SIX1 acts synergistically with TBX18 in mediating ureteral smooth muscle formation

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SUMMARY
Dysfunction of the ureter often leads to urine flow impairment from the kidney to the bladder, causing dilation of the ureter and/or renal pelvis. SIX1 is a crucial regulator of renal development: mutations in human SIX1 cause branchio-oto-renal (BOR) syndrome and SIX1−/− mice exhibit renal agenesis, although the ureter is present. It remains unclear whether SIX1 plays a role in regulating ureteral morphogenesis. We demonstrate here that SIX1 is differentially expressed during ureteral morphogenesis. It was expressed in undifferentiated smooth muscle (SM) progenitors, but was downregulated in differentiating SM cells (SMCs) and had disappeared by E18.5. In SIX1−/− mice, the ureteral mesenchymal precursors failed to condense and differentiate into normal SMCs and showed increased cell death, indicating that SIX1 is required for the maintenance and normal differentiation of SM progenitors. A delay in SMC differentiation was observed in SIX1−/− ureters. A lack of SIX1 in the ureter led to hydroureter and hydrenephrosis without anatomical obstruction when kidney formation was rescued in SIX1−/− embryos by specifically expressing SIX1 in the metanephric mesenchyme, but not the ureter, under control of the Eya1 promoter. We show that SIX1 and Tbx18 genetically interact to synergistically regulate SMC development and ureter function and that their gene products form a complex in cultured cells and in the developing ureter. Two missense mutations in SIX1 from BOR patients reduced or abolished SIX1-TBX18 complex formation. These findings uncover an essential role for SIX1 in establishing a functionally normal ureter and provide new insights into the molecular basis of urinary tract malformations in BOR patients.

KEY WORDS: SIX1, Tbx18, Ureter, Smooth muscle, Proliferation, Apoptosis, Hydroureter, Mouse

INTRODUCTION
Developmental disorders of the ureter constitute a significant portion of CAKUT (congenital anomalies of the kidney and urinary tract anomalies), a major cause of chronic renal failure in children (Hosgor et al., 2005; Kuwayama et al., 2002; Schedl, 2007; Shah et al., 2004). For example, functional impairment of the ureteric smooth muscle (SM) is a common cause of renal dysfunction, leading to a failure of urine transport from the renal pelvis to the bladder, often resulting in dilation of the ureter (hydroureter) and renal pelvis (hydronephrosis), which may ultimately lead to renal failure.

In mammals, ureter and kidney development begins when an epithelial outgrowth (the ureteric bud, UB) appears from the caudal end of the Wolffian duct through inductive interactions with adjacent metanephric mesenchyme (MM) (Dressler, 2006; Saxen et al., 1986). In mice, this process occurs at ~E10.5 and soon after its formation, the nascent UB continues to grow towards the MM and divides into tip and trunk regions in response to local signals from the surrounding mesenchyme (Brenner-Anantharam et al., 2007). Subsequently, the proximal tip region of the UB further invades the MM and undergoes repeated branching morphogenesis to form the intra-renal collecting system (Costantini, 2006; Shah et al., 2004), while the trunk of the UB elongates to form the ureteric epithelium, which is the urothelium that lies outside the kidney.

During elongation, the urothelium recruits cells from the intermediate mesenchyme and tailbud mesenchyme to surround the epithelial tube (Brenner-Anantharam et al., 2007). The ureteral mesenchymal cells are constantly expanded to provide a cellular resource for continuous ureter elongation. Interaction of these mesenchymal cells with urothelium is required for proper morphogenesis of the ureter. In response to signals from the epithelium, the mesenchyme becomes organized into different cell layers (Yu et al., 2002). The subpopulation of mesenchymal cells located immediately adjacent to the urothelium forms a loose stromal cell layer, and these cells express high levels of patched (Pch), which serves as a readout for response to SHH signaling from the epithelium. This layer also expresses BMP4, which promotes elongation of the urothelium and differentiation of the surrounding mesenchyme. Cells in this layer later give rise to laminar propria as well as to stromal cells within the developing ureter. The mesenchymal cells located outside of this stromal cell layer condense to form ureteral SM. Mesenchymal condensation for SM is evident at embryonic day (E) 13.5 and SM cell (SMC) differentiation initiates from ~E15.0 in a proximal-distal wave (Yu et al., 2002). The outermost layer of the mesenchyme matures into a connective tissue coating that contains fibrocytes.

The molecular mechanisms controlling ureter development are only beginning to be elucidated. Epithelial SHH and WNT signaling and mesenchymal BMP4 signaling are known to regulate epithelial-mesenchymal interactions during ureter development (Brenner-Anantharam et al., 2007; Cain et al., 2008; Carroll et al., 2005; Michos et al., 2007; Miyazaki et al., 2000; Raatikainen-Ahokas et al., 2000; Wang et al., 2009; Yu et al., 2002). Recently, two transcription factors, the T-box protein Tbx18 and the zinc-finger protein teashirt 3 (TSHZ3), have been shown to be expressed in SMC precursors and to be important for SMC differentiation (Airik et al., 2006; Caubit et al., 2008). How
these signaling pathways and transcription factors act together to coordinate SMC specification and differentiation is poorly understood. Since these factors and different pathways are unlikely to be linear cascades, it will be necessary to determine how different pathways are organized into complex signaling networks that ultimately generate precise responses within ureteral mesenchymal precursor cells.

