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Developmentally regulated expression of Sema3A chemorepellant in the developing mouse incisor

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Abstract

Objective. Semaphorin 3A (Sema3A) is an essential chemorepellant controlling peripheral axon pathfinding and patterning, but also serves non-neuronal cellular functions. Incisors of rodent are distinctive from molars as they erupt continuously, have only one root and enamel is present only on the labial side. The aim of this study is to address putative regulatory roles of Sema3A chemorepellant in the development of incisor innervation and formation. ***Materials and methods.*** This study analyzed expression of Sema3A mRNAs during embryonic and early post-natal stages of mouse mandibular incisor using sectional radioactive *in situ* hybridization. ***Results.*** Although Sema3A mRNAs were observed in condensed dental mesenchyme during the early bud stage, they were absent in dental papilla or pulp at later stages. Sema3A mRNAs were observed in the dental epithelium including the cervical loops and a prominent expression was also seen in alveolar bone. Interestingly, transcripts were absent from the mesenchymal dental follicle target area (future periodontal ligament) throughout the studied stages. ***Conclusion.*** The expression patterns of *Sema3A* indicate that it may control the timing and patterning of the incisor innervation. In particular, Sema3A appears to regulate innervation of the periodontal ligament, while nerve penetration into the incisor dental pulp appears not to be dependent on Sema3A. Moreover, Sema3A may regulate the functions of cervical loops and the development of alveolar bone. Future study with Sema3A deficient mice will help to elucidate the putative neuronal and non-neuronal functions of Sema3A in incisor tooth development.

Key Words: *axon guidance, incisors, periodontal ligament, radioactive* in situ *hybridization, Semaphorin 3A*

Introduction

Teeth are experimentally proven models to study molecular mechanisms behind morphogenesis [1]. Since teeth are richly supplied with sensory and sympathetic nerve fibers from trigeminal and superior cervical ganglia [2], respectively, they are also advantageous models to study peripheral innervation [3].

Histologically, tooth development has been divided into three partially overlapping phases; initiation, morphogenesis and cell differentiation [3]. Teeth develop as the result of sequential and reciprocal interactions between epithelial and neural crest-derived mesenchymal cells [1,4]. Signaling molecules, in particular belonging to conserved signaling pathways such as Tgfβ (transforming growth factor), Fgf (fibroblast growth factor), Wnt, Hedgehog and Bmp (bone morphogenic protein) have been shown to mediate these inductive tissue interactions. Many of the signals are repetitively used during tooth development and they are modulated by the activators and inhibitors [4,5]. The size and shape of the final molar tooth crown is determined during cap and bell stages by signals from the enamel knots signaling centers [1,4,6].

The development of tooth innervation takes place in a spatiotemporally controlled manner and is tightly linked to the advancing tooth formation [7,8]. Peripheral nerve fibers are guided to their final targets by axon guidance molecules of different families including slits, ephrins and ephs as well as semaphorins and there is increasing evidence that the same molecule families are also involved in regulation of tooth innervation [7,9].

Semaphorin 3A (Sema3A) is a member of secretory semaphorin 3 sub-class (Sema3A- Sema3G) of axon

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guidance molecules [10]. Sema3A repels growth cones of sensory and sympathetic nerve fibers [11,12] by surround repulsion mechanism in which nerve fibers are guided to final target organs and kept fasciculated by Sema3A expressing intervening tissues (exclusion areas) [10]. Extensive defasciculation of cranial and spinal peripheral nerve fibers are seen in Sema3A deficient (*Sema3A*$^{-/-}$) mice [13]. In the developing mouse molar tooth, *Sema3A* is expressed in the mesenchymal exclusion areas avoided by growing dental nerve fibers. In *Sema3A*$^{-/-}$ tooth, dental nerves show abnormal fasciculation as well as premature and ectopic penetration into these areas [7,14]. Thus, Sema3A coordinates both timing and patterning of molar tooth innervation. Because epithelial–mesenchymal interactions and signaling molecules, which regulate tooth formation, also control Sema3A expression, they integrate tooth formation with innervation [14,15].

