

Semaphorin signaling facilitates cleft formation in the developing salivary gland

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Semaphorin signaling plays integral roles in multiple developmental processes. Branching morphogenesis is one such role that has not been thoroughly explored. Here, we show in mice that functional blockage of neuropilin 1 (Npn1) inhibits cleft formation in the developing submandibular gland (SMG) cultured *ex vivo*. This Npn1-dependent morphogenesis is mediated by Sema3A and Sema3C in an additive manner, and can be abolished by decreasing the expression of plexin A2 or plexin D1. VEGF, another known Npn1 ligand, has no apparent effects on SMG development. FGF signaling, which also mediates SMG branching morphogenesis, acts in parallel with semaphorin signaling. Finally, in contrast to the effect of FGF signaling, we find that semaphorins do not stimulate the proliferation of SMG epithelial cells. Instead, the semaphorin signals act locally on the epithelial cells to facilitate SMG cleft formation.

KEY WORDS: Branching morphogenesis, Salivary gland, Cleft formation, Class 3 semaphorin, Plexin, Neuropilin (Npn1; Nrp1), Mouse

INTRODUCTION

Branching morphogenesis is a unique cellular process commonly observed during embryonic organogenesis. Examples of this process during development include the establishments of the airways of the lung, the collecting ducts of the kidney and the excretory tubules of the mammary gland and submandibular gland (Hogan, 1999). Branching morphogenesis involves growth and branching of the tubular epithelial cells from their unbranched precursors and requires regulated interactions between the developing epithelial cells and the surrounding mesenchyme (Affolter et al., 2003; Hogan and Kolodziej, 2002). Several families of molecules have been implicated in regulating branching morphogenesis, but they are thought to act in a tissue-specific manner (Davies, 2002).

The mouse submandibular gland (SMG) has been used as a classical example for studying branching morphogenesis (Grobstein, 1953; Hieda and Nakanishi, 1997). Mouse SMG development begins at embryonic day 11 (E11). At E12, a single epithelial bud surrounded by condensed mesenchyme is formed. By E12.5, small clefts start to appear at the end of the epithelial bud (cleft formation). Meanwhile, the cells in the bud continue to proliferate and cleave repetitively, resulting in bush-like branching patterns. Several branching buds and cords can be seen at E14. Lumenization of the solid cellular cords and buds occurs at E17, and, by postnatal day 1 (P1), the salivary gland is fully developed and starts to secrete mucin.

Molecular regulation of branching morphogenesis has been studied extensively in the lung and the kidney, but the regulation of SMG development has been relatively less well explored (Affolter et al., 2003; Cardoso and Lu, 2006; Hogan, 1999; Hogan and

Kolodziej, 2002; Kuure et al., 2000; Lü et al., 2004; Metzger and Krasnow, 1999; Warburton et al., 2000). To date, some molecules are implicated in the branching morphogenesis of the SMG. For example, fibroblast growth factors (FGFs) and bone morphogenetic proteins (BMPs) have been reported to mediate the formation of the SMG (Hoffman et al., 2002; Steinberg et al., 2005). Interestingly, abnormal salivary gland morphology has also been observed in *Bmp7*-null, *Fgf10*-null, *Fgf8*-null and *Fgfr2b*-null mice (Jaskoll et al., 2004b). In addition, sonic hedgehog (Shh) stimulates the proliferation of branching epithelia by increasing *Fgf8* expression, and *Shh*-null mice have a hypoplastic SMG remnant (Jaskoll et al., 2004a). Despite these results, our understanding of the molecular mechanisms that regulate the development of the salivary gland is still incomplete. In particular, it is not known whether the cleft formation and epithelial proliferation are regulated by the same sets of molecules and how the two essential processes are coordinated to ensure proper branch formation.

Class 3 semaphorins are a family of conserved secreted molecules that play roles in various developmental processes. In the developing nervous system, class 3 semaphorins regulate axon repulsion, axon pruning and neuronal migration (Fujisawa, 2004; He et al., 2002; Huber et al., 2003; Raper, 2000; Waimey and Cheng, 2006). Interestingly, some of these semaphorins have been reported to regulate the formation of the cardiovascular system and the branching formation of the lung (Gitler et al., 2004; Gu et al., 2005; Ito et al., 2000; Kagoshima and Ito, 2001; Torres-Vazquez et al., 2004). Neuropilin is the binding receptor for class 3 semaphorin, but neuropilin has to form co-receptor with plexin to transduce the semaphorin signal inside the cell. In vertebrates, seven class 3 semaphorins, two neuropilins and nine plexins have been identified. In addition to semaphorin, neuropilin binds to vascular endothelial growth factor (VEGF; also known as VEGF-A and Vegfa – Mouse Genome Informatics) and regulates the formation of the vasculature (Fuh et al., 2000; Gu et al., 2003; Soker et al., 1998). Here, we report the role of class 3 semaphorins in branching morphogenesis of the developing SMG. By examining members of vertebrate class 3 semaphorin, neuropilin and plexin, we conclude that semaphorin signaling specifically regulates cleft formation during the development of SMG.

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MATERIALS AND METHODS

SMG cultures

For ex vivo explant culture, SMGs with surrounding mesenchyme were dissected from either E12 or E13 ICR mice and then were cultured on Whatman Nuclepore Track-etch filters (13 mm, 0.1 μ m pore size; VWR) at the air-medium interface. Four SMG explants were evenly placed on one filter in ordered pairs. The filter was then floated on 400 μ l SMG culture medium (DMEM/F12 containing 150 μ g/ml vitamin C, 50 μ g/ml transferrin, 100 U/ml penicillin and 100 μ g/ml streptomycin) and cultured at 37°C in a humidified 5% CO₂/95% air atmosphere. SMGs were photographed after culture for 24, 48 and 72 hours, respectively, and the number of end buds was counted at each time point. Images were adjusted for brightness and contrast, if necessary, for presentation. Each experimental condition was repeated at least four times.

For SMG co-culture experiment, COS cells in a six-well culture dish were lipofectamin-transfected with *Sema3*-expressing plasmids 60 hours before the dissection of SMGs (Zou et al., 2000). The COS cell culture medium was then replaced with SMG culture medium 12 hours before the dissection of SMGs. The SMG explants were dissected as described above and cultured on the conditioned SMG culture media.

Mesenchyme-free SMG explants were obtained by incubation of dissected E13 SMGs with Hanks' balanced salt solution containing 1.6 U/ml Dispase (Roche Molecular Biochemicals) at 37°C for 20 minutes. Epithelia were separated from mesenchyme with fine forceps in Hanks' solution containing 10% BSA. The SMG epithelia were placed on a Nuclepore filter, covered with growth factor-reduced Matrigel (prepared in 1:1 dilution with culture medium; BD Biosciences) and cultured in SMG culture medium.

For dissociated SMG epithelial culture, the mesenchymal-free SMGs (prepared as described above) of 10 to 15 E13 mouse embryos were pooled, minced and then dissociated into single cells by use of Ca²⁺-free Hank's balanced salt solution containing 0.07% collagenase Type II and Type III (1:1 v/v; Sigma) for 45 minutes at 37°C. Triturated cells were centrifuged at 300 g and resuspended with SMG culture medium. Cell clumps and tissue debris were removed by passing the cell suspension through a cell strainer (40 μ m Nylon, BD Falcon). The yielded cell suspension was then seeded onto a 35 mm dish and cultured in SMG medium containing exogenous Fgf7 (100 ng/ml), Fgf10 (200 ng/ml) and Hgf (50 ng/ml).

