Coordination of trigeminal axon navigation and patterning with tooth organ formation: epithelial-mesenchymal interactions, and epithelial Wnt4 and Tgfβ1 regulate semaphorin 3a expression in the dental mesenchyme

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Summary
During development, trigeminal nerve fibers navigate and establish their axonal projections to the developing tooth in a highly spatiotemporally controlled manner. By analyzing Sema3a and its receptor Npn1 knockout mouse embryos, we found that Sema3a regulates dental trigeminal axon navigation and patterning, as well as the timing of the first mandibular molar innervation, and that the effects of Sema3a appear to be mediated by Npn1 present in the axons. By performing tissue recombinant experiments and analyzing the effects of signaling molecules, we found that early oral and dental epithelia, which instruct tooth formation, and epithelial Wnt4 induce Sema3a expression in the presumptive dental mesenchyme before the arrival of the first dental nerve fibers. Later, at the bud stage, epithelial Wnt4 and Tgfβ1 regulate Sema3a expression in the dental mesenchyme. In addition, Wnt4 stimulates mesenchymal expression of Msx1 transcription factor, which is essential for tooth formation, and Tgfβ1 proliferation of the dental mesenchymal cells. Thus, epithelial-mesenchymal interactions control Sema3a expression and may coordinate axon navigation and patterning with tooth formation. Moreover, our results suggest that the odontogenic epithelium possesses the instructive information to control the formation of tooth nerve supply.

Key words: Odontogenesis, Tissue interactions, Tooth, Axon growth, Mouse

Introduction
During development of the peripheral nervous system, growing axons navigate and establish connections to their developing target organs. Regulation of axon growth involves coordinated activity of diffusible and local contact-mediated attractive and repulsive guiding cues including members of for example the netrin, Slit, ephrin and semaphorin families (Dickson, 2002; Tessier-Lavigne and Goodman, 1996; Varela-Echavarria and Guthrie, 1997). Many of the guidance molecules show dynamic and restricted expression patterns in peripheral tissues and organs that correlate with regulation of axon growth. However, how the expression of guidance molecules is regulated during organ formation, and how axon navigation and patterning is coordinated spatiotemporally with organ formation has remained largely unknown.

The developing tooth is a useful model in which to analyze the molecular mechanisms of organ formation. The teeth develop in the oral side of the maxillary and mandibular processes, and their formation is regulated by sequential and reciprocal interactions between the odontogenic epithelium and neural crest derived ectomesenchymal cells (Miletich and Sharpe, 2003; Thesleff, 2003). Signaling molecules have been shown to mediate inductive tissue interactions during odontogenesis. In particular, early oral epithelium- and oral epithelium-expressed signaling molecules regulate dental mesenchymal expression of signaling genes and transcription factors that are essential for tooth formation (Miletich and Sharpe, 2003; Thesleff, 2003). Trigeminal axon pathfinding and nerve fiber patterning, in particular in the murine lower first molar, takes place in a strictly spatiotemporally controlled manner and is tightly linked to tooth formation (Loes et al., 2002; Luukko et al., 1997b; Mohamed and Atkinson, 1983).
The early developing tooth is innervated by nerve fibers originating from the sensory trigeminal ganglion. Because new axons do not emerge from the trigeminal ganglia after E13 (Davies, 1988), axon navigation and their survival around the embryonic tooth germ during the period of programmed cell death (E13-E18) (Davies, 1988) are essential for the proper development of the sensory innervation of the dental pulp and periodontal ligament (Luukko et al., 1997a). The sympathetic innervation of the tooth develops postnatally after the onset of root formation (Fristad et al., 1994).

Some light has been shed on the molecular mechanisms that regulate the development of tooth nerve supply. It appears that this process is regulated by set of neuroregulatory molecules of different families (for a review, see Fried et al., 2000; Luukko, 1998). However, as yet, no gene has been shown to be essential for pioneer dental axon guidance or the establishment of early tooth innervation. The finding that the developing tooth is able to promote its reinnervation when implanted in ectopic locations (Erdelyi et al., 1987) and that the expression of neurotrophins and their receptors persists in vitro, without peripheral nerve fibers cultured tooth explants has suggested that the developing tooth is able to control the formation of its own innervation and that the synthesis of the neuroregulatory molecules is regulated locally and is an intrinsic property of the tooth germ (Luukko et al., 1996; Luukko et al., 1997a).

We have recently reported developmentally regulated mRNA expression of semaphorin 3a (Sema3a) in the developing tooth in sites that are devoid of nerve fibers, suggesting functions in dental axon guidance and tooth formation (Loes et al., 2001). Sema3a, a secreted repulsive axon guidance molecule, shows broad developmentally regulated expression in the peripheral tissues and organs of the embryo including the first branchial arch (BA1) and tooth (Taniguchi et al., 1997; Wright et al., 1995). Targeted inactivation of the Sema3a gene leads to abnormal fasciculation and patterning of a set of peripheral nerves, including the cranial trigeminal, facial and glossopharyngeal nerves, indicating the importance of Sema3a in the establishment of axonal trajectories (Taniguchi et al., 1997; Ulupinar et al., 1999). In addition, Sema3a serves an organogenetic function, e.g. in bone and heart formation (Behar et al., 1996), and controls vascular morphogenesis by inhibiting integrin function (Serini et al., 2003). In the current study, we analyzed the functions and regulation of Sema3a during early, crucial stages of tooth organogenesis and the formation of its trigeminal nerve supply. We found that Sema3a is an essential signal for proper tooth innervation, and that the oral epithelium and dental epithelium expressed Wnt4 induce Sema3a in the mandibular presumptive molar mesenchyme area before the arrival of first nerve fibers. Later, during pioneer dental axon navigation, Wnt4 and Tgfβ1 control Sema3a expression in the dental mesenchyme. Thus, epithelial-mesenchymal interactions may provide a central mechanism for coordination of axon navigation and patterning with the mandibular process and tooth formation.