Incisors of rodents are different from other mammals as they erupt continuously throughout life due to the proliferation of the stem cells in the epithelial cervical loops [16,17]. In contrast to the mandibular molars, which have two roots, mesial and distal ones, the incisors have only one root. Moreover, enamel forms only on the labial side of the tooth crown and the lingual side is covered only with dentine [16,17]. The dental pulp of the incisor also receives sensory and sympathetic nerve fibers from the trigeminal and superior cervical ganglia, respectively [18,19]. However, the dental pulp of incisor is sparsely innervated as compared to that of the molar [2,20,21]. Furthermore, in contrast to molar, incisor periodontal ligament is richly innervated with mechanoceptive Ruffini endings [22].

Due to the apparent differences in the molecular regulation of the tooth types, incisors of rodents are different from molars both in development, structure and innervation. Therefore, the developing incisor is a good model to further dissect out regulatory molecular mechanisms of odontogenesis. To analyze putative functions of Sema3A in the development of incisor innervation and formation, we studied cellular expression patterns of Sema3A mRNAs in embryonic and early post-natal mouse incisor using radioactive *in situ* hybridization.

Materials and methods

The use of the animals was approved by the Department of Biomedicine, Faculty of Medicine and Dentistry, University of Bergen under the surveillance of the Norwegian Animal Research Authority. Outbred stock mice (NMRI) were mated and the presence of vaginal plug was taken as day 0 of embryogenesis (E0). Mice were collected on embryonic days 11, 12, 13, 14 and 16 and post-natal days 0, 4 and 5. The heads of embryos and mandibles of post-natal pups were fixed in 4% paraformaldehyde in PBS, demineralized in ethylenediaminetetraacetic (EDTA) acid and embedded in paraffin. Serial frontal (E11–E16), and sagittal (PN0, 4 and 5) sections were cut with a Leica microtome. Sectional radioactive *in situ* hybridization was done as described in Luukko et al. [23]; 2.9 kb mouse Sema3A cDNAs were used for *in vitro* transcription of 35S-UTP antisense and sense probes [14]. Sections were exposed for 3–4 weeks and no specific hybridization signal was detected in sections hybridized with control sense probes (not shown). All serial sections were observed with high and low magnification objectives and representative bright and dark field images were taken with 5 × 0.15 NA and 10 × 0.3 NA objective using a Zeiss Axioskop 2 Plus microscope. Image plates were made with Adobe Photoshop CS4 software.

Results

At embryonic day 11 (E11) the formation of the incisor tooth germs as epithelial ingrowths was not yet visible. At this stage *Sema3A* expression was seen in the oral epithelium and mesenchyme including the presumptive area of the incisor tooth germs (Figures 1A1 and A2). The middle part of the mandibular process mesenchyme, however, was devoid of signals. One day later (E12), expression of Sema3A mRNAs continued in the oral epithelium but the transcripts were mostly absent from the invaginated early epithelial dental bud. Some transcripts were seen in the presumptive incisor mesenchyme. A prominent *Sema3A* expression was apparent in the mesenchymal condensates of the Meckel's cartilages (Figures 1B1 and B2). At E13 the dental epithelium had reached the bud stage. *Sema3A* showed strong expression in the central part of the epithelial bud and in the alveolar mesenchyme surrounding the tooth germ (Figures 1C1 and C2). In addition, Sema3A mRNAs were seen in condensed dental mesenchyme (future dental pulp), whereas the presumptive dental follicle target area (future periodontal ligament) was devoid of transcripts (Figures 1C1 and C2). At E14 *Sema3A* expression was seen in the outer dental epithelium extending to the cervical loop area, but neither the inner dental epithelium nor the dental papilla showed a positive hybridization signal (Figures 1D1 and D2). Sema3A mRNAs were expressed in the developing alveolar bone around the tooth germ. Of note, no specific expression of *Sema3A* was observed in the dental follicle target area (Figures 1D1 and D2). At E16 *Sema3A* mRNAs were seen in the outer dental epithelia, cervical loops and stellate reticulum. In addition, the developing alveolar bone showed some transcripts (Figures 1E1 and E2). As in earlier stages, Sema3A transcripts were not seen in the dental follicle target area or dental papilla.

