Development 130, 3085-3094

doi:10.1242/dev.00536

Six1 is required for the early organogenesis of mammalian kidney

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Accepted 4 April 2003

SUMMARY

The murine Six gene family, homologous to *Drosophila sine oculis* (*so*) which encodes a homeodomain transcription factor, is composed of six members (*Six1-6*). Among the six members, only the *Six2* gene has been previously shown to be expressed early in kidney development, but its function is unknown. We have recently found that the *Six1* gene is also expressed in the kidney. In the developing kidney, *Six1* is expressed in the uninduced metanephric mesenchyme at E10.5 and in the induced mesenchyme around the ureteric bud at E11.5. At E17.5 to P0, *Six1* expression became restricted to a subpopulation of collecting tubule epithelial cells. To study its in vivo function, we have recently generated *Six1* mutant mice. Loss of *Six1* leads to a failure of ureteric bud invasion into the mesenchyme and subsequent apoptosis of the mesenchyme. These results indicate that *Six1* plays an essential role in early kidney development. In *Six1*$^{-/-}$ kidney development, we have found that *Pax2, Six2* and *Sall1* expression was markedly reduced in the metanephric mesenchyme at E10.5, indicating that *Six1* is required for the expression of these genes in the metanephric mesenchyme. In contrast, *Eya1* expression was unaffected in *Six1*$^{-/-}$ metanephric mesenchyme at E10.5, indicating that *Eya1* may function upstream of *Six1*. Moreover, our results show that both *Eya1* and *Six1* expression in the metanephric mesenchyme is preserved in *Pax2*$^{-/-}$ embryos at E10.5, further indicating that *Pax2* functions downstream of *Eya1* and *Six1* in the metanephric mesenchyme. Thus, the epistatic relationship between Pax, Eya and Six genes in the metanephric mesenchyme during early kidney development is distinct from a genetic pathway elucidated in the *Drosophila* eye imaginal disc. Finally, our results show that *Eya1* and *Six1* genetically interact during mammalian kidney development, because most compound heterozygous embryos show hypoplastic kidneys. These analyses establish a role for *Six1* in the initial inductive step for metanephric development.

Key words: *Six1*, Kidney development, *Eya1*, *Pax2*, *Six2*, *Sall1*, Metanephric mesenchyme, Apoptosis, *Gdnf*, Mouse

INTRODUCTION

The development of permanent kidney starts at around embryonic day (E) 11 in the mouse from the metanephric mesenchyme and the ureteric bud, which both derive from the intermediate mesoderm via inductive interactions between both tissues (Lechner and Dressler, 1997; Kuure et al., 2000; Schedl and Hastie, 2000). The ureteric bud is an epithelial tube produced from the Wolffian duct and it invades the metanephric mesenchyme or blastema. Once the bud and the mesenchyme meet, a series of reciprocal inductive events take place; as a result, the ureteric bud grows and branches to form the urinary collecting system, and the mesenchyme proliferates and differentiates into nephrons. This interactive process continues until a mature kidney is formed. Although it is still unclear whether the metanephric mesenchyme initiates organogenesis by inducing the formation of the ureteric bud, or whether the initial signals derive from the Wolffian duct before budding the ureteric bud, recent genetic and molecular studies have indicated that the metanephric mesenchyme might be specified first and that a mesenchymal signal might promote ureteric bud formation (reviewed by Vainio and Lin, 2002). However, it remains unclear which genes determine the origin of the metanephric kidney and which actual molecules control the establishment of metanephric mesenchyme.

It has been shown that without the metanephric mesenchyme, neither the collecting system nor the nephrons can form (Ashley and Mostofi, 1960). Thus, the formation of a functional metanephric mesenchyme is required for normal renal development. Gene inactivation and in situ hybridization experiments have recently implicated several transcription factors in a role in mediating the formation of the metanephric mesenchyme. The *Foxc1* gene, which encodes a winged helix protein, has been shown to play a role in positioning the mesenchyme, because in *Foxc1*$^{-/-}$ mice, the metanephric mesenchymes form unusually far anteriorly, which causes the ureter to grow too anteriorly or to form more than one ureter (Kume et al., 2000). The homeobox gene *Lim1* is expressed in the intermediate mesoderm from its inception and has been shown to be required for all kidneys (Tsang et al., 2000). The paired box gene *Pax2* is expressed in the intermediate mesoderm from E8.5 and in the metanephric mesenchyme, Wolffian duct and ureteric bud at

E10.5 (Torres et al., 1995). *Pax2*$^{-/-}$ mice fail to form any kidneys and there is no ureteric bud, although the metanephric mesenchyme can be observed morphologically (Torres et al., 1995; Brophy et al., 2001). Recent studies have shown that the absence of *Pax2* causes *Gdnf* expression to be lost from the metanephric mesenchyme, and *Pax2* regulates *Gdnf* transcription in vitro (Brophy et al., 2001). The eyes absent 1 (*Eya1*) gene, which encodes a transcriptional coactivator, is only expressed in the metanephric mesenchyme and *Eya1*$^{-/-}$ mice show renal agenesis and their posterior intermediate mesoderm fails to produce Gdnf (Xu et al., 1999; Buller et al., 2001). *Sall1*, which encodes a zinc finger protein, is also expressed in the metanephric mesenchyme and *Sall1*$^{-/-}$ mice show the failure of tubule formation because of the incomplete ureteric bud outgrowth (Nishinakamura et al., 2001). The transcription factor Wt1 is first expressed in the metanephric mesenchyme before induction, and in *Wt1*-knockout mice the ureteric bud fails to grow out of the Wolffian duct and the metanephric mesenchyme subsequently apoptoses, leading to a complete failure of kidney development (Kreidberg et al., 1993). However, how these regulatory genes function and whether they interact during early metanephric induction is unclear. In addition, the molecular pathway controlling the formation of metanephric mesenchyme is not established.

The glial-derived neurotrophic factor (Gdnf) has been identified as a mesenchyme-derived signal that acts on the receptor tyrosine kinase (Ret) and Gfrα1 coreceptor which are distributed in the ureteric epithelium and induces it to produce a ureteric bud which invades the metanephric mesenchyme (Sainio et al., 1997; Saarma and Sariola, 1999). Indeed, the null mutants of *Gdnf*, *c-Ret* and *Gfrα1* show similar perturbation of ureteric bud outgrowth (Schuchardt et al., 1994; Moore et al., 1996; Pichel et al., 1996; Sanchez et al., 1996; Cacalano et al., 1998). Despite the importance of Gdnf and its receptors c-Ret and Gfrα1 as inductive signals in early kidney morphogenesis, exactly how this signal transduction pathway regulates the development of the ureteric bud and the mechanisms controlling the expression of *Gdnf* in the mesenchyme are not well understood.

The *Six1* gene is homologous to *Drosophila sine oculis* (*so*) gene, an early regulator for *Drosophila* eye formation (Cheyette et al., 1994; Serikaku and O'Tousa, 1994). In *Drosophila*, *so* functions synergistically with the fly *Pax6* gene *eyeless* (*ey*), *eyes absent* (*eya*) and *dachshund* (*dac*) to regulate the eye morphogenesis (reviewed by Treisman, 1999). The mammalian Six gene family consists of six members (*Six1-6*) which share two highly conserved domains, a homeodomain (HD) and a specific Six-domain (SD) crucial for protein-protein interaction (Kawakami et al., 1996; Chen et al., 1997; Pignoni et al., 1997). Besides the eye, the Six genes are widely coexpressed with Pax, Eya and Dach (the mammalian *Dachshund*) genes in many tissues during mammalian organogenesis, suggesting possible interaction between their gene products and the existence of a conserved Pax-Eya-Six regulatory hierarchy (Oliver et al., 1995a; Oliver et al., 1995b; Xu et al., 1997a; Xu et al., 1997b; Xu et al., 1999; Xu et al., 2002). In early mammalian kidney development, *Six2* is expressed in the metanephric mesenchyme before and after induction of kidney organogenesis and its expression in the metanephric mesenchyme is *Eya1*-dependent (Xu et al., 1999). Similarly, we have recently found that *Six1* is also expressed in the metanephric mesenchyme before and after induction. However, the function of Six genes during kidney development has not been established.