The murine homeobox Six gene family, which is homologous to the Drosophila sine oculis, plays essential roles in urinary tract development. Haploinsufficiency for human SIX1 or SIX5 leads to branchio-oto-renal (BOR) syndrome (Hoskins et al., 2007; Ruf et al., 2004), and ~6% of BOR patients show severe renal defects, including hydrourephrosis and hydroureret/megaureter (Izzedine et al., 2004). We and others have previously shown that a lack of Six1 in mice leads to renal agenesis, but that the ureter is still formed in Six1+/− mice (Li et al., 2003; Xu et al., 2003). A recent in vitro analysis indicated that the Six1−/− ureter is capable of expressing α-smooth muscle actin (SMA) when cultured in medium (Bush et al., 2006). However, it remains unclear whether SMA production is normal in the Six1−/− mutant. In addition, the etiology of the hydrourephrosis and hydroureret/megaureter that occur in BOR syndrome is currently unclear.

In this study, we address whether Six1 plays a role during ureter morphogenesis. We found that during ureter development, Six1 is expressed in undifferentiated mesenchymal progenitors but its expression is downregulated in differentiating mesenchymal cells. In Six1−/− ureters, the mesenchymal cells failed to aggregate and differentiate normally and showed increased cell death. Furthermore, we demonstrate that loss of Six1 function specifically in the ureter leads to hydroureret and hydrourephrosis when the kidney is rescued in Six1+/− embryos. Finally, we show that Six1 genetically interacts with Tbx18 during ureter morphogenesis and that their gene products physically interact to form a complex both in vitro and in vivo. Our results uncovered an essential role for Six1 during ureter patterning and provide new insights into the molecular basis for the urinary tract abnormalities that occur in BOR patients.

MATERIALS AND METHODS

Mice and genotyping

The Six1−/− (Six1lacZ+/−) mutation in a 129 strain (Laclef et al., 2003; Xu et al., 2003) and the Tbx18GFP mutation in a CD1 strain (Cai et al., 2008) were used. Genotyping was as described (Cai et al., 2008; Laclef et al., 2003; Xu et al., 2003; Zheng et al., 2003), we stained for β-galactosidase activity in Six1lacZ heterozygotes. Six1 expression was observed in the ureteral mesenchyme from ~E12.5 (Fig. 1A) and was upregulated between E13.5 and E15.5 (Fig. 1B-D). In the ureteral mesenchyme, Six1-expressing cells were distributed in the inner layer that will differentiate into SM and in the outer layer that will mature into connective tissue (Fig. 1C,D). However, its expression was downregulated after E15.5, when the progenitors begin their differentiation (Fig. 1E,F), and became undetectable at ~E18.5 (data not shown) and P0 (Fig. 1G,H). Consistent with our previous observations (Xu et al., 2003), we detected some lacZ-positive cells in the collecting tubules of the kidney at E17.5 (Fig. 1E, arrow), but this expression was transient and had disappeared by E18.5-P0 (data not shown). By contrast, lacZ-positive cells were detected in the ureterum and the proximal tubules of the kidney at E18.5-P0 (Fig. 1G,H and data not shown). Our observation of Six1 expression in the ureteral mesenchyme suggests that it might have a direct role in regulating ureter SMC development.

Six1−/− mice show malformation of ureteral SM

Inspection of freshly prepared specimens at P0 showed that, in most cases, Six1−/− ureters were truncated but their width appeared to be increased when compared with littermate controls (Fig. 2; diameter of the Six1+/− heterozygous ureter, 155±15.5 μm (n=6); diameter of the Six1−/− homozygous ureter, 179±30.9 μm (n=6); P=0.85). Histological analysis revealed that the SM layer of control ureters had developed into a well-organized SM ring surrounding the urothelium, while the outer mesenchymal layer had matured into a connective coating containing sparsely distributed fibrocytes (Fig. 2E). By contrast, in Six1−/− ureters, the SM ring-like structure was formed but the SMCs were less differentiated and were more loosely condensed (Fig. 2D,F), while the outer layer of the mesenchymal cells also appeared to be more loosely distributed than in controls, which was more obvious in the proximal portion (Fig. 2D, arrow). In addition, the multilayered urothelium was noticeably disorganized in the mutant (Fig. 2C-F).

To determine the onset of ureter malformations in the mutant, we analyzed a developmental series of control and Six1 mutant ureters. In Six1+/− controls, condensation of SMC progenitors was observed from as early as E12.5 (Fig. 2G,I,K). In the Six1−/− ureter, the mesenchymal condensation appeared to be delayed from E12.5 (Fig. 2H) and this layer appeared thicker on a radial
than in the controls between E14.5 and 16.5 (Fig. 2J,L). The outer loose mesenchymal layer was also found to be thicker in the mutant than in controls. In addition, disorganization of the ureteric epithelial cells was noticeable from E16.5 (Fig. 2L, compare with 2K), at which stage \textit{Six1} expression is not yet turned on in the epithelium. Since ureter morphogenesis requires mesenchymal-epithelial interactions, the epithelial defect observed at this early stage is most likely caused by defects in these interactions. These results indicate that normal patterning of the ureter requires \textit{Six1} function.

**SMCs fail to differentiate normally in the \textit{Six1}–/– ureter**

To determine the basis for the observed phenotypes, we performed marker gene and morphological analyses. Ureteral mesenchymal progenitors differentiate into SMCs in a proximal-to-distal wave starting at ~E15.0. We first examined whether SM differentiation is delayed in the mutant by analyzing the expression of SMA from E15.5. SMA was present along the entire length of the control ureters at this stage (Fig. 3A). By contrast, SMA was detected in the proximal region but was very faint in the distal ureters of \textit{Six1–/–} littermate embryos (Fig. 3B). By E16.5, SMA was present along the entire length of the mutant ureters (data not shown), which is consistent with previous observations from cultured ureters (Bush et al., 2006). However, we observed an abnormal staining pattern for SMA in the mutant from E16.5 (Fig. 3C,D), which was not reported previously.