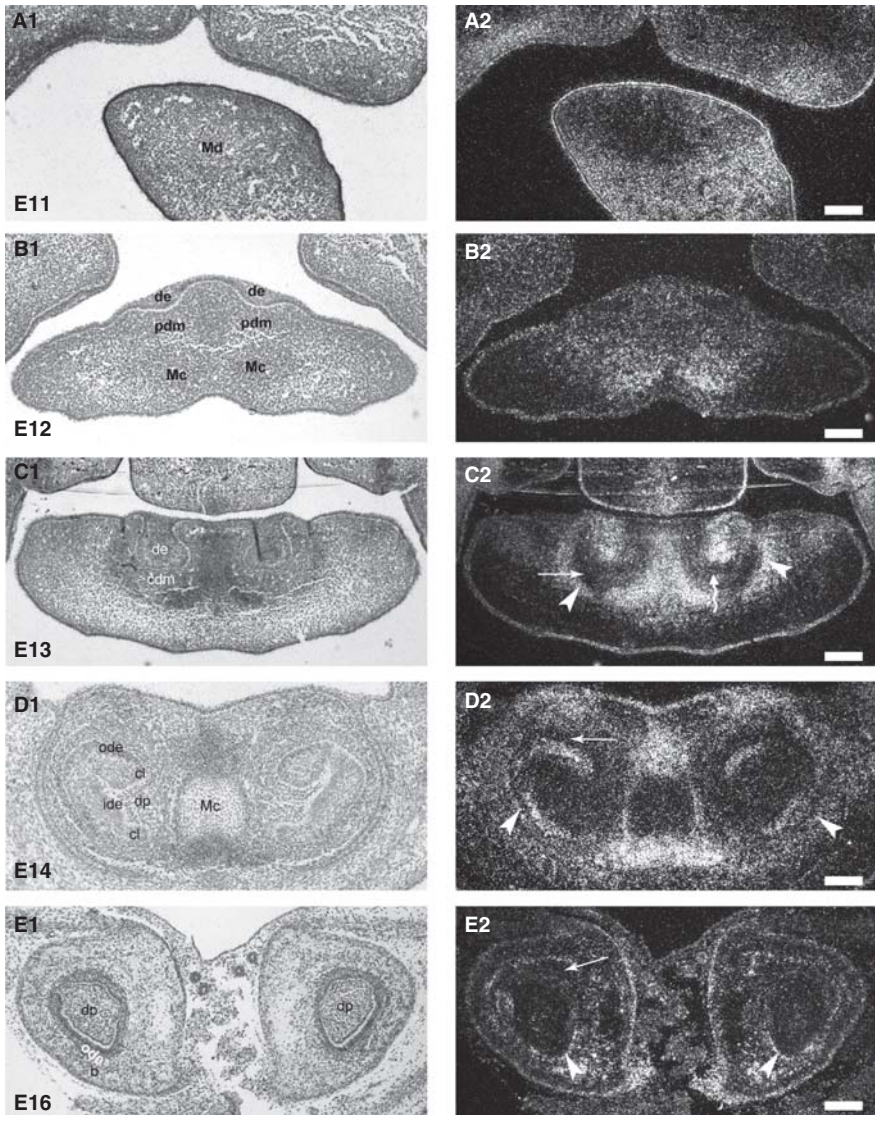

Figure 1. Bright (A1–E1) and dark field (A2–E2) images of frontal sections of heads of embryonic days (E) 11, 12, 13, 14 and 16 mice showing *Sema3A* expression in the incisors and surrounding areas. The plane of the section is slightly tilted in A1 and A2. b, developing alveolar bone; cdm, condensed dental mesenchyme; cl, cervical loop; de, incisor dental epithelium; dp, dental papilla; ide, inner dental epithelium; Mc, Meckel's cartilage; md, mandible; ode, outer dental epithelium; pdm, presumptive dental mesenchyme. Straight arrows in C2, D2 and E2 show absence of *Sema3A* expression in the dental follicle target area; arrow heads show *Sema3A* expression in developing alveolar bone and the curved arrow in C2 shows *Sema3A* expression in condensed dental mesenchyme. Scale bars = 100 μm.

After birth (PN0), *Sema3A* expression continued in the cervical loops and outer dental epithelia both on the labial and lingual side of the tooth germ (Figures 2A1 and A2). A prominent expression was also evident in the surrounding alveolar bone. During later post-natal development, a notable *Sema3A* expression continued in the cervical loops and outer dental epithelium both on the labial and lingual side as visualized for the 4- and 5-day post-natal incisors. Moreover, like at PN0, a prominent expression persisted in the alveolar bone (Figures 2B1–C2). No specific expression of *Sema3A* was seen in the mesenchymal cells of the dental pulp including the area of the primary apical foramen or in the dental follicle target area at any post-natal stages studied (Figures 2B1–C2).

