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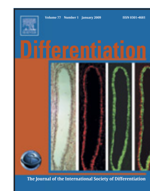

Sema4C-Plexin B2 signalling modulates ureteric branching in developing kidney

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ABSTRACT

Semaphorins, originally identified as axon guidance molecules, have also been implicated in angiogenesis, function of the immune system and cancerous growth. Here we show that deletion of Plexin B2 (Plxnb2), a semaphorin receptor that is expressed both in the pretubular aggregates and the ureteric epithelium in the developing kidney, results in renal hypoplasia and occasional double ureters. The rate of cell proliferation in the ureteric epithelium and consequently the number of ureteric tips are reduced in the kidneys lacking Plexin B2 (*Plxnb2* −/−). Semaphorin 4C, a ligand for Plexin B2, stimulates branching of the ureteric epithelium in wild type and *Plxnb2* +/− kidney explants, but not in *Plxnb2* −/− explants. As shown by co-immunoprecipitation Plexin B2 interacts with the Ret receptor tyrosine kinase, the receptor of Glial-cell-line-derived neurotrophic factor (Gdnf), in embryonic kidneys. Isolated *Plxnb2* −/− ureteric buds fail to respond to Gdnf by branching, but this response is rescued by Fibroblast growth factor 7 and Follistatin as well as by the metanephric mesenchyme. The differentiation of the nephrogenic mesenchyme, its morphology and the rate of apoptosis in the *Plxnb2* −/− kidneys are normal. Plexin B2 is co-expressed with Plexin B1 (Plxnb1) in the kidney. The double homozygous *Plxnb1–Plxnb2*-deficient mice show high embryonic lethality prior to onset of nephrogenesis. The only double homozygous embryo surviving to E12 showed hypoplastic kidneys with ureteric branches and differentiating mesenchyme. Taken together, our results show that Sema4C-Plexin B2 signalling regulates ureteric branching, possibly through modulation of Gdnf signalling by interaction with Ret, and suggest non-redundant roles for Plexin B1 and Plexin B2 in kidney development.

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1. Introduction

The mammalian permanent kidney or metanephros develops by reciprocal interactions between the Wolffian duct-derived ureteric bud (UB) and the metanephric mesenchyme (MM). The tips of the UB induce the surrounding MM to undergo mesenchyme-to-epithelium transition to become secretory nephrons, the functional units of the kidney. The branching ureteric epithelium then differentiates into collecting ducts (Costantini, 2006; Dressler, 2006; Saxén, 1987). If the branching is retarded, the number of nephrons remains small causing renal hypoplasia (Schedl, 2007).

Budding of the UB from the Wolffian duct takes place at embryonic day (E) 11 in mouse. Both the formation of the UB and its subsequent epithelial branching are essentially controlled by Glial-cell-line-derived neurotrophic factor (Gdnf) that is expressed by the cap condensates of the MM. Gdnf binds to and activates the dimeric complex of Ret receptor tyrosine kinase and its co-receptor Gdnf family receptor α1 (Gfrα1) on the Wolffian duct and at the tips of the UB (Costantini and Shakya, 2006). Ureteric branching proceeds in a stereotypic manner and is regulated by several signalling molecules besides Gdnf, including members of the Wnt and fibroblast growth factor (Fgf) families. Negative regulators of branching include Sprouty-1 and members of the bone morphogenetic protein (Bmp) and TGFβ families (Dressler, 2006).

We have now studied the role of Plexin B2 in kidney development, as it is expressed in the ureteric epithelium and condensing MM (Perälä et al., 2005). Plexins (*Plxn*) are the receptors for the semaphorins (Sema) (Tamagnone et al., 1999; Winberg et al., 1998), which were originally described as axon guidance cues (Kolodkin et al., 1992). Plexins are also

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involved in the development of the cardiovascular system (Torres-Vazquez et al., 2004; Toyofuku et al., 2004a, 2004b), in the function of the immune system (Wong et al., 2003; Yamamoto et al., 2008) and in the invasive growth of cancer cells (Giordano et al., 2002). Plexins are divided in four subfamilies based on structural criteria (Tamagnone et al., 1999) and the B-subfamily consists of Plexin B1, B2 and B3. *Plxnb3* is expressed at only low levels during embryogenesis (Artigiani et al., 2004; Perälä et al., 2005). *Plxnb1* and *Plxnb2* are widely expressed by both neuronal and non-neuronal embryonic tissues, including the developing kidney, in partly overlapping patterns (Perälä et al., 2005; Worzfeld et al., 2004). Plexin B1-deficient (*Plxnb1−/−*) mice in the C57BL/6 background are healthy and viable (Deng et al., 2007; Fazzari et al., 2007), although they exhibit a transitory renal phenotype during embryogenesis (Korostylev et al., 2008). From E13.5 to E14.5 the kidneys of *Plxnb1−/−* embryos show more ureteric branches than those of heterozygous and wild type littermates. The ligand of Plexin B1, Semaphorin 4D (Sema4D), inhibits ureteric branching *in vitro* (Korostylev et al., 2008). Plexin B2-deficient (*Plxnb2−/−*) mice die before birth in the C57BL/6 background. They show defects in olfactory bulb development, neuronal proliferation and differentiation as well as neural tube closure (exencephaly) (Deng et al., 2007; Friedel et al., 2007; Hirschberg et al., 2009). In the CD1 background, *Plxnb2−/−* mice survive but exhibit a profoundly altered layering and foliation of the cerebellum (Friedel et al., 2007). Defects outside of the nervous system have not been reported.

