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Six1 regulates *Grem1* expression in the metanephric mesenchyme to initiate branching morphogenesis

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Abstract

Urinary tract morphogenesis requires subdivision of the ureteric bud (UB) into the intra-renal collecting system and the extra-renal ureter, by responding to signals in its surrounding mesenchyme. BMP4 is a mesenchymal regulator promoting ureter development, while GREM1 is necessary to negatively regulate BMP4 activity to induce UB branching. However, the mechanisms that regulate the GREM1-BMP4 signaling are unknown. Previous studies have shown that *Six1*-deficient mice lack kidneys, but form ureters. Here, we show that the tip cells of *Six1*$^{-/-}$ UB fail to form an ampulla for branching. Instead, the UB elongates within *Tbx18*- and *Bmp4*-expressing mesenchyme. We find that the expression of *Grem1* in the metanpehric mesenchyme (MM) is *Six1*-dependent. Treatment of *Six1*$^{-/-}$ kidney rudiments with GREM1 protein restores ampulla formation and branching morphogenesis. Furthermore, we demonstrate that genetic reduction of BMP4 levels in *Six1*$^{-/-}$ (*Six1*$^{-/-}$;*Bmp4*$^{+/-}$) embryos restores urinary tract morphogenesis and kidney formation. This study uncovers an essential function for *Six1* in the MM as an upstream regulator of *Grem1* in initiating branching morphogenesis.

Keywords

SIX1; Gremlin1; BMP4; ureteric bud; ampulla

Introduction

Mesenchymal-epithelial interactions are known to be critical for the growth and patterning of many organs in the human body, including the renal system. The intra-renal collecting system and the extra-renal ureter are derived from the ureteric bud (UB). The UB formation is induced by GDNF (Glial cell line-derived neurotrophic factor), which is secreted by the metanephric mesenchyme (MM) and acts as a ligand that binds to the Ret receptor tyrosine kinase and the Gfrα1 coreceptor expressed in the Wolffian duct (WD), at the level of

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hindlimb at around E10.5 in mice (Costantini, 2006; Davies and Fisher, 2002; Saxen and Sariola, 1987; Vainio and Lin, 2002). The initial outgrowth of the UB is a critical step in the development of the urinary tract and if it fails to occur, the ureter and the kidney will not develop.

Soon after the UB forms, it grows out and develops into tip- and trunk-specific domains. The trunk region normally elongates and differentiates into the ureter, while the tip region swells to form an ampulla, a prerequisite for and the first evidence of UB branching (al-Awqati and Goldberg, 1998). Abnormal regulation of UB morphogenesis can cause a spectrum of congenital birth defects such as urinary tract obstruction (Airik and Kispert, 2007). Depending on whether it is metanephric (around the tip) or more posterior tailbud-derived (around the stalk), the mesenchyme surrounding the ureteric epithelium plays an essential role in promoting epithelial differentiation. BMP4, a paracrinefactor of the TGF-β family, mediates the activity of the tailbud-derived mesenchyme, directs the UB development towards ureter fate, and inhibits branching in explant cultures (Brenner-Anantharam et al., 2007; Miyazaki et al., 2000). The BMP antagonist gremlin1 (Grem1), a member of the CAN domain family that preferentially binds to BMP2/4 in vitro (Hsu et al., 1998), is expressed by the MM (Michos et al., 2004). A recent study has shown that GREM1-mediated reduction of BMP4 activity in the mesenchyme around the nascent UB is essential to initiate UB outgrowth and invasion of the MM by establishing WNT11/GDNF feedback signaling (Michos et al., 2007). However, how GREM1 activity is regulated in the MM and how it acts to locally restrict BMP4 signaling to induce UB tip domain formation and its invasion into the MM are essentially unknown.

We have previously reported that a lack of *Six1* in mice leads to renal agenesis and mutations in the human *SIX1* cause branchio-oto-renal (BOR) syndrome (Ruf et al., 2004; Xu et al., 2003). *Six1* belongs to the murine homeobox Six gene family, which is homologous to the *Drosophila sine oculis* (so). In the developing kidney, *Six1* is expressed in the MM before the initiation of UB branching morphogenesis, and in *Six1*$^{-/-}$ mutants, UB grows out normally and elongates to differentiate into ureter but fails to form collecting system (Bush et al., 2006; Li et al., 2003; Nie et al.; Xu et al., 2003). In the absence of UB branching, the MM is eliminated by apoptosis after E11.0 (Xu et al., 2003). Despite these observations, the mechanism by which SIX1 induces epithelial branching to form the collecting duct system remains unclear.

Here, we have specifically investigated the role of *Six1* in the initiation of UB branching morphogenesis and have identified *Grem1* as a crucial target for upregulation in the MM by SIX1. We provide strong evidence that the extra-cellular BMP antagonist GREM1 is the key player in the *Six1*-regulatory pathway to regulate UB ampulla formation and its subsequent branching morphogenesis, as GREM1-soaked beads when implanted into *Six1*$^{-/-}$ kidney primordia in culture were sufficient to promote branching morphogenesis. Furthermore, we show that inactivation of only one copy of the *Bmp4* gene in *Six1*$^{-/-}$ embryos restored UB patterning and kidney organogenesis. In addition, our results show that the balance between the levels of BMP4 activity in the mesenchyme surrounding the nascent UB tip and GDNF production in the MM appears to be crucial for UB patterning. These results demonstrate that *Six1* is specifically required for upregulating the expression of *Grem1* in the MM to locally restrict BMP4 activity in the mesenchyme to initiate ampulla formation and branching morphogenesis during urinary tract morphogenesis.

Materials and methods

Animal and genotyping

Six1$^{+/-}$ mutant mice in 129/SvEv strain were used for this study. The *Bmp4* gene was inactivated in the germline by intercrossing the floxed *Bmp4*$^{loxP\text{-}lacZ}$ allele (Kulessa and Hogan, 2002) with the *Cre* deleter mouse strain (Schwenk et al., 1995). The resulting *Bmp4–lacZ* allele was used to generate *Six1;Bmp4* compound mutant mice for analysis in a mixed 129/C57BL6 background. The compound mutants carrying the *Hoxb7*-GFP transgene (Srinivas et al., 1999) in a mixed C57BL6/CBA/129 background to mark the ureteric epithelium were used for analysis. PCR genotyping of mice and embryos was done as described previously (Kulessa and Hogan, 2002; Srinivas et al., 1999).

Phenotype analysis, in situ hybridization and immunohistochemistry

Embryos for histology and in situ hybridization were dissected out in PBS, fixed with 4% PFA overnight at 4°C and processed using standard procedures.

Whole-mount and section in situ hybridization were carried out with digoxigenin-labeled riboprobes or with ^{35}S-radioisotope labeled *Bmp4* probe. We used 5 embryos for each genotype at each stage for each probe, and the staining was consistent in each embryo.

Anti-pSMAD1/5/8 (Cell Signaling), HRP-conjugated secondary antibody and DAB was used for detection. After staining, the slides were counter-stained with diluted hematoxylin.

Antibody staining for SMA was done as described previously (Nie et al., 2010).