SMA-positive cells were spindle-shaped and appropriately aggregated to form the SM ring on transverse sections of control ureters at E16.5 (Fig. 3E). In \textit{Six1–/–} ureters, SMA expression was present but the number of SMA-positive cells was greatly reduced (Fig. 3F). At E18.5, SMA was detectable in \textit{Six1–/–} ureters but SMA-positive cells were reduced in number and loosely associated (Fig. 3H, compare with 3G). Similarly, SM myosin heavy chain (SMMHC; \textit{MYH11} – Mouse Genome Informatics) expression was also observed in \textit{Six1–/–} ureters at P0 but the SMMHC-positive cells were greatly reduced in number and disorganized (Fig. 3J) compared with controls (Fig. 3I). TEM further confirmed that SMCs were fewer, disorganized and irregularly shaped in \textit{Six1–/–} ureter transverse sections (Fig. 3L, compare with 3K). In addition, TEM confirmed that there was a thicker outer layer of connective coating in the mutant (Fig. 3K,L). Together, these results demonstrate that \textit{Six1} is required for normal SMC differentiation.
We next examined a set of molecules that are essential for ureter SM development. *Tbx18*, a crucial regulator of the development of SMC precursors, is expressed in the periureteral mesenchymal cells from E11.5, and its expression is downregulated in differentiating SMCs (Airik et al., 2006). At ~E16.0, we found that *Tbx18*-expressing cells were distributed in the SM layer of the control ureter (Fig. 4A). However, more *Tbx18*-positive cells were observed in the SM layer of the *Six1–/–* ureter (Fig. 4B), suggesting that differentiation of the mesenchymal precursors into SMCs is delayed or that a subpopulation of SMC progenitors fails to undergo normal differentiation.

SHH signaling is necessary for normal SMC differentiation by regulating *Bmp4* expression in the mesenchyme (Yu et al., 2002). We found that *Shh* expression in the urothelium, as well as that of its downstream target gene *Pitc1* in the mesenchyme, appeared normal in *Six1–/–* ureters at E14.5-15.5 (Fig. 4C-F). *Bmp4* is expressed in the mesenchymal progenitors adjacent to the epithelium before SMC differentiation (Fig. 4G), and BMP4 signaling promotes SM formation in the ureter and kidney (Brenner-Anantharam et al., 2007; Wang et al., 2009). In the *Six1–/–* ureter, *Bmp4* expression appeared comparable to that of controls at E14.5 (Fig. 4H). We further examined the activity of BMP4 signaling by analyzing the expression of phosphorylated SMAD1/5/8 (pSMAD), which are established mediators of BMP signaling (Massague et al., 2005). No difference in pSMAD antigen staining was observed between control and *Six1–/–* ureters (data not shown). Similarly, the expression of the stromal cell marker retinaldehyde dehydrogenase (*Raldh2*; *Aldh1a2* – Mouse Genome Informatics) in the mesenchymal cell population between the urothelium and the SM layer was not detectably altered in the mutant at E18.5 (Fig. 4I-J). Thus, *Six1* does not appear to be required for the expression of *Shh*, *Pitc1*, *Bmp4* and *Raldh2* during ureter development.

**Increased apoptosis in the developing *Six1–/–* ureter**

Since the mutant ureters often had a thicker mesenchymal layer, especially in the proximal region (Fig. 2), we tested whether cell proliferation is altered by performing BrdU-incorporation experiments. We counted six serial sections of proximal and distal regions of each ureter separately and quantified the number of BrdU-positive cells. Compared with the control, more BrdU-positive cells were observed on transverse sections of the mutant ureter in both proximal and distal regions at E14.5-15.5 (Fig. 5A-D and data not shown). However, as the total number of ureteral mesenchymal cells is increased on a radial plane in the mutant, the proliferation rate in the condensing mesenchymal layer was comparable between control and mutant littermates (0.315±0.03 versus 0.323±0.04), whereas the outer layer of the mutant ureter showed a slightly higher rate of proliferation compared with the *Six1*-heterozygous littermate control (0.263±0.03 versus 0.22±0.01), while the proliferation rate was unaltered in the epithelium in the mutant ureter (Fig. 5E). Since the cell proliferation rate is comparable between control and mutant ureter, and as we often observed truncation of ureters in the mutant, the defect in mesenchymal expansion on a radial plane could be secondary to the elongation defect.

As we have previously found that loss of *Six1* leads to abnormal cell death in the otic vesicle, the olfactory epithelium and in the pharyngeal endoderm-derived organs (Zheng et al., 2003; Zou et al., 2006; Chen et al., 2009), it is possible that a subpopulation of the...
ureteral mesenchymal cells degenerates and thus fails to form a normal SM layer. We therefore investigated whether the mesenchymal cells in the developing Six1−/− ureter undergo abnormal cell death. Transverse sections of E14.5-18.5 normal and mutant ureters (n=6) were processed for the TUNEL method of detecting apoptotic nuclei. No abnormal cell death was observed at E14.5 (data not shown). However, abnormal cell death in the ureteral mesenchymal of Six1−/− embryos was observed in the proximal region of the ureter from E15.5 (Fig. 5G). In addition, increased cell death was also observed in the urothelium (Fig. 5G). At E18.5, apoptotic cells were seen along the entire length of the ureter in Six1−/− embryos (Fig. 5I). By contrast, very few apoptotic cells were detected in the controls at these stages (Fig. 5F,H). Thus, Six1 appears to regulate ureteral mesenchymal cell survival and the defective formation of SM in Six1−/− ureters can be attributed, at least in part, to increased cell death. The early onset of abnormal cell death observed in Six1−/− ureters further indicates that the early mesenchymal Six1 expression is also required for normal development of the urothelium.