**Materials and methods**

**Preparation of tissues**

Animal use was approved by the Department of Biomedicine of the Medical Faculty of the University of Bergen under the surveillance of the Norwegian Animal Research Authority. Production of the Sema3a and Npn1 mutant mice strains and genotyping has been described previously (Behar et al., 1996; Kitsukawa et al., 1997; Taniguchi et al., 1997). The transgenic and NMRI mice were mated overnight and the appearance of the vaginal plug was taken as day E0.5 of embryogenesis. Tissues were processed further for different analyses as described previously (Luukko et al., 1996; Kettunen and Thesleff, 1998). Preparation of postnatal tissues for PGFP.9 immunohistochemistry was performed as described previously (Fristad et al., 1994). NMRI mouse embryos were used for organ culture and tissue recombination experiments. Photographs were taken with a Coolpix 4500 digital camera (Nikon Corporation, Tokyo, Japan) and figures were processed using Adobe Photoshop software (Adobe Systems, San Jose, CA, USA).

**Antibodies and immunohistochemistry**

To detect nerve fibers in paraffin sections, immunohistochemistry with rabbit polyclonal anti-peripherin (Chemicon International, CA, USA) and neuropilin 1 antibodies (Kawakami et al., 1996) (1:150 and 1:1000 dilution) was carried out using the Vectastain pK4001 kit (Vector Laboratories, Burlingame, CA) according to the manufacturer’s instructions (Luukko, 1997; Fristad et al., 1994). Negative control sections were incubated with normal rabbit serum instead of the primary antibody. No specific immunoreactivity was detected.

**In situ hybridization**

For in situ hybridization on sections the 0.6 kb rat Gdnf, 0.1 kb rat Lanr, 0.9 kb mouse Ncam, 0.4 kb rat Ngf and 2.9 kb mouse Sema3a cDNAs were used for in vitro transcription of 35S-UTP- and digoxigenin-labeled and antisense and sense probes. Sectional and whole mount in situ hybridization was performed as described previously (Luukko et al., 1996; Henrique et al., 1995). No specific hybridization signals were detected in tissues hybridized with control sense probes (not shown).

**Three-dimensional reconstruction**

Three-dimensional (3D) computer reconstruction of the tooth germ was generated from 7 µm serial frontal bright- and dark-field photomicrographs (180 sections from each field). The processing of the images was done using custom scripts and programs written with Java Advanced Imaging and Java 3D (Sun Microsystems, CA, USA) (http://java.sun.com). Three-dimensional reconstructions were rendered with a perspective camera view in Visualization Toolkit (Kitware, New York, USA) (http://www.kitware.com). A transparent 3D surface of the inner dental epithelium was generated using the Marching Cubes function in Visualization Toolkit from the outlines of the dental epithelium and the cervical loops. A hybridization signal in the dark-field images with an intensity over 230 was considered to represent positive Sema3a gene expression. The sections were median filtered to reduce the background hybridization signal in the 3D image.

**Organ and tissue culture, recombinant proteins and cell lines**

Organ and tissue cultures were performed as described earlier (Kettunen et al., 1998). Explants shown are representatives of at least three independent experiments. At least six explants of each experimental setup were analyzed. Agarose (BioRad) and heparin acrylic (Sigma) were used. Beads were incubated in recombinant human FGF2 (100 µg/ml) and FGF4 (50 or 100 µg/ml); mouse Fgf8b (50 or 100 µg/ml); human FGF9 (25 or 100 µg/ml); human TGFβ1 (10 or 100 µg/ml); mouse Shh (250 µg/ml); human BMP4 (100 µg/ml) (R&D Systems, Minneapolis, MN); or in BSA (1 mg/ml). All bead experiments were accompanied by positive controls to confirm the activity of the proteins used (Fig. 6, G1-12, P1-Q2; Fig. 7l).
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The Wnt4 and Wnt6 cell lines have been described previously (Kettunen et al., 2000). Quantitative comparison of Wnt protein levels was not performed, but an induction of kidney tubule formation by Wnt4- and Wnt6-producing cells in E11 metanephric mesenchyme explants was used as a positive control in each experiment to confirm the activity of the Wnt proteins. Wnt4 and Wnt6 cell clusters, which were approximately same size had similar tubulogenetic effects on E11 metanephric mesenchyme (Fig. 7G,H) (Kispert et al., 1998). No effects were observed in control NIH3T3 cells.

