INTRODUCTION

Inherited disorders of the kidney and urinary tract are quite common in humans, but their etiology and underlying developmental mechanisms are poorly understood (Mackie and Stephens, 1975; Nishimura et al., 1999; Pope et al., 1999). The kidneys and urinary tract, like the reproductive organs, arise in the intermediate mesoderm between the somites and the lateral plate, and formation of the adult or metanephric kidney is preceded by the transient appearance of the pronephros and mesonephros (Saxen, 1987). In the mouse, the pronephric region gives rise at about the level of somite 5 to an epithelial duct (the nephric or Wolffian duct) which elongates caudally. During this migration the adjacent intermediate mesenchyme of the nephrogenic cord is induced to differentiate into the mesonephric tubules, which in the mouse are most prominent between somites 8 and 15. Shortly before the Wolffian duct reaches the cloaca, the most caudal part of the nephrogenic cord condenses to form the metanephric mesenchyme which, at about the level of somite 25, induces localized outgrowth of a ureter bud from the Wolffian duct. Subsequently, reciprocal interactions between the ureteric bud and the surrounding mesenchyme result in the development of the metanephric kidney.

The caudal end of the Wolffian duct (the common excretory duct) is eventually incorporated into the cloaca, contributing to the trigone of the bladder. Normally, this remodeling results in the orifice of the ureter opening directly into the bladder (Fig. 1). However, clinical analysis of infants with kidney abnormalities such as duplex kidney and/or hydronephrosis has revealed that these structural abnormalities are often associated with a malpositioned ureteral orifice (Mackie and Stephens, 1975). This finding led to the hypothesis that one underlying cause of duplex kidney and hydronephrosis is the abnormal positioning of the primary ureter bud and/or an ectopic ureter bud along the anterior-posterior axis of the Wolffian duct. According to this model (Fig. 1), if an ectopic bud forms anterior to the normal one, then it will induce an anterior ectopic kidney that may fuse with the normal one. However, during the incorporation of the Wolffian duct into the cloaca, the orifice of the ectopic ureter will come to lie in the urethra or sex ducts, resulting in blockage of urine flow and hydronephrosis (Mackie and Stephens, 1975). If this hypothesis is correct, then abnormal or ectopic ureter budding is likely to

SUMMARY

The murine genes, Foxc1 and Foxc2 (previously, Mf1 and Mfh1), encode forkhead/winged helix transcription factors with virtually identical DNA-binding domains and overlapping expression patterns in various embryonic tissues. Foxc1/Mf1 is disrupted in the mutant, congenital hydrocephalus (Foxc1/Mf1ch), which has multiple developmental defects. We show here that, depending on the genetic background, most Foxc1 homozygous mutants are born with abnormalities of the metanephric kidney, including duplex kidneys and double ureters, one of which is a hydroureter. Analysis of embryos reveals that Foxc1 homozygotes have ectopic mesonephric tubules and ectopic anterior ureteric buds. Moreover, expression in the intermediate mesoderm of Glial cell-derived neurotrophic factor (Gdnf), a primary inducer of the ureteric bud, is expanded more anteriorly in Foxc1 homozygous mutants compared with wild type. These findings support the hypothesis of Mackie and Stephens concerning the etiology of duplex kidney and hydronephrosis in human infants with congenital kidney abnormalities (Mackie, G. G. and Stephens, F. G. (1975) J. Urol. 114, 274-280). Previous studies established that most Foxc1lac2 Foxc2tm1 compound heterozygotes have the same spectrum of cardiovascular defects as single homozygous null mutants, demonstrating interaction between the two genes in the cardiovascular system. Here, we show that most compound heterozygotes have hypoplastic kidneys and a single hydroureter, while all heterozygotes are normal. This provides evidence that the two genes interact in kidney as well as heart development.

Key words: forkhead/winged helix gene, Mf1, Mfh1, Foxc1, Foxc2, Mouse, Intermediate mesoderm, Kidney, Hydroureter, Ureter bud, Mesoderm
derived neurotropic factor (Gdnf) produced by the metanephric induction and orientation of the ureteric bud involve glial cell-theureter bud. Recent studies have demonstrated that the nephrogenic mesenchyme, the tissue responsible for inducing budding may, in turn, result from abnormalities in the intermediate mesoderm are still not known.

Mutant mice provide useful models to address the many unresolved questions surrounding congenital defects of the kidney and urinary tract. Here, we report studies on one such model involving the forckhead/winged helix gene Foxc1 (previously Mf1) and the closely related gene Foxc2 (Mfh1).

