Mouse auditory organ development required bone morphogenetic protein signaling

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To investigate roles of bone morphogenetic protein (BMP) signaling on the development of mouse cochlear sensory organ, we isolated initial auditory epithelium from embryo and cultured with agonist or antagonist of BMP signaling. When BMP signaling was blocked with noggin cochlear epithelia were disordered, with hair cells in abnormal arrangement and disheveled hair bundles. The number of myosin VIIA and SRY (sex-determining region Y)-box 2 (SOX2)-positive cells also decreased dramatically. Conversely, an increase of myosin VIIA and SRY (sex-determining region Y)-box 2-positive cells was observed when auditory epithelia were treated with exogenous BMP4. Cell proliferation and cell death were not changed significantly by BMP signaling. Collectively, our results indicated that BMP signaling is essential to cochlear sensory formation and hair cell differentiation. ***NeuroReport*** **22:396–401 © 2011 Wolters Kluwer Health | Lippincott Williams & Wilkins.**

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Introduction

The inner ear's sensory epithelia originate from the ventromedial region of the otocyst, an area that can be defined by the expression of several markers, such as paired box 2 (PAX2), SRY (sex-determining region Y)-box 2 (SOX2), lunatic fringe, cSerrate-1, ISL LIM homeobox 1 (ISL1) (*http://www.ncbi.nlm.nih.gov/gene/3670*) and bone morphogenetic proteins (BMPs) [1–5]. The distinctive temporal and spatial expression patterns of BMP4 in developing chicken and mouse inner ear sensory epithelia have led to the suggestion that this protein may play an important role in the induction of inner ear sensory organs [6–8]. Chicken embryo has been used to study possible roles of BMP4 in the inner ear. For example, noggin, an antagonist of BMPs, has been used to interfere with BMP signaling during chicken inner ear development resulting in defects in semicircular canal formation and otic capsule malformation [9]. Although malformed or missing cristae were observed in these experiments, hair cells developed normally. Thus these results did not clarify why BMP4 is robustly expressed in sensory epithelia primordia, thereby neither confirming nor refuting the hypothesis that BMP signaling is involved in the genesis of inner ear sensory organ. A possible explanation for previously observed lack of sensory epithelia defects after in-ovo application of BMP antagonists is that the antagonists did not penetrate far enough to reach sufficiently high concentrations in developing sensory epithelia to block BMP signaling effectively [7].

As homozygous BMP4 knockout mice die between E6.5 and E9.5 [10], a period before the inner ear has developed [11,12], it is impossible to analyze the role of BMP4 in the genesis of inner ear sensory organs by BMP knockout mice. By using $BMP4^{loxp}$ mouse model, it has been found that BMP4 is required for the formation of vestibular sensory apparatus, proposing that BMP4 regulates Msx1 and Lmo4 activity to drive progenitor cell to sensory fate. Unfortunately, in this condition knockout mouse, BMP4 is still expressed in developing cochlear duct [10,13], therefore the role of BMP4 in cochlear sensory apparatus development could not be investigated with this model.

Our previous study exploited a serum-free floating otocyst culture system, which allowed us to quantitatively analyze progenitor cell proliferation, apoptosis, and cell differentiation in developing otocyst with loss of function and gain of function experiments. We found that BMP4 signaling was involved in the generation of sensory epithelia and hair cell differentiation. However, this otocyst floating culture system could not determine whether hair cells were from auditory compartment or vestibular compartment [7]. To investigate roles of BMP signaling on the development of mouse auditory organ,

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here we isolated initial auditory sensory epithelium from embryo and cultured with agonist or antagonist of BMP signaling. The results indicated that BMP signaling was required for cochlear sensory epithelium formation.

Materials and methods

Tissue culture

Initial cochlear sensory epithelia were dissected from embryos of anaesthetized pregnant C57BL/6J mice (E12.5 or E13.5). The day vaginal plug was found was identified as E0.5. All experiments were approved by Shanghai Medical Experimental Animal Administrative Committee. All efforts were made to minimize animal suffering and reduce the number of animals used. Sensory epithelia were dissected and transferred onto poly-L-ornithine (Sigma-Aldrich, St Louis, Missouri, USA)-treated cover slides. After attachment, epithelia were washed twice with PBS and cultured in serum-free Dulbecco's modified eagle medium/high glucose and F12 medium (mixed 1 : 1) supplemented with N2 and B27 (Invitrogen, Carlsbad, California, USA). BMP4 [10 or 20 ng/ml, (R&D Systems, Minneapolis, Minnesota, USA)] or noggin [0.3 μg/ml, (R&D Systems)] was continuously supplemented. Bromodeoxyuridine (BrdU), (6 μg/ml) was added to the media to label proliferating cells. After 6 (E12.5) or 5 (E13.5) days *in vitro* (DIV), epithelia were harvested and fixed with 4% paraformaldehyde.

