Control of Lineage Decisions in the Embryonic Kidney by Hedgehog Signalling

by

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A thesis submitted in conformity with the requirements for the degree of Master of Science
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Abstract

Foxd1-expressing cells give rise to stromal components in the mammalian embryonic kidney, and are the major source of myofibroblasts during renal fibrosis. The signalling pathways involved in segregation of the stromal and nephrogenic lineages from their common precursor cells is not fully understood. Previous studies in the Rosenblum lab have shown the role of HH signaling in expansion of cortical stromal gene expression and ureteropelvic junction development. I present a stage-specific role for HH signalling in early renal precursors expressing Sall1 and Osrl in promoting stromal cell specification without affecting the nephrogenic lineage. Temporal deletion of PtcI in Sall1+ and Osrl+ cells results in an early increase in stromal cells, disorganization of the cortical stroma, medullary localization of cortical stromal cells, decreased nephron number, and no change in cell proliferation. Mutant kidneys exhibit UPJ obstruction at birth caused by ectopic cortical stromal cells in an activated state of HH signalling.
Acknowledgements

“The same wind that uproots trees makes the grass shine.
The lordly wind loves the weakness and the lowness of grasses.
Never brag of being strong.
The axe doesn’t worry how thick the branches are.
It cuts them to pieces. But not the leaves.
It leaves the leaves alone.” — Rumi, The Essential Rumi

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List of Abbreviations

Anterior-Posterior ........................................................................................................................... AP
Cap Mesenchyme ............................................................................................................................. CM
Chronic Kidney Disease ................................................................................................................... CKD
Congenital Obstructive Nephropathy .............................................................................................. CON
Cubitus Interruptus ............................................................................................................................ Ci
Desert Hedgehog .............................................................................................................................. Dhh
End Stage Renal Disease .................................................................................................................. ESRD
Epithelial To Mesenchymal Transition ............................................................................................ EMT
Fluorescence Activated Cell Sorting ................................................................................................ FACS
Glial Cell-Derived Neurotrophic Factor ........................................................................................... GDNF
Hedgehog .......................................................................................................................................... HH
Indian Hedgehog .............................................................................................................................. IHH
Intermediate Mesoderm ................................................................................................................. IM
Lateral Plate Mesoderm ..................................................................................................................... LPM
Lim Type Homeobox 1 ....................................................................................................................... Lhx1
Metanephric Mesenchyme ................................................................................................................. MM
Nephric Duct ....................................................................................................................................... ND
Odd Skipped Related Gene ............................................................................................................... Osr1
Paraxial Mesoderm ............................................................................................................................ PM
Patched ............................................................................................................................................... Ptc
Quantitative Real-Time PCR ........................................................................................................... qRT-PCR
Red Fluorescence Protein .................................................................................................................. RFP
Renal Vesicle ....................................................................................................................................... RV
Retinoic Acid ........................................................................................................................................ RA
Screted Frizzled-Related Protein ....................................................................................................... Sfrp
Smootherned ....................................................................................................................................... SMO
Sonic Hedgehog .................................................................................................................................... SHH
Tamoxifen ............................................................................................................................................ TAM
T-Box Transcription Factor 18 ........................................................................................................... Tbx1
Unilateral Ureteric Obstruction .......................................................................................................... UUO
Ureteric Bud ......................................................................................................................................... UB
Ureteropelvic Junction ....................................................................................................................... UPJ
Wolffian Duct ......................................................................................................................................... WD
Publication of Thesis Work

Published Book Chapter:


Chapter one of this thesis borrows from published work #1.
Chapter 1: Literature Review
1.1 **Kidney Development**

1.1.1 **Overview**

The mammalian urogenital system, including the kidneys, is derived entirely from the intermediate mesoderm (IM). The nephric duct (ND), also known as the mesonephric or Wolffian duct (WD), arises from the rostral IM and extends caudally towards the hindlimb to connect with the forming bladder (Little et al. 2012). ND-derived signals induce the formation of primitive renal tubules from the adjacent nephrogenic mesenchyme in a distinct temporal sequence along the anterior-posterior (AP) axis of the embryonic trunk, forming the pronephric and mesonephric tubules (Figure 1) (Vainio et al. 1989). These primitive excretory structures degenerate during mammalian embryonic life, with the remnants of the mesonephric tubules contributing to the male gonads.

The developmental potential of the rostral IM becomes progressively restricted while a caudal bean-shaped region of the nephrogenic mesenchyme commits to forming the metanephric mesenchyme (MM) at the hindlimb level (Figure 1) (Vainio et al. 1989). Beginning approximately at day 11 of embryonic life (E11), the MM initiates formation of the permanent functional kidney through a series of reciprocal signalling events with an ND-derived epithelial structure called the ureteric bud (UB) (Wellik et al. 2002). These interactions lead to formation of the collecting ducts, calyces, and pelvis, through the process of branching morphogenesis, as well as formation of the glomeruli and the renal tubules, through the process of nephrogenesis (Figure 1).
A. The pronephros (pro) and the nephric duct (ND) emerge from the cranial intermediate mesoderm. The nephric duct extends and inserts in the forming bladder. Caudal to the pronephros is the mesonephros (meso), which contributes to the male gonad. Metanephric mesenchyme (MM) is distinct by E10.5 and is adjacent to the caudal end of the ND where the ureteric bud (UB) emerges. Signals from the MM induce the UB to undergo a series of branching events to form the collecting system of the kidney B. The cells surrounding the UB are induced to condense and form an aggregate known as the condensing mesenchyme. A subset of cells within the condensing mesenchyme undergo a mesenchymal-to-epithelial transition (MET), and a series of morphological changes to from the renal vesicle, comma-shaped, and S-shaped bodies before maturing into nephrons.
1.1.2 Intermediate Mesoderm Specification and Patterning

Due to the lack of molecular markers specific to the IM at the early post-gastrula stage, defining the early IM remains an area of investigation. Odd-skipped related gene Osrl encodes a zinc-finger transcription factor expressed in the entire AP axis of the embryo and marks the prospective lateral plate mesoderm (LPM) (Figure 2) (Ranghini et al. 2015; James et al. 2006). Expression of Lhx1, a LIM-type homeobox gene, overlaps with that of Osrl in the LPM and is extended to the prospective IM (Dressler 2009; Tsang et al. 2000). Starting from E7.5-E8.75, expression of Pax2/8 is detected exclusively in a band of cells just lateral to the paraxial mesoderm (PM) (Bouchard et al. 2002). It was recently shown that in the absence of Pax2, IM cells assume a paraxial mesodermal pattern of gene expression, suggesting that Pax2 may be involved in defining the boundary between paraxial and intermediate mesoderm (Ranghini et al. 2015). Subsequent to Pax2 expression, Lhx1 becomes restricted to the IM and persists as the ND starts forming and elongating caudally towards the hindlimb (Tsang et al. 2000). Around the same time, Osrl expression becomes restricted to the mesenchyme surrounding the IM and the LPM-derivatives but is excluded from the ND itself (James et al. 2006).

Defects in formation, or absence of the ND in mice lacking either Lhx1, Osrl or Pax2/Pax8 highlight the critical importance of these genes in early specification and patterning of the IM (Bouchard et al. 2002). Yet, relatively little is understood about the hierarchical relationship and the regulatory organization among these genes. Comprehensive analyses of knock-out mice revealed that Pax2/8 form the core of a gene regulatory network together with the transcription factors Lhx1 and Gata3, such that following activation by Pax2/8, the regulatory molecules Gata3 and Lhx1 maintain each other's expression (Ranghini et al. 2015; Boualia et al. 2013; Grote et al. 2006).
Specification of the IM occurs not only along the AP axis, but also along the mediolateral axis of the embryo. Activation of the aforementioned IM-specific genes, particularly Pax2/8, is in part dependent on a balance between signals from the medial tissues, neural tube and somites in addition to signals from the more dorsolateral ectoderm (Reviewed in (Dressler 2009; Takasato et al. 2015). Although low concentrations of BMPs, and a proper balance of both Activin and Retinoic acid (RA) signalling are shown to induce IM at the anterior pole, it is still not clear whether the same set of signals also control IM induction at the posterior end (Kim et al. 2005; Obara-Ishihara et al. 1999). It has been proposed that a specific pattern of Hox gene expression, distinct from the established posterior Hox combination, primes the mesoderm to respond to IM inductive signals at the anterior boundary, which initiates expression of Lhx1, Pax2 and Pax8 (Preger-Ben Noon et al. 2009). By contrast, the Hox11 group of genes is needed to distinguish the posterior mesoderm from the more anterior mesonephric tissue (Wellik et al. 2002). Recent evidence suggests that differential Hox gene expression may contribute to early lineage decisions and AP patterning of the mesoderm through influencing epigenetic mechanisms, and thus the genomic accessibility of anterior and posterior targets of the IM-inducing signals (Soshnikova et al. 2009).
Figure 2 Specification of the Intermediate Mesoderm

Schematic of a flattened E8.5 mouse embryo (dorsal view). A. *Osr1* expression (blue) encompasses the lateral plate mesoderm and the presumptive IM, and extends more anteriorly and more laterally than *Lhx1* (yellow) in (B). B. *Lhx1* (yellow) is expressed just lateral to the paraxial mesoderm (pink). *Pax2* and *Pax8* expression (brown) has an anterior border at approximately the sixth somite, specifically marking the IM as it extends caudally. Following *Pax2* expression, *Lhx1* expression becomes restricted to the IM.
1.1.3 Specification of the Metanephric Lineage

As previously discussed, Osr1 is expressed in the IM before any of the other MM-regulatory genes. Osr1 expression is excluded from the ureteric epithelial lineage at E9.5, while it remains strongly expressed in the differentiating MM from E9.5-E15.5 (James et al. 2006). Moreover, generation of Osr1 knockout mice demonstrated the indispensable role of Osr1 in the initial formation of the MM. Expression of several key regulators of MM development including Six1/2, Pax2 and Sall1 are disrupted in the absence of Osr1, leading the newly formed MM to undergo apoptosis (James et al. 2006). In addition to Osr1, there are a number of genes expressed in the undifferentiated MM including Pax2, Six1, and Sall1, but they are all dispensable for the initial formation of the MM, and are instead required for its subsequent differentiation and/or survival (Gong et al. 2007; Brodbeck et al. 2004; Nishinakamura et al. 2001).

Analysis of transcription factor expression distinguishes two morphologically distinct populations in the induced MM (E11.5): a Six2+ nephrogenic mesenchyme, and an outer layer of Foxd1+ cortical stromal cells that emerge from the Osr1+ caudal IM (Figure 3B). These populations form two mutually exclusive progenitor compartments shortly after the onset of ureteric branching, as there is no compelling evidence suggesting contribution of Foxd1+ cell to the Six2+ pool or vice versa during mammalian kidney development (Mugford et al. 2009; Kobayashi et al. 2014). A lineage tracing study by Mugford et al. (2008) demonstrated the contribution of Osr1+ cells pulse labelled between E8.5- E10.5 to both Six2+ nephron progenitors and Foxd1+ stromal progenitors, while Osr1+ cells labelled at E11.5 onwards were restricted to the nephrogenic lineage (Mugford et al. 2008). Moreover, it was uncovered that Osr1+ cells give rise to Flk1+ endothelial progenitors, and are therefore known as the
multipotent progenitors of the kidney (Mugford et al. 2008). However, utilizing mice lacking OSR1 activity, OSR1 function was found to be crucial for the establishment of the nephron progenitor pool, but not for specification of the stromal lineage. These data suggest that although Osrl+ cells give rise to multiple lineages in the kidney, Osrl expression itself does not regulate lineage decisions within the IM (Mugford et al. 2008).

Investigating the origin of Osrl+ cells is key to understanding the origin of the MM. Until recently, all the different lineages involved in kidney development, including the nephrogenic and stromal progenitors, as well as the ureteric epithelium, were thought to originate from the elongated rostral IM (Dressler 2006). However, investigation of Gata3, a transcription factor expressed in the ND (between E8.5 to E10) and the ND-derived UB, challenged this notion (Figure 4B) (Grote et al. 2006). In the absence of Gata3, the rostral IM fails to elongate caudally and the UB fails to form, while the Pax2-expressing MM persists (Grote et al. 2006). These data suggest that the MM develops independently of the Gata3+ ND-derived UB lineage.

Inducible labeling of caudal IM cells expressing Eya1, a transcription factor essential for the initial formation of the MM, demonstrated the nephron-restricted fate of these cells, and no contribution to ND-derived structures (Figure2B) (Gong et al. 2007; Jinshu Xu et al. 2014). Brachyury (T) is a marker of immature caudal presomatic mesoderm (PSM) that persists in the posterior end of the embryo (Figure 4A). Strikingly, investigation of the fate of T+ cells labelled at E8.5 demonstrated the contribution of these cells to the MM. Labelled cells were not detected in the ND, providing further evidence that the MM arises from late caudal IM marked by T expression (Taguchi et al. 2014). Based on these data, it is now believed that caudal trunk progenitors that lose T expression and start expressing Osrl are the multipotent precursors of the stromal and nephrogenic lineages within the MM field (Takasato et al. 2015).
**Figure 3 Development of the Rostral and Caudal Intermediate Mesoderm**

**A.** A diagram illustrating the elongating anterior IM (orange) and the nascent T+ presomitic or posterior mesoderm (green) in an E8.0 embryo. **B.** Cross section of an E8.5 embryo at the position marked with a dashed line in A, illustrating the *Gata3*+ anterior IM (orange) and the newly formed *Eya1*+ posterior IM (green) derived from the T+ posterior mesoderm. The fate map of the anterior and posterior IM is shown from E8.5 to E11.5. The ND is derived from the elongating anterior IM by E9.5. The ND gives rise to the UB which invades the MM and starts branching at E11.5. *Osr1*+ precursors of the nephrogenic and stromal lineages are located in the forming MM derived from the posterior IM (green). *Tbx18*+ cells (pink) are located adjacent to the ND and the MM, and contribute to both stromal progenitors and the ureteric mesenchyme. At E11.5 *Six2*+ nephrogenic (green) and *Foxd1*+ stromal progenitors (red) (NP and SP, respectively) are specified within the *Osr1*+ progenitor pool.
1.1.4 Induction of Ureteric Bud Outgrowth and Branching Morphogenesis

Glial cell-derived neurotrophic factor (GDNF) is secreted by the MM adjacent to the dorsal ND, and triggers pseudostratification and invagination of the UB (Sainio et al. 1997). Gdnf expression is shown to be under positive regulation of several transcription factors (Eya1, Pax2 and Six1/4) expressed within the MM (Brodbeck et al. 2004). GDNF signalling occurs through the RET receptor tyrosine kinase (RTK) and its co-receptor GFRα1, both of which are expressed on the ureteric epithelia (Gong et al. 2007; Chi et al. 2009). Ret expression is necessary for initial UB formation as well as the subsequent branching morphogenesis, likely because GDNF-driven proliferation at the UB tips is required for the formation and growth of the ureteric branches (Costantini et al. 2006).

Since the receptors required for GDNF signalling are expressed on the entire length of the ND, spatial restriction of Gdnf signalling to the posterior domain of the IM is critical for specification of the UB site and proper UB outgrowth (Sainio et al., 1997). Foxc1 and Foxc2 encode two transcription factors in the uninduced MM which function to restrict cranial expansion of Eya1 and Gdnf signalling domain (Kume et al. 2000). Moreover, SLIT2 is a large secreted protein, strongly expressed in the anterior ND which mediates ROBO2 activation in the nearby nephrogenic mesenchyme (Grieshammer et al. 2004). This intracellular signalling system has been demonstrated to restrict Gdnf signalling to the posterior end through a yet unexplained mechanism (Grieshammer et al. 2004). In addition to the fairly well characterized feed forward mechanism of the interplay between RET and GDNF in initiating UB formation, BMP4 is expressed in the tail bud mesenchyme surrounding the ND and MM, and has been shown to promote UB outgrowth at the correct budding site relative to the ND (Miyazaki et al. 2000).
Once the nascent UB invades the MM, it undergoes a process of iterative branching that generates the urinary collecting system through reciprocal signalling interactions between the ureteric epithelial, nephrogenic and stromal mesenchymal lineages within the kidney. The developing ureteric tree can be segmented into ureteric tips and stalks (Figure 1A). Gdnf is expressed in the cap mesenchyme (CM) surrounding the ureteric tips and is a potent inducer of the Ret signaling pathway, which is essential for branching (Costantini 2010). Wnt11 is expressed in the ureteric tips and maintains Gdnf expression in the metanephric mesenchyme to mediate a feed-forward signaling mechanism, through a yet unknown mechanism (Majumdar et al. 2003; Cain et al. 2009). Ureteric branching requires that UB tips remain responsive to GDNF. β-Catenin (Ctnnb1) is expressed in the surrounding renal stroma and has been shown to mediate canonical Wnt signalling to maintain Ret expression in the UB tips and thereby, elicit GDNF responsiveness (Bridgewater et al., 2008). Furthermore, RA (vitamin A) signalling, via RA receptors RARα and RARβ2, is crucial for regulation of branching morphogenesis through up-regulation of Ret expression in the UB tips (Mendelsohn et al. 1999; Batourina et al. 2001). RA is synthesized from retinaldehyde in the cortical stroma and the UB by the enzymes RALDH2 and RALDH3, implying an additional non-cell autonomous role of stroma in promoting branching (Li et al. 2000; Mendelsohn et al. 1994; Rosselot et al. 2010). Several other signalling pathways including FGF as well as canonical and non-canonical BMP signalling are also required to regulate the branching process (reviewed in (Blake et al. 2014)). Yet, the integration and interplay of these various pathways is not fully understood.