Antisense oligodeoxynucleotides, recombinant peptides and antibodies

Antisense experiments were performed with 2 μ M oligodeoxynucleotides (ODNs) with phosphorothioate modification. The nucleotide sequences used in this report were: *Sema3A* antisense (891-872), 3'-CCTGAA-GTACCCTGCCCTGA-5'; *Sema3B* antisense (2068-2059), 3'-ACC-GACTCCTCTCATCTC-5'; *Sema3C* antisense (589-571), 3'-ATA-CAGACACCCTCACCTCG-5'; *Sema3D* antisense (2359-2341) 3'-ATCTGCTCACAGTACTGGT-5'; *Sema3E* antisense (1962-1943), 3'-TGTCTCACCTACCTCCTTCA-5'; *Sema3F* antisense (549-530), 3'-CTACCGTTACCTCTCACACC-5'; *plexin A1* antisense (5015-4996), 3'-CTGACCTTCTCCGACTTGTG-5'; *plexin A2* antisense (5309-5290), 3'-CCTGTACCTAGACCTACCG-5'; *plexin B1* antisense (1751-1732), 3'-CCTTCTCTCCTCCCTCCAAA-5'; *plexin B2* antisense (3247-3228), 3'-CTGTACACCTCTCCTACG-5'; *plexin B3* antisense (4920-4901), 3'-CTACCACTCCTTCCACCTCA-5'; *plexin C1* antisense (1329-1348), 3'-ACCCTTCTCCTCCTTCTT-5'; *plexin D1* antisense (4809-4790), 3'-CTACCGACGACTCCCTCTT-5'; *plexin D1* sense (4790-4809), 5'-GAGTGGCTGCTGAGGGAGAA-3'; scrambled sequence, 5'-CCG-ACTCTACCACTTGCTC-3'.

Recombinant peptides or antibodies were added into SMG culture medium with concentrations indicated. The human recombinant *SEMA3A* peptide (R&D systems) was added at concentrations of 10, 25, 50, 100 or 150 ng/ml, respectively. Fgf10 (R&D systems) or Fgf7 (R&D systems) was added at concentrations of 250, 500 or 1000 ng/ml, respectively. The neutralizing antibodies to *Npn1* (Calbiochem) or control IgGs (Calbiochem) were added at concentrations of 1, 2, 5 or 10 μ g/ml, respectively.

Semi-quantitative RT-PCR

SMGs were dissected at E13, E17, P1, P4 or in adult mice, respectively. DNase-free RNA was prepared by using an RNAqueous-4 PCR kit and DNA-free DNase removal reagent (Ambion). cDNA was generated with a reverse-transcriptase kit (Invitrogen). Semi-quantitative PCR was performed using specific primers for each transcript.

Whole-mount RNA in situ hybridization and antibody immunostaining

Whole-mount RNA in situ hybridization of the SMG explant cultured in vitro was performed essentially as described (Steinberg et al., 2005). Riboprobes for plexins and semaphorins were used as described (Cheng et al., 2001; Zou et al., 2000). The riboprobes for *Npn1* and *Npn2* were prepared from the mouse *Npn1* cDNA fragment (GenBank accession number BC060129, nucleotide 619-1030) and the mouse *Npn2* cDNA fragment (GenBank accession number NM_01093, nucleotide 1578-2686), respectively. Whole mount immunostaining was performed by application of primary antibodies in M.O.M. blocking reagent (Dako) for 3 hours at room temperature and then of secondary antibodies in PBS-Tween 20 (0.1%) for 2 hours. The antibodies used included Fgfr2 (1:200 dilution, Santa Cruz), Flt1 (1:500, Santa Cruz), Flk1 (1:500, Santa Cruz), VEGF-A (1:1000, AbCam), E-cadherin (1:100, BD Biosciences), fibronectin (1:100, BD Biosciences), alkaline phosphatase-conjugated anti-digoxigenin antibody (1:2000, Roche Molecular Biochemicals) and donkey F(ab)₂ fragments labeled with AlexaFluor 488 or AlexaFluor 594 (Molecular probe).

AP in situ hybridization, BrdU labeling and TUNEL assay

AP-fusion protein was prepared and AP in situ hybridization was performed as described (Cheng and Flanagan, 2001). For labeling of proliferative cells, the cultured SMGs were incubated with 10 μ M 5-bromo-2'-deoxyuridine (BrdU) for 2 hours at 37°C, and were followed by three washes in PBS with 0.1% Tween-20. The SMGs were then fixed with 0.5% Triton X-100 in ethanol/glycine/water (70:20:10, v/v) at pH 2.0 for 1 hour, followed by five washes in PBS. A monoclonal antibody (1:10 dilution) from a BrdU labeling kit (Roche Molecular Biochemicals) was used to detect the BrdU labeling. Pictures were photographed by fluorescence microscope (Leica) and analyzed by MetaMorph Software (Universal Imaging).

Apoptotic cells in SMG explants were examined by performing TUNEL staining, essentially as described in the In Situ Cell Death Detection Kit, POD (Roche Molecular Biochemicals).

RESULTS

Neuropilin 1 is transiently expressed in the epithelial buds of the developing submandibular gland

To test whether class 3 secreted semaphorins play roles in the developing SMG, we first examined whether the binding receptors, neuropilins, were expressed in the developing SMGs. Semi-quantitative RT-PCR analysis of the cDNA prepared from the SMG indicated that neuropilin 1 (*Npn1*; also known as *Nrp1* – Mouse Genome Informatics), but not *Npn2* (also known as *Nrp2*), was detected in the developing salivary glands. *Npn1* transcript was seen in the embryonic SMG, but the expression level was greatly reduced after birth (Fig. 1A). Detailed RNA in situ hybridization analysis further confirmed the transient expression pattern of *Npn1*. *Npn1* transcript was first detectable in the primitive SMG bud at E12.5 (Fig. 1Ba,a'). High expression persisted in the developing SMG epithelial buds at E15.5 (Fig. 1Bb,b',b''), when cleft formation in the bud proceeded actively. After E17.5, *Npn1* transcript decreased (Fig. 1Bc,d,e). Importantly, *Npn1* transcript was mainly present in the epithelial buds as evidenced by colocalization of *Npn1* transcript with the epithelial marker E-cadherin (also known as cadherin 1 – Mouse Genome Informatics), but not with the mesenchymal marker, fibronectin (Fig. 1C). Semi-quantitative RT-PCR analysis of dissected E15.5 SMG tissues also confirmed that *Npn1* existed

