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Redundant functions of Rac GTPases in inner ear morphogenesis

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ABSTRACT

Development of the mammalian inner ear requires coordination of cell proliferation, cell fate determination and morphogenetic movements. While significant progress has been made in identifying developmental signals required for inner ear formation, less is known about how distinct signals are coordinated by their downstream mediators. Members of the Rac family of small GTPases are known regulators of cytoskeletal remodeling and numerous other cellular processes. However, the function of Rac GTPases in otic development is largely unexplored. Here, we show that *Rac1* and *Rac3* redundantly regulate many aspects of inner ear morphogenesis. While no morphological defects were observed in $Rac3^{-/-}$ mice, $Rac1^{CKO}$; $Rac3^{-/-}$ double mutants displayed enhanced vestibular and cochlear malformations compared to $Rac1^{CKO}$ single mutants. Moreover, in $Rac1^{CKO}$; $Rac3^{-/-}$ mutants, we observed compromised E-cadherin-mediated cell adhesion, reduced cell proliferation and increased cell death in the early developing otocyst, leading to a decreased size and malformation of the membranous labyrinth. Finally, cochlear extension was severely disrupted in $Rac1^{CKO}$; $Rac3^{-/-}$ mutants, accompanied by a loss of epithelial cohesion and formation of ectopic sensory patches underneath the cochlear duct. The compartmentalized expression of otic patterning genes within the $Rac1^{CKO}$; $Rac3^{-/-}$ mutant otocyst was largely normal, however, indicating that Rac proteins regulate inner ear morphogenesis without affecting cell fate specification. Taken together, our results reveal an essential role for Rac GTPases in coordinating cell adhesion, cell proliferation, cell death and cell movements during otic development.

Introduction

The mammalian inner ear is a highly complex sensory organ responsible for hearing and balance. All sensory organs are located within a fluid-filled membranous labyrinth, which is subdivided into two functional compartments. The dorsal compartment houses the vestibular apparatus, including three orthogonally positioned semicircular canals for detecting angular motion and the utricle and saccule for detecting linear motion and gravity. The ventral compartment houses the hearing organ, the cochlea, which contains a sensory epithelium (the organ of Corti) responsible for sound detection. Each sensory patch contains hair cells that function as mechanotransducers, as well as other supporting cell types. Malformations of any of the structures of the inner ear due to genetic or environmental factors can lead to deafness and/or balance dysfunction.

During embryogenesis, the inner ear develops from a small patch of thickened ectoderm, the otic placode, which arises adjacent to the dorsal hindbrain at around embryonic day 8.5 (E8.5) in the mouse (Alsina et al., 2009; Bok et al., 2007). This placode invaginates to form the otic cup by E9.0, from which neuroblasts delaminate ventromedially to form the cochleovestibular ganglion. By E9.5, the cup closes and separates from the surface ectoderm to form the otocyst vesicle (otic vesicle). The otocyst is highly patterned at the level of gene transcription as a result of integrated fibroblast growth factor (FGF), Wnt, bone morphogenetic protein (BMP), and Hedgehog signals released from surrounding tissues such as the dorsal hindbrain, notochord, floor plate and periotic mesenchyme. The distinct gene expression domains eventually give rise to specific inner ear structures (Alsina et al., 2009; Bok et al., 2007).

Following its formation, the otocyst grows in size and undergoes elaborate morphogenic changes to form the mature ear (Morsli et al., 1998). Around E10.5, the endolymphatic duct emerges from the dorsal otocyst. Around E11.5, a vertical and horizontal outpouch evaginate from the otocyst to form the vertical (the precursor of the anterior and posterior semicircular canals) and lateral canal plates, respectively. As development proceeds, cells within the opposing walls of the canal plates come together to form fusion plates. After fusion, cells in the central region of the fusion plates undergo resorption leading to the formation of hollow canals by E13.5. The utricle and saccule also emerge from evaginations in the central region of the ear at around E12 and E13, respectively. Simultaneously, the otocyst evaginates ventrally to form the nascent cochlea. Elongation and

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coiling of the cochlea occur between E12.5 and E17.5, by which time it reaches its full length of one and three-quarter turns. These morphogenic changes are directed by highly coordinated changes in cell division, programmed cell death, cell migration and gene transcription. However, the molecular factors that integrate these morphogenic processes are not well understood.

