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Enhanced BMP signaling prevents degeneration and leads to endochondral ossification of Meckel's cartilage in mice

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Abstract

Meckel's cartilage is a transient supporting tissue of the embryonic mandible in mammals, and disappears by taking different ultimate cell fate along the distal-proximal axis, with the majority (middle portion) undergoing degeneration and chondroclastic resorption. While a number of factors have been implicated in the degeneration and resorption processes, signaling pathways that trigger this degradation are currently unknown. BMP signaling has been implicated in almost every step of chondrogenesis. In this study, we used *Noggin* mutant mice as a model for gain-of-BMP signaling function to investigate the function of BMP signaling in Meckel's cartilage development, with a focus on the middle portion. We showed that *Bmp2* and *Bmp7* are expressed in early developing Meckels' cartilage, but their expression disappears thereafter. In contrast, *Noggin* is expressed constantly in Meckel's cartilage throughout the entire gestation period. In the absence of *Noggin*, Meckel's cartilage is significantly thickened attributing to dramatically elevated cell proliferation rate associated with enhanced phosphorylated Smad1/5/8 expression. Interestingly, instead of taking a degeneration fate, the middle portion of Meckel's cartilage in *Noggin* mutants undergoes chondrogenic differentiation and endochondral ossification contributing to the forming mandible. Chondrocyte-specific expression of a constitutively active form of BMPRIa but not BMPRIa leads enlargement of Meckel's cartilage, phenocopying the consequence of *Noggin* deficiency. Our results demonstrate that elevated BMP signaling prevents degeneration and leads to endochondral ossification of Meckel's cartilage, and support the idea that withdrawal of BMP signaling is required for normal Meckel's cartilage development and ultimate cell fate.

Keywords

BMP signaling; Noggin; BmprIa; Meckel's cartilage; development; differentiation

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Introduction

In mammals, Meckel's cartilage acts as a transient support tissue in mandibular arch during early embryogenesis and disappears at late gestation or neonatal stage of development (Dixon, 1997). In mice, the development of Meckel's cartilage, which derives from cranial neural crest (CNC) cells, begins at the first molar bud region at embryonic day 11 (E11), and continues with extension at both the anterior and posterior ends with addition of CNC cells at the chondrogenic front, forming a pair of rod-like hyaline cartilage (Ito et al., 2002). Previous studies have established different ultimate fates of Meckel's cartilage in rodents (Bhaskar et al., 1953; Bernick and Patek, 1969; Savostin-Asling and Asling, 1973; Harada and Ishizeki, 1998; Ishizeki et al., 2001). The distal region of Meckel's cartilage undergoes endochondral ossification contributing to the symphysis of the mandible, whereas the most proximal portion (caudal end) gives rise to malleus and incus through endochondral ossification as well. However, in the middle portion, which counts for the majority of Meckel's cartilage, the chondrocytes become hypertrophic, but never further differentiate and undergo degeneration subsequently, except that the posterior part of this portion is replaced by the sphenomandibular ligament (Harada and Ishizeki, 1998).

Similar to the development of other fetal cartilaginous skeleton, chondrogenesis of Meckel's cartilage progresses via mesenchymal cell condensation, proliferation, and differentiation of chondrocytes. Despite its CNC-origin, the initial chondrogenesis of Meckel's cartilage shares many similar mechanisms with those mesoderm-derived cartilaginous elements. For example, *Sox9* is required for the determination of chondrogenic lineage in axial and appendicular skeletal elements as well as CNC-derived cartilages and endochondral bones (Bi et al., 1999; Mori-Akiyama et al., 2003). In addition, a number of signaling molecules, including bone morphogenetic protein (BMP), connective tissue growth factor (CTGF), fibroblast growth factor (FGF), transforming growth factor β (TGFβ), and Wnt that are known to regulate chondrogenesis of appendicular skeletons, have also been implicated in Meckel's cartilage development (Chai et al., 1994; Nonaka et al., 1999; Ito et al., 2002; Shimo et al., 2004; Terao et al., 2011; Zhang et al., 2011). However, unlike the fate of those mesoderm-derived cartilaginous elements and other CNC-derived cartilages such as cranial base, the majority of Meckel's cartilage does not develop further and becomes degenerated. While previous studies have revealed potential contributions of several factors and cellular processes to the disappearance/resorption (Trichilis and Wroblewski, 1997; Harada and Ishizeki, 1998; Sakakura et al., 2005, 2007a, 2007b; Tsuzurahara et al., 2011; Yang et al., 2012), signaling pathways that prevent further differentiation and trigger degeneration of Meckel's cartilage remain elusive.

BMP signaling acts via binding to heterotetrameric complexes of type I and II receptors, leading to activation of Smad-dependent canonical pathway and Smad-independent noncanonical MAPK pathways that regulate targeted gene expression (Massagué, 2012). BMP signaling plays important roles in multiple steps of chondrogenesis and endochondral bone formation. In the earliest stage of chondrogenesis, BMP signaling promotes mesenchymal cells to differentiate into chondrocytes and stimulates chondrocyte proliferation by inducing *Sox9* expression (Denker et al., 1999; Zehentner et al., 1999; Yoon et al., 2005). BMP signaling also controls chondrocyte differentiation by promoting chondrocyte hypertrophy and is required for endochondral bone formation (Minina et al., 2001; Valcourt et al., 2002; Kobayashi et al., 2005; Retting et al., 2009).

Noggin is a potent BMP antagonist, binding preferentially to BMP2, BMP4, and BMP7 to prevent their signaling (Zimmerman et al., 1996; Groppe et al., 2002; Chen et al., 2004). *Noggin* deficiency in mice, a model of gain-of-BMP function, leads to overgrowth of skeletal elements including the mandible (Brunet et al., 1998; Stottmann et al., 2001).

However, whether or not loss of *Noggin* alters the development of Meckel's cartilage has not been reported. In our and other's previous studies on the effects of overdosed BMP activity on the development of palate, tooth, and temporomandibular joint using *Noggin* mutant mice as a model, the formation of significantly enlarged Meckel's cartilage was observed (He et al., 2010; Lana-Elola et al., 2011; Wang et al., 2011; Hu et al., 2012). In the present study, we followed up our previous observation by detailed analysis of the phenotype in *Noggin* mutants with focus on the middle portion of Meckel's cartilage. We show here that *Noggin* is expressed constantly in Meckel's cartilage throughout the entire embryonic stage, whereas *Bmp2* and *Bmp7* but not *Bmp4* are expressed in developing Meckel's cartilage only at the early stage. In the absence of *Noggin*, the expression of phosphorylated Smad1/5/8 is significantly elevated associated with enhanced cell proliferation rate in Meckel's cartilage, leading to formation of enlarged Meckel's cartilage. Instead becoming arrest of further differentiation and subsequent degeneration, Meckel's cartilage in *Noggin* mutants undergoes endochondral differentiation, unifying with the developing mandibular bone to form a larger mandible. Forced expression of a constitutively active form of BMPRIa in chondrocyte lineage also produces enlarged Meckel's cartilage, mimicking the consequence of *Noggin* deficiency. We conclude that withdrawal of BMP signaling represents an essential step for arrest of chondrogenic differentiation and subsequent degeneration of Meckel's cartilage during development.

