**Hox10 Genes Function in Kidney Development in the Differentiation and Integration of the Cortical Stroma**

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**Abstract**

Organogenesis requires the differentiation and integration of distinct populations of cells to form a functional organ. In the kidney, reciprocal interactions between the ureter and the nephrogenic mesenchyme are required for organ formation. Additionally, the differentiation and integration of stromal cells are also necessary for the proper development of this organ. Much remains to be understood regarding the origin of cortical stromal cells and the pathways involved in their formation and function. By generating triple mutants in the Hox10 paralogous group genes, we demonstrate that Hox10 genes play a critical role in the developing kidney. Careful examination of control kidneys show that Foxd1-expressing stromal precursor cells are first observed in a cap-like pattern anterior to the metanephric mesenchyme and these cells subsequently integrate posteriorly into the kidney periphery as development proceeds. While the initial cap-like pattern of Foxd1-expressing cortical stromal cells is unaffected in Hox10 mutants, these cells fail to become properly integrated into the kidney, and do not differentiate to form the kidney capsule. Consistent with loss of cortical stromal cell function, Hox10 mutant kidneys display reduced and aberrant ureter branching, decreased nephrogenesis. These data therefore provide critical novel insights into the cellular and genetic mechanisms governing cortical cell development during kidney organogenesis. These results, combined with previous evidence demonstrating that Hox11 genes are necessary for patterning the metanephric mesenchyme, support a model whereby distinct populations in the nephrogenic cord are regulated by unique Hox codes, and that differential Hox function along the AP axis of the nephrogenic cord is critical for the differentiation and integration of these cell types during kidney organogenesis.

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**Introduction**

Metanephric kidney development initiates at approximately E10.5 in mice, when the metanephric mesenchyme condenses at the posterior end of the nephrogenic cord adjacent to the hindlimbs. These cells signal to the Wolffian duct to promote evagination of the ureteric bud, which invades the metanephric mesenchyme. Subsequent reciprocal inductive interactions between the nephrogenic mesenchyme and the ureter ultimately lead to the formation of the mature branched kidney [1,2,3]. Initially, Gdf6 expression in the metanephric mesenchyme is recognized by the receptors Ret and Gfrα1 on the ureteric epithelium and causes both the formation and invasion of the UB into the mesenchyme [4,5,6,7,8,9,10,11,12,13,14]. After UB invasion, a number of transcription factors including Pax2, Eya1, Wil1, Sall1, and the Hox11 are necessary to maintain Gdf6 expression in the mesenchyme, promote continued proliferation and expansion of the mesenchyme and to control the further budding and branching of the epithelial ureteric tree [15,16,17,18,19,20,21,22,23]. In turn, the ureteric tree expresses Fig2 and Bmp7 to promote survival and proliferation of the mesenchyme and Wil1b to initiate the formation of cap mesenchyme around the branch tips [24,25,26,27,28]. Undifferentiated cap mesenchyme expresses S6k2, which regulates nephrogenic progenitor cell renewal [29]. Finally, a portion of the cap mesenchyme expresses Wil1 and subsequently undergoes epithelialization to form nephrons [30,31].

In addition to the nephrogenic mesenchyme and ureter epithelium, a third cell type is present during early kidney organogenesis, the stromal cell population. Stromal cells are first observed just after ureteric bud invasion as a group of cells that surround the condensed mesenchyme [32]. A number of genetic studies have shown that stromal cells play critical roles during kidney organogenesis including producing signals that maintain outer and inner zones of differentiation, regulating pathways involved in the differentiation of nephrons, ureter branching morphogenesis, and in regulating the formation of the kidney capsule [32,33,34,35,36,37,38,39]. At present, the transcription factor Foxd1 is the earliest known marker of stromal cells and is
exclusive of these cells as early as E11.5 [34]. Functional analyses have shown that the loss of Foxd1 results in a reduction of nephron number, defects in ureter branching and loss of kidney capsule formation [34,36]. However, much remains to be determined regarding the origin of stromal cells, how they become integrated into the kidney and the signaling networks involved in their function.

Hox genes are expressed along the anterior-posterior (AP) body axis and are necessary for patterning many mesodermal organs. While 28 of the 39 Hox genes are expressed in the kidney [40], only the Hox11 paralogous group genes have been shown to play functional roles during mammalian kidney development [18,22]. Prior to UB invasion, the Hox11 genes are expressed in the condensed metanephric mesenchyme and then later become localized to the nephrogenic cap mesenchyme as nephrogenesis proceeds [41,42,43]. Functional studies have further shown that Hox11 proteins activate Six2 and Gdf1 expression in the metanephric mesenchyme [18,22,44]. As a result of this regulatory role, the UB fails to form in Hox11 mutant mice and the metanephric mesenchyme subsequently undergoes apoptosis [22].

