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Reduction of BMP4 activity by gremlin 1 enables ureteric bud outgrowth and GDNF/WNT11 feedback signalling during kidney branching morphogenesis

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Antagonists act to restrict and negatively modulate the activity of secreted signals during progression of embryogenesis. In mouse embryos lacking the extra-cellular BMP antagonist gremlin 1 (*Grem1*), metanephric development is disrupted at the stage of initiating ureteric bud outgrowth. Treatment of mutant kidney rudiments in culture with recombinant gremlin 1 protein induces additional epithelial buds and restores outgrowth and branching. All epithelial buds express *Wnt11,* and *Gdnf* is significantly upregulated in the surrounding mesenchyme, indicating that epithelial-mesenchymal (e-m) feedback signalling is restored. In the wild type, *Bmp4* is expressed by the mesenchyme enveloping the Wolffian duct and ureteric bud and *Grem1* is upregulated in the mesenchyme around the nascent ureteric bud prior to initiation of its outgrowth. In agreement, BMP activity is reduced locally as revealed by lower levels of nuclear pSMAD protein in the mesenchyme. By contrast, in *Grem1*-deficient kidney rudiments, pSMAD proteins are detected in many cell nuclei in the metanephric mesenchyme, indicative of excessive BMP signal transduction. Indeed, genetic lowering of BMP4 levels in *Grem1*-deficient mouse embryos completely restores ureteric bud outgrowth and branching morphogenesis. The reduction of BMP4 levels in *Grem1* mutant embryos enables normal progression of renal development and restores adult kidney morphology and functions. This study establishes that initiation of metanephric kidney development requires the reduction of BMP4 activity by the antagonist gremlin 1 in the mesenchyme, which in turn enables ureteric bud outgrowth and establishment of autoregulatory GDNF/WNT11 feedback signalling.

KEY WORDS: Antagonist, BMP, gremlin 1, Kidney, Mouse, Signalling

INTRODUCTION

Mammalian kidney organogenesis is regulated by reciprocal epithelial-mesenchymal (e-m) signalling interactions that involve inductive signalling between ureteric bud epithelium and metanephric mesenchyme (Saxén, 1987; Yu et al., 2004). In mouse embryos, the ureteric bud forms around embryonic day (E) 10.5 as an epithelial swelling in the caudal-most part of the Wolffian duct. Development of the definitive kidney is initiated when the ureteric bud elongates to invade and induce the metanephric mesenchyme. In turn, the induced metanephric mesenchyme produces signals that initiate nephrogenesis. During ureteric bud outgrowth, the epithelial tip region thickens to form an ampulla just prior to appearance of the first epithelial branch (Shakya et al., 2005). In addition, this process induces condensation of the metanephric mesenchyme, which is the first morphological sign of nephrogenesis (Saxén, 1987). Many of the human congenital anomalies of the kidney and urinary tract (CAKUT) are caused by defects in these early inductive events, but their aetiology remains poorly understood (Batourina et al., 2002; Pope et al., 1999).

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During the last decade, significant parts of the molecular networks and e-m feedback signalling interactions that regulate mammalian kidney organogenesis have been identified (Costantini, 2006; Vainio and Lin, 2002). It is established that ureteric bud formation and the induction of its branching require GDNF (glial cell line derived neurotrophic factor), a secreted growth factor that is expressed by the metanephric mesenchyme. The GDNF ligand interacts with its cognate receptor RET (ret proto-oncogene), which is first expressed by the Wolffian duct and then by the ureteric epithelial tips as branching morphogenesis progresses (Schuchardt et al., 1996). The current view is that establishment of signalling between GDNF, RET and its co-receptor GFRα1 (glial cell line derived neurotrophic factor family receptor α1) is essential for ureteric bud formation and initiation of outgrowth and branching (Costantini and Shakya, 2006). As the rostral part of the Wolffian duct initially expresses *Ret*, it is competent to form supernumerary buds and branches upon exposure to GDNF (Shakya et al., 2005). Therefore, a mechanism must exist that restricts ureteric bud formation to the caudal-most part of the Wolffian duct. Indeed, supernumerary epithelial buds form in mouse embryos lacking either SLIT2 or ROBO2 functions, the SPRY1 intra-cellular antagonist or the FOXC1 transcriptional regulator. Molecular analysis showed that SLIT2 and/or ROBO2 signalling is required to restrict *Gdnf* expression to caudal mesenchyme (Grieshammer et al., 2004). FOXC1 is also required for caudal restriction of *Gdnf* (Kume et al., 2000), but is not a target of SLIT2/ROBO2 signalling (Grieshammer et al., 2004). By contrast, SPRY1, an intra-cellular antagonist of tyrosine kinase receptors, reduces the sensitivity of the Wolffian duct to GDNF, such that only one ureteric bud forms (Basson et al., 2005; Chi et al., 2004). In *Spry1*-deficient mouse embryos, ectopic epithelial buds form and multiple- and hydro-

ureters are formed, which results in phenotypes identical to the human CAKUT syndrome (Basson et al., 2005). However, ectopic expression of *Spry2* in the Wolffian duct sensitises the epithelium to GDNF signalling, which again results in formation of supernumerary epithelial buds (Chi et al., 2004). Hence, the current view is that the interaction of these different signal transduction cascades restricts GDNF expression and activity such that only one ureteric bud forms in the caudal-most part of the Wolffian duct (Basson et al., 2005; Grieshammer et al., 2004). Metanephric kidney development is then initiated by the onset of ureteric bud outgrowth, and invasion and induction of the metanephric mesenchyme under the influence of GDNF-RET signalling (Costantini and Shakya, 2006).

Wnt11 expression is activated in the epithelial tip of the ureteric bud and WNT11 signalling is in turn required to propagate mesenchymal GDNF signalling, which results in establishment of an autoregulatory e-m feedback signalling loop (Majumdar et al., 2003). In *Wnt11*-deficient mouse embryos, *Gdnf* expression remains lower and the number of epithelial branches is reduced. Conversely, the disruption of GDNF signal reception in *Ret*-deficient embryos reduces *Wnt11* expression. Therefore, autoregulatory GDNF-WNT11 feedback signalling co-ordinately controls the progression of metanephric branching morphogenesis after initiation of ureteric bud outgrowth (Majumdar et al., 2003). During branching, the ureteric epithelial tips secrete additional signals (e.g. WNT9b) (Carroll et al., 2005), which induce nephrogenesis. Nephrogenesis is regulated by transcriptional activation of another WNT signal, WNT4 in the condensing mesenchyme. Mouse embryos that lack *Wnt4* fail to form metanephric kidneys due to disruption of the mesenchyme to epithelial transition of the nephrogenic precursors (Stark et al., 1994).

