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The role of Paraxial Protocadherin in *Xenopus* otic placode development

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Abstract

Vertebrate inner ear develops from its rudiment, otic placode, which later forms otic vesicle and gives rise to tissues comprising the entire inner ear. Although several signaling molecules have been identified as candidates responsible for inner ear specification and patterning, many details remain elusive. Here, we report that *Paraxial Protocadherin* (*PAPC*) is required for otic vesicle formation in *Xenopus* embryos. *PAPC* is expressed strictly in presumptive otic placode and later in otic vesicle during inner ear morphogenesis. Knockdown of *PAPC* by dominant-negative *PAPC* results in the failure of otic vesicle formation and the loss of early inner ear markers *Sox9* and *Tbx2*, suggesting the requirement of *PAPC* in the early stage of otic vesicle development. However, *PAPC* alone is not sufficient to induce otic placode formation.

Keywords: *PAPC*; Otic placode; Otic vesicle; Inner ear; *Sox9*; *Tbx2*; *Xenopus laevis*

In vertebrates, the first morphologically discernible structure during inner ear development is the otic placode, which arises from ectodermal thickenings at the border between the prospective hindbrain and the future epidermis [1,2]. The placode subsequently invaginates to form the otic cup and then the otic vesicle. Cells of otic vesicle undergo a distinct period of intense proliferation and complex morphogenetic movement prior to differentiation into specific cell types of the inner ear [1,3], including the mechanosensory hair cells that transmit balance and auditory information, supporting cells, and the biomineralized otoliths or otoconia that assist in perception [2]. Early inner ear morphogenesis accompanies otic placode specification and patterning.

Classical transplantation experiments have shown that inner ear formation is resulted from interactions between competent epidermal cells and adjacent tissues including the hindbrain and the paraxial mesoderm [1,2]. Studies in various species have implicated that signaling molecules of the FGF family [4–9], *Wnt8C* [9], retinoic acid [10,11], *Shh* [12–14] and the transcription factors like *Dlx* [4], *Pax2* [15], and *Sox9* [16] are involved in the specification and patterning of the inner ear in vertebrate embryos. In the mouse, mutations in some of these genes result in the loss of specific inner ear components, demonstrating the importance of these genes in this process [17].

Protocadherins constitute a large subgroup of the cadherin family, the calcium-dependent cell–cell adhesion molecules, which have functions in tissues of a wide variety of multicellular organisms [18,19]. Many of the protocadherins in mammals are strongly expressed in the central nervous system to modulate synaptic transmission and the generation of specific synaptic connections. Recently, their roles in tissue morphogenesis and formation of neuronal circuits during early vertebrate development have been inferred [18,19].

Xenopus Paraxial Protocadherin (*PAPC*), which was initially identified in a screen for genes present in the Spemann organizer of *Xenopus* embryos, is expressed during gastrulation and somitogenesis [20]. *PAPC* has homophilic adhesion properties and mediates selective cell–cell adhesion and cell sorting, ensuring cell movement during convergent extension (CE) in *Xenopus* and zebrafish [20,21].

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However, like other protocadherins, the cytoplasmic tail of *Xenopus PAPC* was largely different from the highly conserved cytoplasmic domain of the classical cadherins. It also has signaling functions besides adhesion properties. *Xenopus PAPC* acts through its cytoplasmic domain in cooperation with the Wnt/PCP pathway that modulates the activity of the small GTPases Rho A and Rac 1 and c-jun N-terminal kinase (JNK). This novel signaling function of *PAPC* is essential for the CE cell movements in *Xenopus* [22,23]. Moreover, the adhesion property becomes stronger when mutant form of *PAPC* lacking the cytoplasmic domains (*M-PAPC*) was tested, while similar deletions of the intracellular domain of classical cadherins cause loss of adhesion activity or even inhibition of cadherin adhesion [20]. These data strongly suggested a negative regulation of adhesion by the cytoplasmic domains of these protocadherins.

The distinctive feature of *PAPC* in both adhesiveness and signal transduction in gastrulation raises the question whether this also holds true in other developmental events. Here we report our observation of the dynamic *PAPC* expression during *Xenopus* inner ear development. We first show that *PAPC* is initiated in presumptive otic placode at the late neurula stage, prior to any morphological changes in the ectoderm, and later strictly expressed in otic placode throughout the otic vesicle formation. Results from loss-of-function experiments support the requirement of *PAPC* for otic placode specification in *Xenopus*. However, *PAPC* alone is not sufficient to induce otic placode formation.

Materials and methods

Embryo manipulation and microinjection. Xenopus laevis fertilized eggs were obtained, dejellied and cultured as previously described [24], and staged according to Nieuwkoop and Faber [25]. Synthetic RNA mixed with β-galactosidase mRNA was injected into one of the two animal ventral cells to target the otic placode territory at the eight-cell stage [16] or one of the two blastomeres at the two-cell stage [12]. Embryos were collected at stage 32 (tailbud stage) and fixed in MEMFA. After X-gal staining for β-galactosidase acitivity as previously described [26], embryos were transferred to methanol and prepared for in situ hybridization.