In addition to branching morphogenesis, elongation of the epithelial ureteric trunk to the medullary region is essential for formation of a functional collecting duct where urine is concentrated and excreted into the ureter. The cortico-medullary axis of kidney organization and
function is regulated by Wnt7b signaling, whose expression is restricted to the elongating component of the collecting duct system (Yu et al. 2009). Removal of β-catenin from the underlying Wnt-responsive interstitium phenocopies the medullary deficiency of Wnt7b mutants, suggesting a paracrine role for Wnt7b action through the canonical Wnt pathway (Park et al. 2007). In addition to the canonical Wnt signalling pathway, WNT9b and WNT7b act through the non-canonical pathway to mediate the planar-cell-polarity (PCP) process responsible for reorganization and interdigitation of the ureteric epithelial cells, allowing for UB elongation (Karner et al. 2009; McNeill 2009).

1.1.5 Nephrogenesis

The elongating UB extends toward the source of GDNF, and meets the MM cells at around E10.5-E11.0 (Kopan et al. 2014). This induces MM cells to organize a cohesive domain of cells around the UB tips, called the Cap Mesenchyme (CM) (Figure 4). CM contains self-renewing nephron progenitors, characterized by expression of key transcriptional regulators including Six2, Cited1, Osrl and Sall1. Lineage tracing studies have established that Six2+ nephron progenitors contribute to podocytes as well as all the other epithelial cells of the mature nephron (Figure 4). Ex vivo kidney cultures treated with FGF-2 and FGF2-like proteins have demonstrated the importance of FGF signalling through the intracellular mediator RAS in establishment of the CM cells from their precursors in the posterior IM as well as in the self-renewal of nephron progenitors (Brown et al. 2011).

It is well established that a delicate balance of self-renewal and differentiation is required throughout embryogenesis to ensure the formation of the full complement of nephrons. Premature differentiation of CM cells upon deletion of Six2, or signalling factors such as Fgf9 or
Fgf20 leads to depletion of the progenitor pool, and kidney formation is halted (Kobayashi et al. 2008; Self et al. 2006). It has been proposed that relatively high levels of nuclear β-catenin together with reduced Six2 expression in Cited1-negative subset of nephron progenitor cells favors MET over self-renewal (Brown et al. 2013; Park et al. 2012). By contrast, high levels of Six2 in a transcriptional complex with members of the lymphoid enhancer factor/T cell factor (TCF/LEF) family of transcription factors, prevent differentiation induced by β-catenin (Park et al. 2012). Recent studies have uncovered the role of Sall1 upstream of Six2 to maintain an appropriate level of Six2 expression in the CM, so that only a subset of cells are committed to differentiation at each UB tip (Basta et al. 2014; Kanda et al. 2014). SALL1 also acts as a transcriptional repressor recruiting the Mi2/NuRD chromatin remodeling complex in differentiating nephrons to repress β-catenin transcriptional output, thus inhibiting premature differentiation (Ohmori et al. 2015; Denner et al. 2013). Additionally, it was recently demonstrated that OSR1 and SIX2 act synergistically to form a strong repressor complex with TCF/LEF to prevent the activation of WNT/β-CATENIN target genes, such as Wnt4 in the CM (Jingyue Xu et al. 2014).

Once a subset of CM cells at a new branch tip undergo MET in response to WNT9b-dependent signals from the UB renal vesicle (RV) forms at the “arm-pit” of the ureter (Carroll et al. 2005). Here, WNT9b acts through the canonical Wnt signalling pathway and is upstream of all the known markers of nephrogenesis, including Wnt4, and Lhx1 (Karner et al. 2011). Undifferentiated, GDNF-secreting CM surrounds the leading edge of the UB tip towards the kidney periphery, thereby stimulating continued branching (Figure 1B) (Park et al. 2007). In addition, Sall1 expression within the undifferentiated MM restricts Wnt9b expression to the ureteric stalk, to allow for nephron progenitor differentiation without exhausting the progenitor
pool and halting UB branching (Basta et al. 2014; Kanda et al. 2014). Subsequent differentiation of distinct segments of the intermediate structures involves cell proliferation, extensive tubular elongation, and proximal-distal patterning (Kobayashi et al. 2005; Karner et al. 2009; Little et al. 2012; Kanda et al. 2014). Once an RV is formed, its proximal portion elongates and fuses with the UB tip closest to its point of initiation to form the comma-shaped body (Figure 1). The distal portion of the comma-shaped body further elongates and forms the S-shaped body (Figure 1) (Georgas et al. 2009). The molecular mechanisms involved in segmentation and maturation of the S-shaped body into proximal, glomerular, and distal portions are not completely understood. It is known that Notch2 signaling in nephron progenitors is required for establishing proximal-distal patterning of the developing nephron, but is less important in differentiation of the glomerulus itself (Cheng et al. 2007).

1.2 Stroma

1.2.1 Cellular Organization and Function of Renal Stroma

In addition to the nephrogenic mesenchyme and ureteric epithelium, a third major cell type is present during early kidney organogenesis – the stromal cell population (Figure 3). A member of Forkhead transcription factors, Foxd1, is the first gene that distinguishes stromal cells from the nephrogenic mesenchyme as early as E10.5 (Figure 3). It was shown that Foxd1+ cells are first observed anterior to the MM and as development progresses, Hox10 mediates posteriorization and integration of these cells into the periphery of nephrogenic zone (Yallowitz et al. 2011). Foxd1+ cells are also observed interior to the nephrogenic zone, occupying spaces between ureteric bud branches and induced nephrons (Figure 4A). Until recently, the origin and interrelationship between these stromal components were poorly understood. However, fate-map analysis of
*Foxd1*+ cells established that this population contains multipotent self-renewing progenitors giving rise to renal capsule, cortical and medullary interstitial cells, mesangial cells, and pericytes of the kidney (Kobayashi et al. 2014; Hum et al. 2014). It is still not clear whether *Foxd1*+ population in the capsule and nephrogenic zone is a homogenous population or contains different domains with distinct differentiation capacities, similar to the subdomains in the CM (Mugford et al. 2009; Kobayashi et al. 2014).

The embryonic kidney is characterized by an inner medullary and outer cortical zone, both of which contain stromal elements derived from *Foxd1*+ stromal progenitors (Figure 4A). Stromal cells of the renal capsule envelop the kidney as a continuous layer of flattened cells, and strongly express *Sfrp1*, a secreted frizzled-related protein that antagonizes *Wnt4* signalling, preventing the self-renewing nephron progenitors from epithelialization (Levinson et al. 2005; Yoshino et al. 2001). Directly beneath the renal capsule lie round-shaped cortical stromal cells that intercalate and surround the induced nephrogenic structures (Figure 4A). In addition, vascular smooth muscle cells, pericytes, and mesangial cells are essential components of the glomerular and peritubular vessels surrounding endothelial cells throughout the kidney (Figure 4B). Pericytes and mesangial cells are involved in the glomerular vasculature response to various physical stimuli, and in the regulation of renal hemodynamics. Lastly, resident fibroblasts are located in both the outer nephrogenic zone and inner medullary region interspersed between tubules and nephrons, and contain supportive fibroblast cells and ECM components.
Figure 4 Structural Organization of the Prenatal Embryonic Kidney and Development of the Renal Stroma

Stromal cells are present in various locations in the prenatal kidney (E18.5). **A.** A schematic showing the outer nephrogenic zone and inner medullary zone containing stromal elements including cortical stroma (pink), renal capsule (dark purple) and medullary stroma (light purple). A glomerulus is outlined in the dotted black box. **B.** The glomerulus contains stromal derivatives including pericytes (pink), mesangial cells (brown) and endothelial cells of the renal vasculature (black). Abbreviations: NP = nephron progenitors, NZ = nephrogenic zone.
Stromal progenitors play essential roles in nephron progenitor differentiation through non-cell autonomous signalling pathways (Reviewed in (Li et al. 2014)). Homozygous deficiency of Foxd1 causes kidney capsule defects characterized by a thickened, histologically abnormal renal capsule, decreased expression of the stromal/capsule markers Raldh2 and Sfrp1, expansion of the nephron progenitor population, and perturbed nephrogenesis (Levinson et al. 2005; Hatini et al. 1996). It has been suggested that failure to form a discrete nephrogenic zone and juxtamedullary region (as illustrated in Figure 4A) in Foxd1 mutants is causative in delaying nephron differentiation (Levinson et al. 2005). In addition, a more recent study demonstrated the non-cell autonomous role of FOXD1 in BMP7-mediated transition of nephron progenitors to WNT-induced differentiating state by supressing Decorin, a small antagonist of BMP/SMAD signaling (Fetting et al. 2014). Furthermore, loss of capsular expression of Sfrp1, a secreted WNT inhibitor, leads to a slight increase in the number of glomeruli, suggesting that stromal Sfrp1 may also act to restrict WNT4-induced nephron progenitor differentiation to generate the appropriate number of nephrons (Trevant et al. 2008).

Reduced UB branching and abnormal branch patterning in Foxd1-deficient mice suggested a non-cell autonomous role of stromal signalling in regulating renal branching morphogenesis (Hum et al. 2014). Similarly, mice lacking Pbx1, a transcription factor expressed highly in the stroma, share phenotypic characteristics with Foxd1-deficient mice, demonstrating abnormal branching due to the expansion of Ret expression from the ureteric tip to the stalk (Schnabel et al. 2003). Since Foxd1 expression is not affected in Pbx1-deficient mice, it is likely that Pbx1 plays a role in renal branching morphogenesis and ureteric tip specification through mechanisms similar to but independent from Foxd1 (Schnabel et al. 2003). Cortical stromal RA, synthesized by the enzyme RALDH2, is also an important mediator of ureteric branching through Rarβ2-dependent
regulation of Ret expression in the UB. Rarβ2-deficient mice demonstrated a reduction in the number of branches in addition to a mis-patterned capsular layer similar to Foxd1 and Pbx1 deficient mice (Mendelsohn et al. 1999; Batourina et al. 2001). Although, as discussed above, many models of stromal disruption have been shown to display overlapping phenotypes (i.e. expanded nephron progenitor domain), little has been done to investigate coordination or integration of various signalling pathways (Bagherie-Lachidan, Reginensi, Pan, et al. 2015; Ohmori et al. 2015).

### 1.2.2 Developmental Origin of Renal Stroma

The molecular mechanisms underlying differentiation of the distinct stromal and nephrogenic lineages from the common progenitor lineage remain poorly defined. However, studies defining T-box transcription factor 18 (Tbx18) expression in the urogenital region have provided some insight into the spatiotemporal pattern of stromal lineage specification. Tbx18 was previously known as a marker of the undifferentiated ureteric mesenchyme. Careful analysis of Tbx18 expression at several developmental stages demonstrated that it is also present in a narrow band of cells between the mesenchyme surrounding the ND and the metanephros at E11.5 (Figure 3B) (Airik et al. 2006; Bohnenpoll et al. 2013a). Lineage tracing of Tbx18+ cells in addition to the spatial localization of Tbx18+ cells demonstrates a contribution of a spatially restricted population of Tbx18+ cells to the descendents of the Foxd1+ lineage (Bohenepoll et al. 2013b). Hence, it was postulated that at E10.5, in addition to the already specified Foxd1+ and Six2+ committed lineages, there are uncommitted Tbx18+ cells within the Osrl+ MM that can give rise to either stromal or ureteric mesenchymal lineages. According to Bohnenpoll et al (2013), it is likely that Tbx18+ cells that do not differentiate into the mesenchymal cell types of
the ureter lose $Tbx18$ expression between E10.5-E11.5 either die or contribute to the renal stroma through an as yet undefined mechanism (Bohnenpoll et al. 2013b). However, the mechanism by which lineage decisions are made within the common $Osr1+$ IM to separate nephrogenic and ureteric mesenchyme/stromal progenitors remains to be determined.

Studies in the chick and mouse embryo suggest the existence of a distinct progenitor of renal stromal cells residing outside the IM. Retroviral gene transfer techniques followed by fate-mapping analysis in the chick embryo identified the adjacent PM as the principle source of the renal stroma. This was evident by localization of $\beta$-gal+ cells to the $Foxd1+$ stromal zone of the kidney only when $LacZ$-encoding retrovirus was injected to the PM (Guillaume et al. 2009). Moreover, a recent high-throughput gene expression analysis in mice lacking $Pax2$ provided indirect evidence that lower $Pax2$ expression in the mammalian PM might govern the contribution of the PM to the renal stroma (Ranghini et al. 2015). When $Pax2$ is deficient in the IM, IM cells are characterized by a pattern of gene expression that is more consistent with the PM, and genes normally expressed in common derivatives of $Foxd1+$ progenitors are upregulated (Ranghini et al. 2015). Therefore, it was hypothesized that after $Osr1$ expression is restricted to nephron progenitors at E11.5, new stromal progenitors likely arise from the PM, and migrate to the induced MM. Yet, to date, there has been no direct in vivo evidence demonstrating the contribution of PM cells to the renal stroma.

In summary, the published body of work strongly suggests that a $Osr1+$ progenitor pool derived from the $T+$ PSM has the potential to give rise to both stromal and nephron progenitors. Further studies have shown the presence of a subpopulation of $Osr1+$ cells expressing $Tbx18$ that contributes to both ureteric mesenchyme and the renal stromal lineage. It is also likely that stromal progenitors are a heterogeneous population of cells, a subset of which are derived from
the PM. Nevertheless, the molecular mechanisms and signalling pathways regulating the separation of the stromal lineage from either nephrogenic lineage or ureteric mesenchyme has remained poorly understood.

1.2.3 Contribution of the Stroma to Renal Pathologies

1.2.3.1 Horseshoe Kidney

During normal renal development kidneys flank the midline of the embryo before separating from the body wall, rotating and then separating from each other (Fotter 2008). Kidneys maintain their connection to the dorsal body wall through a thin layer of connective tissue until approximately E14.5 (Levinson et al. 2005; Kobayashi et al. 2014). In Foxd1-null mutants where the capsule does not form properly, kidneys remain attached to the body wall and do not ascend to the lumbar region (Hum et al. 2014; Hatini et al. 1996). Given the strong Foxd1 expression in the connective tissue surrounding the kidney and the renal capsule, it is plausible that FOXD1-mediated capsule formation results in kidneys detaching from the dorsal body wall (Levinson et al. 2005). A similar phenomenon occurs in human patients where failure of proper kidney ascension results in ‘horseshoe kidneys’ with an incidence rate of one in 400-600 live births. Horseshoe kidneys are fused at the lower pole, forming a U or horseshoe shape. Failure of ascension also results in the ‘pelvic kidney’ in which the kidney fails to ascend from the pelvis (Nemes et al. 2015; Weizer et al. 2003). In some cases the horseshoe kidneys can present with ureteropelvic obstruction leading to urinary tract infections, abdominal mass, and hematuria (Rodriguez 2014).
1.2.3.2 Renal Fibrosis

Chronic kidney diseases (CKD) regardless of their primary cause, lead to the loss of renal function, and are histologically characterized by fibrosis where functional renal tissue is replaced by permanent interstitial fibrotic tissue (Boor et al. 2012). Fibrotic scar tissue disrupts normal organ structure and hinders regeneration and normal function. The key characteristics of renal fibrosis are the extensive deposition of ECM and expansion of a distinct α-SMA+ population of fibroblasts called myofibroblasts (Grgic et al. 2014; Falke et al. 2015). By definition, myofibroblasts are contractile cells that are the principle source of interstitial collagens, including fibrillar collagens I and III (Grgic et al. 2014).