Several BMP ligands and their receptors are expressed during metanephric kidney organogenesis, but relatively little is known about their essential roles in these processes as loss-of-function mutations often cause early embryonic lethality. However, genetic inactivation of *Bmp7* in mouse embryos leads to premature depletion of the nephrogenic mesenchyme, which manifests itself in a dysplastic kidney phenotype (Dudley et al., 1995; Luo et al., 1995). A recent study shows that BMP4 can compensate for the lack of *Bmp7* during metanephric kidney development, which indicates that BMPs could potentially replace one another during kidney organogenesis (Oxburgh et al., 2005). Furthermore, a small fraction of mice heterozygous for a *Bmp4* loss-of-function allele display CAKUT-like phenotypes and/or multicystic dysplastic kidneys (Miyazaki et al., 2000). This phenotype is likely caused by defects in ureteric stalk elongation, but treatment of metanephric kidney primordia with recombinant BMP4 also interferes with epithelial branching morphogenesis in culture, which indicated that BMP4 activity may require dynamic modulation (Miyazaki et al., 2000; Raatikainen-Ahokas et al., 2000). Gremlin 1 (GREM1) is a member of the CAN domain family of extra-cellular BMP antagonists that binds BMP2 and BMP4 with highest affinity in vitro (Hsu et al., 1998). However, the ligands antagonised by GREM1 in vivo during vertebrate embryogenesis remained elusive to date. We recently showed that *Grem1* is expressed by the meso- and metanephric mesenchyme and that metanephric kidney organogenesis is disrupted in *Grem1*-deficient mouse embryos, resulting in bilateral renal agenesis and neo-natal lethality (Michos et al., 2004). In particular, metanephric kidney development is blocked at the stage of initiating of ureteric bud outgrowth. However, *Gdnf* expression is initially normal in the metanephric mesenchyme and the epithelium continues to express *Ret*. The block in initiating ureteric bud outgrowth causes progressive loss of *Gdnf* expression and elimination of the metanephric mesenchyme by apoptosis.

In the present study, we established that culturing early *Grem1*-deficient kidney primordia in medium supplemented with recombinant GREM1 restores ureteric bud outgrowth and supernumerary epithelial buds are induced. Multiple epithelial buds invade the metanephric mesenchyme and initiate branching morphogenesis. *Wnt11* expression in the epithelial tips and *Gdnf* expression in the metanephric mesenchyme around the epithelium is restored, which is indicative of establishment of e-m feedback signalling in GREM1-treated mutant kidney primordia. We identify excessive BMP activity in the metanephric mesenchyme around the ureteric bud as the primary signalling defect in *Grem1*-deficient mouse embryos. As a consequence, the invasion of the mutant metanephric mesenchyme by the ureteric bud and concurrent establishment of the autoregulatory GDNF/WNT11 feedback signalling loop are disrupted. Therefore, it was important to identify the BMP ligand(s) antagonised by GREM1 in the mesenchyme. BMP4 was identified as a relevant BMP signal by its partially overlapping expression with *Grem1* during initiation of ureteric bud outgrowth and by genetic complementation: inactivation of only one copy of the *Bmp4* gene in *Grem1*-deficient mouse embryos completely restores metanephric kidney organogenesis and functions. We conclude that GREM1-mediated reduction of BMP4 activity in the mesenchyme around the nascent ureteric bud is essential to (1) initiate ureteric bud outgrowth and invasion of the metanephric mesenchyme, and (2) enable autoregulatory e-m feedback signalling that regulates the dynamics of epithelial branching morphogenesis.

MATERIALS AND METHODS

Mutant mouse strains

Grem1 mutant mice were maintained in a 129/SvEv background (RCC, Switzerland), as the bilateral renal aplasia is fully penetrant in this and a mixed 129/C57BL6 genetic background (Michos et al., 2004). The *Bmp4* gene was inactivated in the germline by inter-crossing the floxed $Bmp4^{\text{loxP-}lacZ}$ allele (Kulessa and Hogan, 2002) with the *Cre* deleter mouse strain (Schwenk et al., 1995). The resulting $Bmp4^{\Delta-lacZ}$ allele was used to generate compound mutant mice for analysis in a mixed 129/C57BL6 background. Compound mutants carrying the *Hoxb7*-GFP transgene to mark the ureteric epithelium (Srinivas et al., 1999) were used for analysis. PCR genotyping of mice and embryos was done as described previously (Kulessa and Hogan, 2002; Michos et al., 2004; Srinivas et al., 1999). All animal experiments were performed in accordance with Swiss law and were approved by the regional veterinary authorities.

Molecular and morphological analysis of embryos and organs

Embryos were accurately staged by determining their somite numbers. Whole-mount and section RNA in situ hybridisation and detection of β-galactosidase activity were performed as described previously (Zuniga et al., 2004; Grieshammer, 2004). For histological analysis, 7-10 μm sections were prepared from paraffin-embedded samples fixed in 4% paraformaldehyde. Dewaxed sections were either stained with haematoxylin and Eosin or Periodic Acid Schiff (PAS) to reveal the brush border (microvilli) of the distal and proximal tubules. Phosphorylated SMAD (pSMAD1/5/8; SMAD8 is also known as SMAD9 – Mouse Genome Informatics) proteins were detected on 10 μm sagittal sections of stretched and paraffin-embedded embryos. Dewaxed sections were incubated with pSMAD1/5/8 antibodies (1:250, Cell Signaling) following the manufacturer's instructions, with the exception that endogenous peroxidases were inactivated with 3% H_2O_2 in PBS for 30 minutes. Signal amplification was performed using the appropriate ABC kit (Vector Laboratories) and the immunocomplexes were detected with DAB staining. Sections were counterstained with Hoechst 33258 (5 μg/ml) to reveal the nuclei containing no pSMAD antigen.

Kidney primordia cultures

Kidney primordia were isolated from somite-staged wild-type and mutant embryos and cultured in DMEM supplemented with 10% foetal bovine serum and 0.5% penicillin-streptomycin (Invitrogen) on Nucleopore filters (0.1 μm pore size, Corning) as previously described (Lin et al., 2001) with the following modifications. Experiments were performed using E10.75-11.25 (38-44 somites) embryos instead of the classic T-shape stages (E11.5, 48-50 somites). It is important to realise that it is not possible to efficiently rescue kidney primordia from *Grem1*-deficient embryos older than E11.25. A piece of Gelfoam (Pharmacia) was soaked in medium and the Nucleopore filter with the kidney primordia was placed on top in a 6-well plate and incubated in a humidified atmosphere at 37°C with 5% CO_2. Recombinant GREM1 (R&D Systems) was added to the culture medium at 2-5 μg/ml and changed every 48 hours. noggin-conditioned medium was produced using the B3-CHO cell line (Smith et al., 1993) and used at a dilution of 1:4. Recombinant GDNF (R&D Systems) was used at 100 ng/ml final concentration. After culture, the kidney primordia were fixed in 4% paraformaldehyde (PFA) and processed for molecular analysis. The metanephric mesenchyme was separated from the ureteric bud as described previously (Lin et al., 2001) and isolated mesenchyme was cultured in the same way as metanephric primordia. LiCl (15 μM) treatment was for 72 hours to allow for sufficient early tubulogenesis (Davies et al., 1995; Oxburgh and Robertson, 2002).