In vitro transcription of capped mRNAs and in situ hybridization probes. mRNA for microinjection and probes for in situ hybridization were prepared as previously described [24]. To obtain sense mRNA, *FL-PAPC* (a kind gift of Dr. Eddy M. De Robertis) [20], *M-PAPC* (a kind gift of Dr. Herbert Steinbeisser) [20], *DN-PAPC* (a kind gift of Dr. Herbert Steinbeisser) [20], and β-galactosidase were digested with *Not*I and transcribed with SP6 RNA polymerase. Antisense digoxigenin-labeled probes were synthesized using linearized template encoding *PAPC*[20], *Sox9*[16], and *Tbx2*[27].

RT-PCR. RNA isolation and reverse transcription were performed as described [24]. For each experiment, the quantity of input cDNA was determined by normalization of the *ODC* signal. The primers used for *ODC* were: forward, 5′ GATCATGCACATGTCAAGCC 3′ and reverse, 5′ CAGGGAGAATGCCATGTTCT 3′ (58 °C, 25 cycles) [28]. The primers used for *PAPC* were: forward, 5′ CGTCTAGCAGATACCA AGAATTC 3′ and reverse, 5′ GCTAGTTAGGTGACAGGACAATG 3′ (58 °C, 30 cycles).

Whole-mount in situ hybridization and bleaching. Whole-mount in situ hybridization was carried out with antisense digoxigenin-labeled probes, using a modification of the protocol described by Dietrich et al. [29]. After in situ staining, the embryos were dehydrated in methanol, bleached in methanol/37% H_2O_2 (2:1, V/V) in daylight or under UV irradiation. Samples were then hydrated in 1× PBS and photographed under Leica MZFL-III microscope. For sectioning, samples were overstained by in situ hybridizations and embedded in paraffin, sectioned at 10 μm thickness.

Results

PAPC is expressed in the developing otic placode

In an investigation of *PAPC* function during somitogenesis, we noticed a phenomenon that *PAPC* also had strong expression in otic placode. As the first step toward understanding the role of *Xenopus PAPC* during inner ear development, we used RT-PCR to define its temporal expression in *Xenopus* embryos. In *Xenopus*, the inner ear begins as an otic placode in the stage 23 embryos. This placode invaginates and forms a closed vesicle that is completely separated from the overlying epidermis at stage 28 [30]. Since *PAPC* was also expressed at the paraxial mesoderm in the same stage of otic placode formation, RT-PCR with cDNA derived from head of various stages embryos was performed (Fig. 1B). The *PAPC* transcription level was high from stage 21, before the otic placode is visible, to stage 33, when the otic vesicle formed, while it slightly decreased from stage 37 and still expressed at stage 45 (Fig. 1A).

In *Xenopus* embryos, presumptive otic placode ectoderm is specified to form otic placode by the neurula stage [31]. To visualize the spatial expression patterns of *Xenopus PAPC* in the otic placode and vesicle, we then performed whole-mount in situ hybridization with a digoxigenin-labeled RNA probe for *PAPC*. At stage 17 (late neurula stage), *PAPC* was detected in bilateral patches of cells (red arrow, Fig. 2A) immediately adjacent to the lateral neural crest and corresponding to the prospective otic

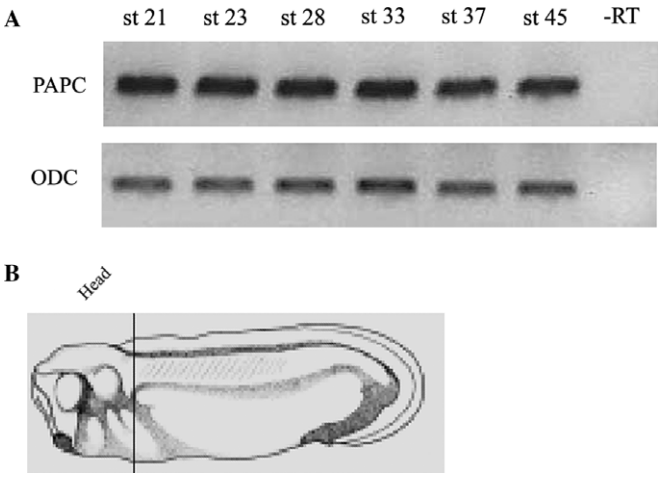

Fig. 1. Expression profile of *PAPC* in *Xenopus* head analyzed by RT-PCR. (A) Total RNA extracted from dissected head of stage 21–45 embryos was reverse transcribed and PCR amplified using *PAPC*-specific primers. *PAPC* transcripts were present at each stage of otic placode development. (B) Vertical line indicates the position to dissect heads of stage 21–45 embryos.