Mechanisms Regulating the Self-Renewal and Differentiation of Nephron Progenitors

Shuang Chen
Washington University in St. Louis
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By

Shuang Chen
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CHAPTER I

Introduction
1. Overview

During development, mammals build kidneys with a large surplus of nephrons from a group of embryonic nephron progenitors called the cap mesenchyme (CM). CM cells are stem-like in many aspects, being able to self-renew and differentiate into nephrons throughout development. However, CM become exhausted shortly after birth and the ability of the kidney to generate new nephrons are lost permanently (Brunskill et al., 2011; Hartman et al., 2007; Rumballe et al., 2011). Therefore, there is a great interest in understanding how these nephron progenitors are maintained during embryonic development and what determines their exhaustion at the end of nephrogenesis.

To gain a better understanding of these mechanisms, I started my thesis work by investigating the role of Fgf signaling in regulating nephron progenitors. Our lab has previously shown that FGF20 expressed from the CM and FGF9 expressed from the UB and CM, are necessary for maintaining nephron progenitors in vivo. My work described in Chapter II extended this finding by showing that FGF9 or FGF20, together with BMP7, is sufficient to maintain purified nephron progenitors in vitro for up to 5 days. Interestingly, the timing of progenitor loss in vitro coincided with that in vivo, indicating a possible intrinsic limit of the CM in retaining a progenitor status. This hypothesis is tested in Chapter III, where I reported the establishment of a new progenitor assay for the developing kidney. Using this system, I discovered that young and old progenitors display differential proliferation rate, adhesion properties and their ability to remain in the niche is inversely correlated with age. These results are consistent with a gradual intrinsic change in the CM preceding the cessation of nephrogenesis and have uncovered an important role for cell intrinsic changes in regulating CM lifespan.

In parallel to investigating how the CM cells are maintained and what leads to their loss, I
studied more downstream event of nephrogenesis, focusing on the role of Notch signaling in nephron formation. Specifically, I investigated why Notch1 and Notch 2, both expressed in the developing nephron have unequal roles. Using quantitative in vitro assay based on luciferase complementation imaging (LCI) system in kidney-derived HEK293 cells, I found that the N2 extracellular domain (ECD) increases Notch protein cell surface localization is cleaved more efficiently upon ligand binding, suggesting that the dominance of Notch2 over Notch1 is due to the unique properties of the N2ECD. The details of this works will be described in Chapter IV.

In this chapter, I will first give a general introduction to mouse kidney development and then introduce what is known about the molecular mechanisms regulating these processes. The second part will be further divided into two segments. The first segment will be an introduction to how the nephrogenic niche regulates the self-renewal and differentiation of CM cells and its implication on why nephrogenesis eventually ends. This is related to work described in Chapter II and Chapter III. In the second part, as related to Chapter IV, I will discuss more downstream events focusing on how CM committed to differentiation generates functional nephrons.

2. **Mouse kidney organogenesis**

The mammalian urogenital system, including the kidney is derived from the intermediate mesoderm (IM). Beginning around embryonic day 9.0 (E9.0) in mouse, cells on the dorsal sides of the IM coalesce into bilateral ducts that elongate from the forelimb region to the cloaca forming the nephric duct (ND) (Saxén and Sariola, 1987). From the rostral to the caudal end, ND induces the formation of three excretory systems in a temporal wave (Vize et al., 1997). Formed first at the most anterior end is the pronephros that completely degenerates in mammals. This is followed by the mesonephros, which transiently participate in filtration and later degenerates or
becomes incorporated into the male reproductive system. The last and most caudal segment, the metanephros, forms the permanent kidneys in mammals (Fig. 1A and 1B) (Schedl, 2007).

The development of the metanephric kidney starts around E10.5 in the mouse when a group of ventral IM cells in the hind limb region aggregate to form the metanephric mesenchyme (MM) (Fig. 1A). MM is characterized by the expression of a distinct group of transcription factors and its ability to secret growth factor GDNF (glial cell-derived neurotrophic factor). The secreted GDNF binds to Ret/Gfrα1 receptor expressing ND cells and induces them to evaginate towards the MM, forming the ureteric bud (UB) (Shakya et al., 2005). The invasion of the UB into the MM marks the establishment of a positive feed back loop between the UB and MM that will drive all subsequent events in nephrogenesis (Cacalano et al., 1998; Moore et al., 1996; Pichel et al., 1996; Sainio et al., 1997; Schuchardt et al., 1996).

As the UB invades the MM, the MM is segregated into a cap mesenchyme (CM) population that is tightly associated with the UB tip and a more loosely associated stromal mesenchyme (SM) surrounding the CM (Levinson and Mendelsohn, 2003) (Fig. 2A). The CM continuously secret GDNF to induce the UB to grow and branch dichotomously, eventually forming the tree-like collecting duct (CD) system of the kidney. Simultaneously, the UB signals back to the CM performing two distinct functions: (a) supporting the survival and proliferation of the CM, such that each newly generated UB tip could be capped by the CM and (b) induce a subpopulation of CM to differentiate into nephrons that connect back to the UB. The interdependence of UB and MM interaction in this process has long been appreciated. Without the UB, the MM would rapidly undergo apoptosis (GROBSTEIN, 1956a). Similarly, the UB would not branch any further in the absence of MM.

The differentiation of CM into nephron epithelia is a classical example of mesenchymal-
to-epithelial transition (MET) in development (Fig. 2B). The induced CM first aggregate at the lateral side of the UB tips forming the pretubular aggregates (PTA). This is a transitory state in which cells induced to differentiate still retains the expression of CM markers (Mugford et al., 2009; Rumballe et al., 2011). Full commitment to the epithelial fate occurs when PTA coalesce into an epithelial vesicle termed renal vesicles (RV) on the ventral side of the UB branch. Each newly formed RV marks the birth of a new nephron. From this point on, the RV further differentiates into comma- and S-Shaped bodies (CSB and SSB). The SSB elongates and segments into different domains of the nephron tubules. The most proximal end of this tubule becomes vascularized to form the glomerulus, which is followed by proximal tubules (PT), loop of Henle (LH) and distal tubules (DT), each having specialized function in filtration and reabsorption. The end of the distal tubule fuses back to the CD forming a continuous lumen, allowing filtrates to be drained into the bladder (Davies, 1995; Georgas et al., 2009; Kobayashi et al., 2008).

Through repetitive branching and new nephron formation, the kidney continues to grow along the radial axis, with the site of UB branching and new nephron formation is at the periphery of the kidney referred to as the nephrogenic zone (Fig. 2A). As a result, the developing kidney contains older nephrons closer to the center (medulla) and younger ones located more peripherally (Fig. 2B). The process of nephrogenesis continues throughout development and ends shortly before or after birth depending on the species, giving rise to 3,000–5,000 nephrons per kidney in the mouse, or the 200,000 to 1.8 million nephrons per kidney in the human (Hughson et al., 2003).

The reiterative nature of nephrogenesis suggests the presence of multipotent progenitors to ensure continuous branching and new nephron formation. Indeed, cell lineage analysis revealed
that the CM is a population of self-renewing progenitors that are capable of giving rise to all nephron cell types (Boyle et al., 2008; Kobayashi et al., 2008). This finding comes from the study of two transcription factors, Six2 (a homeobox transcription factor homologous to the Drosophila *sinus oculis* gene) and Cited1 (Cbp/p300-interacting transactivator), both of which are expressed in the CM throughout kidney development. Lineage tracing studies with both Six2*Cre:GFP*+/tg and Cited1*CreERT2 +/tg mice showed that Six2+ and Cited1+ CM cells are capable of differentiating into all cell types (>20) of an entire nephron. Moreover, once specified, the CM population could sustain itself without contribution from other cell compartments, expanding from ~3,000 cells to ~180,000 cells by the end of nephrogenesis (Kobayashi et al., 2008), highlighting its self-renewal capacity. Beside the CM progenitors, nephrogenesis requires the participation of an additional three progenitor cell types including (a) Ret + UB tip cells (in contrast with stalk cells) required for driving UB growth and branching (Chi et al., 2009; Schmidt-Ott et al., 2005) (b) Foxd1+ stromal progenitors that generates the non-epithelial compartment of the nephron, including interstitial stromal cells, pericytes and mesangial cells (2010a) and (c) Flk1+ endothelial progenitors giving rise to the vasculature and endothelial components of the glomerulus (Mugford et al., 2009; 2005). Together, the nephrogenic zone could be viewed as a progenitor niche supporting multiple progenitor populations, where complex cell-cell interactions drive nephrogenesis.

3. **Molecular regulation of CM self-renewal and differentiation**

Most of our understanding of the molecular mechanisms regulating nephrogenesis came from in vivo loss of function studies completed in the past two decades. These studies revealed key roles for many transcription factors as well as signaling pathways in the proper development
of the kidney. This section will mainly be focused on the molecular regulation of CM population. To understand this regulation, however, one has to place the CM in the context of a highly interactive niche. Therefore, in addition to introducing the basic parameters of CM progenitors (specification, self-renewal and differentiation), I will also discuss how the interaction between CM, UB and stroma regulate the intricate balance between self-renewal, differentiation and its relevance to the cessation of nephrogenesis.

3.1 Specification of the metanephric kidney and the CM

The different types of progenitor cells presented in the nephrogenic niche are all derived from a common IM progenitor expressing the transcription factor Osr1 (odd-skipped related 1) (Mugford et al., 2009). To specify the CM, this population undergoes two rounds of cell fate decisions, first segregating ND from MM, then CM, SM and vascular progenitors within the MM (Mugford et al., 2009; 2005) (Fig. 1C). Proper cell fate decision at each of these stages is most likely not cell autonomous and requires inductive cues from the surrounding tissues. The exact identity of these cues remains unclear. What we do know is that the collaboration of lineage instructive transcription factors plays critical roles in this process.

The specification of ND cells depends on the activity of Osr1, two PAX-family transcriptional regulators, Pax2 and Pax8 (Narlis et al., 2007) and a LIM family member Lhx1 (Tsang et al., 2000) (Pedersen et al., 2005) (Fig. 3A). Pax2 and Pax8 act redundantly to establish the ND. Once formed the development and extension of the ND requires Lhx1. As a result, no UB form when these transcription factors are missing.

The specification of MM requires Osr1, Wt1 (Wilm’s tumor-1) and Hox11 paralogs (homeobox gene11; Hoxa11, Hoxc11 and Hoxd11) (Fig. 3A). Wt1 ensure the survival of IM and
MM (Kreidberg, 2010), whereas the Hox11 genes, known to be essential for anterior-posterior patterning, act more directly to specify a metanephric program (Mugford et al., 2008; Wellik et al., 2002). The subsequent specification of the CM require the continuous expression of Osr1, which is extinguished in the Foxd1+ SM between E9.5 and E10.5 (James, 2006). Osr1 null mice fail to show expression of key CM genes (Pax2, Six2, GDNF, Eya1, and Sall1), putting it on top of the hierarchy of CM induction. Similar to Osr1, the expression of downstream transcription factors such as Eya1 (Eyes Absent Homolog 1), Pax2, Six1, Wt1, Sall1 (Sal-like 1) and Hox11 paralogs persists from the MM to the CM (Dressler et al., 1990; Nishinakamura et al., 2001; Sajithlal et al., 2005; Xu et al., 2003). These factors could directly regulate the expression of key CM genes such as Six2 and GDNF by forming protein complexes such as the Eya/Hox/Pax (Gong et al., 2007) and Eya/Six/Pax (Brodbeck and Englert, 2004) that bind to their promoter. Thus, loss of any of these transcription factors would lead to the absence of Six2 and GDNF expression, which in turn stops UB from invading the MM and lead to kidney agenesis (Dressler et al., 1990; Maas et al., 1999; Wellik et al., 2002). In addition, some of these transcription factors, such as Wt1 and Pax2, have been shown to modify the epigenetic landscape (Cai et al., 2002; Patel et al., 2007) and to interact with the transcriptional machinery (Larsson et al., 1995). The spatiotemporal dynamic expression pattern of many of these genes adds another layer of complexity (Dressler, 2009). Therefore, each of these factors is likely to play diverse roles depending on the cellular context.

Once specified, the CM expresses factors such as Six2 (Self et al., 2006), GDNF (Cebrian et al., 2014) and Cited1(Boyle et al., 2008), marking the entire CM compartment that caps the UB tip (Fig 3B). High-resolution gene expression analysis defined subdomains within the CM, distinguishing between the “capping mesenchyme” (Cited1+Six2+Wnt4−) and ‘induced
mesenchyme’ (Cited1-Six2+Wnt4+) (Fig. 3B). The induced population overlaps with the PTA representing cells already en route to differentiate. In contrast, Cited1+ CM cells never express Wnt4 and was proposed to be confined in the self-renewal phase (Brown et al., 2013). Therefore, the true nephron progenitors are defined as the Six2+Cited1+ cells. However, it was revealed upon more careful study that what appears to be a continuum is actually a heterogeneous population of cells: CM cells are highly motile (unpublished data from Dr. Melissa Little) suggesting that instead of residing in static subdomains, cells might be oscillating between stemness versus commitment as they move around in the CM. The answer to these questions awaits finer maps of the CM at the single cell level.

3.2 Establishing the positive feedback loop between the CM and UB

Although the initial specification of the CM does not rely on the UB, its subsequent maintenance and differentiation requires continuous support from the UB. UB growth and branching is therefore critical in determining the size of the kidney and indirectly regulates nephron endowment.

The induction of UB outgrowth occurs when GDNF secreted by the MM binds to the receptor tyrosine kinase (RTK) complex of Ret/Gfra1 on the ND cells (Fig. 3A). Loss of GDNF, Ret or Gfra1 all leads to kidney agenesis (Jain, 2009). GDNF signaling induces an up-regulation of Ret receptors as well as Wnt11 at the tip of the UB (Costantini and Kopan, 2010). The observation that Wnt11-deficient kidneys have reduced Gdnf expression and number of UB branches suggest that it signals back to the CM to maintain its GDNF expression. Thus, GDNF/Ret/Wnt11 forms an autoregulatory feed-back signaling loop between the CM and UB to coordinate kidney development (Majumdar, 2003). GDNF/Ret is not the only RTK signaling that regulates branching morphogenesis. Fgf signaling, specifically through ligand Fgf10 expressed
in the MM and receptor Fgfr2 expressed in the UB (Bates, 2011), is capable of partially compensating for loss of GFND signaling when an inhibitor of Fgf signaling, sprouty1, is removed (Michos et al., 2010a). For more details of the molecular mechanisms regulating UB branching, the reader is referred to excellent reviews from (Costantini and Kopan, 2010; Dressler, 2006).

3.3 CM survival and self-renewal

Once specified, the CM must survive and self-renew in order to meet the demand of capping new branch tips and generating the vast number of nephrons. The best studies factors related to CM maintenance is Six2. Loss of Six2 does not affect CM specification, but instead leads to premature ectopic differentiation, converting all CM into RV like structures. The depletion of CM results in the loss of GDNF and stalled branching eventually leading to kidney agenesis (Self et al., 2006). The dosage of Six2 may be important for maintaining CM cells, as animals hapolinsufficient for Six2 has reduced nephron numbers (Ben Fogelgren et al., 2009). It is important to note that Six2 expression is not confined to the CM compartment and could be detected in cells induced to differentiate in the PTA and even in the nascent RV (Brunskill et al., 2011; Rumballe et al., 2011). Therefore, Six2 is required but not sufficient to maintain the CM population. Overlapping expression of CM gene Cited1 lead to the speculation that it might work in similar fashion like Six2. Surprisingly, loss of Cited1 alone or together with its homolog Cited2 does not affect nephrogenesis (Boyle et al., 2007). Therefore, despite being a useful CM marker, Cited1 does not appear to have a function in the CM population.

The continuous self-renewal of the CM relies on its ability to survive and proliferate. Several studies have shown a possible role of the bone morphogenic protein-7 (Bmp7) as a key
survival factor for the CM (Dudley et al., 1999; 1995; Luo et al., 1995). Bmp7 is a member of the transforming growth factor beta (TGFβ) signaling pathway family of secreted growth factors and is broadly expressed in the UB and MM in the kidney. Mutants lacking Bmp7 has normal induction of the CM and UB. However, the kidneys would not progress further due to increased cell death amongst CM nephron progenitors which leads to an early arrest of development (Dudley et al., 1995). The ability of BMP7 in promoting CM survival is further supported by experiments where isolated MM could be maintained in vitro for two days in the presence of BMP7 and FGF2 (Dudley et al., 1999). It was later discovered that BMP7 promotes CM survival and proliferation through downstream MAPK signaling pathway (Ulrika et al., 2009). However, the origin of BMP7 responsible for signaling to the CM and upstream signaling required for BMP7 expression is not clear.

The Fgf signaling pathway has been shown to be critical for both the survival and proliferation of CM. Fgf signaling pathway is a highly conserved pathway that regulates diverse developmental processes. In human and mice, a total of 22 different Fgf ligands were identified. All of these ligands requires heparin sulfate proteoglycans to stably bind to four different receptors (Fgfr1-4) (Itoh, 2007). Receptor ligand binding triggers receptor dimerization and activation of downstream signaling events through the MAPK pathway, the PI3K/Akt pathway, the Jak/Stat pathway and the PLCc pathway (Dailey et al., 2005). Expression analysis revealed a complex expression pattern of many of the Fgf family members, including Fgf 1, 7, 8, 9, 10, 12 and 20 and all 4 receptors in the kidney(Brown et al., 2011). MM specific loss of function of Fgfr1 and Fgfr2 showed that the two receptors work redundantly to maintain the CM. In Fgfr1 and Fgfr2 compound mutants, MM specification and UB invasion occur normally, but high rates of apoptosis and low rates of proliferation prematurely depletes the progenitor population.
Several of the Fgf ligands are expressed in the MM and thus emerged as possible ligands for the Fgfr1 and Fgfr2 in the MM. Fgf2, in particular, received much initial interest due to its ability to maintain MM together with BMP7 in vitro. However, deletion of Fgf2 or compound deletion of Fgf1 and Fgf2 resulted in viable, fertile mice without renal defects (Miller et al., 2000). Two other Fgf ligands, Fgf 7 and 10, turned out to act mainly on the UB. Mice with Fgf7 overexpression develops hyperplasia and cystic dilation of the collecting duct system, whereas loss of Fgf10 resulted in a small collecting duct tree and secondarily fewer nephrons (Ohuchi et al., 2000). The MM appears largely intact in both cases. The ligands that act to maintained the CM were recently identified by us as Fgf9 and Fgf20 (Barak et al., 2012). This work will be discussed in details in Chapter II.

3.4 CM differentiation

UB not only supports CM maintenance, but also its differentiation. Early co-culture studies demonstrated that the UB could be replaced by other tissues, including the spinal cord to induce mesenchymal to epithelial transition (GROBSTEIN, 1956b). Later analysis showed that ability to activate canonical Wnt signaling is the common property of these inducers. Activation of canonical Wnt signaling pathway occurs when Wnt ligands secreted from the signaling cell binds to the Frizzled (Fz)/ LRP complex at the cell surface of the target cell. Receptor activation leads to an inhibition of GSK-3/APC/Axin degradation complex in the cytoplasm. This allows β-catenin to accumulate and translocate into the nucleus. Nuclear β-catenin interacts with transcription factors such as lymphoid enhancer-binding factor 1/T cell-specific transcription factor (LEF/TCF) to activate downstream target genes (Nusse, 2012).
During kidney development, two Wnt ligands, Wnt9b and Wnt4, are sequentially required to induce differentiation of nephron progenitors into renal epithelia. Wnt9b is expressed throughout the UB and most prominent on the ventral aspect. Loss of Wnt9b leads to stalled kidney development after the first branching event with no RV formation. When isolated MM are placed in contact with cells producing Wnt9b, RV formation could be rescued (Carroll et al., 2005). Therefore, Wnt9b is both necessary and sufficient to initiate the earliest step of mesenchymal to epithelial transition in the CM. Downstream target analysis showed Wnt4, Pax8 and Fgf8 are important genes in mediating Wnt9b activation. Wnt4 is particularly important as a key gene in relaying the Wnt9b signal. Once induced, it could auto-regulate its own expression and drive epithelialization. Unlike Wnt9b, Wnt4 is not expressed in the UB, but could be detected in the differentiating progenitors as early as the PTA. Its expression persists into the RV and eventually become polarized, playing important role in nephron segmentation. Wnt4 loss of function, similar to Wnt9b, results in in severely hypoplastic kidneys accompanied by limited branching of the ureteric bud. Expression of Pax8 and Fgf8 are absent as well. Importantly, Wnt9b fails to induce differentiation in Wnt4 null metanephric mesenchyme, whereas Wnt4 can induce Wnt9b null mesenchyme to form tubular structures (Carroll et al., 2005). Together, these data supports a model in which Wnt9b from the UB signals in a paracrine manner to induce Wnt4, which then acts in autocrine fashion within the RV to promote epithelialization.

Genetic and pharmacological manipulations suggest that β-catenin/Lef/TCF signaling pathway is activated by both Wnt9b and Wnt4 signaling during CM differentiation (Kispert et al., 1998; Park et al., 2007). Similar to Wnt9b and Wnt4 mutants, CM-specific removal of β-catenin activity completely blocked the formation of RVs and abolished the expression of genes such as Fgf8, Pax8, and Wnt4. In contrast, ectopic expression of stabilized β-catenin in the CM
leads to differentiation of CM in vitro and rescues some differentiation defects in Wnt9b and Wnt4 mutants in vivo. Interestingly, only transient, low-level stabilization of β-catenin could induce epithelization (Ho and Bates, 2011; Park et al., 2012), whereas continuous signal or a higher dosage blocks this process. Some evidence suggests that a non-canonical Wnt signals is required for the final steps of epithelialization which could only occur when canonical signaling is turned off (vandenBerg and Sassoon, 2009). Together, these results indicate that canonical Wnt signaling mediates the majority of signaling activity of Wnt9b during nephrogenesis, but non-canonical Wnt is also involved.

In addition to Wnt signaling, Fgf signaling, including one ligand (Fgf8) and a one Fgf receptor like protein (Fgfrl1), has been shown to play important roles in CM differentiation. Both Fgf8 and Fgfrl1 are expressed in the PTA and RV. Conditional deletion of Fgf8 in the MM or earlier in the entire mesoderm leads to neonatal lethality as a results of severely hypoplastic kidneys (Grieshammer et al., 2005; Perantoni et al., 2005). In these animals, RVs could still form but soon undergoes apoptosis before forming CSB and SSB. Fgfrl1 is most likely a decoy Fgf receptor, since it only contains extracellular domain with ligand binding capacity but lacks protein tyrosine kinase activity at its intracellular domain (Trueb, 2011; Wiedemann and Trueb, 2000). Loss of Fgfrl1 in the kidney leads to stalled differentiation (Gerber et al., 2009), where the expression of genes (Fgf8, Pax8, Wnt4 and Lhx1) essential for MET are missing. It is interesting to note that in Fgf8 and Fgfrl1 mutants, defect in UB and CM survival is observed. However, it is unclear if an RV derived signal could act directly on the CM, or promote survival of CM indirectly by affect UB branching.

3.5 Balancing self-renewal and differentiation of the CM population
As nephrogenesis progresses, a balance between self-renewal and differentiation is the key in maintaining the CM population and supporting continuous nephrogenesis. An emerging network of complex interactions between CM, UB and stroma, has shown to play important roles in striking this balance.

Based on the opposite roles of Six2 and Wnt9b during kidney development, a model was proposed that UB and CM signals counteracts to maintain the balance between self-renewal and differentiation of nephron progenitors (Kobayashi et al., 2008). How could one signaling pathway act in opposing fashion on the same group of cells? To answer this question, Park et al. performed transcriptional profiling and ChIP analysis on CM cells with or without Wnt activation and revealed the ability of Six2 and β-catenin to bind to a common set of enhancers, including both CM specific markers and differentiation genes (Park et al., 2012). Six2 and β-catenin oppose each other through competitive binding to TCF, leading to activation of differentiation genes only in the Six2LO but not Six2HI population. However, both Six2 and β-catenin could activate CM specific genes through TCF independent mechanisms (Park et al., 2012).

Wnt9b is not the only signal that has a bimodal function on the CM. BMP7 signaling, previously found to act through the MAPK pathway to promote CM survival, has an important role in CM differentiation (Brown et al., 2013). Primary cultures of Cited1+ nephron progenitors are refractory to β-catenin induction unless they are treated with BMP7. BMP7 treatment alone does not lead to differentiation; instead it induces a transition from Cited1+Six2+ cell to Cited1−Six2+ cells (Brown et al., 2013). This is consistent with the observation that Cited1 cells are refrained from differentiation and only a few nephrons form in the BMP7 null kidney (Patel and Dressler, 2005). Downstream signal analysis revealed that instead of MAPK signaling, SMAD
signaling is required for this transition. This coincides with the in vivo expression of phosphor-Smad1/5 that is low in the CM and high in the PTA (Brown et al., 2013). The final confirmation of these results awaits CM specific loss of function studies in vivo.

Besides the interaction between CM and UB, the stroma population has emerged as an integral part of the niche in regulating CM self-renewal and differentiation. Loss of SM population (stroma-less) by deletion of FoxD1 resulted in a dramatic expansion of Six2 CM due to stalled differentiation (Hatini et al., 1996; Levinson and Mendelsohn, 2003). Similarly, premature depletion of SM by over-activating Notch signaling in the SM recapitulates this phenotype (Boyle et al., 2011). In an effort to find the link between the presence of stroma and CM size, Fetting et al. compared the expression profile of WT and Foxd1 null kidneys. This revealed an elevated expression of Dcn, a small leucine-rich proteoglycan that could antagonize BMP/SMAD signaling (Iozzo and Schaefer, 2010). Importantly, loss of Dcn could partially rescue the failure of progenitor cell differentiation in the Foxd1 null kidney in vivo (Fetting et al., 2014). Thus, a model has been proposed where stoma regulate CM differentiation permissively by allowing normal BMP7 signaling to occur.

In contrast to this model, an active role of stroma in inducing differentiation has been linked its ability to modulate β-catenin activity in the CM. Supporting evidence came from Das et al. when they found out that a Wnt ligand independent but β-catenin dependent signaling is critical for CM retention in stroma-less mutants (Das et al., 2013). This led to the examination of the Hippo/Warts pathway that has been shown to regulate β-catenin activity independent of Wnt ligand. Hippo and Warts (Mst1/2 and Lats1/2 in mice) are serine/threonine kinases that regulate cell proliferation and differentiation via modifying the phosphorylation status of transcriptional regulator Yap (2009a). In wild-type kidneys, Yap is phosphorylated and remain cytoplasmic. In
contrast, Yap was found to be unphosphorylated and exclusively nuclear in the stromaless mutant. Importantly, nuclear Yap promotes the expression of CM specific genes but represses differentiation genes, linking nuclear YAP to CM retention (Das et al., 2013). How does the stroma regulate the localization of a protein in its neighboring CM? In flies, Fat and Ds family of atypical cadherins have been shown to mediate the activity of the Yap/Taz homologue Yorkie (Willecke et al., 2006). One member of this family, Fat4, is expressed predominantly in the stroma of the embryonic kidney (Mao et al., 2011). Indeed, Fat4 null kidneys have expanded CM with high nuclear YAP similar to other stroma-less mutants. This suggests a model where SM actively induces CM differentiation by producing Fat4 to regulate YAP localization in the CM, which in turn affects β-catenin activity and progenitor status. However, the observation that Yap loss of function does not lead to depletion of CM suggests the input from additional signaling pathways to maintain CM. The prediction that cells closest to the stroma are more inclined to differentiate is also inconsistent with current observations.