Semi-quantitative RT-PCR analysis

Kidney rudiments were collected either directly or after 48 hours in culture into RNA-later (Ambion). RNA was isolated from four kidney rudiments per genotype using the RNeasy kit (Qiagen) and reverse transcribed using Superscript III (Invitrogen). The cDNA contents of all samples were normalised for their *β-actin* (*Actb* – Mouse Genome Informatics) transcripts (primer sequences: forward 5′-ACACTGTGCCCATCTACGAGG-3′; reverse 5′-CATGGATGCCACAGGATTCC-3′). The relative levels of *Gdnf* and *Grem1* transcripts were determined using established primers [*Gdnf* (Towers et al., 1998); *Grem1* (Zuniga et al., 2004)] located in two different exons to avoid potential amplification of genomic DNA (PCR conditions are available upon request). The PCR products were separated on 1.8% agarose gels and visualised by ethidium bromide staining. Digital images were acquired to determine the relative expression levels using the LI-COR Odyssey Imaging Software (version 2.0). Three completely independent series of experiments were performed to assess *Gdnf* expression.

RESULTS

The BMP antagonist gremlin 1 induces supernumerary epithelial buds and restores invasion and branching in *Grem1*-deficient kidney primordia

We have previously shown that metanephric kidney development arrests prior to initiation of ureteric bud outgrowth in *Grem1*-deficient mouse embryos (Michos et al., 2004). To gain insight into the functions of GREM1 during the inductive phase of metanephric kidney organogenesis, wild-type and *Grem1*-deficient metanephric kidney primordia (E11.0-11.25, 40-44 somites; expressing the *Hoxb7*-GFP transgene) (Srinivas et al., 1999) were cultured for up to 96 hours in the presence or absence of soluble recombinant GREM1 protein. In contrast to wild-type controls (Fig. 1A), the development of *Grem1*-deficient kidney primordia arrests at the ureteric bud stage (Fig. 1B, *n*=13/16). Supplementation of the culture medium with GREM1 (5 μg/ml) restores ureteric epithelial outgrowth and branching (Fig. 1C,D; *n*=12/16) and induces several supernumerary epithelial buds (on average two to four; indicated by red asterisks in Fig. 1C,D). Most supernumerary buds appear within 48 hours and by 96 hours the ureteric and ectopic epithelial buds have undergone between two and four branching events (Fig. 1C,D). Epithelial tracings of the GREM1-treated *Grem1* mutants (right-most panels in Fig. 1C,D) reveals that branching of the ureteric bud is delayed by at least 24 hours in comparison to the wild-type (right-most panels in Fig. 1A,E). Ureteric epithelial outgrowth and branching are also restored and ectopic epithelial buds induced when mutant kidneys are cultured in the presence of less recombinant GREM1 (2 μg/ml; data not shown) or the unrelated BMP antagonist noggin (see Fig. S1 in the supplementary material). In wild type, recombinant GREM1 also induces ectopic epithelial buds (red asterisks) and ectopic branches

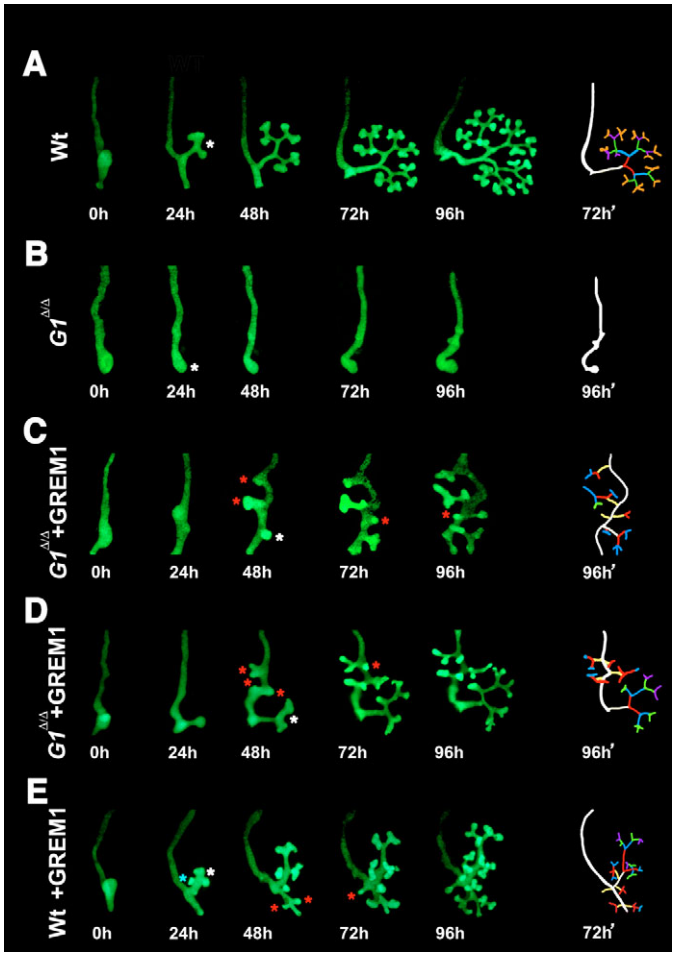

Fig. 1. Recombinant GREM1 protein is able to induce ectopic epithelial buds and restore branching in *Grem1*-deficient kidney primordia. Wild-type and *Grem1*-deficient kidney primordia expressing the *Hoxb7*-GFP transgene in their Wolffian duct and ureteric epithelium (Srinivas et al., 1999) were isolated from mouse embryos of 40-44 somites (E11.0-E11.25) 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 blue asterisks ectopic branches. The right-most panels are schematic tracings of the Wolffian duct and epithelial branching pattern at the developmental time points indicated (96 hours for *Grem1* mutants; 72 hours for wild type). Wolffian duct and ureteric bud are shown in white; ectopic epithelial buds and/or outgrowth in yellow; first branch in red; second branch in blue; third branch in green; fourth branch in purple; fifth branch (A only) in orange. (**A**,**B**) Kidney primordia cultured in control medium. (A) Wild-type control. (B) *Grem1*-deficient metanephros; outgrowth and branching are blocked. (**C-E**) *Grem1*-deficient (C,D) and wild-type (E) kidney primordia cultured in medium supplemented with recombinant GREM1 (5 μg/ml). The *Grem1*-deficient metanephric kidney primordia shown in C and D are representative of the variability observed.

