verified the temporal kinetics of gene expression at each step of the induction process. Wnt signals are important for posteriorization [25, 27]. Indeed, a high concentration of CHIR (a Wnt agonist) was used in combination with BMP4 to maintain the posterior nascent mesoderm in the posteriorization phase [21]. They established the induction of MM from T+ caudal precursors. They also established stepwise protocols for the differentiation of both mouse ESCs and human iPSCs into metanephric nephron progenitors, thus enabling kidney generation using multiple stage-specific growth factors [21]. Coculture of embryoid bodies, containing nephron progenitors, with mouse embryonic spinal cord, a well-established inducer of kidney tubulogenesis, resulted in the formation of 3D tubular structures expressing markers characteristic of renal tubules and glomeruli [21]. Although the authors were unable to confirm urinary production or other kidney functions, this direct differentiation method for kidney regeneration appears to be the most complete and reliable study published to date.

Generating precise renal progenitor cells is essential for the development of a whole kidney *de novo*. The differentiation of induced IM cells into precise renal progenitor cells would allow a complete 3D kidney structure to be constructed from PSCs. However, the means of successfully regenerating a functional vascular system between the regenerated kidney

and the recipient remain unknown. Additionally, the *in vivo* functioning of a regenerated kidney remains unclear. However, further advances in developmental biology and bioengineering may resolve these issues and allow whole kidney regeneration.

## 3. Blastocyst Complementation

Injection of PSCs into blastocysts, the initial embryonic stage after fertilization, synchronizes the development of two line cells, and the combined blastocyst generates a chimeric body. In the first report of this method, normal ESCs were injected into blastocysts of recombination-activating gene 2-deficient mice, which have no mature B or T lymphocytes, to generate somatic chimeras with ESC-derived mature B and T cells [28]. This "blastocyst complementation" system was applied to the reconstruction of several tissues and organs, including thymic epithelium [29], heart [30], yolk sac hematopoiesis [31], germ cells [32], hepatocytes [33], pancreas [34, 35], and kidney [36]. A recent study to generate a functional organ using blastocyst complementation by Kobayashi et al. showed that the injection of rat iPSCs into $Pdx1^{-/-}$ (pancreatogenesis-disabled) mouse blastocysts produced newborn rat/mouse chimeras with a pancreas derived almost entirely from rat iPSCs [34]. The mouse and rat PSC-derived pancreas produced insulin, and the transplantation of PSC-derived pancreas islets improved hyperglycemia in a diabetic rodent model [37]. This study indicated that PSC-derived cellular progeny could occupy and develop in a vacant developmental niche. Furthermore, these results also demonstrated that interspecific blastocyst complementation could be used to generate organs derived from donor PSCs *in vivo* using a xenogeneic environment [36, 37]. This blastocyst complementation system has already been applied to whole kidney reconstruction [36]. Nondeficient murine iPSCs were injected into blastocysts from kidney-deficient mice lacking the SAL-like 1 protein essential for kidney development, and the neonatal mice had kidneys derived almost entirely from injected iPSCs [36]. However, the vascular and nervous systems were not constructed from cells of iPSC origin, and the kidney was therefore not completely complemented. Immunohistochemical analysis of the regenerated kidney indicated that the renal vascular system, including renal segmental, lobar, interlobar, arcuate, and interlobular arterioles, was a chimeric structure originating from both host cells and donor iPSCs [36]. Precise urinary analysis was not carried out and whether or not filtrated and reabsorbed urine was produced is unclear. Moreover, injection of rat iPSCs into kidney-deficient mouse blastocysts failed to generate rat kidneys in mice. This result implies that the key molecules in mice involved in interactions between the mesenchyme and UB do not cross-react with those in rats. The generation of xenogeneic organs using interspecific blastocysts thus requires a host animal strain lacking all renal lineages [36].

The most important problem associated with blastocyst complementation is the ethical issue. It is impossible to exclude the possibility of generating interspecific chimeras containing brain derived from injected PSCs. Although it is difficult to establish a xenogeneic blastocyst complementation system that overcomes the xenogeneic barrier, this strategy appears to be one of the most promising methods for kidney regeneration.

## 4. Adult Kidney Stem/Progenitor Cell Reconstitution

Stem/progenitor cells isolated from many adult organs demonstrate self-renewing ability and can give rise to terminally differentiated cells. This kidney stem/progenitor population disappears in the adult kidney, possibly because of the loss of its niche [37, 38]. However, renal stem/progenitor cells still exist in specific locations in the adult kidney, such as in the renal papilla [39], tubular epithelial cells [40], Bowman's capsule [41], and the S3 segment of the proximal tubules [42, 43]. Kitamura et al. recently reported that adult kidney stem/progenitor cells derived from the S3 segment of adult rat kidney nephrons were able to reconstitute a 3D kidney-like structure *in vitro* [44]. Kidney-like structures were formed when a cluster of kidney stem/progenitor cells was suspended in an extracellular matrix gel and cultured in the presence of several growth factors (combination of glial cell-derived neurotrophic factor, basic FGF, hepatocyte growth factor, epidermal growth factor, and BMP7). The clusters from dissociated S3-segment cells were induced by the hanging-drop method in 3D culture [44], while 2D culture conditions were unable to reconstruct kidney-like structures. Surprisingly, the reconstructed kidney-like structures included all the kidney substructures, including glomeruli, proximal tubules, the loop of Henle, distal tubules, and the collecting ducts, but not the vasculature. They suggested that a cluster of tissue stem/progenitor cells may have the ability to reconstitute the minimum unit of its organ of origin by differentiating into specialized cells in the correct niche. Kidney stem/progenitor cells derived from the S3 segment of adult rat kidney have been shown to express stem cell markers such as Sca-1, c-kit, nestin, and Musashi-1, together with renal lineage markers such as PAX-2 and WT-1 [44]. They assumed that these cells were similar to metanephric mesenchymal cells, based on marker protein expression. However, the clusters can differentiate into collecting-duct-like cells or mesangial-like cells, which are not thought to be derived from MM [44]. In this regard, the question of whether adult kidney stem cells can differentiate into lineages other than UB or MM remains to be answered. The kidney-like structures were not vascularized and did not produce urine. However, adult kidney stem cells remain poorly understood. The potential tumorigenicity of undifferentiated hiPSCs is a critical problem for these cells as a clinical source, while adult stem/progenitor cells have been reported to be nontumorigenic when injected into a mouse model [45–47]. These reports raise the possibility that adult stem cells may represent a safer clinical source than PSCs. If it is possible to establish adult stem/progenitors with multipotency for kidney reconstruction, they may be promising cellular source for kidney repair and regeneration.

## 5. Decellularized Cadaveric Scaffold

Native kidney extracellular matrix (ECM) has been reported to provide a scaffold for cell seeding and a niche for stem cells to differentiate into whole organs [48]. The ECM plays a crucial role in kidney development and repair [48–52]. ECM molecules and their receptors influence organogenesis and repair by providing a scaffold for the spatial organization of cells, by secreting and storing growth factors and cytokines, and by regulating signal transduction [48–53]. ECM scaffolds from whole human-cadaveric and animal organs can be generated by detergent-based decellularization [1, 54]. This strategy was used by Ott et al. [55] to develop a functional rat heart. A whole-heart scaffold with intact 3D geometry and vasculature was prepared by coronary perfusion with detergents into the cadaveric heart. The rat heart was then seeded with neonatal cardiac cells or rat aortic endothelial cells, which subsequently induced the formation of contractile myocardium that performed stroke work [55]. Decellularized cadaveric scaffolds have also been used in several other organ systems, including the liver [56], respiratory tract [57], nerves [58], tendons [59], valves [60], bladder [61], and mammary gland [62]. Furthermore, some studies have used decellularization-recellularization technology for kidney regeneration. Many animals have been used for decellularization studies, including rats [63], rhesus monkeys [64], and pigs [65]. However, regenerated kidneys produced by this method did not have sufficient renal function to produce urine and Epo. Song et al. recently described the successful regeneration of a whole kidney that produced urine after transplantation [66]. Notably, they generated 3D acellular renal scaffolds by perfusion decellularization of cadaveric rat, pig, and human kidneys. Endothelial and epithelial cells were repopulated by perfusion, leading to the formation of viable tissues for renal construction. However, the mechanism whereby the infused cells differentiate and are orchestrated into nephrons with vasculature to produce urine remains unclear. Decellularized cadaveric scaffolds are associated with the problem of massive thrombi, despite strong anticoagulation prophylaxis. Although this strategy still has many obstacles, it demonstrates the impact of regenerative medicine on organ transplantation and its potential as a solution for the shortage of donor organs.

## 6. Tissue Engineering of a Bioartificial Kidney: Renal Tubule Assist Device

The developing field of tissue engineering is an extension of cell therapy, in which biological and engineering science techniques are combined to create structures and devices to replace lost tissue or organ functions [67, 68]. The development of bioartificial kidneys (BAKs) represents the intersection between regenerative medicine and renal replacement therapy [52]. A renal tubule assist device (RAD) containing living renal proximal tubule cells has been successfully engineered, and it demonstrated differentiated absorptive, metabolic, and endocrine functions similar to normal kidneys in animal experiments *in vitro* and *ex vivo* [69]. Briefly, renal proximal tubule segments were harvested from kidneys, and renal tubule progenitor cells were selected and expanded [70]. The tubule progenitor cells were grown in culture dishes with culture medium containing specific additives [68]. A RAD with high-flux hemofiltration cartridges containing polysulfone hollow fibers coated with pronectin-L was used as a scaffold device [68]. Renal proximal tubule cells were then seeded into the hollow fibers and the seeded cartridge was connected to a bioreactor perfusion system, in which the extracapillary space was filled with culture medium and the intracapillary space was perfused with medium. The cell cartridges were used at least 14 days after seeding. The RAD units included confluent monolayers of renal proximal tubule cells with characteristics including microvilli, tight junctional complexes, and endocytic vesicles demonstrated by transmission electron microscopy [68]. The tissue-engineered bioartificial RAD performed differential reabsorption and secretory transport because of the specific active transporters present in the proximal tubule cells *in vivo*. However, these transport functions were less efficient than those in native proximal tubules [68]. The same group reported that the RAD was able to maintain viability in a uremic environment in uremic dogs with acute renal failure when placed in series with a conventional hemofilter and an extracorporeal blood circuit [71]. Furthermore, they also performed clinical trials with BAKs [72–74]. The combination of regenerative medicine and bioengineering thus offers promise for the regeneration of whole kidneys.

## 7. Embryonic Organ Transplantation

We attempted to regenerate a functional, transplantable whole kidney able to produce urine and renal hormones, such as Epo, using a xenoembryo and human mesenchymal stem cells. The embryonic metanephros, which is the mammalian renal anlagen, is thought to represent a potential source for the regeneration of functional whole kidneys [75–83]. An embryonic metanephros transplanted into a host animal (rat) was able to obtain its blood supply from the host [75]. Indeed, the survival of anephric rats was prolonged on the basis of renal function provided by a single transplanted rat renal anlagen, the ureter of which was anastomosed to a host ureter [83]. Furthermore, the transplanted metanephros produced renal hormones including Epo and renin, which elevated the blood pressure of the host animal [77, 78]. Metanephroi from porcine embryos implanted either in the omentum of mice in which costimulation was blocked [79] or under the kidney capsules of immunodeficient mice [81] developed fully functional nephrons. The levels of urea nitrogen and creatinine were higher in cyst fluid produced by the transplanted metanephroi than in sera from the transplanted host animals [81], suggesting urine production. Metanephros transplantation was also shown to reduce vascular calcification in rats with chronic renal failure [79] and to maintain blood pressure in anephric rats with induced acute hypotension [78], implying that the transplanted metanephroi carried out multiple renal functions, other than urine production. These results suggest that metanephros transplantation might be used to overcome the shortage of donor kidneys available for transplantation.

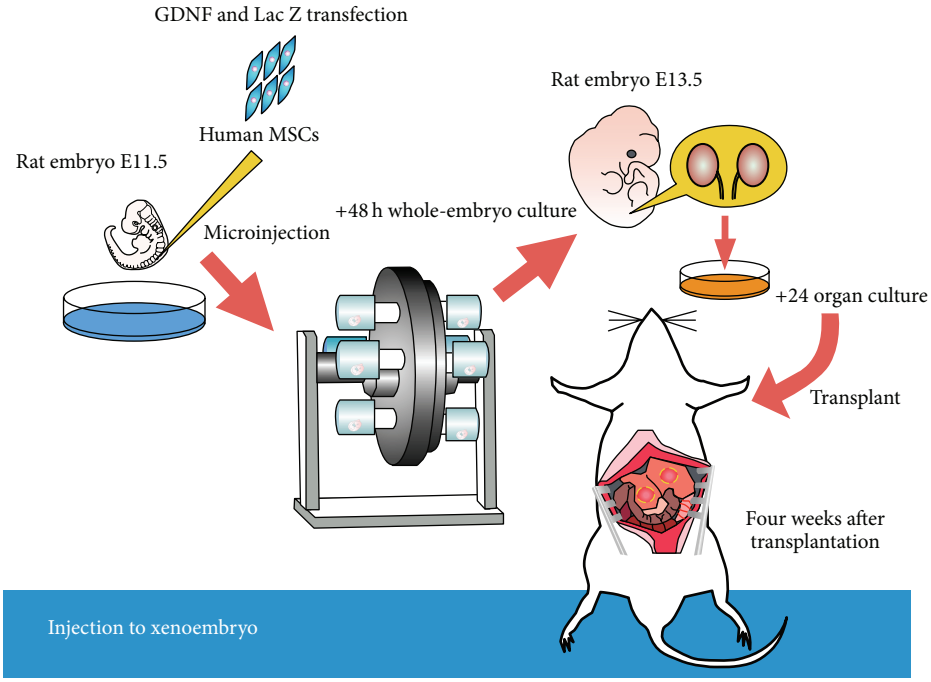



Figure 2: Strategy for nephron regeneration. MSCs were injected at the site of sprouting of the ureteric bud in E11.5 rat embryos. These embryos were then developed in whole-embryo culture for 48 h. Development of a neokidney derived from hMSCs in rat embryos. A kidney anlagen derived from MSCs was transplanted into the adult rat omentum. After culturing, hMSCs formed a neokidney in the rat omentum.

We recently demonstrated that xenotransplanted metanephros could supply endogenous MSCs with a niche for differentiation into Epo-producing tissues [84]. Polymerase chain reaction using species-specific primers and sequence analysis revealed that xenotransplanted metanephroi, either from rat to mouse or from pig to cat, expressed Epo of host animal origin. This indicated that the Epo-producing cells originated in the host animal and developed to produce Epo in the transplanted metanephros. We further showed that the Epo-producing cells did not originate from integrating vessels, but rather from circulating host MSCs mobilized from the bone marrow. Of note, conventional metanephros transplantation requires continuous and strong immunosuppression to avoid humoral rejection associated with the xenogeneic barrier, which can induce adverse effects including carcinogenicity and severe rejection. For safety purposes, the xenotransplant should thus be discarded when it is no longer required, by introducing a cell-fate-regulating system including a suicide gene that can be expressed on demand. To avoid the xenogeneic barrier, we used metanephroi isolated from transgenic ER-E2F1 suicide-inducible mice. E2F1 is a transcription factor that regulates cell proliferation and the ectopic expression of which induces apoptosis. The xenotissue components could therefore be cleared by apoptosis, leaving the autologous Epo-producing tissues [84]. Xenometanephroi *per se* could thus acquire some renal functions in the host omentum, as well as supplying a niche for host stem cells to regenerate renal tissues that can be rebuilt using host-cell components. These techniques may help to reduce the adverse effects of long-term immunosuppressant administration and to address the ethical issues surrounding xenotransplantation [85, 86].

## 8. Use of a Nephrogenic Niche for Growing Xenoembryos

We exploited the developing xenoembryo as a niche for organogenesis using stem cells of renal lineage. Using this strategy, we previously showed that the xenobiotic developmental process for growing xenoembryos allows exogenous human MSCs to undergo epithelial conversion and form a nephron that produces urine and Epo [87–89] (Figure 2). During development of the metanephros, the MM initially forms from the caudal portion of the nephrogenic cord [90] and secretes GDNF, which induces the nearby Wolffian duct to produce a UB [91]. We generated a metanephros in organ culture by microinjecting GDNF-expressing transfected hMSCs into the site of budding, and the recipient embryo was grown in a whole-embryo culture system. Viral-free manipulation was performed using a thermoreversible GDNF polymer [92]. Donor hMSCs were integrated into the rudimentary metanephros and differentiated morphologically into tubular epithelial cells, interstitial cells, and glomerular epithelial cells [88]. We then transplanted the developed metanephros into the omentum to allow vascular integration from the recipient to form a functional nephron. As a result, an hMSC-derived "neokidney" was generated,

which contained a human nephron associated with host vasculature [88]. This neokidney produced urine with higher concentrations of urea nitrogen and creatinine than in the sera of the recipient. This suggests that the neokidney, developed in the omentum, produced urine by hemofiltration [88]. Furthermore, the hMSC-derived neokidney secreted human Epo in response to the induction of anemia in the host animal [93]. This "organ factory" was thus able to preserve normal hormonal regulation to maintain Epo levels. However, the current system was unable to reconstruct a ureter or collecting tubules derived from the UB. To determine if MSCs could differentiate into a UB progenitor using chick embryos, hMSCs expressing PAX2 were injected into the chicken UB progenitor region [90]. The cells migrated caudally with the elongating Wolffian duct, integrated into the Wolffian duct epithelia, and expressed LIM1, demonstrating the ability of hMSCs to differentiate into Wolffian duct cells under the influence of local xenosignals [89]. These results indicate that it might be possible to reconstruct a whole kidney by transplanting renal lineage stem cells at a suitable time and location to regenerate derivatives of the MM and UB. Renal lineage stem cells as MSCs are easy to obtain in large numbers and are relatively cheap to establish. MSCs can be harvested from the patient as adult stem cells. We therefore used hMSCs as a cell source for kidney regeneration and succeeded in differentiating them into a nephron structure by GDNF-transfection and injection into a xenoembryo. Further studies are needed to verify the use of other adult kidney stem cells [42] or nephron progenitor cells [21] induced from iPSCs. With a view to the future clinical application of kidney regeneration, we also validated the effect of exposure of adult stem cells to uremic toxins over long periods and noted some differences in gene expression of stemness markers in MSCs from patients with ESKD. These results suggest that these cells may not be a suitable source for kidney regeneration [94].

Based on our previous promising results, we are currently investigating the possibility of large-scale harvesting of metanephroi from pigs, given that the volume of the kidney in pigs is almost identical to that in humans [92]. The ultimate size of the developed metanephros appears to be imprinted during the early stages of development in the host embryo. Another study also demonstrated that the body size and weight of interspecific chimeras (metanephroi derived from xenoembryos) conformed to the recipient species [34]. We hope that, by overcoming these challenges, this strategy might provide a novel direction for generating donor kidneys with a suitable size and function for transplantation.

## 9. Conclusion

This review has summarized recent research involving the use of renal stem cells and renal bioengineering to regenerate functional whole kidneys *de novo*. Despite significant recent advances, the reconstruction of a complete functional kidney remains difficult, and many problems are still unsolved. Direct differentiation of ESCs/iPSCs into nephron progenitors has not yet succeeded in generating mature functioning tissues *in vivo*. Before regenerated kidneys can be used in clinical practice, a method of generating fully functioning renal tissues that produce urine and Epo needs to be developed. Additionally, the regenerated tissue must be able to survive and function in the long term. Future research in stem cell biology and bioengineering will hopefully resolve these issues and open the door to new therapeutic strategies for kidney regeneration aimed at repairing kidney damage and restoring function.

## Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

## References

[1] S. F. Badylak, D. Taylor, and K. Uygun, "Whole-organ tissue engineering: decellularization and recellularization of three-dimensional matrix scaffolds," *Annual Review of Biomedical Engineering*, vol. 13, pp. 27–53, 2011.

[2] Q. Al-Awqati and J. A. Oliver, "Stem cells in the kidney," *Kidney International*, vol. 61, no. 2, pp. 387–395, 2002.

[3] F. Locatelli, U. Buoncristiani, B. Canaud, H. Köhler, T. Petitclerc, and P. Zucchelli, "Dialysis dose and frequency," *Nephrology Dialysis Transplantation*, vol. 20, no. 2, pp. 285–296, 2005.

[4] M. J. Evans and M. H. Kaufman, "Establishment in culture of pluripotential cells from mouse embryos," *Nature*, vol. 292, no. 5819, pp. 154–156, 1981.

[5] K. Takahashi and S. Yamanaka, "Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors," *Cell*, vol. 126, no. 4, pp. 663–676, 2006.

[6] K. Takahashi, K. Tanabe, M. Ohnuki et al., "Induction of pluripotent stem cells from adult human fibroblasts by defined factors," *Cell*, vol. 131, no. 5, pp. 861–872, 2007.

[7] J. R. Spence, C. N. Mayhew, S. A. Rankin et al., "Directed differentiation of human pluripotent stem cells into intestinal tissue in vitro," *Nature*, vol. 470, no. 7332, pp. 105–110, 2011.

[8] D. C. Hay, D. Zhao, J. Fletcher et al., "Efficient differentiation of hepatocytes from human embryonic stem cells exhibiting markers recapitulating liver development in vivo," *Stem Cells*, vol. 26, no. 4, pp. 894–902, 2008.

[9] H. Basma, A. Soto-Gutiérrez, G. R. Yannam et al., "Differentiation and transplantation of human embryonic stem cell-derived hepatocytes," *Gastroenterology*, vol. 136, no. 3, pp. 990.e4–999.e4, 2009.

[10] S. K. Dhara and S. L. Stice, "Neural differentiation of human embryonic stem cells," *Journal of Cellular Biochemistry*, vol. 105, no. 3, pp. 633–640, 2008.

[11] S. M. Chambers, C. A. Fasano, E. P. Papapetrou, M. Tomishima, M. Sadelain, and L. Studer, "Highly efficient neural conversion of human ES and iPS cells by dual inhibition of SMAD signaling," *Nature Biotechnology*, vol. 27, no. 3, pp. 275–280, 2009.

[12] M. H. Ledran, A. Krassowska, L. Armstrong et al., "Efficient hematopoietic differentiation of human embryonic stem cells on stromal cells derived from hematopoietic niches," *Cell Stem Cell*, vol. 3, no. 1, pp. 85–98, 2008.

[13] K. A. D'Amour, A. G. Bang, S. Eliazer et al., "Production of pancreatic hormone-expressing endocrine cells from human embryonic stem cells," *Nature Biotechnology*, vol. 24, no. 11, pp. 1392–1401, 2006.

[14] D. Zhang, W. Jiang, M. Liu et al., "Highly efficient differentiation of human ES cells and iPS cells into mature pancreatic insulin-producing cells," *Cell Research*, vol. 19, no. 4, pp. 429–438, 2009.

[15] T. Takahashi, B. Lord, P. C. Schulze et al., "Ascorbic acid enhances differentiation of embryonic stem cells into cardiac myocytes," *Circulation*, vol. 107, no. 14, pp. 1912–1916, 2003.

[16] A. Q. Lam, B. S. Freedman, R. Morizane, P. H. Lerou, M. T. Valerius, and J. V. Bonventre, "Rapid and efficient differentiation of human pluripotent stem cells into intermediate mesoderm that forms tubules expressing kidney proximal tubular markers," *Journal of the American Society of Nephrology*, vol. 25, no. 6, pp. 1211–1225, 2014.

[17] P. Gadue, T. L. Huber, P. J. Paddison, and G. M. Keller, "Wnt and TGF-beta signaling are required for the induction of an in vitro model of primitive streak formation using embryonic stem cells," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 103, no. 45, pp. 16806–16811, 2006.

[18] S.-I. Mae, A. Shono, F. Shiota et al., "Monitoring and robust induction of nephrogenic intermediate mesoderm from human pluripotent stem cells," *Nature Communications*, vol. 4, article 1367, 2013.

[19] Y. Xia, E. Nivet, I. Sancho-Martinez et al., "Directed differentiation of human pluripotent cells to ureteric bud kidney progenitor-like cells," *Nature Cell Biology*, vol. 15, no. 12, pp. 1507–1515, 2013.

[20] M. Takasato, P. X. Er, M. Becroft et al., "Directing human embryonic stem cell differentiation towards a renal lineage generates a self-organizing kidney," *Nature Cell Biology*, vol. 16, no. 1, pp. 118–126, 2014.

[21] A. Taguchi, Y. Kaku, T. Ohmori et al., "Redefining the in vivo origin of metanephric nephron progenitors enables generation of complex kidney structures from pluripotent stem cells," *Cell Stem Cell*, vol. 14, no. 1, pp. 53–67, 2014.

[22] J. Blake and N. D. Rosenblum, "Renal branching morphogenesis: morphogenetic and signaling mechanisms," *Seminars in Cell & Developmental Biology*, vol. 36, pp. 2–12, 2014.

[23] T. Araoka, S. I. Mae, Y. Kurose et al., "Efficient and rapid induction of human iPSCs/ESCs into nephrogenic intermediate mesoderm using small molecule-based differentiation methods," *PLoS ONE*, vol. 9, no. 1, Article ID e84881, 2014.

[24] B. G. Herrmann, S. Labeit, A. Poustka, T. R. King, and H. Lehrach, "Cloning of the T gene required in mesoderm formation in the mouse," *Nature*, vol. 343, no. 6259, pp. 617–622, 1990.

[25] V. Wilson, I. Olivera-Martinez, K. G. Storey et al., "Stem cells, signals and vertebrate body axis extension," *Development*, vol. 136, no. 10, pp. 1591–1604, 2009.

[26] P. W. Burridge, G. Keller, J. D. Gold, and J. C. Wu, "Production of de novo cardiomyocytes: human pluripotent stem cell differentiation and direct reprogramming," *Cell Stem Cell*, vol. 10, no. 1, pp. 16–28, 2012.

[27] T. P. Yamaguchi, S. Takada, Y. Yoshikawa, N. Wu, and A. P. McMahon, "T (Brachyury) is a direct target of Wnt3a during paraxial mesoderm specification," *Genes and Development*, vol. 13, no. 24, pp. 3185–3190, 1999.

[28] J. Chen, R. Lansford, V. Stewart, F. Young, and F. W. Alt, "RAG-2-deficient blastocyst complementation: an assay of gene function in lymphocyte development," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 90, no. 10, pp. 4528–4532, 1993.

[29] S. M. Müller, G. Terszowski, C. Blum et al., "Gene targeting of VEGF-A in thymus epithelium disrupts thymus blood vessel architecture," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 102, no. 30, pp. 10587–10592, 2005.

[30] D. Fraidenraich, E. Stillwell, E. Romero et al., "Rescue of cardiac defects in Id knockout embryos by injection of embyonic stem cells," *Science*, vol. 306, no. 5694, pp. 247–252, 2004.

[31] H. Ueno and I. L. Weissman, "Clonal analysis of mouse development reveals a polyclonal origin for yolk sac blood islands," *Developmental Cell*, vol. 11, no. 4, pp. 519–533, 2006.

[32] H. Ueno, B. B. Turnbull, and I. L. Weissman, "Two-step oligoclonal development of male germ cells," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 106, no. 1, pp. 175–180, 2009.

[33] S. Espejel, G. R. Roll, K. J. McLaughlin et al., "Induced pluripotent stem cell - derived hepatocytes have the functional and proliferative capabi lities needed for liver regeneration in mice," *Journal of Clinical Investigation*, vol. 120, no. 9, pp. 3120–3126, 2010.

[34] T. Kobayashi, T. Yamaguchi, S. Hamanaka et al., "Generation of rat pancreas in mouse by interspecific blastocyst injection of pluripotent stem cells," *Cell*, vol. 142, no. 5, pp. 787–799, 2010.

[35] H. Matsunari, H. Nagashima, M. Watanabe et al., "Blastocyst complementation generates exogenic pancreas in vivo in apancreatic cloned pigs," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 110, no. 12, pp. 4557–4562, 2013.

[36] J.-I. Usui, T. Kobayashi, T. Yamaguchi, A. S. Knisely, R. Nishinakamura, and H. Nakauchi, "Generation of kidney from pluripotent stem cells via blastocyst complementation," *The American Journal of Pathology*, vol. 180, no. 6, pp. 2417–2426, 2012.

[37] S. Aggarwal, A. Moggio, and B. Bussolati, "Concise review: stem/progenitor cells for renal tissue repair: current knowledge and perspectives," *Stem Cells Translational Medicine*, vol. 2, no. 12, pp. 1011–1019, 2013.

[38] H. A. Hartman, H. L. Lai, and L. T. Patterson, "Cessation of renal morphogenesis in mice," *Developmental Biology*, vol. 310, no. 2, pp. 379–387, 2007.

[39] J. A. Oliver, O. Maarouf, F. H. Cheema, T. P. Martens, and Q. Al-Awqati, "The renal papilla is a niche for adult kidney stem cells," *The Journal of Clinical Investigation*, vol. 114, no. 6, pp. 795–804, 2004.

[40] A. Maeshima, S. Yamashita, and Y. Nojima, "Identification of renal progenitor-like tubular cells that participate in the regeneration processes of the kidney," *Journal of the American Society of Nephrology*, vol. 14, no. 12, pp. 3138–3146, 2003.

[41] C. Sagrinati, G. S. Netti, B. Mazzinghi et al., "Isolation and characterization of multipotent progenitor cells from the Bowman's capsule of adult human kidneys," *Journal of the American Society of Nephrology*, vol. 17, no. 9, pp. 2443–2456, 2006.

[42] S. Kitamura, Y. Yamasaki, M. Kinomura et al., "Establishment and characterization of renal progenitor like cells from S3 segment of nephron in rat adult kidney," *The FASEB Journal*, vol. 19, no. 13, pp. 1789–1797, 2005.

[43] D. Lindgren, A.-K. Boström, K. Nilsson et al., "Isolation and characterization of progenitor-like cells from human renal proximal tubules," *The American Journal of Pathology*, vol. 178, no. 2, pp. 828–837, 2011.

[44] S. Kitamura, H. Sakurai, and H. Makino, "Single adult kidney stem/progenitor cells reconstitute three-dimensional nephron structures *in vitro*," *Stem Cells*, 2014.

[45] A. A. Simerman, D. A. Dumesic, and G. D. Chazenbalk, "Pluripotent muse cells derived from human adipose tissue: a new perspective on regenerative medicine and cell therapy," *Clinical and Translational Medicine*, vol. 3, article 12, 2014.

[46] Y. Jiang, B. N. Jahagirdar, R. L. Reinhardt et al., "Pluripotency of mesenchymal stem cells derived from adult marrow," *Nature*, vol. 418, no. 6893, pp. 41–49, 2002.

[47] I. Dimomeletis, E. Deindl, M. Zaruba et al., "Assessment of human MAPCs for stem cell transplantation and cardiac regeneration after myocardial infarction in SCID mice," *Experimental Hematology*, vol. 38, no. 11, pp. 1105–1114, 2010.

[48] J. J. Song and H. C. Ott, "Organ engineering based on decellularized matrix scaffolds," *Trends in Molecular Medicine*, vol. 17, no. 8, pp. 424–432, 2011.

[49] J. Oliver, "Correlations of structure and function and mechanisms of recovery in acute tubular necrosis," *The American Journal of Medicine*, vol. 15, no. 4, pp. 535–557, 1953.

[50] F. E. Cuppage and A. Tate, "Repair of the nephron following injury with mercuric chloride.," *The American Journal of Pathology*, vol. 51, no. 3, pp. 405–429, 1967.

[51] B. H. Haagsma and A. W. Pound, "Mercuric chloride-induced tubulonecrosis in the rat kidney: the recovery phase," *British Journal of Experimental Pathology*, vol. 61, no. 3, pp. 229–241, 1980.