Together, these studies suggest that the balance between self-renewal and differentiation of the CM are maintained through crosstalk of various signaling pathways from multiple compartments in the niche.

3.6 Cessation of nephrogenesis

Although an initial balance of CM self-renewal and differentiation is established, the process of nephrogenesis in mammals comes to an abrupt end just before [human ~36 weeks; (Hinchliffe et al., 1991)] or shortly after [mice post-natal day 3; (Rumballe et al., 2011)] birth. Morphologically, a dramatic change in the niche topology could be observed in the post-natal mouse kidney from P1-P4. During this period, the CM shifts to a more lateral position relative to
the UB and is rapidly depleted as many new nephrons appear in a burst. UB tips lose their normal ampulla shape and connect to multiple, instead of one, newly formed nephrons. Stroma and vasculature progenitors become exhausted as well while they differentiate into the interstitium and mature vasculature (Rumballe et al., 2011). Further analysis revealed that the lost of the CM is not due to apoptosis, trans-differentiation or loss of supportive UB tips. Instead, a global commitment into nephrons exhausts the remaining CM (Hartman et al., 2007). Consistent with the loss of CM cells, the expression of CM markers such as Six2 and Cited1 are lost concomitantly and no longer detectable after P2. This loss is permanent, as no reactivation of Six2 and Cited1 could be detected in the adult kidney both during homeostasis and injury repair (Kobayashi et al., 2008). Consequently, no new nephrons could be generated beyond this point.

The molecular mechanism regulating the cessation of nephrogenesis in unclear, but must be involved in shifting the balance of self-renewal and differentiation of nephron progenitors. A parturition-based mechanism was proposed where birth acts as an active trigger for the final commitment of entire CM population into nephrons. A birth trigger is consistent with the timing of the cessation of nephrogenesis in mouse as well as the link between premature birth and low nephron numbers in humans (Faa et al., 2010). Furthermore, analysis of mouse CM population before and after birth revealed change in global gene expression pattern, indicating a change in cell status. Many of these genes are associated with metabolic pathways and could be a results of a shift in tissue oxygen tension after birth (Brunskill et al., 2011). Whether birth could be the trigger in human is still being debated, as the exact timing of the end of nephrogenesis is not clear; early reports suggest it ends before birth (Hinchliffe et al., 1991) while a more recent study argued nephrogenesis continues postnatally in both term and pre-term humans (Faa et al., 2010). These contradicting reports need to be resolved before a conclusion could be made.
An equally possible mechanism is that the lost of CM is a progressive and inevitable process due to the intrinsic property of the niche and does not require a de novo trigger. Recent studies lead us to appreciate that instead a synchronous and reiterating process, nephrogenesis is characterized by structural and temporal discontinuity (Costantini, 2010; Short et al., 2014). Most notably, both the rate of UB branching and CM proliferation decrease progressively over time. The rate of decline in these two compartments are not equal, leading to an overall decrease of CM number per tip from ~6,000 cells at E11.5 to 120 cells at E19.5 (Short et al., 2014). Although still “sandwiched” between the stroma and UB progenitors, the distance between the different compartments changes. This could possibly alter the relative concentration of niche factors, leading to a reduction in maintenance factors (i.e. GDNF, BMP7, FGFs) and increase in differentiation factors (i.e. Wnt9b, Fat4), and eventually depleting all the CM cells.

Understanding the mechanisms leading to the cessation of nephrogenesis has important implications for our goal of increasing nephron endowment in at risk individuals. If only an active trigger is involved, then delaying this trigger may have positive impact on nephron numbers. In contrast, if CM lifespan is an intrinsic property of the niche, we will need to identify what are the intrinsic changes that eventually lead to the depletion of CM and whether this change could be reversed. An investigation of these questions will be discussed in Chapter III.

4. Molecular regulation of nephron segmentation

Formation of an RV is only the first step in generating a nephron. This epithelial vesicle needs to undergo substantial segmentation and elongation before turning into a mature nephron. Each mature nephron contains more than 15 different cell types that need to be properly aligned along the proximal-distal (P/D) axis. The most proximal part of the nephron is called the renal
corpuscle. It is composed of a capillary network (glomerulus) bound by cup-structured epithelia (Bowman’s capsule). As blood enters the glomerulus, the collaboration of two specialized cell types, the fenestrated (with holes) endothelial cells in the glomerulus and the podocytes of the Bowman’s capsule, allows selective filtration to occur. Although the kidneys produce a massive amount of initial filtrate, most of the water, minerals and other essential nutrients are reabsorbed as the filtrate passes through subsequent renal tubules. The proximal tubule is responsible for bulk reabsorption (~90%), whereas loop of Henle further adjust solute composition and distal tubule fine-tunes the final filtrate. Failure of nephron segmentation during development has detrimental consequences and is one of the underlying causes of congenital renal diseases in human.

4.1 Initiation of nephron segmentation

The onset of nephron segmentation occurs very early at the RV stage when no morphological distinctions could be identified in the symmetrical epithelial vesicle. However, molecular analysis revealed polarized expression of many genes in the RV (Fig. 3C). The distal RV (closest to the UB) is characterized by the expression of transcription factors Lhx1 and Brn1 (also known as Pou3f3), Notch ligands (Dll1 and Jag1), Bmp pathway member (Bmp2) and Wnt pathway components (Wnt4, Lef1 and Dkk1). In contrast, the proximal domain (further away from UB) expresses high level of Notch receptor (Notch1 and Notch2), transcription factor Wt1 and Cadherin6 (Cadh6) (Dressler, 2006; Georgas et al., 2009; Kopan et al., 2007; Mugford et al., 2009; Nakai et al., 2003). The signal leading to the establishment of this initial P/D axis is unclear, but the high expression of Wnt targets Lhx1 and Brn1 in the distal RV suggests that a Wnt gradient from the UB might be involved (Kopan et al., 2014b).
As development progresses, the RV starts to unwind by forming two clefts; first at the proximal end and then in the distal region. This transforms the epithelial vesicle into coma- and then S-shaped body (CSB and SSB). By SSB stages, a clear distinction of putative glomerular, proximal and distal domains could be identified based on gene expression patterns (Fig. 3C). Many genes with polarized expression in the RV continue to be expressed in distinct segments in the SSB and are required for the development of that particular segment. For instance, loss of Brn1 leads to loss of LH and DT, whereas Lhx1 mutants only develop podocytes but lack both PT and DT (Kobayashi et al., 2005; Nakai et al., 2003). Notch signaling has emerged as a central player in this process with critical roles in patterning the proximal nephron.

4.2 Notch signaling pathway

Notch signaling pathway is an evolutionarily conserved pathway that plays critical roles in regulating various developmental processes as well as maintaining homeostasis in adult tissues (Artavanis-Tsakonas et al., 1999). There are a total of 4 Notch receptors [Notch1-4] and 5 Notch ligands [Delta-like (Dll) 1, 3 and 4] in mammals. Both Notch receptors and ligands are large, single-pass Type-I transmembrane proteins that mediate communication between neighboring cells (Kopan and Ilagan, 2009; 2007a) (Fig. 4A and 4C).

All 4 Notch receptors share a similar domain structure consisting of an extracellular domain (ECD) followed by transmembrane domain (TMD) and an intracellular domain (ICD) (Kopan and Ilagan, 2009) (Fig. 4A). The N-terminal part of the ECD contains 29-36 epidermal growth factor (EGF)-like repeats, some of which are required for ligand binding (Rao et al., 1995). Following the EGF repeats is the negative regulator region (NRR) that contains three cysteine-rich Lin12-Notch repeats (LNR) and a heterodimerization domain (HD) (Vardar et al.,
The negative regulatory region plays a critical role in preventing receptor activation in the absence of ligands. The ICD of Notch contains a RAM (RBPjk associated molecule) domain, nuclear localization signal (NLS), ANK (seven ankyrin repeats) domain and a PEST (a region rich in Proline, Glutamine, Serine and Threonine) domain (Fryer et al., 2002; Nam et al., 2003; Tamura et al., 1995). The RAM domain is responsible for binding to RBPjk and the ANK domain is required for recruiting Mastermind like (MAML) protein to form the Notch / RBPjk/MAML activation complex. The PEST domain could be ubiquitinated by CyclinC/CDK8, allowing it to be sent for degradation and thus terminating Notch activation (Fryer et al., 2004).

Before emerging on the cell surface, Notch receptors are cleaved at site1 (S1) by furin convertase in the Golgi (1998). Therefore, a mature Notch receptor is a heterodimer composed of Notch ECD and Notch transmembrane and intracellular domain.

The activation of Notch pathway is characterized by multiple key proteolytic cleavage events (Fig. 4B and 4D). Upon ligand binding, ECD undergoes conformational changes that open up the NRR. This allows metalloprotease of ADAM/TACE (a disintegrin and metalloprotease/TNF-α converting enzyme) to gain access at the previously buried domain in the HD and cleaves at site 2 (S2) (Selkoe and Kopan, 2003). This cleavage sheds the extracellular domain and generates a membrane-tethered intermediate recognized by the γ-secretase complex. This is a multi-protein complex containing Presenilin 1 and 2, Nicastrin, Pen-2 and Aph-1, all of which are required for its enzymatic activity (Saxena et al., 2001; Selkoe and Kopan, 2003). γ-secretase complex cleaves the substrate progressively within the transmembrane domain, starting at site 3 (S3) and ending at site 4 (S4). S3-cleavage releases NICD and allows it to translocate into the nucleus (De Strooper et al., 1999; Ray et al., 1999; Schroeter et al., 1998). Upon nuclear translocation, NICD dislocates previously bound co-repressors and assembles a transcriptionally
active complex composed of NICD, RBPjκ and MAML. This complex further recruits transcriptional activation machinery such as CBP/p300 and activates downstream target genes (Fryer et al., 2002; Nam et al., 2006; Tamura et al., 1995). The most well-characterized target genes are bHLH (basic helix loop helix) transcriptional repressors such as Hes-1, Hes-5 and HeyL, all belonging to the hairy enhancer of split (HES) or its related (HESR) family of genes (Davis and Turner, 2001; Iso et al., 2003).

The ability of NICD to directly activate downstream target genes without a signal amplification cascade makes it unique compared to other signaling pathways and indicates a requirement for tight regulation at the level of receptor ligand interaction. This could be achieved by regulating the availability of receptor and ligand at the cells surface or by modifying the efficiency of ligand and receptor engagement (Kopan and Ilagan, 2009).

Receptor and ligand endocytosis plays important roles in regulating their cell surface availability. Both mediated by E3 ubiquitin ligases, ligand endocytosis lead to the presentation of more active ligand on the cells surface, whereas receptor endocytosis results in its degradation in the lysosome or recycling back to the cell surface. More detailed review of this process could be found at (Le Borgne, 2006; Nichols et al., 2007).

The relative strength of receptor-ligand interactions can be modulated by post-translational modifications of Notch receptors (Haines and Irvine, 2003; Rampal et al., 2007; Stanley and Okajima, 2010; Vodovar and Schweisguth, 2008). Glycosylation on the EGF repeats, specifically, the addition of O-glucose or O-fucose and their further modification have critical roles in regulating receptor ligand interaction (Stanley and Okajima, 2010). The addition of O-fucose to Notch receptors by protein O-fucosyltransferase 1 (Pofut1) is not required for generating functional Notch receptors (Okajima et al., 2008) but is necessary for the subsequent
glycosylation of Notch receptors by Fringe proteins [Lunatic fringe (Lfng), Manic fringe (Mfng) and Radical fringe (Rfng) in mammals]. The addition of N-acetylglucosamine (GlcNAc) sugars to the O-fucose moiety by Fringe proteins modulates the differential response of Notch receptors to Dll and Jag ligands (Hicks et al., 2000; Kato et al., 2010). Lfng modification could enhance Delta-to-Notch signaling while limiting Jagged-to-Notch signaling (Visan et al., 2010) although contradicting observation was reported in a different cellular context (Dale et al., 2003). An additional layer of complexity comes from the observation that Fringe modifications do not have the same effects on all receptors; Fringe-modified Notch2 retains its ability to respond to Jagged1, whereas fringe-modified Notch1 does not (Hicks et al., 2000). The speculation is that although highly conserved, paralogs specific glycosylation pattern could result in different efficiency in ligand receptor engagement (Hicks et al., 2000). In addition to Fringe proteins, Notch can also be glycosylated by the glycosyltransferase Rumi (Poglut1) (Acar et al., 2008; Fernandez-Valdivia et al., 2011). Together, fucosylation and glycosylation at critical residues on Notch receptors regulates the ligand receptor interaction and consequently modulate signal strength.

During development, Notch signaling regulates tissue patterning through two general mechanisms: lateral-inhibition and inductive signaling (Artavanis-Tsakonas et al., 1999; Bray, 1998; Greenwald, 1998). Lateral inhibition occurs in a group of initially “equivalent” cells, each expressing comparable levels of the Notch receptor and ligands. Over time, a small stochastic difference leads to random up-regulation of ligand expression in one cell. Elevated ligand expression allows this cells to produce more ligand and fewer receptors and become a signal-sending cell. On the other hand, its neighboring cells with higher Notch signaling began to produce more receptor and less ligand, functioning as signal-receiving cells. This establishes a
feedback loop that further amplifies the initial small difference, inhibiting all neighbors of the signal-sending cell from adopting the same cell fate. A representative example of lateral inhibition would be the differentiation of neuronal progenitors in mammals, where the differentiation of one cell inhibits the neighboring cell from adopting the same fate (la Pompa et al., 1997). In the case of inductive signaling, cells receiving Notch signaling is induced, instead of inhibited from adopting a certain fate (Bray, 1998). This process is employed to establish boarders between different cell populations and often require the aid from Fringe proteins. For example, juxtaposing a Fringe+/Jag+ domain to a Fringe−/Dll+ domain will only allow Notch signaling to occur at the border of two domains. This has been shown to be important in the developing drosophila wing margin (Watson et al., 1994; Wu and Rao, 1999). In contrast, signaling only occur in both Fringe+/Dll+ and the Fringe−/Jag+ domains, but not at the boundary where the two domains meet, a critical step in boundary definition in the ventral spinal cord in mammals (Marklund et al., 2010).

The response to Notch signaling varies greatly between different cell types. This is mainly attributed to the cellular context where the epigenetic landscape and crosstalk with other signaling pathways affects the final output. What is more puzzling is that despite a high homology between the various receptors, the role of each paralog in the same context can be markedly different. For example, loss of Notch1 but not Notch2 or Notch3 leads to skin barrier defects and increases its sensitivity to carcinogenesis. This could not be interpreted as Notch2 and Notch3 not playing a role, as their function could be revealed in a sensitized background when Notch1 level is decreased (Rangarajan et al., 2001; 2009b). In addition, Notch1 is the dominant paralogs in T-cells (Deftos and Bevan, 2000), arterial endothelial cells (Krebs et al., 2000; 2007b), the aortic valve (Garg, 2006), oligodendrocyte (Givogri et al., 2002) and
osteoclast progenitors (2008). In contrast, loss of Notch2 could not be compensated by the presence of Notch1 in the kidney, and possibly in the heart, liver and craniofacial bones (Geisler et al., 2008). The molecular mechanisms underlying paralog dominance in Notch signaling is unclear. Understanding this mechanism(s) will not only be important from a developmental biology point of view but may have important therapeutic implications.

4.3 The complexity of Notch signaling in nephron segmentation

An important role of Notch signaling in kidney development is supported from studies of both human and mice. In human, the link between Notch signaling and kidney development was originally identified in human patients with Alagille syndrome. Alagille syndrome is a congenital, heritable disease that affects multiple organs including the kidney, heart, liver and craniofacial development. Either mutations in Jag1 or Notch2 has been linked to ALGS1 (Li et al., 1997; McDaniell et al., 2006; Oda et al., 1997). It is important to note that both mutations are autosomal haplinsufficient, as only one mutant allele is sufficient to cause the disease. This is in contrast with the mouse, where only a compound loss of Jag1 and Notch2, but not loss of either gene alone, recapitulates Allagile syndrome (McCright et al., 2002). These mice die within 24hrs of birth with hypoplastic kidneys that contain ruptured glomeruli. Further investigation in the developing mouse kidney revealed the expression of multiple Notch family members. This includes the aforementioned receptor Notch1 and Notch2, ligand Jag1 and Dll1 as well as Notch targets Hes1, Hes5, HeyL and modifier Lunatic Fringe (Chen and AL-AWQATI, 2005; Leimeister et al., 2003; Piscione et al., 2004). A polarized expression of Dll1 and Jag1 in the distal RV and the more confined expression of Jag1 and activated Notch1 I the mid-segment of the SSB all indicates a possible role of Notch in nephron segmentation.
To test if Notch is required for these processes, loss of function studies were performed by either treating cultured kidneys with $\gamma$-secretase inhibitor, DAPT (N-S-phenyl- glycine-t-butyl ester), or by genetically removing both copies of Psen1 and 2 (Cheng et al., 2003; Wang et al., 2003). In both cases, MET and the formation of RV is normal. However, the nephrons are truncated lacking the entire proximal domains, including the glomeruli and PT. By treating the cultured kidneys with DAPT at different time points, it was revealed that Notch is only required during a narrow developmental window, perhaps between the formation of RV and SSB, as RVs form normally in all treatments and later treatment did not inhibit the formation of proximal segments (Cheng et al., 2003). In addition, removing DAPT after different length of incubation showed differential recovery ability of podocytes and proximal tubules, with the former being more sensitive to longer inhibition (Cheng et al., 2003).

To further analyze the contribution from each of the two Notch receptors as well as to ensure the phenotype is specific to inhibition of Notch but not other substrates of $\gamma$-secretase, Cheng et al. employed a conditional knockout strategy, deleting either Notch1 or Notch2 in the MM before UB invasion. Results showed that Notch2 deficient MM was able to undergo MET and form RV with normal polarized gene expression pattern, but the subsequent conversion to SSB is defective; The distal segment marked by the expression of Ecadh is correctly formed and connect back to the UB, but the Wt1-expressing podocytes precursors and Cdh6-expressing proximal convoluted tubule precursors are absent (Cheng et al., 2007). This completely phenocopied the kidneys with complete Notch inhibition. Surprisingly, loss of both copies of N1 is inconsequential to the formation of podocytes and proximal tubule, despite its activation at the right time and place. Therefore, Notch2 is absolutely required but Notch1 is dispensable for the formation of proximal segments.
What is the mechanisms underlying the unequal contributions of two highly homologous receptors? It is possible that although N1 is expressed and activated at the right time and place, it is incapable of activating the same set of targets as N2 in specifying proximal fate. This however, is most likely not the case. Overexpression of N1 ICD in the MM is capable of converting all MM to proximal fate (Cheng et al., 2007). In fact, N1 does make a small contribution to the development of nephrons. This is revealed in a sensitized background where delayed removal of N2 in PTA instead of MM allowed some nephron formation. Partial removal of N1 in this background leads to fewer nephrons, whereas complete removal leads to lethal kidney defects (Surendran et al., 2010). This suggests that N1 works synergistically with N2 to promote proximal fate. The progressive loss of nephron formation in these animals lead to the proposal of a new model that proper nephron segmentation requires the activity of Notch to reach a certain threshold, most likely by releasing a sufficient number of NICD molecules. Therefore, a difference in NICD release underlies the dominance of Notch2 in the kidney. However, this is not the only explanation. Subtle differences between Notch1 and Notch2 in expression pattern, cell surface availability and timing of expression could all contribute to its dominant role. All of these possibilities as well as the underlying molecular mechanisms will be investigated in Chapter IV.
Figure legends

Figure 1. The origin and lineage relationship of the developing kidney. (A) Mammalian urogenital system is derived from the intermediate mesoderm “sandwiched” between the paraxial and lateral plate mesoderm. (B) Three excretory systems develop from the IM in a temporal wave from the anterior to the posterior of the embryo. This requires the interaction between the nephric duct (ND-orange) from the dorsal IM and the mesenchyme from the ventral IM (hollow tubules). The permanent kidney in mammals, the metanephric kidney, is formed at the hind limb region through reciprocal interaction between the ureteric bud (UB-orange) and metanephric mesenchyme (MM-green). (C) Lineage relationships between all cell types in the developing kidney. Note that the specification of cap mesenchyme (CM) progenitors involves two steps of fate decision, first separating the ND from MM and then segregation of CM from SM and vascular progenitors within the MM.

Figure 2. Schematic of kidney development through CM-UB interaction and MET. (A) Reciprocal interaction between the UB and CM drives branching morphogenesis as well as CM self-renewal and differentiation. Note that the kidney grows radially and the youngest nephrons are generated in the periphery of the developing kidney call the “nephrogenic zone”, whereas older nephrons as deposited closer to the center (medulla). (B) Differentiation of CM into function nephron is a multistep process. This involves mesenchymal-to-epithelial transition of CM first into renal vesicle (RV) and its further elongation and segmentation into comma- then S-Shapped body (CSB and SSB) and finally a mature nephron. (C). A mature nephron in a continuous epithelial tubule patterned into distinct segments in the proximal-distal axis. This includes glomerulus (G), proximal tubules (PT), loop of Henle (LH) and distal tubule (DT), each having specialized function of filtration and reabsorption.
Figure 3. Key transcription factors and signaling pathways in CM specification, self-renewal and differentiation. (A) Expression of key factors in the UB and MM at the time of UB invasion and outgrowth at E10.5. (B) Expression of key factors in UB, CM and SM for regulating the self-renewal and differentiation of CM cells. (C) Genes involved in RV polarization and nephron segmentation.

Figure 4. Summary of the canonical Notch signaling pathway. (A) All Notch receptors have similar domain structures (see description in text). Although highly conserved, the number of EGF repeats and their modification patterns differ between paralogs. See the distribution of potential fringe (cyan) and Rumi (magenta) sites in the extracellular domain for mNotch1 and mNotch2 (green; same in both receptors) especially in the ligand-binding region of EGF 11-12. (B) Key proteolytic events required for Notch activation. After ligand binding, cleavage at S2 is followed by cleavage at the S3 region. Multiple scissile bonds are cleaved, but only peptides initiating at Val 1744 evade Notch-end rule degradation. Only antibodies that recognize the underlined sequence detect the active form of Notch1. (C) Domain structure of Notch ligands. Ligands of Notch receptors can be divided into two groups based on the length and subtype of EGF-like repeats they contain DSL, DOS, and EGF motifs. (D) Activation of canonical Notch signaling (see text for description). Figure adopted from (Kopan et al., 2014b).
Figure 1. The origin and lineage relationship of the developing kidney.
Figure 2. Schematic of kidney development through CM-UB interaction and MET.
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Figure 4. Summary of the canonical Notch signaling pathway. (Adopted from (Kopan et al., 2014b)
CHAPTER II

The role of FGF9 and FGF20 in maintaining nephron progenitors
This chapter represents a previously published article, entitled “FGF9 and FGF20 Maintain the Stemness of Nephron Progenitors in Mice and Man” which appeared in Developmental Cell, June 2012. Figure 7 and 8 as well as and Fig.3S and 4S represents autonomous work.
FGF9 and FGF20 Maintain the Stemness of Nephron Progenitors in Mice and Man

Hila Barak¹,¹¹, Sung-Ho Huh¹,¹¹, Shuang Chen¹, Cécile Jeanpierre³,⁴,⁹, Jelena Martinovic¹⁰, Mélanie Parisot³,⁴, Christine Bole-Feysot⁵, Patrick Nitschké⁴,⁶, Rémi Salomon³,⁴,⁷,⁹, Corinne Antignac³,⁴,⁸,⁹, David M. Ornitz¹, *, Raphael Kopan¹,², *

¹ Department of Developmental Biology, Washington University School of Medicine, 660 South Euclid Avenue, Campus Box 8103, St. Louis, MO 63110, USA ² Department of Medicine, Washington University School of Medicine, 660 South Euclid Avenue, Campus Box 8103, St. Louis, MO 63110, USA ³ Inserm, U983, Hôpital Necker-Enfants Malades, 75015 Paris, France ⁴ Institut Imagine, Hôpital Necker-Enfants Malades, 75015 Paris, France ⁵ Genomic Plateform, Fondation Imagine, Hôpital Necker-Enfants Malades, 75015 Paris, France ⁶ Bioinformatic Plateform, Université Paris Descartes, Hôpital Necker-Enfants Malades, 75015 Paris, France ⁷ AP-HP, Department of Pediatric Nephrology, Hôpital Necker-Enfants Malades, 75015 Paris, France ⁸ AP-HP, Department of Genetics, Hôpital Necker-Enfants Malades, 75015 Paris, France ⁹ Université Paris Descartes, Sorbonne Paris Cité, 75006 Paris, France ¹⁰ Department of Fetopathology, Laboratoire Cerba, 95310 St. Ouen-l'Aumône and AP-HP, Hôpital Antoine Beclere, 92140 Clamart, France

Corresponding Author:
dornitz@wustl.edu (D.M.O.), kopen@wustl.edu (R.K.) E-mail: Kopan@wustl.edu
Summary

The identity of niche signals necessary to maintain embryonic nephron progenitors is unclear. Here we provide evidence that Fgf20 and Fgf9, expressed in the niche, and Fgf9, secreted from the adjacent ureteric bud, are necessary and sufficient to maintain progenitor stemness. Reduction in the level of these redundant ligands in the mouse led to premature progenitor differentiation within the niche. Loss of FGF20 in humans, or of both ligands in mice, resulted in kidney agenesis. Sufficiency was shown in vitro where Fgf20 or Fgf9 (alone or together with Bmp7) maintained isolated metanephric mesenchyme or sorted nephron progenitors that remained competent to differentiate in response to Wnt signals after 5 or 2 days in culture, respectively. These findings identify a long-sought-after critical component of the nephron stem cell niche and hold promise for long-term culture and utilization of these progenitors in vitro.
**Introduction**

Different mammalian organs deploy different strategies to fulfill their physiological roles in the adult. The gut and the skin rely on stem cells sequestered in specialized niches to maintain homeostasis and recover from injury (Fuchs, 2008; Li and Clevers, 2010). In contrast, the adult kidney has no identifiable stem cells (Little and Bertram, 2009), displays limited repair capacity (Humphreys et al., 2008) and relies instead on a large surplus of nephrons generated from an embryonic pool of stem cells/progenitors during development. Genetic and environmental factors affect the number of nephrons; low numbers correlates with renal diseases and hypertension (Bertram et al., 2011; Keller et al., 2003; Myrie et al., 2011). Improved insight into the nephron progenitors and their environment may help maintain such cells for therapeutic purposes. Therefore, there is growing interest in understanding the mechanisms that regulate these cells during nephrogenesis.