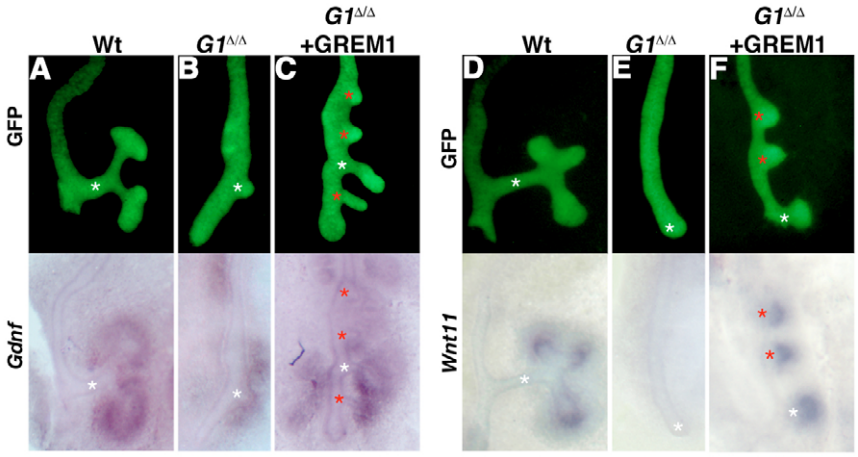

Fig. 2. Treatment with recombinant GREM1 enables upregulation and propagation of *Wnt11* and *Gdnf* expression in *Grem1*-deficient kidney rudiments in culture. Mouse kidney primordia were isolated at E10.75-11.0 (38-42 somites) and cultured for 48 hours either without (**A**,**B**,**D**,**E**) or in the presence of recombinant GREM1 (5 μg/ml, **C**,**F**). All upper panels show the epithelial branching pattern as revealed by the *Hoxb7*-GFP transgene. Lower panels show transcript distributions as revealed by whole-mount in situ hybridisation. Ureteric buds are indicted by white asterisks, ectopic epithelial buds by red asterisks. (A-C) *Gdnf* expression: (A) wild-type control; (B) *Grem1*-deficient kidney primordia, note the smaller size and remaining *Gdnf* expression; (C) *Grem1*-deficient kidney primordia cultured in the presence of recombinant GREM1. Note the ectopic epithelial buds and *Gdnf* expression in the surrounding mesenchyme. (D-F) *Wnt11* expression (D) wild-type control; (E) *Grem1*-deficient kidney primordia, note the complete loss of *Wnt11* expression; (F) *Grem1*-deficient kidney primordia cultured in the presence of recombinant GREM1. *Wnt11* is expressed in the epithelial tips of both ureteric and ectopic buds.

of the ureteric epithelium (blue asterisks, Fig. 1E). In general, treatment with soluble BMP antagonists induces more ectopic epithelial buds in the rostral part of the mutant Wolffian duct (red asterisks, Fig. 1C,D, and see Fig. S1 in the supplementary material) than in wild-type kidney primordia (red asterisks, Fig. 1E). Therefore, the potential for ectopic epithelial bud formation appears increased in the mutant, possibly due to the early developmental arrest of *Grem1*-deficient kidney primordia.

In *Grem1*-deficient embryos, *Ret* continues to be expressed by the Wolffian duct and ureteric epithelium. Also *Gdnf* expression is initially normal, but is downregulated progressively and the mutant mesenchyme is eliminated by apoptosis (Michos et al., 2004). By contrast, the expression of *Wnt11* in the mutant ureteric bud epithelium is lower than in the wild-type bud from the beginning and is completely lost by E11.5 (see Fig. S2 in the supplementary material). Therefore, *Wnt11* and *Gdnf* provide good markers to monitor of e-m feedback signalling in *Grem1*-deficient kidney primordia (Fig. 2). Mutant kidney rudiments (isolated from E10.75-11.0 mouse embryos; 38-40 somites) were cultured for 48 hours to avoid the onset of massive apoptosis (Fig. 2B,E and data not shown) and to allow treatment with recombinant GREM1 to restore ureteric bud outgrowth (white asterisks, Fig. 2C,F) and induce formation of ectopic buds (red asterisks, Fig. 2C,F). Indeed, GREM1 treatment restores *Gdnf* expression in the distal mesenchyme around the invading ureteric bud (white asterisk in Fig. 2C) and expression is also activated around the ectopic buds (red asterisks, Fig. 2C). Semi-quantitative RT-PCR analysis reveals that GREM1 treatment increases *Gdnf* transcript levels by at least twofold (see Fig. S3 in the supplementary material; 1.9±0.4, mean±s.e.m., n=3). In the ureteric epithelium, the expression of *Wnt11* is no longer detected in *Grem1*-deficient kidney primordia after 48 hours (Fig. 2E) in contrast to the wild-type primordia (Fig. 2D). Strikingly, GREM1 treatment of the mutant kidney primordia completely restores *Wnt11* expression in the epithelial tips of the invading ureteric (white asterisk, Fig. 2F) and all ectopic (red asterisks, Fig. 2F) buds. This molecular analysis establishes that recombinant GREM1 is able to restore epithelial WNT11 signalling and thereby autoregulatory e-m feedback signalling and metanephric branching morphogenesis.

The responsiveness of the epithelium and mesenchyme in *Grem1*-deficient kidney primordia was further studied as follows: (1) By forced activation of canonical Wnt signal transduction in culture, nephrogenesis is activated (see Fig. S4 in the supplementary material), thereby establishing that the mutant metanephric mesenchyme remains responsive to inductive signals; (2) By culturing *Grem1*-deficient kidney primordia in an excess of recombinant GDNF (100 ng/ml; Fig. 3) massive epithelial overgrowth and branching are induced along the entire Wolffian duct similar to what occurs in wild type (Fig. 3A,B) (see also Shakya et al., 2005). These results indicate that the block to initiate ureteric bud outgrowth and invasion of the mesenchyme is not caused by defective epithelial signal reception, but rather by defects in signalling, i.e. the failure to upregulate *Gdnf* expression in the mesenchyme and/or *Wnt11* in the ureteric epithelium.

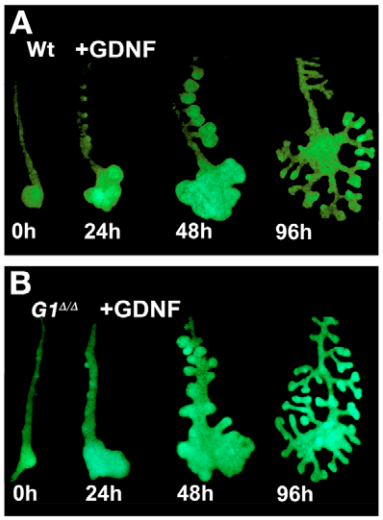

Fig. 3. Recombinant GDNF induces epithelial overgrowth and excessive branching in both wild-type and *Grem1*-deficient mouse metanephric kidney primordia. Kidney primordia (E11.0, 40-42 somites) were cultured for 96 hours in medium supplemented with recombinant GDNF (100 ng/ml). (**A**) Wild type; note the formation of many epithelial buds along the Wolffian duct and overgrowth of the ureteric epithelium within 24 hours, which results in excessive branching. (**B**) *Grem1*-deficient kidney primordia; note the formation of many epithelial buds, overgrowth and excessive branching similar to the wild type (A).