Forkhead/winged helix or Fox (Kaestner et al., 2000) proteins constitute a large family of transcription factors that share an evolutionarily conserved DNA-binding domain and play numerous essential roles in embryonic development, including cell fate determination, proliferation and differentiation (for reviews, Kaufmann and Knochel, 1996). There is, in addition, extensive evidence that these proteins are components of different signal transduction pathways, including those downstream of insulin, activin and TGFβ-related ligands (Chen et al., 1997; Kops et al., 1999; Labbe et al., 1998; Lin et al., 1997; Zhou et al., 1998). In the mouse, mutations in a number of Fox genes have provided evidence for both unique and functionally interactive roles in development (Chen et al., 1998; Kaestner et al., 1997; Kume et al., 1998; Labosky et al., 1997; Winnier et al., 1997; Xuan et al., 1995). Several Fox family members are expressed during kidney development (Hatini et al., 1996; Kume et al., 1998, 2000; Miura et al., 1993; Overdier et al., 1997; Pelletier et al., 1998). For example, Foxd1/Bf2 (Brain factor 2) is expressed specifically in the stromal mesenchymal cells of the kidney and all Bf2-deficient mice die after birth with kidney abnormalities, including hypoplastic kidneys and small ureters (Hatini et al., 1996). We have recently shown that up to 40% of homozygous Foxd2/Mf2 (Mesoderm/mesenchyme forckhead 2) mutant mice have kidney and ureter abnormalities, including hydroreuter, small kidneys and short ureters (Kume et al., 2000). However, very little is known about the roles of other forckhead transcription factors during kidney development.

Our laboratory and others have previously analyzed the forckhead/Mf1 (Mesoderm/mesenchyme forckhead 1) gene, which is expressed at high levels in many tissues including paraxial mesoderm, somites, prechondrogenic mesenchyme, pericardial mesenchyme and the developing cardiovascular system (Hiemisch et al., 1998; Kidson et al., 1999; Kume et al., 1998; Swiderski et al., 1998; Winnier et al., 1999). We now show here that Foxc1/Mf1 is expressed in the intermediate mesoderm and mesonephric and metanephric kidney, where it is required for normal development. Mice homozygous for either a spontaneous mutation in Foxc1/Mf1 (congenital hydrocephalus, Foxc1ch) or an engineered null mutation (Foxc1lacZ) die prenatally and perinatally with multiple abnormalities, including haemorrhagic hydrocephalus and skeletal, ocular and cardiovascular defects (Gruneberg, 1943, 1953; Hong et al., 1999; Kidson et al., 1999; Kume et al., 1998; Winnier et al., 1999). On the CHMU/Le inbred background, Foxc1ch mutants have been reported to have kidney and ureter abnormalities, including hydronephrosis, hydroreuter and double ureters (Green, 1970). We show here that this kidney phenotype is present in the majority (>85%) of mutants on the CHMU/Le × C57BL/6 background but is very rare on the 129 × Black Swiss background. Moreover, on the CHMU/Le × C57BL/6 background, there is anterior expansion or persistence of Gdnf expression in the embryonic intermediate mesoderm of null mutants, and this may be responsible for the induction of ectopic mesonephric tubules and ectopic anterior ureter bud, and the development of duplex kidney and double ureters.

Foxc2/Mfh1 (Mesenchyme forckhead 1) encodes a protein with a virtually identical DNA-binding domain to that of Foxc1/Mf1 (97% identity; 99% similarity). Moreover, the expression patterns of Foxc1/Mf1 and Foxc2/Mfh1 show overlapping domains in many embryonic tissues including, as shown here, the developing kidney (Hiemisch et al., 1998;
Kaestner et al., 1996; Miura et al., 1993; Winnier et al., 1997, 1999). While our laboratory and others have previously shown that Foxc2/Mfh1 null mutants die prenatally and perinatally with skeletal and cardiovascular defects (Iida et al., 1997; Winnier et al., 1997), a possible role for Foxc2/Mfh1 in kidney development has not been investigated in detail.

Our previous studies showed that most embryos that are compound heterozygotes for null mutations in Foxc1/Mf1 and Foxc2/Mfh1 have the same spectrum of cardiovascular defects as seen in each single homozygous null mutant (Winnier et al., 1999), suggesting functional interaction between the two genes in the developing cardiovascular system. We report here that most compound heterozygous mutants on the 129 × Black Swiss genetic background also have hydroureter and renal hypoplasia/agenesis, while heterozygotes are normal. Taken together, these data suggest that Foxc1/Mf1 and Foxc2/Mfh1 play co-operative roles during kidney as well as cardiovascular development.