Within the organ of Corti, hair cell formation begins around E12.5–E13.5 when sensory precursors exit the cell cycle and commit to either a hair cell or supporting cell fate (Chen et al., 2002; Ruben, 1967). Differentiation of hair cells then proceeds in a basal to apical gradient along the length of the cochlea between E15.5 and E17.5, until one row of inner and three rows of outer hair cells are formed (Chen et al., 2002; McKenzie et al., 2004). This hair cell differentiation occurs in parallel with elongation of the cochlear duct. Cochlear extension is thought to be driven by radial and mediolateral intercalations analogous to convergent extension movements during gastrulation (Kelly and Chen, 2007; McKenzie et al., 2004; Wang et al., 2005). This extension occurs in a basal-to-apical direction, and from E14.5 to E18.5, the length of the cochlea increases approximately two-fold and thins from a four- to five-cell layered primordium to two layers of cells. Although the molecular and cellular mechanisms that mediate this morphogenic process are not well understood, genetic studies have implicated an evolutionarily conserved planar cell polarity (PCP) signaling module and non-muscle myosin II activity (Rida and Chen, 2009; Wang et al., 2005; Yamamoto et al., 2009). The downstream effectors of PCP signaling that regulate cochlear extension, however, remain largely unknown.

One important group of signaling proteins that may potentially regulate multiple aspects of inner ear development is the Rac family of small GTPases. Although most noted for their ability to control cytoskeletal remodeling, Rac proteins are known to regulate diverse cellular processes including gene transcription, cell proliferation and growth, cell–cell and cell–matrix adhesion, cell survival, and cell movements (Jaffe and Hall, 2005). Rac proteins have emerged as key participants in multiple signaling pathways during embryonic development including the canonical Wnt/β-catenin, fibroblast growth factor (FGF), non-canonical Wnt/Frizzled, Notch, mitogen-activated protein kinase (MAPK), c-JUN N-terminal kinase/stress activated kinase (JNK/SAPK), nuclear factor κB (NF-κB) and phosphatidylinositol 3-kinase (PI-3K) pathways (Bosco et al., 2009; Dumont et al., 2009; James et al., 2008; Wu et al., 2008).

The mammalian Rac family consists of three members, *Rac1*, *Rac2*, and *Rac3*, which share 88–92% sequence identity. *Rac1* is ubiquitously expressed whereas *Rac2* and *Rac3* are predominantly expressed in hematopoietic and neuronal cells, respectively (Glogauer et al., 2003; Haataja et al., 1997). Previously we found that conditional deletion of the *Rac1* gene ($Rac1^{CKO}$) in the otic epithelium of mice led to defects in temporal bone morphology, cochlear extension and auditory hair cell development, indicating a critical role for *Rac1* in inner ear morphogenesis (Grimsley-Myers et al., 2009). Interestingly, we found that *Rac3* is also expressed in the developing cochlea, suggesting alternate roles for Rac family members (Grimsley-Myers et al., 2009).

Here, we describe redundant functions of *Rac1* and *Rac3* in a series of morphogenetic events in the inner ear, including otocyst morphogenesis, formation of the semicircular canals, cochlear extension and spiral ganglion development. Our results reveal redundant functions of *Rac1* and *Rac3* in coordinating actin assembly and E-cadherin-mediated cell–cell adhesion during otic epithelial morphogenesis.

