Modulation of BMP signaling by Noggin is required for the maintenance of palatal epithelial integrity during palatogenesis

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Abstract

BMP signaling plays many important roles during organ development, including palatogenesis. Loss of BMP signaling leads to cleft palate formation. During development, BMP activities are finely tuned by a number of modulators at the extracellular and intracellular levels. Among the extracellular BMP antagonists is Noggin, which preferentialy binds to BMP2, BMP4 and BMP7, all of which are expressed in the developing palatal shelves. Here we use targeted *Noggin* mutant mice as a model for gain of BMP signaling function to investigate the role of BMP signaling in palate development. We find prominent *Noggin* expression in the palatal epithelium along the anterior-posterior axis during early palate development. Loss of *Noggin* function leads to overactive BMP signaling, particularly in the palatal epithelium. This results in disregulation of cell proliferation, excessive cell death, and changes in gene expression, leading to formation of complete palatal cleft. The excessive cell death in the epithelium disrupts the palatal epithelium integrity, which in turn leads to an abnormal palate-mandible fusion and prevents palatal shelf elevation. This phenotype is recapitulated by ectopic expression of a constitutively active form of BMPR-IA but not BMPR-IB in the epithelium of the developing palate; this suggests a role for BMPR-IA in mediating overactive BMP signaling in the absence of Noggin. Together with the evidence that overexpression of *Noggin* in the palatal epithelium does not cause a cleft palate defect, we conclude from our results that Noggin mediated modulation of BMP signaling is essential for palatal epithelium integrity and for normal palate development.

Keywords

Bmp signaling; Noggin; cleft palate; palatal epithelium; palate development

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Introduction

Cleft palate, one of the most frequent congenital birth defects in the human, results from genetic or environmental perturbations in palate development. Development of the mammalian secondary palate is a multiple staged process, which begins in mice at embryonic day 11.5 (E11.5), when the palatal shelves grow out of the bilateral maxillary processes. The palatal shelves continue to grow vertically along the developing tongue until E14.0, and then bend abruptly to a horizontal position above the tongue. At E14.5, the growing palatal shelves meet each other and fuse in the midline, separating the oral cavity from the nasal cavity. Each step of palate development, like the formation of many other mammalian organs, is directed by reciprocal and sequential epithelial-mesenchymal interactions (Ferguson and Honig, 1984; Ferguson, 1988; Hall, 1992).

The palatal shelves are composed of the epithelial covering and the mesenchymal tissue that is derived largely from cranial neural crest cells, and also from cranial paraxial mesoderm (Ferguson, 1988; Ito et al., 2003). The palatal epithelium, consisting of a basal columnar cell layer and covering periderm cells, is a heterogeneous structure. Based on the cell morphology, position, and genetic markers, the palatal epithelium can be divided into nasal, oral, and medial edge epithelium (MEE) (Ferguson, 1988). Prior to palatal elevation, the nasal portion of the palatal epithelium differentiates into pseudostratified nasal epithelial cells, and the oral portion differentiates into squamous oral epithelial cells. The MEE, which is positioned between the oral and nasal regions, develops into a single layered epithelial seam upon the contact and fusion of two palatal shelves, and ultimately diminishes to form an intact palatal shelf. During palate development, the integrity of palatal epithelium is essential for palate elevation. Disruption of this integrity would usually lead to abnormal adhesion or fusion between the elevating palatal shelves with adjacent structures, such as tongue and mandible, resulting in delayed or failed palate elevation, and consequently, generating a cleft palate defect (Rice et al., 2004; Alappat et al., 2005; Richardson et al., 2006; Xiong et al., 2009).

The Bone Morphogenetic Proteins (BMPs) have been implicated in mammalian palate development (Nie et al., 2006). Several *Bmp* genes, including *Bmp2*, *Bmp4* and *Bmp7*, are expressed in the developing mouse palatal shelves (Lu et al., 2000; Zhang et al., 2002; Nie, 2005; Levi et al., 2006). The requirement for BMP signaling in palate development was initially demonstrated in *Msx1* mutant mice, which exhibit the cleft palate phenotype (Zhang et al., 2002). In the *Msx1* mutant palate, *Bmp4* expression is abrogated and ectopic expression of its human ortholog rescues the cleft palate phenotype in *Msx1* mutant. Furthermore, tissue-specific inactivation of the genes encoding type I BMP I receptors, such as *Bmpr-IA* (*Alk3*) and *ActRIa* (*Alk2*), or overexpression of the BMP antagonist Noggin in the palatal mesenchyme leads to cleft palate formation in corresponding mouse models (Dudas et al., 2004; Liu et al., 2005; Xiong et al., 2009). These lines of evidence indicate an essential role for BMP signaling in palate development. On the other hand, inactivation of the promiscuous TGFβ antagonist *Follistatin* causes a cleft palate phenotype, raising the possibility that an elevated level of BMP signaling also impairs palate development (Matzuk et al., 1995).

During embryonic development, BMP signaling is finely tuned by a number of modulators at different levels (Gazzerro and Canalis, 2006). The intracellular modulators, such as Smad6, Smurf and Tob, can prevent R-Smads activation by interrupting their binding to receptors and Smad4, or mediate R-Smad degradation. At the extracellular level, BMP antagonists modulate BMP signaling activity by blocking selective ligands from binding to their receptors. A number of such extracellular antagonists have been documented, including Noggin, Chordin, Follistatin, and other molecules (Balemans and Van Hul, 2002; Gazzerro

and Canalis, 2006). Among them, Noggin is a secretory polypeptide that binds preferentially to BMP2, BMP4, and BMP7 to prevent their signaling (Zimmerman et al., 1996; Groppe et al., 2002; Chen et al., 2004). *Noggin* (*Nog*) deficient mice exhibit a series of defects in organogenesis (Brunet et al., 1998; McMahon et al., 1998; Bachiller et al., 2000), including a spectrum of craniofacial defects, accompanied with upregulation of BMP activities (Bachiller et al., 2000; Stottmann et al., 2001; Anderson et al., 2006). However, a cleft palate defect in $Nog^{-/-}$ mice has not been reported.

We have previously investigated the role of BMP signaling in palate development using loss-of-function models (Zhang et al., 2002; Xiong et al., 2009). In this study, we used a conventionally gene-targeted *Noggin* mutant line ($Nog^{-/-}$) (McMahon et al., 1998) as a gain-of-BMP function model to further evaluate the role of BMP signaling in palate development. We report here that $Nog^{-/-}$ mice exhibit a cleft palate defect. BMP/Smad signaling is ectopically activated in the $Nog^{-/-}$ palatal epithelium, consistent with the restricted *Noggin* expression pattern in the developing palate. Our results show that palatal epithelium integrity is disrupted in the $Nog^{-/-}$ palate and this disruption leads to an abnormal palate-mandible fusion, preventing the normal palate elevation. This phenotype is recapitulated in a transgenic model in which BMP receptor-IA mediated signaling is ectopically activated in the developing palate. In contrast, overexpression of *Noggin* in the palatal epithelium does not cause a cleft palate defect. We therefore conclude that Noggin-mediated repression of BMP signaling in the palatal epithelium is required for normal palate development.