Mammalian renal progenitors are established in the meta-nephric mesenchyme (MM) following ureteric bud (UB) outgrowth. The MM condenses around UB tips and induces tip branching (Frank Costantini, 2010); the distal aspect of the branched duct is a niche for embryonic multipotent stem/progenitor cells (Boyle et al., 2008; Kobayashi et al., 2008). Nephron progenitors located lateral or proximal to the UB tips form a pretubular aggregate (PTA) before undergoing mesenchymal to epithelial transition generating the renal vesicle (RV). The RV cells grow at different rates to form an S-shaped body (SSB). Nephron progenitors located distal to the UB form a “cap” and express the transcription factors *Six2* and *Sall1*, which play a critical role in maintaining stemness. *Six2*-deficient progenitors differentiate en masse prematurely in a Wnt9b-dependent manner (Kobayashi et al., 2008; Self et al., 2006), causing renal agenesis. The UB is thought to play a dual role. It makes an important contribution to
maintaining progenitors capable of self-renewal and it provides Wnt9b that promotes differentiation of cap metanephric mesenchyme (CMM) (Carroll et al., 2005; Karner et al., 2011; Kobayashi et al., 2008; Park et al., 2007). This stem cell/progenitor population disappears after producing $1.5 \times 10^6$ (human) or $1.3 \times 10^4$ (mouse) nephrons, likely due to the disappearance of their niche (Hartman et al., 2007).

Whether niche signals maintain stemness or block differentiation is unclear. Paradoxically, Wnt9b may constitute part of the niche signal (Karner et al., 2011). However, a niche “stemness” signal, if one exists, is expected to specifically maintain self-renewal while keeping the cells competent to respond to differentiation signals by generating multiple lineages. Such a molecule would have therapeutic potential if it could be used to maintain and expand a population of multipotent renal stem cells in an artificial niche.

FGF ligands are among the candidates for the niche signal required to maintain stemness because inactivation of FGF receptors ($Fgfr$s) in the CMM results in arrested kidney development (Poladia et al., 2006; Sims-Lucas et al., 2010). Supporting the notion that FGF signals are essential for maintenance of the nephron progenitor population is the observation that supplementing isolated MM cells with FGF2 (which is not present in the CMM) and Bmp7 (which is) can maintain their short-term competence in culture (Dudley et al., 1999). Generally, FGF ligands expressed in mesenchymal tissues signal to epithelial splice forms of FGFRs (“b” splice forms), whereas ligands expressed in epithelial cells signal to mesenchymal splice forms of FGFRs (“c” splice forms; (Zhang et al., 2006). Based on this, the ligand may be provided to the CMM by an epithelial source, (i.e., the UB). CMM cells express Fgf7 and Fgf10, which signal to epithelial Fgfr2b in the UB. Targeted deletion of $Fgf7$ leads to a reduction in ureteric
branch number due to decreased signaling through Fgfr2b and secondarily, to fewer nephrons (Bates, 2011; Qiao et al., 1999). Fgf8, expressed in the nascent RV, is required for progression from PTA to RV and for its proliferation (Grieshammer et al., 2005; Perantoni et al., 2005). Loss of Fgf8 results in apoptosis of MM due to loss of a PTA-derived signal, most likely Wnt4 (Grieshammer et al., 2005; Perantoni et al., 2005). Although these studies point to an important role for FGF signaling in maintaining the MM, the ligands that signal to mesenchymal Fgfrs in vivo have not been identified.

Here, we investigate the role of the Fgf9 subfamily in renal development and discovered that Fgf9 acts redundantly with Fgf20 to promote the proliferation, survival and stemness of CMM progenitors in vivo. Moreover, we identified a human missense mutation in FGF20 that results in bilateral renal agenesis. We show that either Fgf9 or Fgf20 is sufficient to maintain differentiation-competent, Six2+ nephron progenitors in vitro for 5 days, whereas cells grown in Fgf8 or Fgf10 lose competence by 48 hr. Sorted Six2+ cells retained competence to form epithelia for at least 48 hr in culture when grown in serum-free media containing Fgf9/BMP7. These studies identify Fgf20 (and Fgf9) as integral parts of the CMM niche where they act to maintain a self-renewing progenitor population competent to respond to an inductive cue. This work lays the foundation for creating an artificial renal stem cell niche.
Results

**Fgf20 and Fgf9 Act Redundantly and Are Essential for Kidney Development**

The *Fgf20* gene was targeted by insertion of a β-Galactosidase (βGal) cDNA into exon 1 (Huh et al., 2012). *Fgf20*βGal/βGal mice are viable, fertile, and have a normal lifespan. They also display several phenotypes, including sensorineural hearing loss due to defects in the development of the organ of Corti (Huh et al., 2012). Examination of E18.5 kidneys from *Fgf20*βGal/βGal embryos revealed a reduction in kidney size (15%) relative to control kidneys (n = 8, p < 0.03). Counts confirmed that fewer Glomeruli formed in *Fgf20*βGal/βGal and *Fgf20*+/βGal kidneys relative to wild-type (15,684 ± 3,035 versus 22,350 ± 2,733; p < 0.001; 19,392 ± 3,070 versus 22,350 ± 2,733, respectively; p < 0.003; Figure 1). The adrenal glands, gonads, and Wolffian duct of *Fgf20*βGal/βGal were indistinguishable from wild-type, as was the gross histology of *Fgf20*βGal/βGal kidneys (Figure 1B; see also Figures 2 and 3). The intermediate steps in nephron development (formation of the RV, SSB and early glomeruli) were comparable to control (white line, Figures 1F–1G, arrow SSB and arrowhead glomeruli). Thus, the overall smaller size was consistent with a smaller progenitor pool that differentiated normally.

Related FGFs have similar biochemical properties and developmental functions (Itoh and Ornitz, 2011; Zhang et al., 2006). We thus tested if the other members of the same subfamily (*Fgf9, Fgf16*) had redundant functions with *Fgf20* in kidney development by generating compound *Fgf9* and *Fgf20* mutant animals. *Fgf20* compound heterozygous (*Fgf9*+/−; *Fgf20*+/βGal and *Fgf9*−/−; *Fgf20*+/βGal) embryos did not exhibit any gross abnormalities (n = 10 and 6, respectively; Figure 1C) *Fgf9*-deficient embryos display aberrant development of the male reproductive tract with no kidney abnormalities (Colvin et al., 2001a; Kim and Capel, 2006), and
die after birth due to lung malformations (Colvin et al., 2001b); therefore, all subsequent analyses were conducted at E18.5 unless stated otherwise. In contrast, all $Fgf9^{+/--}; Fgf20^{βGal/βGal}$ embryos had hypoplastic kidneys ($n = 8$). The defects ranged from mild (15% reduction in 8/15 kidneys, $p = 0.005$; Figure 1D') to severe (60% reduction in 6/15 kidneys, $p < 0.001$; Figure 1D''). One embryo displayed bilateral renal agenesis (similar to Figure 1E). No defects were detected in the adrenal glands, gonads, Mullerian ducts, ductus deferens, or bladder. Significantly, histological analysis revealed that in $Fgf9^{+/--}; Fgf20^{βGal/βGal}$ kidneys, large areas of the nephrogenic zone were missing, replaced by more mature tubular structures (Figures 1H and 1H', arrowheads). Kidney agenesis was observed in all $Fgf9^{−/−}; Fgf20^{βGal/βGal}$ embryos examined ($n = 6$, Figure 1E, yellow arrowhead). In this genetic background, the adrenal glands were reduced in size (Figure 1E, white arrowhead).

These findings indicate that $Fgf9$ and $Fgf20$ function redundantly during kidney development to maintain the nephrogenic zone. Because one allele of $Fgf20$ in $Fgf9$ null embryos is enough to support normal kidney development, whereas $Fgf9^{+/--}; Fgf20^{βGal/βGal}$ mice display a severe phenotype, $Fgf20$ seems to have a more dominant role than $Fgf9$ in the kidney.

**Reduction in Fgf9 and Fgf20 Levels Lead to Loss of Nephron Progenitors and Premature Differentiation**

To better characterize $Fgf9$ and $Fgf20$ deficient kidneys, we examined the expression of several molecular markers of nephron progenitors and their differentiated descendants in control, $Fgf20^{βGal/βGal}$ and $Fgf9^{+/--}; Fgf20^{βGal/βGal}$ dysplastic kidneys. The UB (Cytokeratin 8, Ck8) branched and formed a collecting duct in both control and mutant mice (Figures 2A–2E). Wilms tumor 1
(WT1) was highly expressed in the podocytes, and Lotus tetragonolobus lectin (LTL)-positive proximal convoluted tubules (PT) were readily detected. This and the fact that some Fgf9+/−; Fgf20βGal/βGal mice survive to adulthood, confirmed the presence of mature, functional nephrons, albeit in reduced numbers within smaller, dysplastic kidneys (Figures 2A–2E). Loss of Six2-positive CMM occurred in patches, consistent with an overall reduction in nephron progenitors (see below). As noted before, large areas in the nephrogenic zone of E18.5 Fgf9+/−; Fgf20βGal/βGal kidneys were replaced by mature structures (Figures 2C and 2E, white arrows). Six2-expressing nephron progenitors also express Pax2 (paired-box homeotic transcription factor 2) and low levels of WT1 protein. These progenitors form one or two cell layers distal to each UB tip in both control (Fgf20−/βGal) and in most tips in Fgf20βGal/βGal kidneys (Figures 2A, 2A′, and 2D′, yellow arrowheads) at E18.5. In contrast, in Fgf9+/−; Fgf20βGal/βGal kidneys, three phenotypic categories can be discerned: normal CMM (Figure 2, yellow arrowheads), reduced progenitor numbers (Figures 2B′, 2C′, and 2E′, white arrowheads, also seen near a few tips in Fgf20βGal/βGal kidneys), or loss of progenitors leading to mature structures distal to UB tips (white arrows in Figures 2C, 2C′, and 2E′).

To determine if these defects were intrinsic to the progenitors, we cultured E11.5 metanephroi of various genotypes for 2 days and stained for Ck8 and Six2. Fgf20βGal/βGal metanephroi were smaller than controls. To evaluate progenitor pool size, we counted the number of tips surrounded by Six2+ cells in each genotype (see Experimental Procedures for detail). Whereas UB tips in control animals were surrounded with Six2+ cells, 33.7% of Fgf20βGal/βGal UB tips were surrounded by fewer Six2+ cells (Figure 3C, yellow arrows) and 12.4% lacked Six2+ cells altogether (Figure 3C, blue arrows; quantification in Figure 3M and Table S1). Fgf9+/−; Fgf20βGal/βGal metanephroi displayed a more severe phenotype (41% had fewer and 29.8% had no Six2+ cells;
Figures 3D–3F and 3M). Staining for the RV-SSB differentiation marker Jagged1 (Jag1) (Cheng et al., 2007; 2003) detected numerous Jag1+ epithelial differentiating distal to the UB tip, where Six2-expressing mesenchyme should reside (Figures 3H–3L, white arrows). Consistent with premature differentiation in the niche, Wnt4 was ectopically expressed in cells surrounding UB tips in Fgf9<sup>-/-</sup>; Fgf20<sup>βGal/βGal</sup> metanephroi (Figure 3K, white arrows). These observations are consistent with the hypothesis that Fgf9 and Fgf20 act to prevent premature differentiation of nephron progenitors, reminiscent of Six2-deficient mice (Self et al., 2006) or mice expressing constitutively active Notch in the CMM (Boyle et al., 2011; Cheng et al., 2007; Fujimura et al., 2010).

**Fgf20 Is Expressed Exclusively in Nephron Progenitors, Whereas Fgf9 Signals Mostly from the UB**

To determine the temporal and spatial expression patterns of Fgf20, we analyzed FGF20-βGal activity during kidney development. Whole-mount X-gal staining at E10.5–E12.5 clearly showed activity in mesonephric and metanephric kidneys (Figures 4A–4C, black and red arrowheads, respectively). At E14.5, βGal activity was present in the MM surrounding the UB (Figure 4D). To identify the specific cell population that expressed Fgf20, we stained sections of Fgf20<sup>βGal/βGal</sup> kidneys with antibodies against Ck8, neural cell adhesion molecule (NCAM, marking mesenchymal cells and their epithelial derivatives) and βGal. At E16.5, βGal colocalized with Six2 in CMM cells (Figures 4E and 4F, white arrows). βGal activity persisted in the nascent RV due to expression or perdurance (Figure 4E, yellow arrowhead) but not in the stroma or the UB (Figures 4E and 4F). We therefore conclude that in the metanephric kidney, Fgf20 is expressed exclusively in nephron progenitors. This pattern agrees with the mRNA expression data collected by GUDMAP (2007c) and presented in (Brown et al., 2011). The same resource describes Fgf9
expression as most abundant in the UB, with MM expression detected P0-P2 (see Figure S1 available online). mRNA for both ligands disappears as the remaining progenitors differentiate en masse.

Conditional inactivation of Fgfrs in the CMM caused renal agenesis (Poladia et al., 2006), mimicked by Fgf9/20 compound mutants (Figure 1). Because Fgf9 is expressed in the duct (Barasch et al., 1997; Brown et al., 2011; McMahon et al., 2008), we hypothesized that Fgf9 is a paracrine signal from the duct to the CMM and FGF20 acts in an autocrine manner on the CMM itself. To test this, we inactivated a conditional allele of Fgf9 (Fgf9\textsuperscript{ff}; (Lin et al., 2006)) specifically in the UB, with the HoxB7-Cre BAC transgene (Yu et al., 2002). Removal of both Fgf9 alleles from the UB in Fgf20\textsuperscript{βGal/βGal} embryos permitted normal development (Figures 4G, 4H, 4K, and 4K\textsuperscript{β}). Conversely, when a single allele of Fgf9 was removed from the UB of Fgf20\textsuperscript{βGal/βGal} embryos (HoxB7-Cre\textsuperscript{+/tg}; Fgf9\textsuperscript{+/f}; Fgf20\textsuperscript{βGal/βGal}), dysplastic, smaller kidneys formed (25%–75%; n = 10; Figure 4I). Examination of these kidneys (Figures 4L and 4L\textsuperscript{′}) revealed depletion of nephron progenitors similar to that seen with global loss of an Fgf9 in this background (Figure 2). Fgf20 null embryos lacking both Fgf9 alleles in the UB (HoxB7-cre\textsuperscript{+/tg}; Fgf9\textsuperscript{ff}; Fgf20\textsuperscript{βGal/βGal}) displayed rudimentary kidneys (n = 6) or renal agenesis (n = 4) (Figure 4J), a phenotype slightly milder than the Fgf9/Fgf20 compound null. To ask if Fgf9 was expressed in the MM, we deleted Fgf9 from the mesenchyme using the Pax3-Cre allele (Cheng et al., 2007; Li et al., 2000). Some Pax3-Cre\textsuperscript{+/tg}; Fgf9\textsuperscript{ff}; Fgf20\textsuperscript{βGal/βGal} mice have rudimentary kidneys (Figure S1), consistent with Fgf9 also being expressed in the mesenchyme. These data support a model in which Fgf9 secreted from the UB complements the activity of Fgf20 and Fgf9 produced in the CMM.
FGF20 Loss Is Associated with Renal Agenesis in Humans

Independently, we used a combined approach of homozygosity mapping and exome sequencing to analyze a consanguineous family with several fetuses presenting with isolated bilateral renal agenesis. Autopsy revealed the Potter sequence and bilateral renoureteral agenesis (Figure 5A) in one fetus. Other organs were normal. DNA analysis from this fetus identified homozygous variants in four genes expressed during early kidney development and located in homozygous chromosomal regions. One of these mutations was a single base-pair deletion in exon 2 of FGF20, resulting in a frame shift starting at amino acid 113 and terminating in a stop codon at position 121, before the third heparin-binding domain of the protein (Figures 5B–5D, 1000 genome database, and was not found in our in-house whole exome sequence data.

Moreover, the three other homozygous variants identified in this fetus did not segregate with the kidney defect in this family. Combined with the mouse mutants described above, these data point to FGF20 as an essential protein during mammalian metanephric kidney development whose loss is sufficient to cause bilateral renal agenesis in humans.

In the Absence of Fgf9 and Fgf20, Nephron Progenitors Form but Are Not Maintained Renal epithelia fail to form in humans lacking FGF20 or in mice lacking Fgf9 and Fgf20. We next asked if kidney agenesis reflected failure to establish the CMM (Frank Costantini, 2010) or failure to maintain the progenitors. Because the CMM induces UB outgrowth, we asked if UB induction was initiated properly in mutants by whole-mount Ck8 staining. By E11.5, control embryos had an elongated, T-shaped UB (Figure 6A). Fgf9−/−; Fgf20βGal/βGal embryos initiated UB outgrowth, but in contrast, we only observed a branched UB in two out of seven embryos (Figure 6B). To examine the MM, transverse sections of E11.5
embryos were prepared. In all embryos, the elongating UB contacted the Pax2-positive MM (Figures 6C–6H). Control MM cells condensed around the UB in wild-type embryos (Figure 6C), whereas the Fgf9−/−; Fgf20βGal/βGal MM was significantly smaller and most cells failed to coalesce around the UB (Figure 6D). TUNEL staining detected many apoptotic cells within the MM of Fgf9−/−; Fgf20βGal/βGal embryos at E11.5 (Figures 6E and 6F). By E12.5, only a few Pax2+, Ck8− cells remained in the mesenchyme (Figures 6G and 6H). This analysis suggested that, in the absence of Fgf9 and Fgf20, UB outgrowth was initiated, but the rudimentary MM failed to migrate toward the UB and, in addition to prematurely differentiating (Figures 3K and 3L), underwent high rates of apoptosis reminiscent of isolated MM cells in vitro and in Fgfr-deficient MM in vivo (Poladia et al., 2006). Analysis of Pax2 and Gdnf expression in the MM revealed that their expression correlated with the dose of Fgf9/20 as early as E10.5–E11.5. In contrast, Pax2 (Figure 6) and cRet expression (data not shown) in the UB was unchanged. It cannot be ruled out that Fgf9 has a function in maintaining Etv4 and Etv5 in the UB epithelium (data not shown). The consequence of reduction in GDNF/ret signaling was progressive failure in branching morphogenesis (data not shown), which could be reversed in vitro by the addition of GDNF to metanephroi with limited branching (Figure S2).

Fgf9 Maintains Isolated Progenitors Competent to Respond to Inductive Signals In Vitro

The data presented thus far shows that Fgf20 and Fgf9 together are required for maintenance and survival of nephron progenitors in mice, and infer a similar activity for FGF20 in humans. Moreover, our allelic series indicates that stemness and survival were sensitive to the dose of Fgf9/20 (predicting reduced nephron numbers in humans heterozygous for FGF20). To test if these ligands were sufficient to mimic the niche, we examined the ability of recombinant
FGF9 to preserve the stemness of isolated nephron progenitors in vitro. MM was isolated from E11.5 kidneys and explants were grown in serum free media supplemented with heparin (an important cofactor for FGF signaling; (Yayon et al., 1991)), or heparin plus FGF9. In this and subsequent experiments, the absence of UB was confirmed by Ck8 staining.

To assess survival, we used TUNEL staining; to identify mitotic cells, we used anti phospho-histone H3 (pHH3) antibody. As reported, the majority of isolated MM cells were TUNEL-positive after 4 days in media (Figure 7A). When heparin was added, a greater number of cells survived and some mitotic cells were seen (Figure 7B). The addition of FGF9 and heparin improved both survival and proliferation (Figure 7C). Anti-Cited1, Wt1 (not shown), and Six2 antibody staining 48 hr after MM isolation confirmed that these markers were maintained in ex- plants cultured in heparin/FGF9-containing media (Figures 7E and 7E'), but not with heparin alone. Some Six2-expressing cells were mitotic (Six2+; pHH3+; Figures 7F–7I). These data demonstrate that the addition of FGF9 was sufficient to promote survival and support proliferation of some nephron progenitors in vitro.

Next we asked whether nephron progenitors grown in the presence of FGF9 retained their competence to respond to inductive signals. To avoid introduction of renal tissue, we used freshly dissected dorsal spinal cord segments (dSC; (Saxén and Lehtonen, 1987; Saxén and Sariola, 1987)) as a source of Wnt (Herzlinger et al., 1994; Kispert et al., 1998). After 48 hr in heparin/FGF9 media, dSC was added and the cells were maintained for an additional 2–4 days in serum-free media. All freshly isolated control MM grown in contact with the dSC underwent MET (data not shown). After 2 days in vitro, only FGF9- supplemented MM differentiated into epithelia expressing either early distal (Cdh1) or proximal (Jag1) markers and retaining a few
Six2+ progenitors (Figures 7J and 7K). The fact that these tubular structures contained at least two differentiated cell types (distal and proximal epithelial cells) strongly suggests that progenitor multipotency was retained.

It is clear that Fgf20 plays a more dominant role than Fgf9 in vivo and that any other Fgfs present in the cortex during development (Fgf2, Fgf8, and Fgf10) cannot rescue the niche in the absence of Fgf9 and Fgf20. Fgf9 and Fgf20 share biochemical properties that may be unique among Fgf ligands expressed in the vicinity of the niche. To test this possibility, we cultured isolated E11.5 MM for 48 hr in heparin supplemented media and added FGF9, FGF20, FGF8, or FGF10. First, we established the activity of our recombinant proteins on FGFR2b and FGFR2c splice variants stably expressed in Baf3 cells (Ornitz et al., 1996). Although FGF8, FGF9, and FGF20 all activated the mesenchymal FGFR2c splice form (Figure S3), only FGF9 (7/9 explants) or FGF20 (9/10 explants) could maintain Six2+ cells. Very few Six2-expressing cells (~10 cells/explants) were seen in the presence of FGF8 (7/7 explants); none were detected in eight explants exposed to FGF10 (Figure S3). In summary, these data demonstrated that members of the Fgf9/20 subfamily are necessary and sufficient to promote survival, competence, and multipotency of MM cells in vitro.

**Bmp7 Synergizes with Fgf9 to Promote Survival and Competence of Nephron Progenitors In Vitro**

FGF2 and BMP7 synergized to improve maintenance of nephron progenitors in vitro (Dudley et al., 1999). To test if BMP7 can enhance the effect of the endogenous niche ligand, we cultured isolated MM in media supplemented with BMP7 and FGF9. Whereas isolated MM supplemented with BMP7 alone lost their Six2+ population (Figure 8A), BMP7/FGF9 seemed to
increase the overall number of Six2+ cells and induced nephron progenitors to sort away from Six2- cells and aggregate, confounding efforts to count them (Figures 8B and 8B'). In the presence of dSC, numerous epithelial structures containing distal (Cdh1+) and proximal (Jag1+) cells formed, each surrounded by a tight cluster of Six2+ cells (Figures 8C and 8D). To assess for self-renewal of nephron progenitors in media supplemented with BMP7 and FGF9, EdU (5-ethynyl-2'-deoxyuridine) was added to freshly isolated MM for 4–7 hr; MM cells were then washed and re-fed with BMP7/FGF9 media every 12 hr. After 2 days, many Six2+ cells that remained EdU^bright (Figures 8E–8H, yellow arrows) were detected, suggesting that they only divided once. A few Six2+, EdU^Dimm cells (progenitors that divided once after labeling or labeled late in S phase) were in mitosis after 48 hr (stained by pHH3; Figures 8E–8H, turquoise arrow). Because mammalian S, G2, and M phases are relatively fixed in length, such double labeling indicates that some nephron progenitors were able to divide twice (one immediately after culture, the other 2 days later) in the presence of BMP7 and FGF9.

To ask if Six2+ cells could be maintained for as long as they live in vivo (from E11.5 to P2, or 8 to 10 days), we cultured isolated MM with FGF9 and BMP7 for 8 days in culture. Notably, although some Six2 expressing cells were in mitosis (Six2+; pHH3+; Figures S4A–S4C, yellow arrows), the proportion of Six2+ cells declined after 5 days under these conditions (Figures 8I and 8I'); by day 8, only a few Six2+ cells were detectable (data not shown). Staining for differentiation markers after 5 days confirmed that no Cdh1 or Cdh6 expressing epithelia formed in the absence of inducers (data not shown). Importantly, after 5 days in heparin/FGF9/BMP7 media in vitro, Six2+ nephron progenitors formed epithelial tubules when cocultured with dSC for an additional 4 days in basal media (9/9, three individual MM each in
three repeats). These epithelia expressed either proximal (Cdh6) or distal (Cdh1) markers (Figure 8J).

Finally, to determine if FGF9 acted directly on Six2+ cells or indirectly through FoxD1+ stromal cells, we sorted GFP+ cells by FACS from Six2-GFP::CRE*+/+ kidneys. Small colonies of Six2+ cells, maintained for 48 hr in serum free media supplemented with FGF9, remained competent to differentiate (Figure 7L). Culturing these cells with FGF9+BMP7 for 48 hr resulted in robust expansion of many more Six2+ colonies (Figures 8K and 8K') that maintained competence to respond to differentiation signals and produced epithelia expressing either proximal (Cdh6) or distal (Cdh1) markers after coculture with dSC (Figure 8L). Mitotically active nephron progenitors (Six2+; pHH3+) survived after 5 days (Figures S4E and S4F, yellow arrows). Although when cultured with dSC they could form a few Cdh1- or Cdh6-positive cells, tubular epithelia no longer formed (data not shown). These data demonstrate that Fgf9/Bmp7 act directly on progenitors, promoting their survival while maintaining their competence to respond to inductive signals for at least 2 days.
Discussion

Here we present data demonstrating that members of the Fgf9 ligand family are bona fide secreted niche signals in the developing mammalian kidney. FGF20 acts alone in humans but is redundant with Fgf9 in mouse. In the absence of Fgf9 and Fgf20, UB outgrowth initiates, but because the MM undergoes a high rate of apoptosis, or because Fgf9 signals are needed to maintain Etv4 and Etv5, UB branching is halted and kidney development fails. In human fetuses homozygous for a truncated allele of FGF20, no kidneys form. In mice, a single allele of Fgf20 (Fgf9/−; Fgf20+/βGal) is sufficient to maintain normal kidney development in vivo (however, not in isolated MM), whereas a single allele of Fgf9 (Fgf9+/−; Fgf20βGal/βGal) is insufficient. The redundancy between Fgf9 and Fgf20 produced highly informative variable phenotypes ranging from near normal kidneys to renal agenesis. When Fgf9 and Fgf20 are at limiting doses, the balance between differentiation and self-renewal is disturbed, Six2 expression is reduced, and precocious CMM differentiation can occur suggesting a role for FGF signaling in the maintenance of nuclear factors necessary for stemness (Osafune et al., 2006; Sakaki-Yumoto et al., 2006; Self et al., 2006). Consequently, many Fgf9+/−; Fgf20βGal/βGal kidneys contain regions where nephron progenitors were replaced by differentiating epithelia, reminiscent of MM lacking Six2 (Kobayashi et al., 2008; Self et al., 2006). Members of the Fgf9 subfamily are unique in that they can signal to both b and c splice forms of some FGFRs (Figure S3E; (Ornitz et al., 1996; Zhang et al., 2006)). However, autocrine Fgf9 also regulates epithelial growth and branching (del Moral et al., 2006; White, 2006; Yin et al., 2011; 2008). Although genetic deletion confirmed that mesenchymal Fgf9 makes a measurable contribution to the niche, the bulk of Fgf9 signals are provided from the epithelia (UB) to mesenchyme (MM). In contrast, Fgf20 was expressed exclusively in the CMM, signaling in an unusual autocrine fashion to maintain the mesenchymal
cells in which it is expressed. Interestingly, in the developing organ of Corti, Fgf20 provides an autocrine signal acting within the epithelium to regulate its differentiation (Huh et al., 2012).

