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Joseph M. Chambers & Rebecca A. Wingert

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REVIEW

Advances in understanding vertebrate nephrogenesis

Joseph M. Chambers in and Rebecca A. Wingert in

Department of Biological Sciences, Center for Stem Cells and Regenerative Medicine, Center for Zebrafish Research, Boler-Parseghian Center for Rare and Neglected Diseases, University of Notre Dame, Notre Dame, IN, USA

ABSTRACT

The kidney is a complex organ that performs essential functions such as blood filtration and fluid homeostasis, among others. Recent years have heralded significant advancements in our knowledge of the mechanisms that control kidney formation. Here, we provide an overview of vertebrate renal development with a focus on nephrogenesis, the process of generating the epithelialized functional units of the kidney. These steps begin with intermediate mesoderm specification and proceed all the way to the terminally differentiated nephron cell, with many detailed stages in between. The establishment of nephron architecture with proper cellular barriers is vital throughout these processes. Continuously striving to gain further insights into nephrogenesis can ultimately lead to a better understanding and potential treatments for developmental maladies such as Congenital Anomalies of the Kidney and Urinary Tract (CAKUT).

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Introduction: emergence of the kidney from the intermediate mesoderm

Vertebrate development entails the formation of three germ layers, the ectoderm, mesoderm, and endoderm, which provide cellular blueprints for embryonic organogenesis. Ectoderm gives rise to the central nervous system and skin cells, and endoderm derivatives encompass cells that line the respiratory and digestive tracts. The mesoderm, or middle layer, produces cells that are most abundant in the human body constituting skeletal muscle, cartilage, heart, gonads, and blood, among other tissue types. This review will focus on a member of the mesoderm lineage: the kidney. Much of our understanding about kidney development stems from rodent models, but also has benefited from studies in other vertebrates such as fish, frogs, and birds.²The inception of mesoderm development begins with the differentiation of pluripotent epiblast cells into a transient 'primitive streak' zone.-¹Position along the anterior-posterior embryonic axis and other instructive signals regulate the regionalization of paraxial, intermediate, and lateral plate mesoderm.³

The urogenital system derives from the aforementioned intermediate mesoderm (IM), which is

a narrow section of tissue situated between the paraxial and the lateral plate mesoderm. Early developmental studies are hampered by the limited number of molecular markers that label the emerging IM population. The first IM indicators to appear during embryogenesis are LIM-type homeobox (Lhx1) and the zinc-finger DNAbinding protein odd-skipped related (Osr1).4-7 The expression domains of these two factors intersect indicating the prospective IM and lateral plate mesoderm fields, as Osr1 is expressed across the entire length of the expanding somite tissue. Specific markers solely expressed in the IM do not turn on until about the 4-8 somite stage. The activation of Pax2 and Pax8 within a narrow band early in the IM is speculated to signify that the LPM and IM have assumed separate lineage trajectories. Complementary to these early expression pattern observations, functional assays in mice have demonstrated Lhx1, Osr1, Pax2, and Pax8 are all critical regulators of IM specification. 4,6,8-10 For example, mice lacking either Lhx1 or Pax2/Pax8 fail to form the nephric duct, which is a pair of tubes required for assembly of the urinary system.^{7,11} Interestingly, experiments in chick embryos indicate that the competence to respond

CONTACT Rebecca A. Wingert rwingert@nd.edu Department of Biological Sciences, University of Notre Dame, Notre Dame, IN 46556, USA; Joseph M. Chambers Mchambers@manchester.edu College of Pharmacy, Natural and Health Sciences, Manchester University, Pharmacogenomics Campus, 10627 Diebold Road, Fort Wayne, IN 46845, USA

to these IM patterning factors is conferred by retinoic acid (RA) and Hox gene expression.¹²

