Activated Hedgehog-GLI Signaling Causes Congenital Ureteropelvic Junction Obstruction

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ABSTRACT

Intrinsic ureteropelvic junction obstruction is the most common cause of congenital hydronephrosis, yet the underlying pathogenesis is undefined. Hedgehog proteins control morphogenesis by promoting GLI-dependent transcriptional activation and inhibiting the formation of the GLI3 transcriptional repressor. Hedgehog regulates differentiation and proliferation of ureteric smooth muscle progenitor cells during murine kidney-ureter development. Histopathologic findings of smooth muscle cell hypertrophy and stroma-like cells, consistently observed in obstructing tissue at the time of surgical correction, suggest that Hedgehog signaling is abnormally regulated during the genesis of congenital intrinsic ureteropelvic junction obstruction. Here, we demonstrate that constitutively active Hedgehog signaling in murine intermediate mesoderm–derived renal progenitors results in hydronephrosis and failure to develop a patent pelvic-ureteric junction. Tissue obstructing the ureteropelvic junction was marked as early as E13.5 by an ectopic population of cells expressing Ptch2, a Hedgehog signaling target. Constitutive expression of GLI3 repressor in Ptch1-deficient mice rescued ectopic Ptch2 expression and obstructive hydronephrosis. Whole transcriptome analysis of isolated Ptch2+ cells revealed coexpression of genes characteristic of stromal progenitor cells. Genetic lineage tracing indicated that stromal cells blocking the ureteropelvic junction were derived from intermediate mesoderm–derived renal progenitors and were distinct from the smooth muscle or epithelial lineages. Analysis of obstructive ureteric tissue resected from children with congenital intrinsic ureteropelvic junction obstruction revealed a molecular signature similar to that observed in Ptch1-deficient mice. Together, these results demonstrate a Hedgehog-dependent mechanism underlying mammalian intrinsic ureteropelvic junction obstruction.


Ureteropelvic junction obstruction (UPJO) is the most common form of congenital urinary tract obstruction. UPJO is also a major cause of antenatal dilation of the renal pelvis, identified by ultrasound in 1% of fetuses.1 Most cases of UPJO are caused by intrinsic narrowing of the ureter at the pelvic-kidney junction, with deposition of extracellular matrix, infiltration of inflammatory cells, and smooth muscle hypertrophy.2–4 Yet, underlying pathogenic
mechanisms are largely undefined, particularly because of the absence of experimental models of congenital UPJO. Lack of reliable biomarkers to differentiate UPJO from other forms of kidney dilation limits the ability to predict a need for corrective surgery, currently performed in approximately 50% of affected infants, and the development of specific therapeutic strategies.

The ureteropelvic junction (UPJ) is a component of the renal collecting system, which consists of collecting ducts, calyces, and the pelvis, all derived from the epithelial ureteric bud (UB). After UB outgrowth from the Wolffian duct at 5 weeks human gestation (approximately E10.5 in mice), the distal UB elongates caudally and integrates with the bladder. The rostral tip of the UB invades the metanephric mesenchyme (MM) and undergoes repeated branching events regulated by reciprocal inductive interactions between nephron progenitors surrounding the UB tips, and Foxd1+ cells that surround the nephron progenitors.

Renal calyces and the pelvis are formed from the first several ureteric branch generations. Although the morphogenetic events in pelvicalyceal remodeling are largely undefined, Wnt7b expression in the ureteric stalk is crucial in ureteric tree elongation and pelvis formation. Ureteric smooth muscle development begins at E15.5 with differentiation of Tsh3+ and Tbx18+ smooth muscle progenitors, both of which arise from tail-bud mesoderm.

During renal development, the Hedgehog (HH) ligand, Sonic hedgehog (SHH), and its receptor Patched 1 (Ptc1), are expressed in tubular epithelium and stromal cells adjacent to the pelvis-kidney junction. In vertebrates, HH ligand binds to PTCH and in tubular epithelium and stromal cells adjacent to the pelvic-calycial axis,14 does not differ that is expressed in medullary interstitial cells and regulates renal calyces and the pelvis, all derived from the epithelial ureteric bud (UB). After UB outgrowth from the Wolffian duct at 5 weeks human gestation (approximately E10.5 in mice), the distal UB elongates caudally and integrates with the bladder. The rostral tip of the UB invades the metanephric mesenchyme (MM) and undergoes repeated branching events regulated by reciprocal inductive interactions between nephron progenitors surrounding the UB tips, and Foxd1+ cells that surround the nephron progenitors.

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RESULTS

**Ptc1 Deletion Causes Congenital Intrinsic UPJO**

HH signaling activity, as reported by expression of Ptc1-lacZ, is localized to the UB and the surrounding mesenchyme during murine renal development. Embryonic lethality before metanephric kidney development in Ptc1-null mice provides a basis to investigate Ptc1 functions in a lineage-specific manner.

