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Noggin Is Required for Early Development of Murine Upper Incisors

ABSTRACT

BMP signaling plays crucial roles in the development of many organs, including the tooth. Equally important is BMP signaling homeostasis, as demonstrated by multiple organ defects in mice lacking the extracellular BMP antagonist Noggin. Here, we show that *Noggin* is initially expressed in the maxillary mesenchyme adjunct to the upper incisor at the initiation stage, and then in the developing teeth, including incisors and molars, from the bud stage. *Noggin* mutants develop normal molars and mandibular incisors, but form a single, medially located upper incisor that is arrested at the late bud stage. Histological and molecular marker analyses demonstrated that two distinct upper incisor placodes initiate independently at E11.5, but begin to fuse at E12.5, coupling with elevated cell proliferation rates in the developing tooth germs. We further found that *Chordin* and *Gremlin*, two other BMP antagonists, are co-expressed with *Noggin* in the developing lower incisor and molar teeth. These observations indicate the importance of BMP signaling homeostasis, and suggest a functional redundancy between BMP antagonists during tooth development.

KEY WORDS: BMP antagonist, BMP homeostasis, Chordin, Gremlin, tooth development, tooth fusion.

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INTRODUCTION

BMP signaling is introduced into cells *via* transmembrane serine/threonine kinase of type I and type II BMP receptors (Sieber *et al.*, 2009). The activated type I receptor phosphorylates the receptor-regulated Smads (R-Smad), which then bind to the common Smad (Smad4) and move into the nucleus to regulate gene expression. BMP signaling is modulated by several proteins at different levels (Gazzerro and Canalis, 2006). In the extracellular compartment, secreted BMP antagonists, such as Noggin, Chordin, and Gremlin, and other molecules, such as Wise, modulate BMP signaling activity by binding to selective BMP ligands, thus preventing binding of ligands to their receptors. In the intracellular compartment, BMP signal can be modulated by inhibitory Smads that compete with Smad4 or mediate R-Smad degradation.

While BMP signaling is essential for embryonic development, overactive BMP activities are detrimental, as exemplified in *Noggin* mutant mice. Noggin binds preferentially to BMP2, BMP4, and BMP7 to prevent their signaling (Zimmerman *et al.*, 1996; Groppe *et al.*, 2002). Deletion of the *Noggin* (*Nog*) gene in mice results in a series of defects in organogenesis, including a spectrum of craniofacial defects (Brunet *et al.*, 1998; McMahon *et al.*, 1998; Bachiller *et al.*, 2000; Stottmann *et al.*, 2001; Anderson *et al.*, 2006; He *et al.*, 2010). In addition, mice deficient for both *Noggin* and *Chordin* exhibit more severe defects in several organs, suggesting a functional redundancy among the BMP antagonists (Bachiller *et al.*, 2000; Stottmann *et al.*, 2001; Anderson *et al.*, 2006).

Several *Bmp* genes are expressed in the developing tooth and are implicated in regulating many aspects of tooth development, including the determination of tooth-forming sites and tooth type (Neubüser *et al.*, 1997; Tucker *et al.*, 1998; Zhang *et al.*, 2005), progression and growth (Chen *et al.*, 1996; Zhang *et al.*, 2000; Zhao *et al.*, 2000), terminal differentiation, and tooth eruption (Gluhak-Heinrich *et al.*, 2010; Yao *et al.*, 2010). Disruption of BMP signaling results in an arrest of tooth development (Andl *et al.*, 2004; Liu *et al.*, 2005; Li *et al.*, 2011). Given the importance of BMP signaling in tooth development and the absolute requirement of BMP homeostasis in the development of many other organs, we investigated *Noggin* expression patterns in developing teeth and analyzed tooth phenotypes in mice lacking *Noggin*.

MATERIALS & METHODS

Animals

The generation and genotyping of *Noggin* mutant mice ($Nog^{+/-}$) have been described previously (McMahon *et al.*, 1998). Embryos were collected

from timed-mated pregnant mice, and embryonic ages were determined by the day when the vaginal plug was discovered, which was designated as embryonic day 0.5 (E0.5). Embryonic tails were used for PCR-based genotyping.

