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Development of the Mammalian Kidney

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Structure-function: An overview of the anatomy and function of the kidney

The paired kidneys are the central organs of homeostasis for our body systems (Figure 1A). Around 180 liters of blood are filtered per day by the kidneys, accounting for around 20% of cardiac output. Filtering removes metabolic waste products, and kidney action adjusts water, salt and pH to maintain the homeostatic balance of tissue fluids. The kidneys also regulate blood pressure through the renin-angiotensin-aldosterone system, erythrocyte production through production of erythropoietin, and circulating calcium and phosphate levels, in part through the activation of vitamin D.

The functional unit of the kidney is the nephron (Figure 1B). The mouse kidney has 12 to 16,000 nephrons per kidney, with the numbers varying depending on the strain (Short et al., 2014). Surprisingly, nephron number appears even more variable in man: reports suggest as much as a ten fold difference in nephron number amongst individuals with around 1,000,000 nephrons on average per kidney (Bertram et al., 2011). This large difference may not be trivial as studies link low nephron number to an elevated risk of kidney disease while higher nephron content in the mouse protects against salt-induced hypertension (Hoy et al., 2011; Walker et al., 2012).

Renal filtration takes place within the renal corpuscle at the proximal extent of the nephron (Scott and Quaggin, 2015; Figure 1A–C). Here, the afferent vasculature forms a convoluted knot of leaky blood vessels, the glomerulus. Mesangial cells, a glomerular variant of vascular associated pericytes elsewhere in the body, lie closely opposed to the vascular endothelium, maintaining integrity and porosity of the vasculature. Fluid passes from the glomerular vasculature into the interstitial space of the Bowman's capsule, which is lined by podocytes. The podocytes secrete and sit on top of an unusually thick and highly specialized glomerular basement membrane (Miner, 2011). The glomerular basement membrane acts as a filtration barrier, reducing entry of larger molecular weight serum solutes into the nephron (> 15 kDa) such as serum albumin (Sun et al., 2013).

The podocyte is a spectacular cell. Long, actin-based foot processes extend from the cell body, each resembling the arms of an octopus. Foot processes radiating from adjacent podocyte arms interdigitate to form, the slit diaphragms, narrow openings of around 40

nanometers that regulate fluid flow into the proximal tubular segment of the nephron (Grahammer et al., 2013). Given their unique biosynthetic and structural characteristics, podocytes are readily identified by the expression of a number of genes controlling matrix production, and foot process formation and function (Brunskill et al., 2011). Mutations in genes regulating these structural aspects are linked to renal disease (Bierzynska et al., 2015). Further, podocyte injury has emerged as a key factor in disease progression, most notably diabetic nephropathy, the major cause of end-stage renal disease (Maezawa et al., 2015).

The renal corpuscle and emerging S1 and S2 segments of the proximal tubule are confined to the outer cortex of the kidney (Figure 1B). The final segment of the proximal tubule, the S3 segment, is localized within the outer medullary domain. A key function of a myriad of membrane-embedded channels and transporters within the proximal tubule epithelial membrane is the reabsorption and recovery of vital small molecules such as glucose, amino acids, and essential minerals. Around 70% of the sodium chloride entering the ultrafiltrate is returned to surrounding efferent vascular capillaries. As a consequence, proximal tubule cells have a distinctive transcriptional signature that reflects their heavy lifting role in membrane transport processes (Brunskill et al., 2008; Thiagarajan et al., 2011). Proximal tubule cells are highly metabolically active and, in part because of this, vulnerable to injury. Acute kidney injury, linked to hypoxic episodes, infection or nephrotoxic drugs, is one of the leading causes of death in a hospital setting; the proximal tubule is a shared target of these distinct pathological insults.

The loop of Henle extends the renal tubule deep into the medullar before looping back to become the distal tubule segment within the renal cortex (Figure 1B). The primary role of the loop of Henle is urine concentration, a critical function essential to the radiation and diversification of land colonizing vertebrates (Sand and Layton, 2014). The distal tubule regulates the return of sodium and calcium to the blood in hormone directed processes mediated by aldosterone and parathyroid hormone, and participates in water uptake and pH balance (Subramanya and Ellison, 2014).

The macula densa, a specialized distal tubule component lies just before the convoluted section, close to each glomerulus at the point of entry of afferent arterioles and exit of efferent arterioles (Figure 1C; Bell et al., 2003). Cells of the macula densa monitor salt levels in the distal tubule, and in response secrete local vasoconstrictors restricting adjacent afferent arterioles, reducing fluid flow into the glomerulus. This is a key feedback system to ensure physiologically important filtration rates. The macula densa also communicates with juxtaglomerular cells (Figure 1C), a specialized subset of the smooth muscle surrounding the glomerular arterioles, to regulate renin secretion and systemic blood pressure through renin's catalytic action on the angiotensinogen system. Renin release is also triggered by changes in blood pressure in the afferent arteriole.

The distal tubule connects to the collecting duct, a continuous highly arborized epithelial network with a quite distinct origin from the contiguous renal tubule (Figure 1A–B). The collecting duct system funnels urine from the kidney through the ureter to the bladder. The collecting duct epithelium displays a distinct cortical-medullary axis of branching, growth and cellular organization, the tubular network focusing into a single (mouse) or multiple

(man) renal papillae at its exit point (Figure 1A). The medullary collecting duct is highly water permeable, facilitating water retention, and is a mosaic of two distinct cell types: abundant principal cells, which are critical for sodium retention, and rarer intercalated cells, which regulate pH through acid-base transporters (Al-Awqati, 2014; Pearce et al., 2015).

Intermingled between vascular and non-vascular epithelium, closely opposed to their basal surfaces, are interstitial cell types that show varying degrees of smooth muscle characteristics. *Bona fide* contractile smooth muscle surrounds major blood vessels and the innermost collecting duct epithelium, and contractions propel urine into and along the ureter. In addition to specialized renin-secreting interstitial cell types, an interstitial population associated with proximal tubule segments secretes the hormone erythropoietin, stimulating production of red blood cells. Much of the remaining interstitial cells most closely resemble pericyte-like myofibroblasts, a population linked to renal repair and chronic fibrotic kidney disease (Rabelink and Little, 2013; Kramann and Humphreys, 2015). The kidney is also innervated by the sympathetic nervous system, axons tracking vascular pathways to the glomerulus. Sympathetic neurons counter autoregulatory networks in response to decreased blood pressure from excessive fluid loss, constricting afferent arteriole vesicles and triggering production of renin by juxtaglomerular cells (Grassi et al., 2015).