Immunohistochemistry

Rabbit polyclonal antimyosin VIIA (Proteus Biosciences, Ramona, California, USA), goat polyclonal anti-SOX2 (Santa Cruz Biotechnology, Santa Cruz, California, USA), and monoclonal anti-BrdU (Sigma-Aldrich) were used. Immunohistochemistry was performed as reported previously [14]. In brief, after nonspecific binding was blocked, epithelia were incubated at 4°C overnight with diluted primary antibodies (1 : 200). For BrdU labeling, epithelia were placed in 2N HCl to denature DNA before incubation with antibody. After unbound antibodies were removed, epithelia were incubated with corresponding secondary antibodies conjugated with tetramethyl rhodamine or Alexa Fluor 488 (Invitrogen). Counterstaining with 4′,6-diamidino-2-phenylindole (Sigma-Aldrich) was performed to visualize cell nuclei. Specimens were examined by confocal microscopy (Leica, Heidelberg, Germany). Negative control experiments were performed as above without incubation with diluted primary antibodies.

Quantification of SOX2 and myosin VIIA-positive cells

Cells were counted in whole sensory epithelia. Data were presented as mean ± standard error of mean. Student's *t*-test was used to analyze the difference. Differences among groups were considered significant when a P value of less than 0.05 was obtained.

Results

Exogenous BMP4 promoted hair cell formation and blockage of BMP signaling by noggin reduced hair cell development

Cochlear sensory epithelia were harvested after 6 (E12.5 + 6DIV) or 5 days (E13.5 + 5DIV) of culture, which resembled E18.5 developing cochleae. In normal cochlear epithelia, the number of myosin VIIA-positive cells was 519.75 ± 37.08 (E12.5 + 6DIV) and 1214.38 ± 81.98 (E13.5 + 5DIV). In BMP4 treated sensory epithelia, hair cells increased to 588.50 ± 45.53 (E12.5 + 6DIV, BMP4 10 ng/ml), 650.29 ± 65.48 (E12.5 + 6DIV, BMP4 20 ng/ml), 1269.50 ± 78.43 (E13.5 + 5DIV, BMP4 10 ng/ml), and 1353.25 ±61.85 (E13.5 + 5DIV, BMP4 20 ng/ml). There was significant difference between control and BMP4 (20 ng/ml) groups (E12.5 + 6DIV, Fig. 1k).

When treated with noggin, sensory epithelia were much smaller than BMP4 treated and normal specimens. Hair cells in noggin treated sensory epithelia decreased significantly to 64.14 ± 8.68 (E12.5 + 6DIV) and 231.17 ± 36.77 (E13.5 + 5DIV), compared with control group ($P < 0.01$, Fig. 1k). Moreover, hair cells in noggin treated sensory patches lost their normal arrangement and hair bundles were disheveled (Fig. 2a and b).

To confirm the pathway through which noggin inhibited hair cell formation, we rescued noggin treated cochlear epithelia by adding exogenous BMP4. In rescued epithelia, hair cells recovered to 209 ± 56.07 (E12.5 + 6DIV). A phenotype of reduced hair cell formation by noggin could be rescued by exogenous BMP4 (Fig. 2c), indicating that BMP signaling was essential for cochlear hair cell formation.