Histology, *in situ* Hybridization, Immunostaining, X-Gal Staining, BrdU Labeling, and TUNEL Assay

Standard hematoxylin/eosin staining and non-radioactive *in situ* hybridization were conducted on paraffin sections as described previously (St. Amand *et al*., 2000). For immunostaining, samples were embedded in O.C.T. compound, cryo-sectioned, and subjected to antibodies against pSmad1/5/8 (from Cell Signaling Technology, Danvers, MA, USA) as described previously (He *et al*., 2010). For whole-mount X-Gal staining coupled with whole-mount *in situ* hybridization, samples were fixed in 4% PFA fixative, and subjected to X-Gal staining first, followed by whole-mount *in situ* hybridization without proteinase K treatment. X-Gal staining, BrdU labeling, and TUNEL assay were performed following protocols described previously (He *et al*., 2010).

In vitro Organ Culture and Bead Implantation

E11.5 embryos were collected from intercrosses of *Nog*$^{+/-}$ mice. Each embryo from one litter was labeled and the embryonic tail collected for genotyping. The embryonic head was dissected; the mandible was removed and placed in a Trowell-type organ culture with the oral side facing up. Agarose beads soaked with BSA or Noggin protein (from R&D Systems, Minneapolis, MN, USA) at a concentration of 50 ng/mL was implanted into the midline of, but anterior to, the 2 forming upper incisor germs. Samples were cultured in DMEM supplemented with 10% FCS for 24 hrs prior to being harvested for whole-mount *in situ* hybridization.

