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A functional role for semaphorin 4D/plexin B1 interactions in epithelial branching morphogenesis during organogenesis

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Semaphorins and their receptors, plexins, carry out important functions during development and disease. In contrast to the well-characterized plexin A family, however, very little is known about the functional relevance of B-type plexins in organogenesis, particularly outside the nervous system. Here, we demonstrate that plexin B1 and its ligand Sema4d are selectively expressed in epithelial and mesenchymal compartments during key steps in the genesis of some organs. This selective expression suggests a role in epithelial-mesenchymal interactions. Importantly, using the developing metanephros as a model system, we have observed that endogenously expressed and exogenously supplemented Sema4d inhibits branching morphogenesis during early stages of development of the ureteric collecting duct system. Our results further suggest that the RhoA-ROCK pathway, which is activated downstream of plexin B1, mediates these inhibitory morphogenetic effects of Sema4d and suppresses branch-promoting signalling effectors of the plexin B1 signalling complex. Finally, mice that lack plexin B1 show early anomalies in kidney development in vivo. These results identify a novel function for plexin B1 as a negative regulator of branching morphogenesis during kidney development, and suggest that the Sema4d-plexin B1 ligand-receptor pair contributes to epithelial-mesenchymal interactions during organogenesis via modulation of RhoA signalling.

KEY WORDS: Kidney development, RhoA, ROCK, Epithelial-mesenchymal interaction, Transgenic mouse

INTRODUCTION

During the development of organs, the migration, invasion and differentiation of cells of distinct origins is coordinated and regulated to produce highly ordered structures. Tissue formation requires close interactions between cells of diverse origins, e.g. the mesenchyme and epithelium (Moustakas and Heldin, 2007; Shah et al., 2004). These cell-cell interactions are postulated to be mediated by signals or cues that are secreted or expressed in a locally discrete manner. Several candidates for secreted cues have been described in specific organs [e.g. transforming growth factor beta (TGFβ), bone morphogenetic proteins (BMPs), glial-derived nerve growth factor (GDNF), hepatocyte growth factor (HGF), etc. (reviewed by Moustakas and Heldin, 2007; Shah et al., 2004; Zhang and Vande Woude, 2003)].

Semaphorins constitute a family of secreted or cell-specific cues, the cellular effects of which are achieved via activation of transmembrane proteins called plexins (Tamagnone et al., 1999). Although semaphorins were first described as important axon guidance molecules (Liu and Strittmatter, 2001), several recent studies have established them as key regulators of invasive growth, apoptosis and the immune system (Chédotal et al., 2005; Fiore and Puschel, 2003; Suzuki et al., 2008). Plexin B1, the prototypic member of B-family plexins, and its ligand semaphorin 4D (Sema4d) have been functionally implicated in diverse processes such as migration and proliferation of neuronal, endothelial and tumour cells, and angiogenesis and axonal navigation, among others (Basile et al., 2004; Conrotto et al., 2005; Giordano et al., 2002; Masuda et al., 2004; Suzuki et al., 2008; Swiercz et al., 2002; Swiercz et al., 2004).

In recent studies, we and others have elucidated the composition of the plexin B1 signalling complex. Plexin B1 activation leads to an activation of the RhoGTPase, RhoA (Aurandt et al., 2002; Hirotani et al., 2002; Perrot et al., 2002; Swiercz et al., 2002) and inactivation of R-Ras (Oinuma et al., 2004) and Rac (Vikis et al., 2000). Plexin B1 also physically associates with receptor tyrosine kinases (RTKs), such as Met (Giordano et al., 2002) and Erbb2 (Swiercz et al., 2004), leading to their activation upon binding to Sema4d. Moreover, plexin B1 activation has been recently associated with the activation of diverse intracellular pathways, involving FAK, Src, Pyk2, p190RhoGAP and others (Basile et al., 2005; Basile et al., 2007; Barberis et al., 2005).

Very little is known about the role of plexin B family members in developmental processes in vivo. Using genetically modified mice as tools, we have recently addressed the functions of B-type plexins in neuronal migration and patterning of the brain in vivo (Deng et al., 2007; Friedel et al., 2007). However, not much is known about whether and how B-type plexins modulate the development and maturation of organs outside of the nervous system.

Based upon our finding that plexin B1 and its ligand Sema4d are expressed in a complementary pattern in epithelial and mesenchymal compartments, respectively, over crucial developmental time periods, we hypothesize that this ligand-receptor pair plays a role in organogenesis. Using the developing metanephros as a model system, we have elucidated here the functional contribution of Sema4d and plexin B1 in developmental mesenchyme-epithelial interactions, and we show that plexin B1-mediated RhoA activation is important in shaping the architecture of the developing kidney ex vivo as well as in vivo.

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MATERIALS AND METHODS

mRNA in situ hybridization

The generation of riboprobes and the protocol for mRNA in situ hybridization have been described previously (Worzfeld et al., 2004).

Metanephric organ culture

Mouse embryos were harvested and kidneys were cultured essentially as described by Woolf et al. (Woolf et al., 1995). At 24 hours or 48 hours in vitro, cultured metanephric kidneys were fixed in 100% ice-cold methanol. In some cases, morphology was documented with photomicrographs using a phase-contrast inverted microscope. In a majority of experiments, fixed kidneys were subjected to whole-mount immunofluorescence as described below.

Immunohistochemistry

Following in situ hybridization, some sections were processed for immunohistochemistry with an anti-WT1 antibody (1:300, Santa Cruz Biotechnology) as described before (Worzfeld et al., 2004). For whole-mount staining, cultured mouse kidneys were fixed using 100% ice-cold methanol, blocked in PBS/1% Triton X-100/1% BSA for 1 hour and incubated with primary antibodies (anti-calbindin-D-28K antibody; 1:300; Sigma-Aldrich, Germany and anti-WT1 antibody) overnight at 4°C and stained further using standard procedures.

Confocal analysis and quantification of metanephric morphological parameters: metanephroi stained as wholemounts were imaged using a confocal laser-scanning microscope (Leica TCS AOBS) in stacks spanning the entire thickness of the metanephroi. Ureteric bud branch tips immunostained with the anti-calbindin antibody were counted blindly in each frame and expressed as mean±s.e.m. Sister kidneys were compared using Student's paired *t*-test. When more than two treatment groups were compared, analysis of variance (ANOVA) with random measures followed by post-doc Fischer's test was employed. Metanephric condensates, resulting in comma-shaped bodies were counted and analysed in a similar manner.

Pharmacological reagents

ROCK activity was inhibited via treatment with Y27632 (Sigma Aldrich). The medium was supplemented with K252a (Sigma Aldrich) or a specific inhibitor of Met signalling, PHA-665752 (Tocris) (Christensen et al., 2003). Importantly, experiments comparing the effects of pharmacological inhibitors between mock- and Sema4d-treated kidneys were always carried out on sister kidneys derived from the same embryo.

Preparation of Sema4d

HEK 293T cell were transfected with Sema4d-AP or empty AP expression plasmids in serum-free medium, Sema4d-AP was purified from supernatants and its activity was assessed via alkaline phosphatase activity assays as described previously (Deng et al., 2007). The working concentration of Sema4d-AP was standardized at 150 mU/ml in medium (approximately equivalent to 1 nM).

Bacterial expression and protein purification: TAT-C3 was produced and purified in *Escherichia coli* strain DE3 as GST fusion using standard protocols (Brandt et al., 2003).

Determination of activated Rho: the amount of activated RhoA was determined as described previously (Swiercz et al., 2002). For the determination of activated RhoA in kidney, kidneys were extracted from E14 mice, homogenized in RIPA buffer and GTP-bound RhoA was precipitated and detected via immunoblotting using a monoclonal anti-RhoA antibody (BD, Heidelberg, Germany).

Genetically-modified mice

The generation of mice with constitutive global deletion of the plexin B1-encoding gene, *Plxnb1* (*Plxnb1*$^{-/-}$) has been described previously (Deng et al., 2007). Furthermore, heterozygous knock-in mutant mice expressing a cDNA encoding β-galactosidase (*lacZ*) targeted into the *Plxnb1* locus (*Plxnb1lacZ/+*) were used in studies on expression analysis of plexin B1 via β-galactosidase staining (Friedel et al., 2007).

RESULTS

Expression of Sema4d and plexin B mRNAs during organogenesis

We first analyzed the expression of plexin B1 together with its ligand Sema4d and found that, in a number of developing organs, *Sema4d* mRNA is expressed in mesenchymal cells in pattern complementary to epithelial expression of plexin B1 mRNA (Figs 1, 2). A well known example for mesenchymal-epithelial interactions during development is the metanephros, where the epithelial ureteric buds and the metanephrogenic mesenchyme reciprocally induce each other in a dynamic dialogue (Saxen, 1987; Shah et al., 2004). At E13.5, we found that plexin B1 was expressed in round epithelial structures, which resembled ureteric buds (arrows in Fig. 1A). We reasoned that if these plexin B1-expressing cells indeed represented ureteric epithelium, they ought to be surrounded by a cap of mesenchyme, which is known to condense and aggregate around ureteric buds at E13.5 in mice (Saxen, 1987).