We have recently generated *Six1* null mutant mice and the mice die at birth because of malformations in several organs (Xu et al., 2002; Laclef et al., 2003). We have now examined the role of *Six1* during early kidney development. *Six1* is expressed in the uninduced and induced metanephric mesenchyme and *Six1*$^{-/-}$ embryos lack kidneys because of a failure of metanephric induction. Our analyses show that the epistatic relationship between Pax, Eya and Six in the metanephric mesenchyme during early kidney development is distinct from a genetic pathway elucidated in the *Drosophila* eye imaginal disc. Furthermore, our results show that *Six1* is also required for the expression of *Six2* and *Sall1* in the metanephric mesenchyme. These analyses indicate that *Pax2*, *Eya1*, *Six1*, *Six2* and *Sall1* function in a molecular and genetic pathway during early kidney development, suggesting a role for *Six1* in the establishment of the inductive capacity of the metanephric mesenchyme.

MATERIALS AND METHODS

Animals and genotyping

The *Six1* null mutant allele was created by replacement of the endogenous start codon as well as the exon 1 with a promoterless *E. coli* ATG-*lacZ*-poly(A) cassette and the *PGK-neo* gene (Laclef et al., 2003). Mutant mice carrying *Six1* mutant allele, *Six1*$^{\mathrm{lacZ}}$, were obtained using gene targeting technology. *Eya1*/*Six1* double heterozygous mutant mice were generated by crossing mice carrying mutant alleles of *Eya1* and *Six1* (*Six1*$^{\mathrm{lacZ}}$). Mice heterozygous for a targeted disruption of the *Eya1* or *Pax2* gene were intercrossed to produce embryos of all three possible genotypes, respectively.

Genotyping of mice and embryos was performed as described (Torres et al., 1995; Xu et al., 1999; Xu et al., 2002).

Phenotype analyses and in situ hybridization

Embryos for histology and in situ hybridization were dissected out in PBS and fixed with 4% paraformaldehyde at 4°C overnight. Embryonic membranes were saved in DNA isolation buffer for genotyping. Histology was performed as described (Xu et al., 1999). To visualize *Six1*$^{\mathrm{lacZ}}$ expression, mutant embryos were stained with X-gal and sectioned as described (Xu et al., 2002).

For in situ hybridization, we used four wild type or mutant embryos at each stage for each probe as described (Xu et al., 1997a).

TUNEL analysis

We performed TUNEL assay for detecting apoptotic cell death using the ApopTag detection kit (Intergen). We used six wild type or mutant embryos for this assay.

RESULTS

Six1 is required for kidney development

Six1 is strongly expressed in the metanephric mesenchyme and its expression was not detected in the Wolffian duct or the ureteric bud epithelium at E10.5 (Fig. 1A). To further confirm our observation, we next determined the expression of *Six1* using X-gal staining for *Six1*$^{\mathrm{lacZ}}$. X-gal staining of heterozygous *Six1*$^{\mathrm{lacZ}}$ embryos at E10.5 recapitulated the *Six1*-

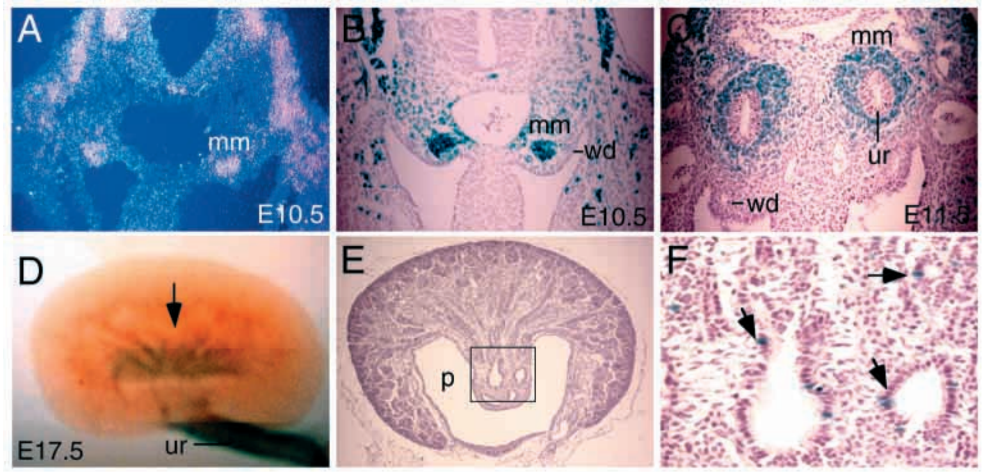

Fig. 1. Expression of *Six1* during kidney development analyzed by in situ and X-gal staining of heterozygous *Six1*^{lacZ} embryos for the *Six1*^{lacZ} allele. (A) In situ hybridization showing *Six1* expression in metanephric mesenchyme (mm) at E10.5. (B) X-gal staining of *Six1*^{lacZ} heterozygous embryos showing strong *Six1*^{lacZ} expression at E10.5, similar to that observed by in situ hybridization (A). (C) At E11.5, *Six1* is expressed in the induced mesenchyme (mm) around the ureteric bud epithelium (ur). (D) X-gal staining of E17.5 *Six1*^{lacZ} heterozygous kidneys showing *Six1*^{lacZ} expression in collecting tubules (arrow). *Six1*^{lacZ} is also expressed in the muscles surrounding the ureter (ur). (E,F) Transverse sections of X-gal-stained *Six1*^{lacZ} heterozygous kidneys at E17.5 revealed that the *Six1*^{lacZ}-expressing cells are localized in the collecting tubules (arrows). F is higher magnification of the boxed area in E. p, renal pelvis; wd, Wolffian duct.

expression pattern obtained by RNA in situ hybridization studies (Fig. 1B). At E11.5, strong X-gal staining was observed in the induced mesenchyme around the ureteric bud (Fig. 1C). From E13.5 to P0, *Six1* expression in the developing kidney was observed in the metanephric tubules as detected by X-gal staining (Fig. 1D and data not shown). Histological sections of X-gal stained E17.5 kidneys revealed that the *lacZ*-positive cells are localized in the collecting tubules (Fig. 1E,F). To study whether *Six1* plays any role during the formation of kidney, we next examined the kidney development in *Six1*$^{-/-}$ mice. Among the 40 *Six1*$^{-/-}$ (*Six1*$^{lacZ/lacZ}$) newborn mice analyzed so far, 39 animals showed renal agenesis (97.5%). Only one animal showed severely hypoplastic or dysplastic kidney rudiments on both sides (data not shown). Other organs that are derived from the embryonic urogenital intermediate mesoderm, including the pro- and mesonephros, the adrenal glands and the genital tracts, appeared normal (Fig. 2A-D and data not shown). Histological examination of *Six1*$^{-/-}$ mice at E12.5, when metanephric kidney forms, demonstrated the presence of a Wolffian duct and sometime the ureter-like structure (Fig. 2B,D).