MATERIALS AND METHODS

Breeding mutant mice and genotyping

Mice heterozygous for the null mutations, Foxc1/Mf1lacZ and Foxc2/Mfh1tm1· (Kume et al., 1998; Winnier et al., 1997), were maintained by interbreeding on the CHMU/Le inbred background were purchased from The Jackson Laboratory. The mutations in Foxc1/Mf1 and Foxc2/Mfh1 are likely to have similar effects, resulting in truncated Foxc1/Mf1 proteins lacking most of the DNA-binding domains (Kume et al., 1998). Because of their poor mating performance on the CHMU/Le genetic background, Foxc1/Mf1 and Foxc2/Mfh1 heterozygous mice were crossed to C57BL/6 mice and maintained by interbreeding on the CHMU/Le × C57BL/6 genetic background. Genotyping was performed by PCR using ch-5’-TA TGAGCGTGTACTCGCACCCT-3’, ch-3’-CGTACCGTTTCGCTGATGTC-3’, followed by Cac8I enzyme digestion. The wild-type and Foxc1/Mf1αθ alleles give (178 and 197 bp) and 375 bp bands, respectively.

Histological analysis

Whole-mount and section in situ hybridization were performed essentially as described (Hogan et al., 1994). The following murine cDNAs were used as templates for [35S]UTP or digoxigenin-labeled antisense RNA probes: 0.8 kb Foxc1/Mf1 cDNA; 1.7 kb Foxc2/Mfh1 cDNA; 1.8 kb c-ret cDNA; 0.3 kb Gdnf cDNA (provided by Dr Yoichi Miyazaki, Vanderbilt University Medical Center); 2.5 kb lim1 cDNA (Barnes et al., 1994); 1.6 kb Eya1 cDNA (provided by Dr Richard L. Maas, Harvard Medical School).

RESULTS

Foxc1/Mf1 and Foxc2/Mfh1 expression during kidney development

At 8.5 days post coitum (dpc), Foxc1/Mf1 transcripts are
present in the presumptive intermediate mesoderm, as well as the presomitic mesoderm and somites (Fig. 2A). This expression continues at 9.5 dpc, when the nephrogenic cord develops and the Wolffian (nephric) duct begins to elongate caudally along the embryo in the intermediate mesoderm. At this time, sections through the posterior of the embryo clearly show that Foxc1/Mf1 is transcribed in an apparent dorsoventral gradient in the mesoderm, with the highest level in the region closest to the neural tube and the lowest levels ventrally, where Bmp4 is expressed (Fig. 2C,F). Gdnf is first detected in the intermediate mesoderm/nephrogenic cord at 8.5 dpc (data not shown) and, in contrast to Foxc1 and Foxc2 (Fig. 2C,D), is in a much more restricted domain at 9.5 dpc (Fig. 2E). Sections through the more anterior trunk at this time show high levels of Foxc1/Mf1 RNA in the mesonephric mesenchyme alongside the Wolffian duct, while only very weak signal is present in the Wolffian duct (Fig. 2G). Later, expression is also seen in the mesonephric tubules (Kume et al., 1998). At 10.5 dpc, formation of the metanephric kidney begins with the outgrowth of the ureteric bud from the Wolffian duct into the metanephric mesenchyme, in which Gdnf is strongly expressed (Fig. 2K). At this time, Foxc1/Mf1 is transcribed in the metanephric mesenchyme, but not in the epithelium of the Wolffian duct and the branching ureter (Fig. 2D). At 12.5 dpc, Foxc1 transcripts continue to be detected in the condensing mesenchyme of the kidney, although transcript levels appear low in the aggregates directly surrounding the ureteric epithelium (Fig. 2L,N).

Foxc2/Mfh1 has a distinct transcription pattern that overlaps with that of Foxc1/Mf1 (Hiemisch et al., 1998; Iida et al., 1997; Kaestner et al., 1996; Miura et al., 1993; Winnier et al., 1997, 1999). Regions of coexpression relevant to kidney development include the intermediate mesoderm at 8.5 dpc, mesonephric mesenchyme at 9.5 dpc, and metanephric mesenchyme at 10.5 and 12.5 dpc (Fig. 2B,D,H,J,M,O). In these regions, some differences are observed. For example, Foxc2/Mfh1 transcripts are present at lower levels than Foxc1/Mf1 in the ventral region of the mesoderm at 9.5 dpc (Fig. 2D) but at higher levels at 10.5 dpc around the ureteric bud (Fig. 2J).

**Foxc1/Mf1ch homozygous mutants have kidney and ureter abnormalities**

Green (1970) described abnormalities of the mesonephric tubules and metanephric kidney and ureters in heterozygous and homozygous congenital hydrocephalus mice. However, the underlying primary defects are still not known. To characterize the nature of these abnormalities, we first examined the kidneys of newborn homozygous Foxc1/Mf1ch and Foxc1/Mf1lacZ mice, since mutations in both alleles are likely to be similar (Kume et al., 1998), although they are carried on different genetic background. Of homozygous Foxc1ch mutants on the CHMU/Le × C57BL/6 genetic background, 15/17 (>85%) had duplex kidneys and double ureters, accompanied by fluid-filled dilation of the kidney and ureter, known as hydronephrosis and hydroureter, respectively. Only 1/15 heterozygous Foxc1ch mutants had this phenotype while all wild types were normal (Table 1: Fig. 3A,B,E). These abnormalities were unilateral (right 54%; left 8%) or bilateral (38%), and occurred in both sexes (male 54% and female 46%). On the 129 × Black Swiss genetic background, only 1/49 (2%) of homozygous Foxc1lacZ mutants showed the same kidney abnormalities, suggesting there are genetic modifiers affecting the penetrance of this phenotype.