Materials and Methods

Animals

The generation and genotyping of $Nog^{+/-}$, *K14Cre* and *pMesNog* mice have been described previously (McMahon et al., 1998; Andl et al., 2004; Xiong et al., 2009). In *Nog* mutant mice, a null *Nog* allele was created by fusing the first 10 amino acids of the *Noggin* coding sequence to the *lacZ* gene so that the *LacZ* expression is under the control of the *Nog* regulatory elements (McMahon et al., 1998). The *pMescaBmpr-IA* and *pMescaBmpr-IB* transgenic mice were generated in a strategy similar to *pMesNog* mice (Xiong et al., 2009). Briefly, a constitutively active form of the chick *Bmpr-IA* (*caBmpr-IA*) with Gln-233 to Asp replacement and a constitutively active form of *Bmpr-IB* (*caBmpr-IB*) with Gln-203 to Asp change (Zou et al., 1997) were cloned into *pMes-IRES-Egfp* vector at the 5' end of the *IRES-Egfp* sequence, and the 3' end of the *LoxP* flanked *STOP* cassette, which is under the control of the chick *β-actin* promoter. Pronuclear injection was performed to generate transgenic founders. Transgene expression in each potential transgenic line was identified by *Egfp* expression and further confirmed by in situ hybridization. Embryos were collected from time-mated pregnant mice and dissected in ice-cold PBS treated with diethyl pyrocarbonate (DEPC). Embryonic head samples were then separated from the trunk, fixed in 4% paraformaldehyde (PFA) overnight at 4°C, and processed for paraffin section or frozen section for immunostaining. A tail sample from each embryo was used for PCR-based genotyping (primer information available upon request). $Nog^{+/-}$ mice were maintained in a C57/B6 background. The *pMesNog*, *pMescaIA*, and *pMesBmpr-IB* transgenic mice were maintained in an outbred CD1 background. All animals and procedures used in this study were approved by the Tulane University Institutional Animal Care and Use Committee.

In vitro organ culture

Paired secondary palatal shelves from individual E13.5 embryos were isolated; the anterior halves of the palatal shelves were dissected and collected. Paired anterior palatal shelves were placed in Trowell type organ culture, and were orientated so that the MEE of each palatal shelf was in contact, as described previously (Taya et al., 1999; Zhang et al., 2002).

Samples were cultured in DMEM media supplemented with 20 % FCS at 37°C in an incubator filled with 5% CO_2 for 3 days, and were then harvested for histological examination.

Histology, in situ hybridization, immunostaining, and X-gal staining

After fixation, staged embryonic head samples were dehydrated through gradient ethanol series, cleared in xylene, and embedded in paraffin. Coronal sections at 10 µm were collected for either Hematoxylin/Eosin staining or non-radioactive in situ hybridization, as described previously (St. Amand et al., 2000). Three independent experiments were carried out for gene expression by in situ hybridization. Immunostaining was performed as described previously (Xiong et al., 2009). Phospho-Smad1 (Ser463/465)/Smad5 (Ser463/465)/Smad8 (Ser426/428) antibody from Cell Signaling (catalog # 9511) was used to detect Smad dependent BMP signaling activities. For X-gal staining, $Nog^{+/-}$ embryos were collected from mating of $Nog^{+/-}$ mice with wild type mice at designated time points, and the tail of each embryo was used for genotyping. Embryonic heads were removed, labeled, and individually fixed in 0.2% glutaraldehyde for overnight at 4°C. After genotyping, $Nog^{+/-}$ embryonic heads were washed in ice cold PBS, washed in 30% sucrose/ PBS solution overnight, and embedded in O.C.T (Tissue-Tek). Cryosections at 10 μm were processed for X-gal staining, as described previously (Ito et al., 2003). For whole mount X-gal staining, samples were fixed for 20 min in 0.2% glutaraldehyde, and subjected to staining according to the standard protocol (Chai et al., 2000).

Cell proliferation and TUNEL assays

Bromodeoxyuridine (BrdU) Labeling and Detection Kit (Roche Diagnostics Corporation, Indianapolis) was used to measure cell proliferation rate. Briefly, BrdU solution was injected intraperitoneally into timed pregnant female mice (1.5ml/100g body weight) one hour before embryos were harvested. Embryonic heads were fixed in Carnoy's fixative and then processed into paraffin sections at 5-μm for immunostaining according to manufacturer's instruction. For BrdU-labeled cell counting, an arbitrary area was defined and both BrdU labeled cells and total cells were counted in that area. The outcome of BrdU labeling was presented as percentage of labeled cells among total nuclei in the fixed region. Collected from nine continuous sections of three individual samples of wild type controls and mutants, respectively, data were subjected to Student's *t*-test to determine the significance of differences. For TUNEL assays, samples were processed into 5 μm paraffin sections and apoptotic cells were detected as described previously (Alappat et al., 2005).

Results

The role of BMP signaling in palate development has been previously studied using various loss-of-function models (Zhang et al., 2002; Liu et al., 2005; Xiong et al., 2009). The cleft palate defects in mice deficient for *Follistatin* further suggest a requirement for a finely tuned level of TGFβ ligand activity in normal palate development (Matzuk et al., 1995), including that of BMPs. Noggin is a specific extracellular antagonist of BMP signaling that modulates BMP activities during embryonic development. *Noggin*-deficient mice therefore provide a gain-of-function model to study the role of BMP signaling in organ development. Besides defects found in many other developing organs, $Nog^{-/-}$ mice also exhibit a spectrum of craniofacial defects, suggesting a role for *Noggin* in palate development (McMahon et al., 1998; Bachiller et al., 2000; Stottmann et al., 2001; Anderson et al., 2006). However, neither expression nor function of *Noggin* has been documented in mammalian palate development.

Expression of Noggin in the developing palate

To investigate the potential role of *Noggin* and BMP signaling in palatogenesis, we began with an examination of *Noggin* expression by in situ hybridization. We found a dynamic *Noggin* expression pattern, primarily encompassing the palatal epithelium (Fig. 1). At E11.5, *Noggin* is expressed in the entire palatal epithelium along the anterior-posterior (A-P) axis (Fig. 1A, B). At E12.5, in the anterior portion of the palatal shelves, *Noggin* expression remains in the epithelium, but is relatively weak in the nasal side and the MEE (Fig. 1C), while in the posterior portion, *Noggin* expression was only detected in the oral side palatal epithelium (Fig. 1D). At E13.5, *Noggin* expression is restricted to the oral side epithelium in both the anterior and posterior palate (Fig. 1E, F). In the craniofacial region, *Noggin* mRNAs were also detected in the oral epithelium, maxillary mesenchyme, tongue and Meckel's cartilage (Fig. 1). We further assayed the *Noggin* expression pattern by X-gal staining in $Nog^{+/-}$ embryos, which carry a *LacZ* knock-in maker (Fig. 1G, H; and Supplemental Fig. 1A-D; McMahon et al., 1998); these results confirmed the expression pattern detected by in situ hybridization. The spatiotemporal profile of *Noggin* expression suggests an involvement of *Noggin* in palate development, prompting us to examine potential palate phenotypes in $Nog^{-/-}$ mice.

$Nog^{-/-}$ mice exhibit complete cleft palate phenotype

Gross examination of $Nog^{-/-}$ mice at postnatal day 0 (P0) identified a complete clefting of the secondary palate with 100% penetrance (Fig. 1J). Histological analyses revealed that the $Nog^{-/-}$ palatal shelves exhibit morphology comparable to the wild type control until E12.5, when the anterior palatal shelves looked normal but posterior palatal shelves appeared slightly smaller than their wild type counterparts (data not shown). At E13.5, the anterior portion of the palatal shelves in the mutants appeared smaller than in the wild type controls, and the posterior palate also appeared slightly shorter (Fig. 2A-D). During normal embryogenesis, the palatal shelves elevate and fuse at the midline at E14.5 (Fig. 2E, G). In $Nog^{-/-}$ embryos, we observed that the anterior palatal shelves elevated to horizontal position above the tongue, but did not meet medially. In contrast, the posterior portion of the palatal shelves fused with the mandible and did not elevate at all (Fig. 2F, H). In addition to the defect in palate development, significantly enlarged Meckel's cartilage was also observed in $Nog^{-/-}$ embryos (Fig. 2), consistent with *Noggin* expression in Meckel's cartilage and its general role in skeletal development (Brunet et al., 1998;McMahon et al., 1998).