Maturation of the urothelium is disturbed in the Six1−/− ureter

We further characterized the defect that might occur during differentiation of the mutant urothelium by immunostaining for uropakin (UPK), a marker for differentiated urothelium (Sun et al., 1999). UPK expression was detected at the apical surface of the urothelium of control ureters at E16.5-18.5 (Fig. 6A,B). In Six1−/− ureters, despite the obvious disorganization of the urothelium, UPK expression was detectable at the apical surface at these stages (Fig. 6C,D). However, its expression was reduced in many areas of the mutant urothelium at E18.5 (Fig. 6D). The alteration in UPK expression could be a secondary effect of increased cell death as detected by the TUNEL assay. Nonetheless, our results suggest that Six1 is not required for the initiation of UPK expression but might be necessary for its maintenance.

Six1−/− mice develop hydrourerter and hydronephrosis when kidney development is rescued by Six1 expression in MM

Although ~22-30% of Six1−/− mice had unilateral hypoplastic kidneys (n=50), no hydrourerter was observed. Histological analysis revealed that the hypoplastic kidneys were extremely rudimentary, with only some tubule-like structures (data not shown), indicating that they were not functional. To determine whether the observed abnormal SM formation would lead to malfunction of the ureter in the presence of a functional kidney, we sought to rescue kidney development in Six1−/− embryos by generating Eya1Six1 knock-in

Fig. 5. Cell proliferation and apoptosis in Six1−/− ureters. (A-D) Cell proliferation analysis by BrdU staining on E14.5 Six1−/− control and Six1−/− mouse ureter transverse sections. (E) Statistical analysis of BrdU-positive cells. BrdU-positive cells and the total number of cells in each layer on each transverse section were counted and data from six sections from each ureter (from four ureters) were normalized as the BrdU labeling index. In the mesenchyme: P=0.1361, control inner layer (il) (0.31±0.03) versus mutant inner layer (0.32±0.04); P=0.0325, control proximal inner layer (prox.) (0.34±0.03) versus mutant proximal inner layer (0.35±0.01); P=0.0782, control distal inner layer (dist.) (0.28±0.04) versus mutant distal inner layer (0.29±0.03); P=0.0964, control outer layer (ol) (0.23±0.01) versus mutant outer layer (0.27±0.02). In E14.5 ureteric epithelium (ue): P=0.1927, control (0.28±0.04) versus mutant (0.29±0.03). P-values were calculated using StatView t-test, error bars indicate s.d. (F-I) TUNEL analysis of E15.5 and E18.5 Six1−/− control and Six1−/− ureters. TUNEL-positive cells (brown nuclei) were counted from five serial sections from each ureter (from at least six ureters). Shown are the average number of TUNEL-positive cells per (10 μm) section for each genotype. sm, smooth muscle layer.
mice (see Fig. S1A,B in the supplementary material). Unlike Six1, Eya1 is exclusively expressed in the MM and its expression is preserved in Six1–/– embryos at E9.5-10.5 (see Fig. S1C in the supplementary material) (Sajithlal et al., 2005; Xu et al., 2003). Thus, expression of Six1 in the MM under the control of the Eya1 promoter may rescue kidney development without affecting the ureter of Six1–/– embryos.

As expected, kidneys were always formed in Eya1Six1/+;Six1–/– embryos (9/9), although they were often smaller than normal. Interestingly, hydronephrosis and hydroureter, either unilaterally or bilaterally, were often observed in the Eya1Six1/+;Six1–/– embryos (~56%, 5/9) from as early as E16.5 (Fig. 7A,B). By contrast, the Eya1lacZ/+;Six1–/– control mice (see Fig. S1A in the supplementary material) had no kidneys (data not shown), similar to what was observed in Six1–/– mice. Histological analysis confirmed dilation of the renal pelvis in the mutant (Fig. 7C,D). Staining with an SM22a in situ probe and anti-SMA antibody on the hydroureter transverse sections confirmed the presence of differentiating SMCs (Fig. 7E-H), although the structural organization of the muscular layer was disrupted. In contrast to the irregularly shaped SMA-positive cells in Six1–/– ureters (Fig. 3D), SMA staining revealed some spindle-shaped SMCs in Eya1Six1/+;Six1–/– ureters (Fig. 7H), which could have resulted from the physical force associated with dilation. Similar to what was observed in Six1–/– mice, early signaling for the development of SMC precursors was not affected in Eya1Six1/+;Six1–/– ureters as judged by the expression of Bmp4 and Pch1 before the onset of hydroureter and hydronephrosis (data not shown).

Analysis of E18.5 mutant urinary tracts by India ink injection revealed no sign of physical obstruction: ink flowed down the ureter into the bladder (Fig. 7I,J), although some regions with a very narrow lumen were observed. Similarly, Six1–/– ureters also had a very thin lumen in some areas as revealed by histological analysis (data not shown). This indicates that hydroureter and hydronephrosis were caused by functional, not anatomical, obstruction, and most likely because of ureteral SM malfunction.

