Murine homolog of SALL1 is essential for ureteric bud invasion in kidney development

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SUMMARY

SALL1 is a mammalian homolog of the Drosophila region-specific homeotic gene spalt (sal); heterozygous mutations in SALL1 in humans lead to Townes-Brock syndrome. We have isolated a mouse homolog of SALL1 (Sall1) and found that mice deficient in Sall1 die in the perinatal period and that kidney agenesis or severe dysgenesis are present. Sall1 is expressed in the metanephric mesenchyme surrounding ureteric bud; homozygous deletion of Sall1 results in an incomplete ureteric bud outgrowth, a failure of tubule formation in the mesenchyme and an apoptosis of the mesenchyme. This phenotype is likely to be primarily caused by the absence of the inductive signal from the ureter, as the Sall1-deficient mesenchyme is competent with respect to epithelial differentiation. Sall1 is therefore essential for ureteric bud invasion, the initial key step for metanephros development.

Key words: Sall1, Kidney, Townes-Brocks syndrome, Mouse

INTRODUCTION

Drosophila sal is the region-specific homeotic gene characterized by unique multiple double-zinc finger motifs (Kuhnlein et al., 1994). sal was first identified by virtue of its capacity to promote terminal differentiation (Jurgens, 1988). It is expressed in anterior and posterior compartments of Drosophila, and mutations in sal cause head and tail segments to develop trunk structures. It also plays a critical role in wing development (de Celis et al., 1996; Nellen et al., 1996). sal is expressed at the anterior/posterior boundary of wing imaginal discs and its expression is controlled by dpp (bone morphogenetic protein 4 ortholog), the expression of which is highest at the boundary, which is in turn controlled by hedgehog expressed in the posterior compartment. Overexpression of dpp broadens the expression domains of sal; hence, sal may be a downstream target of dpp.

Humans have at least three sal-related genes (SALL1, SALL2 and SALL3) (Kohlhase et al., 1996; Kohlhase et al., 1999a). SALL1 is located on chromosome 16q12.1 and heterozygous mutations of SALL1 lead to Townes-Brocks syndrome, an autosomal dominant disease characterized by dysplastic ears, preaxial polydactyly, imperforate anus, and (less commonly) kidney and heart anomalies (Kohlhase et al., 1998). The incidence of kidney abnormality ranges from 20% to 62.5%, depending on the report in question (Kohlhase et al., 1999b; O’Callaghan and Young, 1995). All mutations are localized 5’ to the triple zinc-finger motif and result in premature truncation (Kohlhase et al., 1998; Kohlhase et al., 1999b; Marlin et al., 1999).

The mouse also has three Sal genes. The previously reported msal proved to be a homolog of human SALL3 and was renamed Sall3 (Ott et al., 1996; Kohlhase et al., 1999a). Homologs of SALL1 and SALL2 have also been reported (Buck et al., 2000; Kohlhase et al., 2000).

The kidney develops in three stages: pronephros, mesonephros and metanephros. The nephric duct (Wolffian duct) develops in a cranio-caudal direction from the intermediate mesoderm and acts upon surrounding mesenchyme as an inducer of epithelial transformation to nephric tubules. The pronephric tubules, mesonephric tubules and the anterior portion of the Wolffian duct eventually degenerates; it is the metanephros that becomes the permanent kidney in mammals. Although the pronephros represents a true excretory organ in fish and amphibians, it remains a rudimentary and transient structure in the mouse. The mesonephros appears after and caudal to the pronephros. In metanephros development, the metanephric mesenchyme induces sprouting of the ureteric bud from the caudal region of the Wolffian duct. Signals from the mesenchyme cause further
branching of the ureteric bud, thus forming the kidney collecting system. Reciprocally, the ureteric bud invades the mesenchyme and induces epithelialization and differentiation of the mesenchyme into the nephron (glomerular, proximal tubular and distal tubular epithelium).