From progenitors to products: Assembling the kidney

A general overview

Much of what we understand about kidney development comes from studies of only two mammals: the rat and mouse. Development is comparable in both species, though the mouse is, relatively speaking, 24 hours ahead of its rodent cousin. The new genetics made possible by embryo stem cells and gene targeting have led to the primacy of the mouse as the key mammalian model. A broad overview of kidney development in the mouse is followed by a more detailed discussion of developmentally distinct component parts.

The metanephric kidney derives from intermediate mesoderm in temporally and spatially distinct processes (Taguchi et al., 2014; Takasato and Little, 2015). The first event, around embryonic day 8.75 (e8.75), is the emergence of paired nephric ducts at rostral somite levels. These ducts migrate posteriorly, inducing mesonephric tubular structures laterally until reaching the hindlimb level around e10.5 (Figure 2A). Here, interactions between pre-specified metanephric mesenchyme cells and the adjacent nephric duct trigger the dynamic process of kidney development (Costantini and Kopan, 2010; Little and McMahon, 2012, Combes et al, 2015). The mesenchymal cells induce a single, bilateral, ductal outgrowth, the ureteric bud, which grows into medially positioned metanephric mesenchyme (Figure 2A). The mesenchyme condenses to encapsulate the growing tip of the ureteric bud and, by e11.5, the ureteric bud has branched to generate a T-like structure.

From this time, kidney development takes off. The ureteric-bud-derived epithelium undergoes 12 generations of branching before ceasing 2 days after birth, having established the entire network of the urine trafficking collecting duct system (Short et al., 2014). Initial ingrowth and subsequent branching of the ureteric epithelium is driven by signals supplied by the adjacent capping mesenchyme cells (Figure 2B, 3A). In turn, the branch tips induce

expansion and differentiation of associated capping mesenchyme. At each branching event, a subset of mesenchymal cells forms a tight cluster, the pretubular aggregate, beneath the branch tip. The pretubular aggregate subsequently epithelializes to form a small cyst-like renal vesicle. Each renal vesicle gives rise to a nephron. Nephrogenesis continues along with ureteric branching, though approximately half of all nephrons form in a 2-day window after branching ceases (2–4 days after birth; Short et al., 2014). The final nephron number varies between mouse strains, and progenitor depletion studies show nephron number is dependent on the size of the progenitor pool (Cebrian et al., 2014).

Growth, patterning and morphogenesis over a 24 to 36 hour period transform the renal vesicle into an S-shaped body (Figure 2B, 3A). The distal-most epithelium of the S-shaped body plumbs into the adjacent collecting duct, generating a patent luminal connection essential for fluid trafficking through the kidney (Kao et al., 2012). As the number of nephrons exceeds the number of ureteric branches, multiple nephrons connect to a single branch, or to each other as in the human kidney, generating a cascade of nephrons.

At the proximal-most end, the S-shape body generates the glomerular cleft. A lengthy period of renal corpuscle assembly initiates with podocyte specification in the proximal epithelium and endothelial cell migration into the cleft (Figure 3A). Other nephron segments form from the remainder of the S-shape body. Vasculogenesis and angiogenesis contribute to formation and shaping of the elaborate and highly organized nephron-associated vascular network in conjunction with progressive development of the renal tubule epithelium. Interstitial cell types organize around the vasculature and tubules, and neurons project into the kidney to innervate differentiated target sites.

There is no definitive study on when functional nephrons are first established in the mouse kidney, though some filtering capability is likely by e16.5 to e17.5. Given the continual process of recruitment and differentiation of renal vesicles, the late fetal and early neonatal kidney is a mosaic of nephrons at different stages of the maturation, and full kidney function is not established in the mouse until a few weeks after birth.

The collecting duct network

Abnormal ureteric bud formation often results in a complete failure of kidney development. The fact that this initial event is a particularly vulnerable period in the kidney's developmental program is highlighted by the frequency of unilateral kidney loss in otherwise healthy individuals, estimated at around 1 in 1,000 live births from asymptomatic autopsy (Shapiro et al., 2003). Many lines of evidence highlight the critical importance of *Gdnf* signaling through its *Gfra1/Ret* receptor complex in these early events (Costantini, 2012). Loss of function mutations in any of these components results in complete kidney agenesis (Schuchardt et al., 1994; Sanchez et al., 1996; Pichel et al., 1996; Cacalano et al., 1998; Enomoto et al., 1998).

Gdnf is secreted by the metanephric mesenchyme prior to ureteric bud outgrowth, and *Gdnf* expression is maintained in capping mesenchyme throughout the period of active nephrogenesis. Several positive feedback mechanisms, including the up-regulation of *Ret* by *Gdnf* signaling at branch tips, adding robustness to the receptor tyrosine kinase (RTK)

regulated branching process (Pepicelli et al., 1997; Costantini, 2012). Further, *Gdnf* induces tip-restricted expression of *Wnt11* to maintain normal levels of *Gdnf* through paracrine *Wnt11* action (Figure 2B; Majumdar et al., 2003).

Rudimentary buds form in some *Gdnf* and *Ret* mutants suggesting branch-regulating pathways at play. Mouse genetics have highlighted an overlapping role for *Fgf7* and *Fgf10* from the capping mesenchyme acting through *Fgfr2* in the ureteric epithelium (Qiao et al., 1999; Ohuci et al., 2000; Zhao et al., 2004). As *Ret* and *Fgf* receptors encode RTKs, RTK signaling downstream of ligand-receptor engagement is a convergence point for overlapping signaling activities. Both pathways regulate tip-restricted expression of two highly related ets-domain transcription factors, *Etv4* and *Etv5* (Lu et al., 2009). Together, *Etv4* and *Etv5* are essential for branching (Lu et al., 2009). Sox-domain transcriptional regulators, *Sox8* and *Sox9*, show broadly similar expression to *Etv4* and *Etv5*, and are themselves essential for normal branching outgrowth (Reginensi et al., 2011). *Sox8* and *Sox9* are required for activation of *Etv4/5* and a subset of other tip-expressed *Gdnf* targets, but *Sox8* and *Sox9* are not required for *Ret* expression (Reginensi et al., 2011).