We generated mice in which Ptc1 deficiency is restricted to the metanephric lineage using Ptc1lox/lox mice and an MM-specific Rarb2Cre allele (Ptc1−/−MM). Ureteric bud (UB) mice showed a 60% decrease in expression of Ptc1 exon 3. The target of CRE-mediated excision, in metanephric rudiments at E11.5 (n=3; P<0.05; Figure 1A). qRT-PCR analysis of an unruptured exon of Ptc1 (exon 17) demonstrated a two-fold increase as early as E11.5 (n=3; P<0.05; Figure 1B), consistent with increased HH signaling activity. Compared with control mice, expression of the Ptc1-lacZ reporter allele was increased in the medullary stroma and expanded to the renal cortex in Ptc1−/−MM kidneys (Figure 1, C and D).

Histologic analysis of Ptc1−/MM kidneys at E18.5 demonstrated distention of the renal pelvis (i.e., hydronephrosis) bilaterally and ablation of the renal medulla with no ureteric dilation (80% penetrance, 29 males and 33 females) (Figure 1, E and F). Measurement of pelvic volume in three-dimensional images of E18.5 mutant kidneys generated by magnetic resonance imaging (MRI) revealed a nine-fold increase (n=10; P<0.05; Figure 1, G–I). Histologic and immunofluorescence analysis of the nascent epithelial collecting ducts and distal tubules marked, respectively, by calbindin and E-cadherin, demonstrated comparable formation of the epithelial tubular network within the renal medulla in both wild-type (WT) and mutant kidneys at E15.5 (Supplemental Figure 1, A–F). Further, expression of Lef1, a canonical Wnt pathway effector that is expressed in medullary interstitial cells and regulates the collecting duct cortico-medullary axis, did not differ between Ptc1−/MM kidneys and controls (Supplemental Figure 1, G and H). Intrapelvic dye injection in E18.5 embryos demonstrated that injected dye failed to pass the UPJ in mutant mice (Figure 1, J and K). Together, these data indicate that Rarb2Cre-mediated Ptc1 deletion in the MM causes congenital intrinsic UPJO.

HH signaling was previously shown to control cellular differentiation during ureter formation28 and defects in urethelial differentiation or smooth muscle proliferation cause UPJO in mice.33,34

**Statement of Significance**

Ureteropelvic junction obstruction (UPJO) is a major cause of congenital dilation of the renal pelvis. The pathogenesis of UPJO is undefined, no biomarkers are identified to predict its natural history, and surgical correction is the only treatment. This study demonstrates that increased Hedgehog signaling activity, caused by Ptc1 deficiency within a narrow time window during mouse development, results in ectopic localization of cortical stromal cells to the ureteropelvic junction, causing blockage of urinary outflow and dilation of the renal pelvis. Expression of genes characteristic of cortical stromal cells and Hedgehog signaling activity was increased in obstructive ureteric tissue resected from affected children. These results provide a basis for genetic studies in human UPJO and therapeutic strategies targeting the Hedgehog signaling pathway.
Yet, analysis of the ureter distal to UPJO in 

Ptch1−/−MM mice failed to demonstrate a difference in the expression of ureteric epithelial markers, including uroplakin, cytokeratin, and E-cadherin. Similarly, ureteric smooth muscle cells, marked by smooth muscle actin, expressed transgelin, a differentiation marker, in both WT and mutant tissue at E17.5 (Supplemental Figure 1, I and J). We observed no obvious difference in smooth muscle thickness in the ureter after the full onset of hydronephrosis (Figure 1, L–O). In situ hybridization analysis of Tbx18 expression at E13.5 demonstrated no defect in the ureteric mesenchymal lineage in 

Ptch1−/−MM mutants (Supplemental Figure 1, K and L). Together, these data strongly suggest that UPJO in 

Ptch1-deficient mice is not associated with impaired urothelial differentiation or smooth muscle proliferation.
Increased HH Signaling Activity and Ectopic Ptch2 in the Presumptive UPJO

We investigated the role of HH signaling in UPJO in Ptch1\(^{-/-}\)MM mice. At E18.5, Ptch1-lacZ was strongly expressed in the obstructing tissue located between the renal pelvis and the ureter (Figure 2B). Further, expression of Ptch2, a Ptch1 homolog, was assayed using a Ptch2-lacZ reporter, and was found only in the mutant kidneys in the presumptive UPJ as early as E12.5, with continued expression in UPJ obstructing tissue until E18.5 (Figure 2, D and F, Supplemental Figure 2). To examine the functional contribution of Ptch2 to UPJO, we generated compound mutants with both Ptch1 and Ptch2 deficiency targeted to the metanephric lineage using Rarb2Cre. Histologic analysis at E18.5 revealed no difference between the Ptch1;Ptch2 compound mutants and the Ptch1 mutants alone (Figure 2, G and H). These results demonstrate that the UPJ is an active domain of HH signaling in kidneys of Ptch1\(^{-/-}\)MM mice, and Ptch2 is a specific marker of UPJ obstructing cells.