Organ cultures

Metanephric kidney primordia of E10.5–E10.75 were isolated from wild-type and mutant embryos and laid flat onto the top of a Transwell filter (0.4 μm pore size) for in vitro culture. GREM1 (R&D systems) were added at 2–6 μg/ml into culture medium according to Michos et al. (Michos et al., 2007) and changed every 48 hours. GREM1-soaked beads (1.5 μg/μl) implantation experiments were performed as described (Sajithlal et al., 2005). After culture, the kidney primordia were fixed in 4% paraformaldehyde (PFA) and processed for whole-mount in situ hybridization.

Results

Six1$^{-/-}$ UB tip cells fail to form an ampulla

To address what specific roles SIX1 may play to regulate branching morphogenesis, we performed a detailed analysis to specifically investigate whether *Six1* acts to control the initiation of UB patterning into trunk and tip domains and to induce cells at the UB tip to form an ampulla. The UB is normally formed from the caudal segment of the WD at E10.5, and it grows out and divides into trunk and tip domains, as labeled by differential *c-Ret* expression (Fig. 1A). The trunk elongates to form the ureter, while the tip grows to form an ampulla showing strong *c-Ret* expression at around E11.0 (Fig. 1C). The UB elongates further to invade the mesenchyme and by E11.5, it undergoes branching morphogenesis to form the first T-shaped bud. In *Six1*$^{-/-}$ embryos, the UB grows out normally at E10.5, and its tip domain is initially formed as revealed by the expression of *c-Ret* (Fig. 1B). However, the mutant tip fails to swell and form an ampulla for branching morphogenesis (Fig. 1D,F), and the level of *c-Ret* expression in the tip region becomes largely reduced when compared to wild-type controls. In contrast, the distal trunk region continues to elongate in *Six1*$^{-/-}$ embryos (Fig. 1B,D,F,H). The lack of ampulla formation was further confirmed by staining with *Emx2* and *Gata3* at E10.75–11.25 (Fig. 1G,H and data not shown) and by tracing the

ureteric epithelium expressing *Hoxb7-GFP* transgene (Srinivas et al., 1999) when the metanephric primordia were cultured in vitro (Fig. 1I). Thus, *Six1* appears to have a specific role in inducing ampulla formation at the UB tip.

To further investigate this phenotype, we examined several other UB tip-specific markers. The Wnt family member *Wnt11* is specifically expressed in the UB tips at all stages of ureteric development (Fig. 2A,C) and reciprocal interaction between *Wnt11* and *Gdnf/Ret* regulates UB branching (Kispert et al., 1996; Majumdar et al., 2003). However, in *Six1*$^{-/-}$ embryos, *Wnt11* expression in the tip region is reduced at E10.75 (Fig. 2B) and markedly reduced at E11.0 (Fig. 2D). We then tested whether in the absence of ampulla formation, the cells at the tip region gradually lose its tip-specificity by examining the expression of trunk-specific markers such as *Wnt9b* and *Wnt7b*. *Wnt9b* is expressed in the UB and its expression gradually becomes excluded from the tip domain (Fig. 2E) (Carroll et al., 2005). By contrast, in *Six1*$^{-/-}$ embryos, *Wnt9b* expression was detected throughout the ureteric epithelium at E11.25-11.5 (Fig. 2F and data not shown). At E11.25-11.5, *Wnt7b* is specifically expressed in the trunk region in normal embryos (Fig. 2G,I). However in *Six1*$^{-/-}$ embryos, *Wnt7b* expression was detected throughout the developing ureteric epithelium (Fig. 2H,J). Thus, our results indicate that in the absence of ampulla formation, the mutant UB tip eventually loses its tip-specificity as revealed by reduction of tip-specific gene expression but presence of trunk-specific gene expression at E11.25-11.5.

Failure of the UB invasion into the MM in *Six1*$^{-/-}$ mice results in its elongation within *Tbx18*- and *Bmp4*-expressing mesenchyme

As the mesenchyme surrounding the different regions of the UB plays an instructive role in promoting epithelial differentiation (Brenner-Anantharam et al., 2007), we wished to investigate further if the lack of UB ampulla formation correlated with alterations in the cellular composition and activity of the mesenchyme surrounding the proximal UB tips. We therefore examined the expression of the mesenchymal markers that are important for ureter differentiation. *Tbx18* is specifically expressed in the ureteral mesenchymal progenitors at around E11.25 and in the absence of *Tbx18*, ureteral mesenchymal progenitors fail to differentiate into ureter smooth muscle cells (SMCs) (Airik et al., 2006). Consistent with previous observations (Airik et al., 2006), in wild-type embryos, *Tbx18* is strongly expressed in the ureteral mesenchyme at E11.25-E12.5 but is excluded from the MM at E11.25-11.5 (Fig. 3A,C,E). The UB normally invades the MM (Fig. 1A,C), which is located dorsally to the *Tbx18*-expressing mesenchyme, and undergoes branching morphogenesis to form the collecting duct system (Fig. 3A,C,E). However, in *Six1*$^{-/-}$ embryos, the UB tip is embedded in *Tbx18*-expressing mesenchyme and fails to invade the MM (Fig. 3B). Instead, the UB elongates within the *Tbx18*-expressing mesenchyme (Fig. 3D,F).

In addition to *Tbx18*, BMP4 signaling has been shown to promote ureteric stalk elongation and inhibit branching morphogenesis (Brenner-Anantharam et al., 2007; Miyazaki et al., 2000). *Bmp4* requires *Tbx18* function to maintain its normal expression in the ureteral mesenchyme (Airik et al., 2006). At earlier stages, it is expressed in the mesenchyme surrounding the WD, nascent UB and ureteric stalk (Brenner-Anantharam et al., 2007; Michos et al., 2007). After UB outgrowth, *Bmp4* is not expressed in the MM surrounding the UB tip (Fig. 3G). However, the tips of *Six1*$^{-/-}$ UBs at E10.5-11.5 during which the ampulla formation and its first branching event normally take place are surrounded by *Bmp4*-expressing mesenchyme (Fig. 3H). We also investigated *Bmp4* expression at E12.0–15.5 and found that *Bmp4*-positive cells also surrounded the proximal end of the mutant ureters at these stages (data not shown). Ureteral smooth muscle cells (SMCs) in the mutant ureters were also detected at E15.5 by staining with anti-α-smooth muscle actin (SMA) antibody (Fig. 3I,J), an early marker for differentiating SMCs. Therefore, it appears that in the absence of ampulla formation in *Six1*$^{-/-}$ embryos, the UB tip cells are induced to

differentiate into ureter by responding to signaling from the *Tbx18*- and *Bmp4*-expressing mesenchyme.

Grem1 expression in the MM is *Six1*-dependent

It was previously proposed that GREM1 in the MM antagonizes BMP4 activity to initiate branching by establishing an autoregulatory GDNF/WNT11 feedback signaling loop (Michos et al., 2007). We therefore set out to test whether there is an alteration in *Grem1* expression in the MM of *Six1*$^{-/-}$ embryos. High level of *Grem1* expression was detected in the mesenchyme surrounding the nascent UB tip at around E10.5–10.75 (Fig. 4A) (Michos et al., 2007). By contrast, in *Six1*$^{-/-}$ embryos, *Grem1* expression was markedly reduced at E10.75 (Fig. 4B) and became undetectable at around E11.0 (data not shown). This observation raises the possibility that downregulation of *Grem1* expression in the MM of *Six1*$^{-/-}$ embryos may lead to increase of mesenchymal BMP4 activity.

