

To generate a GST-Zn5 bacterial expression vector, the *SpeI*-*NotI* fragment of Sall1 cDNA (Sato *et al.* 2004) was inserted in-frame into the *SmaI* site of pGEX-4T-3 (GE Healthcare).

### Cell culture and transfection

For immunocytochemistry, E14.1 ES cells were plated on 4-well Lab-TekII chamber slides (Nunc) at a density of  $5 \times 10^3$  cells/well. For localization of GFP-fused Sall1 mutants, NIH3T3 cells were plated on 4-well Lab-TekII chamber slides at a density of  $1 \times 10^4$  cells/well, and 1  $\mu$ g of each plasmid was transiently introduced using Eugene 6 (Roche). For the nuclear extracts used in EMSAs,  $3 \times 10^6$  HEK293 cells were plated in 100-mm dishes.

### Analysis of protein localization in cells

At 24 h after transfection of GFP-fused mutants, NIH3T3 cells were fixed in phosphate-buffered saline (PBS) containing 2% paraformaldehyde, 0.1% Triton X-100 and 2  $\mu$ g/mL DAPI at 4 °C for 20 min, and then washed for  $3 \times 5$  min in PBS at room temperature. For immunocytochemistry, we used a monoclonal anti-Sall1 antibody (Sato *et al.* 2004), an anti-HP1 $\alpha$  antibody (Catalog No. 07-346; Upstate Biotechnology) and an anti-tri-methylated H3K9 antibody (Catalog No. 07-523; Upstate Biotechnology). After fixation, the cells were blocked with 10% goat serum for 30 min at room temperature, incubated with each primary antibody diluted 1 : 100 in PBS containing 1% goat serum for 1 h at room temperature, rinsed with PBS and detected using an Alexa Fluor 488-conjugated anti-mouse IgG secondary antibody (Molecular Probes) or rhodamine-conjugated anti-rabbit IgG secondary antibody (Chemicon). The localization of the labeled proteins was detected using a confocal microscope (Radiance 2100; Bio-Rad).

### Binding site selection

For binding site selection, we employed the CASTing technique as previously described (Morinaga *et al.* 2005) with some modifications. Single-stranded oligonucleotides containing a 20-bp random sequence flanked by a 20-bp PCR primer annealing site were synthesized (5'-GCTCTGGAAGTCTGAGTCC-N20-CGATTCTGTCGACCTCGAAG-3'). A dsDNA library was generated via extension by Taq polymerase primed with a reverse primer (5'-CTTCGAGGTCGACAGAATCG-3'). An aliquot (2 ng) of the dsDNA library was mixed with an excess of GST-Zn5 bound to glutathione-Sepharose beads (GE Healthcare) in 100  $\mu$ L of CASTing binding buffer (20 mM Tris-HCl pH 8.0, 150 mM NaCl, 2 mM MgCl<sub>2</sub>, 25  $\mu$ M ZnCl<sub>2</sub>, 0.2 mM EDTA, 10% glycerol, 0.1% NP-40, 1 mM dithiothreitol (DTT), 1 mg/mL bovine serum albumin (BSA), 100  $\mu$ g/ $\mu$ L poly[dI-dC] and 1% (v/v) protease inhibitor cocktail for mammalian cell extracts (Sigma) and incubated at 4 °C for 30 min with continual rotation. After six washes with binding buffer (without BSA or poly[dI-dC]), the DNA-protein complexes were eluted by incubating the beads with elution buffer (5 mM reduced glutathione, 100 mM Tris-HCl pH 8.0 and 150 mM NaCl). Selected oligonucleotides

were recovered by phenol extraction and ethanol precipitation, and amplified by 20 cycles of PCR with a forward primer (5'-GCTCTGGAAGTCTGAGTCC-3') and the above-described reverse primer. The same procedure was repeated for three additional rounds using 15 cycles of PCR for each. The final PCR products were subcloned into pCRII-TOPO (Invitrogen) and sequenced.

### EMSAs

Bacterially-expressed GST-Zn5 was purified from bacterial lysates by binding to glutathione-Sepharose beads, followed by competitive elution with reduced glutathione. Aliquots (200 ng) of the protein were mixed with <sup>32</sup>P-labeled DNA probes (20 000 cpm) in 15  $\mu$ L of CASTing binding buffer (without BSA), incubated at 30 °C for 30 min and then resolved by 4% polyacrylamide gel electrophoresis in 0.5  $\times$  TBE and 5% glycerol. The gels were dried and autoradiographed. To prepare <sup>32</sup>P-labeled probes, the final CASTing PCR products and dsDNA library before CASTing were labeled by PCR using [ $\alpha$ -<sup>32</sup>P]dCTP. Individual cloned sequences (probes 1, 2, 3, 4, 4-1 and 4-2) were excised from the cloned plasmids by *EcoRI* digestion, labeled using the Klenow large fragment (Takara) and [ $\alpha$ -<sup>32</sup>P]dATP, and purified using MicroSpin G-25 columns (GE Healthcare).

For nuclear extract preparation, HEK293 cells were transiently transfected with 6  $\mu$ g of each plasmid. After 48 h, the cells were washed with PBS, lysed with 600  $\mu$ L of buffer A (10 mM HEPES-KOH pH 7.8, 10 mM KCl, 1.5 mM MgCl<sub>2</sub>, 0.05% NP-40, 0.5 mM DTT and 5% (v/v) protease inhibitor cocktail for mammalian cell extracts) for 10 min on ice and centrifuged at 2 300 g for 1 min. The pellet was suspended in 300  $\mu$ L of buffer B (20 mM HEPES-KOH pH 7.8, 500 mM NaCl, 1.5 mM MgCl<sub>2</sub>, 0.5 mM DTT and 5% v/v protease inhibitor cocktail for mammalian cell extracts), rotated at 4 °C for 30 min and centrifuged at 20 000 g for 30 min. The supernatant was diluted with an equal volume of buffer B containing 50% glycerol. The relative amount of each Sall1 protein was determined by Western blot analysis using the anti-Sall1 monoclonal antibody. The Sall1 protein level was normalized by dilution, with reference to nuclear extracts from cells transfected with the empty pCAG-EGFP vector.

The major satellite DNA probes were amplified by PCR using *Pfu* DNA polymerase, genomic DNA extracted from C57BL/6 mice and the following primers: Major satellite: forward, 5'-GGAATTCGGACCTGGAAATAGGCG-3' and reverse, 5'-GGAATTCCTCAGTGTGCATTTCTCATTTTTTCACG-3'; Major A:

forward, 5'-GAATTCGGACCTGGAAATAGGCGA-GAAAAC-3' and

reverse, 5'-AGTTTTCTCGCCATATTTACAGTCCTAAA-3'; Major B:

forward, 5'-GAGGAAAAGTGGAAAATT-TAG-3' and

reverse, 5'-TCTCATTTCCATAATTATTCAGTTTTCTT-3'; and Major C:

forward, 5'-GAAAATGAGAAACATCCACTTGAAGACTTG-3' and

reverse, 5'-GAATTCCTCAGTGTGCATTTCTCATTT-3'.

Each fragment amplified by PCR was cloned into pCR-Blunt II-TOPO. Major B mutant fragments were derived from synthetic ds oligonucleotides and also cloned into pCR-Blunt II-TOPO.

For EMSAs, 3- $\mu$ L aliquots of the nuclear extracts were diluted with 12  $\mu$ L of EMSA reaction buffer (50 mM HEPES-KOH pH 7.8, 1.5 mM MgCl<sub>2</sub>, 0.5 mM DTT, 0.05 mM ZnCl<sub>2</sub>, 1% (v/v) protease inhibitor cocktail for mammalian cell extracts, 20% (v/v) glycerol, radioisotope-labeled probe (5  $\times$  10<sup>4</sup> cpm)), incubated at 30 °C for 30 min, and then separated by polyacrylamide gel electrophoresis in 4% gels containing 15% glycerol with 0.5  $\times$  TBE as the electrophoresis buffer.

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# *Six1* and *Six4* are essential for *Gdnf* expression in the metanephric mesenchyme and ureteric bud formation, while *Six1* deficiency alone causes mesonephric-tubule defects

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## Abstract

Interaction between the ureteric-bud epithelium and the metanephric mesenchyme is important for kidney development. *Six1* and *Six4* are the mammalian homologs of *Drosophila sine oculis*, and they are coexpressed in the nephrogenic mesenchyme. *Six1*-deficient mice show varying kidney defects, while *Six4*-deficient mice have no apparent abnormalities. Here, we report *Six1/Six4*-deficient mice that we generated in order to elucidate the functions of *Six4* in *Six1*-deficient kidney development. The *Six1/Six4*-deficient mice exhibited more severe kidney phenotypes than the *Six1*-deficient mice; kidney and ureter agenesis was observed in all the neonates examined. The *Six1/Six4*-deficient metanephric mesenchyme cells were directed toward kidney lineage but failed to express *Pax2*, *Pax8*, or *Gdnf*, whereas the expression of these genes was partially reduced or unchanged in the case of *Six1* deficiency. Thus, *Six4* cooperates with *Six1* in the metanephric mesenchyme to regulate the level of *Gdnf* expression; this could explain the absence of the ureteric bud in the *Six1/Six4*-deficient mice. In contrast, *Six1* deficiency alone caused defects in mesonephric-tubule formation, and these defects were not exacerbated in the *Six1/Six4*-deficient mesonephros. These results highlight the fact that *Six1* and *Six4* have collaborative functions in the metanephros but not in the mesonephros.

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## 1. Introduction

In mammals, kidney development occurs in three successive steps – initial pronephros formation followed by mesonephros formation and finally, metanephros formation. In mice, development of the first kidney – the pronephros – is initiated between embryonic day 8.0 and 8.5

(E8.0–E8.5) by signals from the paraxial mesoderm and surface ectoderm. These signals induce the intermediate mesoderm to differentiate into the Wolffian duct (nephric duct). Next, the nephrogenic cord – the mesenchymal tissue – is induced at the ventral side of the Wolffian duct (Obara-Ishihara et al., 1999; Mauch et al., 2000; James and Schultheiss, 2003). The Wolffian duct and nephrogenic cord elongate caudally towards the cloaca, and the Wolffian duct converts the adjacent mesenchyme (nephrogenic cord) into mesonephric tubules between E9.0 and E11.0. When the Wolffian duct reaches the level of the developing

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hindlimb at E10.0, the ureteric bud evaginates from the Wolffian duct and invades the surrounding metanephric mesenchyme, which is formed as a bulge at the caudal-most region of the nephrogenic cord. Subsequently, both the metanephric mesenchyme and ureteric bud undergo reciprocal inductive interactions to form the nephrons and collecting ducts of the metanephros – the third and the adult kidney (Grobstein, 1953; Saxen, 1987). Thus, the two mesenchymal tissues – the nephrogenic cord and metanephric mesenchyme – constitute the nephrogenic mesenchyme.

