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SHH ventralizes the otocyst by maintaining basal PKA activity and regulating GLI3 signaling

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ABSTRACT

During development of the inner ear, secreted morphogens act coordinately to establish otocyst dorsoventral polarity. Among these, Sonic hedgehog (SHH) plays a critical role in determining ventral polarity. However, how this extracellular signal is transduced intracellularly to establish ventral polarity is unknown. In this study, we show that cAMP dependent protein kinase A (PKA) is a key intracellular factor mediating SHH signaling through regulation of GLI3 processing. Gain-of-function experiments using targeted gene transfection by sonoporation or electroporation revealed that SHH signaling inactivates PKA, maintaining a basal level of PKA activity in the ventral otocyst. This, in turn, suppresses partial proteolytic processing of GLI3FL, resulting in a low GLI3R/GLI3FL ratio in the ventral otocyst and the expression of ventral-specific genes required for ventral otocyst morphogenesis. Thus, we identify a molecular mechanism that links extracellular and intracellular signaling, determines early ventral polarity of the inner ear, and has implications for understanding the integration of polarity signals in multiple organ rudiments regulated by gradients of signaling molecules.

1. Introduction

Morphogen gradients are postulated to provide positional signals that are both sufficient and necessary for establishing the dorsoventral (DV) axis of the otocyst, the primordium of the inner ear (Groves and Fekete, 2012; Wu and Kelley, 2012). Dorsalization of the otocyst involves multiple secreted factors, including WNTs, BMPs, and FGFs (Hatch et al., 2007; Ohta et al., 2010; Ohta et al., 2016; Ohyama et al., 2006; Riccomagno et al., 2005). In contrast, only one secreted factor, SHH, released from both the floor plate of the neural tube and underlying notochord, has been reported to be required for its ventralization (Riccomagno et al., 2002). *Shh*$^{-/-}$ embryos fail to form cochlear ducts, whereas dorsal inner ear structures still form (Riccomagno et al., 2002). Moreover, factors downstream of SHH also play essential roles in ventral otocyst development. Mice with inactivating mutations in the seven-transmembrane protein, Smoothened, lack cochlear ducts and saccules (Brown and Epstein, 2011). Similarly, mice with C-terminal deletions of GLI3, which express only the repressor form of GLI3, display severely shortened cochlear ducts (Bok et al., 2007). This, and the analysis of other GLI3 mutants in the same study, provides strong evidence that GLI3 activator function is required for normal formation of the cochlear duct.

cAMP-dependent protein kinase A (PKA) plays an important role in regulation of SHH signaling through the posttranslational modification of GLI. In both invertebrate and vertebrate systems, PKA inhibits HH signal transduction (Epstein et al., 1996; Hammerschmidt et al., 1996; Li et al., 2014, 1995; Tiecke et al., 2007). In *Drosophila,* genetic and biochemical studies showed that in the absence of HH, activated PKA phosphorylates a cluster of serine residues on the C-terminal domain of cubitus interruptus (Ci), the GLI homolog. This results in the partial proteolysis of Ci to generate a transcriptional repressor form of Ci (Price and Kalderon, 2002). Similarly, in vertebrates, two of the three full-length GLI proteins (GLI2FL and GLI3FL) also undergo PKA-dependent phosphorylation and partial proteolytic processing to generate a repressor form (GLIR), with GLI3R being the most prominent (Pan and Wang, 2007; Wang et al., 2000).

Activation of PKA plays a crucial role in polarization of the vertebrate neural tube. Blocking PKA activity in zebrafish embryos ventralizes the neural tube, as shown by dorsal expansion of the ventral marker gene, *axial* (Epstein et al., 1996; Hammerschmidt et al., 1996). Similarly, in PKA-deficient mouse embryos, the number of SHH-dependent ventral cell types in the developing neural tube is expanded (Tuson et al., 2011). These findings suggest that PKA acts in DV polarization of the neural tube by negatively regulating SHH signaling.

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Here, we used western blotting and gene mis-expression to reveal roles for SHH signaling and PKA in establishing the DV axis of the otocyst. We show that PKA forms a DV gradient of activity across both the chick and mouse otocyst, being low ventrally and high dorsally, which parallels a DV gradient in GLI3 protein processing to establish a DV gradient of the ratio of GLI3R to GLI3FL. Overexpression of SHH inhibits PKA and pCREB labeling in the chick dorsal otocyst and alters dorsal gene expression, blocking formation of the semicircular canals. Finally, inhibition of PKA in the chick otocyst with the PKA inhibitor alpha (PKIα), or overexpression of GLI3A, also ventralizes the otocyst. Thus, SHH signaling through inhibition of PKA plays an important role in establishing the ventral patterning of the otocyst.

2. Material and methods

2.1. Embryos

Fertilized White Leghorn chicken eggs were purchased from Merrill's Poultry Farm (Paul, Idaho, USA). Eggs were incubated at 38.5 °C and staged according to the criteria of Hamburger and Hamilton (HH; Hamburger and Hamilton, 1951). CD-1 (Charles River) mouse embryos were collected on the indicated days following detection of a mating plug. The use of mice complied with a University of Utah approved IACUC protocol.

2.2. PKA activity assay

Dissected otocysts (chick: HH stages 20–24; mouse: E10.5–11.0), either kept intact or dissected into dorsal (do), middle (mi), and ventral (ve) fragments, were pooled (5–20 for each lane), enzymatically digested with dispase (GIBCO: working concentration of 0.5 U/ml for 30–45 min at 37 °C), and lysed in buffer consisting of 25 mM Tris–HCl (pH 7.4), 0.5 mM EDTA, 0.5 mM EGTA, and 10 mM β-mercaptoethanol, with one inhibitor cocktail tablet (Complete, Roche #1836153) added per 50 ml. PKA activity was measured using a commercially available kit (PepTag Non-radioactive detection of cAMP-dependent protein kinase assay system, Promega, #V5340), according to the manufacturer's instructions.