It is important to think about developing organisms in a three-dimensional context. This is especially true for understanding kidney ontogeny, as various signal gradients are radiating through the animal in a morphogenic fashion. For example, developmental studies performed in chick embryos found that bone morphogenic protein (BMP) signaling affects IM cell fate in a concentrationdependent manner.^{5,13,14} A variety of studies have revealed RA and activin signaling gradients promote IM marker expression during ontogeny in various vertebrate species. 15-21 Originally, the addition of these factors to culture systems including animal caps, embryoid bodies, and embryonic stem cells supplemented growth and cellular differentiation of kidney fates. A decade later, similar experiments have been refined to generate IM from induced pluripotent stem cells to generate kidney organoids.22

Following IM specification, the progression of vertebrate renal development involves the stepwise generation and degeneration of several kidney forms: the pronephros, mesonephros, and metanephros. Each kidney iteration develops along the anterior-posterior embryonic axis, where each subsequent version becomes more structurally complex than the previous structure. The pronephros emerges first, and while it is vestigial/nonfunctional in mammals, it is functional in other vertebrates such as fish and frogs.²³ The mesonephros is further developed and partially functional in mammals, while serving as the final kidney form in amphibians and fish.²⁴ However, the fully formed and functional version of this vital organ in mammals is the metanephros, which develops through branching morphogenesis events that result in an arborized structure essential for fluid homeostasis. Importantly, all three vertebrate kidney forms share the overall structure of the kidney's functional unit: the nephron. Broadly, the nephron is composed of a blood filter, a segmented tubule, and a collecting duct system to shuttle urine to the bladder.

Mesenchyme induction

The focal point of the remaining sections of this review will cover mechanisms driving

development of nephrons, since these are unifying structures across vertebrate kidney forms. While our primary focus will be centered on recent insights on mechanisms in mammals, we will also highlight some conserved genetic regulators revealed from studies in other vertebrates.

The mammalian metanephros contains two wellcharacterized renal progenitor populations: the metanephric mesenchyme (MM) and the ureteric bud (UB) (Figure 1). The UB derives from the nephric duct and gives rise to the collecting duct system, and the MM is the source of all nephron lineages and contains vascular, stromal, and nephron progenitor cells (NPC). The UB initiates nephron induction by invading the MM and undergoes progressive branching after receiving reciprocal signals from the MM. Occurring simultaneously, UB signals cause the MM to condense around the ureteric tips forming a structure termed the cap mesenchyme (CM), which retains the Six2+ Cited1+ NPC population. The NPCs border the ureteric epithelium and other cell populations are more distant from this site. Recent lineage-tracing studies have begun to appreciate how position, movement, and spatial exposure to differentiation cues can determine self-renewal or differentiation status within NPC pools. For example, a subset of Wnt4-expressing cells was discovered to migrate back to nephron progenitor zone and exhibit plasticity regarding nephron commitment²⁵

In addition to physical location, the reciprocal crosstalk between the CM and UB is essential for nephron formation. The ability to form several thousand to over one million nephrons in mammalian kidneys requires maintaining both a delicate balance of self-renewing NPCs whilst making a sufficient endowment of differentiating nephrons to support renal function. The Six2+ Cited1+ CM possesses the ability to self-renew and sustain ample progenitor cells capable of making the correct number of nephrons. Molecular signals from the UB are responsible for NPC self-renewal or nephron commitment. Factors that are required for maintenance of the progenitor pool include: WT1, SALL1, FGFR1/2, FGF8, WNT11, FGF9, and WNT9B.²⁵⁻³⁸ Without the proper signals the kidney exhausts the NPC pool, which in turn yields insufficient nephron number and can lead to kidney agenesis or predisposition to chronic kidney disease.

