Sall1-dependent signals affect Wnt signaling and ureter tip fate to initiate kidney development

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SUMMARY
Development of the metanephric kidney depends on precise control of branching of the ureteric bud. Branching events represent terminal bifurcations that are thought to depend on unique patterns of gene expression in the tip compared with the stalk and are influenced by mesenchymal signals. The metanephric mesenchyme-derived signals that control gene expression at the ureteric bud tip are not well understood. In mouse Sall1 mutants, the ureteric bud grows out and invades the metanephric mesenchyme, but it fails to initiate branching despite tip-specific expression of Ret and Wnt11. The stalk-specific marker Wnt9b and the β-catenin downstream target Axin2 are ectopically expressed in the mutant ureteric bud tips, suggesting that upregulated canonical Wnt signaling disrupts ureter branching in this mutant. In support of this hypothesis, ureter arrest is rescued by lowering β-catenin levels in the Sall1 mutant and is phenocopied by ectopic expression of a stabilized β-catenin in the ureteric bud. Furthermore, transgenic overexpression of Wnt9b in the ureteric bud causes reduced branching in multiple founder lines. These studies indicate that Sall1-dependent signals from the metanephric mesenchyme are required to modulate ureteric bud tip Wnt patterning in order to initiate branching.

KEY WORDS: Sall1, Spalt, Ureter, Wnt9b, Kidney development, Ureteric bud, Metanephric mesenchyme, β-catenin, Wnt, Tip fate, Mouse

INTRODUCTION
A crucial step in kidney development is the interaction between the outgrown ureteric bud (ub) and the metanephric mesenchyme (mm). Reciprocal signaling triggers the ub to initiate its first T-branch and induces the mm to condense and proliferate. If these tissues fail to interact properly, renal agenesis or severe hypoplasia will occur. Despite the importance of this interaction, defining the precise molecular events that regulate these processes has been particularly difficult because the mm undergoes rapid apoptosis if it is not properly invaded (Donovan et al., 1999). Here, we describe a mouse model in which the initial invasion of the ureter has been captured and can be studied to identify factors that are crucial for this process.

The gene mutated in this mouse model is the transcription factor Sall1 – a multi-zinc-finger transcriptional regulator that is expressed in the mm (Nishinakamura et al., 2001). It is mutated in the autosomal dominant syndrome Townes Brocks (TBS) which is characterized by multi-organ defects (including renal hypoplasia). In this report, we use a Sall1 mouse model that mimics TBS (Sall1−/−ΔZn) and demonstrate that these mice form blind ureters with an overlying undifferentiated cap of mm (Kiefer et al., 2003). The ub grows out and invades the mm, but does not form the first T-branch. The unbranched ub and mm remain associated throughout development, thereby allowing the precise dissection of the initial events in ub morphogenesis that depend on Sall1. We show that Sall1 function in the mm triggers downregulation of the stalk-specific marker Wnt9b and the canonical Wnt downstream gene Axin2 in the outgrown ub. Early ub branching is rescued by a reduction in β-catenin dose supporting the conclusion that ureter arrest is due to altered Wnt signaling in Sall1−/−ΔZn mutants. This theory is further strengthened by findings that ub branching is reduced in Wnt9b transgenic lines and in a stabilized β-catenin mutant. These results demonstrate that downregulation of Wnt9b and canonical Wnt signaling in the ureter tip is required to initiate ub branching.