Molecular mechanisms of kidney development have been revealed mostly by gene targeting. In Pax2 null mutants (paired-box transcription factor), the Wolffian duct develops only partially and metanephric development does not occur (Torres et al., 1995). In mice deficient for a zinc-finger transcription factor WT-1 (Wilm's tumor suppressor; Wt1 – Mouse Genome Informatics), the ureter never reaches the mesenchyme and consequently the mesenchyme undergoes apoptosis (Kreidberg et al., 1993). WT-1 is considered to be a crucial factor in mesonephros and metanephros (Sato et al., 2000). One of the genes we isolated was a member of the murine Sal family from the developing kidney ( chữa et al., 1996). Thus, the null mutants of GDNF and Ret show reduced nephron development with and without a normal collecting system. Reciprocally, the ureteric bud invades the mesenchyme (Moore et al., 1996; Pichel et al., 1996; Sanchez et al., 1996; Schuchardt et al., 1994). Reciprocal signals from the ureteric bud to the mesenchyme remained unidentified for a long period. Recently, LIF (leukemia inhibitory factor) and its related cytokines were reported to be ureter-derided regulators for mesenchymal-to-epithelial conversion, though mice deficient in the common receptor gp130 show perturbation of ureter invasion (Moore et al., 1996; Pichel et al., 1996; Sanchez et al., 1996; Schuchardt et al., 1994).

Many of the genes expressed in the metanephros are also found in the pronephros. We earlier established an in vitro induction system for pronephros in Xenopus (Moriya et al., 1993; Uochi and Asashima, 1996). Animal caps, a presumptive ectoderm of Xenopus embryos at the blastula stage, differentiate into three-dimensional pronephric tubules in three days in chemically defined saline solution upon treatment with activin and retinoic acid. We have used this system to identify molecules expressed in pronephros and potentially in mesonephros and metanephros (Sato et al., 2000). One of the genes we isolated was Xsal-3, a newly identified sal member of Xenopus, which was expressed in the pronephros and the brain (Onuma et al., 1999). We then cloned a member of the murine Sal family from the developing kidney, which proved to be a mouse homolog of human SALL1. We now report cloning, expression patterns and loss-of-function studies of mouse Sall1. Our data show that murine Sall1 is essential for initial inductive events for kidney development.

**MATERIALS AND METHODS**

**Cloning of Sall1**

Degenerate PCR was carried out using as a template 14.5 days post coitus (dpc) fetal kidney cDNA. Primers were designed for conserved regions between Xsal-3, Xsal-1 and Sall3 (EKPFACCTI and WQQVAAI). The resulting 245 bp product was used to screen 14.5 dpc fetal kidney library. Primers and also for the 129SvJ genomic library (Stratagene). The 5’ portion of Sall1 cDNA upstream of the first zinc finger was cloned by screening a random-primed 14.5 dpc embryo cDNA library, and the same sequence was identified in one of the genomic clones we obtained.

**Interspecific mouse backcross mapping**

Interspecific backcross progeny were generated by mating (C57BL/6J × Mus spretus) F1 females and C57BL/6J males as described (Copeland and Jenkins, 1991). A total of 205 N2 mice were used to map the Sall1 locus. DNA isolation, restriction enzyme digestion, agarose gel electrophoresis, Southern blot transfer and hybridization were essentially as described (Jenkins et al., 1992). The Sall1 probe used was a Clal–EcoRI 1.2 kb fragment (probe B). A fragment of 4.7 kb was detected in Spl1 digested C57BL/6J DNA and a fragment of 6.1 kb was detected in Spl1 digested M. spretus DNA. The presence or absence of the 6.1 kb Spl1 M. spretus-specific fragment was followed in backcross mice.

A description of the probes and RFLPs for the loci linked to Sall1, including Chbn1 and Scyd1, has been reported (Kavety et al., 1994; Rossi et al., 1998). Recombination distances were calculated using Map Manager, version 2.6.5. Gene order was determined by minimizing the number of recombination events required to explain the allele distribution patterns.

**Generation of Sall1-deficient mice**

The Sall1–del targeting vector was constructed by incorporating 5′ Snak–EcoRI 5.4 kb fragment and 3′ HindIII–Clal 2.8 kb fragment into a vector that contained the neomycin-resistant (neo+) gene (pMC1-neo polyA) and a diphertheria toxin A subunit (pMC1-DTA) in tandem. The 5′ fragment was subcloned into a Clal site 5′ of the neo+ gene, and the 3′ fragment was cloned into an EcoRV site 3′ of the neo+ gene. For the Sall1–lacZ construct, NotI–XhoI lacZ fragment (3.7 kb) from pcCNlacZ (obtained from RIKEN, Japan) was fused in frame to 5′ Snak–EcoRI 5.4 kb fragment and the resultant Snak–XhoI 9.1 kb fragment was cloned into the Clal–XhoI site of the vector described above. Both constructs were linearized with NotI.