Since phosphorylated SMAD (pSMAD) proteins are established mediators of BMP signaling pathways (Massague et al., 2005), we tested whether the level of pSMAD expression in the mesenchyme is altered in the mutant by performing immunohistochemistry with an antibody that recognizes phosphorylated SMAD1/5/8. pSMAD-positive cells were apparently present in the UB and its surrounding mesenchyme including the MM in both wild-type and *Six1*$^{-/-}$ embryos at E10.5 (Fig. 4C,D and data not shown). We counted five sections from each kidney rudiment (n=3) and quantified the number of pSMAD-positive cells as the ratio of pSMAD-positive cells to the total number of metanephric mesenchymal cells in the control and mutant kidney primordial respectively. Although the total number of cells in the mutant MM is reduced to ~86% of wild-type mesenchymal cells, the percentage of pSMAD-positive cells vs total number of cells in the MM is comparable between wild-type (51.1±7) and mutant (52.2±9) embryos.

The presence of a fraction of pSMAD-positive cells in the MM of wild-type embryos indicates that those cells receive BMP signals. As *Bmp7* is known to be expressed in both the MM and the UB (Fig. 4E), we analyzed its expression in *Six1*$^{-/-}$ embryos. At around E10.5–10.75, *Bmp7* expression was detectable in the MM but the level of its expression appeared to be decreased in *Six1*$^{-/-}$ embryos (Fig. 4F) when compared to wild-type controls (Fig. 4E). However, as a recent report has shown that BMP7 acts through a SMAD-independent signaling mechanism in nephrogenic progenitor cells (Blank et al., 2009), reduction of BMP7 expression in *Six1*$^{-/-}$ MM may not affect the levels of pSMAD expression.

We found that *Six1* is specifically expressed in the MM (Fig. 4I) (Xu et al., 2003), but its expression is transient and disappears soon after the first epithelial branching event (Fig. 4J). Therefore, *Six1* may play a critical role in initiating branching morphogenesis by modulating *Grem1* expression in the MM. To further evaluate the nature of the mutant MM, in addition to *Bmp7*, we analyzed a number of additional MM-specific markers such as *Gdnf, Eya1* and *Wt1*. Consistent with our previous observations (Xu et al., 2003), we found that the levels of *Eya1*, *Wt1* and *Bmp7* expression were normal at E10.5 just before the initiation of UB outgrowth (data not shown). The levels of *Gdnf* expression also appeared to be normal at around E10.5, before UB outgrowth (Fig. 4G,H). It should be noted that the mutant MM appears smaller and not as well condensed as in wild-type controls at E10.5 and E10.75 (Fig. 4F,H, compare with 4E,G). It ultimately degenerates by apoptosis, which was observed from E11.5 (Xu et al., 2003). Thus, while the MM appears morphologically abnormal from E10.5, the clear reduction of *Grem1* expression in *Six1*$^{-/-}$ MM indicates that it may act as a critical player in the *Six1*-regulatory pathway during the initiation of branching morphogenesis.

Exogenous GREM1 restores branching morphogenesis in *Six1*$^{-/-}$ kidney primordia

Since exogenous GDNF can rescue branching morphogenesis in *Grem1*$^{-/-}$ kidney rudiments (Michos et al., 2007), we first tested the responsiveness of the mutant ureteric epithelium to exogenous GDNF by treating *Six1*$^{-/-}$ kidney primordia at different stages with different concentrations of GNDF (100–250 ng/ml). Our results show that the efficiency of exogenous GNDF rescue varies depending upon the GDNF concentration and developmental stages. Addition of GDNF at a higher dose (~200 ng/ml) most efficiently restored branching morphogenesis as well as small kidney formation in *Six1*$^{-/-}$ kidney rudiments at E10.5 (n=15/15) (Fig. 5). Massive epithelial overgrowth and branching were induced along the entire WD in the mutant, similar to what occurred in wild-type (Michos et al., 2007; Shakya et al., 2005). However, GDNF failed to effectively induce UB branching and kidney formation of *Six1*$^{-/-}$ kidney rudiments after E11.25 (data not shown). Very few epithelial budding from the UB tips of the mutant rudiments at around E11.25-11.5 was only observed when GDNF concentration was increased to ~250 ng/ml (data not shown). No kidney formation was observed, and the MM degenerated completely (data not shown). However, as the epithelium of *Six1*$^{-/-}$ kidney rudiments at E10.5, before the onset of cell apoptosis that occurs in the MM after E11.0 (Xu et al., 2003), is able to respond to GDNF to undergo branching morphogenesis, the failure of branching morphogenesis in *Six1*$^{-/-}$ embryos is not caused by an intrinsic defect in the epithelium but rather by defects in mesenchyme-epithelial signaling.

We next performed kidney culture experiment to examine the ability of exogenous GREM1 in rescuing *Six1*$^{-/-}$ UB morphogenesis. E10.5 wild-type and *Six1*$^{-/-}$ kidney primordia expressing the *Hoxb7*-GFP transgene were cultured for up to 96 hours in the presence or absence of soluble recombinant GREM1 protein. The development of *Six1*$^{-/-}$ kidney primordia arrested at the UB stage and the UB elongated into ureter when cultured in medium without GREM1 protein (Fig. 1I, n=13/16). Previous studies have shown that GREM1 is able to induce ectopic bud formation but is less effective in wild-type kidney rudiments than in *Grem1*$^{-/-}$ samples (Michos et al., 2007). Consistent with the previous observation, we found that GREM1 (2–6 μg/ml) was less effective in inducing ectopic UB outgrowth than GDNF and the ectopic buds that underwent two or four branching events by 96 hours were only formed adjacent to the metanephric kidney region (data not shown). Ureteric epithelial tracings of the GREM1-treated *Six1*$^{-/-}$ kidney primordia revealed that the UB tip was dilated to form an ampulla (n=10/10) but only less than half of them proceeded to the first branching event (n=3/10; data not shown).

Our results raise the possibility that UB branching in *Six1*$^{-/-}$ embryos may require locally concentrated GREM1 or additional mesenchymal factors, which are downregulated or missing in *Six1*$^{-/-}$ MM. To address this, microdissected wild-type and E10.5 *Six1*$^{-/-}$ kidney rudiments were implanted with GREM1-soaked beads (1.5 μg/μl). Wild-type kidney rudiments showed ectopic bud formation (red stars, Fig. 6; n=12/12), and majority of them were able to undergo one or more branching (n=10/12). The ectopic buds that formed are all close to the metanephric kidney region. Interestingly, *Six1*$^{-/-}$ rudiments also showed the UB outgrowth from its normal position (white arrows, Fig. 6; n=6/6) and the formation of ectopic buds (red asterisks, Fig. 6; n=6/6). The UBs observed in *Six1*$^{-/-}$ rudiments showed ampulla formation at the tip of the bud that was able to undergo several branching events (arrows, Fig. 6), which was not observed when GREM1 was added into the culture medium (data not shown). No ectopic UB induction and branching were observed when *Six1*$^{-/-}$ rudiments were cultured with beads containing BSA (data not shown). This result establishes that recombinant GREM1 is able to restore epithelial branching morphogenesis in the absence of *Six1*.