Discussion

Semaphorin 3A (Sema3A) signaling is essential for peripheral axon pathfinding and patterning [10,13]. We report for the first time that *Sema3A* shows spatiotemporally regulated expression patterns in the developing mouse incisors.

Sema3A may control dental axon pathfinding and periodontal ligament innervation

Although there is no detailed information available regarding the development of the incisor innervation at different histo-morphological developmental stages, we observed apparent correlations between expression patterns of *Sema3A* and assumed

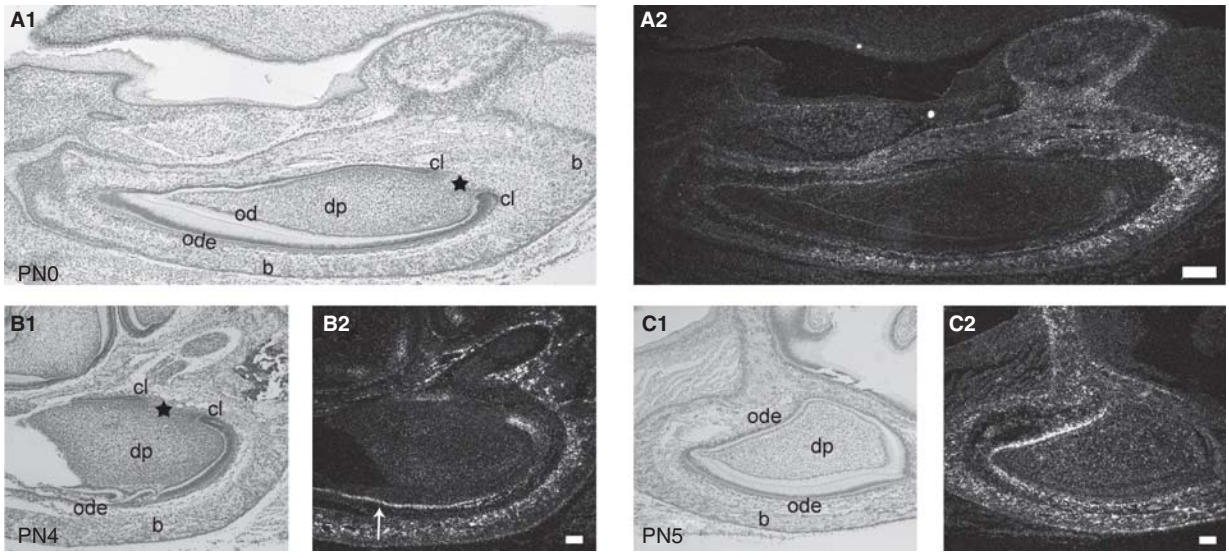

Figure 2. Bright (A1–C1) and dark field (A2–C2) images of sagittal (A and B) and coronal (C) sections of mandibles of post-natal day (PN) 0, 4 and 5 mice showing *Sema3A* expression in the incisors and surrounding areas. b, alveolar bone; cl, cervical loop; dp, dental papilla; od, odontoblasts; ode, outer dental epithelium. Arrow in B2 shows absence of Sema3A expression in dental follicle target area and stars in A1 and B1 show apical foramen. Scale bars = 100 μm.

localization of nerve fibers [20,24]. We found that Sema3A mRNAs were prominently expressed in the mandibular process mesenchyme at E11, whereas the middle part of the mandibular mesenchyme corresponding to the area where inferior alveolar nerve was located [24] was devoid of signals. At the early bud stage (E12) when the pioneer incisor nerve fibers ('the incisor nerve') emerging from the main trunk of the inferior alveolar nerve reach the incisor tooth germ [24], some *Sema3A* expression was present in the presumptive dental mesenchyme. Targeted inactivation of Sema3A expressed in the early molar tooth germ results in premature, ectopic innervation of the tooth germ as well as patterning defects indicating that Sema3A regulates the formation of the 'molar' nerve and channels its growth [14]. Therefore, based on the reported localization of incisor nerve fibers, the *Sema3A* expression we observed and the essential functions of Sema3A in the first branchial arch [13] and molar [7], we suggest Sema3A serves important roles in the establishment of the incisor tooth innervation. Before the formation of incisor nerve Sema3A expressed in the mandibular process guides the growing mandibular nerves and keeps them fasciculated. Later Sema3A is suggested to guide the newly formed incisor nerve towards the tooth germ and keep it fasciculated.