To determine whether *Six1* plays a direct role in early metanephric induction, we next analyzed the kidney development in *Six1*$^{-/-}$ embryos at E10.5-11.5. At E11.5, the ureteric bud invades into the metanephric mesenchyme (Fig. 2E) and subsequent reciprocal interaction between these two

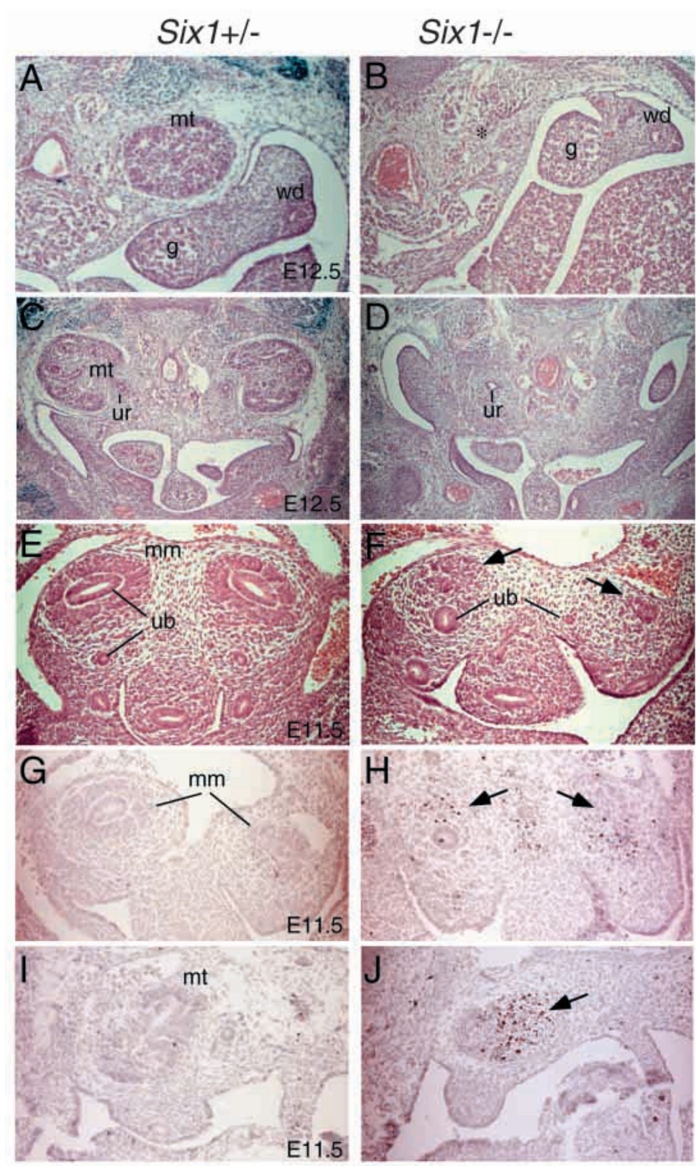

Fig. 2. Kidney development in *Six1*-deficient mice. (A-D) Metanephros (mt) in *Six1*$^{+/-}$ (A,C) and *Six1*$^{-/-}$ (B,D) mice at E12.5. Kidneys are absent in *Six1*$^{-/-}$ mice (*), but ureter (ur) was observed in the left primordium (D). The genital tracts appeared to be normal both in males and females. (E) In *Six1*$^{+/-}$ embryos of E11.5, ureteric bud (ub) grows out from Wolffian duct and invades the mesenchyme and the metanephric mesenchyme (mm) are condensed around the bulging ureteric bud. (F) In *Six1*$^{-/-}$ embryos, although the ureteric buds grow out from Wolffian ducts, they fail to invade the mesenchyme (arrows) completely. (G-J) TUNEL analysis of metanephros in *Six1*$^{+/-}$ and *Six1*$^{-/-}$ embryos at E11.5. Note the apoptotic cells were markedly increased in *Six1*$^{-/-}$ metanephric mesenchyme (arrows). g, gonad; wd, Wolffian duct.

tissues leads to the development of a metanephric kidney. In *Six1*$^{-/-}$ embryos, the metanephric mesenchyme morphologically distinct from the surrounding mesenchyme has formed, albeit reduced in size (Fig. 2F). The ureteric bud also formed but failed to invade the metanephric mesenchyme completely in *Six1*$^{-/-}$ embryos at E11.5 (Fig. 2F). Subsequent mesenchymal condensation and ureteric branching within the mesenchyme did not occur on either side (100%, *n*=20). By TUNEL analysis, apoptotic cells were increased in the mesenchyme of *Six1*$^{-/-}$ embryos at E11.5 (Fig. 2G-J). Thus, loss of *Six1* leads to a failure of ureteric bud invasion into the mesenchyme and subsequent apoptosis of the mesenchyme. These results indicate that *Six1* plays an essential role during early kidney morphogenesis.

Six1 is required for the expression of *Pax2* and *Six2* in the metanephric mesenchyme

To determine the molecular defects in early kidney development of *Six1*$^{-/-}$ animals, we first examined whether the expression of the Pax and Eya gene families depends upon *Six1*. Studies in *Drosophila* indicate that *eya* is epistatic to *so* and both genes reside within the same genetic and molecular pathway downstream of the *Pax6* gene *ey* (Halder et al., 1998). In the kidney, *Pax2*, *Eya1* and *Six1* expression overlaps in the metanephric mesenchyme and all three mutants lack kidney formation (Torres et al., 1995; Xu et al., 1999). To determine whether the *Drosophila* Pax-Eya-Six regulatory hierarchy is conserved during mammalian kidney development, we analyzed whether the expression of *Pax2* or *Eya1* is *Six1*-independent. The paired box gene *Pax2* is normally expressed in the intermediate mesoderm before the formation of metanephric mesenchyme, in uninduced and induced metanephric mesenchyme, Wolffian duct and ureteric epithelium (Torres et al., 1995; Brophy et al., 2001). In *Six1*$^{-/-}$ embryos, no significant difference of *Pax2* expression in the intermediate mesoderm, Wolffian duct and ureteric epithelium was observed at E9.0-10.5 (Fig. 3A-D). However, *Pax2* expression was absent from *Six1*$^{-/-}$ metanephric mesenchyme at E10.5 (arrows, Fig. 3D). *Eya1* is normally expressed in the metanephric mesenchyme before and after induction (Xu et al., 1999). In *Six1*$^{-/-}$ embryos at E10.5, the expression of *Eya1* in the metanephric mesenchyme was observed at normal levels (Fig. 3E,F). Because recent studies demonstrated that *Pax2* expression in the uninduced mesenchyme is independent of induction by the ureteric bud (Brophy et al., 2001), these results indicate that *Six1* is required for the expression of *Pax2*, but not *Eya1* in the metanephric mesenchyme before induction.

Six2, another member of the Six gene family, is also expressed in the uninduced and induced metanephric mesenchyme (Fig. 3G), and its expression was unaffected in the mesenchyme of *Pax2*$^{-/-}$ embryos (Torres et al., 1995). To address whether *Six2* functions redundantly with *Six1* in the mesenchyme during early kidney development, we analyzed the expression of *Six2* in *Six1*$^{-/-}$ metanephric mesenchyme at E10.5-11.5. Interestingly, the expression of *Six2* in the metanephric mesenchyme was markedly reduced in *Six1*$^{-/-}$ embryos at E10.5-11.5 (Fig. 3H), indicating that *Six1* is required for normal expression of *Six2* in the metanephric mesenchyme during early kidney development.

