**Bmp4 Is Essential for the Formation of the Vestibular Apparatus that Detects Angular Head Movements**

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**Abstract**

Angular head movements in vertebrates are detected by the three semicircular canals of the inner ear and their associated sensory tissues, the cristae. Bone morphogenetic protein 4 (Bmp4), a member of the Transforming growth factor family (TGF-β), is conservatively expressed in the developing cristae in several species, including zebrafish, frog, chicken, and mouse. Using mouse models in which Bmp4 is conditionally deleted within the inner ear, as well as chicken models in which Bmp signaling is knocked down specifically in the cristae, we show that Bmp4 is essential for the formation of all three cristae and their associated canals. Our results indicate that Bmp4 does not mediate the formation of sensory hair and supporting cells within the cristae by directly regulating genes required for prosensory development in the inner ear such as Serrat1 (Jagged1 in mouse), Fgf10, and Sox2. Instead, Bmp4 most likely mediates crista formation by regulating Lmo4 and Msx1 in the sensory region and Gata3, p75Ngr, and Lmo4 in the non-sensory region of the crista, the septum cruciatum. In the canals, Bmp2 and Dlx5 are regulated by Bmp4, either directly or indirectly. Mechanisms involved in the formation of sensory organs of the vertebrate inner ear are thought to be analogous to those regulating sensory bristle formation in Drosophila. Our results suggest that, in comparison to sensory bristles, crista formation within the inner ear requires an additional step of sensory and non-sensory fate specification.

**Introduction**

The ability to detect angular head movements in vertebrates lies within the vestibular apparatus of the inner ear [1–3]. This portion of the apparatus consists of three fluid-filled semicircular canals (anterior, lateral, and posterior) that are oriented in nearly orthogonal planes (Figure 1A). Each canal contains an enlarged ampulla that houses the sensory tissue, the crista ampullaris, consisting of sensory hair cells and supporting cells. Within the anterior and posterior canes of many species such as birds and mice, there is a non-sensory structure, the septum cruciatum, which divides the sensory region into two equal halves [4,5]. This septum cruciatum is not present in the lateral crista. Other vestibular sensory organs that are common among all vertebrates are the maculae of the utricle and saccule, which detect head position and linear acceleration. In fishes, the macula of the saccule is used for hearing as well [6].

All the sensory patches within the vestibular inner ear including the presumptive cristae are thought to arise from a common prosensory (neural/sensory competent) region at the otic placode and otocyst stages (Figure 1A, red and blue; [7,8]). This prosensory domain also gives rise to the neurons that innervate various sensory patches of the inner ear. The three semicircular canals are non-sensory structures derived from two epithelial outpouches of the developing otocyst. The vertical outpouch gives rise to the anterior and posterior canals that are joined by the common crus, whereas the horizontal outpouch gives rise to the lateral canal. In the mouse, the morphogenesis of this apparatus starts around 10.5 days post coitum (dpc) and is completed by 13 dpc [9]. In chicken, it starts at embryonic day 3.5 (E3.5) and is completed by E7 [10].

Multiple factors are thought to regulate the formation of the vestibular apparatus [11–15]. For example, Wnt signaling from the dorsal hindbrain is required for the normal patterning of the vestibular structures [12]. Within the inner ear, members of two homeobox containing gene families, Dlx and Hmx, have also been implicated [13,15]. The deletion of one or more members of these gene families results in the lack of canal and crista formation. Notably, the lack of Wnt, Dlx, or Hmx gene functions all result in an early disorganization or absence of Bmp4 expression within the presumptive cristae [12,13,15,16].

The expression of Bmp4 in the presumptive cristae is conserved among several vertebrate species including the zebrafish, frog, chicken, and mouse (Figure 1D; [9,17–19]). Studies in the chicken have shown that the formation of the semicircular canals and cristae is blocked by exogenous Noggin, a Bmp antagonist [20,21]. However, specific roles for Bmp4 in inner ear development cannot be extrapolated unambiguously from these results because other Bmp genes are also expressed in the developing inner ear, including Bmp2 and Bmp7 [22]. In the mouse, the role of Bmp4 in inner ear
The Role of Bmp4 in Vestibular Apparatus Formation

**Author Summary**

Disruption of the sense of balance is highly debilitating, causing vertigo and nausea. Maintenance of proper balance requires sensory inputs from many body parts, including the inner ears and the eyes. Within the inner ear, the vestibular apparatus plays a key role in the sense of balance and is responsible for detecting head orientation and movements. The portion of the vestibular apparatus that detects angular head movements consists of three fluid-filled, semicircular canals oriented at right angles to each other. At one end of each canal is an enlargement that houses the sensory tissue, crista ampullaris, consisting of sensory hair cells and supporting cells. Bone morphogenetic protein 4 (Bmp4), a secreted signaling molecule, is expressed in these sensory regions during development. However, the lack of Bmp4 in mice affects the formation of not only the sensory regions but also their associated canals. These results demonstrate for the first time that a single gene, Bmp4, is required for the formation of the entire sensory apparatus for detecting angular head movements.