Hox10 genes are also strongly expressed in the developing kidney at multiple stages of embryogenesis. Interestingly, while the expression patterns of both Hox10 and Hox11 are largely overlapping, we observe that the anterior boundary of Hox10 expression in the nephrogenic cord is anterior to that of Hox11. Both Hox10 and Hox11 are similarly expressed in the nephrogenic mesenchyme at E13.5, however, we find that Hox10 exhibits additional expression in the cortical stromal cells and suggests that these genes may be playing a unique role in the stroma. The functional data reported herein confirms this novel role and shows that the cortical stromal cells fail to properly differentiate and integrate into the kidney in Hox10 mutants. This cellular defect results in aberrant ureter branching, decreased nephrogenesis and a loss of kidney capsule formation, phenotypes reminiscent of those reported for Foxd1 [34,36]. In summary, our results indicate that the Hox10 genes play a critical role in regulating cortical stromal cell differentiation and integration in the mammalian kidney.

Results

Kidney morphology in Hox10 mutants

There are three Hox10 paralogs in mammals, Hoxa10, Hoxc10 and Hoxd10. While a previous study has shown that Hox10 triple mutants develop severe skeletal defects [45], the potential role(s) these genes play in kidney organogenesis has not been reported. In order to address this, mice with combinations of null mutations for Hoxa10, Hoxc10, and Hoxd10 were generated. Our data indicate that mice with any combination of three mutant alleles within the Hox10 paralogous group have no discernable kidney phenotype. This lack of mutant phenotype is likely due to the functional redundancy that exists among paralogous groups [22,45,46,47,48,49,50]. Consistent with this, combinations of four mutant alleles results in kidney abnormalities and these animals die two to six months after birth. Five-allele mutant mice demonstrate stronger defects and die between zero and eight weeks of age. Finally, Hox10 triple mutants display the most severe phenotypes and die within 24 hours of birth.

Overtly, Hox10 triple mutant kidneys are hypoplastic and morphologically underdeveloped as compared to controls (Fig. 1). Histologically, H/E staining at E18.5 reveals that the outer cortex of Hox10 mutant kidneys extends around only a portion of the kidney, the inner cortex is expanded, and the medulla is greatly reduced (Fig. 1C, D). Notably, the medio-lateral position of the kidneys is reversed, which results in the mispositioning of the ureter-pelvic junction (UPJ) to the lateral instead of medial side of the kidney and aberrant ureter routing from the UPJ to the bladder (Fig. 1B). The latter phenotype appears to be secondary to the failure of Hox10 mutant kidneys to detach from the body wall during embryogenesis (Fig. 1F and 2C and data not shown). As a result, the kidneys cannot properly rotate and ascend from the pelvic to the lumbar region as normally occurs in wild-type animals.

Lotus Tetragonolobus Lectin (LTL) and Dolichos Biloruss Agglutinin (DBA) staining in control and Hox10 mutant kidneys sectioned through the same frontal plane further demonstrate that Hox10 triple mutant kidneys exhibit multiple organizational defects by E16.5 (Fig. 1E, F). Normally, LTL labels the proximal tubules of mature nephrons that are confined to the cortical region, while DBA labels differentiated collecting ducts that extend radially into the medullary region (Fig. 1E). However, as depicted in Fig. 1F, both LTL and DBA staining are reduced and mislocalized in Hox10 mutants, highlighting the significant patterning defects in these mutants.

Hox10 triple mutants exhibit severe ureter branching defects

Subsequent to ureteric bud invasion, the ureter begins to branch within the metanephric mesenchyme and normally undergoes six to seven branching events by E14.5 [51]. Additionally, the kidney detaches from the body wall and ascends within the body cavity. In Hox10 mutants, the kidney fails to detach completely from the body wall and the reproductive tract (asterisks in Figure 1F, and Figure 2A, C). To compare the number of branching events and the number of early nephrons present we performed optical projection tomography (OPT) on dissected kidneys from E14.5 control and Hox10 mutant embryos stained with pan-cytokeratin, to label the ureteric tree, and Cadherin-6 to label early nephrons (Fig. 2B, D, Movies S1 and S2, Table S1) [52]. By E14.5, Hox10 mutant kidneys (n of 4) demonstrate a five-fold reduction in the number of ureteric branches compared to the total number of branches observed in controls (n of 3). There is also a three-fold reduction in the number of Cadherin-6-positive bodies, indicating reduced nephron formation in mutant kidneys.