MATERIALS AND METHODS

Mouse strains
Sall1−/−ΔZn mice have been described elsewhere (Kiefer et al., 2003). Double heterozygous Sall1−/−ΔZn, Hoxb7GFP were mated to Sall1−/−ΔZn to create EGFP-labeled mutant Wolffian ducts and ureters (Srinivas et al., 1999). Sprouty1 (Spry1) mice were a gift from J. Licht (Basson et al., 2005). Reduction of β-catenin activity was achieved by using a β-catenin floxed allele (Ctnnb1fl/+, Jackson Laboratories) that had been first crossed to a β-actin-cre transgenic and then to Sall1−/−ΔZn males. These were mated to Sall1−/−ΔZn−/−; Hoxb7GFP/+ females to analyze Sall1−/−ΔZn−/−; Ctnnb1fl/+ embryos. The presence of β-actin-cre or Hoxb7GFP transgenics had no effect on the phenotypes observed. Activation of β-catenin was achieved by crossing Hoxb7cre/EGFP transgenic mice (Zhao et al., 2004) to β-catenin exon 3 floxed allele (Ctnnb1 ex3fl/ex3f, Jackson Laboratories) (Harada et al., 1999). Conditional deletion of this exon produces a stabilized β-catenin protein in both ureter and Wolffian duct. These studies were performed under the auspices of St Louis University and St Louis VA Medical Center animal care guidelines. Noon on the day of the vaginal plug detection was considered to be E0.5.

Overexpression of Wnt9b in the ub
Transgenic mice were created to conditionally overexpress Wnt9b (ACTWnt9b). A C-terminally epitope-tagged Wnt9b (Wnt9b-FLU) was inserted into the EcoRI site of a previously described transgenic construct [a gift from Yiping Chen, Tulane University (Chen et al., 2004)]. In this construct, expression of Wnt9b-FLU is prevented by an upstream loxed
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RESULTS

Sal1-ΔZn mice exhibit blind ureters that are morphologically normal

Sal1-ΔZn homozygous mice exhibit bilateral renal agenesis (Kiefer et al., 2003). It has been suggested that this phenotype is due to incomplete ureter outgrowth and to mesenchymal apoptosis in Sal1-null mice (Nishikamurra et al., 2001). However, upon closer investigation, we were able to discern outgrown ureters in at least 25 independent E14.5-18.5 Sal1-ΔZn+/- embryos in which there was no evidence of kidney formation (Fig. 1). These blind ureters had grown away from the bladder similar to those in wild-type littermates, despite no evidence of a morphologically distinct metanephric mesenchyme. Blind ureters exhibited normal histological architecture that consisted of urothelium (u) and smooth muscle (sm) layers, as revealed by Hematoxylin and Eosin staining. By E16.5, the smooth muscle layer should have differentiated and begun to express smooth muscle actin; this occurred normally in the mutant ureters (Fig. 1E,F). Both wild-type and mutant ureters also properly expressed a marker that is exclusive to the differentiated epithelium of the urinary tract: uroplakin III (1GJH). Six1+/− and Six1−/− mutants display a very similar phenotype in which renal agenesis is accompanied by growth and differentiation of the ureter (Bush et al., 2006), supporting the conclusion that development of the extra-renal ureter is independent of the proximal branching ub that forms the intra-renal collecting system. However, unlike Six1 (Nie et al., 2010), Sal1 is not required for proper formation of the smooth muscle layer. Sal1 expression is not preserved in Six1−/− mutant umb and it has been suggested that Sal1 is a direct transcriptional target of Six1 regulation (Chai et al., 2006). These findings suggest that Six1 and Sal1 function together in a developmental pathway that regulates initial branching of the ureter.

Blind ureters arrest before the T-stage and maintain persistent mesenchymal cells

To verify the identity of the outgrown ureters and to visualize ureter development at multiple stages, we crossed Sal1-ΔZn mice with the Hoxb7GFP transgenic line (F. Costantini, Columbia University). Visualization of GFP fluorescence verified the existence of blind ureters in Sal1-ΔZn+/- mutant E16.5 embryos (Fig. 2). These ureters had grown at the proper site on either side of the aorta and were morphologically distinguishable from the surrounding tissue. The distal tips appeared dilated and the lengths were often asymmetric. At E11.5, when the wild-type umb has formed its first branch (T-stage), mutant umb remained unbranched (Fig. 2C,D). Both wild-type and mutant umb emerged from the distal Wolffian duct and invaded the metanephric mesenchyme; however, the mutant umb failed to form the T-stage. This unbranched umb remained viable and continued to grow in the absence of proper mesenchymal induction.

