Boxd1-dependent signals control cellularity in the renal capsule, a structure required for normal renal development

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Accepted 25 November 2004

Development 132, 529-539
Published by The Company of Biologists 2005
doi:10.1242/dev.01604

Summary
Development of the metanephric kidney involves the establishment of discrete zones of induction and differentiation that are crucial to the future radial patterning of the organ. Genetic deletion of the forkhead transcription factor, Foxd1, results in striking renal abnormalities, including the loss of these discrete zones and pelvic fused kidneys. We have investigated the molecular and cellular basis of the kidney phenotypes displayed by Foxd1-null embryos and report here that they are likely to be caused by a failure in the correct formation of the renal capsule. Unlike the single layer of Foxd1-positive stroma that comprises the normal renal capsule, the mutant capsule contains heterogeneous layers of cells, including Bmp4-expressing cells, which induce ectopic phospho-Smad1 signaling in nephron progenitors. This mis-signaling disrupts their early patterning, which, in turn, causes mispatterning of the ureteric tree, while delaying and disorganizing nephrogenesis. In addition, the defects in capsule formation prevent the kidneys from detaching from the body wall, thus explaining their fusion and pelvic location. For the first time, functions have been ascribed to the renal capsule that include delineation of the organ and acting as a barrier to inappropriate exogenous signals, while providing a source of endogenous signals that are crucial to the establishment of the correct zones of induction and differentiation.

Key words: Foxd1, Bmp4, Smad1, Fused kidneys, Renal capsule, Mouse

Introduction
The development of the mammalian metanephric kidney involves multiple reciprocal signaling interactions. The best studied of these occurs between the ureteric bud (UB) and the nephrogenic mesenchyme (Burrow, 2000; Dressler, 2002; Grobstein, 1955; Lechner and Dressler, 1997; Saxen, 1997; Vainio and Lin, 2002). The UB eventually gives rise to the mature collecting duct system, while the nephrogenic mesenchyme undergoes a gradual mesenchymal-to-epithelial transition as it converts to nephrons. During this maturation period, the differentiation of renal cell types and renal structures within the kidney occur in a spatially defined manner. At the very edge of the kidney is the capsule and just underneath is the outer cortex, which contains the nephrogenic zone. It is in this region that the early stages of nephron induction and the formation of new generations of ureteric bud branches occur. As the kidney expands, new generations of ureteric buds and nephrons are induced in the nephrogenic zone, displacing older generations to the inner cortex and medulla where they undergo further differentiation. Also within the cortex is a third cell type, the renal stroma (Hatini et al., 1996; Mendelsohn et al., 1999). Although the stroma was initially believed to have a purely supportive function in kidney morphogenesis (Ekblom and Weller, 1991), recent studies have, in fact, revealed that it is crucial for generating signals that control differentiation of surrounding cell types (reviewed by Levinson and Mendelsohn, 2003).

Our previous studies suggest that distinct stromal cell populations occupy the nephrogenic and differentiation zones of the kidney and assist in maintaining their unique signaling environments (Levinson and Mendelsohn, 2003). As an example, Foxd1, a member of the forkhead box (Fox) transcription factor superfamily, is most strongly expressed in the cortical stroma (Hatini et al., 1996). Inactivation of Foxd1 by a genetic ‘knock-in’ approach leads to severe renal malformations, including impaired branching morphogenesis and nephron differentiation (Hatini et al., 1996). In addition, the maturing kidneys in the Foxd1 mutant are fused and remain localized in the pelvis rather than ascending fully to their normal position in the lumbar region (Hatini et al., 1996). The failure of the mutant kidneys to separate from the midline and leave the pelvic region is reminiscent of fused kidneys that sometimes occur in human embryogenesis.

The cause of the pelvic kidneys, as well as the defects in UB branching and nephron formation, in the Foxd1-null mutant embryo has remained unclear. For example, it is still unknown if the ureteric or nephrogenic compartments in the mutant
kidney are capable of receiving inductive signals, and, if they are, why they do not pattern correctly. We have thus analyzed the Foxd1-null mutant in further detail to explain these key phenotypes and to gain a deeper understanding of the role of the stromal cell population in kidney morphogenesis. We report here that it is defects in the maturation of the renal capsule that is the underlying cause of the ectopic kidneys in the Foxd1-null embryo. In addition, we show that Foxd1 plays a crucial role in forming the correct population of cell types within the renal capsule. Thus, although we show that the ureteric and nephrogenic compartments of the mutant kidney are indeed able to receive inductive signals, it is the ectopic presence of cell types within the mutant capsule, such as Bmp4-expressing cells and endothelial cells, that accounts for the impaired branching and delay in nephron differentiation.

Materials and methods

Mouse strains

All mice were maintained in the Swiss-Webster genetic background. Embryonic day 0.5 (E0.5) was considered to be at noon on the day of the plug. Littermates were used for all experiments in which normal and mutant embryos were compared. The Foxd1lacZ mice (Hatini et al., 1996), FoxB7GFP mice (Srinivas et al., 1999) and Bmp4lacZ mice (Lawson et al., 1999) were a generous gift from Eseng Lai, Frank Costantini and Brigid Hogan, respectively. The Foxd1EGFP mice were developed by replacing the lacZ-coding sequence of the homologous recombination targeting vector used to generate the original Foxd1lacZ strain (Hatini et al., 1996) with the EGFP coding sequence from pEGFP-N1 (Clontech).