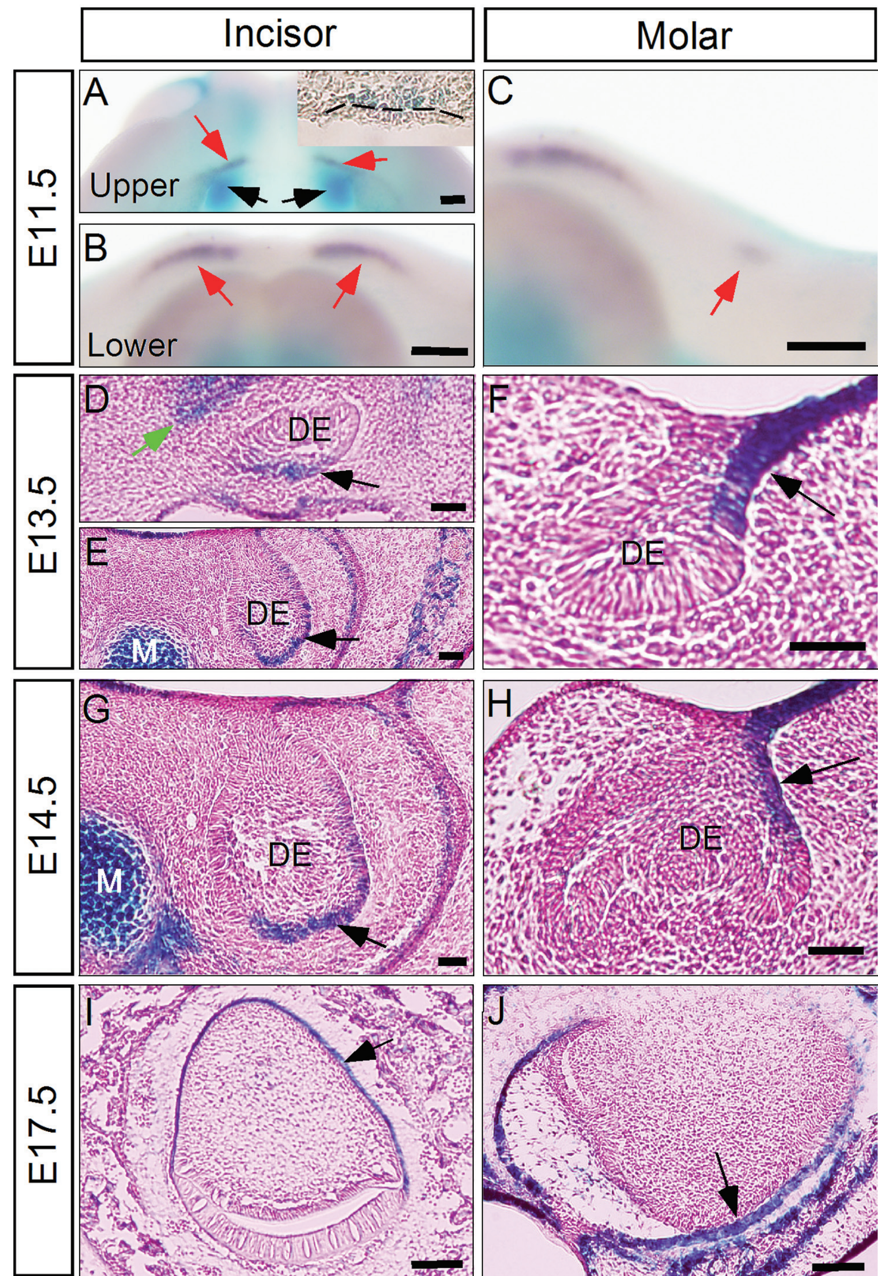

Figure 1. Expression of *Noggin* in the developing tooth. (A-C) At E11.5, X-Gal staining of the *LacZ-Nog* knock-in allele combined with whole-mount *in situ* hybridization reveals absent *Noggin* expression in the upper incisor **(A),** lower incisor **(B),** and lower molar **(C),** as localized by Shh expression (red arrows). However, strong *Noggin* expression, indicated by *LacZ* staining, is seen in the maxillary mesenchyme (black arrows), and in the upper incisor mesenchyme (insert in A). (D-F) At E13.5, *Noggin* expression, as detected by *LacZ* expression, is observed in the epithelium in upper **(D)** and lower **(E)** incisors and molars **(F).** Black arrows point to the expression sites. In the upper incisor at this stage, *Noggin* is also expressed in the adjacent maxillary mesenchyme (green arrow in D). (G, H) *Noggin* is continuously expressed in the epithelium of incisors **(G)** and molars **(H)** at E14.5. (I, J) At E17.5, *Noggin* expression is detected in the ameloblasts of the incisor **(I)** and molar **(J).** Dashed lines outline the dental epithelium. DE, dental epithelium. Scale bar: 100 µm.