The failed palatal shelf contact at the midline in the anterior region of $Nog^{-/-}$ palate could be caused by an impaired palatal fusion. To test this possibility, we used in vitro organ culture as described previously (Taya et al., 1999; Zhang et al., 2002). The anterior halves of the palatal shelves from E13.5 wild type controls and $Nog^{-/-}$ embryos were dissected and placed in contact as pairs with the MEE of each facing the other. After 3 days in culture, paired palatal shelves from both genotypes underwent fusion (5/5 for wild type control, and 6/6 for *Noggin* mutant), as revealed by the disappearance of the midline seam (Supplemental Fig. 2), indicating that failure of fusion is not the cause of cleft palate in $Nog^{-/-}$ embryo. Many factors are known to influence palatal shelf contact, including malformed craniofacial structures. Since micrognathia was rarely found in $Nog^{-/-}$ mutants (Stottmann et al., 2001), the cleft palate is not secondary to micrognathia where the tongue fails to sink down from between the palatal shelves. However, the lack of the primary palate (see below) and the formation of enlarged Meckel's cartilage could also attribute to the failure in palatal shelf contact. Nevertheless, these observations revealed a requirement for *Noggin* in palate development.

In addition to the cleft secondary palate formation, the primary palate was also absent completely in *Noggin* mutants (Fig. 1J), consistent with *Noggin* expression in the forming

site of primary palate and surrounding tissue (Stottmann et al., 2001; Supplemental Fig. 1E, F). However, we did not observe any type of cleft lip formation, although we indeed found defective maxillary incisors that were fused at the midline to form a single tooth bud which was arrested at the late bud stage (unpublished results).

Alteration of BMP signaling in the Nog mutant palate

Noggin functions to antagonize the activities of selective specific BMP ligands, such as BMP2, BMP4, and BMP7, which signal primarily by phosphorylating Smad1/5/8 and activating the Smad4 dependent pathway. Although Smad-independent TGFβ/BMP signaling through p38 MAPK has also been implicated in palate development, this pathway mainly acts in the MEE region to regulate palatal fusion (Xu et al., 2008). We therefore used Smad1/5/8 phosphorylation as an indicator of BMP activated Smad signaling to determine changes in the level of BMP signaling in the $Nog^{-/-}$ palatal shelves. In the wild type palatal shelves, phosphorylated Smad1/5/8 (pSmad1/5/8) was detected in both anterior and posterior portion (Fig. 3A, E). In the anterior palate, we found abundant pSmad1/5/8 staining in the maxillary and palatal mesenchyme, with a few positive signals in the epithelium (Fig. 3A, C). In the posterior palate, pSmad1/5/8 was restricted to the nasal side palatal mesenchyme, with sporadic staining in the oral side palatal mesenchyme and epithelium, and completely negative staining in the Meckel's cartilage (Fig. 3E, G). In $Nog^{-/-}$ embryos, Smad1/5/8 phosphorylation was dramatically elevated in the maxillary mesenchyme and the Meckel's cartilage (Fig. 3B, F). However, in the mutant palate, we detected differential alterations in pSmad1/5/8 staining. In the anterior palatal mesenchyme, the staining was generally reduced (Fig. 3B, D). In contrast, in the posterior palatal mesenchyme, ectopic staining was found in the oral side region, while the nasal half remained comparable staining to the wild type controls (Fig. 3F, H). In the mutant palatal epithelium, we found consistently ectopic pSmad1/5/8 staining in the oral side epithelium along the A-P axis (Fig. 3), in accord with the *Noggin* expression pattern. The ectopic pSmad1/5/8 staining in the mutant epithelium indicates that *Noggin* is required to inhibit Smad dependent signaling in normal palate development. The different alterations in pSmad1/5/8 staining in the mutant palatal mesenchyme, on the other hand, suggest that palatal mesenchyme along the A-P axis responds differentially to the absence of *Noggin*, consistent with the tissue heterogeneity along the A-P axis of the developing palatal shelves (Hilliard et al., 2005; Okano et al., 2006).

Activation of BMP/Smad signaling requires ligand-receptor binding. It has been well documented that *Bmp2* and *Bmp4* are expressed in the developing palate, and it has been shown that exogenous BMP can induce its antagonists expression, and vice versa (Lu et al., 2000; Stottmann et al., 2001; Ashique et al., 2002; Zhang et al., 2002; Nie, 2005). These lines of evidence promoted us to examine if inactivation of *Noggin* could also affect *Bmp2* and *Bmp4* expression in the developing palate. We found that the expression of *Bmp4* is comparable to that in the wild type control (Fig. 4A, B). This observation is consistent with the unaltered expression patterns of *Msx1* and *Shh*, downstream targets of *Bmp4* in the anterior palate (Fig. 4C-F). In the wild type controls at E13.5, *Bmp2* is expressed in the palatal mesenchyme of the anterior portion, and in the MEE as well as the nasal side palatal mesenchyme of the posterior portion (Fig. 4G, I). In the $Nog^{-/-}$ palate, we found that *Bmp2* expression is reduced in the anterior palatal mesenchyme, but is ectopically activated in the oral side of posterior palatal epithelium (Fig. 4H, 4J), suggesting that Noggin regulates *Bmp2* expression through different mechanisms along the A-P axis in the developing palate. In contrast, *Bmp2* expression in the anterior maxillary mesenchyme and the posterior palate mesenchyme of the mutant was not affected (Fig. 4G-J). The down-regulation of *Bmp2* expression in the anterior palatal mesenchyme is accompanied by a reduced expression of *Shox2* (Fig. 4K, L), whose expression is restricted in the anterior palatal mesenchyme and is

dependent on BMP activities (Yu et al., 2005). These results indicate that, in addition to its antagonizing property, Noggin is also able to regulate the expression of *Bmp2*, but not *Bmp4*, in the developing palate. The alteration in *Bmp2* expression could at least partially account for the changes in pSmad1/5/8 activity in the mutant palate along the A-P axis, and a down-regulation of *Shox2* expression in the anterior palatal mesenchyme.

Cellular defects in the Nog$^{-/-}$ palatal shelves

To reveal the underlying cellular mechanisms that lead to a cleft palate formation in the *Nog*$^{-/-}$ palate, we carried out BrdU labeling and TUNEL assays to examine cell proliferation rates and apoptosis levels, respectively. In this study, cell proliferation rate was measured by the ratio of proliferating cells against total nucleus number in a defined area. At E12.5, while slight deviation in cell proliferation rates was found in the both palatal epithelium and mesenchyme along the A-P axis in the wild type controls and mutants (data not shown), the difference was not significant, consistent with morphological analysis results. However, at E13.5 in the mutants, we found that the cell proliferation rate was significantly upregulated in the oral side palatal epithelium, in both the anterior and posterior portion, as compared to the wild type controls (Fig. 5A-E). This elevated cell proliferation rate appears to correlate with an enhanced pSmad1/5/8 staining in the *Nog*$^{-/-}$ palate, suggesting that BMP/Smad signaling positively modulated cell proliferation levels in the palatal epithelium. However, in the mutant mesenchyme, the cell proliferation rate was downregulated in the anterior region, but remained unchanged in the posterior portion despite ectopic pSmad1/5/8 staining in the oral half, as compared to the wild type controls (Fig. 5A-E). This observation is consistent with our previous findings that exogenous BMP induces cell proliferation in the anterior palatal mesenchyme but not in the posterior palatal mesenchyme (Hilliard et al., 2005), and that a reduced level of cell proliferation is observed in the anterior palatal mesenchyme of *Shox2* mutant (Yu et al., 2005). We next carried out TUNEL assay to examine cell apoptosis in the wild type and *Nog*$^{-/-}$ palate at E13.5. We detected no apoptotic cell in the wild type control (Fig. 5F), but in the mutant, on the other hand, we found numerous apoptotic cells in the anterior palatal epithelium on the oral side, where *Noggin* is normally expressed (Fig. 5G). Ectopic apoptotic cells were not found in the nasal side epithelium. However, in the posterior palate of the mutant, we did not find apoptotic cells at this particular stage (data not shown). Thus alterations in cell proliferation rate and enhanced cell apoptosis contribute to abnormal growth and shaping in the mutant palatal shelves.