We further analyzed the responsiveness of the epithelium and mesenchyme in *Six1*$^{-/-}$ kidney primordia by staining with *Wnt11*, *Gdnf* and *Wt1*. The expression of all three markers was detectable in the mutant samples (Fig. 7), although the kidney rudiments appeared to be smaller when compared with controls. This indicates that recombinant GREM1 reconstitutes mesenchyme-epithelial signaling necessary for branching morphogenesis and nephron differentiation. These results show that *Grem1* is a critical mesenchymal regulator downstream of *Six1* in inducing the initiation of UB branching morphogenesis.

Genetic reduction of BMP4 activity in *Six1*$^{-/-}$ embryos rescues kidney organogenesis

Since BMP4 promotes ureter differentiation in zones of the UB normally fated to differentiate into collecting tubules in kidney explant cultures (Brenner-Anantharam et al., 2007; Wang et al., 2009), and GREM1 acts as an antagonist to negatively regulate BMP4 activity in the mesenchyme to initiate UB outgrowth and invasion into the mesenchyme (Michos et al., 2007), we further tested the hypothesis that excessive mesenchymal BMP4 activity blocks the UB tip cells from forming an ampulla in *Six1*$^{-/-}$ embryos by examining *Six1;Bmp4* compound mutant embryos. Because of early lethality of *Bmp4*$^{-/-}$ embryos (Winnier et al., 1995), we tested whether inactivation of one copy of the *Bmp4* gene in *Six1*$^{-/-}$ (*Six1*$^{-/-}$; *Bmp4*$^{+/-}$) embryos restores ampulla formation and its branching morphogenesis. In *Six1*$^{-/-}$*;Bmp4*$^{+/-}$ mutant embryos, ampulla formation and its branching were initiated normally at around E11.25-11.5 (Fig. 8A–D and data not shown). Kidneys did indeed form bilaterally in *Six1*$^{-/-}$*;Bmp4*$^{+/-}$ embryos at E17.5 (6 out of 6) but were smaller (Fig. 8G,H) when compared with normal kidneys (Fig. 7E,F). In contrast, among *Six1*$^{-/-}$*;Bmp4*$^{+/+}$ littermate controls examined (n=9), ~78% (7 out of 9) had no kidneys, while ~22% (2 out of 9) showed unilateral rudimentary kidneys (data not shown). Histological and marker analyses of kidneys confirmed the presence of kidney structures in *Six1*$^{-/-}$*;Bmp4*$^{+/-}$ embryos (Fig. 8I–L). Ureteric epithelial tracing of *Hoxb7-GFP* of *Six1*$^{-/-}$*;Bmp4*$^{+/-}$ kidneys revealed that branching morphogenesis was restored (Fig. 8M,N). This demonstrates that the nephrogenic progenitors in the *Six1*$^{-/-}$*;Bmp4*$^{+/-}$ mutant are able to induce UB branching and undergo nephrogenesis. In addition to the kidney, hydroureter phenotype was observed in *Six1*$^{-/-}$*;Bmp4*$^{+/-}$ animals and the mice die at the end of gestation probably because of multiple defects in several organ systems, as *Six1*$^{-/-}$ mice also lack thymus, parathyroid, ear and olfactory epithelium as well as exhibiting severe defects in craniofacial, muscle and sensory nervous systems (Chen et al., 2009; Laclef et al., 2003a; Laclef et al., 2003b; Xu et al., 2003; Zheng et al., 2003). Taken together, these results demonstrate that the genetic reduction of BMP4 activity in *Six1*-deficient embryos restores UB patterning and metanephric kidney organogenesis (Fig. 8F,H), thus providing the first evidence for the requirement of *Six1* in spatially restricting BMP4 signaling during UB morphogenesis.

Discussion

Although many mesenchymal genes have been implicated in regulating the formation of ampulla at the UB tip, the regulatory relationship among these genes and the cellular and molecular mechanisms controlling the initiation of branching morphogenesis remain largely unknown. A recent study has shown that GREM1 is necessary to negatively regulate BMP4 signaling for establishing mesenchyme-epithelial signaling during UB branching morphogenesis (Michos et al., 2007). However, how the GREM1-BMP4 signaling is regulated during the initiation of UB branching is currently unknown. This study is the first to demonstrate that *Six1* functions as an upstream regulator of *Grem1* to locally restrict and negatively regulate BMP4 activity in the mesenchyme during the initiation of branching morphogenesis. Moreover, we show that reduction of *Bmp4* dosage in *Six1*$^{-/-}$ embryos restores nephron differentiation.

Previous studies have suggested that initiation of UB outgrowth and invasion of the mesenchyme requires antagonism of BMP4 by the extracellular BMP antagonist GREM1 in the mesenchyme (Michos et al., 2007; Michos et al., 2004). Our analyses show that the disruption of UB outgrowth in *Six1*$^{-/-}$ embryos appears less severe than that observed in *Grem1*$^{-/-}$ embryos. In *Grem1*$^{-/-}$ embryos, no proper ureteric tip domain is formed and the ureteric stalk does not elongate but degenerates soon after its outgrowth (Michos et al., 2007). However, in *Six1*$^{-/-}$ embryos, the UB tip domain is initially formed and the UB elongation also occurs normally. This agrees with the fact that *Grem1* is initially expressed in *Six1*$^{-/-}$ kidneys. In contrast to the UB development, the expression of *Gdnf* in the MM is progressively lost, which is likely caused by gradual elimination of the MM because of lack of epithelial-mesenchymal interactions (Xu et al., 2003). This phenotype is similar to that observed in *Grem1*$^{-/-}$ embryos (Michos et al., 2007; Michos et al., 2004). UB invasion into the MM is blocked in both the *Grem1*- and *Six1*-deficient embryos (Figs 1–3 of the present study; (Michos et al., 2007). It has been shown that *Grem1* is only expressed in the WD at E10.0, and its expression becomes evident in the mesenchyme surrounding the UB tip region at around E11.0 (Michos et al., 2007; Michos et al., 2004). Consistent with this observation, we detected *Grem1* expression in the MM from around E10.75 (Fig. 4). In contrast, *Six1* is expressed earlier than *Grem1* in the MM but its expression disappears soon after the initiation of the first branching event at around E11.5 (Fig. 4). This expression pattern of *Six1* further indicates that it may play a key role for the MM to induce initiation of epithelial branching morphogenesis. Our in vivo ChIP analysis performed on embryonic MM extracts has demonstrated direct binding of SIX1 to a region of the *Grem1* promoter containing conserved MEF3 consensus sites, which are recognized by Six family protein (Giordani et al., 2007; Spitz et al., 1998) (Elaine Y-W Wong, Feng Wang and P-X. Xu, unpublished). This suggests that SIX1 exerts its action via a direct interaction with the *Grem1* promoter to specifically upregulate its transcription in the metanephric mesenchymal cells at this critical early stage. This upregulation may be required to locally repress BMP4 activity in the mesenchyme to ensure that UB tip is induced for ampulla formation. This explains why exogenous GREM1 is able to restore ampulla formation and its subsequent branching events in *Six1*$^{-/-}$ kidney primordia. We are currently testing the requirement of each individual binding site for the upregulation of *Grem1* promoter activity by *Six1* in vitro.