β-Galactosidase staining

Frozen sections were post-fixed in 0.2% paraformaldehyde and then placed in lacZ staining solution (1×PBS, 2 mM MgCl2, 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide and 1 mg/ml X-gal) at 30°C overnight in the dark for Bmp4lacZ samples. For Foxd1lacZ samples the incubation times were 3 hours for mutant tissue and 7 hours for heterozygous tissue to normalize for the number of Foxd1lacZ alleles.

In situ hybridization and immunohistochemistry

In situ hybridization on frozen thin sections and whole-mount tissue were performed using DIG-labeled probes as described previously (Mendelsohn et al., 1999). For the probes Pax8, Lim1 and Ret, a similar in situ hybridization protocol was used except that paraffin wax-embedded tissue was used (after deparaffinization) and 33PUTP in situ hybridization protocol was used except that paraffin wax-embedded tissue was used (after deparaffinization) and [33P]UTP (NEN) was used to label the probe. Detection of the labeled probe was achieved by immersion of the slides into emulsion (Kodak), which was then solidified for 3 days at 4°C. Visualization of the exposed emulsion was performed by both dark- and light-field microscopy.

Immunohistochemistry for PECAM ( PharMingen) was preceded by β-galactosidase staining as described above. The anti-phospho-Smad1 antibody (Cell Signaling Tech) and the anti-Ki-67 antibody (Zymed) were used according to manufacturers’ instructions for paraffin-embedded tissue. β-Gal immunofluorescence was performed using goat anti-β-galactosidase antibody (Biogenesis), diluted 1:2000, followed by donkey anti-goat-CY3 (Jackson ImmunoResearch), diluted 1:700.

Lectin histochemistry

Vibratome sections (150 μm) were fixed in 3% paraformaldehyde, permeabilized with saponin and neuraminidase (both from Sigma), and then labeled with TRITC-PNA lectin and FITC-Lotus lectin (Vector Labs). The samples were photographed by confocal microscopy.

Results

Foxd1 is required for the correct formation of the kidney capsule

Mouse mutants lacking Foxd1 were generated previously by replacing the open reading frame of the Foxd1 gene with the lacZ gene (Hatini et al., 1996). In Foxd1lacZ/lacZ embryos just before birth (E18.5), kidneys were small compared with wild-type littermates, were in the pelvic region rather than the lumbar region and failed to separate from the midline (Hatini et al., 1996). It should be noted, however, that the size and positions of other organs (adrenals, gonads and bladder) were normal in the mutant (data not shown). To determine when in development defects occur that lead to the presence of pelvic kidneys, we investigated the changing relationship between the kidney and the body wall in wild-type embryos and in Foxd1-null mutants. Histological analysis at E11.5 revealed that in both wild-type and mutant embryos, the kidneys lie adjacent to one another and in close contact with the dorsal body wall, whereas ureters entered the kidney rudiments ventrally (Fig. 1A,B). At E12.5, kidneys of wild-type embryos had shifted laterally and were separated by the dorsal aorta and tissue of the midline. But, more importantly, the kidneys had already begun to detach from the rest of the body, evidenced by the early formation of loose connective tissue in the dorsolateral aspects of the kidney (yellow arrowheads in Fig. 1C). In Foxd1 mutants at this age, the kidneys remained attached to the dorsal body wall via a thickened layer of cells (yellow arrowheads in Fig. 1D). One interesting result of these abnormalities was that the kidneys fail to separate from each other and rotate. Thus, the ureters are fixed into a position where they enter from the ventral surface rather than from the midline. By E14.5, the renal capsule in wild-type embryos was distinct and comprised a single layer of flattened cells that lie just above the cortical stroma and the condensed mesenchyme of the nephrogenic zone (Fig. 1E). In addition, the kidneys were clearly separated from the surrounding body wall by a loose connective tissue (yellow arrowheads in Fig. 1E). At this age, Foxd1-null kidneys remained in the pelvic region in an immature position, still in close contact with one another, and embedded in mesenchyme (Fig. 1F). The renal capsule was present, but it was histologically abnormal. It was composed of a thick layer of mesenchyme that was continuous with the body wall. Similar results were found for the mutant kidneys at later developmental stages, up to the time of birth (Fig. 2D; data not shown). These findings suggest that the ectopic position of the kidneys in the Foxd1 mutant may be due to defects in the proper specification of the renal capsule.