Independently, other investigators observed that Bmp7, expressed in nephron progenitors, UB and PTA during early kidney development (Dudley et al., 1995), acts to promote survival and proliferation of nephron progenitors (Blank et al., 2009; Dudley et al., 1995; 1999; Dudley and Robertson, 1997; Godin et al., 1998). In Bmp7 null mice, the first round of nephrogenesis is induced, followed later by loss of nephron progenitors, resulting in premature termination of kidney development (Dudley et al., 1995). Moreover, Fgf2, which like Fgf9 is expressed in the UB, can promote survival and maintain competent MM cells in vitro (Barasch et al., 1997; Dudley et al., 1999; Perantoni et al., 1995). However, FGF2 is not necessary in the niche as Fgf2 null mice do not display a kidney phenotype (Zhou et al., 1998). FGF2, FGF8, and FGF10 could not rescue Fgf9/20 null embryos, indicating that their function does not overlap with Fgf9/20 during kidney development. FGF7 and FGF10 signal to the UB and can replace GDNF when the inhibitor Sprouty1 is removed (Michos et al., 2010b). Fgf8 is expressed in the RV and in renal epithelia and is needed for their maintenance (Grieshammer et al., 2005; Perantoni et al., 2005). In vitro analysis confirmed that FGF8 and FGF10 cannot maintain isolated MM, perhaps due to higher affinity of Fgf8 to the decoy receptor Fgfr11, which is expressed in CMM (Brown et al., 2011). Moreover, this is only possible if Fgf9 acted directly on the progenitors and rules out an indirect role via an essential, Fg9-dependent UB or stromal factor. Although restoring the unique cohesion of CMM cells in vitro also requires Bmp7, this cohesion is not needed for retention of competence; Fgf9 alone was sufficient to maintain Six2+ progenitors that are competent to differentiate in response to Wnt. Nonetheless, Bmp7 helps to organize cells into a structure that resembles the CMM niche, perhaps maximizing the benefits of endogenous Fgf20. At present, it
is unclear if conditions that allow robust growth rates are compatible with maintaining competence as highly proliferative Six2+ cells in nephrospheres lost their competence to differentiate (Lusis et al., 2010). It is thought that Wnt9b signals maintain stemness where Six2 is present and drives differentiation where Six2 is absent (Carroll et al., 2005; Karner et al., 2011; Kobayashi et al., 2008; Self et al., 2006). Although it is clear that self-renewing nephron progenitors receive a Wnt9b signal within the CMM (Karner et al., 2011), it is also evident that in the absence of Wnt9b, nephron progenitors survive; they do not undergo apoptosis, and they remain competent to respond to inductive signals as late as E13.5 (Karner et al., 2011). These observations indicate that signals other than Wnt9b maintain competent nephron progenitors in Wnt9b mutant mice, and that in the absence of Wnt9b, proliferation is attenuated and differentiation is lost. Fgf9 and Fgf20 could provide this signal; both are Wnt-responsive genes in some contexts (Chamorro et al., 2004; Karner et al., 2011). The details of the relationships between Wnts and FGFs within the MM remain to be elucidated.

In conclusion, these observations suggest that, at a minimum, Fgf9/20 and Bmp7 organize the nephron progenitor niche and highlight the essential role of FGF20 in human kidney development. Regulating the balance between self-renewal and differentiation is key in determining nephron number, and lower nephron numbers are correlated with higher risk for hypertension, kidney disease, and recovery from injury. Our data indicate that FGF signaling likely regulates multiple important steps in the niche, including survival, proliferation, and competence. The relative contribution of each of these to maintenance of a proper niche is unclear. Because nephron progenitors do not persist in the adult, understanding the mechanisms that regulate this niche is a key step in the development of new therapeutic cell-replacement approaches. The identification of the endogenous niche ligands (Fgf9 and Fgf20) and the
demonstration that they can maintain purified progenitors for 5 days in vitro opens the possibility for long-term maintenance of self-renewing nephron progenitors in culture. The importance of such a culture for research is self-evident; the therapeutic potential of these cells will have to be empirically determined in various injury models.
Material and Methods

Mice

All mice were maintained in the Washington University animal facility according to animal care regulations; Animals Studies Committee of Washington University approved the experimental protocols (protocol 20110027). Lines used here are: Fgf20βGal/βGal (Huh et al., 2012), Fgf9/- (Colvin et al., 2001a), Fgf9ff (Lin et al., 2006), HoxB7-Cre+/tg (Yu et al., 2002), and Six2-GFP::CREtg/+ (Park et al., 2007). Mice and embryos were genotyped using the universal PCR genotyping protocol (Stratman et al., 2003). Primers sequences available upon request.

Organ Culture

Isolation and culture of metanephric kidneys were performed as described (Barak and Boyle, 2011). To manually isolate the MM, E11.5 metanephric kidneys were dissected in cold PBS. Kidneys were treated with 2.25% pancreatin and 0.75% trypsin in Ca-Mg free Tyrode’s solution (pH7.4) (P/T solution) for 30 s, then washed with FBS. MM was dissected from the UB using 26G needles and placed on transwell filters. Explants were cultured at 37°C, 5% CO2 in defined medium (Barak and Boyle, 2011) supplemented with the following as indicated. Heparin (1 mg/ml; Sigma), 8.6 nM FGF9 (PeproTech), 50 ng/ml BMP7 (R&D Systems), and 100 ng/ml FGF2 (PeproTech for positive control, data not shown). FBS was added as carrier in stock solutions (same results were obtained with BSA as carrier). Media was changed daily and activity of FGFs protein was tested by a Baf3 assay as described (Ornitz et al., 1996). To examine competence to respond to inductive signal, dSC was isolated from E11.5–E13.5 embryos. Two fresh dSC segments were placed adjacent to each MM explant. Explants were
then cultured for an additional 2–4 days in unsupplemented media. At least three explants per treatment were prepared for each experiment. Experiments were repeated at least three times on different days. Staining for Ck8 was performed to confirm exclusion of UB remnants from explants.

**Six2+ Cell Sorting**

Kidneys were dissected from E14.5–E17.5 embryos from wild-type females mated with Six2-GFP::CREtg/+ males. Both GFP-positive and -negative kidneys were collected, with negative kidneys serving as sorting controls. To dissociate the cells into single cell suspension, kidneys were treated with pancreatin/trypsin diluted in Tyrode’s solution (Ca+2, Mg+2 free) for 15 min on ice (200 ml per four to eight kidneys), followed by pipetting until clumps were not visible. To stop digestion, 1 ml of cold DMEM with 10% FBS was added to the 200 ml of enzyme solution. Cell suspension was then filtered through a 50 mm filter (CellTrics-Partec disposable filters 04-004-2327), collected in 1.5 ml tubes, and centrifuged for 4 min at 800 rpm. Media was aspirated and cells were resuspended in FACS buffer (3% FCS in PBS). GFP+ cells were sorted using a MoFlo high-speed flow cytometer (Dako Cytomation, Fort Collins, CO). Gating was implemented based on negative control profiles to select for live GFP-positive cells. Sorted cells were collected in 1.5 ml tubes (50,000–100,000 cells per tube) containing kidney media with 5% FBS and kept on ice before plating. Finally, cells were spun down for 4 min at 800 rpm and plated directly on transwell filters in organ culture dishes with kidney media supplemented with heparin containing Fgf9 or Fgf9 and Bmp7. Media was changed every 12 hr.

**Immunohistochemistry**

Immunohistochemistry was performed as detailed in the Supplemental Experimental Procedures.
Statistical Analysis and Imaging

Two-tailed unpaired t tests were performed for statistical analysis. Resulting p values are noted in text. For comparison of kidney sizes, perimeter was measured and used for statistical analysis. Images were acquired with a Zeiss Axioimage Z1 equipped with an ApoTome or with stereomicroscope Leica MZ10F.

Quantification of Nephron Numbers

Kidneys were dissected from P5 pups of Fgf20 WT (+/+), Fgf20 +/βgal (+/-), and Fgf20 βgal/βgal (-/-) mice. To dissociate the tissue, kidneys were chopped into 2 mm2 pieces and digested in 5 ml of 6 N HCL at 37_C for 90 min. Tissue was further dissociated by repeated pipetting. ddH2O (25 ml) was added to each sample, and samples were kept at 4_C. To count the number of nephrons, 100 ml of well-mixed suspension was placed onto a 1 cm 3 1 cm area in a p100 culture dish marked with gridlines. The number of glomeruli was counted under an inverted microscope five times. Total number of glomeruli was estimated as follows: total nephron number per pair of kidneys = average number of glomeruli in 100 ml 3 300.

Patients and Whole Exome Sequencing

The fetus studied by exome sequencing (fetus 2 in Figure 5B) was an 18-week gestation male terminated for recurrence of bilateral renal agenesis with anhydramnios. The couple was consanguineous (first cousins) Caucasian; the mother was a healthy 25-year-old woman with previous history of termination of pregnancy at 26 weeks for anhydramnios. In this family, another consanguineous couple also experienced two terminations of pregnancy for bilateral renal agenesis (fetuses 3 and 4 in Figure 5B). At autopsy, fetus 2 was eutrophic, presented the
Potter sequence including redundant skin, varus feet, and pulmonary hypoplasia. Internal examination showed bilateral renoureteral agenesis (Figure 5A). Other organs were normal. X-ray evaluation showed no anomaly for the gestational age. Neuropathological examination did not detect any abnormality in brain or eyes. DNA was extracted from a frozen liver sample of fetus 2. Genotyping was performed on Illumina Infinium HumanOmni1 beadchip, resulting in the identification of 26 homozygous genomic regions extending from 35 Mb to 700 Kb and spanning the genome (data not shown). Whole exome sequencing was performed using the 50 Mb Agilent SureSelect assay and a SOLiD4 sequencer (50 base fragment reads). The 1.4 Gb of mappable sequence data were aligned to the human genome reference sequence (hg19 build) using BWA aligner (Li and Durbin, 2010). Variants were annotated with in house pipeline based on the Ensembl database (release 61). Known variants from dbSNP132, the 1000 Genomes Project and in-house exome data were excluded from the genetic variants identified in the coding regions or splice sites. Subsequent filtration of the data was performed in order to select variants located in the identified homozygous genomic regions, in genes expressed in the early stages of kidney development (http://www.gudmap.org/) and with a predicted deleterious effect on the protein (Polyphen, Sift). Mutations identified by exome sequencing were validated by Sanger sequencing in all available DNA samples of the family: fetus 2, his parents, a healthy sister, and fetuses 3 and 4. The study was approved by the Comite´ de Protection des Personnes pour la Recherche Biome´dicale Ile de France 2 and informed consent was obtained from the parents.
Figure legends

Figure 1. Fgf20 and Fgf9 Are Required for Kidney Development (A–C) Normal urogenital system formed in control (A), Fgf20\(\beta\text{Gal}/\beta\text{Gal}\) (B), and Fgf9\(+/−\); Fgf20\(+/\beta\text{Gal}\) (C) embryos. Note slightly smaller kidneys in (B) but not in (C). (D–D00) Fgf9\(+/−\); Fgf20\(\beta\text{Gal}/\beta\text{Gal}\) embryos developed mild (D0) to severe (D00) reduction in kidney size. (E) When both alleles were deleted the kidneys were absent (yellow arrowhead) and adrenal glands were smaller (white arrowhead). (F–G0) H&E staining of kidneys from control (F) and Fgf20\(\beta\text{Gal}/\beta\text{Gal}\) (G) mice shows bilateral reduction in kidney size of mutant compared to control. Nephrogenic zone thickness (cyan line) is indistinguishable between Fgf20\(\beta\text{Gal}/\beta\text{Gal}\) and control kidneys. High power image of boxed area in (G) shows normal intermediate steps in nephron development (SSB, arrow; early glomeruli, arrowhead in G0). (H) Kidney section of Fgf9\(+/−\); Fgf20\(\beta\text{Gal}/\beta\text{Gal}\) embryo showed regions where the nephrogenic zone was depleted (arrowheads). (H0 and I) High power image of boxed area in (H). Glomeruli were counted from four control, five Fgf20\(+/\beta\text{Gal}\), and five Fgf20\(\beta\text{Gal}/\beta\text{Gal}\) kidneys. Average count (in thousands with SD) is shown in (I). *\(p = 8.1775 3 10^{-8}\).

B, bladder; G, gonad; K, kidney. Scale bar represents 500 mm.

Figure 2. Loss of Nephron Progenitors in Fgf9/20 Mutants Sections of E18.5 kidneys stained with Ck8 (purple, UB), WT1 (red; low, CMM; high, podocytes), LTL (blue, proximal tubules), Six2 (green, progenitors) or Pax2 (UB, nephron progenitors and nephron epithelia). (A and D) The nephrogenic zone in controls (green) is located distal to the UB and to glomeruli (red) attached to proximal tubules (blue). (A0 and D0) High power image of control nephrogenic zone showed progenitors (yellow arrowheads) packed in two compact cell layers distal UB tips. (B and B0) The nephrogenic zone near some tips in Fgf20\(\beta\text{Gal}/\beta\text{Gal}\) kidneys contains fewer Six2+ cells relative to controls but is otherwise indistinguishable (high power view in B0). (C–E0) Fgf9\(+/−\);
Fgf20^βGal/βGal^ kidneys contained many cortical areas where the nephrogenic zone was replaced by mature structures (white arrows) (C–E). High power image of nephrogenic zone from Fgf9^+/−; Fgf20^βGal/βGal^ kidneys (C0–E0). The cortex contained some UB tips surrounded by two cell layers of nephron progenitors (yellow arrowheads), other tips with fewer Six2^+^ cells (white arrowheads), and some lacking a nephrogenic zone altogether contained mature nephrons instead (white arrows). Scale bars represent 100 mm.

**Figure 3. Reduced Fgf9/20 Levels Allow Premature Differentiation of Nephron Progenitors in the Niche** (A–F) E11.5 metanephroi analyzed after 48 hr in organ culture. Fgf20^βGal/βGal^ metanephroi (B and C) were smaller than controls (A). Some UB tips in Fgf20^βGal/βGal^ metanephroi (B–C) or Fgf9^+/−; Fgf20^βGal/βGal^ metanephroi (D–F) are surrounded by fewer Six2^+^ cells (yellow arrow), whereas others lack Six2 expressing cells (blue arrows). (G–K) Jag1 (epithelial RVs and SSBs), Ck8 (UB), and Six2 (nephron progenitors) demonstrate premature differentiation in the niche in metanephroi distal to some UB tips (white arrows). (L) In situ hybridization of Wnt4 (blue) and antibody staining to Ck8 (brown) shows ectopic Wnt4 expression distal to UB tips in Fgf9^+/−; Fgf20^βGal/βGal^ metanephroi (white arrows). (M) Quantification of tip subtypes (marked with yellow or blue arrows) in metanephroi of various genotypes (Table S1). Normal progenitor numbers in dark green reduced progenitors numbers in light green; no progenitors in pink. Scale bars represent 100 mm.

**Figure 4. Fgf20 Expressed in Nephron Progenitors and Fgf9 Secreted from the UB Collaborate to Maintain the Niche** (A–C) Whole-mount x-gal staining at E10.5-12.5 Fgf20^+/βGal^ embryos detects βGal activity (blue) in the mesonephros (black arrowheads) and metanephric kidneys (red arrowheads). (D) X-gal staining identified activity in the MM of E14.5 Fgf20^+/βGal^ kidneys (black dashed line-UB). (E) Ck8, NCAM (epithelia), and βGal (Fgf20
expression domain) staining of E16.5 Fgf20βGal/βGal kidneys. βGal protein was detected in the CMM (white arrows). The weak signal in the nascent RV (yellow arrowheads) may be due to expression or perdurance. (F) Six2 identified nephron progenitors that contained βGal protein. (G–J) Urogenital system in mice lacking Fgf9 in their UB. Control (HoxB7-Cre+/tg; Fgf20+/βGal) and HoxB7-Cre+/tg;Fgf9f/f; Fgf20+/βGal kidneys appeared normal (G and H). In contrast, HoxB7-Cre+/tg;Fgf9+/f; Fgf20βGal/βGal kidneys are smaller (I). Fully penetrant kidney agenesis (yellow arrowhead) seen in HoxB7-cre;Fgf9f/f; Fgf20βGal/βGal embryos (J). (K and L0) Ck8 (magenta), WT1 (red), LTL (blue), and Six2 (green) staining of HoxB7-Cre; Fgf9f/f; Fgf20βGal/βGal (control, K) or HoxB7-Cre; Fgf9+/f; Fgf20βGal/βGal (L) kidneys. Note large cortical areas where the nephrogenic zone was replaced by mature structures (white arrows, L). High power image of nephrogenic zone of control HoxB7-Cre; Fgf9f/f; Fgf20+/βGal kidney (K0, yellow arrowheads) or HoxB7-Cre; Fgf9+/f; Fgf20βGal/βGal kidney (L0). Note regions lacking Six2 with mature structures (podocytes: red; proximal tubules: blue) located instead at edge of kidney (white arrows). B, bladder; G, gonad; K, kidney. Scale bars represent 50 mm (E and F), 500 mm (G–J), and 100 mm (K–L0). See also Figure S1.

**Figure 5. Identification of a Homozygous Frameshift Mutation in FGF20 that Segregates with Bilateral Renal Agenesis** (A) Bilateral renoureteral agenesis in the 18-week fetus; (a) adrenal glands with flat oval shape due to kidney agenesis. (B) Pedigree of the consanguineous family showing two first cousin couples and four fetuses presenting with anhydramnios/bilateral renal agenesis; segregation of the FGF20 c.337delG mutation is indicated. (C) Nucleotide sequence traces and deduced amino acid sequence within exon 2 shown for wild-type (top) and mutated (bottom) alleles. Deleted guanine at position 337 of the coding sequence is marked with asterisk in the wild-type sequence. The frameshift introduced missense amino acids from
position 113 to 120 (in bold) and created a stop codon at position 121 (numbering as in NCBI sequence AB030648). (D) Wild-type and deduced FGF20 proteins: heparin-binding domains indicated by light gray boxes; position of mutation is marked with red asterisk; the 8 missense amino acids are in black.

**Figure 6. Fgf9/20-Deficient Kidneys Initiate UB Outgrowth But Branching Fails Due to High Rates of Apoptosis in the MM** E11.5 whole-mount Ck8 staining in controls (A) illustrated a branched UB (white arrowhead). In contrast, the UB in Fgf9-/-; Fgf20βGal/βGal E11.5 embryos (B) elongated (arrow) but failed to branch (yellow arrowhead). Sections of E11.5 kidneys stained with Pax2 (UB and MM) showed MM condensing around the UB tips (Ck8+) in controls (C) but not in Fgf9-/-; Fgf20βGal/βGal embryos (D). The mutant MM appeared diffuse and formed a smaller contact zone with the UB. MM in control is Pax2-positive and TUNEL-negative (E); the dying MM cells in Fgf9-/-; Fgf20βGal/βGal embryos are TUNEL-positive (white arrowheads in F). At E12.5, Ck8 and Pax2 outline a rudimentary MM remaining in Fgf9-/-; Fgf20βGal/βGal embryos (H) compared to controls (G) (yellow arrowheads in G–H). See also Figure S2.

**Figure 7. Fgf9 Promotes Survival and Maintains Competence of Nephron Progenitors In Vitro** Isolated E11.5 MM cultured in defined media (M) (A), in heparin-supplemented media (M+H) (B), or in heparin and Fgf9 media (M+H+Fgf9) (C and E–K). Explants were fixed and stained as indicated. After 4 days in media, the majority of MM cells were TUNEL-positive (A). When supplemented with heparin, cell survival improved, some entering mitosis (B). In the presence of Fgf9, survival and proliferation were further increased (C). Isolated E11.5 MM was grown in culture for 2 days with M+H (D) or M+H+Fgf9 (E-I). Six2+ cells were only detected when Fgf9 was present (E and E0). A few progenitors (left-pointing arrowheads) positive for
Six2 (F), pH3 (G), and DAPI (H) underwent mitosis (merged image; I). Nephron progenitors maintained in the presence of Fgf9 for 2 days are competent to respond to Wnt produced by dSC by forming epithelial aggregates expressing both distal (Cdh1+) and proximal (Jag1+) markers (J and J0, higher magnification; see text for detail). Note undifferentiated, Six2+ cells in (K). Colonies of Six2+ cells isolated by FACS were maintained for 2 days in culture in M+H+Fgf9 (L). Scale bars represent 100 mm. See also Figure S3.

**Figure 8. Fgf9 Synergized with Bmp7 to Promote Self-Renewal of Competent Nephron Progenitors In Vitro** Isolated E11.5 MM was grown for 48 hr in media supplemented with Bmp7 (A) or heparin with Fgf9 and Bmp7 (M+H+Fgf9+Bmp7) (B–J). Six2+ cells were not detected in explants grown with Bmp7 (A). In explants cultured in M+H+Fgf9+Bmp7, many Six2+ cells are detected (B and B0). Note that Six2+ cells sorted into a tight cluster with a clear boundary separating them from Six2− cells (B). Adding dSC to cells pretreated by M+H+Fgf9+Bmp7 induced robust differentiation (C). Epithelial aggregates (Cdh1+, Jag+) are surrounded by Six2+ cells (D). Isolated E11.5 MM cultured in M+H+Fgf9+Bmp7 media and EdU (5-ethynyl-20-deoxyuridine) for 4–7 hr, washed, and cultured in M+H+Fgf9+Bmp7 for an additional 44 hr. Explants were stained with EdU detection kit (E and F), pHH3 (E and G), and Six2 (E and H) Many Six2+ cells remained EdU bright (E–H, yellow arrows), suggesting that they did not divide again within 48 hr. A few nephron progenitors Six2+, EDUdim (cells that divided once after labeling), and pHH3+ cells were able to divide at least once within 2 days of culture. Isolated E11.5 MM was grown for 5 days in M+H+Fgf9+Bmp7. Many Six2+ nephron progenitors survived (I, a close-up in I0). Adding dSC after 5 days induced robust epithelial differentiation of either distal (Cdh1+) or proximal (Cdh6+) character (J). Six2-GFP expressing cells were sorted, cultured with M+H+Fgf9+Bmp7 and stained for Six2 after 48 hr (K, close-up
in K0). Adding dSC to these cells for an additional 4-day period induced differentiation of distal (Cdh1+) or proximal (Cdh6+) epithelia (L). Scale bars represent 100 mm. See also Figure S4.

**Figure S3 (related to Figure 7): FGF9 and FGF20 are sufficient to promote survival of nephron progenitors.** Isolated MM from E11.5 kidneys was supplemented with media plus Heparin with either FGF9, FGF20, FGF8 or FGF10 (all expressed in vivo in or near the progenitor population). The absence of UB was confirmed by Ck8 staining (not shown). Survival of nephron progenitors and Six2 expression in culture media was assessed 48hrs after MM isolation. Based on our measurements of Fgf half-life in these experiments (E), media was changed every 12hrs. Six2+ cells were observed in explants cultured in media containing Heparin/FGF9 (in 7/9) or FGF20 (9/10) (A, A’, B, B’). Very few (0-10 cells/explants) Six2 expressing cells were identified in media containing Heparin/FGF8 (in 7/7) (C, C’). No Six2 positive cells were detected in explants cultured with FGF10 (8/8) containing media (D’). Scale bar: 100µm. (E) Comparison of FGF9, 20, 8 and 10 activity using a BaF3 cell mitogenic assay for Fgfr2b (grey bars) and Fgfr2c (black bars). Isolated MM was cultured in the presence of Heparin (1µg/ml) and one of the following FGFs: FGF9 (20ng/ml), FGF20 (40ng/ml), FGF8 (20ng/ml) and FGF10 (20ng/ml). Conditioned media were collected at time 0hr and 12hr and added to BaF3 cells expressing either Fgfr2b or Fgfr2c. Mitogenic activity was then measured in duplicate by the level of [3H] thymidine incorporation as previously described (Ornitz et al., 1996). The percentage of activity was derived by dividing the measured activity by the maximum level of activity based on a standard curve of each Fgf ligand (not shown). Note that after 12hrs of incubation each ligand remained active in the culture media. Error bars represent S.D.

**Figure S4 (related to Figure 8): FGF9 synergized with BMP7 to promote self-renewal of**
competent nephron progenitors in vitro. Isolated E11.5 MM was grown for 5 days in media supplemented with Heparin, FGF9 and BMP7 (M+H+FGF9+BMP7). A-C) Explants were fixed and stained with DAPI (blue), phspho-Histon3 (pHH3; red) and Six2 (green). High numbers of nephron progenitors survived, some entering mitosis (yellow arrows). D-E). Adding dSC to cells pretreated by M+H+FGF9+BMP7 induced differentiation and resulted in epithelial aggregates expressing Cdh1 surrounded by Six2+ cells. E-F) Six2+ sorted cells cultured with M+H+FGF9+BMP7 survived fir 5 days of culture, some entering mitosis (yellow arrows). Scale bars: 100µm.
Figure 1. Fgf20 and Fgf9 Are Required for Kidney Development.
Figure 2. Loss of Nephron Progenitors in Fgf9/20 Mutants.
Figure 3. Reduced Fgf9/20 Levels Allow Premature Differentiation of Nephron Progenitors in the Niche.
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Figure 7. Fgf9 Promotes Survival and Maintains Competence of Nephron Progenitors In Vitro.
Figure 8. Fgf9 Synergized with Bmp7 to Promote Self-Renewal of Competent Nephron Progenitors In Vitro.
Figure S3. (related to Figure 7): FGF9 and FGF20 are sufficient to promote survival of MM
Figure S4. (related to Figure 8): FGF9 synergized with BMP7 to promote self-renewal of competent nephron progenitors in vitro.

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CHAPTER III

The role of cell intrinsic changes in regulating nephron progenitor lifespan
The content of this chapter have been modified from a manuscript in preparation. Additional authors will include Eric Brunskill who conducted the analysis of single-cell RNA sequencing data (Figure 9, 10, 11 and S5), Phillip Dexheimer, Nathan Salomonis and Bruce Aronow, who provided bioinformatics support, and Steve Potter from whom we obtained E12.5 single cell data. Otherwise, the data presented in all figures represent autonomous work.
Summary

Nephrons, the basic functional units of the kidney, are built from an embryonic progenitor population called the cap mesenchyme (CM). Although stem-like in many aspects, these progenitors exhaust before or shortly after birth when nephron numbers reach a species appropriate limit termed “nephron endowment” (Brunskill et al., 2011; Rumballe et al., 2011). The actual number of nephrons varies greatly between individuals, and low nephron numbers have been linked to hypertension and other renal diseases (Bertram et al., 2011; Keller et al., 2003; Vehaskari et al., 2001). However, the mechanisms that lead to the depletion of CM cells remain elusive.

We hypothesized that cell intrinsic changes of the CM actively regulate the lifespan of CM cells. To test this hypothesis, we established a new assay system where the ability of CM to remain as nephron progenitors could be evaluated upon transplantation into an endogenous CM niche. By co-transplanting nephron progenitors isolated from different embryonic stages, we discovered that young and old progenitors display differential proliferation rate, adhesion properties and their ability to remain in the niche is inversely correlated with age. Importantly, an unbiased transcriptome profiling of nephron progenitors at the single cell level revealed distinct transcriptional signature of different age groups, further supporting an intrinsic difference. Interestingly, although most old progenitors exit the niche sooner than young progenitors, a few of them could remain in the niche beyond their endogenous lifespan when completely surrounded by young neighbors, indicating that age-related changes are possibly reversible.

