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Stem cells and kidney organogenesis

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1. ABSTRACT

The discovery of tissue stem cells has launched the current boom in the field of regenerative research, which is tremendously exciting and holds enormous therapeutic potential. Despite such optimism, recent findings have tempered the potential for medical practice. Anatomically complicated organs, such as the kidney, have

proved refractory to stem cell-based regenerative techniques. As the kidney has the capacity to regenerate after renal injury, investigations into the mechanisms underlying kidney organogenesis may provide the clues to solving the puzzle of complex organ regeneration. This article reviews the current understanding of kidney organogenesis and kidney stem cells, and discusses the potential of kidney organogenesis as a therapeutic strategy for renal failure.

2. INTRODUCTION

Over the past decade, stem cell research has advanced significantly. Together with the originally discovered hematopoietic stem cells, other stem cells (or progenitor cells), including endothelial stem cells (1) and neural stem cells (2), are now well characterized. Such research has expanded the potential for organ regeneration using stem cells to replace affected tissues as the ultimate therapeutic strategy. In the field of nephrology, tissue regeneration is gaining considerable attention as the next generation of therapy for renal failure. Prior to the complete loss of renal structure, kidney function may be restored by reactivating quiescent renal stem cells and/or supplying renal stem cells expanded sufficiently *in vitro*. Conversely, in cases in which kidney structure is totally disrupted, the only cure may be the development of a functional whole kidney *de novo*. In this article, we review the recent findings applying kidney stem cell technology, and the accumulated knowledge of kidney organogenesis, to the therapeutic intervention of renal failure.

3. PURSUING KIDNEY STEM CELLS

Bone marrow stem cells have the capacity to contribute to the formation of kidney cells including mesangial cells (3,4), tubular epithelial cells (5), and podocytes (6). Accumulating evidence suggests that kidney stem cells reside in the adult kidney and recent research has focused on the identification and characterization of such stem cells.

3.1. Bone marrow-derived kidney stem cells

Recent findings provide the first evidence that bone marrow stem cells abnormalities are involved in the pathogenesis of renal disease and give rise to the hypothesis that renal progenitor cells reside in the bone marrow (7,8,9). These findings include the observation of mesangial deposits after allogeneic bone marrow transplantation in a patient with IgA nephropathy associated with chronic myeloblastic leukemia (7). Bone marrow exchange from a mouse with IgA nephropathy and a wild-type mouse was found to alter the serum IgA level and induced glomerular damage (8,9). Further evidence for the production of renal stem cells from bone marrow came from the observation of Y chromosome-positive tubular cells in the kidneys of male patients who had received kidney transplants from female donors (6) and the observation that approximately 1% of tubular cells were Y chromosome-positive following recovery from acute tubular necrosis (5). As a result of these preliminary studies many subsequent investigations focused on the identification of extrarenal stem cells within the bone marrow or the circulation by cell lineage analysis using bone marrow transplantation of marked donor cells. Lin *et al* (10) were the first to report the differentiation of bone marrow stem cells into renal proximal tubular cells with the capacity to contribute to renal tubular regeneration when transplanted into female mice with renal ischemia/reperfusion injury. Subsequently, Kale and coworkers reported the therapeutic potential of bone marrow stem cell infusion for the treatment of ischemia/reperfusion injury in mice (11). This report provided the conceptual basis for the development of therapeutic strategies involving exogenous renal stem cells to enhance recovery from acute renal failure (ARF). Subsequent investigations focused on different bone marrow fractions or different experimental models to investigate the potential therapeutic capacity of renal stem cells. These studies employed the transplantation of bone marrow cells marked with LacZ, EGFP, or a genetic marker (Y chromosome), and the detection of marked cells after induction of renal damage using X-gal staining, fluorescence microscopy, or fluorescent *in situ* hybridization for Y chromosome detection, respectively. The detection of marked renal tubular cells suggested that a fraction of transplanted bone marrow cells contributed to renal regeneration following experimental ARF, and confirmed the therapeutic potential of bone marrow transplantation technology. More recently, Duffield *et al* (12) demonstrated that all of the detection systems used in these earlier studies, had the capacity of producing false positives, which could overestimate the contribution of bone marrow-derived cells to renal repair.

Taken together, the data to date indicates that the capacity of adult bone marrow-derived cells to differentiate into, or fuse with, renal tubular cells, is at best an uncommon event without a predominant role in tubular regeneration (13).

3.2. Adult kidney stem cells

Renal stem cells may migrate and populate the kidney during development and remain quiescent, with the capacity for self-renewal and differentiation, until stimulated to differentiate into mature renal residential cells. The conventional method for the isolation of tissue stem cells is the use of cell surface markers. Cells positive for CD133, speculated as a universal cell surface marker of tissue stem cells (14-16), were recently identified in the human adult kidney in the interstitium of the renal cortex (17). These CD133-positive human kidney cells differentiated into renal tissue *in vivo* when injected into immunocompromised SCID mice (17). Gene expression profiling of mesenchymal cells from embryonic kidneys was used to screen for other cell surface markers of renal stem cells (18). Challen *et al* identified 21 genes that were selectively upregulated in cells destined to differentiate into renal tissue. CD24 and cadherin-11 were identified as surface proteins with potential for isolating progenitor cells from the adult kidney. Cells expressing CD24 were incorporated into newly formed tubules, whereas cadherin-11 was expressed primarily in cells forming the interstitium (18). Using CD133 and CD24, Sagrinati *et al* (19) isolated

multipotent progenitor cells with the capacity to differentiate *in vitro* into proximal and distal tubules, osteogenic cells, adipocytes and neuronal cells, and a subset of parietal epithelial cells (PEC) in the Bowman's capsule of the adult human kidney. Intravenous injection of $CD24^{+}CD133^{+}$ PEC into SCID mice with glycerol-induced ARF may regenerate tubular structures in different sections of the nephron, and thereby reduce any associated morphological and functional kidney damage. The stem cell antigen-1 (Sca-1) has also been used to isolate kidney stem cells (20). Populations of Sca-1 positive cells reside in the interstitial space of the kidney and have the capacity to differentiate into several cell lineages and adopt a tubular phenotype when injected directly into the renal parenchyma, shortly after ischemia/reperfusion injury. Comparison studies are required to identify the most appropriate cell surface markers and cell surface marker profiles for the identification of stem cells in the adult kidney.

Gupta *et al* used an approach to isolate multipotent adult progenitor cells from rat kidney (21). The isolated cells have a cell surface marker profile similar to undifferentiated cells and can differentiate into renal tubules when injected under the capsule of an uninjured kidney or intra-arterially after renal ischemia/reperfusion injury. Kitamura *et al* (22) attempted to establish renal progenitor cells using microdissection. This technique is based on the knowledge that one of two distinct areas of cell proliferation during kidney development occur at the corticomedullary junction corresponding to the primitive S3 segment of the proximal tubule (23). Segments of nephron were cultured separately and after simple limiting dilution, the cell line exhibiting the most potent growth was isolated. This technique was used to establish the rKS56 cell line expressing the immature cell markers associated with kidney development and mature cells markers associated with tubules. This cell line has the capacity to differentiate into mature tubular cells *in vitro* and to replace injured tubules and improve renal function following implantation *in vivo*. Investigation of the cell surface marker profile of the rKS56 cell line may lead to the identification of a universal marker of kidney stem cells.

Detection of side-population (SP) cells, which are negative for Hoechst 33342 dye staining, is another common method for identifying stem cells. While the original study used this technique to isolate a population of uncommitted hematopoietic stem cells, subsequent studies revealed that SP cells with stem cell capacity may populate other organs (24,25). Hishikawa *et al* (26) isolated kidney SP cells from two congenital mouse models of renal failure. Microarray analysis revealed that the gene *Musculin/MyoR*, which is usually expressed during muscle development (27), is expressed at high levels in SP cells suggesting the use of this gene as a kidney stem cell marker. Musculin/MyoR-positive SP cells were localized to the interstitial space of the kidney and the therapeutic potential of these cells was demonstrated using the cisplatin-induced ARF model. Although SP cells may be kidney stem cells, it is possible that the therapeutic effect of these cells was not directly due to differentiation of the SP cells and integration into injured tubule cells, but to an indirect paracrine effect on the growth of surrounding cells via HGF, VEGF, and BMP7, since the number of re-distributed SP cells after systemic injection was too low to account for a direct contribution.