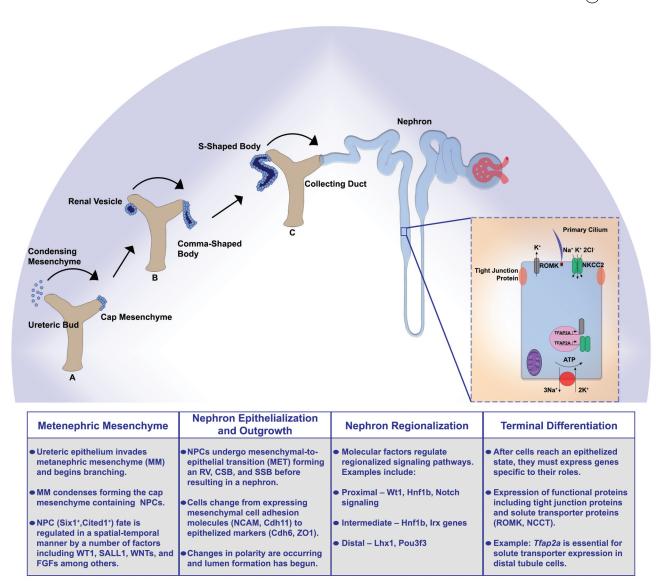


Figure 1. Summary schematic of key nephrogenesis steps. Nephrogenesis steps beginning with ureteric epithelium invading mesenchyme (A, left) and mesenchyme condensing to form the cap mesenchyme (A, right) containing nephron progenitor cells (NPCs). Cap mesenchyme transitions to the renal vesicle (RV) (B, left) and transitions from mesenchymal to epithelized as it continues through the comma-shaped body (CSB) (B, right) and S-shaped body (SSB) (C, left) before ultimately resulting in a complete nephron (C, right). The inset depicts a terminally differentiated thick ascending limb cell with example solute transporters (ROMK, NKCC2) and tight junction proteins. Table with summaries of key steps and examples for each step from the text (bottom).

The spatial location of NPCs is becoming increasingly appreciated as a major determinant of cell-fate decisions. Previously, it was well-accepted that the least committed NPCs were located at the top of the CM, and the most committed NPCs were positioned beneath the ureteric tip and undergo nephron induction and renal vesicle formation.³⁹ It was thought that the derivation of nephron cells from NPCs was a linear progression from the Six2+ Cited1+ self-renewing population, to the Six2+ primed cellular state, and finally the committed state demarcated by Wnt4.^{39–43} However, results from a recent study that

employed lineage tracing and computational modeling support an alternative hypothesis.²⁵ found that NPCs moved randomly around the cap mesenchyme. The authors found that an NPCs can initiate Wnt4 expression, previously believed to indicate 'nephron commitment,' migrate back to the progenitor domain, and regain a self-renewing progenitor status.²⁵ The authors hypothesize that random cell movements influenced by ureteric epithelium result in differing signals that then determine the fate of the NPCs.

A particularly important transitional step during nephrogenesis is when CM cells form a pre-tubular

aggregate (PTA) beneath the ureteric tip. One of the most well-known factors that signals within MM to commence the differentiation process is Wnt9b. The re-expression of Osr1 and Six2 have been documented to halt PTA formation.^{6,44,45} Six2 activity within the CM is believed to antagonize the function of Wnt9b by affecting the stabilization of β -catenin. ^{34,42,43,45–49} Thus, it is essential for PTAs to downregulate Six2 while the Wnt9b/ Wnt4 signaling axis prompts a mesenchymal to epithelial transition (MET) and renal vesicle (RV) polarization.

Nephron epithelization and growth

Renal vesicle (RV) formation is the beginning of many important changes that result in nephron formation (Figure 1). The RV undergoes MET and becomes polarized as it transitions into more developed stages: the comma-shaped body (CSB) and the S-shaped body (SSB). The origins of epithelization are dependent on UB WNT9B signals to initiate WNT4 expression in the PTA.42 This expression leads to β-catenin stabilization and polarity establishment within the RV. 43 Overall MET consists of changing from mesenchymal cell adhesion molecules NCAM, Cdh11, Cdh2, and Cdh4 to a more epithelized status expressing Cdh6, Cdh1, and ZO1, among others.^{50–56}