The aberrant ureter branching in Hox10 mutants is due to intrinsic signaling defects in the developing kidney

In order to test whether the aberrant ureter branching observed in Hox10 mutants might be secondary to its physical inability to properly detach from the body wall and ascend or intrinsic to signaling defects in the kidney, we isolated kidneys from control and Hox10 mutant embryos at E11.5 and cultured them for 72 hours. Initially, the overall size and morphology of the tissue isolated was comparable between control and mutant kidneys (Fig 3A, E). However, the mutant kidneys were noticeably smaller and underdeveloped as compared to controls at the 24- and 48-hour time intervals (Fig 3B-C, F-G). After 72 hours in culture, the kidneys were stained for pan-cytokeratin to label the ureter epithelium and Pax2 to label the nephrogenic mesenchyme. Hox10 mutants develop a distinct morphological patterning defect as compared to controls, similar to what is observed in vivo, with increased length of each ureteric branch and less bifurcations (Fig 3D, H). Hox10 mutants have, on average, a 50% reduction in the number of branches compared to controls (Fig 3I, J). Hence, these results support that the branching defects observed in Hox10 mutants is due to signaling defects in the kidney and not secondary to the failure to detach from the body wall.
Hox10 does not effect the early expression of key nephrogenic mesenchyme markers

Upon ureteric bud induction, the nascent bud invades the metanephric mesenchyme ventrally. At E11.5, this step occurs indistinguishably in Hox10 mutant animals (Fig. 4C–J). Previous studies have shown that Hox11 genes are specifically required for the differentiation of the metanephric mesenchyme [22]. As depicted in Fig. 4A–B, Hox10 and Hox11 are both expressed in the metanephric mesenchyme surrounding the ureter at E11.5. This overlap of expression domains suggests that Hox10 could also play a role in the early patterning of the nephrogenic mesenchyme [18,22,42]. However, the expression patterns of Hox11, Six2, Wt1, and Eya1, all transcription factors that are important for early nephrogenic mesenchyme events [18,19,22,23,29,53], are unaffected in Hox10 triple mutants and remain strongly expressed throughout the condensed nephrogenic mesenchyme (Figs 4C–J). In addition, the fact that the expression patterns of these genes are all unaffected indicates that the nephrogenic mesenchyme, a zone of critical importance for the initial events of kidney development, is both properly established and maintained through E11.5 in Hox10 triple mutants. Therefore, despite the clear overlap of expression (Fig 4A–B),
these data demonstrate no defects in the early metanephric mesenchyme and suggest a unique function for Hox10 paralogous group genes in kidney organogenesis.

Expression of Hox10 and Hox11 in the developing kidney

To obtain a better understanding into the differences between Hox10 and Hox11 function in the kidney, we performed detailed expression analyses on both genes during multiple stages of development and carefully compared the two patterns. As previously described, in-situ hybridization analyses performed on tissue sections from E11.5 show that both Hox10 and Hox11 are expressed in the metanephric mesenchyme surrounding the ureter in similar patterns (Fig. 4A–B). However, in-situ hybridization experiments performed on dissected urogenital tissue from the same stage show that the anterior borders of Hox10 and Hox11 expression are clearly different (Fig. 5A, C arrowheads). As shown in Fig. 5C, the anterior limit of Hox11 expression is at the anterior border of the metanephric mesenchyme. In contrast, Hox10 expression is not limited to the metanephric mesenchyme, but extends into a more anterior region of the nephrogenic cord (Fig. 5A arrowhead). Thus, there are fundamental differences in the expression patterns of Hox10 and Hox11 genes even at these early stages of kidney organogenesis.

In order to directly compare Hox10 and Hox11 expression patterns at later developmental stages, we performed Hox10 in situ hybridization analyses on tissue sections from a Hoxa11eGFP reporter line in which GFP fluorescence has been shown to accurately recapitulate Hox11 expression ([43], Figure S1). At E13.5, both Hox10 and Hox11 are expressed in the nephrogenic mesenchyme (Fig. 5B, B', D, D'). However, Hox10 genes exhibit additional, unique expression in the thin layer of cells surrounding the nephrogenic mesenchyme known as the cortical stromal cells (Compare arrows in Fig. 5B' to 5D'). Thus, Hox10 genes are expressed in both the stromal and nephrogenic mesenchyme compartments of the kidney at this stage, whereas the Hox11 genes are expressed exclusively in the nephrogenic mesenchyme. In addition, the difference in expression domains is consistent with the possibility that Hox10 genes play a unique role in cortical stromal cell development.
Hox10 is required for cortical stromal cell function

Previous studies have shown that the stromal cell population in the developing kidney is critical for proper branching and differentiation of the kidney capsule [35,36]. The earliest known marker for this cell lineage is the transcription factor Foxd1, which is exclusively expressed in the cortical stromal cells as early as E11.5 [34]. Consistent with these data, whole mount in situ hybridization analyses performed on dissected urogenital tissue show that Foxd1 cannot be detected until E11.5. At E11.5, Foxd1-expressing cells are observed in a highly concentrated cap-like pattern that is localized just anterior to the metanephric mesenchyme (Fig 5E). Over approximately the next 12 hours of development, Foxd1-expressing cells are observed in progressively posterior positions, (Fig. 5F, G), and by E13.5, the cortical stromal cells surround and are fully integrated into the developing kidney (Fig. 6A). While Foxd1-expressing cells initiate normally in Hox10 mutants, they do not fully integrate into the kidney in Hox10 mutants (Fig. 6B). Instead, they are observed only in a restricted subset of cells at the periphery of the kidney (Figs. 6A, B). These data demonstrate that Hox10 has a critical function in promoting the proper integration of these cells into the developing kidney.