[52] M. Salvatori, A. Peloso, R. Katari, and G. Orlando, "Regeneration and bioengineering of the kidney: current status and future challenges," *Current Urology Reports*, vol. 15, no. 1, article 379, 2014.

[53] B. Lelongt and P. Ronco, "Role of extracellular matrix in kidney development and repair," *Pediatric Nephrology*, vol. 18, no. 8, pp. 731–742, 2003.

[54] G. Orlando, P. Baptista, M. Birchall et al., "Regenerative medicine as applied to solid organ transplantation: current status and future challenges," *Transplant International*, vol. 24, no. 3, pp. 223–232, 2011.

[55] H. C. Ott, T. S. Matthiesen, S.-K. Goh et al., "Perfusion-decellularized matrix: using nature's platform to engineer a bioartificial heart," *Nature Medicine*, vol. 14, no. 2, pp. 213–221, 2008.

[56] P. M. Baptista, M. M. Siddiqui, G. Lozier, S. R. Rodriguez, A. Atala, and S. Soker, "The use of whole organ decellularization for the generation of a vascularized liver organoid," *Hepatology*, vol. 53, no. 2, pp. 604–617, 2011.

[57] J. J. Song, S. S. Kim, Z. Liu et al., "Enhanced in vivo function of bioartificial lungs in rats," *The Annals of Thoracic Surgery*, vol. 92, no. 3, pp. 998–1006, 2011.

[58] P. M. Crapo, C. J. Medberry, J. E. Reing et al., "Biologic scaffolds composed of central nervous system extracellular matrix," *Biomaterials*, vol. 33, no. 13, pp. 3539–3547, 2012.

[59] T. Martinello, I. Bronzini, A. Volpin et al., "Successful recellularization of human tendon scaffolds using adipose-derived mesenchymal stem cells and collagen gel," *Journal of Tissue Engineering and Regenerative Medicine*, vol. 8, no. 8, pp. 612–619, 2014.

[60] J. L. Honge, J. Funder, E. Hansen, P. M. Dohmen, W. Konertz, and J. M. Hasenkam, "Recellularization of aortic valves in pigs," *European Journal of Cardio-Thoracic Surgery*, vol. 39, no. 6, pp. 829–834, 2011.

[61] Y. Loai, H. Yeger, C. Coz et al., "Bladder tissue engineering: tissue regeneration and neovascularization of HA-VEGF-incorporated bladder acellular constructs in mouse and porcine animal models," *Journal of Biomedical Materials Research—Part A*, vol. 94, no. 4, pp. 1205–1215, 2010.

[62] M. S. Wicha, G. Lowrie, E. Kohn, P. Bagavandoss, and T. Mahn, "Extracellular matrix promotes mammary epithelial growth and differentiation in vitro," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 79, no. 10, pp. 3213–3217, 1982.

[63] E. A. Ross, M. J. Williams, T. Hamazaki et al., "Embryonic stem cells proliferate and differentiate when seeded into kidney scaffolds," *Journal of the American Society of Nephrology*, vol. 20, no. 11, pp. 2338–2347, 2009.

[64] K. H. Nakayama, C. A. Batchelder, C. I. Lee, and A. F. Tarantal, "Decellularized rhesus monkey kidney as a three-dimensional scaffold for renal tissue engineering," *Tissue Engineering—Part A*, vol. 16, no. 7, pp. 2207–2216, 2010.

[65] D. C. Sullivan, S.-H. Mirmalek-Sani, D. B. Deegan et al., "Decellularization methods of porcine kidneys for whole organ engineering using a high-throughput system," *Biomaterials*, vol. 33, no. 31, pp. 7756–7764, 2012.

[66] J. J. Song, J. P. Guyette, S. E. Gilpin, G. Gonzalez, J. P. Vacanti, and H. C. Ott, "Regeneration and experimental orthotopic transplantation of a bioengineered kidney," *Nature Medicine*, vol. 19, no. 5, pp. 646–651, 2013.

[67] S. M. Mackay, A. J. Funke, D. A. Buffington, and H. David Humes, "Tissue engineering of a bioartificial renal tubule," *ASAIO Journal*, vol. 44, no. 3, pp. 179–183, 1998.

[68] H. D. Humes, S. M. MacKay, A. J. Funke, and D. A. Buffington, "Tissue engineering of a bioartificial renal tubule assist device: *in vitro* transport and metabolic characteristics," *Kidney International*, vol. 55, no. 6, pp. 2502–2514, 1999.

[69] J. H. Song and H. D. Humes, "The bioartificial kidney in the treatment of acute kidney injury," *Current Drug Targets*, vol. 10, no. 12, pp. 1227–1234, 2009.

[70] H. D. Humes, J. C. Krauss, D. A. Cieslinski, and A. J. Funke, "Tubulogenesis from isolated single cells of adult mammalian kidney: clonal analysis with a recombinant retrovirus," *The American Journal of Physiology—Renal Fluid and Electrolyte Physiology*, vol. 271, no. 1, pp. F42–F49, 1996.

[71] H. D. Humes, D. A. Buffington, S. M. MacKay, A. J. Funke, and W. F. Weitzel, "Replacement of renal function in uremic animals with a tissue-engineered kidney," *Nature Biotechnology*, vol. 17, no. 5, pp. 451–455, 1999.

[72] H. D. Humes, W. F. Weitzel, R. H. Bartlett et al., "Initial clinical results of the bioartificial kidney containing human cells in ICU patients with acute renal failure," *Kidney International*, vol. 66, no. 4, pp. 1578–1588, 2004.

[73] J. Tumlin, R. Wali, W. Williams et al., "Efficacy and safety of renal tubule cell therapy for acute renal failure," *Journal of the American Society of Nephrology*, vol. 19, no. 5, pp. 1034–1040, 2008.

[74] H. D. Humes, J. T. Sobota, F. Ding, and J. H. Song, "A selective cytopheretic inhibitory device to treat the immunological dysregulation of acute and chronic renal failure," *Blood Purification*, vol. 29, no. 2, pp. 183–190, 2010.

[75] S. A. Rogers, J. A. Lowell, N. A. Hammerman, and M. R. Hammerman, "Transplantation of developing metanephroi into adult rats," *Kidney International*, vol. 54, no. 1, pp. 27–37, 1998.

[76] M. R. Hammerman, "Renal Organogenesis from Transplanted Metanephric Primordia," *Journal of the American Society of Nephrology*, vol. 15, no. 5, pp. 1126–1132, 2004.

[77] K. Matsumoto, T. Yokoo, S. Yokote, Y. Utsunomiya, T. Ohashi, and T. Hosoya, "Functional development of a transplanted embryonic kidney: effect of transplantation site," *Journal of Nephrology*, vol. 25, no. 1, pp. 50–55, 2012.

[78] S. Yokote, T. Yokoo, K. Matsumoto, Y. Utsunomiya, T. Kawamura, and T. Hosoya, "The effect of metanephros transplantation on blood pressure in anephric rats with induced acute hypotension," *Nephrology Dialysis Transplantation*, vol. 27, no. 9, pp. 3449–3455, 2012.

[79] S. Yokote, T. Yokoo, K. Matsumoto et al., "Metanephros transplantation inhibits the progression of vascular calcification in rats with adenine-induced renal failure," *Nephron—Experimental Nephrology*, vol. 120, no. 1, pp. e32–e40, 2012.

[80] S. A. Rogers, M. Talcott, and M. R. Hammerman, "Transplantation of pig metanephroi," *American Society for Artificial Internal Organs*, vol. 49, no. 1, pp. 48–52, 2003.

[81] B. Dekel, T. Burakova, F. D. Arditti et al., "Human and porcine early kidney precursors as a new source for transplantation," *Nature Medicine*, vol. 9, no. 1, pp. 53–60, 2003.

[82] S.-I. Takeda, S. A. Rogers, and M. R. Hammerman, "Differential origin for endothelial and mesangial cells after transplantation of pig fetal renal primordia into rats," *Transplant Immunology*, vol. 15, no. 3, pp. 211–215, 2006.

[83] S. A. Rogers and M. R. Hammerman, "Prolongation of life in anephric rats following de novo renal organogenesis," *Organogenesis*, vol. 1, no. 1, pp. 22–25, 2004.

[84] K. Matsumoto, T. Yokoo, H. Matsunari et al., "Xenotransplanted embryonic kidney provides a niche for endogenous mesenchymal stem cell differentiation into erythropoietin-producing tissue," *Stem Cells*, vol. 30, no. 6, pp. 1228–1235, 2012.

[85] S. Yokote and T. Yokoo, "Organogenesis for kidney regeneration," *Current Opinion in Organ Transplantation*, vol. 18, no. 2, pp. 186–190, 2013.

[86] C. Xinaris and T. Yokoo, "Reforming the kidney starting from a single-cell suspension," *Nephron: Experimental Nephrology*, vol. 126, no. 2, pp. 107–112, 2014.

[87] T. Yokoo, T. Ohashi, S. S. Jin et al., "Human mesenchymal stem cells in rodent whole-embryo culture are reprogrammed to contribute to kidney tissues," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 102, no. 9, pp. 3296–3300, 2005.

[88] T. Yokoo, A. Fukui, T. Ohashi et al., "Xenobiotic kidney organogenesis from human mesenchymal stem cells using a growing rodent embryo," *Journal of the American Society of Nephrology*, vol. 17, no. 4, pp. 1026–1034, 2006.

[89] A. Fukui, T. Yokoo, K. Matsumoto, T. Kawamura, T. Hosoya, and M. Okabe, "Integration of human mesenchymal stem cells into the Wolffian duct in chicken embryos," *Biochemical and Biophysical Research Communications*, vol. 385, no. 3, pp. 330–335, 2009.

[90] L. Saxén and H. Sariola, "Early organogenesis of the kidney," *Pediatric Nephrology*, vol. 1, no. 3, pp. 385–392, 1987.

[91] J. A. Davies and C. E. Fisher, "Genes and proteins in renal development," *Experimental Nephrology*, vol. 10, no. 2, pp. 102–113, 2002.

[92] Y. Gheisari, T. Yokoo, K. Matsumoto et al., "A thermoreversible polymer mediates controlled release of glial cell line-derived neurotrophic factor to enhance kidney regeneration," *Artificial Organs*, vol. 34, no. 8, pp. 642–647, 2010.

[93] T. Yokoo, A. Fukui, K. Matsumoto et al., "Generation of a transplantable erythropoietin-producer derived from human mesenchymal stem cells," *Transplantation*, vol. 85, no. 11, pp. 1654–1658, 2008.

[94] S. Yamanaka, S. Yokote, A. Yamada et al., "Adipose tissue-derived mesenchymal stem cells in long-term dialysis patients display downregulation of PCAF expression and poor angiogenesis activation," *PLoS ONE*, vol. 9, no. 7, Article ID e102311, 2014.

Hindawi Publishing Corporation
Stem Cells International
Volume 2015, Article ID 537204, 7 pages
http://dx.doi.org/10.1155/2015/537204

*Review Article*

# Trophic Factors from Tissue Stem Cells for Renal Regeneration

**Kenji Tsuji and Shinji Kitamura**

*Department of Medicine and Clinical Science, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, 2-5-2 Shikata-cho, Okayama, Okayama 700-8558, Japan*

Correspondence should be addressed to Shinji Kitamura; s-kita@md.okayama-u.ac.jp

Received 3 October 2014; Revised 9 January 2015; Accepted 9 January 2015

Academic Editor: Benedetta Bussolati



Stem cell therapies against renal injury have been advancing. The many trials for renal regeneration are reported to be effective in many kinds of renal injury models. Regarding the therapeutic mechanism, it is believed that stem cells contribute to make regeneration via not only direct stem cell differentiation in the injured space but also indirect effect via secreted factors from stem cells. Direct differentiation from stem cells to renal composed cells has been reported. They differentiate to renal composed cells and make functions. However, regarding renal regeneration, stem cells are discussed to secrete many kinds of growth factors, cytokines, and chemokines in paracrine or autocrine manner, which protect against renal injury, too. In addition, it is reported that stem cells have the ability to communicate with nearby cells via microvesicle-related RNA and proteins. Taken together from many reports, many secreted factors from stem cells were needed for renal regeneration orchestrally with harmony. In this review, we focused on the effects and insights of stem cells and regenerative factors from stem cells.

## 1. Introduction

Renal failure is one of the major healthcare issues. Although medical science has rapidly advanced, there are few effective treatments to rescue kidney injury or to activate kidney regeneration. Recently, stem cell therapies have been proposed for the regeneration toward different kinds of organ failures, such as cardiovascular diseases [1], neurodegenerative diseases [2], and kidney diseases [3]. As to the kidney, many stem cell therapies are reported to be effective in variety of kidney injury models and the therapies are expected to be useful treatment against kidney injury [4].

Nevertheless, the mechanisms of the stem cell-induced regeneration are still controversial. Among these mechanisms, direct differentiation of stem/progenitor cells into renal mature cells have been reported as the direct therapeutic mechanism [5]. Recently, many reports revealed that trophic factors from stem cells might be the important contributor for kidney regeneration [6, 7]. In these reports, there are different kinds of stem cell sources, such as bone marrow-derived mesenchymal stem cells, adipose-derived mesenchymal stem cells, and adult kidney stem/progenitor cells. These secreted factors are reported to regulate the cell proliferation, cell migration, cell differentiation, and immune systems as well as cell-cell interactions and circumstances around the injured space [8].

Among kidney injury, acute kidney injury (AKI) is happening in 2–5% of the hospitalized patients [9]. AKI results from different kinds of factors, such as toxin and renal ischemia [10]. Some cases of AKI are reversible and the recovery of the injured tubules needs the replacement of injured tubular epithelial cells [11]. The cell sources for the replacement of injured tubules have been controversial. Recently, Humphreys et al. reported that most predominant mechanism of repair after ischemic tubular injury is the surviving tubular epithelial cells [12]. In addition, Kusaba et al. reported that fully differentiated kidney epithelial cells repair injured proximal tubule [13]. They suggested that surviving terminally differentiated tubular epithelial cells dedifferentiate, proliferate, migrate, and replace the injured tubules [13]. These reports implied that stem cells such as mesenchymal stem cells (MSC) and adult kidney stem/progenitor cells are not the predominant cell sources for the replacement of injured tubules. And these reports highlighted the possibility

that the role of the stem cells for regeneration might result from the indirect mechanism via secreted factors from these cells. These factors might activate the regenerative process, for example, via regulation of the cell proliferation, tubular cell dedifferentiation, cell migration, and circumstance condition such as inflammation. Actually, stem cells have been reported to secrete a variety of trophic factors and activate the regeneration [14]. Although detail mechanisms are still uncertain, it might open the new strategy to treat AKI or other kidney injury if we can elucidate the detail mechanism of indirect therapeutic mechanism via trophic factors from stem cells and translate into clinical medication treatment.

In this review article, we focused on the recent advances of the therapies related to the trophic factors from these stem cells.

## 2. Mesenchymal Stem Cells

MSCs can be established from adult tissues including bone marrow, adipose tissue, synovial tissue, liver, lung, umbilical cord, placenta, amniotic fluid, and connective tissues [15]. MSCs are the undifferentiated adult cells and have the ability to differentiate into mesenchymal-derived tissues, such as bone, muscle, fat, and other connective tissues [15]. MSCs also have the ability to migrate to damaged and injured space and secrete many kinds of factors which can influence cell characters and change circumstances, which result in tissue repair and regeneration.

MSCs implantation has been reported to be protective for many kinds of kidney injury models, including not only AKI models such as ischemia/reperfusion kidney injury models, cisplatin injury model, glycerol injury model, and mesangioproliferative glomerulonephritis model [16–19] but also CKD models such as 5/6 nephrectomy model, type 1 diabetes model, and unilateral ureteral obstruction (UUO) model [20–22].

Regarding indirect replacement into injured tubules, trophic factors from MSCs have been reported to be protective against kidney injury. Human adipose-derived MSCs inhibited podocyte apoptosis and injury by high glucose mainly via trophic factors [23]. Bone marrow derived MSCs protected against ischemic acute kidney injury via trophic factors [6]. Bone marrow derived MSCs protected the kidney from toxic injury by trophic factors which limited apoptosis and enhanced proliferation of the endogenous tubular cells [7]. These reports indicate that there are variety kinds of factors and therapeutic mechanisms via trophic factors. Stem cells, like MSCs, might activate renal residual cells for regeneration among many kinds of renal diseases. Rota et al. reported that human amniotic fluid stem cells improved cisplatin-induced kidney injury and the effect was mediated through a local paracrine including interleukin-6 (IL-6), VEGF, SDF-1, and IGF-1 [24]. Since different kinds of stem cells secrete specific and different concentrated factors, it seems to be important to elucidate each stem cells contribution for regeneration. And these reports also suggest that cell transplantation of stem cells themselves is not necessary and stem cells do have indirect therapeutic mechanism via secreted factors.

On the other hand, there are some reports which focus on the culture supernatant from stem cells. It is reported with using co-culture system that anti-inflammatory factor HGF and anti-fibrotic factor TSG-6 from MSCs ameliorate albumin-induced tubular inflammation and fibrosis [25]. Low serum cultured adipose tissue derived stromal cells ameliorated AKI and the effect was mainly induced by HGF from these cells [26]. In addition, Human embryonic MSC-derived conditioned medium can rescue kidney function in 5/6 nephrectomy-induced CKD model [27]. These reports focus on the importance of the combination of variety kinds of trophic factors to get more efficient therapeutic effects. Each factor from MSCs is effective for renal regeneration. However, the secreted factors from MSCs are different and the working effect for renal disease is different, too. Therefore, it is supposed that there is the possibility that culture supernatant from stem cells might be optimal condition for renal regeneration because the trophic factors from MSCs might be secreted orchestrally.

MVs from MSCs have been reported to be renoprotective. Human MVs derived from human MSCs can stimulate cell proliferation and inhibit cell apoptosis in vitro and activate recovery of glycerol-induced AKI mice model in vivo [28]. These effects were reversed with RNase treatment, suggesting MVs shuttled mRNA is the main contributor for renoprotection. Interestingly, they implied in the report that MVs from stem cells have the potential to induce dedifferentiation of mature cells which contribute to the tissue repair against injury. In addition, it is reported that human adult MSCs-derived MVs can protect against not only ischemia-reperfusion- (I/R-) induced AKI but also CKD [29]. These factors may open the new strategy to treat kidney injury. Taken together, MSCs contribute not only direct differentiation to kidney but also indirect trophic factors supply (Figure 1).

## 3. Adult Kidney Stem/Progenitor Cells

Adult kidney stem cells have been identified from some research groups in different kinds of technologies [4] (Table 1). These groups also reported that administration of the adult kidney stem cells contributed to renal regeneration. These reports especially focused on the direct therapeutic mechanism via replacement into injured tubules and there are few reports indicating the indirect therapeutic effects via secreted factors from these cells.

Hoechst 33342 was originally used for the isolation of HSC. In the same way, SP cells have been isolated from other organs including kidney [30, 31]. These cells have multilineage capacity and the ability to activate renal regeneration [31]. SP cell administration into kidney injury models, such as cisplatin-induced AKI, adriamycin nephropathy, and CKD models, revealed to be effective for kidney regeneration. In addition, these effects seemed to be mainly induced via

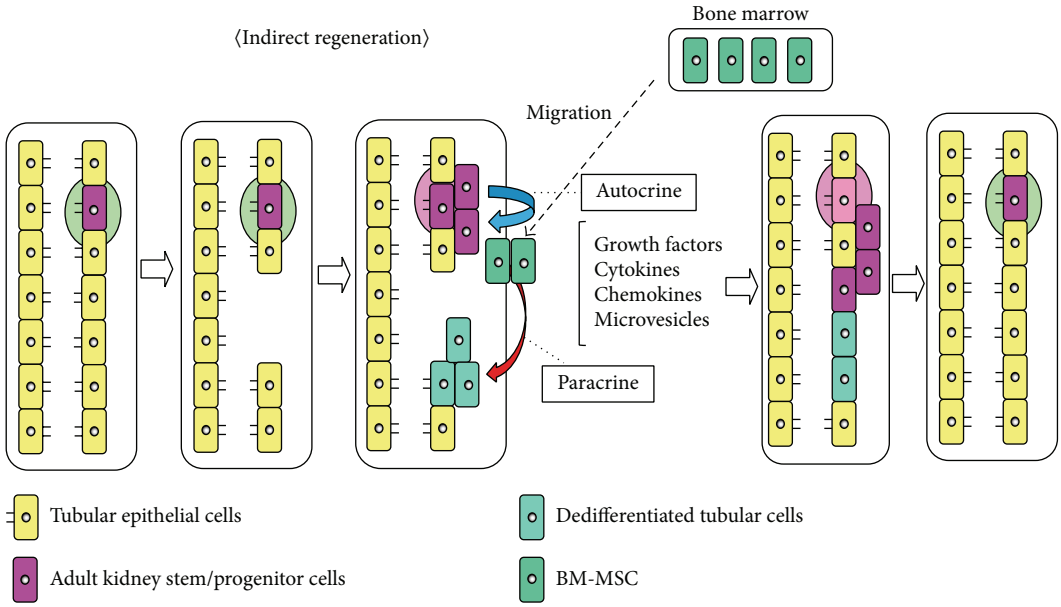



Figure 1: Shema of indirect therapeutic mechanism of stem cells against AKI. Not only the cell proliferation of stem/progenitor cells but also indirect factors such as growth factors, cytokines, chemokines, microvesicles et al. ameliorate injuryed tubular cells.

paracrine effects from SP cells because there are few cell infusions into injured place [32].

Other adult kidney stem cells were isolated with the maker, Sca1$^+$ cells [33]. When they injected these cells directly into the renal parenchyma, they differentiated into tubular phenotype and could contribute to kidney regeneration [33]. Other group isolated the kidney-derived stem cell line which was named multipotent renal progenitor cells (MRPC) [34]. They isolated MRPC using specific cell culture conditions which were similar to those used for the culture of bone marrow derived multipotent adult progenitor cells. These cells had spindle-shaped morphology and have self-renewal ability [34]. They also have the ability to differentiate into cells of all three germ cell layers. When they injected these cells into kidney injury models, they differentiated into renal tubules [34]. Other group reported adult kidney stem/progenitor-like cells by detecting slow cycling cells which represent the stem cell character using DNA labeling with BrdU [35]. They named the cells slow-cycling label-retaining cells (LRCs). LRCs were identified in renal tubular cells, papilla, and renal capsules. Interestingly, all tubular cells have the potential to become LRCs and proliferate after kidney injury, suggesting that differentiated tubular cells could dedifferentiate and have more immature characters when kidney injury occurs. The result suggested that LRCs were regulated via secreted factors at least to some extent. Other group was reported that CD 133$^+$CD24$^+$ adult kidney stem cells locate in the Bowman's capsule [36] and they were recently identified in the tubular compartment [37]. They both were positive for CD133, CD24, and Pax2, but there are some differences between these cells with respect to therapeutic effects. Tubular adult renal stem/progenitor cells (ARPCs) were reported to promote proliferation of surviving tubular cells and inhibited cisplatin-induced apoptosis [38]. These regenerative effects resulted from the ARCs-secreted inhibin-A and decorin mRNA [38]. Interestingly, this protective mRNA was shuttled by MVs. And the transcriptions from MVs differed from those from MSCs. These results suggested that there may be different roles via secreted factors between adult kidney stem cells and MSCs. Bussolati et al. also detected progenitor cells in the inner medullary papilla region [39] and they indicated that CD133$^+$ cells from renal medulla possess higher differentiate ability and stemcellness marker compared to CD133$^+$ cells from proximal tubules. When they evaluated the injection of CD133$^+$ renal cells from human inner medulla in a model of glyceol-induced acute tubular injury model, they found the recovery of renal function and these effects were mediated via prevention of tubular cell necrosis and stimulation of resident cell proliferation and survival which is similar to MSCs [40]. Interestingly, they compared the therapeutic effects to MSCs and revealed that renal progenitor cells showed a high renal localization. Moreover, they also revealed the differences of secreted factors between renal progenitor cells and MSCs. Renal progenitor cells showed higher expression of plated-derived growth factor (PDGF), bFGF, leukemia inhibitory factor, and tumor necrosis factor $\alpha$ compared to MSCs. Taken together, kidney stem cells and MSCs secrete different kinds of trophic factors, which activate renal regeneration together. At the same time, there are important and significant differences between CD133$^+$ renal progenitor cells from different segments, Bowman's capsule, S3 segment of proximal tubules, and inner medullary papilla region. These differences might be occurring from the different niche and environment of these cells. They might have the different roles about renal regeneration and the effects should be induced via trophic factors from these cells, at least to some extent.

Table 1: The summary of trophic effect of secreted factors from tissue stem cells.

| Author | Cell line name | Species | Location |
|---|---|---|---|
| Hishikawa et al. [61] | SP cells | Mouse | Renal interstitial space |
| Challen et al. [62] | SP cells | Mouse | Proximal tubules |
| Inowa et al. [30] | SP cells | Human | Not identified |
| Dekel et al. [33] | Sca1$^+$ cells | Mouse | Nontubular |
| Gupta et al. [34] | MRPCs | Rat | Renal tubules |
| Maeshima et al. [4, 35] | LRC cells | Rat | Renal tubular cells |
| Oliver et al. [63] | Slow-cycling cells | Mouse, rat | Renal papilla |
| Bussolati et al. [36, 39] | CD133$^+$ CD24$^+$ cells | Human | Bowman's capsule, inner medullary papilla, S3 segment of proximal tubules, interstitium |
| Sallustio et al. [37, 38] | CD133$^+$ CD24$^+$ cells | Human | Tubules, glomerulus |
| Sagrinati et al. [64] | CD133$^+$ CD24$^+$ cells | Human | Bowman's capsule |
| Lindgren et al. [65] | CD133$^+$ CD24$^+$ cells | Human | Renal proximal tubules |
| Kitamura et al. [41] | KS cells | Rat | S3 segment of proximal tubules |

SP cells: Side population cells, MRPC cells: Multipotent renal progenitor cells, LRCs: Label-retaining cells, KS cells: kidney stem/progenitor cells.

Our group previously established an adult kidney stem/progenitor cell line (KS cells) from adult rat kidneys [41]. We isolated nephrons and separated into segments and cultured. We could isolate highly proliferative cells from single cell of S3 segment. KS cells showed cobblestone appearance and expressed not only mature tubular markers, such as aquaporin(AQP)-1, 2 and NaCl transporter (NaCl-tr), but also immature cell markers which are related to kidney development, such as paired box-2 (Pax-2), WT-1, and glial cell line-derived neurotrophic factor (GDNF). In vitro, KS cells have the self-renewal ability and can differentiate into mature tubular cells defined by AQP-1, 2 expressions. When we injected KS cells into AKI models, KS cells could differentiate and replaced to renal composed cells in injured tubules [41]. We also suggested the possibility that KS cells could contribute to regeneration by indirect mechanism via secreted factors from KS cells [42].

Table 2: Summary of the adult kidney stem cells.

| Factor | Trophic effect |
|---|---|
| HGF | Cell proliferation, anti-inflammation, antifibrosis |
| EGF | Mitogenesis (renal proximal tubules), anabolism |
| IGF-1 | Mitogenesis (renal proximal tubules) |
| bFGF | Antiapoptosis, epithelial condensation, WT-1 upregulation, cell proliferation, MSC differentiation |
| VEGF | Cell proliferation, matrix remodeling, monocyte chemotaxis, adhesion protein upregulation |
| BMP-7 | Antifibrosis, anti-inflammation, antiapoptosis |
| PGE2 | Anti-inflammation |
| TGF-b | Anti-inflammation |
| MVs | Cell proliferation, antiapoptosis, dedifferentiation (renal mature cells) |
| microRNAs | Kidney development, homeostasis, and renal disease |

## 4. Trophic Factors for Renal Regeneration

Regarding trophic factors from MSCs, it is reported to secrete many kinds of factors such as HGF, EGF, IGF-1, FGF2, VEGF, BMP-7, TGF-b1, IL-6, IL-10, PGE2, granulocyte-colony stimulating factor (G-CSF), and macrophage-colony stimulating factor (M-CSF) [6, 8, 43, 44]. MSC-derived BMP-7 ameliorates diabetic glomerular fibrosis by inhibiting TGF-b signaling [45]. MSCs ameliorate podocyte injury and proteinuria in a type 1 diabetic nephropathy rat model via BMP-7 from MSCs [46]. HGF from MSCs ameliorated DN by inhibiting monocyte chemotactic protein-1 (MCP-1) expression [47]. Since MSCs have been reported to activate regeneration for different kinds of organs and MSCs migrate mainly from bone marrow after renal injury, MSCs should be one of the most important stem cells for renal regeneration.

Trophic factors such as growth factors, cytokines, and chemokines play important roles for many biological functions such as cell growth, migration, differentiation, apoptosis, inflammation, division, and signaling [8] (Table 2). Recently, the use of trophic factors has been advocated to stimulate kidney regeneration [48–50]. Growth factors injections against kidney injury have been reported. Miller et al. first reported that hepatocyte growth factor (HGF) administration against acute ischemia renal injury stimulated the recovery of kidney function and regeneration of proximal tubular epithelium [49]. In addition, they reported that EGF-like growth factor (EGF) or insulin-like growth factor-1 (IGF-1) administration against acute ischemia renal injury activated renal regeneration [49]. It is well known that HGF stimulates the cell proliferation of many kinds of epithelial cells and inhibits inflammation and fibrosis via cognate receptor HGFR/c-Met [50]. IGF-1 and EGF are known to be mitogenic for renal proximal tubules. Another factor, basic fibroblast growth factor (bFGF), is expressed in response to

kidney injury [51]. Recent report revealed that bFGF can reduce functional and structural damage in chronic kidney disease (CKD) rat model [52]. bFGF inhibits apoptosis, activates epithelial condensation, and regulates Wilms' tumor 1 (WT-1) synthesis [53, 54]. In addition, FGF can activate cell proliferation of renal cells and regulate MSC differentiation [55]. Vascular endothelial growth factor (VEGF) is the important factor of the protective effect of MSCs using knockdown of VEGF by siRNA [16]. Human marrow-derived MSC-CM inhibited cisplatin-induced tubular epithelial cell death and these effects were reduced with anti-VEGF antibody [56], suggesting VEGF has the effect on kidney protection against toxic injury. VEGF regulates the survival and proliferation of different kinds of cells especially for endothelial cells and is also important for matrix remodeling, monocyte chemotaxis, and molecule expression related to adhesion. Bone morphogenic protein-7 (BMP7) inhibits transforming growth factor-b (TGF-b) induced fibrosis, reduces inflammation-related cytokines, and reduces cell apoptosis of epithelial tubular cells and podocyte whereas TGF-b and prostaglandin E2 (PGE2) suppress the inflammation by inhibiting lymphocyte activation [25, 26]. Recent report suggested that stroma cell-derived factor 1 (SDF-1) protects tubular epithelial cells against renal ischemia-reperfusion injury [57].

In addition to soluble factors such as growth factors, cytokines, and chemokines, microvesicles (MVs) were recently remarked as the way of cell-cell communication [58]. MVs are released from different kinds of cells [59]. MVs interact with nearby cells by mRNA, miRNA, proteins, transfer surface receptors, and surface ligands [59]. Recently, MVs shuttled RNA from stem cells have been reported to be renoprotective [39]. In addition, microRNAs are focused as new approach for kidney diseases. They are reported to have important roles for kidney development, disease, and regeneration [60]. The detail mechanism will be needed on the interaction between stem cells and micro RNAs for renal regeneration.

These trophic factors are reported to secrete from stem cells such as MSCs and adult kidney stem/progenitor cells. Taken together, trophic factors from stem cells should contribute to renal regeneration against injury and these effects might be induced by various factors orchestrally since stem cells secrete various kinds of factors. Stem cells might contribute to renal regeneration not only through direct differentiation into mature renal cells but also through the secretion of protective factors.