Materials and Methods

Animals

The generation of *Noggin* mutant ($Nog^{+/-}$), *Col2a1-Cre*ER^{T2}, *Rosa26* reporter, *pMes-caBmprIa*, and *pMes-caBmprIb* mice have been described previously (Soriano, 1999; McMahon et al., 1998; Chen et al., 2007; He et al., 2010; Yu et al., 2010). $Nog^{-/-}$ embryos were harvested timed pregnant $Nog^{+/-}$ females mated with $Nog^{+/-}$ males. To achieve chondrocyte-specific expression of constitutively active form of BMPRIa or BMPRIb in developing embryos, *Col2-Cre*ER^{T2} transgenic mice were mated with either *pMes-caBmprIa* or *pMes-caBmprIb* conditional transgenic mice, and timed pregnant females were injected intraperitoneally once with tamoxifen (Sigma) at embryonic day 12.5 (E12.5). Each mouse received a single injection of 200-ul tamoxifen solution, dissolved in corn oil (Sigma), at the final concentration of 1 mg/20 g body weight. Tail sample of each harvested embryo was subjected to PCR-based genotyping. Primer information for PCR-based genotyping for these mouse lines is available upon request. Use of animals in this study was approved by the Institutional Animal Care and Use Committee (IACUC) of Tulane University, and was in strict accordance with the recommendations in the Guide for Care and Use of Laboratory Animals of the National Institutes of Health.

Skeletal preparation, histology, immunostaining, in situ hybridization, X-gal staining, and BrdU labeling assays

Skeletal staining was conducted by Alcian blue/Alizarin red staining for cartilage and bone, as described previously (Zhang et al., 2000). For histological analysis and section in situ hybridization assay, samples were fixed in 4% paraformaldehyde (PFA)/PBS at 4°C for overnight, dehydrated through graded ethanol, paraffin embedded, and sectioned at 10 μm. Sections were subjected to standard Hematoxylin/Eosin staining for histological examination, and to in situ hybridization for gene expression assay using non-radioactive riboprobes, as described previously (St. Amand et al., 2000). For immunohistochemical staining, PFA fixed samples were washed in 30% sucrose/PBS, embedded in O.C.T. compound (Tissue-Tek) and cryo-sectioned at 10 μm. Prior to the application of primary antibody (anti-pSmad1/5/8; from Cell Signaling), slides were treated with acetone for 10-min and air-dried. For immunostaining with antibodies against type II and type X collagens,

samples were processed same as that for histological analysis, and paraffin-embedded sections were subjected to immunostaining, as described previously (Xiong et al., 2009). For X-gal staining, samples were fixed in 0.2% glutaraldehyde at 4°C overnight, washed in ice-cold PBS and subsequently with 30% sucrose/PBS, embedded in O.C.T., and cryo-sectioned at 10 μm. X-gal staining was conducted as described previously (Ito et al., 2003). All experiments were repeated at least three times. To determine cell proliferation rate, we performed Bromodeoxyuridine (BrdU) labeling experiment using BrdU Labeling and detection Kit (Roche Diagnostics Corp.), as described previously (Xiong et al., 2009). BrdU-positive cells and total cell number in a section of Meckel's cartilage were counted on higher magnification image on computer. Data collected from nine sections of three samples (three continuous sections from each sample) of either control or mutant were subjected to statistical analysis. The outcome was presented as percentage of labeled cells among total cells in Meckel's cartilage, and Student's *t*-test was used to determine the significance of difference.

Results

Expression of *Noggin* and *Bmp* genes in developing Meckel's cartilage

To investigate the role of Noggin and BMP signaling in Meckel's cartilage development and degeneration, we examined the expression of *Noggin* and several *Bmp* genes including *Bmp2*, *Bmp4*, and *Bmp7* in the middle portion of Meckel's cartilage. We used the first molar germ as a landmark for our analyses, since the development of Meckel's cartilage initiates at this region. We took advantage of $Nog^{+/-}$ mice in which the *Noggin* coding sequences were replaced with the *LacZ* reporter gene by targeted knock-in approach (McMahon et al., 1998). We assayed *Noggin* expression by X-gal staining from E11.5 at which time Meckel's cartilage condensation appears, to the last day day of gestation (E18.5). As shown in Fig. 1, LacZ activity was detected, though relatively weaker, in forming Meckel's cartilage at E11.5 (Fig. 1A). Strong X-gal staining was observed in Meckel's cartilage at E12.5 (Fig. 1B), and remained there throughout the entire embryonic stage (Fig. 1C, 1D). In situ hybridization assay conformed *Noggin* expression in Meckel's cartilage (insert in Fig. 1C). We performed in situ hybridization assay to examine *Bmp* gene expression. Since all these three *Bmp* genes are expressed in the developing tooth, their expression in tooth germ was therefore used as positive control for in situ hybridization assay. We found that *Bmp2* is expressed in Meckel's cartilage condensation at E11.5, and the expression becomes stronger at E12.5 (Fig. 2A, 2D). However, from E13.5 on, *Bmp2* expression became undetectable in Meckel's cartilage, though lower level of *Bmp2* expression was observed in surrounding mandibular mesenchyme and mandibular bone (Fig. 2G, 2J, 2M). While *Bmp4* expression was never observed in developing Meckel's cartilage in all stages examined, we did detect *Bmp4* transcripts in the surrounding mandibular mesenchyme and mandibular bone at E11.5 to E13.5 as well as E16.5 (Fig. 2B, 2E, 2H, 2K, and 2N). *Bmp7* expression was found in Meckel's cartilage and surrounding mandibular mesenchyme at relatively lower level at E11.5 and E12.5 (Fig. 2C, 2F). However, at E13.5, *Bmp7* expression disappeared, but was detected in the adjacent forming mandibular bone (Fig. 2I). At following E14.5 and E16.5 stages, *Bmp7* expression was not found in Meckel's cartilage as well as surrounding tissues (Fig. 2L, 2O). These results reveal dynamic expression patterns of these *Bmp* genes and implicate potential role of BMP signaling in Meckel's cartilage development.