Studying the exact origin of myofibroblasts during CKD can have implications for anti-fibrotic therapies, but is also challenging due to the lack of markers specific to myofibroblasts. In vitro studies initially suggested that myofibroblasts form via transition of epithelial cells to a mesenchymal phenotype, a process termed epithelial to mesenchymal transition (EMT) (Cheng et al. 2010; Iwano et al. 2002). Expression of αSMA has been detected in tubular and glomerular epithelia in association with disease progression, in both a remnant kidney model and during experimental glomerulonephritis (Iwano et al. 2002). However, lineage tracing studies using an epithelial cell specific γ-glutamyl transpeptidase (GT)-driven GFP and Cadherin(Cdh)16 promoter in a model of obstructive nephropathy failed to detect the contribution of epithelial cells to the myofibroblast pool, raising doubt as to the epithelial origin of the myofibroblasts (Li et al. 2010; Iwano et al. 2002). More recently, studies by Humphreys et al (2010) reported a marked expansion of pulse labelled interstitial cells that expressed α-SMA, following either complete unilateral ureteric obstruction (UUO) or ischemia-reperfusion injury (Grgic et al. 2012; Humphreys et al. 2010). In fact, an elegant lineage-tracing analysis by Humphreys et al (2010)
revealed that majority of myofibroblasts originate specifically from Foxd1+ derived pericytes (Humphreys et al. 2010). Interestingly, lineage tracing of both nephrogenic and UB-derived elements using Six2Cre and Hoxb7Cre demonstrated no contribution to myofibroblasts in both UUO and IR model of fibrosis, contradicting the initially thought contribution of EMT process to the myofibroblast pool (Humphreys et al. 2010).

Although Foxd1+ stromal progenitors are potentially important contributors to the fibrotic response, the factors regulating the differentiation of pericytes and interstitial fibroblasts to myofibroblasts in response to injury are not clear. Recent studies suggest that activated PDGFR signalling in response to injury leads to proliferation and differentiation of the pericytes to myofibroblasts, and that TGFβ1 may be an important contributing cytokine in transducing activated PDGFR signalling (Chen et al. 2011). Moreover, it is shown for the first time in an in vivo model of fibrosis that anti-PDGFR antibody administration markedly decreases the number of α-SMA+ myofibroblasts (Chen et al. 2011). Contrary to the aforementioned findings, other studies using more mature pericyte markers, PDGFRβ- and NG2- promoter–driven mice to functionally deplete pericytes in models of kidney injury did not result in any significant reduction in fibrosis (LeBleu et al. 2013). Similarly, lineage tracing of NG2+ and PDGFRβ+ pericytes did not show a predominant contribution of labeled cells to the myofibroblast population in the fibrotic kidney (LeBleu et al. 2013).

New evidence has implicated Hedgehog (HH) signalling as a major contributor to the fibrotic response. It was shown that pericytes express HH effectors Gli1 and Gli2, and that Gli1+ pericytes proliferation is greatly enhanced (11-fold greater) during fibrosis (Fabian et al. 2012). Further evidence to support HH signalling as a key contributor to fibrosis was shown using lineage tracing of Gli1+ cells in the kidney after acute injury (Kramann, Schneider, et al. 2015).
Genetic ablation of tissue-resident Gli1+ mesenchymal stem cells protected against injury-induced renal fibrosis (Kramann, Schneider, et al. 2015). It was further shown that GLI2 inhibition prevented myofibroblast progression and limited the fibrotic response (Kramann, Fleig, et al. 2015). These data suggest a key regulatory role for GLI2 in inducing myofibroblast formation from pericytes in response to injury. Further studies are needed to fully delineate the mechanism and interactions of HH signalling in the fibrotic response.

Overall, there are discrepancies concerning the origin of myofibroblasts in CKD most likely due to the limitations in the available studies, including the use of overlapping markers, different mouse strains, choice of injury model and the duration and severity of the injury affecting the outcome (Falke et al. 2015). A thorough and detailed analysis of the lineage and signalling mechanisms of the stroma may allow for a greater understanding of the functional significance of this population. These new insights could provide a foundation for the development of more effective treatment options and outcomes of fibrosis in patients.
1.3 Hedgehog Signalling

1.3.1 Hedgehog Signalling Overview

Hedgehog family of proteins are a family of secreted molecules that play an evolutionarily conserved role in patterning and development of many metazoans. Hedgehogs were first identified in the fruit fly *Drosophila melanogaster* (Chen et al. 1996). It is now established through an extensive array of studies that HH is also important in vertebrates for fundamental processes such as patterning the left-right body axis, AP skeletal patterning, angiogenesis, hematopoiesis, chondrogenesis, and development of the cerebellum, craniofacial structures, limbs, lung, kidney and gastrointestinal tract (Reviewed in (Ingham et al. 2001; Briscoe 2013)).

*Drosophila* HH ligand binds to its receptor, Patched (*Ptc*), on the plasma membrane, relieving PTC-mediated inhibition of Smoothened (*Smo*), a transmembrane protein expressed on the cell surface (Hooper et al. 1989; Rohatgi et al. 2007). In the presence of HH, SMO interacts with a molecular complex consisting of Costal-2 (*Cos2*), Fused (*Fu*), and Suppressor of Fused (*SuFu*). Interaction of SMO with COS2 confers FU activity and SUFU inactivity (Méthot et al. 2000). In this state, full-length Cubitus interruptus (*Ci*), the *Drosophila* GLI ortholog, translocates to the nucleus where it acts as a transcriptional activator (Jiang 2006). In the absence of HH, PTC inhibits the interaction between SMO and COS2, allowing COS2 to bind and retains Ci in the cytoplasm. The presence of active SUFU in the COS2-FU-SUFU-Ci complex permits cleavage of Ci to its N-terminal form, which localizes to the nucleus and acts as a transcriptional repressor (Galimberti et al. 2012).

In vertebrates there are three different homologs of Ci protein – GLI1, GLI2 and GLI3 – which are members of the glioblastoma family of transcription factors and contain zinc finger DNA binding domains to activate and repress transcription of target genes (Figure 5) (Mo et al.
Vertebrate GLI proteins are regulated through similar cleavage and truncation processes as Ci protein in *Drosophila*. Moreover, a dynamic interplay between GLI activators and repressors is critical during mammalian organogenesis (Buttitta et al. 2003; Hu et al. 2006). GLI1 and GLI2 exist predominantly as full length proteins in cultured mammalian cells and act as transcriptional activators during murine embryogenesis (Bai et al. 2002). In contrast, GLI3 exists primarily in its N-terminal truncated form which acts as transcription repressor; thus, the predominant activity of GLI3 is to repress expression of HH dependent genes (Bai et al. 2002; Park et al. 2000). Analysis of mice genetically engineered to express the truncated form of GLI3 has demonstrated the pathogenic role of constitutive GLI3 repressor activity during murine embryogenesis (Grotewold et al. 2002). In support of a deleterious role of GLI3R in embryogenesis, studies have reported that genetic elimination of Gli3 in the Shh-null background rescues HH mutant phenotypes in embryonic tissues including neural tube, limb, face, forebrain skin, and kidney (Litingtung et al. 2000; Litingtung et al. 2002; Rallu et al. 2002; Mill et al. 2005; Hu et al. 2006).
Figure 5 Canonical Mammalian Hedgehog (HH) Signalling.

In the presence of HH ligand, SMO translocates to the primary cilium. Activation of SMO inhibits the proteolytic processing of GLI proteins and allows full length GLI proteins to enter the nucleus to activate transcription of HH target genes. GLI1 and GLI2 have predominant activator roles while GLI3 acts as the main transcriptional repressor of HH signalling. In the absence of HH ligand, the receptor PTC represses the activity of the transmembrane protein SMO and its localization to the primary cilium. Full length GLI proteins (GLI FL) associate with the proteolytic complex, which promotes the proteasome-dependent processing or complete degradation of the GLI transcription factors. Truncated forms of GLI proteins (GLI R) translocate to the nucleus and act as transcription inhibitors.
1.3.2 Hedgehog Ligand in Vertebrates

Unlike the single HH gene found in Drosophila (Hh), three different HH genes have been found in vertebrates. These include Desert hedgehog (Dhh), Indian hedgehog (Ihh), and Sonic hedgehog (Shh) (Astorga et al. 2007). Diverse expression patterns of these three HH genes results in their specialized functions via signalling pathway highly similar to that in Drosophila signalling pathway. During early embryogenesis, the role of Dhh is largely restricted to germ cell development, as it is expressed in Sertoli cells in the testes and granulosa cells in the ovaries (Yao et al. 2002). Shh is the most broadly expressed homolog and plays a well-defined role in patterning of the neural tube, as well as in limb bud, olfactory pathway, eye, heart, lung and gut development (Ingham et al. 2001; Jr 2010). Ihh, the most closely related homolog to Shh, is expressed in the primitive endoderm and plays a role in bone and skeletal development.

Of the three mammalian HH members, only Shh and Ihh expression has been identified in the embryonic kidney (Fabian et al. 2012). SHH is found in the urothelium and the presumptive ureter as early as E11.5, and in the distal collecting duct at E14.5 (Yu et al. 2002; Hu et al. 2004). In newborn kidneys, Shh continues to be expressed in the collecting duct in addition to the inner medulla, and renal pelvis (Fabian et al. 2012; Hu et al. 2004). On the other hand, Ihh expression is reported to be restricted to the nephron epithelia in the cortex and outer medulla of the newborn kidney (Yu et al. 2002; Fabian et al. 2012).

1.3.3 Patched Receptor in Vertebrates

*Patched* (Ptc) in *Drosophila* encodes a transmembrane receptor protein containing 12 hydrophobic membrane-spanning domains, with two large hydrophilic extracellular domains where HH ligand binding occurs (Figure 5) (Zaphiropoulos 2004). In zebrafish, mice, and
humans two \textit{Ptc} homologs (\textit{Ptc1}, \textit{Ptc2}) are found, both of which are direct transcriptional targets of HH, and as in \textit{Drosophila}, act to repress the HH signalling pathway (Goodrich et al. 1997; Pearse et al. 2001; Lewis et al. 1999). PTCH1 and PTCH2 proteins share 56\% aminoacid similarity, with the key difference being that PTCH2 is more stable due to a truncated C-terminus (Holtz, K. A. Peterson, et al. 2013). While \textit{Ptc2} expression is not reported in the embryonic kidney, \textit{Ptc1} is detected in the epithelium of the presumptive ureter and the distal collecting ducts as early as E13.5 (Fabian et al. 2012; Cain et al. 2009). Interestingly, unpublished data from Joshua Blake (Previous Graduate Student, Rosenblum Lab) has also demonstrated \textit{Ptc1} expression in the capsular and cortical stroma at E15.5 in mice expressing a \textit{Ptc1}LacZ reporter.

Whether the vertebrate \textit{Ptc1} and \textit{Ptc2} genes serve distinct roles in HH regulation, particularly during embryonic development is not fully understood. Due to the overlapping expression of \textit{Ptc2} and \textit{Dhh} in the testis, it was previously thought that PTCH2 might interact with DHH to control germ cell development. However, germline deletion of PTCH2 demonstrated only a relatively mild phenotype, with the mutants being viable and fertile, suggesting that PTCH2 is dispensable for embryonic development (Zaphiropoulos 2004). In contrast, PTCH1 deletion in mice leads to embryonic lethality at E9.0-10.5, and the embryos were found to have open and overgrown neural tubes (Nieuwenhuis et al. 2006). Interestingly, the combined loss of PTCH1 and PTCH2 results in a yet more significant phenotype and expansion of HH signalling activity domain, indicating that PTCH1 and PTCH2 are not completely redundant (Holtz, K. A. Peterson, et al. 2013). Recent \textit{in vitro} evidence showed that \textit{Ptc1}-null cells remain partly dependent on SHH for activation of the SHH response, supposedly
due to SHH signalling via Ptc2 (Alfaro et al. 2014). However, there is no compelling in vivo evidence in support of the compensatory role of Ptc2 in the absence of Ptc1.

### 1.3.4 Advances in Defining the Role of Hedgehog Signalling in Kidney Development

Mutations that are predicted to generate a truncated protein similar in size to GLI3 repressor have been reported in humans with Pallister-Hall Syndrome (PHS). PHS is characterized by multiple CAKUT phenotypes with variable penetrance (Hall et al. 1980; Kang et al. 1997). Generation of several genetically engineered mouse models has provided a strong basis for understanding the importance of HH signalling in human diseases, particularly congenital renal diseases. Homozygous Gli3Δ699 mutant mice, which express only the truncated form of Gli3, exhibit features similar to human PHS characterized by multi-tissue abnormalities including renal agenesis or dysplasia, renal hypoplasia and hydronephrosis (Kang et al. 1997). Moreover, mice with mutations in the HH signalling pathway (ie. Shh-,Gli1-,Gli2-null mice), exhibit anomalies known to occur in VACTERL syndrome in humans characterized by dysplastic and pelvic kidney (Kim et al. 2001). Specifically, Shh −/− kidneys are characterized by sustained activity of GLI3-repressor and decreased levels of GLI1 and GLI2, such that upon genetic removal of Gli3 in Shh null background, kidney induction is restored (Hu et al. 2006). Together, these studies suggest the critical importance of SHH-GLI pathway in renal health and disease.

A growing body of literature has provided further insight into our understanding of the embryological mechanisms controlled by HH signalling during renal development. The spatial restriction of Shh and its receptor, Ptc1, to the distal ureteric epithelium suggests a role for HH signalling during branching morphogenesis. In fact, Shh mutation targeted to the ureteric lineage
(using *Hoxb7Cre*) causes hydronephrosis in adults, hydroureter and hypoplasia at birth (Yu et al. 2002). Further analysis of the mutants demonstrated that SHH promotes proliferation, establishment and maintenance of sub-epithelial ureteric mesenchyme adjacent to SHH-producing cells of the ureter, while it inhibits smooth muscle differentiation (Yu et al. 2002). Manipulation of HH activity through Ptc1- or Smo-deficient mice suggested that while SHH, emitted by the collecting duct, is not required in the medullary ureteric cells themselves, the absence of the signal is critical for proper ureteric tip-specific gene expression, and also functions in nephron induction and branching morphogenesis (Cain et al. 2009). Moreover conditional expression of GLI3R in nephrogenic mesenchyme and ureteric lineage using *Six2Cre* and *Hoxb7Cre*, demonstrated a cell-autonomous role of GLI3R in controlling self-renewal of nephron progenitors and regulating branching morphogenesis, respectively (Blake et al. 2015).

### 1.4 Ureteropelvic Junction (UPJ) Obstruction

#### 1.4.1 Ureteropelvic Junction Obstruction in Humans

Effective urinary transport from the kidneys to the bladder requires connectivity between the collecting ducts to the renal pelvis and from the pelvis to the ureter. Obstruction of the urinary tract during fetal development causes congenital obstructive nephropathy (CON), which is a common cause of chronic kidney disease (CKD) and end stage renal disease (ESRD) in children. Urinary tract blockage leads to hydronephrosis, a swelling of the kidney due to accumulated urine in the renal pelvis, which is detected by antenatal ultrasound in 1:100 to 1:500 pregnancies (Williams et al. 2007). The underlying etiologies of antenatal hydronephrosis are diverse in nature. While the majority of cases are non-obstructive a significant proportion of cases exhibit obstruction at the junction of the ureter and pelvis (Thom et al. 2013; Rodriguez
The gold-standard therapy for UPJ obstruction remains surgical excision of the strictured UPJ (Klein et al. 2011; Oliveira et al. 2016). Although not all cases of UPJ obstruction require surgical intervention, the ability to define which cases are self-resolving or will benefit from a surgical procedure remains elusive (Oliveira et al. 2016). Moreover, severe UPJ obstruction can result in irreversible modifications of the renal parenchyma (Zhang et al. 2000). Therefore, it is imperative to identify predictive markers for different clinical outcomes in UPJ obstruction and to develop curative non-invasive therapies.