Nephrogenic mesenchyme markers become restricted to regions where Foxd1-expressing cells integrate

We next examined whether the restricted expression domain of Foxd1 in the periphery of the developing kidney in Hox10 mutants might lead to a localized signaling environment in which proper UB branching and nephrogenesis are also restricted. While the ureteric epithelial expression of Pax2 remains intact at E13.5 in Hox10 triple mutants, Pax2 expression in the nephrogenic mesenchyme becomes restricted to the domain in the periphery of the kidney adjacent to the remaining Foxd1-expressing cells (Fig. 6C, D asterisks). Similarly, the expression of the nephrogenic markers Eya1 and Six2 also become restricted to the region immediately adjacent to the Foxd1-expressing cells in the kidney periphery in Hox10 mutants (Fig. 6F, H). These results imply that the continued expressions of these key nephrogenic mesenchymal markers in Hox10 mutants are both dependent upon and reacting to the proper integration of stromal cells.

We next examined the expression patterns of several genes that have reported functions in cortical cell differentiation in both control and Hox10 triple mutant kidneys. At E13.5, Foxd1 expression continues to be restricted in the developing kidney in Hox10 mutants (Fig. 7A, B). Other important markers for stromal cell differentiation include Pbx1 and Raldh2 [39,54,55,56]. Normally, these stromal markers are also expressed around the entire periphery in the developing kidney at E15.5 (Fig. 7C, E). However, in Hox10 mutant kidneys, the expression patterns of both of these markers become restricted to the same regions in the periphery of the kidney as Foxd1 (Fig 7D, F). These data provide further evidence that Hox10 plays an important role in the proper integration of the cortical stromal cells during kidney organogenesis.

Kidney capsule maturation is disrupted in Hox10 mutants

Tenascin-C is a marker for differentiated stromal cells and is normally detected in the kidney periphery within the population of cells that has been postulated to play a role in renal capsule formation [34]. In contrast to the peripheral expression of Tenascin-C in differentiated cortical stromal cells in control embryos, this signal is completely lost in Hox10 mutants and indicates that this population of cells fails to properly differentiate (Fig. 7G, H). In addition to Tenascin-C, previous studies have shown that the secreted frizzled-related protein Sfrp1 is strongly expressed in the renal capsule [36,57,58,59]. Consistent with these reports, we also find that Sfrp1 is expressed at a high level throughout the kidney capsule cell layer and surrounds the periphery of the developing kidney at E14.5 in control embryos (Fig. 7I). However, there is a complete down-regulation of this gene in Hox10 mutants (Fig. 7J arrowheads). Thus, it appears that Hox10 plays a role in the regulation of kidney capsule formation that is similar to what has recently reported for Foxd1 [56].

Figure 4. Ureteric bud formation and early expression of key nephrogenic mesenchymal markers are unaltered in Hox10 mutants. At E11.5, both Hox11 (A) and Hox10 (B) are expressed in the condensed mesenchyme surrounding the ureter. (C, D) Hox11 expression in Hox10 mutant embryos (D) is identical to controls (C). Six2 expression is observed similarly throughout the condensed metanephric mesenchyme of E11.5 control (E) and E11.5 mutant (F) embryos. At E11.5, the expression patterns of Eya1 and WT1 are also unaltered in control (G, I) compared to Hox10 mutants (H, J). doi:10.1371/journal.pone.0023410.g004
A previous study has shown elevated ectopic levels of Bmp4 can inhibit the proper formation of the kidney capsule [36]. Normally, Bmp4 expression is confined to interior regions of the maturing kidney where it inhibits nephron differentiation [36,60,61]. Consistent with the previous studies and loss of kidney capsule in these mutants, Hox10 mutants are also exposed to elevated levels of Bmp4 expression surrounding the mutant kidney (Compare Figs. 8A, D). Hence, our data suggest that the improper formation of the kidney capsule in Hox10 mutants may be due in part to the exposure of ectopic elevated levels of Bmp4.

The loss of proper cortical stromal cell differentiation leads to an improper signaling environment that can secondarily affect ureter branching. Normally, Ret expression is localized in the nephron progenitors at the tips of the UB and provides an inductive signal that stimulates the growth of new ureter branches [Fig. 8B, C; [36]]. However, in Hox10 mutants, Ret expression is not solely localized to the UB tips and instead, can be observed in extended regions along the stalks of the nascent ampulla (Fig. 8E, F arrowheads), consistent with the observed branching defects in Hox10 mutant kidneys.