Lack of *Noggin* leads to enlargement and endochondral ossification of Meckel's cartilage

In our and other's previous studies, the presence of significantly thickened Meckel's cartilage in *Noggin*-deficient embryos was noticed (He et al., 2010; Lana-Elola et al., 2011; Wang et al., 2011). Indeed, skeleton preparations of embryonic heads revealed enlarged

craniofacial cartilages in the mutants at E14.5 including Meckle's cartilage, nasal cartilage, basisphenoid, and presphenoid, as compared to the controls (Fig. 3A, 3B). At E18.5, similar to the control, the enlarged causal end and symphysis of Meckel's cartilage in the mutants remained an unossified status (Fig. 3C–F). To determine the developmental process and fate of the middle portion of Meckel's cartilage in *Noggin* mutants, we made detailed histological analysis. We found that mesenchymal condensations of Meckel's cartilage in $Nog^{-/-}$ embryos formed at the same time as that in the control at E11.5, evidenced by the expression of *Sox9* (Fig. 4A, 4B). Comparing to the controls, the Sox9 expression domain in the mutants appeared larger at this stage, suggesting recruitment of more cells to the blastema of mutant Meckel's cartilage. At E12.5, Meckel's cartilage in both controls and mutants became discernibly larger in diameter (Fig. 4C, 4D). At E14.5, the size of Meckel's cartilage increased only slightly in wild type controls (Fig. 4E), but became significantly larger in mutants (Fig. 4F). At E16.5 when chondrocytes had become hypertrophic and degeneration began in control Meckel's cartilage (Harada and Ishizeki, 1998; Sakakura et al., 2005), the mutant counterpart had undergone extensive endochondral ossification at the first molar level (Fig. 4G, 4H). However, at the posterior region of the middle portion, endochondral ossification had not yet begun at this stage (Fig. 4J). At E18.5 when degeneration of Meckel's cartilage appeared obviously in controls (Fig. 4K), Meckel's cartilage in $Nog^{-/-}$ mutants had almost ossified and integrated into mandibular bone (Fig. 4L). At this time, the posterior middle portion had also begun to ossify (Fig. 4N), indicating delayed chondrogenic differentiation and endochondral ossification in the posterior domain. These observations demonstrate that in the absence of *Noggin*, Meckel's cartilage becomes dramatically enlarged, and the middle portion of Meckel's cartilage continues chondrogenic differentiation process and eventually undergoes endochondral ossification instead of degeneration.

To further confirm that Meckel's cartilage in $Nog^{-/-}$ mice undergoes similar chondrogenic differentiation process as that in long bone formation, we examined the expression of chondrogenic differentiation markers at the first molar level: collagen type II (*Col II*), a marker for chondrocytes, *Ihh*, a marker for prehypertrophic chondrocytes, and collagen type X (*Col X*), a marker for hypertrophic chondrocytes. Our results showed that at E14.5, *Col II* expression was detected in Meckel's cartilage in controls and $Nog^{-/-}$ mice (Fig. 5A, 5B). However, at this stage, expression of *Ihh* and *Col X* was also detected in $Nog^{-/-}$ mutants but was not in the controls (Fig. 5F, 5J), indicating that Meckel's cartilage in the mutant had undergone chondrogenic differentiation. At E16.5, *Col II* expression became barely detectable in the controls (Fig. 5C), but was present in a few undifferentiated chondrocytes in the mutant (Fig. 5D). At this stage, *Ihh* and *Col X* expression was also absent in the control (Fig. 5G, 5K), despite the presence of hypertrophic chondrocytes (Harada and Ishizeki, 1998; Sakakura et al., 2005). This observation of lack of *Col X* expression in hypertrophic chondrocytes in the middle portion of Meckel's cartilage is consistent with the previous report (Chung et al., 1995), suggesting that the cellular hypertrophy of Meckel's cartilage is not necessarily associated with the expression of chondrogenic differentiation markers. In the mutant at this stage, while *Ihh* expression was no longer detectable, *Col X* was found in remaining chondrocytes that underwent final differentiation (Fig. 5J, 5L), consistent with histological evidence that the middle portion of Meckels' cartilage in $Nog^{-/-}$ mutants has undergone extensive chondrogenic differentiation and endochondral ossification at this stage. To examine if there is an invasion of adjacent *Noggin*-negative mandibular mesenchymal cells into mutant Meckel's cartilage that may function to resorb Meckel's cartilage during its chondrogenic differentiation, we made LacZ staining on E14.5 mutant Meckel's cartilage that had undergone chondrogenic differentiation. As shown in the insert in Fig. 5J, all cells in mutant Meckel's cartilage are LacZ positive, suggesting a lack of invasion of cells from adjacent tissues.

Absence of *Noggin* leads to upregulated BMP signaling and elevated cell proliferation in Meckel's cartilage

To determine if the absence of *Noggin* alters BMP signaling activity, we performed immunohistochemical staining to examine the expression of phosphorylated Smad1/5/8 (pSmad1/5/8). In wild type controls, at E12.5 when *Bmp2* is strongly expressed in Meckel's cartilage (Fig. 2), relatively abundant pSmad1/5/8 positive cells were detected (Fig. 6A). However, at E13.5 when *Bmp* genes are no longer expressed in Meckel's cartilage (Fig. 2), the number of pSmad1/5/8 positive cells accordingly decreased dramatically (Fig. 6C). In contrast, as we expected, the number of pSmad1/5/8 positive cells increased significantly in $Nog^{-/-}$ Meckel's cartilage compared to the controls at both stages (Fig. 6B, 6D). At E14.5, wild type Meckel's cartilage remained a level of pSmad1/5/8 positive cells similar to that at E13.5, the amount of pSmad1/5/8 positive cells began to reduce (Fig. 6E, 6F).

We next conducted BrdU labeling experiment to determine if the enlarged Meckel's cartilage is a consequence of increased cell proliferation rate. We found that cell proliferation rate was indeed increased significantly ($P < 0.001$) in mutants as compared with that in controls at E12.5 (Fig. 7A, 7B, 7G). However, at E14.5, we did not detect a significant difference in cell proliferation rates between controls and mutants (Fig. 7C, 7D, 7G), consistent with the reduced amount of pSmad1/5/8 positive cells in mutant Meckel's cartilage. This observation could be attributed to the fact that the chondrocytes in mutant Meckel's cartilage had begun to differentiate at this stage, as assessed by the expression of chondrogenic differentiation markers shown above. Elevated cell proliferation rate was also found in other cranial cartilages such as presphenoid (Fig. 7E, 7F, 7H), suggesting that elevated cell proliferation represents at least one of the mechanisms underlying the enlargement of cranial cartilages in *Noggin* mutants.

