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BMP/SMAD signaling regulates the cell behaviors that drive the initial dorsal-specific regional morphogenesis of the otocyst

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ABSTRACT

During development of the otocyst, regional morphogenesis establishes a dorsal vestibular chamber and a ventral auditory chamber, which collectively constitute the membranous labyrinth of the inner ear. We identified the earliest morphogenetic event heralding the formation of the vestibular chamber, a rapid thinning and expansion of the dorsolateral wall of the otocyst, and showed that this process is generated by changes in otocyst cell shape from columnar to squamous, as opposed to changes in other cell behaviors, such as localized changes in cell proliferation or cell death. Moreover, we showed that thinning and expansion of the dorsolateral otocyst is regulated by BMP/SMAD signaling, which is both sufficient and necessary for localized thinning and expansion. Finally, we showed that BMP/SMAD signaling causes fragmentation of E-cadherin in the dorsolateral otocyst, occurring concomitantly with cell shape change, suggesting that BMP/SMAD signaling regulates cell–cell adhesion during the initial morphogenesis of the otocyst epithelium. Collectively, our results show that BMP signaling via SMADs regulates the cell behaviors that drive the initial dorsal-specific morphogenesis of the otocyst, providing new information about how regional morphogenesis of a complex organ rudiment, the developing membranous labyrinth, is initiated.

Introduction

The developing inner ear provides a unique system in which to analyze the morphogenetic events that transform a simple epithelial embryonic rudiment – the otocyst – into an elaborate three-dimensional, functional organ—the membranous labyrinth. The origin of the inner ear is simple: it arises from the otic placode, a plate-like region of thickened epithelial cells adjacent to the hindbrain, which subsequently invaginates and pinches off from the overlying ectoderm to form a hollow epithelial structure called the otocyst or otic vesicle. In turn, the otocyst gives rise to the membranous labyrinth through regional morphogenesis, a process is which the otocyst is sculpted into a dorsal vestibular chamber, responsible for the perception of motion and body position, and a ventral auditory chamber, responsible for the perception of sound (reviewed by Barald and Kelley, 2004; Mansour and Schoenwolf, 2005; Bok et al., 2007a; Ohyama et al., 2007; Whitfield and Hammond, 2007; Ladher et al., 2010).

Three lines of evidence strongly suggest that stereotypic morphogenesis of the membranous labyrinth is essential for establishing normal auditory and vestibular function—that is, that both form and function are critically *inter*dependent. First, in as many as 30–40% of children with sensorineural hearing loss, imaging reveals significant inner ear malformations (Antonelli et al., 1999; Purcell et al., 2003). For example, in one population of patients with sensorineural hearing loss, inner ear malformations were observed in 38% of the cases, typically including enlarged vestibular aqueduct, Mondini dysplasia, large vestibule, and semicircular canal dysplasia (Wu et al., 2005). Similarly, enlarged vestibular aqueduct has been correlated with vestibular symptoms in children (Grimmer and Hedlund, 2007; Worden and Blevins, 2007), although whether this anomaly represents a defect in morphogenesis or a post-morphogenesis enlargement is unclear. Second, in animal models, experimentally induced changes in otocyst morphogenesis similar to those seen in patients can compromise inner ear function, even when hair and supporting cells are generated (e.g., Hatch et al., 2007; Nichols et al., 2008; Koo et al., 2009). Third, theoretical modeling supported by analytical studies (Manoussaki et al., 2008) demonstrates that cochlear shape is correlated with the cochlea's response to low frequencies, emphasizing the importance of proper cochlear morphogenesis in hearing.