Ret was properly expressed in the mutant umb and became restricted to the ureter tip by E12.5 (Fig. 3C,D). Analysis of Pax2 expression revealed a small cluster of mesenchyme surrounding the
Fig. 1. **Sall1**-ΔZn mutants exhibit blind ureters that properly differentiate into urothelium and smooth muscle layers. (A-D) E18.5 urogenital tracts from wild-type and homozygous mouse embryos demonstrate that **Sall1**-ΔZn mutant mice do not form kidneys (k), but that morphologically distinct ureters are visible. Testes (t) and bladder (bl) are formed. (C,D) Transverse sections at the level indicated by the arrows are shown in the lower panels. Haemotoxylin and Eosin staining reveals a proper urothelium (u) and smooth muscle (sm) layer in cross sections of both wild-type and mutant ureters. The mutant exhibits decreased luminal space that might be secondary to the lack of urine flow. Immunohistochemistry with anti-smooth muscle actin and anti-uroplakin III appears similar to pretubular aggregates in the persistent mesenchymal cells and, with this observation, there was no evidence of formation of a urothelial layer. Transverse sections of both wild-type and mutant ureters by the arrows are shown in the lower panels. Haemotoxylin and Eosin staining reveals a proper urothelium (u) and smooth muscle (sm) layer in cross sections of both wild-type and mutant ureters. 

Fig. 2. Blind ureters are caused by failure to initiate the first branch. (A-D) Hoxb7GFP-labeled genital tracts from wild-type and mutant mice at E16.5 (A,B) and E11.5 (C,D) reveal that the wild-type ureteric bud (ub) properly forms a T-branch at E11.5 and a branched kidney at E16.5. **Sall1**-ΔZn homozygous mutant ureters properly grow out from the Wolffian duct (wd), but fail to branch. Blind ureters persist at E16.5 in the mutant.

Uregulation of GDNF signaling is not sufficient to rescue branching

Our data suggest that **Sall1**-dependent signals from the mm to the ub are required to initiate branching. It has been proposed that a failure to maintain Gdnf expression probably accounts for renal agenesis or for severe hypoplasia in **Sall1** mutants (Schedl, 2007). The requirement for proper Gdnf expression in the critical window of initial ub invasion has been shown through analysis of nephrenotcin (Npt1) and integrin α8 (Itga8) mutant mice (Linton et al., 2007). In these mutants, Gdnf expression appears to be normal at the time of ub outgrowth, but is dramatically reduced at E11.5 when the ub invades the mesenchyme. A genetic reduction of Spry1 gene expression rescued the kidney defect in Itga8 mutants confirming that decreased GDNF-Ret signaling was responsible for renal agenesis in these mice. Therefore, we assayed expression of Gdnf in **Sall1**-ΔZn mutant mesenchyme at E11.5 and found that its expression was significantly reduced when measured using in situ hybridization and quantitative real-time PCR (Fig. 3N, 0.22±0.05 fold change). Mesenchymal Pax2 expression appeared similar to wild-type at the same time point (Fig. 3K,L).