Histological analysis of newborn Foxc1/Mf1ch mice clearly revealed a duplex kidney connecting to double ureters (Fig. 3D). Interestingly, only one of the ureters was always enlarged and fluid filled, while the other was normal (Fig. 3E). This may be related to a model proposed for the etiology of hydronephrosis in humans and outlined in the Introduction and Fig. 1 (Mackie and Stephens, 1975; Stephens and Huston, 1996). We therefore examined histologically the insertion of the ureters in newborn mice. In male homozygotes, the hydroureters, but not the normal ureters, connected aberrantly to Wolffian duct derivatives such as the seminal vesicle or vas deferens (Fig. 3F). In female homozygotes, the hydroureter did not connect with the bladder and ended blindly (Fig. 3G). These data support the idea that, in Foxc1/Mf1ch mutants, on the CHMU/Le × C57BL/6 genetic background, hydroureter results from an ectopic ureter, induced from the Wolffian duct more anteriorly to the normal ureteric bud.

Our analysis also revealed that the position of the newborn gonads was frequently more anterior in Foxc1/Mf1ch homozygous mutants (male 75% and female 100%) than in newborn wild-type mice (Fig. 3A,B) as previously described (Green, 1970). In male mice, the mutant testes were located at the posterior border of the kidney while, in wild-type pups, they were beside the bladder (Fig. 3A). Some mutant ovaries were situated almost at the anterior border of the kidney compared to the wild type (Fig. 3B). The abnormal position of the gonads was unlikely to be secondary to the hydronephrosis and hydroureter since when the renal abnormalities were unilateral we observed the same gonad defects on the side of the normal kidney (data not shown).

**Ectopic ureteric bud and mesonephric tubules in Foxc1/Mf1ch homozygous mutants**

We next asked whether the formation of the ureteric bud is abnormal in Foxc1/Mf1ch mutants since this might be the primary defect leading to a duplex kidney and hydroureter as previously proposed from clinical data (Mackie and Stephens, 1975). Whole-mount in situ hybridization at 10.5 dpc with a c-ret probe showed that the entire posterior end of the Wolffian duct is much broader and expanded in Foxc1ch homozygous mutants compared with wild-type embryos (Fig. 4A,B). By 11.0 dpc, the single normal ureteric bud has extended into the metanephric mesenchyme and has acquired a distinctive shape (Fig. 4C). By contrast, in Foxc1ch mutants, an ectopic ureteric bud is now clearly observed more anteriorly to the normal bud (Fig. 4D). In addition, the Wolffian duct of mutants appears to be kinked and more medial, and additional small buds from the Wolffian duct are often seen more anteriorly to the ectopic bud (Fig. 4B,D).

Gdnf expressed in the nephrogenic mesenchyme is known to be an essential factor for ureter budding, acting through its tyrosine kinase receptor, c-ret and co-receptor, gfrα-1, expressed in the Wolffian duct epithelium (for reviews, Rosenthal, 1999; Sariola and Sainio, 1997). Embryos homozygous for a null mutation in Gdnf lack induction of the ureteric bud, resulting in the complete absence of the metanephric kidney (Moore et al., 1996; Pichel et al., 1996; Sanchez et al., 1996) and Gdnf-soaked beads can ectopically
induce budding of the ureter from the Wolffian duct (Pichel et al., 1996; Sainio et al., 1997). Given the abnormal patterning of the ureteric bud in Foxc1/Mf1 lacZ and Foxc2/Mfh1 tm1 compound heterozygotes compared to expression, an early marker for the intermediate mesoderm, Foxc1ch mesoderm in up to about the level of the 16th somite in the wild type. up to the level of the 23rd somite, while they are present only mesonephric tubules are present and distributed more caudally duct at 10.5 dpc (Fig. 4G,H). In detected in the mesonephric tubules as well as the Wolffian duct while it elongates caudally (Pachnis et al., 1993) and that lack the outgrowth of the ureteric bud, the same phenotype seen in Gdnf mutants. In addition, in Eyal homozygous mutant embryos, Gdnf is not detected in the metanephric mesenchyme, suggesting that Eyal controls the genetic regulatory cascade upstream of Gdnf (Xu et al., 1999). We therefore examined expression of Eyal and found that the Eyal domain also extended more anteriorly in Foxc1/Mf1ch mutants compared to the wild type at 10.5 dpc (data not shown). This suggests that Foxc1/Mf1 regulates either Eyal or more upstream genes in the regulatory cascade in the intermediate mesoderm rather than Gdnf itself.