We further determined whether the hydroureter and hydronephrosis observed in Eya1Six1/+;Six1–/– mice resulted from impaired ureter peristalsis by culturing E14.5 ureters in medium for 3-5 days. Peristaltic contractions were observed after 3 days in culture from the proximal end of the ureter and progressed down the length of the ureter to the bladder in control samples (see Movie 1 in the supplementary material). Consistent with previous observations (Bush et al., 2006), cultured Six1–/– (see Movie 2 in the supplementary material) or Eya1Six1/+;Six1–/– (see Movie 3 in the supplementary material) ureters also had peristaltic contractions, but the contractile waves appeared much weaker and smaller as judged by bright-field microscopy or by tracing the epithelial Hoxb7-GFP (Srinivas et al., 1999) under the microscope. Thus, we obtained direct evidence that abnormal SM formation caused by a lack of Six1

Fig. 6. Uroplakin expression in control and mutant ureters.
Immunostaining for uroplakin IIIa (green) in urothelium (ue) of (A,B) Six1+/– and (C,D) Six1–/– mouse embryos. Lower panels of B and D are counter-stained with Hoechst. Scale bars: 50 µm.

Fig. 7. Lack of Six1 in the ureter leads to hydroureter and hydronephrosis. (A,B) Whole E16.5 urinary tracts from Six1+/– and Eya1Six1+/–;Six1–/– mouse embryos. Arrow points to hydroureter in Eya1Six1+/–;Six1–/–. (C,D) Hematoxylin and Eosin-stained longitudinal sections of the kidney (k) and ureters shown in A and B. Arrow indicates the thin wall of ureteral mesenchyme (um) in the mutant. (E-H) In situ hybridization for SM22a (E,F) and immunostaining for SMA (G,H) on ureter transverse sections. Arrows in the higher magnification image in H point to SMCs. (I,J) E18.5 urinary tracts in which India ink was injected into the renal pelvis. Arrow points to dilated ureter in the Eya1Six1+/–;Six1–/– ureter. bl, bladder; ue, urothelium. Scale bars: 50 µm.
in the ureter impairs ureteric peristalsis, providing a possible explanation for the hydroureter and hydroureter phenotype. In summary, these results provide definitive evidence that Six1 plays an essential role in ureteral SM function.

**Six1 interacts with Tbx18 to synergistically regulate ureter development**

Since Six1 expression overlaps with that of Tbx18 in undifferentiated mesenchymal precursors from early stages (Airik et al., 2006), we examined whether Six1 interacts with Tbx18 to regulate ureter morphogenesis by generating Six1+/–;Tbx18–/– compound mutant mice. Six1+/–;Tbx18+/– (Tbx18-GFP) double-heterozygous mice were of normal appearance and fertile. However, when examined at E16.5, around one-third of these mice displayed mild proximal hydroureter (8/27) (Fig. 8B), which was not observed in the single-heterozygote littermate controls (Fig. 8A). Strikingly, ~54% of Six1+/–;Tbx18+/– mice (13/24) showed unilateral or bilateral hydropoplastic kidneys (Fig. 8E-G), in contrast to only unilateral rudimentary kidneys in ~24% of Six1–/– mice (7/29). When the kidney was present in Six1+/–;Tbx18+/– mice, hydroureter was frequently observed (~81%, 21/26), but never in the Six1–/– single mutant. The hydroureter phenotype, in most cases, was seen throughout the ureter and appeared to be much more severe (Fig. 8E-G) than in the Eya1Six1/+;Six1–/– mutant (Fig. 7B). Moreover, the penetrance of the hydroureter and hydroureter phenotype was significantly higher in Six1+/–;Tbx18+/– than in Eya1Six1/+;Six1–/– embryos (~81% versus ~56%). By contrast, Six1–/–;Tbx18–/– embryos exhibited a similar shortened ureter phenotype to that observed in Tbx18–/– single-mutant embryos (Fig. 8C) (Airik et al., 2006). Double-homozygous embryos at E16.5 (n=4) displayed variable renal defects, including renal agenesis and bilateral hypoplastic kidneys; however, the ureter SM was never formed (data not shown). In summary, these results show that Six1 genetically interacts with Tbx18 during ureter development and one-copy reduction of the Tbx18 gene enhances the ureter defects associated with Six1 deficiency.

We next examined the expression of marker genes to determine whether Six1 and Tbx18 act synergistically to regulate ureteral SM development. SM22α and SM22β staining revealed that SM differentiation was more severely affected in the compound mutant than in the Six1–/– or Eya1Six1/+;Six1–/– ureter before and after the onset of hydroureter. Specifically, SM differentiation as judged by SM22α staining was delayed, being undetectable before E16.0, and the SM layer was very thin and often discontinuous at E16.0, before the onset of hydroureter (Fig. 8I, compare with 8G), and at E16.5 (data not shown). As the Tbx18 mutant allele contains the eGFP reporter, we examined Tbx18 expression by detecting GFP-positive