During metanephros development, the glial cell line-derived neurotrophic factor (*Gdnf*) secreted from the mesenchyme acts on the receptor tyrosine kinase *Ret* and its co-receptor *Gfra1*, both of which are expressed in the Wolffian duct, and it induces ureteric-bud formation from the Wolffian duct. Thus, mice that are deficient in *Gdnf*, *Ret*, or *Gfra1* show similar phenotypes such as failure of ureteric-bud invasion into the mesenchyme (Durbec et al., 1996; Moore et al., 1996; Pichel et al., 1996; Sanchez et al., 1996; Sainio et al., 1997b; Cacalano et al., 1998; Enomoto et al., 1998; Sariola and Saarma, 1999). Several mesenchymal transcription factors also play important roles in metanephros formation (reviewed by Vainio and Lin, 2002). *Pax2* is expressed both in the metanephric mesenchyme and ureteric-bud, and *Pax2*-deficient mice show a reduced mesenchyme and failure in ureteric-bud invasion (Torres et al., 1995). *Pax2* has been shown to bind the *Gdnf* promoter, and this may explain the reduced *Gdnf* expression and the ureteric-bud phenotype in the mutant mice (Brophy et al., 2001). *Sall1* is expressed exclusively in the mesenchyme, and mice lacking this gene show phenotypes similar to those of *Pax2*-null mice (Nishinakamura et al., 2001). However, *Gdnf* and *Pax2* expression remains unaffected in *Sall1*-deficient mice, while *Sall1* continues to be expressed in *Pax2*-mutant mice. This suggests that *Sall1* may function independent of the *Pax2*–*Gdnf* pathway. *Wtl*-deficient mice also exhibit similar phenotypes; however, *Gdnf* expression is not affected in these mice (Kreidberg et al., 1993; Sainio et al., 1997b). Mice lacking *Osr1* do not develop the metanephric mesenchyme, and they do not express *Pax2*, *Gdnf*, or *Sall1*; this implies that *Osr1* is one of the most upstream genes in the metanephric cascade (James and Schultheiss, 2005; Wang et al., 2005; James et al., 2006).

The mesonephros comprises the mesonephric tubules and the Wolffian duct. Two types of mesonephric tubules are formed along the Wolffian duct. The first type comprises a few pairs of cranial mesonephric tubules, which develop as outgrowths from the Wolffian duct and later become epididymal ducts in males. The other type comprises caudal mesonephric tubules, which constitute a majority of the tubules and are formed in the nephrogenic cord – a mesenchymal tissue – upon induction by the Wolffian duct (Sainio et al., 1997a). Further, some of the genes involved in metanephros formation also play important roles in mesonephros development. *Pax2*-deficient mice show defects in mesonephric-tubule formation and Wolffian-duct elonga-

tion (Torres et al., 1995). *Wtl*-deficient mice lack the caudal mesonephric tubules but not the cranial tubules (Kreidberg et al., 1993; Sainio et al., 1997a). *Osr1* deficiency leads to asymmetric defects in Wolffian-duct elongation and to failure in mesonephric-tubule formation (James and Schultheiss, 2005; Wang et al., 2005; James et al., 2006). In contrast to these genes that are expressed in the nephrogenic cord (and also in the Wolffian duct in the case of *Pax2*), *Wnt9b* is expressed exclusively in the Wolffian duct and induces the nephrogenic cord to transform into mesonephric-tubule epithelia. Thus, *Wnt9b*-deficient mice lack mesonephric tubules (Carroll et al., 2005).

The initial specification toward the nephrogenic lineage from the intermediate mesoderm is beginning to be elucidated. *Pax2* and *Pax8* are coexpressed in the Wolffian duct and in the pro- and mesonephric tubules, and *Pax2/Pax8*-deficient mice exhibit complete absence of the pro-, meso-, and metanephros (Bouchard et al., 2002), suggesting redundant roles of the two transcription factors in kidney-lineage commitment. The homeodomain protein *Lim1* is expressed early in the intermediate mesoderm, Wolffian duct, and pro- and mesonephric tubules (Fujii et al., 1994), and is required for kidney development (Kobayashi et al., 2005). In *Lim1*-deficient mice, the intermediate mesoderm is disorganized and fails to express *Pax2* – a prerequisite for kidney development (Tsang et al., 2000).

The *Six* homeobox genes are characterized by conserved six domains and a homeodomain, both of which are required for specific DNA binding. This gene family is essential for compound-eye development, and the prototype of this gene family is *Drosophila sine oculis*. Six members (*Six1*–*Six6*) of the *Six* gene family have been identified in mice and humans (Kawakami et al., 2000). *Six3* and *Six6* are essential for forebrain formation and eye development (Kobayashi et al., 1998; Loosli et al., 1999; Zuber et al., 1999; Carl et al., 2002; Zhu et al., 2002; Lagutin et al., 2003; Lopez-Rios et al., 2003), whereas *Six5* is involved in cataractogenesis and spermatogenesis (Sarkar et al., 2000, 2004). Several papers, including ours, report that *Six1*-deficient mice show anomalies in the development of various organs such as the inner ear, nose, thymus, kidney, and skeletal muscle (Laclef et al., 2003a,b; Zheng et al., 2003; Ozaki et al., 2004; Zou et al., 2004, 2006). During renal development, *Six1* is required for ureteric-bud invasion into the metanephric mesenchyme although variation among animals exists. In *Six1* deficiency, the metanephric mesenchyme is formed, although it is small. Further, expression of *Sall1* and *Six2* is absent, while that of *Pax2* and *Gdnf* is only partially reduced in this condition (Li et al., 2003; Xu et al., 2003). *Eyal*, a murine homolog of the *Drosophila eyes absent* gene, functions as a coactivator of the *Six* family genes. *Eyal*-deficient mice exhibit more severe kidney phenotypes than *Six1*-null mice – absence of the metanephric mesenchyme and ureteric-bud and complete reduction in the expression levels of *Pax2* and *Gdnf* (Xu et al., 1999; Nica et al., 2006). Thus, molecules other

than *Six1* that possibly cooperate with *Eya1* in kidney development might exist.

*Six4*, another member of the *Six* family, is separated from the *Six1* gene on the same chromosome by only 100 kb; its expression overlaps with that of *Six1* in many regions such as the neural placodes, Rathke's pouch, dorsal root ganglia, dermomyotome, myotome, limb bud mesenchyme, and myogenic migrating precursors (Grifone et al., 2005). Interestingly, as we previously demonstrated, *Six4*-deficient mice exhibit no major developmental defects and are fertile (Ozaki et al., 2001). Since *Six1* and *Six4* exhibited a similar binding specificity to the MEF3 site (TCAGGTTC), the roles played by *Six1* and *Six4* in embryogenesis may be redundant (Spitz et al., 1998; Himeda et al., 2004). The first evidence of this assumption was observed during myogenesis; *Six1* and *Six4* contribute to myogenic migration in somites by regulating *Pax3* and *Met* expression (Grifone et al., 2005). By independently generating *Six1/Six4*-deficient mice, we here demonstrate that these two transcription factors (*Six1* and *Six4*) have overlapping functions in kidney development. The *Six1/Six4*-deficient mice exhibited more severe kidney phenotypes than the *Six1*-deficient mice. The expression of *Pax2*, *Pax8*, and *Gdnf* was completely lost in the *Six1/Six4*-deficient metanephric mesenchyme, while it was partially reduced or unchanged in the *Six1*-deficient mice. These results indicate that *Six1* and *Six4* are required for metanephric mesenchyme development.

## 2. Results

### 2.1. *Six1* and *Six4* are coexpressed in the nephrogenic cord and metanephric mesenchyme

To demonstrate the overlapping expression patterns of *Six1* and *Six4* in the mesenchyme of developing renal tissues, we performed in situ hybridization for these two genes by using sections of E9.5 and E10.5 embryos, as described in Fig. 1A and B. In addition to its expression in somites (Oliver et al., 1995), *Six1* was expressed in the nephrogenic cord on E9.5 and in the metanephric mesenchyme on E10.5 but not expressed in the Wolffian duct (Fig. 1C–E). The domains of *Six4* expression were similar to those of *Six1*. *Six4* expression, although weak, was also detected in the nephrogenic cord and metanephric mesenchyme, both of which are mesenchymal components of the developing kidney (Fig. 1F–H).

### 2.2. Kidney phenotypes are exacerbated in *Six1/Six4*-deficient mice

Since *Six1* and *Six4* are on the same chromosome and closely linked, we introduced the *Six1*-targeting vector into *Six4*-heterozygous embryonic stem cells, and we generated strains with mutations in both the genes. As recently described in our paper, the resultant

*Six1/Six4*-heterozygous mice appeared to be normal and fertile. Further, after heterozygous crossing, mice homozygous for *Six1* and *Six4* were born at the expected Mendelian frequency (Konishi et al., 2006). However, the *Six1/Six4*-deficient mice died soon after birth and showed developmental defects in various organs; this finding is consistent with a previous report (Grifone et al., 2005).

Further, by examining the kidney abnormalities in the *Six1/Six4*-deficient mice, we noted that they exhibited more severe phenotypes than the *Six1/Six4*-heterozygous or *Six1*-deficient mice. None of the *Six1/Six4*-heterozygous neonate mice showed developmental defects in the kidneys and ureters (Fig. 2A, Table 1). Of the *Six1*-deficient mice, 30% exhibited uni- or bilateral renal hypoplasia (Fig. 2B), and the remaining 70% exhibited kidney agenesis and had short ureters (Fig. 2C). In contrast, ureters and bilateral kidneys failed to develop in all the *Six1/Six4*-deficient mice, whereas the adrenal glands, urinary bladder, and genital tracts were formed without any apparent defects (Fig. 2D, Table 1).

Next, in the E11.5 embryos, we examined ureteric-bud invasion into the metanephric mesenchyme and the subsequent metanephric-mesenchyme condensation, an essential step for kidney and ureter formation. We stained the kidneys with an antibody against E-cadherin, which is expressed in the Wolffian duct and ureteric bud. A whole-mount view of the E-cadherin staining revealed that the ureteric bud originated from the Wolffian duct and branched in the *Six1/Six4*-heterozygous embryos (Fig. 2E). In the *Six1*-deficient embryos, the ureteric bud was formed but showed defects in elongation and branching (Fig. 2F and G). In contrast, ureteric-bud formation was never detected in the *Six1/Six4*-deficient embryos (Fig. 2H). Hematoxylin-eosin (HE)-stained sections of the E11.5 *Six1/Six4*-heterozygous embryos revealed no developmental defects in the invasion of the ureteric bud and condensation of the metanephric mesenchyme adjacent to the ureteric bud (Fig. 2I). In the *Six1*-deficient embryos, ureteric-bud invasion partially occurred, but variations were observed with regard to ureteric bud development and metanephric-mesenchyme condensation (Fig. 2J). In contrast, none of the *Six1/Six4*-deficient embryos showed ureteric-bud invasion and subsequent metanephric-mesenchyme condensation (Fig. 2K).

Further, prior to ureteric-bud invasion into the metanephric mesenchyme, we examined the metanephric mesenchyme at E10.5 by performing HE staining of the sections. The metanephric mesenchyme appeared as a distinct cell cluster adjacent to the Wolffian duct in the *Six1/Six4*-heterozygous (Fig. 2L and L') and *Six1*-deficient embryos (Fig. 2M and M'), whereas this cell cluster was not observed in the *Six1/Six4*-deficient embryos (Fig. 2N and N'; arrowhead). Thus, the absence of *Six4* exacerbated *Six1*-deficient kidney phenotypes, suggesting that the transcription factors *Six1* and *Six4* cooperatively regulate the early stages of metanephros development and ureteric bud formation.