Development cannot be directly demonstrated either, since Bmp4 null mutant embryos die before significant vestibular development [23]. More recent in vitro experiments of gain- and loss-of Bmp functions in chicken embryos also yielded conflicting results regarding the role of Bmp4 in hair cell formation [24,25]. To overcome these problems, we have exploited the cre/lox approach to generate mice with an inner ear specific deletion of Bmp4. Furthermore, we address the molecular mechanisms by which Bmp4 mediates its effects on crista formation by over-expressing Smad6 or Noggin in the developing anterior crista to knock down Bmp functions. The combined results from these two species demonstrate that Bmp4 in the presumptive cristae is required for the formation of the three cristae and their semicircular canals.

**Materials and Methods**

**Mouse Strains**

The Bmp4loxPallele was generated by first constructing a targeting vector in which flox sites were inserted in introns 2 and 4 of the Bmp4 locus, so that cre recombination excises the entire Bmp4 coding sequence (Figure S1). Bmp4Tm1/+ and Bmp4loxP/loxP mice were maintained on a Black Swiss background and Foxg1cre/cre mice were maintained on a Swiss Webster background. Foxg1cre/+; Bmp4loxP/loxP embryos were generated by crossing male Foxg1cre/+; Bmp4loxP/loxP mice with female Bmp4loxP/loxP mice. For reasons that are unknown, very few Foxg1cre+/-; Bmp4loxP/loxP mice were recovered at birth (Table S1). Therefore, all analyses in this study were conducted by 13.5 dpc, an age when the gross patterning of the canals and ampullae is complete.

**Chicken Embryos and Procedures**

Chicken embryos were staged according to Hamburger and Hamilton [26]. Chicken Noggin cDNA [27] was subcloned into pRES2-EGFP expression vector, in which Noggin is driven by the immediate early Cytomegalovirus promoter (Clontech). Chicken Smad6 cDNA in the pCab-IRES-GFP vector [28] was subcloned into pMES-IRES-GFP expression vector, in which Smad6 is driven under the chicken β-actin promoter and the immediate early enhancer of Cytomegalovirus [29]. pSmad6, pNoggin and their respective control vectors at a concentration of 4 to 6 mg/ml were injected into the lumen of chicken otocysts at E3.5. Plasmids were electroporated into the anterior region of the otocyst using a positive and negative electrode flanking the anterior and posterior poles of the otocyst, respectively. Two 50 milli-second pulses at 10 volts were applied using a CUY21 electroporator.

**Results**

**In Situ Hybridization and Immunostaining**

Pant-fill analyses and in situ hybridizations were performed as described [9]. Chicken and mouse RNA probes were prepared as previously described [9,19,30–32]. Anti-hair cell specific antigen (HCA) antibodies (gift of Guy Richardson) were used at 1:5000 dilution, and staining was performed as previously described [33]. Specimens for antibody staining were fixed overnight at 4°C with 4% paraformaldehyde, except specimens for Mxl1/2 and Gata3 staining were fixed for 30 minutes at room temperature. The following antibody dilutions were used: mouse anti-neurofilament (DSHB, 3A2) 1:2000; mouse anti-Mxl1/2 (DSHB, 4G1), 1:50; rabbit anti-Phospho-Smad1 (gift of Peter ten Dijke), 1:2000; mouse anti-Sox2 (Chemicon, AB3603), 1:2000; mouse anti-Gata3 (Santa Cruz, HG3-31), 1:50; and Goat anti-GFP antibody (GeneTexa, GTX26662), 1:200. For secondary antibody labeling, species-specific antibodies conjugated with Alexa Fluor 488, 564, or 633 were used at 1:500 dilution. Incubations for primary and secondary antibodies were carried out at 4°C overnight and at room temperature for 1 hr, respectively. Total number of double-labeled cells for each specimen was scored using a confocal microscope. Since the total number of cells counted per specimen was different, weighted average percentages (wap) were calculated for each treatment to adjust for the variability of sampling size among specimens (http://mathforum.org/library/drmath/view/57605.html). A total of 70 to 190 cells were counted per treatment.

**Inner Ear Phenotypes of Bmp4 Conditional Knockout Embryos**

To generate conditional Bmp4 null embryos, we used three different mouse lines. The first, Foxg1cre/cre, was made by inserting cre into the endogenous Foxg1 gene which is expressed in tissues such as the embryonic otocysts, eyes, and foregut [34]. The tissue specific recombination activity of this cre allele has been demonstrated by crossing Foxg1cre/+ mice with the Rosa26R reporter line [34]. Bmp4Tm1 is a null allele of Bmp4 [23], whereas the Bmp4loxP conditional allele was generated as described (Figure S1). Foxg1cre/+; Bmp4loxP/loxP; Foxg1cre/+; Bmp4loxP/loxP embryos were obtained at the expected frequency from crossing Foxg1cre/+; Bmp4loxP/loxP mice with Bmp4loxP/loxP mice. Based on morphologies, they can be grouped into three classes: (1) embryos that are severely delayed in development, (2) embryos with eye malformations that are either normal or slightly smaller in their body size, and (3) embryos that are morphologically indistinguishable from Bmp4loxP/loxP littersmates (Table S1). Only the latter two classes were included in subsequent studies.

We evaluated the tissue specificity of Bmp4 deletion in the Foxg1cre/+; Foxg1cre/+; Bmp4loxP/loxP embryos between 9.5 to 10.5 dpc (n = 21) using an RNA probe (B4-del) generated against exons 3 and 4 of Bmp4. Half of the embryos analyzed at 9.5 dpc displayed abnormal Bmp4 expression patterns (n = 4/8). By 10.5 dpc, a higher percentage of Foxg1cre+/-; Bmp4loxP/loxP embryos show no or reduced
Bmp4 expression in tissues such as the eyes and otocysts where Foxg1 is normally transcribed (Figure 1B–1G, arrows; n = 11/13). Significantly, expression patterns are normal in tissues where Foxg1 is not expressed such as the roof of the hindbrain, somites and limb buds (Figure 1B,1C,1H, and 1I, arrowheads). Some of the eleven embryos that display tissue-specific reduction in Bmp4 expression and eye malformations were also slightly smaller in body size (n = 3).

