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BMP-signaling regulates the generation of hair-cells

Cristina Pujades, Andrés Kamaid [1], Berta Alsina, Fernando Giraldez *

Biologia del Desenvolupament, Departament de Ciències Experimentals i de la Salut (DCEXS), Universitat Pompeu Fabra, Parc de Recerca Biomèdica de Barcelona (PRBB) c/Dr. Aiguader 80, 08003-Barcelona, Spain

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Abstract

Bone morphogenetic proteins (BMPs) are diffusible molecules involved in a variety of cellular interactions during development. *Bmp4* expression accompanies the development of the ear sensory organs during patterning and specification of sensory cell fates, yet there is no understanding of the role of BMP4 in this process. The present work was aimed at exploring the effects of BMP-signaling on the development of hair-cells. For this purpose, we studied gene expression, cell proliferation and cell death in isolated chick otic vesicles that were grown in vitro in the presence of recombinant BMP4 or the BMP-inhibitor Noggin. *Cath1* was used as a marker for hair-cell specification. BMP4 reduced the number of *Cath1*-cells and, conversely, Noggin increased the size of the sensory patches and the number of *Cath1*-positive cells. The effect of BMP4 was irreversible and occurred before hair-cell specification. *Lfng* and *Fgf10* were expressed in the prosensory domain before *Cath1*, and their expression was expanded by Noggin. At these stages, modifications of BMP activity did not respecify non-sensory epithelium of the otic vesicle. The expression of *Bmp4* at sensory patches was suppressed by BMP4 and induced by Noggin suggesting an autoregulatory loop. Analysis of BrdU incorporation during 6 and 18 h indicated that the effects of BMP4 were due to its ability to reduce the number of actively proliferating progenitors and inhibit cell fate specification. BMP4 induced cell death within the prosensory domain of the otic vesicle, along with the expression of *Msx1*, but not *Msx2*. On the contrary, BMP-inhibition with Noggin favored hair-cell specification without changes in the overall cell proliferation. We propose that about the stage of terminal division, the balance between BMP and BMP-inhibitory signals regulates survival and specification of hair-cell precursors, the final number of sensory hair-cells being limited by excess levels of BMPs. The final size of sensory patches would hence depend on the balance between BMP4 and opposing signals.

Keywords: BMP; Sensory organ; Cath1; Hair cells; Cell fate; Cell death; Cell progenitors; Cell specification

Introduction

Bone morphogenetic proteins (BMPs) belong to the Transforming Growth Factor (TGFβ) superfamily, which are diffusible molecules involved in a variety of cellular interactions during development (Shi and Massague, 2003). BMPs act through type I and type II serine/threonine kinase receptors that result in phosphorylation of cytosolic R-Smad proteins, which form oligomers with other Smad proteins and regulate transcription of target genes, including the homeobox-containing transcription factors *Msx1* and *2*. The activity of BMPs is regulated by different mechanisms including their inactivation by naturally occurring antagonists like Noggin, Chordin, Dan and other binding proteins that are normally expressed in adjacent spatial domains (Miyazawa et al., 2002; Shi and Massague, 2003). BMPs are important during the development of the nervous system playing specific roles in neural induction (Stern, 2005), patterning of the neural tube and neural crest formation (Aybar and Mayor, 2002; Aybar et al., 2002; Meulemans and Bronner-Fraser, 2004; Chizhikov and Millen, 2004) and induction of neural placodes (Begbie and Graham, 2001; Baker and Bronner-Fraser, 2001).

Cell fate specification in the inner ear proceeds sequentially and in a precise spatial pattern. First, neuroblasts are specified within the proneural domain of the otic placode and otic vesicle, and delaminate to form the cochleo (auditory)-

* Corresponding author. Fax: +34 93 542 2802.
E-mail address: fernando.giraldez@upf.edu (F. Giraldez).
[1] On leave from Dpto. Histología y Embriología, Facultad de Medicina, Universidad de la República, Montevideo, Uruguay.

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vestibular ganglion, the cvg (Alsina et al., 2003). Later on, sensory patches – first vestibular and then cochlear – emerge from specific domains of the otocyst and give rise to hair-cells (HCs) and supporting cells (Barald and Kelley, 2004; Bryant et al., 2002). Specification of both the neuronal and hair-cell lineages requires the ordered activity of Atonal-related proneural genes. The expression of *NeuroD* and *Cath1* is associated with full commitment to neuronal and hair-cell fates, respectively (Bryant et al., 2002; Rubel and Fritzsch, 2002), and particular steps in cell fate acquisition are under the control of cell-to-cell communication signals (Alsina et al., 2004). Several BMPs are expressed during the early development of the inner ear with restricted spatial and temporal patterns that suggest specific developmental functions (Oh et al., 1996; Wu and Oh, 1996). *Bmp4* expression first anticipates and then accompanies the development of the ear sensory organs, and *Bmp4* expression has been adopted as a marker for otic sensory patches in different animal species (Fekete and Wu, 2002; Wu and Oh, 1996). However, the role of BMPs and particularly BMP4 in early development of hair-cells remains intriguing. The functions of BMPs in the development of the chicken inner ear had been explored by blocking BMPs activity in vivo using avian retrovirus encoding *Noggin*, dominant negative or constitutively active forms of BMP receptors, beads carrying Noggin protein and by implantation of Noggin-expressing cell lines (Chang et al., 1999, 2002; Gerlach et al., 2000). The effects reported so far are morphogenetic malformations associated with growth defects of semicircular canals and sensory organs. However, there is no information on the cellular effects of BMPs during the early stages of otic development, and on what specific role(s) they may play in the development of sensory cells.

BMPs are growth suppressors and proapoptotic signals in several developing neural tissues, including sensory organs like the retina (Trousse et al., 2001). The occurrence of programmed cell death in the development of the vertebrate inner ear has been reported for long (reviewed by Leon et al., 2004). During otic placode and otic vesicle stages, cell death coincides with areas of cell proliferation, the closure of the otic pore, the formation of the endolymphatic duct and with the development of sensory organs (Alvarez and Navascues, 1990; Fekete et al., 1997; Lang et al., 2000; Sanz et al., 1999).

In this study, we explored the role played by BMP4 in the generation of sensory hair-cells of the vestibular *cristae*. We analyzed *Bmp4* expression along with *Cath1* during early stages of sensory determination, and then probed for the effects of BMP4 and BMP-inhibition in vitro. The results show that exogenous BMP4 was able to reduce hair-cell precursors, whereas inhibition of endogenous BMP activity with Noggin had the opposite effect. BMP4 induced apoptosis, and the effects of BMP4 were paralleled by induction of *Msx1* expression. We like to propose that the BMP pathway in the ear regulates the hair-cell generation through regulation of survival and specification of hair-cell progenitors.

Materials and methods

Embryos

Fertilized hens' eggs (Granja Gibert, Tarragona, Spain) were incubated at 38°C for designated times and embryos were staged according to Hamburger and Hamilton (1951). Embryos were dissected from the yolk and fixed by immersion in 4% paraformaldehyde in phospate-buffered saline (4%PFA/PBS) overnight at 4°C.

Cryostat sectioning

For cryostat sectioning, embryos were fixed in 4% paraformaldehyde, immersed in 15% sucrose and embedded in 30% gelatin/15% sucrose. Blocks were frozen in isopentane to improve tissue preservation and then sectioned at 20 μm thickness onto Superfrost Plus Slides (Fisher, Pittsburgh, PA) and stored at −20°C. Sections were used either for in situ hybridization or/and anti-BrdU staining.

Whole-mount in situ hybridization

Either in toto or in sections in situ hybridization experiments were performed essentially as described (Nieto et al., 1996), using digoxigenin-labeled riboprobes. Digoxigenin was detected either with anti-DIG-AP and NBT/BCIP (Roche) which gives a purple staining, or anti-DIG-POD and TSA-Cy3 (Perkin-Elmer, NEL752), which gives a fluorescent precipitate. The riboprobes were as follows: *cFgf10* (Ohuchi et al., 1997), *cLfng* (Laufer et al., 1997), *cCath1* (dEST chest49606). *cBMP4*, *cMsx1* and *cMsx2* were kindly provided by Drs. M. Ros and J. Hurlé.

Organotypic cultures of otic vesicles

Otic vesicles were dissected from E3.5-4 embryos corresponding to stage HH20–23, transferred into four-well culture plates (NUNC, Roskilde, Denmark) and incubated in DMEM at 37°C in a water-saturated atmosphere containing 5% CO_2 as described (Leon et al., 1995) unless otherwise stated. Additions of 1% fetal bovine serum (Bio Whittaker Europe), recombinant human BMP4 and Noggin (R&D) were used in culture at concentrations between 1 and 100 ng/ml for BMP4 and 0.1–1 μg/ml for Noggin.

BrdU experiments and TUNEL assay

Otic vesicles were incubated with 10 μg/μl 5-Bromo-2′-deoxyuridine (Aldrich) for 2 h prior to fixation. Otic vesicles were incubated in 2 N HCl for 30 min, three times washed in Sodium Borate pH 8.9 and processed for immunohistochemistry. BrdU mAb BMC9318 (Roche) was used in whole-mount at 1:200. Distribution of apoptotic cells in the otic vesicle was determined by TdT-mediated dUTP nick-end labeling (TUNEL) of the fragmented DNA. Briefly, cultured otic vesicles were fixed for 2 h with 4% paraformaldehyde in PBS and dehydrated by a series of graded methanol steps. After rehydration, otic vesicles were incubated with 10 μg/ml proteinase K (Sigma) for 2 min at room temperature and postfixed with 4% paraformaldehyde and 0.1% glutaraldehyde in PBS. The otic vesicles were then incubated with the terminal deoxynucleotidyl-transferase labeling mix for 30 min at 37°C (Roche) and subsequently with the reaction enzyme terminal deoxynucleotidyl-transferase (Roche) for 2 h at 37°C. The reaction was stopped by incubation with 2 mM EDTA in PBS for 1 h at 65°C. Fluorescein-labeled deoxynucleotides incorporated in apoptotic cells were visualized in a DMR Leica fluorescence microscope. Fluorescent pictures were converted to black and white images for better analysis.