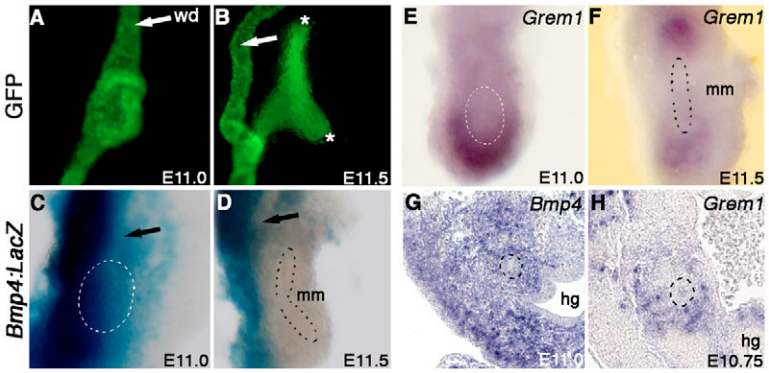

Fig. 4. Dynamic changes in *Grem1* and *Bmp4* expression during initiation of metanephric kidney development in mouse. (**A-D**) *Bmp4* distribution during initiation of metanephric kidney development. (A,B) GFP reveals the morphology of the nascent ureteric bud at E11.0 (40-42 somites; A) and after the first epithelial branching (E11.5, 48-50 somites; B). Asterisks in B indicate the tips of the ureter branches. (C,D) The *Bmp4* transcript distribution was determined using a *lacZ* reporter gene inserted into the endogenous *Bmp4* transcription unit (*Bmp4:lacZ*) in heterozygous embryos (Kulessa and Hogan, 2002), as detection by whole mount in situ hybridisation is not reliable. (C) At E11.0, *Bmp4:lacZ* is expressed by the mesenchyme surrounding the Wolffian duct (arrow) and ureteric bud (see also G). The dotted circle indicates the position of the ureteric bud (compare with A). (D) By E11.5, the *Bmp4:lacZ* expression is retained in the mesenchyme surrounding the Wolffian duct (arrow), while the metanephric mesenchyme is largely devoid of *Bmp4*. The dotted line indicates the position of the first branch (compare with B). (**E**,**F**) *Grem1* expression in the mesenchyme. (E) At E11.0, *Grem1* expression is highest in the mesenchyme around the ureteric bud (indicated by a dotted circle). (F) Subsequently (E11.5), *Grem1* is expressed in the mesenchyme around the tips of first ureteric epithelial branch (indicated by dotted oval). (**G**,**H**) Detection of *Bmp4* and *Grem1* transcripts on transverse sections by in situ hybridisation. Sections are oriented with dorsal to the top. Note that the epithelium (indicated by a dotted line) expresses neither *Bmp4* (G) nor *Grem1* (H). (G) There is abundant *Bmp4* expression in the mesenchyme around the caudal Wolffian duct and ureteric epithelium in agreement with the *Bmp4:lacZ* distribution shown in C. (H) At E10.75 (38 somites), *Grem1* is also expressed in the mesenchyme around the caudal Wolffian duct and ureteric epithelium in agreement with the whole mount in situ hybridisation shown in E. Note that *Bmp4* and *Grem1* are also expressed by the mesenchyme around the hindgut. Hg, hindgut; mm,: metanephric mesenchyme; wd, Wolffian duct.

The *Grem1* deficiency causes aberrant nuclear accumulation of pSMAD proteins in the metanephric mesenchyme around the ureteric bud

The results so far reveal the importance of identifying the primarily affected kidney compartment and relevant BMP ligand(s). With respect to the latter, *Bmp4* (in contrast to *Bmp2* and *Bmp7*) (Michos et al., 2004) is expressed by the mesenchyme along the entire Wolffian duct, including the region where the ureteric bud is forming (Fig. 4C, compare with Fig. 4A, see also Fig. 4G). Therefore, the Wolffian duct and nascent ureteric bud are 'embedded' in *Bmp4*-expressing mesenchyme. By contrast, the nascent metanephric mesenchyme is more or less devoid of *Bmp4* expression during ureteric bud outgrowth and first branching (Fig. 4B,D). Subsequently, *Bmp4* expression is (re-) activated in metanephric mesenchyme enveloping the ureteric stalk, consistent with its proposed functions during stalk elongation (Miyazaki et al., 2000) (and data not shown). Just prior to initiation of ureteric bud outgrowth, the highest levels of *Grem1* transcripts are detected in the mesenchyme around the caudal Wolffian duct and nascent ureteric bud (Fig. 4E,H). Activation of *Grem1* expression does not seem to require GDNF signalling, as *Grem1* remains expressed in the metanephric kidney primordia of *Gdnf* mutant mouse embryos (see Fig. S3 in the supplementary material). After the first epithelial branching event, *Grem1* expression is highest in the metanephric mesenchyme surrounding the ureteric epithelial tips (Fig. 4F). The transcriptional upregulation of *Grem1* just prior to the onset of ureteric bud outgrowth (Fig. 4E,H) would likely inhibit BMP4 locally and generate a region of low mesenchymal BMP activity. Such regional lowering of mesenchymal BMP activity likely relieves repression of ureteric bud outgrowth and enables its invasion into the metanephric mesenchyme in response to GDNF signalling.

To determine if such lowering of BMP signal transduction indeed occurs, the phosphorylation of SMAD proteins (pSMAD1/5/8; established mediators of BMP signal transduction) (Massague et al., 2005) was analysed in wild-type and *Grem1*-deficient mouse embryos (Fig. 5). During ureteric bud formation in the wild type, the Wolffian duct and ureteric bud are largely devoid of nuclear pSMAD proteins (brown stained nuclei, Fig. 5A) as revealed by abundant Hoechst fluorescence (light blue nuclei, Fig. 5A). Whereas the pSMAD antigen is abundant in the ventral mesenchyme, only a few pSMAD-positive nuclei are apparent in the metanephric mesenchyme (demarcated region indicated by 'mm' in Fig. 5A). By contrast, a significant fraction of the cells in the corresponding region of *Grem1*-deficient embryos contain pSMAD proteins in their nuclei and the metanephric mesenchyme appears overall less dense (Fig. 5B). These results indicate that BMP signal transduction is lowered in the wild-type metanephric mesenchyme during initiation of ureteric bud outgrowth (Fig. 5A), whereas it remains excessively high in the mutant mesenchyme (Fig. 5B). In the wild type, the nuclear pSMAD levels remain low in both epithelium and condensing metanephric mesenchyme during invasion and first branching (Fig. 5C,E), while they increase in stromal mesenchyme (Fig. 5C,E). This increase is consistent with a role of BMP4 in ureteric stalk elongation (Miyazaki et al., 2000). In *Grem1* mutant kidneys, the aberrant pSMAD levels persist throughout the mutant metanephric mesenchyme, while the arrested ureteric bud epithelium remains largely devoid of nuclear pSMAD proteins (Fig. 5D,F). The low pSMAD levels in the wild-type metanephric mesenchyme (Fig. 5A,C) overlap well with the area normally expressing *Grem1* (Fig. 4E,F). In summary, the aberrant and persistently high BMP(4) activity in the mutant metanephric mesenchyme reveals a primary defect in *Grem1*-deficient kidney primordia (Fig. 5B,D,F) and sharply contrasts with the dynamic changes of BMP activity in the wild type (Fig. 5A,C,E).