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**Fig. 8. Six1 and Tbx18 genetically interact during ureter development.** (A-F) Urogenital systems in (A) Six1+/–, (B,D,F) Six1+/–;Tbx18+/–, (C) Six1+/–;Tbx18–/– and (E) Six1+/–;Tbx18+/– mouse embryos. Arrows point to dilated ureters. Note the severe dilation in Six1+/–;Tbx18–/– embryos. (G,H) Immunostaining for SMA on Six1+/–;Tbx18–/– (G) and Six1+/–;Tbx18+/– (H) transverse sections. (I,J) Transverse sections of ureters of Six1+/–;Tbx18–/– (Tbx18-GFP) and Six1+/–;Tbx18+/– embryos showing Tbx18-GFP expression in the ureteral mesenchyme. Arrows indicate condensing progenitors. Note that more Tbx18-GFP-positive cells were distributed in the outer layer in the Six1+/–;Tbx18–/– mutant than in the Six1+/–;Tbx18+/– control ureters. (K-P) In situ hybridization for Shh, Ptc1 and Bmp4 on transverse sections. (Q,R) Immunohistochemistry for pSMAD on transverse sections from Six1+/–;Tbx18–/–;Six1+/–;Tbx18+/– embryos. Arrows point to pSMAD-positive nuclei in the condensing mesenchyme. pSMAD-positive cells from five sections from each ureter (three ureters) were quantified as the ratio of pSMAD-positive cells to the total number of condensing mesenchymal cells in the control and mutant ureters. P-values (P=0.1039) were calculated using StatView t-test. (S,T) Transverse sections showing BrdU-positive cells (arrows). Proliferative rate was quantified as the ratio of BrdU-labeled cells to the total number of cells in the condensing mesenchyme from six sections from each ureter (from at least four ureters) on a radial plane. P-values (P=0.003) were calculated using StatView t-test. ue, urothelium.
cells. Section examination at E15.5 showed that in the mutant, more GFP-positive cells were distributed outside of the condensing SM layer (Fig. 8J) than in controls (Fig. 8I), suggesting a recruitment/condensation defect. Examination of Shh expression in the epithelium revealed that in the compound mutant, Shh expression in some regions was reduced, although some epithelial cells still expressed Shh at levels similar to those in controls (Fig. 8K,L). The expression levels of Pichl and Bmp4 in the mesenchyme at E14.5 were decreased in the compound mutant (Fig. SM-P). Further examination of pSMAD expression showed that pSMAD-positive cells were reduced in number in the condensing SM layer in the compound mutant (Fig. 8R) compared with controls (Fig. 8Q). These results suggest that Six1 might act cooperatively with Tbx18 to regulate the SHH and BMP signaling pathways during condensation/aggregation of the ureteral mesenchymal progenitors.

Since reduced proliferation of ureteral mesenchymal progenitors has been observed in Tbx18−/− embryos (Airik et al., 2006), we performed BrdU-incorporation experiments to further examine whether Six1 acts together with Tbx18 to regulate cell proliferation in the ureteral mesenchyme. Our results show that proliferation of ureteral SM progenitors was greatly reduced in Six1−/−;Tbx18+/− ureters at E14.5 (Fig. 8R, arrow; 0.18±0.03 BrdU+ cells/total number of cells) when compared with controls (Fig. 8Q). 0.33±0.02 BrdU+ cells/total number of cells). Thus, Six1 appears to genetically interact with Tbx18 to regulate proliferation of ureteral SM progenitors.

**Six1 and Tbx18 form a complex in cultured cells and developing ureters**

Since Six1 is co-expressed with Tbx18 and these two genes genetically interact during ureter development, we tested whether their gene products physically interact by performing a co-IP analysis. Cell extracts from HEK 293 cells transfected with mouse Six1 and Flag-Tbx18 (FLAG tag added to the N-terminus of Tbx18) or Tbx18-Flag (FLAG tag added to the C-terminus of Tbx18) were incubated with FLAG antibody-coupled agarose beads and analyzed by western blotting with anti-SIX1 antibody. As shown in Fig. 9A, SIX1 co-immunoprecipitated with TBX18. We then examined whether the point mutations that cause single amino acid substitutions in the human SIX1 gene from BOR patients (Ruf et al., 2004) affect SIX1-TBX18 complex formation. Interestingly, the single amino acid substitution of R110W located in the conserved SIX domain decreased SIX1-TBX18 complex formation (Fig. 9B), whereas the single amino acid substitution of Y129C located in the conserved homeodomain completely abolished SIX1-TBX18 interaction. Although the Y129C mutation yielded a slightly smaller product in HEK 293 cells, because this mutant protein can be recognized by the anti-SIX1 antibody (which was raised against the entire homeodomain region) and can also bind to the SIX1 DNA-binding elements at reduced levels (Ruf et al., 2004), we concluded that the Y129C substitution specifically affects the SIX1-TBX18 interaction. These results demonstrate that SIX1 physically interacts with TBX18 and that two missense mutations identified in human BOR patients affect SIX1-TBX18 complex formation.

To further confirm that these two proteins physically interact to form a complex in the developing ureter, we prepared cell extracts from E14.5 mouse ureters and performed a co-IP experiment. Indeed, SIX1 and TBX18 were co-immunoprecipitated with either an anti-SIX1 or anti-TBX18 antibody (Fig. 9C and data not shown). Together, these results demonstrate that SIX1 physically forms a complex with Tbx18 in the developing ureter, thus providing new insights into the molecular mechanisms of the urinary tract defects that occur in BOR patients.

**DISCUSSION**

In this study, we examined the role of Six1 during SM development in the ureter, which has not been previously explored. We have demonstrated that in the absence of Six1, ureter SMCs fail to form a cellular layer with normal SM architecture and function, this leading to hydroureter and hydronephrosis.

**Six1 in ureter patterning and SM formation**

Our results show that Six1 is differentially expressed during ureter morphogenesis. In the developing ureter, Six1 expression was observed in the undifferentiated mesenchymal cells that contribute...
to the SM, the outer connective tissue and the stromal layers. Its expression was downregulated and eventually disappeared in the differentiating mesenchymal cells after E17.5 (Fig. 1). Analysis of Six1+/− ureters indicated that early events, such as condensation/aggregation and survival of the undifferentiated mesenchymal cells, were affected in the absence of Six1. In addition, the onset of SM differentiation was delayed in Six1−/− ureters. Subsequently, we observed abnormal expression of the SM-specific factors SMA, SM22α and SMMHC, indicating that ureteral SM differentiation is affected in Six1−/− embryos. These findings indicate that Six1 has an early role in regulating SM formation.