## 5. Conclusion

In this review paper, we focused that stem cells therapy contributes for regeneration via not only direct differentiation from stem cells to injured cells but also indirect regeneration with trophic factors from stem cell to residual cells. Though the detail movement is still explored, it is very important to resolve this mechanism. To elucidate the mechanism, stem cell therapy will be of clinical use. Now, there are few clinical medications for kidney injury. There are many trophic factors which can stimulate kidney regeneration, such as HGF, EGF, and IGF-1. There should be other unknown factors which can be renoprotective. Besides the soluble factors such as growth factors, microvesicle-related mediators including mRNA, miRNA, and proteins should be other therapeutic factors. These different kinds of trophic factors might contribute to regenerate kidney in orchestration with harmony from stem cells because the effects of each factor were different. It might be useful for culture supernatant of stem cells to regenerate renal injury because it can contain many unknown factors and might be adjusted as optical condition for regeneration. Although there are still much to be explored, we can open the new therapeutic method for kidney diseases when we could elucidate the detail mechanism about renoprotection related to trophic factors.

## Conflict of Interests

The authors declare that they have no conflict of interests regarding the publication of this paper.

## References

[1] A. A. Matar and J. J. Chong, "Stem cell therapy for cardiac dysfunction," *SpringerPlus*, vol. 3, p. 440, 2014.

[2] F. G. Teixeira, M. M. Carvalho, N. Sousa, and A. J. Salgado, "Mesenchymal stem cells secretome: a new paradigm for central nervous system regeneration?" *Cellular and Molecular Life Sciences*, vol. 70, no. 20, pp. 3871–3882, 2013.

[3] H. Asanuma, D. R. Meldrum, and K. K. Meldrum, "Therapeutic applications of mesenchymal stem cells to repair kidney injury," *The Journal of Urology*, vol. 184, no. 1, pp. 26–33, 2010.

[4] A. Maeshima, M. Nakasatomi, and Y. Nojima, "Regenerative medicine for the kidney: renotropic factors, renal stem/progenitor cells, and stem cell therapy," *BioMed Research International*, vol. 2014, Article ID 595493, 10 pages, 2014.

[5] L. G. Cantley, "Adult stem cells in the repair of the injured renal tubule," *Nature Reviews Nephrology*, vol. 1, no. 1, pp. 22–32, 2005.

[6] F. Tögel, Z. Hu, K. Weiss, J. Isaac, C. Lange, and C. Westenfelder, "Administered mesenchymal stem cells protect against ischemic acute renal failure through differentiation-independent mechanisms," *The American Journal of Physiology—Renal Physiology*, vol. 289, no. 1, pp. F31–F42, 2005.

[7] B. Bi, R. Schmitt, M. Israilova, H. Nishio, and L. G. Cantley, "Stromal cells protect against acute tubular injury via an endocrine effect," *Journal of the American Society of Nephrology*, vol. 18, no. 9, pp. 2486–2496, 2007.

[8] A. I. Caplan and J. E. Dennis, "Mesenchymal stem cells as trophic mediators," *Journal of Cellular Biochemistry*, vol. 98, no. 5, pp. 1076–1084, 2006.

[9] K. Nash, A. Hafeez, and S. Hou, "Hospital-acquired renal insufficiency," *The American Journal of Kidney Diseases*, vol. 39, no. 5, pp. 930–936, 2002.

[10] R. Thadhani, M. Pascual, and J. V. Bonventre, "Acute renal failure," *New England Journal of Medicine*, vol. 334, no. 22, pp. 1448–1460, 1996.

[11] M. E. Sabbahy and V. S. Vaidya, "Ischemic kidney injury and mechanisms of tissue repair," *Wiley Interdisciplinary Reviews: Systems Biology and Medicine*, vol. 3, no. 5, pp. 606–618, 2011.

[12] B. D. Humphreys, M. T. Valerius, A. Kobayashi et al., "Intrinsic epithelial cells repair the kidney after injury," *Cell Stem Cell*, vol. 2, no. 3, pp. 284–291, 2008.

[13] T. Kusaba, M. Lalli, R. Kramann, A. Kobayashi, and B. D. Humphreys, "Differentiated kidney epithelial cells repair injured proximal tubule," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 111, no. 4, pp. 1527–1532, 2014.

[14] Y. H. Chou, S. Y. Pan, C. H. Yang, and S. L. Lin, "Stem cells and kidney regeneration," *Journal of the Formosan Medical Association*, vol. 113, no. 4, pp. 201–209, 2014.

[15] M. F. Pittenger, A. M. Mackay, S. C. Beck et al., "Multilineage potential of adult human mesenchymal stem cells," *Science*, vol. 284, no. 5411, pp. 143–147, 1999.

[16] F. Tögel, A. Cohen, P. Zhang, Y. Yang, Z. Hu, and C. Westenfelder, "Autologous and allogeneic marrow stromal cells are safe and effective for the treatment of acute kidney injury," *Stem Cells and Development*, vol. 18, no. 3, pp. 475–485, 2009.

[17] M. Morigi, M. Introna, B. Imberti et al., "Human bone marrow mesenchymal stem cells accelerate recovery of acute renal injury and prolong survival in mice," *Stem Cells*, vol. 26, no. 8, pp. 2075–2082, 2008.

[18] M. B. Herrera, B. Bussolati, S. Bruno, V. Fonsato, G. M. Romanazzi, and G. Camussi, "Mesenchymal stem cells contribute to the renal repair of acute tubular epithelial injury," *International Journal of Molecular Medicine*, vol. 14, no. 6, pp. 1035–1041, 2004.

[19] U. Kunter, S. Rong, P. Boor et al., "Mesenchymal stem cells prevent progressive experimental renal failure but maldifferentiate into glomerular adipocytes," *Journal of the American Society of Nephrology*, vol. 18, no. 6, pp. 1754–1764, 2007.

[20] P. Semedo, M. Correa-Costa, M. A. Cenedeze et al., "Mesenchymal stem cells attenuate renal fibrosis through immune modulation and remodeling properties in a rat remnant kidney model," *Stem Cells*, vol. 27, no. 12, pp. 3063–3073, 2009.

[21] F. E. Ezquer, M. E. Ezquer, D. B. Parrau, D. Carpio, A. J. Yañez, and P. A. Conget, "Systemic administration of multipotent mesenchymal stromal cells reverts hyperglycemia and prevents nephropathy in type 1 diabetic mice," *Biology of Blood and Marrow Transplantation*, vol. 14, no. 6, pp. 631–640, 2008.

[22] H. Asanuma, B. A. Vanderbrink, M. T. Campbell et al., "Arterially delivered mesenchymal stem cells prevent obstruction-induced renal fibrosis," *Journal of Surgical Research*, vol. 168, no. 1, pp. e51–e59, 2011.

[23] D. Li, N. Wang, L. Zhang et al., "Mesenchymal stem cells protect podocytes from apoptosis induced by high glucose via secretion of epithelial growth factor," *Stem Cell Research and Therapy*, vol. 4, no. 5, article 103, 2013.

[24] C. Rota, B. Imberti, M. Pozzobon et al., "Human amniotic fluid stem cell preconditioning improves their regenerative potential," *Stem Cells and Development*, vol. 21, no. 11, pp. 1911–1923, 2012.

[25] H. J. Wu, W. H. Yiu, R. X. Li et al., "Mesenchymal stem cells modulate albumin-induced renal tubular inflammation and fibrosis," *PLoS ONE*, vol. 9, no. 3, Article ID e90883, 2014.

[26] T. Katsuno, T. Ozaki, Y. Saka et al., "Low serum cultured adipose tissue-derived stromal cells ameliorate acute kidney injury in rats," *Cell Transplantation*, vol. 22, no. 2, pp. 287–297, 2013.

[27] A. van Koppen, J. A. Joles, B. W. M. van Balkom et al., "Human embryonic mesenchymal stem cell-derived conditioned medium rescues kidney function in rats with established chronic kidney disease," *PLoS ONE*, vol. 7, no. 6, Article ID e38746, 2012.

[28] S. Bruno, C. Grange, M. C. Deregibus et al., "Mesenchymal stem cell-derived microvesicles protect against acute tubular injury," *Journal of the American Society of Nephrology*, vol. 20, no. 5, pp. 1053–1067, 2009.

[29] S. Gatti, S. Bruno, M. C. Deregibus et al., "Microvesicles derived from human adult mesenchymal stem cells protect against ischaemia-reperfusion-induced acute and chronic kidney injury," *Nephrology Dialysis Transplantation*, vol. 26, no. 5, pp. 1474–1483, 2011.

[30] T. Inowa, K. Hishikawa, T. Takeuchi, T. Kitamura, and T. Fujita, "Isolation and potential existence of side population cells in adult human kidney," *International Journal of Urology*, vol. 15, no. 3, pp. 272–274, 2008.

[31] M. A. Goodell, K. Brose, G. Paradis, A. S. Conner, and R. C. Mulligan, "Isolation and functional properties of murine hematopoietic stem cells that are replicating in vivo," *The Journal of Experimental Medicine*, vol. 183, no. 4, pp. 1797–1806, 1996.

[32] H. Iwatani, T. Ito, E. Imai et al., "Hematopoietic and non-hematopoietic potentials of Hoechstlow/side population cells isolated from adult rat kidney," *Kidney International*, vol. 65, no. 5, pp. 1604–1614, 2004.

[33] B. Dekel, L. Zangi, E. Shezen et al., "Isolation and characterization of nontubular Sca-1$^+$Lin$^-$ multipotent stem/progenitor cells from adult mouse kidney," *Journal of the American Society of Nephrology*, vol. 17, no. 12, pp. 3300–3314, 2006.

[34] S. Gupta, C. Verfaillie, D. Chmielewski et al., "Isolation and characterization of kidney-derived stem cells," *Journal of the American Society of Nephrology*, vol. 17, no. 11, pp. 3028–3040, 2006.

[35] A. Maeshima, S. Yamashita, and Y. Nojima, "Identification of renal progenitor-like tubular cells that participate in the regeneration processes of the kidney," *Journal of the American Society of Nephrology*, vol. 14, no. 12, pp. 3138–3146, 2003.

[36] B. Bussolati, S. Bruno, C. Grange et al., "Isolation of renal progenitor cells from adult human kidney," *The American Journal of Pathology*, vol. 166, no. 2, pp. 545–555, 2005.

[37] F. Sallustio, L. de Benedictis, G. Castellano et al., "TLR2 plays a role in the activation of human resident renal stem/progenitor cells," *The FASEB Journal*, vol. 24, no. 2, pp. 514–525, 2010.

[38] F. Sallustio, V. Costantino, S. N. Cox et al., "Human renal stem/progenitor cells repair tubular epithelial cell injury through TLR2-driven inhibin-A and microvesicle-shuttled decorin," *Kidney International*, vol. 83, no. 3, pp. 392–403, 2013.

[39] B. Bussolati, A. Moggio, F. Collino et al., "Hypoxia modulates the undifferentiated phenotype of human renal inner medullary CD133$^+$ progenitors through Oct4/miR-145 balance," *The American Journal of Physiology - Renal Physiology*, vol. 302, no. 1, pp. 116–128, 2012.

[40] C. Grange, A. Moggio, M. Tapparo, S. Porta, G. Camussi, and B. Bussolati, "Protective effect and localization by optical imaging of human renal CD133$^+$ progenitor cells in an acute kidney injury model," *Physiological Reports*, vol. 2, Article ID e12009, 2014.

[41] S. Kitamura, Y. Yamasaki, M. Kinomura et al., "Establishment and characterization of renal progenitor like cells from S3 segment of nephron in rat adult kidney," *The FASEB Journal*, vol. 19, no. 13, pp. 1789–1797, 2005.

[42] M. Kinomura, S. Kitamura, K. Tanabe et al., "Amelioration of cisplatin-induced acute renal injury by renal progenitor-like

cells derived from the adult rat kidney," *Cell Transplantation*, vol. 17, no. 1-2, pp. 143–158, 2008.

[43] M. E. Groh, B. Maitra, E. Szekely, and O. N. Koç, "Human mesenchymal stem cells require monocyte-mediated activation to suppress alloreactive T cells," *Experimental Hematology*, vol. 33, no. 8, pp. 928–934, 2005.

[44] S. Aggarwal and M. F. Pittenger, "Human mesenchymal stem cells modulate allogeneic immune cell responses," *Blood*, vol. 105, no. 4, pp. 1815–1822, 2005.

[45] S. Lv, G. Liu, A. Sun et al., "Mesenchymal stem cells ameliorate diabetic glomerular fiblosis in vivo and in vitro by inhibiting TGF-$\beta$ signaling via secretion of bone morphogenetic protein 7," *Diabetes and Vascular Desease Research*, vol. 11, no. 4, pp. 251–261, 2014.

[46] S. Wang, Y. Li, J. Zhao, J. Zhang, and Y. Huang, "Mesenchymal stem cells ameliorate podocyte injury and proteinuria in a type 1 diabetic nephropathy rat model," *Biology of Blood and Marrow Transplantation*, vol. 19, no. 4, pp. 538–546, 2013.

[47] S.-S. Lv, G. Liu, J.-P. Wang et al., "Mesenchymal stem cells transplantation ameliorates glomerular injury in streptozotocin-induced diabetic nephropathy in rats via inhibiting macrophage infiltration," *International Immunopharmacology*, vol. 17, no. 2, pp. 275–282, 2013.

[48] S. B. Miller, D. R. Martin, J. Kissane, and M. R. Hammerman, "Hepatocyte growth factor accelerates recovery from acute ischemic renal injury in rats," *The American Journal of Physiology—Renal Physiology*, vol. 266, no. 1, pp. F129–F134, 1994.

[49] S. B. Miller, D. R. Martin, J. Kissane, and M. R. Hammerman, "Insulin-like growth factor I accelerates recovery from ischemic acute tubular necrosis in the rat," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 89, no. 24, pp. 11876–11880, 1992.

[50] S. Neuss, E. Becher, M. Wöltje, L. Tietze, and W. Jahnen-Dechent, "Functional expression of HGF and HGF receptor/c-met in adult human mesenchymal stem cells suggests a role in cell mobilization, tissue repair, and wound healing," *Stem Cells*, vol. 22, no. 3, pp. 405–414, 2004.

[51] S. Villanueva, C. Céspedes, and C. P. Vio, "Ischemic acute renal failure induces the expression of a wide range of nephrogenic proteins," *The American Journal of Physiology—Regulatory Integrative and Comparative Physiology*, vol. 290, no. 4, pp. R861–R870, 2006.

[52] S. Villanueva, F. Contreras, A. Tapia et al., "Basic fibroblast growth factor reduces functional and structural damage in chronic kidney disease," *The American Journal of Physiology—Renal Physiology*, vol. 306, no. 4, pp. F430–F441, 2014.

[53] J. Barasch, J. Qiao, G. McWilliams, D. Chen, J. A. Oliver, and D. Herzlinger, "Ureteric bud cells secrete multiple factors, including bFGF, which rescue renal progenitors from apoptosis," *The American Journal of Physiology—Renal Physiology*, vol. 273, no. 5, pp. F757–F767, 1997.

[54] P. M. Lackie, C. Zuber, and J. Roth, "Polysialic acid and N-CAM localisation in embryonic rat kidney: mesenchymal and epithelial elements show different patterns of expression," *Development*, vol. 110, no. 3, pp. 933–947, 1990.

[55] S. Colleoni, E. Bottani, I. Tessaro et al., "Isolation, growth and differentiation of equine mesenchymal stem cells: effect of donor, source, amount of tissue and supplementation with basic fibroblast growth factor," *Veterinary Research Communications*, vol. 33, no. 8, pp. 811–821, 2009.

[56] N. Eliopoulos, J. Zhao, M. Bouchentouf et al., "Human marrow-derived mesenchymal stromal cells decrease cisplatin renotoxicity in vitro and in vivo and enhance survival of mice post-intraperitoneal injection," *The American Journal of Physiology—Renal Physiology*, vol. 299, no. 6, pp. F1288–F1298, 2010.

[57] G. Stokman, I. Stroo, N. Claessen, G. J. D. Teske, S. Florquin, and J. C. Leemans, "SDF-1 provides morphological and functional protection against renal ischaemia/reperfusion injury," *Nephrology Dialysis Transplantation*, vol. 25, no. 12, pp. 3852–3859, 2010.

[58] J. S. Schorey and S. Bhatnagar, "Exosome function: from tumor immunology to pathogen biology," *Traffic*, vol. 9, no. 6, pp. 871–881, 2008.

[59] O. Morel, F. Toti, B. Hugel, and J.-M. Freyssinet, "Cellular microparticles: a disseminated storage pool of bioactive vascular effectors," *Current Opinion in Hematology*, vol. 11, no. 3, pp. 156–164, 2004.

[60] P. Trionfini, A. Benigni, and G. Remuzzi, "MicroRNAs in kidney physiology and disease," *Nature Reviews Nephrology*, vol. 11, no. 1, pp. 23–33, 2014.

[61] K. Hishikawa, T. Marumo, S. Miura et al., "Musculin/MyoR is expressed in kidney side population cells and can regulate their function," *The Journal of Cell Biology*, vol. 169, no. 6, pp. 921–928, 2005.

[62] G. A. Challen, I. Bertoncello, J. A. Deane, S. D. Ricardo, and M. H. Little, "Kidney side population reveals multilineage potential and renal functional capacity but also cellular heterogeneity," *Journal of the American Society of Nephrology*, vol. 17, no. 7, pp. 1896–1912, 2006.

[63] J. A. Oliver, O. Maarouf, F. H. Cheema et al., "The renal papilla is a niche for adult kidney stem cells," *The Journal of Clinical Investigation*, vol. 114, no. 6, pp. 795–804, 2004.

[64] C. Sagrinati, G. S. Netti, B. Mazzinghi et al., "Isolation and characterization of multipotent progenitor cells from the Bowman's capsule of adult human kidneys," *Journal of the American Society of Nephrology*, vol. 17, no. 9, pp. 2443–2456, 2006.

[65] D. Lindgren, A. K. Bostrom, K. Nilsson et al., "Isolation and characterization of progenitor-like cells from human renal proximal tubules," *The American Journal of Pathology*, vol. 178, no. 2, pp. 828–837, 2011.

Hindawi Publishing Corporation
Stem Cells International
Volume 2015, Article ID 678347, 13 pages
http://dx.doi.org/10.1155/2015/678347

*Review Article*

# Transgenic Strategies to Study Podocyte Loss and Regeneration

**Duccio Lombardi[1] and Laura Lasagni[1,2]**

[1]*Excellence Centre for Research, Transfer and High Education for the Development of DE NOVO Therapies (DENOTHE), University of Florence, 50139 Florence, Italy*
[2]*Department of Clinical and Experimental Medicine, University of Florence, 50139 Florence, Italy*

Correspondence should be addressed to Laura Lasagni; l.lasagni@dfc.unifi.it

Received 6 November 2014; Revised 9 March 2015; Accepted 10 March 2015

Academic Editor: Boon Chin Heng



Podocyte death and regeneration are major topics in kidney research but remain controversial. Data obtained in humans demonstrate the existence of cells sited along Bowman's capsule that behave as podocyte progenitors *in vitro* and in *in vivo* mouse models of podocyte injury xenotrasplanted with this human-derived population. However, this podocyte reservoir still remains elusive in murine models, where it could be more easily studied. Transgenic models can be a powerful tool to identify this population and to better understand its dynamics and hierarchies in both physiological and pathological conditions. Indeed, exploiting transgenic approaches allows detecting, at the single cell level, movements, cell death, and replacement. Moreover, through lineage tracing it is now possible to identify specific population increase and to point out clonal expansions during or after the regenerative processes. However, applying transgenic strategies to study glomerular regeneration requires the search of markers to unequivocally identify this progenitor population. Achieving this aim would lead to a deep comprehension of the biological processes that underlie glomerular regeneration and clarify how different cell pools interface during this phase. Here we discuss strategies that have been used and new approaches in transgenic models finalized to study podocyte loss and subsequent replacement.

## 1. Introduction

A crucial element of the nephron filtration barrier is a specialized epithelial cell known as the podocyte. A vast majority of renal diseases start with the dysfunction or loss of podocytes, resulting in proteinuria that leads to nephron degeneration and to kidney failure. No clinical methods are actually available to heal podocyte damage, and the progressive loss of nephrons leads to failure of kidney function and, finally, to end-stage renal disease (ESRD). Interestingly, several studies have suggested that podocyte stem/progenitor cells exist in humans providing a possible explanation for the podocyte regeneration observed in several clinical and experimental situations [1–3]. The existence of a progenitor reservoir appointed to podocyte replacement has been clearly demonstrated in other vertebrates like zebrafish [4–6]. On the contrary, there are still conflicting opinions on the notion of podocyte regeneration and supporting the identity of the putative progenitor cells in the mouse. Given the consequences of podocyte health for overall renal function, to definitely elucidate the mechanism of podocyte regeneration in mammals is of primary relevance. To this aim, the choice of the most adequate model system and the correct interpretation of the data obtained from a specific model are the main points that researchers must take into consideration. In the last years, new methods that enable us to provide extremely accurate data have been searched and developed. In particular, transgenic mouse models offer important tools for research in the field of podocyte regeneration. For instance, podocyte loss is the trigger stimulus in the chain of events that lead to podocyte regeneration, and now temporally and spatially regulated genetic systems for podocytes ablation are available and allow researchers to strictly control podocyte death. In addition, inducible mouse models of lineage tracing enable us to follow the events of regeneration in real-time by labeling a specific cell type and tracking its fate. In this review, we provide an introduction to transgenic mouse models, an overview of transgenic mouse systems to induce podocyte-specific cell death, discuss how transgenic mouse studies have been used to evaluate podocyte regeneration, and how new

transgenic strategies might help researchers in definitively addressing mechanisms of podocyte regeneration.

## 2. Introduction to Transgenic Models

In the last decades, transgenic animals have been intensively developed and used in order to understand how a determinate gene deletion in a specific cell type correlates with a particular phenotype, which is the role of a certain gene in organ development, or which is the response of organ-specific stem cells to a particular kind of injury [7–9]. Moreover, in transgenic models it is possible to mark certain cell populations, study them at different time points, follow a specific cell pool to understand its role during regeneration, and exploit other benefits that will be discussed in this review.

In the past years, a big setback related to the creation of transgenic animals has been the random genomic integration of the foreign DNA (called transgene). This randomness could result in loss of functionality of endogenous genes or in the disconnection of a gene from the elements that control its expression, leading to genetic deregulation that could alter the physiological state of a cell. On the contrary, transgenes may be inserted in chromosome sites that have been silenced, thus resulting in low or complete loss of transgene expression [7]. Nowadays it is possible to insert the exogenous DNA in a specific *locus* of the animal genome thanks to a process based on homologous recombination, called gene targeting. By gene targeting it is thus possible to introduce, in the animal genome, reporter transgenes which encode for markers that can be used to identify, visualize, and track a desired cell type, or different cellular populations, in pathological as well as in physiological organ contexts. These reporters generally codify for fluorescent proteins and are commonly inserted under the Rosa26 locus by homologous recombination. To label a single cell or to tag a specific population with a fluorophore, without confusing this population with others, it is essential that the genetic mechanisms that lead to the reporter expression are controlled in a cell-specific manner. To achieve this aim, cell-specific promoters, generally genes expressed only by the population of interest, are engineered in order to induce the expression of a recombinase enzyme. This enzyme, in turn, will modify the DNA sequence of the reporter gene to "unlock it" and make it expressible. The most common system used for genetic recombination is the Cre recombinase-LoxP system, in which Cre recombinase, expressed under the control of a tissue- or cell-specific promoter, specifically activates the reporter expression by excising the Stop sequence flanked by two LoxP sites (Figure 1(a)). If the Cre recombinase recognizes two LoxP sites oriented in a head-to-tail manner, it will remove the interposed (called "floxed") sequence, while rejoining the ends together. Otherwise, if the LoxP sites are oriented in a head-to-head manner, the interposed sequence will be simply inverted by the enzyme [10–13]. Cre recombinase is widely employed to tag stem cell populations of various organs and to discern them from other cell pools [8, 14, 15]. Notably, its use combined with the *mT*/*mG* (*membrane-Tomato*/*membrane-Green*) reporter transgene enables us to identify cells that have undergone genetic recombination. Indeed, this system allows us to track a switch from a constitutive expression of the red fluorescent protein tomato red, encoded in a cassette floxed by two LoxP sites, to the expression of the downstream GFP (green fluorescent protein) gene (Figure 1(b)).

The use of reporter transgenes has gained a pivotal role and an increasing relevance in a very important tool for *in vivo* experiments focused on regeneration: lineage tracing analysis. Lineage tracing is defined as the possibility to tag a single desired cell with a reporter and to identify all the progeny derived from this cell. Since the irreversible DNA modification that leads to the expression of the reporter is inherited by all the daughter cells, it is possible to quantify the progeny of a definite founder cell and follow its daughters' behavior in physiologic as well as pathologic conditions [10]. However, lineage tracing analysis is based on a further evolution of transgenic models, the so-called inducible models, which enable a time-based regulation of the Cre recombinase activity. Indeed, in the conditional approaches described above, genetic tracing labels all the cells that, in any moment of their life, activated the cell-specific promoter driving Cre expression. This *in vivo* genetic fate mapping is frequently confused with lineage tracing analysis and can give controversial results. For instance, upregulation or transient activation of the chosen cell-specific promoters in a different or unexpected cell population can lead to erroneous experimental result interpretation. In inducible models, cell-specific promoters induce Cre activity only as a consequence of the administration of exogenous molecules that act as inductors, as in the Tamoxifen-regulated system (CreERT) or in the Tetracycline-regulated system (Figures 1(c) and 1(d), resp.). Thus, the desired cell pool is tagged only during the administration window of the inductor molecule, making it impossible for a cell to undergo genetic recombination upon withdrawal of the molecule, even if promoter-positive.

The introduction, in the last years, of multicolor reporter constructs has increased the data output from tracing analysis. Of particular interest, Livet et al. developed a system, called Confetti [13], that was extensively used to better understand stem cells dynamics during regeneration, to discriminate different cell types contribution in the repair phase and to evidence clonal expansion [14, 16, 17]. This reporter enables the expression of one out of four fluorescent proteins, in a stochastic manner, by using alternating incompatible LoxP variants (LoxP and pLox) and by linking reporter genes *in tandem* with opposite DNA orientation in between these LoxP sites. These variants give rise to mutually exclusive excision of fluorescent reporters, thus inducing the cell to randomly acquire one out of four colors [13] (Figure 2).

The Confetti reporter not only enables the examination of the individual behavior of multiple stem cells in a single niche but also allows us to study how different positional effects in the niche can affect differentiation of stem or progenitor cells [16, 17]. Moreover, the randomness of the recombination events and the possibility to tag cells with different fluorophores allow us to easily visualize clonal expansion of single cells that will appear as continuous clusters of cells of the same color. Cellular hierarchies can thereby be resolved with high definition, without confounding progenitors with cells

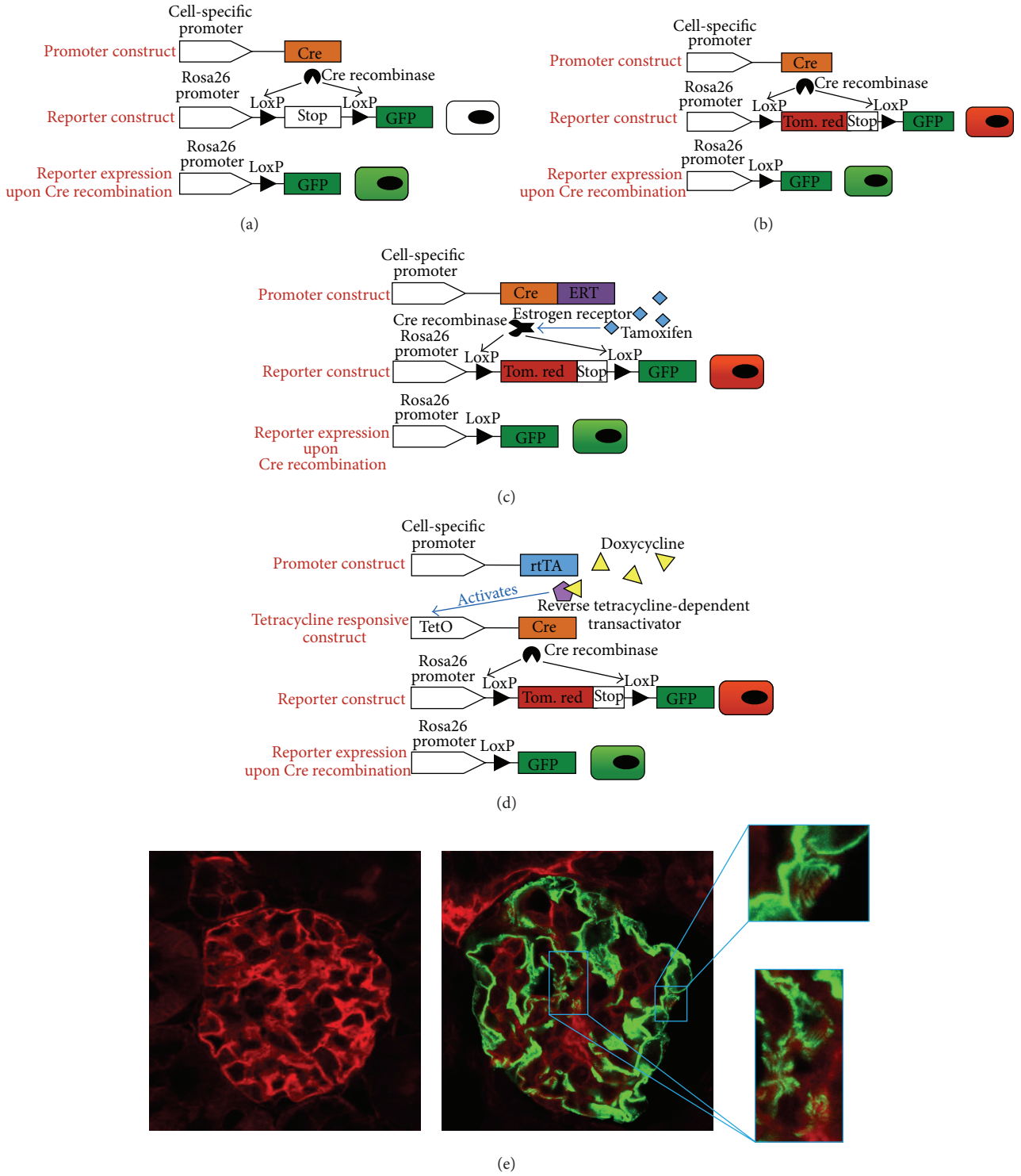



Figure 1: Schematic representation of the principal transgenic solutions. Each line represents a mouse strain, which is crossed with other transgenic strains in order to obtain double or triple transgenic animals. (a) Cell-specific promoter directly controls Cre activity creating a constitutive model. When the promoter gets activated Cre recombinase will be expressed and will gain access to LoxP sites. By removing the LoxP-floxed Stop cassette, the recombinatorial event will lead to GFP expression. (b) *mT*/*mG* (membrane-Tomato/membrane-Green) reporter construct in a constitutive scheme. The cell-specific promoter, in this setting, will change desired population color from ubiquitously expressed tomato red protein to GFP, evidencing cells that had recombinatorial activity. (c) Inducible model based on a fused form of Cre recombinase and estrogen receptor (ERT) proteins. If the chosen promoter is activated this fused protein will always be transcribed. Only following Tamoxifen treatment the ERT will bind the inductor and enter the nucleus, where Cre recombinase will act on the LoxP site of the reporter transgene *mT*/*mG*. (d) Inducible model based on reverse Tetracycline-controlled transactivator (rtTA) protein, which is continuously created by the activated specific promoter. When bound to Tetracycline (Doxycycline form), the transcriptional factor is able to activate Cre transcription controlled by TetO operator sequences. (e) Imaged glomeruli in a podocyte-driven *mT*/*mG* setting prior to (left panel) and following (right panel) induction. The strong fluorescence signal of this reporter line enables the visualization of podocyte primary foot processes (right enlarged box).