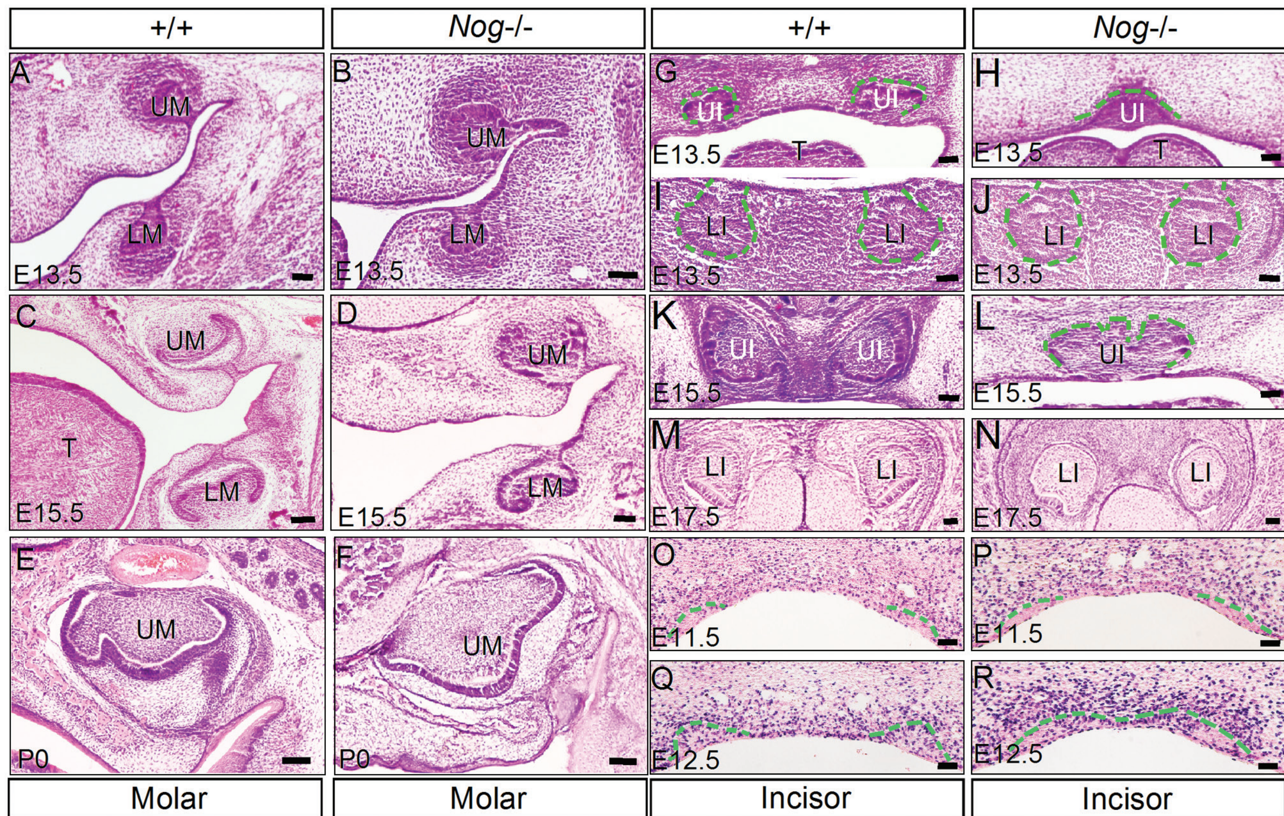

Figure 2. Tooth phenotype in *Noggin* mutants. (A, C, E) Coronal sections show molar development in the wild-type controls at E13.5 **(A)**, E15.5 **(C)**, and P0 **(E)**. **(B, D, F)** Coronal sections show comparable molar development in *Noggin* mutants at each corresponding stage. **(G)** A coronal section shows the formation of 2 maxillary incisor buds in a wild-type embryo at E13.5. **(H)** A coronal section through a $Nog^{-/-}$ embryo at E13.5 shows a single maxillary incisor bud located at the midline. (I, J) Coronal sections show comparable lower incisors in both wild-type **(I)** and $Nog^{-/-}$ **(J)** embryos at E13.5. (K, L) At E15.5, the wild-type upper incisors have developed to the late cap stage **(K),** but the single upper incisor in the mutant remained at the late bud stage **(L).** (M, N) Coronal sections show comparable development of the lower incisor in both wild-type **(M)** and $Nog^{-/-}$ **(N)** mice at E17.5. **(O-R)** BrdU labeling assay shows BrdU-positive cells in the upper incisor regions of both wild-type controls and *Noggin* mutant at E11.5 and E12.5. We measured cell proliferation rates by counting BrdU-positive cells and total cells in defined areas, including dental epithelium, the mesenchyme between the dental placodes, and dental mesenchyme. Three continuous sections from each of 2 individual samples of each genotype were counted, and the outcome was expressed as a percentage of BrdU-positive cells among total cells in each defined area. Student's *t* test was used to determine the significance of difference, and results are presented in the text and detailed in the Appendix Fig. Dashed lines outline the dental epithelium. T, tongue; LI, lower incisor; LM, lower molar; UI, upper incisor, UM, upper molar. Scale bar: 100 µm.

RESULTS AND DISCUSSION

Expression of *Noggin* in the Developing Tooth

To investigate the role of *Noggin* in tooth development, we first examined *Noggin* expression in the developing mouse tooth at several critical stages. We took advantage of the $Nog^{+/-}$ mice in which the *Noggin* coding sequences were replaced by the *LacZ* gene (McMahon *et al*., 1998). Utilizing whole-mount X-Gal staining on $Nog^{+/-}$ embryos, followed by whole-mount *in situ* hybridization on *Shh,* which serves as a molecular marker for the dental epithelium, we failed to detect *Noggin* expression in and around the lower incisor and molar placodes at E11.5 (Figs. 1B, 1C). However, strong *Noggin* expression was seen in the maxillary mesenchyme immediately posterior to the upper incisor placodes (Fig. 1A). *Noggin* expression was also found in some cells in the dental mesenchyme immediately underneath the placode epithelium (insert in Fig. 1A). At the E13.5 bud and E14.5 cap stages, *Noggin* expression was detected in the dental epithelium of the incisors and molars and was also observed in the maxillary mesenchyme adjacent to the upper incisor at the bud stage (Figs. 1D-1H). In the developing molars at both stages (Figs. 1F, 1H), *Noggin* expression was restricted in the dental epithelium on the buccal side, where *Bmp4* is preferentially expressed in the mesenchyme (Zhang *et al*., 2009). At the E17.5 bell stage, in the incisor, *Noggin* expression became localized to the dental epithelium on the lingual side (Fig. 1I), and was seen in the external enamel epithelium of the molar (Fig. 1J). This spatiotemporal *Noggin* expression profile prompted us to examine potential tooth phenotypes in $Nog^{-/-}$ mice.

Phenotypic Analysis of Developing Teeth in *Noggin* Mutants

At E13.5, the mutant molars developed to the bud stage, morphologically comparable with the wild-type controls (Figs. 2A,

2B). While the *Nog*$^{-/-}$ molars at E15.5 appeared slightly delayed in development (Figs. 2C, 2D), at post-natal day 0 (P0), we observed almost identically patterned and grown molars in both *Nog*$^{-/-}$ and wild-type mice (Figs. 2E, 2F), indicating that *Noggin* is dispensable for early molar development, consistent with a previous report (Stottmann *et al*., 2001).