Next, we investigated the functional role of increased HH signaling in the pathogenesis of UPJO by decreasing HH signaling activity. Genetic analyses in mice have established the requirement for a balance of GLI3 repressor and activator during kidney development. Here, we introduced a constitutive GLI3R allele (Gli3\(^{D699}\)) into the Ptch1\(^{-/-}\)MM genetic background to reduce HH signaling activity. In contrast to Ptch1\(^{-/-}\)MM mice, Ptch2-lacZ expression was greatly diminished in the presumptive UPJ of Ptch1\(^{-/-}\)MM;Gli3\(^{D699+/+}\) mice (Figure 3, A–C). Moreover, histologic analysis suggested a partial restoration of normal pelvic morphology (Figure 3, D–F), intra-pelvic dye injection demonstrated rescue of UPJO (Figure 3, G and H), and MRI revealed a marked reduction in pelvic volume in seven out of ten mice (Figure 3I). These results demonstrate that increased HH signaling causes UPJO in Ptch1\(^{-/-}\)MM mice.

PTCH2-Positive Cells Blocking the UPJ Express Genes Characteristic of Stromal Progenitor Cells

We investigated the identity of Ptch2\(^{+}\) cells that block the UPJ in Ptch1\(^{-/-}\)MM mice at E13.5, a...
in Ptch2+ cells by qRT-PCR (Figure 4D). Consistent with these results, in situ hybridization analysis demonstrated expansion and ectopic expression of Foxd1 and Raldh2 at the UPJ of Ptch1−/−MM mice at E13.5 (Figure 4, E–H).

Next, we investigated whether Ptch2+ cells coexpress HH-related and stromal markers or, alternatively, are a heterogeneous group of cells that express either but not both of these markers. We analyzed gene expression at a single-cell resolution using double-florescence in situ hybridization and confocal microscopy of the pelvic-kidney junction in Ptch1−/−MM mice. As expected, Raldh2, and Foxd1 colocalized in the cells blocking the UPJ (Figure 4, J–K”). Remarkably, the vast majority of Ptch2+ cells also expressed Raldh2 and Foxd1 (Figure 4, J–K”). In contrast, coexpression of Bmp4 and Ptch2 was rarely observed (Figure 4, L–M”). Together, these results indicate that Ptch2+ cells that block the presumptive UPJ in Ptch1−/−MM mice are ectopically located cortical stromal cells.

**Ptch1 Deletion in IM-Derived Renal Progenitors Causes UPJO**

Renal stromal progenitors are derived from the Osr1+ population of the IM and the specification of the stromal lineage occurs between E9.5 and E11.0.15,41 Because Rarb2-Cre promoter activity is evident at E9.5,31 we examined whether the ectopic population of Foxd1+ cells emerges as a result of increased HH signaling in Osr1+ cells. To address this possibility, we crossed a Ptch1lox/+ male, followed by tamoxifen injection at E9.5. Histologic examination of kidneys isolated from all four Osr1E2FPCreER2; Ptch1lox/lox mice at E18.5 revealed hydronephrosis similar to that observed in Ptch1−/−MM mice (Figure 5, A and B). Further, in situ hybridization confirmed ectopic Ptch1 expression in cells blocking the UPJ (Figure 5, C and D).

Osr1 is expressed in both anterior and posterior IM, which give rise to the ureteric epithelium and metanephric lineages, respectively.41 To exclude the possibility that increased HH signaling in the UB causes the observed phenotype, we activated HH signaling in Sall1+ daughters of Osr1+ cells found only in the posterior nephrogenic mesenchyme.42 We generated Sall1CreER2;Ptch1lox/lox embryos by crossing a Ptch1lox/lox male with a Sall1CreER2;Ptch1+/+ female, and injected tamoxifen at E9.5.43 A total of 82% of the mutant embryos (n=9 out of 11) demonstrated hydronephrosis with UPJO similar to that observed in Osr1E2FPCreER2;Ptch1lox/lox mice (Figure 5, E–H). Interestingly, we did not observe a similar phenotype after

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Figure 3. Addition of one Gli3R allele in the Ptch1-deficient mice rescues UPJO and pelvis size. (A–C) β-galactosidase staining shows very little Ptch2-LacZ expression in the presumptive UPJ in Ptch1−/−;Ptch22−/-/+;Gli33469/+ compared with that of control mice at E14.5. Scale bars, 200 μm. (D–F) Histologic analysis of E18.5 kidney tissue reveals a partial normalization of the phenotype in Ptch1−/−,Ptch2−/-/+;Gli33469/+ mice compared with Ptch1-deficient mutants. Scale bars, 500 μm. (G and H) Intrapelvic dye injection demonstrates the restoration of UPJ connectivity in Rarb2Cre;Ptch1LacZ/fox/fox, Gli33469/+ mice compared with WT kidneys. Scale bars, 75 μm. (I) Measurement of the pelvis size using three-dimensional reconstructed images created by MRI confirms a significant reduction in pelvis size in Ptch1−/−,Gli33469/+ compared with Ptch1−/− (n=10 per genotype; *P<0.05, **P<0.005).

