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

Odysse Michos1,*,†, Alexandre Gonçalves1,*, Javier Lopez-Rios1, Eva Tiecke1, Florence Naillat2, Konstantin Beier3, Antonella Galli1, Seppo Vainio2 and Rolf Zeller1,‡

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 Gremlin1 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).

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 GFRa1 (glial cell line derived neurotrophic factor family receptor a1) 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-

1Developmental Genetics, DKBW Centre for Biomedicine, University of Basel Medical Faculty, Mattenstrasse 28, CH-4058 Basel, Switzerland. 2Department of Medical Biochemistry and Molecular Biology, Biocenter Oulu, Laboratory of Developmental Biology, Aapistie 5A, PO Box 5000, University of Oulu, F-90570 Oulu, Finland. 3Department of Histology, Anatomy Institute, Pestalozzistrasse 20, CH-4056 Basel, Switzerland.

*These authors contributed equally to this work

Present address: Department of Genetics and Development, Columbia University, HHSC-1416, 701 West 168 Street, New York, NY 10032, USA

‡Author for correspondence (e-mail: Rolf.Zeller@unibas.ch)

Accepted 5 April 2007
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 Shykya, 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 Greml1 is expressed by the meso- and metanephric mesenchyme and that metanephric kidney organogenesis is disrupted in Greml1-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 Greml1-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 Greml1-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 Greml1 during initiation of ureteric bud outgrowth and by genetic complementation: inactivation of only one copy of the Bmp4 gene in Greml1-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

Greml1 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<sup>flx<sup>lox<sup>-</sup></sup> allele (Kulesha and Hogan, 2002) with the Cre deleter mouse strain (Schwenk et al., 1995). The resulting Bmp4<sup>Delta<sup>-</sup>lox<sup>-</sup></sup>-/- 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 (Kulesha 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<sub>2</sub>O<sub>2</sub> 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% CO2. 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 RNasy 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'-ACACTGTGCCCATCTCAGGG-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.
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 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,F). 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.
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 'embryoid' 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 ureter 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 ureter 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
mouse embryos (Winnier et al., 1995) is not rescued by additional inactivation of \textit{Grem1}, which precludes analysis of double homozygous embryos. However, inactivation of one copy of the \textit{Bmp4} gene in \textit{Grem1}-deficient (\(G1^{3/4}\), \(B4^{3/4}\)) mouse embryos restores ureteric bud morphology (Fig. 6C, compare with Fig. 6A,B). In \(G1^{3/4}\), \(B4^{3/4}\) mutant embryos (Fig. 6C,F,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 \textit{Gdnf} in the mesenchyme (Fig. 6F,L, compare with Fig. 6E,K) and \textit{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 \textit{Pax2} transcript underscores the normal progression of kidney organogenesis in \(G1^{3/4}\), \(B4^{3/4}\) embryos by E14.5 (Fig. 7C, compare with Fig. 7A). By contrast, in \textit{Grem1}-deficient embryos at this stage both kidneys have been eliminated by apoptosis (as revealed by the complete lack of \textit{Pax2} expression; Fig. 7B). All \(G1^{3/4}\), \(B4^{3/4}\) 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 \textit{Grem1}-deficient litter mates (\(n=22/25\); the other three manifested unilateral renal aplasia). Histological analysis of \(G1^{3/4}\), \(B4^{3/4}\) 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. 7).

\textbf{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; \(E\)). (\(B,D,F\)) \textit{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.

\textbf{Fig. 6.} Genetic inactivation of one copy of the \textit{Bmp4} gene in a \textit{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 \textit{Ret} receptor (\(A,C,G,I\)) and the \textit{Gdnf} ligand (\(D,F,J,L\)). (\(A,D,G,J\)) Wild-type metanephric primordia. (\(B,E,H,K\)) \textit{Grem1} (\(G1^{3/4}\))-deficient metanephric primordia. Note the complete disruption of ampulla formation (\(E,H\)) and loss of mesenchymal \textit{Gdnf} expression (\(K\)), whereas \textit{Ret} expression remains in the arrested epithelium (\(H\)). (\(C,F,I,L\)) Metanephric primordia of \(G1^{3/4}\), \(B4^{3/4}\) mouse embryos. Note the rescue of ureteric epithelial outgrowth (\(C\)), branching (\(F,I,L\)) and propagation of \textit{Gdnf} expression (\(L\)).
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
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 (Pannman 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 (Pannman 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.

The authors thank F. Costantini for providing the Hobix7-GFP mouse strain and Gdnf mutant mouse embryos, support and helpful suggestions and discussions throughout the project. The authors are grateful to B. Hogan for providing the Bmpdplox-lox mouse strain, K. Cuéllar and G. Hollandé for providing colony founders, R. Harland for the B3-CHO noggin producing cell line and U. Grieshammer for the Gdnf in situ hybridisation probe. We wish to thank A. Rolink for suggesting the use of Gelfoam for kidney culture. I. Ginez, O. Lehmann, N. Lagarde and H. Schaller are thanked for technical assistance and animal care. This research was supported by the Swiss National Science Foundation (Grant 310100-100240 to R.Z.), both Cantons of Basel (to R.Z. and K.B.), the Academy of Finland (107406, 206038 to S.V.), the Sigrid Juselius Foundation and the European Foundation (LSHG-CT-2004-005085 to S.V.), the Fundação para a Ciência e a Tecnologia (FCT; SFRH/BD/24301/2005 to A.G.) and an EMBO long-term and EU Marie Curie long-term fellowship (MEIF-CT-2006-024176 to J.L.-R.). The authors are grateful to M. Affolter, A. Brändli, A. Zuniga and their laboratory colleagues for critical reading and discussions of the manuscript and anonymous reviewers for helpful input.

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

References


Kume, T., Deng, K. and Hogan, B. L. (2000). Murine forehead winged helix genes Foxc1 (Mf1) and Foxc2 (Mfh1) are required for the early organogenesis of the kidney and urinary tract. Development 127, 1389-1395.

Lin, Y., Zhang, S., Rehn, M., Itaranta, R., Tuukkanen, J., Heljasaava, R.,
GREM1-BMP4 antagonism in kidney development

RESEARCH ARTICLE 2405