Like the molar, the lower incisors developed indistinguishably from their wild-type counterparts (Figs. 2I, 2J, 2M, 2N). However, we found the presence of a single upper (maxillary) incisor bud at the midline at the E13.5 bud stage in 13 out of 14 samples examined (Fig. 2H). At E15.5, the single maxillary incisor developed slightly further, but was arrested at the late bud stage, when the control developed to the late cap stage (Figs. 2K, 2L). This residual tooth bud regressed thereafter, because it was not observed in embryos at E17.5 and P0 (data not shown). To investigate cellular alterations that may contribute to defective upper incisor development, we conducted TUNEL and BrdU assays on the *Nog*$^{-/-}$ upper incisor region at E11.5 and E12.5. While no obviously altered level of cell apoptosis was observed (data not shown), significantly increased levels of cell proliferation rates were found in the dental placode epithelium and in the intervening mesenchyme between the placodes of mutants at E11.5, as compared with controls ($p < 0.01$ in both sites) (Figs. 2O, 2P, Appendix Fig.). However, cell proliferation rates appeared similar in the oral epithelium between the dental placodes in both wild-type and mutants at this stage ($p > 0.1$). At E12.5, the cell proliferation rate in the mutant dental mesenchyme was elevated, as compared with that in controls ($p < 0.05$), but the mutant dental epithelium exhibited a decreased level of cell proliferation ($p < 0.05$) (Figs. 2Q, 2R, Appendix Fig.). These observations indicate that the lack of Noggin has a direct impact on the developing upper incisor. As shown in Figs. 2P and 2R, 2 separate upper incisor placodes were distinct at E11.5, but began to fuse at the midline at E12.5 of *Noggin* mutants. While this single upper incisor phenotype might have been an oversight in the previous study (Stottmann *et al*., 2001), this result was reported in a recent study (Lana-Elola *et al*., 2011). This formation of a single large incisor phenotype in the *Noggin* mutant is consistent with the proposed function of BMP signaling in the patterning of incisor placodes (Munne *et al*., 2010).

We next examined the expression of *Shh*, a molecular marker for the dental epithelium. Whole-mount *in situ* hybridization clearly revealed 2 separate upper incisor placodes in the *Nog*$^{-/-}$ embryo at E11.5 (N = 4; Figs. 3A, 3B), which was confirmed by *in situ* section hybridization (Figs. 3C, 3D), despite the lower level of expression. At E12.5, these 2 dental placodes began to fuse at the midline (N = 3; Fig. 3F), consistent with morphological observations. Our results appear to contradict those in the studies by Lana-Elola *et al.* (2011), who reported a single *Shh* expression domain in the upper-incisor-forming region of *Noggin* mutants at E11.5. This difference in results may be due to the transient nature of the separate *Shh* expression domains. The embryos in the other study may have been slightly older, and thus the *Shh* expression domain had already fused. These observations indicate that, in the absence of Noggin, the upper incisors were initially positioned correctly, but fused subsequently. To support this conclusion, we performed a rescue