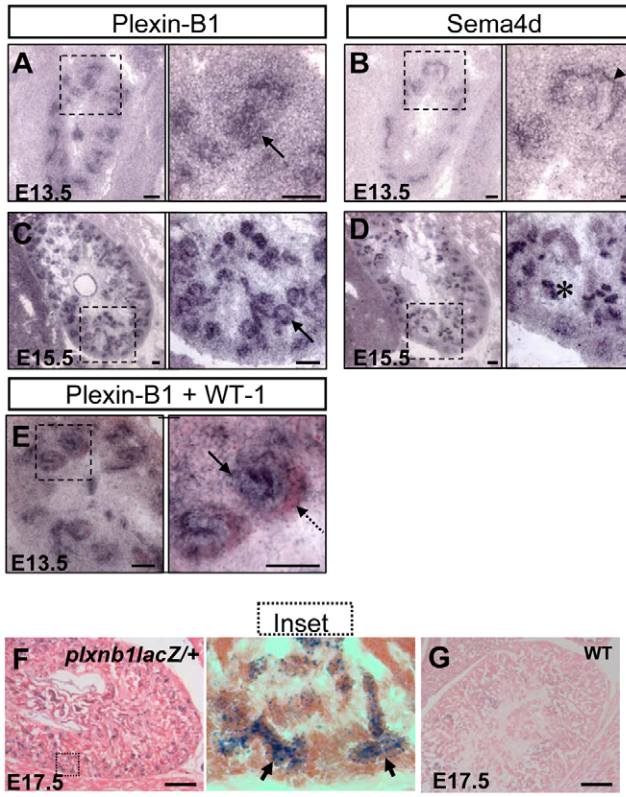

Fig. 1. Reciprocal expression of plexin B1 and Sema4d during early stages of branching morphogenesis during kidney development. Boxed areas are magnified in adjacent panels in A-F. Images represent mRNA in situ hybridization (A-E) or lacZ-staining (F,G). (**A**,**B**) At E13.5, plexin B1 mRNA is expressed in ureteric tips (arrow in A) whereas Sema4d mRNA is expressed in the condensing mesenchyme (arrowhead in B). (**C**,**D**) At E15.5, plexin B1 begins to bt expressed in developing glomeruli (arrow in C) and Sema4d is found in mesenchymal epithelium-derived-S/comma-shaped tubular bodies (asterisk in D). (**E**) In situ mRNA hybridization for plexin B1 (blue-purple stain) and co-immunostaining with an antibody against WT1 (red) reveal expression of plexin B1 in ureteric tips complementary to WT1-stained condensing mesenchyme at E13.5. (**F**) Heterozygous reporter mice expressing *lacZ* via the mouse locus for plexin B1 (*Plxnb1lacZ/+*) reveal β-galactosidase-stained ureteric tips in the outer cortex of developing kidneys at E17.5 (arrows) in addition to tubular structures in the medulla. Kidneys from wild-type littermates do not show β-galactosidase staining (**G**). Scale bars: 10 μm in A-G.

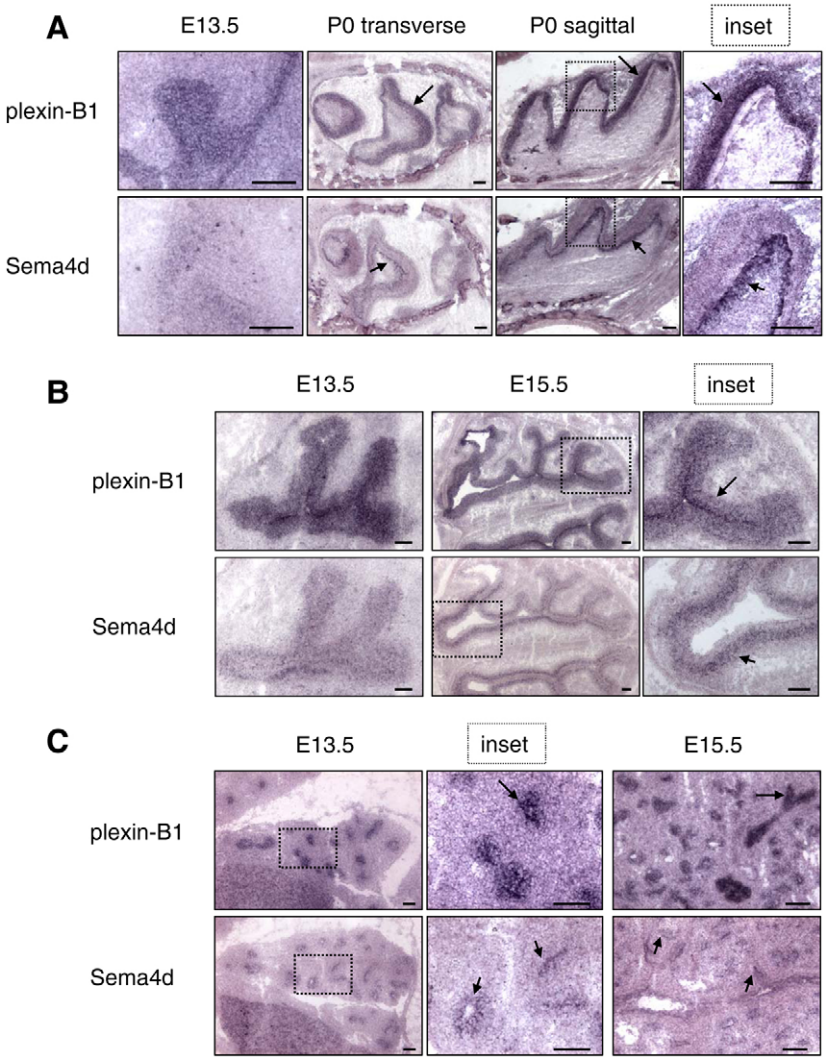

Fig. 2. mRNA in situ hybridization shows complementary expression of plexin B1 and its ligand Sema4d in epithelial and mesenchymal compartments, respectively, during genesis of several, but not all, organs in mice. (**A**) Expression of plexin B1 in the developing tooth cusp at embryonic day 13.5 (E13.5) and at day of birth (P0, long arrows), and of Sema4d in the adjacent mesenchyme (short arrows). (**B**) Expression of plexin B1 in proliferative olfactory epithelium layer (long arrows) and of Sema4d at the border between the olfactory epithelium and mesenchyme (short arrows). Note the reciprocal gradients of plexin B1 and Sema4d expression. (**C**) Expression of plexin B1 in bronchi (E13.5, long arrow) and branching bronchioles (E15.5, long arrow). Sema4d is expressed on the inner aspect of plexin B1-expressing bronchioles (short arrows), but is not found in the surrounding mesenchyme. In all panels, boxed areas are magnified in insets on the right. Scale bars: 10 μm in A-C.

Indeed, using mRNA in situ hybridization for plexin B1 and co-staining for a marker for metanephrogenic mesenchyme, WT1, we found a clear complementarity of plexin B1 expression and WT1-stained condensing mesenchyme (Fig. 1E). To confirm our results further, particularly in the light of a recent contradictory publication (Fazzari et al., 2007) that has reported plexin B1 expression in the mesenchyme, we took advantage of a mouse line expressing the reporter gene product β-galactosidase under the control of the plexin B1 promoter (*Plxnb1lacZ*). This knock-in reporter reflects closely the developmental expression pattern of plexin B1 in the nervous system, because the entire promoter elements of the *Plxnb1* gene are available to direct *lacZ* expression (Friedel et al., 2007). At E17.5, when epithelial-mesenchymal interactions between ureteric buds and condensing mesenchyme still take place in the outer cortex of the kidney, tissue sections of *Plxnb1lacZ* reporter embryos also revealed *lacZ*-staining in ureteric buds in the outer cortex (arrows in the inset of Fig. 1F), thereby entirely corroborating the results obtained by in situ hybridization and co-staining experiments. At E15.5, in agreement with previous results (Perälä et al., 2005), plexin B1 is found in developing glomeruli (arrow in Fig. 1C), indicating that, after the mesenchyme-to-epithelium transition has taken place, plexin B1 starts to be expressed in epithelial cells that had been derived primarily from mesenchymal cells. These observations could be confirmed via β-galactosidase staining of E17.5 embryos derived from *Plxnb1lacZ* reporter mice, which showed expression of plexin B1 in glomerular structures of the inner cortical layer. When analyzing the expression of Sema4d by in situ hybridization at E13.5, we found staining for Sema4d in a complementary pattern to plexin B1 in the metanephrogenic mesenchyme (Fig. 1B). This is consistent with the observation that Sema4d mRNA is found in comma- and S-shaped tubular structures at E15.5, which are of mesenchymal origin (asterisks in Fig. 1D).