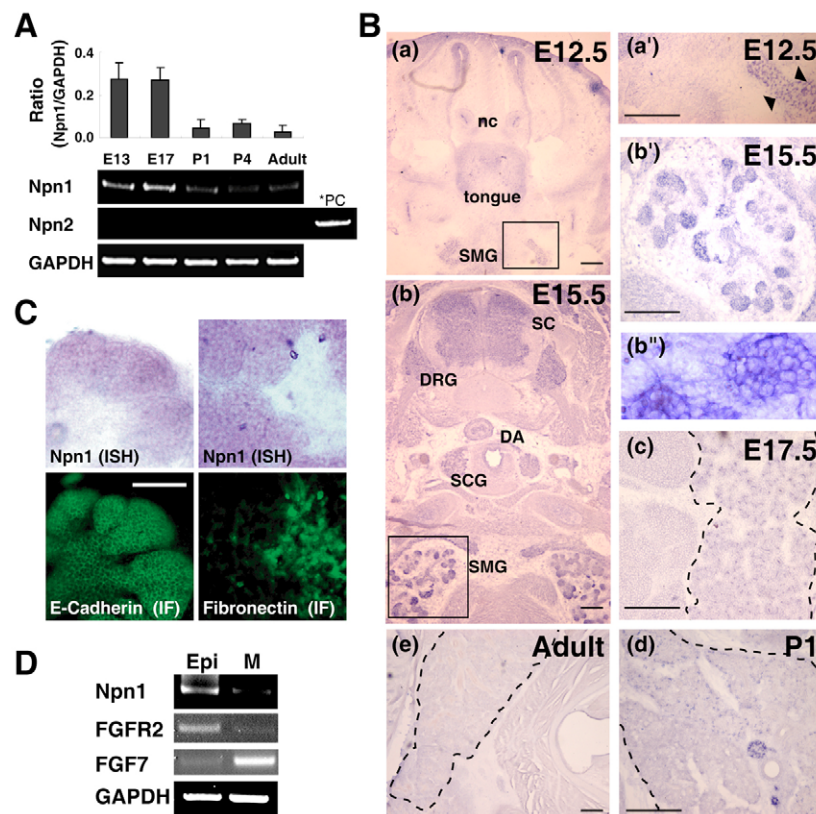


Fig. 1. Neuropilin 1 is transiently expressed in the developing mouse SMG. (A) Semi-quantitative RT-PCR analysis of neuropilins on the cDNA samples prepared from SMGs at the indicated developmental stage. *Npn1*, but not *Npn2*, was transiently detected in the embryonic SMG. Glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) transcript is shown as an internal control. The amount of *Npn1* transcript was normalized against the internal control at each stage for comparison. (B) RNA in situ hybridization analysis of *Npn1* transcript on tissue sections. *Npn1* was detectable in the rudimentary SMG at E12.5 (a). The expression of *Npn1* transcript peaked at E15.5 (b), and was dramatically decreased at E17.5 (c), P1 (d) and adult mouse (e). No signals were detectable in the SMG by using antisense *Npn2* or sense *Npn1* probes. The boxed areas in a and b are magnified and shown in a' and b',b'', (original magnification 400×), respectively. Areas within dashed lines: salivary gland. (C) Colocalization of *Npn1* transcript with E-cadherin, but not with fibronectin, in E15.5 SMG cultured ex vivo. (D) Semi-quantitative RT-PCR analysis of the cDNA samples prepared from either SMG epithelium or the surrounding mesenchyme. *Npn1* was mainly expressed in the SMG epithelium. *Fgfr2* and *Fgf7* were controls for specific expression in Epi and in M, respectively. Scale bars: 50 μ m in B; 100 μ m in C. DA, descending aorta; DRG, dorsal root ganglion; Epi, epithelium; GAPDH, *Gapdh* internal control; IF, immunofluorescence staining; ISH, RNA in situ hybridization; M, mesenchyme; Nc, nasal cavity; PC, positive control; SC, spinal cord; SCG, superior cervical ganglion; SMG, submandibular gland.

mainly in the SMG epithelium (Fig. 1D). The temporal embryonic expression pattern of *Npn1* in the SMG epithelial bud suggests that *Npn1* may play a role in regulating the branching morphogenesis of the developing SMG.

Neuropilin 1 is required for cleft formation in the embryonic SMG cultured ex vivo

To address the function of *Npn1* in SMG development, we turned to an ex vivo SMG explant culture. In this assay, the entire SMG epithelial bud with intact surrounding mesenchyme was excised and cultured so that the bud could continue the branching morphogenesis ex vivo. We first examined whether the *Npn1* neutralizing antibody could prevent the SMG development (He and Tessier-Lavigne, 1997; Chen et al., 1998). The SMG treated with anti-*Npn1* antibody prohibited cleft formation in a dose-dependent manner. The effect could last for 72 hours, and a concentration of 5 μ g/ml completely blocked the activity (Fig. 2Aa,b). Second, we applied ODNs against *Npn1* mRNA into the culture to downregulate the expression of *Npn1* in the SMG epithelial cells. As clearly shown in Fig. 2B, the antisense ODNs specifically inhibited cleft formation (Fig. 2Ba,

upper panels, and b). In situ hybridization and RT-PCR analysis confirmed the diminished *Npn1* transcript after addition of antisense ODNs (Fig. 2Ba, middle panel, and 2C). In addition, we could not detect changes in the extent of BrdU labeling (Fig. 2Ba, lower panels, and c) and TUNEL activity (data not shown) in the SMG explants treated with *Npn1* antisense ODNs compared with the control. Taken together, these data indicate that *Npn1* regulates the SMG branching morphogenesis mainly in the process of cleft formation.

Sema3A and Sema3C promote SMG cleft formation

We next screened the expression patterns of class 3 semaphorins in the developing SMG to identify the candidate semaphorins that were utilized in the *Npn1*-mediated cleft formation. To our surprise, semi-quantitative RT-PCR analysis revealed that almost all class 3 semaphorins were detectable in the embryonic SMGs, although the amounts varied (see Fig. S1A in the supplementary material). Interestingly, RNA in situ hybridization on E15.5 tissue sections revealed that *Sema3A* and *Sema3C* were abundantly expressed at

this stage, whereas others were not (see Fig. S1B in the supplementary material). Thus, expression pattern studies suggest that multiple class 3 semaphorins can be involved in SMG development.

Because class 3 semaphorins are secreted molecules, we developed an SMG co-culture assay in which we seeded semaphorin-transfected COS cells in the culture dish 12 hours before placing the dissected SMGs onto the floating membrane (see Materials and methods). Western blot analysis of the media collected from this co-culture system confirmed the presence of transfected semaphorins at high and equivalent expression levels (data not shown). After co-culture for 48 hours, only Sema3A- and Sema3C-conditioned media exhibited enhancement of branching activity, whereas other members of class 3 semaphorins had no significant effects (Fig. 3A). Besides, a synthetic Sema3A peptide (100 ng/ml) accelerated the SMG branching activity in the SMG explant culture (data not shown). To test whether endogenous semaphorins were required for SMG branching morphogenesis, we knocked down the expression of class 3 semaphorins in the ex vivo SMG culture by adding specific antisense ODNs. Again, only diminished Sema3A and Sema3C significantly reduced the branching morphogenesis of the cultured SMG (Fig. 3B). We therefore conclude that endogenous Sema3A and Sema3C promote the development of the embryonic SMG.

In the SMG co-culture assay, we found that Sema3A and Sema3C promoted branching morphogenesis in a concentration-dependent manner (Fig. 3Ca). At lower concentrations of proteins, Sema3A

and Sema3C enhanced the ability of each other to induce the branching activity in an additive manner (Fig. 3Ca,c). In addition, simultaneous knockdown of endogenous Sema3A and Sema3C by application of antisense ODNs also additively reduced the SMG branching activity (Fig. 3B). Such additive effects were specific to Sema3A and Sema3C, because other class 3 semaphorins had no effects on Sema3A- or Sema3C-promoted branching activity (data not shown).

To address whether cell proliferation is required for Sema3A and/or Sema3C-dependent branching activity, we tested their effects in the SMG co-culture assay at the presence of tunicamycin, a translation inhibitor that inhibits cell proliferation (Spooner et al., 1989). We found that, when the sizes of the epithelial cords were partially reduced, the cleft formation was not affected (see Fig. S2A in the supplementary material). Moreover, the overall proliferation in the SMG explants as measured by BrdU incorporation was not significantly changed in the presence of overexpressed Sema3A (see Fig. S2B in the supplementary material). These results indicate that Sema3A and Sema3C act together to promote cleft formation during SMG branching morphogenesis without obvious effects on cell proliferation.