Cellular polarity is defined as specific proteins localizing to particular regions of the cell boundary denoting certain areas as apical, basal, and lateral. Cellular polarity is particularly important for kidney development as it coincides with lumen formation in the maturing nephron.⁵⁷ The process of apicobasal polarity establishment relies on afadin as without it, kidney tubule cells fail to form correct Par and nectin complexes and do not recruit R-cadherin.⁵⁷ Another factor found to be necessary for polarity in the developing nephron is the Rho GTPase Cdc42. Knock-out studies found severe defects in polarity and lumen formation.⁵⁸ Eventually, the RV lengthens and connects to the UB to form the collecting system. 59,60 This is now the continuous lumen of the nephron. Nephron lumen formation is largely conserved as results from these studies align with findings from a zebrafish lumen formation study focusing on atypical protein kinase C (aPKC).⁶¹ Using the various model organisms available will be beneficial to

continue to understand the process of lumen formation. As we currently understand it, the establishment of apical and basolateral polarity is necessary as collectively the cells need to form the lumen on the apical side of RV cells. As previously mentioned, there are a number of molecular cues that dictate proper polarity and thus lumen formation. One of the main concepts to keep in mind is that there are cell-cell interactions as well as cell-matrix interactions taking place, which have been covered in detail in another review.⁶² Moving forward, a better understanding of these interactions and the molecular cues initiating them is needed.

Additional aspects of nephron growth include planar cell polarity, mechanical stretch, cell migration and proliferation. It is important to understand that many of the events discussed in this review are occurring simultaneously. For example, the events in this section are taking place while transcription factors direct regionalization of nephron tubules, which will be discussed in the following section. Continuing to think of nephrogenesis in a three-dimensional context, planar cell polarity (PCP) is necessary for proper kidney development. PCP is collective tissue polarity or, polarity as it functions perpendicular to the cellular apical-basal polarity. There are numerous examples of the role PCP plays in developmental biology as it controls convergent extension and oriented cell division.⁶³ One elegant example is found as research discovered the PCP-dependent convergent extension resulting in kidney tubule formation.⁶⁴ Additionally, PCP controls oriented-cell division, another process that has been linked to kidney tubule elongation.⁶⁵ While the core PCP components are known to be needed for proper tissue development, other factors and their downstream consequences are being discovered. 65-70 One known factor that regulates kidney PCP is Wnt9b acting via Rho-kinase. 71-73 This is an area of intense research as dramatically increased proliferation can result in disease states such as polycystic kidney disease (PKD).⁶² Hippo signaling has been linked closely with PKD cell proliferation via fat4 a negative regulator of hippo signaling providing one avenue for potential molecular exploration. 74-77 As we continue to learn about PCP and the other effects involved downstream, we can gain better insight to disease states. Specifically, cell proliferation and its relationship with PCP requires a better understanding as it pertains to kidney development.

Interestingly, in zebrafish, there is a concentrated cell proliferation event in the distal portion of the pronephros as cells migrate rostrally to provide the increased cell number necessary for the proximal tubule to undergo coiling morphogenesis.⁷⁸ This nicely supports a study that found distal proliferation of the RV occurs to join the UB as previously mentioned to make the collecting duct system.⁵⁹ Further, collective cell migration occurs in developing and regenerating zebrafish nephrons.⁷⁸⁻⁸¹ Combining these data with recent studies illustrating the unique cellular movement during mammalian kidney development, 25 future studies could be focused on cellular migratory events and their role in nephron development.

Nephron regionalization

As the RV continues to develop, its transitions into the CSB and SSB where regionalization becomes an important concept as this will be vital for a properly segmented, terminally differentiated nephron (Figure 1). Though studies suggest proximo-distal regionalization can be seen in the RV it is especially evident in the later stages of nephrogenesis.^{59,82,83} This is an area of research that has greatly benefitted from the many advantages of the Xenopus and zebrafish model organisms as complements to the single-cell RNA sequencing (scRNA seq) in murine and human kidneys. 84-89 During this time of RV elongation into CSB and SSB a number of signals dictate proximal and distal cell fates. Here, we will discuss some of these signaling factors, but not an exhaustive list.