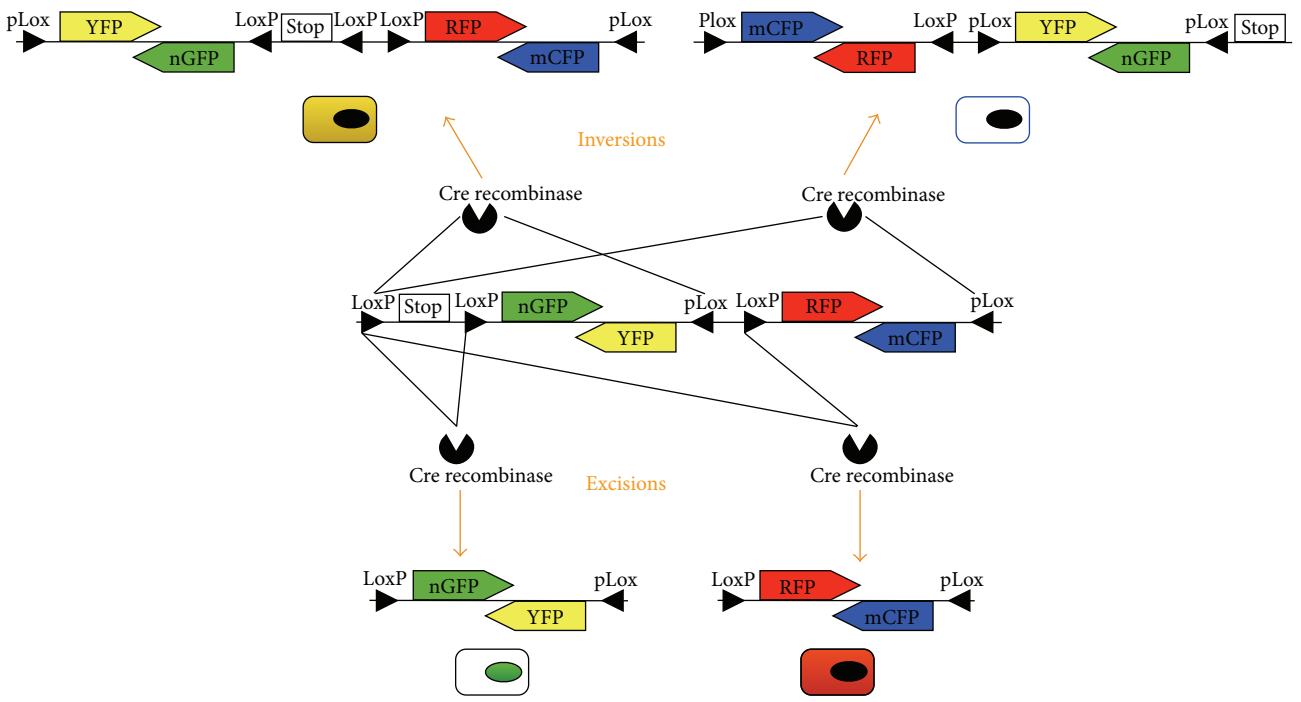



Figure 2: Schematic overview of the Confetti reporter system. In the center, the whole construct. On the bottom, excision eliminates reporter gene thus turning on one cell color. On the top, inversions rely on pLox sites that are used to invert the construct favoring other colors (Cre recombinase inverts segments floxed between two LoxP sites oriented in a head-to-head fashion). Both inverted and excised fragments can continue to invert as long as there is Cre activity, since LoxP sites are excised, while pLox sites are not. Consequently, an inducible model might be best suitable to stabilize cell color following inductor molecule removal. All recombinatorial events that do not involve the LoxP site upstream to the Stop cassette would result in no color acquisition by the cell.

under transient state changes (i.e., dedifferentiation), which complicate accurate cellular identification by antibody-based methods.

All these discussions evidenced how much transgenic strategies would be useful in deciphering regenerative routes. In the following sections, we will focus on transgenic tactics to induce podocyte loss and, consequently, evaluate their *de novo* generation.

## 3. Podocyte Ablation: Transgenic Strategies to Induce Podocyte Loss

The major question related to glomerular regeneration is centered on the podocyte, a cell with an extremely complex cytoskeleton that composes the glomerular filtration barrier and which is the target of a broad range of chemical and physical insults. Indeed, podocytes react to immune- and nonimmune-mediated injury with rearrangement of the complex actin cytoskeleton, spreading of the foot processes along the GBM (glomerular basement membrane), loss of filtration slits, and apical redistribution of slit diaphragm proteins. Consequently, a progressive reduction in podocyte number from podocyte death and/or detachment is observed and leads to the development of glomerulosclerosis, the pathological basis of chronic renal failure. In human, evidence demonstrated that a population of cells localized in the parietal epithelium of Bowman's capsule possesses characteristics of stem/progenitor cells. This population can be expanded as clones in culture and can differentiate into podocyte both *in vitro* and *in vivo* following intravenous administration in a SCID (severe combined immunodeficiency) mice model affected by adriamycin nephropathy (AN), the experimental analogous of human focal segmental glomerulosclerosis (FSGS) [1, 2]. However, the existence of this population in mouse is still an open question whose answer is of paramount importance in regenerative nephrology research. Indeed, a greater understanding of podocyte biology and of the regenerative role of renal progenitors may provide novel therapeutics to treat glomerular disease. Any attempt to identify a progenitor pool able to regenerate lost podocytes relies on experimental procedures to induce podocyte loss. In humans, podocyte damage is the starting event leading to FSGS, a disease histologically characterized by the presence of sclerosis of part of the glomerular capillaries in a minority of glomeruli. Clinically, podocyte loss results in proteinuria, hypoalbuminemia, hypercholesterolemia, and peripheral edema, that is, nephrotic syndrome. FSGS is not a single disease but a lesion as a result of different pathophysiologies that cannot be recapitulated by a single animal model. Thus, different animal models of both primary and secondary FSGS, with specific pros and cons, have been developed to mimic the clinical pathological features of human FSGS [18, 19]. The choice of the model most appropriate to the experimental purpose must be guided by the question that the researcher is trying to solve. In this review we will focus

our attention on models of FSGS obtained using transgenic mice. However, we will provide also a brief description of the two most widely used nontransgenic models: the remnant kidney and adriamycin nephropathy. In the remnant kidney model, 4/6 or 5/6 of the total renal mass is surgically removed by unilateral nephrectomy coupled to ligation of renal artery branches or polectomies in the contralateral kidney. These surgical procedures lead to hypertension, renal damage, and FSGS (which is more severe in the 5/6 model). The remnant kidney model is mainly used in rats. Indeed, the anatomic distribution of the renal artery branches makes it difficult to achieve reproducible 5/6 nephrectomy in mouse; moreover, while 5/6 nephrectomy may be sufficient to evoke development of FSGS in 129/Sv male mice within 1-2 months, most mouse strains, including the C57Bl/6 strain, are resistant to this procedure and do not develop FSGS [20, 21]. In C57Bl/6 mice, it is necessary to treat 5/6 nephrectomized animals with deoxycorticosterone acetate (DOCA) and a high salt diet to induce FSGS. These procedures result in hypertension and nephrosclerosis (an endocrine hypertension model). However, like for the remnant kidney model, the degree of hypertension and hypertensive renal lesions is markedly different between various mouse strains commonly used in research, with 129/Sv strain more susceptible than C57Bl/6 [22]. Surprisingly, in this mouse model, the amount of albumin excretion did not correspond to the degree of glomerular damage and therefore albuminuria is not a good predictor for the degree of glomerular scarring, making the screening of kidney injury for treated mice more challenging [22].

FSGS can also be induced by treating animals with podocyte-toxic drugs, such as adriamycin (Pfizer, Sydney, Australia) (doxorubicin). After the initial toxic injury, mice develop an immune-mediated chronic proteinuric renal disease. The clinic pathological features of AN are nephrotic syndrome, focal glomerulosclerosis, tubular injury, and interstitial compartment expansion with infiltration of mononuclear cells that are composed largely of macrophages and T cells. In mice, AN is associated with acceptable mortality (less than 5%) and morbidity (weight loss) [18]. Nevertheless, several critical points must be taken into consideration when performing adriamycin nephropathy experiments. As first issue, the identification of the optimal regimen of adriamycin administration requires great effort because of the variability between species, strain, gender, and age of the animals and even differences between animals of the same litter. Most rat species are completely sensitive to the renal effects of adriamycin. In male Wistar rats, the dose of adriamycin ranges between 1.5 and 7.5 mg/kg. Instead, male BALB/c mice require 9.8–10.4 mg/kg, [23] while male SCID mice developed on a BALB/c background require only 5.3 mg/kg [24]. C57Bl/6 mice are highly resistant to adriamycin-induced renal injury, but renal injury may be inducible at higher doses (13–25 mg/kg) than those required for BALB/c mice [25–27]. In C57Bl/6-based models, while most studies use a single injection, regimens using multiple injections (i.e., 2 mg/kg × 2 in 20 days, 1 mg/kg/day × 7 days, and 2.5 mg/kg × 6 in 14 days) have also been reported. The choice of the effective dose is further complicated by the variability among drug sources and batches. To overcome these problems, dose-finding tests are usually necessary to ascertain the exact dose necessary to induce the pathological changes required by the investigator. Finally, adriamycin has also effects that are not specific to the kidney such as myelotoxicity, hepatotoxicity, cardiomyopathy, and neurotoxicity as evidenced by lack of animal coordination [18, 28].

Although AN and remnant kidney models both resemble human FSGS, adriamycin nephropathy, which induces podocyte oxidative stress, fostered the concept of primary podocyte injury in the pathogenesis of foot process effacement and glomerulosclerosis. By contrast, renal ablation models pointed to glomerular hypertension (elevated glomerular capillary pressures and flow rates) as the primary pathophysiologic process in the course of adaptive responses to reduced number of functioning nephrons, in turn causing secondary podocyte injury [29, 30].

Advances in transgenic and gene-targeting technologies have elucidated the function of individual genes in glomerular health and disease. Several animal models are now available, in which a transgene insertion or the modification of a gene in podocytes leads to a more or less severe albuminuria, sometimes of nephrotic range, and focal glomerulosclerosis [31–34]. However, conditional cell-targeted ablation constitutes the most promising genetic tool to analyze cell lineage relationships, the role of specific cells during embryogenesis, or physiological processes. Moreover, the ability to control temporally and spatially the tissue damage and to genetically remove a specific cell population has important applications for regeneration studies. The ideal genetic cell ablation tool must be (1) spatially controllable and strictly confined to the target cell population, (2) temporally inducible, (3) germline transmissible, and (4) reversible. In this paragraph we will focus our attention on three of the most commonly used transgenic models in which genetically controlled cell removal ensures the highest precision and consistency in studying the consequences of podocyte damage. As a consequence, studies on mechanisms of podocyte regeneration can be more accurately performed.

*Thy-1.1 Model*. In their endeavor to study the function of the Thy-1 antigen, Kollias et al. [35] generated transgenic mice that ectopically express a mouse-human chimeric Thy-1.1 antigen. Some of these transgenic mice expressed the Thy-1.1 antigen specifically on podocytes, slowly and spontaneously developing albuminuria (from week 7 onward) and focal glomerulosclerosis [35, 36]. After podocyte-specific strain isolation, the model has been extensively used in experimental settings based on the anti-Thy-1.1 monoclonal antibody (mAb) injection. mAb administration induced an acute albuminuria in nonalbuminuric, 5-week-old Thy-1.1 transgenic mice, while the same procedure did not cause albuminuria in nontransgenic mice [36]. Smeets et al. described in detail the dose-dependent effects of anti-Thy-1.1 mAb treatment [37]: high dose injection (1000 mg) induces an albuminuria within 10 min that persisted over a 3-week period. With lower doses (100 $\mu$g) of anti-Thy-1.1 mAb, on the contrary, the developed albuminuria rapidly dropped and returned to baseline levels within 7 days. Glomeruli became increasingly ischemic with

no sign of podocytic hypertrophy or swelling. Electron microscopy revealed reduction in number of foot processes and decrease in diameter of slit pores already 10 minutes after mAb injection, while no large gaps between podocytes or podocyte detachment were observed [37]. In this respect, this model closely resembles human nephropathies such as minimal change disease or FSGS. In contrast to adriamycin that causes severe podocytic injury leading to necrosis and detachment of the podocytes from the GBM, in this model, the podocyte injury is relatively small, with no detachment detectable in the first 24 h after mAb administration. Moreover, in AN the level of proteinuria correlates with the degree of podocyte detachment, while in the anti-Thy-1.1 model podocyte injury is relatively minor despite massive proteinuria. Actually no Rosa26 targeted Thy-1.1 mice line exists that can be exploited with the Cre-LoxP strategy, thus hampering the possibility to specifically control transgene expression by using constitutive or inducible podocyte-specific promoters.

*NEP25 Model*. This model relies on an immunotoxin-mediated cell targeting technology. LMB2 is a chimeric protein composed of the Fv portion of an anti-Tac (human CD25) antibody and PE38, a mutant form of Pseudomonas exotoxin that contains the translocation and ADP ribosylation domains. LMB2 has been used to ablate specific cell types in transgenic mice that express hCD25 in the target cells [38, 39]. Transgenic mouse lines that express hCD25 selectively in podocytes, as the one directed by the nephrin promoter, have been successively developed [40]. Two to three days after LMB2 injection these mice developed nonselective proteinuria, hypoproteinemia, ascites, edema, and renal failure. Because of severe edema and/or renal failure, most transgenic mice died at a time inversely related to the dose of LMB2 injected. With 0.625 ng/g body weight of the toxin, the majority of the transgenic mice showed mild and transient ascites and survived for more than 28 days. Proteinuria peaked at the seventh day and, thereafter, gradually decreased with time, returning nearly to the normal range within 28 days [40]. Light microscopy, transmission and scanning electron microscopy, and immunostaining for podocyte marker proteins demonstrated damage and progressive loss of podocytes, but transferase-mediated dUTP nick-end labeling detected only rare apoptosis in the glomerulus indicating that apoptosis is not the major mechanism of the podocyte loss [41]. In addition to podocytes, glomerular endothelial cells, mesangial cells, parietal epithelial cells (PECs), and, later, tubular cells became damaged. Mice that survived for more than 3 weeks developed segmental or global glomerular sclerosis. It is interesting that, 4 weeks after LMB2 injection of 0.625 ng/g per body weight, the average scores of epithelial injury and mesangial changes were better than those measured at 3 weeks after the damage onset [40]. In addition, urinary protein was almost in normal range 4 weeks after the toxin administration. These suggest that glomeruli that are only mildly injured by LMB2 have the ability to recover. The advantages of this model are that transgenic mice are completely normal unless they receive LMB2 treatment, facilitating colony maintenance and reproduction. Moreover, the damage severity is dose-dependent and controlled by LMB2 quantity injection.

*DTR Model*. The A chain of diphtheria toxin (DTA) catalyzes the ADP-ribosylation of the eukaryotic elongation factor 2, resulting in inhibition of translation, which finally leads to cell death [42, 43]. However, rodents are resistant to DTA because rodent heparin-binding epidermal growth factor-like growth factor does not bind the toxin. On the contrary, the forced expression of the human DT receptor (DTR) in a target cell population is able to render mouse cells susceptible to the toxin effects, triggering cell death [44]. Alternatively, it is possible to induce endogenous expression of DTA in the target cell, as proposed by Breitman et al. [45] and Palmiter et al. [46]. Recently, a conditional mouse line in which the expression of DTA can be controlled by Cre recombinase activity has been generated, thus allowing widespread ablation of different cell types by the use of tissue specific Cre-lines. Lately, Brockschnieder et al. described a mouse line in which a LoxP conditional DTA allele was introduced into the ubiquitously expressed Rosa26 locus [47]. Wharram et al. successfully used this strategy to study the pathological consequences of podocyte injury in adult rats [48], evidencing mesangial matrix expansion, formation of synechiae, and development of sclerosis areas with collapse of glomerular capillaries. Analogously, Jia et al. described a mouse podocyte ablation model in which the podocin (NPHS2) promoter directs Cre-mediated DTA176 (an attenuated DTA gene) production and subsequent podocyte ablation during nephrogenesis [49]. What renders this model so interesting is that *in vitro* studies suggest that internalization of as little as a single molecule causes a cell to die [50]. Recently, a Cre-inducible transgenic mouse strain, in which a LoxP-flanked Stop cassette and the simian diphtheria toxin receptor (iDTR) gene were targeted under the Rosa26 locus of mouse genome, has been generated (iDTR mouse) [51] (Figure 3(a)). iDTR mice can be crossed with any kind of Cre recombinase-expressing mice in which the enzyme is active only in a specific cell pool due to cell-specific promoter activation. Wanner et al. [52] crossed the iDTR strain to hNPHS2.rtTA, TetO.Cre, and *mT*/*mG* mice to specifically target podocyte ablation, in a dose-dependent manner, and determine the influence of acute podocyte loss on podocyte regeneration (Figure 3(b)). High doses (25–100 ng/g body weight) of DT resulted in massive albuminuria and loss of nearly all podocytes, whereas lower doses (2 and 5 ng/g body weight) were sufficient to cause a net loss of approximately 12% of all podocytes after 4 weeks, without imposing any persistent gross damage to the tissue. This injury led to a transient increase in albuminuria, which gradually decreased over 28 days [52].

The strength of the NEP25 and DTR models is that, by targeting specific cell populations, they show no obvious gender differences, as what happens in AN-based models, and that pathological and morphological changes can be modulated simply by adjusting the drug dose. The intrinsic limit of these models is that they cause specific but acute podocyte ablation, which is optimal to study podocyte *de novo* creation, but it does not closely resemble the vast

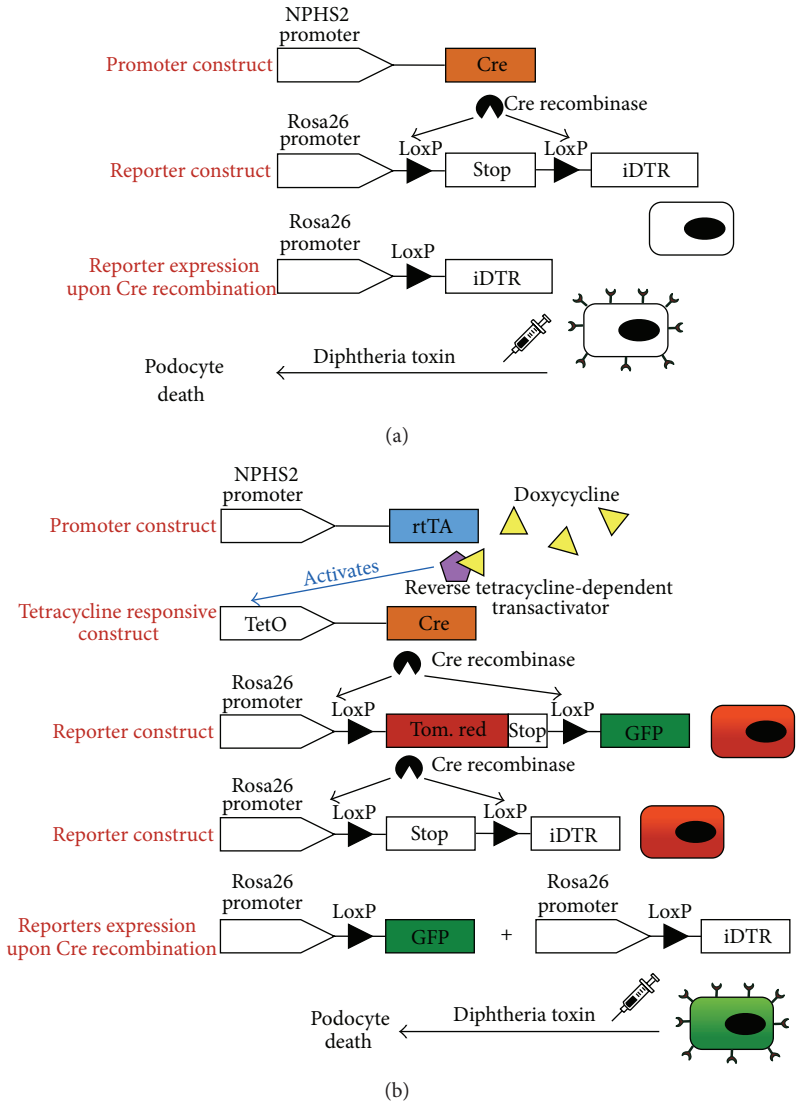



Figure 3: DTR models and strategies. (a) Schematic representation of a floxed Stop cassette followed by DTR transgene targeted under the Rosa26 promoter. The reporter transgene, activated upon Cre recombinase activity induced by podocin promoter activation, forces podocytes to express the DT receptor. The operator can thus easily induce podocyte cell death in a time- and dose-controlled manner. (b) Quadruple transgenic hNPHS2.rtTa; TetO.Cre; *mT*/*mG*; iDTR, in which podocytes can be prompted to turn from red to green while expressing the DTR. Once induced, the authors provoked podocyte death through DT injection [52].

majority of human podocyte-related pathologies, in which podocyte loss is a long-standing and progressive event.

In conclusion, several good models that recapitulate FSGS are available; nevertheless, choosing the correct model to address specific hypothesis is the essential prerequisite for the success of research.

## 4. Podocyte Regeneration: Transgenic Tactics to Identify Progenitor Pool for Podocyte Replacement

Transgenic models have been widely employed, in the last ten years, to identify stem or progenitor populations present in the glomerular compartment and to understand their role in podocyte turnover in both homeostasis and pathological states. One of the first groups that used genetic strategies to identify podocyte reservoir has been Moeller's group that, in 2009, using a fused form of human and rabbit podocalyxin as promoter, tagged parietal epithelial cells of Bowman's capsule [53], already described in humans as podocyte progenitors [2]. By employing this inducible mouse model, the authors were able to specifically label mature PECs with $\beta$-gal, allowing the direct visualization of this compartment for the first time in rodents [53]. This intriguing strategy that later was found to be essential for the discovery of PEC pathological participation in glomerular sclerotic lesions and in crescent formation [54, 55] allowed us to show that PECs labeled at postnatal day 5 migrated to the glomerular tuft and differentiated into podocytes, providing evidence, confirmed

also by Wanner et al., that some podocytes are recruited from PECs at least in juvenile mice [52, 53]. In a more recent paper, the same group confirmed that, during adolescence, generation of podocytes occurs from a subpopulation of PECs that the authors called "podocyte reserve," that is, cells already committed to become podocytes, which shows a transitional phenotype and can be found in Bowman's capsule only in juvenile mice and not in adult mice [56]. Using the same lineage tracing model, podocyte generation from PECs could not be detected in aging mice or in models of glomerular hypertrophy (5/6 nephrectomy and DOCA-salt model) [56]. It is reported that stem/progenitor cells participate not only in regenerative response to acute injury but also in the general maintenance function of replacing cells that are lost during physiologic organ activity, that is, homeostasis. However, some tissues such as muscle and brain exhibit limited turnover. The finding on aging reported by Berger et al. [56] and confirmed also by Wanner et al. [52] suggests that this is true also for the kidney: podocytes have long lifespan and are rarely replaced. It is possible that the aging process in the glomerulus is accompanied by subtle and gradual effects that are compensated during time mostly by nephron surplus [57] and podocyte hypertrophy [57, 58] and thus do not involve the putative progenitor compartment. Interestingly, even in the liver, a tissue with a dramatic regenerative capacity, stem cells are not used during homeostasis but are called into service only upon injury [59, 60]. However, Berger et al. fail to evidence a role for PECs even after injury using two models of glomerular hypertrophy. Indeed, they observed that PECs participate only in the formation of sclerotic lesions, whereas hypertrophied glomeruli did not show migration of genetically tagged PECs to the glomerular tuft, thus arguing against the role of PEC in podocyte regeneration [56]. However, a questionable point in the papers from Appel et al. [53] and from Berger et al. [56] remains the promoter used to tag putative progenitors: can the fused form of human and rabbit podocalyxin genes be used as a promoter to correctly identify progenitor cells?

In a recent paper, Hackl et al. [61], employed a constitutive transgenic mice model controlled by the rat phosphoenolpyruvate carboxykinase promoter (PEPCK), crossed with a GFP reporter strain, to genetically tag PECs. The authors, using a unilateral ureteral obstruction (UUO) disease model, evidenced rare GFP-positive PECs that migrated from Bowman's capsule vascular pole to the glomerular tuft. Nevertheless, this constitutive model does not allow us to unequivocally identify the progenitor pool because of the nonspecificity of the used promoters, which did not mark all PECs, while tagging also other pools [61] with a high grade of recombination in S1–S3 proximal tubular cells [62].

Sakamoto et al. [63] used a constitutive transgenic mouse expressing $\beta$-gal and human CD25 under the control of the nephrin promoter, allowing tagging of podocytes as to induce their specific ablation by LMB2 injection. Following podocyte ablation, the authors investigated phenotypic transition from podocyte to PECs or *vice versa* by double staining for podocyte or PEC markers. The conclusion is that podocytes undergo epithelial phenotypic transition into PEC [63]. However, by this transgenic tactic, they performed a genetic fate mapping of podocyte pool and are not *tracing the podocyte lineage cell* since any cell that might have expressed nephrin, at any moment and even for a very short period, would result as $\beta$-gal$^+$. Thus, $\beta$-gal$^+$ cells could result not only from nephrin expression in fully differentiated podocytes but also from progenitors starting to acquire this podocyte markers upon differentiation into podocyte lineage, as already reported in human [2] and rodents [53] studies of two different groups. This is further corroborated by the findings that, in the paper [63], claudin$^+$ WT1$^+$ cells were reported to mainly localize at the vascular pole, sustaining the idea of PEC differentiation to podocyte while migrating to cover the tuft along the denuded glomerular basement membrane. Furthermore, identification of cells by immunostaining for specific protein markers can lead to misinterpretation of results due to upregulation of markers as consequence of damage or as response to injury, also in more than one particular cell type.

A different hypothesis related to podocyte regeneration has been recently formulated by Pippin et al. who lineage-traced the cells of the juxtaglomerular apparatus by engineering the mouse genome with a modified and inducible form, of the renin gene [64]. The authors were thus able to show that renin-positive cells possess regenerative capacities because of their ability to substitute, in an experimental model of FSGS, not only lost podocytes but also PECs. This suggests that cells of renin lineage may have the capacity to serve as upstream progenitors for both PECs and podocytes [64]. However, this population localizes in the preglomerular vascular wall, and it is actually unclear how it would cross the parietal and the glomerular basement membrane to gain access to the glomerular compartment [65]. Moreover, the number of podocytes generated by renin expressing cells appeared extremely low and its relationship with the outcome of the disease has not been definitively proven. A subsequent study of the same group focused on the role of renin-positive cells in aging nephropathy [66], which is characterized by progressive podocyte loss and consequent focal and global glomerulosclerosis [67, 68]. In the paper, the authors used a constitutive model in which the renin gene drives the recombination that leads to ZsGreen fluorophore expression. Thanks to this genetic fate mapping, it was proved that, at 1 year of age, the intraglomerular compartment exhibited an increment in the number of cells of renin lineage, most of which expressed markers exclusively associated with differentiated podocytes (as podocin and synaptopodin) [66]. However, in this genetic fate mapping strategy based on a constitutive model is not possible to understand if the renin-positive pool can substitute lost podocytes, since any cell (and also podocytes [69, 70]) that had expressed renin, in any moment of the animal life, would be tagged by the reporter. The study by Starke et al. further challenged the involvement of cells of renin lineage in podocyte turnover with an inducible model that enabled tagging of the renin$^+$ population with $\beta$-gal reporter transgene [71]. By setting up a mesangial injury in this transgenic model the authors proved that renin descendant cells never colocalized with podocyte or PEC markers, denying their role as podocyte or even PEC reservoir, evidencing instead a prominent role

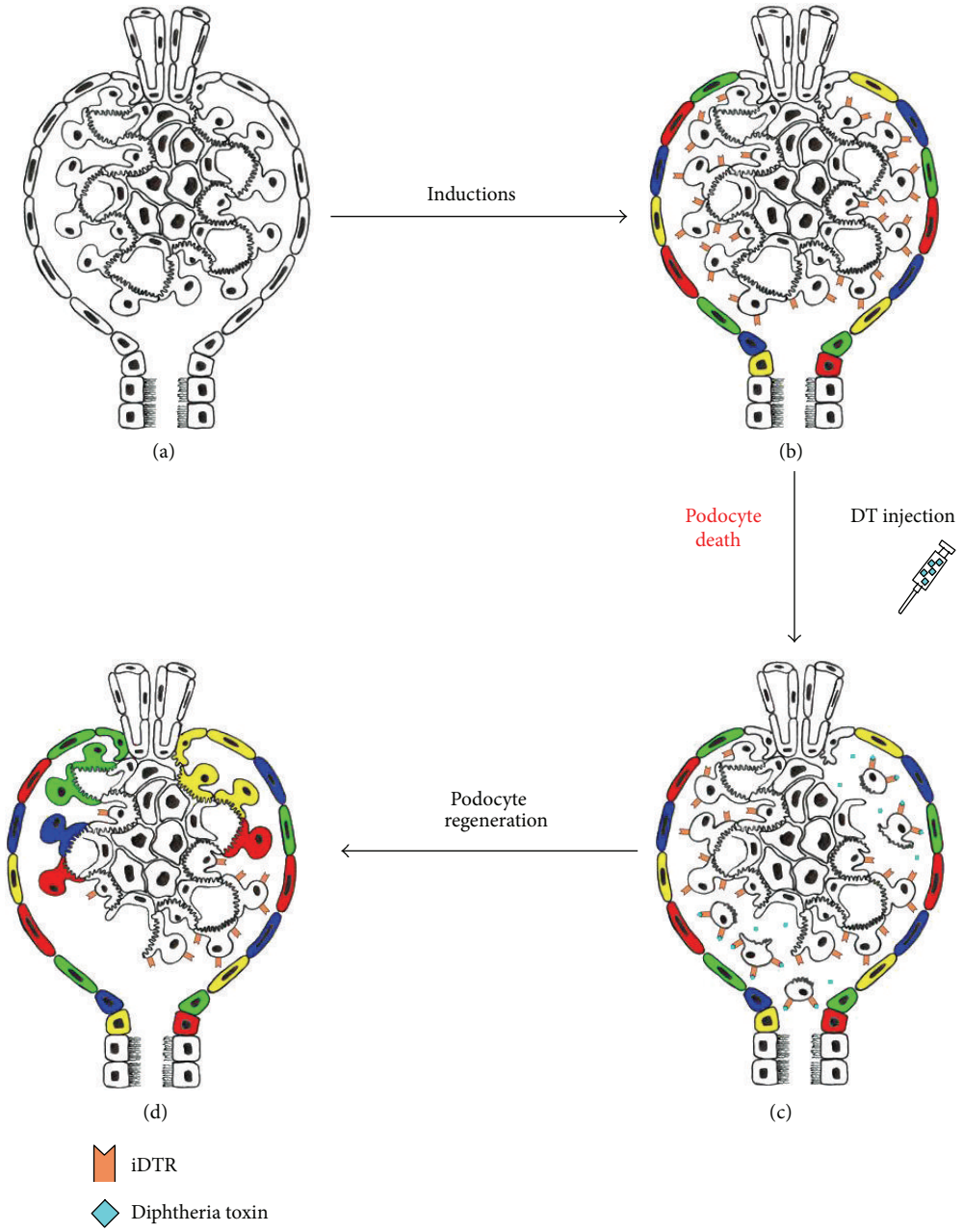



Figure 4: Hypothetical scheme of a combinatorial solution for PEC tagging and podocyte-specific ablation. (a) Glomerulus before induction of the two recombinatorial systems (Cre-LoxP and Flp-FRT), which can be induced independently and at different time points. (b) Once induced, the glomerulus would appear with Confetti-tagged PECs and iDTR-expressing podocytes. (c) At the prespecified time and dose, the operator could specifically ablate only podocytes. (d) *De novo* podocyte generation by the PEC compartment.

in intraglomerular mesangial cell substitution [71]. Thus, controversial findings in the literature still leave the role of renin-positive cells as podocyte progenitors undefined.