Quantitation of results

Quantitation of the size of sensory patches was done from digitalized photomicrographs that were processed in Adobe Photoshop. Whole-mounted otic vesicles were flat-mounted and photographed like shown in Fig. 3. Patches were drawn in Adobe Photoshop and the surface area expressed in

pixels (arbitrary units). In some experiments, the size of the patches was estimated from sections and approximated by measuring the maximum length of the patch from defined sections of the sample (ImageJ, NIH free software). Lengths were measured in pixels, calibrated and converted into microns. Quantitation of cell proliferation was done by counting BrdU-positive cells from photomicrographs of identical magnification. A surface of epithelial section was selected in Photoshop and analyzed for the number of BrdU-positive nuclei. Total nuclear density was measured and the fraction of BrdU-positive nuclei calculated. Quantitation of cell death was done as follows: images were converted to grayscale and inverted in Adobe Photoshop to enhance the apoptotic cells like shown in Fig. 6. Domains of cell death were identified and drawn first in BMP4-treated otic vesicles and equivalent domains in control or Noggin-treated otic vesicles were also drawn and analyzed. Density of apoptotic cells was estimated as the surface occupied by dark-fluorescent spots against background. Signal and background were set equal for all samples by setting identical backgrounds and setting the threshold for signal at 50% of the signal amplitude. This gave values for the fraction of surface area of otic vesicle that was fluorescent and expressed in %.

Values are expressed as means ± SE and Student's *t* test was used for statistics when appropriate.

Results

Bmp4 and prosensory genes in the otic vesicle

Several *Bmp*s are expressed in the early otic placode and vesicle, and *Bmp4* has been shown to foreshadow the prospective sensory patches of the otic vesicle (Cole et al., 2000; Oh et al., 1996; Wu and Oh, 1996). Our first aim was to study the correspondence between the pattern of expression of *Bmp4* and the onset of specification of hair-cells. For this purpose, we carried out experiments by in situ hybridization of chick embryos from E2 to E4, where *Bmp4* expression was analyzed along with the expression of prosensory genes.

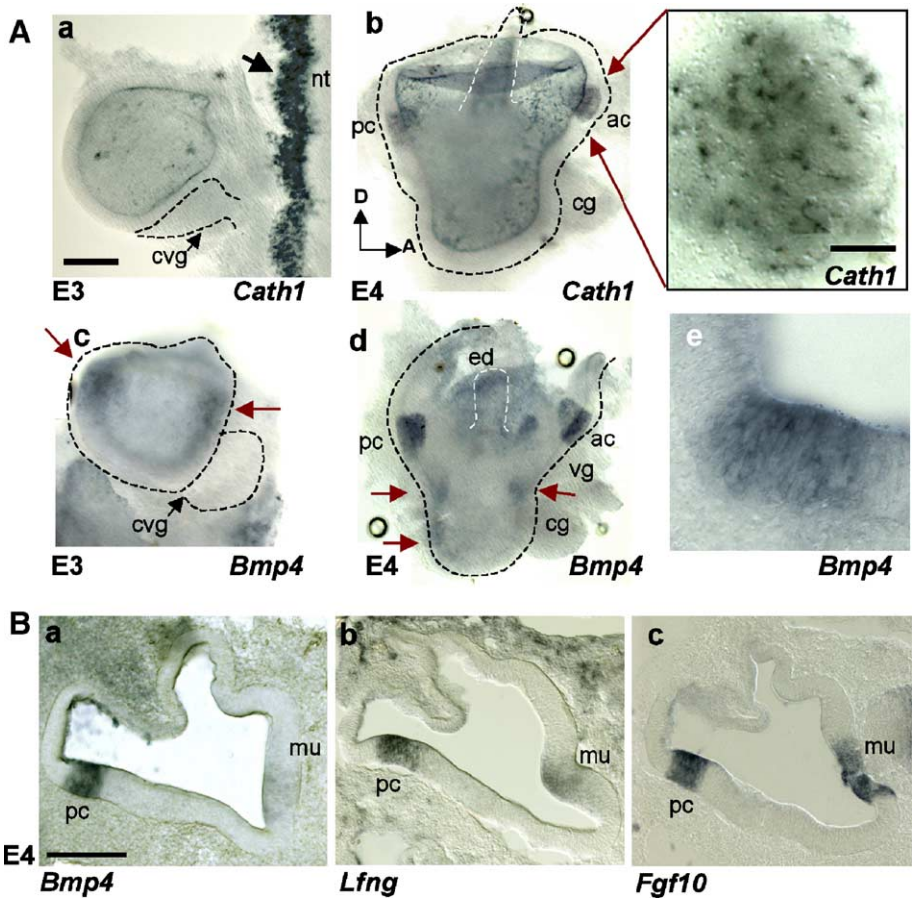

Fig. 1. Expression profile of *Bmp4*, *Cath1* and prosensory genes. (A) Whole-mount in situ hybridization for *Bmp4* and *Cath1* on otic vesicles at E3 and E4. All vesicles are oriented dorsal up and anterior right except in (a) where the otic vesicle was rotated 45° clockwise with respect to bars. (a) Note *Cath1* expression in the dorsal aspect of the neural tube (black arrow) but not in the otic vesicle in E3 (HH19). (b) *Cath1* expression in the anterior and posterior *cristae* (ac, pc) of an E4 (HH24) otocyst. The inset shows the anterior *crista* at higher magnification. Note the salt-and-pepper pattern of expression of *Cath1* on a flat view of the epithelium. (c) *Bmp4* expression in E3 (HH19) otic vesicle showing two early patches of expression (red arrows). (d) *Bmp4* expression in an E4 otocyst, showing labeling in the anterior and posterior *cristae*. Note also the nascent ventral expression domains that correspond to the *maculae* and *papila basilaris* (red arrows). (e) Higher magnification of the posterior *crista* showing that *Bmp4* was ubiquitously expressed within the sensory patch. (B) Expression of *Bmp4*, *Lunatic fringe* (*Lfng*) and *Fgf10* genes in E4 otocysts. Para-saggital sections of E4 otocysts bisecting the vestibular cavity, illustrating the expression of *Bmp4* (a), *Lfng* (b) and *Fgf10* (c) at the posterior *crista* (pc) and the *macula utriculi* (mu). Note that the expression domains of all three genes are similar. ov, otic vesicle; cvg, cochleo-vestibular ganglion; cg, cochlear; vg, vestibular; ac, anterior *crista*; pc, posterior *crista*; mu, *macula utriculi*; bp, *basilar papilla*; ed, endolymphatic duct; nt, neural tube; A, anterior; D, dorsal. Scale bars in panel A: 200 μm, and 20 μm for insets. Scale bar in panel B: 100 μm.

Cath1 is the chick orthologue of *Math1*, it is necessary and sufficient for hair-cell differentiation in vertebrates, and it will be used here as the earliest readout of hair-cell fate (Bertrand et al., 2002; Woods et al., 2004). *Cath1* was not detected until E4 (HH23–25) when it was distinctly expressed in two patches that were located one anterior and the other posterior, dorsal to the equator of the otocyst (Figs. 1Aa, b). They correspond to the nascent anterior and posterior *cristae*, which are the earliest vestibular patches to develop (Cole et al., 2000; Wu and Oh, 1996). *Cath1* transcripts occurred in single cells that were spaced, reflecting the typical arrangement of hair-cells that results from lateral inhibition (inset of Fig. 1Ab). As shown in Figs. 1Ac–d, *Bmp4* anticipated the expression of *Cath1*, and it was expressed in the E3 otic vesicle (HH20) at two distinct anterior and posterior patches (Fig. 1Ac) to then co-localize with *Cath1* by E4 (HH24) (Fig. 1, compare Ab with Ad). The expression of *Bmp4* showed sharp boundaries and extended throughout most cells of the domain (Fig. 1Ae).

Bmp4 and *Lunatic fringe* (*Lfng*) are expressed during the development of prosensory patches (Cole et al., 2000; Sanchez-Calderon et al., 2004). One example of the expression of *Bmp4* and *Lfng* in E4 otocysts is illustrated in Figs. 1Ba and b, and compared with *Fgf10* in Fig. 1Bc. The expression of *Bmp4* and *Lfng* at the posterior *crista* (pc) and *macula utriculi* (mu) is indicated in all three examples. As shown in Fig. 1Bc, *Fgf10* was also expressed at these territories, and all three genes showed similar expression patterns. A detailed study of the *Fgf10* expression will be presented elsewhere, the purpose here being merely to support the use of *Lfng* and *Fgf10* to asses the regional specification of the sensory domain with independence of *Bmp4* expression (see below).