BMP signaling was necessary for cochlear prosensory formation

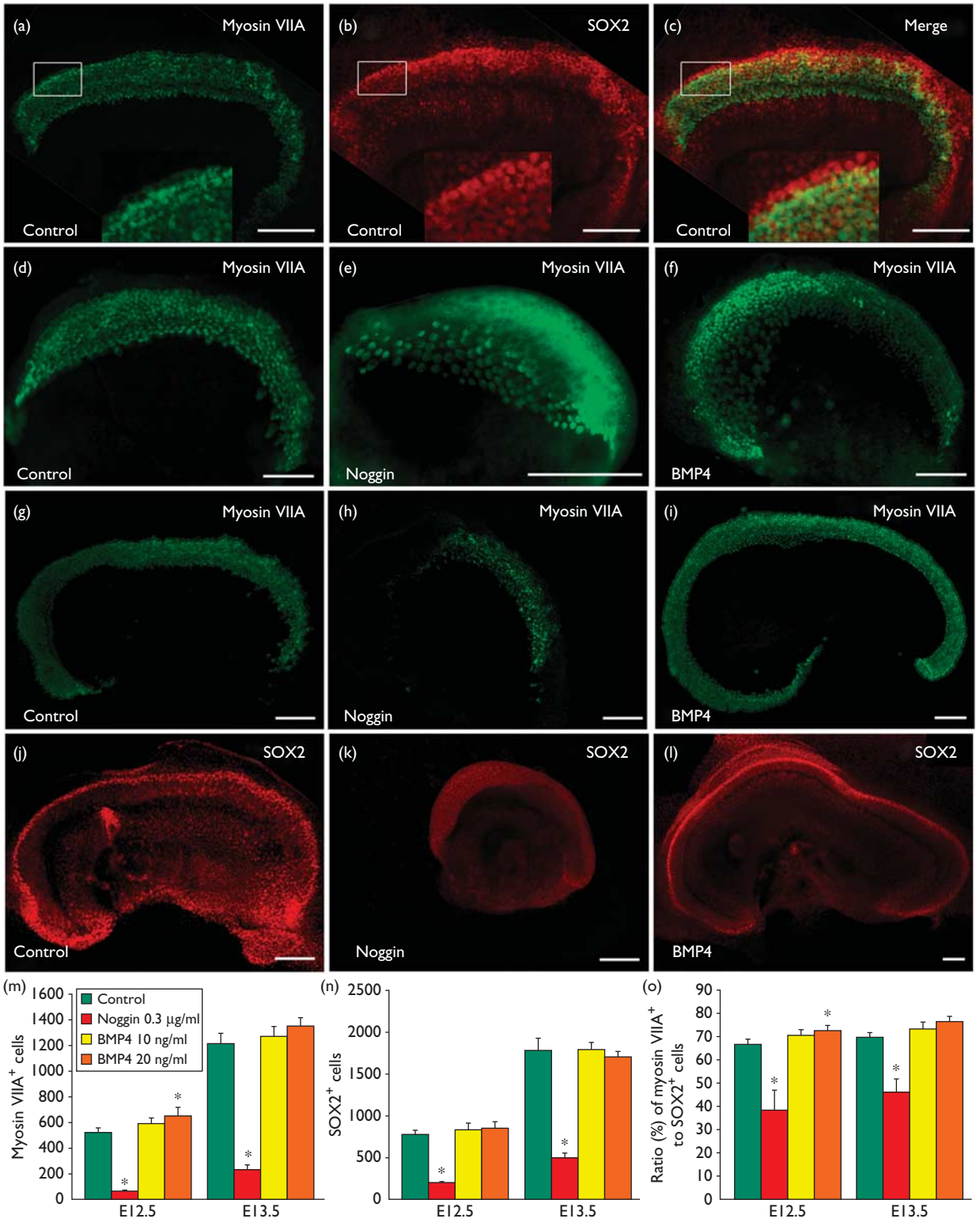
In developing cochlear sensory epithelia, both hair cells and supporting cells were SOX2 positive [15]. SOX2 could be used as a marker for cochlear sensory domain. BMP4 treatment increased SOX2-positive cells in sensory epithelia. When BMP signaling was blocked by noggin, SOX2-positive cells reduced remarkably ($P < 0.01$, Fig. 1), which indicated that BMP signaling was required for cochlear sensory epithelium formation.

The ratio of myosin VIIA-positive cells to total SOX2-positive cells could represent the percentage of hair cells in sensory epithelia. The percentage of hair cells in total SOX2-positive cells increased in BMP4 treated sensory epithelia, whereas it decreased in noggin treated group compared with control group (Fig. 1m), indicating that BMP signaling promoted hair cell differentiation.