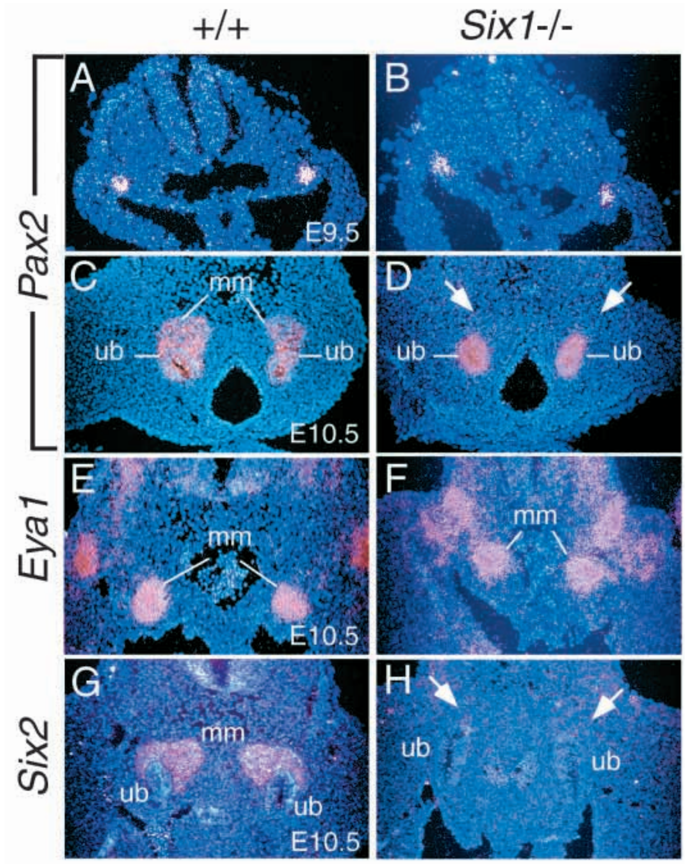

Fig. 3. *Six1* is required for the expression of *Pax2* and *Six2* but not *Eya1* in the metanephric mesenchyme (mm) at E10.5. (A-D) *Pax2* is normally expressed in the intermediate mesoderm (A,B), the metanephric mesenchyme before and after induction and in the ureteric epithelium (ub). In *Six1*$^{-/-}$ embryos, however, its expression in the metanephric mesenchyme at E10.5 is undetectable (arrows in D). (E,F) *Eya1* is expressed in the metanephric mesenchyme before and after induction and its expression is not affected in *Six1*$^{-/-}$ mesenchyme at E10.5. (G,H) *Six2* is also expressed in the metanephric mesenchyme before and after induction and its expression is undetectable in *Six1*$^{-/-}$ mesenchyme at E10.5 (arrows).

Six1 is also required for the expression of *Sall1* in the metanephric mesenchyme

We next analyzed the expression of several other well-characterized molecular markers in metanephric mesenchyme at E10.5 and 11.5. *Bmp4*, a member of the *Tgfβ* superfamily of secreted signals, is expressed in the mesenchymal cells surrounding the Wolffian duct and ureteric stalk (Fig. 4A) and has been implicated in regulating ureteric bud growth and branching (Miyazaki et al., 2000; Raatikainen-Ahokas et al., 2000). *Bmp4*$^{+/-}$ mutant mice show kidney defects that are caused by the misregulated development of the ureteric bud (Miyazaki et al., 2000). Bmp4 protein has also been shown to regulate genes that are expressed by both the ureteric bud and the mesenchyme, including *Gdnf* in organ culture (Miyazaki et al., 2000; Raatikainen-Ahokas et al., 2000). No significant difference of *Bmp4* expression was observed between wild type and *Six1*$^{-/-}$ mesenchyme at E10.5 (Fig. 4A,B), indicating that *Six1* is not required for the expression of *Bmp4* during early

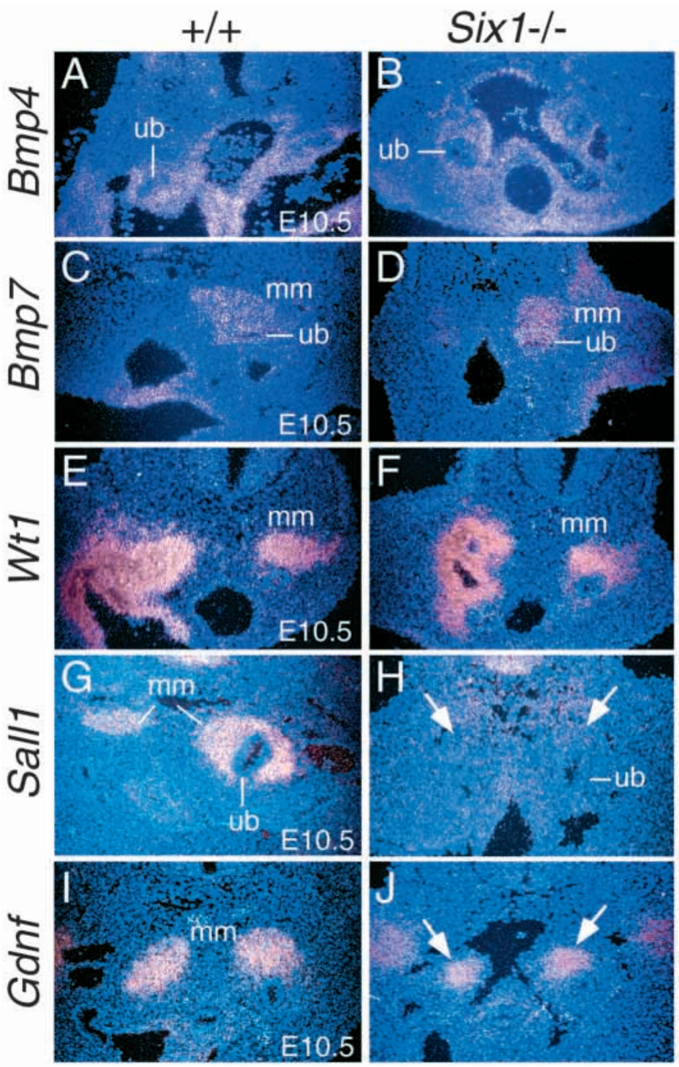

Fig. 4. *Six1* is required for the expression of *Sall1* in the metanephric mesenchyme (mm). (A,B) *Bmp4* is normally expressed in the mesenchyme around the ureteric stalk (ub) and its expression was not affected in *Six1*$^{-/-}$ embryos at E10.5. (C,D) *Bmp7* is normally expressed in the ureteric bud (ub) and metanephric mesenchyme at E10.5 and its expression levels are normal in both structures in *Six1*$^{-/-}$ embryos at E10.5. However, its expression domain is reduced in size in *Six1*$^{-/-}$ metanephric mesenchyme (D). (E,F) *Wt1* is widely expressed in the mesenchyme and urogenital ridge region during kidney development and its expression level is not affected in *Six1*$^{-/-}$ embryos at E10.5. However, its expression domain in the metanephric mesenchyme also appears to be reduced in size in E10.5 *Six1*$^{-/-}$ embryos. (G,H) *Sall1* is expressed in the metanephric mesenchyme before and after induction, however its expression was undetectable in the mesenchyme in *Six1*$^{-/-}$ embryos at E10.5 (arrows). (I,J) *Gdnf* is expressed in the metanephric mesenchyme and its expression levels are normal in *Six1*$^{-/-}$ embryos at E10.5. However, its expression domain appears to be reduced in size in *Six1*$^{-/-}$ embryos at E10.5 (arrows).