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day after the onset of ectopic Ptch2-lacZ expression. We microdissected kidney-ureter junction tissue from Ptch1−/−MM mice obtained from two litters, purified Ptch2+ cells by FACS using antibodies specific for PTCH2, and performed RNA sequencing on Ptch2-positive and -negative cells (Figure 4, A and B). To identify markers for Ptch2+ cells, we inspected differentially expressed genes (GEO accession no. GSE97126) for those that were upregulated and identified the HH signaling effector genes, Ptch1, Gli1, Hhip, and Bmp4. The expression of these genes was validated by qRT-PCR (Figure 4, C and D). Surprisingly, RNA-seq data revealed that Ptch2+ cells highly express genes (Pdgfrα, Tnc, and Sfrp1) that are normally expressed in the embryonic cortical interstitium and capsule (Figure 4C).9,38,39 Because both cortical interstitium and capsule are derived from Foxd1+ stromal progenitors,8 we hypothesized Foxd1+ cells to be the origin of the cells blocking the UPJ. Indeed, a significant increase in expression of both Foxd1 and Raldh2, another marker of stromal progenitor cells, was found in Ptch2+ cells by qRT-PCR (Figure 4D). Consistent with these results, in situ hybridization analysis demonstrated expansion and ectopic expression of Foxd1 and Raldh2 at the UPJ of Ptch1−/−MM mice at E13.5 (Figure 4, E–H).
tamoxifen injection at E8.5 or E10.5 (n=4 per time point, data not shown). Immunofluorescence confirmed that Raldh2+ cells block the UPJ as early as E16.5 when Ptch1 deletion is driven by either Sall1CreERt2 or Osr1CreERt2 (Figure 5, I–L).

To determine the embryonic origin of the cells that block the UPJ, we performed lineage tracing by introducing a Rosa26-ttdTomato reporter allele into a Sall1CreERt2;Ptch1fl/fl;Ptch2fl/+ genetic background. Cre-mediated recombination was induced at E9.5 by tamoxifen treatment of pregnant females. Sall1CreERt2;Ptch1fl/fl;Ptch2fl/+;Rosa26tdT/+ littermates were used as controls. Descendants of Sall1+ cells, indelibly labeled with tdTomato, were identified by high levels of PBX1 expression in stromal cells and low PBX1 levels in nephrogenic cells (Figure 6A). Our results demonstrated that at E9.5 Sall1+ cells contribute to both stromal and nephrogenic lineages. At E16.5, the funnel-shaped pelvis that normally opens into the ureter was blocked by tdTomato-expressing cells in Ptch1-deficient kidneys (Figure 6, B and C). These blocking cells did not express smooth muscle actin, but expressed the mesenchymal protein vimentin (Figure 6, D–F). Vimentin-expressing cells in the UPJO colocalize with ectopic expression of RALDH2 in the mutant kidneys (Supplemental Figure 3). We further confirmed coexpression of tdTomato and vimentin in individual UPJO cells using higher resolution, three-dimensional reconstruction of stacks of single optical sections (Figure 6, G and H). Together, these data demonstrate that the ectopically located cortical stromal cells observed in Ptch1+/−;MM kidneys are derived from Sall1+ progenitor cells.

**Figure 4.** UPJO cells are characterized by ectopic expression of Ptch2 and markers of stromal progenitors. (A and B) Approximately 2500 Ptch2+ and approximately 14,000 Ptch2− cells were isolated from presumptive UPJ of E13.5 kidneys using anti-PTCH2 antibodies followed by FACS. (C) RNA-seq analysis reveals that among the genes upregulated in the Ptch2+ cell population, there were specific markers of cortical stroma and HH signaling targets. (D) qRT-PCR analysis demonstrates a significant increase in Ptch1, Ptch2, and Bmp4 genes downstream of HH signaling, in addition to Foxd1 and Raldh2, specific markers of the cortical stroma (n=4; P<0.05). (E–H) *In situ* hybridization in E13.5 kidney tissues demonstrates an increase in Foxd1 and Raldh2 gene expression at the cortex and ectopic expression in the presumptive UPJO in Ptch1-deficient mice (arrowheads). Scale bars, 200 μm. (I–L) Double-fluorescence *in situ* hybridization on E13.5 demonstrates coexpression of Foxd1 and Raldh2, in addition to coexpression of Ptch2 with both Foxd1 and Raldh2. Bmp4 does not fully colocalize with Ptch2 (asterisks in Ptch2+/Bmp4− cells). Scale bars, 15 μm.
Foxd1 and PtcH2 are Upregulated in Obstructive Ureteric Tissue of a Subset of Children with Congenital Intrinsic UPJO