Cell proliferation and death were not changed significantly by BMP signaling

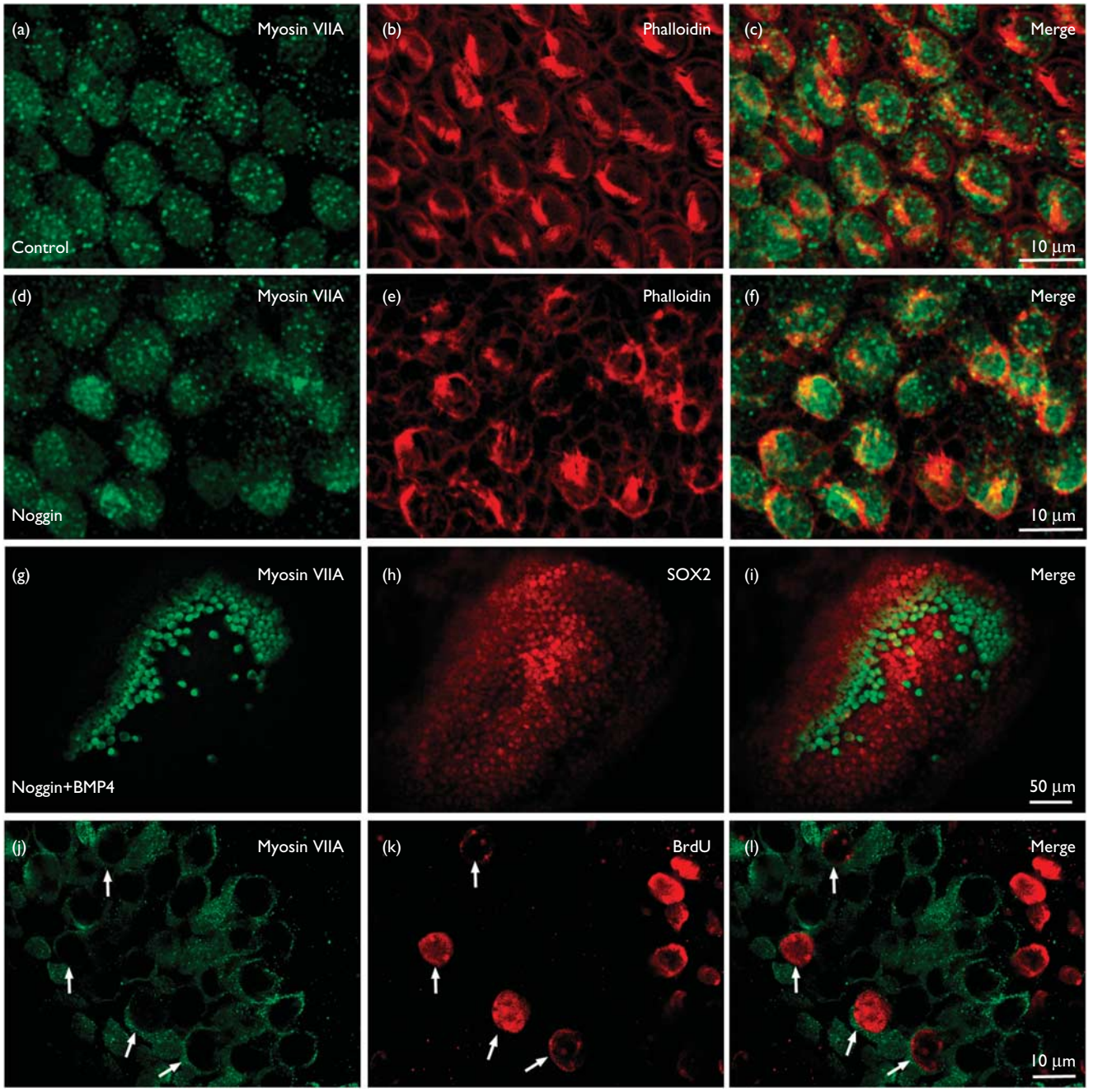
We quantitatively analyzed cell proliferation and apoptosis in the cultured developing cochlear sensory epithelia with loss of function and gain of function experiments.

Fig. 1



(a–c) Cochlear sensory epithelia were viable with normal hair bundles after being cultured for 6 days. (d–l) Representative myosin VIIA or SRY (sex-determining region Y)-box 2 (SOX2)-positive cells in cochlear epithelia in control, noggin, and bone morphogenetic protein (BMP) treated groups were found [E12.5 + 6DIV, (d–f); E13.5 + 5DIV, (g–l)]. Myosin VIIA, green; SOX2, red. (m–o) Statistical data showed that noggin significantly decreased, whereas BMP4 increased the number of myosin VIIA and SOX2-positive cells (*$P<0.05$ vs. control group). As some images were combined from several images, the magnification of each image was not equal, which was corrected with scale bars. All scale bars in this figure represent 100 μm.