Genetic reduction of BMP4 activity in *Grem1*-deficient mouse embryos rescues metanephric kidney organogenesis and postnatal kidney functions

To test the hypothesis that excess mesenchymal BMP4 activity blocks the initiation of ureteric bud outgrowth, compound mutant embryos and mice carrying both *Grem1* and *Bmp4* loss-of-function mutations were generated. The early lethality of *Bmp4*-deficient

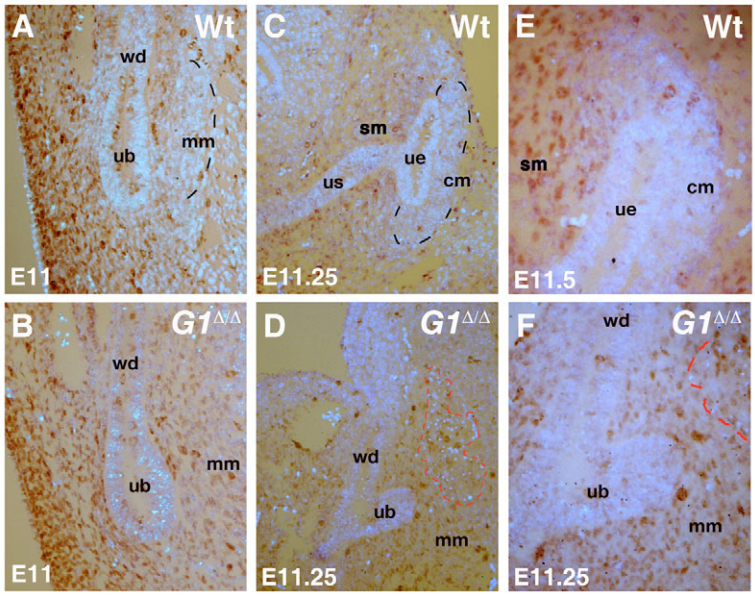

Fig. 5. Detection of phosphorylated SMAD1/5/8 proteins (pSMAD) on tissue sections. The brown precipitate reveals pSMAD-positive nuclei. All sections are counterstained with Hoechst 33258 (light blue fluorescence) to reveal nuclei lacking pSMADs. All sections are sagittal along the primary embryonic axis with ventral to the left and dorsal to the right. Sections shown are representative of the results obtained by analysing serial sections of at least three embryos per genotype. (**A**,**C**,**E**) Wild-type mouse embryos at E11.0 (40 somites; A); E11.25 (47 somites; C) and E11.5 (50 somites, enlargement of tip region; E). (**B**,**D**,**F**) *Grem1*-deficient embryos at E11.0 (42 somites; B) and E11.25 (48 somites; D), an enlargement of the ureteric bud area in D is shown in F. The area within the red dashed line contains many picnotic nuclei indicative of the onset of cellular apoptosis (D,F). cm, condensing metanephric mesenchyme; mm, metanephric mesenchyme; sm, stromal mesenchyme; ub, ureteric bud; ue, ureteric epithelium (first branch); us, ureteric stalk; wd, Wolffian duct.

mouse embryos (Winnier et al., 1995) is not rescued by additional inactivation of *Grem1*, which precludes analysis of double homozygous embryos. However, inactivation of one copy of the *Bmp4* gene in *Grem1*-deficient ($G1^{\Delta/\Delta}$; $B4^{\Delta/+}$) mouse embryos restores ureteric bud morphology (Fig. 6C, compare with Fig. 6A,B). In $G1^{\Delta/\Delta}$; $B4^{\Delta/+}$ mutant embryos (Fig. 6C,F,I,L), ureteric bud outgrowth and branching are initiated with kinetics similar to that in the wild type (Fig. 6A,D,G,J). Consistent with this rescue of epithelial outgrowth and branching, the expression of *Gdnf* in the mesenchyme (Fig. 6F,L, compare with Fig. 6E,K) and *Wnt11* in the ureteric bud epithelium are restored (see Fig. S2 in the supplementary material and data not shown), which is indicative of intact autoregulatory e-m feedback signalling during branching and normal progression of metanephric branching morphogenesis.

Indeed, the distribution of the *Pax2* transcript underscores the normal progression of kidney organogenesis in $G1^{\Delta/\Delta}$; $B4^{\Delta/+}$ embryos by E14.5 (Fig. 7C, compare with Fig. 7A). By contrast, in *Grem1*-deficient embryos at this stage both kidneys have been eliminated by apoptosis (as revealed by the complete lack of *Pax2* expression; Fig. 7B). All $G1^{\Delta/\Delta}$; $B4^{\Delta/+}$ mice are born with two fully developed and functional kidneys (Fig. 7G, compare with Fig. 7D; n=20), whereas complete bilateral renal agenesis is observed in about 90% of all *Grem1*-deficient litter mates (n=22/25; the other three manifested unilateral renal aplasia). Histological analysis of $G1^{\Delta/\Delta}$; $B4^{\Delta/+}$ mice at 1 and 8 months old reveals that their kidneys are morphologically indistinguishable from wild-type kidneys (Fig. 7E-I). No signs of congenital malformations such as CAKUT, glomerulosclerosis or polycystic kidney disease are observed (Fig.