Materials and methods

Mice

The *Rac1* conditional ($Rac1^{CO}$) and knockout ($Rac1^{KO}$) alleles, $Foxg1^{Cre}$ mice, *Pax2-Cre* and *BAT-gal* mice were previously described (Glogauer et al., 2003; Hebert and McConnell, 2000; Maretto et al., 2003; Ohyama and Groves, 2004). $Rac3^{-/-}$ mice (Cho et al., 2005) were kindly provided by Dr. Nora Heisterkamp (Children's Hospital Los Angeles). All strains were maintained on a mixed genetic background. $Foxg1^{Cre}$; $Rac1^{KO/+}$; $Rac3^{+/-}$ males were bred with $Rac1^{CO/CO}$; $Rac3^{-/-}$ females with or without *BAT-gal* to generate $Foxg1^{Cre}$; $Rac1^{KO/CO}$; $Rac3^{-/-}$ (hereafter referred to as $Rac1^{CKO}$; $Rac3^{-/-}$) mutants and littermate controls. *Pax2-Cre*; $Rac1^{CO/+}$; $Rac3^{+/-}$ females were bred with $Rac1^{CO/CO}$; $Rac3^{-/-}$ males to generate *Pax2-Cre*; $Rac1^{CO/CO}$; $Rac3^{-/-}$ mutants. Mice were genotyped for *Cre* and *Rac1* alleles (Grimsley-Myers et al., 2009) and *Rac3* alleles (Cho et al., 2005) as described. For timed pregnancies, the morning of the plug was designated as E0.5 and the day of birth postnatal day 0 (P0). Animal care and use were in accordance with NIH guidelines and was approved by the Animal Care and Use Committee at the University of Virginia.

Paint-fill and in situ hybridization

Paint-fill analyses were performed as described previously (Morsli et al., 1998). Section in situ hybridization was carried out according to standard procedures (Nagalakshmi et al., 2010) using the following digoxigenin-labeled riboprobes: *Lfng* and *Bmp4* (Morsli et al., 1998), *Otx1* (Morsli et al., 1999), *Dlx5* (Depew et al., 1999), *Gbx2* (Bouillet et al., 1995), *Eya1* (Xu et al., 1997), *Gata3, Fgf10, Fgf3, and Six1* (Zheng et al., 2003), *NeuroD1* and *Ngn1* (Ma et al., 1998).

Immunohistochemistry, SEM and X-gal staining

Immunohistochemistry was performed as previously described (Grimsley-Myers et al., 2009). Briefly, dissected temporal bones were fixed in 4% paraformaldehyde overnight at 4 °C and washed in PBS. For whole-mount preparations, cochleae were dissected out of the temporal bones and the anlage of Reissner's membrane removed to expose the sensory epithelium. For sectioning, fixed temporal bones were equilibrated in 30% sucrose, snap frozen in OCT (Tissue Tek), and cryosectioned at 12 μm thickness. Images were collected using a Zeiss LSM 510 Meta or a Zeiss LSM 700 confocal microscope and LSM Image Browser software. Images were then processed in Adobe Photoshop (Adobe Systems). The following primary antibodies were used: anti-BrdU (1:100, Becton Dickinson) anti-phospho-Histone H3 (pHH3, 1:400, Millipore), anti-cleaved Caspase-3 (1:200, with antigen retrieval, Cell Signaling), anti-E-cadherin (1:200, Zymed), anti-Sox2 (1:400, Millipore), anti-Pax2 (1:400, Invitrogen), anti-Myosin VI (1:1000, Proteus BioSciences), rabbit anti-Myosin VIIa (1:1000, Proteus BioSciences), mouse anti-Myosin VIIa (1:100, Developmental Studies Hybridoma Bank), anti-acetylated tubulin (1:500, Sigma), anti-ZO-1 (1:200, Millipore), anti-Fibronectin (1:400, Sigma), anti-Islet-1 (1:200, Developmental Studies Hybridoma Bank), anti-Tuj1 (1:500, Covance), and anti-$p27^{kip1}$ (1:200 with antigen retrieval, Neomarkers). Alexa-conjugated secondary antibodies (1:1000), rhodamine-conjugated phalloidin (1:200) and Hoechst 33342 (1:10,000) were from Invitrogen. Quantitation of hair cell number and organ of Corti length was performed as previously described (Grimsley-Myers et al., 2009). SEM was performed as described previously (Grimsley-Myers et al., 2009). BAT-gal activity was detected by X-gal staining. Briefly, embryos were fixed in 4% paraformaldehyde in PBS at room temperature for 30 min. Whole-mount embryos were then stained overnight at 4 °C with X-gal using standard protocols, postfixed in 4% paraformaldehyde and processed for cryosectioning.

Cell proliferation and apoptosis

To detect proliferating S-phase cells, timed pregnant mice were injected intraperitoneally with 5-bromodeoxyuridine (BrdU) in PBS