To test the hypothesis that Gdnf deficiency accounts for ub arrest in the **Sall1**-ΔZn mutant, we next performed metanephric organ culture with exogenous GDNF and with increased GDNF signaling achieved by reducing the dose of the Spry1 tyrosine kinase inhibitor in vivo. For organ culture, E11.5 Hoxb7GFP wild-type and homozygous **Sall1**-ΔZn-ΔZn metanephric regions were cultured with beads coated with bovine serum albumin (BSA) or recombinant GDNF protein. Recapitulating the in vivo phenotypes, mutant explants failed to branch, whereas wild-type explants underwent several rounds of branching when incubated with BSA-coated beads (Fig. 3O,P). As previously described, exogenous GDNF caused increased branching and altered patterning of the wild-type ub, confirming that GDNF was active in our culture system (Fig. 3Q). Addition of GDNF-coated beads resulted in only a small single branch with abnormal morphology in eight out of 11 mutants (Fig. 3R). Interestingly, mutant ureteric buds did not swell as dramatically as wild-type tissues when treated with exogenous GDNF, suggesting that ampulla formation is abnormal. A similar reduction in Gdnf expression at E11.5 was observed for Grem1-deficient embryos, which arrest prior to ub outgrowth. However, in this mutant, exogenous GDNF induced ub branching of Grem1+/− metanephiroi in organ culture that was comparable with that seen in the wild type (Michos et al., 2007). Thus, the failure of GDNF to rescue the **Sall1**-ΔZn-ΔZn explants is best explained by an inability of the **Sall1**-ΔZn mutant to respond to this branch-promoting factor.
We next tested whether the deficiency in Gdnf could be overcome by reducing Spry1 dose in vivo. Spry1 normally functions to attenuate GDNF activation of the Ret receptor, and loss of Spry1 in Spry1-null embryos produces multiple ectopic buds because of increased Ret signaling (Basson et al., 2005; Basson et al., 2006). Therefore, we postulated that increasing Ret signaling in the presence of low levels of GDNF would allow mutant ureteric buds to branch. In contrast to the rescue observed for *Spry1−/−* mutants examined (*n* = 0/5). Together, these results suggest that although low levels of Gdnf might contribute to ureter arrest in the *Sall1−ΔZn* mutant, reduced signaling through this pathway alone cannot account for this phenotype.

Although *Sall1* is not expressed in the ub, it is expressed in the nephric duct at E9-10, immediately prior to ub outgrowth. Thus, *Sall1* expression in the duct might be required to pattern the ub and enable it to form an ampulla properly. To test this possibility, we performed recombination experiments to determine whether *Sall1−ΔZn* ubs were competent to branch when recombined with wild-type mm. As shown in Fig. 3S,T, mutant ubs underwent several rounds of branching morphogenesis (average = 4.3 ub tips with four out of six recombinations exhibiting branching) when recombined with wild-type mesenchyme; this was comparable with that observed in the wild type → wild type recombination (average = 4.8 ub tips with four out of six recombinations exhibiting branching). Wild-type recombinations were performed prior to the T-stage to verify that unbranched ubs were capable of branching in organ culture. Together, these results indicate that the *Sall1−ΔZn* mutant ub is competent to respond to mesenchymal signals, and suggest that *Sall1*-dependent factors in the mm, independently of GDNF, must be involved in the regulation of ampulla formation and the initiation of ureteric bud branching.

**Wnt9b is ectopically expressed in mutant ureteric bud tips**

We next postulated that *Sall1* might be affecting ureter tip fate. If mutant ub tips are not properly specified, they could adopt a distal ub phenotype and prohibit branching (Caruana et al., 2006; Michael and Davies, 2004; Schmidt-Ott et al., 2005; Shakyta et al., 2005; Sweeney et al., 2008). Tip identity was specified in *Sall1−ΔZn* mutant ubs because both Ret (Fig. 3D) and Wnt11 (Fig. 4D) were properly tip restricted. As the ampulla begins to bifurcate in the wild type, Wnt11 expression is polarized to delineate the emerging tips. However, in the mutant, Wnt11 remains restricted to the single tip, indicating that bifurcation has not been initiated. Wnt11 expression depends on intact GDNF-Ret signaling and is maintained by a Wnt9b/GDNF-Ret pathway during kidney development, as *Wnt9b−/−* embryos arrest when the ub has formed the T-stage (Carroll et al., 2005). *In Sall1−ΔZn* mutant uers, Wnt9b expression was not downregulated and continued to be expressed at the ureter tip (Fig. 4F). Wnt9b is thought to signal through the canonical β-catenin pathway during kidney development, as *Wnt9b−/−* embryos can be rescued by expression of Wnt1 in the ub (Carroll et al., 2005).