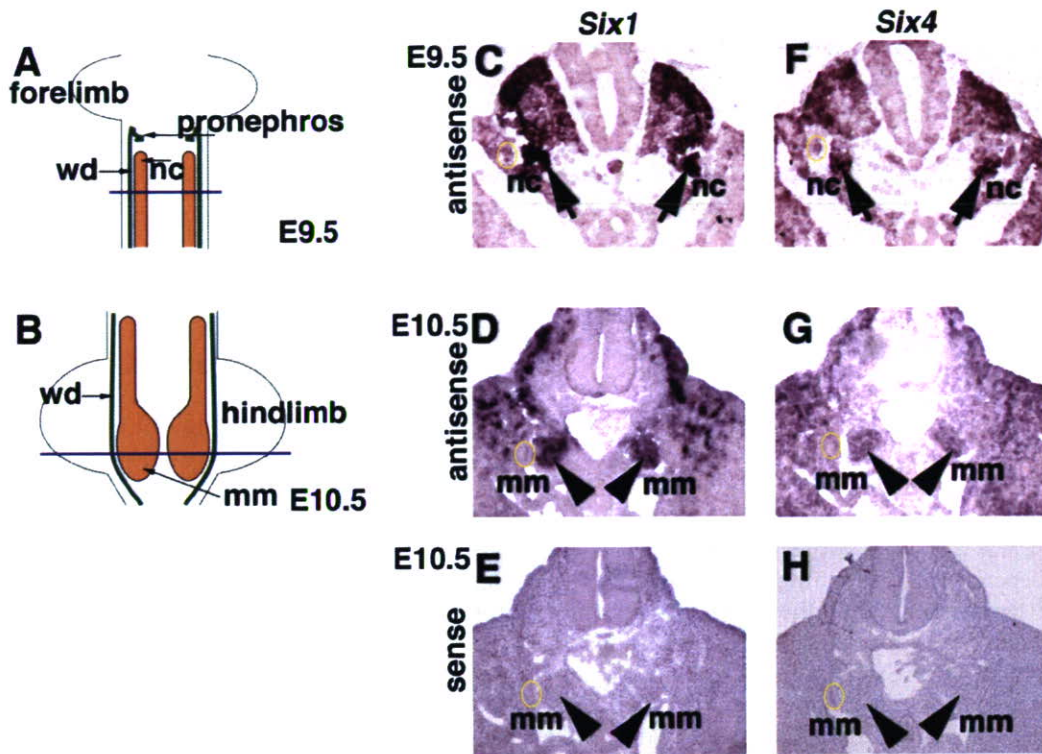


Fig. 1. Overlapping expression patterns of *Six1* and *Six4* in the nephrogenic mesenchyme. (A and B) Nephrogenic cord at E9.5 (A) and metanephric mesenchyme at E10.5 (B). Blue lines in A and B represent the planes along which the sections in C–H were sliced. (C–E) *Six1* expression in the developing kidneys in the E9.5 (C) and E10.5 (D) embryos. The sense probe produced no signals (E). (F–H) *Six4* expression in the developing kidneys in the E9.5 (F) and E10.5 (G) embryos. The sense probe produced no signals (H). *Six1* and *Six4* are coexpressed in the nephrogenic cord (arrow) in E9.5 embryos as well as in the metanephric mesenchyme (arrowhead) in the E10.5 embryos. The yellow circle indicates the Wolffian duct (nc, nephrogenic cord; mm, metanephric mesenchyme).

### 2.3. *Pax2*, *Sall1*, and *Gdnf* were absent in the *Six1/Six4*-deficient metanephric mesenchyme

To examine apoptosis, we carried out a TdT-mediated dUTP-digoxigenin nick end labeling (TUNEL) assay; however, apoptosis-positive cells were not detected in the metanephric mesenchyme of the *Six1/Six4*-heterozygous, *Six1*-deficient, or *Six1/Six4*-deficient embryos (Fig. 3A–C), suggesting that the absence of the mesenchymal cluster in *Six1/Six4* deficiency was not due to increased apoptosis in this region. Furthermore, expression of *EGFP* driven by the *Six1* locus (refer to Section 4), *Osr1*, and *Wtl*, which are known to be expressed at an early stage in kidney precursors (Kreidberg et al., 1993; James et al., 2006), was detected in the metanephric mesenchyme region of the *Six1/Six4*-deficient embryos as well as in the *Six1/Six4*-heterozygous and *Six1*-deficient embryos (Fig. 3D–L). This implied that the *Six1/Six4*-deficient metanephric mesenchymal cells were directed toward kidney lineage. In contrast, *Gdnf*, *Sall1*, and *Pax2* expression was severely affected in the *Six1/Six4*-deficient embryos. *Gdnf* was expressed in the metanephric mesenchyme in the *Six1/Six4*-heterozygous embryos (Fig. 3M), and it was weakly expressed in the *Six1*-deficient embryos (Fig. 3N). However, *Gdnf* expression was absent in the *Six1/Six4*-deficient

embryos (Fig. 3O). These results are consistent with the complete absence of the ureteric bud in the *Six1/Six4*-deficient embryos and with its partial impairment in the *Six1*-deficient embryos because *Gdnf* is a critical regulator of ureteric budding. *Sall1* was expressed in the metanephric mesenchyme in the *Six1/Six4*-heterozygous embryos (Fig. 3P), but its expression was markedly lower in the *Six1*-deficient embryos and absent in the *Six1/Six4*-deficient embryos (Fig. 3Q and R). *Pax2* was expressed both in the metanephric mesenchyme and Wolffian duct in the *Six1/Six4*-heterozygous embryos (Fig. 3S). *Pax2* expression was lowered in the metanephric mesenchyme but not in the Wolffian duct in the *Six1*-deficient embryos, whereas it was completely absent in the *Six1/Six4*-deficient embryos (Fig. 3T and U). These results genetically place *Six1* and *Six4* downstream of *Osr1* and *Wtl* and upstream of *Gdnf*, *Sall1*, and *Pax2*. These results also imply that *Six1* and *Six4* redundantly regulate *Pax2* and *Gdnf* expression in the metanephric mesenchyme.

### 2.4. *Six1* and *Six4* regulate *Pax8* in the metanephric mesenchyme

Together with *Pax2*, *Pax8* is also known to play a role in pro- and mesonephros development from the intermediate

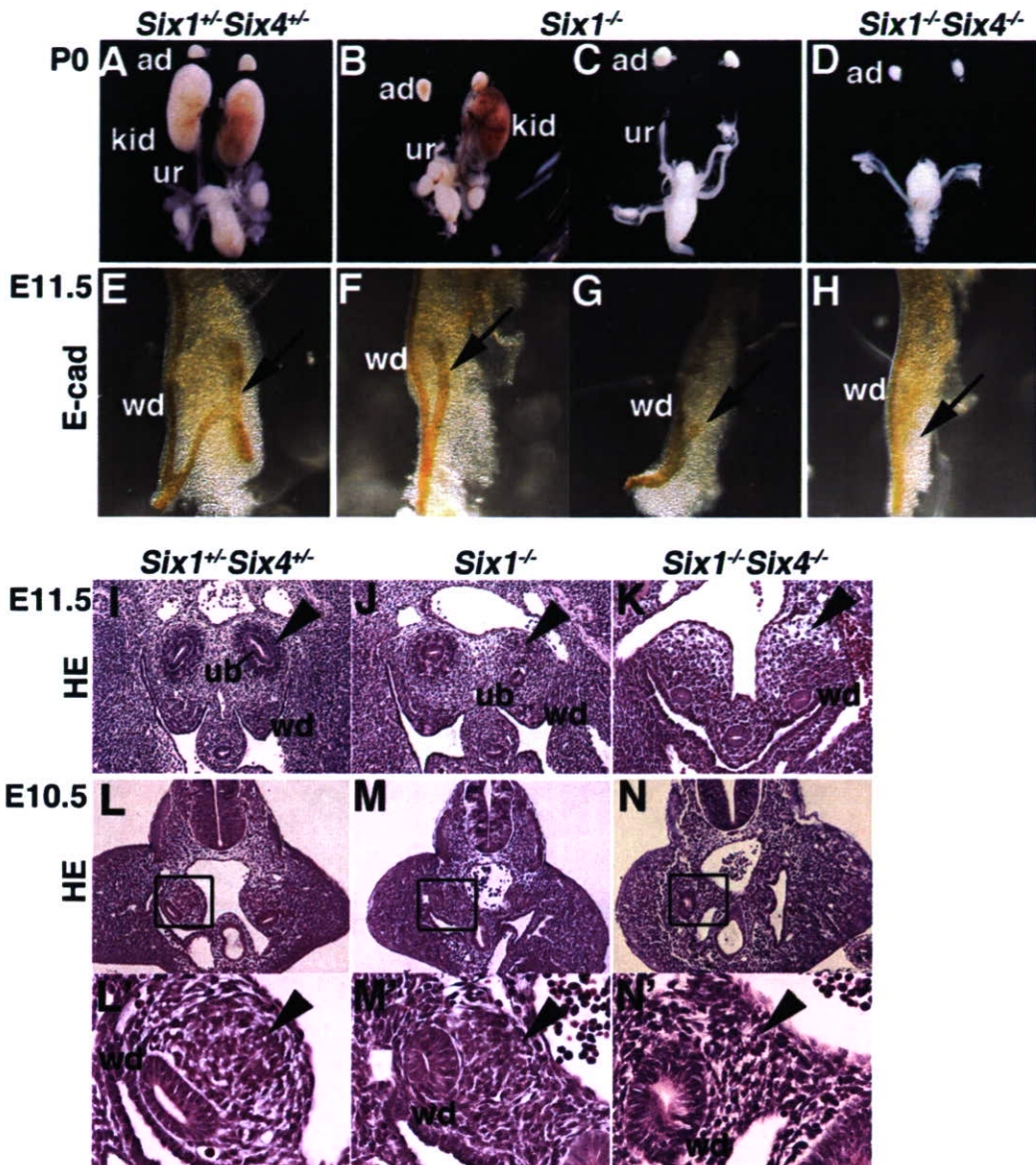


Fig. 2. *Six1/Six4*-deficient mice exhibit more severe renal defects than *Six1*-deficient mice. (A–D) Whole-mount views of urogenital tissues of the *Six1/Six4*-heterozygous (A), *Six1*-deficient (B and C), and *Six1/Six4*-deficient (D) newborn mice. Of the *Six1*-deficient mice, 30% exhibited uni- or bilateral renal hypoplasia (B) and the rest exhibited kidney agenesis (C). In contrast, all the *Six1/Six4*-deficient mice completely lacked kidney and ureter formation (D). (E–H) Whole-mount views of E-cadherin staining in the metanephric region of the E11.5 *Six1/Six4*-heterozygous (E), *Six1*-deficient (F and G), and *Six1/Six4*-deficient (H) embryos. The ureteric buds (arrow) were short and unbranched in the *Six1*-deficient embryos, while they were absent in the *Six1/Six4*-deficient embryos. (I–K) HE staining of the metanephric region of the E11.5 *Six1/Six4*-heterozygous (I), *Six1*-deficient (J), and *Six1/Six4*-deficient (K) embryos. The condensed mesenchyme (black arrowhead) was reduced, and the invasion of the ureteric bud into the mesenchyme was partially impaired in the *Six1*-deficient embryos (J). The mesenchymal cell cluster and the ureteric bud were not detected in the *Six1/Six4*-deficient embryos (K). (L–N) HE staining of the metanephric region of the E10.5 *Six1/Six4*-heterozygous (L), *Six1*-deficient (M), and *Six1/Six4*-deficient (N) embryos. (L'–N') The lower panels are at a higher magnification than the upper panels (L–N). The mesenchymal cell cluster (arrowhead) was not detected in the *Six1/Six4*-deficient embryos (kid, kidney; ur, ureter; ad, adrenal gland; wd, Wolffian duct; ub, ureteric bud).