A recent and very elegant paper by Wanner et al. demonstrated how transgenic animals can be used to study glomerular regeneration and podocyte replacement in aging nephropathy, in a unilateral nephrectomy model and following acute podocyte loss [52]. To study podocyte regeneration the authors induced a severe podocyte depletion thanks to the mentioned transgenic tactics of specific ablation of cells engineered to express the iDTR transgene. To this aim, they created a quadruple inducible transgenic mouse governed by the podocin promoter, which in turn acts on the *mT*/*mG* transgene targeted under one Rosa26 allele and on the iDTR transgene inserted in the other Rosa26 allele. To develop this model, Wanner et al. created two different transgenic lines, both governed by podocin promoter, but with two different reporter transgenes (iDTR in one line and *mT*/*mG* in the other one). When crossed, these lines gave rise to the quadruple transgenic model, with one Rosa26 allele

leading to iDTR expression and with the other Rosa26 allele carrying the *mT*/*mG* transgene (Figure 3(b)). This strategy enabled tagging of podocytes with GFP, while inducing iDTR expression and thus rendering the cells susceptible to toxin-induced ablation. By this it was possible to visualize and quantify by flow cytometry *de novo* generated podocytes, since they can only be red-colored due to the withdrawal of Doxycycline before the onset of the damage. This transgenic route presents intrinsic limits since it is not possible to identify the source of novel podocytes or to individuate progenitor population origin [52]. Intriguingly, a month after DT injection, the authors reported that about 38% of lost podocytes are replaced by newly generated Tomato Red$^+$ podocytes. On the contrary, neither in the aging nephropathy model nor in the unilateral nephrectomy setting, an increase in Tomato Red$^+$ podocytes was evidenced [52]. Data on the aging model have been already discussed above. Similar consideration can be made to explain why podocyte regeneration observed in the iDTR model cannot however be highlighted in the unilateral nephrectomy model, in which the major compensatory mechanism to the nephron loss is the hypertrophy of the survived glomeruli [52, 56]. However, even in the 5/6 nephrectomy and 5/6 nephrectomy + DOCA salt, Berger et al. did not observe podocyte regeneration [56].

In conclusion, from the available experimental data on transgenic mouse models, we can state that cells located in Bowman's capsule give rise to podocytes during the first weeks of age.

The role of PECs or other podocyte progenitors following injury is however still a matter of debate, with discrepancies among studies even on the possibility that podocyte regeneration may occur at all. What account for these discrepancies? One explanation is related to the fact that the renal progenitor cell activity may be more or less marked depending on the nature of injury, as described in liver stem cells [60], or depending on the magnitude of the damage. It is well known that elevated albuminuria impairs PEC differentiation into podocytes, as demonstrated by Peired et al. [72]. It is thus possible that podocyte regeneration could be visualized only in models in which regression of glomerular damage occurs. To this aim, specific podocyte ablation models are more suitable because the degree of podocyte loss could be more finely modulated [48]. In agreement with this hypothesis is the finding that, using the iDTR model, Wanner et al. provide evidence for podocyte regeneration when animals received a DT dose sufficient to cause a net loss of approximately 12% of all podocytes but without imposing any persistent gross damage to the tissue [52].

In addition, all the studies reviewed here evidence the necessity to find a PEC-specific promoter physiologically expressed only in this cell type. This will pave the way for further evolution of transgenic tactics finalized to a deep comprehension of the dynamics between PECs and podocytes. In this context, an important aim would be the creation of a multiple combinatorial inducible transgenic model in which both PECs and podocytes are engineered, the first with a fluorescent reporter and the second with a construct for cell ablation. A possible solution, for instance, could be the development of a Confetti-marked PECs, controlled by the Cre-LoxP system, combined to podocyte-targeted iDTR transgene expression controlled by the flippase recombinase that recognizes flippase recognition target (FRT) (FLp-FRT system) (Figure 4). The creation of a hypothetical transgenic model like this would require great efforts, but similar combinatorial tactics are actually under development in various organisms and organ system and hold great promises for regenerative studies [73, 74].

## 5. Conclusions

Transgenic animal models, recombinatorial strategies, and related lineage tracing analysis are powerful tools that can be widely applied to understand the mechanisms that govern kidney dynamics and regeneration. Transgenic approaches are not only changing our views on kidney regeneration capabilities but also opening to researchers new perspectives in various aspects of kidney physiology that will grant great advantages in understanding this complex organ. Notwithstanding, transgenic-related techniques still need a further development in order to unequivocally identify and characterize stem and progenitor populations involved in podocyte replacement.

## Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

## References

[1] C. Sagrinati, G. S. Netti, B. Mazzinghi et al., "Isolation and characterization of multipotent progenitor cells from the Bowman's capsule of adult human kidneys," *Journal of the American Society of Nephrology*, vol. 17, no. 9, pp. 2443–2456, 2006.

[2] E. Ronconi, C. Sagrinati, M. L. Angelotti et al., "Regeneration of glomerular podocytes by human renal progenitors," *Journal of the American Society of Nephrology*, vol. 20, no. 2, pp. 322–332, 2009.

[3] P. Romagnani and G. Remuzzi, "Renal progenitors in non-diabetic and diabetic nephropathies," *Trends in Endocrinology and Metabolism*, vol. 24, no. 1, pp. 13–20, 2013.

[4] J. Huang, M. McKee, H. D. Huang, A. Xiang, A. J. Davidson, and H. A. J. Lu, "A zebrafish model of conditional targeted podocyte ablation and regeneration," *Kidney International*, vol. 83, no. 6, pp. 1193–1200, 2013.

[5] W. Zhou and F. Hildebrandt, "Inducible podocyte injury and proteinuria in transgenic zebrafish," *Journal of the American Society of Nephrology*, vol. 23, no. 6, pp. 1039–1047, 2012.

[6] P. T. Kroeger and R. A. Wingert, "Using zebrafish to study podocyte genesis during kidney development and regeneration," *Genesis*, vol. 52, no. 9, pp. 771–792, 2014.

[7] L. M. Chaible, M. A. Corat, E. Abdelhay, and M. L. Dagli, "Genetically modified animals for use in research and biotechnology," *Genetics and Molecular Research*, vol. 9, no. 3, pp. 1469–1482, 2010.

[8] N. Barker, J. H. van Es, J. Kuipers et al., "Identification of stem cells in small intestine and colon by marker gene Lgr5," *Nature*, vol. 449, no. 7165, pp. 1003–1007, 2007.

[9] P. Rompolas, K. R. Mesa, and V. Greco, "Spatial organization within a niche as a determinant of stem-cell fate," *Nature*, vol. 502, no. 7472, pp. 513–518, 2013.

[10] K. Kretzschmar and F. M. Watt, "Lineage tracing," *Cell*, vol. 148, no. 1-2, pp. 33–45, 2012.

[11] B. D. Humphreys and D. P. Dirocco, "Lineage-tracing methods and the kidney," *Kidney International*, vol. 86, pp. 481–488, 2014.

[12] R. H. Hoess, A. Wierzbicki, and K. Abremski, "The role of the loxP spacer region in P1 site-specific recombination," *Nucleic Acids Research*, vol. 14, no. 5, pp. 2287–2300, 1986.

[13] J. Livet, T. A. Weissman, H. Kang et al., "Transgenic strategies for combinatorial expression of fluorescent proteins in the nervous system," *Nature*, vol. 450, no. 7166, pp. 56–62, 2007.

[14] A. C. Rios, N. Y. Fu, G. J. Lindeman, and J. E. Visvader, "In situ identification of bipotent stem cells in the mammary gland," *Nature*, vol. 506, no. 7488, pp. 322–327, 2014.

[15] K. Malliaras, Y. Zhang, J. Seinfeld et al., "Cardiomyocyte proliferation and progenitor cell recruitment underlie therapeutic regeneration after myocardial infarction in the adult mouse heart," *EMBO Molecular Medicine*, vol. 5, no. 2, pp. 191–209, 2013.

[16] H. J. Snippert, L. G. van der Flier, T. Sato et al., "Intestinal crypt homeostasis results from neutral competition between symmetrically dividing Lgr5 stem cells," *Cell*, vol. 143, no. 1, pp. 134–144, 2010.

[17] L. Ritsma, S. I. J. Ellenbroek, A. Zomer et al., "Intestinal crypt homeostasis revealed at single-stem-cell level by *in vivo* live imaging," *Nature*, vol. 507, no. 7492, pp. 362–365, 2014.

[18] J. W. Pippin, P. T. Brinkkoetter, F. C. Cormack-Aboud et al., "Inducible rodent models of acquired podocyte diseases," *The American Journal of Physiology—Renal Physiology*, vol. 296, no. 2, pp. F213–F229, 2009.

[19] S. M. L. de Mik, M. J. Hoogduijn, R. W. de Bruin, and F. J. M. F. Dor, "Pathophysiology and treatment of focal segmental glomerulosclerosis: the role of animal models," *BMC Nephrology*, vol. 14, article 74, 2013.

[20] S. Kren and T. H. Hostetter, "The course of the remnant kidney model in mice," *Kidney International*, vol. 56, no. 1, pp. 333–337, 1999.

[21] L.-J. Ma and A. B. Fogo, "Model of robust induction of glomerulosclerosis in mice: importance of genetic background," *Kidney International*, vol. 64, no. 1, pp. 350–355, 2003.

[22] A. Hartner, N. Cordasic, B. Klanke, R. Veelken, and K. F. Hilgers, "Strain differences in the development of hypertension and glomerular lesions induced by deoxycorticosterone acetate salt in mice," *Nephrology Dialysis Transplantation*, vol. 18, no. 10, pp. 1999–2004, 2004.

[23] Q. Cao, Y. Wang, D. Zheng et al., "IL-10/TGF-$\beta$-modified macrophages induce regulatory T cells and protect against adriamycin nephrosis," *Journal of the American Society of Nephrology*, vol. 21, no. 6, pp. 933–942, 2010.

[24] V. W. S. Lee, Y. Wang, X. Qin et al., "Adriamycin nephropathy in severe combined immunodeficient (SCID) mice," *Nephrology Dialysis Transplantation*, vol. 21, no. 11, pp. 3293–3298, 2006.

[25] M. Jeansson, K. Björck, O. Tenstad, and B. Haraldsson, "Adriamycin alters glomerular endothelium to induce proteinuria," *Journal of the American Society of Nephrology*, vol. 20, no. 1, pp. 114–122, 2009.

[26] K. Hayashi, H. Sasamura, K. Ishiguro, Y. Sakamaki, T. Azegami, and H. Itoh, "Regression of glomerulosclerosis in response to transient treatment with angiotensin II blockers is attenuated by blockade of matrix metalloproteinase-2," *Kidney International*, vol. 78, no. 1, pp. 69–78, 2010.

[27] E. Heikkilä, J. Juhila, M. Lassila et al., "$\beta$-Catenin mediates adriamycin-induced albuminuria and podocyte injury in adult mouse kidneys," *Nephrology Dialysis Transplantation*, vol. 25, no. 8, pp. 2437–2446, 2010.

[28] J. Tangpong, S. Miriyala, T. Noel, C. Sinthupibulyakit, P. Jungsuwadee, and D. K. St Clair, "Doxorubicin-induced central nervous system toxicity and protection by xanthone derivative of *Garcinia mangostana*," *Neuroscience*, vol. 175, pp. 292–299, 2011.

[29] M. Ozeki, H. Nagasu, M. Satoh et al., "Reactive oxygen species mediate compensatory glomerular hypertrophy in rat uninephrectomized kidney," *The Journal of Physiological Sciences*, vol. 59, no. 5, pp. 397–404, 2009.

[30] G. Gluhovschi, F. Gadalean, C. Gluhovschi et al., "The solitary kidney—a nephrological perspective," *Romanian Journal of Internal Medicine*, vol. 51, no. 2, pp. 80–88, 2013.

[31] H. Weiher, T. Noda, D. A. Gray, A. H. Sharpe, and R. Jaenisch, "Transgenic mouse model of kidney disease: insertional inactivation of ubiquitously expressed gene leads to nephrotic syndrome," *Cell*, vol. 62, no. 3, pp. 425–434, 1990.

[32] J. B. Kopp, M. E. Klotman, S. H. Adler et al., "Progressive glomerulosclerosis and enhanced renal accumulation of basement membrane components in mice transgenic for human immunodeficiency virus type 1 genes," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 89, no. 5, pp. 1577–1581, 1992.

[33] G. R. Dressler, J. E. Wilkinson, U. W. Rothenpieler, L. T. Patterson, L. Williams-Simons, and H. Westphal, "Deregulation of Pax-2 expression in transgenic mice generates severe kidney abnormalities," *Nature*, vol. 362, no. 6415, pp. 65–67, 1993.

[34] J. B. Kopp and P. E. Klotman, "Transgenic animal models of renal development and pathogenesis," *The American Journal of Physiology—Renal Fluid and Electrolyte Physiology*, vol. 269, no. 5, part 2, pp. F601–F620, 1995.

[35] G. Kollias, D. J. Evans, M. Ritter, J. Beech, R. Morris, and F. Grosveld, "Ectopic expression of Thy-1 in the kidneys of transgenic mice induces functional and proliferative abnormalities," *Cell*, vol. 51, no. 1, pp. 21–31, 1987.

[36] K. J. M. Assmann, J. P. H. F. van Son, H. B. P. M. Dïjkman, S. Mentzel, and J. F. M. Wetzels, "Antibody-induced albuminuria and accelerated focal glomerulosclerosis in the Thy-1.1 transgenic mouse," *Kidney International*, vol. 62, no. 1, pp. 116–126, 2002.

[37] B. Smeets, H. B. P. M. Dijkman, N. A. J. M. te Loeke et al., "Podocyte changes upon induction of albuminuria in Thy-1.1 transgenic mice," *Nephrology Dialysis Transplantation*, vol. 18, no. 12, pp. 2524–2533, 2003.

[38] K. Kobayashi, S. Morita, H. Sawada et al., "Immunotoxin-mediated conditional disruption of specific neurons in transgenic mice," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 92, no. 4, pp. 1132–1136, 1995.

[39] D. Watanabe, H. Inokawa, K. Hashimoto et al., "Ablation of cerebellar Golgi cells disrupts synaptic integration involving GABA inhibition and NMDA receptor activation in motor coordination," *Cell*, vol. 95, no. 1, pp. 17–27, 1998.

[40] T. Matsusaka, J. Xin, S. Niwa et al., "Genetic engineering of glomerular sclerosis in the mouse *via* control of onset and

severity of podocyte-specific injury," *Journal of the American Society of Nephrology*, vol. 16, no. 4, pp. 1013–1023, 2005.

[41] L. Lasagni, L. Ballerini, M. L. Angelotti et al., "Notch activation differentially regulates renal progenitors proliferation and differentiation toward the podocyte lineage in glomerular disorders," *Stem Cells*, vol. 28, no. 9, pp. 1673–1685, 2010.

[42] R. J. Collier, "Understanding the mode of action of diphtheria toxin: a perspective on progress during the 20th century," *Toxicon*, vol. 39, no. 11, pp. 1793–1803, 2001.

[43] T. Honjo, Y. Nishizuka, I. Kato, and O. Hayaishi, "Adenosine diphosphate ribosylation of aminoacyl transferase II and inhibition of protein synthesis by diphtheria toxin," *Journal of Biological Chemistry*, vol. 246, no. 13, pp. 4251–4260, 1971.

[44] M. Saito, T. Iwawaki, C. Taya et al., "Diphtheria toxin receptor-mediated conditional and targeted cell ablation in transgenic mice," *Nature Biotechnology*, vol. 19, no. 8, pp. 746–750, 2001.

[45] M. L. Breitman, S. Clapoff, J. Rossant et al., "Genetic ablation: targeted expression of a toxin gene causes microphthalmia in transgenic mice," *Science*, vol. 238, no. 4833, pp. 1563–1565, 1987.

[46] R. D. Palmiter, R. R. Behringer, C. J. Quaife, F. Maxwell, I. H. Maxwell, and R. L. Brinster, "Cell lineage ablation in transgenic mice by cell-specific expression of a toxin gene," *Cell*, vol. 50, no. 3, pp. 435–443, 1987.

[47] D. Brockschnieder, C. Lappe-Siefke, S. Goebbels, M. R. Boesl, K.-A. Nave, and D. Riethmacher, "Cell depletion due to diphtheria toxin fragment A after Cre-mediated recombination," *Molecular and Cellular Biology*, vol. 24, no. 17, pp. 7636–7642, 2004.

[48] B. L. Wharram, M. Goyal, J. E. Wiggins et al., "Podocyte depletion causes glomerulosclerosis: diphtheria toxin-induced podocyte depletion in rats expressing human diphtheria toxin receptor transgene," *Journal of the American Society of Nephrology*, vol. 16, no. 10, pp. 2941–2952, 2005.

[49] Q. Jia, B. W. McDill, B. Sankarapandian et al., "Ablation of developing podocytes disrupts cellular interactions and nephrogenesis both inside and outside the glomerulus," *The American Journal of Physiology—Renal Physiology*, vol. 295, no. 6, pp. F1790–F1798, 2008.

[50] M. Yamaizumi, E. Mekada, T. Uchida, and Y. Okada, "One molecule of diphtheria toxin fragment A introduced into a cell can kill the cell," *Cell*, vol. 15, no. 1, pp. 245–250, 1978.

[51] T. Buch, F. L. Heppner, C. Tertilt et al., "A Cre-inducible diphtheria toxin receptor mediates cell lineage ablation after toxin administration," *Nature Methods*, vol. 2, no. 6, pp. 419–426, 2005.

[52] N. Wanner, B. Hartleben, N. Herbach et al., "Unraveling the role of podocyte turnover in glomerular aging and injury," *Journal of the American Society of Nephrology*, vol. 25, no. 4, pp. 707–716, 2014.

[53] D. Appel, D. B. Kershaw, B. Smeets et al., "Recruitment of podocytes from glomerular parietal epithelial cells," *Journal of the American Society of Nephrology*, vol. 20, no. 2, pp. 333–343, 2009.

[54] B. Smeets, C. Kuppe, E.-M. Sicking et al., "Parietal epithelial cells participate in the formation of sclerotic lesions in focal segmental glomerulosclerosis," *Journal of the American Society of Nephrology*, vol. 22, no. 7, pp. 1262–1274, 2011.

[55] B. Smeets, S. Uhlig, A. Fuss et al., "Tracing the origin of glomerular extracapillary lesions from parietal epithelial cells," *Journal of the American Society of Nephrology*, vol. 20, no. 12, pp. 2604–2615, 2009.

[56] K. Berger, K. Schulte, P. Boor et al., "The regenerative potential of parietal epithelial cells in adult mice," *Journal of the American Society of Nephrology*, vol. 25, no. 4, pp. 693–705, 2014.

[57] W. Kriz, "Podocyte hypertrophy mismatch and glomerular disease," *Nature Reviews Nephrology*, vol. 8, no. 11, pp. 618–619, 2012.

[58] J. E. Wiggins, M. Goyal, S. K. Sanden et al., "Podocyte hypertrophy, 'adaptation' and 'decompensation' associated with glomerular enlargement and glomerulosclerosis in the aging rat: prevention by calorie restriction," *Journal of the American Society of Nephrology*, vol. 16, no. 10, pp. 2953–2966, 2005.

[59] D. Zipori, "The nature of stem cells: state rather than entity," *Nature Reviews Genetics*, vol. 5, no. 11, pp. 873–878, 2004.

[60] B. Z. Stanger, "Cellular homeostasis and repair in the mammalian liver," *Annual Review of Physiology*, vol. 77, no. 1, pp. 179–200, 2015.

[61] M. J. Hackl, J. L. Burford, K. Villanueva et al., "Tracking the fate of glomerular epithelial cells in vivo using serial multiphoton imaging in new mouse models with fluorescent lineage tags," *Nature Medicine*, vol. 19, no. 12, pp. 1661–1666, 2013.

[62] E. B. Rankin, J. E. Tomaszewski, and V. H. Haase, "Renal cyst development in mice with conditional inactivation of the von Hippel-Lindau tumor suppressor," *Cancer Research*, vol. 66, no. 5, pp. 2576–2583, 2006.

[63] K. Sakamoto, T. Ueno, N. Kobayashi et al., "The direction and role of phenotypic transition between podocytes and parietal epithelial cells in focal segmental glomerulosclerosis," *The American Journal of Physiology—Renal Physiology*, vol. 306, no. 1, pp. F98–F104, 2014.

[64] J. W. Pippin, M. A. Sparks, S. T. Glenn et al., "Cells of renin lineage are progenitors of podocytes and parietal epithelial cells in experimental glomerular disease," *The American Journal of Pathology*, vol. 183, no. 2, pp. 542–557, 2013.

[65] F. Grahammer, N. Wanner, and T. B. Huber, "Podocyte regeneration: who can become a podocyte?" *American Journal of Pathology*, vol. 183, no. 2, pp. 333–335, 2013.

[66] J. W. Pippin, S. T. Glenn, R. D. Krofft et al., "Cells of renin lineage take on a podocyte phenotype in aging nephropathy," *American Journal of Physiology—Renal Physiology*, vol. 306, no. 10, pp. F1198–F1209, 2014.

[67] N. Perico, G. Remuzzi, and A. Benigni, "Aging and the kidney," *Current Opinion in Nephrology and Hypertension*, vol. 20, no. 3, pp. 312–317, 2011.

[68] J. Wiggins, "Podocytes and glomerular function with aging," *Seminars in Nephrology*, vol. 29, no. 6, pp. 587–593, 2009.

[69] M. C. Liebau, D. Lang, J. Böhm et al., "Functional expression of the renin-angiotensin system in human podocytes," *The American Journal of Physiology—Renal Physiology*, vol. 290, no. 3, pp. F710–F719, 2006.

[70] J. C. Q. Velez, A. M. Bland, J. M. Arthur, J. R. Raymond, and M. G. Janech, "Characterization of renin-angiotensin system enzyme activities in cultured mouse podocytes," *The American Journal of Physiology—Renal Physiology*, vol. 293, no. 1, pp. F398–F407, 2007.

[71] C. Starke, H. Betz, L. Hickmann et al., "Renin lineage cells repopulate the glomerular mesangium after injury," *Journal of the American Society of Nephrology*, vol. 26, no. 1, pp. 48–54, 2015.

[72] A. Peired, M. L. Angelotti, E. Ronconi et al., "Proteinuria impairs podocyte regeneration by sequestering retinoic acid,"

*Journal of the American Society of Nephrology*, vol. 24, no. 11, pp. 1756–1768, 2013.

[73] M. Yamamoto, N. A. Shook, O. Kanisicak et al., "A multifunctional reporter mouse line for Cre- and FLP-dependent lineage analysis," *Genesis*, vol. 47, no. 2, pp. 107–114, 2009.

[74] L. E. Fenno, J. Mattis, C. Ramakrishnan et al., "Targeting cells with single vectors using multiple-feature Boolean logic," *Nature Methods*, vol. 11, no. 7, pp. 763–772, 2014.

Hindawi Publishing Corporation
Stem Cells International
Volume 2015, Article ID 362562, 7 pages
http://dx.doi.org/10.1155/2015/362562

*Review Article*

# Human Urine as a Noninvasive Source of Kidney Cells

**Fanny Oliveira Arcolino,[1] Agnès Tort Piella,[2] Elli Papadimitriou,[3] Benedetta Bussolati,[3] Daniel J. Antonie,[2] Patricia Murray,[2] Lamberthus van den Heuvel,[1] and Elena Levtchenko[1]**

[1]*Department of Development and Regeneration, Catholic University Leuven, Herestraat 49, 3000 Leuven, Belgium*
[2]*Institute of Translational Medicine, University of Liverpool, Crown Street, Liverpool L69 3BX, UK*
[3]*Center for Molecular Biotechnology, Department of Molecular Biotechnology and Health Sciences, University of Turin, Via Nizza 52, 10126 Turin, Italy*

Correspondence should be addressed to Elena Levtchenko; elena.levtchenko@uzleuven.be

Received 7 November 2014; Accepted 3 December 2014

Academic Editor: Akito Maeshima



Urine represents an unlimited source of patient-specific kidney cells that can be harvested noninvasively. Urine derived podocytes and proximal tubule cells have been used to study disease mechanisms and to screen for novel drug therapies in a variety of human kidney disorders. The urinary kidney stem/progenitor cells and extracellular vesicles, instead, might be promising for therapeutic treatments of kidney injury. The greatest advantages of urine as a source of viable cells are the easy collection and less complicated ethical issues. However, extensive characterization and *in vivo* studies still have to be performed before the clinical use of urine-derived kidney progenitors.

## 1. Introduction

Currently, dialysis and kidney transplantation are the only successful therapies for patients suffering from chronic renal failure. Increasing shortage of donor organs for orthotopic kidney transplantation worldwide urges the need of alternative therapies. Using kidney progenitor cells might be an alternative approach of treatment in different kidney diseases [1]. Cells isolated from kidney tissue samples have the advantage of coming from a defined origin. However, they are only available in limited amounts and the life span of mature cells in culture is short, while repeated isolations of cells from the same donor are not allowed. On the other hand, kidney epithelia are exposed to continuous passage of filtrate, and thousands of living cells from healthy humans are excreted daily [2]. These exfoliated cells from urinary sediment can be isolated and cultured and include epithelial cells shed from different parts of the nephrons, ureters, bladder, and urethra [3] representing a limitless source of noninvasively harvested viable cells.

The main types of exfoliated kidney cells in urine demonstrated so far are podocytes, proximal tubular cells, and undifferentiated cells called kidney stem/progenitor cells. Extracellular vesicles are also present in urine and can be an interesting source for studying the disease mechanisms and prognosis, as well as a potential regenerative stimulus through their paracrine effect [4] (Table 1 and Figure 1).

The use of urinary cells entails less ethical concerns and, most importantly, reduces immune response and rejection when applied in an autologous manner. As for all types of cells, prior to clinical use, further studies need to be performed to improve the isolation, culture, and differentiation steps to deliver cells with consistent number, quality, and stability.

## 2. Differentiated Kidney Cells Isolated from Urine

*2.1. Podocytes.* Podocytes are mature epithelial cells with a complex cellular organization consisting of a cell body, major processes, and foot processes. Interdigitating foot processes of neighboring podocytes form the slit diaphragm, and together they cover the outer part of the glomerular basement membrane playing a major role in establishing

Table 1: Types of kidney cells exfoliated in urine and their current applications.

| Urine-derived kidney cell | Markers of disease activity | Disease modeling | Studying cell biology/physiology | Therapeutic effects |
|---|---|---|---|---|
| Podocytes | (i) Diabetic nephropathy [13, 17]<br>(ii) Membranous nephropathy [61]<br>(iii) Focal and segmental glomerulosclerosis [11]<br>(iv) Henoch-Schönlein nephritis<br>(v) IgA nephritis<br>(vi) Lupus nephritis [9, 15]<br>(vii) Preeclampsia [18]<br>(viii) D + HUS [62]<br>(ix) Diffuse mesangial sclerosis [63] | (i) Lupus nephritis [16] | (i) Characterization [22]<br>(ii) Function [64, 65] | Not studied |
| PTECs | (i) Acute tubular necrosis [66, 67]<br>(ii) Diabetes mellitus [67] | (i) Cystinosis [23, 24, 27]<br>(ii) Diabetes mellitus [67]<br>(iii) Hyperoxaluria [26]<br>(iv) Dent disease [29]<br>(v) Lowe syndrome [68] | (i) Characterization [30]<br>(ii) Function [28, 30, 69] | (i) Paracrine effects of conditioned medium [70] |
| Stem/ progenitors | Not studied | Not studied | (i) Characterization [37, 38, 42, 43, 71, 72] | (i) Differentiation into glomerular cells [38, 73]<br>(ii) Genitourinary tissue reconstruction [74–76]<br>(iii) Skeletal muscle regeneration [50, 77, 78]<br>(iv) Neurologic tissue reconstruction [79] |
| Extracellular vesicles | (i) Focal and segmental glomerulosclerosis [80, 81] | Not studied | (i) Characterization [53, 81–84]<br>(ii) Function [53] | (i) Kidney transplantation [57] |

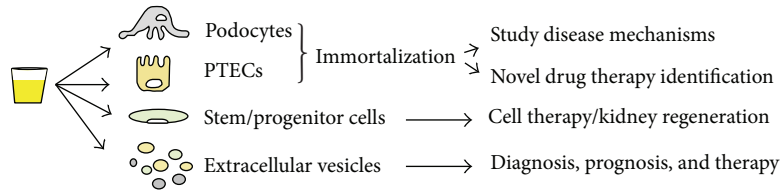



Figure 1: Urine as source of specific kidney cells and extracellular vesicles: applications and future perspectives.

the selective permeability of the glomerular filtration barrier, which explains why podocyte injury is typically associated with marked proteinuria [5].

Since the 70s different techniques have been used for the isolation of podocytes directly from the glomeruli [6, 7]. However, podocytes can also be isolated from human urine, both from healthy subjects or patients with glomerulopathies, representing a noninvasive source of viable cells [8]. The quantification of podocytes in urine can be performed by immunofluorescence using specific antibodies such as anti-podocalyxin [9–11] or by isolation of the podocyte specific mRNA products (e.g., podocin or nephrin) [12].

Usually, higher numbers of podocytes are found in urine of patients with glomerular diseases compared to healthy subjects and those cells show faster *ex vivo* proliferation rate [11, 13]. For example, in patients with focal and segmental glomerulosclerosis (FSGS), podocyte loss increases in accordance to the level of injury and might be a marker of disease progression [11, 14]. Besides FSGS, urinary podocytes have been detected during the acute phase of other diseases, such as Henoch-Schönlein nephritis [10], IgA nephropathy [10], lupus nephritis [9, 15, 16], and also diabetic nephropathy [17] and preeclampsia [18]. Moreover, it has been suggested that urinary excretion of podocytes might be helpful to discriminate between acute and chronic stages of glomerular damage [19].

The loss of podocytes in urine has been also demonstrated in healthy individuals [14]. Interestingly, in healthy subjects most of the shed podocytes are senescent, while in experimental or human disease conditions a lot of viable podocytes are excreted [20].

Because of the limited proliferation rate and short life span of podocytes in culture, the immortalization step is instrumental for their maintenance. In 2002, Saleem et al. [21] developed a human conditionally immortalized podocyte cell line expressing specifically nephrin and podocin and later, in 2010, Sakairi et al. [22] created long-term urinary cell cultures from FSGS patients and healthy volunteers, showing that

both cell lines present similar podocyte features [22]. These immortalized podocytes are transformed by insertion of a temperature-sensitive mutant of the proto-oncogene, SV40 large T antigen, so that they dedifferentiate and replicate under permissive conditions at 33°C, allowing unlimited turnover of cells, and regain a podocyte phenotype under nonpermissive conditions at 37°C [20].

*2.2. Proximal Tubule Epithelial Cells (PTECs).* The proximal tubules are the primary targets in numerous inherited and acquired conditions such as in ischemic or toxic kidney injury or genetic Fanconi syndromes [23].

In 1991, Racusen et al. [24] were the first to show that viable PTECs could be isolated from human urine of patients with nephropathic cystinosis, a lysosomal storage disease causing renal Fanconi syndrome. Dörrenhaus et al. [3], in 2000, described proximal tubules urine cell colonies designated as type-2 colonies with a cobblestone-like morphology. These cells were able to form domes caused by the transepithelial fluid transport from the medium to the area between the culture plate surface and the cells monolayer [3]. Later on, three-dimensional collagen gel cultures were established using PTECs from human urine to drive them to a highly polarised state. And because the cells still had some proliferative potential and became polarized, they were able to form organised structures resembling the *in vivo* tubules [25].