In order to resolve more clearly the spatial relationship of Foxd1+ cells to the developing kidney capsule we stained tissue sections from Foxd1lacZ/lacZ and Foxd1lacZ/lacZ embryos for lacZ expression at various developmental stages (Fig. 2A-G). At E13.5 there are numerous Foxd1-β-gal+ cells that surround both the heterozygous and mutant kidneys, especially in the ventrolateral aspects of the dorsal body wall (yellow arrowheads in Fig. 2A,B). By E16.5 in the heterozygote, there remains a smaller population of Foxd1-β-gal+ cells just outside the dorsal surface of the kidneys. These cells appear to comprise a thin layer of connective tissue that may act to keep the kidneys loosely attached to the dorsal body wall (yellow arrowheads in Fig. 2C,C'). Similar pictures of the mutant
Loss of Foxd1 expression results in alterations in the cell types of the renal capsule

As loss of Foxd1 expression results in abnormalities in the morphology of the mutant capsule, we wished to investigate further if this defect correlated with alterations in the cellular composition and activity of this structure. We therefore examined the expression of molecular markers that define the normal renal capsule. In addition to Foxd1, Raldh2 [whose protein product is a dehydrogenase required for retinoic acid synthesis (Hsu et al., 2000; Niederreither et al., 1999)] and Sfrp1 [which encodes for a secreted frizzled-related protein that antagonizes Wnt signaling (Yoshino et al., 2001)] are both strongly expressed in the renal capsule (black arrowheads in Fig. 3A,C,E). In wild-type embryos, Raldh2 expression was at high levels in the capsule and the cortical stroma and at lower levels in the nephrogenic mesenchyme and epithelia (Fig. 3C), whereas Sfrp1 was highly expressed in the capsule and at barely detectable levels in the cortical stroma that populate the nephrogenic zone (Fig. 3E). In Foxd1 mutants, however, Raldh2 expression was undetectable in the capsule (compare expression underneath black arrowheads in Fig. 3B with those in Fig. 3D). Likewise, Sfrp1 expression was also practically undetectable in the mutant capsule (Fig. 3F). Thus, molecular markers normally expressed in the capsule are undetectable in the capsule of the Foxd1-mutant kidney, suggesting that Foxd1 may be required for either establishing or maintaining the normal cellular activity of this structure.

It is important to point out, however, that the mutant renal capsule contained numerous cells negative for β-gal expression, whereas in the heterozygous kidney almost all the cells in the capsule were Foxd1-β-gal+. This difference raises the possibility that either these β-gal-negative cells are derived from the Foxd1+ lineage (but they lost their ability to transcribe from the Foxd1 promoter) or they are distinct cell types that are ectopically present in the mutant capsule. In either case, an alteration in the cell population of the mutant capsule may result in an aberrant signaling environment in this structure that, in turn, may ultimately explain the defects in the patterning of the ureteric and nephrogenic compartments displayed by the mutant kidney. To distinguish between these possibilities, we analyzed tissue from mutant and wild-type kidney for expression of a number of other molecular markers to determine the identity of these β-gal-negative cells.

Examination of two markers of the medullary stroma, Pod1 (Quaggin et al., 1998) and p57 (Hiromura et al., 2001), revealed that, although there was abundant expression of these two genes in the Foxd1-mutant kidneys, there was no detectable expression in the mutant capsule (data not shown). We, therefore, next examined markers of other cell lineages, such as the nephrogenic mesenchyme (Gdnf) (Durbec et al., 1996) and nephrogenic precursors (Pax2, Wnt4, Lim1, Pax8, Sfrp2 and Hoxa11) (Dressler et al., 1990; Fujii et al., 1994; Leimeister et al., 1998; Plachov et al., 1990; Sariola and Sainio, 1998; Wellik et al., 2002). The expression of these markers...
were present at similar levels to wild type in the nephrogenic structures of the mutant. However, they were not detectable in the capsule of either wild-type or Foxd1-mutant kidneys (data not shown). These findings suggest that cells that populate the Foxd1-null capsule are likely to be neither medullary stroma nor nephron progenitors.

The loss of Sfrp1 expression in the mutant renal capsule led us to investigate for the presence of endothelial cells in this structure because it is known that sFRP1 regulates endothelial migration (Dufourcq et al., 2002). Normally, endothelial cells are localized to the interior of the kidney at E16.5 (Fig. 3G), as revealed by expression of PECAM (Pecam1 – Mouse Genome Informatics), a marker of this cell type (Sheibani et al., 1999). In Foxd1lacZ/lacZ kidneys, however, Pecam1 expression was also strongly displayed in the capsule, though expression did not overlap with lacZ (Fig. 3H). This difference in expression of Pecam1 was also observed at E14.5 and E18.5 (data not shown). Likewise, the expression of Flk1, another marker of endothelial cells (Robert et al., 1996), revealed an identical expression pattern to Pecam1 at E14.5 in wild-type and mutant tissue (data not shown). These results indicate that, indeed, a cell type not normally observed in the renal capsule is abnormally present in the mutant capsule. Finally, the lectin peanut agglutinin (PNA) displayed ectopic reactivity with the mutant capsule at E15.5 (Fig. 3J), further indicating an altered composition of the mutant capsule.