Cell proliferation assay

The effect of exogenous proteins on dental mesenchymal cell proliferation was analyzed as described earlier (Kettunen et al., 1998). The explants were labeled for 1.5 hours with 10 mM BrdU (Sigma) after 24 hours’ culture. The incorporated BrdU was detected by indirect immunoperoxidase method with monoclonal antibody against BrdU (Sigma, CA) and the biotinylated goat anti-mouse secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) in wholembolts and tissue sections.

Results

Sema3a is absent from the mesenchymal dental axon pathway and dental follicle target field

To address possible neuronal functions of Sema3a we first compared its mRNA expression with the localization of nerve fibers in the embryonic mouse mandibular first molar tooth germ using peripherin antibodies from the serial paraffin sections. Sema3a was first observed in the presumptive dental mesenchyme between the thickened dental epithelium and buccal nerve at E11.5 (Fig. 1A1,B1). At the early bud stage (E12.5) Sema3a expression was observed in the condensing dental and deep jaw mesenchyme (Fig. 1A2) adjacent to the trigeminal nerve branch, the ‘molar nerve’, which had extended from the inferior alveolar nerve next to the tooth germ (Fig. 1B2). At the bud stage, Sema3a continued in the condensed dental mesenchyme under which the molar nerve had diverged into the buccal and shorter lingual branches (Fig. 1A3,B3). At the cap stage (E14.5), nerve fibers extended to the buccal side of the tooth germ and were located in the area between the Sema3a-expressing mesenchymal dental follicle and forming alveolar bone (Fig. 1A4,B4). Later during the bell stage (E16.5 and E18.5) nerve fibers were located in the mesenchymal dental follicle target field between Sema3a-expressing outer dental epithelium and alveolar bone, as well as in the base of the dental papilla mesenchyme between the epithelial cervical loops, which also expressed transcripts as shown for the E18 tooth (Fig. 1A5,B5). Thus, Sema3a is expressed in sites that harbor the mesenchymal dental axon pathway and target field around the developing tooth.

Fig. 1. Sema3a regulates the timing of tooth innervation and dental axon guidance and patterning. Expression of Sema3a in the embryonic mandibular molar tooth germ (A1-A5) at the epithelial thickening (E11.5) (A1), early bud (E12.5) (A2), bud (E13.5) (A3), cap (E14.5) (A4) and bell (E16.5) (A5) stages compared with the localization of nerve fibers in corresponding stages of wild-type (Sema3a+/+) (B1-B5) and Sema3a null mouse embryos (Sema3a−/−) (C1-C5) using peripherin antibodies. (A1-A5) The ‘molar nerve’ is indicated by arrows in B2. b, developing alveolar bone; cdm, condensing dental mesenchyme; cm, condensed dental mesenchyme; de, dental epithelium; dp, dental papilla mesenchyme; df, dental follicle; tn, trigeminal inferior alveolar nerve; pm, presumptive dental mesenchyme. Arrowheads mark nerve fibers. Scale bars: 100 µm.
Sema3a regulates timing and patterning of tooth innervation

To investigate whether Sema3a regulates dental axon guidance, we studied the localization of nerve fibers in tooth of the Sema3a mutant mouse embryos (Tanguchi et al., 1997). Ectopic nerve fibers were found prematurely in the mesenchyme next to the dental epithelium at the epithelial thickening (E11.5) and early bud stage (E12.5) (Fig. 1C1,C2). At the bud stage (E13.5), axons were ectopically present in the condensed dental mesenchyme, and some had reached the epithelial bud (Fig. 1C3). Although many nerve fibers showed largely proper localization in the mesenchymal dental follicle target field around the cap and bell stage tooth germs (E14.5, E16, E18.5), disoriented nerve fibers were present next to the outer dental epithelium and in the dental papilla mesenchyme (Fig. 1C4-C5). No defects were observed in tooth formation in the studied Sema3a+− embryos (Fig. 1C1-C5) or in embryos of another Sema3a mutant mouse strain (Behar et al., 1996), which suffers from non-neuronal defects and dies after birth (not shown). Thus, Sema3a regulates the timing of tooth innervation as well as dental axon navigation and patterning but is not needed for early tooth organ formation. Moreover, Sema3a is the first signaling molecule shown to be necessary for the development of tooth innervation.

Npn1 mutant mice show defects in axon navigation

The receptor neuropilin 1 (Npn1) mediates Sema3a signaling by forming receptor complexes with plexins (Bagri and Tessier-Lavigne, 2002). Experimental and genetic analyses have provided evidence that Npn1 mediates in vivo effects of Sema3a on trigeminal axons during their pathfinding (Kitsukawa et al., 1997; Rochlin and Farbman, 1998). To analyze whether Npn1 is a signaling receptor for Sema3a in dental axons, we first analyzed its mRNA expression in trigeminal sensory ganglion. In situ hybridization revealed a prominent Npn1 expression in the ganglion cells during E12.5-E14.5, i.e. during the period when the dental axons are navigating to and around the tooth germ as shown for E13.5 ganglion, while hardly any neuropilin 2 (Bagri and Tessier-Lavigne, 2002) expression was seen (Fig. 2A1-B2). Immunohistochemical analysis from the E12.5-E13.5 head sections using Npn1 antibodies showed that dental axons expressed the protein (Fig. 2C), which is in agreement with the recent report showing Npn1 in dental axons at E15 (Lillesaar and Fried, 2004). To investigate the in vivo roles of Npn1 in tooth, we analyzed Npn1−/− mutant mouse embryos (Kitsukawa et al., 1997). Apparently owing to the defects in the cardiovascular system, the embryos die at about E12.5 when the molar tooth germ is at the early bud stage. No defects in tooth formation in the E11.5-E12.5 mutant embryos were detected compared to corresponding wild-type embryos. Immunohistochemical analysis of 12.5 Npn1−/− embryos revealed that nerve fibers were prematurely present next to the dental epithelium, and some were also ectopically localized in the condensing dental mesenchyme area (Fig. 2D). This phenotype resembled the defects observed in corresponding stages of Sema3a−/− embryos but appeared to be less severe.