So far, only two other transcription factors, TBX18 and TSHZ3, have been shown to be expressed in the undifferentiated ureteral mesenchyme. Loss-of-function studies in mice have indicated that Tbx18 is required for progenitor cell development, whereas Tshz3 is dispensable in the ureter before SM differentiation (Airik et al., 2006; Caubit et al., 2008). Deletion of Tbx18 leads to failure of not only SM formation but also urothelium maturation, as well as to the absence of stromal marker expression (Airik et al., 2006). Inactivation of Tshz3 results in a more specific defect of SMC differentiation without affecting the urothelium or the stromal cell layer (Caubit et al., 2008). How the ureteral SM fate is determined remains poorly understood. In this study, we demonstrated that Six1 and Tbx18 act synergistically to regulate ureter morphogenesis.

During ureter development, Tbx18 is specifically expressed in the ureteral mesenchyme from E11.5 and its expression remains high until E14.5, but is downregulated concomitantly with SM differentiation from E15.5 (Airik et al., 2006). This expression pattern overlaps with that of Six1 (Fig. 1). However, as the onset of Tbx18 expression appears to occur earlier than that of Six1, Tbx18 might have a unique early role in the ureteral mesenchyme. This would explain why the ureter phenotype was much more severe in Six1−/− ureters. Nonetheless, the overlapping expression patterns of Six1 and Tbx18 in the ureteral mesenchyme suggests several possibilities as to their actions in regulating SM development. First, Six1 might act synergistically with Tbx18 in specifying or maintaining the ureteral mesenchymal progenitors. In the absence of the mesenchymal progenitors fated to become SMCs, no SM will form. This would explain why there was no SM formation in Six1;Tbx18 double homozygotes (data not shown). Since our data show that the number of SM progenitors appeared to be increased on radial planes as labeled by Tbx18 (Fig. 4), but that the number of differentiated SMCs was reduced in the Six1−/− ureter (Fig. 3), we speculate that some of these progenitors fail to acquire a SM fate. Upon failure to differentiate into SMCs, the progenitor cells undertake the cell death pathway, as detected by the TUNEL assay.

During ureteral SM formation, the mesenchymal progenitor cells proliferate longitudinally along with UB elongation. In Six1−/− ureters, more mesenchymal cells were observed on the radial axis and the shape of SMCs was irregular and disorganized. However, because the cell proliferation rate in the mutant was similar to that in control ureters, this might indicate that the expansion of mesenchymal cells on the radial plane is a secondary defect to the lack of a growing and ascending kidney in the Six1−/− mutant, which is the likely cause for the truncation of the ureters. Indeed, cell proliferation on the radial plane of Eya1Six1+/−,Six1−/− ureters that were of normal length appeared comparable to that in controls at E14.5-15.5 (data not shown). Although we failed to observe any obvious reduction in cell proliferation in the ureteral mesenchyme in Six1−/− or Eya1Six1+/−,Six1−/− ureters at E14.5-15.5, because reduced proliferation has been observed in several other organ systems of Six1−/− single-mutant embryos (Laclef et al., 2003; Xu et al., 2003; Zheng et al., 2003; Zou et al., 2006), it is possible that Six1 cooperates with Tbx18 to modulate cell proliferation during SM development. Indeed, proliferation of ureteral SM progenitors in Six1−/−;Tbx18−/− embryos was reduced to ~54.5% of that of Six1−/− heterozygous controls at E14.5 (Fig. 8). Thus, Six1 appears to act synergistically with Tbx18 in regulating the proliferation of ureteral mesenchymal precursors.

During mesenchymal aggregation, a process that is disrupted in both Tbx18−/− (Airik et al., 2006) and Six1−/− (this study) mutants, the orientations and shapes of SMCs are rearranged resulting in spindle-shaped cells surrounding the epithelium, a process most likely regulated by subepithelial mesenchyme or Bmp4-expressing cells. In Six1−/− ureters, SHH and BMP4 signaling were normal, as assessed by the expression of Shh, Ptch1, Bmp4 and pSMA (Fig. 4 and data not shown). In addition, the expression of Raldh2 in the stromal cells located between the SM and urothelium was also normal, suggesting that the abnormal SM formation observed in Six1 mutants is a cell-autonomous defect. Thus, the mutant ureteral mesenchymal cells are probably incompetent to perceive signals from neighboring cells that are required for regulating SMC aggregation and maturation.

In contrast to Six1−/− ureters, loss of Tbx18 in the ureteral mesenchyme not only affects the SMCs but also the stromal cells, as Bmp4 and Ptc1 expression was downregulated in the proximal region in the Tbx18 mutant ureters at E12.5 (Airik et al., 2006). This is consistent with the idea that Tbx18 has a unique early role in regulating ureter development. Our observation of a reduction in Ptc1, Bmp4 and pSMA expression in Six1−/−;Tbx18−/− ureters suggests that Six1 might cooperate with Tbx18 in regulating epithelial-mesenchymal interactions during ureter patterning. Interestingly, loss of both genes in the mesenchyme also appears to affect urothelium differentiation (Figs 5, 6) (Airik et al., 2006). Unlike Tbx18, Six1 expression was also observed in the urothelium from E18.5, which suggests that it might have a cell-autonomous role during urothelium maturation from around the newborn stage. However, because we observed abnormal cell death and disorganization of the urothelium from E15.5, which is almost 3 days before the onset of Six1 expression in the urothelium, defects in the early events of ureteral mesenchyme development are most likely to affect urothelium development. We are currently using a conditional knockout approach to specifically delete Six1 from the epithelial component or mesenchyme to investigate its roles in urothelium maturation and SM formation. Nonetheless, the Six1 mutants provide a new mouse model for congenital ureter malformations and help us to understand the mechanisms that control ureter morphogenesis and SM formation. Because we found that these two proteins form a complex not only in cultured cells but also in developing ureters, Six1 and Tbx18 are likely to act together in the ureteral mesenchymal progenitors to regulate signaling pathways that control mesenchymal-epithelial interactions during ureter patterning.