In a normal otocyst, Bmp4 is transcribed in an anterior streak of tissue and a posterior focus (Figure 1D; [9]), whereas the posterior focus demarcates the location of the posterior cristae (Figure 1D, arrowhead). Among the 11 affected Foxg1cre/+; Bmp4loxP/Tm1 embryos, Bmp4 transcripts are absent from the posterior region of the otocyst and are either absent or reduced in the anterior (Figure 1E, arrow). Similar results were obtained from affected Foxg1cre/+; Bmp4loxP/Tm1 specimens at 11.5 dpc (see below). By this age, Bmp4 is also expressed in the non-sensory region of the growing cochlear duct [9].

**Figure 1. Schematic representations of mouse inner ear development from 11.5 to 13 dpc.** (A) Upper panel shows schematic cross-sections through the prospective or definitive anterior and posterior canals at the level of the lines. Blue marks the three Bmp4-positive presumptive cristae, while red marks the other three sensory tissues—the maculae utriculi and sacculi, and the organ of Corti. An enlargement of a mature anterior cristal at 15.5 dpc or later is shown. (B–I) Inner ear phenotypes of Bmp4 conditional null embryos. Wholemount in situ hybridization of Bmp4loxP/+ (B,D,F,H) and Foxg1cre/+; Bmp4loxP/Tm1 (B4cko, C,E,G,I) embryos at 10.5 dpc hybridized with Bmp4 RNA probe specific for exons 3 and 4 (B4-del). (B, C) Arrows point to the down-regulation of Bmp4 expression in the eyes and otocysts of Foxg1cre/+; Bmp4loxP/Tm1 (C), compared to Bmp4loxP/+ embryos (B). Arrowheads point to unaffected Bmp4 expression in limb buds and somites. (D) and (E) are higher magnifications of the otocysts shown in (B) and (C), respectively. Arrow and arrowhead in (D) point to Bmp4 hybridization signals in the anterior streak (encompassing anterior and lateral cristae) and the posterior cristae of the otocyst, respectively. An arrow in (E) points to the residual Bmp4 expression in the anterior streak of Foxg1cre/+; Bmp4loxP/Tm1 embryos. (F–I) Higher magnifications of Bmp4 expression domains in the eyes (F, G) and hindbrain (H,I) in Bmp4loxP/+ (F, H) and Foxg1cre/+; Bmp4loxP/Tm1 (G, I) embryos. Arrows point to the reduction of Bmp4 expression, and the malformation of the eyes, whereas arrowheads point to the normal Bmp4 expression in the hindbrain. Scale bar in (C) applies to (B); scale bars in (D), (G) and (I) equal 100μm and apply to (E), (F), and (H), respectively. Abbreviations: aa, anterior ampulla; ac, anterior crista; asc, anterior semicircular canal; cc, common crus; cd, cochlear duct; ed, endolymphatic duct; fp, fusion plate; hp, horizontal canal pouch; la, lateral ampulla; lc, lateral crista; lsc, lateral semicircular canal; oc, organ of Corti; pa, posterior ampulla; pc, posterior crista; psc, posterior semicircular canal; rd, resorption domain; s, saccule; u, utricle; vp, vertical canal pouch.

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Paint-Filled Analyses of Bmp4 Conditional Knockout Inner Ears

The gross anatomy of the Foxg1cre/+; Bmp4loxP/Tm1 inner ears at 13.5 dpc was examined by paint filling the membranous labyrinth. Consistent with the variable Bmp4 expression patterns, the paint-filled Foxg1cre/+; Bmp4loxP/Tm1 specimens also show a range of inner ear phenotypes (Figure 2A–2D). In the most severe cases, there is no discernible ampulla or semicircular canal, and the utricle and saccule are malformed. Only an intact endolymphatic duct is evident in the dorsal region of the inner ear (Figure 2B and 2C; n = 8/14). The remaining specimens are either indistinguishable from Bmp4loxP/+ (n = 3/14) embryos, or display only a lateral canal truncation (Figure 2D; n = 3/14). A percentage of the Bmp4loxP/Tm1 also display similar defects in the lateral canal (n = 5/10). Therefore, this milder phenotype observed in Foxg1cre/+; Bmp4loxP/Tm1 embryos is probably due to insufficiency of Bmp4 caused by the presence of both the Tm1 and the un-recombined floxed Bmp4 allele rather than an incomplete penetrance of the cre activity. Cochlear ducts of Foxg1cre/+; Bmp4loxP/Tm1 embryos show some variability in length (Figure 2B–2D). We attributed this variability to a slight difference in staging or global growth defects of the ear.