The ectopic presence of Pecam1+/Flk1+ cells in the mutant capsule was intriguing to us because a recent report has shown that migration of endothelial cells is promoted by Bmp signaling (Valdimarsdottir et al., 2002). Normally, Bmp4+ cells are present in the interior regions of the maturing kidney (Dudley and Robertson, 1997; Miyazaki et al., 2000) and, thus, probably guide the migrating endothelial cells into the future capillary tufts of the developing glomeruli. The presence of endothelial cells in the mutant capsule, therefore, suggested to us that cells in the mutant capsule could be positive for Bmp4 expression. We investigated this possibility by examining Bmp4 expression (as measured by lacZ expression from a Bmp4lacZ allele) in tissue from Bmp4lacZ/Foxd1GFP/+ and Bmp4 lacZ/Foxd1GFP/GFP embryos. Indeed, at E14.5 Bmp4 expression was at a high level in the capsule of the Foxd1-mutant kidney (Fig. 4B), but not in the capsule of the wild-type kidney (Fig. 4A). We also investigated the expression pattern of Bmp4 at E12.5: Bmp4lacZ-expressing cells completely surrounded the mutant kidney (Fig. 4D), an expression pattern that was not observed in wild-type littermates (Fig. 4C) at this age. These results could reflect the difference in location within the pelvis between the wild-type and mutant kidneys at this age, and their positions relative to the abundant levels of Bmp4+ cells that exist in the caudal aspects of the embryo, especially near the cloaca (data not shown).

The presence of Bmp4+ cells in the capsule of the mutant kidney is an abnormal occurrence. However, it is not clear whether the Bmp4+ cells of the mutant capsule are derived from the Foxd1 lineage (that is, Bmp4 expression is upregulated in the absence of Foxd1) or they are a separate cell type (a subset of the β-gal-negative cells) that are now ectopically located in the mutant capsule due to alterations in its structure. To address this question, we performed double-labeling experiments using immunofluorescence with an anti-β-gal antibody coupled with GFP visualization on E12.5 tissue from Bmp4lacZ/Foxd1GFP/+ and Bmp4lacZ/Foxd1GFP/GFP embryos. From these results (Fig. 4E,F), it was clear that Bmp4 and Foxd1 are not expressed in the same cells, which strongly implies that the β-gal-negative cells

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**Fig. 2.** Expression of Foxd1lacZ in heterozygous and mutant embryos. At E13.5 (A,B) there is strong Foxd1lacZ expression in the dorsal body wall and kidneys, as well as a trail of β-gal+ cells that leads into the capsule of the kidney (arrowheads). At E16.5 (C,D), this trail is thinner (left arrowheads) and a gap between the normal kidney and the dorsal body wall exists (C, right arrowheads), while the mutant kidney is still attached (D, right arrow). In wild type (C'), there are both round β-gal+ cells in the loose connective tissue between the kidney and the dorsal body wall (yellow arrowheads) and flattened β-gal+ cells in the renal capsule (red arrowheads). In the mutant (D'), there is a mixture of β-gal+ and β-gal- cells in the capsule (red arrowheads). At E18.5, the wild-type capsule (E) still consists of a single layer of β-gal+ cells (red arrowheads), which persists into early adulthood (G). At E18.5, the mutant capsule is thickened (F,F') and still contains a mixture of β-gal+ (blue arrowheads) and β-gal- cells (red arrowheads). Higher magnification of bracketed areas in C, D, and F are displayed in (C',D',F'), d, dorsal; v, ventral; cs, cortical stroma. Scale bar: 400 μm for C,D; 250 μm for A,B; 120 μm for C',D'; 75 μm for E,F; 20 μm for G,F.
Foxd1 is required for renal capsule formation

in the capsule of the E14.5 Foxd1^{lacZ/lacZ} kidney are a mixture of Bmp4+ and Pecam1+/Flk1+ cells and these cells are most probably not derived from the early Foxd1+-cell lineage. However, it should be noted that by E18.5 in the Foxd1 mutant, expression of Bmp4 is limited to the convoluted tubules (Fig. 4H), a pattern of expression similar to the wild-type kidney (Fig. 4G). Thus, the ectopic presence of Bmp4+ cells in the capsule of the Foxd1-mutant kidney was transient, diminishing at late stages of development.

The ectopic presence of Bmp4+ cells in the capsule of the mutant kidney results in aberrant signaling to the nephrogenic mesenchyme

As in vitro experiments have shown that Bmp4 can inhibit nephron differentiation (Raatikainen-Ahokas et al., 2000), we wished to determine if the ectopic presence of Bmp4+ cells in the mutant capsule led to abnormal signaling in Foxd1-null kidneys. Binding of Bmp4 to its cell surface receptors induces cytoplasmic phosphorylation of Smad1 (phospho-Smad1) in order to propagate its signal (Dale and Jones, 1999). We, therefore, performed immunohistochemistry of phospho-Smad1 on sections of wild-type and Foxd1-mutant embryonic kidneys in order to determine the targets of Bmp4 signaling. In the wild-type kidney at E12.5, phospho-Smad1 expression is in the condensed mesenchyme, particularly in the pre-tubular aggregates that lie on the side of the UB tip opposite of the forming kidney capsule (Fig. 4I'). However, there is a distinct lack of phospho-Smad1 expression in the nascent capsule of the wild-type kidney. In the Foxd1-mutant kidney at this age, there is also phospho-Smad1 expression in the condensed mesenchyme (Fig. 4J'), and in some cells in the thick layer of mesenchyme that forms the abnormal capsule of the mutant kidney.

