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Murine forkhead/winged helix genes *Foxc1* (*Mf1*) and *Foxc2* (*Mfh1*) are required for the early organogenesis of the kidney and urinary tract

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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/Mf1*^{ch}), 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 hydroureter 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 *Foxc1*^{lacZ} *Foxc2*^{tm1} 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

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 hydroureter 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 hydroureter 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 hydroureter (Mackie and Stephens, 1975). If this hypothesis is correct, then abnormal or ectopic ureter budding is likely to

precede development of congenital hydroureter. Ectopic budding may, in turn, result from abnormalities in the nephrogenic mesenchyme, the tissue responsible for inducing the ureter bud. Recent studies have demonstrated that the induction and orientation of the ureteric bud involve glial cell-derived neurotropic factor (Gdnf) produced by the metanephric mesenchyme, and its receptors, c-ret and gfrα-1, expressed in the ureteric epithelium (for reviews, Rosenthal, 1999; Sariola and Sainio, 1997). However, the mechanisms controlling the expression of *Gdnf* (Sanchez et al., 1996; Xu et al., 1999) 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 forkhead/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 forkhead 2*) mutant mice have kidney and ureter abnormalities, including hydroureter, small kidneys and short ureters (Kume et al., 2000). However, very little is known about the roles of other forkhead transcription factors during kidney development.

Our laboratory and others have previously analyzed the *Foxc1/Mf1* (*Mesoderm/mesenchyme forkhead 1*) gene, which is expressed at high levels in many tissues including paraxial mesoderm, somites, prechondrogenic mesenchyme, periocular mesenchyme and the developing cardiovascular system (Hiemisch et al., 1998; Kidson et al., 1999; Kume et al., 1998; Swiderski et al., 1999; 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*, $Foxc1^{ch}$) or an engineered null mutation ($Foxc1^{lacZ}$) 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, $Foxc1^{ch}$ mutants have been reported to have kidney and ureter abnormalities, including hydronephrosis, hydroureter 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 forkhead 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;

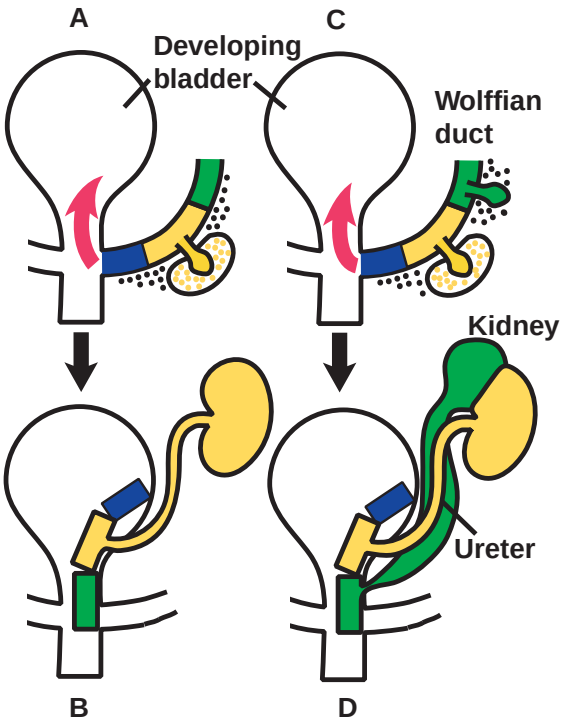

Fig. 1. Schematic representation of the model proposed by Mackie and Stephens (1975) for the development of duplex kidney and hydronephrosis in human infants. (A,B) Normal kidney development in which a single ureter bud is induced by and grows into the adjacent nephric mesenchyme (small dots). As development proceeds, the caudal end of the Wolffian duct (the common excretory duct) is incorporated into the developing bladder (red arrow). The region marked yellow gives rise to the trigone while the green and blue regions contribute to the urethra and lateral bladder, respectively. The ureter has its orifice within the trigone and urine flows normally. (C,D) It is hypothesized that, in some congenital abnormalities, an ectopic ureter bud is induced more anteriorly than normal (green region of Wolffian duct). Reciprocal interactions between the ectopic bud and the adjacent nephrogenic mesenchyme give rise to an ectopic kidney that fuses with the normal kidney, giving a duplex kidney. Ultimately, the ectopic ureter opens into the urethra and not the trigone, leading to abnormal outflow of urine and development of hydroureter. (Adapted from Fig. 5 of Mackie and Stephens, 1975).