Activation of BMPRIa-mediated signaling in Meckel's cartilage resembles effect of *Noggin*-deficiency

Because *Bmp2* and *Bmp7* are expressed in developing Meckel's cartilage only at early stage (E11.5 and E12.5), but are no longer expressed there from E13.5 on, despite continuous expression of these two *Bmps* as well as *Bmp4* in the surrounding mesenchyme and developing mandubular bones at late stage, we wondered if the withdrawal of BMP signaling in developing Meckel's cartilage represents a critical step for the arrest of further chondrogenic differentiation and subsequent degeneration. To address this question, we generated conditional transgenic mice that expressed a constitutively active form of either BMPRIa (*caBmprIa*) or BMPRIb (*caBmprIb*) in chondrocyte lineage. The inducible transgenic allele *Col2a1-Cre*ER^{T2}, upon administration of tamoxifen, gives rise to high specificity and strong efficiency of Cre recombination activity in chondrocytes including Meckel's cartilage (Chen et al., 2007; Fig. 8A). We found that the expression of *caBmprIb,* activated by administration of tamoxifen at E12.5, did not produce a phenotype in Meckel's cartilage (Fig. 8B; N = 5), despite previous evidence for the functional efficiency of this transgenic allele in rescuing tooth defects in mice lacking *BmprIa* and in causing severe ichthyosis-like skin phenotype in mice expressing this transgenic allele in the skin (Yu et al., 2010; Li et al., 2011). However, when *caBmprIa* was activated in chondrocytes after tamoxifen administration, enlarged Meckel's cartilage was found in *Col2a1-Cre*ER^{T2};*pMes-caBmprIa* mice (Fig. 8C, 8D; N = 5). The enlarged Meckel's cartilage also underwent endochondral differentiation, as assessed by the expression of *Ihh* and *Col X*, mimicking the phenotype observed in $Nog^{-/-}$ mice (Fig. 8E, 8F; N = 2).

Discussion

As a transient embryonic structure, Meckel's cartilage disappears through different ultimate fates of the chondrocytes depending on their position (Bhaskar et al., 1953; Frommer and Margolies, 1971; Richman and Diewert, 1988; Harada and Ishizeki, 1998). It has been well established that the majority of Meckel's cartilage, the middle portion, undergoes degeneration. Despite that the chondrocytes in this portion become hypertrophic, they do not express chondrogenic differentiation markers, such as *Col X* and *Ihh* (Chung et al., 1995; this study), suggesting an arrest of chondrogenic differentiation prior to degeneration. Previous studies have implicated multiple factors and cellular processes in degeneration and resorption of Meckel's cartilage, including several matrix metalloproteinases (Ishizeki and Nawa, 2000; Sakakura et al., 2007a, 2007b), macrophages (Harada and Ishezeki, 1998; Sakakura et al., 2005; Tsuzurahara et al., 2011), and possible autophagy and apoptosis (Trichilis and Wroblewski, 1997; Yang et al., 2012). However, the underlying mechanism that is responsible for the arrest of chondrogenic differentiation and subsequent degeneration of Meckel's cartilage remains unknown.

In this study, we investigated the role of BMP signaling in Meckel's cartilage development using $Nog^{-/-}$ mice as a gain-of BMP function model. We focused our studies on the middle portion of Meckel's cartilage because of its distinct ultimate fate. We found that in the absence of *Noggin*, which leads to elevated BMP signaling and cell proliferation rate, the fate of chondrocytes in this portion is changed. Meckel's cartilage becomes enlarged and undergoes further chondrogenic differentiation and endochondral ossification. This phenotype is resembled by expression of a constitutively active form of *BmprIa* in chondrocyte lineage, indicating that suppression of BMP activity is essential for normal Meckel's cartilage development and the ultimate fate of the chondrocytes. Interestingly, the caudal end and the symphysis of Meckel's cartilage, both undergo endochondral differentiation eventually in wild type animals, also became enlarged in *Noggin* mutants. However, they exhibited similar chondrogenic differentiation status in the mutants at the end of gestation as compared to the controls, suggesting that loss of *Noggin* may not alter the fate of chondrocytes in these two compartments.

BMP signaling has been shown to play critical roles in multiple steps of chondrogenesis and endochondral ossification. In developing Meckel's cartilage, *Bmp2* and *Bmp7* are expressed in the mesenchymal condensation and chondrocytes of Meckel's cartilage at early stage, consistent with the role of BMP signaling in promoting differentiation of mesenchymal cells into chondrocytes and stimulating chondrocyte proliferation (Denker et al., 1999; Zehentner et al., 1999; Yoon et al., 2005). However, the expression of these two genes is abruptly inhibited in Meckel's cartilage at E13.5, and never comes back. In accord with this inhibition of *Bmp* gene expression is the dramatically reduced BMP signaling activity in Meckel's cartilage after this stage. The residual BMP signaling activity present in Meckel's cartilage from E13.5 on could be the consequence of the expression of *Bmp2*, *Bmp4*, and *Bmp7* in the surrounding mesenchyme and the developing mandibular bone. Since BMP signaling also induces/stimulates chondrocyte hypertrophy and is required for endochondral bone formation (Valcourt et al., 2002; Kobayashi et al., 2005; Retting et al., 2009), the suppression of *Bmp* expression appears to responsible for the arrest of chondrogenic differentiation and subsequent endochondral ossification. Interestingly, although cellular hypertrophy is seen in the chondrocytes of Meckel's cartilage, these hypertrophic chondrocytes never express chondrogenic differentiation markers including *Ihh* and *Col X*, suggesting an abnormal hypertrophic process. It was shown previously that *Col X* expression is closely associated with the hypertrophic chondrocyte differentiation that undergoes endochondral ossification (Solursh et al., 1986). Thus the absence of *Col X*

expression in Meckel's cartilage could be responsible, at least partially, for the lack of endochondral ossification.

BMP signaling is known to be transduced by a type II receptor together with two originally identified type I receptors (BMPRIa and BMPRIb) as well as activin receptor type IA (ActRIa). These type I BMP receptors appear to have distinct but overlapping function during embryogenesis. In the developing chick limb, expression of *caBmprIa* but not *caBmprIb* promotes chondrogenic differentiation (Zou et al., 1997). Similarly in mice, expression of *caBmprIa* in chondrocytes accelerates chondrogenic differentiation in the axial and appendage skeletal elements (Kobayashi et al., 2005). Consistent with these previous findings, in our current study, we show that expression of *caBmprIa* but not *caBmprIb* in chondrocyte lineage induces formation of significantly enlarged Meckel's cartilage and enhances chondrogenic differentiation, indicating that BMPRIa and BMPRIb have distinct function in regulating chondrogenesis of CNC-derived cells in mice. Interesting, tissue-specific inactivation of *BmprIa* in the CNC lineage did not affect the size of Meckel's cartilage (Li et al., 2011), suggesting potential functional redundancy between *BmprIa* and other type I BMP receptors such as *BmprIb* whose knockout did not give rise to any obvious craniofacial defect (Baur et al., 2000; Yi et al., 2000). On the other hand, in the developing chicken face, mis-expression of either *caBmprIa* or *caBmprIb* similarly produced enlarged cranial cartilages including Meckel's cartilage (Ashique et al., 2002), suggesting a species specific function of BMPRIB-mediated signaling in the regulation of chondrogenesis during craniofacial development. Nevertheless, these observations support the idea that despite different origins of germ layer and different species, BMPRIa-mediated signaling has a conserved role in promoting chondrogenesis.