At E14.5 in the wild-type kidney, phospho-Smad1 expression is in the ureter and the stalks of the UB, but not in the region of the tips facing the capsule (Fig. 4K,K'). There is also expression in the pre-nephrogenic structures of the induced mesenchyme that exist under the arms of the bifurcated UB tips. These expression results correspond well with the previously determined expression pattern of the Bmp receptors in the developing kidney (Martinez et al., 2001). At this age in the Foxd1 mutant, phospho-Smad1 expression is present in the nephron progenitors that lie between the ureteric bud tips and the outside edge of the kidney (Fig. 4L,L'). Thus, ectopic location of Bmp4+ cells in the capsule of the Foxd1-null kidney induces ectopic Bmp4 signaling in the nephrogenic compartment. In addition, there is also phospho-Smad1 expression in the UB but only in the side of the UB that is in direct contact with the phospho-Smad1+ pre-tubular aggregates (Fig. 4L',L').

Defects in the proper specification of the mutant capsule result in aberrant patterning of the nephrogenic compartment

The initial study suggested that nephron formation was nearly absent in Foxd1 mutants (Hatini et al., 1996). Thus, it is possible that the ectopic formation of the pre-nephrogenic structures in regions just below the edge of the kidney in the Foxd1-null embryos may place them in an inappropriate signaling environment that prevents their further differentiation. To begin to address this issue and to determine when a possible block in differentiation occurs, we first wanted to verify that the enlarged condensates that are displayed in the Foxd1-mutant kidney expressed an appropriate marker of condensed mesenchyme. Pax2, which is strongly expressed in condensed mesenchyme (Dressler et al., 1990), is localized in

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Fig. 3. Genetic markers reveal that the renal capsule in the Foxd1 null is aberrant. Arrowheads indicate the position of the capsule. β-Gal+ cells populate the capsule of the heterozygote (A) and mutant (B). Raldh2 expression in the heterozygote (C) is at high levels in the capsule and the cortical stroma (cs) and is at lower levels in the mesenchyme of the nephrogenic zone. In the mutant (D), Raldh2 is undetectable in the capsule, though a low level of expression remains in the mesenchyme. Sfrp1 expression in wild-type (E) is at high levels in the capsule, but almost undetectable in the mutant (F). Normally (G), Pecam1 expression (brown cells) is localized to the interior of the kidney, away from the capsular stroma (blue cells), but is in high abundance in the capsule of the Foxd1 null (H). Reactivity with peanut agglutinin (PNA) normally is not detectable in the capsule (I), but there is ectopic reactivity in the Foxd1 null at this age (J). Scale bar: 100 µm for A-D; 150 µm for E-H; 200 µm for I,J.

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Development
nephron progenitors in both wild-type and Foxd1-null kidneys (Fig. 5A,B). Second, we also wanted to verify that the subpopulation of condensed mesenchyme that is induced to form pre-tubular aggregates and renal vesicles in Foxd1 mutants express appropriate markers of these structures. Thus, we examined the expression of Wnt4 (Sariola and Sainio, 1998) and Sfrp2 (Leimeister et al., 1998), which in wild-type embryos are expressed in pre-tubular aggregates and renal vesicles, respectively, and are exclusively localized beneath ureteric bud branches in a ratio of one pre-tubular aggregate per bud tip (Fig. 5C,E and Fig. 4K'). In Foxd1 mutants, Wnt4 and Sfrp2 were expressed in similar structures as wild type, but they were found at the very edge of the kidney facing the renal capsule (Fig. 5D,F), a location where they were never found in wild-type littermates. In addition, multiple pre-tubular aggregates (2-4) were observed per UB ampulla (Fig. 5D; Fig. 4L). Examination of other markers of pre-nephrogenic structures, such as Lim1 (Fujii et al., 1994), Pax8 (Plachov et al., 1990) and Hoxa11 (Patterson et al., 2001; Wellik et al., 2002), revealed similar results (data not shown). Interestingly, the mislocation of the pre-nephrogenic structures in the mutant kidney corresponds to the region of ectopic phospho-Smad1 expression. Thus, abnormal Bmp4 expression in the capsule may have a directly negative effect on nephron progenitors, as
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previously suggested (Raatikainen-Ahokas et al., 2000). Third, we wanted to examine the proliferation of the cells in the pre-nephrogenic structures in the mutant kidney to determine if lack of growth was the reason for the loss in future nephron formation. However, immunohistochemistry with the antibody Ki-67, which selectively binds to proliferating cells (Gerdes et al., 1991), revealed that both wild-type and mutant kidneys displayed similar levels of proliferation in the condensed mesenchyme (Fig. 5G,H). Similar results were also observed with phospho-histone H3 and BrdU (data not shown).

Although the nephrogenic mesenchyme of the mutant kidney was able to condense and form pre-nephrogenic structures, it was not clear at which subsequent stage of development nephron differentiation might be blocked. To investigate this, we used fluorescently labeled peanut agglutinin (PNA) and lotus lectin to identify glomeruli and proximal tubule segments, respectively, in vibrotome sections from wild-type and Foxd1-mutant kidneys at various embryonic ages. At E15.5, wild-type embryos displayed numerous PNA+-glomeruli and lotus lectin+-tubules in the juxtamedullary region of the kidney, but almost none of these structures were displayed in the Foxd1 null (Fig. 5I,J), although there is no discrete nephrogenic zone. At E18.5, both the wild-type kidneys (M) and the Foxd1-null (N) display numerous glomeruli and proximal tubules, but they are poorly organized in the mutant. We counted 100 nephrons in M and 85 nephrons in N. Scale bar: 250 µm in A,B,D-F,I,J; 150 µm in G,H,K,L; 300 µm in M,N.