We also conditionally deleted Bmp4 in the inner ear using a transgenic mouse strain, TgPax2cre. The inner ear phenotypes obtained using this cre strain are also variable. Ten out of 15 TgPax2cre; Bmp4loxP/Tm1 specimens have inner ear defects. Those with a milder phenotype show defects in the three ampullae and canals, in addition to lateral canal truncation (Figure 2E; n = 5). The more severe phenotypes include utricle and saccule malformations (Figure 2F; n = 5). Similar to the Foxg1cre/+; Bmp4loxP/Tm1 inner ears, the cochlear duct is relatively normal, consistent with the presence of Bmp4 expression in this region (data not shown). Taken together, inner ear-specific deletion of Bmp4 using two independent cre lines indicates that Bmp4 is required for the formation of the three cristae and semicircular canals, and possibly the utricle and saccule.

Some of the Bmp4loxP/Tm1 embryos generated by breeding TgPax2cre; Bmp4loxP/Tm1 with Bmp4loxP/loxP mice also display lateral...
canal truncation as well (n = 4/7), suggesting that a combination of Tm1 andloxP alleles can generate hypomorphs depending on the genetic background. Notably, our Bmp4loxP/Tm1 mice in Black Swiss background do not circle but a small percentage of Bmp4 heterozygous mice in C57BL/6 background do [35].

Gene Expression Analyses of Foxg1cre/+; Bmp4loxP/Tm1 Embryos

Analysis of paint-filled ears of younger embryos indicates that the vestibular defects in Foxg1cre/+; Bmp4loxP/Tm1 are already apparent at 11.5 dpc (Figure 2G and 2H). To better understand the underlying molecular mechanisms of the phenotypes, we first investigated the expression patterns of a number of genes associated with the prospective cristae such as Fgf10, Gata3, Foxg1, Lmo4, Msx1, and Sox2, in Foxg1cre/+; Bmp4loxP/Tm1 embryos. At 11.5 dpc, the expression domains of these genes normally overlap with that of Bmp4 in the presumptive crista (Figure 3A; data not shown). Conditional mutant ears that have smaller canal pouches consistently in the posterior crista of Embryos [36], and its expression pattern in the canal pouches of mice is shown). Conditional mutant ears that have smaller canal pouches associated markers as well (Figure 3B–B’, n = 28/42 ears). Conversely, in all conditional mutants with residual Bmp4 expression in the anterior region (n = 13/42), other crista markers are also present.

Lack of Bmp4 expression in the inner ear also resulted in the absence of semicircular canal formation. The overall size of the vertical canal pouch is usually smaller than normal, particularly in the posterior region (Figure 2G and 2H). This finding is in agreement with the observation that Bmp4 expression is lost most consistently in the posterior crista of Foxg1cre/+; Bmp4loxP/Tm1 ears. Bmp2 has been implicated in canal formation in the chicken inner ear [36], and its expression pattern in the canal pouches of mice is similar to that of chickens (Figure 3D and 3F). At 11.5 dpc, Bmp2 expression in Foxg1cre/+; Bmp4loxP/Tm1 inner ears is often reduced and sometimes absent in the canal pouches (Figure 3E; arrowheads, n = 8; Figure 3G; n = 4/6, missing posterior signal).

Other genes such as Dlx5, Hmx2, and Hmx3 have also been implicated in canal development. Dlx5 and Hmx are expressed in the otic placode and later in the entire canal pouch (Figure 3H–H’; [37–40]). In Foxg1cre/+; Bmp4loxP/Tm1 embryos with affected inner ears, Dlx5 expression is down-regulated in the canal pouch, but the expression of Hmx3 is unaltered at least up to 11.5 dpc (Figure 3I–I’; n = 4). In contrast, Dlx5 expression in the endolympathic duct is normal (Figure 3I). These results suggest that Bmp4 is required for the maintenance of Dlx5 expression only in the canal pouch, and that the regulation of Hmx3 in the canal pouch is independent of Bmp4 and Dlx5.

Gene Expression Patterns in the Differentiating Chicken Crista

Our results suggest that absence of Bmp4 affects the expression patterns of many genes in the presumptive cristae of mice. However, it is not clear whether these changes are direct or indirect due to the loss of sensory tissues in the conditional mutants. To address this question, we analyzed the short-term effects of down-regulating Bmp signaling on seven known crista-associated genes-Fgf10, Gata3, Lmo4, Msx1, p75Ngfr, Ser1, and Sox2-in chicken inner ears. First, we examined in more detail the expression profiles of these genes during normal cristal development (Figure 4). In a mature anterior or posterior crista, the sensory patch is saddle-shaped, consisting of sensory hair cells and supporting cells. A non-sensory region, the septum cruciatum, is located in the middle of the saddle (Figure 4, schematic diagrams).

Initially, the expression pattern of each of the seven investigated genes largely overlaps with the expression domain of Bmp4 in the presumptive anterior or posterior crista (Figure 4A, A’, B; [30]; [19]; data not shown). After E3.5, the expression patterns of these genes start to segregate (Figure 4C–C’, D–D’). At E5.5, genes such as Bmp4, Sox2, Ser1, and Fgf10 are expressed in two separate domains associated with the sensory patches (Figure 4C–C’, D, double arrows; data not shown). In between the two sensory patches is the p75Ngfr- and Gata3-positive region that eventually develops into the septum cruciatum (Figure 4C–C’, arrow). In contrast, Lmo4 is expressed in both sensory (Figure 4D’, double arrows in pc) and non-sensory regions (Figure 4D’, arrow) of the presumptive crista, and this expression pattern is maintained at E10 (Figure 4G’). By E10, Bmp4, Fgf10, Msx1, Sox2, and Ser1 are associated with supporting cells of the sensory region (Figure 4E’, F, F’, G’), The expression patterns of these genes are qualitatively different from that of Bdnf, which is associated with sensory hair cells (Figure 4E’, insert ii). Gata3 and p75Ngfr expression domains remain outside of the sensory tissue proper, in the septum cruciatum (Figure 4E–E’, G, G’, arrows; [32]) as well as in the transitional zone beyond the crista (Figure 4G, G’, arrowheads).