1.4.2 Underlying Causes of UPJ Obstruction

Human renal biopsies of patients with intrinsic UPJ obstruction demonstrate dilation of the proximal and distal tubules and collecting ducts, chronic tubulointerstitial injury, glomerulosclerosis, fibrosis, and aberration of glomerular development (Ekinci et al. 2003). Classical analyses of neonatal UPJ tissues with intrinsic obstruction, attempting to determine developmental causes of UPJ obstruction, have associated defective UPJ with abnormal smooth muscle arrangement. Moreover, comparison of surgically removed obstructed UPJ specimens with autopsies from age-matched healthy cadavers revealed an increased index of smooth muscle apoptosis and a higher level of elastin and collagen in the obstructed UPJ tissue (Reviewed in Williams et al. 2007)). Yet, these studies do not determine whether smooth muscle apoptosis is primary or secondary to the obstruction. More recently, a number of genetic mutations (such as Tbx18 and ATGR2) that are likely to cause delayed apoptosis of the undifferentiated ureteric mesenchyme have been identified in association with UPJ obstruction (Vivante et al. 2015; Nishimura et al. 1999).

The two mouse models of obstructive hydronephrosis published to date are both characterized by developmental defects in urothelial and smooth muscle differentiation.
Urothelium-specific deletion of Sec10, the central unit of exocyst complex, causes disruption of urothelial cell differentiation and formation of the hydrophobic barrier in the ureter (Fogelgren et al. 2015). The resulting urinary damage to ureteric mesechencymal and smooth muscle cells was suggested to cause over proliferation of the surrounding cell population, eventually blocking urine outflow (Fogelgren et al. 2015). In a separate study, it was demonstrated that loss of the core component of TGFβ signalling pathway, Smad4, in the lower urinary tract mesenchyme initially results in disruption of smooth muscle differentiation, and lack of peristalsis (Yan et al. 2014; Vrljicak et al. 2004). This functional impairment was shown to progress into a permanent physical obstruction of the UPJ and hydronephrosis, due to a kink in the ureter and epithelial crowding.

Unpublished work performed by Lijun Chi (Previous Fellow, Rosneblum Lab) and Marian Staite (Previous Graduate Student, Rosenblum Lab) has uncovered a novel role of HH signalling in UPJ development in mice. HH signalling functions in MM were investigated by Rarb2Cre-mediated deletion of the HH cell surface receptor Ptc1 in the metanephric lineage including stromal and nephrogenic cells. The resultant increase in HH signalling activity in the MM produced an expanded cortical stromal domain marked by Foxd1 and Raldh2, and ectopic expression of stromal markers at the presumptive UPJ as early as E13.5 (Figure 6A-D). Moreover, an ectopic cell cluster expressing Ptc2, a homolog of Ptc1, causes physical obstruction of the UPJ and marked hydronephrosis prior to birth (Figure 6E-J). Remarkably, constitutive expression of GLI3 repressor in Ptc1-deficient background rescues stromal expansion in the outer cortex as well as the discontinuous UPJ phenotype, indicating the essential role of GLI3R in UPJ development (data not shown). On the contrary, Ptc2 deficiency in Ptc1-null background does not reduce the severity of the phenotype suggesting that Ptc2 does
not play a compensatory role to inhibit HH signalling activity. Together, these data demonstrate that elevated HH signalling in the MM results in increased in cortical stromal gene expression and prenatal UPJ obstruction caused by cells of stromal origin. These results raise the possibility that HH signalling acts in the precursors of cortical stromal cells to influence their specification and provide a basis for understanding the role of HH signalling in early lineage decisions as well as pathogenesis of obstructive hydronephrosis.
**Figure 6** Activated HH signalling in metanephric mesenchyme affects stromal gene expression and leads to obstructive hydronephrosis

In situ hybridization demonstrates that Foxd1 and Raldh2 mRNA expression domain is broader in the cortex of Ptc1 mutant kidneys (A-D). At E18.5, Ptc1 mutant kidneys exhibit a dilated renal pelvis and thinning of the cortex. Intrapelvic dye injections demonstrate a blockage of flow at E18.5 compared to WT, illustrating an absence of a patent lumen connecting the ureter and pelvis. (G-J). Beta-galactosidase staining at E13.5 using LacZ as a reporter of Ptc2 expression in WT and Ptc1 mutant kidneys shows ectopic Ptc2 expression between the ureter and the presumptive pelvis (E-F).
1.5 Concluding Remarks, Hypothesis and Objectives

Stromal cells are one of the earliest lineages specified in the mammalian embryonic kidney. Embryonic stromal progenitor cells are marked by expression of the transcription factor Foxd1 as early as E 10.5-E11.5, and play crucial roles in normal renal development. The published body of work strongly suggest that an Osr1+ progenitor pool derived from the T+ PSM has the potential to give rise to both stromal and nephron progenitors. Further studies have shown the emergence of a subpopulation of Osr1+ cells expressing Tbx18 that contributes to both ureteric mesenchyme and the renal stromal lineage. It is also likely that stromal progenitors are a heterogeneous population of cells, a subset of which is derived from the PM. The molecular mechanisms and signalling pathways regulating the separation of the stromal lineage from either nephrogenic or ureteric mesenchymal lineages has remained poorly understood.

Understanding the temporal pattern of specification, and identifying novel molecular markers of the common precursor population of the stromal and nephrogenic lineages is likely to lead to a more comprehensive view of the origin of stromal cells. Sall1, a zinc-finger transcription factor functioning downstream of Osr1, is highly expressed in both nephrogenic and stromal progenitors in the developing kidney (Osafune et al. 2006a; James et al. 2006). Sall1 is expressed in the posterior trunk mesoderm (ie. PSM), and was recently shown to be actively involved in the transcriptional network that regulates stem cell pluripotency during embryoid body formation (Buck et al. 2001; Karantzali et al. 2011). Yet, whether early Sall1 expression is restricted to any of the earliest committed populations or represents common precursors of stromal and nephrogenic lineages remains to be elucidated.

Moreover, previous work conducted in the Rosenblum lab demonstrated that constitutively active HH signalling during renal development (through Rarb2Cre; Ptc1$flox^-$)
results in an expanded stromal gene expression and prenatal UPJ obstruction caused by ectopically located stromal cells. However, Rarb2-Cre transgenic mice were generated using a 4.8kb Rarb2 sequence fused with Cre that does not faithfully mimic the endogenous activity of Rarb2 promoter (Kobayashi et al. 2005). Therefore, the exact origin of the stromal cell types observed in this model remained elusive. Delineating the spatial and temporal role of HH signalling in early renal development will help us gain further insights into the mechanisms by which stromal versus nephrogenic cell fate decisions can be modulated, and will in turn reveal how early activation of HH-GLI pathway leads to congenital UPJ obstruction.

Based on our previous unpublished data and the available literature, I hypothesized that HH signalling controls the specification of nephrogenic and stromal progenitors within the uncommitted multipotent precursor compartments marked by Osrl and Sall1. This hypothesis is examined here through the three objectives outlined below:

1. Determine the multipotent potential of Sall1+ cells prior to complete segregation of the stromal and nephrogenic lineages at the onset of ureteric branching.

2. Examine the time dependent effects of activated HH signalling in Sall1+ and Osrl+ cells on nephrogenic and stromal cell specification.

3. Define the contribution of stromal cells and HH signalling to the formation of UPJ obstruction in mouse and human models of the disease.
Chapter 2: Activated HH Signalling in Multipotent Renal Precursors Promotes Stromal Cell Specification
2.1 Introduction

Studies have identified an abundant presence of Sall1-expressing cells in both condensing mesenchyme, and nephrogenic structures (Nishinakamura et al. 2005). Colony formation studies have shown that isolated E11.5 Sall1+ cells give rise to all the tubular structures of the nephron in vitro (Osafune et al. 2006a). Although molecular markers of the stromal lineage (Foxd1 and Raldh2) were not detected in the colonies formed, data from microarray analysis on Sall1+ cells indicated expression of Foxd1 and Raldh2 in this population (Osafune et al. 2006b). Therefore, it is likely that Sall1+ cells are multipotent in nature and are not fully committed to the nephrogenic lineage prior to the onset of kidney development.

Osr1 is a definite marker of the nephrogenic mesenchyme with broader differentiation capacity, giving rise to stromal, nephrogenic and endothelial progenitors of the kidney, as well as the ureteric mesenchyme between E9.5-E11.5 (Mugford et al. 2008). Previous results generated by Marian Staite demonstrated that aberrant HH activity in the MM leads to the expansion of cortical stromal gene expression. Whether HH signalling activation specifically plays a role in perturbing the fate of the Sall1+ cells and/or their upstream precursors to become a stromal or nephrogenic progenitor requires further investigation.

Here I addressed the lineage of Sall1+ cells using a temporal fate-mapping approach. Cell fate analysis reveals the contribution of Sall1+ cells at E9.5 to all the stromal and nephrogenic cells, whereas Sall1+ cells at E10.5 are restricted to nephrogenic and capsular stromal cell fate. Temporal activation of HH signaling in Sall1+ cells and their upstream Osr1+ cells at E9.5 leads to an early increase in stromal cells, disorganization of the cortical stroma and ectopic presence of cortical stromal cells in the medulla. Since there is no change in the number of nephron progenitors or Six2 expression, our findings suggest a stage- and lineage- specific effect of HH
signaling in promoting stromal cell specification from their common precursor cells marked by either Sall1 or Osr1.
2.2 Experimental Methods

2.2.1 Mouse Models

Mice used in all experiments were housed at the Toronto Center for Phenogenomics (TCP) animal facility (Toronto, Canada). All experimental protocols using mice were approved by the Animal Committee at TCP and were carried out in accordance with the regulations of the Canadian Council on Animal Care (CCAC). For staging of embryos, the morning of vaginal plug was designated as embryonic day 0.5 (E0.5). Littermates were used for all experiments in which normal and mutant embryos were compared.

$Osr1^{EGFPCreERt2}$ and $Sall1^{CreERt2}$ were mated to $Ptcf1^{+/-}$ mice to generate $Sall1^{CreERt2};Ptcf1^{+/-}$ or $Osr1^{EGFPCreERt2};Ptcf1^{+/-}$ males, which were then crossed with homozygous $Ptcf1$ conditional ($Ptcf1^{flox/flox}$) mice to generate $Sall1^{CreERt2};Ptcf1^{flox/-}$ or $Osr1^{EGFPCreERt2};Ptcf1^{flox/-}$ mutant progeny, respectively (Mugford et al. 2008; Inoue et al. 2010; Ellis et al. 2003). $Ptcf1$ is specifically mutated in $Sall1^{+}$ or $Osr1^{+}$ cells of the progeny when Cre recombinase is activated within 24 hours after tamoxifen (TM) administration to a pregnant female. TM (Sigma) was dissolved in Sesame oil (Sigma) and administered interaperitoneally at a single dose of 1mg/40mg body weight at noon of the desired embryonic day.

Analysis of HH activity was achieved by mating $Sall1^{CreERt2}$ mice to $Ptcf1^{LacZ/+}$ mice to generate $Sall1^{CreERt2};Ptcf1^{LacZ/+}$ males (Goodrich et al. 1997). These males were crossed with homozygous $Ptcf1$ conditional mice to generate $Sall1^{CreERt2};Ptcf1^{LacZflox}$ mutant progeny carrying a LacZ reporter allele. Cre-dependent recombination is activated in $Sall1^{+}$ cells upon TM injection according to the above procedure.
Temporal fate mapping was performed by crossing the \textit{R26-tdTomato} reporter strain with \textit{Sall1}^{creER\textsuperscript{2}} mice. \textit{tdTomato} heterozygote progeny express a bright red fluorescence protein (RFP) upon TM-induced Cre-recombination (Kobayashi et al. 2012).

Table 1. List of primers for PCR analysis of mouse genomic day

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<thead>
<tr>
<th>Gene</th>
<th>Primer Sequence (5' to 3')</th>
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<tr>
<td>\textit{Osr1Cre} WT Rev</td>
<td>CTG GCT TAG GGT GAA TGA CG</td>
</tr>
<tr>
<td>\textit{Osr1Cre} Common</td>
<td>CCC TCT TCC TGT CTT TGC AG</td>
</tr>
<tr>
<td>\textit{Osr1Cre} MUT Rev</td>
<td>GTA GGT CAG GGT GGT CAC GA</td>
</tr>
<tr>
<td>\textit{Sall1Cre} 1</td>
<td>AGC TAA AGC TGC CAG AGT GC</td>
</tr>
<tr>
<td>\textit{Sall1Cre} 2</td>
<td>CAA CTT GCG ATT GCC ATA AA</td>
</tr>
<tr>
<td>\textit{Sall1Cre} 3</td>
<td>GCG TCG GCT ACC CGT GAT AC</td>
</tr>
<tr>
<td>\textit{Pt1} Fwd</td>
<td>CCA CCA GTG ATT TCT GCT CA</td>
</tr>
<tr>
<td>\textit{Ptc1} Rev</td>
<td>AGT ACG AGG CAT GCA AGA CC</td>
</tr>
<tr>
<td>\textit{Ptc1} Del Rev</td>
<td>TGA CTG CAA ACT TTC CCA TCT</td>
</tr>
<tr>
<td>\textit{Ptc1- LacZ} Fwd</td>
<td>TTC ATT GAA CCT TGG GGA AC</td>
</tr>
<tr>
<td>\textit{Ptc1- LacZ} Rev</td>
<td>AGT GCG TGA CAC AGA TCA GC</td>
</tr>
</tbody>
</table>

### 2.2.2 Whole Mount B-Galactosidase Staining

Whole kidneys were briefly fixed in lacZ fix solution (25% gluteraldehyde, 100 nM EGTA, 1 M MgCl\textsubscript{2}, 0.1 M sodium phosphate) and rinsed in wash buffer (0.1 M sodium phosphate buffer, 2% nonidet-P40), 1 M MgCl\textsubscript{2}). Kidneys were then placed in lacZ staining solution (25 mg/ml X-gal, potassium ferrocyanide, potassium ferricyanide) at 37° C overnight in the dark. Once staining had occurred, the reaction was terminated in wash buffer and tissue was post-fixed in 10% buffered formalin at 4° C. Stained tissues were then processed for embedding in paraffin wax followed by midsagittal sectioning at intervals of 5 µm. Sections were then counterstained with eosin.
2.2.3 Histology and Immunohistochemistry

To harvest embryos from timed pregnancies, pregnant mice were sacrificed using a CO₂ chamber. Embryonic tissues were fixed in 4% Paraformaldehyde (PFA) at 4°C overnight, and were dehydrated in 70% ethanol prior to paraffin embedding and further processing. Midsagittal tissues sections were generated in 5µm intervals by the CMHD’s Core Pathology Lab at TCP, followed by hematoxylin and eosin (H&E) staining using standard methods.

To visualize tdTomato expression, fixed kidneys were cryopreserved using a sucrose gradient (gradual increase from 10 to 30%), embedded in OCT (Sakura Finetek, Torrance, CA), and sectioned at 8 µm. Cryosectioning of the OCT embedded tissue was conducted by the TCP.

Immunofluorescence staining was carried out on either paraffin embedded or frozen sections according to the following procedure. Sections were first deparaffinised in xylene and rehydrated through ethanol gradient. Subsequently, antigen retrieval was performed on paraffin-embedded tissues using citrate buffer (pH=6) in a pressure cooker. Non-specific antigens were blocked at room temperature using DAKO serum-free protein block prior to incubation with primary antibodies. Primary antibodies were diluted in protein block (DAKO) and incubated overnight at 4°C. Primary antibodies were: R&D Mouse monoclonal anti-SALL1 (PP-K9814-002), Abcam Rabbit polyclonal anti-SALL1 (ab31526), Cell Signalling Rabbit polyclonal anti-PBX1 (4342), Santa Cruz Mouse anti-PBX1 (sc-889x), Abcam Rabbit polyclonal anti-RALDH2 (ab96060), Rockland Rabbit polyclonal anti-RFP (600-401-379), Cell Signalling mouse monoclonal anti- PHH3 (Ser10) (9706), Sigma mouse monoclonal anti- NCAM (C9672), Proteintech rabbit polyclonal anti-SIX2 (11562-1-AP), and Dako mouse monoclonal anti- WT1 (Clone 6F-H2). Secondary antibodies used for immunofluorescence application were Alexa-Fluor 488 goat anti-mouse and Alexa-Fluor 568 goat anti-rabbit (Molecular Probes,
1:1000 dilution). Nuclei were visualized using DAPI counterstain (Life Technologies, 1:2000). Slides were mounted using liquid mounting medium (Vectashield) and imaged using an epifluorescence microscope (Zeiss). Images were captured at the same exposure for all samples, and 3 sections per kidney were used in the assessments.