The complementary expression of plexin B1 in ureteric buds and of Sema4d in metanephrogenic mesenchyme prompted us to analyze the mRNA expression of this ligand-receptor pair in other developing organs that rely on epithelial-mesenchymal interactions during their genesis, e.g. mammalian teeth (Tucker and Sharpe, 2004). In mouse, at E13.5 (bud stage), the oral epithelium invaginates into the underlying condensing mesenchyme. During this period, we found plexin B1 to be strongly expressed in the ectoderm-derived epithelial layer (Fig. 2A). Strikingly, Sema4d was selectively expressed in the mesenchymal layer, which is aggregated directly under the epithelial layer (Fig. 2A). This expression pattern persisted during the cap stage and bell stage (not shown), and was also seen at later stages of progressive differentiation, where plexin B1 was found in the enamel epithelium (arrows in Fig. 2A) and Sema4d was expressed in odontoblasts (arrowheads in Fig. 2A).

During the development of the olfactory primordia, the interaction between the olfactory placodal epithelium and the associated mesenchyme is essential to guide olfactory patterning, morphogenesis and differentiation (Balmer and La Mantia, 2005). At E13.5 and E15.5, plexin B1 was found to be strongly expressed throughout the olfactory epithelium (arrows in Fig. 2B), supporting previous results (Perälä et al., 2005). Complementary to the distribution of plexin B1, we found an expression of Sema4d in the mesenchymatous shelves that are covered by the olfactory epithelium (arrowheads in Fig. 2B).

In contrast to above, the mesenchymal versus epithelial distribution of the Sema4d/plexin B1 ligand-receptor pair did not hold true in some organs, such as the lung. During lung development, the endoderm-derived epithelial lung buds invaginate into the surrounding mesenchyme and form the bronchi and bronchioles through a series of dichotomous divisions (Cardoso and Lü, 2006). At E13.5, when segmental bronchi progressively branch, plexin B1 was strongly expressed in the bronchial epithelium (Fig. 1C). Plexin B1 mRNA continued to be highly expressed in the epithelium after E15.5 during the formation of terminal bronchioles (arrows in Fig. 1C, E15.5). Sema4d was found on the inner aspect of plexin B1-expressing epithelium, rather than in the lung mesenchyme (Fig. 1C).

Thus, plexinB1 and Sema4d were observed to show complementary expression patterns in several but not all developing non-neuronal organs, with plexin B1 being typically found in the epithelium and Sema4d in the surrounding mesenchymal tissue, suggesting a role for this ligand-receptor pair in epithelial-mesenchymal interactions during organogenesis.

Effects of Sema4d on metanephroic development

To directly test this hypothesis, we used a well-established mammalian model of mesenchyme-epithelial interactions: mesenchyme-induced epithelial branching in the developing urinary collecting duct (Shah et al., 2004). We employed the ex vivo organ culture system of kidneys derived from mice at E12, which enables testing effects of individual molecules on branching morphogenesis and nephron formation over 2-4 days (Saxen, 1987) (Fig. 3A-C). Three-dimensional reconstruction of anti-calbindin-stained ureteric trees following confocal microscopy on whole cultured metanephroi revealed normal development in terms of number of ureteric tips and the length and area of developing kidneys, consistent with previous reports (Saxen, 1987; Woolf et al., 1995) (Fig. 3D-G). Concentrated supernatants of HEK293T cells expressing AP-tagged Sema4d were used to test the effects of plexin B1 activation via exogenously supplied Sema4d on developing kidneys, and supernatants from vector-transfected HEK293 cells served as controls (mock), as described previously (Deng et al., 2007). Sema4d or mock treatments were always performed on sister kidneys derived from individual embryos at E12 (Fig. 3H,I), in order to circumvent differences arising from dissimilar development and maturation of embryos within and between litters. The functional integrity of Sema4d-enriched medium was confirmed by testing its ability to activate RhoA in a RBD-GST-based pull-down assay (Fig. 3J) (Swiercz et al., 2002).

E12 kidneys cultured with Sema4d over 48 hours showed a striking reduction in the extent of ureteric branching when compared with sister kidneys cultured in the presence of mock medium (see Fig. 3H,I for typical examples and Fig. 3K for summary). Both the number of the developed, calbindin-positive ureteric tips, as well as the number of branch points in the ureteric tree were found to be consistently reduced upon Sema4d treatment when compared with

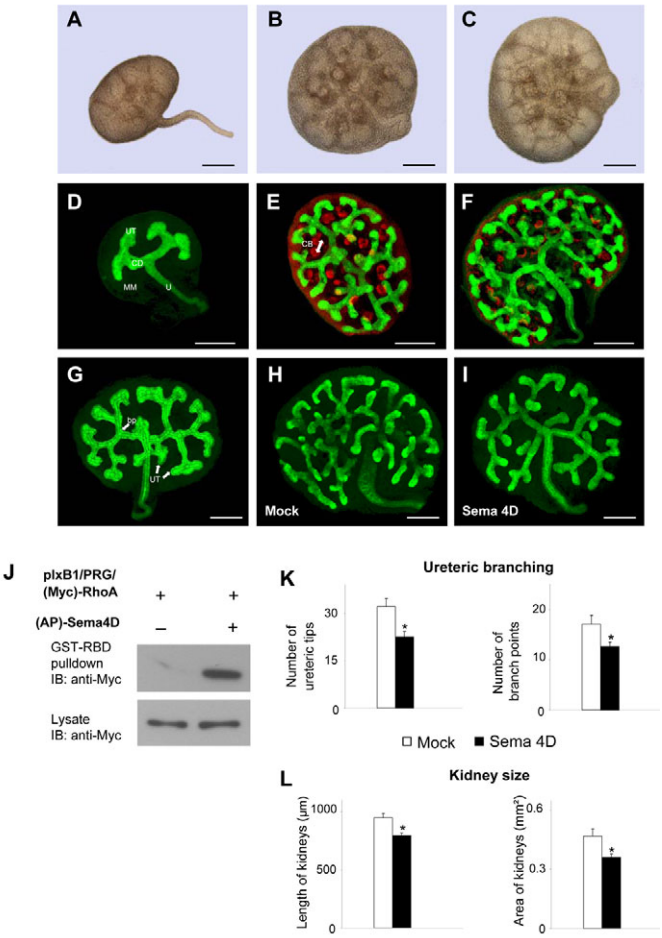

Fig. 3. Development of mouse metanephroi in whole-organ cultures and its modulation by Sema4d. (**A-C**) Phase-contrast images of a kidney rudiment isolated at E12 (A) and of the same kidney after 24 hours (B) and 48 hours (C) in culture. (**D-F**) Maximal projections of confocal images of whole cultured metanephroi showing the ureteric tree stained with an anti-calbindin antibody (green, D) and the condensing mesenchyme stained with an anti-WT1 antibody (red, E,F) at E12 (D) and after 24 hours (E) and 48 hours (F) in culture. UT, ureteric tip; CD, collecting duct; MM, metanephrogenic mesenchyme; CB, comma-shape bodies, two examples are indicated by the double arrow. (**G**) Illustration of the method used for quantifying ureteric branch points (bp) and ureteric tips (arrows, UT) in maximal confocal projections. (**H**,**I**) Typical examples of E12 metanephroi cultured for 24 hours in medium supplemented with concentrated medium of mock-transfected HEK293T cells (H) or Sema4d-AP-transfected HEK293T cells (I). The ureteric tree is labelled with an anti-calbindin antibody (green). (**J**) Western blot analysis demonstrating the efficacy of Sema4d in inducing RhoA activation in a GST-RBD-based assay in HEK293T cells transfected with plexin B1 (PlxnB1) and PDZRhoGEF (PRG). Anti-Myc-positive bands in western blotting serve as loading controls. (**K**) Sema4d treatment reduces the number of ureteric tips and branch points over mock treatment in sister cultures of metanephroi. (**L**) Sema4d treatment decreases the size of the developing kidney over mock treatment. Only sister kidneys cultured for 48 hours were taken for analysis. **P*<0.001 in comparison with mock, Student's paired *t*-test. Scale bars: 200 µm in A-I.

mock treatment (P=0.001 and P=0.01, respectively; Fig. 3K). Sema4d-induced deceleration of kidney growth was also evident as a decrease in the length ($P \leq 0.001$) and area (P=0.002) of developing kidneys (Fig. 3L). To address whether activation of plexin B1 also

affects the differentiation of metanephrogenic mesenchyme, we addressed the effects of Sema4d treatment on the number of comma-shaped bodies identified via WT1 staining, which result from the transition of metanephric mesenchyme into tubular epithelium. This was not affected by Sema4d (see Table S1 in the supplementary material), suggesting therefore that activation of plexin B1 leads to selective changes in the branching morphogenesis of ureteric tips in the developing kidney.