Labeling cells with the DNA marker bromodeoxyuridine (BrdU) is also an established technique to identify tissue stem cells and is based on the assumption that stem cells cycle slowly and differentiate only as demanded by tissue turnover. Maeshima *et al* (28) report some success with renal tissue by injecting BrdU intraperitonealy into adult rats once a day for 7 days, and inducing ischemic/reperfusion injury after 14 days. BrdU-positive cells were observed in the tubules, but not in the glomeruli or capillary vessels. Quantitative analysis showed a two-fold increase in the number of BrdU-positive cells after reperfusion, suggesting that the majority of proliferating cells in the recovering kidney following renal ischemia were derived from BrdU-positive renal progenitor cells. In similar experiments, Oliver *et al* (29) injected 3-day-old rats subcutaneously twice a day with BrdU for 3.5 days and after 2 months identified BrdU-positive cells. During this stage of rat development, the kidney is growing and residential cells are proliferating, thus the slow-cycling stem cells are readily distinguished from other cells. Numerous BrdU-positive cells were found in the renal papilla and only small numbers were found in the outer-cortex, mid-cortex, and medulla, localized mainly within the interstitial area. The FACS-sorted BrdU-positive cells developed epithelial characteristics *in vitro* and were shown to migrate and incorporate into mature tubules *in vivo*. Following a transient episode of ischemia, the BrdU-positive cells quickly entered the cell cycle and disappeared from the papilla, thereby implicating these cells in renal repair (29). This study suggests that the renal papilla contains a population of stem cells involved in kidney maintenance and repair, although the signals and mechanism underlying this process is unclear. Vegetseder *et al* recently published data that may exclude the contribution of stem cells in tubular regeneration in healthy rat kidneys (30). By definition, stem cells are undifferentiated and slow-cycling and the division of stem cells leads to the generation of slowly cycling stem cells (self-renewal) or rapidly cycling transit-amplifying (TA) cells. The study by Vegetseder *et al* provided evidence that slow-cycling cells were well-differentiated after cell division and that the rapidly cycling TA population was absent from the S3 segment in healthy rat kidneys (30). This result suggests that tissue stem cells do not contribute to normal cell turn-over. Further investigations are required to elucidate the role of kidney stem cells in each disease state.

A recent study by Lin *et al* (31) confirmed the involvement of stem cells in renal repair. This study provided direct evidence that regenerating-tubule cells are derived from renal tubular epithelial cells using chimeric mice in which the mature renal tubular epithelial cells and their progeny are permanently labeled with EGFP. The relative contribution of intrinsic versus exogenous populations of cells to renal repair after

ischemic/reperfusion injury was determined by quantitative analyses of regenerating BrdU-positive cells and bone marrow-derived Y-positive cells. It was revealed that 89% of proliferating epithelial cells originated from the host cells and the remaining 11% originated from the donor bone marrow cells. In agreement with the earlier work of Duffield *et al* (12), this data demonstrated that extrarenal bone marrow-derived cells can be incorporated into renal tubules after ischemic injury, and that intrarenal cells are the major source of tubular regeneration. The current focus in this field is the application of bone marrow-derived cells to the treatment of renal injury. Recent evidence suggests that liver progenitor cells, known as oval cells, that transdifferentiate into hepatocytes or biliary epithelial cells, may originate not only from the point where the terminal bile ducts meet the periportal hepatocytes, but also from the bone marrow (32). It is equally likely that the origin of other stem cells, including those in the kidney, is not restricted to one location but may be supplied from different locations according to the severity, location, and duration of damage. Resident stem cells constitute only a small percentage of the cells comprising an organ (33), implying that tissue-specific stem cells are not sufficient in number for therapeutic regeneration after tissue injury and must either be expanded *in vivo* or supplied on demand from the circulation. Future studies are required to provide a better understanding of the mechanism underlying control of the contribution of renal stem cells in a given pathophysiological setting for the therapeutic application of renal stem cells to human disease.

3.3. The molecular phenotype of kidney stem cells

According to the findings described thus far, renal stem cells exist within the adult kidney; however, the question remains as to whether enough stem cells are present for therapeutic kidney regeneration after injury. One way to answer this question experimentally is to identify the molecules that recruit and/or stimulate quiescent renal stem cells, particularly in response to renal damage. Growth factors are thought to act as renotropic factors with the capacity to activate resident stem cells, accelerating the regeneration of tubular parenchymal components after acute injury (34,35). The growth factors mediating renal development have been studied intensively on the premise that renal regeneration and development share a common underlying mechanism (36,37). Hepatocyte growth factor (HGF) is of particular interest as during perinatal kidney development, HGF induces branching morphogenesis of the ureteric bud (UB) (38,39) and stimulates the epithelial differentiation of metanephric mesenchymal cells (40). The *bcl*-2, *bax*, and *pax-2* genes, which will be described in more detail later, are also of interest as these genes play important roles in metanephric and urogenital development (41-43) and are also re-expressed in proximal tubular cells after acute tubular damage (44,45). These examples are consistent with the hypothesis that tissue regeneration activates a cascade of developmental gene pathways. A number of growth factors participate in renal development (46), and several are identified as renotropic factors important in tubular regeneration of the kidney, including HGF, epidermal growth factor (EGF), insulin-like growth factor-I (IGF-I), bone morphogenetic protein-7 (BMP-7) (47), and leukemia inhibitory factor (LIF) (48). These factors are potent regulators of kidney organogenesis (46,49), and administration of these growth factors promotes tubular regeneration after a variety of insults (47,50). For example, the facilitation of renal regeneration by BMP-7 involves reversal of the transforming growth factor (TGF)-β1-induced epithelial-to-mesenchymal transition, a process associated with enhanced E-cadherin expression that appears to mimic the role of BMP-7 in embryogenesis (50). Another example is the acceleration of renal regeneration after renal injury caused by follistatin induced inhibition of activin A, a negative regulator of branching morphogenesis during kidney development (51,52).

This work was further enhanced by a recent finding that the homozygous deletion of *Sall1* impairs complete UB outgrowth and tubule formation in the mesenchyme, implicating this gene in the initial step of the mesenchymal-to-epithelial conversion (53). *Sall1* is distinct from the known regulators of this process. It is also expressed in the subventricular zone of the central nervous system and progress zones of limb buds, where neural and mesenchymal stem cells reside, respectively, leading to speculation that *Sall1* is associated with stem cells in several organs, including the kidney. Subsequently, Osafune *et al* (54) established a novel colony-forming assay system using NIH3T3 fibroblast cells expressing Wnt4 and identified renal progenitors in metanephric mesenchyme using *Sall1* as a marker. Only cells strongly expressing *Sall1* formed colonies from which a three-dimensional kidney structure was reconstituted in an organ culture setting. This assay system has the potential to identify renal stem cells in the adult kidney. Further research into these molecules may reveal novel renotropic factors with the capacity to specifically induce the differentiation of tissue stem cells into mature cells for therapeutic applications.

4. KIDNEY ORGANOGENESIS

4.1. Evolutionary change in kidney organogenesis

4.1.1. The three stages of amniotic kidney development

The urogenital system encompassing the kidneys and gonads and their respective duct systems are derived from intermediate mesoderm (IM) located between the paraxial mesoderm and the lateral plate. The kidney of amniotes (reptiles, birds, and mammals) develops through three stages; the transient pronephros and mesonephros, and the permanent metanephros. These stages develop successively, from the rostral to the caudal, overlapping in time. Investigation of the similarities and differences between the pronephros, mesonephros and metanephros reveal the evolutionary conservation of patterns of development.

4.1.1.1. The pronephros

The first kidney, the pronephros, is usually a transient embryonic stage in vertebrates. The pronephros is usually poorly developed in the amniotes and appears as a rudimentary structure without obvious excretory function during embryonic development. By comparison, most fish