2.2.4 **In situ mRNA Hybridization**

In situ hybridizations were performed on paraffin embedded embryonic sections using digitoxin-labelled RNA probes encoding *Ptc1* as described (Mo et al. 1997).

2.2.5 **RNA Isolation and Quantitative Real-Time Reverse Transcriptase- PCR**

To evaluate Cre-recombinase efficiency after TM administration, forelimb tissues were harvested from embryos and preserved in RNAlater® (Sigma) at 4°C. RNA was isolated from the ruptured tissue using a RNeasy micro kit (Qiagen). cDNA was subsequently synthesized from the extracted RNA using oligo-dT primers and superscript(II) (Invitrogen). Real-time PCR reaction mix contained 1ng of cDNA sample, SYBR green master mix (Invitrogen) and primers to a total volume of 10 µl. All primers were designed using Primer Blast and verified through UCSC *in silico* PCR (Table 2). Quantitative real-time PCR (qRT-PCR) amplification was carried out using the Applied Biosystems 7900 HT fast RT-PCR system. Relative levels of mRNA expression were determined using the ΔΔCT method, where individual expression values were normalized to *Gapdh*. 
Table 2 Primer sequences used for qRT-PCR (Mus-musculus genes)

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
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<tr>
<td>Foxd1</td>
<td>TGG ACT ACG GCT AAA GG</td>
<td>CTG TGC GAG GTT TCG</td>
</tr>
<tr>
<td>Six2</td>
<td>GCC CAA GGA AGA GAA CA</td>
<td>GAA CTT ACC ACC TAC TT</td>
</tr>
<tr>
<td>Ptc1- Exon3</td>
<td>CCC GTC AGA AGA TAG GAG AAG AGG</td>
<td>GCA GGA GAG CCT TCG TGG TC</td>
</tr>
<tr>
<td>Osr1</td>
<td>GAG CGA CCT TAC TCC TGC GA</td>
<td>GTC TGG ACA GCA AGA GT</td>
</tr>
<tr>
<td>Gapdh</td>
<td>CGA CTT CAA CAG CAA CTC CCA TTC TCC</td>
<td>TGG GTG GTC CAG GGT TTC TTA CTC CTT</td>
</tr>
</tbody>
</table>

2.2.6 Flow Cytometry

To analyse gene profiles of Osr1+ cells in a state of increased HH signalling, Osr1<sup>EGFPCreER<sup>2</sup></sup> transgenic mice was used to isolate GFP+ cells via fluorescence activated cell sorting (FACS). Osr1+ specific conditional knock outs (Osr1<sup>EGFPCreER<sup>2</sup>; Ptc1<sup>lox/lox</sup>) and heterozygote littermate controls (Osr1<sup>EGFPCreER<sup>2</sup>; Ptc1<sup>lox/+</sup>) were collected at E11.5 and processed individually for FACS as below. Isolated metanephric rudiments were dissected in HBSS:BSA and digested in 2 mg/mL of Collagenase (Roche) in DEPC/Ringer’s solution for 15 minutes at 37°C. Kidneys were dissociated into a single-cell suspension by triturating the sample with a 1mL pipette. After centrifuging the cell suspension for 5 minutes at 4000rpm, cells were re-suspended in sorting media (HBSS/BSA containing 1% heat inactivated FCS and 1 mM EDTA). The cell suspension was filtered through a 40 μm nylon mesh cell strainer (BD Falcon) into 5mL polystyrene round bottom tubes (Falcon). Propidium Iodide (Sigma) was added at a final concentration of 0.5mg/mL to label dead cells. FACS analysis was carried out using the FACS Vantage (Becton Dickinson Immunocytometry Systems) in the flow cytometry facility at the Sickkids Research Institute. The gate for the Osr1-GFP+ population was set to exclude all the cells falling into the fluorescing range of the negative control cells and to include only the highly fluorescing population. Cells were directly collected in 350 μL of buffer RLT (Qiagen)
and frozen on dry-ice. RNA extraction from sorted cells was performed according to the RNeasy® Micro-kit instructions (Qiagen).

2.2.7 Automated Cell Quantification in Tissue Sections

To quantitate the number of cells, ImageJ® “analyze particles” command was used according to published protocols (Gering et al. 2004). In summary, the image was first converted to 8-bit black and white, followed by threshold level adjustment to mark only the cells to be counted. The optimal size of the particles to be counted was set to be 0.01 to infinity, with no preference for shape, and the counted particles were outlined with dashes. The area of the kidney section was measured by ImageJ® and the total number of cells in each section was normalized to the area of the corresponding section prior to any comparison. Wherever only the cortical stromal or nephrogenic cells were counted, the number of cells was normalized to the number of UB tips in the cortex rather than the area of the whole section.

2.2.8 Data Analysis

All statistical and mathematical analyses of data were done using Microsoft Excel. Student’s t-test (with unequal variances) was used to determine the statistical significance of any changes observed. A p-value of less than 0.05 was considered statistically significant.
2.3 Recombination in Sall1$^{CreERt2}$ Mice is Tamoxifen Dependent and Specific to Sall1+ Cells

I first confirmed that without TM, there is no leakiness of Cre activation by assessing tdTomato expression in Sall1$^{CreERt2};$R26-tdTomato mice that were not exposed to TM at any time in their development (data not shown). In the developing kidney, Sall1 has been shown to be expressed in multipotent nephron progenitor cells and CM-derived differentiating structures (PTA, RV, comma and S-shaped bodies), as well as the cortical stromal cells (Osafune et al. 2006a; Ohmori et al. 2015). I confirmed Cre specificity in Sall1$^{CreERt2};$R26-tdTomato by demonstrating that when TM is injected after the onset of ureteric branching (E11.5), tdTomato expression is detected in nephron progenitors, nephrogenic intermediates and the cortical stroma, but not in the epithelial UB-derived cells (Figure 7).
Figure 7 Recombination Specificity in $Sall1^{CreERt2}$ Mice

A-B. Immunostaining for $tdTomato$ (red) and $Sall1$ (green) expression in E16.5 $Sall1^{CreERt2},tdTomato$ kidneys upon tamoxifen treatment at E11.5. C. Merged image showing yellowish color in cells co-expressing $Sall1$ and $tdTomato$. Inset shows the outlined region in higher magnification showing the overlap of $tdTomato$ expression with that of $Sall1$, including in the cap mesenchyme (CM), intermediate nephrogenic structures, such as S-shaped nephrons, and cortical stroma (CS). White arrowheads point to the $Sall1+$ cells in the CM and the intermediate nephrogenic structures that are not marked by $tdTomato$. 
2.4 Sall1+ Cells in the Caudal IM Contribute to Stromal and Nephrogenic Lineages

Previous lineage tracing studies have demonstrated the multipotent capacity of Osr1+ cells between E7.5 and E10.5 to give rise to all the cortical progenitor compartments of the metanephric kidney, including both stromal and nephron progenitors (Mugford et al. 2008). Osr1 has been shown to be functionally upstream of a number of genes required for initial MM establishment including Sall1 (James et al. 2006). In the absence of Sall1, initial ureteric branching fails to occur and the MM undergoes apoptosis (Nishinakamura et al. 2001; Nishinakamura et al. 2005). Due to the expression of Sall1 in both nephrogenic and stromal progenitors in the developing kidney, Sall1 expression prior to the onset of UB induction is likely to mark the precursor of these two lineages. I addressed this hypothesis, and determined the developmental time window during which Sall1+ cells have multipotent capacity, using a temporal fate mapping approach. I inter-crossed Sall1CreER2 with R26-tdTomato mice to permanently label descendants of the Sall1+ population via TM (1mg) –mediated induction of Cre recombination (Kobayashi et al. 2008). I traced the fate of Sall1+ cells marked at 24-hour intervals from E9.5 through E11.5.

I first marked Sall1+ cells by injecting TM at E9.5, and subsequent analysis was performed at E14.5-E16.5 –a stage at which full spectrum of nephrogenic and stromal cells are developed to detect the cell types that are descended from Sall1+ cells marked at E9.5-E10.5. TdTomato-labelled cells were found in both cortex and medulla. Immunostaining for Pbx1, which has a low expression in nephron progenitors and high expression in stromal cells (Schnabel et al. 2003), demonstrated that nephron progenitors and their derivatives, including podocytes and tubular structures, were tdTomato+ (Figure 8A-C). In addition, tdTomato
expression co-localized with cortical stromal cells marked by high expression of *Pbx1* (Figure 8A-C). I also found traces of *tdTomato* expression in the medullary stroma intercalating the renal tubules and collecting ducts (Figure 8A-C). These data suggest that *Sall1*+ cells between E9.5 and E10.5 have the potential to contribute to the stromal progenitor compartment in addition to the nephrogenic lineage.

Surprisingly, when TM was injected at E10.5, *tdTomato*+ cells were exclusively found in the CM surrounding the UB tips, and the flat layer of capsular stroma (Figure 8D, white arrowheads). No *tdTomato* expression was detected in other cortical and medullary stromal cells (Figure 8D, white arrows). Together, the results of our lineage tracing experiments suggest a gradual restriction in the fate of *Sall1*+ precursors between E9.5 and the onset of ureteric branching at E11.5, such that these cells initially have the capacity to contribute to both nephrogenic and stromal lineages, but lose their ability to contribute to the majority of stromal cells (except for the capsular stroma) at E10.5.
Figure 8 Sall1+ cells transiently contribute to cortical stromal cells between E9.5 and E10.5

A-C. Immunostaining of E16.5 Sall1\textsuperscript{CreER\textsubscript{T2}};\textit{tdTomato} kidneys for \textit{tdTomato} and \textit{Pbx1} after Cre activation at E9.5. Low Pbx1 expression marks nephron progenitors (yellow arrows). \textit{tdTomato} expression colocalizes with Pbx1 expression, in the capsule, sub-capsular stroma and the medullary stroma (white arrowheads) upon tamoxifen administration at E9.5 (n=4). D-F. Immunostaining of E16.5 Sall1\textsuperscript{CreER\textsubscript{T2}};\textit{tdTomato} kidneys for \textit{tdTomato} and \textit{Pbx1} after Cre activation at E10.5. \textit{tdTomato} expression is found in the nephron progenitors and their derivatives. Pbx1 expression colocalizes with \textit{tdTomato} expression only a subset of cells in the inner layer of capsular stroma (arrows). There is no tdTomato expression in the medullary stroma and the cortical stromal cells in between nephron progenitors (asterisks) (n=3).
2.5 Increased Hedgehog Signalling in Sall1+ Cells at E9.5

To investigate the role of increased HH signalling in lineage specification within Sall1+ cells I used Sall1^{CreER2};Ptc1^{+/−} mice and crossed them with Ptc1^{flox/flox} mice to conditionally delete Ptc1 in a temporally controlled fashion between E9.5 and E10.5. Following loxP site recombination, Exon 3 of the Ptc1 gene is excised to generate a non-functional PTC1 protein (Ellis et al. 2003). Since Sall1 is also strongly expressed in the developing limb bud (Buck et al. 2001), I used RNA extracted from the limb tissue of each embryo to confirm that a single dose TM injection activated Cre recombination. Results of qRT-PCR analysis using Ptc1 primers designed for the disrupted region of Ptc1 demonstrated a 50% decrease in Ptc1 mRNA in E13.5 mutants (Sall1^{CreER2};Ptc1^{flox/−}) compared to the heterozygote controls after TM injection at E9.5 (Figure 9B). Next, I confirmed that Ptc1 inactivation in Sall1+ precursor cells results in increased HH signalling activity in mutant mice by analyzing Ptc1-lacZ expression as a readout of HH signalling. In the embryonic kidney, Ptc1 is normally expressed in the mesenchyme surrounding the presumptive ureter, and the urothelium. Up-regulated Ptc1 mRNA expression as a consequence of activated HH signalling in Sall1+ precursor cells is predicted to result in ectopic lacZ expression in the cortex. Our data confirmed that while Ptc1-lacZ expression was maintained in the urothelium and medullary stroma (similar to controls), it was ectopically expressed, albeit in a mosaic pattern, in the condensing mesenchyme and the stroma surrounding the UB tips in mutant kidney tissue (Figure 9C-F).
Figure 9 Schematic of Ptc1 gene and confirmation of Ptc1 deletion and HH activity

A. Schematic of murine Ptc1 gene demonstrating the location of loxP allele in relation to Exon 3. B. At E3.5 kidneys were dissected from ureteric tissue after tamoxifen administration at E9.5, and mRNA levels of the excised region of Ptc1, exon3, were measured. Ptc1-mutant kidneys exhibited a significant decrease in Ptc1 (50%) compared to the heterozygote controls. C-F. β-galactosidase staining at E13.5 and E16.5 for LacZ as a reporter of Ptc1 expression shows persistent HH activity in the medullary stroma around the ureter and ectopic activity in the cortex, compared to control expression only in the medullary stroma surrounding the ureter.
Histological examination followed by TAM administration at E9.5 did not show a marked phenotypical difference in mutant kidneys compared to the wild-type littermate controls at E13.5. However, immunostaining for CM marked by Sall1, and cortical stroma marked by co-expression of Pbx1 and Sall1, revealed an expansion of Sall1+ cells and aberrant localization of cortical stromal cells at E14.5 (Figure 10A,B). In wild-type kidneys, cortical stromal cells are arranged in a very organized pattern consisting of a layer of capsular stroma and 1-2 layers of sub-capsular cells (Figure 10A). In contrast, in mutant mice some stromal cells surrounded the CM in an unusual multi-layer pattern, and some formed a single layer in between multiple Sall1+ cell layers (Figure 10B). To further investigate the disrupted arrangement of cortical stromal cells, I examined the expression of Raldh2, a specific marker of cortical stromal cells through immunostaining. In mutant kidneys, Raldh2 was expressed ectopically in the junction of the ureter and pelvis, an area normally occupied by the medullary interstitial cells at both E13.5 and E16.5 (Figure 10C-E). These data raise the possibility that the disrupted arrangement and ectopic localization of cortical stromal cells may be due to abnormal expansion of stromal progenitors.
Figure 10 Ptc1 mutant kidneys display disorganized cortical stroma

Ptc1 mutant kidneys display disruption in arrangement of the cortical stromal cells marked with Pbx1 (red) and Sall1 (green) compared to the typical 1-2 organized cell layers around the CM. Insets show the higher magnification of the dashed areas (A-B). Raldh2, a specific marker of cortical stromal cells is ectopically expressed at the junction of the ureter and the presumptive pelvis at both E16.5 (C-D) and E13.5 (E-F).
To determine whether there is a difference in the number of stromal cells in mutant kidneys, three midsagittal sections (80 μm apart), from four different E13.5 mutants and littermate controls, were immunostained to mark stromal cells by high Pbx1 expression (Pbx1\textsuperscript{Hi}) (Figure 11A,B). Low Pbx1 expression marks the nephron progenitor population. Next, the average number of Pbx1\textsuperscript{Hi} cells across different kidney layers were counted using ImageJ\textsuperscript{®} software. This analysis indicated a 78% increase in the total number of Pbx1+ cells, including the cortical and medullary interstitial cells, per unit area in Sall1\textsuperscript{CreER\textsuperscript{2}},Ptc1\textsuperscript{flox/-} kidneys (n=4, p<0.005) (Figure 11C). Next, I examined the number of stromal progenitors, by exclusively counting Pbx1+ cells in the renal cortex, including the capsular and sub-capsular stroma, and the cells intercalating the UB tips. The number of cortical stromal cells per UB tip was found to be increased by 20% in the mutants (n=4, p<0.05) (Figure 11D). Taken together, our quantitative analyses demonstrated that HH signalling activation in Sall1+ precursors at E9.5 leads to a significant increase in the number of stromal progenitors and their derived medullary stromal cells at E13.5.
Figure 11 Increased number of cortical and medullary stromal cells in Ptc1 mutant kidneys

A-B. Immunostaining of E13.5 kidneys for stromal cells marked by Pbx1 expression (dashed area marks renal cortex). Both cortical and medullary zones, separated by dashed line, are populated with more Pbx1Hi cells in mutants. C. Quantitation of the number of Pbx1Hi stromal cells in sections across the kidney shows a 78% increase in total number of stromal cells per unit area in mutants compared to controls. (n=4, P<0.005). D. Quantitation of the number of Pbx1Hi stromal cells exclusively in the cortex (outlined area), reveals a modest but significant increase in the number of stromal progenitors in mutants compared to controls (n=4, P<0.05). CS=Cortical Stroma, MS=Medullary Stroma, UB=Ureteric Bud.
Next, I examined whether the increased number of stromal cells in mutant kidneys is caused by increased cell proliferation in the stromal compartment, since HH signalling was previously shown to promote proliferation of the ureteric and medullary mesenchyme (Yu et al. 2002). I used two different cell cycle markers, PHH3 and Ki-67, to mark proliferating cells at E13.5, when an increase in stromal cells is observed (Figure 12A-E). Ki-67 marks cells in all active phases of cell cycle, whereas PHH3 specifically marks mitotic cells in G2-M phase at the time of analysis. Statistical analysis of the proportion of Pbx1+ cells expressing Ki-67, or PHH3 per unit area did not indicate a significant difference between mutants and controls (n=4). These results indicate that Pbx1+ stromal cells do not undergo increased proliferation in mutant kidneys, and that increased cell proliferation does not underlie the expansion of stromal cells observed in the mutants.
Figure 12 Proliferative capacity of Pbx1+ cells is not changed in Ptc1 mutants at E13.5

Cell proliferation was measured by immunostaining for PBX1, co-localizing with Ki-67, a specific nuclear marker for proliferating cells (red) (A-B), or PHH3 (red) (D-E). Quantitation of both markers shows that proliferative index in mutant stromal cells is comparable to that of the WT controls (C-F).
In summary, our data showed that activation of HH signalling in Sall1+ cells at E9.5 leads to an increased number of stromal progenitors, disorganization of the progenitor compartments (nephrogenic and stromal) around the UB tips, and ectopic localization of the cortical stromal cells in the medullary kidney. At the same time, the Sall1CreER\textsuperscript{2}; Ptc1\textsuperscript{flox/–} model has limitations since the earliest stage when immunofluorescence staining allows for reliable quantitation of the number of Pbx1\textsuperscript{Hi} cells across serial sections is E13.5. At E13.5, stromal progenitors have already started to contribute to differentiated stromal derivatives. Thus, the magnitude of the effect of HH signalling activation in Sall1+ precursors on initial stromal progenitor specification may have been masked. Also, the fact that Sall1+ cells in Sall1CreER\textsuperscript{2}; Ptc1\textsuperscript{flox/–} mice are not marked with a reporter allele in this model hinders our ability to examine whether the ectopic stromal cells descend from the precursor population within which HH signalling was activated. It could be possible that non-cell-autonomous effects of HH signalling perturb the fate of the surrounding Sall1-negative cells such that they gain stromal characteristics.