Sema3A and Sema3C induce cleft formation through functional Npn1

If Npn1 mediated the Sema3A and Sema3C signals in the developing SMG, blockage of Npn1 function should abolish the branching activity enhanced by Sema3A or Sema3C in the SMG co-

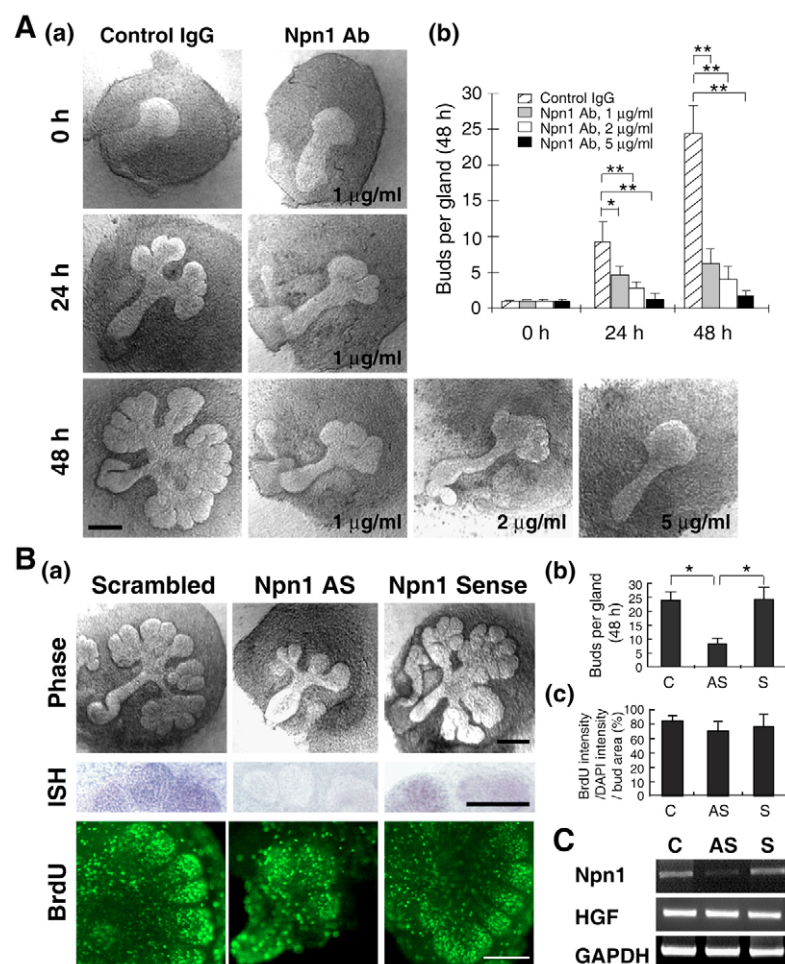


Fig. 2. Neuropilin 1 is required for SMG branching morphogenesis in mice.

(A) Npn1-neutralizing antibody inhibited SMG branching in a concentration-dependent manner. (a) Representative photographs at the indicated time point of the SMG explants treated with either control antibody or different concentrations of anti-Npn1 antibodies. (b) The numbers of terminal buds in each cultured SMG explant were counted and summarized (from five independent experiments). Paired *t*-test: *, $P < 0.05$; **, $P < 0.01$. (B) ODNs against *Npn1* mRNA specifically blocked SMG branch formation. (a) Top panels: representative photographs of the SMG explants cultured 48 hours after treatment with 2 µM *Npn1* antisense ODNs, scrambled ODNs or *Npn1*-sense ODNs. Middle panels: RNA in situ hybridization indicated that the *Npn1* transcript decreased in the explant treated with *Npn1* antisense ODNs. Bottom panels: BrdU (bromodeoxyuridine) labeling revealed that the rates of cell proliferation were not significantly changed in each experimental condition. (b) The numbers of terminal buds in each SMG explant cultured for 48 hours were counted and are summarized as a bar graph (from five independent experiments). Paired *t*-test: *, $P < 0.05$. (c) Quantification of the proliferative activity labeled by BrdU is shown by bar graph as the green fluorescence intensity relative to DAPI-stained blue fluorescence intensity per unit area analyzed by MetaMorph software ($n = 12$). (C) Semi-quantitative RT-PCR indicated that *Npn1* transcripts were significantly reduced in the SMG treated with *Npn1* antisense ODNs. Scale bars: 100 µm. AS, *Npn1* antisense ODN; C, scrambled sequence; HGF, hepatocyte growth factor; S, *Npn1*-sense ODN.

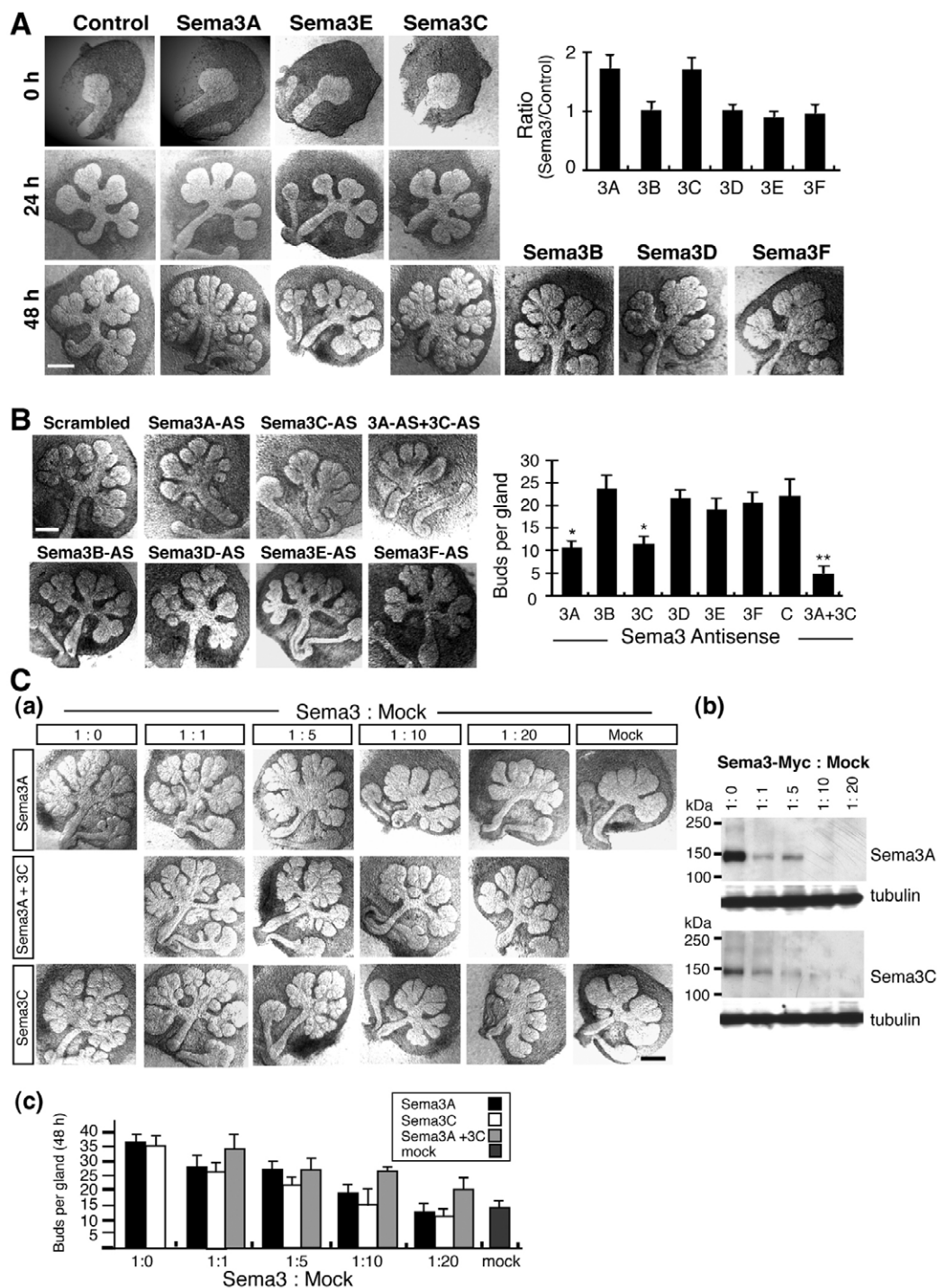


Fig. 3. Sema3A and Sema3C additively promote SMG cleft formation. (A) Sema3A and Sema3C are the only class 3 semaphorins that could promote cleft formation in the SMG co-culture assays. Representative photographs of the growth of SMG explants co-cultured with class 3 semaphorins were shown. The bar graph summarizes the ratios of the number of terminal buds in each co-culture to the number of the terminal buds in the control culture ($n \geq 7$). The number of terminal buds at the presence of Sema3A or Sema3C was almost doubled. (B) Treatment of antisense ODNs against Sema3A or Sema3C specifically reduced the number of terminal buds in the SMG cultured ex vivo. Representative explants are shown. The bar graph summarizes five independent experiments. The terminal bud number was significantly further reduced when both Sema3A antisense ODNs and Sema3C antisense ODNs were added together into the culture. Paired *t*-test: *, $P < 0.05$; **, $P < 0.01$. (C) Sema3A and Sema3C additively promoted bud formation in a concentration-dependent manner. In the SMG co-culture experiments, Sema3-transfected COS cells were serially diluted to test the synergistic effects. Fold dilution in the co-culture is indicated as the Sema3-transfected COS cells diluted with mock-transfected COS cells. (a) Additive effects of Sema3A and Sema3C were most obvious at the lower concentrations of semaphorins. The effects were saturated at 1:1 dilution. (b) The amounts of semaphorin proteins present in each condition were assayed by western blotting. Gradual decreases of Sema3A or Sema3C in the serial dilutions were observed. Tubulin: internal loading control. (c) The bar graph summarizes four independent experiments. Scale bars: 100 μm . C, scrambled sequence.