Although the molecular mechanism of mesonephros development remains poorly understood, especially in mammals, it has been shown that c-ret is expressed throughout the Wolffian duct while it elongates caudally (Pachnis et al., 1993) and that c-ret-deficient mice have a reduced number of mesonephric tubules (Schuchardt et al., 1996). This evidence suggests that the Gdnf-c-ret signaling pathway regulates interactions between mesenchymal and epithelial cells during development of both the mesonephros and metanephros in the mouse embryo (Sainio and Raatikainen-Ahokas, 1999). Given the presence of an ectopic ureteric bud and abnormal expression of Gdnf in Foxc1/Mf1ch mutant mice, we sought to determine whether the mesonephros might be defective as well. We therefore examined the expression of lim1, which is detected in the mesonephric tubules as well as the Wolffian duct at 10.5 dpc (Fig. 4G,H). In Foxc1ch mutant embryos, more mesonephric tubules are present and distributed more caudally up to the level of the 23rd somite, while they are present only up to about the level of the 16th somite in the wild type.

One possible explanation for the abnormalities described above is an expansion of the domain of the intermediate mesoderm in Foxc1ch homozygous embryos early in development, at the time when the dorsoventral domains of the mesoderm are being established (see Discussion). However, Foxc1ch homozygotes showed no obvious difference of lim1 expression, an early marker for the intermediate mesoderm, compared to Foxc1ch heterozygotes at 8.0 dpc (Fig. 4I,J).

Kidney and ureter abnormalities in Foxc1/Mf1lacZ and Foxc2/Mfh1tm1 compound heterozygotes

Since Foxc2/Mfh1 expression overlaps with that of Foxc1/Mf1 in the intermediate mesoderm and developing kidney, we also examined the kidneys of newborn homozygous Foxc2/Mfh1 mutants and compound heterozygotes of Foxc1/Mf1lacZ and Foxc2/Mfh1tm1 (Fig. 5; Table 1). On the 129 × Black Swiss genetic background, 5/8 homozygous null Foxc2/Mfh1 mutants had smaller than normal kidneys with a single ureter. Of the compound heterozygotes, 7/19 had hypoplastic kidneys and 13/19 had a single hydrourater, but one had a duplex kidney and double ureters. Hydroureter was either unilateral (85%) or bilateral (15%), and hypoplastic kidneys were either unilateral (71%) or bilateral (29%). In severe cases, hydroureter was accompanied by hydrenephrosis (Fig. 5A) (26%) or the complete absence of the kidney (agenesis) was observed (5%) (Fig. 5B). By contrast, each single heterozygote alone had no abnormality (Table 1).

Given the early phenotype of Foxc1/Mf1ch mutants described above, we also examined the formation of the ureteric bud in double heterozygous embryos. In compound heterozygotes, the outgrowth of the bud was much broader (2/3) (Fig. 5D) than in the wild type (Fig. 5C) at 10.5 dpc and 3/4 had an ectopic ureteric bud 11.0 dpc (Fig. 5E,F). In addition, more outgrowth of the bud was much broader (2/3) than in the wild type, as discussed above. We also examined the formation of the ureteric bud in double heterozygous embryos. In compound heterozygotes, the outgrowth of the bud was much broader (2/3) (Fig. 5D) than in the wild type (Fig. 5C) at 10.5 dpc and 3/4 had an ectopic ureteric bud 11.0 dpc (Fig. 5E,F). In addition, more mesonephric tubules were distributed caudally in compound heterozygotes between somites 16 and 23 compared to the wild type (Fig. 5G,H).

**DISCUSSION**

In spite of exciting progress in identifying signaling factors involved in cell-cell interactions during kidney morphogenesis, a great deal remains to be learnt about the molecular mechanisms underlying the etiology of congenital kidney and urinary tract abnormalities in humans. We show here that Foxc1/Mf1 homozygous mutants have abnormalities in kidney and ureter development, including duplex kidneys and double ureters accompanied by hydrenephrosis and hydroureter and preceded by ectopic mesonephric tubules and anterior ureter bud. Similar to other tissues, we find that the forkhead/winged helix genes, Foxc1/Mf1 and the closely related gene Foxc2/Mfh1, exhibit similar expression patterns in the developing kidney. Moreover, we provide evidence for a