Sema3a expression in the postnatal tooth

The first trigeminal nerve fibers from the dental follicle penetrate the dental papilla (future dental pulp) of the mouse mandibular first molar at about three days postnatally (P3) when the shape of the tooth crown is largely ready, crown calcification has started, and the formation of the mesial and distal roots, which attach the tooth to the alveolar bone, starts (Mohamed and Atkinson, 1983). Closer observation of E18 teeth revealed that Sema3a was expressed in the middle part of the base of the dental papilla mesenchyme, while the mesial and distal regions were mostly devoid of transcripts. In situ hybridization analysis from the sagittal sections and 3D-reconstruction of PN1 tooth confirmed Sema3a expression in the area of the future pulp floor and mesenchymal cells adjacent to epithelial cervical loops (Fig. 3A1,A2,B). Later, at P4, Sema3a was expressed in preodontoblasts next to the epithelial cells of the pulp floor as well as next to the epithelial root sheaths, which form the mesial and distal roots of the molar (Fig. 2C1-C2). Thus, Sema3a-free areas mark the sites...
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of the secondary apical foramina through which the sensory and sympathetic nerve fibers enter the dental pulp. However, most probably owing to the fact that ectopic nerve fibers were present in the dental papilla of Sema3a mutant mice already during embryonic development (see Fig. 1C1-C5), we were not able to observe obvious defects in timing or patterning of dental papilla innervation, or in tooth formation in Sema3a mutant molars at P5 when the first nerve fibers are normally present in the dental pulp from tissue sections using PGP9.5 antibodies (not shown).

Ngf, Gdnf, Lanr, Ncam and Net3 expression is not altered in Sema3a mutant tooth

The finding that some of the nerve fibers showed normal localization within the tooth target field in Sema3a\(^{-/-}\) mice, in particular at the later morphogenetic cap and bell stages, suggested that other neuroregulatory molecules might guide dental axons and partially rescue the tooth innervation phenotype in Sema3a mutant embryos. To study this, we analyzed mRNA expression of molecules implicated in dental axon guidance and patterning. Nerve growth factor (Ngf), which is essential for tooth innervation (Matsuo et al., 2001; Qian and Naftel, 1996), is expressed in the mesenchymal axon pathway, and, later, together with glial cell line-derived neurotrophic factor (Gdnf), in the dental follicle target area around the tooth germ (Luukko et al., 1997a; Luukko et al., 1997c; Mitsiadis et al., 1992; Nosrat et al., 1998). Similarly, the neural cell adhesion molecule (Ncam) and low-affinity neurotrophin receptor (Lanr), which may sequester neurotrophins, are present in the dental follicle target field during axon navigation (Luukko et al., 1996; Mitsiadis et al., 1992; Obara and Takeda, 1993). In addition, mRNAs for netrin 3 (Net3) are expressed in the mesenchymal axon pathway and dental follicle region and may therefore be involved in the regulation of dental axon growth (Loes et al., 2003). In situ hybridization analysis of the late bud/early stage tooth germs (E14–) revealed no differences between the expression patterns of Ngf, Gdnf, Lanr, Ncam and Net3 in Sema3a\(^{-/-}\) and wild-type mice (Fig. 4A1-J2).

Epithelial-mesenchymal interactions regulate Sema3a expression

The observation that Sema3a mRNAs appeared in the presumptive dental mesenchyme at E11.5 under the thickened
dental epithelium before the arrival of the pioneer dental nerve fibers suggested that Sema3a expression in tooth is not controlled by growing axons but by a local mechanism. Tissue separation and recombination studies have demonstrated that tooth formation is regulated by epithelial-mesenchymal interactions (Lumsden, 1988; Mina and Kollar, 1987). To analyze whether tissue interactions regulate mesenchymal Sema3a expression, we first separated epithelial and mesenchymal tissue components from E10.5 and E11.5 mandibular processes, as well as E11 and E12 molar tooth germs, and cultured the isolated mesenchymes in a Trowell-type culture for 24 hours. The explants were serially sectioned and analyzed by in situ hybridization. The removal of the epithelium resulted in a loss of almost if not all Sema3a expression in the E10.5 mandibles, while in the intact cultured mandibles Sema3a expression persisted (Fig. 5B1-C2). In E11 mandibular mesenchyme explants, some Sema3a transcripts persisted in the proximal deep aboral part, whereas little if any endogenous Sema3a was found in the presumptive molar mesenchyme area (Fig. 5F1,F2), as also confirmed by culturing isolated E11 presumptive molar mesenchyme explants alone (not shown). At E12, however, more endogenous expression of Sema3a persisted in the isolated cultured dental mesenchymes (Fig. 5J1,J2), while transcripts were not observed in the E12 dental epithelia (not shown). Thus, Sema3a expression in the E10.5 mandibular mesenchymes and E11 presumptive dental mesenchyme is dependent on epithelial signaling.