CCE embryonic stem cells were used for the Sall1–del construct and E14.1 cells (provided by Dr N. Yoshida) were used for the Sall1–lacZ construct. CCE and E14.1 cells were plated in mitomycin C-treated STO cell lines and primary embryonic fibroblasts, respectively, and clones resistant to G418 (250 μg/ml) were screened using lacZ blots or genomic PCR. The primer sequences used for PCR were as follows: GTACAGCTTCCTCAGGAC and TCTCCAGTTGGA-GTTCTCTCG for neo r (420 bp). The sequence was also used to verify that only one copy of the vector was integrated into the genome. Of 118 clones, eight were correctly targeted for Sall1–del, and 15 of 116 were targeted for Sall1–lacZ.

Recipient blastocysts were from C57BL/6N mice. Chimeric animals were bred with C57BL/6N females. Mutant animals studied were of F2 and F3 generation. Mice were genotyped using Southern blots or genomic PCR. The primer sequences used for PCR were as follows: GTACAGCTTCCTCAGGAC and TCTCCAGTTGGA-GTTCTCTCG for Sall1 (200 bp); and AAGGGACTGCTGCTXT-TGG and ATATCAGGGTAGCCAACGC for neo r (420 bp).
Sall1 is essential for kidney development

Many Sall1-lacZ heterozygotes died in the perinatal period without any apparent histological abnormalities. We assume that death was probably due to the toxicity of lacZ, as such lethality was not observed in Sall1-del heterozygotes. The surviving Sall1-lacZ heterozygotes bred normally and resultant homozygous mice showed phenotypes identical to those of Sall1-del homozygous mice.

Histological examination

Samples were fixed in 10% formalin and processed for paraffin-embedded sectioning (6 μm), followed by double staining with Hematoxylin and Eosin. X-gal staining was as described (Koseki et al., 1991). All of the X-gal staining patterns
of heterozygous Sall1-lacZ mice described were reconfirmed by in situ hybridization. For detection of apoptosis, ApopTag Fluorescein Direct Kits (Intergen) were used for paraffin-embedded sections and counterstained with Propidium Iodide. Omission of terminal deoxynucleotidyl transferase gave no background signals.

**In situ hybridization**

In situ hybridization was carried out using digoxigenin-labeled antisense riboprobes. Samples were fixed overnight in 4% paraformaldehyde (PFA), then in sucrose and embedded with OCT compound. Sections were cut, air-dried and then fixed in 4% PFA for 15 minutes at room temperature. After washing with PBS, sections were treated with proteinase K (5 µg/ml for 15 minutes), refixed with PFA, washed with PBS, treated with 0.2 M hydrochloride for 10 minutes and washed again with PBS. Sections were acetylated for 10 minutes, washed with PBS and dehydrated with ethanol. Hybridization mixture (50% formamide, 10 mM Tris-HCl pH 7.6, 200 µg/ml tRNA, 1x Denhardt’s solution, 10% dextran sulfate, 600 mM NaCl, 0.25% SDS, 1 mM EDTA, and the probe) was then applied on top of the section, overlaid with parafilm, and incubated overnight at 70°C in a humidified chamber. Sections were washed with 30 minutes at 70°C, treated with RNase for 30 minutes at 37°C, washed with 2xSSC for 20 minutes at 70°C and washed twice with 0.2xSSC for 20 minutes each at 70°C. Sections were rinsed with buffer 1 (100 mM Tris-HCl pH 7.5, 150 mM NaCl), blocked with 10% sheep serum in buffer 1 for 1 hour and incubated with alkaline phosphatase-conjugated sheep polyclonal antidigoxigenin antibody (Roche) diluted 1:4000 in buffer 1 for 30 minutes. After washing twice with buffer 1, alkaline phosphatase activity was detected in the presence of NBT/BCIP (Roche).