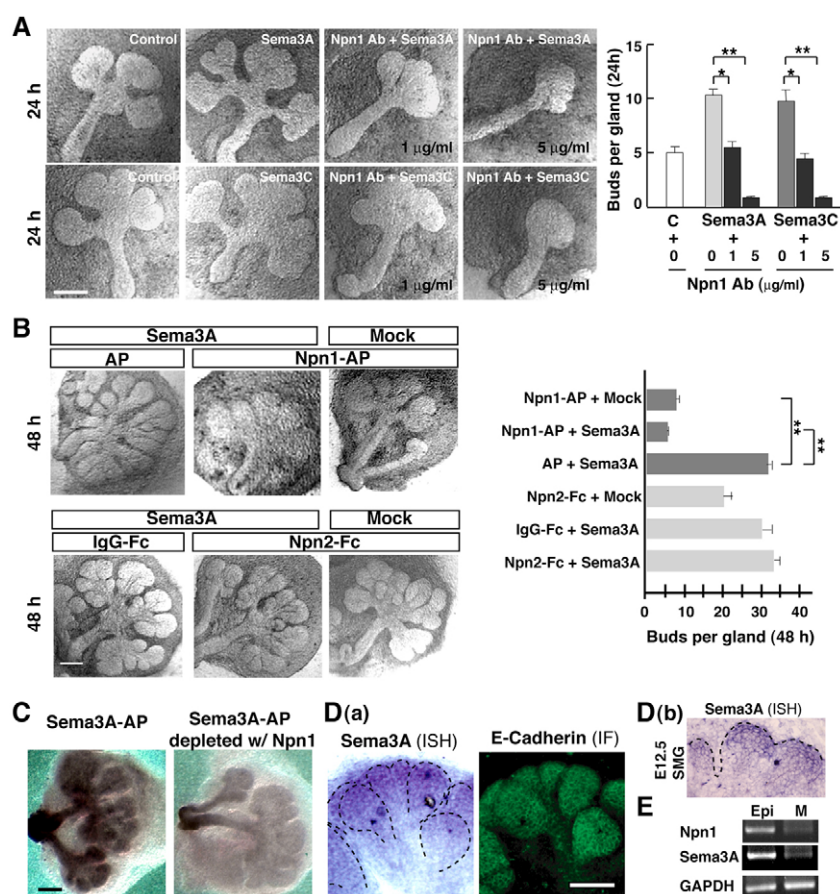


Fig. 4. Functional Npn1 is required for the Sema3A- and Sema3C-mediated SMG branching morphogenesis in mice. (A) Anti-Npn1 antibody dose-dependently abolished SMG cleft formation promoted by Sema3A or Sema3C in the SMG co-cultures. Complete inhibition could be achieved in the presence of 5 μ g/ml neutralizing antibody. Representative explants are shown and the summary of six independent experiments is shown in the bar graph. Paired *t*-test: *, $P < 0.05$; **, $P < 0.01$. (B) Npn1-AP fusion proteins, but not AP proteins, blocked Sema3A-mediated branching activity in the SMG co-cultures. Note that Npn1-AP fusion protein alone could block the endogenous branching activities (Mock) (upper panels). By contrast, application of Npn2-Fc fusion proteins in the co-cultures had no effects on the SMG branching activity (lower panels). Representative explants were shown and the summary of five independent experiments is shown in the bar graph. Paired *t*-test: *, $P < 0.05$; **, $P < 0.01$. (C) Sema3A-AP bound the epithelial buds in the SMG cultured ex vivo for 24 hours. As a control, when the Sema3A-AP conditioned medium was depleted of the AP-fusion proteins by pre-incubation with Npn1-transfected COS cells, the binding activity on the epithelial buds was greatly reduced. (D) Sema3A mRNA was detected in the SMG epithelial buds cultured ex vivo (a) and in E12.5 embryonic SMG (b). The expression was distributed as a gradient with the highest level at the front end of the terminal bud. Immunofluorescence staining of E-cadherin highlighted the epithelial buds in a. Area within dashed line, epithelial bud. (E) Semi-quantitative RT-PCR analysis confirmed that Sema3A transcript was mainly in the SMG epithelium. Scale bars: 100 μ m. C, mock-transfected COS cells; Epi, epithelium; IF, immunofluorescence; ISH, RNA in situ hybridization; M, mesenchyme.

culture assays. Indeed, the SMGs treated with anti-Npn1 antibodies or Npn1 antisense ODNs in the Sema3A (or Sema3C) co-cultures exhibited reduced enhancement of branching morphogenesis that would otherwise be promoted by Sema3A or Sema3C (Fig. 4A). Again, at a concentration of 5 μ g/ml, the anti-Npn1 antibody completely blocked the cleft formation of the SMGs, even though the epithelial cells still proliferated, as evidenced by the expansion of the epithelial cords (Fig. 4A). Two additional fusion protein experiments were performed to corroborate the requirements. First, we added a soluble protein containing the extracellular domain of neuropilin into the SMG cultures to block the semaphorin-neuropilin interactions. In the presence of Npn1-AP (alkaline phosphatase) fusion proteins, Sema3A no longer enhanced the branching activity in the co-culture assay (Fig. 4B, upper panels). By contrast, the presence of Npn2 fusion proteins in the culture could not inhibit the Sema3A-mediated branching activity (Fig. 4B,

lower panels). Note that addition of Npn1-AP alone could suppress branching activity of the SMG, presumably by disrupting the interactions between the endogenous semaphorin ligands and Npn1 (Fig. 4B). Second, to test the direct binding of Sema3A to the SMG, we incubated the SMG explant with Sema3A-AP-conditioned media and showed that AP binding activity was apparent in the epithelial buds. However, this binding activity disappeared if the Sema3A-AP proteins in the conditioned media were depleted by pre-incubating the conditioned media with COS cells expressing Npn1 (Fig. 4C). Taken together, these results indicate that the SMG branching morphogenesis stimulated by Sema3A and Sema3C is mainly mediated by Npn1.