Together, these data is consistent with a gradual intrinsic change in the CM preceding the cessation of nephrogenesis. Manipulating age-related changes may open the door to increasing nephron endowment in at-risk individuals.
Introduction

The mammalian kidney is an essential excretory organ responsible for filtering metabolic wastes from the blood, maintaining pH/solute balance, regulating blood pressure and bone densities. All of these complicated functions are accomplished by the nephrons, which are the basic functional units of the kidney. Despite its critical roles, the adult kidney does not have the capacity to generate new nephrons (neo-nephrogenesis) and instead relies on a large pool of nephrons generated during embryonic development (Humphreys et al., 2008; Rinkevich et al., 2014). Nephron loss is a normal process of aging but could be accelerated by disease such as diabetes, leading to end-stage renal disease (ESRD) (Bertram et al., 2011; Hoy et al., 2006; Keller et al., 2003; Schreuder, 2012). Therefore, a robust nephron endowment is critical for long-term renal function.

During development, nephrons are generated through the reciprocal interaction between the ureteric bud (UB) and metanephric mesenchyme (MM), both derived from the intermediate mesoderm (Frank Costantini, 2010; Hendry et al., 2011). The MM promotes growth and repetitive branching of the UB, leading to the formation of the collecting duct of the kidney. Simultaneously, UB signals back to the MM and orchestrate nephron formation during each round of branching. Cell lineage analysis revealed that the cap mesenchyme (CM), a subpopulation of the MM, represents self-renewing, multipotent nephron progenitors (Boyle et al., 2008; Kobayashi et al., 2008; Mugford et al., 2009). The mouse CM is formed around midgestation (E11.5, embryonic day 11.5) when the MM segregates into a group of highly condensed mesenchyme “capping” the UB tip (CM) and more loosely associated stromal mesenchyme (SM). Characterized by the expression of transcription factors such as Six2 and Cited1 (Boyle et al., 2007; Self et al., 2006), the CM is situated in a special niche environment
under the influence of signals from the UB, SM as well as themselves. Upon induction by Wnt9b expressed from the UB, CM cells first aggregate at the lateral side of the UB forming the pretubular aggregate (PTA). This is a transition state where progenitors specified to differentiate (Wnt4+) retains the expression of CM marker Six2. PTA then undergoes mesenchymal to epithelial transition (MET) to form renal vesicles (RV) that will develop into nephrons. Each nephron is patterned along the proximal distal axis with a glomerulus at the most proximal end followed by proximal tubules, loop of Henle and distal tubules, which connect back to the UB. At the same time, niche growth factors such FGF9/20, BMP7 and Wnt9b allows the CM to self-renew and proliferate, ensuring repetitive rounds of nephrogenesis (Barak et al., 2012; Carroll et al., 2005; Courtney M Karner, 2011; Das et al., 2013). Although stem-like in many aspects, CM differentiate en masse shortly after birth and are lost permanently thereafter (Brunskill et al., 2011; Hartman et al., 2007; Rumballe et al., 2011). However, the mechanistic basis for their depletion remains unclear.

The prevailing view is that nephron progenitors are passively regulated by cell extrinsic signals coming from the surrounding niche environment, including the UB and stroma, which dictates the timing of their final commitment. This extrinsic trigger could simply arise due to alterations in growth factor concentrations as a result of changes in niche topology over time (Costantini, 2010; Hartman et al., 2007; Rumballe et al., 2011; Short et al., 2014) or involve additional physiological input such as birth (Brunskill et al., 2011). However, recent studies showed that the CM is an integral part of its own niche, secreting at least one factor (FGF20) to maintain CM cells in a progenitor state (Barak et al., 2012). Thus, an equally possible mechanism exists where CM intrinsic changes actively regulate its choice of self-renewal vs. differentiation and determine when nephrogenesis end. Stated otherwise, the key question is
whether the CM represents true stem cells that are forced to differentiate by the end of nephrogenesis or resembles a transient amplifying population with limited self-renewal capacity that are eventually exhausted. If CM were forced to differentiate, then delaying the extrinsic trigger may have a positive impact on nephron numbers. In contrast, if CM lifespan is an intrinsic property, we will need to identify what are these intrinsic changes whether they could be reversed. Determining which of these mechanisms is at play has important implications for our goal of increasing nephron endowment in at risk individuals.

We hypothesized that cell intrinsic changes of the CM regulate the lifespan of CM cells. However, there is no readily available method to test this hypothesis. The renal field lags behind other stem cell fields in the lack of definitive progenitor assays (Hendry et al., 2011; Little and McMahon, 2012). Moreover, an intrinsic change in the progenitor cells could not be easily revealed with traditional genetic methods without altering the overall niche environment (Barak et al., 2012). Competitive repopulation assays proved extremely powerful in dissecting the stem cell hierarchy in hematopoiesis (Morrison and Weissman, 1994) permitting studies of the role played by various genes in self renewal and differentiation (Maillard et al., 2008; Scott et al., 1997). Assays alike have been used to tease out the relative contribution of intrinsic and extrinsic cues in regulating progenitors cells in solid organs as well (Barbe and Levitt, 1991; 1995; O'Leary and Stanfield, 1989; Schlaggar and O'Leary, 1991).

In this study, we established new kidney progenitor assay on the same principle, where the ability of CM to remain as nephron progenitors could be evaluated upon transplantation into an endogenous CM niche. Using this tool, we performed heterochronic transplantation of nephron progenitors isolated from kidneys of different ages and discovered that their ability to remain in the niche is inversely correlated with age, consistent with a gradual intrinsic change
preceding the cessation of nephrogenesis. Analysis of the cellular basis for this intrinsic difference revealed a change in their cell proliferation and adhesion properties, where young progenitors are more proliferative and motile while old progenitors prefer to aggregate and exit the niche. This functional readout was further supported by an unbiased approach where single-cell RNA sequencing revealed distinct transcriptional profiles of nephron progenitors at different developmental stages. Interestingly, these intrinsic changes in old cells are likely reversible when completely immersed in a young environment.

Together, these data suggest that CM intrinsic, age-dependent changes precede the cessation of nephrogenesis and actively regulate CM lifespan. Dissection of the underlying basis of these intrinsic differences will and have important implications for understanding the regulation of nephron endowment in vivo.
Results

FACS Sorted Nephron Progenitors Engraft Into The CM Niche

To ask if nephron progenitors can survive transplantation into a host niche, we FACS purified nephron progenitors from Six2\textsuperscript{TGC}+/tg, Rosa +/tdTomato mice and transplanted them into a host metanephroi cultured in vitro. All nephron progenitors are GFP positive and their fate could be followed by the lineage label tdTomato. To obtain highly concentrated cell suspension with limited number of sorted progenitors (~10\textsuperscript{5} cells per experiment), the initial suspension were concentrated through multiple steps (see material and methods) to achieve a final density of ~1.5*10\textsuperscript{5} cells/µl. Using a manual microinjector (Narishige IM-6), these cells were transplanted into the CM of an E12.5 Pax2\textsuperscript{Cre}+/tg; Rosa +/eYFP (hence Pax2-eYFP) recipient kidney explant under a fluorescent stereoscope (Figure 1A). Recipient of this age was chosen as its CM contained more nephron progenitors per niche compared to older kidneys (~2000 cells vs. 100 cells (Short et al., 2014)) and retained its integrity better after isolation and injection than younger (E11.5) kidneys (data not shown). Kidneys imaged right after the injection showed that each niche contained around 50-100 injected cells (Figure 4B and data not shown).

The explant was cultured for another 4 days post injection and immunostaining was performed to assess self-renewal (retention in the CM) vs. differentiation (formation of nephron epithelia) of injected progenitors and their decedents (Figures 1A-C). By acquiring Z-stack images on a confocal microscope, we obtained 3D images of each injection site where cell numbers could be quantified. The analysis clearly demonstrated that transplanted nephron progenitors were able to engraft into the CM niche (Six2+) (Figures 1B and 1C, arrow), proliferate (EdU+) (Figure 1D) and differentiate into nephron epithelia (Figures 1B and 1C, arrowhead). Extending the culture to 7-days allowed further maturation of the nephron epithelial,
where injected cells could be found in all segments of a nephron including podocytes precursors (Wilm’s Tumor 1-WT1), proximal tubules (Lotus tetraglobus lectin-LTL), loop of Henle (Tamm-Horsfall Protein, THP), distal tubules (Ecadherin- ECadh) (Figures 2A-2C). No contributions were made to the UB (Cytokeratine8- Cyto8) (Figure 2C). This distribution indicated that the differentiation capacity of progenitors was unaffected by the isolation and injection protocol.

Combined, these data demonstrated that we have established a novel assay to evaluate the engraftment of FACS purified nephron progenitors in an intact CM niche.

**Heterochronic Transplantation Indicates That Young Six2+ Progenitor Engraft Better Than Old Ones**

We then asked if we could detect age-related intrinsic changes of nephron progenitors using this system. Instead of injecting young or old Six2+ cells into different niches, we FACS purified E12.5 $Six2^{TGC+/tg}$; Rosa $+/tdTomato$ (red, young) and P0 $Six2^{TGC+/tg}$; CAG-$eCFP$ $+/tg$ (blue, old) nephron progenitors (Figure S1) and injected their 1:1 mixture into the CM of an E12.5 $Pax2-eYFP$ recipient kidney explant. The advantage of a co-injection experiment is that it allows us to compare the ratio of young and old cells remaining in the same niche, thus excluding confounding variations in the location and number of cells injected. The “intrinsic change” hypothesis predicts that all old progenitors will exit the CM by the end of 4 days (corresponding to endogenous P4) whereas young progenitors will both engraft and send their descendants to differentiate. By contrast, if old and young Six2+ progenitors were intrinsically the same, all cells introduced into a young niche would follow similar engraftment/exit kinetics regardless of age (Figure 3A).
4 days post injection, the metanephroi were fixed, stained and processed for imaging as described in the methods. We quantified the number of Six2+ red and blue cells in Z-stacks acquired by confocal imaging using Imaris™ software (Figures 3B’ and 3C’). The analysis identified a significant difference in the behavior of E12.5 and P0 Six2+ progenitors: in 37 independent niches injected on 4 different days, on average, 5.7-fold more young cells remained in the niche after 4 days in culture relative to the old cells (mean 85%±7% vs. 15%±7%; p<10^{-23}, Figure 1D). Both young and old progenitors produced descendants that differentiated into nephron epithelia (Figure 3B’ and 3C’). Importantly, the results were the same when the lineage labels (tdTomato and CFP) were switched between young and old cells (compare Figure 3B and B’ and 3C and C’). The fact that lineage label has no effect on engraftment was further confirmed by co-injection of progenitors of the same age (young and young or old and old), where cells of different colors behave the same. The only difference is that most of the old cells had exit the niche by the end of 4 days (Figure S2).

Together, heterochronic transplantation uncovered an engrafting advantage of young progenitors, with more young cells remaining in the CM niche than old cells. However, this age-dependent difference may simply reflect the inherent heterogeneity within the Six2+ population: while Six2 marks CM progenitor, its expression persists in the PTA, marking CM cells that are induced to differentiate (Figure S3). At P0, perhaps a higher percentage of Six2+ cells were induced, explaining the reduced engraftment in the niche.

**Cited1+ Nephron Progenitors Display Age-Related Decrease In CM Engraftment**

Previous studies showed that Cited1 expression is confined to the more distal region of the cap and some investigators proposed that this population is refractory to Wnt9b induced
differentiation (Brown et al., 2013; Mugford et al., 2009). Indeed, Cited1 never overlaps with Wnt4 expression, a marker for commitment to MET (Hendry et al., 2011; Mugford et al., 2009; Rumballe et al., 2011). To exclude the possibility that the aging of nephron progenitors we observed was due to an altered ratio of progenitors/committed cells in the Six2+ population, we analyzed the Cited1+ progenitors. We confirmed that GFP expression in the Cited1CreERT2-GFP+/tg mouse overlapped perfectly with staining of the Cited1 protein [Figures 3S and 4A; (Boyle et al., 2008)], labeling a more distal subpopulation of Six2+ cells. We sorted the cells based on high GFP expression to avoid low levels of GFP that may persist in committed, Cited1- cells (Figure S1). Indeed, co-injection of E14.5 Cited1CreERT2-GFP+/tg; Rosa+/tdTomato and E14.5 Six2TGC+/tg; CAG-eCFP+/tg progenitors revealed a higher engraftment of the red Cited1+ cells in the CM (mean 69%±8% vs. 31%±8%; n=28, p<10⁻²⁵).

Sorting for Cited1+ cells led to the enrichment of a population with higher engraftment rate relative to the Six2 cells. On the assumption that these are purified, uninduced nephron progenitors, we asked how Cited1+ cells isolated from different stages of embryonic development behave relative to E12.5 Six2+ cells. If Cited1 cells were refractory to induction until forced to differentiate at P2, then P0 Cited1+ cells would be expected to engraft better than E12.5 Six2+ population that contain a fraction of cells already committed to differentiation. Conversely, if Cited1+ cell age, they will progressively lose their ability to engraft. We co-injected Cited1CreERT2-GFP+/tg; Rosa+/tdTomato cells of three different ages (E14.5 or E18.5 or P0) with E12.5 Six2TGC+/tg; CAG-eCFP+/tg into a young niche. Each experiment contains at least 25 data points from two independent experiments with reversed labeling. The results were unequivocal, showing a continuous decline in the percentage of Cited1+ cells engrafting in the CM relative to the young, E12.5 Six2+ cells. At E14.5, 65% of engrafted cells came from the
Cited1+ population (Figure 4B; n=24, $p<10^{-22}$); at 18.5, this percent declined to around half of the engrafted (45%, $p<10^{-7}$ n=40) and at P0, only 27% of engrafted cells were from the Cited1+ population ($p<10^{-34}$; n=37). Thus, old Cited1+ cells behaved similarly to old Six2+ cells, with 60% less number of cells remaining in the niche compared to the young Six2 population.

These results determined that age-dependent changes precede the cessation of nephrogenesis, and these changes occur within the “uninduced”, Cited1+ population.

**Young And Old Nephron Progenitors Differ In Their Proliferation Rate And Adhesion Properties**

We then asked what is the cellular basis for the intrinsic difference between the young and old nephron progenitors. In principle, the number of progenitors remaining in the niche depends on the initial number of cells injected, their rate of proliferation and apoptosis and the fraction committed to differentiation. Since the initial numbers are identical, we examined the percentage of cells undergoing apoptosis. We found very low level of apoptosis in both young and old injected cells (Figures 5A-C), consistent with previous observation that apoptosis usually do not occur in wild type CM (Hartman et al., 2007). Next, we investigated if the engraftment percentage reflected proliferation rates by incubating the explants for 3hr in EdU-containing media prior to fixation at 24 and 48hrs post-injection. Results showed that the percentage of young and old cells that incorporated EdU are not significantly different by the end of day one, with 38% ± 8% and 31% ± 11% being EdU+ respectively. By the end of day two, the proliferation rate of young cells was 1.6 fold higher than that of old cells (46% ± 7% vs. 28% ± 8%, $p<10^{-3}$). This is in agreement with a recent study showing that the proportion of cells that incorporated EdU are lower in older CM in vivo (Short et al., 2014).
To further analyze the distribution of mitotic figures within the injected population, we performed time-lapse imaging with a confocal microscope to follow the injected progenitors over time. Unfortunately, an optimal resolution with high magnification is associated with high photo toxicity. We were only able to capture the injected metanephroi for the first 20hr at 15min interval with medium resolution, which did not allow us to capture individual cell division. However, we were able to follow the pattern of cell movements over time. This revealed that the young progenitors was highly motile while the old population were more inclined to stay with other old cells, exiting the niche as a group (Supplemental movie 1).

We hypothesized that this reflects adhesion differences that promotes old-old but minimizes old-young association, enforcing old cells to exit via community-effect (Gurdon, 1988; Gurdon et al., 1993; Stüttem and Campos-Ortega, 1991). To test if this is the case, we cultured the 1:1 mixture of FACS sorted young and old Cited1+ nephron progenitors in vitro in the presence of heparin, Fgf9 and BMP7 (Barak et al., 2012). After 48hr in culture, the cells formed aggregates with cells retaining the expression of Six2 (Figure 6). When the aggregates contained cells of the same age, the colored cell mixed randomly (Figures 6H-H”). In contrast, when we mixed young and old Cited1+ cells we noticed that they sorted away from each other; young cells mainly stay at the periphery while old cells remained in the center (Figures 3F-F’’’ and 3G-G’’’), consistent with differences in adhesion properties.

Together, young and old progenitors are intrinsically different at the cellular level with distinct proliferation rate and adhesion properties, both of which could potentially affect their decision to stay or exit the niche.

Old Progenitors Can Engraft If They Associate With Young Cells
Although most of the old cells exit the niche by the end of the 4-day culture period, a few of them were able to remain as Six2+ cells in the CM beyond their normal lifespan. The biggest difference here is that old cells could come into contact with young cells, which does not occur in their endogenous environment. Therefore, we hypothesized that the ability of the old progenitors to remain in the CM depends on contact with young cells (niche). If this were the case, then the distribution of old cells remaining in the CM would not be random. Only cells that are alone or in small clusters could make contact with the young niche and stay. In contrast, the majority of the old cells will be surrounded by other old cell due to preferential adhesion (as shown in the previous experiment) and are insulated from the young environment, leading to their early exit via “community effect” (Gurdon, 1988; Gurdon et al., 1993; Stüttem and Campos-Ortega, 1991).

To analyze the neighborhood of old cells remaining within the niche we used Imaris™ software to calculate the distance between CFP or tdTomato-labeled resident progenitors in the CM at the end of 4-day culture. We classified cells into two groups: those within less than one cell diameter (~12μM) from at least one neighbor, and those with no neighbor within 12μM distance (Figure 7). To exclude the confounding effects of cell division in generating clusters, we only counted groups containing 4 or more cells as clusters and all the rest as “single” cells (Figure 7D). The analysis showed that ~50% of young cells were found in clusters in 35 of 38 injected niches (Figure 7G, exception 35-37). Strikingly, 100% of old cells engrafted as in “single” cell in 36 of 38 niches (Figure 7G, exception 11 and 38). This result may simply reflect the reduced numbers of P0 cells remaining in the niche. To address this, we asked what was the distribution only in niches that had similar number of cells (Table 1). The number of old cells in niche #10 (33) (Table 1) was similar to the number of young cells in 13 niches (yellow label); yet
100% of the old cells in niche #10 engrafted as single cells. Similar observation is found with niche #30 (20), where all old cells were found as single cells, while fewer young cells are still capable of remaining in clusters. Together, these data showed that most of the old progenitors remaining in the CM are found as single cells or in small clusters and implies that the decision of nephron progenitors to stay or exit the niche is reinforced by cell-cell contacts.

Next, we asked if the long-lived old cells are simply staying in the CM as quiescent cells, or are actively participating in nephrogenesis, with the latter indicating possible “rejuvenation” by the young niche. We examined metanephroi co-injected with young and old cells and cultured for 7 days. The fact that we could detect old cells and their decedents in the CM (Figure S4, red cells indicated by arrowheads), a newly formed S-Shaped body (Figure S4, white arrow) and a more mature nephron epithelia towards the medullar region (Figure S4, yellow arrow, stained with mature proximal tubule marker LTL) indicated that these cells are actively proliferating and producing differentiated descendent during the entire culture period.

Finally, we tested if Fgf20 is required for old cells to stay in the niche by co-injecting wild type young and old nephron progenitors into Fgf20 null recipients. We found that a mutant niche environment in the recipient did not abrogate the ability of old progenitors to stay. However, all of the ones that did stay were in contact with the co-injected wild type young progenitors (Figure 7H), again supporting the importance of a young progenitor in maintaining old progenitor in the CM. Nevertheless, this experiment does not distinguish whether it is FGF20 or some other factors secreted by the young cells that are important for retention. A definitive test would require the co-injection of wild type old cells and Fgf20 null young cells into an Fgf20 null niche.
Combined, these data suggest the niche environment created by the CM themselves play an important role in affecting their decision to stay or exit the niche. The intrinsic changes associated with old progenitors are likely reversible when surrounded by a young niche.

**Single cell RNA sequencing revealed distinct pattern of gene expression profile in nephron progenitors of different ages**

Our data suggests that there are cell intrinsic changes within nephron progenitors that control the ability of progenitors to self-renew and differentiate during the course of embryonic development. However, the molecular basis for this functional difference is unclear. We hypothesized that one potential difference for the age-related differences is manifested at the transcriptional level.

To test this hypothesis, we performed an unbiased transcriptional profiling on nephron progenitors isolated at different embryonic stages using single-cell RNA sequencing. We chose this method as it has the potential to reveal biologically meaningful heterogeneity that could be easily masked on a population level. For example, a profile of 18 single dendritic cells revealed that genes important for immune regulation were expressed either at high level or not at all, a feature never observed on the whole population (Shalek et al., 2013). Furthermore, single-cell RNA-SEQ was used to study the developing embryos, where sampling of 33 single cells allowed the identification of transcripts specific to multiple developmental stages and revealed timing differences in gene expression between early human and mouse embryos (Xue et al., 2013). The power of this analysis is especially valuable to us, as we are more likely to find subtle changes within the same cell type over time but not dramatic differences in transcriptional profiles between different cell types.
To perform a single-cell RNA-Seq analysis, we FACS purified Cited1+ nephron progenitors from E14.5, E18.5 and P0 kidneys that expressed single or double reporter genes (GFP+ or GFP+tdTomato+ or GFP+CFP+) respectively, where each reporter combination serves as a barcode for age. A cell suspension containing equal numbers of the three cell populations were captured onto a 10uM-17uM Fluidigm C1 Chip using the single-cell AutoPrep system. We visually inspected the Fluidigm chip using a fluorescent microscope to confirm the presence of a single cell in each well. The Fluidigm AutoPrep system then generated cDNA libraries using Clontech UltraLow Smarter Amplification chemistry and the cDNA was converted into sequencing libraries using the Illumina NexteraXT DNA Sample Preparation kit. A total of 91 single cells were processed by the Fluidigm C1 and paired-end sequencing was carried out with Hiseq2500 to an average depth of ~4 million reads per cell. The sequencing data was processed using Illumina sequence analysis software and the CCHMC barcode deconvolution pipeline where it was mapped to the mouse genome (MM10) to generate gene counts and splice form detection with summarized RPKM (reads per kilobase of gene locus summarized mRNA per million reads). In addition, each cell was mapped to unique sequences found in each of the reporter genes to determine cell age.

We first analyzed the RNA-SEQ data using Genespring software. RNA-SEQ data generated from the CCHMC pipeline (Bam files) from each cell were loaded into Genespring and the data was normalized using DeSeq normalization algorithm. After normalization, we confirmed that the sequencing data of all 91 cells passed quality control using PCA (principle component analysis) (Figure S5). The RNA-Seq data was filtered (>10RPKM) to exclude transcripts with extremely low reads, resulting in a list of 10784 genes that are expressed across the entire population. Unsupervised hierarchical clustering identified at least 3 distinct gene
expression patterns among all cells, with each group containing some numbers of E14.5, E18.5 and P0 cells (Figure 8). This cursory analysis indicated that the Cited1 population displays a certain degree of heterogeneity at all ages examined, with some of the heterogeneity introduced by technical noise. To further analyze the single-cell data, we next performed more stringent analysis of the data by performing a one-way Anova (p>0.05) without multiple testing correction, which yielded a list of 1261 differentially expressed genes. A heat map generated from the 1261 differentially expressed genes identified distinct expression signatures for each age group (Figure 9). A GO term analysis of these 1261 genes using TopGene showed enrichment of gene/pathways regulating RNA binding, ribosomal components, cell cycle, chromatin organization, microtubule cytoskeleton and cell division, consistent with a change in cell status.

This age-dependent expression pattern was further confirmed in the 91-cell pool using the Monocole analysis tool kit. Monocole is specifically designed for differential expression analysis for single cell RNA-Seq, where it learns the process of differentiation and places cells in order according to their progress through it (pseudo-time analysis). Monocle identified 5 distinct groups of cells based on their expression profile. Moreover, the progression from group 1 to 5 matches well with the progression of age (Figure 10).

As the sequencing data became available to us only recently, we are still in progress of performing more in depth analysis. However, our preliminary results are consistent with our results that nephron progenitors of different ages are intrinsically different at the transcriptional level, providing an independent confirmation of our functional read out.
Discussion

Our understanding of the molecular and cellular mechanisms that control nephrogenesis is growing rapidly in recent years (Frank Costantini, 2010; Hendry et al., 2011; Kopan et al., 2014a; Little and McMahon, 2012). However, the mechanisms that terminate this process at the end of development remain elusive. It is unclear how stem-like cap mesenchyme cells capable of self-renew and differentiation throughout development loses this balance and becomes exhausted shortly after birth. This is a particularly important issue as the lifespan of these nephron progenitors affect final nephron numbers, which is linked to long-term renal function (Hoy et al., 2006; Keller et al., 2003; Luyckx and Brenner, 2005; Schreuder, 2012).

In this study, we asked if cell intrinsic changes play a role in regulating CM lifespan. We first established a new assay system where the ability of CM to remain as nephron progenitors could be evaluated upon transplantation into an endogenous CM niche. By co-transplanting nephron progenitors isolated from different embryonic stages, we discovered that young and old progenitors display differential proliferation rate, adhesion properties and their ability to remain in the niche is inversely correlated with age. This functional readout is further supported by our results from unbiased transcriptional profiling of nephron progenitors at the single cells level, where cells of each age group are characterized by their unique expression profile. Many of the differentially expressed genes trend with age, showing a gradual increase or decrease over the course of development. Importantly, the genes/pathways that are different between age groups, overlapping with our finding at the cellular level and revealed many other potential regulators including RNA binding proteins, ribosomal components and chromatin modifiers, indicating possible changes in cell translation and epigenetic states. Combined, these data suggest that a gradual intrinsic change occurs in the CM preceding the cessation of
nephrogenesis and these changes could affect the choice of nephron progenitors to stay or exit the niche.

Our results offers important insights to a long-standing question in the kidney field, that is, are nephron progenitors true stem cells, or do the majority of them more closely resemble a transient amplifying population? The finding that nephron progenitors only have a finite lifespan even when placed in a supportive environment suggests that the majority of them are transient amplifying cells and argues against a model where all nephron progenitors remain the same until forced to differentiate by the end of nephrogenesis. It is important to note here that cell intrinsic and extrinsic mechanisms are not mutually exclusive. Therefore, our experiment does not rule out possible roles of extrinsic triggers, such as changes in niche topology and birth, which could work in parallel to cell intrinsic changes in regulating progenitor lifespan.