Because the whole organ culture model does not enable testing the effects of exogenously added Sema4d independently of Sema4d, which is endogenously expressed by the mesenchyme, the effects of Sema4d on branching morphogenesis described above probably represent an underestimation. Therefore, we addressed the contribution of endogenous Sema4d in shaping the architecture of the developing kidney by using an anti-Sema4d antibody, which has been shown to neutralize endogenous Sema4d in previous studies (Masuda et al., 2004). When compared with pre-immune serum (control), treatment with the anti-Sema4d antibody led to a partial reduction in Sema4d-induced RhoA activation in HEK293T cells (Fig. 4A), showing that this approach can at least partially attenuate Sema4d-induced activation of plexin B1. E12 kidneys cultured with the anti-Sema4d antibody showed a small, but significant, increase in the number of ureteric tips (P=0.004), as well as in the number of branch points (P=0.04), when compared with sister kidneys cultured with pre-immune serum (Fig. 4B-D). The length and area of anti-Sema4d-treated kidneys showed only a trend towards increase, which did not reach statistical significance (P=0.15 and P=0.11 for length and area, respectively; Fig. 4E). Albeit the magnitude of the observed differences in ureteric branching was small, given that the anti-Sema4d antibody may have only partially impeded Sema4d-plexin B1 signalling, the above results suggest that Sema4d, synthesized and released endogenously by the metanephrogenic mesenchyme at E12, functions to repress branching morphogenesis of ureteric tips.

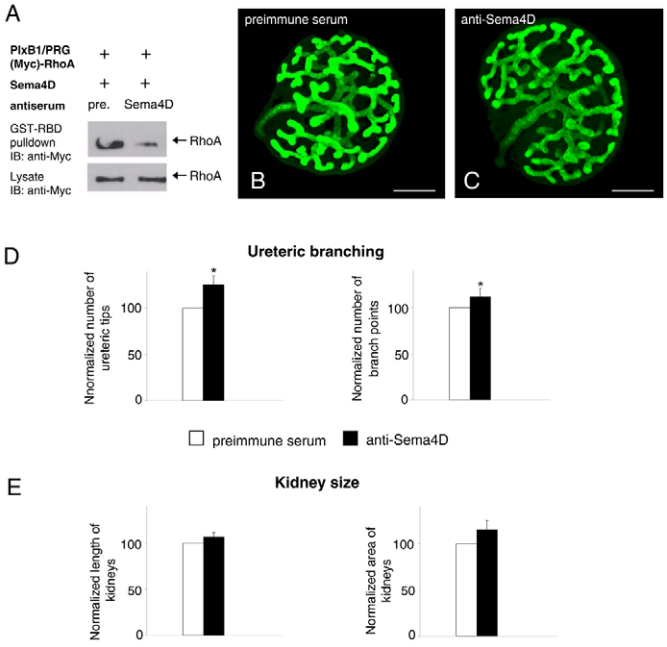

Fig. 4. Effects of blockade of endogenous Sema4d on metanephroic development. (**A**) Western blot analysis demonstrating the efficacy of a Sema4d function-blocking antibody (anti-Sema4d), but not preimmune serum (pre.; control), in attenuating Sema4d-induced RhoA activation in a GST-RBD-based assay in HEK293T cells transfected with plexin B1 (plxnB1) and PDZRhoGEF (PRG). Anti-Myc-positive bands in western blotting serve as loading controls. (**B**,**C**) Typical examples of the anti-calbindin-immunoreactive ureteric tree (green) in sister metanephroi from E12 embryos cultured in the presence of an anti-Sema4d antibody (C) or preimmune serum (B, control). (**D**,**E**) E12 metanephroi cultured with the anti-Sema4d antibody showed significantly more branching (D), but were the same size (E), when compared with metanephroi cultured with preimmune serum (control). *P<0.05, Student's paired t-test. Scale bars: 250 μm.

Signalling mechanisms mediating effects of Sema4d on ureteric branching

The nature of molecular pathways that mediate the inhibitory effects of Sema4d on ureteric branching raises an important and complex issue. Among the plexin B1 signalling mediators, RTKs and, in particular, Met have been best studied with respect to kidney development (Woolf et al., 1995; Santos et al., 1994; Zhang and Vande Woude, 2003). To assess their potential involvement in plexin B-mediated cellular effects in the developing kidney, we cultured sister kidneys in the presence of pharmacological inhibitors with either Sema4d-containing medium or control medium (see Fig. 5A-D for typical examples; see Fig. 5E,F for a summary). K252a is known to block RTKs such as Met and Trks (Morotti et al., 2002). Consistent with previous transgenic studies reporting a requirement for RTKs such as Met and Ret in ureteric branching, we observed that 200 nM K252a in the absence of Sema4d led to an inhibition of ureteric branching: the numbers of branch points and ureteric tips, as well as the length and area of the metanephroi, were significantly reduced (Fig. 5E,F). Furthermore, the number of comma-shaped bodies arising from the mesenchyme-derived epithelium was also significantly reduced (see Table S1 in the supplementary material). Importantly, however, inhibition of Met via K252a did not change the nature or the magnitude of the effects of Sema4d on developing metanephroi (Fig. 5C-F). Thus, inhibition, rather than activation, of RTKs mimicked the effects of Sema4d, suggesting that Sema4d-induced repression of ureteric branching is not associated with the activation of RTKs via plexin B1.

Because K252a can block several kinases in addition to inhibiting Met (e.g. Morotti et al., 2002), we used PHA-665752, which has been recently identified as an active-site competitive inhibitor of Met with over 50-fold selectivity for Met when compared with a broad panel of diverse tyrosine- and serine-threonine kinases (Christensen et al., 2003). We observed that pre-treatment with PHA-665752 (1 μM) significantly reduced ureteric branching (Fig. 5I,J) and decreased area and length in mouse metanephroi cultured at day E12 (Fig. 5K,L). Importantly, treatment with PHA-665752 did not alter Sema4d-induced reduction in ureteric branching (Fig. 5I-L). We therefore inferred that Met activation is not required for Sema4d-induced inhibition of branching morphogenesis in the developing metanephros.

Using a Rhotekin-GST-based pull-down assay, we observed that, similar to developing neurons, application of Sema4d indeed leads to an activation of RhoA in developing metanephroi at E12 when compared with treatment with mock medium (Fig. 6A). To assess the role of Rho-dependent mechanisms in Sema4d-induced effects on developing metanephroi, we used lower concentrations of Y27632 (1 μM and 3 μM), which dose-dependently and specifically inhibit the Rho kinase (ROCK) (Narumiya et al., 2000; Davies et al., 2000). Treatment with 1 μM Y-27632 tended to increase the number of ureteric tips in mock-treated kidneys (P=0.01) and produced a small, but statistically significant, increase in the number of branch