Our observation that the UB tip of *Six1*$^{-/-}$ embryos is wrapped up by the *Tbx18*- and *Bmp4*-expressing mesenchyme suggests that the block of UB ampulla formation at the UB tip may be caused by responding to the signaling in the *Tbx18*- and *Bmp4*-expressing mesenchyme, which are cellular sources for ureteral smooth muscle (Airik et al., 2006; Wang et al., 2009). Our recent observation that reduction of one-copy of the *Tbx18* gene dosage in *Six1*$^{-/-}$ (*Tbx18*$^{+/-}$;*Six1*$^{-/-}$) embryos, which show reduced *Bmp4* expression in the ureteral mesenchyme, also rescues UB branching morphogenesis and kidney formation (Nie et al., 2010) further indicates that reducing BMP4 activity in the *Tbx18*-expressing mesenchyme surrounding the UB tip of *Six1*$^{-/-}$ embryos reconstitutes mesenchyme-epithelial signaling crosstalk to enable branching morphogenesis and nephron formation. As previous lineage-tracing study has shown that Bmp4+ cells are derived from tail-bud mesenchyme (Brenner-Anantharam et al., 2007) and there is no evidence that *Grem1* is expressed in the same cell lineage as *Bmp4*, GREM1 is less likely to directly repress BMP4 activity in the same cells. Although lineage tracing of *Grem1*-expressing cells and colocalization studies are important to address this issue, GREM1 may act as an extracellular signaling molecule to locally antagonize BMP4 activity in the *Bmp4*-expressing mesenchyme (Fig. 9), which would normally act on the UB epithelium to induce its differentiation into the ureter (Brenner-

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Anantharam et al., 2007). It is possible that the BMP4 signaling may repress *Wnt11* expression in the UB tip, which normally signals back to the mesenchyme either directly or indirectly to upregulate the expression of *Gdnf* in the MM. Nonetheless, GREM1 is likely to play an indirect role in establishing the WNT11-GDNF signaling to an optimal level for branching morphogenesis to occur in *Six1*$^{-/-}$ kidney primordia.

Recent studies have suggested that BMP4 in the mesenchyme may utilize ActRIIA and its co-receptor Dragon, both are highly expressed in the ureteric stalk, for signaling (Samad et al., 2005; Xia et al.); however the downstream pathway that mediates the function of BMP4/Dragon/ActRIIA remains to be determined. In *Grem1*$^{-/-}$ embryos, BMP4 signaling transduction in the MM appears to be increased because the levels of pSMAD expression are increased in the MM of *Grem1*$^{-/-}$ embryos (Michos et al., 2007). However, this does not appear to occur in *Six1*$^{-/-}$ MM, as we failed to detect obvious changes in the levels of pSMAD expression (Fig. 4). Since BMPs are known to activate both SMAD1/5/8 and mitogen-activated protein kinase (MAPK) p38, JNKs and Erk1/2 pathways (Derynck and Zhang, 2003; Yu et al., 2008), it is possible that BMP4 may utilize SMAD-dependent and SMAD-independent pathways as recently demonstrated for BMP7 in the nephron progenitor cells (Blank et al., 2009).

Our analysis also shows that the balance between the levels of BMP4 activity in the *Bmp4*-expressing mesenchyme surrounding the nascent UB tip and GDNF production specifically in the MM appears to be crucial for UB patterning. When the BMP4 activity is lowered in the *Tbx18*- or *Bmp4*-expressing mesenchyme enveloping the UB tip of *Six1*$^{-/-}$ embryos, the level of GDNF produced in the MM becomes sufficient to induce the UB tip cells to proliferate and initiate branching (Fig. 9). Alternatively, when excessive GDNF is added in culture medium, it becomes sufficient to restore branching morphogenesis in *Grem1*$^{-/-}$ (Michos et al., 2007) or *Six1*$^{-/-}$ kidney rudiments at earlier stages (Fig. 5). Thus, BMP4 and GDNF may antagonize each other's effect in inducing ampulla formation. In support of this, BMP4 has been shown to suppress the effects of GDNF in inducing ectopic buds in vitro (Brophy et al., 2001). As *Gdnf* expression appears to be normal in rescued *Six1*$^{-/-}$ kidney primordia, *Six1* is unlikely to play a direct role in modulating *Gdnf* expression in the MM during early epithelial branching.

In summary, our results presented here identify *Six1* as an upstream regulator of *Grem1* in the MM, which locally restricts BMP4 signaling in the mesenchyme surrounding the nascent UB (Fig. 9). Our findings that genetically lowering BMP4 activity in *Six1*$^{-/-}$ mice restores UB branching and kidney organogenesis in vivo provide strong evidence that *Six1* is indeed required for spatially restricting BMP4 activity to ensure ampulla formation and its invasion into the MM during kidney development. After the initiation of branching morphogenesis, *Six1* is no longer expressed in the MM (Fig. 4), it therefore seems to be only required for the initiation of branching morphogenesis, but not for repeated branching events to occur and for the maintenance of *Grem1* expression in the MM.

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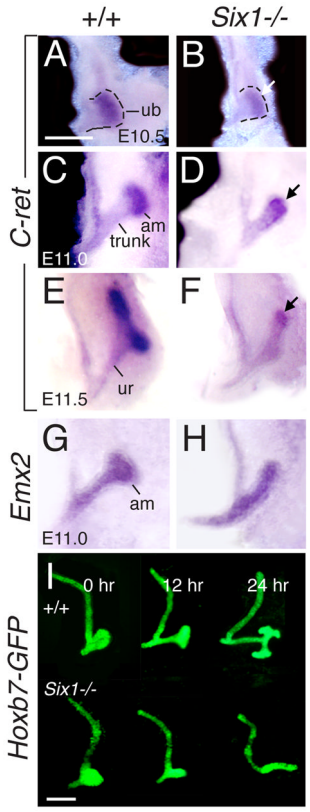

Fig. 1.

Six1$^{-/-}$ UB tip fails to form ampulla. (A–H) The metanephric region was dissected out from E10.5 (A,B), E11.0 (C,D,G,H) and E11.5 (E,F) embryos and stained for *c-Ret* or *Emx2* by whole-mount in situ hybridization. (A) Normal UB outgrowth and the formation of tip domain as labeled by *c-Ret* expression. (C,E) The UB tip domain swells to form an ampulla, which undergo branching to the T-bud stage at E11.5 (E), while the trunk region elongates to form the ureter. (G) E11.0 metanephric region showing *Emx2* expression in ureteric stalk and ampulla. (B, D, F, H) In *Six1*$^{-/-}$ embryos, the UB outgrowth, its subdivision into the trunk and tip domains and elongation appear to occur normally, but its tip region is not dilated for the formation of ampulla. (I) Kidney rudiments expressing *Hoxb7-GFP* transgene were dissected from E10.5 wild-type and *Six1*$^{-/-}$ embryos and cultured in vitro for 0, 12 or 24 hour, further confirming no ampulla formation in the mutant. Abb.: am, ampulla, ub, ureteric bud, ur, ureter. Scale bars: 100 μm in A–H; 50 μm in I.