To specifically address whether Sema3a expression in the oral side of the E11.5 proximal jaw mesenchyme containing the presumptive dental mesenchyme area is induced by the overlying epithelium, we placed E10.5 oral epithelium, which includes presumptive dental epithelium as well as E11 and E12 dental epithelia, onto the proximal presumptive molar area of the E10.5 mandibular mesenchyme, which is devoid of Sema3a (Fig. 5A1,A2). In situ hybridization revealed Sema3a expression in the mesenchyme adjacent to E10.5 oral and E11 dental epithelium after 24 hours and 2 days culture respectively (Fig. 5D1,D2,G1,G2). Similarly, a prominent expression was observed in the mesenchyme next to the E12 dental epithelium, which had reached the bud stage after 3 days culture (Fig. 5L1,L2). In addition, in homochronic E11 and E12 tooth

![Fig. 4. Ngf, Lanr, Gdnf, Ncam and Net3 expression is not altered in the Sema3a+/− tooth. Bright- and dark-field images of frontal sections of the E14- late bud/early cap stage wild-type (A1-A2,C1-C2,E1-E2,G1-G2,I1-I2) and Sema3a mutant (B1-B2,D1-D2,F1-F2,H1-H2,J1-J2) upper and lower first molar tooth germs. Abbreviations: de, dental epithelium; dp, dental papilla. Scale bar: 100 μm.](image-url)
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recombinants, which were cultured for 24 hours, Sema3a expression in the mesenchyme correlated to the expression in in vivo developed teeth (compare E1,E2 with I1,I2). No specific Sema3a expression is seen in the E10.5 mandibular mesenchyme cultured alone without epithelium (compare also images in Fig. 5J1-L2 cultured with BSA-soaked beads and Wnt6 and NIH3T3 cells), while in the cultured intact mandible a prominent mesenchymal Sema3a expression is present (C1-C2). (D1-D2) E10.5 oral epithelium has induced Sema3a in the underlying E10.5 molar area of mandibular mesenchyme, which is devoid of Sema3a (arrows in A1). (F1,F2) In cultured E11 mandibular mesenchyme, Sema3a transcripts are largely absent from the presumptive molar area (arrow), whereas the remaining Sema3a is seen in the deep aboral mesenchyme. (G1-G2,L1-L2) Sema3a induction is observed next to the E11 and E12 dental epithelia, which were placed onto the proximal, molar region of E10.5 mandibular mesenchyme. (H1-K2) A prominent Sema3a expression is seen in the E11 jaw and E12 dental mesenchyme under the E11 and E12 dental epithelia, respectively, while some endogenous Sema3a is observed in the dental mesenchyme explant. The expression patterns of Sema3a in these homochronic recombinant explants correlate with the Sema3a expression in in vivo developed teeth (compare E1,E2 with I1,I2). de, dental epithelium; dm, dental mesenchyme; m, mandibular mesenchyme; Md, mandibular process, Mx, maxillary process; oe, oral epithelium. Scale bars: 100 µm (200 µm for D2).

Fig. 5. Epithelial-mesenchymal interactions regulate Sema3a expression in the mandibular and dental mesenchyme.

Expression of Sema3a in the E10.5 head (A1,A2) and E11.5 (E1,E2) and E12.5 (H1-H2) mandibular molar tooth germs, as well as in cultured mandibular and dental mesenchymes and tissue recombinants analyzed by in situ hybridization from frontal sections after 24 hours (B1-D2,F1,F2,H1,H2,J1,J2,K1,K2), 2 days (G1-G2) and 3 days (L1-L2) of culture. Bright- and dark-field images. (B1-B2) No specific Sema3a expression is seen in the E10.5 mandibular mesenchyme cultured alone without epithelium (compare also images in Fig. 5J1-L2 cultured with BSA-soaked beads and Wnt6 and NIH3T3 cells), while in the cultured intact mandible a prominent mesenchymal Sema3a expression is present (C1-C2). (D1-D2) E10.5 oral epithelium has induced Sema3a in the underlying E10.5 molar area of mandibular mesenchyme, which is devoid of Sema3a (arrows in A1). (F1,F2) In cultured E11 mandibular mesenchyme, Sema3a transcripts are largely absent from the presumptive molar area (arrow), whereas the remaining Sema3a is seen in the deep aboral mesenchyme. (G1-G2,L1-L2) Sema3a induction is observed next to the E11 and E12 dental epithelia, which were placed onto the proximal, molar region of E10.5 mandibular mesenchyme. (H1-K2) A prominent Sema3a expression is seen in the E11 jaw and E12 dental mesenchyme under the E11 and E12 dental epithelia, respectively, while some endogenous Sema3a is observed in the dental mesenchyme explant. The expression patterns of Sema3a in these homochronic recombinant explants correlate with the Sema3a expression in in vivo developed teeth (compare E1,E2 with I1,I2). de, dental epithelium; dm, dental mesenchyme; m, mandibular mesenchyme; Md, mandibular process, Mx, maxillary process; oe, oral epithelium. Scale bars: 100 µm (200 µm for D2).