Fig. 5. Nephrogenesis is delayed and disorganized in the Foxd1 null. (A) Wild type displayed Pax2 expression in condensed mesenchyme (cm) around the UB tips. The Foxd1 null (B) displayed much larger condensates. Wild type (C) and Foxd1 null (D) displayed Wnt4 expression in pre-tubular aggregates (pa) and both displayed expression of Sfrp2 in renal vesicles (rv) (E,F). Cell proliferation was determined by Ki-67 reactivity (G,H). Histochemistry with TRITC-PNA and FITC-lotus lectin revealed the presence of glomeruli (red spheres) and proximal tubules (green structures), respectively. At E15.5 and E16.5, wild-type kidneys (I,K) displayed glomeruli (gl) and proximal tubules (pt) in the juxtamedullary region, just below the nephrogenic zone. The Foxd1 null displayed no glomeruli or tubules at E15.5 (J), but did so at E16.5 (L), although there is no discrete nephrogenic zone. At E18.5, both the wild-type kidneys (M) and the Foxd1-null (N) display numerous glomeruli and proximal tubules, but they are poorly organized in the mutant. We counted 100 nephrons in M and 85 nephrons in N. Scale bar: 250 µm in A,B,D-F,I,J; 150 µm in G,H,K,L; 300 µm in M,N.
earlier in the mutant kidney. It should be noted, however, that in Foxd1-null kidneys, nephrons were present both at the outside edge of the kidney as well as in the center, regions that normally do not contain nephrons. Thus, the normal radial patterning of the nephrogenic compartments is highly disrupted in the mutant kidney. This result is consistent with the ectopic Bmp4 signaling that results from the abnormalities in the formation of the mutant renal capsule. In addition, it is interesting to note that the mutant kidney has lost its ectopic PNA reactivity by E16.5 (Fig. 5L), as well as its expression of Bmp4 in the capsule by E18.5 (Fig. 4H), which may indicate that the delay in nephrogenesis in the mutant kidney is relieved when the alterations in the capsule are sufficiently diminished. Nonetheless, contrary to what was reported earlier (Hatini et al., 1996), Foxd1 is not required for nephrogenesis, though it is indirectly required for its proper temporal and spatial patterning.

Aberrations in the patterning of the mutant ureteric tree result indirectly from defects in capsule formation
As previously reported, ureteric bud patterning is abnormal in the Foxd1 mutant, which led to the suggestion that Foxd1-regulated signals from stromal cells could be required for branching morphogenesis (Hatini et al., 1996). In the present study, we observed a threefold difference in ureteric bud branching (as determined by the number of bud tips) at E13.5 (data not shown), which increased to ~40-fold by E18.5 (Fig. 6A,B). In addition, the overall ureteric patterning in the Foxd1 mutant was significantly altered. The collecting system of the mutant kidney was composed of elongated tubules with very few ampulla at the surface of the kidney, which is in striking contrast to the wild type (Fig. 6A,B), suggesting that bifurcation but not elongation was impaired – a phenotype also seen in Bmp4-treated kidney rudiments (Miyazaki et al., 2000).

In wild-type embryos, nephron progenitors are associated only with UB tips, where Ret is selectively localized (Fig. 6C,E,E′). However, in Foxd1 mutants the branches of the UB are mostly enclosed by condensed nephrogenic mesenchyme, and Ret is expressed ectopically throughout these portions of the enclosed ureteric buds (Fig. 6D,F,F′). It is interesting to note, however, that areas of the UB that are not in contact with condensed mesenchyme (such as the regions indicated by the arrowheads in Fig. 6F′) do not express Ret, which is analogous to the cleft in the normal UB (yellow arrowhead in Fig. 6E′). This result suggests that the UB in the Foxd1-mutant is still competent to respond to signals.

Discussion
Loss of Foxd1 expression results in fused kidneys due to capsule malformations
The Foxd1−/− embryo represents an important model with which to study the function of renal stroma in kidney development. In this study, we examined the cause of the phenotypes displayed by the Foxd1−/− kidney, including pelvic kidneys and disorganization of the ureteric and nephrogenic compartments. Indeed, the presence of fused kidneys in the Foxd1−/− embryo is of particular interest because of the prevalent urological condition this represents in humans – about one in 400-600 live births (Nation, 1945; Weizer et al., 2003). We propose a novel explanation for kidney fusion: defects in the stromal cell population result in the inability of the kidneys to detach from the body wall or separate from the midline. Normally, the early metanephros flank the midline, in close proximity to each other, and are embedded in body tissue. But very rapidly (by
E12.5), the kidneys separate from the midline, rotate and begin to detach from the body wall. By E14.5, the kidneys are further detached from the surrounding tissue, though they maintain a connection to the dorsal body wall through what appears to be a loose connective tissue. In addition, by this age, the renal capsule is more clearly defined, consisting of a single, continuous layer of flattened cells at the outside edge of the kidney. Interestingly, the roundish cells that comprise the loose connective tissue and the flattened cells that comprise the renal capsule strongly express Foxd1. Given this expression pattern, it is very likely that the Foxd1+ cell population of the renal stroma is important for both the proper maturation of the renal capsule and detachment of the kidney from the dorsal body wall. This notion is supported by the results of our examination of the Foxd1-null embryo. We have shown in this study that loss of Foxd1, which is uniquely expressed in the stromal compartment, results in malformation of the renal capsule, which most probably explains the inability of the mutant kidneys to detach from the body wall and ascend into the lumbar region of the embryo.