Tip-specific expression domains are also regulated by retinoic acid (RA) synthesized and released by interstitial progenitors (Rosselot et al., 2010). In ureteric tip explants, RA signaling is required for *Gdnf*-mediated activation of *Ret* (and most likely *Ret*-dependent targets) in the ureteric epithelium (Rosselot et al., 2010). Wnt signaling is a third major signaling pathway regulating branch tip programs. Wnt pathway reporters show high endogenous levels of canonical Wnt signaling within branch tips, and *Ret* expression and branching growth are lost following genetic or biochemical perturbation of the canonical Wnt pathway (Marose et al., 2008; Bridgewater et al., 2008; Karner et al., 2010). However, the biologically relevant Wnt-ligand(s) have not been identified.

Outgrowth promoting activities are countered by the action of several other pathways (Costantini, 2012). Collectively, their actions ensure the formation of a single ureteric bud of the appropriate size from each nephric duct. As a result, a single ureter will connect each kidney to the bladder. Kidneys with duplex ureters are prone to blockage-related kidney damage with a subsequent loss of kidney function. Factors constraining the outgrowth process act by either limiting the domain of *Gdnf* expression within the intermediate mesoderm (*Slit2/Robo2*; *Foxc1/Foxc2*), or by inhibiting (*Bmp4*) or attenuating (*Spry1*) RTK pathway action within the ureteric bud (Grieshammer et al., 2004; Kume et al., 2000; Michos et al., 2004; Wainwright et al., 2015). Importantly, ureteric branching is rescued in *Ret/Gdnf* mutants when *Spry1* action is also removed: branching now becomes highly dependent upon cap mesenchyme-derived *Fgf10* signaling (Michos et al., 2010).

The mouse kidney contains around 3,400 branch tips at the cessation of branching two days after birth (Short et al., 2014). The continued expression of RTK, Wnt and RA target genes in branch tips throughout the branching period argues for the continued action of these pathways throughout the branching process. However, the relative importance of each pathway at each stage has not been determined. Genetic analysis also indicates even greater pleiotropy in RTK usage throughout the branching period with evidence for both *Met* and *Egf* receptor engagement (Ishibe et al., 2009). New signaling centers may also feed into

ongoing branch regulation. Early studies in kidney explants suggested additional signaling from forming nephrons (Saxen, 1987). In support of this model, branching ceases early in *Wnt4* mutants that fail at the earliest stage of the nephrogenic program (Stark et al., 1994).

The branch tip harbors the progenitor pool for the entire collecting duct network, and is the mitogenic and motogenic center for branching growth. How each uretic tip-directed pathway controls distinct tip properties is not entirely clear. When Ret signaling, or *Etv4* and *Etv5* activity, is removed, tip cells rapidly lose tip properties, exit the tip niche and adopt stalk fates (Chi et al., 2009; Kuure et al., 2010). Thus, competition to remain in the tip niche requires active signaling and, at this level, RTK signaling has an executive function for all tip properties. Gdnf/Ret signaling activates MAP kinase, PI3 kinase and PLC gamma pathways (Costantini, 2012). Of these PI3 kinase action is essential for *Etv4* and *Etv5* expression and branching growth (Tang et al., 2002; Lu et al., 2009). Genetic removal of MAP kinase pathway components blocks branching, but the ureteric epithelium continues to elongate, highlighting a direct role for MAP-kinase signaling in the branching process (Ihermann-Hella et al., 2014). Analysis of *Ctnnd1* (β -catenin) mutants suggests canonical Wnt signaling maintains the epithelial progenitor pool, as loss of *Ctnnd1* results in premature differentiation of progenitor cells (Marose et al., 2008; Bridgewater et al., 2008).

RTK signaling underpins epithelial branching in many organ systems. Modeling suggests Turing mechanisms can account for early branching patterns in both the lung and kidney, pointing to similar regulatory processes at play (Menshykau et al., 2012; Menshykau and Iber, 2013). However, each organ's epithelial network has a distinct shape and form. RTK signaling is likely important not simply in directing branching growth but in directing the branching pattern. Gdnf deficiency is rescued on removing Spry1-mediated antagonism of RTK action, leading ureteric tips to branch extensively; however, their branching pattern is abnormal (Michos et al., 2010). Further, different Fgf ligands stimulate quite distinct modes of epithelial outgrowth: some trigger elongation while others stimulate budding or active branching (Qiao et al., 2001). For the most part, dichotomous branching is the major branching mode throughout kidney development, though trichotomous branching is observed immediately after the T-bud stage (Majumdar et al., 2003). A self-avoidance process, potentially mediated through Bmp7 action, has been implicated in directing spatial outgrowth of each branch tip (Davies et al., 2014). Branch angles are not fixed but change over the course of kidney development and likely contribute to the overall shape of the organ (Short et al., 2014).

Cell organization, cell shape, cell division and cell adhesion have been explored in the context of branching growth. Initial outgrowth of the ureteric bud is preceded by a cuboidal to pseudostratified rearrangement of the epithelium, but this type of remodeling appears restricted to this time (Chi et al., 2009). Extensive filopodial outgrowth at branch tips is thought to drive branching of many epithelial networks. Filopodial formation is associated with the terminal extension of the nephric duct to the cloaca in a Gdnf/Ret dependent process that completes the interconnection of the urinary drainage system (Chia et al., 2011). However, there is no evidence of filopodia-directed outgrowth of ureteric epithelial branch tips. Genetic modification of actin depolymerizing factors indicates that actin remodeling is critical for the branching process (Kuure et al., 2010). Further, enhanced epithelial adhesion

following removal of MAP kinase signaling suggests adhesive remodeling is necessary for normal branching (Ithmann-Hella et al., 2014). Altering adhesive properties may also impinge on proliferative programs. Tip growth occurs with a mitosis-associated dispersal of epithelial cells. Following cell division, one of the mitotic products inserts back into the original location of the parent cell while the other inserts a few cell diameters away (Packard et al., 2013). The significance of cell dispersal to the branching process is not understood. However, the process may help in maintaining an active tip environment, facilitating a rapid sorting from the tip niche of cells no longer responsive to tip signals or those initiating differentiated programs.