2.6 Increased Hedgehog Signalling in Osr1+ Cells at E9.5

Osrl expression has been shown previously to mark multipotent renal progenitors, whose developmental potential is less restricted than that of Sall1+ cells, and can give rise to nephrons, stroma and capillary endothelial cells between E9.5 to E11.5 (Mugford et al. 2008). To address the cell-autonomous function of activated HH signalling within the common precursor of the nephrogenic and stromal lineages, I took advantage of endogenous GFP expression in Osrl\textsuperscript{EGFPCreER\textsuperscript{2}} mouse strain, and continuous Osrl expression in the IM, stromal and nephrogenic progenitors between E9.5- E11.5 (Mugford et al. 2008). Fluorescence-activated cell sorting (FACS) was used to isolate Osrl+ cells from E11.5 Osrl\textsuperscript{EGFPCreER\textsuperscript{2}; Ptc1\textsuperscript{flox/–}} and
**Osr1**<sup>EGFPCreER<sup>2T;Ptc1<sup>flox<sup>+</sup>(Ptc1 heterozygote control) MM after TM administration at E9.5 (Figure 13A). This allowed us to quantitatively measure the contribution of activated HH signalling in promoting specification of stromal progenitors from their Osr1+ precursor population at the onset of renal development.

After optimization of flow-sort conditions, sample collection and subsequent RNA extraction from small cell volumes, I isolated an average of ~13,800 GFP+ MM cells per pair of E11.5 metanephric rudiment (Figure 13B). qRT-PCR analysis was performed to confirm the relative purity of the FACS-isolated Osr1-GFP+ population by comparing Osr1 mRNA levels in GFP+ to that in the GFP- cell fraction. Results of our analysis on 10 pairs of metanephroi demonstrated 12-fold greater Osr1 expression in GFP+ cells compared to the GFP- cell fraction (Figure 13C). I subsequently confirmed that Cre-induced recombination resulted in deletion of Ptc1 (73% decrease) in **Osr1**<sup>EGFPCreER<sup>2T;Ptc1<sup>flox<sup>+</sup> cells using qRT-PCR with primers specific for the loxP-flanked exon 3 of the Ptc1 gene (Figure 13D). Next, I compared the mRNA expression levels of stromal and nephron progenitors specific markers, Foxd1 and Six2 respectively, in control (Ptc1 heterozygous) and Ptc1-null Osr1+ cells. qRT-PCR analyses demonstrated a 60% increase in Foxd1 expression in the Ptc1-null Osr1+ cells (n=5, p<0.05), while Six2 expression remained relatively unchanged (Figure 13E,F). These data in conjunction with our previous findings from Sall1<sup>CreER<sup>2 mice suggest that HH signalling activation within the common precursors of stromal and nephrogenic cells at E9.5 promotes specification of the stromal cell lineage.
**Figure 13 Foxd1 is increased in Ptc1 null Osrt+ cells**

A. The inset shows a representative Osrt\[^{EGFPcreER\(2\)}^\] + metanephros at E11.5, where GFP expression marks Osrt+ cells. The plot represents FACS analysis of GFP labeled Osrt+ MM cells B. The table represents the average number of Osrt+ cells sorted from 10 pairs of metanephroi (5 mutants and 5 controls). C. Quantitative real-time PCR (qRT-PCR) of E11.5 FACS-isolated cells confirmed significantly higher expression of Osrt in GFP+ compared to GFP- cell fractions. D. qRT-PCR analysis showed a 70% decrease in Ptc1 exon3 of mutant Osrt+ cells compared to controls, confirming Cre dependent recombination upon tamoxifen administration at E9.5. E-F. Foxd1 expression is increased by 60% in Ptc1 null Osrt+ cells, whereas Six2 expression is relatively unchanged in mutant and control cells.
To investigate how the increase in stromal gene expression influences development of the kidney in Osr1<sup>EGFP<sub>CreER<sub>2</sub></sup>;Ptc1-null mice, I examined stromal and nephrogenic progenitors using immunostaining at advancing stages in development (Figure 14 A-H). Consistent with our finding in Sall1<sup>CreER<sub>2</sub></sup>;Ptc1-null kidneys, immunostaining for nephron progenitors marked by Sall1<sup><sub></sub></sup>, and cortical stroma marked by co-expression of Pbx1 and Sall1, revealed an expansion of Sall1<sup>+</sup> cells and abnormal localization of cortical stromal cells at E14.5 in mutants (Figure 14B). In addition to the ectopic Raldh2<sup>+</sup> cells in the medullary region similar to Sall1<sup>CreER<sub>2</sub></sup>;Ptc1-null mutants, E16.5 kidneys exhibited an ectopic population of Raldh2<sup>+</sup> cells within the ureteric lumen, more distal to the kidney (Figure 14C,D). Previous studies have suggested the establishment of a Tbx18<sup>+</sup> population within the Osr1<sup>+</sup> metanephric field at E10.5, that lies in close proximity to the ND, and contains both ureteric mesenchyme and renal stromal progenitors, distinct from the already specified Foxd1<sup>+</sup> cells (Bohenpoll et al. 2013a). It is likely that aberrant activation of HH signalling in Osr1<sup>+</sup> cells impact the fate of Tbx18<sup>+</sup> cells to further contribute to the stromal progenitor population in the kidney-proper/ureter.
Figure 14 Stromal progenitor marker, Raldh2, is ectopically expressed in Osr1EGFPCreERT2; Ptc1-null mutants

A. Histological comparison of E16.5 mutant (OsrtCreERT2;Ptc1\textsuperscript{flox/-}) with control (Ptc1\textsuperscript{flox/+}) shows the onset of renal pelvis and tubular dilation in addition to the aberrant presence of cell clusters inside the ureteral lumen (black arrows).

B. Immunostaining of E14.5 kidneys shows disruption in regular organization of nephron progenitors, marked by Sall1 (green) and stromal cells, marked by Sall1 and Pbx1 (yellow) around the UB tips in mutants compared to controls.

C. Immunostaining for Raldh2 (red) shows ectopic expression in the medullary stroma in E15.5 mutants (dashed circles).

D. Immunostaining of E16.5 kidneys demonstrates that the cell clusters inside the ureteral lumen also display Raldh2 (red) expression (white arrow).
2.7 Defect in Nephrogenesis in Ptc1 Mutant Kidneys

Various studies have established a molecular signalling crosstalk between cortical stromal cells and nephron progenitors, important for regulating nephron number (reviewed in (Li et al. 2014)). Since lineage tracing results showed that Sall1+ cells in the IM and un-induced MM contribute to both stromal cells and nephron progenitors, I investigated how HH signalling activation in Sall1+ cells at E9.5 affects nephron progenitors and their differentiation.

Analysis of nephron number, marked by WT1 immunostaining, revealed a 24% decrease in the number of mature glomeruli in Sall1CreERT2;Ptc1floxt/mutant kidneys compared to littermate controls (n=4, p<0.01) (Figure15). To investigate the underlying mechanism, I first determined whether activated HH signalling in Sall1+ progenitors leads to an increase in the number of stromal progenitors with the concomitant negative effect on nephron progenitors. Quantitation of the number of Six2+ cells surrounding each UB tip demonstrated no significant difference between the mutants and controls at E13.5, the stage at which I measured 20% increase in cortical stromal cells (Figure 16A-C). This finding is consistent with the qRT-PCR analysis of Osr1+ cells FACS-isolated at E11.5, which demonstrated no significant difference in Six2 expression in mutant and heterozygote controls (Figure 13F). Next, I examined the patterning of the CM surrounding UB tips at E18.5 through immunofluorescence staining of PAX2 and SALL, additional markers of nephron progenitors. All the 5 E18.5 mutants exhibited a continuous CM along the outer cortex comparable to the CM arrangement in WT kidneys (Figure 16D-G). These data suggest that the lower number of mature glomeruli measured at birth are likely not the result of defects in nephron progenitor specification or maintenance.
Figure 15 *Ptc1* mutant kidneys demonstrate a significant decrease in the number of glomeruli at E18.5.

**A,B.** Immunostaining of E18.5 kidneys for WT1 (green) marking mature glomeruli (grey arrows). **C.** Quantification of the number of WT1+ structures demonstrates a significant decrease in the number of glomeruli in *Ptc1* mutant kidneys (B) compared to controls (A).

Figure 16 *Ptc1* mutant kidneys exhibit no marked defect in nephron progenitor specification and maintenance

**A,B.** SIX2 staining of the nephron progenitors does not show any marked defect in nephron progenitor domain at E13.5. **C.** Quantitation of the Six2+ cells confirms that the number of nephron progenitors per UB tip in mutants is comparable to controls (n=4, p>0.05). **D-G.** Staining with PAX2 and SALL1 shows an apparently normal-sized nephron progenitor compartment (arrowheads) later in development (E18.5) (n=5).
To determine whether reduction in glomeruli number is due to a defect in nephron formation, I quantified the number of developing nephrons marked by NCAM immunostaining at E13.5. Interestingly, our analysis demonstrated a 28% decrease in the intermediate nephrogenic structures in mutants (n=5, p<0.005) (Figure 17), which closely corresponds to the observed 24% decrease in mature glomeruli at E18.5 (reported above). A growing body of literature as well as unpublished studies by Winny Li, Hovhannes Martyrosian and Chris Rowan from the Rosenblum lab have established the non-cell autonomous effects of stromal population on the MET process and nephron differentiation (Mao et al. 2015; Bagherie-Lachidan, Reginensi, Zaveri, et al. 2015; Fetting et al. 2014; Phua et al. 2015). Thus, it is likely that perturbations of the stromal lineage discussed above are the underlying cause of the observed defects in nephrogenesis.
Figure 17 Nephron formation is modestly decreased in *Ptc1* mutant kidneys

**A, B.** Immunostaining of E13.5 kidneys for NCAM (green) marking the intermediate nephrogenic structures including, renal vesicles, comma-shaped and S-shaped nephrons. **C.** Quantitation of the NCAM+ structures reveals a modest (28%) but significant decrease in the number of nephrogenic intermediate structures per UB tip.
2.8 Discussion

The majority of adult kidney cellular components are derived from two distinct self-renewing progenitors: the stromal and nephron progenitors. Although these two populations are spatially and molecularly distinct at the onset of metanephric development, they arise from a common Osrl+ precursor population. Yet, labeling studies have suggested that the Osrl+ IM mesenchyme is predominantly a heterogeneous population of cells already committed to either cap mesenchyme or interstitial progenitor cell. Other lineage tracing studies have indicated that Eya1+ cells contribute only to the metanephric nephron-forming cell fate, but not stroma as early as E8.75 (Jinshu Xu et al. 2014). Despite the in vivo and in vitro advances made in understanding the signalling mechanisms involved in establishing the nephron progenitor compartment, the regulation of stromal cell fate from the Osrl+ progenitor pool has remained unknown (Taguchi et al. 2014). Neither Osrl nor Foxd1 expression are required for specification of stromal progenitors (Yallowitz et al. 2011; Hum et al. 2014). Thus, identification of other genes marking the common precursor population and a detailed understanding of temporal fate restriction in precursors of the stromal and nephron progenitors will allow deeper insight the origin of stromal cells.

2.8.1 Sall1 is expressed in the Precursors of Stromal and Nephron progenitors Between E9.5 and E10.5.

Results of our lineage tracing experiments suggest a gradual restriction of Sall1 expression in renal precursors between E9.5 and the onset of ureteric branching at E11.5, such that Sall1+ cells initially have the capacity to contribute to both nephrogenic and stromal lineages, but lose their capacity to contribute to the majority of stromal cells (except for the capsular stroma) at E10.5. It is likely that Sall1 expression is transiently turned off in the
majority of stromal progenitors and their precursors between E10.5-E11.5, and is maintained only in the progenitors of the renal capsule, supporting the idea that stromal progenitors are a heterogeneous population of cells with distinct differentiation capacities, previously proposed by Kobayashi et al. (Kobayashi et al. 2014). These data also suggest a potential temporally-specific role for Sall1 in development of the renal capsule, upstream of Foxd1 and Hoxd11. This can be examined in more detail using Sall1<sup>CreER<sup>2</sup>/flo<sup>x</sup></sup> mice upon TM injection at E10.5 (Levinson et al. 2005; Yallowitz et al. 2011; Inoue et al. 2010).

Moreover, SALL1 in the nephrogenic mesenchyme has previously been shown to be involved in initial UB outgrowth (E10.5-E11.5) possibly through binding to a nucleosome remodeling and deacetylase (NuRD) complex, and repressing unknown inhibitors of UB outgrowth (Nishinakamura et al. 2001; Kiefer et al. 2010). Our findings raise the possibility that lack of Sall1 expression in the precursors of stromal cells between E10.5 and E11.5 may be required for disinhibition of genes involved in establishing the initial signalling interaction between the MM and the growing UB. Overexpressing Sall1 in Sall1<sup>+</sup> cells at E10.5 using R26Sall1 mice, which allow exogenous Sall1 expression in a Cre-dependent manner, will be of interest for future investigation (Jiang et al. 2010).

2.8.2 Activation of HH Signalling in Precursors of Stromal and Nephron Progenitors Promotes Stromal Cell Specification

The requirement for HH signalling during murine kidney development has previously been established by analyzing the renal phenotype in Shh-null mice, 50% of which display bilateral renal aplasia as early as E14.5 (Hu et al. 2004). Moreover, Marian Staite previously showed the importance of the SHH effector protein, GLI3R in regulating stromal progenitors gene expression. To specifically address the role of HH signalling in early precursors of stromal
progenitors, I first investigated whether constitutively active HH signalling (via Ptc1 deletion) in Sall1+ cells had an effect on stromal and nephrogenic progenitor populations. Activation of HH signalling at E9.5, a developmental stage prior to emergence of Foxd1+ cells anterior to the MM (Yallowitz et al. 2011), led to disorganization of the cortical stroma at E14.5 and ectopic expression of Raldh2, a specific marker of cortical stromal cells, at the junction of the ureter and the presumptive pelvis at E16.5. This finding led us to examine earlier developmental stages for the possibility of an abnormally higher number of stromal cells in the mutants. Results of our cell quantitation analysis demonstrated a significant increase in the number of cortical stromal cells, containing self-renewing stromal progenitors, as well as the total number of stromal cells marked by Pbx1. Proliferation assays (using PHH3 and Ki-67 staining) confirmed that the increase in stromal cells is not a consequence of increased proliferation in these cells. However, the earliest stage at which the number of stromal cells and stromal progenitors can be reliably examined in serial sections through immunostaining with Pbx1, without encountering sectioning bias, is E13.5. Since stromal progenitors at this stage have already begun to differentiate to their derivative components our results may not truly reflect the change in stromal progenitors caused by activation of HH signalling in their precursors at E9.5. Moreover, using Sall1CreERT2, it is not possible to examine whether the extra stromal cells in the mutants originate from Sall1+ precursor cells with activated HH signalling. It is possible that paracrine effects of HH signalling perturb the fate of a Sall1-negative population such that it gains stromal characteristics and integrate into the developing kidney.