Table 1  
Kidney abnormalities in newborn mutants

Genotype	Normal kidney	Hypoplastic kidney <sup>a</sup>	Kidney agenesis <sup>b</sup>	Kidney & ureter agenesis
<i>Six1</i> <sup>+/-</sup> <i>Six4</i> <sup>+/-</sup>	8/8	0/8	0/8	0/8
<i>Six1</i> <sup>-/-</sup>	0/20	6/20	14/20	0/20
<i>Six1</i> <sup>-/-</sup> <i>Six4</i> <sup>-/-</sup>	0/15	0/15	0/15	15/15

<sup>a</sup> Bilateral hypoplastic kidneys or unilateral agenesis.

<sup>b</sup> A lack of bilateral kidneys but not ureters.



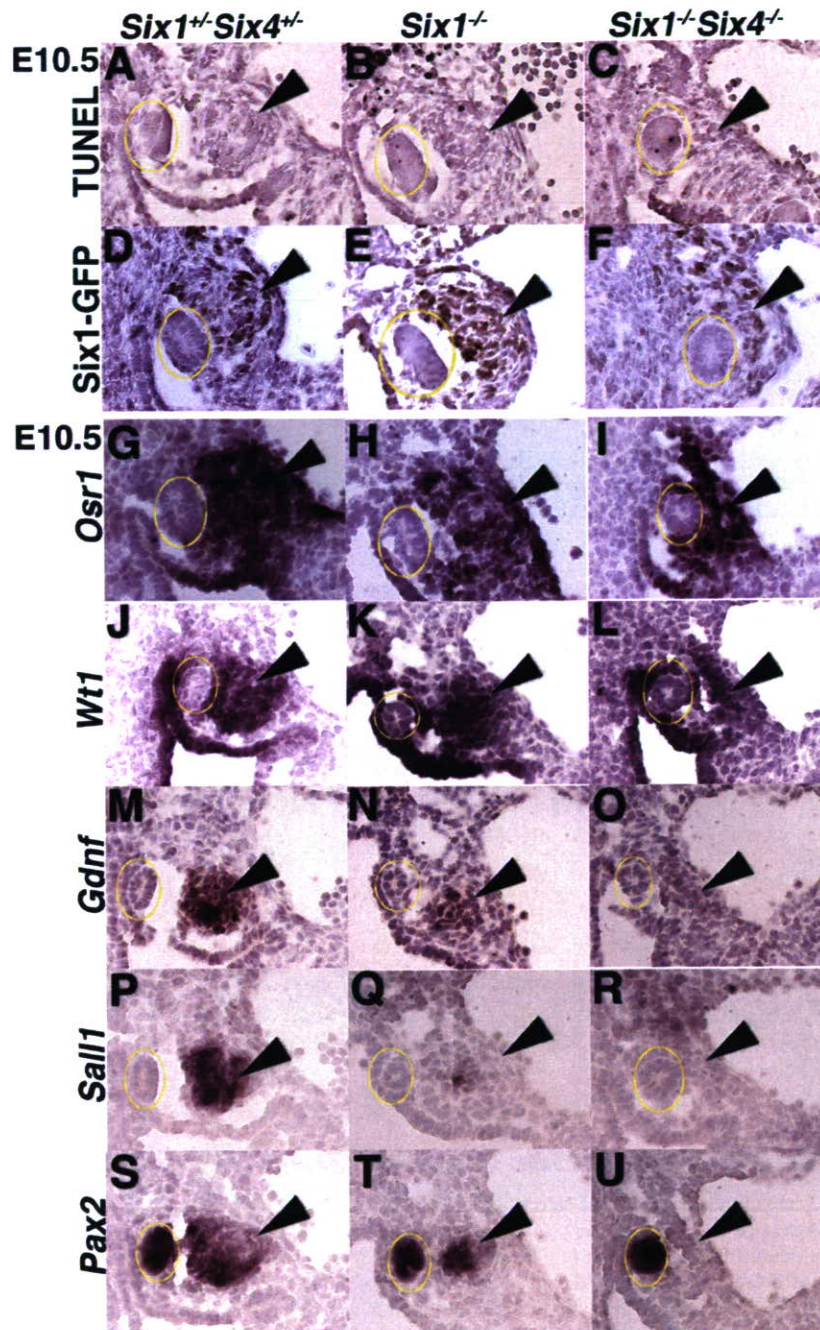


Fig. 3. *Pax2*, *Sall1*, and *Gdnf* expression is absent in the *Six1/Six4*-deficient metanephric mesenchyme. (A–C) TUNEL analysis of the metanephric region in the E10.5 *Six1/Six4*-heterozygous (A), *Six1*-deficient (B), and *Six1/Six4*-deficient (C) embryos. TUNEL-positive cells were not detected in the *Six1/Six4*-deficient metanephric mesenchyme (arrowhead). (D–F) EGFP staining in the metanephric region in the E10.5 *Six1/Six4*-heterozygous (D), *Six1*-deficient (E), and *Six1/Six4*-deficient (F) embryos. EGFP was knocked in the *Six1* locus. EGFP-positive cells were detected in the *Six1/Six4*-deficient metanephric mesenchyme. (G–I) In situ hybridization of *Osr1* in the metanephric region of the E10.5 *Six1/Six4*-heterozygous (G), *Six1*-deficient (H), and *Six1/Six4*-deficient (I) embryos. *Osr1* expression in the metanephric mesenchyme was unaffected in the *Six1/Six4*-deficient embryos. (J–L) *Wt1* expression in the metanephric region of the E10.5 *Six1/Six4*-heterozygous (J), *Six1*-deficient (K), and *Six1/Six4*-deficient (L) embryos. *Wt1* expression was intact in the mutant mice. (M–O) *Gdnf* expression in the metanephric region of the E10.5 *Six1/Six4*-heterozygous (M), *Six1*-deficient (N), and *Six1/Six4*-deficient (O) embryos. *Gdnf* expression was reduced in the *Six1*-deficient embryos and absent in the *Six1/Six4*-deficient mesenchyme. (P–R) *Sall1* expression in the metanephric region of the E10.5 *Six1/Six4*-heterozygous (P), *Six1*-deficient (Q), and *Six1/Six4*-deficient (R) embryos. *Sall1* expression was reduced in the *Six1*-deficient embryos and absent in the *Six1/Six4*-deficient mesenchyme. (S–U) *Pax2* expression in the metanephric region of the E10.5 *Six1/Six4*-heterozygous (S), *Six1*-deficient (T), and *Six1/Six4*-deficient (U) embryos. *Pax2* expression was reduced in the *Six1*-deficient mesenchyme and absent in the *Six1/Six4*-deficient mesenchyme. *Pax2* expression in the Wolffian duct remained intact in the mutant mice. The yellow circle indicates the Wolffian duct.

mesoderm (Bouchard et al., 2002). *Pax8* expression was not detected in the metanephric region at E9.5 or E10.5, while it was detected in the mesonephric region (Fig. 4A–F). However, at E10.0, *Pax8* was expressed in the metanephric mesen-

chyme and Wolffian duct (Fig. 4E, arrowhead) as well as in the nephrogenic cord and Wolffian duct in the mesonephric region (Fig. 4B, arrow). These results show that *Pax8* was transiently expressed in the metanephric region at E10.0.