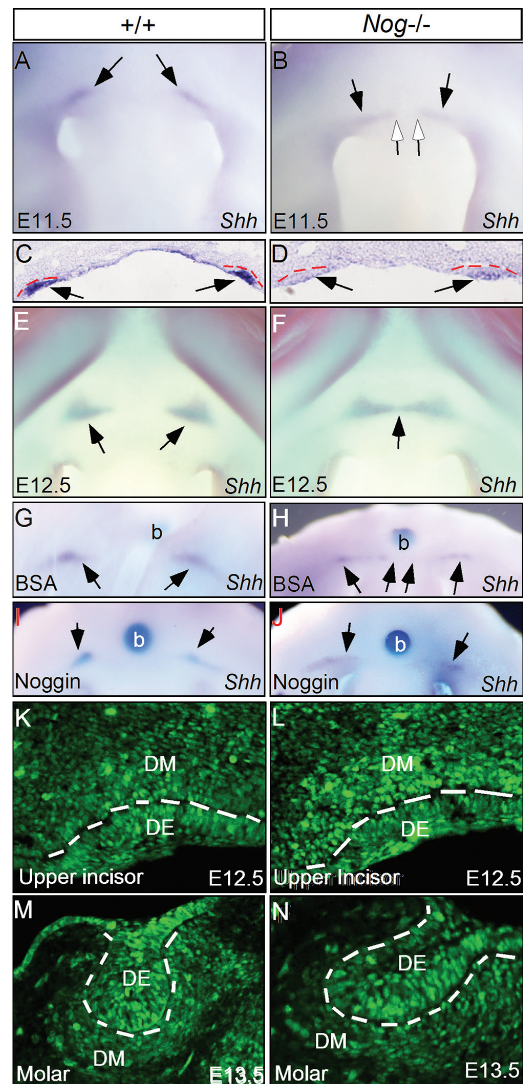

Figure 3. Lack of Noggin causes fusion of upper incisor placodes. (A-F) *Shh* expression indicates upper incisor placodes at E11.5 and E12.5. At E11.5, 2 separate *Shh* expression domains were seen in the maxillary region of control **(A, C)** and *Nog* mutant **(B, D)**. At E12.5, the 2 separate *Shh* expression domains remain in the control **(E)**. However, in the mutant, the 2 *Shh* expression domains have fused **(F)**. Black arrows points to *Shh* expression domains and open arrows in (B) point to the medial boundary of *Shh* expression domains. **(G)** An E11.5 wild type head implanted with a BSA bead shows 2 separate *Shh* expression domains after 24 hrs in organ culture. **(H)** An E11.5 *Nog*$^{-/-}$ head implanted with a BSA bead shows extension of *Shh* expression from the upper incisor placodes towards the midline after 24 hrs in organ culture. **(I)** An E11.5 wild type head implanted with a bead soaked with Noggin protein shows 2 separate *Shh* expression domains after 24 hrs in organ culture. **(J)** An E11.5 *Nog*$^{-/-}$ head implanted with a bead soaked with Noggin protein shows wild type like *Shh* expression domains after 24 hrs in organ culture. Black arrows point to *Shh* expression domains. **(K, L)** Immunohistochemical staining shows an enhanced level of pSmad1/5/8 staining in the mesenchyme and epithelium of the *Nog*$^{-/-}$ upper incisor (L), as compared to the wild type control (K). (M, N) Immunostaining shows comparable level of pSmad1/5/8 in the molar of control **(M)** and Noggin mutant **(N)**. Dash lines demarcate the dental epithelium. b, bead; DE, dental epithelium; DM, dental mesenchyme.