Discussion

Interestingly, Hox10 and Hox11 mutant mice yield distinct kidney phenotypes despite largely overlapping expression patterns throughout kidney development. Previous studies have shown that the Hox11 genes play a critical role in the formation and invasion of the UB via the upregulation of Gdnf and Six2 in the metanephric mesenchyme [18,22]. As a result of the loss of Gdnf, kidneys do not form in Hox11 mutant mice. In contrast, the initial stages of kidney development are unaffected in Hox10 triple mutants. These mice undergo normal UB induction, and initiate the proper expression of several key transcription factors essential for the formation, proliferation and survival of the nephrogenic mesenchyme. These data indicate that, unlike the situation observed in Hox11 mutants, the nephrogenic mesenchyme is properly formed in Hox10 triple

Figure 5. Expression patterns of Hox10, Hox11 and Foxd1 in the early nephrogenic cord. At E11.5, the Hox10 genes (A) exhibit a more anterior boundary of expression in dissected urogenital mesenchyme compared to Hox11 in control mice (Compare arrowheads in A and C). By E13.5, Hox10 (B) and Hoxa11eGFP (D) are both expressed in the nephrogenic cap mesenchyme, but Hox10 genes (B') are additionally expressed in the cortical stroma cells (black arrow), whereas Hox11 (D') is expressed in the renal vesicles but not in the cortical stroma (white arrow). (E) At E11.5, Foxd1 expression forms a highly concentrated cap-like pattern localized just anterior to the metanephric kidney. (F–G) As development progresses, these Foxd1-expressing cells are observed in progressively posterior positions (F, G). Panels (B–B') and (D–D') show light microscopy and fluorescent images of the same section, respectively. In situ hybridization analyses for Hox10 were performed using probes to all three paralogs (Hoxa10, Hoxc10, Hoxd10). doi:10.1371/journal.pone.0023410.g005
Figure 6. Nephrogenic mesenchyme markers become restricted to regions of Foxd1 cell integration. (A–F) Serial sections through control (A, C, E) and Hox10 mutants (B, D, F) at E13.5. (A) Foxd1 expression is observed in the cortical stromal cells fully integrated into the periphery of the kidney at E13.5 in controls. (B) Foxd1 signal is regionally restricted in Hox10 mutants. (C–D) Expression of the nephrogenic marker Pax2 in control (C) and Hox10 mutant (D) embryos. In Hox10 mutants, Pax2 (D) is restricted to the domain in the kidney that is selectively expressing Foxd1. Red asterisks depict nephrogenic mesenchymal expression of Pax2 in (C, D). Similar patterns are observed with both Eya1 (E, F) and Six2 (G, H) in which the normal expression in the mesenchymal condensations in the in the nephrogenic zone (E, G) becomes positionally restricted to regions of Foxd1-expressing cells in the Hox10 mutant (F, H).

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mutants. It is not until later stages of organogenesis that the primary defect of Hox10 triple mutants is observed. The cortical stromal cells fail to properly differentiate and integrate into the developing kidney. The defect in cortical stromal cell development in Hox10 mutants leads to additional phenotypes including aberrant ureter branching and decreased nephrogenesis (Fig. 9).

Further support for a role for Hox10 genes in cortical stromal cell function comes from the fact that many of the structural and molecular defects in Hox10 mutants are highly reminiscent of those that have been reported for Foxd1. Specifically, loss of Foxd1 function results in hypoplastic kidneys, impaired branching morphogenesis and reduction of nephron number [34,36]. In addition, Foxd1 mutant kidneys fail to detach from the body wall during embryogenesis and as a result, do not ascend from the pelvic to lumbar region. All of these characteristics are phenocopied in Hox10 triple mutants and therefore provide further support for Hox10 genes playing a critical role in the development and integration of cortical stromal cells. It is interesting to note however, that the initial stromal cell precursor population is retained in both Foxd1 and Hox10 mutants. Additionally, we show that Hox10 does not regulate Foxd1 expression in the cortical stromal cells. Thus, Hox10 does not regulate the initial formation of the cortical stromal cell population, but rather, governs the proper integration and subsequent differentiation of this lineage in the developing metanephric kidney.

A previous study has shown that the cortical stromal cells play an important role in regulating the formation of the kidney capsule [36]. Both Hox10 and Foxd1 mutants demonstrate defects in capsule development, as evidenced by the loss Sfppl and Tenascin-C expression in the periphery of the developing kidney [34,36]. Discerning the mechanisms by which Hox10 genes regulate the differentiation and integration of this population will be the focus of future studies.