Nog$^{-/-}$ mice exhibit abnormal fusion between the posterior palate and the mandible

Palate elevation defects could arise from multiple etiologies, such as intrinsic elevation force deficiency, extrinsic blockage by the tongue, or abnormal fusion between palate shelf and adjacent structures (Gritli-Linde, 2007). As shown in Fig. 2, while the anterior portion of the mutant palate elevated normally, the posterior palate failed to elevate, apparently due to an aberrant palate-mandible fusion. To reveal the process of the abnormal fusion, we examined serial histological sections of the *Nog*$^{-/-}$ palatal shelves at E14.5 when they would have elevated to the horizontal position above the tongue (Fig. 6A). We observed an initial adhesion of the palatal epithelium with the mandibular epithelium, followed by an elimination of the adhered epithelia, forming a mesenchymal continuity between the palatal shelf and the mandible. Similar abnormal palate-mandible fusion phenotype has been reported in *Fgf10*$^{-/-}$ mice (Rice et al., 2004; Alappat et al., 2005). In *Fgf10* mutants, cell apoptosis induced by ectopic *Tgfβ3* expression was thought to attribute to the disruption of palatal epithelial integrity and abnormal fusion (Alappat et al., 2005). The phenotypic similarity between *Nog*$^{-/-}$ and *Fgf10*$^{-/-}$ mice promoted us to examine if the expression of *Fgf10* and its downstream effectors was altered in the *Nog*$^{-/-}$ palate. We did not see a down-regulation of *Fgf10* expression in the *Nog*$^{-/-}$ palate (data not shown). However, we indeed observed an ectopic *Tgfβ3* expression in the oral side epithelium of the mutant posterior

palatal shelves prior to adhesion (Fig. 6H), although *Tgfβ3* expression in the mutant MEE remained comparable to that in wild type controls where palatal shelves just met at the midline (Fig. 6G, H). Associated with this ectopic activation of *Tgfβ3* expression is aberrant induction of apoptosis in the periderm cells prior to adhesion (Fig. 6I). Together with our pSmad1/5/8 immunostaining results, these results suggest that *Noggin* functions downstream of or in parallel to *Fgf10* in inhibiting *Tgfβ3* expression in the oral side palatal epithelium, and an elevated BMP/Smad signaling in the posterior palate can activate *Tgfβ3* expression and induce epithelial cell apoptosis, which consequently disrupts palatal epithelium integrity and leads to abnormal palate-mandible fusion.

Ectopic activation of BMP signaling in the palate epithelium recapitulates the abnormal palate-mandible fusion phenotype in Nog^{-/-} mice

To test if ectopic activation of BMP signaling in the developing palatal epithelium could recapitulate the abnormal palate-mandible fusion phenotype observed in *Nog*^{-/-} mice, we generated conditional transgenic mice that express a constitutively active form of BMP receptor-IA (*pMescaBmpr-IA*) or a constitutively active form of BMP receptor-IB (*pMescaBmpr-IB*) upon crossing to a *Cre* mouse line. These mutated forms of BMP receptors have been demonstrated to activate BMP signaling pathway in the absence of BMP ligands (Zou et al., 1997; Zhang et al., 2000). Using a *K14-Cre* mouse line (Andl et al., 2004; Xu et al., 2008), we ectopically expressed *caBmpr-IA* or *caBmpr-IB* in embryonic epithelium, confirmed by assessing expression of an *Egfp* transgene integrated into the transgenic vector (Fig. 7C). As shown in Fig. 7B, this *K14-Cre* transgenic allele was able to activate *Rosa26* reporter expression uniformly in craniofacial region as early as E11.5, including the developing palatal shelves. We found that mice carrying the *K14-Cre* and *caBmpr-IB* double transgenic alleles (*K14Cre;caBmpr-IB*) did not develop a cleft palate defect (data not shown). However, these mice in fact developed severe ichthyosis-like skin phenotype postnatally, indicating a functional transgenicc allele (Yu, X. et al., unpublished results). The *K14Cre;pMescaBmpr-IA* mice, however, exhibited a cleft palate phenotype (data not shown). Histological analyses revealed an abnormal palate-mandible fusion in the posterior palate region at E14.5, resembling the phenotype found in *Nog*^{-/-} mice (Fig. 7D, F). We have shown previously that transgenic overexpression of *Noggin* in the palatal mesenchyme causes a cleft palate defect (Xiong et al., 2009). Using the same conditional *Noggin* transgenic line (*pMesNog*), we overexpressed *Noggin* in the palatal epithelium by compounding the *K14-Cre* allele with the *pMesNog* conditional transgenic allele (Supplemental Fig. 1G). While mice carrying the *K14Cre* and *pMesNog* transgenic alleles exhibited an arrest of tooth development at the lamina/early bud stage (data not shown), they did not develop a cleft palate defect (Fig. 7E), consistent with the previous report that mice carrying the *K14-Noggin* transgenic allele survived to adulthood (Plikus et al., 2005). These results support the idea that repression of BMP activities by Noggin in the palatal epithelium is required for the maintenance of palatal epithelium integrity and is essential for normal palatogenesis.

Discussion

In this paper, we report the expression pattern of the BMP antagonist *Noggin* in the mouse developing palate, and show that inactivation of *Noggin* causes a cleft palate formation. We demonstrate that both cell proliferation and cell apoptosis are deregulated in the *Nog*^{-/-} palate. Particularly, we found abnormal cell apoptosis in periderm cells in the oral side of the posterior palate, which leads to aberrant palate-mandible fusion and blocks the normal palatal elevation. We further show that ectopic activation of BMP signaling in the palatal epithelium recapitulates the abnormal palate-mandible fusion phenotype observed in *Nog*^{-/-}

mice. Our studies thus demonstrate that repression of BMP activities by Noggin is required for palate epithelium integrity, which is essential for normal palate development.