Our findings in genetic mouse models, described above, raise the possibility that aberrant HH signaling activity in Foxd1+ cells could contribute to the genesis of human UPJO. To address this possibility, we obtained obstructed and normal ureter tissue from eight infants and young children at the time of surgical repair of congenital intrinsic UPJO. Children with a history of urinary tract infection were excluded to control for secondary effects on gene expression. RNA was isolated from a segment of both the obstructive and normal ureter and was subjected to qRT-PCR for gene expression analysis. Contiguous tissue was used for in situ examination of protein expression by immunohistochemistry. We controlled for variations across individuals by normalizing gene expression in the obstructed UPJ segment to that in the adjacent normal ureter for each individual analyzed.

We identified five patients in whom FOXD1 was increased by an average of six-fold in the obstructive ureteric tissue segment compared with contiguous normal ureter (Figure 7A). In contrast to our findings in PtcH1-deficient mice, expression of RALDH2 was not significantly altered in affected patients (data not shown). HH signaling activity in obstructing ureteric tissue was assessed by analyzing the expression of PTCH2, PTC1, GLI1, and HHIP. PTC2 expression was increased by an average of three-fold in four of the five patients with increased FOXD1 (Figure 7B, Table 1). GLI1 and HHIP were significantly increased in three of these four patients (three of the five patients with increased FOXD1) (Figure 7B, Table 1). PTC1 was increased in a single patient (U5) who did not exhibit an increase in either PTC2 or FOXD1 (summarized in Table 1). Patient U5 also exhibited increased expression of GLI1 and HHIP (Figure 7, C and D). Immunohistochemical analysis confirmed increased expression of both FOXD1 and PTC2 protein in the muscular layer of the obstructing tissue in patients U2, U4, and U7 (Figure 7, E–H, representative samples from patient U7). Overall, our data supports a role for increased HH signaling and renal stromal cells in the pathogenesis of human UPJO.
DISCUSSION

The molecular mechanisms underlying congenital intrinsic UPJO are poorly understood. Here, we demonstrated that constitutive activity of HH-GLI signaling in IM-derived renal progenitors induces formation of an ectopic population of Ptch2+ cortical stromal cells that obstruct the UPJ during embryogenesis. In addition, we identified a molecular signature characteristic of stromal progenitors and activated HH signaling in the obstructed UPJ in a subset of children with congenital UPJO. These results demonstrate a HH-GLI–dependent mechanism causing murine intrinsic UPJO and implicate dysregulated HH signaling in the pathogenesis of human UPJO.

Role of Stromal Cells in the Pathogenesis of UPJO

Although it has been established that the majority of Foxd1+ stromal progenitors originate from the Osr1+ posterior IM, how stromal lineage segregation occurs is not clear. Previous studies have shown that neither Foxd1 nor Osr1 regulate specification and differentiation of the stromal lineage. Here, we provide the first evidence that HH signaling may play a role in stromal cell specification from precursor cells. Induction of Ptc1 deficiency in both Osr1+ and Sall1+ cells at E9.5 caused obstructive hydronephrosis with ectopic Raldh2 expression in the presumptive UPJ. Lineage tracing analysis after activation of HH signaling in Sall1+ cells confirmed that the ectopically located stromal cells in the UPJ are direct descendants of IM-derived renal progenitors. Previous published work has

tdTomato+ cells are distinct from smooth muscle actin-expressing smooth muscle lineage (green). (F) Higher resolution confocal image showing vimentin (dark blue) in the UPJ obstructing tdTomato+;SMA+ cells. Scale bar, 25 μm, inset shows a cross-section of z-stacks. (G and H) Three-dimensional reconstructed image from stacks of single optical sections of the UPJ obstructing cells showing colocalization of tdTomato and vimentin (dark blue) in individual cells. *indicates UPJO; dashed lines indicate kidney periphery or individual cells; arrows indicate obstructing tdTomato+ cells.