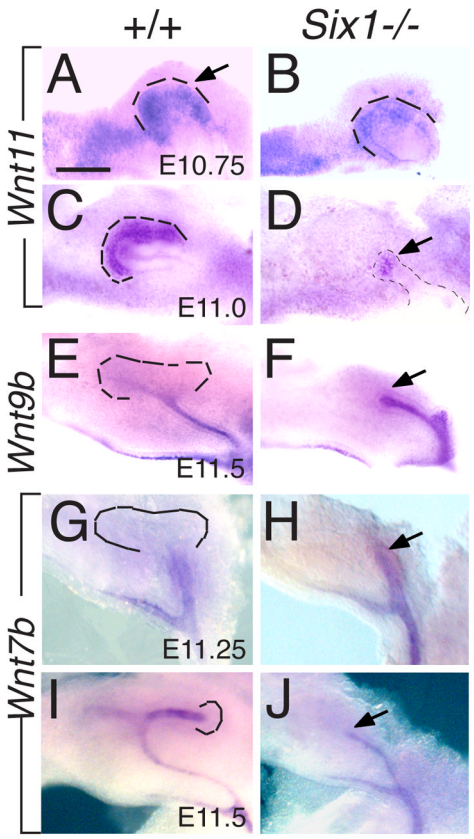

Fig. 2. Altered *Wnt11* expression in *Six1*$^{-/-}$ UB tips

The metanephric region was dissected out from E10.5–11.5 embryos and stained for *Wnt11*, *Wnt9b* or *Wnt7b* by whole-mount in situ hybridization. (A–D) *Wnt11* expression in the most proximal tip domain at E10.75 (A,B) and E11.0 (C,D) in wild-type embryos and *Six1*$^{-/-}$ embryos. (E,F) *Wnt9b* expression in the WD and the ureteric stalk in controls and *Six1*$^{-/-}$ embryos at E11.25-11.5. (G–J) *Wnt7b* expression in WD and UB trunk region at E11.25 (I,J) and E11.5 (K,L) in control and *Six1*$^{-/-}$ embryos. Arrows in J and L point to the proximal end of ureteric epithelium. Dashed lines outline the UB tip regions. Scale bar: 50 μm in A–D; 80 μm in E–J.

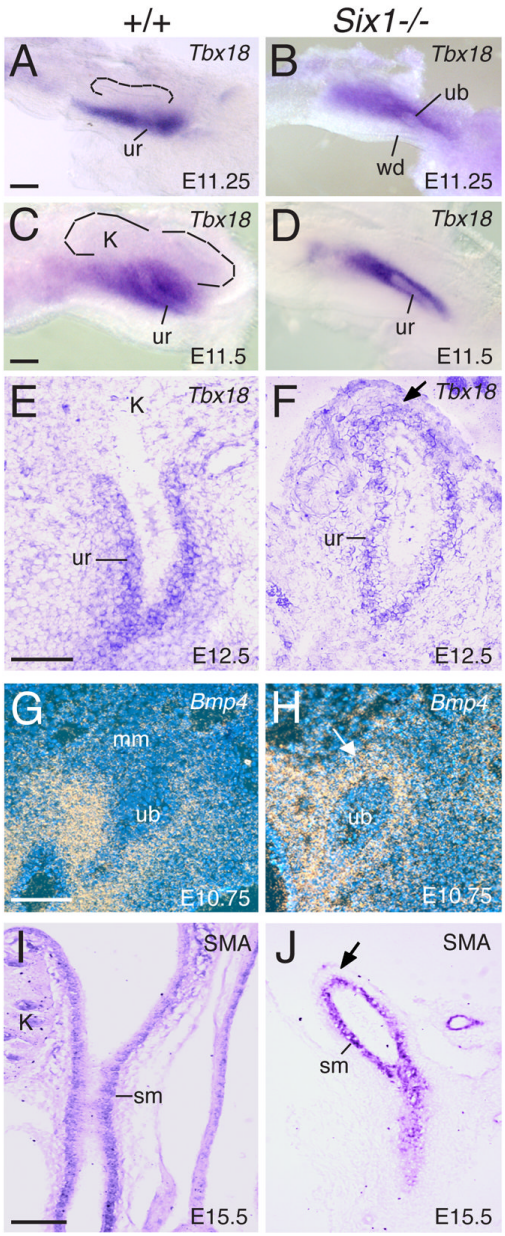

Fig. 3. *Six1*$^{-/-}$ UB elongates within *Tbx18*- and *Bmp4*-expressing mesenchyme

(A,C) Whole mount in situ hybridization of the metanephric region showing the strong *Tbx18* expression domain surrounding the UB trunk region, which is ventral to the MM. Dashed lines point to UB branching within the MM. (B,D) In *Six1*$^{-/-}$ embryos, the elongating ureter is embedded within the *Tbx18*-expressing mesenchyme. (E,F) Section in situ at E12.5 showing *Tbx18* expression surrounding the ureteral mesenchyme in control and *Six1*$^{-/-}$ embryos. Arrow points to the proximal end of the ureter in the mutant. (G,H) Radioisotope section in situ hybridization showing normal *Bmp4* expression in the mesenchyme surrounding the ureteric stalk and cloaca but is absent in the metanephric mesenchyme in wild-type control (G), whereas in *Six1*$^{-/-}$ embryos, the UB tip is wrapped up by *Bmp4*-expressing mesenchyme (arrow). (I,J) Longitudinal sections showing SMA-positive cells surrounding the ureter in control and *Six1*$^{-/-}$ embryos at E15.5. Abb.: k,

kidney, mm, metanephric mesenchyme, sm, smooth muscle, ub, ureteric bud, ur, ureter, wd, Wolffian duct. Scale bars: 50 μm in A-D; 100 μm in E–J.

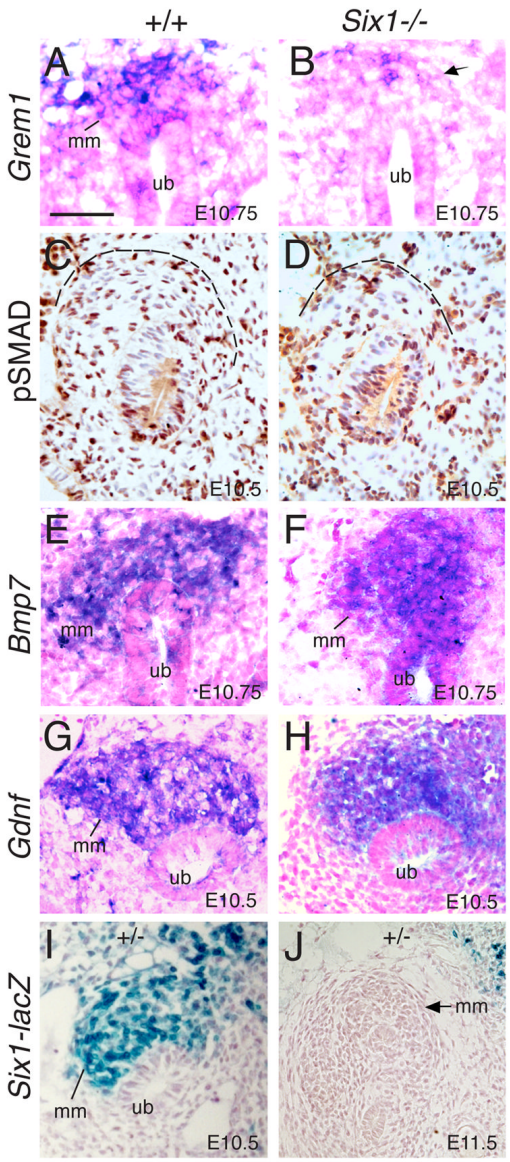

Fig. 4. *Grem1* expression in the mesenchyme surrounding the UB tips is *Six1*-dependent