During later stages, the incisor nerve divides into smaller branches and innervates the presumptive dental follicle target area (future periodontal ligament) [20,24]. Most of these nerve fibers reside in the periodontal ligament as Ruffini mechanoreceptors, but some of them enter into the incisor dental pulp [20,21,25]. We found no specific expression of *Sema3A* in the presumptive dental follicle target area starting from the late bud stage (E13) up to post-natal stages. We also noticed a high level of *Sema3A* expression in the adjacent developing alveolar bone during this period. These results suggest that the absence of Sema3A expression in the dental follicle target area allows nerve fibers to grow and innervate this area, i.e. the future periodontal ligament. *Sema3A* expressed in the adjacent developing alveolar bone is suggested to exert surround repulsion [10] and channel the growth of nerve fibers within the developing periodontium, as has been proposed for the molar tooth germ [7]. In addition, Sema3A expressed in the alveolar bone may prevent its innervation. Innervation of the tissues and organs is not only regulated by axon repellants but also attractants as well as survival factors, which sustain nerves after they have reached their targets. Therefore, it is apparent that other locally produced neuroregulatory factors such as neurotrophins are involved in innervation of dental follicle target area of the incisor [22,26].

Sema3A is not needed for timing of pulp innervation

Whereas mouse mandibular molar has two roots, the incisor is a single rooted tooth. We have shown recently that nerve fibers enter the molar pulp through secondary apical foramina, i.e. the sites where the future mesial and distal roots develop [3]. We have also found that *Sema3A* is expressed at the base of the dental pulp mesenchyme, but it is specifically absent from the sites of the secondary apical foramina prior to and during nerve fiber penetration into the dental pulp [14]. This suggests that repellant signaling activity of Sema3A forces sensory nerve fibers to enter the dental pulp specifically through the secondary apical

foramina before there is any histological evidence of pulp floor formation [3,14]. In line with these findings, we observed *Sema3A* transcripts in the condensed dental mesenchyme of the E13 bud stage incisor tooth germ, but they were absent from the dental papilla and pulp at later stages. This suggests that down-regulation of Sema3A from the apical foramen is needed to allow nerve fiber penetration into the incisor dental pulp. However, because nerve fibers appear to penetrate into the mouse incisor dental pulp post-natally long after [20] the down-regulation of the Sema3A from the dental papilla mesenchyme, Sema3A appears not to regulate the exact timing of the incisor pulp innervation.

Sema3A may serve non-neuronal roles in incisor formation

We also found Sema3A mRNAs in sites which did not show any apparent correlation with development of tooth innervation such as the outer dental epithelium and cervical loops. In contrast to molars, incisors of rodents are different from other mammals as they erupt continuously throughout life due to the proliferation of the epithelial stem cells in the cervical loops [16]. The proliferation and fate of the stem cells are proposed to be regulated by the different signaling molecules such as Fgf10, Fgf3, Follistatin and Sprouty [4,17,27]. Although Sema3A was first characterized as an axon repellant molecule [11], it has been shown to serve broader developmental and cellular functions such as in cell proliferation, migration, adhesion and cell death [10,28]. Therefore, it is tempting to speculate that Sema3A might also play a role in the regulation of the cellular functions in the cervical loops. Previously bone and cartilage abnormalities were reported in *Sema3A*$^{-/-}$ mice [29]. We indeed observed a prominent *Sema3A* signal in the developing mandible and alveolar bone already from the early stages of their histological formation. Thus, Sema3A may also function as a regulator of tooth supporting bone.

In conclusion, the temporal and spatial expression pattern of *Sema3A* in rodent incisor suggests neuronal and non-neuronal functions in the development of the tooth proper and its supporting tissues. Future loss-of-function studies are needed to elucidate the *in vivo* functions of Sema3A signaling in incisor development.

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