Our understanding of the cellular and molecular mechanisms underlying regional morphogenesis of the otocyst is highly limited. Formation of the vestibular chamber is complex and involves several morphogenetic events, including initial dorsolateral outgrowth of a primordial canal pouch that is remodeled into the anterior and posterior semicircular canals, followed by lateral outgrowth of a second canal pouch that is remodeled into the lateral semicircular

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canal (Chang et al., 1999; 2004a). Only the remodeling stage of vestibular chamber morphogenesis has been analyzed at the level of cell behaviors: it involves focal apoptosis in the chick and *Xenopus* (Haddon and Lewis, 1991; Fekete et al., 1997), and cell rearrangements in the mouse (Martin and Swanson, 1993). Formation of the auditory chamber involves the ventromedial outgrowth of the otocyst wall to form the cochlear bud, and the rapid elongation of this bud to form the cochlear duct (Bissonnette and Fekete, 1996; Morsli et al., 1998). The cell behaviors driving the latter event are better understood and include a mediolateral cell–cell intercalation that results in a convergent extension movement, which concomitantly narrows and lengthens the duct as cells merge toward the center (Yamamoto et al., 2009).

Not only is our understanding of the cell behaviors that drive regional morphogenesis of the otocyst limited, so are the signaling pathways that directly control the particular cell behaviors driving morphogenesis. Several signaling pathways regulate development of the vestibular and auditory chambers of the inner ear, including the WNT (Dabdoub et al., 2003; Montcouquiol and Kelley, 2003; Montcouquiol et al., 2003, 2006; Dabdoub and Kelley, 2005; Riccomagno et al., 2005; Wang et al., 2005; Kelly and Chen, 2007; Qian et al., 2007; Rida and Chen, 2009; Yamamoto et al., 2009), BMP (Chang et al., 1999, 2002, 2004b, 2008; Gerlach et al., 2000; Bok et al., 2007b; Hammond et al., 2009; Hwang et al., 2010), SHH (Riccomagno et al., 2002, 2005; Bok et al., 2007c; Hammond et al., 2010; Sapède and Pujades, 2010), and FGF (Mansour et al., 1993; Léger and Brand, 2002; Pauley et al., 2003; Ohuchi et al., 2005; Hatch et al., 2007; Zelarayan et al., 2007) pathways. However, in only one case do we know the cell behaviors directly regulated by signaling: namely, non-canonical WNT signaling controls cell–cell intercalation and convergent extension in the elongating cochlear duct (Dabdoub et al., 2003; Dabdoub and Kelley, 2005; Kelly and Chen, 2007; Rida and Chen, 2009).

In this study, we examined the molecular signaling regulating the initial morphogenetic event heralding formation of the vestibular chamber: rapid thinning and expansion of the dorsolateral wall of the otocyst to form the primordial canal outpouch. We show that thinning and expansion is driven by changes in otocyst cell shape from columnar to squamous, and not by other cell behaviors such as localized cell proliferation or death, and that change in cell shape is accompanied by E-cadherin fragmentation, suggesting that changes in cell–cell adhesion are required for this morphogenesis to occur. We further show that BMP signaling, acting through SMADs, is both sufficient and necessary for thinning and expansion. In summary, our studies demonstrate an essential role for BMP signaling in initiating regional morphogenesis of the dorsal otocyst to form the vestibular chamber of the inner ear.

Materials and methods

Embryos

Fertilized White Leghorn chicken eggs were purchased from Merrill's Poultry Farm (Idaho, USA). Eggs were incubated at 38.5 °C and staged according to the criteria of Hamburger and Hamilton (1951; HH; reprinted as Hamburger and Hamilton, 1992). CD-1 (Charles River) mice were mated to obtain embryos at desired stages, indicated as embryonic (E) days following detection of a vaginal plug. All animal studies complied with protocols approved by the University of Utah Institutional Animal Care and Use Committee.

In situ hybridization for gene expression analysis

Whole mount in situ hybridization was performed using standard procedures with chicken (c) probes for *Bmp2*, *Bmp4*, *Bmp7*, and *Smad6* (kindly provided by T. Nohno, Kawasaki Medical School, Okayama, Japan; C. Tickle, University of Dundee, UK; B. Houston, University of Dundee, UK; and E. Laufer, Columbia University, USA). In a subset of embryos, the otocyst was first opened with a tungsten needle to avoid trapping of probes and detection reagents.