Epithelial Wnt4 and Tgfβ1 induces mesenchymal Sema3a expression

Early oral and dental epithelium expressed signals in particular members of the fibroblast growth factor, bone morphogenetic protein, Wnt and Hedgehog families control gene expression in the underlying mesenchyme (Miletich and Sharpe, 2003; Thesleff, 2003). To identify the epithelial signal(s) that induce(s) and regulate(s) Sema3a expression, we applied protein-soaked beads and protein-producing cells of different members of these families to the mandibular and dental mesenchyme explants and cultured them for 24 hours (Kettunen and Thesleff, 1998). The explants were serially sectioned and analyzed for Sema3a expression by in situ hybridization.

Fgf8 mRNAs are expressed in the E10.5 proximal oral epithelium and later in the early dental epithelium (Kettunen and Thesleff, 1998) and are essential for the outgrowth and patterning of the BA1 and for tooth formation (Trumpp et al., 1999). As reported earlier (Neubuser et al., 1997), Fgf8 induced a prominent Pax9 expression in the presumptive molar area of E10 mandibular processes, whereas no Sema3a induction was observed around the Fgf8 beads (Fig. 6A1-B2). Similarly, beads soaked in Bmp4, Fgf9 and Shh, mRNAs of which are expressed in the presumptive and early dental epithelium (Hardcastle et al., 1998; Kettunen and Thesleff, 1998; Vainio et al., 1993), had no effect on Sema3a expression in the molar area of E10.5 or E12.5 mandibular mesenchymes as shown for Fgf9 at E12.5 (Fig. 6N1,N2; not shown). Furthermore, protein-soaked beads for the enamel knot expressed Fgf4 (Jernvall et al., 1994) did not have effects on Sema3a expression in the molar region of E12 mandibular mesenchyme explants (not shown).

The finding that the E10.5 oral epithelium was able to induce Sema3a suggested that the expression of the putative signaling molecule(s), which induce(s) the initial mesenchymal Sema3a expression, is or are not limited to the presumptive dental
The Wnts form a large family of conserved secreted signaling molecules that regulate neuronal and non-neuronal development. Several Wnts are expressed in the lower jaw and dental epithelia (Sarkar and Sharpe, 1999). In developing limb, epithelial Wnt4 induces neurotrophin 3 (Nt3) expression in the adjacent mesenchyme (Patapoutian et al., 1999), the development of which is dependent on epithelial-mesenchymal interactions. Moreover, Wnt4 and Wnt5 regulate commissural axon guidance (Lyuksyutova et al., 2003; Yoshikawa et al., 2003). To investigate whether Wnt factors control Sema3a, we placed clusters of Wnt4-producing cells, mRNAs of which are present in the oral and dental epithelium during E10.5-E14.5 (Sarkar and Sharpe, 1999), onto the presumptive molar mesenchyme area of E10.5 and E11.5 lower jaws as well as onto isolated E12.5 dental mesenchyme. Wnt4-producing cells upregulated Sema3a expression in the adjacent mesenchymal cells at all stages studied (Fig. 6, C1-E2), while cells producing Wnt6, mRNAs of which are present in the oral epithelium (Sarkar and Sharpe, 1999), showed no effect on mesenchymal Sema3a expression at E10.5 or E11.5 (Fig. 6J1,J2 and not shown). mRNAs for Tgfβ1 appear in the epithelial dental bud at E12.5 (not shown), and later at the cap stage they also appear in the dental mesenchyme (Vaahtokari et al., 1991). Tgfβ1 regulates Ngf and Nt3 mRNA levels in the epithelial and mesenchymal cells of the maxillary process in culture (Buchman et al., 1994). When Tgfβ1-soaked beads...
were placed onto the molar area of E12.5 mandibular jaw mesenchyme, a prominent Sema3a expression was observed in the surrounding cells (Fig. 6G1,G2). No effects on Sema3a were observed in the mesenchyme cultured with control NIH3T3 cells or beads soaked in BSA (1 mg/ml) from E10 to E12.5 (Fig. 6K1-M2; not shown).

Wnt4 stimulates Msx1 expression in the jaw mesenchyme

Because Wnt signaling is essential for tooth formation (Andl et al., 2002; van Genderen et al., 1994), we investigated whether Wnt4 is involved in odontogenesis by analyzing its effects on the expression of Msx1 and Pax9 transcription factors. Msx1 and Pax9 are necessary for tooth morphogenesis beyond the bud stage and their expression in the early mandibular mesenchyme is induced by and dependent on epithelial signaling (Ferguson et al., 2000; Neubuser et al., 1997; Peters et al., 1998; Satokata and Maas, 1994; Vainio et al., 1993). We found that Wnt4-producing cells were able to stimulate endogenous Msx1 in E10 mandibular mesenchyme explants, whereas no Msx1 was observed around the control NIH3T3 cell clusters (Fig. 6H1-I2). No effects on Pax9 expression in E10.5 presumptive dental mesenchyme around the cells were observed (not shown).