Importantly, epithelial rearrangements and regulated growth processes continue to shape the ureteric network beyond the branch tips. A Wnt7b directed epithelial remodeling process is essential for initiating formation of the medullary ureteric architecture around e15.5 to 16.5 (Yu et al., 2009). Wnt7b secreted by the ureteric epithelium signals to underlying interstitial cells to induce production of additional Wnt signals. These are thought to bias the plane of ureteric epithelia cell divisions directing epithelial growth and remodeling to establish the medullary region (Yu et al., 2009). These events are likely to involve extensive rearrangement of the first few branch events of the initial ureteric tree (Short et al., 2014). Wnt9b-mediated planar polarity signaling through the JNK pathway also underlies elongation of the ureteric network (Karner et al., 2009). The critical importance of regulating the plane of cell division for normal epithelial growth is exemplified by a spectrum of cystic kidney diseases in which this process is perturbed (Schnell and Carroll, 2014)

The collecting duct differentiates along a cortico-medullary axis, generating the majority of the two differentiated cells types, principal cells and intercalated cells, in the medullary component. Principal cells, readily distinguished by expression of several water channel membrane proteins including *Aqp2/3/4* are the most abundant differentiated cell type. Intercalated cells come in A- and B-types, displaying distinct ion exchange proteins, *Slc4a1* and *Slc26a4*, respectively (Al-Awqati and Gao, 2011; Wall, 2005). Notch signaling promotes principal cell fate, whereas a mature intercalated cell fate is dependent on the transcriptional regulator *Foxi1* (Jeong et al., 2009; Bloqvist et al. 2004). Although each cell type has a clear, distinct physiological activity, there is evidence for considerable plasticity and interconversion amongst these cell types, at least in cell culture (Fejes-Toth et al., 1992)

The main body of the nephron

The main body of the mouse nephron - from the podocytes of the renal corpuscle to the nephron's interconnection with the collecting duct - derives from a self-renewing, multi-potent *Six2*+ progenitor population established by e10.5, prior to ureteric bud outgrowth (Kobayashi et al., 2008; Figure 2B). In this, *Six2* is essential for maintaining the progenitor state; the absence of *Six2* leads to premature and ectopic commitment of the entire progenitor pool to renal vesicle fates by e12.5 (Self et al., 2006; Kobayashi et al., 2008).

Several transcriptional regulators have been identified in the specification or maintenance of the *Six2* progenitor pool, including *Six1*, *Hox11* paralogs, *Pax2*, *Wt1*, and *Sall1* (Little and McMahon, 2012; O'Brien and McMahon, 2013). Genetic analyses suggest complex hierarchical relationships rather than a similar linear pathway of action for these regulatory

factors. Further, protein-protein interaction studies indicate complex formation by several of these components with *Six2* that are linked to maintenance of the nephron progenitor state (Park et al., 2012; Xu et al., 2014; Kanda et al., 2014).

With the exception of the three *Hox11* paralogs (*Hoxa11*, *Hoxc11* and *Hoxd11*), all the other transcriptional regulatory factors are expressed within the mesonephric kidney (Mugford et al., 2008). Despite the loss of all *Hox11* paralogs, a condensed mass of Pax2+, Eya1+ mesenchyme is formed at the appropriate axial level for the metanephric kidney anlagen (Wellick et al., 2003). Thus, specification of the metanephric mesenchyme is independent of the activity of Hox11 genes. However, the combined activity of Hox11 members is essential for expression of *Gdnf* and other features of the metanephric progenitor program, suggesting that Hox11 paralogs determine metanephric specific kidney outcomes for associated mesenchymal cell derivatives (Wellick et al., 2003). In support of this model, mesonephric tubules ectopically expressing *Hoxd11* adopt metanephric features of regional gene expression (Mugford et al., 2008).

Ingrowth of a ureteric bud into the metanephric mesenchyme is accompanied by the activation of *Cited1* in uncommitted nephron progenitors (Boyle et al., 2008). Whereas *Six2* expression persists transiently into proximal regions of epithelializing nephron precursors, *Cited1* is only expressed within uncommitted nephron progenitors (Mugford et al., 2009; Park et al., 2012). Consequently, *Cited1*'s expression has become the “gold standard” for identifying the nephron progenitor compartment. Surprisingly given the specificity of *Cited1* expression, *Cited1* activity is not required for kidney development (Boyle et al., 2007). Recent studies examining *Six2* levels and proliferation rates have identified regional heterogeneity within the nephron progenitor niche (Short et al., 2014). Whether these temporally and spatially distinct progenitor pools play distinct roles in the kidney's developmental program is not clear (Short et al., 2014).

Generating the full complement of nephrons is dependent on balancing expansion of the nephron progenitor compartment with their commitment to epithelial renal vesicles. Quantifying multiple cellular metrics within the developing mouse kidney has provided a broad overview of these events (Short et al., 2014; Combes et al., 2014). The size of the starting nephron progenitor pool at e10.5 is unclear, but a recent analysis has suggested around 2,000 cells (Wainwright et al., 2015). Extrapolating using cell cycle estimates from later stages with numbers determined at e11.5 by cell sorting (12,000 progenitors; Kobayashi et al., 2008) and confocal imaging (8,000 progenitors, A. Combes and M. Little, personal communication) gives a somewhat larger initiating population of 4,000 to 6,800 (A. Combes and M. Little, personal communication). Using these numbers, nephron progenitors undergo a 174- to 600-fold expansion over the course of mouse kidney development to generate more than 1,000,000 progenitors (A. Combes and M. Little, personal communication).

At the level of the mouse tip niche, there is a steady decline in the ratio of nephron progenitors to tip niche until birth when nephrogenesis markedly accelerates (Short et al., 2014). It is unlikely birth is an actual trigger of these events as kidney development is completed at different times relative to birth in different mammalian species: for example,

prior to birth, around 36 weeks in man (Hinchliffe et al., 1991). All nephron progenitors disappear from the mouse kidney by postnatal day 3 to 4. Consequently, the population of nephrons generated during the developmental period is the population for life.