Previous lineage tracing and gene expression analysis has demonstrated Osr1 expression in both stromal and nephrogenic progenitors at E11.5, after which Osr1 is exclusively expressed in the nephrogenic lineage (Mugford et al. 2008). I took advantage of GFP expression in Osr1+
cells of the $Osr^{1EGFPcreER^{2}}$ mice to address the cell-autonomous effect of constitutively active HH signalling on specification of $Foxd1^{+}$ stromal progenitors from $Osr1^{+}$ precursors. In support of our previous findings, qRT-PCR analysis of the FACS isolated GFP-tagged $Osr1^{+}$ cells at E11.5 demonstrated a significant increase in expression of $Foxd1$ in $Osr1^{+}$ cells in an activated state of HH signalling. Moreover $Osr1^{EGFPcreER^{2}};Ptc1^{flx/}$ mutants exhibited disorganization of cortical stroma and ectopic $Raldh2^{+}$ cells in the medulla and the presumptive UPJ area at later stages of development. These data suggest that the increased $Foxd1$ expression in $Osr1^{+}$ cells at E11.5 is reflective of an aberrant increase in stromal progenitor cells caused by HH signalling activation in either $Osr1$ or $Sall1^{+}$ precursor cells. Analyzing the number of cells in the stromal fraction isolated from E11.5 $Osr1^{+}$ cells would add further validation to our findings (Ohmori et al. 2015).

2.8.3 Nephron Progenitor Specification and Maintenance are not affected by Induction of Hedgehog Signalling Activity at E9.5

In addition to defects in arrangement and organization of stromal progenitors in the nephrogenic zone, ectopic presence of stromal progenitor markers in the renal medulla and abnormal increase in the number of stromal cells, $Ptc1^{-}$-null kidneys also exhibited lower glomerular number at E18.5. However, both qualitative and quantitative analyses of nephron progenitors in the developing kidney did not show any marked decrease in this cell population, suggesting that nephron progenitors survival and self-renewal is not affected by activated HH signalling. This data implies that the number of stromal cells in our mutants is not increased at the expense of a reduction in $Six2^{+}$ nephron progenitors, and perturbation of HH signalling does not change the fate of $Osr1^{+}Sall1^{+}$ cells already committed to nephrogenic fate. This is in
agreement with previous published studies indicating the segregation of nephrogenic lineage, marked by *Eya1* expression, from the *Osr1*+ IM as early as E8.75 (Jinshu Xu et al. 2014).

Quantifying the number of nephron intermediate structures marked by NCAM revealed a significant decrease in these structures. However, whether the decrease in NCAM+ structures results from increased apoptosis of the developing nephrons or a defect in nephron differentiation requires further investigation. Thus, it is necessary to examine the expression of genes involved in nephron differentiation, such as *wnt4*, *wnt9b* and *Lim1*, and to determine the apoptotic index within the nephron forming structures (Yu et al. 2009; Kobayashi et al. 2005). Regardless of the mechanism underlying reduced nephron formation, it is very likely that the changes in stromal compartment caused by activation of HH signalling affect the process of nephrogenesis in a non-cell autonomous manner (Review of the signalling mechanisms in (Li et al. 2014)).
2.9 Conclusion

In recent years, there have been remarkable advances in our knowledge of the functional role of stromal cells during kidney development. This has changed the historical view of the stroma as merely a supportive cell compartment to recognize embryonic stromal cells as active regulators of ureteric branching morphogenesis and nephron differentiation (Hatini et al. 1996; Fetting et al. 2014; Bagherie-Lachidan, Reginensi, Pan, et al. 2015; Levinson et al. 2005). In the adult kidney, the stromal lineage contributes to fibrosis in models of kidney injury, indicating the critical role of stromal-derived cells beyond development (Humphreys et al. 2010; Gomez et al. 2014; Fabian et al. 2012). Despite the critical importance of stromal cells in normal renal development, and the knowledge that Foxd1+ self-renewing stromal progenitors have the same origin as nephron progenitors in Osrl+ posterior IM, there exists a lack of knowledge regarding how the stromal lineage segregates from its common precursor population (Kobayashi et al. 2014; Mugford et al. 2008).

In this thesis, I present a novel stage-specific role for HH signalling in early renal precursor cells expressing Sall1 and Osrl. Winny Li and Hovhannes Martyrosyan from the Rosenblum lab have previously demonstrated the requirement for HH signalling in stromal cells for proper renal capsule formation and regulation of nephrogenesis through a crosstalk with nephron progenitors. Moreover, constitutive HH signalling activity in the metanephric field resulted in an increase in stromal gene expression. The body of work presented here demonstrates the effect of aberrant HH signalling activity in Sall1+ and Osrl+ renal precursors between E9.5 and E10.5 in promoting stromal cell specification without affecting cell proliferation/self-renewal in either the stromal or nephrogenic progenitor pools. I propose the following model of stromal cell specification: an uncommitted pool of Sall+Osrl+ precursor
cells exist in the posterior IM that either contributes to the stromal progenitor lineage or undergoes apoptosis (or contributes to cell types other than nephrogenic or stromal). Activation of HH signalling in this precursor population favors specification of more stromal cells. The abnormally high number of stromal progenitors leads to disruption in their normal cortical arrangement around the CM, and some of these cells migrate to the ectopic medullary location close to the ureter. Yet, the body of work presented here does not fully define the molecular mechanisms regulated by HH signalling that could potentially be involved in the specification of stromal cells from their uncommitted precursor population.

Much information is yet to be revealed about the molecular pathways involved in segregation of various renal lineages, particularly stromal progenitors from their common precursors in the IM and un-induced MM. Considering the critical role of stromal cells in regulating nephron differentiation and ureteric branching, understanding the pathways involved in their initial specification have important implications for multiple renal disease processes and for ongoing attempts at de novo kidney tissue regeneration, all of which have failed to generate native stromal tissue thus far.
2.10 Future Directions

To further verify the role of activated HH signalling in promoting stromal progenitor specification from the $Osr1^+$ precursor pool, it is necessary to complement our qRT-PCR analysis on FACS-isolated $Osr1^+$ cells by quantifying the number of stromal cells to that of nephron progenitors through FACS analysis of control and HH-activated mutants. It was recently shown by Ohomori et al. (2015) that stromal cells can be specifically isolated from nephron progenitors through fractioning the FAC-sorted $Osr1^+$ cells based on $Pdgfr\beta$ and $Integrin\alpha8$ expression, where the $Pdgfr\beta^+ /Integrin\alpha8^-$ contains stromal cells, while $Pdgfr\beta^- /Integrin\alpha8^+$ population contains nephron progenitors (Ohmori et al. 2015; Taguchi et al. 2014).

To begin to elucidate the molecular mechanisms through which HH signalling activation in the renal precursors enhances stromal lineage specification, it is necessary to perform a high-throughput gene expression analysis on FACS-isolated $Osr1^+$ cells. RNA sequencing can be performed on total RNA extracted from $Osr1^+$ cells sorted from caudal nephrogenic mesenchyme of the $Osr1^{EGFPCreER2;Ptc1^{floxed}}$ and heterozygous controls ($Osr1^{EGFPCreER2;Ptc1^{floxed/+}}$) as early as E10.5, 24 hours after TM injection to the pregnant dam. This will generate a validated set of differentially regulated genes that provides the basis for functional analysis in controlling stromal cell specification and migration in vitro. A novel study by Taguchi et al., 2014 established a protocol to derive MM from pluripotent stem cells (PSC) in vitro, by using information about early renal development in vivo (Taguchi et al. 2014). These progenitors were reported to reconstitute the 3D structures of the kidney in vitro, including glomeruli with podocytes and renal tubules, but they completely lacked $Foxd1^+$ stromal compartment (Taguchi et al. 2014). Using data from the proposed RNA sequencing experiment,
it is possible to generate novel protocols that direct cells toward both nephron and stromal progenitor lineages.
Chapter 3: Activated Hedgehog Signalling in Multipotent Renal Progenitors Leads to Prenatal Hydronephrosis
3.1 Introduction

Although congenital UPJ obstruction is a major cause of neonatal hydronephrosis, little is known about in utero development of this disease. Previous work done in the Rosenblum lab has demonstrated that increased HH signalling in the MM using a Rarb2Cre mouse model, leads to the formation of a Ptc2+ ectopic cell mass at the junction of the ureter and pelvis as early as E16.5. UPJ obstruction in these mice leads to antenatal hydronephrosis and death of the embryos following birth. Remarkably, gene expression analysis on the isolated ectopic cell mass showed a significant increase in cortical stromal markers in addition to downstream targets of HH signalling. Yet, the early cellular origin of the ectopic cell mass, and the embryonic stage at which HH signaling activity in the cells of origin leads to UPJ obstruction and hydronephrosis remained elusive.

Here, I addressed the temporal role of HH signalling specifically within Sall1+ and Osrl+ precursors of the kidney lineages in pathogenesis of UPJ obstruction. Activation of HH signaling in these precursor population between E9.5-E10.5 leads to prenatal hydronephrosis caused by physical obstruction of the UPJ by Raldh2+ cells. Analysis of Ptc1 expression as a readout of HH signaling activity demonstrated that the obstructing cell mass in these models are also HH responsive. Moreover, analysis of biopsies from human neonates with UPJ obstruction indicated a remarkable increase in Ptc2, and Foxd1 expression in a subset of patients. Although the true etiology of UPJ obstruction is multifactorial, our findings suggest a stage-specific pathogenic role of HH signalling in the early precursor cells of the kidney marked by Osrl and Sall1 to cause UPJ obstruction by a cortical stromal cell mass.
3.2 Experimental Methods

3.2.1 Intrapelvic Dye Injections

Visualization of the ureteropelvic lumen was performed as previously described (Airik et al. 2006). Briefly, bromo-phenol blue dye was injected into the renal pelvis of isolated urogenital systems at E18.5 using a pulled-out Pasteur glass pipette.

3.2.3 Patients’ Specimens Handling

For the patient group, I used excisional UPJ biopsies from 11 patients with the mean age of 7.8 months (2 months- 3 years) who underwent pyeloplasty at the Hospital for Sick Children, Toronto due to congenital UPJ obstruction. No patients had a known urinary tract infection prior to the surgery. Studies were approved by the Research Ethics Boards (REB), and clinical data was stored according to REB-approved procedures.

Specimens removed from patients were kept in normal saline on ice prior to being sectioned into two identical halves. Half of the tissue was preserved in RALater®(Sigma) for subsequent RNA extraction, and the other half was fixed in 4%PFA for histological examination. The samples were then processed for paraffin-embedded sectioning and H&E staining as discussed in the previous chapter. RNA extraction from the samples stored in RALater® was carried out by combining TRIzol® RNA extraction protocol with QIAGEN RNeasy® micro kit for RNA purification. Detailed description of cDNA synthesis and qRT-PCR methods can be found in the previous chapter, while the primer sequences are listed in table 3.
### Table 3. Primer sequences used for qRT-PCR (*Homo Sapiens*)

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### 3.2.4 Chromogenic Immunohistochemistry (IHC)

Immunohistochemical staining of human specimens were performed according to published protocols (Orlando et al. 2013). Briefly, after tissue rehydration, antigen retrieval and protein blocking steps, slides were incubated with primary antibodies, including rabbit polyclonal anti-human PTCH2 antibody (LS-B301) or goat polyclonal anti human FOXD1 antibody (sc-47585). Endogenous peroxidase activity was blocked by incubating in hydrogen peroxide for 15 minutes. For horseradish peroxidase (HRP) detection, sections were incubated with appropriate biotinylated- secondary antibody (Vector) for 1 hour at room temperature. Next, in a separate reaction, avidin and biotinylated peroxidase (Vector ABC Kit) were mixed according to manufacturer instructions such that some of the binding sites on avidin were left unoccupied. This complex was then incubated with the tissue sections. The unoccupied biotin-binding sites on the complex bind to the biotinylated secondary antibody, amplifying the signal. The brown product of the enzyme was developed using 3,3’- Diaminobenzidine (DAB) substrate (Vector). Cell nuclei were counterstained with hematoxylin.

### 3.2.5 Quantification of IHC Staining

Intensity of the DAB staining, was quantitated and analyzed using ImageJ® software following the instructions below (Jensen 2013; Safadi et al. 2010):
In each image, the FOXD1 positive area (brown stain) and total area in the analyzed field were automatically measured using color deconvolution plugin (Ruifrok AC, 2001). In the present study, color deconvolution was used to separate the DAB stain from hematoxylin which represents the whole nucleated area. Each image was changed into an 8-bit type (gray) and then processed into binary (black and white) image. The measurement criteria was set to calculate the area of the field (total nucleated area) and area fraction (area of black color representing DAB stain). A score for FOXD1 expression in the analyzed field was calculated by dividing the positively stained area over the total tissue area. For each immunostained UPJ sample, 5-6 different areas were analyzed independently to reach an average FOXD1 expression score.

3.2.6 Acknowledgement of Contributing Colleagues

The majority of the work discussed in this thesis was done by the author. However, I would like to acknowledge technical assistance of Lijun Chi, who guided me through and conducted the intrapelvic dye injection experiment (Figure 18K,L).

3.3 UPJ Obstruction and Hydronephrosis in Ptc1 Mutant Mice

When TM was administered to a Ptc1<sup>lox/lox</sup> dam bred with a Sall1<sup>CreER<sub>T2</sub>;Ptc1<sup>+/−</sup></sup> male at E10.5, no marked abnormalities were observed in kidneys at birth (Figure 18A,B). In contrast, TM administration at E9.5 caused marked bilateral hydronephrosis and a thin renal cortex, mimicking the phenotype observed in Rarb2-Cre; Ptc1-null mutants (Figure 18C,D). Moreover, histological analysis revealed that the renal pelvis is not developed in Sall1<sup>CreER<sub>T2</sub>;Ptc1<sup>lox/lox</sup></sup> mice at E16.5, whereas it is fully formed and connected to the ureter in the littermate controls (Figure 18E, F). To determine whether a physical obstruction of the urinary tract was associated
with hydronephrosis in mutant mice, blue dye was injected into the renal pelvis of the dissected urogenital region. In control $Ptc1^{\text{flo}}+/+$ kidneys (n = 9), dye traversed the length of the ureter and entered the bladder (Figure 18K). However, in all the mutant kidneys (n = 4), dye accumulated in the renal pelvis, failing to pass this structure (Figure 18L). Together, these data demonstrated that deletion of $Ptc1$ in $Sall1^+$ cells starting at E9.5 causes an obstruction at the junction of the ureter and pelvis.
Figure 18 Activation of HH signalling in Sall1+ cells at E9.5 leads to abnormal pelvis and UPJ development