Interestingly, the number of urinary PTECs does not always correspond to the degree of kidney injury, as for example, in primary hyperoxaluria; a higher number of exfoliated PTECs in urine were not detected [26]. Anyhow, lots of viable proximal tubule cells are voided in human urine and they can be exploited for physiological studies such as analysing the transport of drugs and different substances in the proximal tubules. Again, the limitations of using PTECs in culture are the limited cells number and short life span that can be overcome by the conditional immortalization of the cells. The first immortalized proximal tubule cell line was generated by Racusen et al. in 1995 [27]. The next cell line was only established in 2010, when Wilmer et al. [28] developed conditionally immortalized PTECs from urine of healthy subjects. These cells expressed multiple endogenous organic ion transporters, mimicking renal reabsorption and excretion. Shortly afterwards, another conditionally immortalized PTECs line was established from the urine of cystinotic patients [23].

Conditionally immortalized PTECs are useful models to explore the mechanisms involved in specific proximal tubular renal pathologies. Gorvin et al. [29] have used urinary PTECs lines of patients with Dent's disease to study receptor mediated endocytosis and endosomal acidification depending on the type of mutation in the *CLCN5* gene causing this condition. Urinary PTECs have been also applied for *in vitro* studies of cell physiology and toxicology, including the influx and efflux of drugs [28], and might represent a promising step towards a bioartificial kidney device [30]. In 2004, an FDA-approved phase I/II clinical trial that was performed in 10 patients using PTECs harvested from human kidneys in a bioartificial kidney demonstrated that the addition of human PTECs to replacement therapy improves metabolic activity with systemic effects in patients with acute renal failure and multiorgan failure [31]. Later, in 2008 a phase II randomized trial using the same device with nonautologous PTECs showed more rapid recovery of kidney function in critically ill patients with acute renal failure [32]. Presenting similar genetic and functional characteristics as kidney harvested PTECs [30], the urinary cells might have great advantage of possible autologous cell therapy that has to be further evaluated.

## 3. Undifferentiated Kidney Cells Isolated from Urine

During nephrogenesis, stem/progenitor cells are located in the cap mesenchyme and behave as true committed stem cells, capable of self-renewing and differentiation into different types of nephron epithelia [33, 34]. These cells express specific renal progenitor cells markers as SIX2 [34], Cited1 [33], NCAM, ep-CAM, and FZD7 [35] and have been extensively characterized. As amniotic fluid (AF) is mainly composed of fetal urine and lung exudates [36], it is believed that some subpopulations of AF cells are of kidney origin and are committed to renal fates. Indeed, amniotic fluid is believed to be an important source of stem cells in an intermediate stage between embryonic stem cells and adult stem cells. Da Sacco et al. [37] have successfully isolated a subpopulation of metanephric mesenchyme-like cells from AF and later showed that these cells are committed to nephron lineages, being capable to differentiate into functional podocytes [38]. These cells represent a new model to study podocyte cell biology and development. Additionally, the isolated renal committed stem cells from AF can be an attractive source of cells to repair kidney injury.

It is known that, in humans, nephrogenesis is completed at about 34–36 weeks of gestational age. Thus, the presence of stem/progenitor cells in the adult human kidney is highly discussed, as well as their possible origins. Initially, CD133+ cells presenting progenitor cells characteristics were isolated from adult renal cortex [39]. Subsequently, these cells were found in different segments of the nephrons as the urinary pole of the Bowman's capsule [40], in the proximal tubules and the inner medullary papilla region, including Henle's loop and the S3 limb segment [41]. In agreement with the idea of an existent stem cell-like population in mature kidneys, it has been demonstrated that freshly voided urine [42] and urine from the upper urinary tract [43] contain stem cells capable to reconstruct urological tissues. Contrasting these results, other groups have shown that the repair of acute injured renal tubules does not involve specialized kidney progenitors [44] but occurs from resident differentiated tubular cells that had survived the injury and underwent dedifferentiation [45] in response to damaging factors that may give them a higher proliferation capacity, the ability to redifferentiate and reintegrate the injured site. Regarding podocyte regeneration, it has been shown using linage fate tracing that cells of renin lineage from juxta-glomerular apparatus might represent progenitor cells in glomerular disease [46, 47].

The effectiveness of kidney stem/progenitor cell transplantation in animal models of kidney injury has been described using tissue progenitor cells from embryonic [48] and adult kidneys [47, 49]. Human embryonic nephron progenitor NCAM+ cells were engrafted and integrated in diseased murine kidneys and had beneficial effects on renal function halting disease progression in the 5/6-nephrectomy kidney injury model [48]. The injection of adult kidney CD133+CD24+PDX− cells in an adriamycin-induced nephropathy mouse model showed reduced proteinuria and improved chronic glomerular damage [47], while CD133+CD24+CD106+ cells injected in SCID mouse with acute tubular injury were able to generate novel tubular cells and improve renal function [49]. Moreover, the therapeutic effect of urine-derived stem cells was tested on athymic mouse model and VEGF-expressing urine-derived stem cells combined with human umbilical venous endothelial cells were used for treating vesicoureteral reflux and stress urinary incontinence [50]. These cells have also been effective in the development of a multilayer mucosal structure similar to that of native urinary tract tissue when seeded on 3D porous small intestinal submucosa scaffold and may serve as an alternative cell source in cell-based tissue engineering [51]. However, the therapeutic potential of urinary KSPCs in renal injury still has to be studied.

Altogether, these studies confirm the importance of urine as a noninvasive source of viable cells with potential for regenerative medicine and tissue engineering, in addition to cytotoxicity and pharmacological studies.

## 4. Urinary Extracellular Vesicles (EVs)

Extracellular vesicles (EVs) are small particles (100–1000 nm) secreted by all types of cells under both physiological and pathological conditions. They are composed by a lipid bilayer, which encloses several cytoplasmic proteins, lipids as well as nucleic acids, comprising their biological "cargo" [52–54]. Due to their apparently important role in cell-cell communication, EVs have gained an increasing interest during the last decades whereas numerous studies demonstrate their isolation from various body fluids, including urine [53]. As the content of EVs may reflect both the cell of origin and its pathophysiological state, urinary EVs represent a unique source of information for diagnostic purposes and may possibly display therapeutic functions along with stem/progenitor cells.

Recent studies provide evidence that uEVs present in the preurine may transfer information within the nephron segments, thus representing a mechanism of intranephron communication [55, 56]. Urinary EVs derive from every epithelial cell of the kidney, including renal progenitor cells. In this regard, Dimuccio et al. showed absence of CD133+ urinary EVs in patients suffering from end stage kidney disease, underlying the possible exhaustion of CD133+ progenitors in these patients [57]. Moreover, lower levels of CD133+ uEVs were present in delayed graft compared to early graft recovery, suggesting a possible correlation between levels of CD133+ vesicles in urine and the renal homeostasis or recovery after injury [57].

Finally, EVs are known to recapitulate the therapeutic effect of stem cells, due to their paracrine effects, resulting in a horizontal transfer of mRNA, microRNA, and proteins [52, 58–60]. In particular, mesenchymal stem cell-derived EVs stimulated proliferation and apoptosis resistance of tubular epithelial cells *in vitro* [59] and accelerated the morphological and functional recovery *in vivo* in different experimental animal models of renal injury [58, 60]. Within the urinary EVs, it is therefore possible that progenitor-derived EVs may be involved in local paracrine effect on neighboring cells, directing differentiation or regenerative programs.

Therefore, considering that every epithelial cell of the kidney may secret vesicles into the urinary space, urinary EVs may be used as markers of prognosis, diagnosis, and therapy of several kidney diseases.

## 5. Advantages, Limitations, and Future Challenges

The use of urine as source of kidney cells has great advantages compared to tissue harvesting due to the noninvasive methods of collection; it raises less ethical concerns, once urine is a excreted product of the body; many samples can be collected from the same individual, allowing investigation of disease progression and its treatments; cells could be used for autologous therapy avoiding immune rejection after transplantation due to antigenic differences; and very importantly, the cells collected in urine are viable, are able to proliferate in culture, and present similar features of cells harvested from kidney tissue.

However, culture of kidney cells presents some limitations because of the maturity of the cells. If urinary kidney stem/progenitor cells were able to differentiate into fully mature and functional kidney cells, they could overcome this problem.

In the future though, human renal cells isolated from urine might play a role in tissue engineering for personalized medicine in patients suffering from nephropathies or chronic renal disease. Bioartificial kidneys in combination with autologous kidney cells could help to improve kidney function and its outcomes. However before that, a full characterization and a rigorous selection of the cells should be done for safe implementation in clinical applications.

## Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

## Authors' Contribution

Fanny Oliveira Arcolino and Agnès Tort Piella contributed equally to the work.

## Acknowledgments

The authors acknowledge funding support from the FP7 NephroTools ITN (289754). Elena Levtchenko is supported

by the Research Foundation-Flanders (F.W.O. Vlaanderen), Grant 1801110N.

## References


[1] B. Dekel and Y. Reisner, "Engraftment of human early kidney precursors," *Transplant Immunology*, vol. 12, no. 3-4, pp. 241–247, 2004.

[2] L. F. Prescott, "The normal urinary excretion rates of renal tubular cells, leucocytes and red blood cells," *Clinical Science*, vol. 31, no. 3, pp. 425–435, 1966.

[3] A. Dörrenhaus, J. I. F. Müller, K. Golka, P. Jedrusik, H. Schulze, and W. Föllmann, "Cultures of exfoliated epithelial cells from different locations of the human urinary tract and the renal tubular system," *Archives of Toxicology*, vol. 74, no. 10, pp. 618–626, 2000.

[4] K. Sabin and N. Kikyo, "Microvesicles as mediators of tissue regeneration," *Translational Research*, vol. 163, no. 4, pp. 286–295, 2014.

[5] A. Greka and P. Mundel, "Cell biology and pathology of podocytes," *Annual Review of Physiology*, vol. 74, pp. 299–323, 2012.

[6] H. Burlington and E. P. Cronkite, "Characteristics of cell cultures derived from renal glomeruli," *Proceedings of the Society for Experimental Biology and Medicine*, vol. 142, no. 1, pp. 143–149, 1973.

[7] R. J. Quigg, A. V. Cybulsky, J. B. Jacobs, and D. J. Salant, "Anti-Fx1A produces complement-dependent cytotoxicity of glomerular epithelial cells," *Kidney International*, vol. 34, no. 1, pp. 43–52, 1988.

[8] P. Mundel, J. Reiser, and W. Kriz, "Induction of differentiation in cultured rat and human podocytes," *Journal of the American Society of Nephrology*, vol. 8, no. 5, pp. 697–705, 1997.

[9] T. Nakamura, C. Ushiyama, S. Suzuki et al., "Urinary podocytes for the assessment of disease activity in lupus nephritis," *The American Journal of the Medical Sciences*, vol. 320, no. 2, pp. 112–116, 2000.

[10] M. Hara, T. Yanagihara, T. Takada et al., "Urinary excretion of podocytes reflects disease activity in children with glomerulonephritis," *The American Journal of Nephrology*, vol. 18, no. 1, pp. 35–41, 1998.

[11] M. Hara, T. Yanagihara, and I. Kihara, "Urinary podocytes in primary focal segmental glomerulosclerosis," *Nephron*, vol. 89, no. 3, pp. 342–347, 2001.

[12] Y. Sato, B. L. Wharram, K. L. Sang et al., "Urine podocyte mRNAs mark progression of renal disease," *Journal of the American Society of Nephrology*, vol. 20, no. 5, pp. 1041–1052, 2009.

[13] A. L. Al-Malki, "Assessment of urinary osteopontin in association with podocyte for early predication of nephropathy in diabetic patients," *Disease Markers*, vol. 2014, Article ID 493736, 5 pages, 2014.

[14] S. U. Vogelmann, W. J. Nelson, B. D. Myers, and K. V. Lemley, "Urinary excretion of viable podocytes in health and renal disease," *American Journal of Physiology: Renal Physiology*, vol. 285, no. 1, pp. F40–F48, 2003.

[15] J. J. Bollain-Y-Goytia, M. González-Castañeda, F. Torres-Del-Muro et al., "Increased excretion of urinary podocytes in lupus nephritis," *Indian Journal of Nephrology*, vol. 21, no. 3, pp. 166–171, 2011.

[16] T. Nakamura, C. Ushiyama, N. Shimada et al., "Effect of cyclophosphamide or azathioprine on urinary podocytes in patients with diffuse proliferative lupus nephritis," *Nephron*, vol. 87, no. 2, pp. 192–193, 2001.

[17] T. Nakamura, C. Ushiyama, S. Suzuki et al., "Urinary excretion of podocytes in patients with diabetic nephropathy," *Nephrology Dialysis Transplantation*, vol. 15, no. 9, pp. 1379–1383, 2000.

[18] V. D. Garovic, S. J. Wagner, S. T. Turner et al., "Urinary podocyte excretion as a marker for preeclampsia," *American Journal of Obstetrics & Gynecology*, vol. 196, no. 4, pp. 320.e1–320.e7, 2007.

[19] D. Yu, A. Petermann, U. Kunter, S. Rong, S. J. Shankland, and J. Floege, "Urinary podocyte loss is a more specific marker of ongoing glomerular damage than proteinuria," *Journal of the American Society of Nephrology*, vol. 16, no. 6, pp. 1733–1741, 2005.

[20] L. Ni, M. Saleem, and P. W. Mathieson, "Podocyte culture: tricks of the trade," *Nephrology*, vol. 17, no. 6, pp. 525–531, 2012.

[21] M. A. Saleem, M. J. O'Hare, J. Reiser et al., "A conditionally immortalized human podocyte cell line demonstrating nephrin and podocin expression," *Journal of the American Society of Nephrology*, vol. 13, no. 3, pp. 630–638, 2002.

[22] T. Sakairi, Y. Abe, H. Kajiyama et al., "Conditionally immortalized human podocyte cell lines established from urine," *The American Journal of Physiology—Renal Physiology*, vol. 298, no. 3, pp. F557–F567, 2010.

[23] K. Peeters, M. J. Wilmer, J. P. Schoeber et al., "Role of P-glycoprotein expression and function in cystinotic renal proximal tubular cells," *Pharmaceutics*, vol. 3, no. 4, pp. 782–792, 2011.

[24] L. C. Racusen, B. A. Fivush, H. Andersson, and W. A. Gahl, "Culture of renal tubular cells from the urine of patients with nephropathic cystinosis," *Journal of the American Society of Nephrology*, vol. 1, no. 8, pp. 1028–1033, 1991.

[25] C. N. Inoue, N. Sunagawa, T. Morimoto et al., "Reconstruction of tubular structures in three-dimensional collagen gel culture using proximal tubular epithelial cells voided in human urine," *In Vitro Cellular & Developmental Biology—Animal*, vol. 39, no. 8-9, pp. 364–367, 2003.

[26] K. L. Price, S.-A. Hulton, W. G. van'T Hoff, J. R. Masters, and G. Rumsby, "Primary cultures of renal proximal tubule cells derived from individuals with primary hyperoxaluria," *Urological Research*, vol. 37, no. 3, pp. 127–132, 2009.

[27] L. C. Racusen, P. D. Wilson, P. A. Hartz, B. A. Fivush, C. R. Burrow, and E. T. Philip, "Renal proximal tubular epithelium from patients with nephropathic cystinosis: immortalized cell lines as in vitro model systems," *Kidney International*, vol. 48, no. 2, pp. 536–543, 1995.

[28] M. J. Wilmer, M. A. Saleem, R. Masereeuw et al., "Novel conditionally immortalized human proximal tubule cell line expressing functional influx and efflux transporters," *Cell and Tissue Research*, vol. 339, no. 2, pp. 449–457, 2010.

[29] C. M. Gorvin, M. J. Wilmer, S. E. Piret et al., "Receptor-mediated endocytosis and endosomal acidification is impaired in proximal tubule epithelial cells of Dent disease patients," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 110, no. 17, pp. 7014–7019, 2013.

[30] J. Jansen, C. M. S. Schophuizen, M. J. Wilmer et al., "A morphological and functional comparison of proximal tubule cell lines established from human urine and kidney tissue," *Experimental Cell Research*, vol. 323, no. 1, pp. 87–99, 2014.

[31] H. D. Humes, W. F. Weitzel, R. H. Bartlett et al., "Initial clinical results of the bioartificial kidney containing human cells in ICU

patients with acute renal failure," *Kidney International*, vol. 66, no. 4, pp. 1578–1588, 2004.

[32] J. Tumlin, R. Wali, W. Williams et al., "Efficacy and safety of renal tubule cell therapy for acute renal failure," *Journal of the American Society of Nephrology*, vol. 19, no. 5, pp. 1034–1040, 2008.

[33] S. Boyle, A. Misfeldt, K. J. Chandler et al., "Fate mapping using Cited1-CreERT2 mice demonstrates that the cap mesenchyme contains self-renewing progenitor cells and gives rise exclusively to nephronic epithelia," *Developmental Biology*, vol. 313, no. 1, pp. 234–245, 2008.

[34] M. Self, O. V. Lagutin, B. Bowling et al., "Six2 is required for suppression of nephrogenesis and progenitor renewal in the developing kidney," *The EMBO Journal*, vol. 25, no. 21, pp. 5214–5228, 2006.

[35] S. Metsuyanim, O. Harari-Steinberg, E. Buzhor et al., "Expression of stem cell markers in the human fetal kidney," *PLoS ONE*, vol. 4, no. 8, Article ID e6709, 2009.

[36] M. A. Underwood, W. M. Gilbert, and M. P. Sherman, "Amniotic fluid: not just fetal urine anymore," *Journal of Perinatology*, vol. 25, no. 5, pp. 341–348, 2005.

[37] S. Da Sacco, S. Sedrakyan, F. Boldrin et al., "Human amniotic fluid as a potential new source of organ specific precursor cells for future regenerative medicine applications," *The Journal of Urology*, vol. 183, no. 3, pp. 1193–1200, 2010.

[38] S. da Sacco, K. V. Lemley, S. Sedrakyan et al., "A novel source of cultured podocytes," *PLoS ONE*, vol. 8, no. 12, Article ID e81812, 2013.

[39] B. Bussolati, S. Bruno, C. Grange et al., "Isolation of renal progenitor cells from adult human kidney," *The American Journal of Pathology*, vol. 166, no. 2, pp. 545–555, 2005.

[40] C. Sagrinati, G. S. Netti, B. Mazzinghi et al., "Isolation and characterization of multipotent progenitor cells from the Bowman's capsule of adult human kidneys," *Journal of the American Society of Nephrology*, vol. 17, no. 9, pp. 2443–2456, 2006.

[41] B. Bussolati, A. Moggio, F. Collino et al., "Hypoxia modulates the undifferentiated phenotype of human renal inner medullary CD133$^+$ progenitors through Oct4/miR-145 balance," *The American Journal of Physiology—Renal Physiology*, vol. 302, no. 1, pp. 116–128, 2012.

[42] Y. Zhang, E. McNeill, H. Tian et al., "Urine derived cells are a potential source for urological tissue reconstruction," *The Journal of Urology*, vol. 180, no. 5, pp. 2226–2233, 2008.

[43] S. Bharadwaj, G. Liu, Y. Shi et al., "Characterization of urine-derived stem cells obtained from upper urinary tract for use in cell-based urological tissue engineering," *Tissue Engineering Part A*, vol. 17, no. 15-16, pp. 2123–2132, 2011.

[44] B. D. Humphreys, S. Czerniak, D. P. DiRocco, W. Hasnain, R. Cheema, and J. V. Bonventre, "Repair of injured proximal tubule does not involve specialized progenitors," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 108, no. 22, pp. 9226–9231, 2011.

[45] T. Kusaba, M. Lalli, R. Kramann, A. Kobayashi, and B. D. Humphreys, "Differentiated kidney epithelial cells repair injured proximal tubule," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 111, no. 4, pp. 1527–1532, 2014.

[46] J. W. Pippin, M. A. Sparks, S. T. Glenn et al., "Cells of renin lineage are progenitors of podocytes and parietal epithelial cells in experimental glomerular disease," *The American Journal of Pathology*, vol. 183, no. 2, pp. 542–557, 2013.

[47] E. Ronconi, C. Sagrinati, M. L. Angelotti et al., "Regeneration of glomerular podocytes by human renal progenitors," *Journal of the American Society of Nephrology*, vol. 20, no. 2, pp. 322–332, 2009.

[48] O. Harari-Steinberg, S. Metsuyanim, D. Omer et al., "Identification of human nephron progenitors capable of generation of kidney structures and functional repair of chronic renal disease," *EMBO Molecular Medicine*, vol. 5, no. 10, pp. 1556–1568, 2013.

[49] M. L. Angelotti, E. Ronconi, L. Ballerini et al., "Characterization of renal progenitors committed toward tubular lineage and their regenerative potential in renal tubular injury," *Stem Cells*, vol. 30, no. 8, pp. 1714–1725, 2012.

[50] G. Liu, X. Wang, X. Sun, C. Deng, A. Atala, and Y. Zhang, "The effect of urine-derived stem cells expressing VEGF loaded in collagen hydrogels on myogenesis and innervation following after subcutaneous implantation in nude mice," *Biomaterials*, vol. 34, no. 34, pp. 8617–8629, 2013.

[51] S. Wu, Y. Liu, S. Bharadwaj, A. Atala, and Y. Zhang, "Human urine-derived stem cells seeded in a modified 3D porous small intestinal submucosa scaffold for urethral tissue engineering," *Biomaterials*, vol. 32, no. 5, pp. 1317–1326, 2011.

[52] L. Biancone, S. Bruno, M. C. Deregibus, C. Tetta, and G. Camussi, "Therapeutic potential of mesenchymal stem cell-derived microvesicles," *Nephrology Dialysis Transplantation*, vol. 27, no. 8, pp. 3037–3042, 2012.

[53] F. T. Borges, L. A. Reis, and N. Schor, "Extracellular vesicles: structure, function, and potential clinical uses in renal diseases," *Brazilian Journal of Medical and Biological Research*, vol. 46, no. 10, pp. 824–830, 2013.

[54] G. Camussi, M.-C. Deregibus, S. Bruno, C. Grange, V. Fonsato, and C. Tetta, "Exosome/microvesicle-mediated epigenetic reprogramming of cells," *American Journal of Cancer Research*, vol. 1, no. 1, pp. 98–110, 2011.

[55] J. M. Street, W. Birkhoff, R. I. Menzies, D. J. Webb, M. A. Bailey, and J. W. Dear, "Exosomal transmission of functional aquaporin 2 in kidney cortical collecting duct cells," *The Journal of Physiology*, vol. 589, part 24, pp. 6119–6127, 2011.

[56] J. J. Gildea, J. E. Seaton, K. G. Victor et al., "Exosomal transfer from human renal proximal tubule cells to distal tubule and collecting duct cells," *Clinical Biochemistry*, vol. 47, no. 15, pp. 89–94, 2014.

[57] V. Dimuccio, A. Ranghino, L. P. Barbato et al., "Urinary CD133+ extracellular vesicles are decreased in kidney transplanted patients with slow graft function and vascular damage," *PloS ONE*, vol. 9, no. 8, Article ID e104490, 2014.

[58] S. Bruno, C. Grange, F. Collino et al., "Microvesicles derived from mesenchymal stem cells enhance survival in a lethal model of acute kidney injury," *PLoS ONE*, vol. 7, no. 3, Article ID e33115, 2012.

[59] S. Bruno, C. Grange, M. C. Deregibus et al., "Mesenchymal stem cell-derived microvesicles protect against acute tubular injury," *Journal of the American Society of Nephrology*, vol. 20, no. 5, pp. 1053–1067, 2009.

[60] S. Gatti, S. Bruno, M. C. Deregibus et al., "Microvesicles derived from human adult mesenchymal stem cells protect against ischaemia-reperfusion-induced acute and chronic kidney injury," *Nephrology Dialysis Transplantation*, vol. 26, no. 5, pp. 1474–1483, 2011.

[61] A. T. Petermann, R. Krofft, M. Blonski et al., "Podocytes that detach in experimental membranous nephropathy are viable," *Kidney International*, vol. 64, no. 4, pp. 1222–1231, 2003.

[62] L. de Petris, J. Patrick, E. Christen, and H. Trachtman, "Urinary podocyte mRNA excretion in children with D+HUS: a potential marker of long-term outcome," *Renal Failure*, vol. 28, no. 6, pp. 475–482, 2006.

[63] Y. Ikezumi, T. Suzuki, T. Karasawa et al., "Glomerular epithelial cell phenotype in diffuse mesangial sclerosis: a report of 2 cases with markedly increased urinary podocyte excretion," *Human Pathology*, vol. 45, no. 8, pp. 1778–1783, 2014.

[64] J. Eyre, K. Ioannou, B. D. Grubb et al., "Statin-sensitive endocytosis of albumin by glomerular podocytes," *American Journal of Physiology: Renal Physiology*, vol. 292, no. 2, pp. F674–F681, 2007.

[65] E. Dobrinskikh, K. Okamura, J. B. Kopp, R. B. Doctor, and J. Blaine, "Human podocytes perform polarized, caveolae-dependent albumin endocytosis," *American Journal of Physiology—Renal Physiology*, vol. 306, no. 9, pp. F941–F951, 2014.

[66] L. C. Racusen, B. A. Fivush, Y.-L. Li et al., "Dissociation of tubular cell detachment and tubular cell death in clinical and experimental "acute tubular necrosis"," *Laboratory Investigation*, vol. 64, no. 4, pp. 546–556, 1991.

[67] C. J. Detrisac, R. K. Mayfield, J. A. Colwell, A. J. Garvin, and D. A. Sens, "In vitro culture of cells exfoliated in the urine by patents with diabetes mellitus," *Journal of Clinical Investigation*, vol. 71, no. 1, pp. 170–173, 1983.

[68] M. Vicinanza, A. Di Campli, E. Polishchuk et al., "OCRL controls trafficking through early endosomes via PtdIns4,5P 2-dependent regulation of endosomal actin," *The EMBO Journal*, vol. 30, no. 24, pp. 4970–4985, 2011.

[69] C. N. Inoue, Y. Kondo, S. Ohnuma, T. Morimoto, T. Nishio, and K. Iinuma, "Use of cultured tubular cells isolated from human urine for investigation of renal transporter," *Clinical Nephrology*, vol. 53, no. 2, pp. 90–98, 2000.

[70] R. Moghadasali, H. A. M. Mutsaers, M. Azarnia et al., "Mesenchymal stem cell-conditioned medium accelerates regeneration of human renal proximal tubule epithelial cells after gentamicin toxicity," *Experimental and Toxicologic Pathology*, vol. 65, no. 5, pp. 595–600, 2013.

[71] S. Bharadwaj, G. Liu, Y. Shi et al., "Multipotential differentiation of human urine-derived stem cells: potential for therapeutic applications in urology," *Stem Cells*, vol. 31, no. 9, pp. 1840–1856, 2013.

[72] R. Lang, G. Liu, Y. Shi et al., "Self-renewal and differentiation capacity of urine-derived stem cells after urine preservation for 24 hours," *PLoS ONE*, vol. 8, no. 1, Article ID e53980, 2013.

[73] E. Lazzeri, C. Crescioli, E. Ronconi et al., "Regenerative potential of embryonic renal multipotent progenitors in acute renal failure," *Journal of the American Society of Nephrology*, vol. 18, no. 12, pp. 3128–3138, 2007.

[74] S. Wu, Z. Wang, S. Bharadwaj, S. J. Hodges, A. Atala, and Y. Zhang, "Implantation of autologous urine derived stem cells expressing vascular endothelial growth factor for potential use in genitourinary reconstruction," *The Journal of Urology*, vol. 186, no. 2, pp. 640–647, 2011.

[75] A. Bodin, S. Bharadwaj, S. Wu, P. Gatenholm, A. Atala, and Y. Zhang, "Tissue-engineered conduit using urine-derived stem cells seeded bacterial cellulose polymer in urinary reconstruction and diversion," *Biomaterials*, vol. 31, no. 34, pp. 8889–8901, 2010.

[76] B. Ouyang, X. Sun, D. Han et al., "Human urine-derived stem cells alone or genetically-modified with FGF2 improve type 2 diabetic erectile dysfunction in a rat model," *PLoS ONE*, vol. 9, no. 3, Article ID e92825, 2014.

[77] W. Chen, M. Xie, B. Yang et al., "Skeletal myogenic differentiation of human urine-derived cells as a potential source for skeletal muscle regeneration," *Journal of Tissue Engineering and Regenerative Medicine*, 2014.

[78] G. Liu, R. A. Pareta, R. Wu et al., "Skeletal myogenic differentiation of urine-derived stem cells and angiogenesis using microbeads loaded with growth factors," *Biomaterials*, vol. 34, no. 4, pp. 1311–1326, 2013.

[79] J. J. Guan, X. Niu, F. X. Gong et al., "Biological characteristics of human-urine-derived stem cells: potential for cell-based therapy in neurology," *Tissue Engineering Part A*, vol. 20, no. 13-14, pp. 1794–1806, 2014.

[80] H. Zhou, A. Cheruvanky, X. Hu et al., "Urinary exosomal transcription factors, a new class of biomarkers for renal disease," *Kidney International*, vol. 74, no. 5, pp. 613–621, 2008.

[81] S. Keller, C. Rupp, A. Stoeck et al., "CD24 is a marker of exosomes secreted into urine and amniotic fluid," *Kidney International*, vol. 72, no. 9, pp. 1095–1102, 2007.

[82] M. C. Hogan, K. L. Johnson, R. M. Zenka et al., "Subfractionation, characterization, and in-depth proteomic analysis of glomerular membrane vesicles in human urine," *Kidney International*, vol. 85, no. 5, pp. 1225–1237, 2014.

[83] T. Pisitkun, R.-F. Shen, and M. A. Knepper, "Identification and proteomic profiling of exosomes in human urine," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 101, no. 36, pp. 13368–13373, 2004.

[84] P. A. Gonzales, T. Pisitkun, J. D. Hoffert et al., "Large-scale proteomics and phosphoproteomics of urinary exosomes," *Journal of the American Society of Nephrology*, vol. 20, no. 2, pp. 363–379, 2009.

Hindawi Publishing Corporation
Stem Cells International
Volume 2015, Article ID 391043, 11 pages
http://dx.doi.org/10.1155/2015/391043

*Research Article*

# WNT/$\beta$-Catenin Signaling Is Required for Integration of $CD_{24+}$ Renal Progenitor Cells into Glycerol-Damaged Adult Renal Tubules

**Zhao Zhang,[1] Diana M. Iglesias,[1] Rachel Corsini,[1] LeeLee Chu,[1] and Paul Goodyer[1,2]**

[1]*Department of Paediatrics, Montreal Children's Hospital Research Institute, McGill University, Montreal, QC, Canada*
[2]*Department of Human Genetics, Montreal Children's Hospital Research Institute, McGill University, Montreal, QC, Canada*

Correspondence should be addressed to Paul Goodyer; paul.goodyer@mcgill.ca

Received 5 November 2014; Accepted 5 January 2015

Academic Editor: Laura Lasagni



During development, nephron progenitor cells (NPC) are induced to differentiate by WNT9b signals from the ureteric bud. Although nephrogenesis ends in the perinatal period, acute kidney injury (AKI) elicits repopulation of damaged nephrons. Interestingly, embryonic NPC infused into adult mice with AKI are incorporated into regenerating tubules. Since WNT/$\beta$-catenin signaling is crucial for primary nephrogenesis, we reasoned that it might also be needed for the endogenous repair mechanism and for integration of exogenous NPC. When we examined glycerol-induced AKI in adult mice bearing a $\beta$-catenin/TCF reporter transgene, endogenous tubular cells reexpressed the NPC marker, CD24, and showed widespread $\beta$-catenin/TCF signaling. We isolated $CD_{24+}$ cells from E15 kidneys of mice with the canonical WNT signaling reporter. 40% of cells responded to WNT3a *in vitro* and when infused into glycerol-injured adult, the cells exhibited $\beta$-catenin/TCF reporter activity when integrated into damaged tubules. When embryonic $CD_{24+}$ cells were treated with a $\beta$-catenin/TCF pathway inhibitor (IWR-1) prior to infusion into glycerol-injured mice, tubular integration of cells was sharply reduced. Thus, the endogenous canonical $\beta$-catenin/TCF pathway is reactivated during recovery from AKI and is required for integration of exogenous embryonic renal progenitor cells into damaged tubules. These events appear to recapitulate the WNT-dependent inductive process which drives primary nephrogenesis.