Fig. 2



(a–c) Normal cochlear epithelia remained viable with well-orientated hair bundles. (d–f) Noggin treatment resulted in abnormal arrangement of hair cells and disheveled bundles. (g–i) The phenotype of reduced hair cell formation by noggin could be rescued by exogenous bone morphogenetic protein 4. (j–l) Cell proliferation was not significantly changed by BMP signaling. Newborn hair cells that are double-labeled by BrdU and Myosin VIIA (white arrows). Myosin VIIA, green; phalloidin, SOX2, and bromodeoxyuridine, red. Scale bars: 10 μm (a, b, and d) and 50 μm (c).

Double immunofluorescence of BrdU and myosin VIIA was performed to label newly generated hair cells (Fig. 2d). There was no significant difference of BrdU-positive cells among BMP4 treated, noggin treated, and normal sensory epithelia. Apoptotic cells did not change significantly in each group (data not shown).

Discussion

BMP signaling is essential for inner ear sensory apparatus formation

BMP4 mRNA expression in all sensory organ primordia of developing inner ear has led to the hypothesis that this signaling protein plays a role in the induction or

differentiation of inner ear sensory epithelia [3]. Here we show that loss of BMP signaling by treatment of BMP antagonist noggin markedly reduce the number of hair cells in the developing cochlear epithelia, which strongly supports the hypothesis that BMP signaling is required for cochlear hair cell generation. In previous study, we used serum-free floating culture technique, which allowed us to quantitatively test the function of BMP4 on avian inner ear sensory epithelium formation in a controlled environment. It has been found that BMP signaling was essential for avian hair cell formation [7].

SOX2 is required for prosensory formation in the inner ear [16–18]. Both newly formed hair cells and supporting cells are SOX2 positive [15]. When BMP signaling was blocked in E12.5 cochlear sensory epithelium, SOX2-positive cells were markedly reduced, suggesting that BMP signaling is required for cochlear sensory cell fate decision. The role of BMP signaling on cochlear sensory fate decision is consistent with results from BMP4 conditional null mouse, which showed that BMP4 was required for vestibular crista formation [13].

The effect of BMP4 on promoting differentiation of sensory progenitor cells is consistent with its temporal and spatial expression in developing inner ear sensory organ [2,3,6,19]. BMP4 mRNA was expressed in all sensory organ primordia before hair cells and supporting cells differentiation. At this crucial period of sensory organ formation, progenitor cells downregulate PAX2, exit cell cycle, and initiate differentiation into hair cells and supporting cells [20,21]. These events seem to be correlated with the presence of BMP4 in sensory patches, supporting a possible role of BMP4 on specifying cell differentiation in cochlear sensory epithelia.

BMP4 promotes hair cell differentiation

It was proposed from BMP4 conditional null mutants that BMP4 drives progenitor cells to sensory fate by regulation of Msx1 and Lom4, and to nonsensory fate by regulating Gata3, p75 Ngfr, and Lmo4, suggesting BMP4 has a global role in organizing crista structure into sensory and non-sensory domains rather than just promoting or inhibiting hair cell fate [10,13].

Three mechanisms could account for hair cells increase by exogenous BMP4: (i) promotion of sensory progenitor cell proliferation, (ii) prevention of progenitor cell apoptosis, or (iii) promotion of progenitor cell differentiation to hair cells. Our results suggest that BMP4 does not affect cell proliferation and apoptosis in developing cochlear epithelia, implying that a plausible mechanism of BMP4's effect on increasing cochlear hair cells is to drive more progenitor cells to take a sensory cell fate, as confirmed by the increment of myosin VIIA and SOX2-positive cell ratio in BMP4 treated group.

Another line of evidence for BMP4's effect on sensory epithelium progenitor cells arises from our loss-of-function analysis with noggin. We found that noggin treatment considerably reduced both myosin VIIA and SOX2-positive cells, as well as the ratio of myosin VIIA-positive cells to SOX2-positive cells. Our previous work showed that noggin treatment did not affect the number of PAX2-positive progenitor cells, whereas the number of hair cells was considerably reduced [7]. These results imply that inner ear progenitors fail to differentiate into hair cells when BMP signaling is blocked.

Conclusion

We concluded that BMP signaling was essential for cochlear sensory apparatus formation and differentiation of sensory progenitor cells to hair cells.