Figure 6. Sall1+ renal precursor cells give rise to UPJ obstructing cells. (A) Immunofluorescence staining in Sall1CreERt2;Ptch1+;Rosa26tdTomato+ kidneys at E16.5, showing high PBX1 expression (green) in stromal cells and low PBX1 expression in nephron progenitors. After Cre-mediated recombination in Sall1+ cells at E9.5, tdTomato-labeled cells localize to both stromal and nephron progenitors, and are excluded from the ureteric lineage (light blue). Scale bars, 25 μm. (B and C) tdTomato+ cells localize in and block the UPJ in mutant mice. Scale bars, 200 μm. (D and E) UPJ obstructing tdTomato+ cells are distinct from smooth muscle actin-expressing smooth muscle lineage (green). (F) Higher resolution confocal image showing vimentin (dark blue) in the UPJ obstructing tdTomato+;SMA+ cells. Scale bar, 25 μm, inset shows a cross-section of z-stacks. (G and H) Three-dimensional reconstructed image from stacks of single optical sections of the UPJ obstructing cells showing colocalization of tdTomato and vimentin (dark blue) in individual cells. *indicates UPJO; dashed lines indicate kidney periphery or individual cells; arrows indicate obstructing tdTomato+ cells.


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shown that Ptch1 deletion in the MM using Tcf21Cre, which is not active at the IM stage, results in glomerular and tubular cysts but not hydronephrosis. Our findings further suggest that renal progenitors are responsive to the effect of HH signaling before establishment of the nephrogenic and stromal lineage boundaries. HH signaling has been shown to regulate Foxd1 expression in the context of craniofacial and optic vesicle development. It is likely that constitutive HH signaling activity during stromal cell specification promotes stromal cell fate by upregulating Foxd1. A differential analysis of the genetic profile in Osr1+ or Sall1+ cells is likely to reveal mechanisms involved in stromal lineage specification.

What mechanism could underlie the ectopic location of Ptch1-deficient stromal progenitors? Foxd1+ cells initially form in a caplike pattern anterior to the MM, and subsequently integrate into the kidney periphery in a Hox10-dependent manner. As the kidney grows, a subset of differentiated stromal cells localize within the glomerular capillary loops and medulla, whereas self-renewing Foxd1+ cells are maintained in the cortex. Our data suggests that Ptch1 in the MM is not crucial for the initial integration of Foxd1+ cells into the cortex because renal capsule and cortical interstitium patterning was unaffected in Ptch1-deficient mice. RNA-seq of Ptch2+ cells demonstrated enrichment for a SHH receptor, Boc, which is essential in SHH-dependent commissural neuron guidance in the spinal cord. Thus, SHH generated in renal medulla may act as a chemoattractant for BOC-expressing stromal cells, resulting in ectopic localization in the UPJ.

HH-GLI Mutations and Control of Human Renal-Urinary Tract Development

Little is known about the role of PTCH2 in development. Similar to Ptch1, transcriptional activation of Ptc1 occurs in response to SHH, and Ptc1 is downregulated in the absence of GLI. Although medullary stroma is a domain of high HH signaling activity, Ptc1 is not expressed in the medullary stroma in WT kidneys. Upon removal of functional PTCH1 from the MM, Ptc1 is markedly upregulated in the obstructing cells at the presumptive UPJ as early as E12.5. This finding illustrates a requirement for PTCH1 to downregulate Ptc1 expression, which is consistent with increased expression of Ptc1 in both human basal cell carcinoma and medulloblastoma with PTCH1 mutations. Loss of Ptc2 in Ptc1-null mice worsens the

Figure 7. A subset of children with congenital UPJO demonstrates changes in expression of FOXD1 and PTCH2. (A) The expression of FOXD1 in the obstructive UPJ tissue resected from children with congenital UPJO relative to the nonobstructive ureteric tissue is significantly higher in patients 2, 4, 6, 7, and 8. (B) In patients 2, 4, 7, and 8, the expression of PTCH2 in the obstructive UPJ tissue relative to the nonobstructive ureteric tissue is significantly higher than that of the rest of the patients. (C and D) U2, 4, 6, 7, and 5 demonstrate significantly higher relative expression of GLI1 compared with the other patients. U2, 4, 7, and 5 demonstrate significantly higher relative expression of HHIP compared with the other patients. (E–H) Immunohistochemical staining of the obstructive UPJ and nonobstructive ureteric tissue from patient U7 using anti-FOXD1 and anti-PTCH2 antibodies shows a higher intensity of expression of both FOXD1 and PTCH2 in the UPJO (Original magnification, ×40).
Table 1. Summary of the transcriptional analysis in children with congenital UPJO

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Resected obstructive ureteric tissue from patients 2, 4, and 7 demonstrates an increase in the levels of FOXD1, PTCH2, GLI1, and HHIP transcripts relative to the adjacent nonobstructive ureteric tissue. Patients 6 and 8 have increased FOXD1 along with GLI1 and PTCH2, respectively. Patient 5 demonstrates increased PTCH1, GLI1, and HHIP but not FOXD1 or PTCH2. In patients 1 and 3, expression of the analyzed genes were comparable in obstructive tissue to that of the adjacent nonobstructive ureter. +, upregulated; —, not upregulated.

mutant phenotype, suggesting that the two homologs have overlapping functions.53,54 However, genetic removal of Ptch2 in our Ptch1+/−;Mi mice did not affect the severity of the observed phenotype. This suggests that Ptch2 does not play a functional role in regulating HH signaling activity during kidney development, but serves as a specific marker of the onset of UPJO.