In summary, our analyses indicated that while most of the crista-associated genes initially overlap in their expression domains within the presumptive anterior and posterior cristae, their expression patterns segregate into either sensory and/or non-sensory regions of the crista as development proceeds.

Genes Affected by Down-Regulation of Bmp Signal Transduction in the Crista

Next, we investigated whether the expression of each of these crista-associated genes is affected by down-regulation of Bmp signaling. Vectors (pSmad6 and pNoggin) encoding Smad6 or Noggin translationally coupled to GFP were electroporated into the developing anterior crista region in ovo at E3.5, a time when these crista-associated genes are co-expressed in the presumptive crista. Smad6 is an intracellular inhibitor that competes with Smad4 for binding to phosphorylated Smad1/Smad5/Smad8 proteins, thus preventing their subsequent translocation to the nucleus and activation of Bmp target genes [41]. Ectopic Smad6 expression has been used successfully to address the roles of Bmps in neural induction and placode formation [29,42]. Figure 5A and B illustrate Gfp signals in the Bmp4-positive anterior crista region (Figure 5C) within 14 hrs after electroporation with pSmad6 and pGfp, respectively.

Electroporation of pSmad6 results in the down-regulation of genes that are eventually associated with the non-sensory, septum cruciatum, such as Gata3 (Figure 5D; n = 10/10) and p75Ngfr (Figure 5F; n = 10/10), whereas expression levels of genes associated with the sensory regions such as Sox2 (n = 0/6), Fgf10 (n = 0/6), Lmo4 (n = 0/9) and Ser1 (n = 0/5) are not affected (Figure 5D”, F”, and data not shown). Electroporation of a control vector expressing Gfp alone does not result in gene expression changes in most cases (Figure 5E–E’, G–G’; n = 42/44). Down-regulation of Msx1 in response to pSmad6 is variable (n = 7/14; data not shown), but quite consistently seen in response to pNoggin (Figure 6A; n = 6/6). The expression of Lmo4, which is associated with both sensory and non-sensory regions, is down-regulated by pNoggin (Figure 6C; n = 11/11), but this is not observed with pSmad6 (n = 9; data not shown). Since Noggin is a secreted molecule, down-regulation of Gata3 and Msx1 in the mesenchyme near the site of electroporation is also observed (Figure 6A’, B’, E’, double arrowheads; n = 6/6). Genes that are not down-regulated by pSmad6, such as Bmp4, Fgf10, Ser1 and Sox2, remain unaffected by pNoggin treatments (Figure 6A”, C”, E”, data not shown).
Electroporation of a control vector, pIRES-Gfp, usually causes no change in these gene expression patterns (Figure 6B, 6D, 6F; \( n = 26/28 \)).

Blocking Bmp Signaling Down-Regulates Msx and Gata3 Immunoreactivities in the Crista

The changes in gene expression were verified at the protein level by double staining the electroporated cells for GFP and translated gene products. The levels of phosphosmad1 were used to evaluate the effects of Smad6 inhibition on Bmp signal transduction. Cells electroporated with \( pSmad6 \) show a down-regulation of phosphosmad1 staining (Figure 7A, A'; \( w_{ap} = 98\% \), \( n = 7 \); see Materials and Methods), whereas cells electroporated with \( pGfp \) do not (Figure 7B; \( w_{ap} = 23\% \), \( n = 7 \)). Moreover, \( pSmad6 \)-electroporated cells also show a down-regulation of Msx (Figure 7C, C'; \( w = 94\% \), \( n = 4 \)) and Gata3 immunoreactivities (Figure 7E, E'; \( w = 87\% \), \( n = 8 \)), whereas Sox2 levels are barely

Figure 3. Gene expression analyses of \( Foxg1^{cre/+} \); \( Bmp4^{loxP/Tm} \) inner ears at 11.5 dpc. (A–A') Adjacent sections of a \( Bmp4^{loxP/+} \) control showing the \( Bmp4 \)-positive lateral crista region (A, A'), which is also positive for Gata3 (A') and Msx1 (A''). (B,B',B'') Adjacent sections of a \( Foxg1^{cre/+} \); \( Bmp4^{loxP/Tm} \) (B4cko) embryo showing the lack of crista-associated expression of \( Bmp4 \) (B), Gata3 (B') and Msx1 (B''). (C) A schematic diagram showing the expression domains of \( Bmp2 \) and \( Bmp4 \) in the canal pouch at 11.5 dpc, and the approximate level of section for each panel. (D–G) Wholemount (D,E) and section (F,G) in situ hybridization showing the reduction of \( Bmp2 \) expression in the canal pouch (outlined in D, E) of \( Foxg1^{cre/+} \); \( Bmp4^{loxP/Tm} \) (E,G), compared to \( Bmp4^{loxP/+} \) (D,F) inner ears. (F) \( Bmp2 \) expression is associated with the prospective posterior and lateral canals (vp and hp) in \( Bmp4^{loxP/+} \) embryos but only in the anterior region of the canal pouch in \( Foxg1^{cre/+} \); \( Bmp4^{loxP/Tm} \) embryos (G, arrow) where residual \( Bmp4 \) expression is sometimes present. (H–I') Dlx5 (H, I) and Hmx3 (H', I') expression domains in the canal pouch of \( Bmp4^{loxP/+} \) (H, H') and \( Foxg1^{cre/+} \); \( Bmp4^{loxP/Tm} \) (I, I') embryos. The endolymphatic duct (ed) is Dlx5-positive (H,I) and Hmx3-negative (H',I'). Canal pouches of \( Foxg1^{cre/+} \); \( Bmp4^{loxP/Tm} \) inner ears are Dlx5 negative (I) and Hmx3 positive (I'). Orientations: A, anterior; L, lateral. Orientations in (I') apply to all panels except (D) and (E). Scale bars = 100 \( \mu m \). Scale bar in (F) applies to (A–B) and (G); scale bars in (E) and (I) apply to (D) and (H–I'), respectively.