In conclusion, in this study, we provide evidence that precisely regulated *Bmp* gene expression and BMP signaling activity is essential for normal development of the middle portion of Meckel's cartilage and its ultimate chondrocyte fate. Withdrawal of BMP signaling leads to arrest of chondrogenic differentiation and subsequent degeneration of this portion of Meckel's cartilage during embryogenesis. Elevation BMP signaling activity in the absence of *Noggin* or maintenance of BMP signaling activity by expression of *caBmprIa* enhances chondrogenic differentiation, leading to endochondral ossification of the middle portion of Meckel's cartilage.

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Research Highlights

1. *Bmp* genes and Noggin are expressed in developing Meckel's cartilage.
2. *Noggin* mutants exhibit enlarged craniofacial cartilages and Mekcel's cartilage.
3. $Nog^{-/-}$ Meckel's cartilage undergoes chondrogenic differentiation.
4. Elevated BMP signaling in phenocopies the fate of $Nog^{-/-}$ Meckel's cartilage.
5. BMP signaling withdraw is required for normal Meckel's cartilage development.

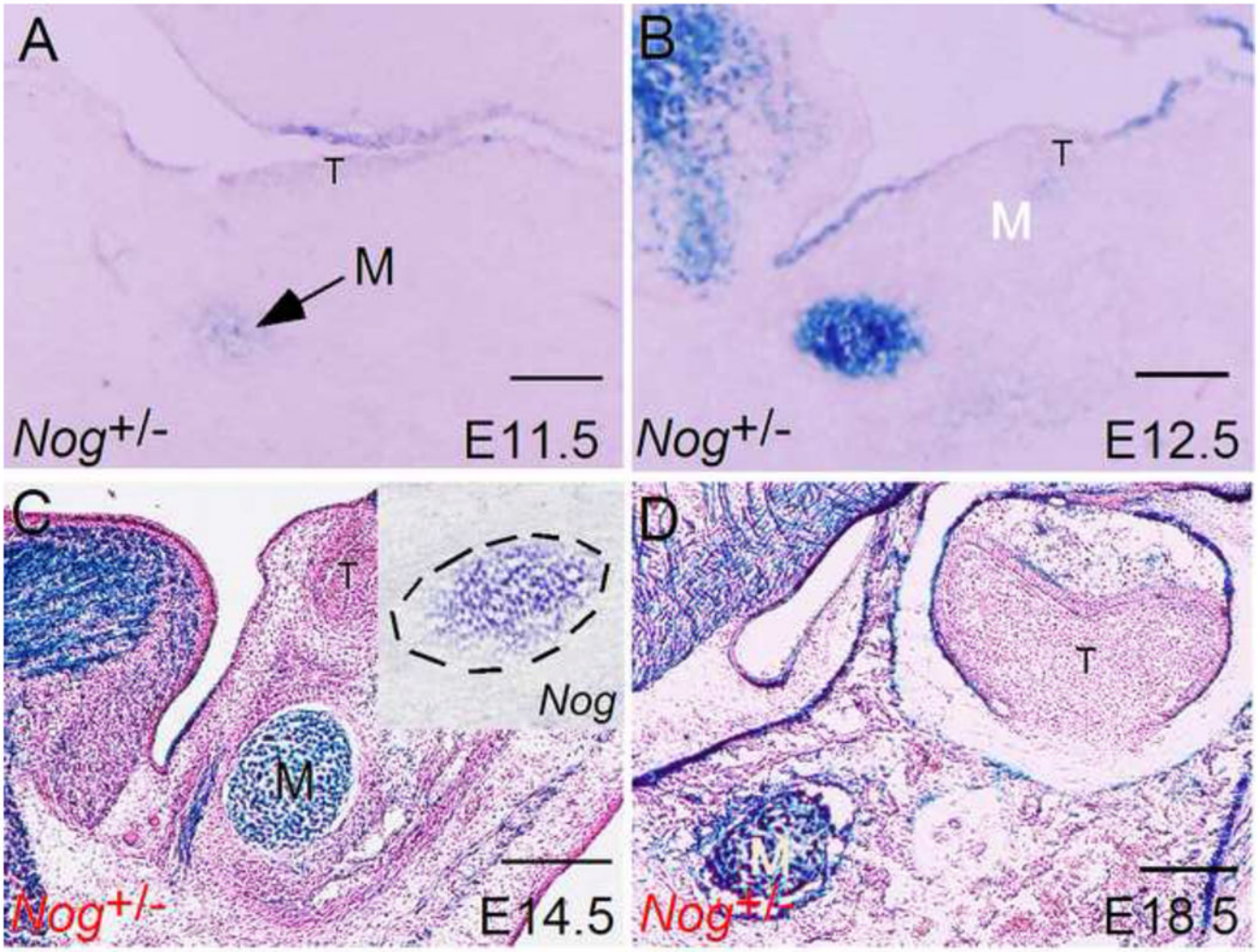

Figure 1. Expression of *Noggin* in developing Meckel's cartilage

Noggin expression, as assessed by LacZ activity, is seen in the initial mesenchymal condensation of Meckel's cartilage at E11.5 (A), and in Meckel's cartilage through subsequent stages of embryogenesis (B–D). Insert in (C) shows in situ hybridization detection of *Noggin* expression in Meckel's cartilage M, Meckel's cartilage. Scale bar = 100-μm.

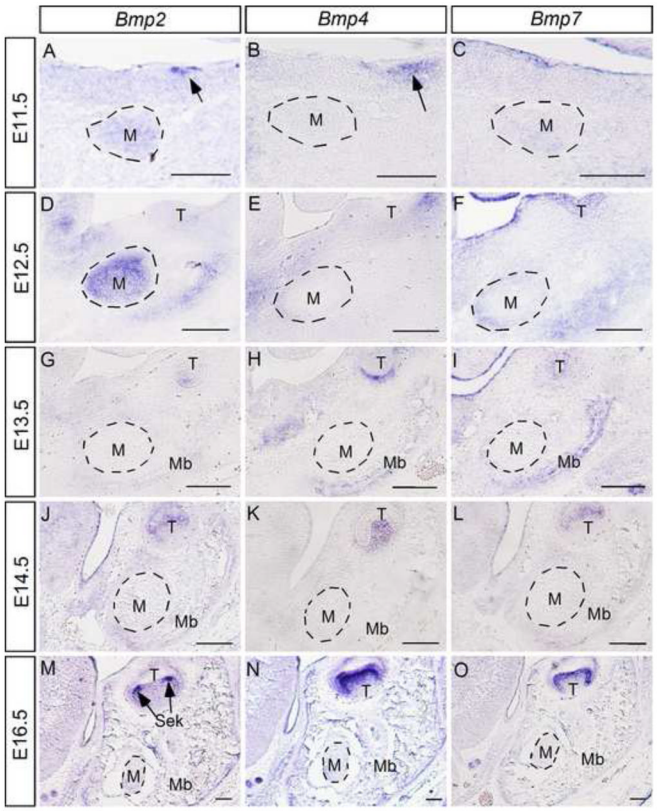

Figure 2. Expression of *Bmp2*, *Bmp4*, and *Bmp7* in developing Meckel's cartilage