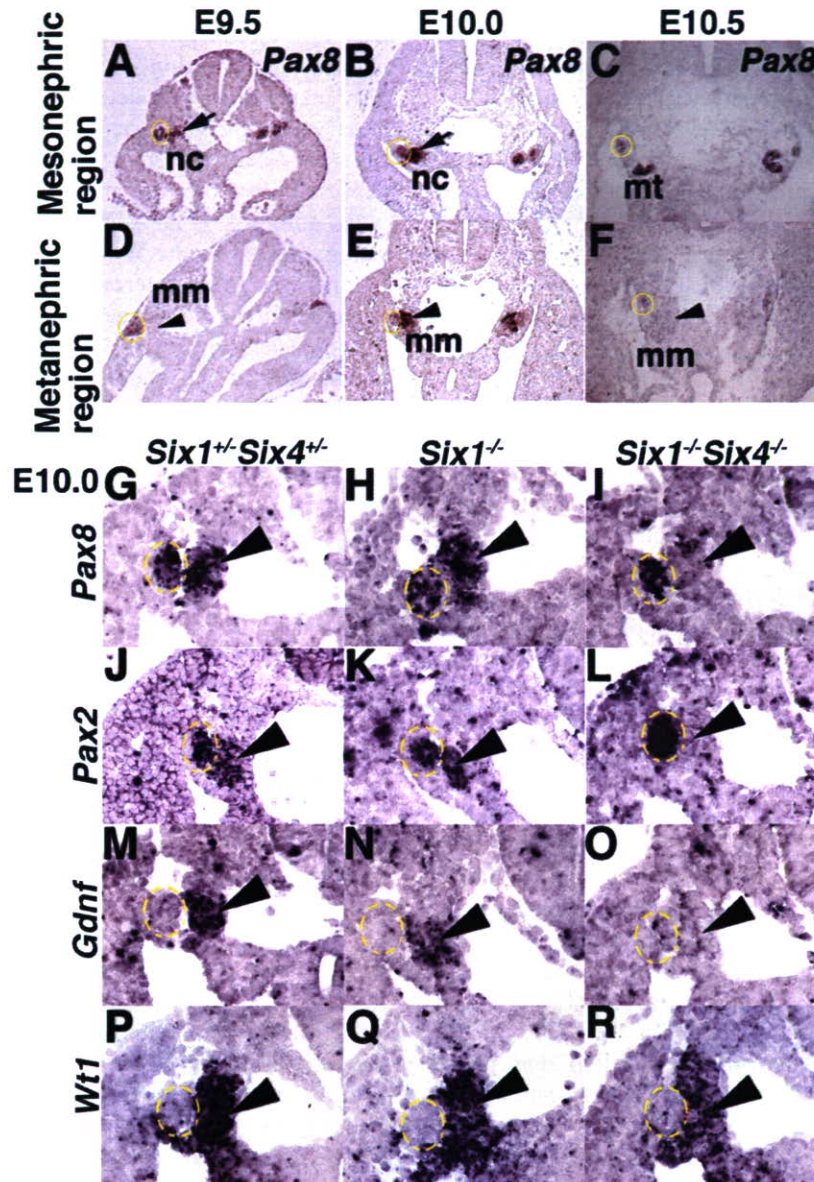


Fig. 4. *Pax8* expression is absent in the metanephric mesenchyme in *Six1/Six4*-deficient mice. (A–F) Transient expression of *Pax8* in the metanephric mesenchyme. *Pax8* expression in either the mesonephric (A–C) or the metanephric region (D–F) of the wild-type embryos at E9.5 (A and D), E10.0 (B and E), and E10.5 (C and F). *Pax8* expression was detected in the nephrogenic cord (arrow) and Wolffian duct (yellow circle) in the E9.5 (A) and E10.0 (B) embryos, and it was also detected in the mesonephric tubules and Wolffian duct in the E10.5 embryos (C). *Pax8* was not expressed in the metanephric mesenchyme (arrowhead) at E9.5 (D) and E10.5 (F), while it was expressed in the metanephric mesenchyme at E10.0 (E). (G–I) *Pax8* expression in the E10.0 *Six1/Six4*-heterozygous (G), *Six1*-deficient (H), and *Six1/Six4*-deficient (I) embryos. *Pax8* was expressed in the metanephric mesenchyme (arrowhead) and Wolffian duct (yellow circle) in the *Six1/Six4*-heterozygous (G) and *Six1*-deficient (H) embryos but not in the mesenchyme in the *Six1/Six4*-deficient (I) embryos. (J–L) *Pax2* expression in the E10.0 *Six1/Six4*-heterozygous (J), *Six1*-deficient (K), and *Six1/Six4*-deficient (L) embryos. *Pax2* was expressed in the metanephric mesenchyme and Wolffian duct in the *Six1/Six4*-heterozygous (J) and *Six1*-deficient (K) embryos but not in the mesenchyme in the *Six1/Six4*-deficient embryos (L). (M–O) *Gdnf* expression in the E10.0 *Six1/Six4*-heterozygous (M), *Six1*-deficient (N), and *Six1/Six4*-deficient (O) embryos. *Gdnf* expression was detected in the metanephric region in the *Six1/Six4*-heterozygous (M) and *Six1*-deficient (N) embryos but not in the *Six1/Six4*-deficient (O) embryos. (P–R) *Wt1* expression in the metanephric region of the E10.0 *Six1/Six4*-heterozygous (P), *Six1*-deficient (Q), and *Six1/Six4*-deficient (R) embryos. *Wt1* expression was maintained in the mutant mice (nc, nephrogenic cord; mt, mesonephric tubules; mm, metanephric mesenchyme).

Next, we examined *Pax8* expression in the mutant embryos at E10.0. Similar to *Pax8* expression in the wild type, it was expressed in the Wolffian duct and metanephric mesenchyme in the *Six1/Six4*-heterozygous and *Six1*-deficient embryos (Fig. 4G and H). In contrast, *Pax8* expression was absent in the mesenchyme but not in the Wolffian duct in the *Six1/Six4*-deficient embryos (Fig. 4I). Similar to the *Pax2* and *Gdnf* expression detected in the embryos examined at E10.0, the expression of these genes, although low, was also detected in the *Six1*-deficient embryos (Fig. 4K and N). However, it was completely absent in the *Six1/Six4*-deficient embryos (Fig. 4L and O). In contrast, *Wt1* expression was unaffected in both the *Six1*-deficient and *Six1/Six4*-deficient embryos (Fig. 4P–R). These results suggest that *Six1* and *Six4* regulate *Pax8* as well as *Pax2*, which may possibly explain the exacerbated metanephric phenotypes of the *Six1/Six4*-deficient embryos.

### 2.5. *Six1* deficiency alone causes defects in the caudal mesonephric tubules

*Pax2* and *Pax8* are known to be expressed in the mesonephric tubules of mouse embryos (Bouchard et al., 2002; Carroll et al., 2005). Therefore, we examined mesonephric-tubule formation by performing HE staining of sagittal sections of the E10.5 embryos. Mesonephric tubules were histologically detected in the *Six1/Six4*-heterozygous embryos (Fig. 5A). In contrast, they were not detected in the *Six1*- and *Six1/Six4*-deficient embryos (Fig. 5B and C). To confirm these findings, we performed whole-mount in situ hybridization to detect *Lim1*, which is expressed in the mesonephric tubules and Wolffian duct (Fujii et al., 1994). Mesonephric tubules and the Wolffian duct were detected in the *Six1/Six4*-heterozygous embryos (Fig. 5D). In contrast, caudal mesonephric tubules (black arrow), which are formed in the nephrogenic cord on induction by the Wolffian duct, could not be detected in the *Six1*- and *Six1/Six4*-deficient embryos (Fig. 5E and F); however, cranial tubules (white arrowhead), which develop as an outgrowth from the Wolffian duct, were detected in these embryos. To eliminate the possibility that mesonephric-tubule formation is merely delayed, we also examined the embryos at E11.5. Although mesonephric tubules were detected in the *Six1/Six4*-heterozygous embryos (Fig. 5G), only few tubules were detected in the *Six1*- and *Six1/Six4*-deficient embryos (Fig. 5H and I). To confirm these results, we performed staining for E-cadherin, which is expressed in the mesonephric tubules and Wolffian duct. Mesonephric tubules were detected along the Wolffian duct in the *Six1/Six4*-heterozygous embryos (Fig. 5J). In contrast, caudal mesonephric tubules (black arrow) were not formed in the *Six1*- and *Six1/Six4*-deficient embryos, while cranial mesonephric tubules (white arrowhead), which are derived from the Wolffian duct, were present (Fig. 5K and L). These results indicate that *Six1* deficiency alone causes mesonephric-tubule defects

and that the *Six1/Six4*-deficient embryos exhibit a phenotype comparable to that of the *Six1*-deficient embryos.

### 2.6. *Pax2* expression is absent in the nephrogenic cord in *Six1*-deficient mice

Next, we investigated the expression of the genes essential for mesonephric-tubule formation by performing in situ hybridization in the nephrogenic cord at E9.5. *Osr1* expression was detected in the *Six1/Six4*-heterozygous embryos and was maintained in the *Six1*- and *Six1/Six4*-deficient embryos (Fig. 6A–C). *Wt1* was also expressed in both the mutants and *Six1/Six4*-heterozygous embryos (Fig. 6D–F). In contrast, *Pax2*, which was expressed in the Wolffian duct and nephrogenic cord in the *Six1/Six4*-heterozygous embryos, could not be detected in the nephrogenic cord of the *Six1*- and *Six1/Six4*-deficient embryos. However *Pax2* expression was maintained in the Wolffian duct (Fig. 6G–I). *Pax8* was also detected in the Wolffian duct and nephrogenic cord in the *Six1/Six4*-heterozygous embryos and the *Six1*-deficient embryos retained *Pax8* expression in the nephrogenic cord, while the *Six1/Six4*-deficient cords lacked the expression (Fig. 6J–L). These results suggest that *Pax2* and *Pax8* are regulated by *Six1* and *Six4* in the nephrogenic cord, similar to the metanephric mesenchyme. However, *Six1*-mediated *Pax2* activation but not *Six1/Six4*-dependent *Pax8* upregulation may play a central role in mesonephric tubule formation, unlike the situation in the metanephros. This is because *Six1* deficiency alone is sufficient to affect *Pax2* expression and cause mesonephric-tubule defects. *Wnt9b*, which was expressed in the Wolffian duct, was unaffected in both mutant embryos (Fig. 6M–O); this indicates that the kidney phenotypes could not be accounted for by *Wnt9b* deficiency in the Wolffian duct.

## 3. Discussion

In this paper, we demonstrate that *Six1* and *Six4* have redundant roles in kidney development, and that inactivation of both genes leads to more severe metanephric phenotypes than those of *Six1*-deficient mice. In the *Six1/Six4*-deficient mice, a distinct cell cluster is absent in the metanephric mesenchyme, and the ureteric bud is not formed. However, both these components are present in the *Six1*-deficient mice, although they are impaired. In contrast, mesonephric tubules are absent in both the *Six1*- and *Six1/Six4*-deficient mice, suggesting that *Six4* plays a minor role in mesonephros formation.

The expression of *Pax2* and *Gdnf* is partially lowered in the *Six1*-deficient mice and absent in the *Six1/Six4*-deficient mice. In contrast, *Pax8* expression is absent only in the double mutant mice. These results indicate that *Pax2* and *Gdnf* are regulated mainly by *Six1* and partially by *Six4*, while *Pax8* expression is maintained when either *Six1* or *Six4* is present. *Sall1* is almost absent in the