BMP4 inhibits hair-cell output in the otic vesicle

Otic neurons and sensory hair-cells are born sequentially, but within a domain that is in both cases positive to *Lfng* and *Fgf10*, hair-cells being anticipated by *Bmp4* expression (see above). This poses the question of what may be the role of BMP4 in the specification of sensory fate. We analyzed this problem by studying the effects of exogenous BMP4 and inhibition of BMP-signaling on *Cath1* expression – as the earliest readout of hair-cell development – using isolated otic vesicles grown in culture for 18 h. Note that these experiments mimic an ectopic gain of function of BMP4 in the whole otic vesicle and the inhibition of endogenous BMP activity. Figs. 2a–c show *Cath1* expression in the anterior *crista* in E4 (HH22) otic explants grown in control (a), in the presence of exogenous BMP4 (b) or Noggin (c). The frame and hence the surface area were identical for all three photographs. Otic vesicles in control conditions (a) developed *Cath1*-positive patches that were very similar to those developed in vivo (compare (a) with Fig. 1Ab). Otic vesicles incubated with BMP4 did also develop sensory patches, but surprisingly those patches were smaller than in control. Note that patches although smaller, still expressed *Cath1* with a salt-and-pepper pattern (Figs. 2b, e). The incubation with Noggin, on the contrary, produced very large *Cath1*-positive patches (Figs. 2c, f). The bar diagram of Fig. 2d compare the size of *Cath1*-expressing domains in control, BMP4 and Noggin. The increased size of *Cath1*-positive patches occurred at a constant cell density indicating an increase in the number of *Cath1*-expressing cells. The photomicrographs in Figs. 2e–f are from optical sections after flat mounting the explants and show that the spacing of *Cath1*-positive cells within the plane of the epithelium was similar in both

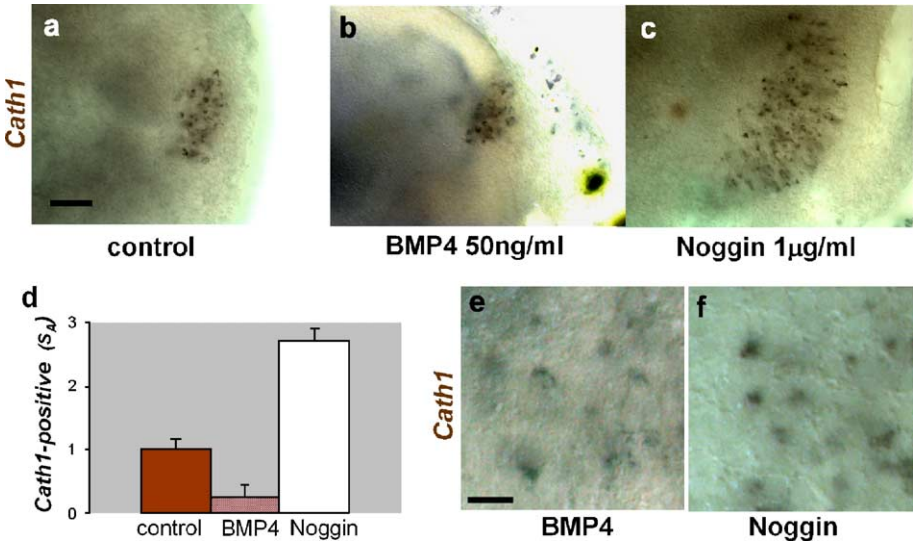

Fig. 2. BMP4 reduced sensory hair-cell output. BMP4 and Noggin modified the number of *Cath1*-expressing cells. E4 (HH22) otic vesicles were isolated and incubated for 18 h in control (a), 50 ng/ml BMP4 (b) and 1 µg/ml Noggin (c), and processed for *Cath1* in situ hybridization. Photomicrographs correspond to identical magnifications of the anterior sensory patch as identified by *Cath1*-expression. Note the reduction of the size of the patch with BMP4 and its enlargement with Noggin. Photomicrographs in panels (e) and (f) show a detail of two representative *Cath1*-positive patches from otic vesicles incubated with BMP4 and Noggin, respectively. The size of the patches was measured planimetrically and plotted in panel (d). Values are control:BMP4:Noggin = 1 ± 0.2 (n = 9):0.2 ± 0.2 (n = 10):2.7 ± 0.2 (n = 9). Difference BMP4 against control $p \leq 0.08$, and Noggin against control $p \leq 0.01$. Scale bar: 50 µm in panel (a), 10 µm in panels (e) and (f).

conditions. This suggests that neither BMP4 nor Noggin interfered with the cellular or molecular mechanisms that generate the singling out and spacing of hair-cells. We further studied the reversibility of the effects of BMP4 by incubating otic vesicles first with BMP4 and then returning to control medium. Under these conditions, BMP4 reduction of *Cath1*-positive cells persisted after returning to control medium, suggesting that the effect of BMP4 was irreversible (size of the patches normalized to control were: control = 1.00 ± 0.25; BMP4 = 0.04 ± 0.36; n = 5). Summarizing, these results indicate, first, that BMP4 decreases the number of hair-cells. Secondly, that there is an endogenous BMP activity that negatively regulates the number of *Cath1*-positive cells. Thirdly, that the effect of BMP4 occurs probably at early stages of hair-cell generation. And finally, that BMP4 acts in an irreversible manner (see below cell death).

The experiments that follow studied the effects of BMP4 on *Lfng*, *Fgf10* and *Bmp4* genes that mark early the otic prosensory domain (Fig. 3). Cultured E4 (HH22) otic vesicles expressed *Lfng* and *Fgf10* at the anterior and posterior *cristae*, and at the ventral domain that anticipates *macula* and *papila basilaris* (Figs. 3a, d). When incubated with BMP4, the expression of *Lfng* was also present at the anterior and posterior *cristae* (see red arrows); however, the *cristae* were much smaller with BMP4 than in control (Fig. 3b, n = 9/11 otic vesicles). On the contrary, incubation with Noggin expanded *Lfng* at the *cristae* (see red arrows in Fig. 3c, n = 9/9). Similarly, the expression of *Fgf10* at the *cristae* (Figs. 3d–f, red arrows) was either very small or suppressed by BMP4 (Fig. 3e), and the size of the *cristae* increased dramatically with Noggin (Fig. 3f, red arrows, n = 10/10 for BMP4 and 6/6 for Noggin). Experiments done on E3 otic vesicles gave similar effects, and *cristae* did not develop at all with BMP4 (not shown). The ventral expression domain of *Lfng* and *Fgf10* persisted after BMP4 (Fig. 3e) and also in E3 otic vesicles. Recall that, at these stages (E3–E4, HH19–22), *Lfng* and *Fgf10* are also expressed within the proneuronal

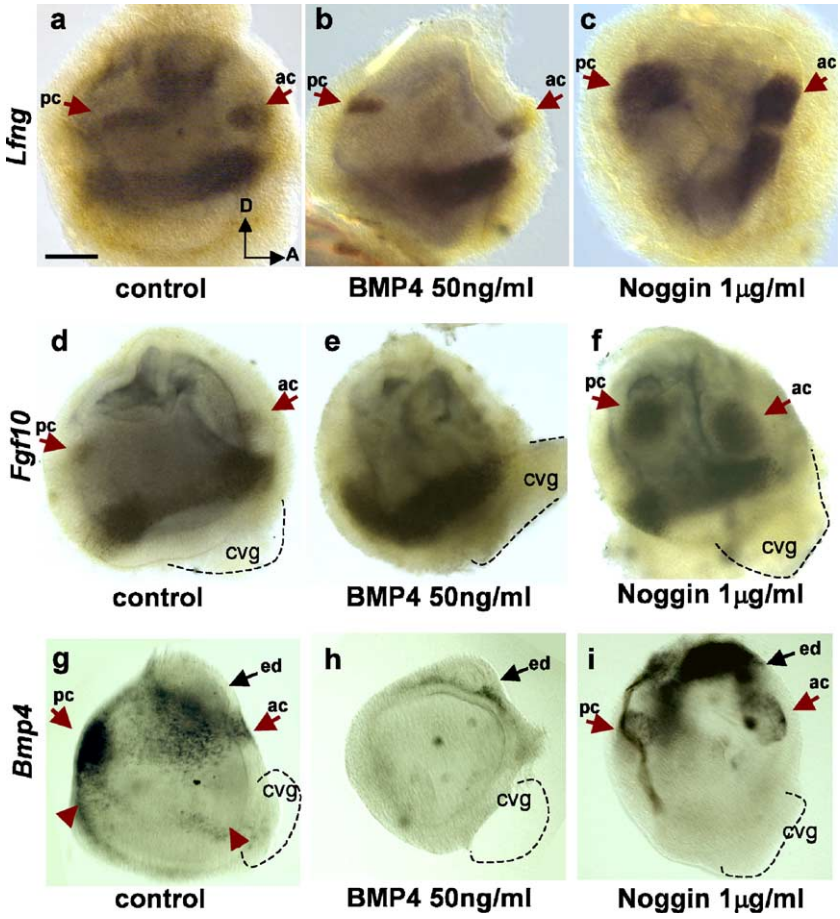

Fig. 3. Effects of BMP4 and Noggin on prosensory genes *Lfng*, *Fgf10* and *Bmp4*. (a–c). Expression of *Lfng* after addition of BMP4 or Noggin in E4 (HH22) otic vesicles. (a) *Lfng* expression in control medium showing the two *cristae* (red arrows) and the ventral expression domain (*maculae* and *papila basilaris*, see Cole et al., 2000). (b) Incubation with BMP4 (50 ng/ml) reduced the size of the *cristae*. (c) Noggin (1 μg/ml) induced the expansion of *Lfng*-positive patches. (d–f) A similar experiment probed for *Fgf10*. (d) In control, the anterior and posterior *cristae* expressing *Fgf10* just appeared to be visible (red arrows). (e) BMP4 maintained an intense expression of *Fgf10* at the ventral domain, but was weaker or absent at the *cristae*. (f) On the contrary, Noggin exaggerated *Fgf10* expression at the *cristae* (red arrows). (g–i) BMP4 reduced *Bmp4* expression in the otic vesicle. (g) Control otic vesicles showed two characteristic domains of *Bmp4* expression (red arrows). Note also that weaker expression was detected at more ventral aspects corresponding to the initiation of *maculae* (anterior arrowhead) and *papila basilaris* (posterior arrowhead). (h) BMP4 resulted in a complete suppression of *Bmp4* expression in the otic vesicle and (i) the opposite result was found with Noggin (red arrows). Note also the expression at the endolymphatic duct (ed, black arrow) (Cole et al., 2000). A, anterior; D, dorsal; cvg, cochleo-vestibular ganglion; ac, anterior cristae; pc, posterior cristae; ed, endolymphatic duct/sac. Scale bar: 100 μm.

domain (Cole et al., 2000; Alsina et al., 2004), and on the other hand, *Bmp4* is strongly expressed at the *cristae* but its expression delayed at the ventral domain (Fig. 1 and Cole et al., 2001). Since there was no evidence for ectopic sensory patches after BMP4 or Noggin, the indication is that neither the gain nor the loss-of-function of the BMP-signaling resulted in respecification of otic epithelium at the stages under study.