(A–C) In situ hybridization shows *Bmp2* and *Bmp7* but not *Bmp4* expression in mesenchymal condensation of Meckel's cartilage at E11.5. Note *Bmp2* expression in the dental epithelium (arrow in panel A) and *Bmp4* expression in the dental mesenchyme (arrow in panel B). (D–F) At E12.5, strong *Bmp2* expression and relatively weaker *Bmp7* expression but not *Bmp4* is detected in Meckel's cartilage. In addition, all three *Bmp* genes are expressed in the surrounding mesenchymal tissues. (G–O) At E13.5 and subsequently developmental stages at E14.5 and E16.5, these *Bmp* genes are no longer expressed in Meckels' cartilage but are expressed at lower levels in the surrounding mesenchymal tissues and developing mandibular bone. M, Meckel's cartilage; T, tooth; Mb, mandibular bone; Sek, secondary enamel knot. Scale bar = 100-μm.

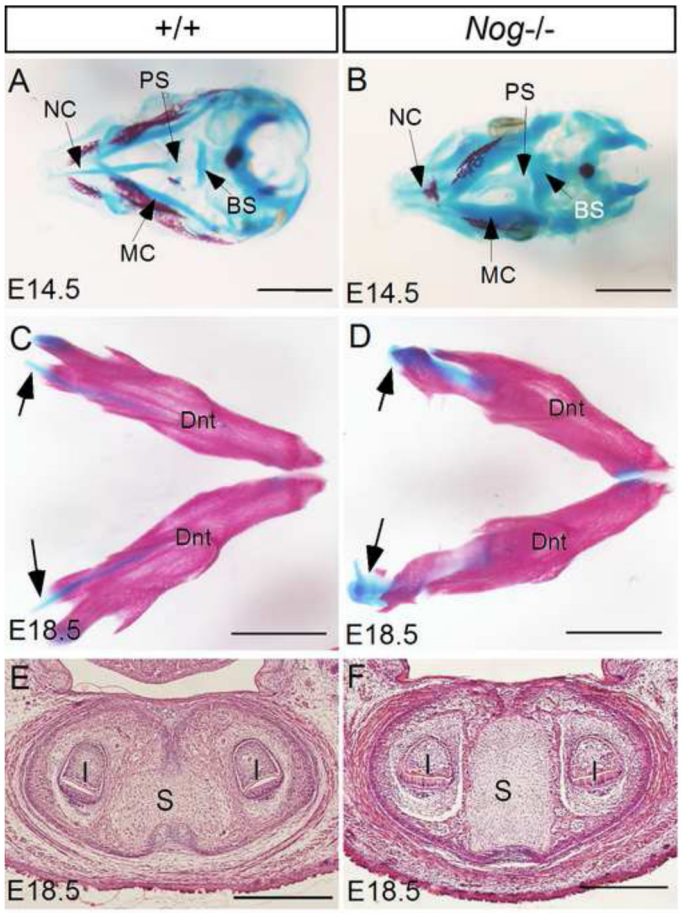

Figure 3. Enlarged craniofacial cartilages in in Noggin mutants

(A, B) Skeleton preparations of control (A) and *Nog*$^{-/-}$ (B) heads at E14.5 reveal generally enlarged craniofacial cartilages in the mutants. (C, D) Skeletal staining of mandibular arches of E18.5 control (C) and mutant (D) shows enlarged but unossified caudal end (black arrows) of Meckel's cartilage in mutant. (E, F) Coronal sections of E18.5 control (E) and *Nog*$^{-/-}$ mutant (F) reveal enlarged and unossified symphysis in the mutant. I, incisor; S, symphysis; BS, basisphenoid; MC, Meckel's cartilage; NC, nasal cartilage; PS, presphenoid; Dnt, dentary. Scale bar = 2-mm (A–D); 500-μm (E, F).

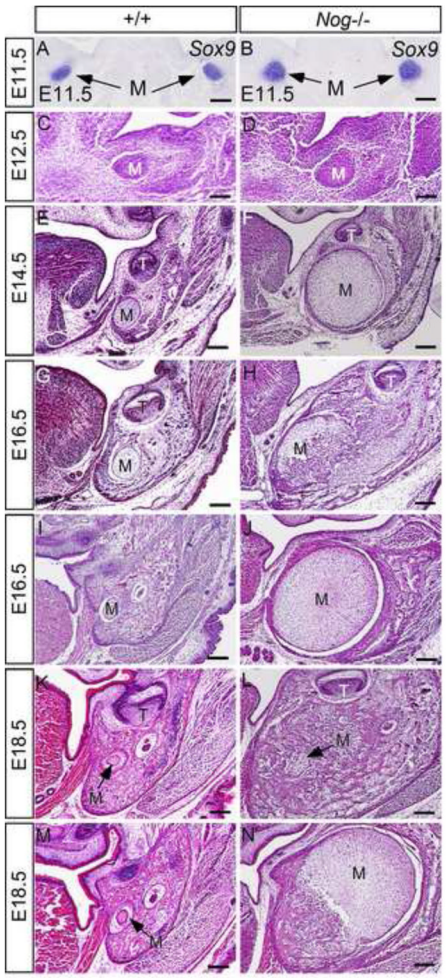

Figure 4. Lack of *Noggin* leads to formation of enlarged Meckel's cartilage and endochondral ossification

(A, B) In situ hybridization shows *Sox9* expression in mesenchymal condensations of Meckel's cartilage in control and $Nog^{-/-}$ embryos at E11.5. (C–F) Histological sections through the middle portion of Meckel's cartilage of E12.5 and E14.5 control and $Nog^{-/-}$ embryos show enlarged Meckel's cartilage in mutants. (G, H) Histological sections through the first molar level of mandible show Meckel's cartilage with hypertrophic chondrocytes in E16.5 control mice (G) and Meckel's cartilage in E16.5 $Nog^{-/-}$ mice that are undergoing endochondral ossification (H). (I, J) histological sections through the posterior part of middle portion of E16.5 control (I) and $Nog^{-/-}$ mutant (J) show enlarged Meckel's cartilage in the mutant that has not yet started endochondral ossification process. (K–N) Coronal sections through the first molar region (K, L) and the posterior region (M, N) of the middle portion of Meckel's cartilage of E18.5 control (K, M) and mutant (L, N) show degenerating Meckel's cartilage in controls and almost completely ossified Meckel's cartilage at the first molar level and Meckel's cartilage undergoing endochondral ossification in the posterior region in mutants. M, Meckel's cartilage; T, tooth; Scale bar = 100-μm.