Another key question regards the cellular origins of the cortical stromal cell lineage. Previous studies have suggested that stromal cells may be derived from the metanephric blastema, either as cells that do not undergo nephron differentiation like the cap mesenchyme, or from a unique cell lineage present in the blastema [32,62,63]. More recently, a lineage trace with an Osr1-Cre provided evidence that the initial population of Foxd1-expressing cells are derived from the intermediate mesoderm [64]. Alternatively, a recent fate map study in chick suggests that the kidney stromal cells may be derived from paraxial mesoderm progenitors in this species [65]. We report here that Foxd1-expressing cells are initially observed as a highly concentrated cap-like pattern that is localized just anterior to the metanephric mesenchyme. As development progresses, these cells are observed in progressively posterior positions, becoming integrated into the periphery of the kidney to form the cortical stroma. These data are consistent with the cortical stromal cell lineage arising from the nephrogenic mesenchyme in a region just anterior to the metanephric mesenchyme. The more anterior border of Hox10 expression as compared to Hox11 is consistent with a unique role for Hox10 in the formation of this cell type. In support of this hypothesis, Mugford et al (2008b) showed that while the stromal cell lineage was labeled strongly when Osr1-Cre was activated at early developmental stages, while only the nephrogenic mesenchyme and not the cortical stroma was labeled when Osr1-Cre was activated at later stages. This result provides additional support for our hypothesis that the cortical stromal cell lineage arises from a population of cells that is anterior to the metanephric mesenchyme. Hence, the combined data suggest that the general AP patterning functions of Hox genes directly translates into the differentiation of the distinct cell populations in the kidney. Future experiments removing Hox10 (or Foxd1) in specific cell populations will allow for a more definitive determination of when and where
these early regulatory signals are required for the appropriate differentiation and integration of the kidney cortical stroma cells.

**Materials and Methods**

**Animals and histology**

Generation of *Hox10* mutant embryos was previously described [45]. Embryos and kidneys were dissected in PBS, fixed in formalin for one to three hours, and dehydrated through graded alcohols and stored in 70% ethanol at 4°C. Embryos were vacuum-embedded in paraffin, sectioned at 5 μm and stained with hematoxylin and eosin. A piece of tissue from the embryo was used for genotyping. All animal experiments performed in this report were reviewed and approved by the University of Michigan’s Committee on Use and Care of Animals, Protocol #08787, under Animal Welfare Assurance #A3114-01 on file with the NIH Office of Laboratory Animal Welfare.

**In situ hybridization**

Whole mount *in situ* hybridization was performed as previously described [22,66]. For section *in situ* hybridization, embryos were collected in PBS and fixed overnight in 4% paraformaldehyde in PBS (PFA) at 4°C. Embryos were vacuum-embedded in paraffin, sectioned at 5 μm and stained with hematoxylin and eosin. A piece of tissue from the embryo was used for genotyping. All animal experiments performed in this report were reviewed and approved by the University of Michigan’s Committee on Use and Care of Animals, Protocol #08787, under Animal Welfare Assurance #A3114-01 on file with the NIH Office of Laboratory Animal Welfare.

**Immunohistochemistry (IHC)**

For IHC on sections, embryos were processed and sectioned as described above for section ISH. IHC using antibodies to E-cadherin (R&D Systems) and smooth muscle actin (Cy3-conjugated mouse monoclonal, Sigma) were performed as previously described [71]. Anti-E-cadherin was used in a dilution of 1:100 and detected by a 1:500 dilution of donkey anti-goat Alexafluor 488 (Invitrogen). Anti-smooth muscle actin was used in a dilution of 1:100. Immunohistochemical (IHC) localization of pan-cytokeratin (Sigma) on whole embryonic kidneys at E12.5 and E13.5 was performed as previously described [68,69,70].

**Lectin immunohistochemistry**

Lectin immunohistochemistry was performed as previously described [37]. After washing in PBS, slides were incubated in...
50 mM NH₄Cl in PBS at room temperature for 20 minutes, followed by a 20 minute incubation with GSP solution (0.2% gelatin+0.075% saponin (S4521, Sigma) in PBS) at 37°C. Slides were then incubated with 1:250 rhodamine labeled DBA (Vector Labs) and 1:400 fluorescein labeled LTA (Vector Labs) at 4°C overnight in the humidifying chamber. Slides were then incubated with GSP solution at 37°C two times for 20 minutes and rinsed in PBS+0.05% tween at room temperature. After washing for 20 minutes with PBS, slides were mounted with Pro-Long Gold, antifade reagent (Invitrogen).

OPT imaging of embryonic kidneys

E14.5 kidneys were collected in ice cold PBS and fixed in 4% PFA for 10 minutes. They were washed two times in cold PBS and then washed in TBS for 20 minutes. After washing the kidneys in TBS+1% triton (TBSTr) for 20 minutes, they were blocked in TBSTr+1% BSA at room temperature for one hour. They were then incubated overnight at 4°C rocking with anti-pan-cytokeratin (1:100) and anti-Cadherin-6 (1:200) in TBSTr+1% donkey serum. The next day they were washed in TBSTr three to four times at room temperature and one time overnight at 4°C. Kidneys were then incubated overnight at 4°C rocking with donkey anti-mouse Alexafluor 555 (Invitrogen, 1:100) and goat anti-rabbit Alexafluor 488 (Invitrogen, 1:200) diluted in TBSTr+1% donkey serum. After washing four to five times at room temperature and one time overnight at 4°C in TBSTr, the tissue was fixed for 15 minutes in 4% PFA and washed three times 10 minutes in TBS. Kidneys were then stored in TBS+0.1% sodium azide prior to embedding.