(A,B) Section in situ with *Grem1* probe showing its expression in the mesenchyme surrounding the UB tip in normal and *Six1*$^{-/-}$ embryos. (C,D) Immunohistochemistry for pSMAD on sections from kidney region of normal and *Six1*$^{-/-}$ embryos. pSMAD-positive nuclei (brown) are present in both the mesenchyme and the epithelium of wild-type and *Six1*$^{-/-}$ embryos. pSMAD-positive cells from five sections from each MM (three kidneys) were quantified as the ratio of the pSMAD-positive cells to the total number of metanephric mesenchymal cells in the control and mutant ureters. *P*-values (*P*=0.304) were calculated using StatView *t*-test. Dashed lines outline the peripheral boundary of the MM. (E,F) Section in situ showing *Bmp7* expression in the MM and UB in normal and *Six1*$^{-/-}$ embryos at E10.75. (G,H) Section in situ showing *Gdnf* expression in the MM at E10.5 normal and *Six1*$^{-/-}$ embryos. Note that the mutant MM is not as well condensed as in controls. (I) A section stained by X-gal showing *Six1* expression in the MM at E10.5, (J) but its expression in the MM disappears at E11.5. Abb.: mm, metanephric mesenchyme; ub, ureteric bud. Scale bars: 50 μm.

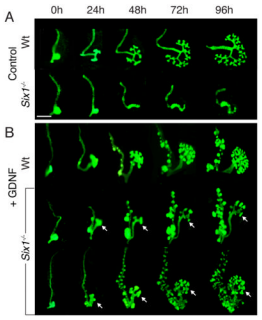

Fig. 5. Excessive GDNF is sufficient to restore not only UB branching but also tubulogenesis of the mesenchyme in *Six1*-deficient kidney primordia in culture

(A) Wild-type and *Six1*-deficient kidney primordia cultured in control medium showing normal UB outgrowth and branching and no branching in *Six1*$^{-/-}$ rudiments. (B) Control and *Six1*$^{-/-}$ kidney primordia at E10.5 were cultured in the presence of GDNF at ~200 ng/ml, many epithelial buds along the WD and overgrowth of the ureteric epithelium within 24 hours, which results in excessive branching in both wild-type and *Six1*-deficient kidney primordia. Note the branching and the small kidneys in GDNF-treated *Six1*-deficient kidney primordia. Scale bars: 100 μm.

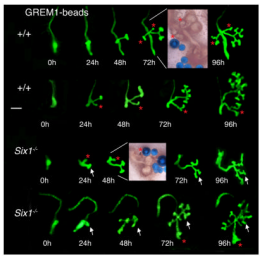

Fig. 6. Recombinant GREM1 protein is able to induce ampulla formation and branching morphogenesis in *Six1*$^{-/-}$ kidney primordia

Wild-type and *Six1*$^{-/-}$ kidney primordia expressing the *Hoxb7*-GFP transgene in their WD and ureteric epithelium were isolated from mouse embryos at E10.5–11.0, implanted with GREM1-soaked beads and cultured for up to 96 hours. Panels show from left to right: cultures at 0 hours, 24 hours, 48 hours, 72 hours and 96 hours (time: ±2–3 hours). White asterisks indicate ureteric buds, red asterisks indicate ectopic epithelial buds and ectopic branches. Note the branching and the small kidneys in *Six1*-deficient kidney primordia (white arrows). Scale bar: 100 μm.

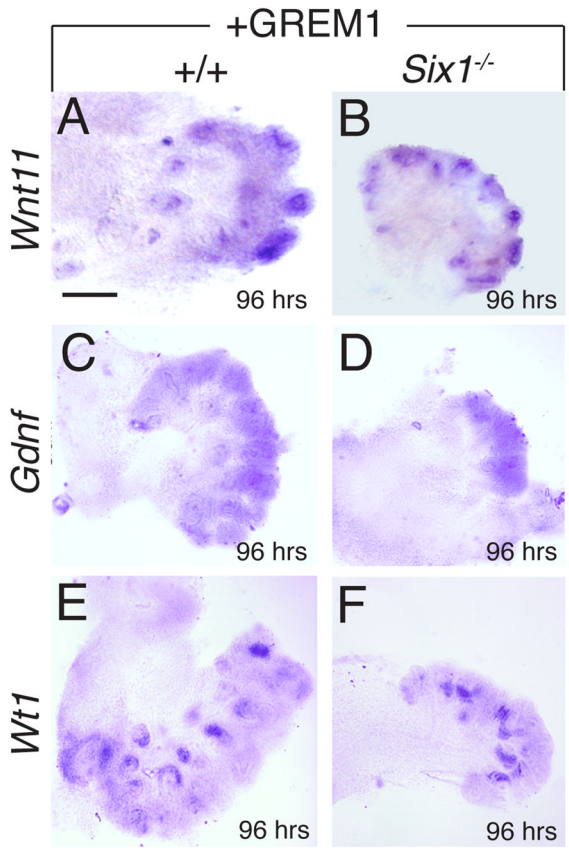

Fig. 7. Recombinant GREM1 is able to restore *Wnt11* and *Gdnf* expression in *Six1*-deficient kidney rudiments in culture

Mouse kidney primordia were isolated at E10. 5–11.0, cultured for 96 hours in the presence of recombinant GREM1-soaked beads and then whole-mount stained with *Wnt11* (A,B), *Gdnf* (C,D) and *Wt1* (E,F) probes. Obvious *Wnt11*, *Gdnf*, and *Wt1* expression was observed in the mutant rudiments implanted with GREM1-soaked beads and culture for 96 hours. Scale bars: 25 μm.