A,B. H&E staining showing when TM is injected at E10.5, Sall1CreERT2;Ptc1\textsuperscript{lox/+} kidneys look comparable to WT controls E18.5. C, D. When TM is injected at E9.5 mutant kidneys exhibit a dilated renal pelvis and thinning of the cortex. E,F. Incomplete formation of renal pelvis, and an absent connection with the ureter in mutants at E16.5 (marked by stars). G,H. Whole mount preparations of E18.5 kidneys demonstrates comparable morphology of the mutants and controls when TM is injected at E10.5. I,K. When TM is injected at E9.5 there is a marked hydronephrosis in mutants, characterized by the accumulation of urine in the pelvis. Intrapelvic dye injection demonstrates a blockage of flow in E18.5 mutant kidneys at the junction of the ureter and pelvis in mutant mice.
*Osr1*+ cells are functionally upstream of *Sall1*+ cells in the IM and have a broader differentiation capacity. When I examined renal phenotypes of *Osr1*\(^{EGFPCreER}^{2};Ptc1^{lox/−}\) embryos at E18.5 following TM injection at E9.5, a phenotype similar to *Sall1*\(^{CreER}^{2};Ptc1^{-}\)-null model, with marked hydronephrosis and a thin renal cortex was observed (Figure 19A-D). Strikingly, in *Osr1*\(^{EGFPCreER}^{2};Ptc1^{lox/−}\) mice, hydronephroses had a unilateral occurrence, with the abnormal kidney on the left side (n=5). This is consistent with the left-sidedness of the UPJ obstruction occurrences in the majority of the patients diagnosed with congenital hydronephrosis (Zhang et al. 2000). Immunostaining for *Raldh2*, a marker of cortical stroma, demonstrated ectopic presence of *Raldh2*+ cells at the obstructed UPJ of the hydronephrotic mutants. In contrast, *Raldh2* expression was restricted to the renal cortex in the control kidneys (Figure 20E, F). This data agrees with the expanded *Raldh2* expression domain to the medullary stroma and ectopic localization of *Raldh2*+ cells in the ureteral lumen observed at E15.5 (Figure 14). These results confirm that increased HH signalling specifically during lineage segregation of stromal progenitors leads to congenital UPJ obstruction associated with other renal abnormalities (discussed before). Although the mechanisms underlying migration of the expanded and disorganized stromal progenitors (shown in Figure 10) to the UPJ area remains to be elucidated, it is likely that continuous activity of HH signalling plays an important role in this process.
Figure 19 Activated HH signalling in Osr1+ cells at E9.5 leads to unilateral hydronephrosis

A,B. Whole mount preparations of E18.5 kidneys demonstrates unilateral(left-sided) hydronephrosis in Osr1<sup>EGFP<sub>CreER<sub>2</sub>;Ptc1<sup>flox</sup></sub> mutants. C,D. Mutant kidneys display a dilated renal pelvis and thinning of the renal cortex at E18.5. E,F. Higher magnification of the ureteropelvic junction (UPJ) area immunostained for Raldh2 (red), a cortical stromal marker, and DAPI (blue), marking nuclei. There is a disconnection between the ureter (u) and pelvis in the mutants, with ectopic expression of Raldh2 at the UPJ (arrow).
3.4 HH Signalling is Active in UPJ Obstruction in Mutant Mice

*Ptc1* is directly downstream of HH signalling, and is reported as a robust readout of HH transcriptional activity (Pearse et al. 2001). To further explore HH signalling activity in UPJ development, I examined *Ptc1* expression in *Osr1<sup>CreER<sup>2</sup>;Ptc1* mutant kidneys using RNA *in situ* hybridization at E18.5, when hydronephrosis and UPJ obstruction is observed. *Ptc1* in control kidneys is largely expressed in the medullary stroma surrounding the pelvis, and in the developing nephrons (Figure 20C)(Yu et al. 2002). In mutant mice, *Ptc1* expression was increased in the expression domain observed in control mice, but was also expanded to the nephrogenic zone, confirming the activation of HH signaling in *Osr1*+ cells descendants (Figure 20D). Closer examination of *Ptc1* expression in mutants, revealed abnormal presence of *Ptc1*-expressing cells at the UPJ area in the tissue between the ureter and pelvis (Figure 20E, F). This suggests that the obstructing cells in UPJ are in activated state of HH signalling. Analysis of other genes downstream of HH signalling such as *Gli1*, and *Ptc2*, the *Ptc1* homolog exclusively expressed in the obstructed UPJ in *Rarb2Cre;Ptc1*-null mice, will further validate this finding.
Figure 20 Ectopic Ptc1 expression in the obstructed UPJ

*In situ* hybridization of *Ptc1* RNA probe on E17.5 control and mutants with *Ptc1* mutation targeted to their *Osr1+* cells at E9.5. **A, B.** *Ptc1* is expressed in the inner and outer medullary stroma, and in the glomeruli of the control kidney. *Ptc1* is upregulated in the medullary stroma and is highly expressed in the cortex of the mutant kidney. The circled area shows open UPJ in the control, and the obstructed UPJ in the mutant. **C, D.** Higher magnification of the cortex, showing no *Ptc1* expression in the nephrogenic zone (arrowheads) of the control kidney. *Ptc1* is highly expressed in the nephrogenic zone (arrowheads) of the mutant. **E, F.** Higher magnification of the ureter showing *Ptc1* expression in the cell layer adjacent to the urothelium in both controls and mutants. There are *Ptc1* expressing cells in the ureteric lumen at the UPJ in the mutant (black arrows)
3.5 Stromal Progenitors and Hedgehog Signalling Contribute To UPJ Obstruction in a Subset of Human Neonates

Congenital UPJ obstruction is the most common cause of neonatal obstructive hydronephrosis, with the prevalence of 1 in 1000 to 2000 neonates (Klein et al. 2011). However, the molecular pathogenesis of this disease is poorly understood. Unpublished data from Lijun Chi demonstrated higher mRNA expression of Ptc1, and Ptc2, two distinct readouts of HH signalling, in addition to Foxd1, a stromal progenitor marker, in the UPJ obstructing cell mass isolated from Rarb2-Cre;Ptc1\textsuperscript{flox/-} kidneys. This, together with the findings discussed in the preceding sections, raises the possibility that aberrant HH signalling activity may similarly lead to congenital UPJ obstruction in human neonates through a defect in migration/differentiation of cells of cortical stromal origin. To examine this hypothesis I first asked whether genes differentially expressed in the obstructed UPJ in our mouse models are also mis-regulated in human neonates diagnosed with congenital UPJ obstruction.

Due to extensive genetic heterogeneity and variable expressivity among individuals with respect to clinical features, and perhaps also with age, I compared the expression of stromal progenitor markers in UPJ tissue from each patient to that of the adjacent normal ureter from the same patient by qRT-PCR (Vivante et al. 2015). Gene expression analysis demonstrated between a 2.2-fold and a 12-fold increase in Foxd1 mRNA in the obstructed UPJ tissue, compared to the adjacent control tissue, in patients 3, 5, 7, 10 and 11 (Figure 21A). Tbx18 is a marker of the ureteral mesenchyme, and is essential for ureteral smooth muscle cell development (Airik et al. 2006). Moreover, lineage tracing studies has previously shown the contribution of Tbx18\(^+\) cells to a subset of Foxd1\(^+\) stromal progenitors in mice (Bohnenpoll et al. 2013a). Our qRT-PCR analysis did not show a marked change in Tbx18 expression in UPJO tissue compared to the
ureter in 4 out of 5 patients with increased Foxd1, suggesting that ureteral mesenchymal cells most likely do not contribute to the UPJ obstruction in these patients (Figure 21A). I further validated our qRT-PCR results in patients 3 and 7, with the biggest change in Foxd1 expression, by performing IHC on paraffin-embedded UPJ and their adjacent ureteric tissues (Figure 21 D-H). Moreover, our IHC results demonstrated a significant increase in FOXD1 in patient 9, for whom I did not obtain enough RNA to perform qPCR analysis (Figure 21H). On the contrary, quantitative analysis of our IHC results did not show a significant change in FOXD1 in patients 5 and 7, possibly due to the low sensitivity of IHC assay to quantitatively detect smaller changes.
Figure 21 Expression of stromal progenitors markers in the obstructed UPI compared to the adjacent normal ureter in human neonates.

A. Foxd1 mRNA is increased in the obstructed UPJ compared to the normal ureter in patient number 3, 5, 7, 10 and 11 (Individual patients labelled U1-U10). B,C. H&E staining of a representative midsagittal section of a UPJ specimen and its adjacent ureteric tissue. D-G. Immunohistochemical staining using anti-FOXD1 antibody. U2 is a representative sample in which there is no increase in FOXD1 (D,E), whereas U3 demonstrates increased FOXD1 expression (F,G). Yellow arrowheads point to the FOXD1 expressing nuclei stained with both DAB and haematoxylin. Orange arrowheads point to the nuclei not expressing FOXD1. H. Semi-quantitative analysis of immunohistochemical staining for patients with increased Foxd1 mRNA. There is a significant increase in DAB intensity in U3, U7 and U9, for which I did not have enough RNA to perform qPCR. No significant change was detected in U5 and U7.
As discussed above, the obstructing cells in\(Rarb2\text{Cre;}Ptc1^{\text{floxed}}\) mutants exhibited higher\(\text{Ptc1}\) and\(\text{Ptc2}\) mRNA expression in compared to the non-obstructing cells, in response to HH signaling activity. When I examined expression of\(\text{Ptc1}\) and\(\text{Ptc2}\) using qRT-PCR analysis, the obstructed UPJ in patients 3, 5 and 10 were found to show a 5.6-, 2- and 3- fold increase in\(\text{Ptc2}\) expression respectively (Figure 22). Interestingly,\(\text{Ptc1}\) was not detected in patient 4 with highest change magnitude in\(\text{Ptc2}\) expression, whereas it was up-regulated in patient 6 with no change in\(\text{Ptc2}\) expression. Thus, it is likely that\(\text{Ptc1}\) and\(\text{Ptc2}\) expression provide distinct readouts of HH signalling depending on particular genetic makeup of the patient. I further assessed transcriptional activity in response to HH signalling by analyzing two other HH target genes,\(\text{Gli1}\) and\(\text{Hhip}\) (Holtz, K. a Peterson, et al. 2013). Based on our qRT-PCR analyses,\(\text{Gli1}\) and/or\(\text{Hhip}\) were increased by different magnitudes in patients with increased\(\text{Ptc2}\) or\(\text{Ptc1}\) (Figure 22).

Taken together, our data demonstrate that the obstructed UPJ in patients 3, 5 and 10 exhibit an increase in both\(\text{Ptc2}\) and\(\text{Foxd1}\) expression. Notably, although there was no increase in\(\text{Foxd1}\) or\(\text{Ptc2}\) expression in patient number 6,\(\text{Ptc1}\) expression was dramatically increased. Overall, although there is a high variation in gene expression among different patients, our findings indicate a preliminary correlation between activated HH signalling or\(\text{Foxd1}\) expression with UPJ obstruction, at least at the transcription level, in a subset of the patients analyzed here.
Figure 22 Expression of HH signalling response genes in the obstructed UPI compared to the normal adjacent ureter in human neonates.

A. The graph shows an increase in Ptc2 expression in the obstructed UPJ compared to the normal ureter in patient number 3, 5 and 10 (Individual patients labelled U1-U10). In patient number 6, there is an increase in Ptc1 expression, but no change in Ptc2.

B-E. Immunohistochemical analysis of PTCH2 expression in the obstructed UPJ. Yellow arrowheads point to the Ptc2-expressing cells stained with DAB in brown in conjunction with haematoxylin in purple. U6 represents a sample with no change in PTCH2 (B,C), whereas U10 demonstrates an increase in PTCH2 protein (D,E).
3.6 Discussion

HH signalling pathway is crucial for development, and regulates cell fate determination, proliferation and tissue patterning (Goodrich et al. 1997; Litingtung et al. 2000; Litingtung et al. 2002). HH signalling is particularly important in the urogenital system development as Shh deficiency in the ureteric bud lineage, and truncating mutations in Gli3, are shown to cause renal agenesis and hypoplasia (Hu et al. 2004; Grotewold et al. 2002; Blake et al. 2015). Moreover, it was shown that aberrant activation of HH signalling, through targeting Ptc1 deficiency to the ureteric epithelium, causes renal hypoplasia due to abnormal branching morphogenesis (Cain et al. 2009). Work done by Lijun Chi from our lab demonstrated the pathogenic role of constitutively active HH signalling in the MM induced by Rarb2Cre, resulting in prenatal UPJ obstruction and hydronephrosis. However, the Rarb2 promoter is active in the nephrogenic mesenchyme beginning at least from E9.5, and maintained in the MM-derived cells, excluding the medullary stroma. Therefore, the spatial and temporal effect of Ptc1 deficiency cannot be delineated using this model (Kobayashi et al. 2005). Here, I reported that that activation of HH signalling in the renal precursor population marked by either Sall1 or Osrl at E9.5 leads to the same phenotype as Rarb2-Cre:Ptc1-null mutants. Yet, conditional activation of the pathway at E10.5 does not lead to the same phenotype. Also, Ptc1 deletion in MM after the onset of ureteric branching using Tcf21Cre/Pod1Cre has been previously shown to only cause mild cystic kidneys and no prenatal hydronephrosis (Maezawa et al. 2012). Together, these data suggest that abnormal activation of HH signalling prior to fate restriction in the renal precursors is a contributing factor to pathogenesis of UPJ obstruction.

I showed the presence of Ptc1+Raldh2+ cells in the obstructing UPJ tissue in the mutants. This coupled with the findings discussed in the previous chapter indicating the effect of activated
HH signalling in promoting stromal lineage specification, further explains the contribution of renal stromal progenitors to the pathogenesis of UPJ obstruction. There are currently two major models of obstructive hydronephrosis that are both associated with developmental defects in urothelial and smooth muscle differentiation (Fogelgren et al. 2015; Yan et al. 2014). Yet, Marian Staite previously demonstrated no defect in the smooth muscle cells (marked by SMA) and ureteric epithelium (marked by UPKIII) in Rarb2-Cre;Ptc1-null hydronephrotic kidneys, which is consistent with the absence of hydroureter in these mutants. Moreover, since there is no evidence in favor of contribution of Foxd1+ cells to ureteric smooth muscle cells, it is unlikely that smooth muscle cells are affected in our models of UPJ obstruction (Kobayashi et al. 2014). Thus, in cases where there are no defects in ureteric epithelium or smooth muscle cells, I propose that an abnormally expanded stromal progenitor population (caused by aberrant activation of HH signalling in their precursor compartment) could explain the pathogenesis of UPJ obstruction and hydronephrosis.

Evidently, the true etiology of this complicated congenital abnormality in humans is multifactorial and therefore has remained largely unknown. Here, I examined our proposed model in 11 neonates who underwent pyeloplasty for constricted UPJ removal. I compared the expression of Ptc1, Ptc2 and other HH signalling response genes as well as Foxd1, a marker of stromal progenitors, through IHC and qRT-PCR. Our results demonstrated increased expression of Ptc2 and Foxd1 in 3 patients at least at the RNA level. Although, I were not able to quantitatively compare the expression of these genes at the protein level, our semi-quantitative IHC measurements validates the increase in Foxd1 expression in least 2 of the patients identified through qRT-PCR. Analyzing a larger patient sample population and using more sensitive protein expression assays are necessary to confirm the contribution of migration/differentiation
defects in stromal progenitors under abnormally activated HH signalling condition in human neonates.
3.7 Conclusion and Future Directions

Despite many rodent genetic models with adult-onset hydronephrosis, there are very few models with prenatal development of congenital obstructive nephropathy, and among the latter group conditional knockouts have mainly targeted the smooth muscle cells surrounding the ureter or urothelium (Klein et al. 2011). Here, I present two UPJ obstruction models with over-activation of HH signalling targeted to the renal precursors and their descendants within the kidney proper. UPJ obstruction and severe hydronephrosis in these models are observed secondary to the renal abnormalities discussed in the previous chapter. In addition, I identified cells expressing markers of renal stromal progenitors in the obstructing UPJ, suggesting potential migration/ differentiation defect in this lineage caused by over-activity of HH signalling in their precursors. Future studies should focus on candidate molecular mechanisms involved in proper migration of stromal progenitors and their cell-cell interactions with the surrounding mesenchyme that might be regulated by HH signalling.

Interestingly, our mutants exhibited abnormal positioning of the gonads, suggesting potential abnormalities in the entire urogenital system including the mesonephros (Figure 1). Although the anatomy and gene expression patterns in the mesonephros are well characterized, the mechanisms that regulate gonadal development, including the interactions of early mesodermal tissues, are poorly understood (Murashima et al. 2014). It has recently been shown that midline structures including the notochord and floor plate are the source of SHH essential for mesonephros development (Murashima et al. 2014). Further investigation of our newly identified mouse models in which the timing of HH signaling activation can be temporally regulated would provide deeper insight into the role of HH signalling in mesonephros and gonadal development.
Building on the foundation of the work described in this thesis, future work should focus on whole exome sequencing to discover mutations associated with abnormal HH signalling activity or specification of renal stromal cells from the IM in patients with congenital UPJ obstruction and hydrenephrosis. This will provide further insights into the underlying causes of human UPJ obstructions, characterization of the progression of the disease in utero, and perhaps novel bio-markers for UPJO progression and severity.
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