---

**Table 1. Kidney and urinary abnormalities in newborn single mutants and compound heterozygotes of Foxc1lacZ and Foxc2tm1**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>n</th>
<th>Duplex kidney</th>
<th>Small kidney</th>
<th>Hydroureter</th>
<th>Double ureters</th>
</tr>
</thead>
<tbody>
<tr>
<td>4/+*</td>
<td>5</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>4/+‡</td>
<td>6</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Foxc1ch+/-</td>
<td>17</td>
<td>15</td>
<td>0</td>
<td>15 (15)§</td>
<td>15</td>
</tr>
<tr>
<td>Foxc1ch+/-</td>
<td>49</td>
<td>1</td>
<td>0</td>
<td>7 (1)§</td>
<td>1</td>
</tr>
<tr>
<td>Foxc2tm1+/-</td>
<td>8</td>
<td>0</td>
<td>5</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Foxc1ch+/-</td>
<td>15</td>
<td>1</td>
<td>0</td>
<td>1 (1)§</td>
<td>1</td>
</tr>
<tr>
<td>Foxc1ch+/-</td>
<td>7</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Foxc2tm1+/-</td>
<td>7</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Foxc1 lacZ; Foxc2tm1+/+</td>
<td>19</td>
<td>1</td>
<td>7 (1)**</td>
<td>13 (5)§</td>
<td>1</td>
</tr>
</tbody>
</table>

*CHMU; LexC57BL/6 genetic background.
‡129× Black Swiss genetic background.
§Bilateral or unilateral traits.
§Hydroureter accompanied by hydrenephrosis.
||Less than 3/4 wild-type length.
**Renal agenesis.
Fig. 3. Kidney and ureter abnormalities in newborn mice homozygous for Foxc1<sup>ch</sup>. (A,B) Wild-type and mutant newborn kidneys. (A) Male mutant kidneys have hydroureters (asterisks). Note that mutant testes (arrowheads) are located more anteriorly compared to wild-type testes (arrowheads). (B) Female mutant kidneys have hydroureters (asterisks) and normal ureter (white arrow) behind it. Yellow arrows indicate the oviducts of mutant. Note that mutant ovaries are located more anteriorly (arrowheads) compared to the wild type. (C,D) Sections of newborn wild-type (C) and mutant (D) kidneys. Mutant has a duplex kidney showing a clear boundary of the peripheral metanephrogenic mesenchyme (arrow). Note that the upper part of the kidney connects to the hydroureter (asterisk). (E) Dorsal view of newborn mutant kidneys with double ureters. Normal ureters (arrows) and ectopic hydroureters (asterisks). (F,G) Sections selected from serial sections showing abnormal position of the hydroureters in newborn Foxc1<sup>ch</sup> mutants. (F) Male mutant hydroureter (arrow) does not connect to the bladder (b), but aberrantly to a derivative of the Wolffian duct (arrowhead). (G) Female mutant hydroureter (arrow) ends blindly, while the normal ureter (arrowhead) connects to the bladder (b). Scale bar: C,D, 800 µm; E,F, 400 µm.

A genetic interaction between the two genes in kidney development since most compound heterozygous embryos have hydroureter with single ureters and renal hypoplasia/agenesis, while single heterozygotes are normal.

Models for the formation of an ectopic ureteric bud and excess mesonephric tubules

The striking phenotype in Foxc1/Mf1<sup>loc2</sup> and Foxc1/Mf1<sup>loc2</sup> homozygotes is the presence of duplex kidneys and double ureters, one of which is fluid-filled. Both defects most likely result from an ectopic anterior ureteric bud from the Wolffian duct. Induction of this bud is, in turn, probably due to anterior expansion or persistence of Gdnf expression in mutant embryos. This conclusion is supported by previous experimental data showing induction of an ectopic ureter bud by Gdnf-soaked beads (Pichel et al., 1996; Sainio et al., 1997). It is also likely that the presence of ectopic mesonephric tubules in the region between about somites 16 and 25, which is normally devoid of obvious tubules, results from abnormal expression of Gdnf. Taken together, these findings provide strong support for the ideas previously proposed for the etiology of some congenital defects of the kidney and urinary tract in humans (Mackie and Stephens, 1975).