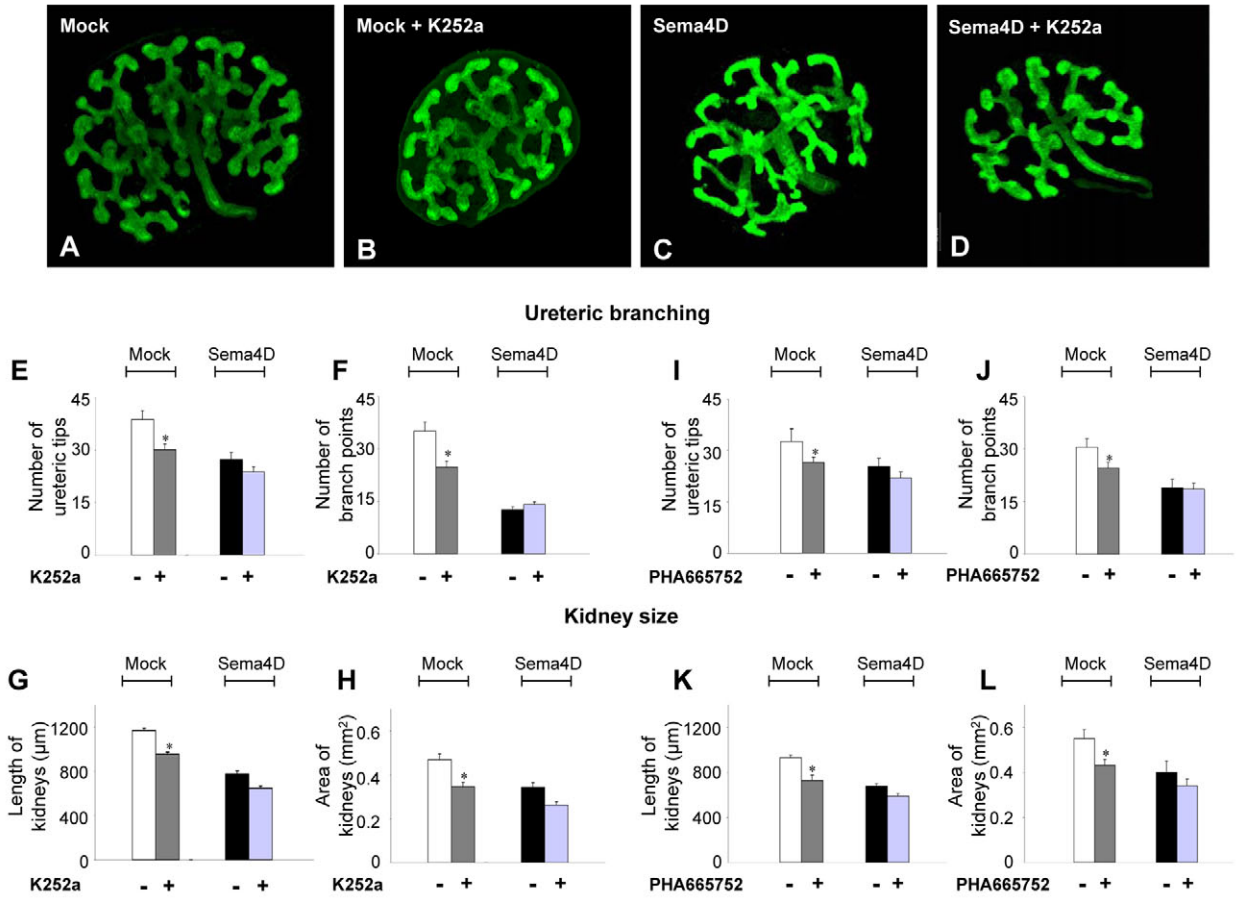

Fig. 5. Pharmacological blockade of receptor tyrosine kinases (RTK) by K252a or by the selective Met inhibitor PHA665752 does not affect Sema4d-induced inhibition of ureteric branching in metanephroi cultured at E12. (**A-D**) Typical examples of anti-calbindin-stained mouse metanephroi cultured with either Sema4d or mock medium in the presence or absence of the RTK inhibitor, K252a (200 nM). (**E-H**) Blockade of RTKs by K252a significantly reduced ureteric branching (E,F) and size (G,H) in mock-treated kidneys, but did not affect Sema4d-induced modulation of these parameters in developing kidneys. (**I-L**) Similar results were obtained when using the selective Met inhibitor PHA665752. *P<0.001 when compared with mock or Sema4d alone; Student's paired t-test.

points (P=0.013), suggesting that endogenous ROCK mediates an inhibitory tone on ureteric branching during development under the conditions used in this study (typical examples are in Fig. 6B,C; see summary in Fig. 6J). Importantly, 1 μM Y27632 fully reversed the inhibitory effects of Sema4d on ureteric branching (Fig. 6D,J). Furthermore, Sema4d produced a striking increase in the ureteric branching in the presence of 1 μM Y27632 (84.4±2.6% increase in branch points and 29.8±3.4% increase in ureteric tips), which was significantly higher than that observed in metanephroi treated with 1 μM Y27632 and mock medium (36.3±2.7% increase in branch points and 6.0±3.1% increase in ureteric tips; Fig. 6D,J) (P<0.001 in both cases).

Because recent studies have implicated ROCK in modulating the morphology of ureteric buds at several distinct stages during ureteric development (Meyer et al., 2006; Michael et al., 2005), we used doses of Y27632 that specifically block ROCK (Davies et al., 2000) to study whether structural changes are linked with ureteric branching and how these are modulated by Sema4d-plexin B1 interactions. We observed that both 1 μM and 3 μM concentrations of Y27632 were associated with a rapid increase in the size of the metanephroi (Fig. 6C,F,K). However, unlike 1 μM Y27632, a concentration of 3 μM Y27632 produced a blatant deformation of the developing ureteric tree with a pronounced swelling of ureteric tips (see Fig. 6E for typical examples). Staining of the actin cytoskeleton with TRITC-labelled phalloidin revealed a flattening of wedge cells, as well as an increase in the lumen of ureteric tips in metanephroi cultured in the presence of 3 μM Y27632 (Fig. 6G,H). Furthermore, unlike 1 μM Y27632, treatment with 3 μM Y-27632 did not increase ureteric branching over mock medium (Fig. 6B,E,J). Interestingly, addition of Sema4d to the medium could rescue some, but not all, the effects of 3 μM Y27632. Swelling of ureteric tips, as well as an increase in the size of lumen, was less prominent in Sema4d + 3 μM Y27632-treated metanephroi (Fig. 6F,I). Furthermore, Sema4d produced a striking increase in the ureteric branching in the presence of 3 μM Y27632 (55.7±3.1% increase in branch points and 15.1±3.1% increase in ureteric tips), which was significantly higher than that observed in metanephroi treated with mock medium, Sema4d medium alone or 3 μM Y27632 with mock medium (see Fig. 6F for a typical example; see Fig. 6J for a summary) (P≤0.05 in all cases). However, kidney hyperplasia elicited by 3 μM Y27632 was not changed by Sema4d treatment (Fig. 6F,K), suggesting that mechanisms underlying Y27632-induced metanephroic hyperplasia differ from those associated with deformation of ureteric tips and changes in ureteric branching in the developing kidney. Furthermore, we observed that treatment with a TAT-fusion protein of recombinant C3 exoenzyme (TAT-C3, 0.5 μM), a known specific direct inhibitor of RhoA, reversed the inhibitory