kidney development. *Bmp7*, another member of the *Tgfβ* superfamily, has been proposed to function as a survival signal that prevents mesenchymal cells from undergoing apoptosis during kidney development (Dudley et al., 1999; Reddi, 2000; Al-Awqati and Oliver, 2002). *Bmp7* is normally expressed in the metanephric mesenchyme and ureteric epithelium and its expression level was unaffected in both structures in *Six1*$^{-/-}$ embryos at E10.5 (Fig. 4C,D). However, its expression domain in *Six1*$^{-/-}$ metanephric mesenchyme is reduced in size (Fig. 4D). *Wt1* is expressed in the metanephric mesenchyme and its absence leads to failure of mesenchymal induction (Kreidberg et al., 1993). In E10.5 *Six1*$^{-/-}$ embryos, although the expression level of *Wt1* in the mesenchyme is normal, its expression domain became smaller than that in wild-type embryos (Fig. 4E,F). *Sall1*, which encodes a zinc finger protein, is expressed in the kidney mesenchyme (Fig. 4G) and its inactivation in mice leads to incomplete ureteric bud growth and failure of tubule formation (Nishinakamura et al., 2001), similar to that seen in *Six1*$^{-/-}$ animals. Interestingly, *Sall1* expression in *Six1*$^{-/-}$ metanephric mesenchyme was reduced to background level at E10.5-11.5 (arrows, Fig. 4H), indicating that *Sall1* expression in the mesenchyme is *Six1*-dependent.

Gdnf has been shown to act as a mesenchymal signal regulating ureteric bud outgrowth through its receptors c-Ret and Gfrα1 expressed in the ureteric epithelium (Vega et al., 1996; Sainio et al., 1997). The null embryos of *Gdnf* lack induction of the ureteric bud, resulting in the complete absence of the metanephric kidney, and Gdnf-soaked beads can ectopically induce budding of the ureter from the Wolffian duct (Pichel et al., 1996; Sainio et al., 1997). No significant difference of *Gdnf* expression level in the metanephric mesenchyme was observed between wild type and *Six1*$^{-/-}$ embryos at E10.5 (Fig. 4I,J). However, its expression domain is also reduced in size when compared to that in wild-type embryos (arrows in Fig. 4J). This result is consistent with the observation that the ureteric bud outgrows from Wolffian duct, but fails to invade mesenchyme completely in *Six1*$^{-/-}$ embryos (Fig. 2). In summary, our results show that *Six1* is required for the expression of *Pax2*, *Six2* and *Sall1* in the mesenchyme at E10.5-11.5. In addition, our data show that *Six1* inactivation led to size reduction of *Bmp7*, *Wt1* and *Gdnf* expression domains in the mesenchyme.

Our results also show that both *Pax2* and *Bmp7* expression in the ureteric epithelium was unaffected in *Six1*$^{-/-}$ embryos (Fig. 3C,D and Fig. 4C,D). To determine whether the failure of kidney development in *Six1*$^{-/-}$ mice is also caused by a defect in the ureteric epithelium, we next examined several other epithelial factors that are known to be important for early kidney formation, including c-Ret, Gfrα1 and Lim1. Our results show that the expression of these markers in the ureteric bud epithelium was also unaffected in the absence of *Six1* (data not shown).

Eya1, *Six1* and *Sall1* expression in the metanephric mesenchyme is *Pax2*-independent

To further clarify the genetic relationship between Pax, Eya, Six and *Sall1* in the metanephric mesenchyme during early kidney development, we next examined the expression of *Sall1*, *Eya1* and *Six1* in *Pax2*$^{-/-}$ embryos. *Pax2* mutant mice do not have a ureteric bud, however the metanephric mesenchyme can be observed morphologically (Torres et al., 1995; Brophy et al., 2001). As shown in Fig. 5, the expression levels of all three genes in the metanephric mesenchyme were unaffected in *Pax2*$^{-/-}$ embryos at E10.5. This result is consistent with previous observation that *Six2* expression was also unaffected in *Pax2*$^{-/-}$ mesenchyme at E10.5 (Torres et al., 1995). In addition, similar to the expression of *Six2* in *Eya1*$^{-/-}$ embryos at E10.5 (Xu et al., 1999), *Six1* expression was also reduced to background level in *Eya1*$^{-/-}$ mesenchyme at E10.5 (Fig. 5G,H). These results together with previous observations further indicate that *Eya1*, *Six1* and *Six2* function upstream of *Pax2* in the metanephric mesenchyme during early kidney development. Therefore, the genetic relationship between these genes in the metanephric mesenchyme before induction differs from that observed in *Drosophila* eye imaginal disc.

Eya1 and *Six1* genetically interact during kidney development

Because *Eya1* and *Six1* expression overlaps in the metanephric mesenchyme in the developing kidney and *Eya1* and *Six1* physically interact in vitro and in cultured cells (Buller et al., 2001), to further test whether these genes interact in a molecular pathway during mammalian kidney development, we examined the kidneys of newborn compound heterozygotes of *Eya1*$^{+/-}$/*Six1*$^{+/-}$ (Table 1 and Fig. 6). On 129 background, 15 of 21 (15/21) compound heterozygous mice had smaller than normal kidneys (Table 1 and Fig. 6A). Hypoplastic kidneys were either unilateral (6/15) or bilateral (9/15). In severe cases, complete absence of the kidney (agenesis) was observed (28.6%). Similar observation was obtained in C57BL6 background (Table 1). Renal agenesis was either unilateral or bilateral (Table 1 and Fig. 6B-D). In some compound heterozygous animals that show renal agenesis, ureters that end blindly were observed (arrow, Fig. 6B,C). In contrast, each single heterozygote alone had no or mild kidney abnormalities (Table 1). These data suggest that there is a genetic interaction between *Eya1* and *Six1* during mammalian kidney development.

To analyze the developmental basis of the renal hypoplasia associated with *Eya1*$^{+/-}$/*Six1*$^{+/-}$ heterozygotes, we compared histological sections of *Eya1*$^{+/-}$/*Six1*$^{+/-}$ heterozygous and control kidneys at various stages. Transverse or longitudinal sections of E17.5 *Eya1*$^{+/-}$/*Six1*$^{+/-}$ hypoplastic kidneys confirmed that the volume of the renal parenchyma is reduced and there are fewer nephrons, but that normal developing structures are present (Fig. 6E,F). In *Six1*$^{+/-}$ kidneys, the differentiating metanephric cap tissues (vesicles) in the peripheral nephrogenic zone, in which ureteric bud branching and induction of new nephrons takes place, are morphologically apparent (arrow, lower panel of Fig. 6E). However, in the hypoplastic *Eya1*$^{+/-}$/*Six1*$^{+/-}$ kidneys, the differentiating metanephric vesicles in the peripheral nephrogenic zone were largely reduced in number (arrow, lower panel of Fig. 6F). Therefore, the reduction of nephrons in the hypoplastic kidneys may result from a reduced induction between the ureteric bud and the metanephric cap tissue in the

