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Research paper

The connexin 30.3 of zebrafish homologue of human connexin 26 may play similar role in the inner ear

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ABSTRACT

The intercellular gap junction channels formed by connexins (CXs) are important for recycling potassium ions in the inner ear. CXs are encoded by a family of the *CX* gene, such as *GJB2*, and the mechanism leading to mutant connexin-associated diseases, including hearing loss, remains to be elucidated. In this study, using bioinformatics, we found that two zebrafish *cx* genes, *cx27.5* and *cx30.3*, are likely homologous to human and mouse *GJB2*. During embryogenesis, zebrafish *cx27.5* was rarely expressed at 1.5 −3 h post-fertilization (hpf), but a relatively high level of *cx27.5* expression was detected from 6 to 96 hpf. However, zebrafish *cx30.3* transcripts were hardly detected until 9 hpf. The temporal experiment was conducted in whole larvae. Both *cx27.5* and *cx30.3* transcripts were revealed significantly in the inner ear by reverse transcription polymerase chain reaction (RT-PCR) and whole-mount in situ hybridization (WISH). In the HeLa cell model, we found that zebrafish Cx27.5 was distributed intracellularly in the cytoplasm, whereas Cx30.3 was localized in the plasma membrane of HeLa cells stably expressing Cx proteins. The expression pattern of zebrafish Cx30.3 in HeLa cells was more similar to that of cells expressing human CX26 than Cx27.5. In addition, we found that Cx30.3 was localized in the cell membrane of hair cells within the inner ear by immunohistochemistry (IHC), suggesting that zebrafish *cx30.3* might play an essential role in the development of the inner ear, in the same manner as human *GJB2*. We then performed morpholino knockdown studies in zebrafish embryos to elucidate the physiological functions of Cx30.3. The zebrafish *cx30.3* morphants exhibited wild-type-like and heart edema phenotypes with smaller inner ears at 72 hpf. Based on these results, we suggest that the zebrafish Cx30.3 and mammalian CX26 may play alike roles in the inner ear. Thus, zebrafish can potentially serve as a model for studying hearing loss disorders that result from human CX26 mutations.

1. Introduction

Intercellular communication is one of the most essential properties for maintaining the homeostasis of tissues and organs in multicellular organisms (Meşe et al., 2007; Maeda et al., 2009). Changes in the regulation of gap junction channels can cause developmental defects, physiological defects, and disease (Peracchia et al., 2000; Minekawa et al., 2009; Smyth et al., 2010). However, the molecular mechanisms underlying gap junction deregulation remain largely unknown.

Gap junctions exist as plaque to mediate the electrical and biochemical coupling conduits between adjacent cells that permit

Abbreviations: AW, the widths of the anterior otolith; CXs, connexins; dpf, days post-fertilization; ef1α, elongation factor 1α; GJ, gap junction; hpf, hours post-fertilization; IHC, immunohistochemistry; MOs, morpholino oligonucleotides; OD, the distances between the two otoliths; ORF, open reading frame; PW, the widths of the posterior otolith; RT-PCR, reverse transcription polymerase chain reaction; WISH, whole-mount in situ hybridization

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the exchange of ions, small molecules, second messengers, and small interference RNAs from cell to cell (Saez et al., 2003; Valiunas et al., 2005). Each gap junction is formed by two end-to-end hexameric structures, referred to as connexons or hemichannels, each of which spans the plasma membrane. In chordate animals, gap junction channels are encoded by a family of genes called *connexins* (*CXs*), which encode the transmembrane subunits of connexons. To date, at least 21 connexin isoforms have been described in the human genome. These isoforms have approximately 40% overall sequence identity, with prominent identity in the transmembrane and extracellular domains (Goodenough, 1974; Harris, 2002; Sohl and Willecke, 2004; Meşe et al., 2007). Connexons may be formed by oligomerization between CXs of either a single type or more than one type, leading to the formation of either homomeric or heteromeric hemichannels, respectively (Marziano et al., 2003).

The crucial role of gap junction-mediated intercellular communication in auditory functions has been confirmed by numerous studies (White and Bruzzone, 1996; Evans and Martin, 2002; Meşe et al., 2007). In the cochlea, it has been hypothesized that gap junction networks provide pathways by which potassium ions are rapidly removed and recycled in order to maintain high concentrations of potassium ions in the endolymph, as well as possible pathways for nutrient passage. This K^+ recycling pathway is required for sensory hair cell transduction (Kelsell et al., 1997; Steel and Kros, 2001; Ahmad et al., 2003). Multiple subtypes of Cxs are prominently expressed in the mouse cochlea; for instance, Cx26 and Cx30 are expressed in the cochlear supporting cells, fibrocytes in the spiral limbus and spiral ligament, and stria vascularis of humans and rodents (Lautermann et al., 1998; Xu and Nicholson, 2013).