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/Mf1*^{lacZ} and *Foxc2/Mfh1*^{tm1} (Kume et al., 1998; Winnier et al., 1997), were maintained by interbreeding on the 129 × Black Swiss genetic background. Genotyping was performed as described (Kume et al., 1998; Winnier et al., 1997). *Foxc1/Mf1*^{ch} heterozygous mice on the CHMU/Le inbred background were purchased from The Jackson Laboratory. The mutations in *Foxc1/Mf1*^{ch} and *Foxc1/Mf1*^{lacZ} 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*^{ch} 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′ primer (5′-TATGAGCGTGTACTCGCACCCT-3′) and ch-3′ primer (5′-CGTACCGTTCTCCGTCTTGATGTC-3′), followed by *Cac*8I enzyme digestion. The wild-type and *Foxc1/Mf1*^{ch} alleles give (178 and 197 bp) and 375 bp bands, respectively.

Histological analysis

Histological analysis was performed as described previously (Kume et al., 1998).

In situ hybridization

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 [α-^{35}S]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

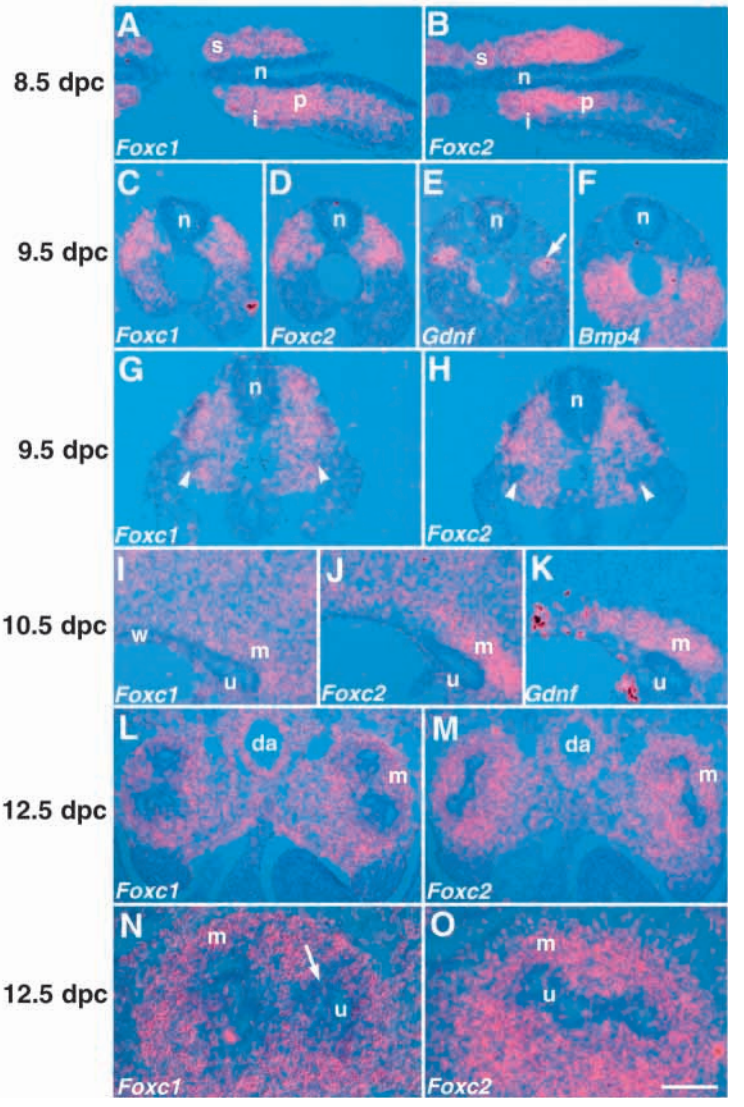

Fig. 2. Overlapping expression of *Foxc1/Mf1* and *Foxc2/Mfh1* in the developing kidney as revealed by section in situ hybridization. (A,B) Adjacent sections through the posterior region of 8.5 dpc embryo. Expression of both *Foxc1* (A) and *Foxc2* (B) are seen in the intermediate mesoderm (i), as well as the presomitic mesoderm (p) and somites (s). (C-F) Transverse sections at the level of the presomitic mesoderm of 9.5 dpc embryos. *Foxc1* shows a gradient expression pattern in the mesoderm with highest levels closest to the neural tube (C), while *Foxc2* is not expressed in the ventral region of the mesoderm (D). (E) *Gdnf* is expressed in the nephrogenic cord (arrow). (F) *Bmp4* is strongly expressed in the ventral mesoderm. (G,H) Transverse sections through the more anterior trunk of a 9.5 dpc embryo. Colocalization of *Foxc1* (G) and *Foxc2* (H) in the mesonephric mesenchyme alongside the Wolffian duct (arrowheads). (I-K) Sagittal sections showing the region of the metanephric mesenchyme at 10.5 dpc. Coexpression of *Foxc1* (I) and *Foxc2* (J) are seen in the metanephric mesenchyme. (K) *Gdnf* is localized to the metanephric mesenchyme (m). (L-O) Transverse sections showing the developing metanephric kidney at 12.5 dpc. Both *Foxc1* and *Foxc2* are expressed in the condensing mesenchyme, while *Foxc1* appears to be expressed at lower levels in the mesenchymal aggregates (L). (N,O) High magnifications of L and M, respectively. Abbreviations: da, dorsal aorta; n, neural tube; w, Wolffian duct. Scale bar: A-G 100 μm; G and H, 80 μm; I-K, 100μm; L and M, 140μm; N and O, 60 μm.

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. 2I). 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/Mf1^{ch} 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/Mf1^{ch}* and *Foxc1/Mf1^{lacZ}* 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 *Foxc1^{ch}* 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 *Foxc1^{ch}* 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 *Foxc1^{lacZ}* mutants showed the same kidney abnormalities, suggesting there are genetic modifiers affecting the penetrance of this phenotype.