## 1. Introduction

During early mammalian embryogenesis, uncommitted mesenchymal stem cells that will form the metanephric kidney downregulate genes marking pluripotentiality (e.g., *Oct4*, *Nanog*) and begin to express a transcription factor, OSR1, that specifies the pool of renal progenitor cells (RPC) in intermediate mesoderm [1]. Fate-mapping studies in embryonic mice suggest that, by E9, some RPC give rise to the nephric ducts and their derivative ureteric buds [1]; others, expressing WT1, are committed to a nephron progenitor cell (NPC) phenotype and are primed for responsiveness to the inductive WNT signal. After embryonic day E11.5, WNT9b signals from the arborizing ureteric bud (UB) begin to induce NPC to cluster at the tip of each UB branch tip, exhibit robust $\beta$-catenin/TCF pathway signaling activity [2, 3], and then differentiate into epithelial cells of the emerging nephrons. WNT9b activation of the canonical WNT signaling pathway is essential for induction of RPC; ectopic WNT9b can substitute experimentally for the UB signal [3]. At the S-shaped body stage, canonical $\beta$-catenin/TCF signaling activity is sustained by autocrine expression of WNT4 in the differentiating cells [3], but pathway activity is downregulated as nephrons undergo terminal differentiation and renal development comes to an end [2].

Sagrinati et al. used the stem cell surface marker CD24 to track the fate of RPC in adult human and mouse kidney [4]. They found $CD_{24+}$ cells both in the nephrogenic mesenchyme and in the ureteric bud, paralleling fate-mapping studies by Mugford which showed that a common early progenitor pool of *Osr1* (+) cells gives rise to both lineages [1]. In embryonic human kidney, $CD_{24+}$ cells acquire CD133 at the cell surface when committed to the NPC phenotype. CD24/CD133 expression is sustained in nephrogenic cells of the early renal

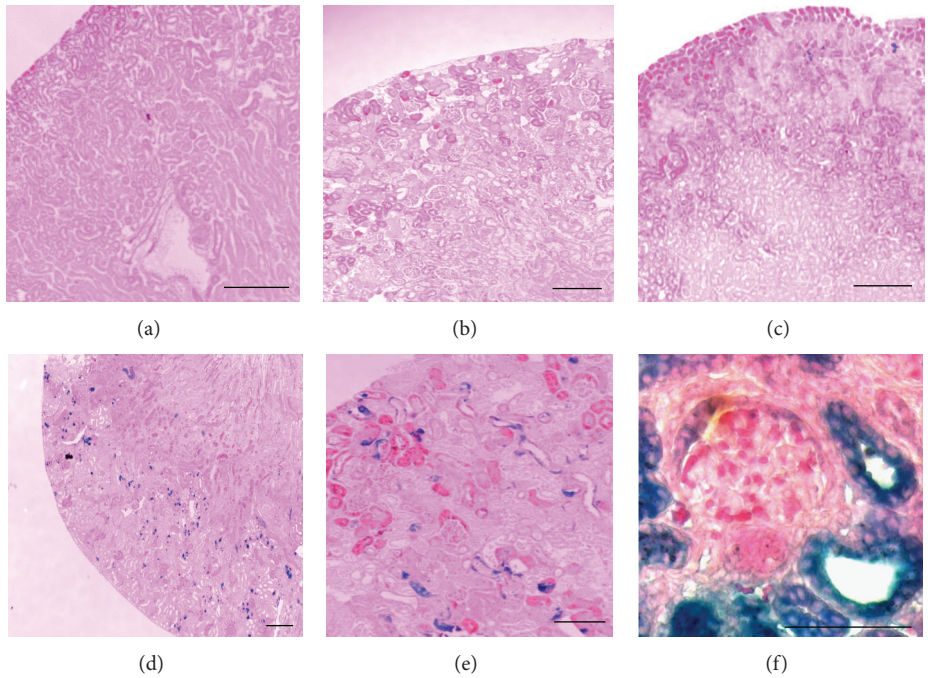



Figure 1: Endogenous $\beta$-catenin/TCF reporter transgene activity following glycerol-induced renal tubular injury in adult mice. (a and b) No $\beta$-galactosidase signal is seen in uninjured or glycerol-treated mouse kidneys of CD1 wild-type mice. (c) $\beta$-galactosidase signaling is minimal in the uninjured $\beta$-catenin/TCF reporter mouse kidney. (d–f) Strong $\beta$-catenin/TCF reporter activity (X-Gal, blue) is seen in renal tubules of adult mouse kidney 3 days after glycerol-induced renal injury. Scale bars: 50 $\mu$m.

vesicle and S-shaped body [5]. The two surface antigens mark 35–50% of all cells in the human fetal kidney at 8-9-week gestation but only 10–20% of kidney cells by 12–17 weeks [6]. Interestingly, Lazzeri et al. isolated $CD_{24+}/CD_{133+}$ cells from human embryonic kidney and infused them into SCID mice at the peak of glycerol-induced acute kidney injury; this improved renal function and improved renal histology compared to controls (Lazzeri). Remarkably, after 2 weeks, labeled exogenous NPC constituted about 15% of proximal tubular cells and were continuing to proliferate within the tubular wall [6].

The mechanism by which exogenous embryonic RPC are integrated into the damaged adult renal tubule is unknown. However, we reasoned that the process might recapitulate events during kidney development and might also be related to the endogenous tubular repair mechanism in adult kidney. If so, regenerative events must recruit the canonical WNT/$\beta$-catenin/TCF signaling pathway that is crucial for primary nephrogenesis. To test this hypothesis, we induced glycerol-mediated proximal tubular injury in adult mice and then examined $\beta$-catenin/TCF signaling in endogenous cells and in infused exogenous embryonic nephron progenitor cells during their integration into damaged tubules.

## 2. Results

*2.1. A Subset of Endogenous Renal Cells Reactivate Canonical β-Catenin/TCF Signaling after Glycerol-Induced Tubular Injury.* During recovery from glycerol-induced injury, damaged proximal tubules are repopulated by proliferating endogenous cells. To ascertain whether this endogenous repair process engages the canonical WNT signaling pathway, we induced proximal tubular injury with 50% glycerol (8 $\mu$L/g i.m.) in adult mice bearing a $\beta$-catenin/TCF-lacZ reporter transgene or wild-type CD1 mice [2]. No $\beta$-galactosidase signal was seen in uninjured (Figure 1(a)) or glycerol-treated (Figure 1(b)) wild-type mouse kidney or in uninjured $\beta$-catenin/TCF-lacZ reporter mice (Figure 1(c)) after three days. In contrast, we noted a strong reporter signal in tubular cells within glycerol-injured areas of the renal cortex among $\beta$-catenin/TCF-lacZ reporter mice (Figures 1(d)–1(f)). Most proximal tubular cells with a strong $\beta$-galactosidase signal also showed coexpression of the renal progenitor cell marker, CD24 (Figures 2(a)–2(d)).

*2.2. $CD_{24+}$ Renal Progenitor Cells from Embryonic Kidney Exhibit Canonical β-Catenin/TCF Signaling in Response to WNT3a In Vitro.* At embryonic day E15, the CD24 surface antigen is predominantly expressed in putative RPC of the nephrogenic zone (Figure 3(a)). To refine the distribution of these $CD_{24+}$ cells, we examined E18 kidneys from mice bearing the *Hoxb7*-GFP transgene to mark the ureteric bud (Figures 3(b)–3(e)). $CD_{24+}$ cells are seen in the cap mesenchyme surrounding ureteric bud tips (Figures 3(b) and 3(c)) and in the comma-shaped (Figure 3(d)) and S-shaped bodies (Figure 3(e)) of emerging nephrons. Some $CD_{24+}$ cells are also seen scattered within the *Hoxb7*-GFP (+) ureteric bud trunk (Figure 3(f)).

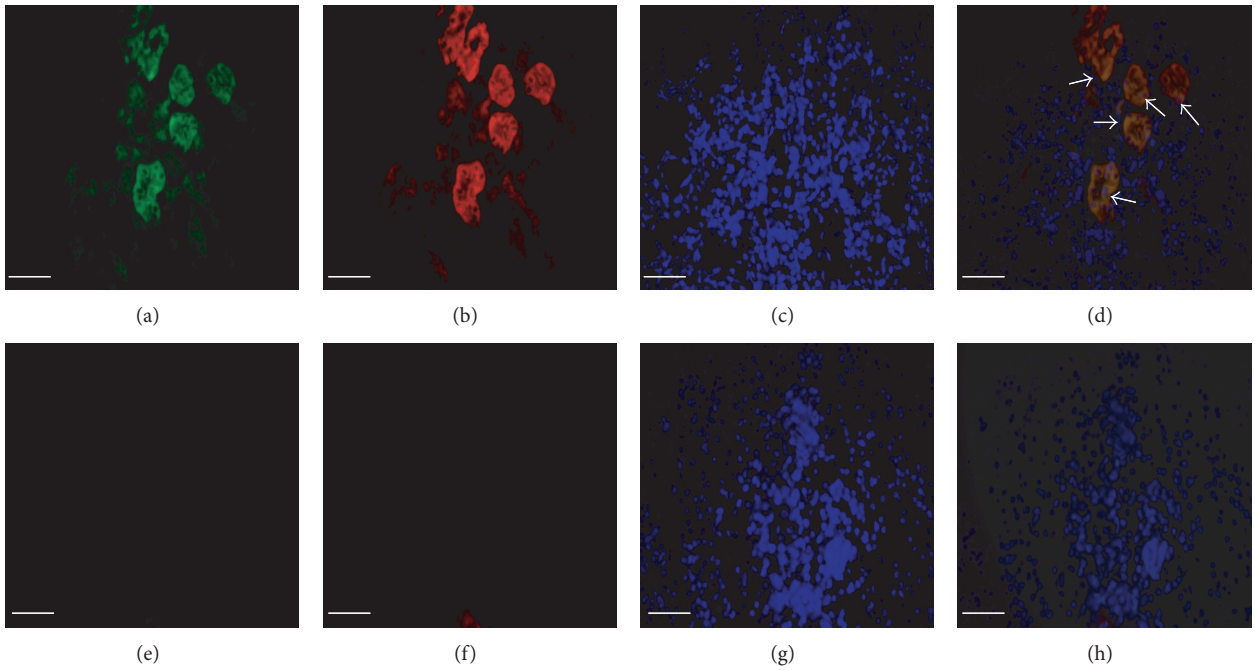


Figure 2: Endogenous coexpression of CD24 and $\beta$-catenin/TCF reporter transgene following glycerol-induced renal injury in adult mice. (a–d) Kidney sections showing endogenous tubular cells of glycerol-treated mice: (a) immunostaining for reporter $\beta$-galactosidase (green); (b) immunostaining for CD24 (red); (c) nuclear staining with DAPI; (d) merged images showing coexpression of $\beta$-galactosidase and CD24 in tubular cells (white arrows). (e–h) Kidney sections showing endogenous tubular cells in control (PBS injected) mice: (e) $\beta$-galactosidase reporter (green); (f) CD24 (red); (g) DAPI; (h) merge. Scale bars: 50 $\mu$m.

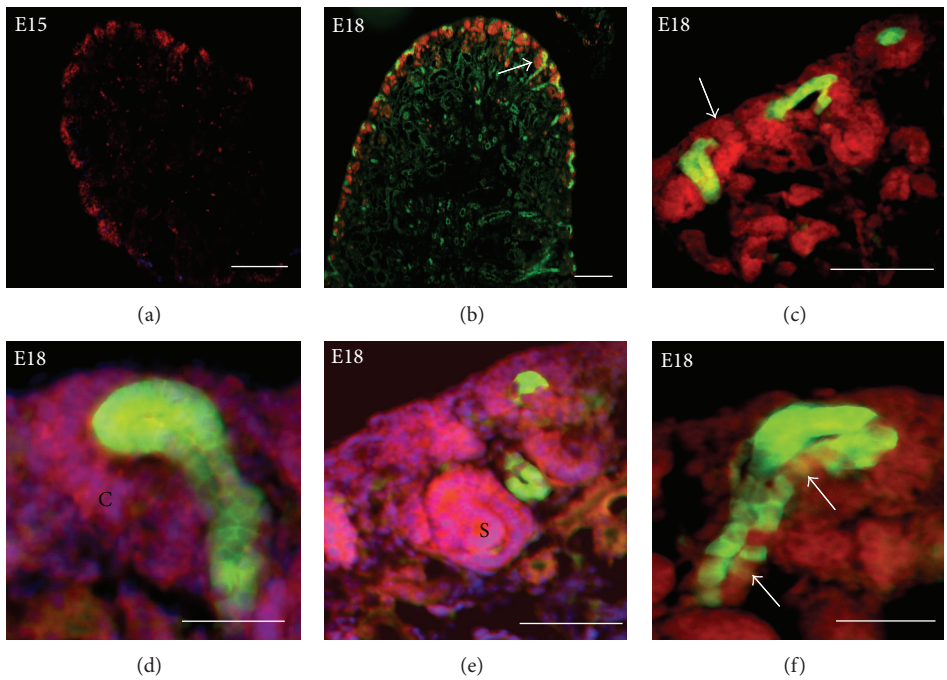



Figure 3: CD24 expression in embryonic mouse kidney. (a) PE-red-tagged $CD_{24+}$ cells are seen predominantly in the nephrogenic zone of E15 mouse kidney. (b-c) In E18 kidney from *Hoxb7*-GFP transgenic mice, $CD_{24+}$ cells (red) are seen in the cap mesenchyme associated with ureteric bud branch tips (green). (d-e) At high power, $CD_{24+}$ cells (red) are seen within comma-shaped (C) and S-shaped (S) bodies of emerging nephrons. (f) Occasional $CD_{24+}$ cells (red) are seen within the ureteric bud trunk (green) of E18 *Hoxb7*-GFP mice. Scale bar: 50 $\mu$m.

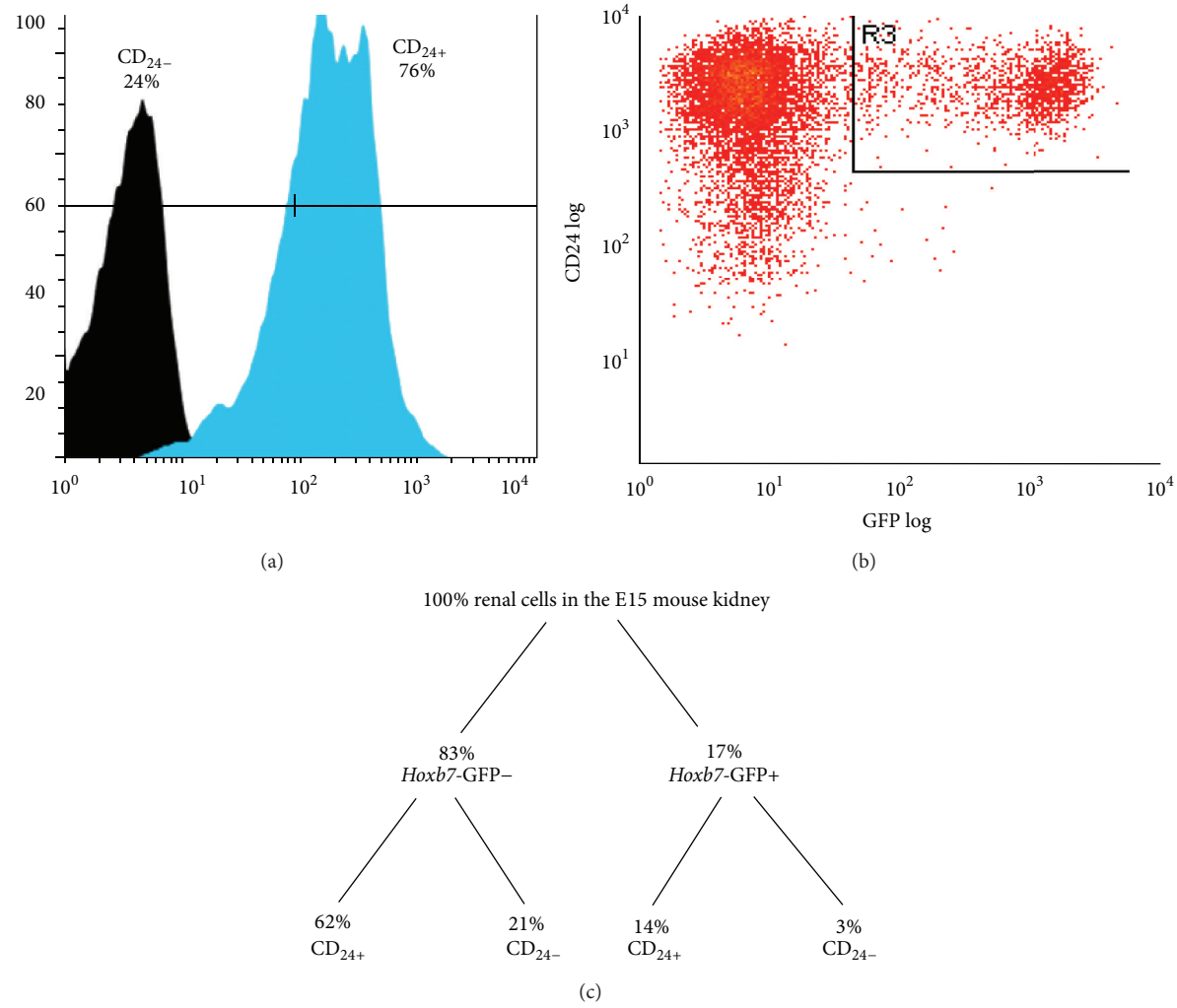
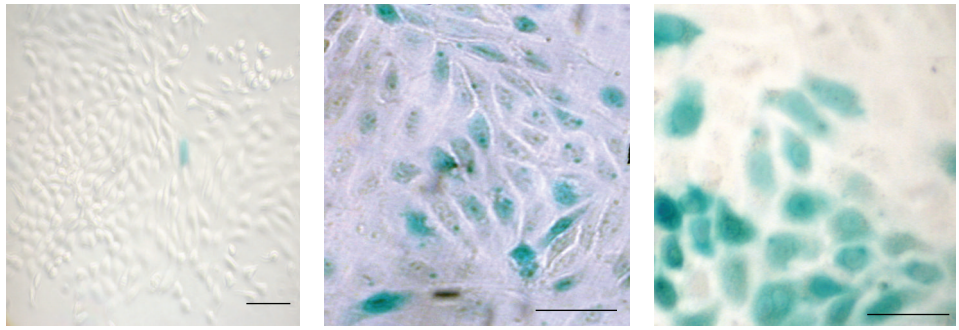



Figure 4: FACS isolation of $CD_{24+}$ cells from E15 mouse kidney. Single-cell suspensions were prepared from E15 *Hoxb7*-GFP mouse kidneys and analyzed for expression of CD24 and *Hoxb7*-GFP by fluorescence-activated cell sorting (FACS). (a) $CD_{24+}$ cells represent about 76% of cells in the E15 mouse kidney. (b) 14% of $CD_{24+}$ cells coexpress the *Hoxb7*-GFP ureteric bud marker. (c) Percentages of E15 mouse kidney cells expressing CD24/*Hoxb7*-GFP.

To characterize the responsiveness of $CD_{24+}$ cells to canonical WNT signals, we excised kidneys from embryonic day E15 *Hoxb7*-GFP mice and isolated $CD_{24+}$ cells by fluorescence-activated cell sorting (FACS, blue peak) (Figure 4(a)). About 14% of the $CD_{24+}$ cells also expressed GFP (Figures 4(b) and 4(c)). To characterize the $CD_{24+}$ cells, we replated them in monolayer culture for 48 hours and examined expression of various developmental genes by RT-PCR. We identified transcripts for multiple markers of the metanephric mesenchyme (*Wt1*, *Osr1*, and *Gdnf*), cap mesenchyme (*Six2*, *Cited1*), and S-shaped body (*Wnt4, Pax8)*. We also identified expression of genes associated with the UB trunk (*Wnt7b*) but not the UB tip (e.g., *Wnt9b*, *Ret*, and *Wnt11*).

To examine activation of the canonical WNT signaling pathway, we isolated $CD_{24+}$ cells by FACS from pooled E15 kidneys of progeny from mice bearing the $\beta$-catenin/TCF reporter transgene. After 24–48 hours in monolayer culture, few of the cells exhibited baseline reporter transgene activity (Figure 5(a)). However, when the $CD_{24+}$ cell monolayer was cocultured with inserts containing L-cells expressing WNT3a or GFP(+) cells isolated from E15 *Hoxb7*-GFP mice, about 40% of cells showed canonical $\beta$-catenin/TCF signaling activity (Figures 5(b) and 5(c)).

*2.3. Infused $CD_{24+}$ Cells from E15 Mouse Kidney Are Integrated into the Damaged Renal Tubules of Adult Mice with Glycerol-Induced Proximal Tubular Injury.* To confirm that embryonic $CD_{24+}$ cells can function as RPC and are integrated into acutely damaged renal tubules of adult mice, we first isolated $CD_{24+}$ cells by FACS from kidneys of wild-type embryonic day E15 mice and stained them with PKH26 red fluorescent dye. We then induced proximal renal tubular injury with intramuscular injection of 50% glycerol (8 $\mu$L/g body weight) in normal 6-month-old mice [6]. Control or glycerol-injured mice were twice infused (via the tail vein) with



(a) (b) (c)

Figure 5: $\beta$-catenin/TCF signaling in $CD_{24+}$ embryonic kidney cells *in vitro*. (a) $CD_{24+}$ cells isolated from E15 kidney of mice bearing the $\beta$-catenin/TCF-lacZ transgene show minimal $\beta$-galactosidase activity (blue) in baseline monolayer culture. (b) About 40% of $CD_{24+}$ cells exposed to murine L-cells expressing Wnt3a showed canonical $\beta$-catenin/TCF signaling. (c) $CD_{24+}$ cells exposed to ureteric bud cells isolated by FACS from E15 *Hoxb7*-GFP mice showed similar canonical $\beta$-catenin/TCF signaling. Scale bar: 50 $\mu$m.

0.5 million $CD_{24+}$ PKH26 red-stained embryonic kidney cells (or cell supernatant as a control) three and four days after glycerol injection. Kidneys were examined by immunofluorescent microscopy after an additional 3 days. In uninjured mice, proximal tubules showed normal staining for *Lotus tetragonolobus* agglutinin (LTA) and there was no uptake of exogenous $CD_{24+}$ PKH26 red-stained cells into the kidney (Figure 6(a)). In glycerol-injured mice which had no infusion of cells, we noted extensive tubular dilatation, patchy flattening, or necrosis of proximal tubular epithelial cells (Figure 6(b)). No nonspecific uptake of dye was seen in glycerol-injured mice infused with the supernatant from PKH26-stained $CD_{24+}$ cells (Figure 6(c)). However, there was widespread integration of the exogenous embryonic $CD_{24+}$ PKH26 red-stained cells into renal tubules of glycerol-injured mice (Figure 6(d)). At high power, red-stained exogenous $CD_{24+}$ cells were integrated into the proximal tubular wall and exhibited a polarized epithelial phenotype (Figures 6(e) and 6(f)). LTA expression is seen both in the exogenous PKH26 red-stained $CD_{24+}$ cells and in the adjacent endogenous proximal tubular cells (Figure 6(h)).

*2.4. $CD_{24+}$ Cells from Embryonic $\beta$-Catenin/TCF Mice Activate the Canonical WNT/$\beta$-Catenin Signaling Pathway When Infused into Glycerol-Injured Mice.* To determine whether exogenous $CD_{24+}$ cells activate the canonical WNT signaling pathway during integration into damaged tubules, we isolated $CD_{24+}$ cells from E15 embryonic $\beta$-catenin/TCF reporter mice and infused them into adult wild-type mice with glycerol-induced proximal tubular injury. No exogenous reporter signal is seen in kidney of uninjured mice (Figure 7(a)), but robust canonical WNT signaling activity is evident in exogenous cells integrated into many (but not all) tubules of the injured kidney (Figures 7(b)–7(f)). In some sections, the signaling is clearly evident in the S1 segment of proximal tubules at the junctions with renal glomeruli (Figures 7(b)–7(d)). Occasionally, the WNT signaling activity is seen in exogenous cells lining the urinary pole of Bowman's capsule (Figure 7(f)). Exogenous $CD_{24+}$ cells expressing the $\beta$-catenin/TCF reporter also showed strong staining for the cell proliferation marker, PCNA (Figures 7(g) and 7(h)).

Table 1: *Wnt* mRNA expression in mouse embryonic and adult kidney.

| | Wnt4 | Wnt7b | Wnt9b | Wnt11 |
|---|---|---|---|---|
| E15 mouse kidney | + | + | + | + |
| $CD_{24+}$ cells (E15) | + | + | No | + |
| Adult kidney (no glycerol) | No | + | + | No |
| Adult kidney (with glycerol) | + | + | + | No |

Transcripts for each *Wnt* were identified by RT/PCR in E15 mouse kidney, CD24 cells isolated by FACS from E15 mouse kidney, normal adult mouse kidney, and adult mouse kidney 3 days after induction of glycerol-induced proximal tubular injury.

*2.5. WNT4 Is Activated in Exogenous $CD_{24+}$ Cells during Renal Regeneration.* During nephrogenesis, the canonical WNT signaling pathway is initially activated in renal progenitor cells by WNT9b released from the UB; WNT signaling is then sustained in RPC by endogenous WNT4 expression as the cells undergo the mesenchyme-to-epithelial transition and form the S-shaped body [7, 8]. We reasoned that the $\beta$-catenin/TCF-lacZ reporter activity seen after acute glycerol-induced tubular injury might be driven by reexpression of an autocrine or paracrine WNT signal. As seen in Table 1, E15 embryonic mouse kidney expresses WNTs 4, 7b, 9b, and 11. Of these, *Wnt4* mRNA is detected in $CD_{24+}$ cells and in glycerol-injured adult kidney, but not in uninjured kidney. Wnt9b was not expressed in $CD_{24+}$ cells but was detectable by RT/PCR both in control and in glycerol-injured adult mice. *Wnt11* mRNA was noted in the pool of $CD_{24+}$ cells isolated from embryonic kidney but not in control or glycerol-injured adult kidney. Wnt7 transcripts were detected in all samples.

*2.6. Activation of the Canonical WNT/$\beta$-Catenin Pathway Is Required for Integration of $CD_{24+}$ Cells into Damaged Proximal Tubules.* To inhibit the WNT/$\beta$-catenin pathway, we isolated

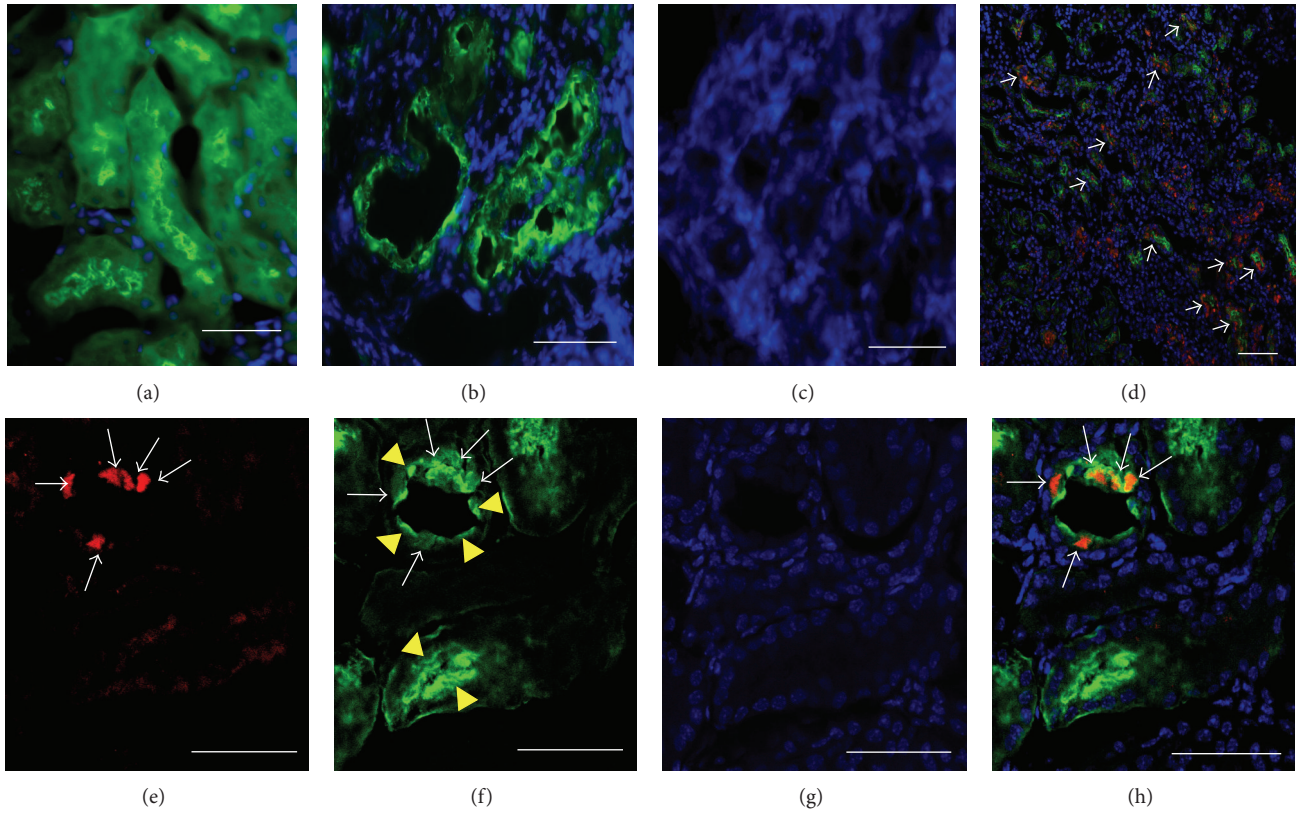



FIGURE 6: Exogenous renal embryonic $CD_{24+}$ cells stained with PKH26 red integrate into the renal tubules of adult mice with glycerol-induced proximal tubular injury. (a) *Lotus tetragonolobus* agglutinin (LTA) staining of normal control renal proximal tubules (green). (b) LTA staining of kidney (green) 3 days after induction of acute tubular injury with 50% glycerol 8 $\mu$L/g shows tubular dilatation, flattening, and detachment of proximal tubular cells. (c) No uptake of red dye is seen in glycerol-injured kidneys following infusion of the supernatant from the PKH26 red staining procedure for $CD_{24+}$ cells. (d) Following infusion of PKH26 red-stained $CD_{24+}$ cells into glycerol-injured mice, exogenous cells are seen within the damaged renal tubules (white arrows). (e–h) Confocal immunofluorescent microscopy examining integration of $CD_{24+}$ cells into the wall of glycerol-damaged proximal tubules. (e) Patchy integration of PKH26 red $CD_{24+}$ cells into the proximal tubular wall (white arrows). (f) Exogenous $CD_{24+}$ cells (white arrows) have a polarized appearance and stain for LTA (green) and are intercalated among the endogenous LTA(+) epithelia (yellow arrow heads). (g) The same section stained with DAPI alone. (h) Merged image of (e), (f), (g). Scale bars: 50 $\mu$m (f) and 70 $\mu$m (g–j). LTA: green; DAPI: blue; PKH26: red.