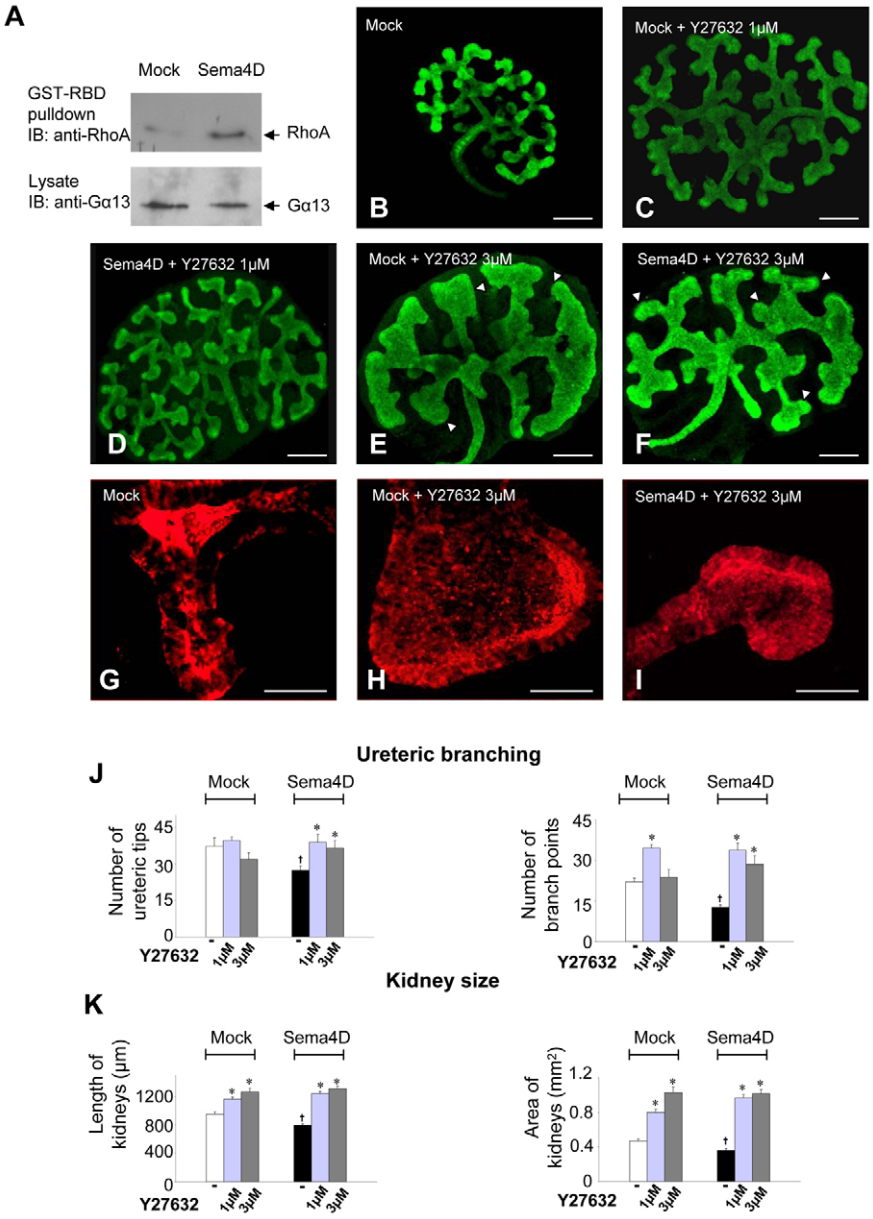

Fig. 6. Sema4d-induced inhibition of ureteric branching morphogenesis requires activation of the Rho-ROCK pathway. (**A**) Treatment with Sema4d leads to activation of RhoA in mice metanephroi cultured at E14.5. Lysates of kidneys treated with either Sema4d or mock medium for 5 hours were incubated with RBD-coupled sepharose and the amount of RhoA was analysed by western blotting with an anti-RhoA antibody. The amount of Gα13 was comparable in the two samples (internal control). (**B-D**) Typical examples of anti-calbindin-stained metanephroi cultured with either Sema4d or mock medium in the presence or absence of the ROCK inhibitor Y27632 (1 μM). In the presence of 1 μM Y27632, Sema4d did not attenuate ureteric branching. (**E**,**F**) Y27632 (3 μM) led to a hyperplasia and distortion of ureteric structures (arrowheads), to a lesser extent in Sema4d-treated kidneys than in mock-treated kidneys. (**G-I**) Higher magnification views of phalloidin-stained ureteric tips in Sema4d- or mock-treated kidneys cultured in the presence of 3 μM Y27632. The Y27632-induced distortion of apical wedge cells and actin bundles and the increase in tip lumen occurs to a lesser extent in Sema4d-treated kidneys in comparison with mock-treated kidneys. (**J**,**K**) Quantitative summary of the effects of Y27632 at either 1 μM or 3 μM on Sema4d- or mock-treated metanephroi. †P<0.001 when compared with mock; *P<0.05 when compared with mock or Sema4d alone; ANOVA followed by post-hoc Fisher's test. Scale bars: 200 μm in B-F; 70 μm in G-I.

effects of Sema4d and increased ureteric branching over control levels (see Fig. S1 in the supplementary material; P<0.001), which is consistent with effects of Y27632 at doses of 1 μM and 3 μM.

Modulation of metanephroic development by plexin B1 in vivo

In kidneys derived at E12, when compared with heterozygous littermates, mice with a constitutive global deletion of *Plxnb1*, the gene encoding plexin B1 (*Plxnb1*$^{-/-}$), demonstrated a complete loss of expression of plexin B1 mRNA (Fig. 7A, upper panels), whereas the expression of plexin B2 mRNA remained unchanged (Fig. 7A, lower panels). Interestingly, although *Plxnb1*$^{-/-}$ and *Plxnb1*$^{+/-}$ embryos were comparable in size, kidneys of *Plxnb1*$^{-/-}$ embryos at E13.5 were obviously larger than their corresponding *Plxnb1*$^{+/-}$ littermates, showing a significant increase in the both length (P<0.001) and area (P=0.01) (see Fig. 7B,C for typical examples and Fig. 7D,E for quantitative summary). Furthermore, *Plxnb1*$^{-/-}$ kidneys showed ~36% more ureteric tips than their *Plxnb1*$^{+/-}$ littermates (Fig. 7F). In order to ascertain whether this truly reflects enhanced ureteric branching, we analyzed the number of ureteric tips in a region of interest of a defined area (white boxes in Fig. 7B,C). The number of ureteric tips per unit area was consistently higher in *Plxnb1*$^{-/-}$ mice than in their corresponding *Plxnb1*$^{+/-}$ littermates at E13.5 (Fig. 7G). At E14.5, *Plxnb1*$^{-/-}$ embryos continued to show larger kidneys with a significantly greater area than *Plxnb1*$^{+/-}$ embryos (Fig. 7H,I). However, starting from E15.5, the kidneys of *Plxnb1*$^{-/-}$ embryos did not differ from those of *Plxnb1*$^{+/-}$ embryos (Fig. 7H,I). Furthermore, analysis of Nissl-stained paraffin sections showed that the number of developed nephrons did not differ between *Plxnb1*$^{-/-}$ and *Plxnb1*$^{+/-}$ embryos at E17.5 (Fig. 7J). Taken together, these data suggest that an activation of plexin B1 is indeed functionally linked to the inhibition of epithelial branching morphogenesis during early stages of ureteric development, which is compensated over later stages of kidney development in mice that lack plexin B1.

Interestingly, in E12 metanephroi isolated from mice lacking plexin B1, Sema4d failed to inhibit ureteric branching, although inhibition was observed in metanephroi of wild-type littermate

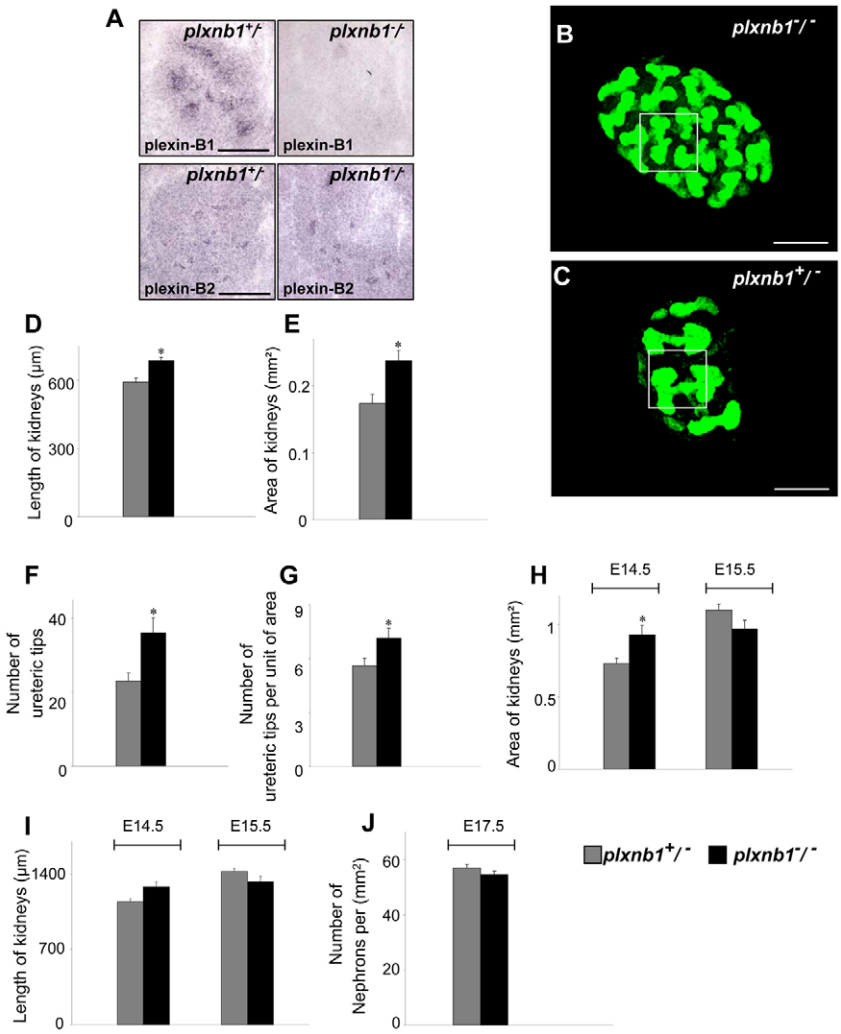

Fig. 7. Early defects in kidney development in mice constitutively lacking plexin B1 expression (*Plxnb1*$^{-/-}$). (**A**) When compared with heterozygous control littermates (*Plxnb1*$^{+/-}$), *Plxnb1*$^{-/-}$ mice show a loss of plexin B1 mRNA, whereas the expression of plexin-B2 mRNA remains unchanged. (**B**,**C**) Typical examples of kidneys dissected out of *Plxnb1*$^{-/-}$ (B) and *Plxnb1*$^{+/-}$ (C) littermates at E13.5 and stained with an anti-calbindin-28K antibody. (**D-G**) In comparison with heterozygous littermates, *Plxnb1*$^{-/-}$ kidneys are larger in size (D,E) and have increased ureteric branching (F,G) at E13.5. (**H**,**I**) At E14.5, *Plxnb1*$^{-/-}$ mice show a larger area of kidneys than heterozygous littermates, but at E15.5 no differences can be detected. (**J**) The number of developed nephrons is not significantly different between *Plxnb1*$^{-/-}$ and *Plxnb1*$^{+/-}$ littermates at E17.5. **P*<0.001, Student's *t*-test. Scale bar: 200 μm.

embryos. Furthermore, in these ex vivo culture experiments, we observed that metanephroi derived from plexin B1 knockout mice were consistently larger than those from wild-type littermates, and they failed to respond to Sema4d with respect to kidney size (Fig. 8), which is entirely consistent with our in vivo analysis of plexin B1 knockout mice (Fig. 7). These results thus show that plexin B1 can fully account for the observed effects of Sema4d on branching morphogenesis in the kidney.