Consistent with this interpretation, a β-catenin pathway member and target gene (*Jho et al., 2002; Asimina*, 2002). *Axin2* was also ectopically expressed in the ub tip in the *Sall1−ΔZn* mutant (Fig. 4G,H). *Axin2* is a gene shown to be modulated by β-catenin signaling in the kidney (Schmidt-Ott et al., 2007), also occurred in mutant rudiments (2.3±0.11 fold change). These data suggest that *Sall1*-dependent signals in the mm cause the downregulation of canonical Wnt signaling in the ub tip resulting in initiation of branching.
Reduction in β-catenin dosage rescues early branching

Since Wnt9b and at least two canonical Wnt-responsive genes were upregulated in Sall1-ΔZn<sup>+/–</sup> mutant ureters, we hypothesized that ub branching could be rescued by lowering β-catenin activity. In contrast to reduced Spry1 dose, which had no effect on the Sall1-ΔZn<sup>+/–</sup> phenotype, lowering the dose of β-catenin rescued branching in 50% of Sall1-ΔZn<sup>+/–</sup>;Ctnnb1<sup>+/+</sup> E12.5-13.5 embryos. Seven out of 14 mutant kidneys branched to the T-stage and initiated at least a second round of branching from each tip, resulting in a statistically significant rescue of the blind ureter phenotype (Fisher’s exact test P<0.005, Fig. 4K,N,O). Sall1-ΔZn<sup>+/–</sup>;Ctnnb1<sup>+/+</sup> littersmates (21 out of 22) and the remainder of the Sall1-ΔZn<sup>+/–</sup>;Ctnnb1<sup>+/+</sup> kidneys arrested with blind ureters (Fig. 4J,M,O). Sall1-ΔZn<sup>+/+</sup>;Ctnnb1<sup>+/+</sup> control kidneys branched normally (Fig. 4L), verifying that lowered β-catenin dose alone has no effect on kidney branching. This rescue supports the idea that increased β-catenin signaling is a major component of the Sall1-ΔZn<sup>+/–</sup> blind ureter phenotype.

Ectopic Wnt9b or β-catenin activity in the ureter inhibits branching

Tight control of β-catenin is required during nephrogenesis in both the ureter and mesenchyme (Bridgewater et al., 2008; Carroll et al., 2005; Iglesias et al., 2007; Kuure et al., 2007; Marose et al., 2008; Park et al., 2007). Therefore, we next sought to understand whether increased β-catenin signaling in the ureter tip could be responsible for defective initiation of ub branching. To test this hypothesis, we created a new transgenic mouse to conditionally overexpress Wnt9b (ACTWnt9b) and used an existing conditionally active
As the effects of overexpression of Wnt9b alone were less severe than the Sall1-ΔZn\(^{\Delta 3}\) blind ureter phenotype, we increased Wnt signaling more dramatically in the ub using a stabilized β-catenin allele. A significant number of E11.5-12.5 Hoxb7creEGFP; Ctnnb1\(^{ex3\Delta 3}+\) embryos (35 out of 60) exhibited unbranched ureters that were remarkably similar to those seen in Sall1-ΔZn\(^{\Delta 3}\) embryos (Fig. 5L). The remaining mutants were able to form the T-branch, but arrested shortly thereafter, and often exhibited abnormal branch patterns (data not shown). Hoxb7creEGFP; Ctnnb1\(^{+}\) littersmates branched normally at all stages, confirming that the transgene alone had no deleterious effects. The high frequency of renal agenesis for this cross has recently been reported, but no detailed analysis was provided (Karner et al., 2010). Similarly, inhibition of ureteric bud branching was observed in organ culture when β-catenin signaling was activated using an inhibitor of GSK-3: LiCl (n=8/8, data not shown). Gene maker analysis demonstrated the presence of blind ureters that properly express tip restricted Wnt11 (Fig. 5O,P). Wnt11 expression was expanded in Hoxb7creEGFP; Ctnnb1\(^{ex3\Delta 3}\) kidneys compared with Sall1-ΔZn mutants (compare Fig. 4D with Fig. 5P) and this pattern probably indicates sites that could initiate abnormal branching later in development in these embryos. Wnt9b expression was preserved in ub stalks and was properly downregulated in wild-type and Ctnnb1\(^{ex3\Delta 3}\) mutant ub tips, demonstrating that dominant β-catenin has no effect on Wnt9b gene expression (Fig. 5M,N). Pax2 was expressed in the ureter and the overlying mm in a very similar pattern to that seen in Sall1-ΔZn embryos (compare Fig. 5R with Fig. 3F). Sall1 expression in the mesenchyme was not altered, suggesting that its role in affecting ureter branching occurs upstream of β-catenin signaling. Overall, the strong correlation between the phenotypic consequences of loss of Sall1 in the mm and increased Wnt9b-β-catenin signaling in the ub suggests that increased β-catenin signaling at ub tips is an important mechanism for the Sall1-ΔZn blind ureter phenotype.