Palatal epithelium integrity and palate development

Epithelial-mesenchymal interaction controls each step of palate development. Palatal epithelium thus plays an essential role in palate shelf outgrowth, elevation, fusion and further differentiation (Ferguson, 1988). In addition to these aspects, recent studies have demonstrated that the integrity of palatal epithelium is particularly required for palate elevation (Alappat et al., 2005; Casey et al., 2006; Jiang et al., 1998; Richardson et al., 2006; 2009; Xiong et al., 2009). In normal palate development, the palatal shelves may contact the adjacent tissues, including tongue and mandible. However, a clear epithelial boundary is always maintained at the contact interface, preventing abnormal adhesion and fusion between palate and these structures. In pathological conditions, when the epithelium integrity is disrupted, abnormal adhesion or fusion occurs. Abnormal adhesion is observed in *Irf6* heterozygous mice: the palatal epithelium adheres to the mandible and the tongue; however, the palatal shelves still manage to elevate and fuse to form an intact structure despite the abnormal epithelial adhesion (Richardson et al., 2006; 2009). In the event that adhesion is further deregulated, with elimination of the epithelial seam, (i.e., when a mesenchymal continuity is formed between palatal shelves and adjacent structures), palatal shelf elevation is hampered, as observed in *Irf6* homozygous mice (Richardson et al., 2006; 2009). Similar abnormal palate-mandible or palate-tongue fusion phenotypes have also been reported in *Jagged2* mutant, *Fgf10* mutant and *Hand2* hypomorphic mice (Alappat et al., 2005; Casey et al., 2006; Xiong et al., 2009). In these animal models, ectopic cell apoptosis is a common cause of disruption in epithelium integrity. However, since adhesion/fusion always occurs between two epithelial surfaces, disruption of either surface could lead to abnormal adhesion/fusion. In *Jagged2* mutants, the abnormal palate-tongue fusion phenotype results from an abnormal tongue epithelial differentiation, despite normally developed palatal shelves as assessed at histological, cellular, and molecular levels (Casey et al., 2006). In *Hand2* hypomorphic and *Fgf10* mutant mice, the abnormal palate-mandible fusion has been attributed to ectopic cell apoptosis within the palatal epithelium (Alappat et al., 2005; Xiong et al., 2009). As shown in this study, in E13.5 *Noggin* mutants, ectopic apoptotic cells were found in the oral side epithelium of the anterior palate and in the mandibular epithelium, but not in the posterior palate (Fig. 5G). Since the anterior palatal shelves do not make contact with the mandible, an abnormal palate-mandible fusion does not occur. However, in the posterior palate at E14.5, we observed ectopic cell death in the oral side palatal epithelium, but not in the mandibular epithelium, where abnormal fusion occurred (Fig. 6I). Here, aberrant apoptosis was initially detected in periderm cells in the abnormal fusion region prior to making contact with the mandibular epithelium. A similar phenotype was also observed in *Hand2* hypomorphic mice (Xiong et al., 2009). During normal palate development, periderm cells in the MEE region are removed prior to palatal shelf contact and subsequent fusion (Fitchett and Hay, 1989), and artificial removal of periderm cells causes degradation of the basal layer cells in the MEE (Cuervo and Covarrubias, 2004). Together, these results support an essential role for periderm cells in the maintenance of palatal epithelial integrity.

Differential cellular and gene expression responses to the absence of Noggin along the A-P axis of developing palate

It has been well documented that the developing secondary palatal shelf is an asymmetric structure along the anterior-posterior axis (Zhang et al., 2002; Alappat et al., 2005; Yu et al., 2005; He et al., 2008). The anterior and posterior portions of the palatal shelves differ not only at the cellular level, but also at the molecular level (reviewed in Hilliard, 2005; Okano et al., 2006; Gritli-Linde, 2007). *Noggin* is expressed in the oral side palatal epithelium in

both anterior and posterior palate shelves and *Noggin* deficiency leads to ectopic activation of BMP/Smad signaling in these structures. Associated with this enhanced/ectopic BMP/Smad signaling is ectopic cell death in both anterior and posterior palatal epithelium. However, it is interesting to note that the timing of the apoptosis occurring varies along the A-P axis. In the anterior palatal epithelium of $Nog^{-/-}$ embryo, ectopic cell death was detected as early as E13.5, while in the posterior palate, ectopic cell death was not detected until E14.5, suggesting that molecular mechanisms underlying the cell death are different along the A-P axis. This idea is supported by the fact that *Tgfβ3*, which is required for cell death in the MEE during normal fusion of palate, is ectopically expressed in the posterior palatal epithelium, but not in the anterior counterpart. These results suggest that the ectopic *Tgfβ3* expression may mediate the cell death in the posterior palate. The ectopic cell apoptosis found in the anterior palatal epithelium in *Noggin* mutant is mediated by a different mechanism.

In addition, the palatal mesenchymal cells of the anterior and posterior palate also respond differentially to the loss of *Noggin*. At the cellular level, the anterior palatal mesenchyme exhibits a down-regulated cell proliferation rate; while in the posterior palate, the cell proliferation rate is not affected (Fig. 5E). In terms of gene expression, *Bmp2* expression was down-regulated in the anterior palatal mesenchyme, but remained unaltered in the posterior palatal mesenchyme (Fig. 4). It has been shown previously that *Bmp2* acts downstream of *Bmp4* and *Shh* to stimulate cell proliferation in the anterior palatal mesenchyme (Zhang et al., 2002). This down-regulation of *Bmp2* expression appears to be responsible for the reduction in Smad1/5/8 phosphorylation in the anterior palatal mesenchyme of *Noggin* mutant, which contributes to the reduced cell proliferation rate. While Smad1/5/8 phosphorylation was enhanced in the posterior palatal mesehcyme in the absence of Noggin, the cell proliferation rate was unaltered, consistent with the previous finding that neither BMP2 nor BMP4 regulate cell proliferation in the posterior palatal mesenchyme (Zhang et al., 2002;Hilliard et al., 2005). In the posterior palate of *Noggin* mutant, *Bmp2* expression was not changed in the mesenchyme, but was upregulated in the oral side epithelium. Although we cannot rule out the possibility that the expression and/or activity of other BMPs is upregulated in the posterior palate in the absence of *Noggin*, the upregulated *Bmp2* expression in the oral side epithelium could count for, at least partially, the ectopic Smad1/5/8 phosphorylation in the oral side mesenchyme of the $Nog^{-/-}$ posterior palate.

It was shown previously that application of exogenous Noggin protein to the globular process of the frontonasal mass of developing chick embryo induces clefts of the primary palate (Ashique et al., 2002). However, in $Nog^{-/-}$ mice, the absence of *Noggin* leads to a complete lack of the primary palate structure (Fig. 1J). These observations indicate that BMP homeostasis is also essential for development of the primary palate. There are many lines of evidence showing requirement of BMP signaling and BMP homeostasis in palate development (Matzuk et al., 1995;Zhang et al., 2002;Dudas et al., 2004;Liu et al., 2005;Xiong et al., 2009). Interestingly, while overexpression of *Noggin* in the palatal mesenchyme leads to cleft palate formation (Xiong et al., 2009), *Noggin* overexpression in the palatal epithelium does not cause a cleft palate defect (Fig. 7E, and Supplemental Fig. 1F), despite of the fact that *K14Cre;pMesNog* mice show an arrest of tooth development at the lamina/early bud stage (data not shown). This is likely due to different amounts of transgenic Noggin produced in the palatal mesenchyme and the palatal epithelium. The different responses of the developing palate and tooth to Noggin overexpression in the epithelium could be attributed to differential sensitivity to altered BMP signaling.