The zebrafish (*Danio rerio*) has several advantages that make it an attractive animal model for investigating the development and function of the vertebrate inner ear. One of these properties is that, unlike the mouse model, zebrafish embryos are optically clear and are produced in large numbers (Postlethwait et al., 1994; Whitfield, 2002). Although the inner ear of the zebrafish does not contain a specialized hearing organ to manage hearing and balance, many genetic mechanisms of inner ear development and function are similar to those of other vertebrate species (Haddon and Lewis, 1996; Haffter et al., 1996; Whitfield et al., 2002). The zebrafish and the closely related goldfish (*Carassius auratus*) have three otic endorgans, the saccule, lagena, and utricle, which primarily mediate auditory and vestibular function (Sokolowski and Popper, 1988; Lanford et al., 2000; Kwak et al., 2006). The nascent otic vesicle contains two sensory epithelia corresponding to the utricular (anterior) and saccular (posterior) maculae (Kwak et al., 2006; Stooke-Vaughan et al., 2012). Thus, the zebrafish can provide an excellent platform for investigating the development of sensory epithelia and the structure–function relationships in the vertebrate inner ear (Lanford et al., 2000). Eastman et al. identified 37 zebrafish connexin genes, 23 of which are zebrafish cognates of 16 mammalian connexins, and 14 of which are apparently unique to zebrafish (Eastman et al., 2006).

Many reports have indicated that mutations in connexin genes are associated with recessive and dominant forms of nonsyndromic hearing loss in different human populations, as well as various skin phenotypes linked to deafness (Xia et al., 1998; Grifa et al., 1999; Ahmad et al., 2003). In addition, our previous studies have reported that mutations in the *GJB2* (*CX26*) gene frequently occur in Taiwanese patients with nonsyndromic hearing loss (Yang et al., 2007, 2010). Tao et al. (2010) first identified and characterized that zebrafish *cx30.3* is closely related to both mammalian *CX26* and *CX30*. The sequence homology, mRNA expression in embryos and tissues, and similar functional properties show that zebrafish *cx30.3* might play similar roles in skin development, hearing, and balance in zebrafish (Tao et al., 2010). Despite remarkable progress in genetic linkage studies regarding deafness, the auditory functions of gap junction proteins and the mechanisms leading to mutation-mediated disease remain largely unknown. In this study, we identified another zebrafish homolog of the mammalian *GJB2* (*CX26*) gene, referred to as *cx27.5*. Analysis of mRNA expression showed that *cx30.3* and *cx27.5* transcripts were expressed not only during embryogenesis, but also in multiple organs of adult zebrafish. Immunostaining analysis showed that zebrafish Cx30.3 was located intracellularly in the plasma membrane between adjacent Cx30.3-EGFP-expressing HeLa cells and in the cell membrane of sensory hair cells in the inner ear of adult zebrafish. Using antisense morpholino oligonucleotides (MOs) directed against zebrafish *cx30.3*, we demonstrated that *cx30.3* morphants exhibit abnormal development of otic vesicles. Phylogenetic analysis, mRNA expression, cellular localization and morpholino analysis indicate that zebrafish *cx30.3* is more similar to human *GJB2* than is *cx27.5*.

2. Materials and methods

2.1. Fish maintenance

All zebrafish (*D. rerio*) studies were conducted with AB wild-type larvae. Zebrafish larvae were maintained and raised at 28.5 °C in a continuous flow-through system with 10 h-dark and 14-h light day-light cycle. Embryos were reared in fresh egg water (60 μg/ml sea salts) at 28.5 °C and were staged according to hours post-fertilization (hpf). For some experiments, embryos were incubated in 0.003% 1-phenyl-2-thiourea (PTU; Sigma) to block pigmentation (Kimmel et al., 1995).

2.2. Phylogenetic analysis and cloning of zebrafish connexin gene

Protein blast queries with human connexin 26 (hCX26) protein sequence were used to identify the zebrafish connexin protein sequence, and those protein sequences were aligned using ClustalX 2.1. A phylogenetic tree was then constructed using the neighbor joining method (Saitou and Nei, 1987). To obtain the zebrafish *connexin* expression plasmids, primers based on *cx27.5* or *cx30.3* sequences were used to amplify the full-length open reading frame (ORF) from whole larvae cDNA. In addition, the restriction endonuclease sites *Xho*I and *Hin*dIII were added to the 5′ and 3′ ends of the sequence by PCR-based amplification using a primer pair. Primer sequences used for amplification are as follows: *zf-cx27.5-001-Xho*I-F 5′-AGCCTCGAGATGCCACTAACACCACCTG-3′; *zf-cx27.5-002 -Xho*I-F 5′-GGCCTCGAGATGAACTGGGCGTCATTTTATGC-3′; *zf-cx27.5-Hin*dIII-R 5′-CGCAA GCTTGAAGGAGCAGAGTTTTTGG-3′; *zf-cx30.3-Xho*I-F 5′AGCCTCGAGATGAGTTGGGGAG CACTT-3′; *zf-cx30.3-Hin*dIII-R 5′-CGCAAGCTTAACAGTCTTATTGCTCGATG-3′. Above underlined sequences are restriction sites for *Xho*I and *Hin*dIII respectively. The PCR fragments were then fused in-frame to the N-terminus of autofluorescent reporter proteins EGFP (pLEGFP-N1 vector; Clontech, Palo Alto, CA) for fusion protein generation and sequenced on an ABI PRISM 3730 using the fluorescent dideoxy terminator method to confirm the DNA sequence of all constructs. In addition, the *cx30.3* PCR fragments were also cloned upstream and in-frame with autofluorescent reporter proteins TagRFP in pTagRFP-N vector (Evrogen). This fusion protein construct Cx30.3-TagRFP was used for morpholino knockdown experiments.

2.3. Production of specific polyclonal antibody against zebrafish Cx30.3

To generate the specific polyclonal antibody against zebrafish Cx30.3, we aligned the amino acid sequences between Cx30.3 and