We examined also the effects of BMP4 on *Bmp4* expression and, surprisingly, the observation was that BMP4 reduced *Bmp4* endogenous expression (Figs. 3g–i). Control otic vesicles expressed *Bmp4* at the two anterior and posterior patches, as well as at other dorsal domains related to the endolymphatic sac (black arrow in Figs. 3g–i). Incubation with BMP4 dramatically suppressed *Bmp4* expression in otic vesicles (Fig. 3h). The expression of *Bmp4* in the presence of Noggin, however, was intense and distinct at the two patches that correspond to the anterior and posterior *cristae* (Fig. 3i, red arrows). Note that *Bmp4* was also induced at the endolymphatic duct (ed) by Noggin (black arrow in Fig. 3i). Taken together, these results indicate that the regulation of BMP4 activity modulates the size of the prosensory domain, the expression of *Bmp4* being negatively regulated by its own activity (see discussion).

BMP4 reduced the number of progenitors within the prosensory domains

The reduction in the number of *Cath1*-positive cells along with that of the size of the prosensory patches induced by BMP4, and the converse effect of Noggin, could be related in principle to the rate or the number of dividing sensory

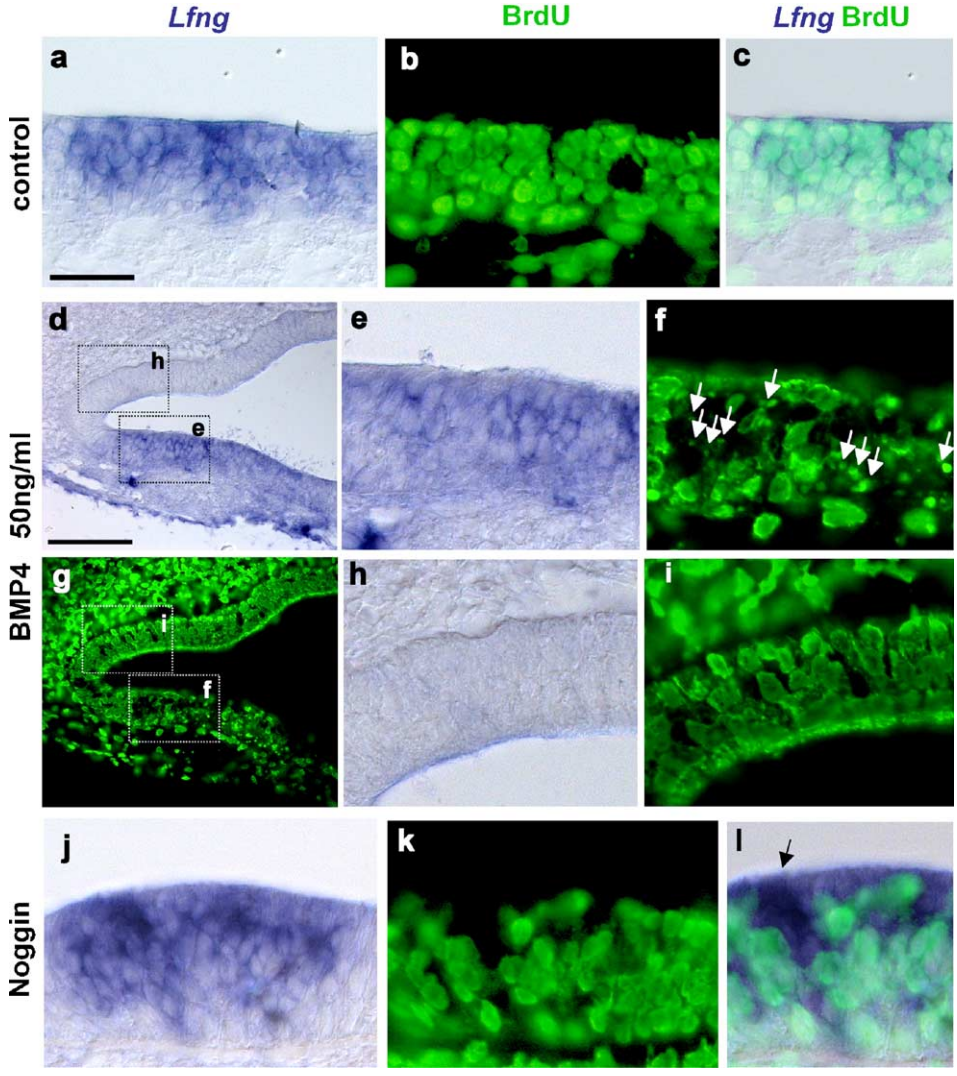

Fig. 4. BMP4 reduced cell proliferation within the prosensory patches. HH22 otic vesicles were grown in control culture medium, with BMP4 (50 ng/ml) or Noggin (1 μg/ml) for a period of 18 h and exposed throughout the culture to BrdU. Prosensory patches were identified by the expression of *Lfng*. (a) Section of a control otic vesicle expressing *Lfng* at the prosensory patch and (b) the corresponding section showing the BrdU-labeling. (c) Overlay image. Most *Lfng* cells have incorporated BrdU in culture. (d–i) *Lfng* expression and BrdU-labeling after BMP4 treatment. (e) High magnification of the *Lfng* prosensory epithelium. (f) Disperse BrdU-labeled cells were detected in the corresponding prosensory patch. Note that some of the BrdU labeled cells show fragmentation of the nuclei (white arrows). (h) Magnification of a non-sensory epithelium. (i) In the non-sensory epithelium, as in control, there was a high density of BrdU-positive cells. (j–l) *Lfng* expression and BrdU-incorporation after incubation with Noggin. (k) High density of BrdU-labeled cells. (l) Most *Lfng* cells show BrdU-incorporation in the overlay, but with a stripe of silence. Scale bar in panel a corresponds to 25 μm and holds for panels a–c, e–f, h–i, and j–l. Scale bar in panels (d) and (g) is 50 μm.

progenitors. This possibility was investigated by examining cell proliferation with bromo-deoxi-uridine (BrdU) incorporation within the prosesory domain. Fig. 4 illustrates the effects of BMP4 and Noggin upon continuous 18-h exposure to BrdU in HH22 otic vesicles. This allowed us to analyze DNA-synthesis and assess the spatial distribution of proliferating cells within the prosensory domain as labeled with *Lfng*. In control otic vesicles, the proliferative activity was intense along the otic epithelium as it was within the prosensory domain (Figs. 4a–c). Figs. 4b–c show also that in control conditions *Lfng*-positive cells incorporated BrdU, indicating that they were actively proliferating throughout the incubation period. Incubation with BMP4 (50 ng/ml) induced a strong and restricted loss of BrdU-incorporation into nuclei within the prosensory patches labeled by *Lfng* (Figs. 4d–i). Density of BrdU-positive nuclei measured as per-cent of labeled nuclei was: control = 58 ± 8.1 (n = 4); BMP4 = 34 ± 4.9 (n = 3), $p < 0.02$; Noggin = 45 ± 7.1 (n = 3) $p < 0.16$). Note that neighboring domains that were negative to *Lfng* showed intense proliferative activity (Figs. 4h–i, compare f and i), indicating that the effect of BMP4 was selective to prosensory domains. After BMP4 addition, prosensory patches contained condensed nuclei and nuclear debris that were also positive for BrdU (Fig. 4f), indicating that the loss of proliferative activity was related to apoptotic death of cellular progenitors. Noggin, on the other hand, did not change the overall proliferative activity within sensory patches (Figs. 4j–l). However, upon Noggin treatment, groups of cells within *Lfng*-positive patches seemed to be silenced and to arrest DNA replication (Figs. 4k–l), suggesting that BMP-inhibition would favor cell-cycle withdrawal at particular spots (see below). In summary, BMP4 reduced cell proliferation within prosensory patches.

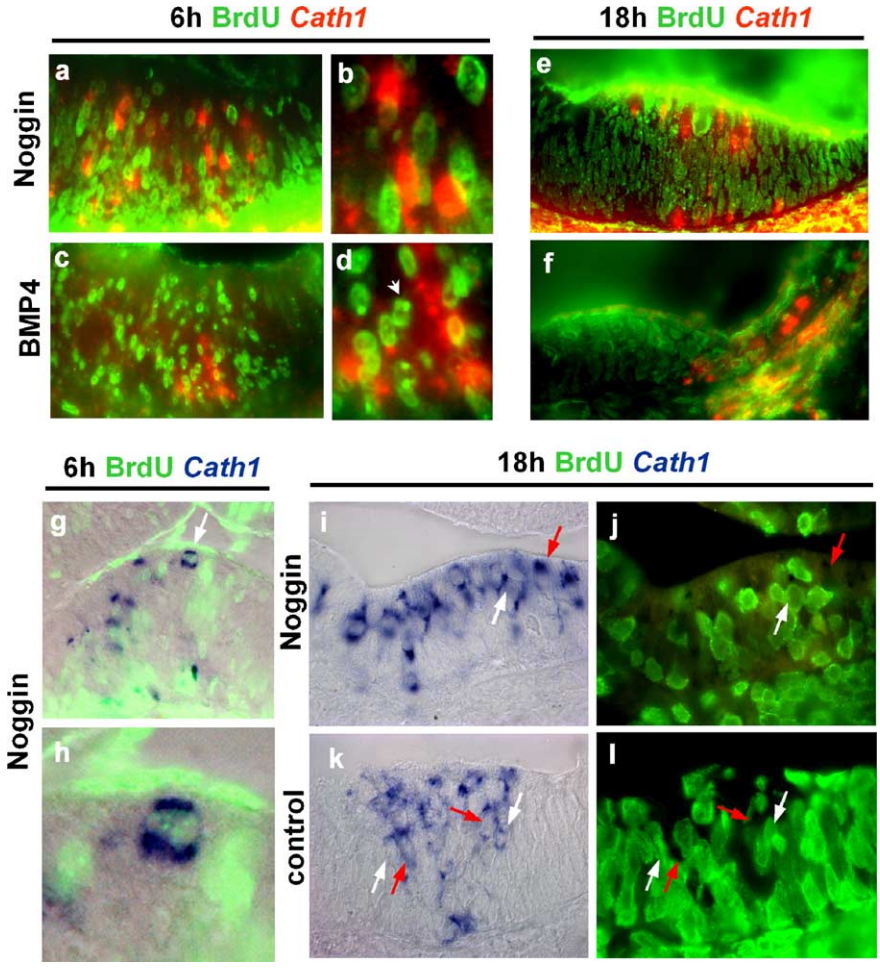

Fig. 5. BMP4 and Noggin caused opposite effects on the determination of hair-cell precursors. Cell proliferation determination of hair-cell precursor fate measured by BrdU incorporation and the expression of *Cath1* in HH22 otic vesicles. (a) A patch corresponding to an otic vesicle after 6 h with Noggin. Note the high number of *Cath1*-positive cells (fluorescent red) in between BrdU-positive cells (green). (b) Higher magnification image of panel (a). (c) *Cath1*-positive patch after 6 h with BMP4. (d) At high magnification, condensed BrdU-positive nuclei and apoptotic bodies were apparent (white arrowhead). (e, f) Photomicrographs of patches from otic vesicles incubated with BMP4 or Noggin for 18 h. (g) Same experiment than in panel (a) but *Cath1*-expression was detected with purple chromogenic substrate to better identify co-localization of in situ hybridization reaction and BrdU-incorporation. (h) Higher magnification of the cell labeled with an arrow in panel (g). Note the expression of *Cath1* and BrdU nuclear staining. (i, j) Section of a sensory patch with *Cath1* expressing cells after 18 h of culture with Noggin (i), and the corresponding BrdU-incorporation image in panel (j). (k, l) Similar but for a patch corresponding to a control otic vesicle. White arrows point to a *Cath1*- and BrdU-positive and red arrow points to *Cath1*-expressing cells that did not incorporate BrdU during the culture.