Capsule abnormalities in the Foxd1-null embryo may lead to aberrant patterning of the nephrogenic compartment

In the normal kidney, the renal capsule, in addition to Foxd1, also expresses Sfrp1 and Raldh2. In the Foxd1-null kidney, however, the maturation of the capsule is aberrant. Instead of a single, homogeneous layer of cells, there is a thicker, heterogeneous layer that is histologically abnormal, displays undetectable levels of Sfrp1 and Raldh2 expression, and contains ectopic cell types. These include Bmp4-expressing cells and endothelial cells, though other cell types that we have not identified may also be present. Normally, the exclusion of these non-capsular cell types from the capsule may be based on expression of inhibitory signals or on differential adhesive properties. For example, one defect in the mutant capsule may be a change in the composition of the extracellular matrix (ECM), as there is an ectopic reactivity of PNA and lectins adhere to sugar moieties of glycoproteins that are often expressed in the ECM (Faraggiana et al., 1982). Indeed, a change in lectin histochemistry is a valid criterion of disease in the study of pathology (Danguy et al., 1998; Silva et al., 1993).

The early alterations in capsule cellular composition and morphology suggest that this defect may be the cause of the abnormal nephrogenic and ureteric patterning that occurs later. However, as renal patterning is reciprocal, it is also possible that loss of Foxd1 expression in stroma generates signals that act on non-stromal cell types, which in turn regulate normal capsule differentiation. Nonetheless, the ectopic presence of Bmp4-expressing cells in the capsule of the mutant kidney is of particular importance. The normal localization of Bmp4-expressing cells is consistent with its proposed role in promoting nephron differentiation (Raatikainen-Ahokas et al., 2000) and maturation of the collecting duct system (Miyazaki et al., 2000). Indeed, our current studies support the conclusions of these in vitro experiments by showing that the targets of Bmp4 signaling, as judged by phospho-Smad1 expression, are the pre-tubular aggregates of the induced mesenchyme and the pre-nephrogenic epithelia that they form, as well as the UB stalks. In Foxd1-null kidneys, however, the establishment of unique zones of induction (the nephrogenic zone) and differentiation (the juxtamedullary region) never occurs. Instead, there is an absence of a clearly defined nephrogenic zone, nephron development is delayed and when glomeruli do appear, they occupy all regions of the kidney, including the edges. Our findings suggest that this aberrant patterning is due to the inappropriate presence of Bmp4-secreting cells in the mutant renal capsule. This ectopic localization results in an alteration in the normal pattern of Bmp4 signaling, such that signaling to both peripheral and interior nephron progenitors occurs. Thus, it appears likely that in wild-type embryonic kidneys all nephrogenic mesenchyme can respond to Bmp4 signals, but the establishment of unique zones of nephron induction and differentiation is highly dependent on the appropriate positioning of Bmp4-secreting cells. Therefore, defects in the formation of the renal capsule in the Foxd1-null mouse, which allows inappropriate accumulation of Bmp4-expressing cells in an important signaling environment, could disrupt the normal patterning of the nephrogenic compartment.

Foxd1-signaling in cortical stroma is not required directly for nephron differentiation

In spite of this mislocalization of pre-nephrogenic structures in the mutant, the fact that Bmp4 could still signal to the pre-tubular aggregates and the pre-nephrogenic epithelia reveals that the defect in the Foxd1 mutant is not an inability of the condensed mesenchyme to respond to inductive signals. Indeed, in addition to displaying phospho-Smad1 expression, there is also expression of Wnt4, Lmi1, Fax8, Sfrp2 and Hoxa11 in the mutant. Furthermore, although the initial report describing the Foxd1-null mouse mentioned a severe reduction in the differentiation of the condensed mesenchyme into nephrons (Hatini et al., 1996), our studies revealed only a delay in nephrogenesis up to E15.5, which was overcome by E16.5. In fact, near the time of birth (E18.5) the mutant kidney displayed the presence of nephrons (on a per-volume basis) at levels nearly approaching wild type. This result is surprising as the number of UB tips is drastically reduced in the mutant kidney and it has previously been thought that nephron number is determined by the number of UB tips (Clark and Bertram, 1999; Oliver, 1968). This paradox may be explained by the observation that, unlike the situation in wild-type embryos, where each UB tip is associated with one pre-tubular aggregate, UB ampullae in Foxd1-null kidneys are associated with two to four pre-tubular aggregates each. Presently, it is still unclear whether this change in the UB:pre-tubular aggregate ratio in the mutant is due to a general defect in the patterning of the nephrogenic compartment or, rather, due to a misregulation of signals in a potential Foxd1-regulated mesenchyme-stroma-UB signaling axis that dictates this ratio. What is clear, however, is that nephron differentiation in the Foxd1-null mutant is independent of the defects in UB branching and that the defect in the Foxd1-mutant kidney is not so much a reduction in its nephrogenic capacity, but, rather, in its ability to correctly organize this compartment.