Chick *Bmp* type-I and type-II receptor probes were obtained by RT-PCR using the following primers:

BmpRIA: 5′-AGCGATTGCTTGGAGCCTATCT-3′ and 5′-AGCTGGCTTCTTCTGTGGTGAA-3′
BmpRIB: 5′-GACACTCCTATTCCACACCA-3′ and 5′-GAGCTTAATGTCCTGCGACT-3′
BmpRII: 5′-GGTCGATACGGAGCAGTGTACA-3′ and 5′-CTGCTCCTTCAAGCACTTCTGG-3′

Mouse (m) *Bmp* type-I and type-II receptors were obtained by RT-PCR using the following primers:

BmpRIA: 5′-GTGAGCATCAAGTGGCATTGG-3′ and 5′-GACACAAGAGAAGAGGGGAGAGTCG-3′
BmpRIB: 5′-AAAGCATCCCTCTGTGTTTCACTC-3′ and 5′-CCAAGGACGCTTTTTGCCCCTCTT-3′
BmpRII: 5′-AACCACCACAAACACCACCG-3′ and 5′-GATACTTACCACACCGTCCATCTTC-3′

Immunohistochemistry

Paraffin sections of chick and mouse embryos fixed with 4% paraformaldehyde (PFA) were prepared with a microtome (Leica LM2255) and either stained with hematoxylin and/or eosin or subjected to immunostaining using standard procedures. For immunohistochemistry, specimens were incubated at a 1:100 dilution in PBST (phosphate-buffered saline plus 0.1% Tween20) containing 0.2% fetal bovine serum (FBS) either for 2 h at room temperature or overnight at 4 °C with the following primary antibodies: anti-GFP (mouse monoclonal, Roche, Cat. #11814460001), anti-E-cadherin (mouse monoclonal, BD Bioscience, Cat. #610182), anti-pSMAD1/5/8 (rabbit polyclonal, Cell Signaling Technology, Cat. #9511), and anti-laminin (rabbit polyclonal, SIGMA, Cat. #L9393). After three washes with PBST, specimens were incubated with the following secondary antibodies diluted 1:200 in a solution of PBST containing 0.2% FBS: Alexa Fluor 488 goat anti-mouse IgG or Alexa Fluor 594 goat anti-rabbit IgG (Molecular Probes). In many cases, sections were then counterstained with Hoechst stain to mark cell nuclei.

Terminal dUTP Nuclear End Labeling (TUNEL) was done according to the manufacturer's procedures (In Situ Cell Death Detection Kit, Fluorescein: Cat. #11684795910, Roche). Sections were counterstained with Hoechst stain.

5-Ethynyl-2′-deoxyuridine (EdU) labeling was done according to the procedure described by Warren et al. (2009). Chick eggs were windowed, the vitelline membranes were opened, and 100–200 μl of 500 μM EdU in 0.9% NaCl was added onto the underlying embryos. After 2 or 24 h of incubation, embryos were harvested and fixed in 4% PFA for 1–2 h at 4 °C. EdU labeling was detected following the manufacturer's procedures (Click-iT® EdU Alexa Fluor® 488 Imaging Kit for 50 coverslips: Cat. #C10337, Invitrogen). In addition, cell embryos were labeled with an antibody to phosphorylated Histone H3 (pHH3: rabbit polyclonal, Upstate Biotechnology, Inc., Cat. #06570), as a second means to assess cell division.

In ovo sonoporation

HH 11–14 chick embryos were sonoporated in ovo. To prepare the DNA–microbubble mixture, 10 μl of a plasmid DNA solution (concentration 2.0–4.0 μg/μl), (pCAGGS-GFP, pCAGGS-m*Noggin*, or pCAGGS-m*Bmp4*; the GFP vector was co-sonoporated with the gene of interest), was added to 10 μl of SonoVue (BRACCO, Milan, Italy, purchased from Protech, Texas, USA). Eggs were windowed, the vitelline and amniotic membranes were opened, and the DNA–microbubble mixture was