Nephron progenitors occupy a distinct position within the tip niche. Progenitors maintain close contact with the tip throughout the branching process and are surrounded by a pool of interstitial progenitors (see later). Given the importance of distinct signals issuing from both the underlying ureteric epithelium and overlying interstitial progenitors, this physical arrangement may have developmental significance. The mechanisms controlling distinct niche progenitor cell layering are not known, though *Itga8* in the cap mesenchyme and its receptor *nephronectin* on the adjacent ureteric epithelium are required for interactions promoting Gdnf-dependent ureteric bud outgrowth (Muller et al., 1997; Linton et al., 2007). However, the potential action of this pathway in regulating progenitor niche organization has not been directly addressed. A *Sall1-Kif26b* pathway has also been linked to adherence of cap mesenchyme to the ureteric bud (Uchiyama et al., 2010).

Fgf, Bmp, Wnt and Fat signaling all play important roles in regulating nephron progenitors. Fgf9 produced by the ureteric tips, and Fgf9 and Fgf20 in the CM regulate the proliferative expansion of nephron progenitors through Fgfr1 and Fgfr2 stimulation of RAS and PI3K pathways (Barak et al., 2012; Poladia et al., 2006; Brown et al., 2011). *Bmp7* is expressed broadly in the ureteric epithelium, cap mesenchyme and nephron derivatives (Dudley et al., 1997). Bmp7 signaling promotes the maintenance and proliferation of progenitors through a JNK mechanism (Blank et al., 2009; Tomita et al., 2013), and progenitor commitment through Smad-directed processes (Brown et al. 2013).

Wnt9b's action is complex: Wnt9b is essential for both the proliferation and commitment of progenitor cells (Carroll et al., 2005; Karner et al., 2011). Both processes require the activity of Ctnnd1, a co-activator in the transcriptional complex formed in response to canonical Wnt signaling (Park et al., 2007; Karner et al., 2011). Application of small molecule modulators of Ctnnd1 levels suggests that different levels of Wnt signaling may underpin different actions of the canonical pathway: signaling^{low}/b-catenin^{low} proliferation and signaling^{high}/b-catenin^{high} nephron induction (Brown et al., 2015).

Other signaling pathways are thought to also modulate the response to canonical Wnt signaling. Bmp7-directed Smad action is postulated to convert nephron progenitors from a Cited1+/Six2+ progenitor state refractory to Wnt9b/Ctnnd1 inductive signaling to a Cited1-/Six2+ responsive cell state (Brown et al., 2013). Further, atypical cadherin signaling originating from interstitial progenitors has been suggested to promote adjacent nephron progenitor cell differentiation. Independent studies agree that interstitial cell-derived Fat4 signaling acting through Dchs partners is required to promote progenitor commitment; undifferentiated progenitors accumulate in the absence of Fat4 and Dchs1 (Das et al., 2013; Reginensi et al., 2013; Bagherie-Lachidan et al., 2015). However, there are conflicting views of Yap/Taz and Wnt9b/Ctnnd1 action in these events.

One study suggests that Fat4/Dchs1 directed nuclear accumulation of Yap/Taz promotes progenitor differentiation. A comparison of distinct transcriptional targets of Wnt9b's

progenitor renewal and differentiation promoting activities provided evidence that nuclear Yap/Taz acts in concert with Wnt9/Ctnnd1 to promote differentiation (Das et al., 2013). A second study indicates Fat4/Dchs1 action is independent of Yap/Taz and does not alter canonical Wnt signaling responses but likely has a parallel role in progenitor differentiation (Bagherie-Lachidan et al., 2015). Additional work will be required to resolve these mechanistic differences. In addition to Fat4, Decorin, produced by interstitial progenitors, also promotes nephron progenitor differentiation, inhibiting Bmp7 action and thereby sensitizing nephron progenitors to inductive Wnt signaling (Fetting et al., 2014).

In a significant recent study, conditions have been established that support extensive progenitor replication while preserving the ability of progenitors to differentiate under appropriate conditions through applying lessons learned from normal kidney development (Brown et al., 2015). Signaling networks connect with distinct transcriptional regulatory programs to control cell state. Here, several groups have reported chromatin immunoprecipitation studies on target interactions of key transcriptional components, including Sall1, Wt1, Six2 and Ctnnd1 (Hartwig et al., 2010; Park et al., 2012; Kanda et al., 2014). These studies point to overlapping and unique targets of progenitor maintenance by Sall1 and Six2 (Park et al., 2012; Kanda et al., 2014). Further, they highlight an unexpected molecular interaction between Six2 and canonical Wnt transcriptional complexes on enhancers driving expression of genes regulating progenitor and differentiating cell states mediated by both protein-protein and direct DNA binding interactions (Park et al., 2012). Interestingly, engagement of Ctnnd1 in the complexes is not observed until progenitor commitment. These findings argue against a direct transcriptional role for Ctnnd1-engaged, canonical signaling induced transcriptional complexes in regulating the uncommitted progenitor state, but support their role in initiating progenitor differentiation (Park et al., 2012).

The first cellular evidence of progenitor commitment is a clustering of cells into a pretubular aggregate beneath ureteric branch tips (Figure 2B). The cellular mechanisms determining movement and coalescence of this cohort of cells, and their precise origin from within the progenitor pool are not understood. *De novo* activation of *Fgf8*, *Wnt4* and *Pax8* distinguishes the pretubular aggregate (Plachov et al., 1990; Stark et al., 1994; Grieshammer et al., 2005; Perantoni et al., 2005). Both *Fgf8* and *Wnt4* signals are essential for the continued transition of progenitors to an epithelial renal vesicle (Stark et al., Grieshammer et al., 2005; Perantoni et al., 2005). In this pathway, *Fgf8* acts upstream of *Wnt4*, though *Wnt4* is required to maintain both its own and *Fgf8* expression (Stark et al., Grieshammer et al., 2005; Perantoni et al., 2005).