Histological analysis of newborn *Foxc1/Mf1^{ch}* 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 hydroureter 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/Mf1^{ch}* 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/Mf1^{ch}* 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/Mf1^{ch}* homozygous mutants

We next asked whether the formation of the ureteric bud is abnormal in *Foxc1/Mf1^{ch}* 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 *Foxc1^{ch}* 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 *Foxc1^{ch}* 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^{ch}* mutants, we examined the expression of *Gdnf* at 10.5 dpc just prior to normal bud formation. As shown in Fig. 4E,F, *Gdnf* transcripts extend much more anteriorly in *Foxc1^{ch}* mutants compared to wild-type embryos. In addition, from analysis of serial sections, its expression appears to be more medial (data not shown). Taken together, these findings suggest that the ectopic anterior ureteric bud(s) result from anterior extension or persistence of the normal *Gdnf* expression domain.

Mutations of the human *EYA1* gene, a homologue of the *Drosophila eyes absent* (*eya*) gene, are associated with the dominant inherited disorder, branchio-oto-renal (BOR) syndrome in which very variable defects in kidney and urinary tract development are seen (Abdelhak et al., 1997). Recent experiments show that *Eya1* expression overlaps with that of *Gdnf* during kidney development and mice homozygous for a null mutation in *Eya1* lack the outgrowth of the ureteric bud, the same phenotype seen in *Gdnf* mutants. In addition, in *Eya1* homozygous mutant embryos, *Gdnf* is not detected in the metanephric mesenchyme, suggesting that *Eya1* controls the genetic regulatory cascade upstream of *Gdnf* (Xu et al., 1999). We therefore examined expression of *Eya1* and found that the *Eya1* domain also extended more anteriorly in *Foxc1/Mf1^{ch}* mutants compared to the wild type at 10.5 dpc (data not shown). This suggests that *Foxc1/Mf1* regulates either *Eya1* 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/Mf1^{ch}* 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 *Foxc1^{ch}* 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 *Foxc1^{ch}* homozygous embryos early in development, at the time when the dorsoventral domains of the mesoderm are being established (see Discussion). However, *Foxc1^{ch}* homozygotes showed no obvious difference of *lim1* expression, an early marker for the intermediate mesoderm, compared to *Foxc1^{ch}* heterozygotes at 8.0 dpc (Fig. 4I,J).

Kidney and ureter abnormalities in *Foxc1/Mf1^{lacZ}* and *Foxc2/Mfh1^{tm1}* 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/Mf1^{lacZ}* and *Foxc2/Mfh1^{tm1}* (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 hydroureter, 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 hydronephrosis (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).