DISCUSSION

The most interesting finding of this study is that Sema4d-plexin B1 signalling represses branching morphogenesis in a classical model for developmental epithelial-mesenchymal interactions, namely the developing metanephric ureteric tree. Class III semaphorins have been implicated in inhibition of branching morphogenesis in the developing lung (Ito et al., 2000) and, very recently, also in the kidney (Tufro et al., 2008). In the developing salivary gland, however, signalling via Class III semaphorins has recently been reported to facilitate, rather than to inhibit, cleft formation (Chung et al., 2007). A functional role for Class IV semaphorins in branching morphogenesis during organogenesis had not been experimentally demonstrated so far. We observed that both endogenously expressed as well as exogenously applied Sema4d suppresses the branching morphogenesis of the ureteric bud epithelium, which is the first and crucial important step in the establishment of a collecting duct system for enabling kidney function. Entirely consistent with this finding, we observed that mice lacking plexin B1 demonstrate exaggerated ureteric branching and increased kidney size. Importantly, these processes were detected over early stages of kidney development, i.e. between E12.5-E14.5, during which crucial functional interactions between the ureteric bud epithelium and metanephrogenic mesenchyme take place (Saxen, 1987).

The finding that the major functional outcome of the activation of the Sema4d-plexin B1 axis in the developing kidney entails an inhibition, rather than stimulation, of branching morphogenesis is unexpected in the light of the ability of Sema4d-plexin B1 to signal via RTKs, such as Met, which are recognized as activators of branching morphogenesis (Santos et al., 1994; Woolf et al., 1995; Zhang and Vande Woude, 2003). Indeed, in several developing organs we studied, such as the kidney, lung and teeth, the observed expression pattern of plexin B1 closely matches the reported distribution of Met (Ohmichi et al., 1998; Tabata et al., 1996; Thewke and Seeds, 1996; Woolf et al., 1995). Similarly, there are many parallels between the pattern of expression of Sema4d reported here and that of the classical Met ligand HGF reported in previous studies in developing tissues such as the lung, teeth and olfactory conchae (Ohmichi et al., 1998; Tabata et al., 1996; Thewke and Seeds, 1996; Woolf et al., 1995). Furthermore, there are many similarities between their cellular functions. For example, both HGF

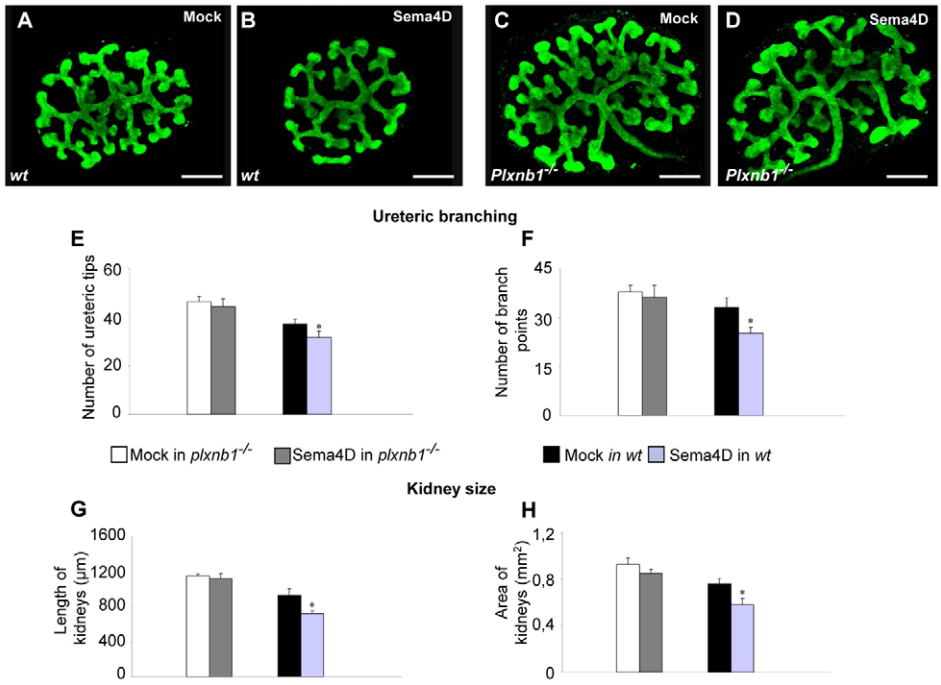

Fig. 8. Effects of Sema4d on metanephroic development are mediated by plexin B1. (**A-D**) Typical examples of anti-calbindin-stained metanephroi derived from wild-type (wt) or plexin B1-knockout embryos (*Plxnb1*$^{-/-}$) cultured with either Sema4d or mock medium. (**E-H**) The Sema4d-induced inhibition of ureteric branching (E,F) and kidney size (G,H) in wild-type kidneys is lost in kidneys that lack plexin B1. **P*<0.05 when compared with mock; Student's paired *t*-test.

and Sema4d have been reported to trigger invasive growth in epithelial cells (Giordano et al., 2002), a process that uses the basic mechanisms of branching morphogenesis, with considerable on-going crosstalk between the two ligand-receptor pairs (Conrotto et al., 2004). Similarly, both Sema4d and HGF have been reported to stimulate axonal branching and growth in developing sensory neurons (Maina et al., 1997; Masuda et al., 2004). Collectively, these findings indicate that the net modulation of branching morphogenesis in biological systems by B-type plexins is highly context dependent and that signalling via RTKs may play roles of varying prominence in the different systems. Indeed, in addition to activating Erbb2 and Met, B-type plexins can trigger multiple signalling events in parallel by modulating the activity of Rho family GEFs and GAPs, which govern the activation status of RhoA, R-Ras and several cytosolic kinases, including Pyk2, Src, FAK, PI3K and Akt (Aurandt et al., 2002; Basile et al., 2005; Basile et al., 2007; Giordano et al., 2002; Hirotani et al., 2002; Oinuma et al., 2004; Perrot et al., 2002; Swiercz et al., 2002; Swiercz et al., 2004; Vikis et al., 2000). Furthermore, the cellular functions of several of these diverse mediators can be mutually contradictory. For example, recent evidence suggests that, depending upon whether Met or Erbb2 become recruited, plexin B1 elicits entirely opposite effects upon cell motility via inactivation or activation of RhoA, respectively (Swiercz et al., 2008). These aspects can confer a tremendous level of complexity and versatility to the biological functions of Sema4d-plexin B1 interactions in developing organs.

Importantly, we found a prominent role for the RhoA-ROCK pathway in mediating the functions of Sema4d-plexin B1 in the developing kidney. Several lines of evidence from cell and organ culture experiments suggest that RhoA participates in diverse aspects of epithelial development, such as tubulogenesis, cyst formation, epithelial barrier function and epithelial polarity (Bruewer et al., 2004; Eisen et al., 2006; Hasegawa et al., 1999; Meyer et al., 2006; Micheal et al., 2005; Rosário and Birchmeier, 2003). Consistent with the above, blockade of the RhoA effector ROCK, via Y27632, in developing kidneys is associated with a diverse set of phenotypic changes, and ROCK has been implicated in several distinct steps during kidney development (Meyer et al., 2006; Michael et al., 2005). Here, we used doses that have been shown to block ROCK specifically (Davies et al., 2000; Narumiya et al., 2000) and observed that partial blockade of ROCK, which did not cause a marked deformation of ureteric tips, fully blocked the inhibitory effects of Sema4d and increased ureteric branching. These results were corroborated by experiments on RhoA inhibition via the C3 exoenzyme. This suggests that RhoA signalling via ROCK acts as an endogenous brake on branching morphogenesis during kidney development and that this function is distinct from other functions of ROCK in maintaining the cytoskeletal structure of the ureteric tree. This is supported by reports on the expression of RhoA in the developing kidney in a pattern suggestive of a functional role in epithelial-mesenchymal interactions (Bianchi et al., 2003). Furthermore, RhoA and ROCK have been associated with the inhibition of branching morphogenesis in other model systems, such as the developing lung (Moore et al., 2005) and neurites of hippocampal neurons (Luo, 2000). Consistent with previous studies (Michael et al., 2005; Meyer et al., 2006), we observed that a higher degree of ROCK blockade led to rapid structural deformities in the entire developing metanephros, including swelling and increased lumen in ureteric tips. The latter may be associated with a loss of epithelial barrier function, which has previously been suggested to be regulated by Rho GTPases (Fujita et al., 2000; Hasegawa et al., 1999). Interestingly, Sema4d was observed to partially rescue some of these structural anamolies, either via an increase in RhoA activity and the consequential drop in the degree of ROCK blockade, or through the actions of other signalling mediators activated downstream of plexin B1.