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affected (Figure 7G; wap = 2.2%, n = 8). Down-regulation of Msx (Figure 7D, D’; wap = 8.6%, n = 5), Gata3 (Figure 7F, F’; wap = 0%, n = 5) and Sox2 (data not shown, wap = 11%, n = 4) immunoreactivities are minimal in cells electroporated with pGfp. Similar results are observed with pNoggin, except down-regulation of Gata3 staining is also observed in the mesenchyme (Figure 7H;
n = 6), whereas Gata3 expression is normal in specimens electroporated with the control plasmid, pIRES-Gfp (Figure 7I; n = 6). Taken together, our results suggest that the down-regulation of Bmp signal transduction appears to preferentially affect genes associated with non-sensory rather than sensory region of the crista.

Inner Ear Phenotypes after Down-Regulation of Bmp Signal Transduction

To investigate whether the knock down of Bmp signal transduction has a long-term effect on crista or canal formation, we harvested some electroporated embryos at E7 and processed them for paint-fill analyses or at E8.5 for sensory hair cell staining using anti-HCA antibody. More than half of the inner ears electroporated with pGfp have normal canals (Figure 8A; n = 12/21), and the rest show non-resorption of the anterior canal (Figure 8B,F, arrow; n = 9/21). However, specimens in which the canal pouch fails to resorb, the anterior crista is usually normal showing a saddle-shaped pattern with anti-HCA staining (Figure 8F, I; n = 4/5), similar to controls (Figure 8E, H). Most of the pSmad6 electroporated specimens either lack the anterior canal or show a canal pouch that is not resorbed (Figure 8C; n = 15/19), and the anterior ampulla is malformed (Figure 8C,G, small arrows; n = 15/19). Within the ampulla, the crista is usually much smaller in size, lacks the cruciatum, and thus lacks the saddle- or W-shaped staining pattern (Figure 8G; n = 10/12). However, some sensory hair cells remain within the malformed cristae based on the punctate staining pattern with anti-HCA antibodies (Figure 8J). These results indicate that down-regulating Bmp signal transduction in the presumptive anterior crista cell-autonomously causes patterning defects in the crista. Inner ears electroporated with pNoggin instead of pSmad6 show a much more severe phenotype involving all three canals and ampullae (Figure 8D; n = 7/8).

The high percentages of specimens with canal defects in the pGfp specimens suggest that canal formation is particularly sensitive to electroporation. Furthermore, since the electroporated region often includes some of the canal pouch epithelium, the canal phenotypes observed in pSmad6 and pNoggin specimens could be due to a direct down-regulation of Bmp2 signaling, originating within the canal epithelium [36], rather than down-regulation of Bmp4 signaling generated from the crista.
**Discussion**

**Cell Fate Specification in the Crista**

The developmental program for the generation of sensory patches within the vertebrate inner ear is thought to be similar to that required for sensory bristle formation in *Drosophila*, in which Notch signaling generates cell type diversity [43–45]. The prevailing concept is that neural fate is specified within the prosensory epithelia of the developing inner ear via Delta-Notch signaling, whereas sensory fate is maintained within the prosensory domain by positive feedback of Ser1-Notch signaling [46,47]. Eventually, lateral inhibition mediated by Notch signaling dictates that cells within the sensory patches differentiate into either hair cells or supporting cells. There is no direct evidence for neural fate specification in the crista-prosensory regions [48]. Notably, in the organ of Corti, there are two rows of p75Ngf-positive pillar cells that are specialized non-sensory cells located between the one row of inner and the first row of outer hair cells [49]. Interestingly, p75Ngfr is also broadly expressed in the prospective organ of Corti initially, and its expression becomes restricted to the pillar cells at later stages [50]. Thus, it is likely that similar cell fate decisions proposed here for the cristae also apply to the organ of Corti.

**Bmp4 in Crista Formation**

The requirement of Bmp4 for crista formation is clearly indicated by results obtained from Bmp4 conditional null mutants. Our down-regulation of Bmp signaling in presumptive cristae of chicken embryos reveals several interesting insights concerning the possible roles of Bmp4 in crista formation. First, genes that are known to be required for prosensory formation in the inner ear such as Sox2, Jag1, and Fig10 are not affected by either *pSmad6* (cell-autonomous) or *pNoggin* (non-cell autonomous) treatments [51–55]. Determining whether these prosensory genes function in parallel or directly upstream of Bmp4 will require further investigation (Figure 9C). Second, genes in both sensory and
null mutants. A while, yet it is not clear if there is a crista phenotype in the expression of cruciatum by regulating Bmp4 could also mediate the formation of the non-sensory pathways of Bmp signaling. Notably, presumably due to the more extensive and/or non-cell autonomous inhibiting hair cell fate. This organizing role could involve interacting with the Notch signaling pathway in cell type specification.