The expression of Sema3A was further examined in detail in the developing SMG. Interestingly, the Sema3A mRNA was mainly detected in the epithelial buds (Fig. 4D,E). The expression pattern of Sema3A transcript, either in the SMG explant culture (Fig. 4Da)

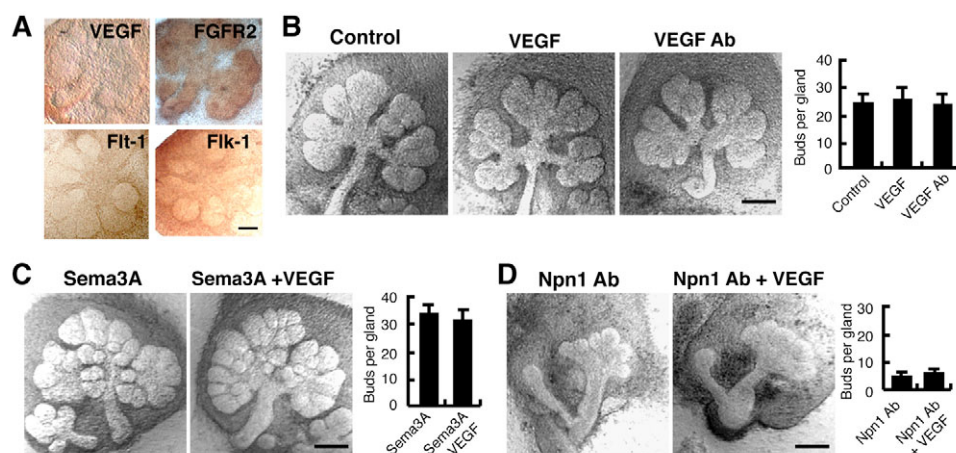


Fig. 5. VEGF is not required for Npn1-mediated cleft formation in mice. (A) Whole-mount immunohistochemistry showed that VEGF and its receptors, Flt1 and Flk1, did not express in the epithelial buds of the SMG. By contrast, Fgfr2 was expressed in the SMG epithelial buds as previously reported. (B) Neither exogenous VEGF nor VEGF antibody (VEGF Ab) affected normal branching morphogenesis of the SMG cultured ex vivo. The bar graph summarizes five independent experiments. (C) VEGF had no effects on the branching activities promoted by Sema3A. The bar graph summarizes four independent experiments. (D) VEGF could not change effects caused by application of Npn1-neutralizing antibody at sub-optimal concentration (2 $\mu\text{g/ml}$) in the SMG ex vivo cultures. The bar graph summarizes five independent experiments. Scale bars: 100 μm .

or in the E15.5 SMG tissues (Fig. 4Db), exhibited a graded expression pattern, with the highest level at the advancing end and the lowest at the base of the bud. As a comparison, the *Npn1* mRNA was evenly expressed in the developing SMG epithelial buds (Fig. 1C). We thus conclude that both Npn1 and Sema3A are expressed in the SMG epithelial cells, and that they may function in an autocrine (or paracrine) manner within the epithelium to mediate SMG cleft formation.

VEGF is not required for Npn1-mediated cleft formation

Npn1 is also known to form a receptor complex with VEGF-high affinity receptor tyrosine kinase, VEGFR1 (Flt1) or VEGFR2 (Flk1; also known as Kdr1 – Mouse Genome Informatics) to mediate VEGF signals (Fuh et al., 2000). To address whether VEGF plays a role in the Npn1-mediated SMG branching morphogenesis, we first examined the expression patterns of VEGF and its receptors, Flt1 and Flk1 in SMGs. Immunostaining of the cultured SMG explants showed that VEGF was only detectable in the mesenchyme near the stalk of the SMG, whereas Flt1 and Flk1 were not expressed in the epithelial buds of developing SMGs. As a control, Fgfr2 was shown mainly expressed in the SMG epithelial buds (Fig. 5A).

The functional role of VEGF and VEGFR was further examined in the SMG explant cultures. Addition of recombinant VEGF into the SMG cultured ex vivo had no apparent effects on SMG development (Fig. 5B). Likewise, blocking the endogenous VEGF by adding anti-VEGF antibody also had no effects on the SMG (Fig. 5B). As VEGF might compete with Sema3A for Npn1 binding (Miao et al., 1999), we tested whether adding VEGF could have effects on the branching activity promoted by Sema3A in the SMG co-culture assay. Addition of VEGF to the cultures, even at a very high concentration (1.75 $\mu\text{g/ml}$), had no detectable effects (Fig. 5C). Alternatively, we pre-treated the SMG explants with VEGF 1 day before the application of 2 $\mu\text{g/ml}$ anti-Npn1 antibody, which only partially inhibited the branching activity. If VEGF had any Sema3A-competitive binding effect toward Npn1, the pre-added exogenous VEGF should enhance Npn1-mediated inhibition in the assay.

Again, no differences were observed (Fig. 5D). These results taken together, we conclude that VEGF plays no role in the Sema3A (3C)/Npn1-mediated SMG branching morphogenesis.

Plexin A2 and plexin D1 are required for Sema3-mediated cleft formation

We then investigated which plexins are required for Npn1-mediated cleft formation in the SMG. Semi-quantitative RT-PCR analysis showed that only plexin A1, A2 and D1 were relatively abundantly expressed in the E13 SMG (see Fig. S3A in the supplementary material). Other plexins were either absent in the SMG or not expressed until late embryonic stages. RNA in situ hybridization of plexins on the E15.5 SMG sections showed similar patterns (see Fig. S3B in the supplementary material). In the SMG explant cultures, we found that, among all plexins, only loss of plexin A2 or plexin D1 had a significant reduction in the branching morphogenesis (Fig. 6A). Additional expression pattern studies on these two plexins revealed that their transient high expressions in the SMGs from E13.5 to E17.5 corresponded well to the period of active branching of the developing SMG (see Fig. S4A,B in the supplementary material). Therefore, plexin A2 and plexin D1 are likely the candidates for mediating semaphorin signals in the developing SMG.

We next asked whether plexin A2 and plexin D1 played roles in the branching activity enhanced by Sema3A or Sema3C in the SMG co-culture assay. Clearly, the effects of Sema3A (or Sema3C)-inducing SMG cleft formation were ameliorated by application of either anti-plexin A2 or anti-plexin D1 ODNs (Fig. 6Ba). It is interesting to note that application of the two antisense ODNs against plexin A2 and plexin D1 in the SMG co-cultures only partially enhanced the effects. In addition, the inhibitions reached by adding these two specific ODNs were not as complete as those caused by adding anti-Npn1 antibodies (Fig. 6Bb, Fig. 2Ab). These observations could be explained by the presence of residual plexin A2 or plexin D1 in the SMG. Alternatively, additional molecules might participate in the Npn1-mediated branching activity. However, it is sufficient to conclude that Npn1, at least partially, forms receptor complexes with either plexin A2 or plexin D1 to transduce the Sema3A or Sema3C signal to mediate SMG cleft formation.