Several models can be considered for the role of Foxc1/Mf1 in the formation of ectopic anterior ureter buds and mesonephric tubules. The first is that both phenotypes are the result of the persistence of Gdnf transcription in nephrogenic mesenchyme cells that normally only transiently express the gene. During normal development, Gdnf is first expressed in the nephrogenic cord at 8.5 dpc and then in the mesonephric and metanephric mesenchyme as they differentiate alongside the Wolffian duct (Sanchez et al., 1996; Xu et al., 1999; Fig. 2E,K). According to our model, Gdnf expression and/or Gdnf-expressing cells are normally lost from the region between somites 16 and 25 in the mouse so that, by 10.5 dpc, expression is only seen in the metanephric mesenchyme around the region of the future ureter bud. The more anterior expression of Gdnf seen in Foxc1/Mf1 homozygous mutants (Fig. 4F) suggests that these mutants have defects in the mechanism(s) that normally downregulates Gdnf expression anterior to the region around somite 25. Possible mechanisms for the programmed suppression of Gdnf include the withdrawal of a positive factor normally inducing or maintaining gene expression, or the activation of a negative factor actively repressing Gdnf. If such mechanisms exist, then Foxc1/Mf1 might function upstream or downstream of the factor(s) normally regulating Gdnf expression in posterior mesonephric mesenchyme. In the above model, we have not distinguished between direct or indirect regulation of Gdnf. The finding that expression of Eya1 is also seen more anteriorly in Foxc1/Mf1 homozygous mutants than the wild type, raises the possibility Foxc1 negatively regulates Eya1 rather than Gdnf. Evidence that Eya1 is upstream of Gdnf comes from the recent finding that Gdnf is not detected in Eya1 mutant embryos, while regulation of Eya1 is not known (Xu et al., 1999).

A second, equally plausible, model is that the absence of mesonephric tubules between the levels of somites 16 and 25 in the mouse is normally the result of an active posterior migration or condensation of nephrogenic mesenchyme away from this region into the region of the future metanephric mesenchyme. According to this model, in Foxc1/Mf1 homozygous mutants the mesenchyme condenses in a posterior direction more slowly or incompletely than in wild type, leading to the more anterior and diffuse distribution of Gdnf-
expressing cells. In support of this hypothesis, abnormal condensation of skeletogenic mesenchyme has been reported in congenital hydrocephalus homozygotes (Gruneberg, 1953; Kume et al., 1998).

Fig. 4. An ectopic ureteric bud and extra mesonephric tubules in Foxc1ch homozygous embryos. Anterior is to the left in A-H. (A-D) Whole-mount in situ hybridization showing c-ret expression in the Wolffian duct (w) and the outgrowth of the ureteric bud (arrows). The Wolffian duct is kinked and additional small bud (arrowhead) is seen in the mutant embryo. (C,D) At 11.0 dpc, the ureteric bud (arrow) has formed as a distinct shape in the wild type (C), while an ectopic ureteric bud (arrowhead) is seen more anteriorly to the normal bud in the mutant embryo (D). (E,F) Sagittal sections showing Gdnf expression at 10.5 dpc. Gdnf is strongly expressed in the metanephric mesenchyme (m) in the wild type (E), while Gdnf expression is extended more anteriorly (arrow) in mutant embryo (F). (G,H) Whole-mount in situ hybridization showing lim1 expression in the Wolffian duct (w) and mesonephric tubules (arrowhead) at 10.5 dpc. Approximate positions of the ureteric bud are indicated by yellow arrows. More mesonephric tubules (arrowheads) are distributed caudally in mutant embryo (H) compared to the wild type (G). (I) Ventral view of embryos at 8.0 dpc showing lim1 expression in the intermediate mesoderm. Arrows indicate expression in the node. Abbreviation: g, gut; u, ureteric bud. Scale bar: E,F, 100 μm.

Fig. 5. Abnormal development of the intermediate mesoderm in compound heterozygotes for Foxc1lacZ and Foxc2tm1. (A,B) Newborn wild-type and compound heterozygous mutant kidneys. (A) Compound heterozygote showing a hypoplastic kidney on the left and hydronephrosis and hydrourerter (asterisk) on the right. (B) Compound heterozygote showing hydrourerter (asterisk) on the left and renal agenesis (arrowhead) on the right. (C-H) Whole-mount in situ hybridization using a c-ret probe (C-F) and a lim1 probe (G,H). Anterior to the left. (C,D) c-ret expression showing the Wolffian duct (w) and the ureteric bud (arrow) at 10.5 dpc. While the wild-type embryo has a single distinct bud growing out from the Wolffian duct (C), the bud is much broader in compound heterozygous embryo (D). (E,F) Compound heterozygous embryos showing an ectopic anterior ureteric bud (arrowhead) in addition to the normal bud (arrow) at 11.0 dpc. (G,H) lim1 expression showing the Wolffian duct (w) and the mesonephric tubules (arrowhead) at 10.5 dpc. Yellow arrows indicate approximate positions of the ureteric bud. More mesonephric tubules (arrowhead) are distributed caudally in mutant embryo (H) compared to the wild type (G). (I) Section selected from serial sections showing the abnormal position of the hydrourerter in newborn male compound heterozygote. While the normal ureter (asterisk) connects to the bladder (b), the hydrourerter (arrow) aberrantly connects to a derivative of the Wolffian duct (arrowhead). Scale bar: I, 400 μm.