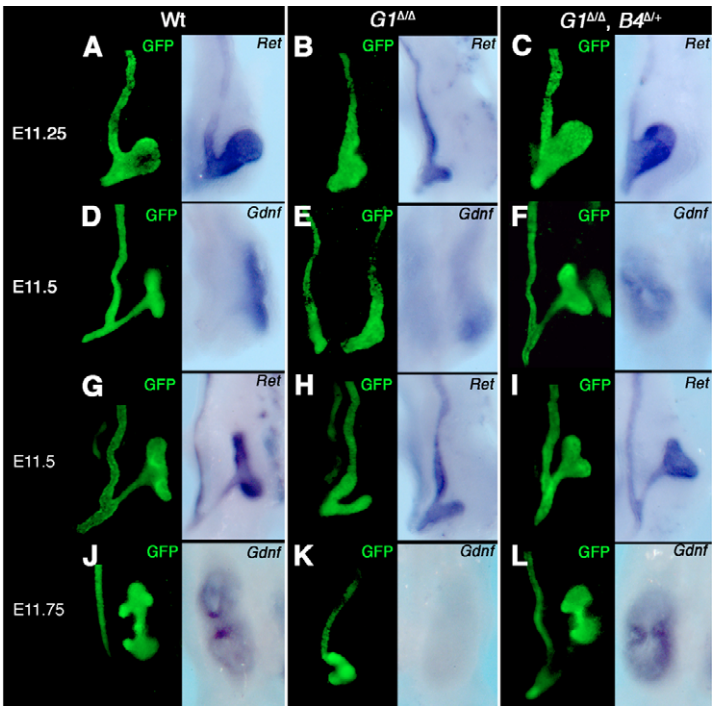

Fig. 6. Genetic inactivation of one copy of the *Bmp4* gene in a *Grem1* mutant background rescues the kinetics of ureteric epithelial growth and branching. Left panels: GFP reveals the morphology of the epithelium before fixation. Right panels: whole-mount in situ detection of the expression of the *Ret* receptor (**A-C**,**G-I**) and the *Gdnf* ligand (**D-F**,**J-L**). (A,D,G,J) Wild-type metanephric primordia. (B,E,H,K) *Grem1* ($G1^{\Delta/\Delta}$)-deficient metanephric primordia. Note the complete disruption of ampulla formation (E,H,K) and loss of mesenchymal *Gdnf* expression (K), whereas *Ret* expression remains in the arrested epithelium (H). (C,F,I,L) Metanephric primordia of $G1^{\Delta/\Delta}$; $B4^{\Delta/+}$ mouse embryos. Note the rescue of ureteric epithelial outgrowth (C), branching (F,I,L) and propagation of *Gdnf* expression (L).

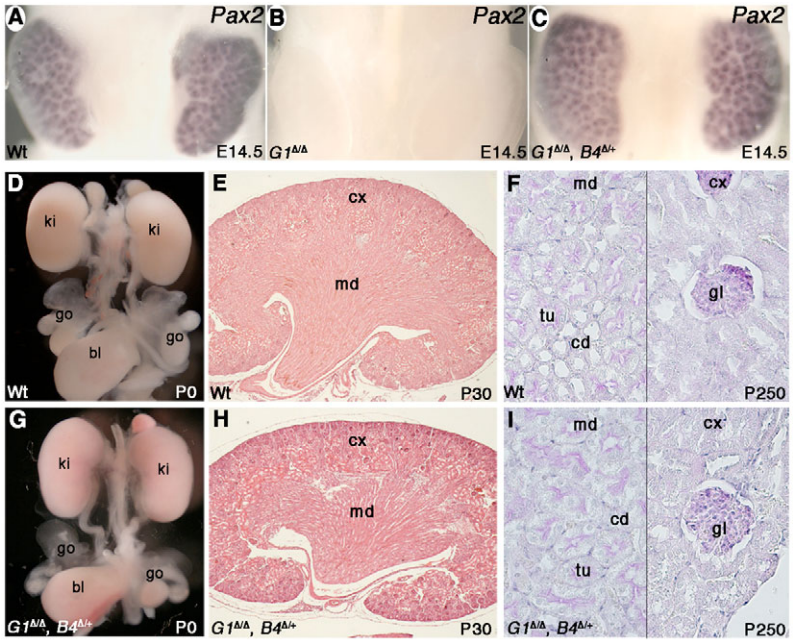

Fig. 7. Inactivation of one copy of the *Bmp4* gene in a *Grem1*-deficient mouse embryo results in formation of two normal kidneys. (**A-C**) Whole-mount in situ hybridisation to detect *Pax2* expression at E14.5. (A) Wild-type *Pax2* distribution. (B) The absence of *Pax2* expression in a *Grem1*-deficient embryo is due to the complete renal aplasia at this stage. (C) The *Pax2* distribution in kidneys of a *Grem1*$^{\Delta/\Delta}$; *Bmp4*$^{\Delta/+}$ embryo is indistinguishable from a wild-type littermate (A). (**D**) Gross morphology of the wild-type urogenital system at birth (postnatal day P0). (**E**) Haematoxylin and Eosin staining of a histological section of a wild-type kidney 30 days after birth (P30). (**F**) PAS staining reveals the morphology of the medulla (collecting ducts) and cortex regions of a wild-type kidney at about 8 months of age (P250). (**G**) Gross morphology of the urogenital system of a *Grem1*$^{\Delta/\Delta}$; *Bmp4*$^{\Delta/+}$ mouse at birth (P0). Note that both kidneys are of normal size and shape (compare with D). (**H**) Haematoxylin and Eosin staining of a kidney section from a *Grem1*$^{\Delta/\Delta}$; *Bmp4*$^{\Delta/+}$ mouse at P30. (**I**) PAS staining of a kidney from a *Grem1*$^{\Delta/\Delta}$; *Bmp4*$^{\Delta/+}$ mouse at P250. Medulla, cortex and glomeruli appear normal. Bl, bladder; cd, collecting duct; cx, cortex; gl, glomerulus; go, gonad; ki, kidney; md, medulla, tu, tubules.

7G,H,I). Taken together, these results show that the genetic reduction of BMP4 activity in *Grem1*-deficient mouse embryos completely restores metanephric kidney organogenesis and functions.

DISCUSSION

Our study establishes genetically that the extra-cellular BMP antagonist GREM1 is required to reduce BMP4 activity in the mesenchyme surrounding the nascent ureteric bud in mouse embryos. The reduction of BMP4 signal transduction in the metanephric mesenchyme is necessary to initiate ureteric bud outgrowth and establish autoregulatory feedback signalling between GDNF-RET and WNT11, which in turn regulates epithelial branching morphogenesis (Fig. 8). In *Grem1*-deficient kidney primordia, mesenchymal BMP signal transduction is increased, as revealed by the increased nuclear accumulation of pSMAD proteins in the metanephric mesenchyme, which blocks initiation of ureteric bud outgrowth. Indeed, general inhibition of BMP activity in *Grem1*-deficient kidney primordia, by addition of recombinant GREM1, induces supernumerary epithelial buds and restores outgrowth and branching. At the molecular level, GREM1 treatment (re-) activates *Wnt11* expression in the epithelial buds and enables upregulation and propagation of *Gdnf* expression in the mesenchyme. Most importantly, genetic reduction of BMP4 activity in the context of a *Grem1* deficiency completely restores metanephric kidney organogenesis and functions. Therefore, we can conclude that local reduction of BMP4 activity by GREM1 is vital to initiate ureteric bud outgrowth and thereby metanephric kidney organogenesis (Fig. 8).