Inactivating mutations in Gli3 and Ptch1 have been identified in humans with Greig cephalopolysyndactyly syndrome and Nevado basal cell carcinoma syndrome (Gorlin syndrome), respectively.55,56 Currently, no known association between human Gli3 loss of function or Ptch1 mutations and renal malformation exists. Previous studies have shown a significant decrease in Gli3 expression in UPJO specimens from neonatal patients compared with that in ureters from patients with Wilms tumor.57 Our data extend these observations and demonstrate a significant increase in Ptch2 expression in the obstructive UPJ segments in a subset of patients with congenital UPJO.

Ptch1-Deficient Mice as a Novel Genetic Model of Congenital UPJO

Our results uncover a mechanism distinct from previous studies that implicated abnormal ureteric smooth muscle or urothelial development in UPJO. Conditional deletion of the TGF-β/BMP signaling effectors, Smad4 and Id2, in the Tbx18+ ureteric mesenchyme has been shown to cause UPJO, secondary to a functional obstruction caused by a reduction in smooth muscle cell number and ureteric contractility.58 Although HH signaling has been shown to regulate smooth muscle development,28 we did not observe disruption in ureteric smooth muscle even after the onset of hydronephrosis in mice with Ptch1 deficiency driven by Rarb2Cre or Sall1Cre. This is consistent with previous studies showing intact smooth muscle cells in mice with targeted deletion of smoothed in the MM,25 and confirms the nonoverlapping domain of Tbx18 and Rarb2Cre.15 Although Osr1, Sall1, and Tbx18 have overlapping expression domains, Ptch1 deficiency induced by Sall1Cre or Osr1Cre does not cause gross defects or overgrowth of the ureteric mesenchyme. In another UPJO model, targeted inactivation of Sec10 in Ksp1.3+ ureteric epithelium resulted in decreased ureteric expression of uroplakin, followed by overgrowth of ureteric mesenchymal cells.34 In contrast, in Ptch1-deficient mice reported here, the urothelial layer is physically intact and exhibits normal expression of urothelial markers distal to the UPJ. This provides further evidence that the MM and UB behave as distinct lineages after E8.5.41,59

CONCISE METHODS

Mice

Rarb2Cre mice (provided by R. Behringer, University of Texas, Houston, TX) were mated with Ptch1+/− or Ptch1loxP/loxP (provided by C.C. Hui, University of Toronto, Toronto, Canada) mice to generate Rarb2Cre;Ptch1+/− or Rarb2Cre;Ptch1LacZ/+ males. Ptch1loxP/loxP (provided by B. Wainwright, University of Queensland, Queensland, Australia) females were used to generate Rarb2Cre;Ptch1loxP/loxP or Rarb2Cre;Ptch1loxP/LacZ (Ptch1+/−;MM) progeny. Sall1CreERT2, (a gift from R. Nishinakamura43) and Osr1EGFPCreERT2;Ptch1loxP/loxP embryos were generated by a similar approach. Osr1EGFPCreERT2 and Rosa26tdTomato mice were purchased from Jackson Laboratories. For mouse breeding, noon of the day of a vaginal plug was considered as E0.5. To activate Cre recombinase in the ERT2-carrying embryos, pregnant dams were injected intraperitoneally with 3 mg of tamoxifen dissolved in sesame oil. All of the animal procedures were approved by the Animal Ethics Committee at the Toronto Center for Phenogenomics (Toronto, Ontario, Canada) and in accordance with the Canadian Council of Animal Care.

Patient Specimens

This study was approved by the Research Ethics Board of the Hospital for Sick Children in Toronto, Canada (approval no. 100043163). After informed consent, excisional UPJ biopsies and a short segment of the adjacent unaffected ureter were obtained during reparatory surgery at the Hospital for Sick Children.