Tgfβ1 stimulates dental mesenchymal cell proliferation

Because Tgfβ1 is prominently expressed in the highly proliferative cells in the cervical loops and dental papilla mesenchyme during the cap stage (Vaahtokari et al., 1991) when dental axons are growing around the tooth germ, we analyzed the effects of Tgfβ1 on dental cell proliferation. Tgfβ1-soaked agarose beads were applied onto the molar area of isolated dental mesenchyme at E12 when Tgfβ1 is expressed in the epithelial bud but not in the underlying mesenchyme. The explants were cultured for 24 hours, the last 1.5 hours with 5-bromo-2-deoxyuridine (BrdU). Whole-mount and sectional immunohistochemical analysis showed that E12 dental mesenchymal cells around Tgfβ1 and positive control Fgf2 (Kettunen et al., 1998)–releasing beads had incorporated BrdU markedly (Fig. 7A-D). This mimicked the effects of E12 dental epithelia, which also stimulated the proliferation of adjacent dental mesenchymal cells in homochronic recombinants (Fig. 7C,D). No elevated BrdU incorporation was seen in explants cultured with BSA-soaked beads (Fig. 7E). Thus, besides controlling the establishment of tooth innervation, Tgfβ1 may regulate tooth morphogenesis by stimulating dental cell proliferation.

Discussion

Sema3a regulates timing of tooth innervation and dental axon navigation and patterning

Teeth are essential for survival, and the presence of a sensory innervation is of great importance for their function and protection. To investigate the regulatory mechanisms of peripheral axon guidance and patterning, we studied the functions and regulation of Sema3a in the formation of the mouse lower molar, which is tightly linked with the development and patterning of the mandibular process.

Analysis of Sema3a knockout mice revealed that Sema3a is an essential signal for the establishment of early tooth innervation, though not for its formation, and that its effects appear to be mediated by the Npn1 receptor expressed in the dental axons. That the nerve fibers prematurely innervate the Sema3a−/− tooth already at the epithelial thickening stage indicates that Sema3a, by forming exclusion areas in the jaw and presumptive dental mesenchyme, regulates the timing of tooth innervation by apparently by preventing ingrowth of other trigeminal nerve fibers such as buccal nerve. At the early bud stage, Sema3a signaling appears to regulate the formation of the single ‘molar’ nerve and channels the growth of pioneer dental axons to the restricted mesenchymal pathway towards the tooth. During subsequent morphogenetic bud, cap and bell stages, Sema3a restricts axon growth into the mesenchymal target field around the tooth and prevents their ingrowth to the condensed dental mesenchyme and the dental papilla. The absence of Sema3a from the sites of the developing secondary apical foramina during E18-PN4 suggests that Sema3a...
exclusion areas in the base of the dental papilla do not determine the timing of nerve fiber penetration to the dental papilla but are involved in regulation of the sites through which the nerve fibers are able to enter, i.e. through the forming root canals.

We also noticed that many nerve fibers showed largely normal localization within the tooth target field in Sema3a−/− embryos, and that errors in dental axon patterning became increasingly corrected as tooth morphogenesis proceeded, which is in line with the corrections of other sensory axon projections in Sema3a−/− mice (White and Behar, 2000). We found that mRNA expression of Ngf, Gdnf, Larr, Ncam and Net3 was not affected in the dental follicle target area of the Sema3a−/− teeth. This indicates that their expression is not regulated or dependent on Sema3a, and that they appear to partially rescue tooth innervation phenotype in Sema3a−/− mice. Thus, these results provide genetic evidence for the model that axon guidance and establishment of tooth nerve supply involves redundant and independent signaling of neuroregulatory genes of different families.

Epithelial-mesenchymal interactions regulate the establishment of tooth nerve supply

The finding that precisely regulated expression domains of Sema3a are crucial to the timing of tooth innervation as well as dental axon guidance and patterning led us to use Sema3a as a marker gene for analysis of the basal regulatory mechanisms behind the establishment of tooth nerve supply. By performing tissue recombination experiments, we found that the oral epithelium is necessary for Sema3a expression in the E10.5 and E11 mandibular mesenchyme, and that E10.5 oral epithelium as well as E11 and E12 dental epithelia are able to induce Sema3a expression in the presumptive molar mesenchyme area of the lower jaw lacking Sema3a. Furthermore, we showed that later at E12 when the first dental axons are about to or are navigating to the developing tooth, dental epithelium controls Sema3a in the dental mesenchyme. Thus, these results suggest that local epithelial-mesenchymal interactions control Sema3a expression and the establishment of tooth innervation. As tooth formation has been shown to be controlled by interactions between epithelial and mesenchymal tissues (Lumsden, 1988; Mina and Kollar, 1987), tissue interactions may therefore provide a mechanism to coordinate axon navigation and patterning spatiotemporally with tooth formation.