**DISCUSSION**

Sall1 has been implicated as a regulator of canonical Wnt signaling, but it is not clear whether this is a direct or indirect effect (Bohn et al., 2006; Chihara and Hayashi, 2000; Ma et al., 2006; Onai et al., 2004; Ribeiro et al., 2004; Sato et al., 2004; Uez et al., 2008). As Sall1 is a transcription factor expressed in the mm, it is likely to regulate expression of membrane bound or secreted factors that would signal to the ub to undergo initial branching. Based on our results, Sall1 would be predicted to repress Wnt9b expression and β-catenin signaling in ureteric bud tip cells. As Sall1 acts as a potent transcriptional repressor through binding to a nucleosome remodeling and deactylase (NuRD) complex, an attractive hypothesis is that Sall1 is necessary to repress a branch inhibitor that is secreted by the mm (Kiefer et al., 2002; Lauberth and Rauchman, 2006). Whereas branch inhibitors normally function to restrict ub outgrowth to a single bud at the proper location, ectopic expression of these factors could disrupt branching. An alternative possibility is that there is a deficiency in branch-promoting factors in the Sall1-ΔZn mutant. In addition to GDNF, FGFs and other growth factors are thought to positively regulate ub branching (Costantini, 2006). Further studies are needed to determine which of these factors might regulate initial branch formation by the ub via modulation of Wnt9b-β-catenin activity in ub tip cells.

Although our studies point to an important mechanism by which Sall1-dependent signals from the mm regulate the ub, it is likely that this transcription factor also has cell-autonomous functions in the mm. Progenitors derived from Sall1 mutant mm can be induced...
to differentiate into nephron precursors but form smaller colonies than wild-type cells, suggesting that Sall1 is required for maintenance or proliferation of metanephric progenitors (Osafune et al., 2006). Recently, it has been suggested that Sall1 also has a role in modulating compact adhesion of mm cells adjacent to the ub tip through direct regulation of the kinesin family gene Kif26 (Uchiyama et al., 2010). Identification of Sall1 target genes in the kidney will better elucidate its multiple roles in the mm.

Several key studies have addressed the role of canonical Wnt signaling in the developing kidney using both loss- and gain-of-function approaches (Bridgewater et al., 2008; Carroll et al., 2005; Marose et al., 2008; Park et al., 2007). A general conclusion from these studies is that kidney development is extremely sensitive to the level or duration of β-catenin signaling both in the mm and in the ub. This tight control of β-catenin activity appears to be crucial at the ureteric bud tip/cap mesenchyme region in order to balance the need to induce nephrons yet maintain the pool of progenitor cells (Park et al., 2007). Our results suggest that tight control of β-catenin activity in the ub tip cells is also required to initiate normal cells (Park et al., 2007). Spatial gene expression in the T-stage mouse metanephros. Gene Expr. Patterns 6, 807-825.