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References

1 Li H, Liu H, Sage C, Huang M, Chen ZY, Heller S. Islet-1 expression in the developing chicken inner ear. *J Comp Neurol* 2004; **477**:1–10.
2 Oh SH, Johnson R, Wu DK. Differential expression of bone morphogenetic proteins in the developing vestibular and auditory sensory organs. *J Neurosci* 1996; **16**:6463–6475.
3 Morsli H, Choo D, Ryan A, Johnson R, Wu DK. Development of the mouse inner ear and origin of its sensory organs. *J Neurosci* 1998; **18**:3327–3335.
4 Chatterjee S, Kraus P, Lufkin T. A symphony of inner ear developmental control genes. *BMC Genet* 2010; **11**:68.
5 Burton Q, Cole LK, Mulheisen M, Chang W, Wu DK. The role of Pax2 in mouse inner ear development. *Dev Biol* 2004; **272**:161–175.
6 Ohyama T, Basch ML, Mishina Y, Lyons KM, Segil N, Groves AK. BMP signaling is necessary for patterning the sensory and nonsensory regions of the developing mammalian cochlea. *J Neurosci* 2010; **30**:15044–15051.
7 Li H, Corrales CE, Wang Z, Zhao Y, Wang Y, Liu H, *et al.* BMP4 signaling is involved in the generation of inner ear sensory epithelia. *BMC Dev Biol* 2005; **5**:16.
8 Blauwkamp MN, Beyer LA, Kabara L, Takemura K, Buck T, King WM, *et al.* The role of bone morphogenetic protein 4 in inner ear development and function. *Hear Res* 2007; **225**:71–79.
9 Gerlach LM, Hutson MR, Germiller JA, Nguyen-Luu D, Victor JC, Barald KF. Addition of the BMP4 antagonist, noggin, disrupts avian inner ear development. *Development* 2000; **127**:45–54.
10 Winnier G, Blessing M, Labosky PA, Hogan BL. Bone morphogenetic protein-4 is required for mesoderm formation and patterning in the mouse. *Genes Dev* 1995; **9**:2105–2116.
11 Cantos R, Cole LK, Acampora D, Simeone A, Wu DK. Patterning of the mammalian cochlea. *Proc Natl Acad Sci U S A* 2000; **97**:11707–11713.
12 Radde-Gallwitz K, Pan L, Gan L, Lin X, Segil N, Chen P. Expression of Islet1 marks the sensory and neuronal lineages in the mammalian inner ear. *J Comp Neurol* 2004; **477**:412–421.
13 Chang W, Lin Z, Kulessa H, Hebert J, Hogan BL, Wu DK. Bmp4 is essential for the formation of the vestibular apparatus that detects angular head movements. *PLoS Genet* 2008; **4**:e1000050.

14 Li H, Liu H, Corrales CE, Risner JR, Forrester J, Holt JR, *et al.* Differentiation of neurons from neural precursors generated in floating spheres from embryonic stem cells. *BMC Neurosci* 2009; **10**:122.
15 Bok J, Chang W, Wu DK. Patterning and morphogenesis of the vertebrate inner ear. *Int J Dev Biol* 2007; **51**:521–533.
16 Kiernan AE, Pelling AL, Leung KK, Tang AS, Bell DM, Tease C, *et al.* Sox2 is required for sensory organ development in the mammalian inner ear. *Nature* 2005; **434**:1031–1035.
17 Dabdoub A, Puligilla C, Jones JM, Fritzsch B, Cheah KS, Pevny LH, *et al.* Sox2 signaling in prosensory domain specification and subsequent hair cell differentiation in the developing cochlea. *Proc Natl Acad Sci U S A* 2008; **105**:18396–18401.
18 Puligilla C, Dabdoub A, Brenowitz SD, Kelley MW. Sox2 induces neuronal formation in the developing mammalian cochlea. *J Neurosci* 2010; **30**:714–722.
19 Chang W, ten Dijke P, Wu DK. BMP pathways are involved in otic capsule formation and epithelial-mesenchymal signaling in the developing chicken inner ear. *Dev Biol* 2002; **251**:380–394.
20 Li H, Liu H, Corrales CE, Mutai H, Heller S. Correlation of Pax-2 expression with cell proliferation in the developing chicken inner ear. *J Neurobiol* 2004; **60**:61–70.
21 Christophorou NA, Mende M, Lleras-Forero L, Grocott T, Streit A. Pax2 coordinates epithelial morphogenesis and cell fate in the inner ear. *Dev Biol* 2010; **345**:180–190.