How do cell intrinsic changes affect a progenitor’s choice to stay or exit the niche? One possibility is that nephron progenitors contain an “internal-clock” that only allow these cells to remain in the niche for a certain amount of time. Consistent with such a possibility, a “cell-division counting mechanism” has been proposed to exists in mouse glial precursors, pancreatic progenitors and *Xenopus* retinoblasts, in which the progenitor cells can divide only a fixed number of times before undergoing terminal differentiation, although the molecular nature of such a clock remains unclear (Durand and Raff, 2000; Stanger et al., 2007). In the case of nephron progenitors, such a “clock” could be embedded not only in the regulation of cell division, but in many of the other genes/pathways uncovered by our transcriptional analysis, such as cell translation and chromatin modifiers, all representing interesting candidates to be tested in future experiments. It is important to keep in mind that unlike more typical stem/progenitor niche that is established by a separate population of niche support cells and
stem/progenitor cells, the CM is an integral part of its own niche (Barak et al., 2012). Therefore, cell intrinsic changes could alter CM behavior not only on a strictly cell-autonomous manner, but also by modulating how a progenitor respond to its environment as well as the environment established by the CM itself. Supporting the first possibility is the striking observation that young and old nephron progenitors sort away from each other in cell aggregates when cultured in vitro. This is highly reminiscent of the “cell sorting-out” phenomenon seen in other developmental contexts, where cells in a single-cell suspension automatically organize themselves according to their developmental origin, or more precisely, their distinct adhesion properties. Separately, GO term analysis of GUDMAP data identified differences between young and old Crym+ cap mesenchyme cells with the expression of proteinaceous extracellular matrix as the top hit (p<10-5) (data not shown), indicating that cell intrinsic changes in the CM could alter niche environment. Therefore, CM intrinsic changes could modulate the CM itself, its niche environment and how it responds to environmental changes, all of which could potentially affect the decision of progenitor to stay or exit the niche.

It is important to take into consideration of the roles CM in regulating its own niche when interpreting another intriguing observation in our experiment; that is, how could a few old progenitors remain as nephron progenitors beyond their endogenous life span? The fact that old cells could only be found as single cells surround by the young niche suggests that direct contact of old cells, either with the young cells or the young niche environment they create, is important for extending their lifespan. Moreover, these old cells are not simply staying in the niche as quiescent cells but actively contributing to nephrogenesis, indicating possible “rejuvenation”. Our experiment, however, could not distinguish whether this “rejuvenation” reflects a preexisting heterogeneity in the old progenitor population or a trait all old cells are capable of
acquiring when immersed in a young environment. As indicated by our single-cell RNAseq data, heterogeneity exists among cells of the same age group. For example, in the E18.5 groups, cells could be found in a state similar to E14.5, or P0 or somewhere in between. Although some of this heterogeneity could be attributed to technical noise that await to be identified with more rigorous analysis, the fact that it is reported by two different algorithms (clustering in Genespring and Monocole analysis) indicates that it is likely true and is important to take into consideration when analyzing age related phenomenon. In this regard, a time course analysis identifying the step-wise signature of “rejuvenation” as well as the cell states that are capable of being “rejuvenated” will be important future experiments. This will allow us to better understand the gene network that regulate progenitor lifespan and possible ways to modulate this network in order to prolong progenitor lifespan.

Finally, based on all these findings, we propose a new model where cell intrinsic changes actively contribute to the cessation of nephrogenesis (Figure 11). In this model, nephron progenitors is established as a heterogeneous population of transient amplifying cells with different niche retention capacity. While some cells are able to self-renew without differentiating until the last round of UB branching (long-retention), others leave the niche somewhere in between the commencement and cessation of nephrogenesis (medium- and short-retention). Regardless of their initial difference, the niche retention capacity of all progenitors decreases with age due to cell intrinsic changes, such that long-retention cells gradually become medium-retention cells and medium-retention cells become short-retention cells as nephrogenesis progresses. As a result, progenitors become exhausted when all the long retaining cells lost their self-renewal capacity and differentiate.
In conclusion, this study uncovered a previously unknown role of cell intrinsic changes in regulating nephron progenitor lifespan. The functional study and unbiased transcriptome analysis supported each other and provided important insights into the dynamic nature of nephron progenitors during nephrogenesis. Some of the players uncovered in this initial characterization function may have important functions in regulating progenitor lifespan and are promising candidates that remain to be functionally tested in future studies (see Chapter V). The new protocol I established would be useful for studying the role of these players in individual CM cells while preserving an intact niche.
Material and methods

Animals

The starting colony for this experiment include the following lines: Six2$^{TGC+/tg}$ (Kobayashi et al., 2008), Rosa $^{Ai9/Ai9}$ [or Rosa tdTom/tdTom, (Madisen et al., 2009)], CAG-eCFP/eCFP (Hadjantonakis et al., 2002), Rosa eYFP/eYFP (generated by Frank Costantini, Columbia University, obtained from JAX), Cited$^{CreErt2GFP+/tg}$ (Boyle et al., 2008), Pax2$^{Cre+/tg}$ (Ohyama and Groves, 2004) and the constitutively activated version of Rosa tdTom/tdTom, Rosa Tom-A/Tom-A, where the stop codon were floxed out, was generated by mating male Rosa tdTom/tdTom with female Msx2Cre$+/tg$. In order to obtain embryos with different transgenic labels of different ages on the same experiment day, we generated the males of the following genotypes: Six2$^{TGC+/tg}$, Rosa tdTom/tdTom, Six2$^{TGC+/tg}$, CAG-eCFP$^{tg/tg}$, Cited$^{CreErt2GFP+/tg}$, Rosa tdToma-A/tdTom-A, Cited$^{CreErt2GFP+/tg}$, CAG-eCFP$^{tg/tg}$ and Pax2$^{Cre+/tg}$, Rosa eYFP/eYFP. These males are then used to set up time mating with CD1 females at the pre-estrous or estrous stage. Multiple males of the same genotype are used to ensure plugs on a certain day.

Kidney organ culture

Mouse metanephric organ cultures were performed as described by Barak et al. (Barak and Boyle, 2011). Briefly, kidneys were removed from E12.5 days post-coitum (dpc) mouse embryos and cultured on trans-well filters (35-3102, Falcon, pore size 1μm) at an air- fluid interface in a serum-free kidney medium consisting of equal volumes of Dulbecco’s modified Eagle medium and Ham’s F12 medium, 25 mM HEPES, sodium bicarbonate (1.1 mg/ml), 10 nM Na2SeO3.5H2O, 10$^{-11}$ M prostaglandin E1, and iron-saturated transferrin (5 ig/ml). The medium was changed every 24 hrs. Each individual cultured metanephros was photographed under a
Leica fluorescent stereoscope microscope daily to record their growth.

**FACS sorting of nephron progenitors**

_E12.5, E14.5, E18.5 and P0 kidneys_ were dissected from control (no transgene) or Six2TGC +/-tg or Cited1-CreERT2 +/-tg mice containing either Rosa tdTomato or Actin-CFP reporter and placed into ice cold PBS. After removal of PBS, pancreatin/trypsin solution [2.25g of pancreatin (P3292 Sigma) and 0.7g trypsin powder (T4799 Sigma) into PBS pH=7] was added (500ul per litter of kidneys <E14.5 and 1ml per 6 kidneys >E18.5) to dissociate the kidneys. To facilitate the dissociation process, kidneys order than E18.5 were cut into smaller pieces and run through pipettes with progressively smaller pipette tips (p1000- p10) during its 15min incubation on ice. When no large clumps were visible, enzyme digestions was stopped by addition of kidney media containing 10% FBS. The dissociated cells were filtered through 50um cell strainer (04-0042-2317, Partec) and spun down for 4min at 500G. Following media aspiration, cell pellets were resuspended in appropriate volumes of FACS buffer (PBS with 3% FBS) and kept on ice until FACS sorting. Sorting was performed by BD FACSDiva flow cytometer. Gating was implemented based on negative control profiles to select for live, single cell that was GFP+tdTomato+ or GFP+CFP+ double positive. Sorted cells were collected in 1.7 ml eppendorf tubes containing 900ul of kidney media with 5% FBS and kept on ice before plating. Finally, cells were spun down for 4 min at 500G and resuspended into 20ul before being plated on trans-well filters in organ culture dishes. Depending on the downstream application, each organ culture dish could contain (a) ice cold kidney media if used for transplantation or (b) 37C kidney media supplemented with Heparin (1 mg/ml; Sigma), 8.6 nM FGF9 (PeproTech), 50 ng/ml BMP7 (R&D Systems) if used for in vitro culture.
Glass capillary needles for transplantation

Glass capillaries (TW-100, World Precision Instruments) were cleaned with acetone (4hrs), 100% ethanol (4hrs), deionized water (soak overnight followed by flowing 10ml through each capillary tube) and allowed to dry overnight in an oven (80 °C). Clean glass capillaries were pulled with a Flaming/Brown type micropipette puller (P-97, Sutter Instrument) with a heat of 820, pull force of 150, velocity of 150 and a time/delay of 150. Using the needle-on-needle method (Pipette cookbook, Sutter) the needles were further trimmed to have an opening of 25-35um at the needle tip (measured with a micrometer). Trimmed needles were fire-polished at the tip with a micro-forg (MF-900, Narishige) at heat level 60 for 2 sec. To prevent cells from sticking to the wall of the needle, the needle tip were silanized in Sigmacote (SL2 Sigma) for at least 2hrs at room temperature and followed by 3 times washes in H2O, 70% ethanol and H2O.

To ensure sterility, washed needles were autoclaved before injections.

Transplantation of nephron progenitors

E12.5 recipient kidneys (Pax2Cre +/tg, Rosa +/eYFP) were dissected prior to FACS sorting and cultured as previously described (Barak and Boyle, 2011) until ready to be injected. The injection set includes a fluorescent stereomicroscope (Leica, FM-10), a manual microinjector (IM-6, Narishige) and a micromanipulator (Narishige) for holding and adjusting the position of the microinjector. To perform the injection, a capillary needle was loaded onto the microinjector and backfilled with H2O form the injector reservoir to the point where the needle starts to tapper. To load FACS purified progenitors into the needle, a negative pressure was applied to take up, FACS sorted cells concentrated on the trans-well filter until the media surface came close to the H2O surface, leaving a small air bubble in between. Once inside the needle, progenitors aggregate at the air-liquid interface at the air bubble, minimizing the amount of liquid in between.
cells. By gradually applying a positive pressure, concentrated progenitors are pushed towards the
tip of the capillary and injected into the CM niche of recipient kidneys by using the eYFP
fluorescence as a guide. Each metanephroi were injected at 2-4 different niche locations. Injected
kidneys were then cultured for another 4-7 days with media changed once every 24hrs.

**Immunofluorescence**

Recipient kidneys were fixed in 4% PFA for 6hrs at 4C followed by wash with PBS and blocked
in PBS-BB (PBS containing 1% BSA, 0.2% powdered skim milk and 0.3% Triton-100)
overnight at 4°C. After blocking, kidney cultures were incubated with primary antibodies in the
same buffer for 48hrs to allow sufficient penetration. Primary antibodies used includes Six2
(1:300, 11562-1-AP, Proteintech), Cytokeratin 8 (1:50, Troma-1, Hybridoma Bank), LTL-Biotin
(1:300, B-1325, Vector Lab), Ecadh (1:300, 610182, Transduction Lab), Wt1(1:250, sc-192,
Santa Cruz Biotechnology.) Primary antibodies were washed off for 4hrs at room temperature
followed by overnight secondary antibody incubation and another 4hrs of wash at room
temperature. Stained kidneys were mounted with ProLong® Gold Antifade Reagent (Life
Technologies) and #1.5 coverslips to stand overnight before imaging.

**EdU pulse-labeling**

EdU stock solution (1mg/ml in PBS) is added to the media to reach a final concentration of
10µM at the end of 24 or 48hrs post-injection. Pulse labeling is allowed to last for 3hrs or 7hrs
before fixation of culture 4% PFA on ice for 2hrs. Cells are then stained for EdU incorporation
according to the Click-iT® EdU imaging kit protocol (Life Technologies, C10338) and followed
by antibody labeling of Six2. Staining with an incomplete kit on EdU-containing cells was
included as negative controls.
Confocal imaging

Confocal imaging was performed on a Nikon A1Rsi inverted confocal microscope. 3D sectioning was optimized to minimize capture time but retain data sufficient resolution for Imaris (Bitplane) identifying individual cells. For each injection site, samples were imaged on a 40X water-immersion lens using a pinhole of 1.9 um on all channels. The z-stacks were taken with an interval of 1.5 um to produce a stack of consecutive images with 1/3 overlaps. For time-lapse imaging, filters with kidneys were places at the bottom of a 35mm MatTek glass bottom dish, with 1mm media in between the filter and the glass. To avoid minor movements on the Z-axis, an autofocus function was used find the filter before acquiring images at each time point. Each injection site was imaged at a 15min interval for a total of 24hrs.

Single cell RNA sequencing and data analysis

Single nephron progenitors obtained from FACS sorting were loaded onto a Fluidigm C1 Chip by captured using a 10uM-17uM filter using the single-cell AutoPrep system. cDNA libraries were generated on the same platform using Clontech UltraLow Smarter Amplification chemistry. The cDNA was converted into sequencing libraries using the Illumina NexteraXT DNA Sample Preparation kit. A total of 91 single cells were processed by the Fluidigm C1 and single-end 50 sequencing was carried out with Hiseq2500 to an average depth of ~4 million reads per cell. The sequencing data was processed using Illumina sequence analysis software and the CCHMC barcode deconvolution pipeline where it was mapped to the mouse genome (MM10) to generate gene counts and splice form detection with summarized RPKM (reads per kilobase of gene locus summarized mRNA per million reads). In addition, each cell was mapped to unique sequences found in each of the reporter genes to determine cell age. To analyze sequencing data, Bam files from each cell were loaded into Genespring and single-cell data was normalized using DeSeq
normalization algorithm. A filter of >10RKPM was applied to exclude transcription with extremely low expression. Unsupervised clustering was performed to identify genes differentially expressed among all entities. Supervised cluster with one-way anova was used to find genes expressed differently across age groups.
Figure legends

**Figure 1. FACS sorted nephron progenitors engraft into the CM niche.** (A) E12.5 *Pax2-eYFP* recipient kidney right after injection with *E12.5 Six2 TGC +/tg; Rosa +/tdTomato* nephron progenitors at two different sites. Dotted line marks the branching UB. (B) The same recipient kidney 96hrs after injection. Six2 antibody (green) labels the progenitor cells in the periphery. Cytokeratine-8 (Cyto8) antibody labels the collecting duct. (C). Enlarged image of the injection site in yellow frame in (B). Note injected progenitors in both the CM (arrow) and differentiated nephron epithelia below the UB branch (arrowhead). (D). 3hr Pulse-labeling of injected progenitors with EdU by the end of 48hrs. EdU stain pseudo-colored in white. Magenta arrows point at triple positive (Six2+tdTom+EdU+) cells. Scale bar, 100um in A and B, 30um in C and D.

**Figure 2. Transplanted nephron progenitors contribute to different segments of developing nephrons.** (A). tdTomato+ progenitors differentiated into podocytes precursor [green, antibody staining of Wilm’s tumor 1 (Wt1)], proximal tubule [white, staining of Lotus Tetragonolobus Lectin (LTL)], (B) distal tubule (white, antibody staining of E-Cadherin and (C) thick ascending limb of loop of Henle [green, antibody staining of Tamm-Horsfall Protein (THP)] but not UB (white, antibody staining of Cyto8). Scale bar, 30um.

**Figure 3. More young Six2+ progenitor remained in the CM niche after 4-day culture.** (A) Schematic diagram of the experiment design. An even distribution of old and young cells in the CM 4-days after injection would predict the lack of intrinsic difference (left), whereas an uneven presence would indicate variation in intrinsic properties (right). (B-C) Transplantation of 1:1
mixture of FACS sorted Six2+ cells into the CM niche of E12.5 Pax2-eYFP recipient kidney. The placement of transplanted cells into the CM is aided by the YFP reporter. (B’-C’) Immunostaining of injected explants after 4-day culturing. Six2 antibody marks the CM and Cytokeratin8 labels the UB. Note that both young and old Six2+ cells contributed to the differentiated nephron epithelia (arrows), while the majority of injected cells that remained in the CM are young (arrowheads). Switching the lineage label does not alter the results. (D) Quantification of the percentage of young and old cells to the total number of injected cells remaining in the CM after 4 days. 5.7-fold more young cells stayed compared to old cells (stdev=±8%, p<.52). Scale bars in (B-C) 1mm and (B’-C’) 30um.

**Figure 4.** The ability of Cited1+ cells to remain as nephron progenitors is inversely correlated with age. (A-B) Antibody staining of transgenic GFP (green, under the control of Cited1 promoter), endogenous Cited1 protein (red) and Cytokeratin-8 (white, marking the UB). Note that the GFP label completely overlaps with that of endogenous protein (arrowhead). (B) Percentage of Cited1+ and Six2+ cells remaining in the CM niche 4 days after co-transplantation of E14.5, E18.5 and P0 Cited1+ nephron progenitors with E12.5 Six2+ nephron progenitors respectively (65% vs. 35%, stdev=6%, n=24, p<10-22; 45% vs. 55%, stdev=7%, n=40, p<10-7; 27% vs. 73%, n=37, p<10-34). Scale bar, 30um.

**Figure 5.** Comparison of apoptosis and proliferate rate of young and old progenitors. (A) TUNEL staining of a kidney explant 24hrs after transplantation. Six2 antibody stains all cap mesenchyme (white), tdTomato and CFP labels P0 and E12.5 Six2+ cells respectively, TUNEL positive cells are labeled in green. Note that the injected cells along with host CM are TUNEL-.
All cells immediately boarding CM (most likely stromal cells) are also TUNEL-. All TUNEL positive cells are located outside of the forming kidney capsule (dotted line). (B) Close up view of one site 24hrs post injection. (C) Close up view of one site 48hrs post injections. No injected cells are TUNEL+. (D) Percentage of EdU+ cells present in total number of tdTomato or CFP positive cells after a 3hr EdU pulse at the end of 24hr and 48hr post injection. Differences observed on day 1 (38% ± 8% and 31% ± 11%) is non-significant (n.s.). Differences on day 2 is significant (46% ± 7% vs. 28% ± 8%, p<10^-3). Scale bar, 30um.

**Figure 6. Young and old progenitors display differential adhesion properties when cultured in vitro.** (A-D) A 1:1 mixture of FACS sorted E12.5 and P0 Six2+ cells imaged live with a fluorescent stereoscope immediately after plating on top of a filter (A) and 48hr culture with heparin, FGF9 and BMP7 (B-D). (E-H) High magnification image of fixed heterochronic co-cultures. Antibody staining of Six2 is in green. Note that the initial culture is a monolayer of evenly distributed cells (E-E’’’). By the end of 48hrs, all cells form aggregates. Cells of different age segregate from each other (F-F’’’ and G-G’’’). Cells of the same age mix evenly within the aggregate (H-H’’’). Scale bar, 30um.

**Figure 7. Old cells remaining in the niche are single cells surrounded by young neighbors.** (A-C) A representative example of cell number quantification using Imaris software. Antibody staining labels CM (Six2, green) and UB (Cyto8, white). E12.5 tdTomato+ cells (red) and P0 CFP cells (blue). All bright green dots mark Six2+ nuclei that are positive for either tdTomato (B) or CFP (C). (D) Criteria for defining single cell vs. cluster. (E-F) Counting the number of single cell in a representative niche (G) Quantification of the percentage of single cell to total
cells that remained in the niche by the end of 4-day culture in 38 injected niches (H) Staining of Fgf20null niche injected with Fgf20null P0 (red) and wild-type E12.5 (blue) progenitors. Note that all Six2+red cells are in contact with blue cells (arrowheads). Scale bars, 30um.

**Figure 8. Unsupervised hierarchical clustering of the transcription profile of 91 single progenitors from E14.5, E18.5 and P0 kidneys.** Cited1+ Cells are labeled according to age. Red (E14.5), Blue (E18.5) and Brown (P0). Heat map is generated by clustering was based on the entire gene expression pattern. Red, blue and yellow indicate high, low and intermediate expression levels, respectively. Dendrogram indicates the degree of similarity in gene expression between cells.

**Figure 9. Supervised clustering of 91 single cell expression profile based on age and GO term analysis of the differentially expressed genes.** Heat map of gene expression pattern based of age groups (left, E14.5; middle, E18.5; right, P0) Red, blue and yellow indicate high, low and intermediate expression levels, respectively. Genes differentially expressed in each block is analyzed by TopGene. Gene/pathways with top hits are listed on the right.

**Figure 10. The advancement of progenitor age matches with the predicted progression of cell differentiation by Monocle.** Top panel, pseudo-time analysis places cells in order according to the predicted differentiation sequences. Lower panel, the progression of differentiation matches the advancement of cell ages.
Figure 11. Model for cell intrinsic changes in regulating progenitor lifespan. CM is composed of a heterogeneous population of cells with long, medium and short niche capacity. The niche retention capacity of all cells decreases with age, such that long-retention cells become medium- then short-retention cells. All nephron progenitors are exhausted when all short-retention cells differentiate.

Table 1. The percentage of old and young cells that are found as single cells in the CM niche after 4-day culture. The total number of cells remaining in the niche (column 3 and 5) as well as the percentage of single cells out of total number (column 2 and 4) are quantified for a total of 38 niches (column 1) 4 days after co-injection with old (P0) and young (E12.5) progenitors. Cells labeled with the same color (yellow and yellow or orange and orange) contain similar number of cells. While young population include single cells as well as clusters (column 4) old population (column 2) are entirely found as single cells.

Figure S1. Gating criteria for sorting double positive nephron progenitors. Gating applied to select for live, single cells that are either tdTomato+GFP+ or CFP+GFP+.

Figure S2. Co-transplantation of nephron progenitors of the same age. Transplantation of 1:1 mixture (E12.5 vs. E12.5, A) or (P0 vs. P0) FACS sorted Six2+ cells into the CM niche of E12.5 Pax2-eYFP recipient kidney. The placement of transplanted cells into the CM is aided by the YFP reporter. (A’-B’) Immunostaining of injected explants after 4-day culturing. Six2 antibody marks the CM and Cytokeratin8 labels the UB. Note that red and blue cells showed similar behavior (A’ and B’). Scale bars in (B-C) 1mm and (B’-C’) 30um.
Figure S3. Cited1+ cells represent uninduced nephron progenitors. (A) Antibody staining of GFP (green, reporting Cited1+) labels a more distal population of nephron progenitors than Six2 (red). Note that Six2 but not Cited1 could be detected in the PTA. (B) Quantification of the niche engraftment rate of E14.5 Cited1 and E14.5 Six2 cells after co-injection. Cited1 showed a higher engraftment rate (69% vs. 31%, stdev=8%, $p<10^{-25}$).

Figure S4. Old progenitors remaining the CM niche actively contribute to nephron formation. (A-E) antibody staining of injected metanephroi cultured for 7 days. E12.5 (Blue) and P0 (Red) are both found in the CM (weak green, Wt1 labeling), a newly forming S-Shaped body (arrow) and more mature proximal tubule segment (white, LTL).
Figure 1. FACS sorted nephron progenitors engraft into the CM niche.
Figure 2. Transplanted nephron progenitors contribute to different segments of developing nephrons.
Figure 3. More young Six2+ progenitor remained in the CM niche after 4-day culture.
Figure 4. The ability of Cited1+ cells to remain the niche as progenitors is inversely correlated with age.
Figure 5. Comparison of apoptosis and proliferate rate of young and old progenitors.
Figure 6. Young and old progenitors display differential adhesion properties when cultured in vitro.
Figure 7. Old cells remaining in the niche are single cells surrounded by young neighbors.
Figure 8. Unsupervised hierarchical clustering of the transcription profile of 91 single progenitors from E14.5, E18.5 and P0 kidneys.
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Table 1. The percentage of old and young cells that are found as single cells in the CM niche after 4-day culture.

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Supplemental Figure 1. FACS sorting GFP+tdTomato+ or GFP+CFP+ nephron progenitors
Supplemental Figure 2. Young/young or old/old progenitors behave similarly when co-injected.

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Supplemental Figure 3. Cited1+ cells are uninduced nephron progenitors and have higher engraftment rate than Six2+ cells of the same age.
Supplemental Figure 4. Old progenitors actively contribute to nephrogenesis during the 7-day culture period.
Supplemental Figure. Quality control of single cell RNA sequencing data of 91 cells.
CHAPTER IV

The Mechanism of Notch Paralog Dominance in Nephron Segmentation
This chapter represents a previously published article, entitled “The Extracellular Domain of Notch2 Increases its Cell-Surface Abundance and Ligand Responsiveness during Kidney Development” which appeared in Developmental Cell, June 2013. Figure 5 and Fig.5S represents autonomous work.
The Extracellular Domain of Notch2 Increases its Cell-Surface Abundance and Ligand Responsiveness during Kidney Development

Zhenyi Liu¹, Shuang Chen¹, Scott Boyle¹, Yu Zhu¹, Andrew Zhang¹, David R. Piwnica-Worms¹,²,³, Ma. Xenia G. Ilagan¹, Raphael Kopan¹,⁴,⁵

¹Department of Developmental Biology ²Molecular Imaging Center, Mallinckrodt Institute of Radiology ³BRIGHT Institute ⁴Division of Dermatology, Department of Medicine
Washington University School of Medicine, 660 South Euclid Avenue, St. Louis, MO 63110, USA ⁵Current address: Cincinnati Children’s Research Foundation, Division of Developmental Biology; 3333 Burnet Avenue, ML 7007, Cincinnati, OH 45229, USA

Corresponding Author:
Raphael Kopan, Ph.D. Tel: 314-747 5520 Fax: 314-747 5503
E-mail: Kopan@wustl.edu

Key Words Kidney, Alagille Syndrome, Notch, Paralog Dominance, Domain Swap
Summary

Notch2, but not Notch1, plays indispensable roles in kidney organogenesis and Notch2 haploinsufficiency is associated with Alagille syndrome. We proposed that proximal nephron fates are regulated by a threshold that requires nearly all available free Notch intracellular domains (ICDs), but we could not identify the mechanism explaining why Notch2 (N2) is more important than Notch1 (N1). By generating mice that swap their ICDs, we establish that overall protein concentration, expression domain, or ICD amino acid composition does not account for the differential requirement for these receptors. Instead, we find that the N2 extracellular domain (ECD) increases Notch protein localized to the cell surface during kidney development and is cleaved more efficiently upon ligand binding. This context-specific asymmetry in NICD release efficiency is further enhanced by Fringe. Our results indicate that elevating N1 surface level could compensate for the loss of N2 signal in specific cell contexts.
Introduction

The kidney is an essential organ with growing clinical importance in the aging western population. It regulates excretion of soluble waste, maintains pH and electrolyte balance, and controls blood pressure and vitamin D levels. Its functional unit, the nephron, consists of a filtration apparatus called the glomerulus, followed by renal tubules made up of specialized epithelial cells that modify the filtrate, which eventually flows into the collecting duct system and drains into the bladder.

During development, nephrons form as the outcome of reciprocal interactions between the metanephric mesenchyme (MM) and the ureteric bud (UB) (Frank Costantini, 2010). GDNF, secreted by the MM, induces UB branching; Wnt9b, secreted by the UB, induces a few MM cells to undergo mesenchymal to epithelial transition (MET) and form a renal vesicle (RV), which grows into the S-shaped body (SSB) after fusing with the ureteric stalk (Figure 1A; Georgas et al., 2009). Together with endothelial and mesangial cells, the proximal third of the SSB forms the glomerulus; the rest of the SSB gives rise to the various segments and cell types that link the glomerulus to the collecting duct.