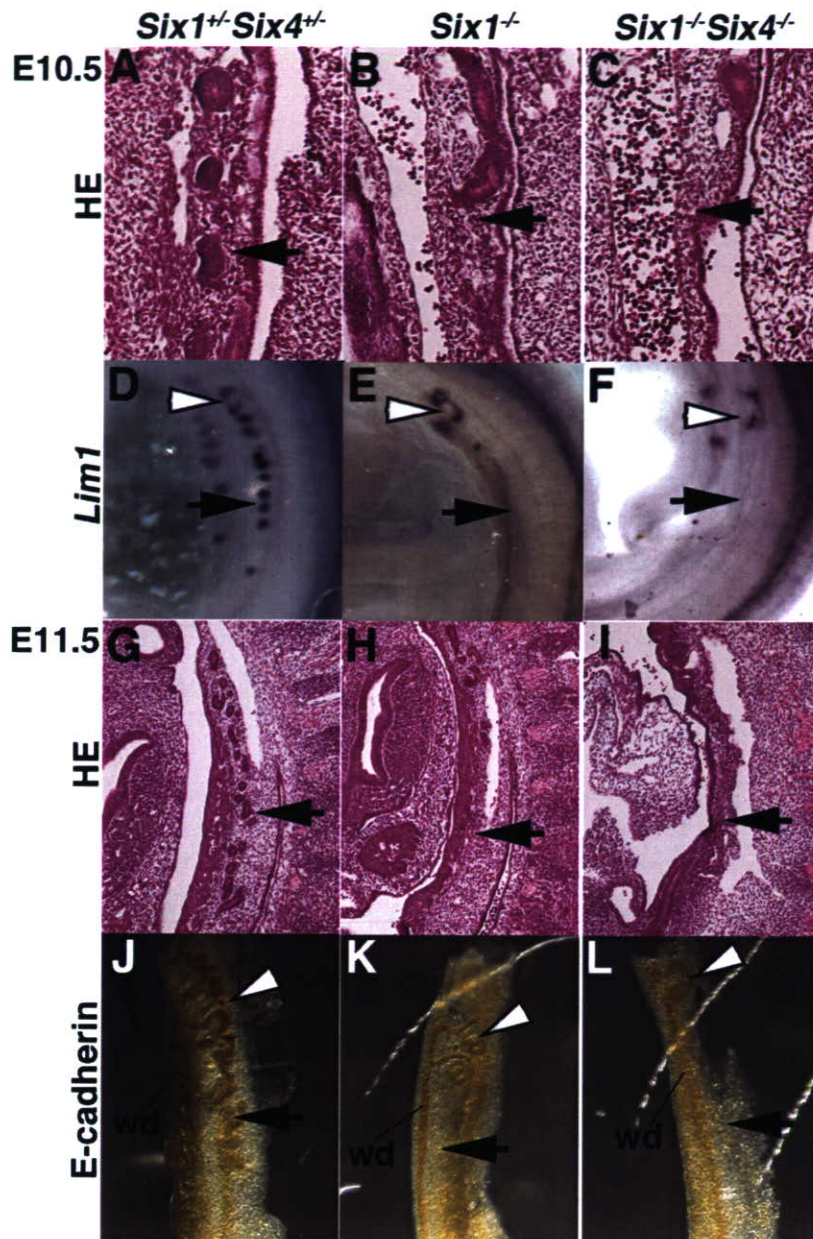


Fig. 5. Caudal mesonephric tubules are not formed in the *Six1*- and *Six1/Six4*-deficient embryos. (A–C) HE staining of the sagittal sections of the mesonephric region of the E10.5 *Six1/Six4*-heterozygous (A), *Six1*-deficient (B), and *Six1/Six4*-deficient (C) embryos. Several mesonephric tubules (arrow) were observed in the *Six1/Six4*-heterozygous embryos (A) but they were fewer in the *Six1*- (B) and *Six1/Six4*-deficient embryos (C). (D–F) Whole-mount in situ hybridization of *Lim1* in the mesonephros of the E10.5 *Six1/Six4*-heterozygous (D), *Six1*-deficient (E), and *Six1/Six4*-deficient (F) embryos. *Lim1*-positive mesonephric tubules were observed lined in the craniocaudal direction in the *Six1/Six4*-heterozygous embryos (D), while *Lim1*-positive caudal mesonephric tubules (black arrow) were not observed in the *Six1*- (E) and *Six1/Six4*-deficient (F) embryos. (G–I) HE staining of the sagittal sections of the mesonephric region of the E11.5 *Six1/Six4*-heterozygous (G), *Six1*-deficient (H), and *Six1/Six4*-deficient (I) embryos. Many mesonephric tubules were observed in the *Six1/Six4*-heterozygous embryos (G), while few were observed in the *Six1*- (H) and *Six1/Six4*-deficient embryos (I). (J–L) Whole-mount immunostaining of E-cadherin, which is expressed in the mesonephric tubules and the Wolffian duct, in the mesonephros of the E11.5 *Six1/Six4*-heterozygous (J), *Six1*-deficient (K), and *Six1/Six4*-deficient (L) embryos. Many mesonephric tubules were observed lined in the craniocaudal direction in the *Six1/Six4*-heterozygous embryos (J), while caudal mesonephric tubules were not observed in the *Six1*- (K) and *Six1/Six4*-deficient embryos (L). The white arrowhead indicates the cranial mesonephric tubules (wd, Wolffian duct).

*Six1*-deficient mice, and *Six4* may play a minimal role in the expression of this gene. This differential regulation could result either from the difference in the amount of the two corresponding gene products in the tissue or from

the binding specificity of the DNA-binding domains to the target sequences. Previously, we reported that the promoter region of the *sodium-potassium-chloride cotransporter 1 (Scl1 2a2)* gene is a common target of *Six1* and *Six4*,

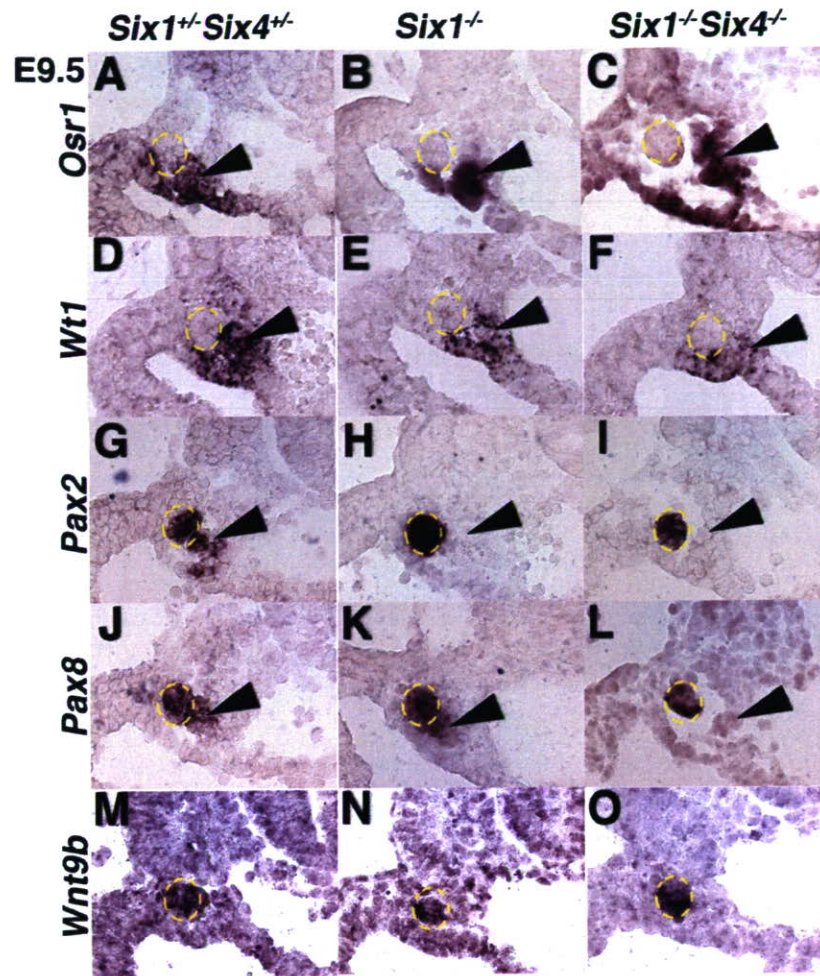


Fig. 6. *Pax2* expression is absent in the nephrogenic cord in *Six1*-deficient embryos. (A–C) In situ hybridization of *Osr1* in the mesonephric region of the E9.5 *Six1/Six4*-heterozygous (A), *Six1*-deficient (B), and *Six1/Six4*-deficient (C) embryos. *Osr1* expression in the nephrogenic cord (arrow) remained intact in the *Six1*- (B) and *Six1/Six4*-deficient embryos (C). (D–F) *Wt1* expression in the mesonephric region of the *Six1/Six4*-heterozygous (D), *Six1*-deficient (E), and *Six1/Six4*-deficient (F) embryos at E9.5. *Wt1* expression in the nephrogenic cord (D) remained intact in the *Six1*- (E) and *Six1/Six4*-deficient (F) embryos. (G–I) *Pax2* expression in the mesonephric region of the E9.5 *Six1/Six4*-heterozygous (G), *Six1*-deficient (H), and *Six1/Six4*-deficient (I) embryos. *Pax2* expression in the nephrogenic cord was absent in the *Six1*- (H) and *Six1/Six4*-deficient (I) embryos. (J–L) *Pax8* expression in the mesonephric region of the *Six1/Six4*-heterozygous (J), *Six1*-deficient (K), and *Six1/Six4*-deficient (L) embryos at E9.5. *Pax8* expression in the nephrogenic cord is absent in the *Six1/Six4*-deficient embryos (L) but not in the *Six1*-deficient embryos (K). (M–O) *Wnt9b* expression in the mesonephric region of the E9.5 *Six1/Six4*-heterozygous (M), *Six1*-deficient (N), and *Six1/Six4*-deficient (O) embryos. *Wnt9b* expression in the Wolffian duct (yellow circle) was unaffected in the mutant embryos.

and that this region contains multiple *Six1*-binding sites and one common binding site for *Six1* and *Six4* (Ando et al., 2005). A similar mechanism may also underlie the differential regulation of common target genes during kidney development.

The *Six* gene family has a consensus binding site, and *Six1* is reported to bind to the first intron of *Gdnf* (Li et al., 2003) – a key attractant for ureteric budding. Further, *Pax2* binds to the 5'-untranslated region of *Gdnf* exon 1 (Brophy et al., 2001). *Six2* also binds to the *Gdnf* promoter region and activates *Gdnf* expression (Brodbeck et al., 2004). However, *Six2* expression is absent in *Six1*-deficient mouse embryos (data not shown; Xu et al., 2003). These results are consistent with the findings that

both the *Six1*- and *Pax2*-deficient mice show incomplete ureteric-bud invasion into the mesenchyme (Brophy et al., 2001; Li et al., 2003). Though the mechanism underlying the direct binding of *Six4* to the promoters remains to be elucidated, *Six1* and *Six4* may regulate *Gdnf* expression in two ways – directly or indirectly. The former is executed by binding to the *Gdnf* promoter and the latter, via *Pax2* and *Pax8*. Thus, inactivation of *Six1* and *Six4* should lead to a complete absence of *Gdnf*, a critical factor for ureteric bud formation. This reveals a redundancy in the *Six*-*Pax*-*Gdnf* pathway. *Eyal* is a cofactor for *Six1*-dependent transcriptional activation, and the loss of *Eyal* in mice leads to a metanephric phenotype similar to that of *Six1/Six4*-deficient mice and more severe than that of

the *Six1*-deficient mice; *Pax2* and *Gdnf* expression is completely absent in this phenotype (Xu et al., 1999; Nica et al., 2006). Hence, it is possible that both *Six1* and *Six4* cooperate with *Eya1*, thus regulating *Pax2/Pax8* and *Gdnf* expression.

Mesonephric tubules were absent in both the *Six1*- and *Six1/Six4*-deficient mice. Although several researchers have reported metanephric abnormalities in *Six1*-null mice (Laclef et al., 2003a; Li et al., 2003; Xu et al., 2003), this is the first report of mesonephric phenotypes in these mice. *Pax2* was absent in the *Six1*- and *Six1/Six4*-deficient mice, while *Pax8* remained undetected only in the *Six1/Six4*-deficient mice. This resembled a cascade similar to that occurring during metanephros formation. However, the absence of *Six4* did not exacerbate the defects observed in *Six1* deficiency; this suggests that a reduction in *Pax8* expression does not contribute to the lack of mesonephric tubules and that *Pax2* expression may play a major role in this process. Consistent with this finding, Torres et al. (1995) reported that mesonephric-tubule formation is impaired in *Pax2*-deficient mice. Thus, *Six1*-dependent *Pax2* activation in the nephrogenic cord (a mesenchymal tissue) could be a prerequisite for mesonephric-tubule formation, which involves a mesenchymal-to-epithelial transition induced by signals, including *Wnt9b* from the Wolffian duct (Carroll et al., 2005).