Within the sensory pathway, both Msx1 and Lmo4 are affected by the reduction of Bmp signaling. Msx1 has been shown to be downstream of Bmp4 in several other tissues. A similar relationship has also been suggested in the inner ear. No crista phenotype in Msx1 null mutants has been reported so far, but there could be functional redundancy between Msx1 and Msx2. Lmo4 is one of the Lim domain-only containing genes expressed in the inner ear and is thought to be required for crista and canal formation in mice as well (Lin Gan, unpublished results). Therefore, both Msx1 and Lmo4 could be important mediators of Bmp4 signaling. Notably, pNoggin treatments appear to down-regulate these genes more effectively than pSmad6, presumably due to the more extensive and/or non-cell autonomous effects of Noggin.

In addition to regulating Msx1 and Lmo4 in the sensory region, Bmp4 could also mediate the formation of the non-sensory cruciatum by regulating p73Ngfr, Gata3, and Lmo4 activities. The expression of p73Ngfr in the developing cruciatum has been known for a while, yet it is not clear if there is a crista phenotype in p73Ngfr null mutants. Gata3 is an important gene in inner ear development as evident by the rudimentary inner ear structure of Gata3-/ mouse embryos. In humans, mutations in GATA3 are associated with HDR (hypoparathyroidism, sensorineural deafness, and renal anomaly) syndrome. Given the importance of a GATA factor (pannier) in activating the achaete-scute proneural complex in Drosophila, Gata3 may have a more global effect on cell fate specification in vertebrate crista beyond formation of the cruciatum (see below). Furthermore, the conserved Gata3 expression in the mesenchyme surrounding the presumptive cristae between chicken and mouse, may also contribute to the proper formation of the crista. We speculate that the observed down-regulation of Gata3 expression in the mesenchyme by pNoggin, but not by pSmad6, may contribute to the more severe phenotype caused by pNoggin.

In other systems, Bnp and Gata pathways are thought to interact. For example, during Drosophila embryogenesis, panner (homolog of Gata1) is induced by dpp (decapentaplegic, homolog of Bmp4) in the dorsal embryo. Later in development, regulation of dpp becomes dependent on panner. Within the inner ear, Gata3 expression appears to begin before that of Bmp4. However, the relatively normal Bmp4 expression in Gata3 null inner ears does not support Gata3 functioning upstream of Bmp4. Nevertheless, our results here show that the maintenance of Gata3 in the cristae is dependent on Bmp4.

Furthermore, while we have classified genes as sensory and non-sensory in the above discussion according to their expression domains in a mature crista, their earlier developmental functions may not be limited to the cell types that they are expressed in at maturity. This notion is based on the ubiquitous expression of these genes in the presumptive anterior and posterior cristae. The lateral crista does not contain a cruciatum in either chicken or mouse. Yet, Gata3, considered to be a non-sensory gene, is
expressed in the prospective lateral crista of the mouse (Figure 3A). It is not known if Gata3 is also expressed in the prospective lateral crista of the chicken. Nevertheless, based on the limited number of crista-associated genes analyzed here, the classic non-sensory genes appear to be more readily affected than the so-called sensory genes when Bmp signal transduction is down-regulated. Therefore, an attractive hypothesis that remains to be tested is that the non-sensory genes such as Gata3 and p75Ngfr are key players in mediating the early organizing roles of Bmp4.

In addition, based on the inner ear phenotypes in Bmp4 conditional knockout embryos, Bmp4 is also required for the formation of the utricle and saccule. Since little expression of Bmp4 is detected in their presumptive tissues using in situ hybridization [9], further study is needed to determine whether cristae are the source of Bmp4 that is required for their development.

Bmp4 in Canal Formation

We have proposed that the sensory cristae may induce the formation of their associated semicircular canals [1]. Bmp4 is strongly expressed in the presumptive crista but not the canal pouch [9]. Therefore, the canal phenotypes of Bmp4 conditional null mutants also lend support to the hypothesis that crista regulates canal formation. Recent fate mapping data in chicken indicate that there is a canal genesis zone located adjacent to each crista that gives rise to majority of the cells in the canal [36]. The expression domain of Bmp2 in the canal pouch corresponds to this canal genesis zone, and experimental evidence suggests that Fgfs secreted from the presumptive crista induce the formation of the canals by regulating the expression of Bmp2 [36]. The absence of all three canals in Fgf10 null mice is consistent with this hypothesis [54]. It is not clear, though, whether the effect of Bmp4 on canal formation is direct, or is indirectly mediated through Fgfs in the crista. The use of SU5402 (inhibitor of Fgf receptors) to block canal formation in chicken embryos also affects Bmp4 expression in the crista (Chang and Wu, unpublished results). Therefore, both Fgfs and Bmp4 could be involved in mediating canal formation.