Effects of BMP-signaling on hair-cell production and hair-cell specification

Given that BMP4 reduced the number of *Cath1*-positive cells along with the loss of active progenitors within prosensory domains, we wanted to further analyze these effects and to test whether the expansion of *Cath1* after BMP-inhibition was a result of an increased output of progenitors, a favored cell specification, or both. Otic vesicles were exposed to continuous BrdU-labeling and analyzed for BrdU-incorporation and *Cath1* expression. Otic vesicles (HH22) were incubated with BrdU and BMP4 or Noggin for periods of 6 h (Figs. 5a–d, g–h) or 18 h (Figs. 5e, f, and i–l). Incubation with BMP4 during 6 h resulted in small patches with few *Cath1*-positive cells accompanied by condensed nuclei and apoptotic bodies (Figs. 5c, d, see arrowhead and Torchinsky et al., 1999). In the presence of Noggin, patches were large with rounded BrdU-positive nuclei indicating actively proliferating cells intermingled with cells expressing *Cath1* (Figs. 5a, b). Interestingly, after the 6 h incubation with Noggin, we were able to find cells that were positive to *Cath1* and to BrdU (see Figs. 5g, h), suggesting that either *Cath1*-positive cells are able to proliferate, or that *Cath1* is expressed after S-phase and about cell division. Comparison of the maximum diameter of *Cath1*-positive patches after 6 h (see methods) gave an increase of about 60% for Noggin above control levels (control = 40.2 ± 10; Noggin = 70.2 ± 8; (μm), $n = 7$; $p < 0.01$). Similarly, although not statistically significant, BMP4 tended to reduce the size of the patches within the 6-h period (BMP4 = 20.9 ± 6; (μm), $n = 5$; $p < 0.28$). This suggests that, at least in part, the effects of BMP and BMP-inhibition on hair-cell production were very rapid and did not require completion of cell-division (<6 h).

This notion was supported further by the following experiment in which we compared the percentage of *Cath1*-positive cells that incorporated BrdU in 18-h incubations in control medium and with Noggin (Figs. 5i–l). This should give an estimate of the fraction of hair-cells that were born in culture (both *Cath1*- and BrdU-positive) and of those that have been specified in the explant without cell division (*Cath1*-positive, BrdU-negative). After 18 h in culture with BMP4, it was difficult to find *Cath1*-positive cells in sections of isolated otic vesicles (Fig. 5f), picnotic nuclei and epithelial disorganization being similar to that shown above. Noggin-treated vesicles, on the other hand, showed the typical array of ordered *Cath1*-positive cells and cell proliferation activity within the epithelium (Fig. 5e). Double staining for *Cath1* and BrdU-incorporation was clearer when *Cath1* was visualized with a purple precipitate and BrdU with immunofluorescence rather than with double fluorescence (Figs. 5i–l). In control medium, 35% ± 5 of the cells were positive both for *Cath1* and BrdU ($n = 6$ sections from 3 patches), indicating that only one third of the cells was actually born in culture. With Noggin, this figure was slightly lower, but not significantly different, 21 ± 13% ($n = 6$ sections from 3 patches) (Figs. 5i–l), suggesting that at least in part the resulting increase in *Cath1*-positive cells after Noggin must arise from cell-specification, and not from newly nascent precursors.

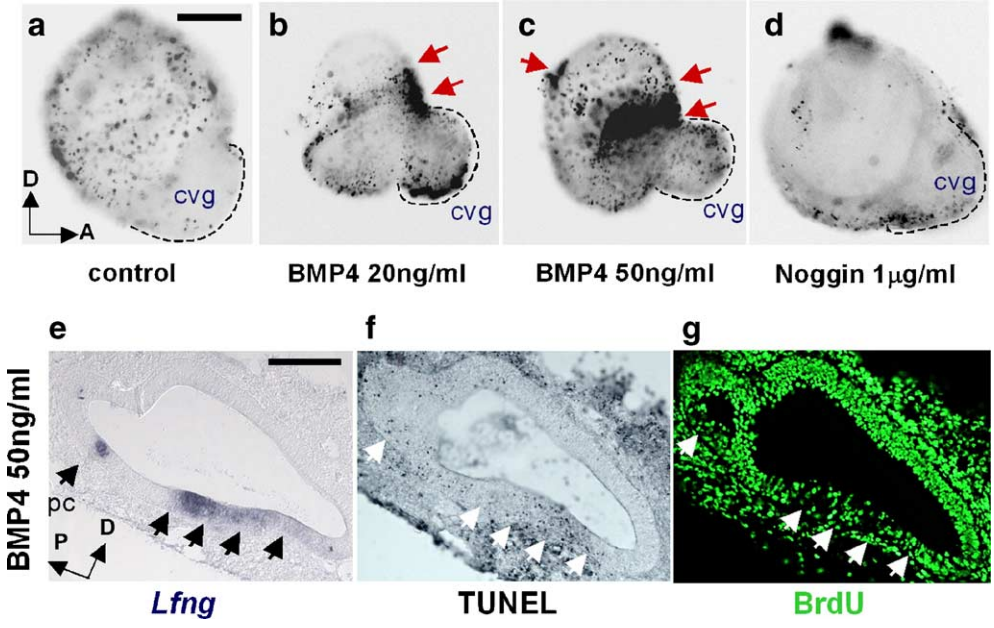

Fig. 6. BMP4 induced cell death in the prosensory domain of the otic vesicle. HH19 otic vesicles were isolated and incubated for 18 h in control (a), 20 ng/ml BMP4 (b), 50 ng/ml BMP4 (c) and 1 μg/ml Noggin (d), and processed for TUNEL assay. Arrows in panels (b) and (c) indicate areas of high density of TUNEL labeled cells after BMP4 treatment. Alternate serial sections of E3.5 otic vesicles after 18 h of culture with BMP4 and BrdU were processed for either *Lfng* expression or TUNEL. (e) Para-sagittal section of an otic vesicle incubated with BMP4 showing two patches of *Lfng* expression (pc and *papila basilaris* arrowheads). (f) The corresponding alternate serial section processed for TUNEL. Note positive cells within the *Lfng* expressing domains (arrowheads). (g) BrdU incorporation of the same section as shown for TUNEL. Note that BrdU incorporation was reduced at the TUNEL-positive domains (arrowheads). A, anterior; D, dorsal; P, posterior; cvg, cochleo-vestibular ganglion; pc, posterior *cristae*. Scale bar = 200 μm.

BMP and cell death

The reduction of cell proliferation induced by BMP4 could be related to the impaired survival of progenitors. To explore this possibility, we analyzed cell death using the TUNEL technique, after BMP4 and Noggin-incubation. Culture experiments were also performed in presence of BrdU. A typical experiment is shown in Figs. 6a–d that illustrates TUNEL-positive cells in control (a), and in the presence of 20 and 50 ng/ml BMP4 (b and c) or 1 μg/ml Noggin (d). A small but detectable apoptotic cell death occurred in control otic vesicles (a), as it does in normally developing otic vesicles (Leon et al., 2004). The incubation with BMP4 (b and c) induced an increase in cell death at the equator of the otic vesicle, particularly strong at two domains located anterior and posterior (arrows in c). Noggin reduced cell death in cultured otic vesicles (d). The effects of BMP4 on cell death were prominent within the sensory domains, as shown in Figs. 6e–g. Serial sections of BMP4-treated E3.5 (HH22) otic vesicles were assayed for either *Lfng* or TUNEL. As shown with arrowheads in Figs. 6e–g, the *Lfng*-positive domains (Fig. 6e) correlated with cell death domains (Fig. 6f), and along with the decreased BrdU incorporation (Fig. 6g). BMP4-induced apoptosis required cells to enter the cell-cycle, as revealed by studying the density of TUNEL-positive cells induced by BMP4 in the presence of 10 mM hydroxy-urea (HU), a compound that reduces DNA-synthesis by inhibiting the ribonucleoside diphosphate reductase. BMP4-induced apoptosis was reduced by 10 mM HU (BMP4 = 1.0 ± 0.23 n = 10, BMP4 + HU = 0.21 ± 0.12 n = 8, $p < 0.01$; control values were 0.06 ± 0.10 n = 8). These results reinforce the idea that the deleterious effects of BMP4 occurred on actively proliferating cells.