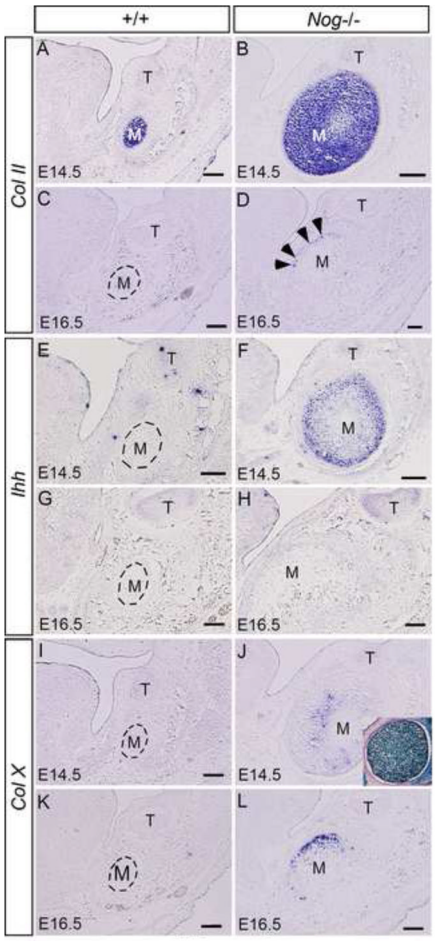

Figure 5. Expression of chondrogenic differentiation markers developing Meckel's cartilage of *Nog*$^{-/-}$ mutants

(A–D) Expression of *Col II* is seen in Meckel's cartilage of wild type control and *Nog*$^{-/-}$ embryos at E14.5 (A, B), but is barely detectable in wild type Meckel's cartilage at E16.5 (C). *Col II* expression is also observed in some undifferentiated chondrocytes (arrowheads) of *Nog*$^{-/-}$ Meckel's cartilage at E16.5 (D). (E–H) The prehypertrophic chondrocyte marker *Ihh* is never expressed in control Meckel's cartilage at E14.5 (E) and E16.5 (G). In contrast, *Ihh* is strongly expressed in *Nog*$^{-/-}$ Meckel's cartilage at E14.5 (F) but not at E16.5 (H). (I–L) *Col X* expression is not detected in the middle portion of Meckel's cartilage in wild type embryos at E14.5 (I) and E16.5 (K), but is seen in *Nog*$^{-/-}$ Meckel's cartilage at both stages (J, L). Insert in (J) shows that all cells in E14.5 *Nog*$^{-/-}$ Meckel's cartilage are LacZ-positive. M, Meckel's cartilage; T, tooth. Scale bar = 100-μm.

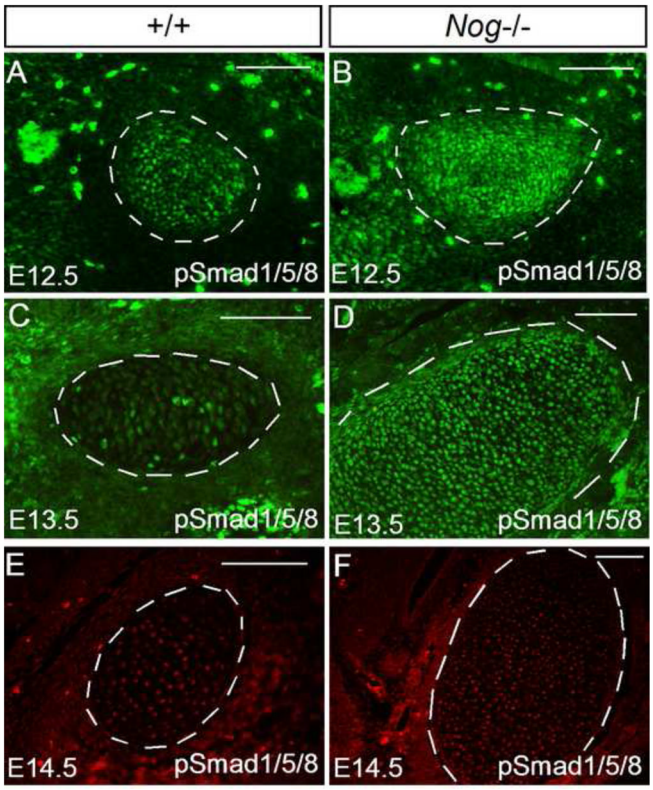

Figure 6. Elevated pSmad activity in *Nog*$^{-/-}$ Meckel's cartilage

(A–F) Immunohistochemical staining show pSmad1/5/8 positive cells in control and *Nog*$^{-/-}$ Meckel's cartilage. At E12.5, control Meckel's cartilage has relatively high number of pSmad1/5/8 positive cells (A), but at E13.5 and E14.5, it contains only a few positive cells (C, E). In contrast, the number of pSmad1/5/8 positive cells increases significantly in *Nog*$^{-/-}$ Meckel's cartilage at E12.5 (B) and E13.5 (D). However, at E14.5, the number of pSmad1/5/8 positive cells is reduced in *Nog*$^{-/-}$ Meckel's cartilage (F). Scale bar = 100-μm.