*Pax2* and *Pax8* are expressed in the intermediate mesoderm, and these two genes must play a major role in directing the intermediate mesoderm toward nephrogenic lineage because mice deficient in both these genes lack Wolffian-duct formation (Bouchard et al., 2002). In contrast, in *Six1/Six4*-null mice, direction toward kidney lineage occurs, although *Pax2* and *Pax8* expression is abolished. This apparent inconsistency can be explained by studying the expression domains of these genes. *Six1* and *Six4* are expressed in the nephrogenic mesenchyme (nephrogenic cord and metanephric mesenchyme) but not in the Wolffian duct, while *Pax2* and *Pax8* are expressed in each of these intermediate mesoderm-derived components. In the absence of *Six1* and *Six4*, *Pax2* and *Pax8* expression is reduced in the nephrogenic mesenchyme but not in the Wolffian duct (Fig. 6T, L). Therefore, *Six1/Six4*-null mice tend to exhibit mesenchyme-specific lowering of *Pax2* and *Pax8* expression; thus, they exhibit a milder phenotype than conventional *Pax2/Pax8*-deficient mice. In the mesenchyme, *Six1* and *Six4* are located upstream of *Pax2* and *Pax8*, as mentioned in this paper. This cascade does not exist in the Wolffian duct. Instead, *Pax2/Pax8*-dependent *Gata3* activation is reported to regulate migration of the Wolffian duct toward the cloaca. In *Gata3*-null mice, mesenchymal migration is maintained, and *Pax2* expression in the mesenchyme (and possibly the expression of its upstream regulators *Six1* and *Six4*) is not affected (Grote et al., 2006). Thus, the *Six-Pax* cascade in the mesenchyme and the *Pax-Gata3* pathway in the Wolffian duct may operate independently.

Our data place *Six1* and *Six4* upstream of *Pax2* and *Pax8* in the mesenchymal tissues in both the metanephros and mesonephros. In addition, our data further elaborates on the findings of Xu et al., revealing that *Six1* is located upstream of *Pax2* during metanephric development (Xu et al., 2003). This *Six-Pax* cascade is distinct from the situation in *Drosophila*, in which *sine oculis* (*Six* homolog) is located downstream of *eyeless* (*Pax* homolog). However, the *Six-Pax* cascade is conserved during mammalian muscle development, where *Six1* and *Six4* are located upstream of *Pax3* and *Met* and regulate myogenic migration in somites (Grifone et al., 2005). Then how are *Six1* and *Six4* regulated in the nephrogenic mesenchyme? A recent study predicted the TCF4- and Gli-binding sites in the promoters of *Six1* and *Six4* by using computational methods, suggesting that Wnt and hedgehog may regulate the *Six* genes (Hallikas et al., 2006). In fact, *Six1* expression in the limb buds is reduced in *Shh* mutants (Bonnin et al., 2005). In the metanephros, *Shh* deficiency leads to a reduction in *Pax2* and *Sall1* genes downstream of *Six1* (Hu et al., 2006). Thus, an analysis using mutant mice that lack the candidate soluble factors may further reveal the details of the *Six-Pax* cascade that controls kidney formation.

Finally, *Six1* mutation is reported to be associated with the branchio-oto-renal syndrome, which leads to kidney or urinary tract malformation (Ruf et al., 2004). In addition, a deletion at 14q22–23 that overlaps the *SIX1* and *SIX4* loci causes multiple abnormalities, including renal hypoplasia (Bennett et al., 1991). Thus, an analysis of *Six1/Six4*-deficient mice may be useful for elucidating the mechanism underlying these diseases.

## 4. Experimental procedures

### 4.1. Generating mutant mice and confirming their genotype

The procedure for generating *Six1*- and *Six1/Six4*-deficient mice has been previously described (Ozaki et al., 2004; Konishi et al., 2006). *Six1/Six4*-deficient mice carry a targeted in-frame fusion of the *EGFP* gene into the first coding exon of the *Six1* gene and that of the *LacZ* gene into the first coding exon of the *Six4* gene. Mice and embryos from subsequent generations were genotyped by PCR. The *Six1* mutant allele was detected using the primers WtmSix1F (5'-GCGCCCGGGCCCGTGC GCCCC-3') and KOmSix1R (5'-TGCCCCAGGATGTTGCCGTCC-3'), and the wild-type allele was detected using the primers WtmSix1F and WtmSix1R (5'-GCTTTCAGCCACAGCTGCTGC-3'). The length of the PCR products was 323 and 470 bp in the wild-type and mutant alleles, respectively. The *Six4* mutant allele was detected using the primers WtmSix4F (5'-ACATCAAGCAGGAGAATGGGATGG-3') and KOmSix4R (5'-CCGTAATGGGATAGGTTACGTTGG-3'), and the wild-type allele was detected using the primers WtmSix4F and WtmSix4R (5'-AGAAGTTCGAGTGGAGTTGTACC-3'). The length of the PCR products was 212 and 445 bp in the wild-type and mutant alleles, respectively.

### 4.2. Histological examination

The embryos were fixed in 10% formalin in phosphate-buffered saline (PBS). The dehydrated specimens were embedded in paraffin wax and subsequently cut into 6- $\mu$ m thick serial sections, de-waxed, and then stained with HE.

### 4.3. RNA in situ hybridization

The embryos were fixed by incubating them at 4 °C overnight in 10% formalin in PBS. After washing the samples with PBS containing 30% sucrose, they were embedded in OCT compound (Tissue-Tek), frozen, and cut into 10- $\mu$ m thick serial cryosections. In situ hybridization was performed using digoxigenin-labeled antisense riboprobes, as described previously (Nishinakamura et al., 2001). We used the TSA-amplification kit (PerkinElmer) when required. For whole-mount in situ hybridization, the dissected urogenital tissues were fixed by incubating them at 4 °C overnight in 10% formalin in PBS; they were then dehydrated using a methanol series. Prior to hybridization, the specimens were treated with 20  $\mu$ g/ml proteinase K (Roche) for 15 min, followed by hybridization with a labeled RNA probe at 68 °C overnight (1  $\mu$ g/ml). Post hybridization, the specimens were washed twice with 0.1% CHAPS/2 $\times$ SSC at 68 °C for 20 min each time; they were then incubated with RNase A (20  $\mu$ g/ml; 2 $\times$ SSC; 37 °C; 30 min) and washed twice with 0.1% CHAPS/0.2 $\times$ SSC at 68 °C for 20 min each time. Further, the specimens were blocked with PBS containing 10% sheep serum and 0.1% Triton X-100 and incubated with alkaline phosphatase-conjugated anti-digoxigenin antibody (1:2000 in PBT, Roche) overnight at 4 °C. The samples were developed by using NBT and BCIP. We used the cDNA of the following genes as in situ hybridization probes: *Pax2*, *WT1*, *Sall1*, *Gdnf* (Nishinakamura et al., 2001), and *Wnt9b* (a kind gift from A.P. McMahon). The cDNA for other probes was isolated by PCR, subcloned into pBluescript KS(-), and sequenced. None of the sense probes produced signals.

### 4.4. Whole-mount immunohistochemistry

The dissected urogenital tissues were fixed in 10% formalin in PBS and treated with 0.3% H<sub>2</sub>O<sub>2</sub> in methanol for 60 min. After washing the specimens with PBT (PBS with 1% Triton X-100), they were incubated at 4 °C for 2 h in a blocking solution containing 1% skim milk and 1% normal goat serum in PBT. The specimens were then incubated at 4 °C for 16 h with a mouse primary antibody against E-cadherin (1:1000 in PBT, Becton Dickinson). They were then washed with PBT and incubated at 4 °C for 16 h with a peroxidase-conjugated goat secondary antibody against rabbit IgG (1:500 in PBT, KPL). The samples were developed by incubating them for 30 min with 0.2 mg/ml DAB and 0.01% H<sub>2</sub>O<sub>2</sub> in PBS. The specimens were fixed in 10% formalin in PBS and cleaned with 75% glycerol for microscopic observation.

### 4.5. TUNEL analysis

The cryosections were fixed in 1% paraformaldehyde in PBS and permeabilized with 30% acetic acid in ethanol for 5 min at -30 °C. TUNEL analysis was performed by using the Apoptag In Situ Cell Death Detection Kit (Chemicon). In brief, fragmented DNA in apoptotic cells was end-labeled with digoxigenin, and the labeled DNA was detected using a peroxidase-conjugated anti-digoxigenin antibody, stained using a metal-enhanced DAB kit (Pierce), and counterstained with hematoxylin.

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# Stem cells in the embryonic kidney

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The mammalian kidney, the metanephros, is formed by a reciprocally inductive interaction between two precursor tissues, the metanephric mesenchyme and the ureteric bud. The ureteric bud induces the metanephric mesenchyme to differentiate into the epithelia of glomeruli and renal tubules. Multipotent renal progenitors that form colonies upon *Wnt4* stimulation and strongly express *Sall1* exist in the metanephric mesenchyme; these cells can partially reconstitute a three-dimensional structure in an organ culture setting. *Six2* maintains this mesenchymal progenitor population by opposing *Wnt4*-mediated epithelialization. Upon epithelial tube formation, *Notch2* is required for the differentiation of proximal nephron structures (podocyte and proximal tubules). In addition, the induction methods of the intermediate mesoderm, the precursor of the metanephric mesenchyme, begin to be elucidated. If derivation of metanephric mesenchyme becomes possible, we will be closer to the generation and manipulation of multiple cell lineages in the kidney.

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## STRATEGY TOWARD KIDNEY RECONSTITUTION USING PROGENITOR CELLS

Stem cells are defined by two criteria: self-renewal and multipotency. Few reports in the kidney field have addressed both of these criteria at a clonal level, so it is better to use the term 'progenitor' rather than 'stem cells.' In this review, renal progenitors in the embryonic kidney, not those in the adult kidney, from the viewpoint of developmental biology and stem/progenitor cell biology will be discussed. To generate multiple cell lineages for kidney regeneration, the identification of renal progenitors is a prerequisite. Furthermore, there exist three obstacles to be overcome: (1) derivation of the renal progenitors; (2) expansion of the renal progenitors; and (3) control of lineage commitment of the renal progenitors toward differentiated cell types. Recent studies are beginning to elucidate the molecular bases of these processes and will hopefully be able to get closer to the goal of kidney regeneration.

## METANEPHRIC MESENCHYME AS A PROGENITOR CELL POPULATION

The mammalian kidney, the metanephros, is formed by a reciprocally inductive interaction between two precursor tissues, the metanephric mesenchyme and the ureteric bud. Upon induction by the ureteric bud, the metanephric mesenchyme differentiates into the epithelia of glomeruli and renal tubules. Mesenchymal cells sequentially form condensates, renal vesicles, comma (C)- and S-shaped bodies, and terminal epithelia of glomeruli and renal tubules. A previous report retrospectively suggested the presence of multipotent cells in embryonic kidneys, demonstrating that cells in several portions of the nephron were derived from a single stem cell, using *LacZ* gene transduction with a retrovirus into a single cell of the mesenchyme.<sup>1</sup> However, no one has isolated renal progenitor cells with multilineage differentiation potential from the embryonic kidney or has examined their differentiation mechanisms in a single cell culture. There has been a lack of assay systems that specifically identify renal progenitors, as in the cases of the neurosphere method for neural stem cells and the colony assay for hematopoietic progenitors.