Bmp4 is unable to compensate for the reduction of Bmp2 expression in the anterior palate in vivo

While it remains to be determined how *Bmp2* expression is differentially regulated in the developing palate along the A-P axis in the *Noggin* mutant, it is interesting to note that *Bmp4* expression is unaltered. BMPs, including BMP2, BMP4 and BMP7, have been shown to function as mitogens in developing craniofacial structures (Barlow and Francis-West, 1997; Wang et al., 1999; Ashique et al., 2002; Zhang et al., 2002). *Bmp2* and *Bmp4* are expressed in a partially overlapping pattern in the anterior palate mesenchyme. Although exogenously applied BMP2 or BMP4 can equally induce cell proliferation in the anterior palatal mesenchyme in vitro (Zhang et al., 2002; Hilliard et al., 2005), the unaltered *Bmp4* expression in the *Nog$^{-/-}$* palate suggests that *Bmp4* fails to compensate for the reduction of *Bmp2* expression, in terms of activating BMP/Smad signaling and regulating cell proliferation, in the anterior palatal mesenchyme in vivo. Similar results have also been previously demonstrated in an in vitro study in which blocking Shh signaling did not affect *Bmp4* expression but eliminated *Bmp2* expression and subsequently inhibited cell proliferation in the anterior palatal mesenchyme (Zhang et al., 2002). Thus, *Bmp4* appears to maintain *Msx1* expression in the mesenchyme and *Shh* expression in the MEE, while *Bmp2* is primarily responsible for the regulation of cell proliferation within the anterior palatal mesenchyme. This hypothesis is supported by the fact that expression pattern of pSmad1/5/8 in the developing palate is almost identical to that of *Bmp2* in both the anterior and posterior portion (Fig. 3; Fig. 4). In addition, we also observed a significantly reduced *Shox2* expression in the anterior palatal mesenchyme of *Noggin* mutants. We have also demonstrated previously that BMP activities are not sufficient to induce ectopic *Shox2* expression, but are necessary for *Shox2* expression in the anterior palatal mesenchyme (Yu et al., 2005). Given the fact that *Bmp4* expression remains unaltered in the *Nog$^{-/-}$* anterior palate, it cannot maintain *Shox2* expression at the wild type level. BMP4 and BMP2 indeed have differential potency in activating BMP/Smad signaling (Upton et al., 2008), and BMP2/BMP4 heterodimers are more potent than homodimers of any of the proteins in activating BMP/Smad signaling. This functional difference is likely due to different binding affinities between specific BMP ligands and receptors, or availability of BMP receptors in specific cell context. Currently, three type I receptors (BMPR-IA, BMPR-IB, and ActRIa) and three type II receptors (BMPR-II; ActRIIA, and ActRIIB) have been identified to bind to BMP ligands with different affinities (Sieber et al., 2009). Expression of BMP receptors and their functions in palate development warrant future investigation.

BMPR-IA likely mediates overactive BMP signaling in disruption of palatal epithelial integrity

During development and disease, BMP signaling is involved in the regulation of multiple cellular processes, including proliferation and apoptosis. In the palatal epithelium of *Noggin* mutants, in which BMP signaling is overactive, we observed the simultaneous occurrence of elevated level of cell proliferation and excess apoptotic cells. This simultaneous elevation of cell proliferation rate and apoptosis was also observed in the palatal mesenchyme in mice bearing inactivation of *Alk5* in neural crest cells and their derivatives (Dudas et al., 2006). In the posterior palate of *Noggin* mutant, the excess cell apoptosis in the palatal epithelium resulted in abnormal palate-mandible fusion. This phenotype was recapitulated by ectopic expression of a constitutively active form of BMPR-IA in the palatal epithelium. In contrast, ectopic expression of a constitutively active form of BMPR-IB did not produce any obvious palate defect. Since *Bmp2* expression and Smad1/5/8 phosphorylation were elevated in the region where excess cell death and abnormal fusion occurred, it appears that overexpressed BMP2 acts through BMPR-IA and Smad-dependent pathway in this context, leading to the disruption of palatal epithelial integrity. This view is supported by the observations of high binding affinity of BMP2 to BMPR-IA (Knaus and Sebald, 2001), and *Bmpr-IA* but not

Bmpr-IB is strongly expressed in the palatal epithelium during palatogenesis (Li, L. and Chen, Y.P., unpublished results).