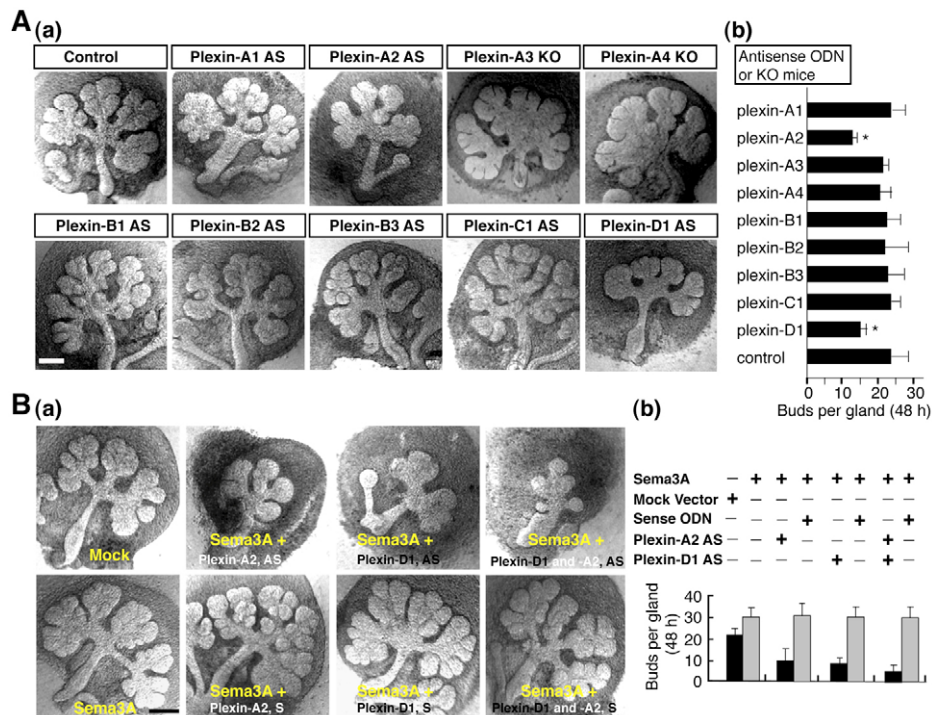


Fig. 6. Plexin A2 and plexin D1 are required for class 3 semaphorin-mediated cleft formation. (A) SMG cleft formation was reduced in the SMG ex vivo cultures treated with antisense ODNs against plexin A2 or plexin D1 for 48 hours. No effects were observed in the SMG explants dissected from knockout mouse mutants of plexin A3 or plexin A4, or in the SMG explants treated with antisense ODNs against other plexins. Representative explants are shown in **a** and the summary of five independent experiments is shown as a bar graph in **b**. Paired *t*-test: *, $P < 0.05$ while compared with the controls. (B) SMG cleft formation promoted by Sema3A in the SMG co-cultures was partially abolished by the treatment of antisense ODNs against plexin A2 or plexin D1, but not by sense ODNs. Representative photographs were taken from 24-hour co-cultures (a). A summary of seven independent experiments is shown in **b**. Scale bars: 100 μ m. AS, antisense ODNs; KO, knockout mutants; S, sense ODNs.

Npn1-mediated semaphorin signaling acts in parallel with FGF signaling in the developing SMG

The FGF family members, including Fgf1, 7 and 10, are shown to stimulate SMG branching morphogenesis through activation of their receptor – Fgfr2b (Steinberg et al., 2005). To test whether inactivation of Npn1 would affect FGF signaling in the developing SMG, we first examined the expression level of FGFs and Fgfr2 in the cultured SMG explants pre-treated with Npn1 antisense ODNs. Semi-quantitative RT-PCR analysis showed that their expressions were not changed with the treatment (Fig. 7A). Moreover, in the SMG explant culture, Fgf7 or Fgf10 effectively

stimulated SMG branching morphogenesis in the presence of Npn1 antisense ODNs (Fig. 7Ba,b). Interestingly, Fgf7 specifically promoted SMG cleft formation with short ductile formation, as has been reported (Steinberg et al., 2005), even in the absence of Npn1 (Fig. 7Ba, upper panels). Likewise, the specific effects of Fgf10 on ductal elongation and branch number were independent of Npn1 (Fig. 7B, lower panels). These results indicate that the Npn1-mediated semaphorin signaling pathway functions in parallel, perhaps in an independent but cooperative manner, with the FGF signaling pathway to mediate the development of salivary glands.

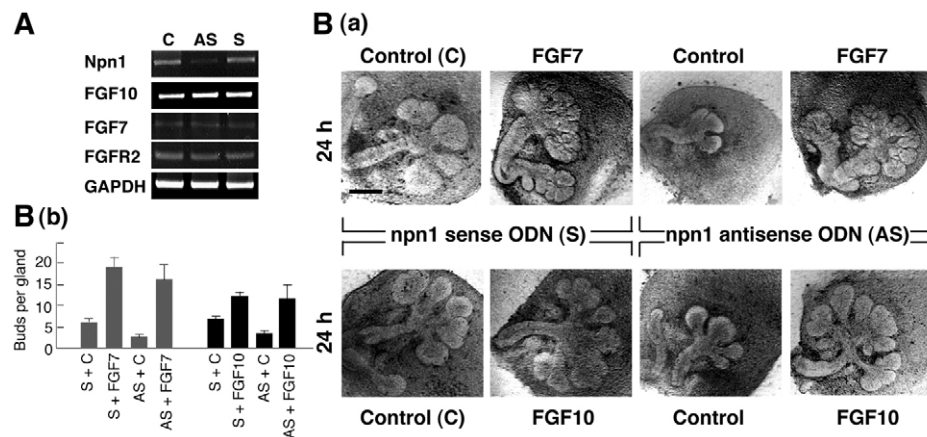


Fig. 7. FGF-induced SMG branching morphogenesis is independent of Npn1 function in mice. (A) Semi-quantitative RT-PCR analysis indicated that the expression of Fgf10, Fgf7 and Fgfr2 in the SMG explants were not altered when the SMG cultures were treated with Npn1 antisense ODNs. *Gapdh* is an internal control. C, no treatment; S, SMG cultures treated with Npn1 sense ODNs. (B) Fgf7 (500 ng/ml) and Fgf10 (1000 ng/ml) still effectively promoted SMG branching formation in the presence of Npn1 antisense ODNs (a). Representative photographs were taken from 24-hour cultures. A summary of five independent experiments is shown in **b**. Scale bar: 100 μ m. AS, treated with Npn1 antisense ODNs; C, control peptide; S, treated with Npn1 sense ODNs.

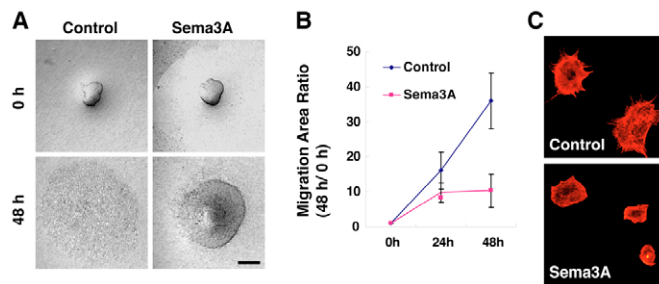


Fig. 8. Sema3A restricts migration of cultured SMG epithelial cells. (A) The migration of mouse epithelial cells in mesenchyme-free SMG explant culture was restricted by synthetic Sema3A peptide (100 ng/ml). (B) The extent of epithelial cell migration is summarized at three time points, as indicated. The migration ratios were obtained from dividing the area measured at each time point by the area measured at 0 hours. (C) Sema3A caused cytoplasmic constriction of the SMG epithelial cells. Dissociated epithelial cells from E13.5 SMG were cultured for 2 days and then treated with synthetic Sema3A or scrambled peptide for another 1 or 2 days. Cells were fixed with paraformaldehyde and stained with rhodamine phalloidin. Scale bar: 100 μ m.

Sema3A restricts the movement of the cultured SMG epithelial cells

Because Npn1 and Sema3A are expressed mainly in the SMG epithelial cells, we tested whether the surrounding mesenchyme is required for Sema3A/Npn1-mediated cleft formation. We isolated the epithelial rudiments free from mesenchyme and cultured them in Matrigel with reduced growth factor. Under this culture condition, the epithelial rudiments were unable to grow and form branches (Hosokawa et al., 1999). Interestingly, addition of Sema3A into this culture did not stimulate branching of the SMG epithelia (Fig. 8A), whereas application of Fgf10 or Fgf7 did stimulate growth and branching of mesenchyme-free SMG rudiments as reported before (data not shown) (Steinberg et al., 2005). These results indicate that the Sema3A signal per se does not stimulate the proliferation of SMG epithelial cells. In addition, growth-promoting signals from the mesenchyme are required for Sema3A-mediated cleft formation in the developing SMG.

In the Matrigel culture, the epithelial cells migrated out of the rudiments to form a circular cell sheet (Hosokawa et al., 1999). The degree of migration could be quantified by measuring the ratio of the final spreading area to the initial area covered by the epithelial rudiment. Adding Sema3A (100 ng/ml) to the culture significantly reduced the cell migration (Fig. 8A,B). To examine the effects of Sema3A on single epithelial cells, we added Sema3A to the dissociated SMG epithelial cells and found that the cytoplasmic expansion in each cell collapsed after the treatment of Sema3A for 24 hours (Fig. 8C). In these Sema3A-treated cells, partial dissolution of actin fiber was observed when stained with rhodamine phalloidin (Fig. 8C). These data suggest that Sema3A may collapse the epithelial cells locally and thus induce cleft formation of the developing SMG.