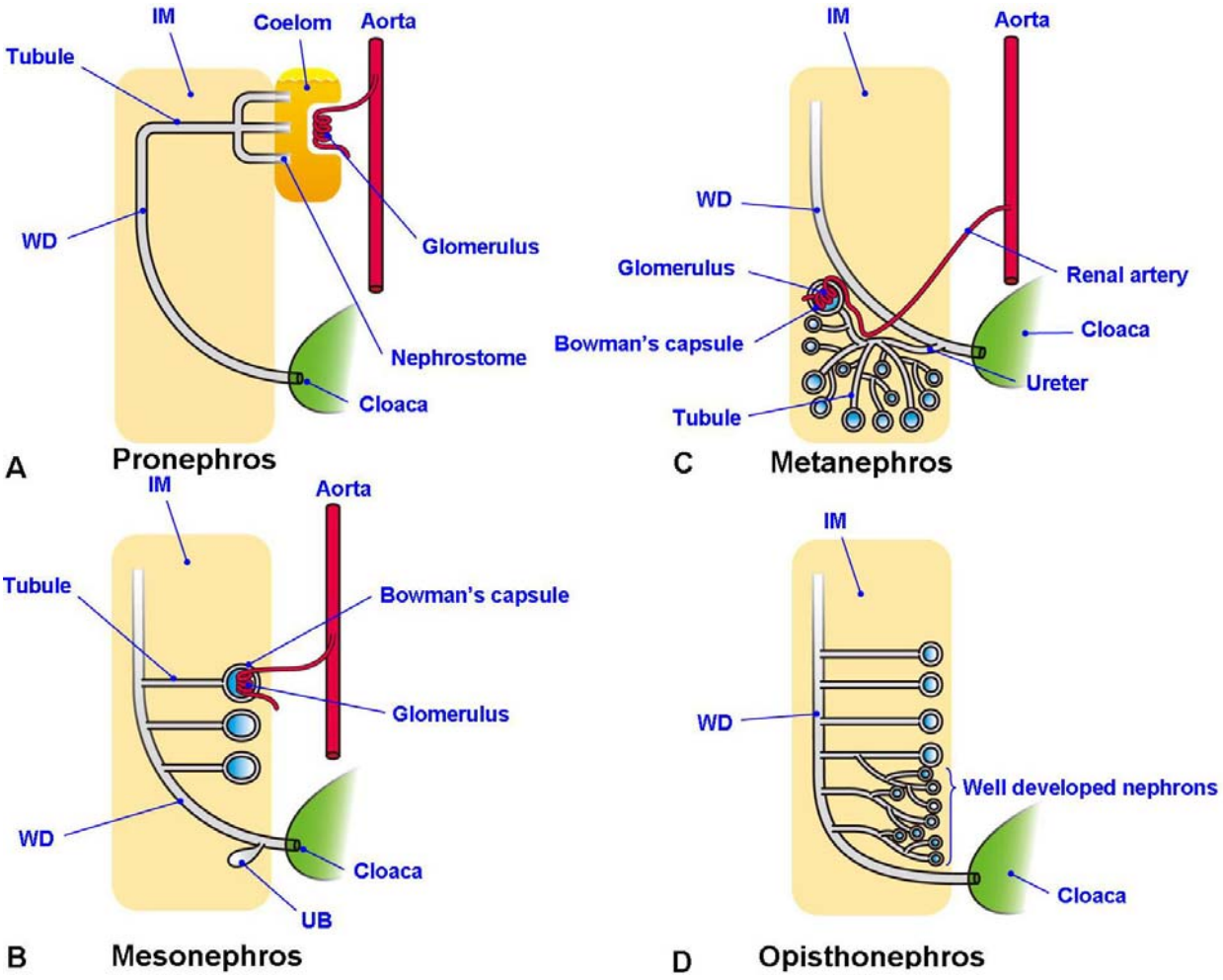

Figure 1. Development of the vertebrate kidney. A. Pronephros: The pronephros arises from the anterior section of the IM. The glomeruli are referred to as external glomeruli and are not located within the Bowman's capsule. Waste is deposited into the coelom then excreted outside the body. B. Mesonephros: The mesonephros arises from the middle section of the IM. The ducts are referred to as WDs and the glomeruli, referred to as internal glomeruli, are located within the Bowman's capsule. Waste is drained directly into the ducts. C. Metanephros: The metanephros is the permanent kidney of amniotes and arises from posterior section of the IM. A new duct, the ureter, has arisen and the collecting duct system has developed an extra order of complexity by branching and allowing many nephrons to form in a compact space. The internal glomeruli are supplied by a renal artery extending from the aorta. D. Opisthonephros: The opisthonephros is the adult kidney of fish and amphibians. It arises from the middle and posterior section of the IM. Although the main duct is a WD, the tubules of the posterior region of the opisthonephros are well developed resembling those of the metanephros.

and amphibians have a well-developed pronephros that functions transitionally during larval stages.

The pronephros arises just caudal to the head (Figure 1A) and usually contains one nephron. The pronephric glomerulus, which is supplied by capillaries branching from the dorsal aorta, is referred to as the external glomerulus (Figure 1A) as it is not within Bowman's capsule but protrudes into the roof of the coelom (body cavity). Filtrate from the glomerulus passing into the coelom is driven into the pronephric tubules via ciliated nephrostomes and finally flows into the pronephric duct as urine. The pronephric tubules have distinct proximal and distal segments with one end opening into the coelom and the other opening into the duct. Thus, waste is deposited into the coelom and then excreted outside the body.

4.1.1.2. The mesonephros

In most vertebrates, the pronephros degenerates and disappears as the second kidney, the mesonephros, develops caudal to the pronephros (Figure 1B). The duct of the mesonephros is an extension of the pronephric duct and both ducts are often regarded as a single duct, the Wolffian duct (WD), also referred to as the archinephric duct. The WD continues to elongate and eventually opens to the cloaca, with the induction of IM in the mesonephric tubules occurring caudally along the WD. The morphology of the

mesonephros varies considerably between species. In mammals, the mesonephros is comprised of many nephrons, although the number is variable, ranging from almost no nephrons in rodents to 34 in humans and 80 in pigs (55). The degree of mesonephric development appears to be linked to the extent of placental development (55). Although the more caudal mesonephric tubules tend to be well developed, they have no loop of Henle or juxtaglomerular apparatus. Also, the mesonephros usually consists of a linear sequence of nephrons as the mesonephric tubules seldom branch, unlike the tubules of the metanephros.

Unlike the pronephros, the mesonephric glomerulus is contained entirely within Bowman's capsule and as such is referred to as the internal glomeruli (Figure 1B). While the mesonephric tubules are not connected to the coelom tubules in the anterior mesonephros sometimes have connections to the coelom via ciliated tubules, called peritoneal funnels. The filtrate from the glomerulus, which is also supplied by the aorta, is forced into Bowman's capsule as a result of high blood pressure and flows into the mesonephric tubules without the aid of ciliated tissues, nephrostomes or peritoneal funnels. Finally, the fluid is drained into the WD as urine. These tubules also have distinct proximal and distal segments. Thus, by losing connections to the coelom, the nephrons can receive blood directly and excrete waste more effectively. It is likely that the pronephric tubules connect to the coelom so that fluid in the coelom can act like a balloon to maintain body shape at a stage in development when bone and muscle formation is immature.

The mesonephros contributes to the adult state of development as it is the source of hematopoietic stem cells necessary for blood cell development (56, 57). Moreover, in male, the caudal section of the WD becomes the male genital duct (vas deferens) for sperm transport by connecting the testis to the anterior region of the kidney, whereas the rostral section of the WD coils and forms the epididymis (58). In female mammals, the WD regresses and the müllerian duct arises next to the WD before regression is complete and forms the oviduct, uterus, and vagina.

4.1.1.3. The metanephros

In amniotes, the mesonephros degenerates as the last kidney, the metanephros, develops caudal to the mesonephros (Figure 1C). In order to form the metanephros, the UBs, formed at the caudal end of the WDs, and the metanephrogenic mesenchyme (MM), at the caudal region of the IM, undergo a process of reciprocal induction (59). The UBs first extend dorsally then rostrally, passing parallel to the WDs. Then, the UBs invade the IM, inducing it to become MM, which in turn forms the nephrons or other tissues. Reciprocally, the UBs are induced by the MM to branch and differentiate into the collecting duct system. The elongated stalk of the UB at the caudal region develops into the ureters. After induction by the UB, the MM aggregates at the tip of the UB. The aggregated mesenchyme differentiates successively into vesicles, comma-shaped bodies, S-shaped bodies, and finally nephrons. This process is referred to as the mesenchymal-to-epithelial transition (MET). Thus, the metanephros consists of derivatives of two different tissues: 1) The derivatives of the MM (nephrons; from the glomeruli to the distal tubules, stromal, neuronal, endothelial, and smooth muscle cells, as well as the juxtaglomerular apparatus and perhaps capillaries. 2) The derivatives of the UB (the collecting duct system; the collecting ducts, calyces, pelvis, and the ureters).

Instead of the linear structure of the mesonephros, the metanephros has a branched organization due to the branching of the UBs, leading to a large number of nephrons and a collecting duct system in a much more compact structure (60). The final size of the adult kidney is variable. The number of nephrons in a normal human kidney ranges from 600,000 to 1,000,000, whereas in the mouse it ranges from 1,600 glomeruli at birth to about 11,000 in the adult (61). The glomeruli of the metanephros receive blood from the renal artery, running from the aorta to the hilum of the metanephros. The renal artery branches into smaller arteries and finally terminates in afferent glomerular arterioles (Figure 1C). The metanephric nephrons in mammals and a few species of birds possess the loop of Henle, which aids urine concentration and thus conservation of water (62). The metanephros has an excretory duct, the ureter, which was not present in the mesonephros. As a result the dual use of the WD is not required in male amniotes as urine is transported exclusively by the ureters and sperm is transported exclusively by the WDs (vas deferens).

4.1.2. Influence of the environment on the development of kidney function

The main functions of the kidney are osmoregulation (the regulation of water and electrolyte balance) and excretion (the elimination of metabolic waste containing nitrogen). Kidney function has modified during evolution according to changes in the environment. The presence of the three kidneys in amniotes and the absence of the metanephros in fish and amphibians may reflect adaptation to life on land (63). This section discusses environmental factors leading to physiological and anatomical differences in the kidney among vertebrates.