BMP4 induces Msx1 but not Msx2 in the sensory cristae

There is no direct evidence for a link between the BMP-signaling cascade and cell death. However, it has been postulated that *Msx* genes may mediate different aspects of BMP-signaling during development (see Discussion). Fig. 7 shows experiments where the expression of *Msx1* and *Msx2* was explored after a 6-h incubation of otic vesicles with BMP4 or Noggin. *Msx1* was expressed in the vestibular *cristae* (arrows) and in the endolymphatic duct (ed) in control vesicles (Fig. 7a). Its expression was restricted to the otic epithelium (Figs. 7d–f). *Msx1* was induced by

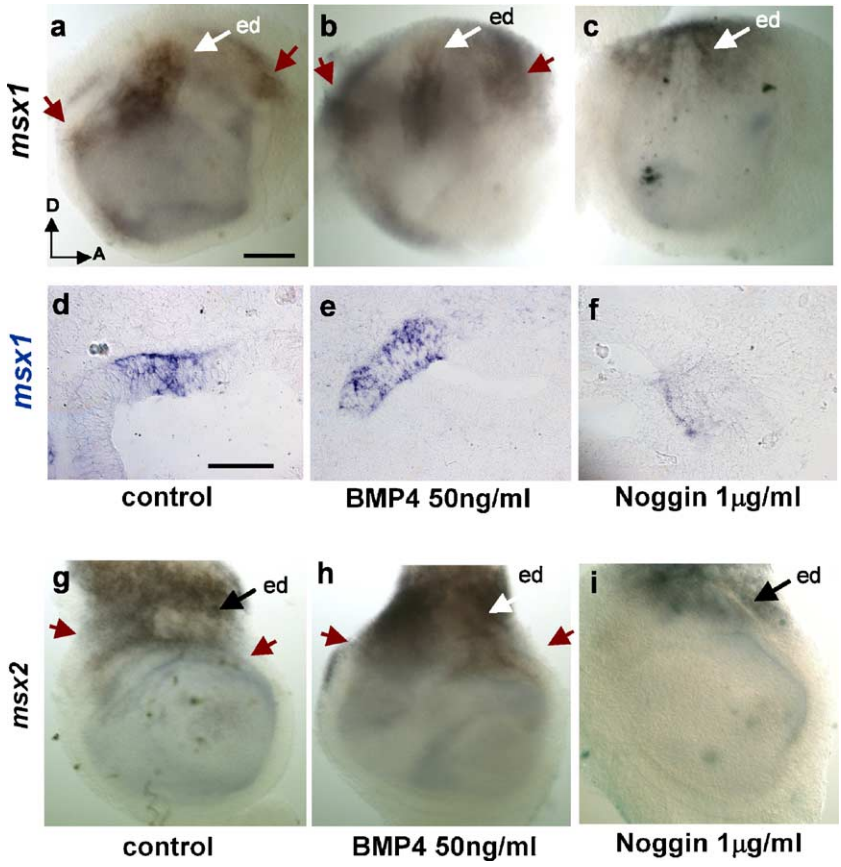

Fig. 7. Effects of BMP4 and Noggin on *Msx1* and *Msx2*. (a–c) E3.5 otic vesicles were isolated and grown in culture for 6 h with control medium (a), or with addition of 50 ng/ml BMP4 (b) or 1 μg/ml Noggin (c) and assayed for *Msx1* expression. (a) Low levels of *Msx1* expression were detected in the two *cristae* (red arrows) and the endolymphatic duct (ed). (b) Otic vesicle with increased expression of *Msx1* at the *cristae* and the endolymphatic duct (ed) after incubation with BMP4. (c) Noggin abolished *Msx1* expression at *cristae* and endolymphatic duct. (d–f) Representative sections of otic vesicles showing that in all conditions *Msx1* expression was restricted to the otic epithelium. (g–i) Experiment probed for *Msx2*. (g) In control conditions, *Msx2* was faintly expressed at the *cristae* (red arrows). (h) *Msx2* expression was not substantially increased by BMP4 (red arrows), (i) Noggin reduced *Msx2* expression in all domains. cvg, cochleo-vestibular ganglion; ed, endolymphatic duct/sac. Scale bar = 100 μm (a–c and g–i) and 50 μm (d–f).

BMP4 at the presumptive *cristae* (Fig. 7b upper arrows, and Fig. 7e) and the basilar *papilla* (Fig. 7b) and it was suppressed by Noggin (Figs. 7c, f). Expression of *Msx2*, however, was fainter than that of *Msx1* in control medium (Fig. 7g) and there were no signs of increased expression after BMP4 (Fig. 7h). An effect of BMP4 could be detected, however, in the mesenchymal tissue attached to the dorsal aspect of the otocyst (Fig. 7h), and Noggin, like with *Msx1*, was able to suppress *Msx2* expression. These experiments show that *Msx1* but not *Msx2* correlates with the activity of BMP-signaling in the otic vesicle.

Discussion

The experiments described in this paper were aimed at studying the function of BMP4 during early otic development by analyzing the effects of BMP4 and BMP4-inhibition on gene expression, cell proliferation and cell death. The results show that: (1) *Bmp4* anticipates *Cath1* expression in sensory patches; (2) BMP4-inhibition by Noggin expanded sensory patches and increased the number of *Cath1*-positive cells revealing the existence of an endogenous BMP4 activity, BMP4 inducing opposite effects; (3) BMP4 reduced cell proliferation and induced cell death within prosensory patches; (4) Noggin expanded the population of *Cath1*-positive cells without requiring cell proliferation. The results suggest that BMP4 and BMP-inhibition regulate the generation of hair-cells: a high BMP4 activity would drive progenitors out of specification and into cell-cycle and apoptosis, whereas suppression of BMP-activity would allow hair-cell specification and cell-cycle withdrawal (Fig. 8). We like to speculate that the balance between BMP4 and BMP-inhibition is important at terminal division, by selecting between hair-cell specification and the exhaustion of precursors.

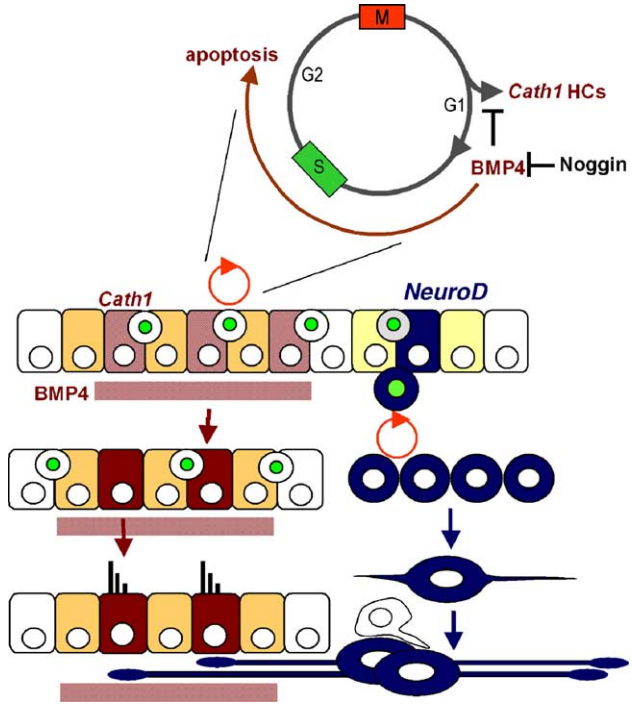

Fig. 8. Cell division and cell fate: the role of BMP4 on of hair-cell production. Progenitors have the ability to proliferate and incorporate BrdU (green nuclei exemplifying proliferation cells) and produce neuronal precursors (*NeuroD* depicted in blue), and sensory precursors. Hair-cell clusters that express *Cath1* are depicted in stripped red that turns red after specification and further differentiation (ciliated hair-cell). The brown line illustrates the presence of BMP4 expression within the prosensory territory. As exemplified in the cell-cycle schematic drawing, BMP4 allows the progenitors to enter S-phase and drives them to die. Antagonism of BMP4 signaling by Noggin induces cell cycle exit and hair-cell fate specification.

Expression of BMP4 and prosensory genes during hair-cell specification

The basic functional unit of the ear consists of mechano-transducing hair-cells (HCs), supporting cells and primary afferent neurons. Those elements develop in a stereotyped manner with variations among animal species. Cell fate determination in the inner ear is sequential and coordinated in a precise spatial pattern. First cells to be determined are the neuroblasts that delaminate from the otic epithelium, at otic cup and placode stages. Then sensory patches emerge and give rise to hair-cells (HCs) and supporting cells.

Gene expression studies showed that all presumptive sensory organs initially express *Bmp4* and members of the Notch signaling system, a pattern that although not exactly is conserved in mouse and fish (Cole et al., 2000; Mowbray et al., 2001; Oh et al., 1996; Sanchez-Calderon et al., 2004; Wu and Oh, 1996). Before studying the functions of BMP4, we studied the expression pattern of *Bmp4* along with that of unambiguous markers of early specification of hair-cells like *Cath1*. Proneural genes are crucial in the specification and differentiation of neuronal and sensory precursors (Bertrand et al., 2002). *Cath1* is the chick homologue of mammalian *Math1*, a proneural gene that belongs to the Atonal-like family of basic-helix–loop–helix transcription factors (Bertrand et al., 2002). Using gain- and loss-of-function approaches in mouse and chick embryos, it has been well established that *Math1* is necessary and sufficient to generate hair-cells from otic sensory domains (Woods et al., 2004). We analyzed the expression of *Cath1* in order to get an early readout of hair-cell specification and to describe its relation with BMP4 signaling. The results show that *Cath1* expression first appears at the anterior and posterior *cristae* between days 3.5 and 4 of development (HH22 to HH24), and it is preceded by about 1 day by the expression of *Bmp4*, *Lfng* and *Fgf10*.

BMP4 regulates survival of prosensory progenitors and hair-cell specification

The experiments reported in the present paper show that BMP4 reduced the expression of prosensory-expressed genes – like *Lfng*, *Fgf10* and *Bmp4* – and the proneural hair-cell determination gene *Cath1*, and, in parallel, BMP4 increased cell death. On the contrary, BMP4-inhibition expanded sensory patches and the number of *Cath1*-positive cells. The question now arises on what is the role for such localized and timed expression of *Bmp4* during hair-cell specification? Our experiments point to the notion that BMP4 regulates the

generation of hair-cells by acting through a mechanism that regulates survival and specification of hair-cell progenitors. BMP4 reduces the number of precursors by inducing cell death on actively proliferating cells, whereas Noggin favors hair-cell specification. The fact that Noggin induced more hair-cells suggests that endogenous BMP4 is maintaining a negative pressure on the normal generation of hair-cells. In this connection, Wang et al. (2004) showed that null mice for *Hmx2* and *Hmx3* display altered expression of inner ear markers at E11.5 such as expansion of *Bmp4* and *Dlx5*, and the number of apoptotic bodies and mitotic figures in the utricular and saccular maculae at E15.5 is much higher in mutant embryos. *Bmp4* expansion, although with reduced expression in mutants, coincides with reduction of hair-cell population. It is likely that BMP4 and BMP4-inhibition by Noggin regulate the final size of sensory patches through the regulation of the pool of progenitors.