A 800 bp Xhol-Spel fragment of Sall1 cDNA was subcloned into pBluescript II KS- (Stratagene) and a transcript was generated with T7 polymerase. Wnt4 was a gift from Dr Andrew P. McMahon. cDNA for other probes was isolated by PCR, subcloned into pCRII (Invitrogen) and sequenced. None of the sense probes produced any signals.

**Organ culture of metanephric mesenchyme**

Metanephric rudiments are dissected from 11.5 dpc embryos and cultured at air-fluid interface on Transwell (0.4 µm) (Corning) supplied with DMEM plus 10% fetal calf serum. For recombination experiments with spinal cord, mesenchyme at 11.25 dpc was used to minimize apoptosis in the mutants. The mesenchyme was removed from the ureteric bud after 5 minutes incubation with 0.2% collagenase (Sigma) and cultured in direct contact with spinal cord on Transwell. As a specific size of the mesenchyme is required for the efficient response to spinal cord, left and right mesenchyme were combined and cultured.

**GenBank Accession Number**

The DDBJ/EMBL/GenBank Accession number for the Sall1 cDNA sequence reported in this paper is AB051409.
RESULTS

Cloning of a mouse homolog of SALL1 (Sall1)

We isolated a murine sal cDNA encoding 1323 amino acids from a fetal kidney cDNA library (Fig. 1A). There were four characteristic double zinc-finger motifs, the second of which had triple zinc fingers, and it also had a single zinc-finger motif at its N terminus. It had a putative nuclear localization signal in the C-terminal region, and GFP fusion protein was localized exclusively in the nucleus when expressed in COS cells (Fig. 1A and data not shown). This gene was 90% identical at the amino acid level to human SALL1, a causative gene for Townes-Brocks syndrome. Interspecific backcross analysis showed that this mouse gene was localized in the central region of mouse chromosome 8 linked to Cbln1 and Scyd1 (Fig. 1B). This region is homologous to human chromosome 18q and 16q, the latter of which is the location of SALL1, further supporting the assumption that it is a murine homolog of SALL1. Fluorescent in situ hybridization analysis also confirmed that it was localized on mouse chromosome 8 (data not shown). Recently, Buck et al. have reported a murine homolog of SALL1; their clone is practically identical to ours, albeit with minor nucleotide differences (Buck et al., 2000).

Generation of Sall1-deficient mice

To examine developmental functions of this mouse homolog of SALL1, we inactivated it in the mouse using embryonic stem cells (Fig. 2A, B). Sall1 gene consists of three exons, the first intron being approximately 9 kb. Most zinc-finger domains are located in exon 2 and the last 10th zinc-finger domain is separated by a short second intron. We generated two types of targeting constructs: one deleted the entire coding sequence, except for the N-terminal 52 amino acids, thus eliminating all the zinc-finger motifs (Sall1-del); the other deleted the same region and lacZ was fused in frame to N-terminal 52 amino acids (Sall1-lacZ). Homologous recombinants were obtained from both constructs and chimeras from both transmitted the mutations through the germline. Mutant mice from the two constructs showed essentially the same phenotypes. Mice were genotyped by Southern blots and genomic PCR (Fig. 2C, D). RT-PCR revealed the absence of the Sall1 transcript in the homozygous mutant mice (Fig. 2E). In situ hybridization also confirmed the absence of the Sall1 transcript, but not Sall2, in the mutant mice (data not shown).