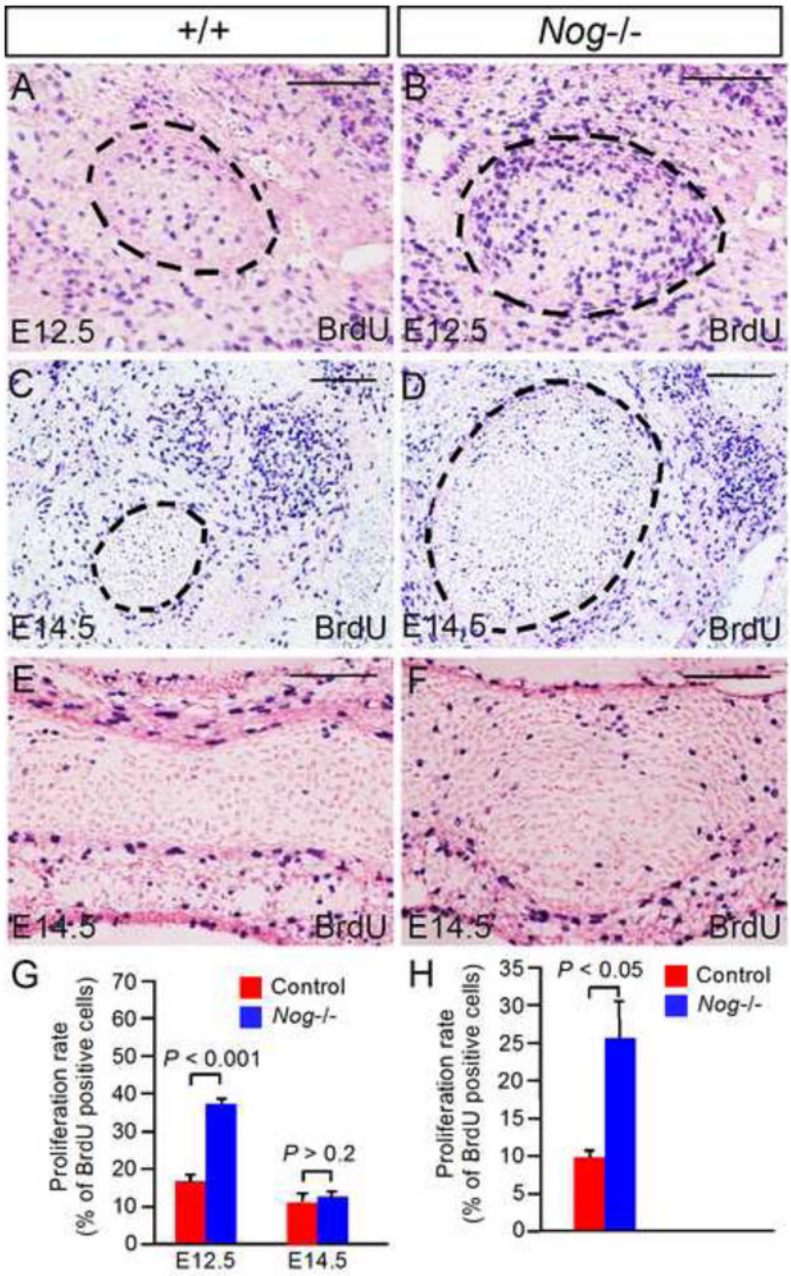

Figure 7. Lack of Noggin leads to elevated cell proliferation rate in Meckel's cartilage

(A–D) Coronal sections show BrdU labeled cells in control and *Nog*$^{-/-}$ Meckel's cartilage at E12.5 and E14.5. (E, F) Coronal sections show BrdU labeled cells in presphehoid of E14.5 control (E) and mutant F). (G) Statistical analysis shows significantly increased cell proliferation rate in *Nog*$^{-/-}$ Meckel's cartilage at E12.5 ($P < 0.001$) but not at E14.5 ($P > 0.2$) as compared to controls. (H) Statistical analysis shows significantly increased cell proliferation rate ($P < 0.05$) in the presphenoid in E14.5 *Nog*$^{-/-}$ embryo as compared to that in control. Standard errors are indicated. Scale bar = 100-μm.

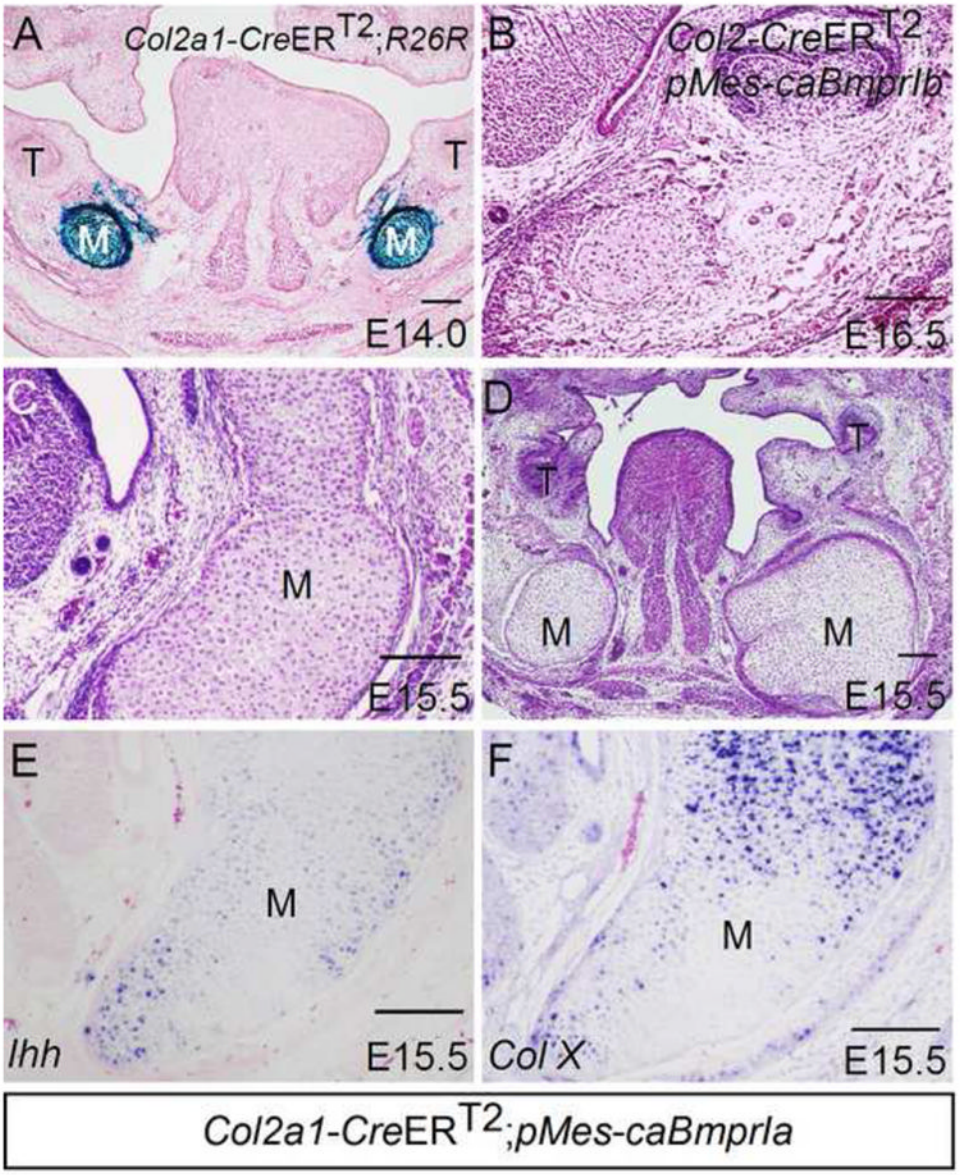

Figure 8. Activation of BMPRIa-mediated BMP signaling in chondrocyte-lineage resembles *Nog*$^{-/-}$ Meckel's cartilage phenotype

(A) X-gal staining shows Cre activity in chondrocytes of Meckel's cartilage of an E14.0 *Col2a1-Cre*ER^{T2};*R26R* embryo that received an administration of tamoxifen at E12.5. (B) A coronal section of an E16.5 *Col2a1-Cre*ER^{T2};*pMes-caBmprIb* embryo shows unaffected Meckel's cartilage. (C, D) Coronal sections E15.5 *Col2a1-Cre*ER^{T2};*pMes-caBmprIa* embryos show significantly enlarged Meckel's cartilage. (E, F) In situ hybridization assay shows *Ihh* expression (E) and *Col X* expression (F) in enlarged Meckel's cartilage of E15.5 *Col2a1-Cre*ER^{T2};*pMes-caBmprIa* embryo. M, Meckel's cartilage; T, tooth. Scale bar = 100-μm.