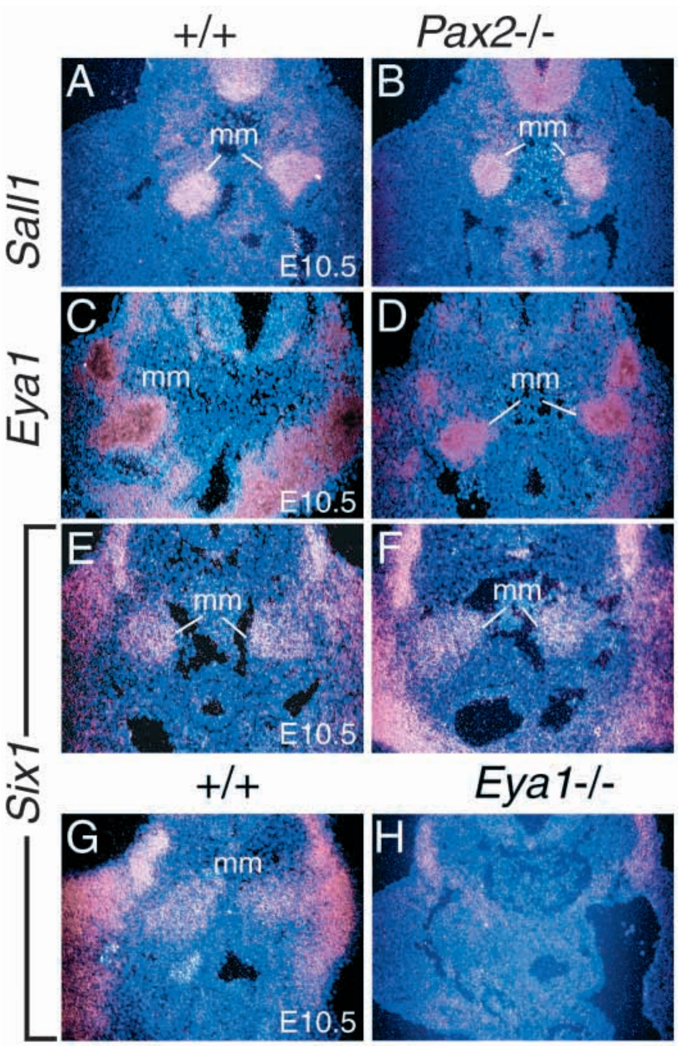

Fig. 5. *Sall1*, *Eya1* and *Six1* expression is not affected in *Pax2*$^{-/-}$ metanephric mesenchyme (mm). (A,B) *Sall1* is expressed in the metanephric mesenchyme and its expression is not affected in *Pax2*$^{-/-}$ mesenchyme at E10.5. (C,D) *Eya1* is expressed in the metanephric mesenchyme and its expression level is unaffected in *Pax2*$^{-/-}$ embryos at E10.5. (E,F) *Six1* is expressed in the metanephric mesenchyme and its expression level is also unaffected in *Pax2*$^{-/-}$ embryos at E10.5. (G,H) However, *Six1* expression in *Eya1*$^{-/-}$ mesenchyme is significantly reduced when compared to that in wild-type embryos at E10.5. Its expression in the limb bud and somites is also significantly reduced. Six homozygous embryos were used for each probe.

Table 1. Kidney abnormalities in newborn compound heterozygotes of *Eya1* and *Six1*

Genotype	*n*	Small kidney	No kidney
Wild type 129	13	0	0
Eya1$^{+/-}$ 129	17	3*	0
Six1$^{+/-}$ 129	19	2†	One bilateral
Eya1$^{+/-}$/*Six1*$^{+/-}$ 129	21	15‡	Five bilateral, one unilateral
Wild type C57BL6	16	0	One unilateral
Eya1$^{+/-}$ C57BL6	13	1§	0
Six1$^{+/-}$ C57BL6	12	0	0
Eya1$^{+/-}$/*Six1*$^{+/-}$ C57BL6	14	10¶	Four unilateral

n, number of animals

*Three *Eya1*$^{+/-}$ 129 animals showed unilaterally smaller kidney with reduction of ~10% in weight.

†Two *Six1*$^{+/-}$ 129 animals showed smaller kidneys on one side, with reduction of ~10-15% in weight.

‡Fifteen *Eya1*/*Six1* 129 compound heterozygotes showed small kidneys either bilaterally (*n*=9) or unilaterally (*n*=6), with reduction of ~75-22% in weight.

§One *Eya1*$^{+/-}$ C57BL6 animal showed smaller kidney on one side, with reduction of ~15% in weight.

¶Ten *Eya1*/*Six1* C57BL6 compound heterozygotes showed small kidneys either unilaterally (*n*=6) or bilaterally (*n*=4). Weight reduction was from 20 to 30%, less severe than that observed in 129 background.

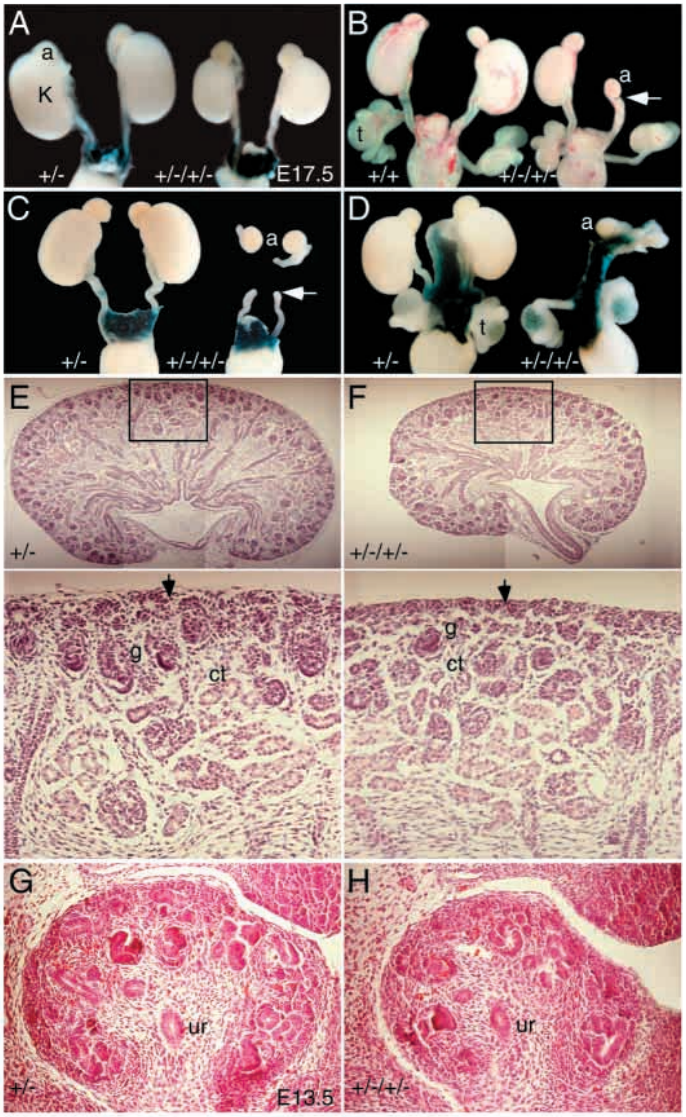

Fig. 6. Renal hypoplasia or agenesis in *Eya1/Six1* double heterozygous animals. (A-D) E17.5 wild type, *Six1*$^{+/–}$ or *Eya1*$^{+/–}$/*Six1*$^{+/–}$ kidneys. Samples shown in A, C and D were X-gal stained for *Six1*^{lacZ} and it is expressed in the testis (t) and muscles surrounding the ureters and kidneys (D). *Eya1*$^{+/–}$/*Six1*$^{+/–}$ animals show either smaller kidneys bilaterally (A), a small kidney on one side and no kidney on the other side (B), or complete absence of kidneys (C,D). In some *Eya1*$^{+/–}$/*Six1*$^{+/–}$ animals that show renal agenesis, ureters that end blindly are observed (arrow in B,C). Adrenal glands and the genital tracts appeared to be normal in all compound heterozygous animals analyzed so far. (E,F) Histological analysis of kidneys of E17.5 *Six1*$^{+/–}$ and *Eya1*$^{+/–}$/*Six1*$^{+/–}$ animals. The number of nephrons was markedly reduced in the double heterozygous kidneys, but normal developing structures are present. The lower panels are higher magnification of the boxed areas. In *Six1*$^{+/–}$ animals, the differentiating metanephric cap tissue (vesicles) in the peripheral nephrogenic zone, in which ureteric bud branching and induction of new nephrons takes place, are morphologically apparent (arrow in E). However, in the hypoplastic *Eya1*$^{+/–}$/*Six1*$^{+/–}$ kidney, the differentiating metanephric vesicles in the peripheral nephrogenic zone were largely reduced in number (arrow in F). (G,H) A reduced number of ureteric bud branches is detected in the compound heterozygous animals at E13.5. ct, convoluting tubule; g, glomerulus; ur, ureter.