Wnt4 regulates the transition of mesenchymal pretubular aggregates to epithelial renal vesicles (Kispert et al., 1998). In this, the analysis of *Wnt4*-dependent actions suggests distinct pathways at play. Activation of *Lhx1* in the renal vesicle requires both *Wnt4* signaling and Ctnnd1 activity, indicative of canonical Wnt signaling (Park et al., 2007). However, canonical Wnt signaling clearly inhibits epithelial formation (Park et al., 2007), and biochemical and cellular studies point to non-canonical, Ca²⁺/NFAT dependent signaling in the epithelial transition (Burn et al., 2011; Tanigawa et al., 2011). The cellular mechanisms underlying epithelial formation have not been determined. Epithelial formation

commonly requires cell-to-cell surface interactions mediated by the cadherin-family of homophilic cell adhesion molecules. However, the predominant epithelial cadherin, *Cdh1*, is not present until after renal vesicle formation (Georgas et al., 2009), and *Cdh6*, which is present within the forming renal vesicle, is not essential for epithelial formation, although *Cdh6* mutants display a weak kidney phenotype (Mah et al., 2000). Lumen formation within the epithelial vesicle and derived structures is dependent on the action of afadin and non-muscle myosin action (Yang et al., 2013).

Distinct domains of gene activity segregate the newly formed renal vesicle into proximal and distal domains suggestive of early regional patterning (Figure 3A; Mugford et al., 2009; Georgas et al., 2009). Conclusive evidence that the observed domains correspond to distinct fates in the adult nephron awaits precise fate-mapping studies from early nephron stages. Notch2 signaling is critical for patterning of proximal fates in renal vesicle derivatives (podocytes and proximal tubule), while graded canonical Wnt signaling has been implicated in specification of the remaining medial and distal segments (Cheng et al., 2007; Lindstrom et al., 2015). Emerging polarity within renal vesicle derivatives correlates with the proximity of nephron forming epithelium to the ureteric epithelium: distal markers are activated in cells closest to the ureteric epithelium. Polarizing Wnt signals from the ureteric epithelium (including *Wnt9b*) are attractive candidates in regulating renal vesicle polarity. However, patterned nephron structures can form when isolated metanephric mesenchyme receives an appropriate inductive signal in the absence of ureteric epithelium (Saxen, 1987). These disparate results could be explained if renal vesicles self-organize polarity stochastically while ureteric epithelium-derived signals act to bias and effectively orientate this process.

The cellular dynamics underlying early nephron morphogenesis are not well understood. Growth and dramatic epithelial reorganization accompany the transition of a renal vesicle to an S-shaped body (Figure 3A). Uneven proliferation may play a role in extending distal elongation (Georgas et al., 2009). As there has been no extensive 3-dimensional comparison of different stages in the transition to an S-shaped body, it is not clear if there is a reproducible stereotypical process at play. However, by the time an epithelial connection is established between the distal S-shaped body and the ureteric epithelium, there is considerable molecular heterogeneity evident along the proximo-distal axis of the nascent nephron (Georgas et al., 2009). Limited fate-mapping studies point to some spatial coherence between restricted domains of gene expression in the S-shaped body and adult nephron anatomy (Figure 3B; Barker et al., 2012; www.gudmap.org).

Proximally, expression of the transcriptional regulators *Math*, *Wt1* and *Lmx1b* mark the onset of podocyte specification and regulate later podocyte programs (Mundlos et al., 1993; Rohr et al., 2002; Sadl et al., 2002; Miner et al., 2002; Moriguchi et al., 2006). The mid-section of the S-shaped body is demarcated by expression of genes encoding two Notch ligands, *Jag1* and *Dll1*, and *Lfng*, a Notch receptor modifying enzyme, suggesting Notch-signaling may regulate proximal nephron fates (Leimeister et al., 2003). Consistent with this view, Notch signaling through the Notch2 receptor regulates podocyte and proximal tubule fates (Cheng et al., 2007). As Notch ligands are not freely diffusible, the curvature of the S-shape body may facilitate Notch ligands produced in the mid-segment contacting Notch2+ responsive cells in the proximal epithelium. Interestingly, Notch2-responsiveness is not

conferred by earlier Wnt signaling (Boyle et al., 2011). More likely, Wnt-dependent epithelialization establishes a tissue context for appropriate spatial signaling to direct the proximal nephron patterning process.

The transcriptional regulators Pou3f3, Hnf1b, Irx1 and Irx2 overlap Dll, Jag1 and Lfng producing domains in the S-shaped body (Blumenfeld et al., 1993; Nakai et al., 2003; Massa et al., 2013; Heliot et al, 2013). Precise comparative spatial mapping has not been performed to determine the temporal and spatial overlap in their expression domains and consequently, the degree of molecular heterogeneity in the mid-section of the S-shaped body. However, Hnf1b appears to extend more proximally than these other genes. Both Pou3f3 and Hnf1b are independently required for loop of Henle development. Hnf1b is also required for the proximal tubule, while a Pou3f3 requirement extends distally to the macula densa and distal convoluted tubule (Nakai et al., 2003; Massa et al., 2013; Heliot et al, 2013). The phenotype of Hnf1b mutants is more severe than Pou3f3 mutants: glomeruli connect to the collecting duct through a vestigial epithelial tube, and *Pou3f3*, *Irx1/2*, *Dll1*, *Jag1* and *Lfng* are all down-regulated in *Hnf1b* mutants (Massa et al., 2013; Heliot et al, 2013).

Several transcriptional regulators, including Lef1, Sox9 and Lhx1, extend into the distal-most cells of the S-shaped body (Kobayashi et al., 2005; Mugford et al., 2009; Reginensi et al., 2009). Of these, Lhx1 and Sox9 have been implicated in distal nephron development (Kobayashi et al., 2005; Kumar et al., 2015). Surprisingly, even highly abnormally patterned S-shaped bodies fuse to the collecting duct suggesting this critical interconnection may not be dependent on distal nephron patterning events (Kobayashi et al., 2005; Massa et al., 2013; Heliot et al., 2013).

Development from the S-shaped body to the functioning nephron sees the emergence of mature cell types coupled to clonal expansion of nephron domains (Barker et al., 2012; Rinkevich et al., 2014). Podocyte maturation has been extensively characterized, driven in part by the clear connection of podocyte biology to kidney disease (Schell et al., 2014). However, the regulatory controls driving maturation of other nephron components are less well understood, though there are likely continuing roles for some of the early factors such as Hnf1b in driving late differentiation programs of channel and transporter gene expression in the proximal tubule (Naylor and Davidson, 2014). Development of nephron segments occurs in concert with the development of other kidney structures. For example, the elongation of the loop of Henle is prevented in *Wnt7b* mutants where the medullary ureteric system fails to form (Yu et al., 2009). As in other organ systems, it is likely that function will play a role in regulating tubular maturation; as an example, active fluid transport maintains epithelial polarity of membrane proteins in cells of the adult nephron (Jang et al., 2013).