Bmp4 signaling determines UB patterning indirectly via nephron progenitors

Previous reports have suggested that Bmp4 inhibits budding of the UB while promoting its elongation (Bush et al., 2004;
Miyazaki et al., 2000). Likewise, Bush et al. (Bush et al., 2004) have also suggested that the tips of the growing UB are not exposed to high concentrations of Bmps, but rather the region of the UB destined to become the stalk sees high concentrations of these factors and is thus inhibited from branching. Our results reveal that phospho-Smad1 expression in the wild-type kidney is in the stalks of the UB with no detectable expression in the region of the UB tips facing the capsule. This result correlates well with this earlier suggestion by Bush et al. as Bmp4 signaling is in the appropriate region of the UB to promote its elongation (that is, in the stalks) without interfering with its ability to branch (that is, lack of signaling in the UB tips facing the capsule).

In the Foxd1 mutant, there is Bmp4 signaling to the UB, though only in those regions that are in contact with mesenchyme and pre-nephrogenic structures that also display Bmp4 signaling (see Fig. 4L,L’). Nonetheless, the branching pattern of the ureteric tree is aberrant. After E12.5, it does not display ‘Y-shaped’ branches typical of the wild-type ureteric tree but instead amorphous branches, with thickened ampullae, and they are few in number. In addition, unlike the wild-type UB, which is enclosed by nephron progenitors only at its tips, many of the UB branches of the mutant kidney are enclosed in a thick layer of nephron progenitors, which we hypothesize is a consequence of ectopic Bmp4 signaling in the capsule. Thus, in the Foxd1-null kidney the UB cleft and stalk are not distinguished from the tips and Ret expression remains at a high level throughout this region of the UB, unlike the wild-type UB in which Ret expression becomes selectively localized in the tips when they become capped by condensation of the nephrogenic mesenchyme. This ectopic domain of Ret expression in the UB of the mutant may be a result of abnormal contact with nephron progenitors or it may be due to failure of these domains to access signals that control branching and elongation. Consistent with these possibilities, the regions of the mutant UB that are not in contact with nephron progenitors do not display Ret expression, indicating that the mutant UB may still be competent to respond to signals but is prevented from doing so by the presence of the enlarged condensates. Indeed, the eventual differentiation in the mutant of the nephron progenitors into mature nephrons at E16.5, probably allows further UB branching because by E18.5 there are tips at the surface of the kidney, though ~40-fold less than wild type. Thus, unlike what was previously thought (Hatin et al., 1996), it appears that the architecture of the mature ureteric tree in the mutant kidney – long spindly branches with very few tips at the surface of the kidney – is a consequence of the defects in the patterning of the nephrogenic compartment, rather than from a direct defect in signaling from the stroma. This result in vivo correlates very well with in vitro organ cultures treated with exogenous Bmp4 that also display defects in ureter patterning (and also without a loss of Ret expression) but are indirectly due to interference in the differentiation of the nephrogenic mesenchyme (Raatikainen-Ahokas et al., 2000).

The capsule provide critical functions required for the normal patterning of the developing kidney

Although additional studies will be necessary to determine further signals affected by the loss of Foxd1 expression, the unique expression of Foxd1 in the capsular stroma and the severe affects that occur upon its absence highlight the importance of this cell type in the overall, normal patterning of the kidney. In particular, the results in our study suggest that in the wild-type kidney a very careful balance of signals emanating from the capsular stroma, the cortical stroma, and the interior pattern the nephrogenic and ureteric compartments of the nephrogenic zone. Signals from the capsule, such as sFRP1, may keep the mesenchyme from differentiating too quickly, as well as indirectly promoting UB branching while orientating the growth of the ampullae toward the kidney surface (Schumacher et al., 2002). Meanwhile, signals from the interior of the kidney, such as Bmp4, result in the induction of the mesenchyme and elongation of the UB stalks, followed by signals from the stroma that influence the balance between glomerulogenesis and tubulogenesis (Yang et al., 2002). In this way, a controlled radial expansion of the kidney occurs, while maintaining its proper compartmentalization. In the Foxd1 mutant, this careful balance of signals is significantly disturbed because of an aberration in the maturation of the kidney capsule; thus, illustrating both the capacity and the importance of renal capsule signaling that is necessary for the proper positioning and patterning of the developing kidney.

We thank Qais al-Awqati, Elizabeth Lacy, Jonathan Barasch, Frank Costantini and Brigid Hogan for critically reading the manuscript; Frank Costantini, Brigid Hogan and Eseng Lai for the HoxB7-GFP, Bmp4-lacZ and the Foxd1-lacZ mouse strains, respectively; the Core Histology facility for their excellent technical help; Greg Dressler (Pax2), Peter McCaffery (Raldh2), Jeanine D’Armiento (Sfrp1), Jeremy Nathans (Sfrp2) and Andy McMahon (Wnt4) for in situ probes. This work was supported by grants from the National Institutes of Health to R.S.L. (F32 DK10072), M.V. (5T32 DK07328) and C.L.M. (R01 61459.01).

References


Foxd1 is required for renal capsule formation