In both man and mouse, proper renal organogenesis requires the Notch signaling pathway. This pathway is comprised of four Notch receptors, N1-4, and five canonical ligands, Dll1, Dll3, Dll4, Jag1 and Jag2 (Kopan and Ilagan, 2009). As all receptors and ligands are type I transmembrane proteins, the Notch pathway mediates communications between adjacent cells. Binding of the ligand to the Notch extracellular domain (NECD) exposes the S2 cleavage site that is, by default, masked by the negative regulation region (NRR). Cleavage at the S2 site is followed by intramembrane proteolysis at the S3 site by γ-secretase, which releases the Notch intracellular domain (NICD) from the cell membrane. Subsequently, NICD translocates to the
nucleus and forms a transcriptional activation complex with RBP and Mastermind on specific DNA sites to turn on the expression of target genes, including Hes/Hey family members. In addition to these core pathway components, various other factors can modulate the strength of the Notch signaling pathway (Kopan and Ilagan, 2009).

Despite the presence of N1, N2, Dll1 and Jag1 in the developing nephron, only haploinsufficiency in either JAG1 or N2 causes Alagille syndrome in humans, a disease characterized by craniofacial abnormalities, and heart, liver and kidney malformations (Penton et al., 2012). To model Alagille syndrome in mice, simultaneous reduction in both N2 and Jagl is required (McCright et al., 2002). Moreover, whereas removal of N1 from the nephron progenitors is well tolerated, conditionally removing N2 alone from nephron progenitors in the intermediate mesoderm (with Pax3-Cre) results in complete loss of the proximal nephron and death of newborn pups within 48 hours (Cheng et al., 2007). The contribution of N1 could only be revealed in a sensitized genetic background in which N2 levels were reduced (Surendran et al., 2010). Thus far, a molecular explanation for the unequal role of N2 (vs N1) and JAG1 (vs other ligands) in human and mouse kidney development and disease has remained elusive.

To address this question, we used multiple approaches to determine if differences in the spatial expression domains, the expression level or the amino acid composition accounted for the unequal contributions of N1 and N2 to nephron development. We demonstrated that expression levels of N1 and N2 proteins are equivalent within the renal epithelia, and that differential expression outside of this domain did not contribute to the functional differences. To address the role of amino acid composition, we seamlessly swapped the entire N1ICD and N2ICD genomic coding regions to create two new strains of mice harboring genes we call N12 and N21. These mice provide a unique platform in which to distinguish NICD dose-dependent phenomena from
NICD composition-dependent ones in various tissues and disease models (Chu et al., 2011; Fan et al., 2004; Graziani et al., 2008; Parr et al., 2004; Rangarajan et al., 2001). Using these new tools, we demonstrated that N1ICD and N2ICD are fully interchangeable during kidney development; nephrogenesis occurs normally in each of the 10,000 nephrons as long as the N2 ECD controls ICD release, but fails to complete any nephrons when the N1 ECD controls ICD release. This confirmed the existence of a threshold, a developmental switch that is controlled by the concentration, but not the composition, of Notch ICDs. The switch determines if an individual nephron will develop its proximal elements (Cheng et al., 2007).

To gain more insight into how the ECD controls the free NICD concentration, we determined whether N1 and N2 ECDs differed in efficiency of ICD release in vitro using the Notch luciferase complementation imaging assay (Notch LCI; Ilagan et al., 2011). We show that when present at similar levels on the surface of HEK293 cells, the N2 ECD is consistently, but only marginally (~2 fold), better than the N1 ECD at releasing ICD in response to either Jag1 or Dll1. We further found that in RVs and SSB cells, N2 is more abundant on the cell surface than N1. Using N12 and N21 strains we could demonstrate that this uneven distribution is determined by ECD. Finally, a series of ligand loss-of-function alleles revealed a dose-dependent effect for ligands and a dominant requirement for Jag1 in the kidney context relative to that of Dll1. This may be amplified by ECD glycosylation by one of the three Fringe genes, Lunatic Fringe (Lfng), whose expression overlaps with N1 in the developing nephron. We propose that the combined effects of these factors make the N2 contribution critical to kidney development. The importance of ECD in the kidney epithelial cell is also reflected in the labeling frequencies of N1::CreLO and N2::CreLO reporter mice (2007b; 2010b; 2011), in which the release of Cre recombinase is solely determined by the Notch ECD. In summary, these data
imply that the number of NICD molecules in the nucleus of RV cells is near the amount needed to promote proximal nephron development, thereby explaining why loss of one $N_2$ or $Jag1$ allele causes a developmental syndrome in humans. Because the ICDs are interchangeable, investigating N1 trafficking in organs affected by Alagille syndrome may lead to therapeutic benefit without the risk associated with agonist use.
Results

N1 and N2 have similar expression patterns in developing renal epithelia

We reasoned that the functional difference between the two Notch paralogs during metanephric kidney development could be explained by one or a combination of several possible mechanisms: (1) differences in promoters/enhancers, which give rise to differential temporal or spatial expression domains by controlling mRNA levels; (2) differences in the 3’UTRs, which may affect the stability/translation of mRNAs of Notch paralogs and therefore protein abundance; (3) differences in ECD composition, which lead to differential responses to ligands and, consequently, different numbers of NICD molecules released, resulting in different signal “strength”; (4) differences in ICD composition, which lead to differential associations with distinct binding partners and activation of unique downstream targets (Spitz and Furlong, 2012).

A careful examination of the N1 and N2 expression patterns in the developing kidney has not been possible before due to the lack of appropriate antibodies. After confirming the specificity of newly developed antibodies against the N1ICD and the N2ICD (see below), we analyzed the expression patterns of these receptors at E17.5 using immunofluorescence on wild type kidneys (Figure 1). Both receptors are expressed in an overlapping cell population in the RV and the SSB that is thought to give rise to proximal tubules and podocytes (Figure 1E-L). In addition to the renal epithelia, N1 is expressed in endothelial precursor cells within the kidney anlagen (Figure 1B, arrowheads). In contrast, N2 is broadly expressed in the MM, vascular smooth muscle cells (VSMC), and the UB, but is absent from endothelial cells (Figure 1C,D).

The exclusive expression of N2 in the MM may explain why this protein is indispensable (Fujimura et al., 2010). However, because progenitor maintenance and MET proceed normally in the absence of N2 (Cheng et al., 2007; McCright et al., 2002), N2 activation in MM is unlikely to
perform a significant function there (Boyle et al., 2011). To directly test whether N2 is activated in MM cells, we examined the labeling pattern of a N2 activation-dependent reporter line, N2::CreLO (Figure S1) (2007b; 2011). In this reporter line, one copy of the N2 ICD is replaced with Cre recombinase, which is released upon N2 activation. In the presence of the reporter allele RosaCAG-EYFP (Madisen et al., 2009), the released Cre will excise the floxed “stop” cassette between the Rosa/CAG promoter and EYFP reporter and activate EYFP expression, indelibly marking cells that have experienced N2 activation (and their progeny) (2007b). The N2::CreLO labeling pattern in E17.5 kidneys revealed only a few EYFP-positive cells in Six2-positive MM cells (Figure 1M,O). The few MM cells experiencing N2 activation will most likely exit the stem cell niche (Boyle et al., 2011; Cheng et al., 2007; Fujimura et al., 2010). If N2 receptors were activated in cells as they underwent MET, most RV cells would be labeled. Instead, only a few EYFP-positive cells are detected in RVs. Consistent with Notch activation in the RV, many labeled cells are seen in the SSBs, proximal tubules and podocytes. N2 activation thus occurs in the epithelial cells and not in their mesenchymal precursors (Figure 1M-O). In summary, both receptors are expressed in the domain where Notch proteins impact the decision to make proximal nephron cells and the differential expression of N2 in the MM does not explain why N2 is essential for kidney development while N1 is not.

ICD swap between N1 and N2 creates N12 and N21 chimeric receptors

All NICD paralogs form transcriptional activation complexes with RBPjk and Mastermind on target promoters. Although Notch proteins can activate similar targets and can act redundantly in vivo (Riccio et al., 2008), in vitro and in vivo studies suggest that in some contexts, these complexes are distinct as NICD paralogs can have different or even opposite functions (Chu et
Amino acids not conserved between N1 and N2 ICD are located at the solvent-accessible surface of the Ankyrin domain and could therefore participate in unique interactions with putative co-activators or co-repressors, contributing to their functional differences (Spitz and Furlong, 2012). To investigate this, we used galK-selection-based BAC recombineering (Warming et al., 2005) to swap the entire genomic regions coding the ICDs between the N1 and N2 loci in B6-derived ES cells (Figure 2A; Figure S2). The swapped region ranged from Exon 28, coding for the transmembrane domain (TMD), to the stop codon in Exon 34. We did not swap the 3’UTRs in order to retain transcript-specific regulation of mRNA stability and translation. We designated the new alleles N12 (N2ICD in the N1 locus) and N21 (N1ICD in the N2 locus) (Figure 2A). To facilitate ES cell screening and post-recombination analysis with pyrosequencing based methods (2009c; 2010c), we introduced silent single nucleotide variations (SNVs) into the TMD coding regions (G38066C for N12 and G125011C for N21), as well as an SNV in the ICD coding region of N12 (G38129A) (Figure 2A). After germline transmission was obtained, the frt-flanked neomycin/G418 selection cassette was removed by mating with flippase deleter mice (Rodríguez et al., 2000). This left a 34bp frt sequence between the stop codon and the 3’UTR in mice with N12 and N21 chromosomes (Figure 2A; Figure S2).

PCR amplification confirmed the presence of the hybrid exon 28 in the genome (Figure S3A, B). Loss of sequences from N1 exon 30 or N2 exon 34 respectively identified N112/12 and N221/21 homozygous mice, which are both viable (Figure S3A, B; Detailed phenotypic analysis of other organs will be described elsewhere). The loss of N1ICD in N112/12 or N2ICD in N221/21 mice was also confirmed by Western blot with N1ICD- and N2ICD-specific antibodies, respectively (Figure 2B). The introduced SNVs allowed us to compare the mRNA levels
transcribed from the \(N12\) chromosome to \(N1\) and \(N21\) chromosome to \(N2\) in various heterozygous tissues of \(N1^{+/12}\) and \(N2^{+/21}\) mice, respectively, with pyrosequencing (Figure 2C). This analysis revealed that the shorter transcript was slightly more abundant in all tissues examined (Figure 2C). Western blot and immunostaining with either anti-Notch ICD or anti-Notch ECD antibody confirms the expression of chimeric proteins (Figure 2B, Figure S3C, D). To assess whether the chimeric Notch receptors could reach the cell surface as efficiently as the endogenous receptors, we isolated RV and SSB cells from Lfng-GFP mice, in which EGFP is expressed under the control of LFringe regulatory sequences. Double staining of E17.5 kidneys with either N1 or N2 ICD antibodies shows extensive overlap with EGFP (Figure 2D-G; Figure S3E). To exclude the epithelial cells from differentiated tubules, we isolated GFP+ cells from E13.5, Lfng-GFP; \(N1^{+/+};\ N2^{+/+}\) (denoted as WT), Lfng-GFP; \(N1^{12/12};\ N2^{+/+}\) (denoted as \(N1^{12/12}\)) and Lfng-GFP; \(N1^{+/+};\ N2^{21/21}\) (denoted as \(N2^{21/21}\)) kidneys before tubule formation (Figure 3H), and stained them with anti-N1 ECD or anti-N2 ECD specific antibodies (Fiorini et al., 2009). Flow cytometry analysis confirmed that the cell surface distribution of N12 and N21 is similar to N1 and N2, respectively (Figure 3I).

**N1ICD and N2ICD are interchangeable in the kidney**

We have shown previously that conditional deletion of N2 from the intermediate mesoderm (with \(Pax3-Cre\)) produced mice with non-functional, hypoplastic kidneys lacking podocytes and proximal tubules (Cheng et al., 2007). We found that \(N2^{21/21}\) and compound heterozygous \(N1^{+/+};\ N2^{21/2+}\) mice (both lacking N2ICD) formed functional nephrons in normal numbers (Figure 3). This result demonstrates that even a single copy of N1ICD can fully rescue the loss of N2ICD when expressed from the N2 locus. In contrast, when the endogenous \(N2\) alleles are conditionally
deleted, even the presence of two N2ICD expressed from the *N1* locus (*Pax3-Cre; N2^{ff}; N1^{12/12}*) cannot rescue a single nephron (Figure 3). These mice were indistinguishable from *Pax3-Cre; N2^{ff}* mice in their kidney morphology and died within 24 hours of birth (Figure 3). These data demonstrate that N1ICD and N2ICD are fully interchangeable, and that the functional differences between N2 and N1 are determined by differences in their ECDs and/or their corresponding protein levels during kidney development.

**N2 and N1 promoters/3’UTRs deliver similar levels of protein in the RV and SSB cells**

We next sought to determine if N1 is less abundant than N2 protein within RVs and/or SSBs, thereby explaining the differences in their function during kidney development. This is a technically challenging question to answer: First, it proved impractical to isolate enough RV and SSB cells for Western blot analysis. Second, different antibodies recognizing unique epitopes in Notch paralogs may have different affinities, making the comparison difficult. Fortunately, the domain swap offered us the opportunity to examine N2ICD protein levels by immunostaining in wild type (where N2ICD production is under the control of the endogenous *N2* locus) and in *N1^{12/12}; N2^{21/21}* double-homozygous mice where N2ICD production is under the control of the *N1* locus.

To perform this experiment, we first confirmed the specificity of the anti-N1ICD and anti-N2ICD antibodies on kidney sections from either *N1^{12/12} or N2^{21/21}* mice (Figure 4A, B). Next, we used the anti-N2ICD antibody and analyzed immunostained kidneys from *N2^{+/−}, wild type, and N1^{12/12}; N2^{+/−}* mice, which have 1, 2 and 4 copies of the N2ICD antigen, respectively (Figure S4A-C). Pixel intensity correlated well with gene dose (Figure S4D), confirming that this assay is sensitive enough to quantitatively compare N2ICD levels in wild type and *N1^{12/12}; N2^{21/21}*
mice. Finally, we compared the abundance of N2ICD protein levels in kidneys from wild type and N1<sup>12/12</sup>; N2<sup>21/21</sup> by immunostaining (Figure 4). Anti-N2ICD antibody staining of N1<sup>12/12</sup>; N2<sup>21/21</sup> kidneys confirmed that N2ICD recapitulated the N1 expression pattern, including its strong expression in the developing RVs, SSBs and all endothelial precursor cells, and its absence from the MM (Figure 4C,D). Importantly, the expression levels within RVs and SSBs are comparable in the two samples (Figure 4E,F). Therefore, the functional differences between N1 and N2 could not be attributed to differential expression levels, miRNA targeting of their 3’UTRs, or their ICD composition.

**Receptors containing the N2 ECD are more abundant on the plasma membrane of RV and SSB cells**

Since only receptors on the cell surface could engage with ligands for activation, we determined whether the cell surface level of N1 and N2 are comparable by flow cytometry, using LFng-GFP kidneys (Figure 2H). To rule out antibody-based artifacts, we employed two different sets of monoclonal anti-N1 and anti-N2 ECD antibodies: one set was raised in Armenian hamster (Moriyama et al., 2008); the other set in rat (Fiorini et al., 2009). The geometric mean fluorescence intensity (GMFI) of antibodies against N2 in EGFP+ epithelial cells isolated from E13.5 Lfng-GFP kidneys is about four fold (4.5±1.0) higher than that generated by anti N1 antibodies (Figure 4G). To ensure that this result did not reflect differential affinity, we sorted stable HEK293 cell lines in which surface biotinylation assays confirmed that the amounts of N1 ECD and N2 ECD on the cell surface are similar (described in the next section and in Figure S5A-C). The results show that the differences in affinity between N1 and N2 antibodies (Figure S5D) could account for only a fraction of distribution difference seen in RV and SSB. Therefore,
N2 is more abundant than N1 on the surface of renal epithelial cells in the developing nephron. Most importantly, because N21 has the same surface abundance as N2 (Figure 2I), the ECD, but not the ICD, determines the surface level of N2 and N1.

**The N2 ECD releases more N1ICD than the N1 ECD does in response to ligands**

Considering that only one allele of N2 is sufficient for normal kidney development whereas two alleles of N1 could not (Figure 3), a small difference in surface distribution alone may not explain the functional dominance of N2. Therefore, we asked whether differences in ECD composition could also impact the amount of ICDs released in response to ligand. To address this, we used a quantitative *in vitro* assay based on luciferase complementation imaging (LCI) system in kidney-derived HEK293 cells (Figure 5A; (Ilagan et al., 2011)). We first fused the carboxy-terminal half of luciferase (CLuc) to the N-terminus of RBP and generated two parental CLuc-RBP-expressing HEK293 Flp-In™ cell lines by random integration. Then we fused the N-terminal half of luciferase (NLuc) to the C-terminus of full length N1 and N21 respectively, and targeted them into the same genomic locus in the two parental cell lines using the Flp-In system (Figure 5A). In these Notch Flp-In cells, the isogenic expression of N1-NLuc and N21-NLuc minimizes positional effects and ensures similar expression levels (Figure S5A-C). In the absence of ligand binding, N1-NLuc and N21-NLuc fusion proteins are anchored to the cell membrane, whereas CLuc-RBP fusion protein is segregated into the nucleus and no luciferase activity is detected. The binding of ligands to the ECD (or unfolding of the NRR by calcium chelation with EGTA) triggers receptor proteolysis and the release of the N1ICD-NLuc fragment, which then translocates into the nucleus and interacts with CLuc-RBP to reconstitute a quantifiable luciferase activity. The amount of light emitted is directly proportional to the
amount of N1ICD released and is therefore a measure of signal strength (Ilagan et al., 2011). To control for cell-line specific variations, we tested a total of 10 N1-NLuc sub-clones and 10 N21-NLuc sub-clones for each of the two CLuc-RBP parental cell lines.

To compare signal strength of N1 and N21 in these cells, we co-cultured the Notch Flp-In cells with either ligand-presenting cells (CHO-Dll1 or -Jag1) or control CHO cells. After 24hrs of co-culturing, significantly more light is emitted from N21-NLuc than N1-NLuc cells (p<10^{-6}, Student T-test, Figure 5B; similar results were obtained with sub-clones derived from the other CLuc-RBP-expressing parental cell line, not shown). Considering that the released N1ICD-NLuc fragments from N21-NLuc and N1-NLuc differ by six amino acids at their N-termini (VLLSRK for N1-NLuc vs. VIMAKR for N21-NLuc), we tested whether differential stability accounted for the apparent difference in bioluminescence between N1-NLuc and N21-NLuc. After activating the reporter cells overnight on immobilized ligand, we added a γ-secretase inhibitor (DAPT) to block the release of additional N1ICD-NLuc fragments and followed the decay of bioluminescence as a function of time (Figure 5C). The N1ICD-NLuc^{VLLSRK} proved to be as stable as the N1ICD-NLuc^{VIMAKR}, allaying the concern that we were detecting differences in protein stability. Finally, as mentioned above, the amounts of N1 and N21 on the cell surface are similar (Figure S5), suggesting that the difference in luminescence is not simply due to unequal amounts of surface receptors. Collectively, these experiments suggest that N2ECD is more efficient in eliciting ligand-mediated receptor activation in kidney cells.

The activation of Notch receptors requires the unfolding of the NRR domain within the ECD to expose the S2 cleavage site (Kopan and Ilagan, 2009). We therefore tested if differences in the dynamics of NRR unfolding may contribute to the differences between the two ECDs. We monitored the kinetics of N1 and N21 activation in our Flp-In lines in the presence of the
calcium chelator EGTA for one hour. After 30 minutes of EGTA treatment, more bioluminescence was detected with N21-NLuc than with N1-NLuc (Figure 5D), suggesting that subtle differences in NRR unfolding may contribute to the higher activation probability of N21.

**Dll1 and Jag1 contribute differentially to nephron segmentation**

Two major Notch ligands, Dll1 and Jag1, are expressed in the developing renal epithelia (Chen and AL-AWQATI, 2005; Leimeister et al., 2003); co-immunostaining of SSBs shows that their expression domains largely overlap with each other (Figure 6A), with LFng (Figure 2 E, G and Figure S3E) and Notch receptors in the middle part of the SSBs (Figure 6B-D). To assess the contribution of each ligand to nephron development, we created an allelic series of conditionally deleted ligands in the MM using *Six2-Cre*<sup>tg</sup>/*+* (Kobayashi et al., 2008) (Figure 6E-P). Because *Six2-Cre* is strongly expressed in metanephric mesenchymal cells from which all renal epithelial cells are derived, near complete deletion of floxed Jag1 and Dll1 is achieved at the genomic DNA level in these cells (Fig. S6A-D). Staining with the proximal tubule marker LTL revealed mildly disrupted nephron development in *Dll1* mutants (Figure 6H) but a drastic reduction in the number of nephrons in *Jag1* mutants (Figure 6J). Interestingly, in the presence of one *Jag1* allele (*Six2-Cre*<sup>tg</sup>/*+*; *Dll1*<sup>ff</sup>; *Jag1*<sup>+f</sup>), nephron number was severely compromised, but some WT1+ podocytes formed (compare Figure 6N to 6M); the presence of one *Dll1* allele could not support production of podocytes, despite the presence of some proximal tubules (Figure 6O). Simultaneous deletion of both ligands led to near complete loss of nephrons (Figure 6L, P), approaching the drastic phenotype seen in N2 mutants where both glomeruli and proximal tubules are missing (Cheng et al., 2007). These data collectively demonstrate that although both
ligands contribute to the normal development of the nephrons, Jag1 plays a dominant role in general and in the development of podocytes in particular.

Fringe family members can modulate the response of N1 and N2 to Dll1 and Jag1 ligands. In most contexts, fringe modification renders N1 more responsive to Dll1 ligand and less responsive to Jag1. In contrast, fringe modification of N2 could potentiate, reduce or have no effect on ligand-mediated signaling, depending on the context (Stanley and Okajima, 2010). We therefore examined the expression pattern of the three fringe family members by in situ hybridization in E17.5 kidneys (Figure 7A-C). Only Lfng was detected; it was expressed in a pattern similar to EGFP from Lfng-GFP mice with strong signal in some epithelial cells of RVs and the middle segment of SSBs and weak signal in differentiated tubules (Figure 7A). We next tested NICD release from N1-NLuc and N21-NLuc cell lines co-cultured with Dll1 or Jag1-expressing CHO cells in the presence and absence of LFng. Overexpression of Lfng significantly enhanced the N1-NLuc response to Dll1 and suppressed its response to Jag1. In contrast, its effects on N21-NLuc were minimal (Figure 7D; only 3 line of 20 shown). Although the net loss in response to Jag1 may be offset by the gain in response to Dll1 in vitro (Figure 7D), these data suggest that fringe could contribute to the unequal contribution of N1 and N2 in vivo.

**N2 ECD released Cre more efficiently than N1 ECD in developing nephrons of N::Cre mice**

All the data presented thus far ascribes the difference between N1 and N2 to their ECDs. Unfortunately, we have not yet identified antibodies that specifically recognize activated N2ICD. Therefore, we used a surrogate assay to compare N1 and N2 cleavage in vivo by comparing the effectiveness of Cre release in two activation-dependent Notch reporter mice, *N1::CreLo* (Liu et al., 2011; 2007b) and *N2::CreLo* (Figure S1). The release of Cre in both lines is under the control
of a Notch ECD, and Cre activity will provide an estimate of the efficiency of its release. Many labeled epithelial cells were seen in RVs and SSBs of N2::Cre\textsuperscript{Lo}; Rosa\textsuperscript{CAG-EYFP} mice (Figure 1M-O, Figure 7E). In contrast, we could not find any labeled epithelial cells in RVs or SSBs of N1::Cre\textsuperscript{Lo}; Rosa\textsuperscript{CAG-EYFP} mice, although endothelial cells are very efficiently labeled (Figure 7F-H), as are cells in many other tissues (2007b). Because we obtained immunohistological and genetic evidence that N1ICD complements N2 activity in a sensitized background (Cheng et al., 2007; Surendran et al., 2010), these observations are consistent with a model that in renal epithelia N2 is more abundant at the cell surface, where it undergoes proteolysis more efficiently than N1 in response to available ligands. Like Notch ICDs, only Cre6MT released by the N2 ECD, but not the N1 ECD, reached a concentration threshold needed to excise the floxed stop allele in renal epithelial cells.
Discussion

Human patients and mouse models of Alagille syndrome support the idea that kidney development is particularly sensitive to N2 dosage even in the presence of N1. We investigated several possible mechanisms that could explain the dominant contribution of N2 over N1 to nephrogenesis (Cheng et al., 2007; Surendran et al., 2010). A precise mechanistic understanding will not only enhance our knowledge of how Notch signaling contributes to kidney organogenesis, but more importantly, could offer insights into therapeutic options for kidney defects seen in Alagille syndrome (Penton et al., 2012) and perhaps other Notch-related congenital disorders. Furthermore, such understanding may prove generally applicable to many other organs and signaling pathways.

Although N2 is expressed in the MM, none of the known Notch ligands or targets are expressed there (Boyle et al., 2011; Chen and AL-AWQATI, 2005; Leimeister et al., 2003; Ong et al., 2006). Consistent with a ligand-poor environment, N2 activation is an infrequent event in the MM. Genetic analyses confirmed that Notch proteins function in nascent renal epithelial cells (this study, (Cheng et al., 2007; Wang et al., 2003)), where the \(N1\) and \(N2\) expression domains are indistinguishable (Chen and AL-AWQATI, 2005; Leimeister et al., 2003). These results rule out enhancer evolution as the mechanistic explanation for the functional importance of N2.

We therefore focused on two alternative hypotheses: either N1ICD is a weak activator of key target(s) regulated normally by N2ICD due to its amino acid composition, or N1ICD concentration is insufficient to functionally compensate for N2 deficiency. To differentiate between these possibilities, we generated two new alleles of Notch (\(N12\) and \(N21\)) in which we swapped the entire genomic sequences coding for Notch ICDs, in contrast to a previous study that established the equivalence of the domain C-terminal to the ankyrin repeats of Notch.
The availability of mice in which the same epitope is transcribed and translated from different loci enabled comparison of protein abundance with the same ICD-specific antibody. This analysis demonstrated that the two paralogs are expressed at similar levels in the developing renal epithelium and, therefore, differences in overall protein concentration cannot explain the dominant role of N2.