Table 1. Kidney and urinary abnormalities in newborn single mutants and compound heterozygotes of *Foxc1^{lacZ}* and *Foxc2^{tm1}*¶

Genotype	*n*	Duplex kidney	Small kidney‖	Hydroureter	Double ureters
+/+*	5	0	0	0	0
+/+‡	6	0	0	0	0
*Foxc1$^{ch-/-}$**	17	15	0	15 (15)§	15
Foxc1$^{lacZ-/-}$‡	49	1	0	7 (1)§	1
Foxc2$^{tm1-/-}$‡	8	0	5	0	0
*Foxc1$^{ch+/-}$**	15	1	0	1 (1)§	1
Foxc1$^{lacZ+/-}$‡	7	0	0	0	0
Foxc2$^{tm1+/-}$‡	7	0	0	0	0
Foxc1$^{lacZ+/-}$; Foxc2$^{tm1+/-}$‡	19	1	7 (1)**	13 (5)§	1

*CHMU/Le×C57BL/6 genetic background.
‡129×Black Swiss genetic background.
¶Bilateral or unilateral traits.
§Hydroureter accompanied by hydronephrosis.
‖Less than 3/4 wild-type length.
**Renal agenesis.

Given the early phenotype of *Foxc1/Mf1^{ch}* 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 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 hydronephrosis 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

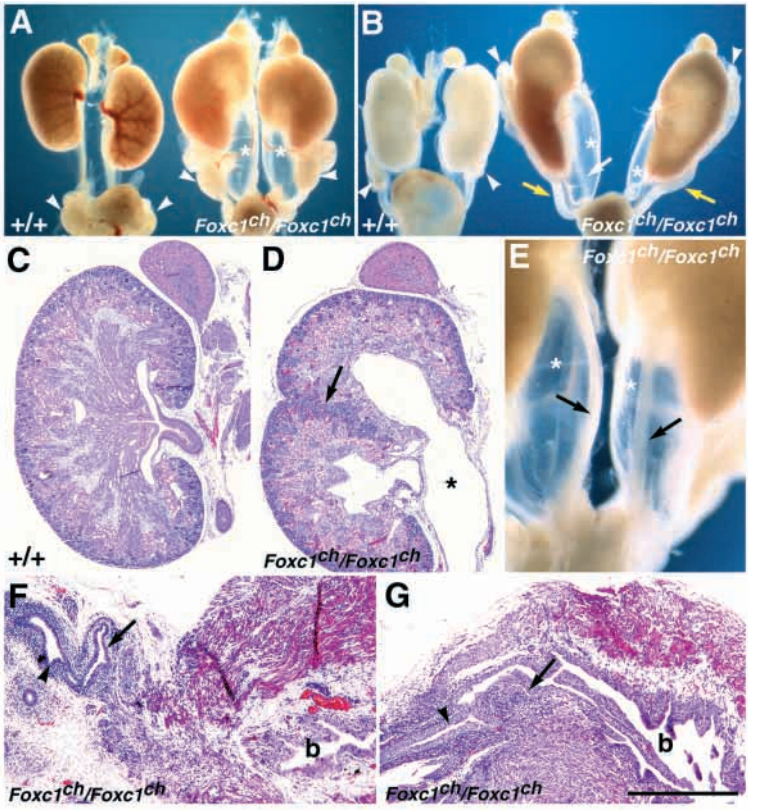

Fig. 3. Kidney and ureter abnormalities in newborn mice homozygous for *Foxc1*^{ch}. (A,B) Wild-type and mutant newborn kidneys. (A) Male mutant kidneys have hydroureters (asterisks). Note that mutant testes (arrowheads) are located more anteriorly compared to wild-type testes (arrowheads). (B) Female 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*^{ch} 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; F,G, 400 μm.

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*^{ch} and *Foxc1/Mf1*^{lacZ} 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*-