In this context, another interesting observation was that pharmacological blockade of ROCK unmasked stimulatory effects of Sema4d on ureteric branching in developing metanephroi. This suggests that, upon plexin B1-mediated activation, the RhoA-ROCK pathway inhibits branch-promoting signalling events that are concurrently triggered by plexin B1. This finding is very interesting because, so far, branching patterns are generally thought to be regulated by a precise temporal and spatial balance between

stimulatory branching morphogens, such as HGF, and inhibitory morphogens, such as members of the TGFβ superfamily, e.g. specific TGFβ isoforms and BMPs (Shah et al., 2004; Zhang and Vande Woude, 2003). Via their unique ability to trigger activation of the branch-promoting RTKs, as well as of inhibitory RhoA-dependent pathways, plexin B proteins harbour the potential to fine-tune branching morphogenesis in a novel manner. Furthermore, recent studies suggest that plexin B1 can switch between molecular states that favour the activation of either Met or RhoA in a context-dependent manner (Basile et al., 2004; Conrotto et al., 2005; Swiercz et al., 2008). It is plausible, therefore, that plexin B proteins inhibit branching morphogenesis at a specific stage of ureteric development and counterbalance this via activation of alternative signalling pathways when inhibition ceases to be required. Indeed, it has been hypothesized that, during kidney development, negative-feedback processes between the metanephrogenic mesenchyme and the ureteric buds are timed to a specific point during mesenchyme-derived tubule formation and cease to exist following the fusion of the metanephric tubule with the lateral ureteric branch as nephrogenesis proceeds (Shah et al., 2004).

The functional significance of inhibition of branching morphogenesis in kidney development in vivo has been difficult to elucidate, as deletion mouse mutants of the typical inhibitory morphogens or their receptors have yielded mixed phenotypes that range from early embryonic lethality to no overt renal anomalies (Shah et al., 2004; Zhang and Vande Woude, 2003). Although the phenotype of plexin B1 knockout mice closely matched our ex vivo results at early stages in ureteric development, it appears that compensatory mechanisms can overcome the effects of disturbances in the Sema4d-plexin B1 in early development, consistent with the notion of functional redundancy of branching-regulatory factors during embryonic kidney development (Shah et al., 2004), probably arising as a result of a convergence on common effector systems. Furthermore, plexin B2, which can function as a low-affinity receptor for Sema4d, is also expressed in the developing kidney.

In summary, this study extends the functional repertoire of the Sema4d-plexin B1 axis to modulation of branching morphogenesis during organogenesis outside the nervous system. Our results identify Sema4d as a novel negative morphogen in kidney development and suggest that activation of the RhoA-ROCK pathway by plexin B1 constitutes an important regulatory mechanism in the fine-tuning of epithelial branching via mesenchyme-derived cues.

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Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/135/20/3333/DC1

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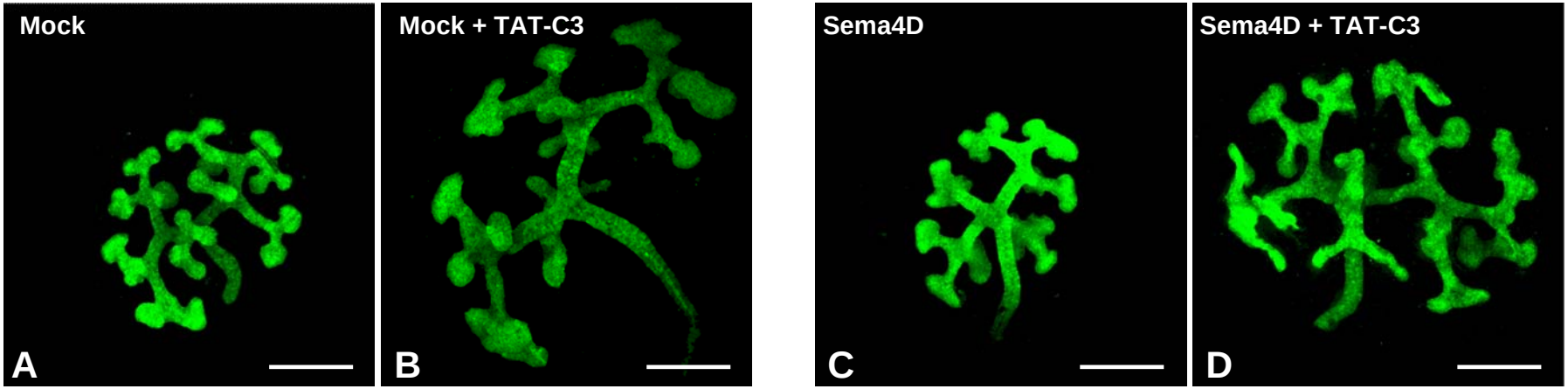
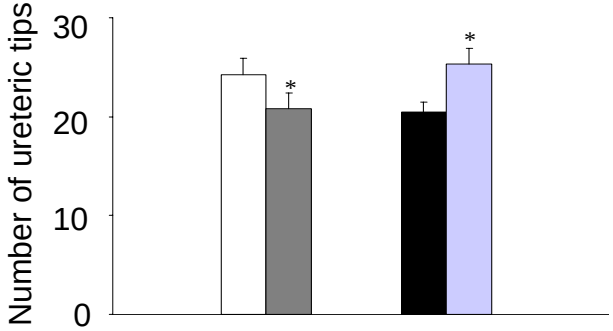
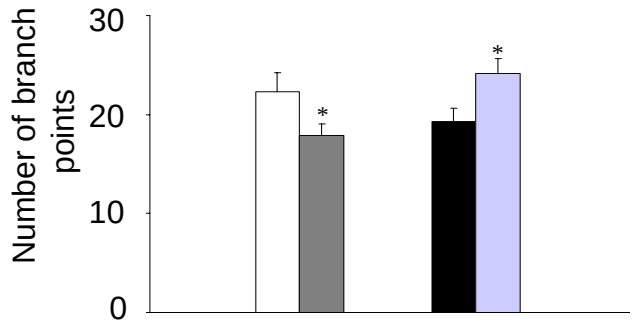


Ureteric branching

E



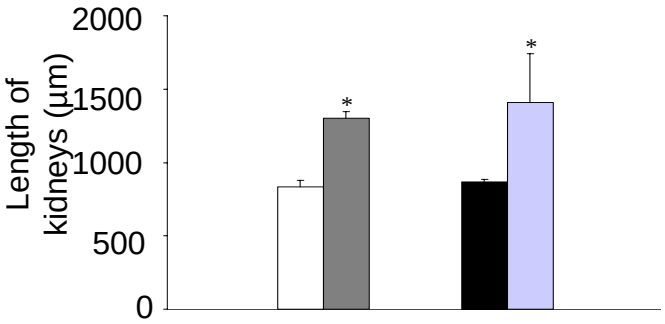
F



Mock Mock + TAT-C3 Sema4D Sema4D + TAT-C3

Kidney size

G



H

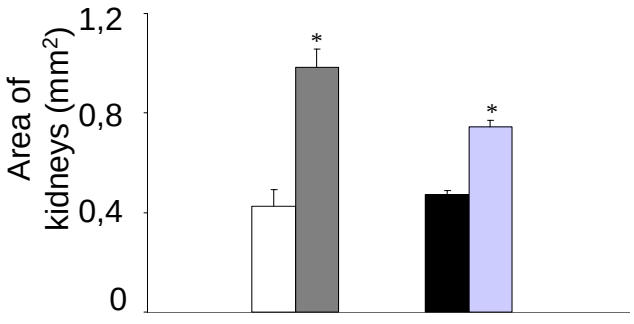


Table S1. Quantitative summary of the development of comma-shaped bodies in developing cultured metanephroi over different treatment groups

Treatment group	Number of comma-shaped bodies
Mock	14.50±1.08
Sema4d	13.00±1.06
Preimmune serum	15.33±1.18
Anti-Sema4d	14.00±1.12
Mock	18.25±1.58
Mock + K252a	13.25±0.80*
Sema4d	12.45±1.06
Sema4d + K252a	13.18±0.88
Mock	13.58±1.38
Mock + 1 μM Y27632	13.69±0.89
Mock + 3 μM Y27632	11.33±1.06
Sema4d	13.00±1.07
Sema4d + 1 μMY27632	14.57±5.50
Sema4d + 3 μM Y27632	11.44±1.26

*$P<0.05$, Student's *t*-test.