4.1.2.1. Three routes for the elimination of ammonia

The metabolism of proteins, amino acids, or nucleic acids produces nitrogen, usually in the reduced form of ammonia. Since ammonia is highly toxic, it must be rapidly removed from the body. In vertebrates, ammonia is eliminated via three routes including: 1) Ammonotelism, direct excretion of ammonia; 2) Uricotelism, excretion of nitrogen in the form of uric acid and; 3) Ureotelism, excretion of nitrogen in the form of urea. Ammonotelism is common in animals living in water due to the water soluble nature of ammonia and the large volumes of water required for flushing ammonia from body tissues. Animals living in water eliminate ammonia through gills, skin, or kidneys. Most of the nitrogenous waste of fish is excreted directly into the surrounding water by the gills. Many amphibian larvae can change the route of nitrogen excretion according

to the availability of water. Amphibian larvae eliminate ammonia in water and excrete urea on land after metamorphosis.

Vertebrates living on land do not use large quantities of water for excretion; instead they have evolved mechanisms to conserve water. Furthermore, the gills, which are the major route for ammonia excretion in fish, have been lost in amniotes. For this reason, ammonia is converted into uric acid or urea, which is less toxic. Birds and reptiles, which are oviparous (egg-laying), depend primarily on uricotelism, the conversion of ammonia into uric acid. Since uric acid is only slightly soluble in water, it exists in a semi-solid or solid form allowing its excretion in relatively small volumes of water. Moreover, since uric acid does not exert osmotic pressure within embryos, it can be safely sequestered within the egg until hatching. By comparison, mammals depend largely on ureotelism, the conversion of ammonia into urea, which is highly soluble in water. Mammals conserve water by excreting urea as highly concentrated urine via the well-developed loop of Henle in the metanephros. In this way, the metanephros has addressed the related problems of water conservation and nitrogen elimination.

4.1.2.2. Why does the mesonephros develop rather than the pronephros?

The mesonephros develops in the adult in most fish and amphibians that use the pronephros as a transient kidney in the larval stage. We speculate that more nephrons are required for the higher waste load of adults. In general, high levels of metabolism due to an increase in body size lead to a greater production of waste. The mesonephric nephrons are more highly developed in amphibians than in fish. This is consistent with the lack of gills in adult amphibians to excrete toxic nitrogen waste. The more highly developed mesonephric nephrons in adult amphibians are most likely an adaptation to the loss of gills and have the capacity to excrete waste efficiently as the glomeruli are connected directly to the high blood pressure of the aorta. Thus, we speculate that the mesonephros develops nephrons caudally along the aorta to acquire a greater flow of blood.

4.1.2.3. What is the opisthonephros?

In animals that do not possess a metanephros, such as most adult fish and amphibians, the caudal regions of the mesonephroi are more highly developed (Figure 1D). The mesonephros is the functional kidney during embryogenesis and is modified in the adult by incorporation of additional tubules caudal to the original mesonephros. This extended mesonephros is sometimes referred to as the opisthonephros (64). The opisthonephros of adult amphibians has a large number of nephrons due to a highly branched collecting duct system, similar to that of the metanephros. For example, the frog *Rana* has around 2,000 opisthonephric nephrons and although the opisthonephros has distinct proximal and distal segments, it does not possess a loop of Henle (65).

4.1.2.4. Why does the metanephros develop instead of the opisthonephros?

The amniotes have developed the metanephric kidney, although it is unclear if it is necessary only in amniotes. An increase in waste elimination and water conservation is achieved in the opisthonephros by an increase in the number of nephrons and in the metanephros by an increase in branching of the collecting ducts (66).
Among the amniotes, mammals and birds are blood-warmed animals and control body temperature through internal means such as muscle shivering, fat burning, and panting, which are very energy-intensive. The energy required for the homeothermic control of body temperature comes from food, leading to more waste production. Moreover, as vertebrates have become more terrestrial, water economy has become more important due to the threat of dehydration. In mammals and a few species of birds the loop of Henle has developed as an adaptation to conserve water (67). The loop of Henle creates an environment around the tubules that encourages the reabsorption of water before it is excreted from the body. As a consequence, urine becomes highly concentrated.

4.1.2.5. What does it mean that male amniotes stop the dual use of their WDs?

In male amniotes that do not possess a metanephros, the WD in the mesonephros or opisthonephros transports both sperm and urine. In the metanephros, the ureter transports urine exclusively, while the WD transports sperm exclusively. It is unclear why these functions are divided in male amniotes.
External fertilization, in which eggs and sperm meet outside the body, is common among invertebrates and many aquatic vertebrates. However, many vertebrates live and mate in dry environments or fast-moving streams, which is disadvantageous to external fertilization. Internal fertilization increases the success rate of sperm transfer under these conditions. Indeed, within amniotes, fertilization is usually internal, with sperm transfer occasionally aided by a male intromittent organ.

Sharks also reproduce by internal fertilization and many fish and amphibians reproduce by external fertilization. Interestingly, in male sharks, the WDs may be involved exclusively in sperm transport due to the presence of male specific accessory urinary ducts that drain urine from the opisthonephros (68). Thus, male sharks do not have dual function WDs, despite the absence of a metanephros. Although the mechanisms underlying the development of accessory urinary ducts are unknown, we speculate that the opisthonephros may be a prototype of the metanephros. Thus, it is interesting that male amniotes and male sharks, which copulate via an intromittent organ, have ducts only for sperm transport. The enhanced success rate of fertilization resulting from more concentrated sperm may be one reason why the metanephros develops instead of the opisthonephros.

4.1.2.6. Why can not nephrons regenerate in mammals?

In mammals, the formation of new nephrons, neo-nephrogenesis, is terminated shortly after birth (69). In contrast, it is probably a common feature in

elasmobranches and teleosts. Elger *et al.* (70) demonstrated that neo-nephrogenesis, not hypertrophy, is observed in the skate, *Leucoraja erinacea*, after partial nephrectomy. They also demonstrated that the adult skate kidney, which is the opisthonephros, contains stem cell-like mesenchymal cells. We speculate that even though the skate possesses stem cell-like mesenchymal cells, neo-UBs may also be required for neo-nephrogenesis. In this aspect, the metanephros of adult amniotes, including mammals, has no more neo-UBs because metanephric UBs have already differentiated into the collecting duct system and because branching of the UB arise exclusively in the tip of the UB in the metanephros. By comparison, entire part of the WD in the opisthonephros may have ability to sprout neo-mesonephric buds for interaction with renal stem cells (see 4.3). Thus, a potential of the opisthonephros and the metanephros for neo-nephrogenesis seems to be different. However, the reasons why neo-nephrogenesis is not observed in the newt, *Notophthalmus viridescens*, a urodelic amphibian, are unclear (71).

4.1.3. The tripartite and holonephric concept of kidney organization

The holonephric concept of kidney formation is that the three types of kidney arise as part of one organ, the holonephros, which produces tubules rostral to caudal. An alternative view, the tripartite concept, is that each kidney arises within the anterior, middle, or posterior region of the IM, giving rise to the pronephros, mesonephros, or metanephros, respectively. Although we have reviewed different types of kidney, they are all derived from the same band of the IM, which runs caudally along the somites (72). Moreover, histological structures and physiological functions are similar among the different kidney types. Thus, both concepts can be applied to the current understanding kidney development.

Based on the holonephric concept, it is easy to consider the pronephric duct and the mesonephric duct as a single duct. The WD arises from the rostral domain of the IM and eventually opens to the cloaca, passing through the lateral domain, then the ventral and medial domains of the IM. Therefore, the UB is formed dorsally to invade the IM at the caudal end (Figure 1C) (73). Based on the tripartite concept, the opisthonephros arises from the middle and posterior region of the IM. We speculate that the posterior region of the IM may be designated for generation of the opisthonephros in fish and amphibians when the new duct does not arise and for the generation of the metanephros in amniotes only when the UB arises (Figure 1D).

4.2. Developmental process

This section reviews the essential steps during development of the kidney. Most developmental studies are performed using the metanephros of the mouse as a model of the mammalian permanent kidney. However, it may be useful to choose the appropriate model for studying kidney development as similar developmental events are likely to occur in all three kidneys (74).

4.2.1. Wolffian duct specification

The formation of the kidney is likely to initiate with the induction of the mesenchyme by the WD. Later, the WD also forms the UB, which is the lead for development of the metanephros, at the caudal end of the WD. Since the WD plays a central role in kidney development, it is known as the organizer.

WD morphogenesis occurs very early in development, for example, in mouse embryos, the WD is formed at embryonic day (E) 9.0 (75) and the UBs are formed at E10.5 (76). Since mouse embryos are very small at these early stages, experimental manipulation is difficult. It is also difficult to culture manipulated viviparous embryos outside of the uterus. For these reasons, WD morphogenesis has been extensively studied using chicken embryos (77-79). The advantages of using chicken embryos include: 1) Manipulation is relatively easy as the embryos are large readily accessed. 2) Manipulated embryos may be cultured *in ovo*. In this review, based on holonephric concept, we regard the pronephric duct and the mesonephric duct as a single duct, the WD.