Histologic Analyses

Paraffin-embedded kidney or ureter were prefixed in 4% paraformaldehyde overnight at 4°C and sectioned at 5 μm. OCT-embedded frozen kidneys were prefixed in 4% paraformaldehyde, cryopreserved using sucrose gradient, and sectioned at 8 μm. Immunofluorescence and immunohistochemical staining was performed on paraffin-embedded or frozen sections using standard protocols (see Supplemental Material for protocol and antibodies used). For β-galactosidase staining, 10 μm frozen sections were postfixed in 0.2% paraformaldehyde, washed in LacZ rinse buffer, and stained by immersion in β-galactosidase staining solution overnight at 37°C in the dark (see Supplemental Material for solution composition). The X-gal–stained sections were counterstained with eosin. Immunofluorescence imaging was done using a Zeiss epi-fluorescence microscope and Olympus spinning disk confocal microscope, and Z-stack processing was done using Velocity software. Other images were captured using Leica light microscope.
Intrapelvic Dye Injections
Briefly, bromophenol blue dye was injected into the renal pelvis of isolated whole urogenital systems at E18.5 using a pulled-out Pasteur glass pipette.16

MRI Preparation, Imaging, and Renal Pelvis Measurement
Kidney specimens were fixed for 24 hours in MRI fixative solution containing 0.2 ml/50 ml Prohance (Bracco, Quebec, Canada)/4% paraformaldehyde. Kidneys were then placed in 0.2 ml/50 ml in Prohance/PBS for 1 week. Image acquisition was performed at the Mouse Imaging Centre on a 7.0T Magnex magnet equipped with a multichannel Varian DirectDrive Console (Varian, Inc., Palo Alto, CA) using multiple-mouse MRI with solenoid coils (14 mm diameter, 19 mm length).61 The imaging sequence was a gradient-echo (parameters: TR= 50 milliseconds; TE= 3.2–4.8 milliseconds; 4–6 averages; 35 μm isotropic voxels; scan time, 10 hours 22 minutes–15 hours 33 minutes). Renal pelvis measurements were obtained using MRI and three-dimensional volumetric visualization software (Amira v5). Kidney volumes were serially segmented semiautomatically, and then volumes of the renal pelvis were compared between groups.62,63

FACS and Quantitative RT-PCR
Tissue from E13.5 mutant kidneys in each litter was pooled and dissociated into single cells using 1 mg/ml collagenase B (Sigma-Aldrich) at 37°C for 10 minutes. Cells were incubated with an anti-PTECH2 antibody (1:100, rabbit, G1191; Cell Signaling) followed by staining with Alexa-488 goat anti-rabbit secondary antibody (1:100, rabbit, G1191; Cell Signaling) followed by staining with Alexa-488 goat anti-rabbit secondary antibody (1:1000; Molecular Probes, Invitrogen). Cells were sorted using FACS Aria SORP (BD Biosciences) at Sickkids-UHN flow cytometry facility. Total RNA from sorted cells, and whole kidneys were isolated using the Qiagen RNeasy Micro kit. RNA from human UPJ specimens was isolated using TRizol RNA extraction protocol, followed by purification with QIAAGEN RNeasy micro kit. First-strand cDNA was synthesized using SuperScript (II) First-Strand Synthesis System (Invitrogen). Primers for specific transcripts were designed using RNA blast and verified through UCSC in silico PCR. Quantitative PCR was carried out using Applied Biosystems 7900 HT fast RT-PCR system.

RNA Sequencing
RNA purity and yield was determined using Nanodrop analysis followed by integrity analysis with Agilent RNA 6000 Pico Lab Chip analysis before cDNA library preparation. All samples had RIN>8. RNA samples were used for TruSeq cDNA library preparation and sequencing on the Illumina HiSeq 2500 system. The RNA-seq datasets comprised 17,000 paired-end reads. Reads were aligned to the mouse genome (mm9) using TopHat v2.0.5, and quantified with cufflinks v2.0.0, using ENSEMBL 65 genes. Subsequent analysis of differentially expressed genes was carried out using GeneSpring software, filtering out duplicate reads, reads that align to more than one place in the genome, and removing genes that did not have at least three RPKM expression in two samples. The raw data are deposited in GEO with accession number GSE97126.

In Situ Hybridization
In situ hybridizations were performed on paraffin-embedded kidney sections using digoxin-labeled RNA probes encoding Pch1, Pch2, Bmp4, Foxd1, and Raldh2 as described.64 Double-florescence in situ hybridization was carried out following the published protocols.65

Statistical Analyses
Statistical analysis was performed using GraphPad Prism software (version 5.0c). Data were analyzed using two-tailed t test. A probability of <0.05 was considered statistically significant and is designated with an asterisk in all figures.

ACKNOWLEDGMENTS
This work was supported by operating grants by the Canadian Institutes of Health Research and the Kidney Foundation of Canada (to N.D.R.), a Tier I Canada Research Chair in Developmental Nephrology (to N.D.R.), a Tier I Canada Research Chair in Imaging (to R.M.H.), and an Alexander Graham Bell Canada Graduate Scholarship (to S.S.-D).

DISCLOSURES
None.

REFERENCES


This article contains supplemental material online at http://jasn.asnjournals.org/lookup/suppl/doi:10.1681/ASN.2017050482/-/DCSupplemental.