While the RV is usually subdivided into proximal and distal domains, the SSB gains a medial (or intermediate) region. Expression of unique factors in combination with loss of function experiments have determined much of what we understand to be segmentation of the nephron during development. Recently, mostly due to the advances in scRNA seq, there has been an approach at understanding the role timing and location play in combination with gene expression for the development of individual nephron cells.^{25,90}

Beginning with the proximal portion of the SSB, Wilm's Tumor 1 (Wt1) continues its proximal expression observed in the RV, and this expression continues into mature podocytes. 91,92 Next, there have been

a number of studies to better understand the role Notch signaling plays in proximal segmentation. Losing components of Notch signaling results in loss of proximal segments. These changes appear first during the SSB stage, coinciding with expression of Notch signaling components. 83,93-96 Interesting evidence has shown that members of the Iroquois (Irx) transcription factor family play roles in the medial SSB development. Studies in Xenopus and zebrafish have shown that loss of Irx genes have consequences for the development of intermediate segments of the nephron. 97–99 The transcription factor Hnf1b is expressed in mouse SSB and disruption of its expression has drastic phenotypes in Xenopus and zebrafish. Xenopus deficient in *hnf1b* do not properly form proximal or intermediate sections of the nephron, 100 while zebrafish deficient in *hnf1b* do not form any nephron segments. ¹⁰¹ The previously mentioned transcription factor Lhx1 is needed for proper distal formation. 102 Additionally, downstream of Lhx1 the POU-domain containing transcription factor POU3F3 (also called Brn1) is needed for distal tubule and Loop of Henle formation. 103

Recently, there have been several large-scale efforts to catalog factors that are expressed during nephron segmentation, such as scRNA sequencing of mice and human kidneys. These data repositories provide a wealth of information that will be useful to design future genetic studies. Additionally, genetic studies such as forward and chemical screens using other vertebrate models have generated new insights about mechanisms that control nephron segmentation in different species.⁸⁶ One premiere system has been the zebrafish embryonic kidney, or pronephros, where a growing list of genes and signaling pathways has been identified. 104-114 Recent evidence from human kidneys strongly suggests potential conservation in gene expression and function with their zebrafish counterpart. 104,115 Combining the large data sets of mice and human scRNA sequencing with the relative ease of loss of function studies in zebrafish could streamline an evolutionarily conserved pipeline of essential factors for nephrogenesis.

Nephron terminal differentiation

By the time the nephron is fully developed, it will contain a number of unique cell-types that each need to have the appropriate gene expression to complete

their vital functions (Figure 1). The nephron begins with the blood filter, or renal corpuscle encompassing the glomerulus and Bowman's capsule. This contains a number of cell types including capillaries, mesangium, podocytes, and parietal cells. Next, the tubule contains the proximal convoluted tubule, proximal straight tubule, the Loop of Henle (including descending limb, thin ascending limb, thick ascending limb), distal convoluted tubule, and connecting tubule. The proximal tubule functions in absorption and secretion in an effort to regulate pH of the filtrate. Largely, the Loop of Henle functions to concentrate the filtrate by reabsorbing water. The distal tubule ensures proper ion transport occurs to fine-tune the filtrate by regulating potassium, sodium, and calcium levels. Each unique segment is needed to maintain blood homeostasis by completing these functions. Nephron cells must acquire a number of features to be generally considered terminally differentiated, including proper epithelization, cilia formation, and expression of functional proteins such as tight junctions and solute transporters.

We have previously discussed nephron epithelization during the RV to SSB stages so in this section we will focus on the remaining steps of terminal differentiation beginning with cilia. Cilia are hairlike structures projecting from the apical surface of cells that have essential roles in various organs signal transduction. 110 Cilia including a unique role in kidney development and function as ciliogenesis is an essential step as kidney tubule cells differentiate while also playing a role in signaling to properly form the kidney. The earliest observed cilia formation is at the RV stage as the developing nephron undergoes MET, establishes apical-basal polarity, and begins to form a lumen. 116 Cilia length are largely dynamic as they respond to stimuli to carry out their tasks. Cilium length increases as nephrons mature, this is speculated to indicate that they may be playing important roles that are currently not understood. 110,116 Without proper cilia form and/or function disease states, termed ciliopathies, occur. One of the most common ciliopathies is polycystic kidney disease which is the result of mutations in cilia localized proteins such as polycystin-1 and -2. This is an area of nephrogenesis that is also greatly complemented by the advantages of zebrafish and Xenopus as model organisms. Several studies have found cilia-related

genes affecting kidney development including Wnt/ PCP genes, *hnf1b*, and PKD2, among others. 118–120 One potential interesting avenue of future research could focus on multiciliated cells, or cells with multiple cilia projecting from the cell surface that function in fluid propulsion in zebrafish and *Xenopus.* Though currently not believed to be present in normal adult healthy kidneys, there have been reports of multiciliated cells present in kidneys during certain disease states. 121