However, this is not the only effect of BMP4 in hair-cell production since BMP-inhibition by Noggin is able to increase the number of *Cath1*-positive cells without the need of cell proliferation, as shown in both 6-h and 18-h incubation experiments. This indicates that BMP-inhibition also favors cell specification. The simplest explanation for these results is that BMP4 tends to prevent cell specification and drive cells to enter the cell-cycle and die, whereas anti-BMP-signaling would allow cell-specification as illustrated in the diagram of Fig. 8. Otic progenitors undergo several cycles of cell division and progressive specialization to generate first neurons and then sensory cells. A connection must exist between cell-division and cell fate in one hand, and extrinsic and intrinsic factors in the other so to generate different cell types at the appropriate time and locations (Cayouette et al., 2003; Livesey and Cepko, 2001). The balance between BMP4 and inhibitory signals, and probably other growth factors, would set a steady-state whereby progenitor activity and cell specification are coupled to generate the adequate number of hair-cells. The function of BMP4 described here is reminiscent of that described in dorsal telencephalic progenitors, where exogenous BMP4 reduces the production of interneurons in dorsolateral wall explants and inhibited their increase by Shh. Here also BMP-signaling inhibition with a dominant-negative BMP receptor virus increased the production of interneurons, even if Shh was blocked. The control of progenitor cell populations by BMPs has been also reported in the neural tube by Liu et al. (2004) (see below).

An alternative explanation for the observed effects of BMP4 and Noggin would be that they occur in different domains, so that BMP4 would induce cell-death *within* the sensory domain and inhibit the surrounding epithelium to become sensory. Hence, Noggin would act by recruitment of neighboring epithelium into sensory fate. Further knowledge on the specific properties of sensory-competent and sensory-determined domains is necessary to further analyze this possibility.

In addition, it may be possible that BMP4 may have other effects beyond those analyzed here and not excluded by our experiments. The instructive role for BMP4 for the prosensory domain has not yet direct supporting evidence. Our experiments indicate that BMP4 is not able to respecify the otic epithelium to sensory fate, but they also suggest that the proapoptotic effect occurs at the prosensory domains. Studies by Lim et al. (2005) with BMPR dominant-negative expression show that it does not disrupt diencephalic patterning, but caused craniofacial, eye and neural tube closure defects, suggesting that patterning and regulation of cell survival by apoptosis may be dissociated for the BMP signal and at particular stages of development. We are aware that our experiments do not address the possible effects of BMP4 on early patterning or on later occurring cell differentiation, which are not exclusive with our observations and indeed very possible.

Bmp4 and BMP4 expression

Exogenous BMP4 was able to down-regulate *Bmp4* expression after 24 h in the presence of recombinant BMP4. This opens the question of whether the observed effects of exogenous BMP4 or Noggin may be caused by the opposite changes in the transcription of the *Bmp4* gene. This possibility is unlikely because recombinant BMP4 or Noggin is acting ubiquitously on the explants, independently on *Bmp4* transcription. Therefore, the effects observed in our experiments are probably not related to endogenous transcription but to the added exogenous factors. The diminished expression of *Bmp4* could be caused by down-regulation of expression and/or by death of *Bmp4*-expressing cells, but this problem was not further explored. In the mouse retina, forced expression of *Msx2*, a target of BMP-signaling, induces suppression of *Bmp4* expression and cell death (Wu et al., 2003).

On the other hand, there are several secreted BMP-binding proteins that sequester and antagonize the biological effects of BMPs. Noggin is expressed transiently in the tissue surrounding the otic cup and vesicle, but does not co-express with *Bmp4* in the epithelium (Chang et al., 1999). In developing organs, BMPs and BMP antagonists are often detected in adjacent domains (Brunet et al., 1998; Reshef et al., 1998). Therefore, both autoregulatory loops and neighboring inhibition may balance BMP4 activity in the sensory patches, not excluding the possible action of Noggin on other BMPs.

BMP4 and apoptosis

BMPs are involved in proliferation and differentiation during development (Hogan, 1996; Mehler et al., 1997), as well as in programmed cell death, including the neural tube and the retina (Coucouvanis and Martin, 1999; Furuta et al., 1997; Golden et al., 1999; Liu et al., 2004; Trousse et al., 2001). Apoptosis is important for eliminating regressing tissue regions during embryonic development and several *Bmp* genes are involved in the regulation of apoptosis in the developing limb (Chen and Zhao, 1998; Merino et al., 1998; Pizette and Niswander, 1999). The effects of BMPs on precursor cell populations and its potential role in regulating the size of specific cell populations have not been discovered until very recently. BMP-signaling at early stages of chick neural tube development induces roof-plate cell fate, accompanied by an increase of programmed cell

death and a repression of neuronal differentiation. At later stages, however, dorsal progenitor cells lose their competence to generate roof-plate cells in response to BMP-signaling and generate dorsal interneurons (Liu et al., 2004).

Normally occurring cell death during the development of the vertebrate inner ear has been known for a long time (reviewed by Leon et al., 2004). During otic placode and otic vesicle stages, cell death coincides with areas of cell proliferation, the closure of the otic pore, the formation of the endolymphatic duct and with the development of sensory organs (Alvarez and Navascues, 1990; Fekete et al., 1997; Lang et al., 2000; Sanz et al., 1999). Our results propose a causal connection between *Bmp4* expression and cell death that occur at the sensory patches (Alvarez and Navascues, 1990; Cole et al., 2000; Lang et al., 2000), and suggest that it is part of a mechanism required for establishing the appropriate size of the sensory patches and the number of hair-cells. BMP4 regulation of programmed cell death has been also documented in cranial neural crest development and limb development (Graham et al., 1994, 1996; Merino et al., 1999; Zuzarte-Luis and Hurle, 2002).

Very little is known about the mechanisms that couple BMPs with cell death. It has been postulated that different members of the *Msx* genes may mediate different aspects of BMP-signaling during development of the neural tube, including dorsal cell fates and cell death, which in this case was associated with *Msx1* (Liu et al., 2004). *Msx1* promotes cell death in neural folds, neural crest and animal cap assays (Tribulo et al., 2003). A study on ventricular zone progenitor cells has suggested that *Msx2* and the cyclin-dependent kinase inhibitor p21 mediate cell death induced by BMP4 (Israsena and Kessler, 2002). $p21^{WAF1/Cip1}$ activity results in hypo-phosphorylation of Rb (retinoblastoma tumor suppressor protein) and arrest of the cells in the G0/G1 phase. The expression patterns of *Msx1* and *Msx2* coincide in the otocyst and are consistent with the local activation of BMP4, but they also suggest a differential role of *Msx1* or *Msx2*. Exogenous BMP4 increased *Msx1* transcription in the *cristae* and the endolymphatic duct, but not *Msx2*, suggesting that *Msx1* could be one direct target of BMP4 involved with the effects described in the paper.

In conclusion, we propose that BMP4 has a specific role in controlling the number of hair-cells within the sensory patches. This function is exerted through the regulation of the fate of cell progenitors that populate prosensory domains, which are induced to die by BMP4, or allowed to commit to sensory fate when BMP-signaling is blocked. The experiments probably reflect the behavior of the system at two extremes, BMP4 suppression and BMP4 excess. Normal development, most probably, requires a delicate balance between BMP4 in one side, and counteracting factors like BMP-inhibitors and other growth factors in the other, in order to maintain the balance between uncommitted progenitors and cell determination.

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References

Alsina, B., Giraldez, F., Varela-Nieto, I., 2003. Growth factors and early development of otic neurons: interactions between intrinsic and extrinsic signals. Curr. Top. Dev. Biol. 57, 177–206.

Alsina, B., Abello, G., Ulloa, E., Henrique, D., Pujades, C., Giraldez, F., 2004. FGF signaling is required for determination of otic neuroblasts in the chick embryo. Dev. Biol. 267, 119–134.

Alvarez, I.S., Navascues, J., 1990. Shaping, invagination, and closure of the chick embryo otic vesicle: scanning electron microscopic and quantitative study. Anat. Rec. 228, 315–326.

Aybar, M.J., Mayor, R., 2002. Early induction of neural crest cells: lessons learned from frog, fish and chick. Curr. Opin. Genet. Dev. 12, 452–458.

Aybar, M.J., Glavic, A., Mayor, R., 2002. Extracellular signals, cell interactions and transcription factors involved in the induction of the neural crest cells. Biol. Res. 35, 267–275.

Baker, C.V., Bronner-Fraser, M., 2001. Vertebrate cranial placodes: I. Embryonic induction. Dev. Biol. 232, 1–61.

Barald, K.F., Kelley, M.W., 2004. From placode to polarization: new tunes in inner ear development. Development 131, 4119–4130.

Begbie, J., Graham, A., 2001. The ectodermal placodes: a dysfunctional family. Philos. Trans. R. Soc. Lond., Ser. B Biol. Sci. 356, 1655–1660.

Bertrand, N., Castro, D.S., Guillemot, F., 2002. Proneural genes and the specification of neural cell types. Nat. Rev., Neurosci. 3, 517–530.

Brunet, L.J., McMahon, J.A., McMahon, A.P., Harland, R.M., 1998. Noggin, cartilage morphogenesis, and joint formation in the mammalian skeleton. Science 280, 1455–1457.

Bryant, J., Goodyear, R.J., Richardson, G.P., 2002. Sensory organ development in the inner ear: molecular and cellular mechanisms. Br. Med. Bull. 63, 39–57.