Formation of the ureteric bud, initiation of its outgrowth and branching appear as distinct processes (Fig. 8). In the metanephros, a single ureteric bud forms in the caudal part of each of the bilateral Wolffian ducts at the level of the mid-hind limb bud. GDNF is essential for ureteric bud formation, as ureteric buds fail to form in most *Gdnf*-deficient mouse embryos (Pichel et al., 1996). As *Grem1* remains expressed in *Gdnf* mutant metanephric kidney rudiments, the activation of its expression does not depend on GDNF signalling and seems not to require the presence of an intact ureteric bud. Interestingly, recombinant GREM1 is itself able to induce supernumerary epithelial buds in *Grem1*-deficient kidney primordia. These results indicate that in spite of the early developmental arrest, the competence of the rostral Wolffian duct to form supernumerary epithelial buds remains intact in *Grem1*-deficient embryos. In wild-type embryos, the signalling interactions mediated by SLIT2/ROBO2 and FOXC1 restrict *Gdnf* expression to the caudal

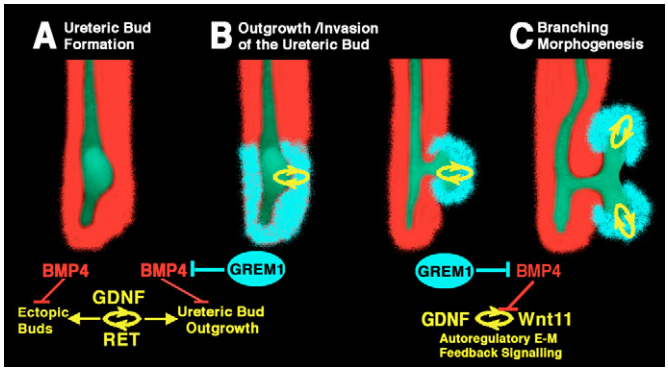

Fig. 8. Reduction of BMP4 activity by gremlin 1 in the mesenchyme around the ureteric bud is essential to enable ureteric epithelial outgrowth, GDNF-RET and WNT11-mediated e-m feedback signalling and branching morphogenesis. (**A**) In mouse, the ureteric bud forms in the caudal-most part of the Wolffian duct under the influence of GDNF-RET signalling. During this inductive period, *Bmp4* is expressed by the mesenchyme enveloping the Wolffian duct. High levels of mesenchymal BMP4 activity inhibit the formation of ectopic epithelial buds and epithelial branching at this stage (prior to E11.0). At this early stage only low levels of *Grem1* transcripts are detected (not shown). (**B**) Expression of the BMP antagonist *Grem1* is upregulated in the mesenchyme around the nascent ureteric bud thereby locally reducing BMP4 signal transduction (around E11.75-11.0). This reduction of BMP4 activity by GREM1 enables initiation of ureteric bud outgrowth and its invasion into the metanephric mesenchyme. (**C**) GREM1 is required to maintain and propagate expression of *Wnt11* in the ureteric epithelial tip(s) and *Gdnf* in the mesenchyme via e-m feedback signalling. For details see Discussion.

Wolffian duct (see Introduction and references therein). We provide evidence that BMP4 signalling by the mesenchyme enveloping the Wolffian duct is part of a safeguard mechanism that inhibits formation of supernumerary epithelial buds (Fig. 8A). Local upregulation of *Grem1* expression reduces pSMAD-mediated BMP signal transduction in the mesenchyme and relieves this repression around the ureteric bud, which enables ureteric bud outgrowth and invasion of the metanephric mesenchyme (Fig. 8B). Taken together, the successful initiation of ureteric bud outgrowth likely requires both antagonism of BMP4 by GREM1 in mesenchyme and signalling by GDNF from the metanephric mesenchyme to RET in the ureteric epithelium (Fig. 8A,B).

Subsequently, autoregulatory feedback signalling between GDNF in the mesenchyme and WNT11 in the epithelial tips is established to regulate branching morphogenesis (Fig. 8C) (Majumdar et al., 2003). As GREM1 is required for upregulation of *Wnt11* in the ureteric epithelium and *Gdnf* expression in the mesenchyme, it appears to be crucial for establishment of e-m feedback signalling (Fig. 8C). As BMP signal transduction is increased in the metanephric mesenchyme of *Grem1*-deficient embryos, one might expect a direct effect of GREM1 and/or BMP4 on mesenchymal *Gdnf* expression. However, treatment of isolated metanephric mesenchyme with either GREM1 or BMP4 did not alter *Gdnf* expression significantly within 18-24 hours (O.M. and R.Z., unpublished). Therefore, we favour an alternative explanation, by which the primary defect (elevated BMP4 activity) in the *Grem1*-deficient metanephric mesenchyme blocks initiation of ureteric epithelial outgrowth and signalling as evidenced by the loss of *Wnt11* expression. GREM1-mediated reduction of BMP activity in the mesenchyme may act via epithelial signalling to propagate mesenchymal *Gdnf* expression, analogous to the requirement of GREM1 for *Shh* expression in the limb bud mesenchyme (see below). Further (genetic) studies are required to understand how excess BMP signal transduction in the mesenchyme blocks initiation of ureteric bud outgrowth. In agreement with our studies, others have already shown that (1) addition of recombinant BMPs to metanephric kidney primordia in culture partially inhibits epithelial branching morphogenesis (Bush et al., 2004; Piscione et al., 1997), and (2) overexpression of the BMP receptor type 1A (ALK3; also known as BMPR1A – Mouse Genome Informatics) in the ureteric epithelium causes renal aplasia or dysplasia in a fraction of mice (Hu et al., 2003).

Last but not least, the present study reveals striking mechanistic similarities in the way GREM1-mediated e-m feedback signalling controls limb and kidney organogenesis. During limb bud development, GREM1-mediated BMP4 antagonism is key to establishing and propagating the feedback signalling loop between SHH (expressed by the posterior mesenchyme) and FGF (in the apical ectodermal ridge, AER), which enables progression of limb bud morphogenesis (Panman et al., 2006; Michos et al., 2004; Zuniga et al., 1999). Our previous studies showed that GREM1 is not required to initiate SHH signalling by the limb bud organiser in the posterior mesenchyme, but is essential to initiate the dynamic phase of SHH/FGF e-m feedback signalling. In particular, GREM1-mediated SHH/FGF feedback signalling regulates the temporally and spatially coordinated propagation of both signalling centres during limb bud outgrowth and patterning (Panman et al., 2006). Similarly, GREM1 is not required to activate GDNF signalling in the metanephric mesenchyme and for formation of the ureteric bud, but for initiation of epithelial outgrowth and establishment of autoregulatory GDNF/WNT11 e-m feedback signalling. In both the limb and kidney primordia, GREM1 acts in the mesenchyme to reduce BMP4 signal transduction and thereby relieve the inhibitory effect on the ureteric and AER epithelium (this study and J.-D. Bénazet and R. Z., unpublished). As a consequence, the expression of *Wnt11* in the ureteric bud and *Fgfs* in AER are upregulated and this epithelial signalling in turn propagates GDNF in the metanephric, and SHH in the limb bud, mesenchyme, respectively.

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Supplementary material

Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/134/13/2397/DC1

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