Tight junction proteins can be found along the entire nephron structure from the slit diaphragm in the glomerulus and along the tubule in various segments. 122-124 The function of the numerous tight junction proteins differs depending on the spatial expression, but overall tight junctions provide a controlled blockade to paracellular transfers of water and ions. 122 Overall, tight junctions comprise proteins including occludins, claudins, and junctional adhesion molecules. 123,125-127 These proteins vary in the function and thus their expression along the nephron. Tight junction proteins are conserved across vertebrate species, further illustrating their importance to the general role of the kidney. 124

Another suite of proteins essential to nephron function are the solute transporters found along the nephron tubule. To ensure they are able to modify the filtrate, each segment will express a suite of genes that act specific to their location along the tubule. Many of these genes that specify segments are solute transporters. Interestingly, many of these segment-specific genes are largely conserved from, zebrafish, Xenopus, rodents, and humans. 2,21,86,99,128 enables a robust host of opportunities to study segmentation and terminal differentiation of the nephron. Several factors previously discussed, including *Hnf1b*, are needed to reach epithelial status while others have been identified to push the epithelial fate to terminal differentiation of the unique segment cell types. 101,129 A number of specific examples can be tracked across species, such as the decrease in the distal solute transporter NCCT or Slc12a3 in mice and zebrafish deficient in ppargc1a. 106,130 One recent study from our laboratory identified the transcription factor AP-2 (tfap2a) as the key determinant in the terminal differentiation of the zebrafish distal early segment, analogous to the mammalian thick ascending limb. 104 In the absence of Tfap2a, the nephron cells were found to reach an epithelialized point of

development in which they were primed to be distal tubule cells but failed to express the solute transporter suite of genes necessary for proper function of this particular segment. 104 Further, tight regulation of Tfap2a expression was found to be essential, and controlled by the paralogs potassium channel tetramerization domain containing 15a and 15b (kctd15a, 15b). 105 Interestingly, a study found similar localizations of TFAP2A and KCTD15 expression during human nephrogenesis suggesting possible conserved roles in development. 115,131,132

Conclusions and future directions

While there have been substantial advances in understanding the molecular mechanisms dictating the intricate events resulting in kidney development, there remain a number of challenges. Clinically, patients resort to dialysis or transplantation when faced with kidney ailments. Neither of these treatments are particularly reassuring to the many people facing kidney dysfunction. Two areas of research that are blossoming and resulting in the hope of newer treatments are single-cell RNA sequencing and organoids. Both fields are advancing quickly and can be combined with high throughput loss of function studies to continue to identify necessary genes to enhance kidney development and function.

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Writing-original draft preparation, J.M.C.; writing-review and editing, R.A.W.; funding acquisition, J.M.C. and R.A.W. Both authors have read and agreed to the published version of the manuscript.

Abbreviations

CM	cap mesenchyme
CSB	comma-shaped body
IM	intermediate mesoderm
MET	mesenchymal to epithelial transition
MM	metanephric mesenchyme
NPC	nephron progenitor cell
PCP	planar cell polarity

(Continued)

CM	cap mesenchyme
CSB	comma-shaped body
IM	intermediate mesoderm
MET	mesenchymal to epithelial transition
MM	metanephric mesenchyme
NPC	nephron progenitor cell
PCP	planar cell polarity
PKD	polycystic kidney disease
PTA	pre-tubular aggregate
RV	renal vesicle
scRNA seq	single cell RNA sequencing
SSB	S-shaped body
UB	ureteric bud

Disclosure of potential conflicts of interest

The authors declare no conflict of interest.

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ORCID

Joseph M. Chambers http://orcid.org/0000-0002-6333-

Rebecca A. Wingert http://orcid.org/0000-0003-3133-7549

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