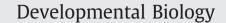
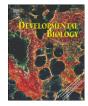
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Modulation of BMP signaling by Noggin is required for the maintenance of palatal epithelial integrity during palatogenesis

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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.

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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

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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

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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 TGFB 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 genetargeted 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 Nogginmediated 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 icecold 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, pMescalA, 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% CO₂ 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 (1.5 ml/100 g body weight) 1 h before embryos were harvested. Embryonic heads were fixed in

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