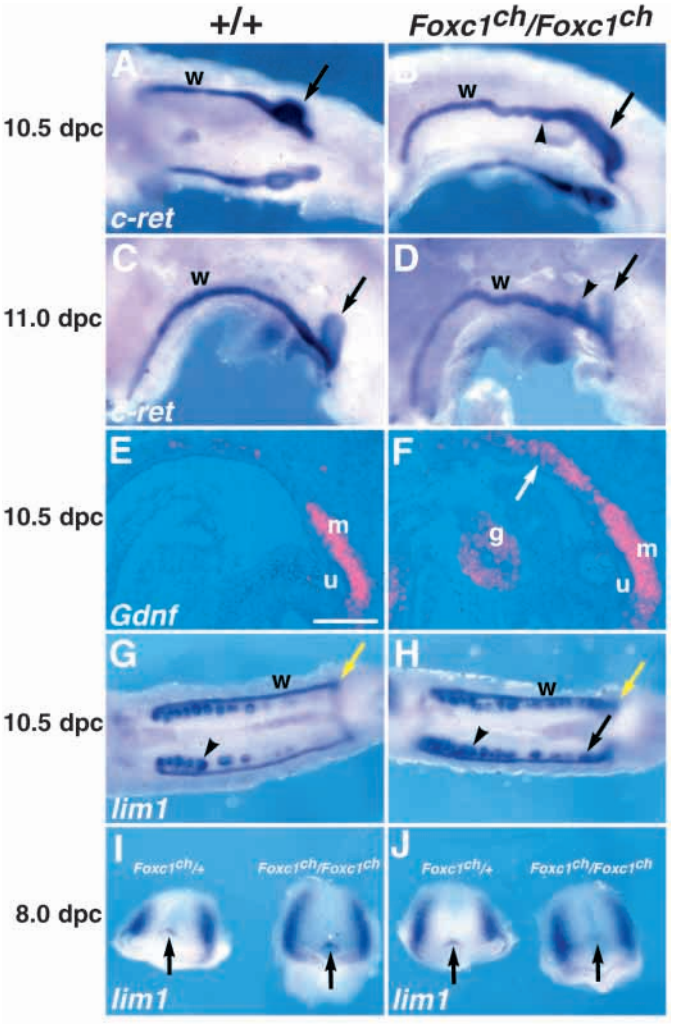

Fig. 4. An ectopic ureteric bud and extra mesonephric tubules in *Foxc1*^{ch} homozygous embryos. Anterior is to the left in A-H. (A-D) Whole-mount in situ hybridization showing *c-ret* expression in the Wolffian duct (w) and the outgrowth of the ureteric bud (arrows). (A,B) The outgrowth of the ureteric bud in the mutant embryo (B) is much broader than the wild type (A). 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,J) 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.

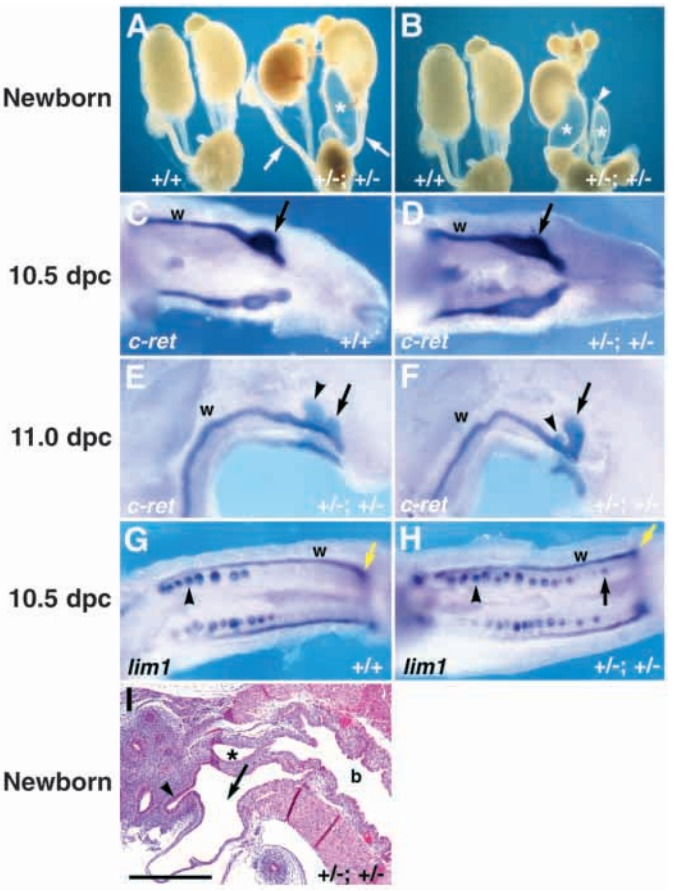

Fig. 5. Abnormal development of the intermediate mesoderm in compound heterozygotes for *Foxc1*^{lacZ} and *Foxc2*^{tm1}. (A,B) Newborn wild-type and compound heterozygous mutant kidneys. (A) Compound heterozygote showing a hypoplastic kidney on the left and hydronephrosis and hydroureter (asterisk) on the right. Arrows indicate the oviducts. (B) Compound heterozygote showing hydroureter (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 (arrow) are distributed caudally in compound heterozygous embryo (H) compared to the wild type (G). (I) Section selected from serial sections showing the abnormal position of the hydroureter in newborn male compound heterozygote. While the normal ureter (asterisk) connects to the bladder (b), the hydroureter (arrow) aberrantly connects to a derivative of the Wolffian duct (arrowhead). Scale bar: I, 400 μm.

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).

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^{ch}* mutant embryos compared to heterozygotes at 8.0 dpc (Fig. 4I,J), 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 ureter 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^{ch}* 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).

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