$CD_{24+}$ cells from E15 $\beta$-catenin/TCF reporter mice and exposed them to IWR-1, an inhibitor of tankyrase proteins that destabilize the derivative axin complex [9]. Previously, investigators have demonstrated that IWR-1 (100 $\mu$M) blocks canonical WNT signaling in E11.5 embryonic kidney explants [10]. After 24-hour exposure to 100 $\mu$M IWR-1, WNT3a-stimulated $\beta$-catenin/TCF reporter activity was reduced to 33% of control (Figure 8(a)). To confirm that IWR-1 inhibition persisted during the period during which exogenous cells are integrated into damaged tubules, we exposed $CD_{24+}$ cells bearing the $\beta$-catenin/TCF reporter to 100 $\mu$M IWR-1 for 12 hours and then transferred the cells to control culture medium for various periods of time. After 72 hours, WNT3a-stimulated $\beta$-catenin/TCF reporter activity was still inhibited to 43% of control (Figure 8(b)).

To examine the effect of IWR-1 pretreatment on exogenous cell integration into damaged adult tubules, we isolated $CD_{24+}$ cells from wild-type embryonic E15 mouse kidney, cultured them for 12 hours in the presence or absence of IWR-1 (100 $\mu$M), and then infused 0.5 million PKH26 red-labeled cells into mice three days after glycerol-induced proximal tubular injury. Whereas control $CD_{24+}$ cells are widely integrated into damaged proximal tubule segments, integration of IWR-1 pretreated $CD_{24+}$ cells was strikingly reduced (Figure 9(b)) compared to untreated cells (Figure 9(a)). The percentage of proximal tubules exhibiting exogenous $CD_{24+}$ cell integration fell from 34% (untreated cells) to 11% (IWR-1 pretreated cells) (Figure 9(c)).

## 3. Discussion

There are over 300 human clinical trials of adult mesenchymal stem cells registered at the NIH clinical trial registry (http://clinicaltrials.gov/). These studies are predicated on the ability of infused adult bone marrow stem cells to home to sites of acute tissue injury and exert a number of salutary effects on the tissue injury/repair process. However, although kidney is derived from stem cells within the embryonic mesenchyme, adult mesenchymal stem cells lack the capacity

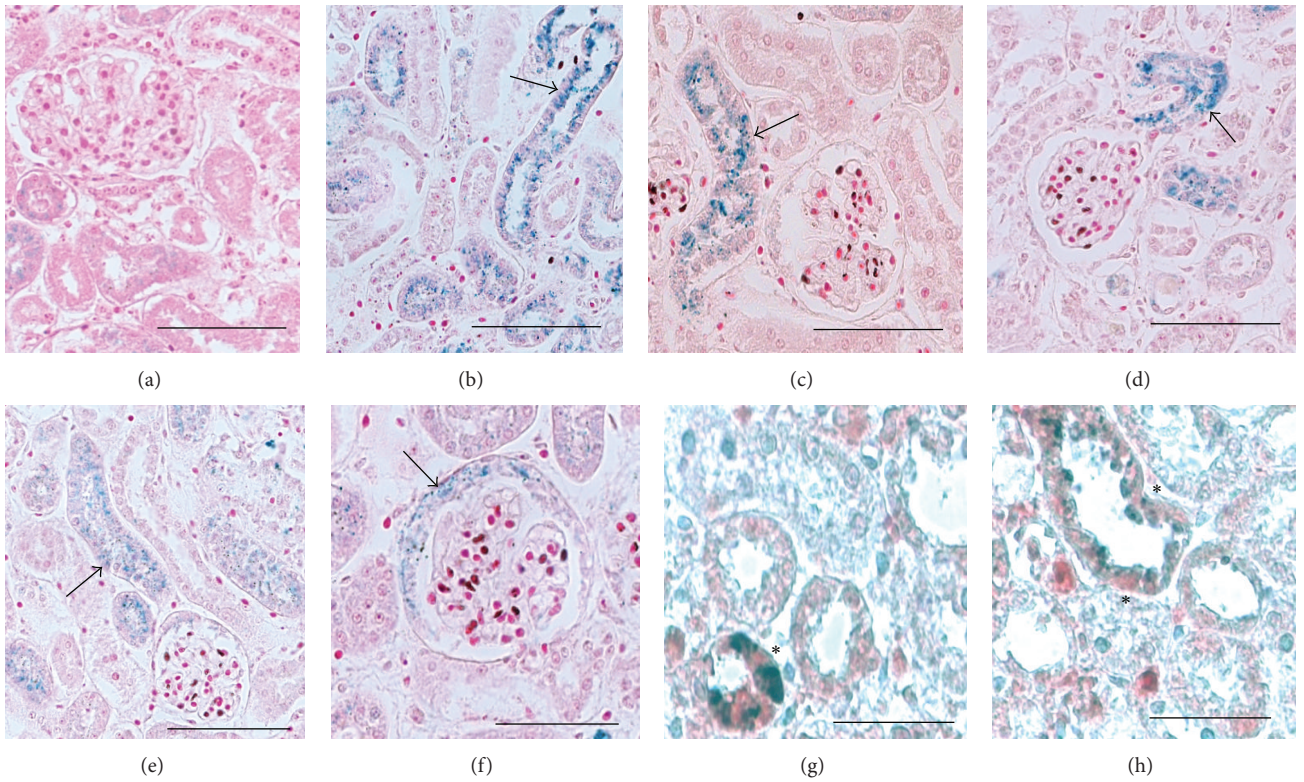



FIGURE 7: $\beta$-catenin/TCF pathway reporter activity in exogenous $CD_{24+}$ cells during integration into the glycerol-damaged adult renal tubule. (a–h) $CD_{24+}$ cells (0.5 million) were isolated by FACS from embryonic E15 mice bearing the $\beta$-catenin/TCF reporter and infused into CD1 wild-type adult mice 3 days after i.m. administration of glycerol 8 $\mu$L/g or PBS (control). Kidney sections were stained with X-Gal to identify $\beta$-catenin/TCF reporter activity (blue) after an additional 3 days. (a) Minimal $\beta$-catenin/TCF reporter activity is seen after infusion of $CD_{24+}$ cells into control mice. (b-c) Strong $\beta$-catenin/TCF reporter activity (arrow) is seen in exogenous cells integrated into glycerol-damaged renal tubules. (d-e) $\beta$-catenin/TCF reporter activity (arrow) is seen in exogenous cells integrated into the S1 segment of glycerol-damaged proximal tubules. (f) $\beta$-catenin/TCF reporter activity (arrow) is seen in exogenous cells integrated into the urinary pole of Bowman's capsule. (g-h) Exogenous $CD_{24+}$ cells integrated into glycerol-damaged renal tubules exhibit both $\beta$-catenin/TCF reporter activity and strong staining for the marker of cell proliferation, PCNA (red) (asterisk). Scale bars: 50 $\mu$m.

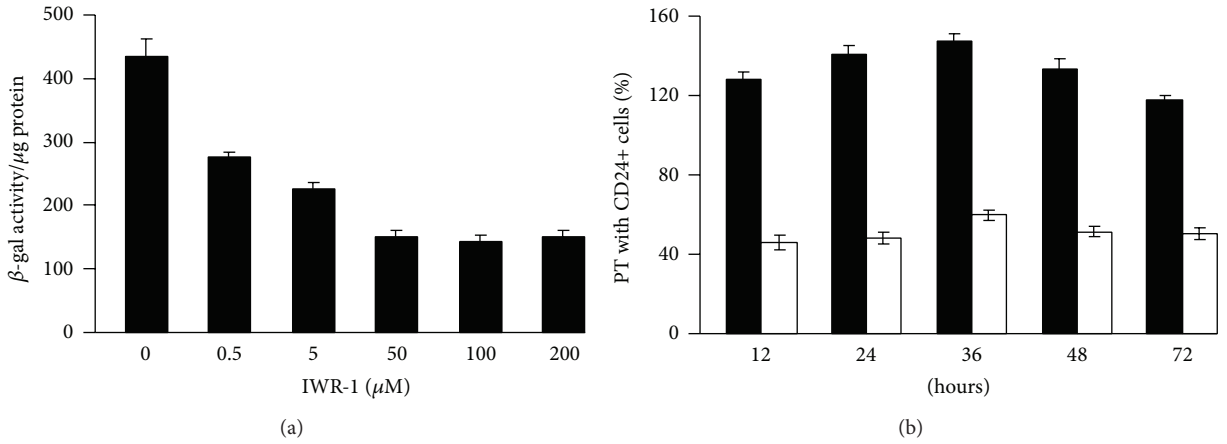



FIGURE 8: IWR-1 inhibits WNT3a-stimulated $\beta$-catenin/TCF signaling activity in embryonic $CD_{24+}$ cells from E15 mouse kidney. $CD_{24+}$ cells from mice bearing the $\beta$-catenin/TCF reporter transgene were isolated by FACS from E15 mouse kidneys and cultured for 12 hours. IWR-1 was then added and $CD_{24+}$ cells were exposed to L-cells expressing WNT3a for 12 hours to assess $\beta$-catenin/TCF signaling activity. (a) $\beta$-galactosidase activity per $\mu$g cell protein was maximally inhibited to 34% of control by IWR-1 concentrations 100 $\mu$M (24 hours). (b) $CD_{24+}$ cells were exposed to control medium (black bars) or IWR-1 (100 $\mu$M) (white bars) for 12 hours and then transferred to control medium for various periods of time to track persistence of WNT3a-stimulated $\beta$-galactosidase activity; significant inhibition was evident after 12 hours (36% of control) and 72 hours (43% of control) of washout in control medium.

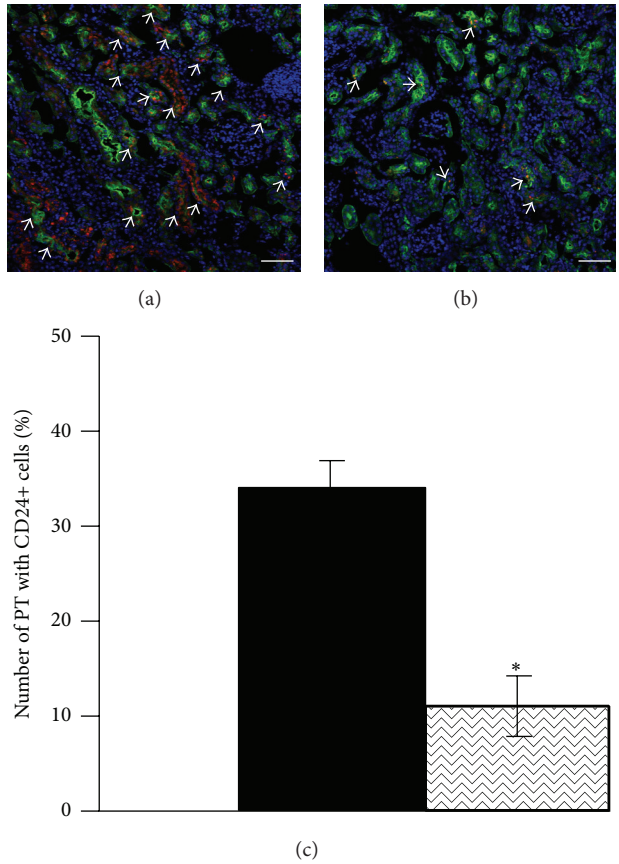



FIGURE 9: IWR-1 pretreatment of exogenous $CD_{24+}$ cells reduces integration into glycerol-damaged renal proximal tubules. $CD_{24+}$ cells were isolated by FACS from embryonic E15 mouse kidney and cultured for 12 hours in the presence or absence of IWR-1 (100 $\mu$M). 0.5 million $CD_{24+}$ cells were then labeled with PKH26 red dye and infused into mice three days after glycerol-induced proximal tubular injury. (a) Control $CD_{24+}$ cells (white arrows) are widely integrated into damaged proximal tubules stained with LTA (green); (b) proximal tubular integration (white arrows) of $CD_{24+}$ cells after IWR-1 pretreatment is strikingly reduced (a-b, scale bars: 50 $\mu$m). (c) The number of LTA-stained proximal tubules (PT) showing integration of PKH26 red $CD_{24+}$ cells was estimated in 310–340 tubules from 10 different kidney sections. Pretreatment of exogenous $CD_{24+}$ cells with IWR-1 (100 $\mu$M) reduced the percentage of tubules showing $CD_{24+}$ cells integration from 34% (control) to 11% (IWR-1). $P < 0.001$.

for integration into the intrinsic epithelial structures of adult organs. Infusion of bone marrow MSC hastens the recovery from experimental kidney injury, but the exogenous cells take up an interstitial position adjacent to renal tubules and are rarely incorporated into the tubular wall [11, 12]. Similarly, infusion of bone marrow hematopoietic stem cells into CTNS knockout mice with nephropathic cystinosis can reverse pathologic cystine accumulation and ameliorate progressive renal dysfunction [13]. Again, however, these cells were noted to take up a peritubular position [13] and the effect on adjacent mutant cells was recently attributed to paracrine transfer of wild-type cystinosin via microvesicle shedding [14].

In contrast to the above, Lazzeri et al. infused human kidney embryonic $CD_{24+}/CD_{133+}$ cells into glycerol-injured adult SCID mice and demonstrated accelerated renal recovery associated with widespread integration of the exogenous cells into damaged renal tubules [6]. Thus, while nephron progenitor cells in the metanephric mesenchyme may have lost some of the plasticity exhibited by embryonic stem cells, they appear to have acquired special characteristics which facilitate their integration into nephrons during primary nephrogenesis. Although stem cells from bone marrow also arise from embryonic mesenchyme, they have not acquired (or have lost) these characteristics. In our studies, embryonic $CD_{24+}$ cells show responsiveness to WNT3a *in vitro* and when infused into mice with glycerol-induced injury, they exhibit robust activation of the pathway during integration into the tubular wall. Importantly, IWR-1 inhibition of the $\beta$-catenin/TCF pathway sharply reduces integration of exogenous NPC into the damaged tubules. Taken together, these observations suggest that embryonic NPC have been primed to activate the $\beta$-catenin/TCF pathway in response to an inductive WNT signal and that this capacity is crucial for their ability to regenerate damaged adult renal tubules.

We used FACS for the CD24 surface marker to capture a population of cells from embryonic kidney with the capacity to integrate into nephrons. In E10.5 embryonic mice (prior to nephrogenesis), *Cd24* mRNA is strongly and specifically expressed in the metanephric mesenchyme [15]. As reported

by others at E15, we noted CD24 protein expression in cells of the cap mesenchyme surrounding each ureteric bud tip, in cells undergoing the mesenchyme-to-epithelium transition in the S-shaped body, and in a few cells of the branching ureteric bud [16, 17]. Thus, our E15 $CD_{24+}$ cells comprise a heterogeneous mix of uninduced RPC (expressing *Osr1*, *Wt1*, and *Six2*) and cells in transition to the epithelial phenotype of proximal tubules (*Wnt4* and *Pax8*) and collecting ducts (*Wnt7b*). Few E15 $CD_{24+}$ cells from embryos with the $\beta$-catenin/TCF reporter exhibited basal activity of the canonical WNT signaling pathway. However, about 40% displayed $\beta$-catenin/TCF reporter activity in response to WNT3a or cocultured UB cells. Several days after infusion into adult mice with glycerol-induced renal injury, a substantial number of $CD_{24+}$ cells are seen within the tubular wall, displaying a polarized epithelial phenotype and luminal expression of LTA, a marker of proximal tubular cell differentiation. Thus, the pool of $CD_{24+}$ cells isolated from E15 kidney contains a subset of functional NPC that respond to canonical WNT signals, express genes involved in tubular differentiation, integrate into to the nephron wall, and exhibit properties of the mature tubular cell *in vivo*. In humans, the progenitor cell pool can be further refined by coselection for the CD133 antigen, noted in the cap mesenchyme and S-shaped body [6], but an antibody for the murine homolog of CD133 has not been developed. In mice, it may be possible to distinguish compartments of uninduced self-renewing RPC (Six2+/Cited1+) from WNT-inducible NPC (Six2+) in the cap mesenchyme [18]. However, our studies do not attempt to characterize the specific subset of NPC that integrate into damaged tubules but, rather, focus on the $\beta$-catenin-dependent mechanism involved in integration of these cells.

During primary nephrogenesis, it is thought that NPC in the cap mesenchyme begin to differentiate in response to WNT9b released from ureteric bud tips [3]. However, during progression to the S-shaped body stage, autonomous expression of WNT4 is required to sustain canonical pathway activity and complete nephrogenesis [7]. WNT7b is expressed in the ureteric bud trunk and WNT11 at its tip. However, mammalian nephrogenesis comes to an end in the perinatal period and embryonic WNT expression is downregulated in adult kidney. Thus, it is unclear how the canonical WNT/$\beta$-catenin signaling pathway is activated in exogenous or endogenous $CD_{24+}$ cells that can repopulate the damaged renal tubule. Of the four potential canonical WNT ligands expressed in embryonic kidney (WNTs 4, 7b, 9b, and 11), transcripts for all except *Wnt9b* were detectable by RT/PCR in embryonic $CD_{24+}$ cells. Interestingly, *Wnt4* mRNA is induced by glycerol injury in adult kidney. Although our studies do not implicate any one particular WNT, it is clear that both embryonic $CD_{24+}$ cells and damaged adult kidney express several ligands that could account for pathway activity.

Our studies demonstrate $\beta$-catenin/TCF reporter activity not only in the exogenous embryonic $CD_{24+}$ cells which are taken up by damaged renal tubules, but also by endogenous tubular cells that express CD24 after glycerol injury. The origin of these endogenous cells is unclear. Humphreys has argued that recovery from acute tubular necrosis cannot be explained by new tubular cells arising from a single stem cell focus in each nephron [19]. However, Angelotti et al. has identified subsets of apoptosis-resistant $CD_{24+}/CD_{133+}/CD_{106+}$ cells retained in putative stem cell niches at the urine pole of Bowman's capsule and related $CD_{24+}/CD_{133+}/CD_{106-}$ cells scattered along the proximal and distal nephron [20]. When isolated from adult kidney by FACS, these cells express *OSR1* [21], exhibit self-renewal *in vitro*, and can be induced to express a variety of markers of the proximal tubule, loop of Henle, and distal convoluted tubule [4]. These observations favor the view that adult mammalian nephrons retain a population of quiescent NPC that participate in regeneration of the damaged nephron. Following glycerol-induced tubular injury in adult $\beta$-catenin/TCF reporter mice, we noted striking endogenous $\beta$-catenin/TCF signaling activity both in the crescent of cells lining the urinary pole of Bowman's capsule and in the proximal tubule. Although this mirrors the two subsets of cells described by Angelotti et al. [20], formal fate-mapping studies are needed to ascertain whether discrete subsets of adult NPC repopulate the damaged adult tubule or whether all tubular cells can dedifferentiate, express CD24, and reactivate the WNT4/$\beta$-catenin/TCF pathway when injured.

In summary, our observations indicate that a crucial distinction between the committed nephron progenitor cell in metanephric mesenchyme and the mesenchymal stem cell from adult bone marrow is the capacity to activate the $\beta$-catenin/TCF pathway in response to canonical WNT signals. We suggest that this property may be essential for any cell-based regenerative therapy of the damaged mammalian kidney.

## 4. Materials and Methods

*4.1. Isolation and Culture of Embryonic E15 $CD_{24+}$ Cells.* To obtain embryonic $CD_{24+}$ cells, kidneys were resected from embryonic day E15 wild-type or transgenic mice bearing a $\beta$-catenin/TCF-responsive beta-galactosidase reporter transgene ($\beta$-catenin/TCF-lacZ) [2]. Embryonic mouse kidneys were minced and digested with 1 mg/mL collagenase type B (Roche), 2.5 mg/mL Dispase II (Roche), and 30 $\mu$g/mL DNase I in F12/DMEM culture medium with 10% FBS at 37°C for 45 minutes under 5% $CO_2$. The cell suspension was filtered through a 35 $\mu$m cell strainer in a 15 mL Falcon tube, washed three times with cold 1X PBS containing 2% FBS, and pelleted at 1000 rpm/min for 5 min (4°C). The cells were then placed in monolayer culture (F12/DMEM + 10% FBS at 37°C under $CO_2$) for 24–48 hours. The adherent E15 monolayer culture was then detached with trypsin, and the single-cell suspension was incubated with CD24 antibody conjugated to Alexa Fluor 647 (Biolegend) in the dark. $CD_{24+}$ cells were isolated by fluorescence-activated cell sorting (FACS) with forward and side scatter width/height gating to ensure isolation of singlet cells. Cell sorting was performed on a MoFlo cell sorter (DakoCytomation, Carpinteria, CA). In some experiments, the $CD_{24+}$ cells were stained with PKH26 red (Sigma-Aldrich) according to manufacturer's instructions and washed three times in chilled PBS prior to use. In other

experiments, they were exposed to various concentrations of the tankyrase inhibitor IWR-1 (Enzo Life Science).

*4.2. β-Galactosidase Activity of $CD_{24+}$ Cells In Vitro.* β-galactosidase activity generated by the TCF/β-gal reporter transgene was determined using the Tropix Galacto-Star system chemiluminescent reporter gene assay system (Applied Biosystems). The signal was measured in a GLOMAX 96 microplate luminometer (Promega, San Luis Obispo, CA, USA).

*4.3. Activation of Canonical WNT Signaling Pathway in Cultured $CD_{24+}$ Cells.* To activate the canonical WNT signaling pathway *in vitro*, monolayer cultures of E15 $CD_{24+}$ cells bearing the β-catenin/TCF reporter were exposed for 24 hours to coculture inserts (0.4 μm pore size) containing (1) mouse fibroblast L-cells (ATCC); (2) L-cells expressing WNT3a (ATCC); (3) *Hoxb7*-GFP(+) ureteric bud cells isolated by FACS from E15 *Hoxb7*-GFP C3H mice. These mice express the *Hoxb7*-GFP transgene exclusively in the ureteric bud lineage in embryonic kidney [22, 23].

*4.4. Analysis of β-Galactosidase Activity in Transgenic Mice.* Whole kidneys or monolayer cultures of E15 $CD_{24+}$ cells from mice bearing the β-catenin/TCF-LacZ reporter transgene were fixed and stained as previously described [2]. Kidneys were washed in PBS and visualized directly or embedded in paraffin for sectioning, counterstained with hematoxylin and eosin, and visualized by light microscopy.

*4.5. Glycerol-Induced Acute Renal Tubular Injury in Mice.* Proximal renal tubular injury was induced with intramuscular injection of 50% glycerol (8 μL/g body weight) (Sigma-Aldrich) into the inferior hind limbs of normal 6-month-old CD1 mice (Charles River Lab, USA) under anesthesia as described by Lazzeri et al. [6]. Control mice were injected with the same volume of phosphate buffered saline. Control or glycerol-treated mice were twice infused via the tail vein with 0.5 million embryonic $CD_{24+}$ cells three and four days after glycerol injection: Group 1 ($n = 8$) received two intravenous infusions of embryonic $CD_{24+}$ cells labeled with PKH26 red fluorescent dye; as a control in a parallel experiment, mice were injected with supernatant from the third saline wash of PKH26-stained cells; Group 2 ($n = 8$) received two intravenous infusions of $CD_{24+}$ cells obtained from E15 β-catenin/TCF transgenic embryonic mice [2].

To study the effect of acute renal injury on endogenous canonical WNT signaling pathway activity, we induced acute proximal tubule injury with glycerol in six-month-old CD1 mice ($n = 8$) bearing the β-catenin/TCF reporter transgene. Three days after the first infusion (day 6), mice were killed and kidneys were harvested for analysis.

All animal procedures followed the guidelines established by the Canadian Council of Animal Care and were approved by the Animal Care Committee from the Research Institute from the McGill University Health Center.

*4.6. Reverse Transcriptase PCR.* Total RNA was isolated from cells using Qiagen RNeasy Mini-Plus Kit with gDNA eliminator column (Qiagen, Mississauga, ON, Canada). Two-step reverse transcriptase-PCR was performed; first-strand cDNA was primed with random hexamers and TaqMan MultiScribe Reverse Transcriptase according to the manufacturer's instructions (Applied Biosystems, Foster City, CA).

*4.7. Immunohistochemistry.* Paraffin-embedded sections (5 μm) of embryonic or adult kidneys were incubated in 5% $H_2O_2$ to quench endogenous peroxidase activity, followed by 30 min incubation with normal horse serum. Tissue sections were then incubated with rabbit polyclonal antibody against murine PCNA (1 : 100) (Santa Cruz, CA) at 4°C overnight and then incubated with a universal biotinylated secondary antibody (Vector Laboratory, Burlingame, CA). Staining was developed using ACE (Vector Laboratory).

*4.8. Immunofluorescent Staining.* Staining was performed on 14 μm frozen sections of embryonic mouse kidney. Briefly, sections were rinsed in PBS for 10 min and fixed in ice-cold acetone for 10 min. Sections were blocked with horse serum for 1 h at room temperature and then incubated with anti-CD24 PE conjugated antibody (1 : 200), counterstained with LTA and DAPI, and mounted with aqueous gel mount (Sigma). Confocal microscopy was performed with a Zeiss LSM780 Laser Scanning Confocal Microscopy (Carl Zeiss, Jena, Germany).

*4.9. Statistical Analysis.* The results are expressed as the mean ± s.d. Statistical significance between experimental groups was assessed by Student's unpaired *t*-test.

## Conflict of Interests

All the authors declared no conflict of interests.

## Acknowledgments

This work was supported by operating grants (MOP-119571, MOP-82904) from the Canadian Institutes of Health Research (CIHR), an operating grant (232129) from Génome Québec, and an infrastructure support grant to the McGill University Health Centre Research Institute from the Fonds de Recherches en Santé du Québec (FRSQ). Dr. Goodyer is the recipient of a CIHR James McGill Research Chair.

## References

[1] J. W. Mugford, P. Sipilä, J. A. McMahon, and A. P. McMahon, "Osr1 expression demarcates a multi-potent population of intermediate mesoderm that undergoes progressive restriction to an Osr1-dependent nephron progenitor compartment within the mammalian kidney," *Developmental Biology*, vol. 324, no. 1, pp. 88–98, 2008.

[2] D. M. Iglesias, P.-A. Hueber, L. Chu et al., "Canonical WNT signaling during kidney development," *The American Journal of*

*Physiology—Renal Physiology*, vol. 293, no. 2, pp. F494–F500, 2007.

[3] T. J. Carroll, J.-S. Park, S. Hayashi, A. Majumdar, and A. P. McMahon, "Wnt9b plays a central role in the regulation of mesenchymal to epithelial transitions underlying organogenesis of the mammalian urogenital system," *Developmental Cell*, vol. 9, no. 2, pp. 283–292, 2005.

[4] C. Sagrinati, G. S. Netti, B. Mazzinghi et al., "Isolation and characterization of multipotent progenitor cells from the Bowman's capsule of adult human kidneys," *Journal of the American Society of Nephrology*, vol. 17, no. 9, pp. 2443–2456, 2006.

[5] P. Romagnani, "Toward the identification of a 'renopoietic system'?" *Stem Cells*, vol. 27, no. 9, pp. 2247–2253, 2009.

[6] E. Lazzeri, C. Crescioli, E. Ronconi et al., "Regenerative potential of embryonic renal multipotent progenitors in acute renal failure," *Journal of the American Society of Nephrology*, vol. 18, no. 12, pp. 3128–3138, 2007.

[7] K. Stark, S. Vainio, G. Vassileva, and A. P. McMahon, "Epithelial transformation metanephric mesenchyme in the developing kidney regulated by Wnt-4," *Nature*, vol. 372, no. 6507, pp. 679–683, 1994.

[8] J. P. Lyons, U. W. Mueller, H. Ji et al., "Wnt-4 activates the canonical beta-catenin-mediated Wnt pathway and binds Frizzled-6 CRD: functional implications of Wnt/beta-catenin activity in kidney epithelial cells," *Experimental Cell Research*, vol. 298, no. 2, pp. 369–387, 2004.

[9] S.-M. A. Huang, Y. M. Mishina, S. Liu et al., "Tankyrase inhibition stabilizes axin and antagonizes Wnt signalling," *Nature*, vol. 461, no. 7264, pp. 614–620, 2009.

[10] C. M. Karner, C. E. Merkel, M. Dodge et al., "Tankyrase is necessary for canonical Wnt signaling during kidney development," *Developmental Dynamics*, vol. 239, no. 7, pp. 2014–2023, 2010.

[11] P. V. Hauser, R. de Fazio, S. Bruno et al., "Stem cells derived from human amniotic fluid contribute to acute kidney injury recovery," *The American Journal of Pathology*, vol. 177, no. 4, pp. 2011–2021, 2010.

[12] M. B. Herrera, B. Bussolati, S. Bruno et al., "Exogenous mesenchymal stem cells localize to the kidney by means of CD44 following acute tubular injury," *Kidney International*, vol. 72, no. 4, pp. 430–441, 2007.

[13] K. Syres, F. Harrison, M. Tadlock et al., "Successful treatment of the murine model of cystinosis using bone marrow cell transplantation," *Blood*, vol. 114, no. 12, pp. 2542–2552, 2009.

[14] D. M. Iglesias, R. El-Kares, A. Taranta et al., "Stem cell microvesicles transfer cystinosin to human cystinotic cells and reduce cystine accumulation in vitro," *PLoS ONE*, vol. 7, no. 8, Article ID e42840, 2012.

[15] A. P. McMahon, B. J. Aronow, D. R. Davidson et al., "GUDMAP: the genitourinary developmental molecular anatomy project," *Journal of the American Society of Nephrology*, vol. 19, no. 4, pp. 667–671, 2008.

[16] P. Romagnani and R. Kalluri, "Possible mechanisms of kidney repair," *Fibrogenesis & Tissue Repair*, vol. 2, article 3, 2009.

[17] G. A. Challen, G. Martinez, M. J. Davis et al., "Identifying the molecular phenotype of renal progenitor cells," *Journal of the American Society of Nephrology*, vol. 15, no. 9, pp. 2344–2357, 2004.

[18] A. C. Brown, S. D. Muthukrishnan, J. A. Guay et al., "Role for compartmentalization in nephron progenitor differentiation," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 110, no. 12, pp. 4640–4645, 2013.

[19] B. D. Humphreys, S. Czerniak, D. P. DiRocco, W. Hasnain, R. Cheema, and J. V. Bonventre, "Repair of injured proximal tubule does not involve specialized progenitors," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 108, no. 22, pp. 9226–9231, 2011.

[20] M. L. Angelotti, E. Ronconi, L. Ballerini et al., "Characterization of renal progenitors committed toward tubular lineage and their regenerative potential in renal tubular injury," *Stem Cells*, vol. 30, no. 8, pp. 1714–1725, 2012.

[21] Z. Zhang, D. Iglesias, N. Eliopoulos et al., "A variant *OSR1* allele which disturbs *OSR1* mRNA expression in renal progenitor cells is associated with reduction of newborn kidney size and function," *Human Molecular Genetics*, vol. 20, no. 21, pp. 4167–4174, 2011.

[22] S. Srinivas, M. R. Goldberg, T. Watanabe, V. D'Agati, Q. Al-Awqati, and F. Costantini, "Expression of green fluorescent protein in the ureteric bud of transgenic mice: a new tool for the analysis of ureteric bud morphogenesis," *Developmental Genetics*, vol. 24, no. 3-4, pp. 241–251, 1999.

[23] T. Watanabe and F. Costantini, "Real-time analysis of ureteric bud branching morphogenesis in vitro," *Developmental Biology*, vol. 271, no. 1, pp. 98–108, 2004.