peripheral nephrogenic zone. To analyze the onset of the phenotype during development, we analyzed the kidneys at earlier stages. Although the first stages of metanephric kidney development, including evagination of the ureteric bud and its initial branching between E10.5 and 12.5 appeared to occur normally in all *Eya1*$^{+/–}$/*Six1*$^{+/–}$ embryos (n=12), a reduction in the number of ureteric bud branches was first observed at E13.5 (Fig. 6G,H). Taken together, the results suggest that kidney hypoplasia observed in *Eya1*$^{+/–}$/*Six1*$^{+/–}$ animals resulted from abnormal nephrogenesis during late stages of embryogenesis.

Six1$^{–/–}$ metanephric mesenchyme is incompetent for tubulogenesis in organ culture

To further demonstrate that kidney development is arrested at the initial step in *Six1*$^{–/–}$ embryos, kidney rudiments were isolated from *Six1*$^{–/–}$ embryos at E11.0 and cultured in vitro. Five days after culture, all wild type or heterozygous rudiments developed into a fully branched kidney structure showing strong *Pax2* expression (n=5 and n=10, respectively; Fig. 7A). In contrast, *Six1*$^{–/–}$ kidney rudiments formed no kidneys (n=6, Fig. 7B). We next examined whether *Six1* mutant mesenchyme could respond to inductive signals by culturing E11.0 *Six1*$^{–/–}$ mesenchyme with wild type or heterozygous spinal cord. Five days after culture, 100% (11/11) of the *Six1*$^{+/–}$ mesenchymal cultures exhibited characteristic tubules showing *Pax2* mRNA expression (Fig. 7C). In contrast, none of the *Six1*$^{–/–}$ mesenchymes (0/6) exhibited any sign of tubule formation (Fig. 7D). The *Six1*$^{–/–}$ mesenchyme left in the cultures showed no expression of *Pax2* (arrow, Fig. 7D). *Pax2* mRNA

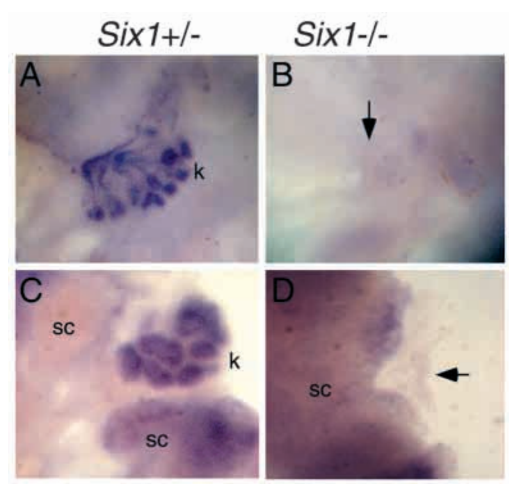

Fig. 7. *Six1* mutant mesenchyme is unresponsive to induction. (A) E11.0 *Six1*$^{+/–}$ kidney rudiments cultured for 5 days and stained with *Pax2* in situ probe. After 5 days of culture, they developed into a fully branched kidney structure (k) showing *Pax2* expression in the collecting tubules and in the nephrons. (B) E11.0 *Six1*$^{–/–}$ metanephric rudiments cultured for 5 days and stained with *Pax2* in situ probe. No kidney formation was observed (arrow). (C) E11.0 *Six1*$^{+/–}$ metanephric mesenchyme cultured with spinal cord (sc) for 5 days and stained with *Pax2* in situ probe. The *Six1*$^{+/–}$ mesenchymal cultures exhibited characteristic tubules (k) showing *Pax2* expression. (D) E11.0 *Six1*$^{–/–}$ metanephric mesenchyme cultured with hetorozygous spinal cord for 5 days and stained with *Pax2* in situ probe. None of the *Six1*$^{–/–}$ mesenchymes exhibited any sign of tubule formation. Note the disappearance of *Six1* mutant mesenchyme (arrow), which shows no *Pax2* expression.

expression was detected in the spinal cord, which was used as a heterologous inducer. Thus, *Six1* mutant mesenchyme was unresponsive to inductive signals.

DISCUSSION

Despite exciting progress in elucidating important genes involved in inductive events during early kidney development, the molecular mechanisms governing the inductive processes of kidney organogenesis remain largely unknown. We show here that the homeobox gene *Six1* is expressed in the metanephric mesenchyme before and after metanephric induction and inactivation of this gene results in renal agenesis. Moreover, we show that *Eya1*, *Six1*, *Six2*, *Pax2* and *Sall1* may function in a molecular pathway and provide evidence for a genetic interaction between *Eya1* and *Six1* in kidney development.

The formation of mammalian kidney involves three distinct processes: first, establishment of the metanephric mesenchyme from posterior intermediate mesoderm; second, outgrowth and branching of the ureteric bud; and third, transformation and differentiation of the metanephric mesenchyme to renal epithelial cells. Our data indicate that in the absence of *Six1*, kidney development was arrested at the second step of these three processes. Although the ureteric bud is present in *Six1*$^{-/-}$ embryos, it fails to invade the mesenchyme completely and the mesenchymal cells undergo abnormal apoptosis from E11.5. Subsequent branching morphogenesis of the ureteric bud and tubule differentiation in the mesenchyme do not occur. It is known that Gdnf and its receptors, c-Ret and Gfrα1, are essential for normal growth and branching morphogenesis of the ureteric bud during kidney development. Indeed, Gdnf can function as a chemoattractant for Ret-expressing epithelial cells and stimulate branching morphogenesis of the ureteric bud (Vega et al., 1996; Tang et al., 1998). Consistent with the observation that the ureteric bud has formed in *Six1*$^{-/-}$ animals, we have detected *Gdnf* expression in *Six1*$^{-/-}$ metanephric mesenchyme. This result demonstrates that the initial expression of *Gdnf* at mRNA level does not require *Six1*. Although we were unable to directly determine whether GDNF protein is produced by *Six1*$^{-/-}$ metanephric mesenchyme, our results demonstrate that whatever amount is made in *Six1*$^{-/-}$ embryos, is insufficient to ensure invasion of the ureteric bud into the metanephric mesenchyme. This evidence also suggests that some other factors that are under the control of a *Six1*-regulatory pathway may be important for fully supporting ureteric bud invasion of the metanephric mesenchyme. They could be, for example, cell atrix components that mediate interaction between the epithelium and mesenchyme. Further expression studies in *Six1* mutant embryos are required to test this hypothesis.