Cayouette, M., Barres, B.A., Raff, M., 2003. Importance of intrinsic mechanisms in cell fate decisions in the developing rat retina. Neuron 40, 897–904.

Chang, W., Nunes, F.D., Jesus-Escobar, J.M., Harland, R., Wu, D.K., 1999. Ectopic noggin blocks sensory and nonsensory organ morphogenesis in the chicken inner ear. Dev. Biol. 216, 369–381.

Chang, W., ten Dijke, P., Wu, D.K., 2002. BMP pathways are involved in otic capsule formation and epithelial–mesenchymal signaling in the developing chicken inner ear. Dev. Biol. 251, 380–394.

Chen, Y., Zhao, X., 1998. Shaping limbs by apoptosis. J. Exp. Zool. 282, 691–702.

Chizhikov, V.V., Millen, K.J., 2004. Mechanisms of roof plate formation in the vertebrate CNS. Nat. Rev., Neurosci. 5, 808–812.

Cole, L.K., Le Roux, I., Nunes, F., Laufer, E., Lewis, J., Wu, D.K., 2000. Sensory organ generation in the chicken inner ear: contributions of bone morphogenetic protein 4, serrate1, and lunatic fringe. J. Comp. Neurol. 424 (3), 509–520.

Coucouvanis, E., Martin, G.R., 1999. BMP signaling plays a role in visceral endoderm differentiation and cavitation in the early mouse embryo. Development 126, 535–546.

Fekete, D.M., Wu, D.K., 2002. Revisiting cell fate specification in the inner ear. Curr. Opin. Neurobiol. 12, 35–42.

Fekete, D.M., Homburger, S.A., Waring, M.T., Riedl, A.E., Garcia, L.F., 1997. Involvement of programmed cell death in morphogenesis of the vertebrate inner ear. Development 124, 2451–2461.

Furuta, Y., Piston, D.W., Hogan, B.L., 1997. Bone morphogenetic proteins (BMPs) as regulators of dorsal forebrain development. Development 124, 2203–2212.

Gerlach, L.M., Hutson, M.R., Germiller, J.A., Nguyen-Luu, D., Victor, J.C., Barald, K.F., 2000. Addition of the BMP4 antagonist, noggin, disrupts avian inner ear development. Development 127, 45–54.

Golden, J.A., Bracilovic, A., McFadden, K.A., Beesley, J.S., Rubenstein, J.L., Grinspan, J.B., 1999. Ectopic bone morphogenetic proteins 5 and 4 in the chicken forebrain lead to cyclopia and holoprosencephaly. Proc. Natl. Acad. Sci. U. S. A. 96, 2439–2444.

Graham, A., Francis-West, P., Brickell, P., Lumsden, A., 1994. The signalling molecule BMP4 mediates apoptosis in the rhombencephalic neural crest. Nature 372, 684–686.

Graham, A., Koentges, G., Lumsden, A., 1996. Neural crest apoptosis and the establishment of craniofacial pattern: an honorable death. Mol. Cell. Neurosci. 8, 76–83.

Hamburger, V., Hamilton, H., 1951. A series of normal stages in the development of the chick embryo. J. Morphol. 88, 49–92.

Hogan, B.L., 1996. Bone morphogenetic proteins in development. Curr. Opin. Genet. Dev. 6, 432–438.

Israsena, N., Kessler, J.A., 2002. Msx2 and p21(CIP1/WAF1) mediate the proapoptotic effects of bone morphogenetic protein-4 on ventricular zone progenitor cells. J. Neurosci. Res. 69, 803–809.

Lang, H., Bever, M.M., Fekete, D.M., 2000. Cell proliferation and cell death in the developing chick inner ear: spatial and temporal patterns. J. Comp. Neurol. 417, 205–220.

Laufer, E., Dahn, R., Orozco, O.E., Yeo, C.Y., Pisenti, J., Henrique, D., Abbott, U.K., Fallon, J.F., Tabin, C., 1997. Expression of Radical fringe in limb-bud ectoderm regulates apical ectodermal ridge formation. Nature 386, 366–373.

Leon, Y., Sanchez, J.A., Miner, C., Ariza-McNaughton, L., Represa, J.J., Giraldez, F., 1995. Developmental regulation of Fos-protein during proliferative growth of the otic vesicle and its relation to differentiation induced by retinoic acid. Dev. Biol. 167, 75–86.

Leon, Y., Sanchez-Galiano, S., Gorospe, I., 2004. Programmed cell death in the development of the vertebrate inner ear. Apoptosis 9, 255–264.

Lim, Y., Cho, G., Minarcik, J., Golden, J., 2005. Altered BMP signaling disrupts chick diencephalic development. Mech. Dev. 122, 603–620.

Liu, Y., Helms, A.W., Johnson, J.E., 2004. Distinct activities of Msx1 and Msx3 in dorsal neural tube development. Development 131, 1017–1028.

Livesey, F.J., Cepko, C.L., 2001. Vertebrate neural cell-fate determination: lessons from the retina. Nat. Rev., Neurosci. 2, 109–118.

Mehler, M.F., Mabie, P.C., Zhang, D., Kessler, J.A., 1997. Bone morphogenetic proteins in the nervous system. Trends Neurosci. 20, 309–317.

Merino, R., Ganan, Y., Macias, D., Economides, A.N., Sampath, K.T., Hurle, J.M., 1998. Morphogenesis of digits in the avian limb is controlled by FGFs, TGFbetas, and noggin through BMP signaling. Dev. Biol. 200, 35–45.

Merino, R., Rodriguez-Leon, J., Macias, D., Ganan, Y., Economides, A.N., Hurle, J.M., 1999. The BMP antagonist Gremlin regulates outgrowth, chondrogenesis and programmed cell death in the developing limb. Development 126, 5515–5522.

Meulemans, D., Bronner-Fraser, M., 2004. Gene-regulatory interactions in neural crest evolution and development. Dev. Cell 7, 291–299.

Miyazawa, K., Shinozaki, M., Hara, T., Furuya, T., Miyazono, K., 2002. Two major Smad pathways in TGF-beta superfamily signalling. Genes Cells, 1191–1204 (Dec. 7).

Mowbray, C., Hammerschmidt, M., Whitfield, T.T., 2001. Expression of BMP signalling pathway members in the developing zebrafish inner ear and lateral line. Mech. Dev. 108, 179–184.

Nieto, M.A., Patel, K., Wilkinson, D.G., 1996. In situ hybridization analysis of chick embryos in whole mount and tissue sections. Methods Cell Biol. 51, 219–235.

Oh, S.H., Johnson, R., Wu, D.K., 1996. Differential expression of bone morphogenetic proteins in the developing vestibular and auditory sensory organs. J. Neurosci. 16, 6463–6475.

Ohuchi, H., Nakagawa, T., Yamamoto, A., Araga, A., Ohata, T., Ishimaru, Y., Yoshioka, H., Kuwana, T., Nohno, T., Yamasaki, M., Itoh, N., Noji, S., 1997. The mesenchymal factor, FGF10, initiates and maintains the outgrowth of the chick limb bud through interaction with FGF8, an apical ectodermal factor. Development 124, 2235–2244.

Pizette, S., Niswander, L., 1999. BMPs negatively regulate structure and function of the limb apical ectodermal ridge. Development 126, 883–894.

Reshef, R., Maroto, M., Lassar, A.B., 1998. Regulation of dorsal somitic cell fates: BMPs and Noggin control the timing and pattern of myogenic regulator expression. Genes Dev. 12, 290–303.

Rubel, E.W., Fritzsch, B., 2002. Auditory system development: primary auditory neurons and their targets. Annu. Rev. Neurosci. 25, 51–101.

Sanchez-Calderon, H., Martin-Partido, G., Hidalgo-Sanchez, M., 2004. Otx2, Gbx2, and Fgf8 expression patterns in the chick developing inner ear and their possible roles in otic specification and early innervation. Gene Expression Patterns 4, 659–669.

Sanz, C., Leon, Y., Canon, S., Alvarez, L., Giraldez, F., Varela-Nieto, I., 1999. Pattern of expression of the jun family of transcription factors during the early development of the inner ear: implications in apoptosis. J. Cell Sci. 112, 3967–3974.

Shi, Y., Massague, J., 2003. Mechanisms of TGF-beta signaling from cell membrane to the nucleus. Cell 113, 685–700.

Stern, C.D., 2005. Neural induction: old problem, new findings, yet more questions. Development 132, 2007–2021.

Torchinsky, C., Messana, E.P., Arsura, M., Cotanche, D.A., 1999. Regulation of p27Kip1 during gentamicin mediated hair cell death. J. Neurocytol. 28, 913–924.

Tribulo, C., Aybar, M.J., Nguyen, V.H., Mullins, M.C., Mayor, R., 2003. Regulation of Msx genes by a Bmp gradient is essential for neural crest specification. Development 130, 6441–6452.

Trousse, F., Esteve, P., Bovolenta, P., 2001. Bmp4 mediates apoptotic cell death in the developing chick eye. J. Neurosci. 21, 1292–1301.

Wang, W., Grimmer, J.F., Van De Water, T.R., Lufkin, T., 2004. Hmx2 and Hmx3 homeobox genes direct development of the murine inner ear and hypothalamus and can be functionally replaced by *Drosophila* Hmx. Dev. Cell 7, 439–453.

Woods, C., Montcouquiol, M., Kelley, M.W., 2004. Math1 regulates development of the sensory epithelium in the mammalian cochlea. Nat. Neurosci. 7, 1310–1318.

Wu, D.K., Oh, S.H., 1996. Sensory organ generation in the chick inner ear. J. Neurosci. 16, 6454–6462.

Wu, L.Y., Li, M., Hinton, D.R., Guo, L., Jiang, S., Wang, J.T., Zeng, A., Xie, J.B., Snead, M., Shuler, C., Maxson Jr., R.E., Liu, Y.H., 2003. Microphthalmia resulting from MSX2-induced apoptosis in the optic vesicle. Invest. Ophthalmol. Visual Sci. 44, 2404–2412.

Zuzarte-Luis, V., Hurle, J.M., 2002. Programmed cell death in the developing limb. Int. J. Dev. Biol. 46, 871–876.