Initially undifferentiated IM is specified to the WD. As previously reported, the pronephric progenitors of the chick can first be identified at the 6-7 somite stages by coexpression of transcription factors *Pax2*/*Lim1*/c-*Sim1*. In amniotes, by the 9-10 somite stage, *Lim1* (80,81) and *c-Sim1* (82) become restricted to a dorsal subpopulation of the *Pax2* (83) expressing progenitors, which gives rise to the WD. By comparison, *Pax2* expressing cells ventral to the WD coexpress *WT1* (84), suggesting that they are the mesenchyme that gives rise to the nephrons. Thus, *Pax2* is expressed in the WD progenitors and in the mesenchyme. Obara-Ishihara *et al* demonstrated using lineage tracing with DiI and replication–defective retrovirus, that the IM adjacent to somite 10 at the 10-somite stage includes the WD progenitors (77). Moreover, we found that when the WD progenitors are labeled with DiI, the DiI-labeled cells are located not only in the WDs, but also in the UBs in the later stages of kidney development (A. Fukui, unpublished data). Together, these data demonstrate that the population of WD progenitors also includes the UB progenitors.

4.2.2. Wolffian duct and ureteric bud formation

Moreover, in order to examine how the WD and UB are formed, we traced the WD and UB progenitor cells with DiI. If DiI-labeled cells proliferate, the signals weaken because DiI stains the plasma membrane of cells. When the WD and UB progenitor cells are labeled with DiI, the signals do not weaken during caudal elongation of the WD (A. Fukui, unpublished data). The DiI labeled cells in the UB weaken rapidly after the UB begins to elongate dorsally as a duct, while the DiI signals in the WDs remain strong (A. Fukui, unpublished data). This result suggests that while the cells comprising the WD do not proliferate during caudal elongation the WD progenitors do. This data also suggests that cells comprising the UB proliferate actively *in situ* to generate the well-branched collecting duct system. Moreover, we found that epithelialization of the WD arises in a successive manner from the anterior to the posterior after it reaches the cloaca (A. Fukui, unpublished data). Thus, WD development involves the caudal migration of mesenchymal cells derived from the WD progenitor cells followed by epithelialization, as previously discussed (77).

By comparison, the formation of the UB involves elongation and branching of the epithelialized duct. Thus, the formation of the WD and UB occurs by different developmental processes.

4.2.3. Budding and branching of the ureteric bud

The UB induces the MM to form nephrons and other tissues. The MM, in turn, induces the UB to bud and branch and then to form the collecting duct system. These two-way, sequential interactions are known as reciprocal inductions. The reciprocal induction between the epithelium (the UB) and the mesenchyme (the MM) is the first step in generating the overall architecture of the kidney (59). The *GDNF/c-Ret* (*GDNFRα*) signaling is essential for promoting the budding of the UB (85,86). The UB sprouts from the caudal end of the WD primarily in response to *GDNF*, which is secreted by the MM. Conversely, the MM is induced to condense and then aggregate around the tip of the UB by the UB. These signals also control branching of the UB, which is likely to involve repeats of the budding process. The elongated stalk of the UB develops into the ureters. Ectopic budding causes congenital abnormalities of the kidney and urinary tract (CAKUT), leading to hypoplastic kidney disease, ectopia of the ureterovesical orifice, urinary outflow obstruction and/or reflux (87). *BMP4* inhibits ectopic budding and branching morphogenesis. *BMP4* also can promote tubule elongation (88).

4.2.4. Nephron development

Signals released from the UB induce differentiation of the MM into the nephrons. As the UB invades the MM, some loose cells of the MM are induced to form epithelial aggregates around the newly branched tip of the UB and begin the mesenchymal-to-epithelial transition (MET) process. By comparison, other MM cells generate the interstitial stroma. The aggregated mesenchyme forms a polarized renal vesicle, one end of which maintains contact with the UB epithelium. Then, a single cleft is formed in the vesicle to generate the comma-shaped body, followed by a second cleft to generate the S-shaped body. As the nephrons grow, the distal ends of the S-shaped bodies fuse with the UB-derived collecting ducts, and the proximal ends form the glomeruli. Thus, in the metanephros, since the UB continues to branch and induce new nephrons around the tip of the UB along the radial axis, the outer nephrons are younger during development.

4.2.5. Glomeruli development

The glomerulus is a complex structure comprised of cells that are both epithelial and endothelial in origin. At the S-shaped body stage, when glomerular development is first evident, the polarized vesicle elongates and forms two visible clefts, the proximal of which is infiltrated by endothelial precursor cells to generate a primitive vascular tuft. The podocyte precursors, located at the most proximal end of the S-shaped body, contact the endothelial cells and begin to differentiate. *VEGF* (vascular endothelial growth factor) is likely to help attract endothelial cells into the proximal cleft, eventually giving rise to the glomerular capillary loops (89). Developing podocytes are a key source of *VEGF*. These infiltrating endothelial cells may also generate the mesangial cells in response to stimulation by *PDGF-B* (platelet-derived growth factor B) (90). *PDGF-B* and its receptor *PDGFR-β* are expressed in the vasculature of the kidney. While *PDGF-B* is expressed in endothelial cells, its receptor is expressed in the smooth muscle and later the mesangial cells. Signaling from the endothelial cells to the smooth muscle appears to result in the recruitment of smooth muscle cells into the mesangial cells and capillaries of the glomerulus. As endothelial cells migrate and proliferate, they interact with differentiating podocytes to lay down the glomerular basement membrane (GBM). The GBM is formed at the boundary between podocyte and endothelial cells (63).

4.3. Key molecules in kidney development

Six key genes, *Lim1, Pax2, WT1, GDNF, c-Ret*, and *Wnt4,* appear to be involved in the essential steps of kidney development. Several studies have investigated the role of these genes by inducing the differentiation of undifferentiated cells into kidney cells.

4.3.1. Characteristics of key molecules

4.3.1.1. Lim1

Lim1 encodes a homebox protein containing a LIM domain that is expressed early in the IM. *Lim1* encodes a LIM domain containing a homeobox protein that is expressed early in the IM. *Lim1* is expressed in the WD and UB, developing tubules and collecting duct system, but not in the mesenchyme (80). Since, *Lim1* is required for correct patterning of all epithelial structures derived from the IM, including the WD, UB, and tubules, mice lacking *Lim1* lack all IM derivatives, including the pro-, meso-, and metanephros (81).

4.3.1.2. Pax2

Pax2 encodes a paired box-containing transcription factor and is expressed in multiple embryonic tissues. Like *Lim1, Pax2* expression is first detected in the IM, prior to formation of the WD. *Pax2* is expressed in the WD, UB, and early condensates, is downregulated in mature epithelia, and not expressed in glomeruli (83). Mutations in the *Pax2* gene cause the renal-coloboma syndrome (RCS), a rare syndrome characterized by optic nerve colobomas and renal abnormalities, sometimes with vesico ureteric reflux (VUR), high-frequency hearing loss, central nervous system anomalies, or genital anomalies. In *Pax2* null embryos, the WD forms but there are no pronephric or mesonephric tubules, no UB, and no metanephros. This suggests that *Pax2* is required for the mesenchyme to respond to inductive signals (91).

Although *Pax2* mutant mice do form the WD, this may be due to redundancy with the closely related gene *Pax8* (92). In the mouse, *Pax2/Pax8* double homozygous null embryos fail to generate epithelial structures and do not exhibit *Lim1* expression by E9.5 (93). By comparison, the ectopic expression of *Pax2* in chick embryos is sufficient to induce *Lim1*-expressing nephric structures in the IM, suggesting that *Pax2* expression is sufficient to specify renal tissue from the undifferentiated IM (63). *Pax2* has also been implicated in a number of cellular processes, including cell proliferation, apoptosis, and MET.

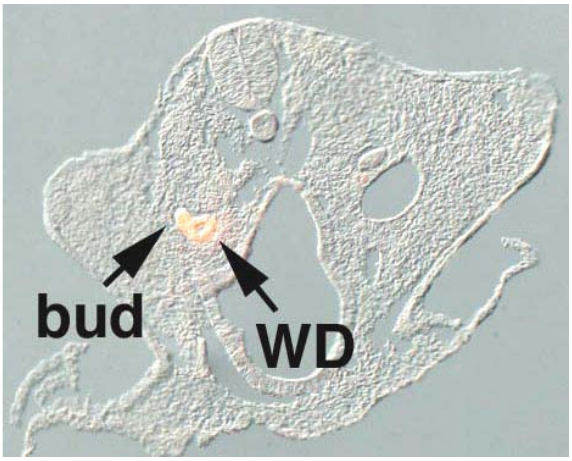

Figure 2. The bud of the mesonephros (confocal and DIC image of a frozen section, X100). DiI-labeled cells are located in the WD and in the bud of the mesonephros approximately 36 hours after labeling WD progenitor cells (E3.0).