Stained kidneys were embedded in warm 1% low melting point agarose and left until set. Slices containing the specimen were excised, and glued to aluminium-magnetic mounts. Specimens were then dehydrated in 100% methanol for 6 hours with 3 changes of methanol, and then cleared overnight in Benzyl Alcohol Benzyl Benzoate mixed at a ratio of 1:2. Once clear, samples were imaged in a Bioptonics 3001 OPT scanner (Bioptonics, UK), at maximum resolution of 3.2 μm per pixel zoom. Images were acquired at 0.9 degree intervals, with each frame averaged over 4 images. Quantification/Skeletonisation was done using a Kidney Analysis Application [72]. Rendering/Visualization was performed using Drishti http://anuss.anu.edu.au/Vizlab/drishti/.

Supporting Information

Figure S1 Hox11 mRNA expression overlaps with Hoxa11eGFP reporter expression. Hox11 in situ analysis (A) was done on tissue sections from an E13.5 Hoxa11eGFP

Figure 8. Hox10 kidneys demonstrate aberrant branching defects and are exposed to elevated levels of Bmp4. (A, D) Bmp4 is normally down-regulated in the body wall around the kidney by E12.5 (A), but this expression is ectopically maintained in Hox10 mutants (D). (B, C) Ret is normally expressed in the tips of the UB, providing an inductive signal that stimulates the growth of new ureter branches. (E, F) Ectopic Ret expression is observed in extended regions along the stalks of the ampulla (arrowheads) in Hox10 mutants.

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Figure 9. Model depicting the role of the Hox10 genes in the proper differentiation and integration of the cortical stromal cells during mammalian kidney development. In control animals, the cortical stromal cells are initially concentrated in a cap-like region just anterior to the metanephric kidney. As development progresses, these cells become integrated into the periphery of the kidney. Once integrated, the cortical stromal cells promote proper ureter branching and nephron differentiation. In Hox10 mutants, while the formation of the initial cortical stromal population is unaffected, these cells fail to properly integrate into the kidney periphery. The absence of cortical stromal cells in parts of the developing kidney creates distinct areas of signaling defects that secondarily results in aberrant ureter branching and decreased nephrogenesis.

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heterozygous embryo (B). Hox11 mRNA and Hox11-eGFP are both expressed in the nephrogenic cap mesenchyme (white arrows in A' and B') and not in the cortical stroma cells. (THF)

Movie S1  Movie rendering of OPT analysis of a control kidney, immunostained with pan-cytokeratin (red) and cadherin 6 (green). The brown color represents unstained space filling of the rest of the dissected tissue. (MOV)

Movie S2  Movie rendering of OPT analysis of two Hox10 triple mutant kidneys, immunostained with pan-cytokeratin (red) and cadherin 6 (green). The brown color represents unstained space filling of the rest of the dissected tissue. Of note, in mutant kidneys, some ureteric tips are not associated with nephrogenic mesenchyme. (MOV)

References
1. Dressler GR (2006) The cellular basis of kidney development. Annu Rev Cell Dev Biol 22: 509–529.
2. Kuure S, Vuotienranta R, Vainio S (2000) Kidney morphogenesis: cellular and molecular regulation. Mech Dev 92: 31–45.
3. Sainio K, Suvanto P, Davies J, Wartiovaara J, Wartiovaara K, et al. (1997) Glial-nerve interactions. Mech Dev 54: 95–103.
4. Moor MW, Klein RD, Farinas I, Sauer H, Armanini M, et al. (1996) Renal and neuronal abnormalities in mice lacking GDNF. Nature 382: 70–73.
5. Enomoto H, Araki T, Jackman A, Heuckeroth RO, Snider WD, et al. (1998) The Hox10 promoter contains enhancers required for retinal and kidney development. Proc Natl Acad Sci U S A 95: 10707–10712.
6. Schuchardt A, D'Agati V, Larsson-Blomberg L, Costantini F, Pachnis V (1994) The Glial-Cre transgene causes a dominant negative effect on glial lineage development in mice. EMBO J 13: 347–354.
7. Kuure S, Vainio S, Vainio R, Vainio S (1998) Kidney morphogenesis: cellular and molecular regulation. Mech Dev 92: 31–45.
8. Kuure S, Vuotienranta R, Vainio S (2000) Kidney morphogenesis: cellular and molecular regulation. Mech Dev 92: 31–45.
9. Kuure S, Vainio S, Vainio R, Vainio S (1998) Kidney morphogenesis: cellular and molecular regulation. Mech Dev 92: 31–45.
10. Kuure S, Vainio S, Vainio R, Vainio S (1998) Kidney morphogenesis: cellular and molecular regulation. Mech Dev 92: 31–45.