The interstitial cell population

The bulk of interstitial cell types, with the exception of smooth muscle lining the renal pelvis and ureter, originate from a self-renewing, multipotent progenitor pool demarcated by the transcriptional regulator Foxd1 (Figure 2B; Kobayashi et al., 2014). Though definitive clonal mapping has not been possible, distinct Six2⁺ and Foxd1⁺ nephron and interstitial progenitor pools, respectively, likely emerge from an Osr1⁺ progenitor prior to e10.5 (Mugford et al., 2008b). At e10.5, a bolus of Six2⁺ metanephric mesenchyme is surrounded

by a halo of Foxd1+ cells. Lineage tracing indicates nephron fate is fixed within Six2+ cells at this time. Interstitial fate is also fixed in most Foxd1+ cells. However, a small percentage (1 to 2%) remain capable of transitioning to Six2+/Foxd1– nephron progenitors until e11.0 (Kobayashi et al., 2014). Foxd1 is not essential for the interstitial progenitor fate, and currently fate-specifying mechanisms are unclear. A number of transcriptional regulators display a readily identifiable interstitial progenitor profile in whole kidney *in situ* expression studies (Yu et al., 2012). Of these, Pbx1 plays a role in maintaining the progenitor state by blocking premature maturation of the progenitor pool (Hurtado et al., 2015).

Fate mapping shows a continuous commitment of Foxd1+ progenitors to Foxd1– interstitial cell types in parallel with the commitment of neighboring Six2+ nephron progenitors (Figure 2B; Kobayashi et al., 2014). For the most part, descendants of the Foxd1 pool are closely associated with vascular endothelium forming the mesangial cells of the glomerulus and functionally related pericytes regulating the remainder of the kidney vasculature. Foxd1 descendants also generate smooth muscle and smooth muscle derivatives associated with the arterial system, notably renin secreting juxtaglomerular cell types (Figure 1C). The extent to which cellular diversity reflects pre-patterning or local responses of a common interstitial cell type to a specific target tissue have not been critically examined, though there is evidence, particularly within arteriole-associated smooth muscle, for regional plasticity.

The interplay of interstitial cells and vascular cells is most evident in formation of the vascular knot critical for normal glomerular function. This consists of multiple loops supported by mesangial cells. Both Notch and Pdgfrb signaling are required for mesangial cell programs and, in their absence, the vasculature tuft collapses (Lindahl et al., 1998; Lin EE et al., 2014; Boyle et al., 2014). Further, loss of Foxd1 or Foxd1 interstitial progenitor derivatives demonstrates a requirement for normal interstitial cell types in forming the renal arterial tree (Sequeira-Lopez et al., 2014). What is less clear is the potential interplay between interstitial cell types and the renal tubular component of the nephron. Here the shape, size and close apposition of key cellular players makes resolving cell-type specific interactions a particular challenge.

Vascular and neural development

Analysis of organ explants and the tracing of vascular development *in vivo* support multiple routes to vascularizing the mouse kidney (Abrahamson et al., 1998). Glomerular vascular progenitors descendant from Osr1+ precursors are present within the initial kidney anlagen, and Flk1+ vascular progenitors collect between bifurcating, branch tips in the tip niche at later stages (Figure 2B; Hyink et al., 1996; Mugford et al., 2008b; Mugford et al., 2009). However, vascular ingrowth into the developing kidney is also possible on grafting of kidneys to new tissue sites (Sariola et al., 1984; Abrahamson et al., 1998). While this may suggest distinct developmental origins for the glomerular vasculature and main arterial tree, definitive experiments to settle this issue, without a potential for graft-induced anomalies, have not been possible to date.

The first organization into nephron-associated vasculature is seen with the convergence of vascular cells on the glomerular cleft of the forming S-shape body (Figure 3A). This coincides with and is likely driven by Vegfa: Vegfa is produced by podocyte precursors and

continues into mature podocytes where Vegfa signaling maintains the adjacent glomerular vasculature and promotes endothelial fenestration (Eremina et al., 2003). A second signaling axis essential for the glomerular vasculature is the Cxcl12/Cxcr4 pathway, where Cxcl12 secreted by podocytes and mesangial cells regulates endothelial Cxcr4 activity (Takabatake et al., 2009). Vascular promoting activities within the glomerulus may be countered by Sem3a inhibition of vascular cell numbers (Reidy et al., 2009). Recent experiments also highlight a role for renal tubule derived Vegfa in maintenance of the peritubular vasculature (Dimke et al., 2015). Though critical for renal function and renal health, the renal vasculature is poorly understood from a developmental perspective. The molecular programs establishing renal vasculature architecture, and endothelial cell differentiation and function have not been extensively characterized.

Sympathetic innervation of the kidney has attracted considerable interest given the potential for reducing hypertension by surgical deinnervation of the sympathetic input (Grassi et al., 2015). However, though the adult physiological action of sympathetic neural regulation has been extensively studied, the developmental programs generating the neural network have not been comprehensively addressed with modern tracing, fate mapping and molecular approaches (Sariola et al., 1988; Ferguson and Bell, 1993). Non-renal studies have highlighted guidance pathways shared by vascular and neural cells in a variety of animal models (Carmeliet and Tessier-Lavigne, 2005). In the kidney, sympathetic axonal processes closely track the main arterial input into the kidney, given precedents in other systems, it seems reasonable to speculate that the physical linkage of arterial and sympathetic systems may reflect coordinated sensing of shared guidance cues.

Man versus mouse

How similar is the developing mouse kidney to our own (Cullen-McEwen et al., 2016)? Given that the principal drive in studying the mouse is to understand something of us, this is an important and pertinent question. Mutations in genes for many key regulators of the developing mouse kidney associate with congenital anomalies of the urinary tract (CAKUT) syndromes in the patient population (Vivante et al., 2014; Nicolaou et al., 2015). Indeed, by taking a set of genes educated by mouse studies to examine patient DNA samples, headway has been made in identifying potential genetic factors underlying developmental anomalies of the human kidney in families with isolated CAKUT (Hwang et al., 2014).