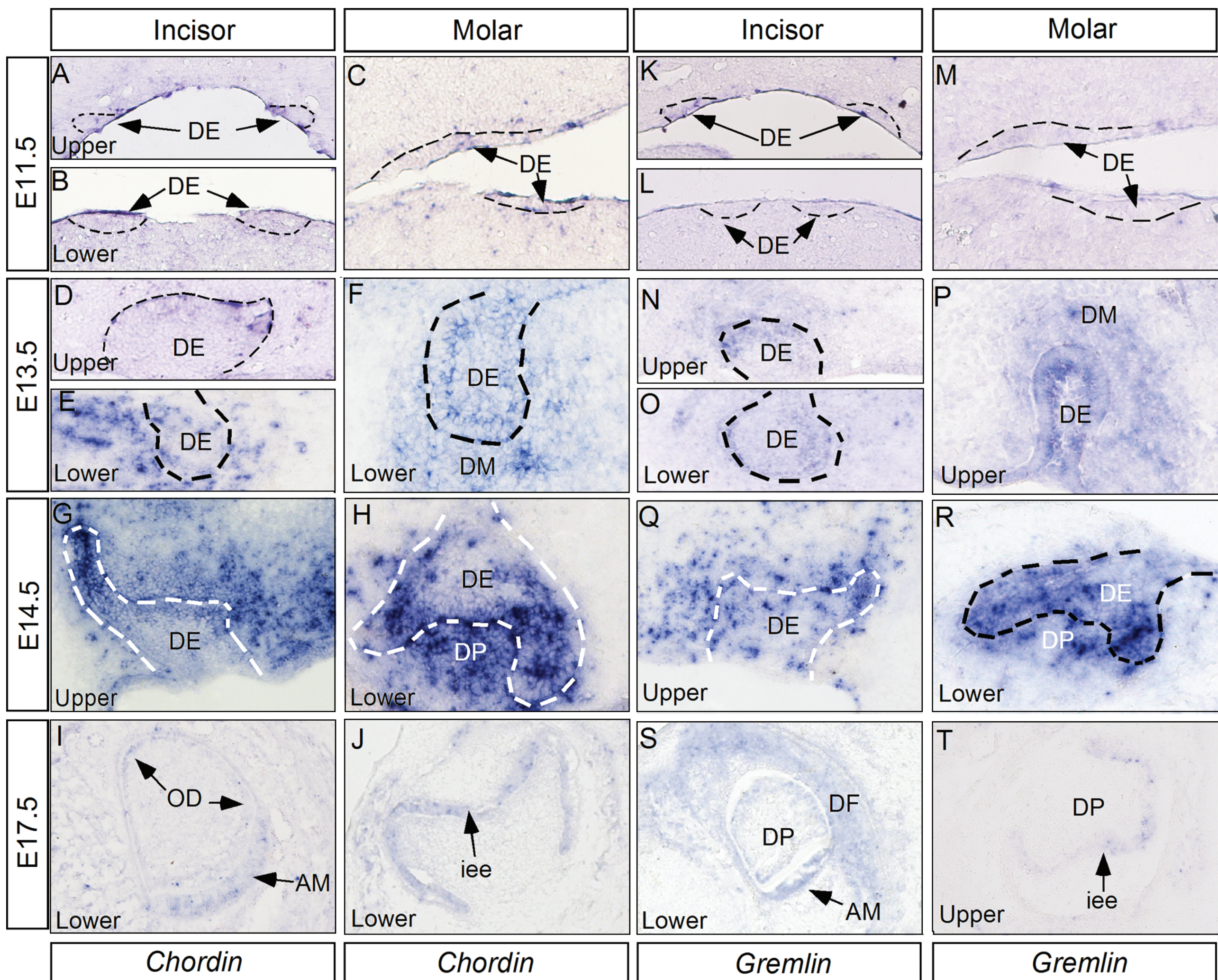

Figure 4. Expression of *Chordin* and *Gremlin* in the developing tooth. (A-C) *Chordin* expression is not seen in the tooth germs of upper incisors **(A),** lower incisors **(B),** and molars **(C)** at E11.5. (D-F) *Chordin* expression becomes detectable in the epithelium of upper incisors **(D),** and the epithelium and mesenchyme of lower incisors **(E)** and molars **(F)** at E13.5. (G, H) At E14.5, strong *Chordin* expression appears in the dental epithelium and mesenchyme of incisors **(G)** and molars **(H)**. (I, J) Weak *Chordin* expression is observed in the enamel epithelium of incisors **(I)** and molars **(J)** at E17.5. A lower level of *Chordin* expression is also seen in the odontoblasts. (K-M) *In situ* hybridization failed to detect *Gremlin* expression in the upper **(K)** and lower incisors **(L)**, and in molars **(M)** at E11.5. (N-P) *Gremlin* expression is detected in the epithelium and mesenchyme of upper **(N)** and lower incisors **(O),** as well as molars **(P)** at E13.5. (Q, R) At E14.5, *Gremlin* expression remains in the epithelium and mesenchyme of incisors **(Q)** and molars **(R)**. (S, T) Weak *Gremlin* expression is detected in the enamel epithelium of both incisors **(S)** and molars **(T)**. Dashed lines demarcate the dental epithelium. AM, ameloblasts; DE, dental epithelium; DM, dental mesenchyme; DP, dental papilla; OD, odontoblast; iee, inner enamel epithelium.

experiment. We implanted beads soaked with either BSA or Noggin protein close to the forming upper incisors of E11.5 *Nog*$^{-/-}$ heads that were placed in organ culture. In the samples implanted with BSA beads after 24 hrs in culture, *Shh* expression had extended from the incisor placodes toward the midline (N = 3; Fig. 3H), indicating the progression of placode fusion. However, this fusion process was prevented by exogenously applied Noggin protein, as evidenced by 2 separate *Shh* expression domains (N = 3; Fig. 3J). Consistent with the defective upper incisor is the elevation of BMP signaling activity at E12.5, as determined by immunostaining on phosphorylated Smad1/5/8 (pSmad1/5/8), an indicator of BMP-activated Smad signaling (Figs. 3K, 3L). However, the activity of BMP signaling appeared unaltered in the *Nog*$^{-/-}$ molars, as compared with the controls (Figs. 3M, 3N).