A third more speculative model that we have considered is that, during gastrulation, Foxc1/Mf1 plays a role in regulating cell fate along the dorsoventral axis, with high levels promoting dorsal, paraxial mesoderm fates (somites) and lower levels promoting lateral plate or ventral mesoderm (Fig. 2C). The size and extent
of the initial intermediate mesoderm domain may then depend on the counteracting action of dorsalizing factors (mediated in part by the action of Foxc1/Mf1) and ventralizing factors such as Bmp4, which are expressed at higher levels ventrally (Fig. 2C,F). According to this model, Foxc1/Mf1 mutant embryos may have an expanded domain of intermediate mesoderm and this may ultimately result in an expanded domain of Gdnf expression. However, we observed no obvious change of spatial expression of lim1, an early marker for the intermediate mesoderm, in Foxc1/Mf1/− mutant embryos compared to heterozygotes at 8.0 dpc (Fig. 4L), arguing against this hypothesis.

Possible models for the role of Foxc1/Mf1 and Foxc2/Mfh1 in kidney development

Both Foxc1/Mf1 and Foxc2/Mfh1 are coexpressed in the developing mesonephric and metanephric kidneys (Fig. 2). We show here that compound heterozygotes of Foxc1/Mf1 and Foxc2/Mfh1 have hydroureter and hypoplastic kidney, while heterozygotes are normal on the same genetic background. Moreover, the penetrance of the renal abnormalities in the compound heterozygotes on the 129 × BlackSwiss background is higher than for the homozygous Foxc1 mutants. Although hydroureter in newborn compound heterozygotes was associated with single ureters (except in one case), an ectopic ureteric bud was observed in embryos. Moreover, histological analysis of newborn compound heterozygotes revealed that, in the female, the single hydroureter failed to connect to the bladder and ended blindly (data not shown), and that, in the male, the hydroureter either connected to the bladder more ventrally than normal or connected to a derivative of the Wolffian duct (Fig. 5I). These anatomical findings support the idea that the fluid accumulation in the ureter is a secondary effect due to the abnormal position of the urerter orifice. This, in turn, supports the idea that in the majority of double heterozygotes only the most anterior ectopic ureter bud continues to grow and is subsequently abnormally positioned in relation to the bladder while, in Foxc1/Mf1 homozygous mutants, both ureteric buds grow. At present, we have no simple explanation for the difference in phenotype (two ureters versus one) between homozygous Foxc1/Mf1 mutants and double heterozygotes. According to the models of Mackie and Stephens (1975), one possibility is that, in compound heterozygotes, the prospective metanephric mesenchyme proliferates and condenses posteriorly much more slowly or incompletely than in Foxc1/Mf1/− homozygotes, leading to the formation and continued growth of ureter buds anterior to their normal position. The poorly condensed and sparse nature of the nephrogenic mesenchyme might also account for the small size of the kidney that develops in response to signals from the anteriorly positioned bud. Further studies will be needed to explore this hypothesis and the reason for the different penetrance of the homozygous and compound heterozygous phenotypes on the same genetic background. Whatever the explanations, it is clear that compound heterozygotes have kidney abnormalities while single heterozygotes on the same genetic background do not. We therefore conclude that Foxc1/Mf1 and Foxc2/Mfh1 show a similar genetic interaction in the development of the kidney as in the development and remodelling of the aortic arch (Winnier et al., 1999) and the development of the anterior chamber of the eye (Smith et al., 2000).

We thank Drs Ikuni Ichikawa and Yoichi Miyazaki for helpful and stimulating discussions. We also thank Dr Maureen Gannon for critical reading of the manuscript. B. L. M. H. is an Investigator of the Howard Hughes Medical Institute.

REFERENCES


Kume, T., Deng, K. and Hogan, B. L. M. (2000). Minimal phenotype of mice
Forkhead genes in kidney and ureter development

homozygous for a null mutation in the forkhead/winged helix gene, Mf2.

Labbe, E., Silvestri, C., Hoodless, P. A., Wrana, J. L. and Attisano, L.

Labosky, P. A., Winnier, G. E., Jetton, T. L., Hargett, L., Ryan, A. K.,

Rosenfeld, M. G., Parlow, A. F. and Hogan, B. L. M. (1997). Duplex kidneys: a correlation of

Moore, M. W., Klein, R. D., Farinas, I., Sauer, H., Armanini, M., Phillips,

Nishimura, H., Yerkes, E., Hohenfellner, K., Miyazaki, Y., Ma, J., Hunley,

Sanchez, M. P., Silos-Santiago, I., Fri sen, J., He, B., Lira, S. A. and


Smith, R. S., Zabala, A., Kume, T., Savinova, O. V., Kidson, S. H.,


Sviderski, R. E., Reiter, R. S., Nishimura, D. Y., Alward, W. L., Kalenak,


Winnier, G. E., Kume, T., Deng, K., Rogers, R., Bundy, J., Raines, C.,