It should be noted that Six1 and Tbx18 are unlikely to act in a linear cascade by regulating each other, as loss of either gene does not affect the expression of the other (Fig. 8J and data not shown). TBX18 and SIX1 are likely to act in parallel by forming a complex in the mesenchymal progenitors to regulate the expression of certain downstream genes. In future studies it will be important to identify their common downstream targets in the ureteral mesenchyme in order to understand their mode of action in controlling ureteral SM development.
**SIX1 and hydroureter: new insight towards understanding the pathogenesis of the renal defects that occur in BOR syndrome**

In this study, we demonstrated that a lack of *Six1* in the ureter leads to either bilateral or unilateral hydroureter from as early as E16.5 in the presence of a kidney restored by specifically expressing *Six1* in the MM (Fig. 7). Hydrourephrosis was also observed, but this is likely to be a secondary defect of hydroureter as urinary flow blockage is thought to dilate the renal pelvis. Analysis of ureter morphology in *Six1*–/– or *Eya1*+/–;*Six1*–/– embryos at E18.5 revealed a continuous lumen along the entire ureter length and no physical obstruction was observed (Figs 2-7). Thus, it is very likely that the observed abnormal morphology and organization of SMCs is the major cause of hydroureter in the mutant. SMCs mediate the peristaltic movements that conduct the urine from the renal pelvis to the bladder, and *Six1*–/– ureters exhibited peristaltic movements (see Movies 1-3 in the supplementary material), consistent with previous observations (Bush et al., 2006). However, the peristaltic movements were much weaker in the *Six1*–/– ureters compared with controls. Such impaired peristalsis will lead to functional urine flow blockage. It is of interest to note that haploinsufficiency for human SIX1 results in BOR syndrome, an autosomal dominant developmental disorder characterized by branchial clefts, hearing loss and renal anomalies (Abdelhak et al., 1997; Ruf et al., 2004). The renal defects observed in BOR patients include duplication or absence of the ureter, hydroureter or megaureter and hydronephrosis (Heimler and Lieber, 1986; Izzedine et al., 2004). Thus, the newly generated *Eya1*+/–;*Six1*–/– mice will serve as a new animal model for understanding the etiology of malformation of the ureter SM.

Previous studies have shown that loss of *Tbx18* leads to a severe form of hydroureter/hydronephrosis caused by a functional and partially physical ureter obstruction (Airik et al., 2006). Our results show that the two *SIX1* missense mutations identified from BOR patients that cause single amino acid substitutions reduced or abolished *Six1*-TBX18 interactions. These results are also consistent with the observation that the two genes act synergistically during ureter morphogenesis, as severe hydroureter or megaureter was also observed in some *Tbx18*+/–;*Six1*–/– mice. Therefore, our results strongly suggest that the *SIX1* mutations in BOR patients are likely to influence the formation of the SIX1-TBX18 complex, thus causing the disease phenotype by affecting the expression of downstream genes.

Finally, an interesting finding of this work is that one-copy reduction of *Tbx18* substantially rescues *Six1*–/– kidney development. Previous work described that in addition to expression in the mesenchyme surrounding the ureteric stalk from ~E11.5, *Tbx18* is also expressed in the mesoderm next to the MM (Airik et al., 2006). We speculate that *Tbx18* modulates a signaling pathway that negatively regulates ureteric branching morphogenesis. As BMP4 signaling has a role in inhibiting ureteric branching but promoting ureter elongation, we hypothesize that loss of *Six1* in the MM leads to upregulation of BMP4 activity in the MM, which in turn inhibits UB branching but promotes its differentiation into ureter. Indeed, we found that one-copy reduction of *Bmp4* in *Six1*–/– embryos could restore kidney organogenesis but that hydroureter is also observed from as early as E16.5 (X.N., A. El-Hashash, J. Xu and P.-X.X., unpublished), similar to that observed in *Tbx18*+/–;*Six1*–/– mice. As *Bmp4* expression is eventually lost in *Tbx18*-null mice (Airik et al., 2006), it is possible that *Tbx18* is required for the maintenance of BMP4 activity in the mesenchyme, and one-copy reduction of *Tbx18* might cause a reduction in BMP4 activity in the mesenchyme that surrounds the proximal end of the UB, thus restoring kidney organogenesis by promoting branching morphogenesis in *Six1*–/–,*Tbx18*+/– embryos. In support of this view, BMP4 activity appears to be reduced in the ureteral mesenchyme in *Six1*–/–,*Tbx18*+/– embryos at E14.5 (Fig. 8).

In summary, in this work we provide definitive evidence that *Six1* has an essential role in ureter development. Our results strongly suggest that SIX1 acts as a co-transcription factor for TBX18 in ureteral mesenchyme development, thus providing new insights into the molecular basis of the urinary tract malformations in BOR patients.

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**Competing interests statement**

The authors declare no competing financial interests.

**Supplementary material**

Supplementary material for this article is available at http://dev.biologists.org/supplemental/10.1242/dev.045757/D1C1

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**References**