## RENAL PROGENITORS IN THE *SALL1*-HIGH POPULATION

Targeted disruption of *Wnt4* results in kidney agenesis and impairs mesenchymal-to-epithelial transition,<sup>2</sup> and co-culture of the mesenchyme with NIH3T3 cells expressing

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*Wnt4* induces tubulogenesis in an organ culture,<sup>3</sup> suggesting both essential and sufficient roles of *Wnt4* in the epithelial differentiation of the metanephric mesenchyme. More recently, *Wnt9b* expressed in the ureteric bud was shown to function upstream of *Wnt4*.<sup>4</sup> Thus, we attempted to set up an assay using *Wnt4*, which could identify and characterize the progenitor cells with multipotent differentiation potential from uninduced metanephric mesenchyme. We previously generated mice in which the green fluorescence protein (*GFP*) gene was knocked into the locus of *Sall1* (*Sall1-GFP* mice), a zinc-finger nuclear factor that is expressed in the metanephric mesenchyme and is essential for kidney development.<sup>5,6</sup> *Sall1* is also expressed in the subventricular zone of the central nervous system and progress zones of limb buds, where neural and mesenchymal stem cells reside, respectively, leading to speculation that *Sall1* might have some association with stem/progenitor cells in several organs, including the kidney. When we plated dissociated mesenchymal cells at a low density in serum-free conditions onto NIH3T3 cells stably expressing *Wnt4*, a single cell formed colonies consisting of several types of epithelial cells that exist in glomeruli and renal tubules.<sup>7</sup> This indicates that the single cell plated initially may be a multipotent renal progenitor, which differentiates into glomerular podocytes and the epithelial cells of renal tubules. We also found that only cells strongly expressing *Sall1*, isolated from *Sall1-GFP* mice, formed colonies and that they partially reconstituted a three-dimensional kidney structure, which contains glomeruli- and tubule-like components in an organ culture setting (Figure 1). Thus, multipotent renal progenitors of epithelial cells do exist and they reside in the *Sall1*-high population of the mesenchyme. The *Sall1-GFP* high fraction constitutes 20–30% of the mesenchymal cells during embryonic development and is distinct from stromal cells and hemangioblasts, as assessed by gene expression patterns. We next generated colonies from *Sall1*-deficient mice to address the role of *Sall1* in the renal progenitors. The numbers of colonies formed were not significantly different among wild-type, heterozygous, and homozygous mesenchyme, suggesting that colony-forming progenitors do exist in the absence of *Sall1*. *Sall1*-null colonies were positive for E-cadherin and marker genes for terminally differentiated epithelia, suggesting that differentiation (including mesenchymal-to-epithelial transformation) may not be impaired in the absence of *Sall1*. By contrast, the size of *Sall1*-deficient colonies was significantly smaller than that of heterozygous or wild-type colonies. Thus, *Sall1* is not required for the generation or differentiation of renal progenitors, but it may be required for the proliferation/survival of the kidney progenitors. As *Sall4*, another member of the *Sall* family of genes, is essential for proliferation of embryonic stem (ES) cells, the *Sall* family may have a common role of stem cell/progenitor proliferation.<sup>8</sup> Thus, our colony-forming assay, which identifies multipotent progenitors of epithelial cells in the embryonic mouse kidney, can be used to examine molecular mechanisms functioning in kidney development. Moreover, the

colony assay will possibly be useful for the identification of renal progenitors induced from a variety of cell sources, such as ES cells.

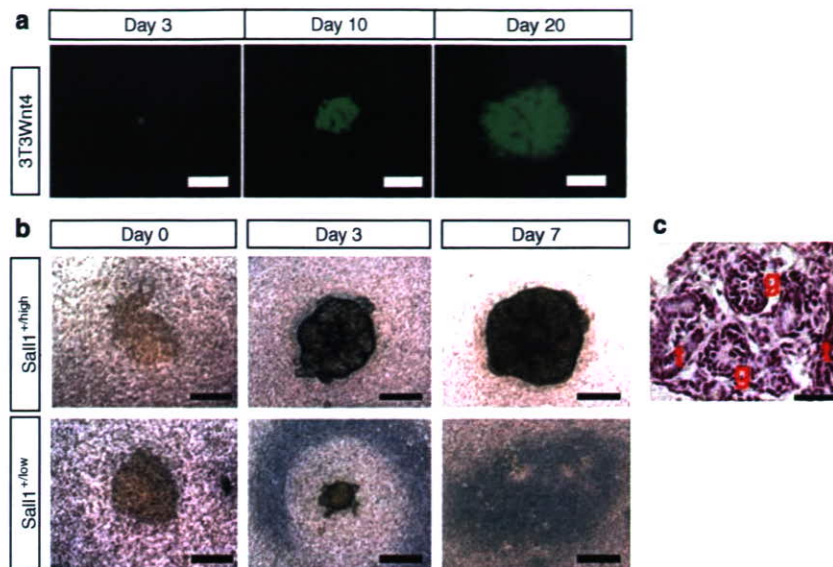
#### IDENTIFICATION OF GENES EXPRESSED IN PROGENITORS BY MICROARRAY ANALYSIS

In the embryonic kidney, *Sall1* is expressed abundantly in mesenchyme-derived structures from undifferentiated mesenchyme, comma-, S-shaped bodies, and renal tubules. We isolated the *GFP*-positive population from embryonic kidneys of *Sall1-GFP* mice by fluorescein-activated cell sorting (FACS) and compared the gene expression profiles of *GFP*-positive (mesenchyme) and *GFP*-negative (ureteric bud) populations using microarray analysis, followed by *in situ* hybridization.<sup>6</sup> We detected many genes known to be important for metanephros development, including *Sall1*, *GDNF*, and *Six2* (which will be discussed later), and genes expressed abundantly in the metanephric mesenchyme, such as *Unc4.1* and *Osr2*. Therefore, the combination of microarray technology and *Sall1-GFP* mice is useful for the systematic identification of genes expressed in the developing kidney.

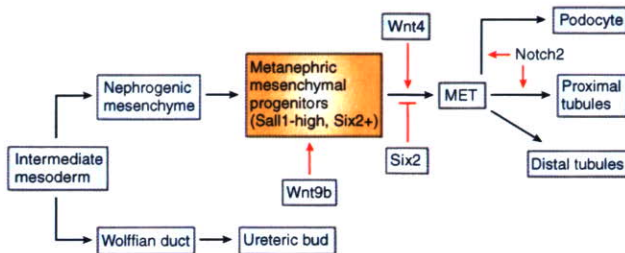
Identification of surface markers expressed in the mesenchymal progenitor population is useful, as it is possible to isolate progenitors from mice that have not been genetically manipulated, and potentially also from humans. Challen *et al.*<sup>9</sup> performed microarray analysis using undifferentiated mesenchyme and found that *CD24* and *cadherin-11* are preferentially expressed in the mesenchyme. Therefore, these may prove useful in the purification of mesenchymal progenitors by FACS.

#### MAINTENANCE OF PROGENITORS BY *Six2*

The *Six* homeobox genes are characterized by conserved Six domains and a homeodomain, both of which are required for specific DNA binding. The prototype of this gene family is *Drosophila sine oculis*, which is essential for compound eye formation. Six members (*Six1–Six6*) of the *Six* gene family have been identified in mice and humans. Among these, *Six2* is expressed in the undifferentiated mesenchyme that caps the ureteric bud, and its expression pattern is reciprocal to that of *Wnt4*, which is expressed near the ureteric stalk. *Wnt4* and the Wnt agonist *sFRP2* are upregulated in the cap portion of the mesenchyme in *Six2*-deficient mice, and ectopic tubulogenesis is observed.<sup>10</sup> In addition, overexpression of *Six2* in mesenchymal cells prevents their epithelial differentiation in an organ culture assay. These results suggest that *Six2* is required to maintain the mesenchymal progenitor population by opposing *Wnt4*-mediated epithelialization. As *Six2* is expressed in *Sall1*-high mesenchymal cells, as described in the previous section, the *Sall1*-high and *Six2*-positive mesenchyme may be a progenitor population in the kidney, and *Six2* might maintain the mesenchyme in an undifferentiated state (Figure 2). It would be interesting to test whether sustained expression of *Six2* maintains the progenitors in our colony assay and also *in vivo*.



**Figure 1 | Metanephric mesenchymal progenitors.** (a) *In vitro* colony formation from the metanephric mesenchyme on feeder cells expressing Wnt4. (b) Only Sall1-GFP<sup>high</sup> cells (upper panels) differentiated into kidney structures in organ culture, whereas Sall1-GFP<sup>low</sup> cells (lower) disappeared. (c) Hematoxylin–eosin staining of sections of Sall1-GFP<sup>high</sup> aggregates at day 10. Tubule- (t) and glomerulus-like structures (g) are seen. Bars: (a) 50, (b) 500, (c) 25  $\mu$ m.



**Figure 2 | Kidney development in view of stem cell biology.** See the text for details. MET, mesenchymal-to-epithelial transition.

### TRANSITION FROM THE PROGENITOR TO TUBULES BY WNT SIGNALING

As described above, *Wnt9b* and *Wnt4* are essential for the epithelial transformation of the mesenchymal progenitor into renal tubules. *Wnt9b* is secreted from the ureteric bud, and consequently *Wnt4* is induced in the mesenchyme where it functions in an autocrine manner on the mesenchyme itself. Downstream of Wnts,  $\beta$ -catenin is likely to play an important role. Removal of  $\beta$ -catenin from *Six2*-positive progenitors blocks the epithelial conversion of the mesenchyme.<sup>11</sup> By contrast, activation of  $\beta$ -catenin in the same cell population partially replaces the requirement for *Wnt9b* and *Wnt4*. However, complete epithelial conversion of mesenchymal progenitors does not occur by  $\beta$ -catenin activation, suggesting that further modulation of canonical signaling will be required for the complete progression of tubulogenesis.

Microarray analysis of cultured rat mesenchyme also shows that  $\beta$ -catenin-dependent signaling is activated and directly regulates *cyclin D1*, *Pax8*, and *Emx2*.<sup>12</sup>  $\beta$ -Catenin activation leads to the survival and proliferation of epithelial

progenitors, but not epithelial conversion, suggesting again that additional pathways downstream of Wnt signaling may cooperate with  $\beta$ -catenin. Indeed Rac- and c-Jun N-terminal kinase-dependent planar cell polarity pathways downstream of *Wnt4* positively regulate the size of the colonies formed from the mesenchyme.<sup>7</sup> Nonetheless, these results enforce the importance of Wnt signaling in the epithelial conversion of the mesenchyme.

### NEPHRON SPECIFICATION BY NOTCH2

The mesenchyme differentiates into epithelia after Wnt stimulation, but a fate decision is required for further differentiation toward glomerular podocytes and proximal or distal renal tubules. *Notch2* is required for the differentiation of proximal nephron structures (podocyte and proximal tubules), as mesenchyme-specific *Notch2* deletion leads to impaired formation of these proximal structures.<sup>13</sup> Although proximodistal polarity is initiated in the absence of *Notch2*, the latter is essential for the final establishment of proximal fates. It remains to be solved whether *Notch2* functions through lateral inhibition or other mechanisms, but elucidation of this process could lead to the cell fate manipulation of kidney progenitors, which should be a useful technology for kidney regeneration: stimulation of progenitors in the mesenchyme by Wnts followed by *Notch2* ligand may result in the generation of the necessary lineages in patients (Figure 2). Interestingly, *Notch1* cannot compensate for *Notch2* deficiency, although *Notch1* activity is present in the kidney and increasing its activity enhances the formation of proximal tubules at the expense of podocytes and distal nephrons.

Of course, there are likely to be many more genes involved in lineage commitment and the differentiation of the many