4.3.1.3. WT1

The Wilms' tumor suppressor gene *WT1* encodes a zinc finger transcription factor. *WT1* is expressed at low levels in the mesenchyme before induction by the UB and at higher levels in aggregates, comma-shaped bodies, simultaneously with the repression of other genes, such as *Pax2 and Lim1*. Expression of WT1 is excluded from the WDs, UBs and tubules (84). *WT1* was originally identified as the gene involved in the pathogenesis of Wilms' tumor or nephroblastoma, which consists of undifferentiated mesenchymal cells, poor organized epithelium, and surrounding stromal cells. Since the neoplastic cells of the tumor are able to differentiate into a wide variety of cell types, the genes are thought to be important regulators of early kidney development. In humans, mutations in *WT1* are not only responsible for Wilms' tumors but also for the WAGR, Denys-Drash, and Frasier syndromes, all of which are characterized by the presence of kidney and genital abnormalities. *WT1* germline mutations are found in only 10% of patients with Wilms' tumors. Once mesenchymal cells have been induced, *WT1* expression is upregulated and the mesenchymal cells condense around the UB and then aggregate to undergo MET. *WT1* also becomes upregulated in the differentiating podocytes at the proximal region of the S-shaped body, which persist in the podocytes into adulthood (94). In *WT1*-null mice, although the WD forms, the UB never invades the MM. The MM then undergoes rapid apoptosis and the kidney does not form. *WT1* is essential in the MM for regulation of *GDNF* expression and the promotion of survival of uninduced cells (84).

4.3.1.4. GDNF/c-Ret

GDNF (glial cell-line-derived neurotrophic factor) is essential for budding and branching of the UB. *GDNF* is a peptide growth factor originally isolated from media conditioned by glial cells and has been widely studied due to its potent neurotrophic activity and its effect on cell survival. The MM expresses *GDNF,* which stimulates the budding of the UB via a receptor complex that includes *c-Ret* (a receptor tyrosine kinase) and the *GDNFRα* (GDNF receptor α) (85,86). GDNF is a direct transcriptional target of *Pax2* in the MM (91). Since the entire region of the WD, including the UB, initially expresses the *GDNF* receptor complex, the WD is capable of forming of the UB in response to a local source of *GDNF*. After invasion of the UB, *GDNF* expression is maintained in the condensed mesenchyme, but is downregulated in differentiating vesicles and stroma. *c-Ret* is localized to the branching tips of the UB. Mice mutants for *GDNF*, *GDNFRα*, or *c-Ret* lack kidneys due to the failure of UB formation. The phenotypes are variable, suggesting that redundant or modifier genes are included in the system.

4.3.1.5. Wnt4

Wnt4 encodes a secreted glycoprotein related to the *Drosophila wingless* gene and the mammalian *int-1* proto-oncogene. As a first marker of the induction*, Wnt4* is expressed in the early mesenchymal aggregates and comma-shaped bodies on either side of the UB, in which MET arises (95). In the absence of *Wnt4*, few aggregates form, and polarized renal vesicles and nephrons are absent due to failure of MET. Thus, *Wnt4* acts in an autocrine loop, driving its own expression. In *Wnt4* mutant embryos there is normal condensation of the MM and normal expression of *Pax2, WT1,* and *c-Ret* observed (95). This suggests that the initial inductive events are not *Wnt4* dependent. Interestingly, the UB undergoes significant branching in *Wnt4* mutant embryos (95).

4.3.2. Similar genes are expressed in all three kidneys

The six genes *Lim1, Pax2, WT1, GDNF, c-Ret*, and *Wnt4* are expressed in a similar manner in the mesonephros and metanephros in chicken embryos (A. Fukui, unpublished data). Vize *et al* previously reported that many genes are expressed in all three kidneys in the mouse, suggesting that similar pathways of development, function, and induction have been conserved (74). The mechanisms underlying development of the mesonephros and the metanephros are likely to be similar. When WD/UB progenitor cells are labeled with DiI, DiI-labeled cells are found in the WD, the UB in the metanephros, and also in the UB-like structures that sprout from the WD in the mesonephros (Figure 2) (A. Fukui, unpublished data). We also found that the tips of the UB-like structures express *c-Ret*, as in the UB and that the adjacent mesenchyme expresses *GDNF,* as in the MM (A. Fukui, unpublished data). These results strongly suggest that the reciprocal interaction with mesenchyme occurs in buds in a similar manner as in the metanephros. These results suggest that histological structures and developmental mechanisms are highly conserved among all kidneys.

Investigations on lower or non-viviparous vertebrates would further an understanding of the developmental mechanisms underlying kidney development. A comparison of genes involved in the development of other organs with those of the kidney would also be useful. For example, the lung represents another organ that undergoes extensive epithelial

branching, a common feature of organogenesis. The branching morphogenesis of the lung begins when epithelial buds form from preexisting epithelium, as in the kidney. Many of the genes involved in lung branching morphogenesis, such as Hedgehog, *BMP*, and *FGF* family members, are also expressed in the developing kidney (96). This suggests that genes involved in the essential mechanisms of development are conserved among organs.

4.3.3. Studies using key genes

For the generation of the 'perfect' kidney *in vitro*, it is necessary to regulate a number of developmental processes by controlling the molecular milieu. Although the signaling and phenotypes of single gene mutations have been extensively studied, it would be difficult to examine all genes involved in kidney development. The challenge now is to generate a three-dimensional, functional kidney derived from human mesenchymal stem cells (hMSCs) of patients with chronic renal failure (CRF) as the ultimate therapy to avoid hemodialysis or allotransplantation (97). To this end, the transplantation of a functional kidney is more important than aiming for the ideal kidney shape or size.

Indeed, several groups, including us, have succeeded in artificially differentiating undifferentiated cells into nephric cells by manipulating the expression of key genes. Osafune *et al* found that when isolated MM cells strongly expressing *Sall1*, a zinc finger nuclear factor essential for complete UB invasion and MM induction, are cultured on *Wnt4*-expressing feeder cells following aggregation, they differentiate into glomeruli and renal tubules (54). This suggests that exogenous *Wnt4* promotes MET and that *Wnt4* is sufficient to induce the differentiation of nephrons from isolated MM. Kobayashi *et al* found that *Wnt4*-transformed mouse embryonic stem (ES) cells differentiate into renal tubular cells (98). In a previous study we reported the differentiation of *GDNF*-expressing hMSCs into podocytes, tubular epithelial cells, and interstitial cells following transplantation into the MM of developing rat embryos (99). *GDNF* was chosen to induce the differentiation of hMSCs as the MM excretes *GDNF*, which is essential for the reciprocal interaction between the MM and the UB.

5. THERAPEUTIC ORGANOGENESIS

5.1. Generation of a functional transplantable kidney

The rebuilding of a kidney *de novo* faces many challenges. In 1999, Chan *et al* (100) reported the first attempt to establish a functional renal unit, by developing a transplantable pronephros in Xenopus. The presumptive ectoderm (animal cap) of Xenopus contains pluripotent stem cells that can be differentiated into multi-lineage tissue cells under specific culture conditions (101). Pronephric tubule-like structures can be induced from animal caps after only 3 hours using a combination of activin and retinoic acid. Transplantation of these pronephros-like units partially corrects the edema of bilaterally nephrectomized tadpoles allowing survival for up to 1 month (101). To our knowledge, this study remains the only one to establish a transplantable functional kidney unit *in vivo*, although the pronephros structure formed is too primitive for any clinical application in humans.

Lanza *et al.* (102) attempted to establish a self-kidney unit to eliminate the disadvantages of immune-response and the adverse effects of immunosuppressants. A histocompatible kidney for artificial organ transplantation was generated by a nuclear transplantation technique in which dermal fibroblasts isolated from an adult cow were transferred into enucleated bovine oocytes and nonsurgically transferred into progestin-synchronized recipients. After 6-7 weeks, metanephri were isolated from embryos, digested using collagenase, and expanded in culture until the desired cell number was obtained. These cells were then seeded on a specialized polymer tube followed by implantation into the same cow from which the cells were cloned. Remarkably, this renal device seeded with cloned metanephric cells produce urine-like liquid, whereas those without cells or seeded with allogeneic cells did not. Histological analysis of the explant revealed a well-developed renal structure comprised of organized glomeruli-like, tubular-like, and vascular elements that were clearly distinct but continuous within the renal structure. The renal tissues of the implanted renal device appeared integrally connected in a unidirectional manner to the reservoirs, resulting in the excretion of urine into the collecting system. Although it is not clear how the cultured cells obtained from the donor metanephros gained polarity and self-assembled into glomeruli and tubules, this technique demonstrates the successful use of nuclear transplantation for renal regeneration and avoids the risks and long-term effects of immunosuppression.