Expression patterns of Sall1

Whole-mount X-gal staining of Sall1-lacZ heterozygous embryo at 11.5 dpc showed abundant expression of Sall1 in limb buds and a relatively weaker signal in the spinal cord and the brain (Fig. 3A). Staining of 14.5 dpc embryo showed expression in limb buds, anorectal region, developing olfactory bulb, nose and eye (Fig. 3B). Transverse section of 10.5 and 11.5 dpc showed Sall1 staining in ventromedial parts of otic vesicles and endocardium (Fig. 3C,D). In kidney development, Sall1 expression was observed in the nephrogenic primordium at 10.5 dpc (Fig. 3E). At 11.5 dpc, Sall1 was expressed in mesonephric tubules and Wolffian ducts, and in the metanephric mesenchyme surrounding the ureteric bud (Fig. 3F,G), findings that were reconfirmed by in situ hybridization (Fig. 3H). Expression of Sall1 was reduced in the caudal area of the Wolffian duct and was undetectable in ureteric buds. At 14.5 dpc, Sall1 expression was observed in the mesenchyme around the ureteric buds and weakly in comma-shaped bodies of metanephric tubules, but not in glomeruli, in the cortical regions of the developing kidney (Fig. 3I,J). In the newborn, Sall1 staining was observed in kidneys, hearts, livers, brain regions surrounding ventricles and olfactory bulbs (data not shown). All this evidence was reconfirmed by in situ hybridization. Thus, Sall1-lacZ mice serve as useful tools to...
monitor \textit{Sall1} expression and \textit{Sall1} was shown to be expressed in tissues affected in Townes-Brocks syndrome, namely limb, ear, anus, heart and kidney. This expression pattern partly overlaps that of mouse \textit{Sall2} (data not shown) and \textit{msal} (\textit{Sall3}) (Ott et al., 1996; Ott et al., 2001).

**Kidney defects in \textit{Sall1}-deficient mice**

Homozygous mice were born at Mendelian frequency but all died within 24 hours after birth without suckling. Kidney agenesis or severe dysgenesis were present (Fig. 4). Nine out of 28 mice (32.1\%) had no kidneys or ureters, bilaterally (Fig. 4B). Eight mice (28.6\%) had unilateral kidney agenesis and hypoplasia on the other side. Eleven mice (39.3\%) had two small remnant kidneys (Fig. 4C). In either case, the bladder contained no urine. Histological examination of the remnant kidneys showed a disorganized cortical structure, shrunken glomeruli, necrotic proximal tubules and multiple cysts (Fig. 4F,G). Development of other organs including brain, adrenal glands, bladder, testis or ovary was normal. There was no limb deformity, anorectal anomaly or ear anomaly, all characteristic of Townes-Brocks syndrome. The layer structure of olfactory bulbs in \textit{Sall1} mutant newborn mice was slightly disorganized, though adult phenotypes of \textit{Sall1} mutation could not be determined, owing to the lethality of the mutant mice (data not shown).

At day 11.5 of gestation, the ureteric bud invades into metanephric mesenchyme and subsequent reciprocal interaction between these two tissues leads to development of a metanephric kidney (Fig. 5A). In the \textit{Sall1}-null mice, mesonephros development was normal and morphologically distinct metanephric mesenchyme was formed, albeit reduced in size (Fig. 5B). By contrast, the ureteric bud formed but failed to invade the metanephric mesenchyme. Subsequent differentiation of mesenchyme and branching of the ureteric bud did not occur on either side (44.4\%, \( n=18 \)). Some mutants showed invasion of uroteric bud on one side (38.9\%) or both (16.7\%), but mesenchymal condensation and ureteric branching were significantly poorer than in wild-type littermates (Fig. 5C). Consequently, at day 12.5 and 14.5 of gestation, kidneys in mutant mice were absent or small, with poorly differentiated tubules and ureteric components (Fig. 5D-I). Furthermore, several apoptotic cells with dark nuclear fragments were found in the mesenchyme at 11.5 dpc and TUNEL analysis confirmed apoptosis in the mesenchyme at this stage (Fig. 5J,K). Thus, loss of \textit{Sall1} leads to a failure of ureteric bud invasion into mesenchyme and subsequent apoptosis of the mesenchyme. This phenotype is more severe than findings in mutant mice deficient in Wnt4, or BMP7, in which ureter-mesenchyme interaction does occur to some extent. The variability of the phenotypes may be due to the mixed genetic background of the animals analyzed or to residual activity of other \textit{Sall} genes. \textit{Sall2} and \textit{Sall3} were expressed in developing kidney (data not shown).

Heterozygous mice were apparently normal and did not show phenotypes of Townes-Brocks syndrome, such as dysplastic ears, preaxial polydactyly, imperforate anus and renal anomaly.

**Downregulation of metanephric mesenchymal genes in \textit{Sall1}-deficient mice**

To examine kidney phenotypes in more detail, we next examined expression patterns of several well-characterized
Sall1 is essential for kidney development

molecular markers of either mesenchyme or ureteric bud-derived cells.