In addition to the postulated role of Bmp2 in canal formation, Dlx5 is also a key player in canal formation, and its activity is directly or indirectly regulated by Bmp4 as well. Even though the canal phenotypes in Dlx5-/- mutants are milder than those in Bmp4 conditional mutants, the phenotypes in Dlx5-/-; Dlx6-/- double mutants appear to be more severe [16]. It is possible that there is a positive feedback loop between Dlx and Bmp4 in canal formation, such that Dlx proteins induce Bmp4, and in turn, their activities are maintained by Bmp4. It is also interesting that the expression of Dlx5 in the canal pouch is more susceptible than

Figure 8. pSmad6 and pNoggin-induced inner ear phenotypes. Paint-filled inner ears at E7 after electroporation with pGfp (A, B), pSmad6 (C), or pNoggin (D) at E3.5. Inner ears electroporated with pGfp are either normal (A), or show non-resorption of the anterior canal (B, arrow) and absence of a distinct anterior ampulla (B, small arrow). (C) A pSmad6-treated inner ear showing a malformed anterior ampulla (small arrow) and absence of the anterior canal. The common crus is wider than normal (arrow). (D) A pNoggin-treated inner ear showing the absence of the three ampullae, anterior and lateral canals. The posterior fusion plate is not resorbed (arrow). (E–G) Anti-HCA staining of partially dissected inner ear of controls (E) or inner ears electroporated with pGfp (F) or pSmad6 (G) at E3.5 and harvested at E8.5. (E) Anterior crista shows a typical saddle or W-shaped pattern with anti-HCA staining. (F) pGfp-treated inner ear with a canal pouch that is not resorbed (arrow), but the anterior crista appears normal. (G) pSmad6-treated inner ear showing a malformed and reduced anterior crista and no anterior canal. Arrows point to the outline of the ampulla. (H, I, J) Flattened anterior cristae from (E, F, G), respectively. Arrows in (H) and (I) point to the location of the septum cruciatum, and punctate staining represents stereocilia bundles on top of the sensory hair cells. Orientations: M, medial. Scale bars in (C), (E), and (H) apply to (A–D), (F–G), and (I–J), respectively. doi:10.1371/journal.pgen.1000050.g008
Hmx3 to the lack of Bmp4. Even though both Dlx and Hmx pathways are required for canal formation, regulation of these two pathways appears distinct. This notion is also supported by studies of Gbx2-/- and Wnt1-/-; Wnt3a-/- mutant embryos, in which Dlx5 expression is down-regulated but Hmx3 expression is relatively normal [12,67].

Regulators of Bmp4 Expression

Given the importance of Bmp4 in forming the vestibular apparatus, it is not surprising that many genes such as Dlx, Hmx, Tbx1, Eya1 and Six1 regulate its activity, directly or indirectly (Figure 9C). Within the presumptive crista, Bmp4 expression does not seem to be dependent on Bmp signaling (Figures 5 and 6). In contrast, Bmp4 expression is thought to be maintained by Notch signaling [68]. When Notch signaling is blocked by DAPT, a gamma-secretase inhibitor, Bmp4 expression in the crista is drastically reduced [68], whereas ectopic expression of activated Notch causes ectopic sensory patches, some of which are Bmp4 positive [46]. This Notch signaling is thought to be mediated by Ser1. Consistently, mice with conditional knockout of Jag1 fail to form cristae [31,32].

Taken together, our results unequivocally demonstrate the importance of Bmp4 in patterning and cell fate specification of cristae and canals. Depending on the genetic background, some Bmp4+/- mice also display mild vestibular and auditory defects [35]. Given the multiple effects of Bmp4 in the inner ear, it is not surprising that paradoxical results were obtained from various in vitro experimental conditions [24,25]. Blocking Bmp signal transduction in the presumptive crista of chicken reveals several potential pathways by which Bmp4 could mediate its functions. The relationships among the proteins regulated by Bmps including Gata3, Lmo4, Msx1, and p75Ngr, will require further investigations. In erythroid cells, Gata1 and Lmo2 are known to directly interact and form a complex with other transcription factors [69]. Therefore, it is conceivable that activation of Bmp downstream genes in the crista also require Gata3 and Lmo4 to form a transcriptional complex. Future studies will focus on deciphering the relationships among these proteins in the formation of an important sensory organ, the crista.

Supporting Information

Figure S1  Generation of a floxed Bmp4 allele in which loxP sites are inserted in introns 2 and 4. A FRT flanked Pkg-neo cassette was placed downstream of the 3’ loxP site and removed in vivo by crossing mice carrying the floxed Bmp4lox-Neo allele with ACTB-FLP transgenic mice [70]. The targeting vector was electroporated into Tl1 ES cells, and clones carrying the correctly targeted Bmp4loxP allele were identified by Southern blot analysis of genomic DNA digested with the XbaI restriction enzyme and injected into blastocysts. Homozygous Bmp4loxP mice on a 129/Black Swiss background are fully viable and show no obvious abnormalities. Compound mutant mice with Bmp4loxP and Bmp4Tm1 alleles show reduced viability with only 72% of the expected number of animals reaching weaning age. The surviving
BmploxP/Tm1m animals appear normal. The cause of the reduced viability is unknown.

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**Table S1** Summary of phenotypes.

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**Author Contributions**

Conceived and designed the experiments: WC. Performed the experiments: WC ZL DW. Analyzed the data: WC DW. Contributed reagents/materials/analysis tools: HK JH BH. Wrote the paper: DW. Partially contributed to writing the paper: WC. Performed critical editing of the paper: BH.

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