5.2. Ectopic kidney generation from the xeno-metanephros

Dekel and coworkers (103) investigated the use of the metanephros of early embryos as a source of a transplantable regenerated kidney to address the shortage of organs for kidney transplantation. The authors used porcine embryos due to the ethical issues associated with the use of human embryonic tissue. When implanted under the kidney capsule of immunodeficient mice the metanephros of porcine embryos underwent significant growth and developed highly differentiated kidney structures, suggesting that the embryonic kidney contains renal progenitor cells with the capacity to generate many cell types. The cyst fluid arising from the transplant had higher average levels of urea nitrogen and creatinine than the normal body sera of the mouse, showing that the transplant had the capacity to filter host blood and produce urine. This was the first demonstration of urine production from an artificial kidney. Considering the current shortage of transplantable kidneys, these findings demonstrate the feasibility of utilizing pig kidney precursors for renal transplantation. A potential problem with this system is the suitability of the renal capsule for dialysis patients as a transplant site. In dialysis patients the vasculature of this area has been significantly disrupted and the limited space beneath the renal capsule may restrict the growth of transplants. These concerns may be overcome by the approach established by Rogers *et al* (104) employing the metanephros as a source of transplantable artificial kidney

and transplanting the graft into the host omentum, a site not confined by a tight organ capsule and not disturbed by dialysis. This experiment was based on previous studies showing minimal immunogenicity in tissues harvested at earlier gestational stages with a metanephros (105). In both allo- and xeno-transplantation (rat metanephros to rat omentum and pig metanephros to rat omentum), transplants assume a kidney-like shape *in situ* and histologically are found to contain well-differentiated kidney structures. In this study, successful xeno-transplantation required the use of immunosuppressants to avoid the loss of transplants soon after transplantation. Interestingly, the grafted pig metanephros was slightly larger in volume (diameter and weight) than the normal rat kidney. The transplanted tissue produced urine and after intact ureteroureterostomy with the ureter of the kidney that was removed, anephric rats commenced voiding and had a prolonged lifespan (106). These findings successfully demonstrate the potential of a new and practical therapeutic strategy for the treatment of CRF which establishes a functional renal unit by implanting a xeno-metanephros and utilizes immunosuppressants.

5.3. Xenobiotic organogenesis for chronic renal failure.

5.3.1. Mesenchymal stem cell to nephron

Due to the anatomical complexity of the kidney and the need for each residential cell to communicate with each other to produce urine, the artificial kidney structure must include glomeruli, tubules, interstitium, and vessels. On this basis we investigated the potential for reconstructing an organized and functional kidney structure using the developing heterozoic embryo as an 'organ factory'. During embryogenesis, a single fertilized cell develops into a whole body within 266 days in the human and 20 days in the rodent. The neonate has every organ positioned correctly, indicating that a single fertilized ovum contains the blueprints to build the body, including the kidney. The aim of our investigation was to utilize the programming of the developing embryo combined with the use of stem cells placed in the appropriate embryonic niche.

As outlined in chapter 3, during development of the metanephros (the permanent kidney), the metanephric mesenchyme forms from the caudal region of the nephrogenic cord (69) and secretes glial cell line-derived neurotrophic factor (GDNF), which subsequently induces the nearby WD to produce a UB (107). The metanephric mesenchyme consequently forms the glomerulus, proximal tubule, loop of Henle, and distal tubule, as well as the interstitium, due to reciprocal epithelial-mesenchymal induction between the UB and metanephric mesenchyme (108). We hypothesized that GDNF-expressing mesenchymal stem cells may differentiate into a kidney structure if positioned at the budding site and stimulated spatially and temporally by the numerous factors found in the developmental milieu.

To investigate this hypothesis, hMSCs were injected into the developing metanephros *in vitro*. This was not sufficient to achieve kidney organogenesis or integration of the hMSCs into the developing rodent metanephros. No kidney structure was established and no kidney-specific genes were expressed (99), indicating that before the metanephros begins to develop the hMSCs must be placed in a specific embryonic niche to allow exposure to the full repertoire of nephrogenic signals required to generate the organ. This can best be achieved by implanting hMSCs into the nephrogenic site of a developing embryo. Therefore, we established a culture system involving whole embryo culture followed by organ culture of the metanephros. This 'relay culture' was designed to allow development of the metanephros from structures present prior to budding up until complete organogenesis *ex utero*. Embryos were isolated before commencement of budding and cultured until the formation of a rudiment kidney that was then removed from the embryo and developed further by organ culture *in vitro* (99). Using the relay culture system kidney rudiments continued to grow *in vitro*, indicating that the metanephros can develop completely *ex utero* following dissection from the embryo prior to sprouting of the UB. In subsequent relay culture experiments hMSCs, labeled with the LacZ gene and DiI and engineered to temporally express GDNF using adenovirus, were microinjected into embryos at the site of budding prior to whole embryo culture. Following relay culture, X-gal-positive cells were scattered throughout the metanephric rudiment and were morphologically and genetically identical to tubular epithelial cells, interstitial cells, and glomerular epithelial cells (99). These findings demonstrated that a xenobiotic process for culturing embryos allows endogenous hMSCs to undergo an epithelial conversion and transform into a functional nephron consisting of glomerular epithelial cells linked with tubular epithelial cells. It is also known that hMSCs can differentiate into renal stroma after renal development.

5.3.2. Integration of host vessels for urine production

Development of a functional transplanted kidney requires integration of host vessels for urine production. For this reason, the relay culture system must allow for vascular integration of transplant recipient vessels to form a functional nephron. Hammerman *et al* (106) demonstrated that a metanephros can grow and differentiate into a functional renal unit with integration of recipient vessels if it is implanted into the omentum. Based on our confirmation that the metanephros from rat embryos past E13.5 will continue to develop once implanted in the omentum, the relay culture system was modified to terminate organ culture within 24 hours, thereby allowing sufficient time for the metanephros to develop and allowing the kidney primordia to be transplanted into the omentum. The modified relay culture system allows the successful development of an hMSCs-derived 'neo-kidney' shown to be equivalent to a human nephron.

LacZ-transgenic rats were used as recipients to examine the origin of the neo-kidney vasculature (109). Following modified relay culture, several vessels from the omentum were found to have integrated into the neo-kidney and most of the peritubular capillaries were of recipient origin. Furthermore, electron microscopy revealed red blood cells in the glomerular vasculature. These data demonstrate that the vasculature of the neo-kidney in the omentum originates from the host and communicates with

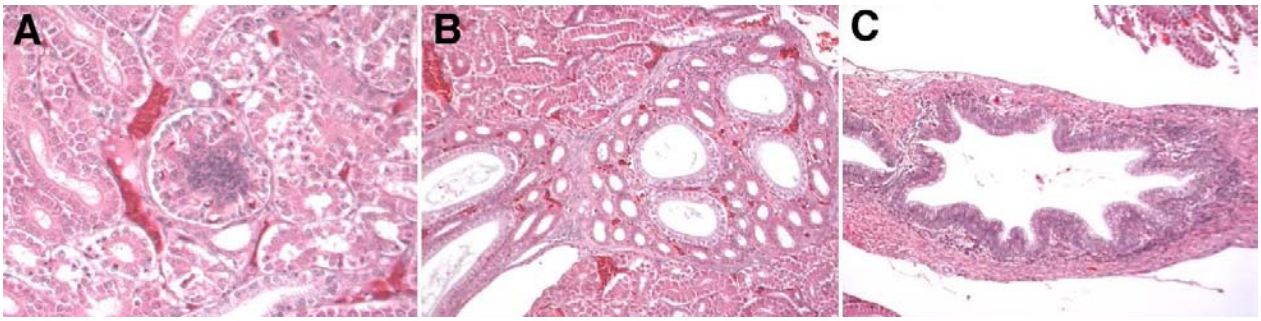

Figure 3. Histological examination of the adult chicken kidney (HE stain). A Glomerulus and tubules (X400) B Collecting ducts (X200) C Ureters (X200).

the host circulation, thereby highlighting the capacity of the neo-kidney to collect and filter host blood to produce urine. If left for 4 weeks in the omentum the neo-kidney develops hydronephrosis due to the production of urine if the ureter is buried under the fat of the omentum with no egress for the urine. Analysis of the liquid from the expanded ureter shows a higher level of urea nitrogen and creatinine than the recipient sera that is comparable with native urine (110). In conclusion, the development of a neo-kidney in the omentum has the capacity to produce urine by filtration of the recipient's blood.