However, there has been little direct comparative molecular analysis of the developing mouse and human kidney which display distinct anatomical features to their organization. The human kidney is multi-lobed, forming 8 to 15 independent renal calyces, while the mouse kidney makes just one (Treuting and Kowalewska, 2011). This may suggest a fundamental difference at the very earliest stage in branching and organization of nephron progenitor pools, or the secondary remodeling of the medullary ureteric regions. Whereas the mouse ureteric network undergoes dichotomous branching as a standard routine to extend the cortical territory, branching appears to be limited to the first 14- to 15-week period of human development; thereafter, ureteric tips continue to outgrow extensively but fail to branch (Osathanondh and Potter, 1963). Given the coupling of renal vesicle induction to ureteric branching, and the specific location of renal vesicle formation beneath branch

tips, the human observations raise additional questions about the conservation of ureteric growth programs and nephron inductive processes.

If the same mechanisms are at play, how do they operate at different phases of human kidney development? The observation that different Fgf signals induce elongation versus branching growth mediated through the MAP kinase pathway is especially intriguing in this regard (Qiao et al., 2001; Ihermann-Hella et al., 2014). Arcading is a prevalent mode of hooking up nephrons in the human kidney following the period of branching growth. In this, nephrons interconnect to other nephrons as well as to the ureteric epithelium. Consequently, the connection process is flexible to epithelial origin (Cullen-McEwen et al., 2016). Importantly, the final number of nephrons differs greatly between mouse and man. Does this reflect species differences in the size of the initiating progenitor pool, or different rates of progenitor expansion and commitment? Mechanisms regulating organ size across different species are generally poorly understood; the kidney is no exception in this regard.

Detailed and extensive molecular analysis of the mouse kidney by the Genitourinary Molecular Anatomy Project (GUDMAP; www.gudmap.org) has generated exceptional insights into the developing mouse kidney (McMahon et al., 2008). The high-resolution developmental anatomy produced by GUDMAP participants provides a molecular framework to extend comparative analysis to the human kidney. Efforts are already underway in a human GUDMAP initiative (www.gudmap.org). Early studies have revealed some striking differences in *Six1* and *Six2* regulation within the nephron progenitor compartment (O'Brien et al., 2015). Given the critical role of Six-factors in maintaining nephron progenitors, these results may have relevance to differences in the duration of nephrogenesis and the final nephron count which differ dramatically between mouse and human kidney

In addressing species differences, a better understanding of the underpinnings of mouse nephrogenesis is essential. A more detailed comparative molecular anatomy of nephron morphogenesis coupled with extensive regional fate mapping can provide a much clearer view of how early domains of gene expression relate to mature structures of the adult kidney (Figure 3A–B). The cellular complexity of the mammalian kidney is not well understood. Twenty-six cell-types have been postulated to form from cap mesenchyme and ureteric bud derivatives (Al-Awquati and Oliver, 2002). However, in the absence of extensive molecular characterization, this figure likely underestimates the number of distinct cell types. Detailed studies of proximal tubule development suggests considerable spatial heterogeneity in gene expression patterns (Thiagarajan et al., 2011) though direct comparative expression studies, co-labelling tissue sections with multiple gene probes, are essential to determine the actual extent of cellular diversity.

In the mouse, the majority of nephrons form after birth while in the human kidney nephrogenesis ceases around 36 weeks so that nephrogenesis is complete at birth (Osathanondh and Potter, 1963; Short et al., 2014). Consequently, the final phases of kidney development occur in very different contexts between these species. This begs the question of whether there are species differences regulating these terminal programs? And further, whether premature birth, now survivable as early as 22 to 26 weeks, may influence

subsequent development of the human kidney? Premature birth may significantly impact kidney function (Sutherland et al., 2014). Notably, hypoxia associated with premature birth has damaging effects on multiple organ systems and can influence nephron number in experimental mouse models (Wilkinson et al., 2015).

Translating development biology: Regenerative approaches to kidney disease

Given the limits of dialysis, a shortage of kidneys for transplantation and an absence of other therapeutic options, new solutions for treating end stage renal disease are a high priority. The last few years have seen some important enabling developments with the first reports of directed differentiation of mouse and human pluripotent stem cells to distinct kidney progenitor types (Takasato et al., 2014; Taguchi et al., 2014; Xia et al., 2015). Depending on the induction conditions, cultures can self organize to generate kidney-like organoids with both ureteric-like and nephron progenitor-like cell types (Takasato et al., 2014). In other procedures, isolated progenitors can be generated independently for each of these lineages (Taguchi et al., 2014; Xia et al., 2014), and nephron progenitor-like cells differentiated into distinct nephron fates (Taguchi et al., 2015). Further, differentiated nephron types can be reprogrammed to earlier nephron progenitor-like states through the ectopic action of a small number of transcriptional regulators associated with normal progenitor programs (Hendry et al., 2013). In addition, *in vitro* culture conditions have been identified that enable the long-term expansion of nephron progenitors while maintaining their competence to initiate nephrogenesis under appropriate conditions (Brown et al., 2015).

Strikingly, all of these advances come from a rigorous application of knowledge gained from studying kidney development in model systems (Takasato and Little, 2015; Taguchi and Nishinakamura, 2015; Vanslambrouck and Little, 2015). While there is room for methodological improvements and a firmer grasp on the cell types generated in these procedures, applied developmental biology has opened the door for regenerative approaches to treat human kidney disease (Little and Takasato, 2015).

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Figure 1. Schematic illustrations of the adult mouse metanephric kidney

A). Gross regional organization of the adult kidney. B). Anatomy of the nephron. C). High-resolution overview of the renal corpuscle and associated structures (region boxed in B).

Figure 2. Schematic illustrations of the developing mouse kidney

A) Overviews of the mesonephric and metanephric kidney anlagen pre (E10.5) and post (E11.5) ureteric bud invasion into the metanephric mesenchyme (MM). Anterior is to the top and medial to the right. B). Cellular and molecular organization of collecting duct ($Wnt11^+$), nephron ($Six2^+$), interstitial ($Foxd1^+$) and vascular progenitors within a ureteric tip niche.

Figure 3. Schematic illustrations of nephron patterning process

A). Transition of polarized renal vesicle to patterned S-shape body. B). Speculative color-encoded map of the cell fate relationship of progenitor sub-domains in S-shaped body in (A) to adult nephron anatomy. Verification requires more extensive cell fate mapping studies.