Pax2-deficient mice do not develop mesonephric tubules and lack ureteric buds (Torres et al., 1995). In metanephros at 11.5 dpc, Pax2 is expressed both in ureteric bud and condensed mesenchyme surrounding the ureteric bud (Fig. 6A). In Sall1 mutant mice, expression of Pax2 was unaltered in the ureteric bud, though no bulging of ureteric bud tip was observed (Fig. 6B). Expression domain size of the mesenchymal component of Pax2 was significantly reduced, thereby reflecting the size of the mesenchyme. Thus, Pax2 expression in the mutant was consistent with the histological characteristics: failure of the ureteric bud to invade and reduced size of the mesenchyme.

Mice deficient in the tyrosine-kinase type receptor, Ret, as well as its ligand GDNF, show failure of ureteric bud invasion and subsequent failure of mesenchymal differentiation (Moore et al., 1996; Pichel et al., 1996; Sanchez et al., 1996; Schuchardt et al., 1994). Ret was exclusively expressed in the ureteric bud in the wild type, and its expression in Sall1 mutant mice was unaltered, though bulging of ureteric bud tip was not evident (Fig. 6C,D). This result, together with that of ureteric bud component of Pax2, indicates that markers of ureteric bud were not affected in the absence of Sall1.

GDNF is a ligand for Ret and is expressed in the metanephric mesenchyme. GDNF is weakly expressed in the uninduced mesenchyme and strongly upregulated upon ureteric bud invasion (Fig. 6E). Sall1 mutant mice showed a reduced expression of GDNF at 11.5 dpc (Fig. 6F). GDNF expression before ureteric bud invasion (10.5 dpc) was, however, unaffected in the mutant mice (data not shown). Expression of Eya1, a putative upstream molecule of GDNF (Xu et al., 1999), was not significantly affected in the mutants at 10.5 or 11.5 dpc, except for the reduced size of the expression domain at 11.5 dpc (data not shown).

BMP7-deficient mice show normal initial metanephric induction, though the size of the kidney is reduced (Dudley et al., 1995; Luo et al., 1995). BMP7 is expressed both in the ureteric bud and in the mesenchyme (Fig. 6G). In Sall1 mutant mice, BMP7 expression was reduced in the mesenchyme and relatively unaffected in the ureteric bud (Fig. 6H).

Wnt4 is required for epithelialization of the induced mesenchyme but not for the initial induction by ureter (Stark et al., 1994). It is expressed in mesenchymal cells on sides of the ureteric bud at 11.5 dpc and correlates to the site where the first pretubular aggregates form (Fig. 6I). Sall1-deficient mice showed significantly reduced Wnt4 expression (Fig. 6J).

WT-1 is expressed in the metanephric mesenchyme and its
absence leads to failure of mesenchymal induction (Kreidberg et al., 1993). Expression domain size of WT-1 was also significantly reduced in the absence of Sall1 (Fig. 6K,L). Thus, Sall1 deficiency led to remarkable downregulation of some mesenchymal markers (GDNF, BMP7, Wnt4) and reduced size of expression domain of others (Pax2 and WT-1). These data are consistent with histological findings that Sall1 mutant metanephric mesenchyme was significantly reduced in size and was not invaded by the ureteric bud. Downregulation of the markers suggests that the mutant mesenchyme is not induced properly by signals from the ureteric bud. None of the mesenchymal markers was, however, completely absent, but was reduced, thus indicating that specification of the mesenchyme occurs in Sall1 deficiency.

### Tubule induction in Sall1-deficient metanephric mesenchyme

To further demonstrate that kidney development is impaired in Sall1-deficient mice at the initial stage, kidney rudiments were isolated from Sall1 mutant mice at 11.5 dpc and cultured in vitro (Fig. 7A-C). All wild-type or heterozygous rudiments developed into a fully branched kidney structure in 3 days (n=7 and n=20, respectively). Sall1 mutants, however, formed no (n=9) or only small numbers (n=5) of branched structures (if any), findings that are consistent with phenotypes in vivo.