In the mammalian kidney, *Pax2*, *Eya1*, *Six1* and *Six2* expression overlaps in the metanephric mesenchyme and the null mutants of *Pax2*, *Eya1* and *Six1* lack kidney formation (Torres et al., 1995; Xu et al., 1999). Because *Pax2* expression in the intermediate mesoderm was unaffected in *Eya1*$^{-/-}$ embryos at E9.5 and *Six2* expression was lost in *Eya1*$^{-/-}$ metanephric mesenchyme at E10.5, we previously suggested that the *Drosophila* Pax-Eya-Six regulatory hierarchy has been conserved in mammalian kidney development (Xu et al., 1999). Although we previously did not detect *Pax2* expression in *Eya1*$^{-/-}$ metanephric mesenchyme at E10.5, we concluded that it was because of deficient ureteric bud outgrowth and failure of metanephric induction. This interpretation was based on previous analyses in *Danforths' Short tail* (*Sd*) mutants suggesting that *Pax2* expression in the metanephric mesenchyme requires inductive interaction between the mesenchyme and the ureteric bud (Phelps and Dressler, 1993). However, recent expression studies in *Ret* mutants have demonstrated that *Pax2* is expressed in the metanephric mesenchyme before induction and its expression in the mesenchyme is independent of ureteric bud outgrowth (Brophy et al., 2001). Here we show that during mouse kidney development, *Pax2* expression in the metanephric mesenchyme before induction is *Eya1*- and *Six1*-dependent. Consistent with our observation, it has been previously shown that *Six2* expression is also preserved in *Pax2*$^{-/-}$ mesenchyme (Torres et al., 1995). In contrast, we have found that *Six1* expression in the mesenchyme was lost in *Eya1*$^{-/-}$ embryos, similar to that of *Six2* (Xu et al., 1999). Interestingly, we have found that *Six2* expression in the metanephric mesenchyme is also *Six1*-dependent. Therefore, our results together with previous observations suggest that there is an *Eya1*-Six-*Pax2* regulatory hierarchy controlling early mammalian kidney development, distinct from the Pax-Eya-Six regulatory pathway elucidated in *Drosophila* eye imaginal disc. Detailed examination of kidneys in *Pax2*/*Six1* or *Eya1/Six1/Pax2* compound knockouts will enhance our understanding of the possible molecular and genetic interactions between these transcription factors during early mammalian kidney morphogenesis.

Pax2 has recently been proposed to be a direct positive regulator of *Gdnf*, because *Pax2*$^{-/-}$ embryos do not express *Gdnf* in the uninduced mesenchyme and *Pax2* regulates the expression of *Gdnf* in vitro (Brophy et al., 2001). However, our result shows that *Pax2* is not required for the expression of *Gdnf* in the metanephric mesenchyme. We propose two hypotheses to explain these observations. First, because *Pax2* expression in the intermediate mesoderm was unaffected in *Six1*$^{-/-}$ embryos, we hypothesize that *Pax2* expression in the posterior intermediate mesoderm is required for the initiation of *Gdnf* expression during the specification of metanephric mesenchyme. Once *Gdnf* is turned on in the mesenchyme, *Pax2* expression in the mesenchyme may not be required for the maintenance of *Gdnf* expression as metanephric development proceeds. This could explain why *Gdnf* expression was absent in *Pax2*$^{-/-}$ embryos. Consistent with this hypothesis, *Gdnf* expression was also observed in *Wt1*$^{-/-}$ metanephric mesenchymes which do not express Pax2 protein, although *Pax2* mRNA expression was observed in *Wt1*$^{-/-}$ metanephric mesenchyme (Kreidberg et al., 1993; Donovan et al., 1999). Second, because *Pax2* is expressed normally in the Wolffian duct and ureteric bud in *Six1*$^{-/-}$ embryos, it is possible that the expression of Pax2 in the Wolffian duct and ureteric bud epithelium is required for the maintenance of *Gdnf* expression in the mesenchyme. This could also explain the reduction of *Gdnf* expression observed in *Pax2*$^{-/-}$ metanephric mesenchyme. In support of this hypothesis, a greatly reduced level of *Gdnf* mRNA in the metanephric mesenchyme at E11.5 has also been seen in mice defective for *Emx2*, a homeobox gene expressed primarily in the ureteric bud, whose disruption inhibits ureteric bud growth and branching (Miyamoto et al., 1997). Interestingly, *Pax2* expression was also significantly reduced in *Emx2*$^{-/-}$ ureteric bud at E11.5, whereas its expression in *Emx2*$^{-/-}$ metanephric mesenchyme was apparently normal at this stage (Miyamoto et al., 1997).

Our results also show that *Sall1* functions downstream of *Six1*. *Sall1* is a mammalian homolog of the *Drosophila* region-specific homeotic gene *spalt* (*sal*). Inactivation of murine *Sall1* results in renal agenesis or severe dysgenesis because of incomplete ureteric bud outgrowth and the failure of tubule formation, similar to that seen in *Six1*$^{-/-}$ embryos. It has been shown previously that *Gdnf*, *Eya1*, *Pax2* and *Wt1* are expressed in *Sall1*$^{-/-}$ metanephric mesenchyme at E10.5, indicating that *Sall1* may function downstream of or independent from these genes. Because our results show that *Sall1* expression is also unaffected in E10.5 *Pax2*$^{-/-}$ metanephric mesenchyme, it is possible that *Sall1* and *Pax2* function in parallel during early kidney development. Heterozygous mutations in the human *SALL1* lead to Townes-Brocks syndrome, which shows phenotypic overlap with Branchio-Oto-Renal (BOR) syndrome, a deficiency for the human *EYA1* gene. Interestingly, *Sall1* expression was also undetectable in *Eya1*$^{-/-}$ mesenchyme (data not shown). Therefore, it is probable that *Eya1*, *Six1*, *Six2*, *Pax2* and *Sall1* function in a genetic and molecular pathway in the metanephric mesenchyme during early kidney morphogenesis.

Wt1 is also expressed in the metanephric mesenchyme and its absence leads to failure of ureteric bud outgrowth and apoptosis of the mesenchyme. Our results show that *Six1* is not required for the expression of *Wt1*. It has been shown previously that *Six2* is expressed in *Wt1*$^{-/-}$ metanephric mesenchyme (Donovan et al., 1999) and *Wt1* is expressed in *Eya1*$^{-/-}$ mesenchyme (Xu et al., 1999). Thus, it is possible that *Wt1* functions in a pathway independent from *Eya1* and Six genes for metanephric development. It is also possible that *Wt1* functions in parallel or synergistically with *Eya1* and Six genes for metanephric development.

Finally, it should be noted that during late embryonic mouse kidney development, *Six1* expression was only observed in collecting tubules, but not in renal epithelia which are derived from metanephric mesenchyme. Although it is generally accepted that metanephric mesenchyme is committed to differentiating into nephrons whereas the ureteric bud is restricted to forming the renal collecting system, several in vitro cell fate studies demonstrated that metanephric mesenchyme differentiates into portions of the renal collecting system, in addition to nephron epithelia (Koseki et al., 1991; Herzlinger et al., 1992; Qiao et al., 1995). The observation of *Six1* expression in a subpopulation of collecting tubule epithelial cells during kidney development is consistent with this finding. Therefore, it is possible that the *Six1*-expressing metanephric mesenchymal cells at E11.5 are pluripotent renal epithelial stem cells and a subpopulation of those cells are recruited into collecting tubule epithelia during renal collecting system morphogenesis. Our results indicate that in addition to its early function in the initiation of mammalian kidney development, *Six1* may also play a role in the morphogenesis of the renal collecting system.

We thank P. Gruss for the *Pax2* mutant mice, R. Nishinakamura for the *Sall1* probe, B. Tang for technical assistance and L. Ross for helpful comments. Photomicroscopy and image analysis was made possible by equipment purchased with a grant from the M. J. Murdock Charitable Trust. This work was supported by NIH P20RR 12345-02 (to P.-X.X.).

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