DISCUSSION

We have systemically studied the roles of class 3 semaphorins and their receptors in the developing SMG. Here we report that during SMG branching morphogenesis, Sema3A and Sema3C function together to promote cleft formation. These two semaphorins are produced mainly by growing epithelial cells, and they act in an autocrine (or paracrine) fashion. Neuropilin 1, as well as plexin A2

and plexin D1, are the receptors that are expressed on the same type of cells in the epithelial bud to receive Sema3A and Sema3C signals. Additionally, other factors such as FGFs that come from the neighboring mesenchymal cells act in parallel with semaphorins to control SMG branching morphogenesis.

Specific roles for class 3 semaphorins in regulating SMG cleft formation

The roles of class 3 semaphorins in branching morphogenesis have recently been studied in the lung, mammary gland and kidney (Hinck, 2004). In particular, Sema3A and Sema3C have been proposed to regulate the branching of the lung bud via a push-pull mechanism: Sema3A is expressed in the mesenchyme to push (or repel) the developing lung epithelial cells that express Npn1, while the epithelial cells at the tip of the lung bud secrete Sema3C to pull (or attract) themselves, perhaps through Npn2, in an autocrine fashion (Hinck, 2004; Ito et al., 2000). In addition, other signaling pathways such as FGFs and Shh are involved in the lung branching morphogenesis (Cardoso and Lu, 2006), and all of these factors function together in a complicated, yet coordinated, manner to ensure the normal development of the lung.

Previous studies on the development of embryonic SMG have already shown that FGFs, BMPs and Shh are involved in SMG branching morphogenesis (Jaskoll et al., 2004b; Steinberg et al., 2005) (see discussion below), but the roles of semaphorins in this process have not been demonstrated. In this report, we find that Sema3A and Sema3C are required in the developing SMG, but, surprisingly, the way they work seems quite different from that reported in the developing lung bud: these two semaphorins are secreted from the developing epithelial cells and function additively, instead of antagonistically, to promote SMG cleft formation. Moreover, both Sema3A and Sema3C signals are mediated by Npn1. Only Npn1, but not Npn2, is detected in the developing SMG epithelial cells, and we have demonstrated that the cleft formation activity promoted by either semaphorin requires the presence of functional Npn1. Our results reveal an unexpected functional interaction between semaphorins and their receptors. The cooperative behavior of Sema3A and Sema3C through the same binding receptor, Npn1, in SMG development may be mechanistically different from the reported antagonistic effects of Sema3C and Sema3A observed in the developing lung bud (Kagoshima and Ito, 2001) or in the repulsion of the dorsal root ganglion axons (Takahashi et al., 1998).

The plexin receptors utilized in the developing SMG to mediate the semaphorin signals are also unique. In the projections of peripheral sensory neurons, plexin A3 and plexin A4 are required for Sema3A function (Cheng et al., 2001; Yaron et al., 2005). In the embryonic vasculogenesis, plexin D1 directly binds Sema3E to mediate its signal (Gu et al., 2005). But in the development of the heart, plexin D1 is reported to form co-receptor with Npn1 to mediate Sema3C function (Gitler et al., 2004; Torres-Vazquez et al., 2004). In the developing SMG, however, we show that plexin D1 and plexin A2 are the main plexin receptors that form co-receptors with Npn1 to mediate Sema3A and Sema3C signals. Sema3E does not affect the plexin D1-mediated SMG branching, indicating that direct functional binding between plexin D1 and Sema3E may not occur during SMG development. The SMG explants from plexin A3-null or plexin A4-null mutant mice grow normally, indicating that these two plexins are dispensable. Taken all together, these results suggest that molecular interactions within the same ligand-receptor families can be distinct, depending on the cellular contexts and developmental stages. We still do not know whether plexin D1

and plexin A2 function independently of each other as separate Npn1-co-receptor complexes or whether they aggregate together with Npn1 as huge protein complexes for downstream signaling. Given the abundance and diversity of the semaphorin ligand-receptor families, one can expect that many other specific interactions will be identified in different tissues or developmental processes.

We have demonstrated in this study that *Sema3A* or *Sema3C* collapses and constricts dissociated embryonic SMG epithelial cells, indicating that these semaphorins act as autocrine inhibitory cues. Recently, an elegant study on the branching morphogenesis of the mammary gland epithelia shows that the branching of the epithelial buds is regulated by local concentration of autocrine inhibitory factors (Nelson et al., 2006). Interestingly, during the development of embryonic SMG, the highest expression of *Sema3A* transcript is found at the front tip of the epithelial buds. This expression pattern is consistent with its role as a local regulator for cleft formation, as primitive indentations for subsequent cleft formation are often initiated at the tip of the epithelial bud. It is thus quite plausible that, in the developing SMG, the autocrine semaphorins exert a pushing force locally to separate neighboring epithelial cells through collapsing the cells that express the receptors, and consequently promote cleft formation.

Multiple signaling pathways control SMG branching morphogenesis

Branching morphogenesis requires coordinated interactions between epithelial and mesenchymal cells to promote cell proliferation, differentiation and migration. Cleft formation is a very distinct event during the early phase of branching morphogenesis. Essentially all the developing epithelial buds repeatedly undergo cleft formation until the final tree-like or bush-like structure is formed. Several morphogens, including FGFs, Hgf, BMPs and Shh, are required for SMG branching morphogenesis. Previous studies suggest that, during embryonic development, these factors are mainly derived from the neighboring mesenchyme. They may stimulate cell proliferation and/or regulate cleft formation (Steinberg et al., 2005). Here we show that semaphorin signaling is also required for SMG branching morphogenesis, but its action seems to be more specific: semaphorins seem not to stimulate cell proliferation; they promote cleft formation.

How semaphorins are coordinated with other factors to regulate SMG branch formation is still not clear, but our results suggest that semaphorins function in parallel with some FGFs without much crosstalk between their signaling pathways. Moreover, it has been reported that the SMG epithelial rudiments without mesenchyme developed normally in the presence of FGFs. In SMG co-culture assays, we observed that the autocrine function of semaphorins required additional signals from the mesenchyme. Therefore, it is likely that at the early stage of SMG morphogenesis, the surrounding mesenchymal cells secrete morphogens such as FGFs to stimulate the proliferation of the SMG epithelial cells. Later, when the growing epithelial bud is ready for cleft formation, the cells at the front end secrete semaphorins, perhaps with the existing FGFs, to promote the local cleft formation.

Cleft formation also requires conversion of cell-cell adhesive interactions to cell-matrix interactions (Hosokawa et al., 1999; Kadoya and Yamashina, 2005). It has recently been shown that the stimulation of integrin receptor on the epithelial cell by fibronectin deposited in the matrix is essential for SMG cleft formation (Sakai et al., 2003). It would therefore be interesting to know whether semaphorin signaling interacts with integrin signaling in the

developing epithelial cells. Among all semaphorins, *Sema7A* has been shown to directly bind to integrin subunit β_1 and promote neurite outgrowth (Pasterkamp et al., 2003). However, as class 3 semaphorin proteins lack the integrin-binding motif, RGD, it is unlikely that *Sema3A* (or *Sema3C*) can bind integrin receptors. In addition, although semaphorin signaling has been associated with integrin activities, the interactions seem to be diverse and context-dependent. For example, *Sema3C* can increase integrin activity in glomerular epithelial cells (Banu et al., 2006), while semaphorin/plexin signaling can also disrupt integrin-based adhesion, leading to inhibition of lamellipodia extension and cell motility in NIH3T3 and COS cells (Barberis et al., 2004). It has also been shown that autocrine *Sema3A* can inhibit integrin function to control morphogenesis of the vasculature (Serini et al., 2003). Apparently, further experiments are required to understand how semaphorin signals can contribute to the changes of cell adhesion during SMG cleft formation.

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Supplementary material

Supplementary material for this article is available at <http://dev.biologists.org/cgi/content/full/134/16/2935/DC1>

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