5.3.3. Recapitulation of renal function other than urine production

The kidney plays an important local role in removing uremic toxins and excess fluid by producing urine and also contributes systemically to the maintenance of homeostasis through hematopoiesis, blood pressure control, and calcium/phosphorus balance. We investigated the capacity of the neo-kidney to produce human proteins involved in renal function and to participate in human homeostasis, focusing particularly on erythropoietin production. Erythropoietin (EPO) functions to stimulate red blood-cell production and is produced mainly in the kidneys. Administration of recombinant human EPO (rHuEPO) has been used to treat and mitigate renal anemia in patients with CRF (111), improving quality of life, and reducing the risk of mortality and morbidity (112,113). We found that i) human EPO is produced in rats harboring a neo-kidney derived from human bone marrow cells; ii) human EPO production is stimulated by induction of anemia, suggesting that regulation by physiological inducers of EPO levels, such as conditions of anemia, may be intact in this system; and iii) EPO generated by the neo-kidney in response to anemia is at sufficient levels to restore the rate of recovery from anemia in rats with native EPO suppression to a rate similar to that of control rats (data submitted for publication). Using mouse models of hereditary renal diseases, focusing on Fabry disease, we confirmed that the neo-kidney produced by our system was biologically viable. Fabry disease is an X-linked lysosomal storage disease caused by a deficiency of α-galactosidase A (α-gal A). Fabry disease leads to the abnormal accumulation of glycosphingolipid with terminal α-galactosyl residues (Gb3) in various organs including the kidney, leading to CRF (114). In relay culture experiments hMSCs were transfected with GDNF and injected into E9.5 Fabry mouse embryos. Following relay culture to generate neo-kidneys, the basal level of α-gal A activity in the Fabry mouse metanephros was low compared to wild type, while the metanephros regenerated with hMSCs expressed significantly higher levels of α-gal A (99). Gb3 started to accumulate within the UB and S shape bodies in the metanephros of the Fabry animals and this accumulation was markedly less following replacement with the hMSCs-derived nephron expressing normal levels of α-gal A activity (99). These findings indicate that the regenerated neo-kidney has the capacity to maintain the local environment. Taken together, these results suggest that the development of a neo-kidney in the omentum fulfills the normal renal functions in addition to urine production.

5.3.4. Mesenchymal stem cell to collecting ducts and ureters

In our current culture system, transplanted hMSCs are unable to differentiate into derivatives of the UB, such as the collecting ducts and the ureters. This suggests that the fate of the UB is already determined and that chimeric UB must be generated by transplanting hMSCs to the UB progenitor cells at an early stage during embryogenesis. This lead us to focus on identifying UB progenitor cells using chicken embryos (see 4.2).

In situ hybridization revealed similarities in the expression profile of several key molecules mediating kidney morphogenesis in chicken and mammalian embryos (A. Fukui, unpublished data). Chicken kidneys have glomeruli, containing mesangium cells and podocytes, tubules with the loop of Henle, juxtaglomerular apparatus (115), and a collecting duct system (Figure 3). Arginine vasotocin, an avian antidiuretic hormone, increases diffusional water permeability in the collecting duct, leading to a reduced rate of glomerular filtration and reduced urine flow, as in mammals (116). The high level of conservation of the molecular milieu for kidney development, and similarities in histological structures and functions between chickens and mammals, suggests that chicken embryos have the capacity for the differentiation of hMSCs into a functional kidney. To investigate this possibility future experiments include examination of the capacity of hMSCs to differentiate into WDs and UBs in chicken embryos when transplanted into the progenitor cell region. If this is achieved it may then be possible to generate whole kidneys derived exclusively from hMSCs,

which can be transplanted to patients without immune rejection.

5.4. Cell sources for kidney regeneration

Apart from adult kidney stem cells, other stem cells may be used as a source of cells for kidney regeneration. This section reviews the possibility of utilizing ES cells and MSCs.

5.4.1. Embryonic stem cells

ES cells are undifferentiated pluripotential stem cells isolated from the inner cell mass of blastocysts (117,118). ES cell have the capacity to differentiate into several cells types of mesodermal, endodermal and ectodermal lineage, according to culture conditions, and are a potential source of cells s for tissue regeneration. The application of ES cells for regenerative medicine has been approved in many other disease models, including Parkinson disease and diabetes (119,120). Since human ES cells have the capacity to differentiate into kidney structures when injected into immunosuppressed mice (121,122), studies have focused on identifying the precise culture conditions allowing the differentiation ES cells into renal cells *in vitro*. Schuldiner *et al* showed that human ES cells cultured with eight growth factors, including HGF and activin A, differentiated into cells expressing WT-1 and renin (123). More recently, it was reported that mouse ES cells stably transfected with Wnt4 (Wnt4-ES cell) differentiate into tubular-like structures expressing AQP2 when cultured in the presence of HGF and activin A (98). Using such *in vitro* techniques it may eventually be possible to identify the key molecules (123) that determine the fate of ES cells, although it may be difficult to establish a whole functional kidney *in vitro* for clinical use with this technology. An *ex vivo* culturing system in which ES cells (or ES derived cells) were cultured in the developing metanephros, was investigated to determine the capacity of the ES cells to differentiate into kidney cells integrated into the kidney structure. ROSA26 ES cells were stimulated with developmental signals in the microenvironment of a developing kidney following injection into a metanephros cultured *in vitro*. ES cell-derived, β-galactosidase-positive cells were identified in epithelial structures resembling tubules with efficiency approaching 50% (124). Based on this phenomenon, Kim and Dressler attempted to identify the nephrogenic growth factors inducing the differentiation of ES cells into renal epithelial cells (125). When injected into a developing metanephros, ES cells treated with retinoic acid, Activin-A, and Bmp7 contribute to tubular epithelia with near 100% efficiency (125). Furthermore, Vignearu *et al* showed that ES cells expressing brachyury, which denotes mesoderm specification, may become a renal progenitor population in the presence of Activin A (126). After injection into a developing metanephros, these cells may incorporate into the blastemal cells of the nephrogenic zone. Also, after a single injection into developing live newborn mouse kidneys, these cells stably integrated into proximal tubules with normal morphology and polarization for 7 months without teratoma formation (126). Taken together, these data highlight ES cells as a potential source of renal stem (or progenitor) cells for regenerative therapy.

5.4.2. Mesenchymal stem cells

Studies in mice monitoring the fate of MSCs or MSC-like populations after intravenous or intraperitoneal transplantation demonstrated the presence of donor cells in bone marrow, spleen, bone, cartilage, and lung up to 5 months later (127,128). More recently, Liechty *et al* reported that human MSCs xeno-transplanted intraperitoneally into sheep embryos are capable of engraftment in multiple tissues including chondrocytes, adipocytes, myocytes, cardiomyocytes, bone marrow stromal cells, and thymic stroma (129). These findings suggest that MSCs have the capacity for site-specific differentiation into various tissue types.

In our experiments investigating the generation of the neo-kidney, we utilized primary hMSCs obtained from the bone marrow of healthy volunteers. hMSCs were shown recently to retain plasticity and the capacity to differentiate into several cells types according to the microenvironment (130). As discussed above, ES cells are an ideal candidate as a source of cells for kidney regeneration. Unlike ES cells (131), hMSCs injected into established metanephroi may not integrate into renal structures during organ culture providing a disadvantage over ES cells in the feasible of forming functional renal structures. Our finding that that hMSCs do not express WT1 or Pax2 (our unpublished data), suggests that hMSCs do not possess the complete set of nephrogenic molecular features. An advantage of hMSCs over ES cells is the ease with which adult MSCs can be isolated from autologous bone marrow and applied to therapeutic use without serious ethical issues or the use of immunosuppressants.

6. PERSPECTIVE

The establishment of a functional whole kidney *de novo* has not received a great deal of public attention due to the challenging and lengthy nature of advances in this field of research. This has changed recently with publication of the catastrophic Medicaid costs for dialysis-related diseases. This has highlighted the need for innovative therapy to treat CRF to provide a substitute for dialysis. Regenerative medicine is the great hope for realizing this goal. In cases in which the kidney structure is totally disrupted, a reality in patients with end-stage renal damage undergoing long-term dialysis, the only strategy for a cure may be the development of a functional whole kidney *de novo*. As reviewed in this article, the challenges faced heading towards the development of a functional whole kidney have been met step by step. It should be noted that regenerative medicine for renal disease is in the development phase and a long way from being established. Our current aim is to test larger host embryos to establish larger organs more suited for use in humans. It was reported recently that pig metanephroi transplanted into rat omentum grew to a larger volume than a normal rat kidney (132). For this reason we are examining the applicability of pig in the relay culture system, although our understanding of the renal development processes extends to only a few animal models. Since the current form of the neo-kidney is chimeric and derived from both hMCS and the host embryo tissues, we are attempting to eliminate xenogenic cells from

the graft before transplantation into the omentum by using a transgenic host carrying a regulated suicide gene, and also by exchanging the posterior section of the WD with a human form during elongation to ensure that the collecting system in the neo-kidney is of host origin. Technical developments such as these would allow our approach to path the way for long-term renal replacement therapy in the future. Although many hurdles remain before the clinical application of our approach is realized, these investigations are important early steps for the ultimate relief of patients from dialysis.

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