Earlier tissue recombination studies have shown that E10-E11 mouse oral and dental epithelium possesses instructive information to control tooth formation as well as the potential to determinate tooth type (Lumsden, 1988; Mina and Kollar, 1987; Tucker et al., 1998). Our tissue recombination experiments showing that the E10.5 oral and E11 dental epithelia induce mesenchymal Sema3a expression indicate that, besides the odontogenic information, the presumptive dental epithelium also possesses the instructive information to control the formation of early tooth nerve supply and possible tooth-specific sensory innervation that is distinct, in some aspects, from the adjacent cutaneous sensory system (Fried et al., 2000; Kvinnsland et al., 2004). Furthermore, because the formation of teeth of all types is controlled by the epithelial-mesenchymal interactions, the interactions also appear to provide a rationale for the fact that the timing and pattern of tooth innervation in different teeth and species correlate better to the developmental stage of the individual tooth than the chronological age of the animal.

Epithelial-mesenchymal interactions mediated by Wnt4 and Tgfβ1 may coordinate trigeminal axon navigation and patterning with tooth formation

Early oral and dental epithelium expressed signaling molecules have been implicated in the mediation of organogenetic tissue interactions. We found that oral and dental epithelium expressed Wnt4 induces Sema3a in the mandibular

![Fig. 8. Schematic model for coordination of early tooth organogenesis and establishment of nerve supply by epithelial-mesenchymal interactions. The Sema3a exclusion areas (in red) regulate timing of tooth innervation and the innervation pattern. Prior to the histological onset of tooth formation (E10.5), the odontogenic oral epithelium, which instructs tooth formation and also possesses information to control tooth-specific nerve supply, induces (mediated by Wnt4) Sema3a in the presumptive dental mesenchyme. During subsequent morphogenesis epithelial signaling and Wnt4 and Tgfβ1 continue to control Sema3a expression domains in the dental mesenchyme target area. Wnt4 and Tgfβ1 contribute to the regulation of tooth morphogenesis by maintaining Mst1 (the effect of Wnt4 on Mst1 expression at E11.5 is hypothetical) and stimulating dental mesenchymal cell proliferation, respectively. The trigeminal molar nerve located in the mesenchymal axon pathway and tooth target fields are indicated in black.](image-url)
Development

Results show that β expression in the dental mesenchyme. Thus, Wnt4 and Tgfβ1 may act as in vivo epithelial signals that control mesenchymal Sema3a expression. Of particular interest is the observation that Wnt signaling has been shown to be essential for tooth formation, as evidenced by the finding that overexpression of the Wnt inhibitor Dickkopf1 in the BA1 ectoderm and targeted inactivation of Lef1 transcription factor (which is needed for Fgf4 expression in the primary enamel knot signaling center) in transgenic mice result in arrest of tooth formation prior to the bud and cap stages, respectively (Andl et al., 2002; van Genderen et al., 1994; Kratochwil et al., 2002). Epithelial Fgf4 induces Fgf3 expression in the dental mesenchyme (Kettunen et al., 2000), which is required for Shh expression in the future enamel knot (Kratochwil et al., 2002). In addition, it has been suggested that interactions between epithelial expressed Wnt7 and Shh determine the position of tooth initiation (Sarkar et al., 2000). We have found that Wnt4 maintained mesenchymal expression of Mxl1 transcription factor, which is essential for tooth morphogenesis (Satokata and Maas, 1994) in the early jaw mesenchyme, whereas Tgfβ1, the expression of which correlates with tooth morphogenesis, stimulated the proliferation of the dental mesenchymal cells. Thus, besides regulating Sema3a, Wnt4 and Tgfβ1 appear to be involved in the regulation of tooth formation. We propose that they may act as signals that mediate epithelial-mesenchymal interactions and coordinate trigeminal axon growth and patterning with tooth formation (Fig. 8).

Epithelial-mesenchymal interactions may coordinate establishment of the peripheral nerves with outgrowth and patterning of the mandibular process During development of the nervous system, peripheral axons establish nerve tracts and contacts to target tissues and organs (the development of most of which is regulated by epithelial-mesenchymal interactions) very accurately in place and time. Developmentally regulated signaling of Sema3a is shown to serve crucial functions for trigeminal axon pathfinding and patterning in the mandibular process (Dillon et al., 2004; Taniguchi et al., 1997), which grows out, undergoes patterning and develops into teeth, various tissues and skeletal elements. Like the formation of the tooth, the development of the BA1 is regulated by reciprocal epithelial-mesenchymal interactions, and epithelial signaling controls the expression of transcription factors involved in the establishment of the polarity and pattern of the BA1 (Cobourne and Sharpe, 2003). Furthermore, epithelial signaling controls the expression of Nt3, which promotes trigeminal axon growth, in the maxillary process mesenchyme (O’Connor and Tessier-Lavigne, 1999). Our results show that Sema3a expression in the E10.5-E11 early mandibular mesenchyme is dependent on the overlying epithelium and that epithelial Wnt4 induces the expression of Sema3a and maintains Mxl1 when epithelial signaling controls the patterning of the process (Ferguson et al., 2000) and nerve branches in the mandibular process are being established (Lumsden, 1982). Hence, these results suggest that tissue interactions may link the establishment and patterning of the peripheral nerves to the outgrowth and patterning of the lower jaw. Thus, given the remarkable similarities of the developmental processes, it is tempting to propose that epithelial-mesenchymal interactions may provide an important mechanism for coordinating tissue and organ formation, and establishment of the peripheral nerve supply.

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