In vitro, both Plexin B1 and Plexin B2 act as receptors for Sema4D (Masuda et al., 2004). The preferable ligand for Plexin B2 is Sema4C (Deng et al., 2007). The binding of Sema4D and Sema4C to Plexin B1 and Plexin B2, respectively, leads to the phosphorylation of ErbB-2 and the plexin protein itself (Deng et al., 2007; Swiercz et al., 2004). Ligand binding to plexins also modifies the adhesion of cells to the extracellular matrix through conformational change and activation of the cytoplasmic GAP domain, leading to the inactivation of R-Ras and thereby integrins (Oinuma et al., 2004; Oinuma et al., 2006). Plexins regulate the actin cytoskeleton through interactions with PDZ-RhoGEF and leukemia-associated Rho GEF (LARG) and activation of RhoA, causing for example the collapse of the axonal growth cone (Aurandt et al., 2002; Driessens et al., 2002; Hirotani et al., 2002; Perrot et al., 2002; Swiercz et al., 2002).

In this study we show that *Plxnb2−/−* embryos in the C57BL/6 background exhibit renal hypoplasia with occasional double ureters. In *Plxnb2−/−* kidneys, the branching of the ureteric epithelium and cell proliferation in the ureteric tips are reduced. The response of the isolated ureteric epithelium to Gdnf is compromised and co-immunoprecipitation experiments show an interaction between Plexin B2 and Ret. Sema4C enhances branching of E11.5 *Plxnb2+/+ and Plxnb2+/−* kidneys in culture, but not of *Plxnb2−/−* kidneys. Our data show that Sema4C-Plexin B2 signalling is a new regulator of the ureteric branching and a potential regulator of Gndf-Ret signalling, and that the functions of the closely related plexins, Plexin B1 and Plexin B2, seem to be different during kidney development.

2. Materials and methods

2.1. Animals

Wild type mouse (*Mus musculus*) embryos were collected from NMRI and C57BL/6 mice. The day of vaginal plug appearance was defined as the embryonic day 0.5 (E0.5). The Plexin B2-deficient mice (Plxnb2^{tmlMat1}, here defined as *Plxnb2−/−*) in the C57BL/6 background (Friedel et al., 2007) were genotyped as previously described. Plexin B1-deficient (*Plxnb1−/−*) mice in the C57BL/6 strain were genotyped as previously described (Fazzari et al., 2007). Embryos were collected at stages E11.5–E16.5. The permit for the animal experiments was received from the State Provincial Office of Southern Finland.

2.2. Tissue culture

Kidney explants were dissected from E11.5 or E12.5 embryos and cultured on Nuclepore filters (0.1 μm, Whatman) in DMEM supplemented with 10% fetal bovine serum, Glutamax and penicillin–streptomycin as previously described (Sainio, 2003). For induction of supernumerary budding, E11.5 explants were cultured in the presence of either 15 or 50 ng/ml of recombinant rat Gdnf (R&D Systems).

To isolate the UB epithelium, E12.5 kidneys were treated with pancreatin-trypsin solution for 1 min at room temperature (Sainio, 2003). Thereafter the UB epithelium was mechanically separated from the mesenchyme in DMEM+10% FCS, fragments of UB epithelium were collected under microscope and transferred into growth media. For mesenchyme-free 3D cultures, UBs were transferred into polymerized Matrigel (BD Biosciences) matrix using pulled glass capillaries. Matrigel matrix was prepared on ice by 1:2 dilution of Matrigel with UB culture medium, which composed of DMEM/F12 supplemented with 10% charcoal/dextran treated FCS (Hyclone), 200 nM all-trans- and 9-cis retinoic acid (Sigma) and either 50 ng/ml recombinant rat Gdnf, 100 ng/ml Fgf7 (R&D Systems) and 100 ng/ml Follistatin (R&D Systems) or Gdnf only or Fgf7 and Follistatin. 50 μl of the matrix was used in 96-well plates and 150 μl of UB culture medium was added to the wells the next day. The adhesion of *Plxnb2−/−* UB cells to the extracellular matrix molecules was analyzed by letting the isolated E12.5 UB cells adhere onto chamber-slides coated with laminin 0.5 μg/cm^2 (Sigma), 0.1% fibronectin (Sigma), collagen (Millipore) or Matrigel. The cells were fixed after 24 h with 4% PFA and stained.

In vitro culture of metanephric mesenchymes from E11.5 embryos was done as described previously (Kuure et al., 2007). The isolated MMs were differentiated by a transient (48 h) pulse of 5 μM of 5′-bromoindirubin-3′-oxime (BIO; Calbiochem) and subsequent culture in standard medium 3 days before fixation with ice-cold methanol.

2.3. Production of Plexin B2 antibody

A polyclonal antibody, TI-2B, was raised in rabbits against a peptide specific for the intracellular part of mouse Plexin B2 (1572-EDSQQDLPGERHALLEEENR-1591) (Washington Biotechnology, Inc.) and affinity purified. For immunohistochemistry, TI-2B was used at 1:100 and in Western blotting at 1:1000.The specificity of the antibody was tested by Western blotting with lysates of COS7 cells transfected with cDNA expression constructs (Artigiani et al., 2003) to overexpress either Plexin B1 or Plexin B2.

2.4. Production of purified recombinant Sema4C

The secreted form of Sema4C was purified from the conditioned media of cells transfected with a cDNA expression construct, a kind gift of Dr. Steven Strittmatter (Yale University). Sema4C was affinity purified using metal-ion chromatography (Ni-NTA agarose, Qiagen), then analyzed and quantified by SDS-PAGE followed by gel staining with Coomassie blue.

2.5. Antibodies and in situ hybridization probes

The primary antibodies used for immunohistochemistry were HRP-conjugated rabbit anti-Actin (Santa Cruz) (1:1000), mouse anti-pan-cytokeratin (Sigma) (1:400), rabbit anti-brush border

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