To determine if the kidney abnormality in Sall1-deficient mice was due to an autonomous cell defect of metanephric mesenchyme, or to the incomplete invasion of the ureteric bud that is a normal inducer, the mutant mesenchyme was recombined with the spinal cord. The mesenchyme cultured in vitro degenerates when removed from the ureteric bud. When recombined with the spinal cord, however, 11 out of 11 wild-type and 15 of 16 heterozygous mesenchymes formed renal tubules within 5 days, as shown in Fig. 7D (Saxen, 1987). Wnt family members expressed in spinal cord bypass the inductive signal from the ureter by mimicking the normal mesenchymal action of Wnt4 (Kispert et al., 1998). Eleven out of 12 metanephric mesenchymes from Sall1-deficient mice formed renal tubules when recombined with the wild-type or heterozygous spinal cord – independent of the severity of the ureteric invasion impairment (Fig. 7E). This indicates that the Sall1-deficient mesenchyme is competent with respect to epithelial transformation. Induced mutant tubules were consistently smaller than those of wild type or heterozygotes, perhaps because of reduced cell number of the mutant mesenchyme at the time of dissection. These data demonstrate that there is no inherent defect in the ability of Sall1-deficient metanephric mesenchyme to make tubules upon induction; thus, the kidney phenotype of the Sall1-deficient mice is likely to be primarily caused by the lack of invasion of the ureteric bud that is a normal inducer.

### DISCUSSION

Our data indicate that Sall1 is required for initial interaction between the ureter and the metanephric mesenchyme, though phenotypes of the mutants were variable, as in cases of some knockouts with kidney phenotypes. For example, only one-third of the newborn Ret mutants show complete absence of ureter and kidney, and one-tenth have bilateral kidney rudiments (Schuchardt et al., 1994). Ret mutants also show a range of defects in formation, growth and branching of the ureteric bud at the initial stage of metanephros formation (Schuchardt et al., 1996). Some of the GDNF mutants do develop ureteric buds that invade the mesenchyme (Moore et al., 1996; Pichel et al., 1996; Sanchez et al., 1996). The phenotype variability may be due to redundant functions of related molecules or to a mixed genetic background of the animals examined.

Based on impaired invasion of the ureteric bud in Sall1-deficient kidney (Fig. 5) and responsiveness of Sall1-null mesenchyme to spinal cord-derived signal in organ culture (Fig. 7), loss of Sall1 expression in the mesenchyme is likely to lead to failure to attract the ureteric bud that is a normal inducer, and to subsequent failure of tubule differentiation in...
the mesenchyme. Reduced but persistent expression of early mesenchymal markers is also consistent with this model.

The most severe cases of \textit{Sall1} knockout were similar to those of GDNF or WT-1 deficient mice. GDNF is expressed in the metanephric mesenchyme and is a key molecule for attracting ureteric bud, which acts through the Ret receptor. Thus, GDNF knockouts show failure of ureteric bud invasion. GDNF expression in the mesenchyme is reduced in \textit{Sall1} knockout at 11.5 dpc, but not at 10.5 dpc (before ureteric bud invasion). This indicates that \textit{Sall1} is not absolutely required for GDNF expression. Furthermore, expression of Eya1, an upstream molecule of GDNF, is relatively unaffected in the mutants. Therefore, GDNF reduction in \textit{Sall1} mutants, which could lead to incomplete ureteric bud invasion, is not likely to be caused by a direct effect of \textit{Sall1} on GDNF expression.

WT-1 is also expressed in the metanephric mesenchyme, and its absence leads to failure of ureteric bud invasion and apoptosis of the mesenchyme. WT-1 expression in \textit{Sall1}-deficient mice was, however, weakly detected, though reduced, thus indicating that \textit{Sall1} is not essential for WT-1 expression. Furthermore WT-1 knockout show extrarenal phenotypes such as abnormal heart, lung and gonad development, which were not observed in the \textit{Sall1} knockouts. Therefore, \textit{Sall1} is unlikely to be upstream of WT-1. It is still possible that \textit{Sall1} is a downstream effector of WT-1, or that \textit{Sall1} and WT-1 are synergistic for metanephric development.