We next addressed the role of Notch amino acid composition. We demonstrated that N1ICD and N2ICD are fully interchangeable during kidney development: even one copy of N1ICD expressed under the N2 ECD control is sufficient to produce a normal kidney. If neither overall protein concentration nor NICD composition could explain the unequal roles of N1 and N2 in the developing kidney, ECD control over NICD nuclear concentration is likely the differentiating factor between N2 and N1. We discovered that the N2 ECD indeed generates more NICD than the N1 ECD does in the renal epithelial cell context by a combination of two mechanisms. First, N2 is more abundant at the cell surface than N1. Accounting for the differences in affinity between anti-N1 and anti-N2 antibodies, the difference is between 2 and 3 fold. Importantly, N21 and N2 are equally abundant at the cell surface indicating that surface distribution is determined by sequences in the Notch ECD, not ICD. Although other transmembrane proteins may contain trafficking signals in their ECD (Albu and Constantinescu, 2011; Steiner et al., 2008; VandenBussche et al., 2009), the only indication that the Notch2 ECD may play a role in its trafficking comes from a study on the importance of S1 cleavage to the exocytosis of N1, but not N2 (Gordon et al., 2009). Second, in luciferase complementation imaging assays (Ilagan et al., 2011) that quantified the amount of NICD released from N21 and N1 in cultured human embryonic kidney cells in response to ligand or EGTA, N21 consistently released more NICD. In the EGTA paradigm, all surface receptors are activated; given that
surface biotinylation confirmed that N1 and N21 are present at equal amounts, and the NICDs have the same half-life, we conclude that the N2 NRR must be easier to activate than N1 NRR in kidney epithelia, and since it contains the S1 site, may be regulating exocytosis as well. However, where the trafficking signals reside within the ECD, and whether they only functions in the developing kidney, remains to be investigated. Supporting the conclusion that the ICD passively reflects the advantages provided by a specific ECD, the N2 ECD is more potent than the N1 ECD in Notch::Cre reporter mice, where the amount of Cre released directly reflects the activation frequency by the respective Notch ECD.

These two factors (surface density and ease of activation) may work in synergy or simply be additive, but other factors may serve to further amplify the effectiveness of the N2 ECD in vivo. As reported before (Hicks et al., 2000), the response of N1 to Jag1 is significantly inhibited by Lfng modification, whereas that of N2 is not; considering that Lfng is co-expressed with N1 and N2 in the epithelial cells of developing nephrons, it may be promoting a potent N2-JAG1 signaling axis during nephrogenesis. Indeed, analysis of ligand loss-of-function alleles showed that, although Dll1 and Jag1 are co-expressed with N1 and N2, Jag1 plays a dominant role. This is consistent with reports that only mutations in JAG1, but not DLL1, cause Alagille syndrome (Piccoli and Spinner, 2001).

The data herein indicates that a NICD nuclear concentration threshold must be met to define the identity of the proximal renal epithelia, and this threshold acts as a digital (all or nothing) switch (Figure 7I). This is very reminiscent of the intestinal differentiation program in C. elegans (Raj et al., 2010). In that system, mRNA levels must rise above a threshold to activate expression of a master regulatory gene. The relative strength, or penetrance, of various alleles reflects the number of cells reaching the ON decision. In the Notch system, variability in the
number of NICDs released to the nucleus might regulate an ON/OFF decision to form the proximal nephron. The advantage of the N2 ECD/NRR and the large impact of these differences suggest that the overall numbers of NICD in the nucleus are just above the ON state in RV cells and thus highly prone to perturbations. Whether a master regulator lies downstream of NICD and the identity/number of targets that must be activated to promote proximal development remain unknown.

In summary, our experiments produce strong evidence of functional equivalence between the N1 and N2 ICDs in vivo, despite apparent differences in multiple assays based on overexpression, including our own (Ong et al., 2006). A higher surface level coupled with greater responsiveness of the N2 ECD to ligand translates into a higher probability of NICD release during a critical step in kidney development. These data illustrate the binary nature of a critical step in nephron segmentation, where nephrons with NICD concentration falling below a threshold fail completely to produce proximal structures, and highlight an underappreciated importance for the ECD/NRR in controlling surface distribution. In contrast to binary response to NICD levels, the outcome of ligand reduction is graded: nephrons can form proximal tubules without podocytes when onlyDll1 is present, suggestive of a second Notch-dependent decision. Finally, investigation into Notch paralogs trafficking to cell surface may provide leads for treating the renal (and perhaps all) manifestations of Alagille syndrome.
Material and Methods

Mice
Generation, genotyping strategy and PCR primers related to N12 and N21 mice and the source of other mouse lines are described in detail in Supplemental Experimental Procedures. All mice were housed in the Washington University animal facility and all experimental procedures were approved by Washington University Animals Studies Committee.

Pyrosequencing
Total RNA was purified, reverse transcribed and used for pyrosequencing as described (2010c).

Immunohistochemistry
Kidneys were dissected and fixed in 4% PFA overnight, washed extensively with 1XPBS, soaked overnight in 30% sucrose and embedded in Optimal Cutting Medium for frozen sections, or dehydrated through a 30%, 50%, 70% ethanol series and embedded for paraffin sections. For frozen sections, antigen retrieval was achieved by permeabilization in 1XPBS, 0.1% Triton X-100 for 20 minutes at room temperature. For paraffin sections, this was achieved by boiling in 10 mM Sodium Citrate (pH 6.0) for 20 minutes. For both frozen and paraffin sections, 1XPBS containing 3%BSA and 0.1% Tween was used for blocking. Detailed information on primary antibodies and their dilution were described in Supplemental Experimental Procedures. FITC-, Cy3-, and Cy5-conjugated secondary antibodies or streptavidin (Jackson ImmunoResearch) was used for visualization.

Flow Cytometry
Flow cytometry were performed as described (2011). Briefly, E13.5 embryonic kidneys were dissected, mechanically disrupted and digested with 1mg/ml collagenase at 37°C for 15 minutes to get single cell suspensions. Cells were further washed and stained with PE- or APC-
conjugated anti-N1 or -N2 ECD antibodies (eBioscience and Biolegend) in staining buffer (1XPBS +3% BSA) on ice for 20-30 minutes, followed by flow cytometry analysis. Cultured 293 cells were mechanically removed from culture plates and analyzed in a similar manner. Data were collected on BD FACScan with FlowJo Collectors’ Edition and analyzed with FlowJo software (TreeStar).

In situ Hybridization, Western blot and Nephron Number Quantification

Conventional methods were used for in situ hybridization. Probes for lunatic, radical and manic fringe were labeled with Digoxigenin and detected with alkaline phosphatase-conjugated anti-Digoxigenin antibody (Roche). Kidneys from newborn pups or cultured cells were used for Western blot. Nephron number was determined as described (Godley et al., 1996). Details are described in Supplemental Experimental Procedures.

Generation and maintenance of isogenic N1 and N21 LCI reporter lines

Flp-In™TRex™293 host cells (Invitrogen, R780-07), which were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 1% Pen-Strep (henceforth, media) and 100 µg/ml ZeoCin™, were cotransfected with pcDNA3-(Click beetle green) CBG CLuc-RBP and a Puromycin expression construct using FuGENE6 (Roche). Puromycin-resistant clones were selected in media containing 0.6 µg/ml puromycin to generate Flp-In™TRex™ 293 CBG CLuc-RBP parental cell lines. LCI imaging was performed on CBG CLuc-RBP expressing clones by transiently transfecting a constitutively active N1ΔE-NLuc plasmid to verify luciferase complementation. Two LCI positive clones, D10 and D6, were selected as the CLuc-RBP parental cell lines and expanded in media containing 0.4µg/ml puromycin and 100µg/ml ZeoCin™. Stable expression of CLuc-RBP after different passages was confirmed by Western blot. To generate N1-NLuc and N21-NLuc Flp-In cells, D10
and D6 clones were co-transfected with pcDNA5/FRT expression vector (Invitrogen, V6010-20) containing either N1-CBG NLuc or N21-CBG NLuc and pOG44 vector at a 1:9 ratio. Positive clones, identified by selection for hygromycin-resistance (150µg/ml) and gain of Zeocin™ sensitivity, were tested for their ability to reconstitute luciferase activity upon EGTA treatment. 20 sub-clones were subsequently maintained in media containing 0.4µg/ml puromycin (to maintain CBG CLuc-RBP) and 100 µg/ml hygromycin (to maintain Notch CBG NLuc).

**Luciferase Complementation Imaging (LCI) Assays**

LCI assays are described in detail in (Ilagan et al., 2011). For ligand-dependent activation, 10⁴ ligand-presenting cells (CHO-Dll, CHO-Jag or CHO control cells) were seeded into each well of uncoated 96-well black plates 24 hours prior to the seeding of 4X10⁴ N1-NLuc or N21-NLuc cells. After another 24 hours, co-cultured cells were imaged in phenol red-free culture medium containing 150 µg/ml D-luciferin. For the ligand-independent activation assays, black 96-well plates were coated with 0.1mg/ml poly-lysine at room temperature overnight, washed twice with PBS and air-dried for 30 minutes before 4X10⁴ cells were seeded into each well. Twenty-four hours later, an initial image (t=0) was obtained of the cells using Hank’s Balanced Salt Solution (HBSS) containing 150 µg/ml D-luciferin (100µl/well). Then, another 100µl of HBSS/D-luciferin solution containing 2X EGTA (200µM) was added per well and images were obtained every 5 minutes for 1 hour.

To monitor the rate of NICD degradation, black 96-well plates were coated with 5µg/ml anti-Fc antibody (Jackson Immunoresearch) for 6 hours at 4°C. Unbound antibodies were washed off twice with PBS and conditioned media containing Fc control or Dll1-Fc/Jag-Fc were added (50µl/well) and incubated overnight at 4°C. Excess ligands were washed off twice with PBS before cells were seeded. Reporter cells were plated on the immobilized ligands as described in
After 24 hours, an initial image was obtained (t=0), after which DMSO or DAPT (5μM) was added to the wells to stop NICD production, and images were taken every hour for 6 hours. Additional, detailed methods for ligand conditioned media preparation, IVIS imaging and photon flux quantification can be found in (Ilagan et al., 2011).

**Surface Biotinylation Assay**

Surface biotinylation was used to compare the surface level of N1-NLuc and N21-NLuc in Flp-In cells. Surface Biotinylation Assay 6-well plates were pre-coated with 0.1mg/ml poly-lysine and then seeded with 4X10⁵ cells per well. After cells grew to near confluence (to cover up most of the poly-lysine coating and therefore minimize its competition against cell surface proteins for biotin), culture medium was removed and cells were washed three times each for 5 minutes with 3ml ice-cold 1XPBS supplemented with 1mM MgCl₂ and 0.1mM CaCl₂, pH7.0 (PBS++).

All surface proteins were then biotinylated by incubating cells with 1.2 ml freshly prepared 0.5mg/ml Sulfo-NHS-SS-Biotin in PBS++ on ice. After 30 minutes, the reaction was stopped by adding an equal volume of 100 mM glycine in PBS++ for 15 minutes on ice. This was followed by 2 washes with 3ml 50mM glycine in PBS++ and one wash with 3ml PBS++, each for 5 minutes. After the PBS++ wash buffer was completely aspirated, cells were lysed in 500µl RIPA buffer on ice for 30 minutes and the genomic DNA sheared by passing the lysate through a 27 1/2G needle 5 times. The lysate was then cleared by centrifugation at 13,000 rpm at 4°C for 10 minutes. A 50µl aliquot of the supernatant was saved for comparing the total Notch protein level on a western blot and the rest was incubated with 50 µl streptavidin-coupled agarose beads at 4°C on a rocker for 90 minutes. The beads were collected by centrifugation and washed 3 times in RIPA buffer and another 3 times in PBS++ buffer at 4°C. After the last wash, as much buffer was removed as possible and the beads were boiled in 50µl Laemmlll buffer to extract the
surface protein. The surface and total protein from N1-NLuc and N21-NLuc cell lines were separated on SDS-PAGE, immunoblotted, and their relative amounts determined by densitometry.
**Figure legends**

**Figure 1.** Variation in the expression pattern of N1 and N2 does not explain their functional difference. (A) Diagram showing major structures of developing nephrons. MM, metanephric mesenchyme; RV, renal vesicle; SSB, S-shaped body; UB, ureteric bud. The presumptive distal and proximal tubules, as well as podocyte precursor cells, are denoted in purple, green and red, respectively. (B-L) Comparison of N1 and N2 expression in different structures of an E17.5 kidney. CD31 marks endothelial cells; SMA (smooth muscle actin) marks vascular smooth muscle cells; CK8 (Cytokeratin 8) marks UB and its derivatives; NCAM marks all epithelial cells. Arrowheads denote endothelial cell precursors. (I-L) show the double staining with N1 ICD and N2 ECD antibodies. (M-O) Labeling pattern of N2::Cre reporter in E17.5 kidney. All scale bars are 10µm except for O, which is 500µm.

**Figure 2.** Generation of the N12 and N21 alleles. (A) Schematic illustration of N1 (blue) and N2 (red) loci before and after the ICD swap. The N1 ICD encompasses 5,926bp on chromosome 2, ranging from nucleotide +38,103 to +44,028 (A in ATG is +1) and encoding amino acid 1,750 to 2,531; for N2, the ICD encompasses 8,699bp on chromosome 3, ranging from nucleotide +125,048 to +133,746 and encoding amino acid 1,705 to 2,473. Amino acids in black denote the S3 cleavage sites. Green triangle denotes FRT site. (B) Western blot analyses with ICD-specific antibodies of kidney extracts from newborn pups with designated genotypes (WT, N1/12 and N2/21; two different individuals per genotype). (C) mRNA level comparisons between chimeric N12 and N21 and their corresponding endogenous alleles in various tissues of wild type (WT), N1+/12 and N2+/21 newborn pups. Allele ratios were calculated by determining the G/C ratio at SNVs G38066C and G125011C introduced into the targeting constructs with pyrosequencing. Error bars represent standard deviation. (D-G) Double staining of EGFP and N1 (D and E) or N2
(F and G) on E17.5 Lfng-GFP kidneys. Asterisks (*) denote EGFP+ tubules. (H) EGFP labeling patterns in E13.5 Lfng-GFP kidney. (I) E13.5 Lfng-GFP kidneys with wild type (WT) or single homozygous (N1^{12/12} or N2^{21/21}) Notch alleles were dissociated into single cells, stained with PE-conjugated N1 or N2 ECD-specific antibodies (eBioscience) and analyzed with flow cytometry. The cell surface levels of wild type (N1, N2) and chimeric (N12, N21) were compared in EGFP+ cells. Scale bars in D-G are 10µm and the one in H is 100µm.

**Figure 3.** Notch ICDs can functionally replace each other in kidney development. Kidney phenotypes were characterized in newborn mice with the indicated genotypes. Scale bars: 500µm for whole kidneys; 20µm for the magnified windows showing WT1 and LTL staining. Standard deviations of nephron number are shown in the parentheses.

**Figure 4.** Comparison of the Total and Surface Levels of N1 and N2 in Developing Nephron Epithelia. (A, B) Confirmation of the specificity of anti-N1 and -N2 ICD antibodies on kidney sections from N1^{12/12} (A) and N2^{21/21} (B) mice. (C, D) Anti-N2 ICD antibody staining on kidney sections from N1^{12/12}; N2^{21/21} double-homozygous mice, in which all N2ICD is expressed from the N1 locus. (E, F) The levels of protein expressed from the N1 and N2 loci in developing RVs and SSBs were compared by immunostaining with N2 ICD specific antibodies on N1^{12/12}; N2^{21/21} mice (the N1 locus, E) and wild type (the N2 locus, F). The secondary antibody was used without signal amplification and exposure times were identical for the red channel to allow quantitative comparisons. Arrowhead denotes endothelial cells. MM, metanephric mesenchyme; RV, renal vesicle; UB, ureteric bud. (G) Flow cytometry analysis on EGFP+ live cells from E13.5 Lfng-GFP kidneys with two different sets of anti-Notch ECD antibodies. All scale bars: 10µm.
Figure 5. N2 ECD is more potent than N1 ECD in mediating ligand-induced ICD release. (A) The Notch LCI strategy for comparing the potency of N1 and N2 ECDs: NLuc is fused to the C-terminus of N1 or N21. These two constructs were expressed from the same genomic locus in parental cell lines that stably express CLuc-RBP. For both N1 and N21, activation releases N1ICD-NLuc. The subsequent interaction of N1ICD-NLuc with CLuc-RBP reconstitutes luciferase. The amount of NICD released is proportional to the light produced. (B) and (D) show LCI results for 10 independent cell lines in the presence of either co-cultured ligand-expressing cells (CHO-Dll1 and CHO-Jag1) (B) or 100µM EGTA (D) (* denotes p<10^{-6}, Student T-test). (C) The stability of N1ICD-NLuc fragments released from N1 and N21 fusion proteins, which differ by 6 amino acids at N-terminus (VLLSRK and VIMAKR, respectively), was determined by the luminescence lifetime measurements after blocking NICD-NLuc release with the γ-secretase inhibitor DAPT. Thick lines in (C) and (D) represent the average of N1 and N21 cell lines. All scale bars represent standard deviation. See also Figure S5.

Figure 6. Jag1 is the dominant ligand of N2 in the kidney. (A-D) Comparison of N1, N2, Dll1 and Jag1 expression in the developing nephron. (E-P) Phenotypes of newborn kidneys after ligand deletion. Scale bars: (A-D, M-P), 10µm; (E-L), 500µm.

Figure 7. Modulation by Lfng contributes to the dominance of N2 in the developing kidney. (A-C) In situ hybridization of three fringe genes in the developing kidney. (D) Effects of Lfng modification on Notch1 and Notch21 activation in HEK293 cells (* denotes p<0.05, Student T-test). All scale bars represent standard deviation. (E-H) Comparison of the labeling pattern between N2::Cre^{LO} (E) and N1::Cre^{LO} (F-H) in vivo in developing nephrons. Arrowheads denote endothelial cells. (I) A model proposing a NICD-dependent switch that regulates proximal nephron development and explaining how N2 achieves its dominant roles over N1. See
discussion for details. The weight of lines indicates the weight of effects. Scale bars: (A-C), (G-H) 10µm; (E, F) 500µm.

**Figure S5** (related to Figure 5): N21-NLuc and N1-NLuc cell lines have similar total (B) and surface (C) receptor levels. Cell surface proteins were isolated via surface protein biotinylation followed by pull down with streptavidin beads. (A) The lack of nuclear protein CLuc-RBP (film over-exposed on purpose) and the enrichment of the membrane protein Na+/K+ ATPase α1 chain serve as controls for the surface biotinylation process. (B) β-actin and (C) transferrin receptor were used as loading controls for total and membrane proteins, respectively. Three independent N2-1-NLuc and three independent N1-NLuc cell lines derived from the same CLuc-RBP parental cell line were randomly chosen. Similar results were obtained for another six cell lines derived from a different CLuc-RBP parental cell line (data not shown). All scale bars represent standard deviation. (D) Anti-N1 and N2 ECD antibodies from Biolegend and eBioscience show similar affinity to each other. These antibodies were tested on N1-NLuc and N21-NLuc cell lines that expressed similar levels of receptors on the cell surface. Representative data from one set of cell lines are shown. Similar results were obtained from six pairs of cell lines.
Figure 1. Variation in the expression pattern of N1 and N2 does not explain their functional difference.
Figure 2. Generation of the N12 and N21 alleles.

A

Notch1

Notch12

Notch21

Notch2

Exon 28

FVGC GVLLSRK K RKRGRH

G38066C

G38128A

FILLGVIMAKRRRQ RH

G12501C

ICD

3'UTR

ECD

TMD

B

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C

Relative mRNA levels

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D

E17.5 Lfng-GFP Kidney

Notch1

Notch1/ GFP/ NCAM

F

Notch2

Notch2/ GFP/ NCAM

G

H

E13.5 Lfng-GFP Kidney

GFP/CK8/NCAM/DAPI

I

E13.5 Kidney GFP+ Cells

% of Max vs. 10^5

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<th>N2 ECD Ab</th>
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Figure 3. Notch ICDs can functionally replace each other and thus do not contribute to the functional difference of N1 and N2 in kidney development.

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**Gross Morphology**

**WT1 LTL**

**Podocyte (WT1)**

**Proximal Tubules (LTL)**

(Enlarged)

| Nephron # Per kidney at P28 (n=6) | 9860 (±853) | NA | NA | NA | 9628 (±900) |

**Summary**

(WT1/LTL)
Figure 4. Comparison of the Total and Surface Levels of N1 and N2 in Developing Nephron Epithelia.
**Figure 5.** N2 ECD is more potent than N1 ECD in mediating ligand-induced ICD release.
Figure 6. Jag1 is the dominant ligand of N2 in the kidney.
Figure 7. Modulation by Lfng contributes to the dominance of N2 in the developing kidney.
Figure S5 (related to Figure 5): N21-NLuc and N1-NLuc cell lines have similar total (B) and surface (C) receptor levels.
CHAPTER V

Conclusions and Future Directions
In this thesis, I presented work on three projects, each examining a different aspect of kidney development. Although seemingly independent, the earlier projects have helped me to narrow down the focus of my research and prepared me with the knowledge and tools to answer the later questions.

My work started with the project described in Chapter IV, where I examined the mechanistic basis for unequal role of Notch 1 and Notch 2 in the developing nephron. By establishing an in vitro system that allows me to quantify Notch signaling strength, I discovered that the dominant role of Notch2 maps to its extracellular domain (ECD), with N2 ECD being more efficient in release of Notch intracellular domain (ICD) upon ligand binding. I further showed that this difference in NICD release is likely determined by the negative regulatory region (NRR) on the ECD, where subtle differences in NRR unfolding contribute to the higher activation probability of N2 ICD. Together with our in vivo analysis, this data provided important mechanistic insights into the phenomenon of Notch paralog dominance in the developing kidney.

With a clear result and limited immediate leads from this first study, I turned to another interesting but less well characterized process in the developing kidney, that is the regulation of the self-renewal and differentiation of nephron progenitors. Our lab has shown that FGF9 and FGF20 are necessary for maintaining nephron progenitors in vivo. In my second project described in Chapter II, I extended this finding by showing that FGF9 and FGF20 are sufficient to rescue nephron progenitors from undergoing apoptosis in vitro. Moreover, I found that FGF9 are able to act synergistically with BMP7 to support FACS purified nephron progenitors to proliferate while maintaining their differentiation potential for up to 5 days.
In an effort to extend the our culture beyond 5 days, I realized that the limit we and many others face may not simply indicate the need of additional growth factors in the culture media, but instead reflect some intrinsic properties of the nephron progenitors themselves. This led to a more in-depth analysis of the transient nature of nephron progenitors as described in Chapter III. Here I report the establishment of a kidney progenitor assay system that allowed me to evaluate the self-renew and differentiation capacity of CM upon transplantation. Using this system, I discovered that nephron progenitors isolated from different developmental stages display differential proliferation rate, adhesion properties and their ability to remain in the niche is inversely correlated with age. Importantly, an unbiased transcriptome profiling of nephron progenitors at the single cell level revealed distinct transcriptional signature of different age groups, further supporting an intrinsic difference.

Together, these results suggest that cell intrinsic changes occur before the cessation of nephrogenesis and actively regulate to the decision of nephron progenitors to stay or exit the niche. These changes must be understood for prolonging nephrogenesis in vivo or realizing the goal of culturing these cells in vitro or prolonging nephrogenesis in vivo.

**Future directions**

1. Defining genes that drive age related changes in nephron progenitors

   Our preliminary analysis of the single cells RNA sequencing data has revealed many genes and that are differentially expressed in young and old nephron progenitors. An important next step is to identify which ones of these genes are functionally important for nephron progenitor aging.
We are currently in the progress of performing more rigorous bioinformatics analysis to further tease out real signal from technical noise, which is one of the major challenges in single cell RNAseq analysis. We are also fortunate to obtain 37 E12.5 Six2+ single cell RNAseq data from Dr. Steve Potter. Added to our current set, this will help to increase our temporal range and better identify genes with functional importance. After all, the biggest difference in engraftment was observed between E12.5 and P0 cells. Similar to previous experiments, we will perform GO term analysis to identify functionally relevant gene/pathways that are changed from E12.5 to P0.

Once this is complete, we will further narrow down our list by filtering genes that trend with age. Since the functional readout reported a gradual decline in the ability of nephron progenitors to remain in the niche, we reasoned that genes/pathways that change in this same pattern, either decreasing or increasing over time, are more likely to be functionally relevant. In fact, one candidate gene that fits these criteria has already emerged from our initial analysis. By screening a list of nephron progenitor marker genes, we found that Osr1, one of the earliest key transcription factors required for the specification and maintenance of nephron progenitors gradually declines over time in the Cited1+ population. With Osr1 conditional gain- and loss-of-function mouse available (Osr1 f/f and Rosa-STOP-Osr1, Rulang Jiang lab, CCHMC), we could use our transplantation system to test if Osr1 is necessary and/or sufficient to regulate the niche retention capacity of nephron progenitors. If Osr1 indeed promotes CM cells to remain in a progenitor state, we will then follow up by identifying its downstream targets genes. This will be achieved by filtering genes that (a) expression trend with Osr1 and (b) contain Osr1 binding motif in their enhancers and (c) change in expression level when Osr1 level is decreased or increased. Moreover, it would be interesting to test if these enhancers as well as Osr1 itself were regulated on the chromatin level, as it has been one of the major signatures that came out of our
analysis. By combing FAIRE (Formaldehyde-Assisted Isolation of Regulatory Elements) and qPCR, we could find out if the enhancer of particular gene is in an open (active) or close (inactive) state. Other candidate genes coming out of our in silico analysis could be analyzed in a similar fashion. Together, these efforts will help us gain a better understanding of the cell intrinsic regulatory network that is critical for nephron progenitor lifespan.

2. Define rejuvenation associated changes

Our initial results showed that age-dependent changes precede the cessation of nephrogenesis. We also observed that old cells could remain in the niche if they form cell-cell contacts with younger neighbors, but exit from the niche if in contact with older neighbors. This could reflect a pre-existing variation among the progenitors or a progressive change brought about by the young environment (rejuvenation). An interesting future aim would be to distinguished between these mechanisms. Our preliminary experiments have shown it is feasible to isolate single old progenitors after they are injected (data not shown). Therefore, a time-course single cell RNAseq experiment on old cells isolated from cultures 1, 2, 3 and 4 days after injection would likely be informative. A pre-existing signature would further confirm the heterogeneity we uncovered in our preliminary analysis and should be functionally tested to confirm its requirement for better engraftment. If rejuvenation is an acquired trait, then we will need to definite what are the genes that were changed in this process and whether we could manipulate these genes to prolong the lifespan of nephron progenitors.

3. Broader use of the new nephron progenitor assay
The kidney field lags behind other stem/progenitor field in the lack of a definitive progenitor assay (Hendry et al., 2011). Nephron progenitors could only be cultured for a limited amount of time in vitro precluding the evaluation of their self-renewal capacity (Barak et al., 2012; Dudley et al., 1999; Lusis et al., 2010). Moreover, differentiation assay often employ exogenous tissues or injured environment where inappropriate context only reveal some but not the full potential of these nephron progenitors (Bussolati et al., 2005; Gupta et al., 2006). The new progenitor assay I reported here is the first functional assay system that allows one to assess the self-renewal and differentiation of nephron progenitors in a native environment. Not only can we use this system to compare cells of different age and origin, but also to interrogate the function of individual genes and their role in regulating nephron progenitors. Traditional genetic methods mutating gene of interest often lead to the collapse of the entire niche due to the fact that these progenitors are part of their own niche. This current method, however, allows one to test gene functions in an intact niche.

Another application is related to adopting nephron progenitors for therapeutics uses. Although this population does not persist in the postnatal kidney, recent successes in directed differentiation of human iPS cells to the nephron progenitor phenotype represents an attractive possibility for the creation of a cell source either able to elicit de novo nephrogenesis or be differentiated further into multiple renal cell types (Hendry et al., 2013; Taguchi et al., 2014). A common difficulty in these experiments is to define when during a nephron progenitor state is reached and even more challenging, how to maintain nephron progenitors in such a state, all of which could be evaluated by using our transplantation system.
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