In conclusion, our studies show an absolute requirement for Noggin in normal palate development. The absence of Noggin alters the levels of BMP signaling, causing aberrant cell proliferation and cell death in the developing palate. Repression of BMP activities by Noggin in the palatal epithelium is essential for the maintenance of epithelium integrity. Our results highlight the importance of fine-tuned BMP signaling in palate formation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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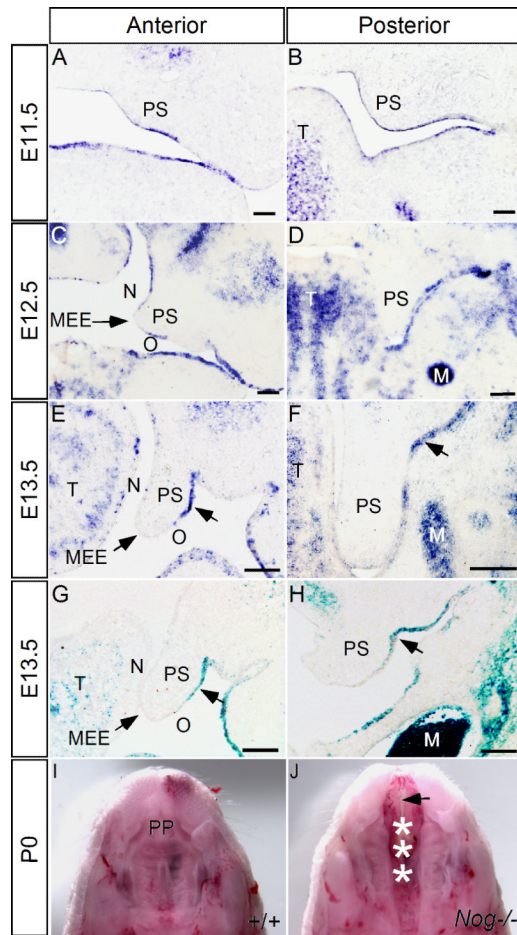
Figure 1.
Expression of *Noggin* in the developing mouse palatal shelves. (A, B) At E11.5, *Noggin* mRNA is detected in the epithelium of the oral-nasal cavity, including the anterior (A) and posterior (B) palatal shelves. (C, D) In the anterior palatal shelves at E12.5, *Noggin* expression is detected in the nasal side and oral side palatal epithelium, but not in the MEE (C), while in the posterior palate, *Noggin* mRNA is restricted to the oral side palatal epithelium (D). (E-H) At E13.5, *Noggin* expression (arrows) is only detected in the oral side palatal epithelium (arrows) in both the anterior (E) and posterior (F) palatal shelves. *LacZ* reporter expression in the *Nog*$^{+/-}$ palate recapitulates the expression pattern of *Noggin* mRNA (G, H). (I, J) At P0, the wild type mouse develops an intact palatal shelf (I), while *Nog*$^{-/-}$ mouse exhibits a complete cleft of the secondary palate (asterisks in J) and a lack of the primary palate (arrow in J). M, Meckel's cartilage; MEE, medial edge epithelium; PP, primary palate; PS, palatal shelf; T, tongue. N designates nasal side of the palatal shelf, and O designates oral side of the palatal shelf. Scale bars represent 100 μm.
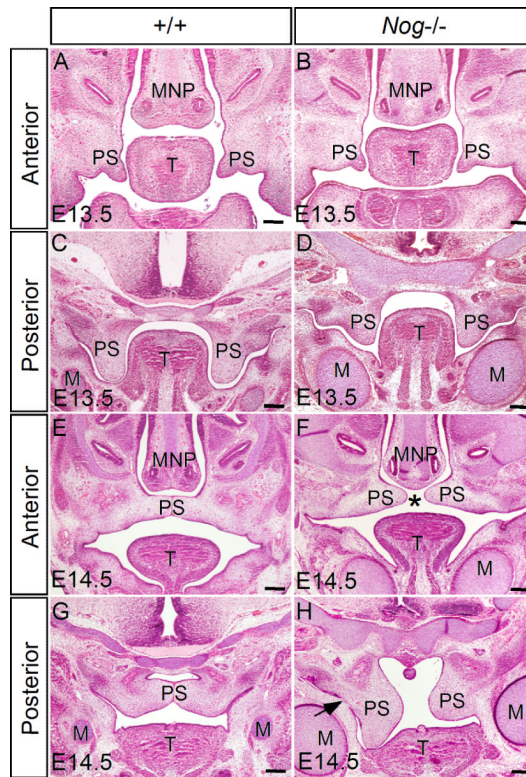
Figure 2.
Nog^{-/-} embryos show defective palate development. (A, C) At E13.5, the wild type palatal shelves grow vertically along the tongue in the oral-nasal cavity. (B, D) In E13.5 mutant, the anterior palatal shelves appear smaller than the wild type controls (B); in the posterior portion, the mutant palatal shelves grow shorter but slightly wider than the wild type counterparts, accompanied by ectopic cartilage formation and hyperplastic Meckel's cartilage (D). (E, G) At E14.5, the palatal shelves in wild type embryos have elevated and fused to form an intact structure above the tongue. (F, H) In the mutant at E14.5, however, the palatal shelves fail to contact each other, forming a palatal cleft (asterisk). Note that in the mutant, the anterior palatal shelves have elevated but did not make contact (F), while the posterior palatal shelves remain in a vertical position, and show abnormal fusion (arrow) with the mandible (H). M, Meckel's cartilage; MNP, medial nasal process; PS, palatal shelf; T, tongue. Scale bars represent 200 μm.
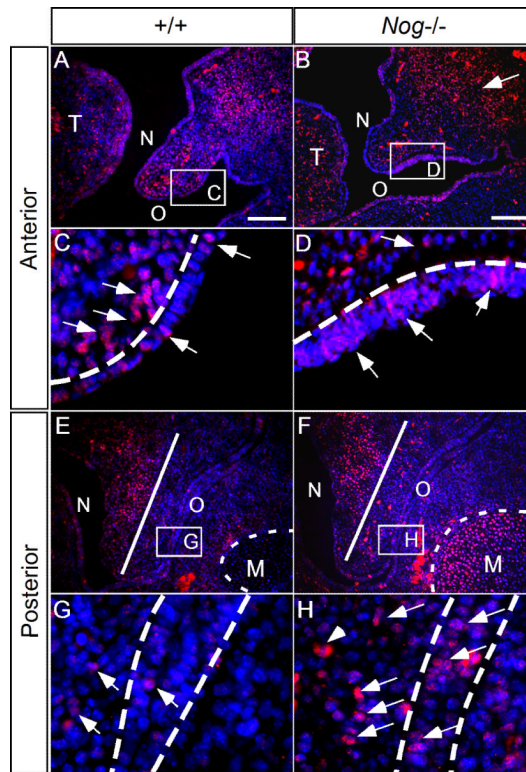
Figure 3.
Noggin deficiency alters BMP/Smad signaling activity in the developing palate. (A, C, E, G) In the wild type control at E13.5, pSmad1/5/8 is detected in both the anterior and posterior palate. In the anterior palate, pSmad1/5/8 signal is found at high levels in the mesenchyme but is sparse in the epithelium (A, C); in the posterior palate, pSmad1/5/8/ activity is mainly restricted in the nasal side palatal mesenchyme, with a few positive signals in the oral side palatal epithelium (E, G). (B, D, F, H) In the *Nog*$^{-/-}$ palate at the comparable stage, Smad1/5/8 phosphorylation is enhanced in the oral side palatal epithelium, in both the anterior and posterior palate. As compared to the wild type control, the mutant anterior palatal mesenchyme exhibits downregulated pSmad1/5/8 signal, while the posterior palatal mesenchyme shows ectopic pSmad1/5/8 activity in the oral side. Note that the pSmad1/5/8 signal is significantly enhanced in the *Nog*$^{-/-}$ Meckel's cartilage (F). The straight white line in (E) and (F) divides the palatal mesenchyme into nasal and oral halves. The dash white lines in (C, D, G, H) demarcate the epithelial boundary. M, Meckel's cartilage; T, tongue. N designates nasal side of the palatal shelf, and O designates oral side of the palatal shelf. Scale bars represent 100 μm.
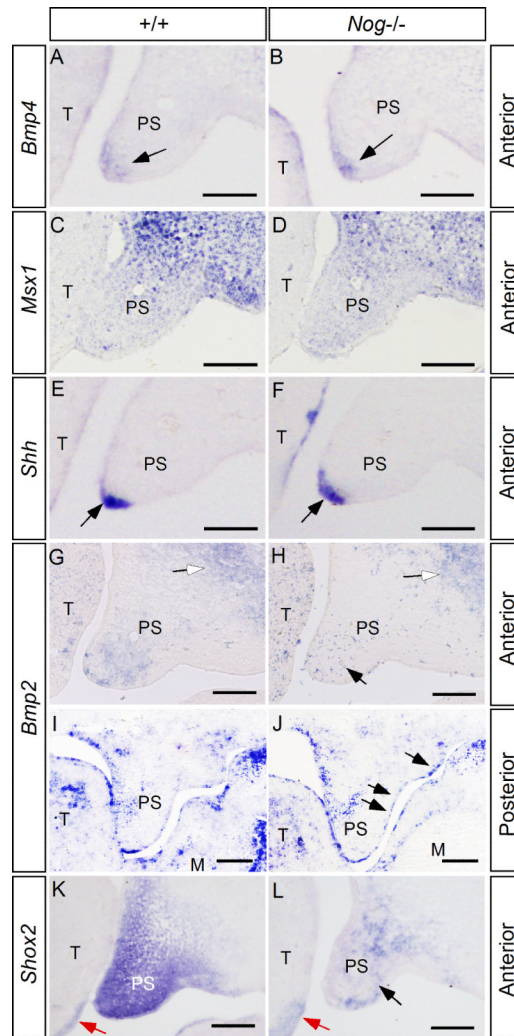
Figure 4.