Identification of downstream direct targets of \textit{Sall1} is needed to fully explain kidney phenotypes of \textit{Sall1}-deficient mice. \textit{Sall1} contains 10 zinc-finger motifs, most of which are clustered in dupplex or triplet. It is not known which zinc-finger domain is involved in DNA binding or in transactivation. Some sets of zinc fingers may bind to DNA and other sets may serve for protein-protein interactions, as in the case of Olf-1 associated zinc finger (OAZ), which has 30 zinc fingers (Hata et al., 2000). OAZ uses different zinc fingers depending on target promoters and \textit{Sall1} may use similar mechanisms. \textit{Drosophila} \textit{spalt-related (salr)} binds to an A/T-rich consensus sequence through its triplet zinc fingers, but endogenous targets have not been identified (Barrio et al., 1996). \textit{Drosophila} \textit{sal/salr} is reported to regulate the expression of \textit{knirps} and \textit{iroquios} in wing veins, but direct binding of \textit{sal/salr} to their promoters was not seen (Lunde et al., 1998; de Celis and Barrio, 2000). A search for \textit{Sall1} target genes is under way in our laboratory.

In \textit{Drosophila} wing imaginal discs, \textit{sal} is located downstream of \textit{dpp}, which is a BMP4 ortholog (de Celis et al., 1996; Nellen et al., 1996). BMP7 is the major BMP family gene expressed in mouse developing kidney and is essential for kidney development (Dudley et al., 1995; Luo et al., 1995). It is tempting to speculate that \textit{Sall1} expression is controlled by BMP7. The kidney phenotype of \textit{Sall1} knockout is, however, more severe than that of BMP7 knockout. In addition BMP7 deficient mice show eye abnormality, which is not observed in \textit{Sall1}-deficient mice. Therefore, \textit{Sall1} is unlikely to be a downstream signaling component of BMP7.

Recently, \textit{sal} was found to be downstream of the \textit{wingless} signal in the \textit{Drosophila} tracheal system and \textit{sal} deletion results in absence of dorsal trunks of the trachea (Chihara and Hayashi, 2000; Llimargas, 2000). Though Wnt4 is essential for kidney development, a normal ureter-mesenchyme interaction occurs in its mutants, which is different from phenotypes of \textit{Sall1}-deficient mice (Stark et al., 1994). Furthermore \textit{Sall1} is not expressed in trachea and lungs, and \textit{Sall1}-deficient mice apparently have no lung defects. Therefore, the simple analogy of \textit{Drosophila} does not apply to mammals.

LIF and related cytokines are ureter-derived regulators for mesenchymal-to-epithelial conversion (Barasch et al., 1999). \textit{Sall1} expression was, however, unaffected in mice deficient in gp130, the common receptor for LIF family cytokines (data not shown). Furthermore the kidney phenotype of gp130-deficient mice is much milder than that of \textit{Sall1}-deficient mice (Barasch et al., 2000).
et al., 1999). Thus, Sall1 is unlikely to be downstream of the LIF/gp130 signal. Identification of molecules that govern the expression of Sall1 in mesenchymal cells will be important for understanding mechanisms of kidney development: Sall1-lacZ heterozygous mice represent powerful tools with which to achieve this.

We have found almost no abnormality in Sall1 mutant mice except for that in the kidney, despite abundant expression in various tissues. Expression of GDNF, BMP7, Pax2, Wnt4 in other regions, such as in the brain and spinal cord, remained intact in the Sall1 mutant mice (data not shown). As some of expression patterns of Sall2 and Sall3 overlapped those of Sall1, they may compensate for each other. Elimination of these other Sall gene family members would elucidate their roles in other tissues as well as in the kidney.

Another intriguing point is that heterozygous mutant mice do not show similar phenotypes of Townes-Brocks syndrome therefore cannot serve as a disease model for human disease. Even phenotypes of homozygous mutant mice differed from those of the human disease. The relative importance of SALL1 over SALL2 and SALL3 may be higher in humans than in mice, and Sall1 deficiency may be compensated for by Sall2 and Sall3 in mice. Alternatively, heterozygous mutations in humans, which result in premature truncation 5′ to the triple zinc-finger motif, may serve in a dominant-negative fashion and eliminate functions of all SALL genes. To test these hypotheses, generating transgenic mice carrying a truncated Sall1, as well as deleting all Sall genes in mice, would be necessary. Nonetheless, the kidney phenotypes observed in Townes-Brocks syndrome are likely to be explained by the essential role of SALL1 in the initial key step of kidney development. Elucidation of upstream and downstream molecular events will lead to better understanding of kidney development, as well as to finding potential therapy targets for individuals with this disease.

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