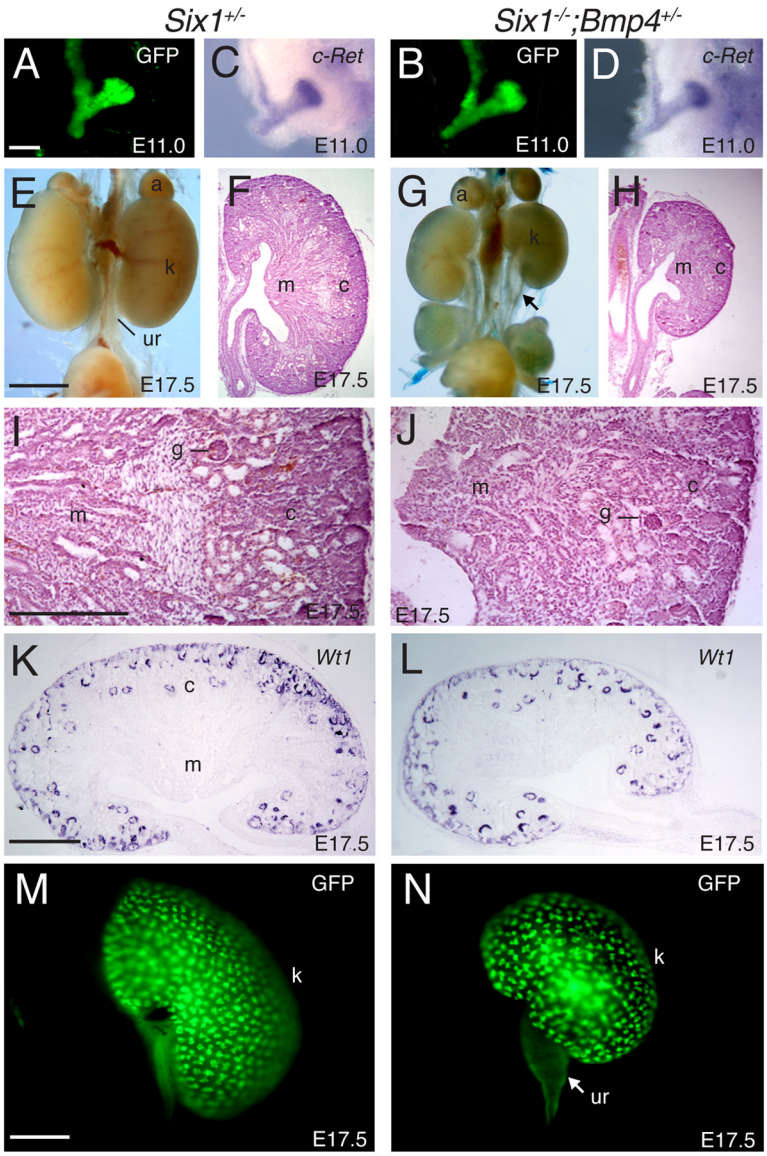

Fig. 8. Inactivation of one copy of the *Bmp4* gene in *Six1*$^{-/-}$ embryos results in formation of two small kidneys

(A,C) GFP reveals the morphology of the epithelium before fixation and (B,D) whole-mount in situ detection for the expression of the *Ret* receptor in control and *Six1*$^{-/-}$;*Bmp4*$^{+/-}$ embryos at E11.25. (E,G) Gross morphology of urogenital system and (F,H) H&E staining of histological sections of kidneys of *Six1*$^{+/-}$ control (E,F) and *Six1*$^{-/-}$;*Bmp4*$^{+/-}$ (G,H) embryos at E17.5. (I,J) Higher magnification of boxed area of F and H respectively. Developing kidney structures such as glomeruli, tubules and collecting ducts are present in the compound mutant (J). However, the patterning of the medulla (m) and cortex (c) appeared abnormal. (K,L) Kidney section of control and *Six1*$^{-/-}$;*Bmp4*$^{+/-}$ kidney stained with *Wt1* probe showing the developing nephrons. (M,N) Epithelial tracing of *Hoxb7-GFP* of E17.5 kidney in control and *Six1*$^{-/-}$;*Bmp4*$^{+/-}$ animals. Arrow points to hydroureter phenotype in the compound mutant. Abb.: a, adrenal gland; c, cortex; g, glomeruli; k, kidney; m, medulla; ur, ureter. Scale bars: 50 μm in A–N; 25 μm in I,J.

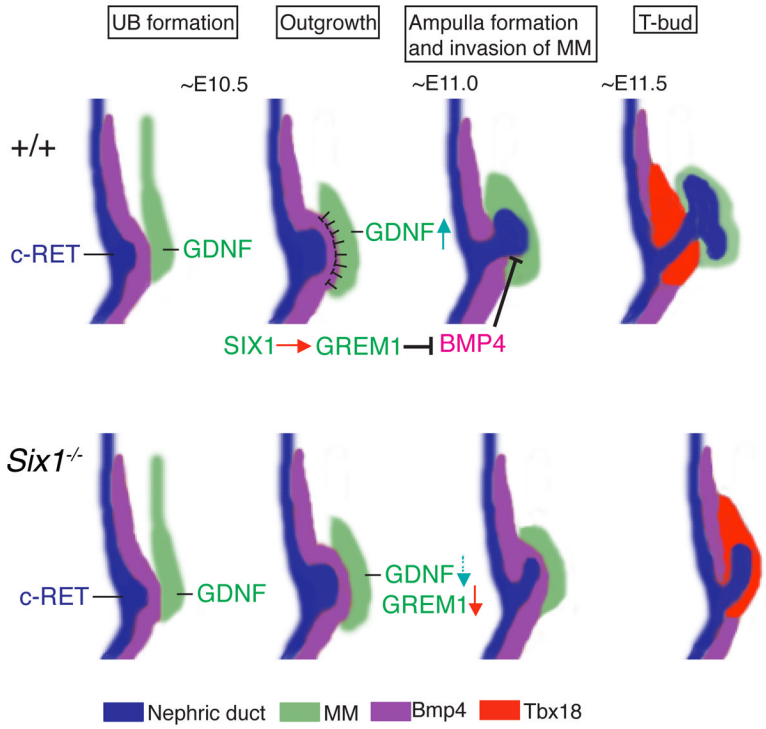

Fig. 9. Upregulation of *Grem1* by *Six1* in the MM is essential to locally restrict BMP4 signaling for the initiation of branching morphogenesis

In mice, the UB formation and outgrowth is induced by GDNF-RET signaling, which is initially expressed at normal levels in *Six1*$^{-/-}$ embryos. During this inductive period, *Six1* is specifically expressed in the MM (green). *Bmp4* (purple) is expressed by the mesenchyme enveloping the WD (blue) and nascent UB (blue). *Grem1* transcripts are detected in the MM around the tip region of the UB, thereby locally antagonizing BMP4 in the *Bmp4*-expressing mesenchyme (purple) (around E10.75-11.0). This reduction of BMP4 activity by GREM1 establishes epithelial-mesenchymal interactions to enable ampulla formation of the UB and its invasion into the MM. *Gdnf* expression is also upregulated during this period to stimulate ampulla formation and establish WNT11-GDNF signaling for branching of the ampulla. Our results indicate that *Six1* plays an essential role in lowering BMP4 activity by upregulating *Grem1* expression.

In *Six1*$^{-/-}$ embryos, the UB outgrowth is normal but the tip cells fail to be induced for ampulla. Instead, the UB tip is wrapped up by *Tbx18*- and *Bmp4*-expressing mesenchyme, which is the cellular source for ureteral mesenchyme (Airik et al., 2006). By responding to the signaling in the *Tbx18*- and *Bmp4*-positive mesenchyme, the tip is induced for ureter differentiation. Our results show that the balance between the levels of BMP4 activity in the *Tbx18*$^{+}$ and *Bmp4*$^{+}$ mesenchyme and GDNF production in the MM may also be critical for UB patterning, as excessive GDNF can restore branching as well as kidney formation in *Six1*$^{-/-}$ kidney rudiments in culture and lowering BMP4 activity in vivo can rescue branching morphogenesis and nephron formation.