Table S1  Ureteric Tree Branch Analysis. (DOCX)

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Author Contributions
Conceived and designed the experiments: ARY SMH IMS DMW. Performed the experiments: ARY SMH KMS. Analyzed the data: ARY SMH IMS DMW. Contributed reagents/materials/analysis tools: ARY SMH IMS DMW. Wrote the paper: ARY SMH IMS DMW.
Leimeister C, Bach A, Gessler M (1998) Developmental expression patterns of Hox11 in vivo. Mech Dev 33: 312–323.

Nelson LT, Rakhit S, Sun H, Wellik DM (2008) Generation and expression of a Hoxa11-eGFP targeted allele in mice. Dev Dyn 237: 3410–3416.

Yallowitz AR, Gong KQ, Swinehart LT, Nelson LT, Wellik DM (2009) Non-homoeodomain regions of Hox proteins mediate activation versus repression of Six2 via a single enhancer site in vivo. Dev Biol 335: 156–165.

Wellik DM, Capecci MR (2003) Hox10 and Hox11 genes are required to globally pattern the mammalian skeleton. Science 301: 363–367.

Condie BG, Capecci MR (1994) Mice with targeted disruptions in the paralogous genes hoxa-3 and hoxd-3 reveal synergetic interactions. Nature 370: 304–307.

Davis AP, Witte DP, Hsieh-Li HM, Potter SS, Capecci MR (1995) Absence of retinaldehyde dehydrogenase type 2 (RALDH-2) gene during mouse development and urogenital differentiation. Genesis 37: 123–130.

Fromental-Ramain C, Warot X, Messadecq N, LeMeur M, Dolle P, et al. (1996) Hoxa-13 and Hoxd-13 play a crucial role in the patterning of the limb autopod. Development 122: 2997–3011.

Green JM, Paetke J, Thomas KR, Capecci MR (2000) Maintenance of functional equivalence during paralogous Hox gene evolution. Nature 403: 661–665.

McIntyre DC, Rakhit S, Yallowitz AR, Loken L, Jeannotte L, et al. (2007) Hox gene expression patterns Kidney Cortical Stroma.

Short KM, Hodson MJ, Smyth IM (2010) Tomographic quantification of bone morphogenetic protein family members potentially account for limited tissue defects in BMP7 deficient embryos. Dev Dyn 239: 549–562.

Miyazaki Y, Oshima K, Fogo A, Hogan BL, Ichikawa I (2000) Bone morphogenetic protein 4 regulates the budding site and elongation of the mouse ureter. J Clin Invest 105: 863–873.

Al-Awqati Q, Oliver JA (2002) Stem cells in the kidney. Kidney Int 61: 317–395.

Ekbloem P, Weller A (1991) Ontogeny of tubulointerstitial cells. Kidney Int 39: 394–400.

Mugford JW, Sipila P, McMahon JA, McMahon AP (2008) Osr1 expression demarcates a multi-potent population of intermediate mesoderm that undergoes progressive restriction to an Osr1-dependent nephron progenitor compartment within the mammalian kidney. Dev Biol 324: 88–98.

Guillaume R, Bressan M, Herzlinger D (2009) Paraxial mesoderm contributes stromal cells to the developing kidney. Dev Biol 329: 169–175.

Huppert SS, Bagan MX, De Strooper B, Kops R (2005) Analysis of Notch function in presomitic mesoderm suggests a gamma-secretase-independent role for presinilins in somite differentiation. Developmental Cell 8: 677–688.

Di Giacomo G, Koss M, Capellini TD, Belgakana A, Popperl H, et al. (2006) Spatio-temporal expression of Fsh3 during mouse organogenesis. Gene Expr Patterns.

Sengonuguz I, Schofield PR (1993) The sequence of a mouse homologous to the human BMP-7 gene is conserved. Nature 365: 417–419.

Benson GV, Nguyen TH, Maas RL (1995) The expression pattern of the murine Hoxa-10 gene and the sequence recognition of its homeodomain reveal specific properties of Abdominal B-like genes. Mol Cell Biol 15: 1591–1601.

Holland E, Karsten SL, Kudo L, Geschwind DH, Carpenter EM (2004) Identification of a Hox10-regulated transcriptional network and combinatorial interactions with Hoxa10 during spinal cord development. J Neurosci Res 75: 367–379.

Houikka SL, Capecci MR (1998) The mouse Hoxc11 gene: genomic structure and expression pattern. Mech Dev 70: 133–145.

Brenner-Anantharam A, Cebrail C, Guillaume R, Hurtado R, Sun TT, et al. (2007) Tailbud-derived mesenchyme promotes urinary tract segmentation via BMP4 signaling. Development 134: 1967–1975.

Short KM, Hodson MJ, Smyth IM (2010) Tomographic quantification of branching morphogenesis and renal development. Kidney International In press.