Gene expression in the wild type and *Nog*^{-/-} palate. (A, B) At E13.5, *Bmp4* is expressed in the anterior palatal mesenchyme (arrow) underlying MEE in the wild type palate (A); in the *Nog*^{-/-} palate, comparable *Bmp4* expression (arrow) is observed (B). (C, D) Comparable Msx1 expression is observed in the anterior palatal mesenchyme of E13.5 wild type (C) and *Nog*^{-/-} embryo (D). (E, F) *Shh* expression (arrows) in the MEE of the anterior palatal shelves is not affected in *Nog*^{-/-} embryo. (G, I) In E13.5 wild type controls, *Bmp2* expression is detected in the anterior palatal mesenchyme and maxillary region (open arrow) (G); in the posterior palate, it is expressed in the nasal side palatal mesenchyme and the MEE region (I). (H) In the *Nog*^{-/-} anterior palate, *Bmp2* expression is downregulated in the palatal mesenchyme (black arrow), but is not affected in the maxillary region (open arrow). (J) In the posterior palate of the mutant, *Bmp2* expression is not altered in the palatal mesenchyme and the MEE, but is ectopically activated in the oral side palatal epithelium (arrows). (K, L) *Shox2* expression is significantly down-regulated in the anterior palatal mesenchyme of the mutant (L), as compared to the wild type control (K). Note comparable *Shox2* expression at the ventral-lateral side of the developing tongue (red arrows). PS, palatal shelf; T, tongue. Scale bars represent 100 μm.
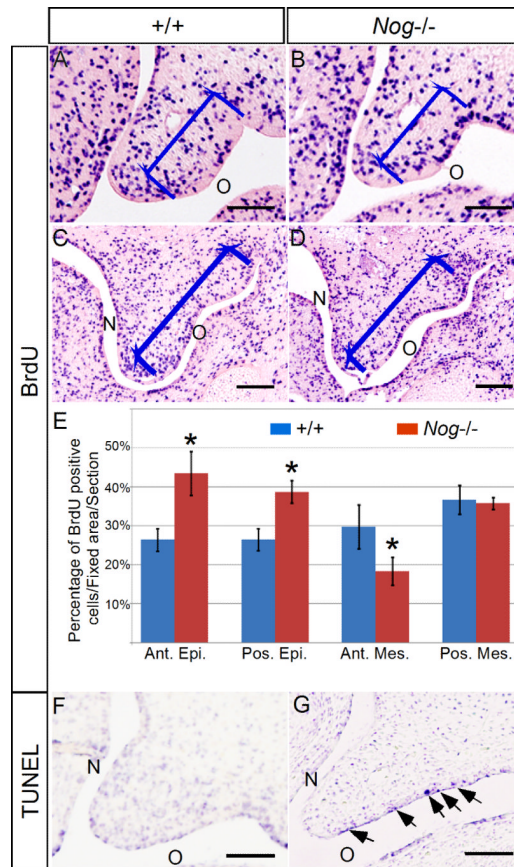
Figure 5.
The *Nog*^{-/-} palatal shelves exhibit defective cell proliferation and excessive apoptosis. (A-D) Coronal sections of BrdU labeled palatal shelf in the wild type and *Nog*^{-/-} palate at E13.5. The blue bracket defines the regions used for cell proliferation ratio statistics. (E) Statistical data analysis shows cell proliferation rate is upregulated in the mutant palatal epithelium (both anterior and posterior). Cell proliferation rate is downregulated in the mutant anterior palatal mesenchyme, but remains unaltered in the posterior palatal mesenchyme. (F, G) At E13.5, the anterior palate of mutant exhibits excessive apoptotic cells in the oral side epithelium, but not in the nasal side (G), as compared to the wild type control (F). Ant. Epi., epithelium of anterior palate; Ant. Mes., mesenchyme of anterior palate; Pos. Epi., epithelium of posterior palate; Pos. Mes., mesenchyme of posterior palate; *, $P < 0.01$. Scale bars represent 100 μm.
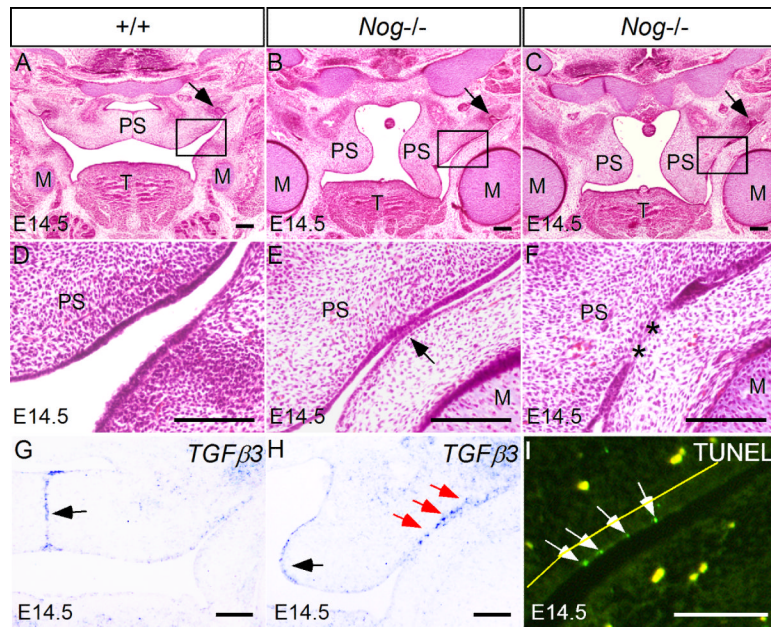
Figure 6.
Nog^-/- embryo shows abnormal palate-mandible fusion in the posterior palatal region. (A, D) At E14.5, the wild type palatal shelves have elevated to a position above the tongue and merged at the midline. (B, C, E, F) In the *Nog*^-/- embryo at the comparable stage, the palatal shelves remain in a vertical position, showing progressive adhesion (E) and fusion (F) with the mandible. The asterisks denote a confluence of mesenchyme. The presence of the maxillary molar (arrows in A, B and C) marks the level of the sections. (G, H) *Tgfβ3* is expressed in the MEE of the wild type palatal shelves (G); in the *Nog*^-/- palate, *Tgfβ3* expression is not affected in the MEE (black arrow in H), but is ectopically activated in the oral side epithelium (red arrows in H) where aberrant palate-mandible adhesion occurs. (I) TUNEL assay detects ectopic apoptotic periderm cells (arrows) in the oral side palatal epithelium prior to abnormal palate-mandible adhesion. No apoptotic cells are observed in the adjacent mandible epithelium. The yellow fluorescent dots in (I) represent autofluorescence from blood cells. The yellow line marks the basement membrane between palatal mesenchyme and the basal epithelial layer. M, Meckel's cartilage; T, tongue; PS, palatal shelf. Scale bars represent 100 μm.
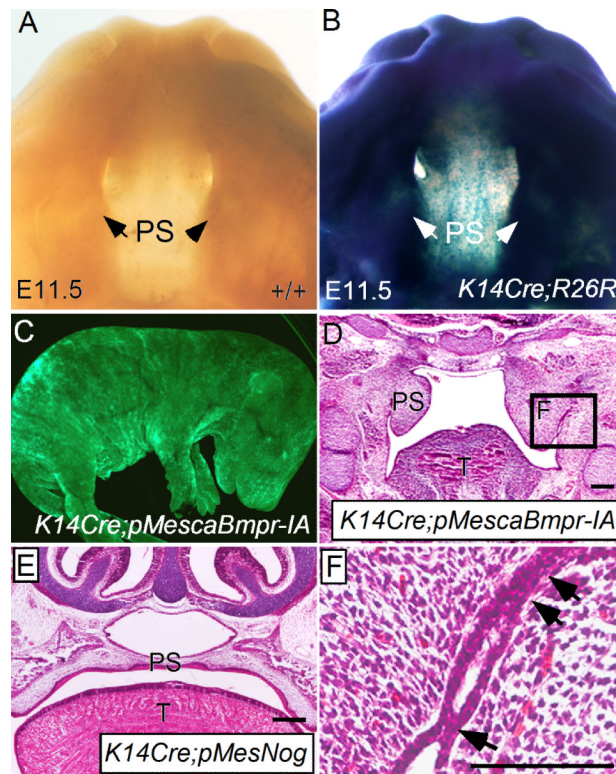
Figure 7.
Ectopic activation of BMP signaling in the palatal epithelium recapitulates the palate-mandible fusion phenotype observed in *Nog*$^{-/-}$ mice (A) An E11.5 wild type embryonic head shows negative staining of β-galactosidase. (B) An E11.5 *K14Cre;R26R* embryonic head shows staining of β-galactosidase in the craniofacial region, including the palatal shelves. (C) An E17.5 *K14Cre;pMescaBmpr-IA* embryo shows GFP expression throughout the entire embryo. (D) An E14.5 *K14Cre;pMescaBmpr-IA* embryo exhibits abnormal palate-mandible fusion and failure in palatal shelf elevation in the posterior portion. (E) An E16.5 *K14Cre;pMesNog* embryo shows a normally formed palate. (F) Higher magnification of the defined area in (D), showing an abnormal palate-mandible adhesion/fusion sites (arrows). T, tongue; PS, palatal shelf. Scale bars represent 100 μm.