2.3. pCREB staining and quantification of signal intensity

We detected pCREB in tissue sections by using a phospho-specific primary antibody (rabbit polyclonal, MILLIPORE, cat. # 06-519) diluted 1:1000 in blocking solution (0.1% FBS). A Vectastain ABC HRP kit (cat# PK-4001) was used according to the manufacturer's manual for enhancing the signal with HRP visualized with 3,3′-diaminobenzidine (DAB, SIGMA, cat# D5905).

To quantify pCREB signal intensity in tissue sections, we used an ImageJ macro, kindly developed by Dr. H. Otsuna, VoxelPress (https://dl.dropboxusercontent.com/u/4774326/VoxelPress/Products.html). The signal to noise (background) labeling of pCREB was variable from embryo to embryo, even in groups of the same type of embryo at the same stage and processed identically in the same batch. Hence, to ensure that accurate measurements of pCREB labeling could be made independently of observer bias, we used the macro to automatically normalize the signal intensity of pCREB labeling in the wall of the otocyst in multiple sections through approximately its mid-craniocaudal extent. To detect regional differences in control otocysts (dorsolateral, ventromedial, etc.) at different stages, or in otocysts from embryos into which *Shh* was sonoporated, masks were manually created in ImageJ to segment the analysis. To compare electroporated (i.e., GFP-positive) cells in control and experimental otocysts, the macro automatically created masks to restrict the analysis to fluorescent cells.

2.4. In ovo *gene transduction*

Chick eggs were windowed using standard techniques, and embryos at HH stages 15–16 were either sonoporated (Ohta et al., 2003) or electroporated (Ohta et al., 2010) *in ovo* to transfect expression vectors. For sonoporation, a DNA-microbubble mixture was prepared by adding 10 µl of a plasmid DNA solution (concentration 2.0–4.0 µg/µl), consisting of pCAGGS-*Gfp* alone or pCAGGS-*Gfp* and pCAGGS-c*Shh* to 10 µl of SonoVue (BRACCO, Protech, Texas, USA), a microbubble solution used for gene transfection. The DNA-microbubble mixture was then injected into the head mesenchyme adjacent to the developing otocyst using a glass micropipette (GD-1.2: Narishige, Tokyo, Japan). Injected embryos were immediately exposed to ultrasound using a 3-mm diameter ultrasound probe (Sonitron 2000 N, Nepagene, Japan) with an input frequency of 1 MHz, an output intensity of 2.0 W/cm^2, and a pulse duty ratio of 20% for a duration of 60 s.

For electroporation, the plasmid solution contained 0.1% fast green to enable visualization of the injection site and consisted of either 2.0 µg/µl of pCAGGS-*Gfp* alone or 2.0 µg/µl of pCAGGS-*Gfp* and pCAGGS-h*Pkiα* or 2.0 µg/µl of pCAGGS-h*Gli3A*^{HIGH}. DNA was injected into the lumen of the developing otocyst using a fine glass micropipette with positive and negative electrodes positioned accordingly. Two 50-ms pulses at 10 V were then applied using a CUY21 electroporator. Because the wall of the otocyst was impaled during electroporation, localized damage of the wall was sometimes detected, with a small cluster of dying cells present in the lumen. However, the damaged region and dying cells were excluded from our subsequent analyses.

Transfected cells were identified in tissue sections by using an antibody directed against GFP (mouse monoclonal, Roche, #11814460001). AlexaFluor 488-labeled donkey anti-mouse IgG (Invitrogen, cat. #A21202), diluted 1:1000, was used as the secondary antibody.

2.5. Expression vectors

pCAGGS-*Gfp* was kindly provided by Dr. H. Ogawa (Ogawa et al., 1995) and pCAGGS-*Gli3A*^{HIGH} by Dr. J. Briscoe (Persson et al., 2002; Stamataki et al., 2005). pCAGGS-c*Shh* was described previously (Ohta et al., 2010, 2003). Full-length human cDNA encoding protein kinase inhibitor alpha (h*Pkiα*) was purchased from DNAFORM (cDNA clone ID: 4288676, http://www.dnaform.jp/) and inserted into a pCAGGS vector.

2.6. Three-dimensional (3D) reconstruction of the inner ear

To visualize the 3-D structure of the chick inner ear, paraffin serial transverse sections of chick embryos at HH stage 30 were stained with hematoxylin and eosin. Sections were then photographed, and control and experimental inner ears were reconstructed using Amira 5.1 software (FEI Company, FEI.com).

2.7. In situ *hybridization on paraffin sections*

Chick and mouse embryos were fixed with 4% paraformaldehyde and embedded in paraffin, and transverse sections were subjected to *in situ* hybridization (ISH) using standard procedures with chick (c) probes for c*Pax2, cOtx2, cNkx5.1,* and *cDlx5* (kindly provided, respectively, by Drs. D. Henrique, L. Bally-Cuif, E. Bober, W. Upholt). *cPax2* was used to mark the ventromedial otocyst (Sanchez-Calderon et al., 2005); *cOtx2,* the ventrolateral otocyst (Morsli et al., 1999); and *cNkx5.1* and *cDlx5,* the dorsolateral otocyst (Brown et al., 2005; Hadrys et al., 1998).