Expression of *Chordin* and *Gremlin* in the Developing Tooth

Since *Noggin* is expressed in the developing molar and lower incisor from the bud stage on (Fig. 1), we wondered why these teeth develop normally in the absence of *Noggin*. There exist several

other secreted BMP antagonists. While these BMP antagonists bind preferentially to selective BMPs and with different affinities, Chordin has been demonstrated to have a redundant role with Noggin in the development of several organs (Bachiller *et al*., 2000; Stottmann *et al*., 2001; Anderson *et al*., 2006). It is possible that other BMP antagonists have redundant functions to compensate for the loss of Noggin in the development of molars and mandibular incisors. To address this possibility, we examined the expression of *Chordin* and *Gremlin*, because Chordin and Noggin are classified in the same family of BMP antagonists (Sieber *et al*., 2009). While Gremlin belongs to the Cerberus/Dan family of modulators for TGF-β/BMP proteins and for Wnt proteins, it also has a role similar to that of Noggin in the inhibition of BMP activity, as demonstrated by the fact that overexpression of *Gremlin* or *Noggin* in the chick limbs exhibits similar effects in inhibiting chondrogenesis (Capdevila and Johnson, 1998; Merino *et al*., 1999). Almost identical expression patterns of both genes were found in the developing teeth (Fig. 4). The expression was barely detectable in all types of tooth germs at E11.5 (Figs. 4A-4C, 4K-4M), but was observed in both the epithelial and mesenchymal components of all tooth germs at E13.5 (Figs. 4D-4F, 4N-4P). At E14.5, the expression remained in the dental epithelium and mesenchyme, particularly in the epithelial tips (Figs. 4G, 4H, 4Q, 4R). The expression became down-regulated at E17.5, but was detectable in the enamel epithelium of both molars and incisors (Figs. 4I, 4J, 4S, 4T).

The strength of BMP activity, which is crucial for biological responses, depends on the concentration of BMP ligands and the availability of receptors and intracellular effectors, and is regulated by extracellular and intracellular modulators. The presence of BMP antagonists is essential for the maintenance of BMP homeostasis. This explains why BMPs and their antagonists are often co-expressed in developing organs or tissues and the existence of a Noggin-BMP autoregulatory loop, as evidenced by the fact that BMP and Noggin induce their mutual expression (Kameda *et al*., 1999; Nifuji and Noda, 1999; Stottmann *et al*., 2001; Ashique *et al*., 2002). Loss of a BMP antagonist in a developing embryo would disrupt the finely tuned BMP activity, leading to aberrant responses of tissues/cells to overactive BMP signaling.

The fact that *Noggin* is expressed in the developing tooth suggests a role for Noggin in modulating BMP activity during tooth development. However, despite the fact that loss of *Noggin* causes a series of defects in many organs, the early development of teeth (except the maxillary incisor) appears normal. One possibility is that the developing tooth has a higher tolerance to an enhanced level of BMP activity, but the fusion and an early developmental arrest of the maxillary incisor would argue against it. Alternatively, a plausible interpretation is the functional redundancy by other BMP antagonists. In fact, we showed that both *Chordin* and *Gremlin* are co-expressed with *Noggin* in the developing tooth from the bud stage until at least the E17.5 bell stage. In contrast to *Noggin* expression in the dental mesenchyme and the maxillary mesenchyme immediately adjacent to the upper incisors at the initiation stage (E11.5), expression of *Chordin* and *Gremlin* was detected neither in the developing tooth nor in the surrounding tissues at this stage. *Noggin* expression appears to have a direct impact on upper incisor development by regulating BMP activity. The absence of *Noggin* leads to overactive BMP signaling, which may induce an odontogenic fate in the epithelium between the dental placodes, as manifested by ectopic *Shh* expression. Meanwhile, the increased cell proliferation rate in the dental epithelium at E11.5 could cause encroachment of dental epithelium on the midline. These events together would contribute to the fusion phenotype. The application of exogenous Noggin to the mutants could have reversed these altered molecular and cellular events in the tissues between the placodes, leading to the increased spacing between the incisors. The expression of *Chordin* and *Gremlin* in the developing tooth from the bud stage could compensate for the absence of *Noggin*, allowing for normal development of molars and lower incisors in $Nog^{-/-}$ mice. The lack of potentially functional compensation from *Chordin* and *Gremlin* at the tooth initiation stage could contribute to the defectively developed maxillary incisor in $Nog^{-/-}$ mice, providing additional evidence for a requirement of BMP homeostasis in tooth development. Noggin is not required for the initial positioning of the upper incisor placodes, but is essential for the maintenance of their position and subsequent development. Taken together, our results support the idea that Noggin, Chordin, and Gremlin have functionally redundant roles in mouse tooth development.

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