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Connexin26 gap junction mediates miRNA intercellular genetic communication in the cochlea and is required for inner ear development

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Organ development requires well-established intercellular communication to coordinate cell proliferations and differentiations. MicroRNAs (miRNAs) are small, non-coding RNAs that can broadly regulate gene expression and play a critical role in the organ development. In this study, we found that miRNAs could pass through gap junctions between native cochlear supporting cells to play a role in the cochlear development. Connexin26 (Cx26) and Cx30 are predominant isoforms and co-express in the cochlea. Cx26 deficiency but not Cx30 deficiency can cause cochlear developmental disorders. We found that associated with Cx26 deletion induced the cochlear developmental disorders, deletion of Cx26 but not Cx30 disrupted miRNA intercellular transfer in the cochlea, although inner ear gap junctions still retained permeability after deletion of Cx26. Moreover, we found that deletion of Cx26 but not Cx30 reduced miR-96 expression in the cochlea during postnatal development. The reduction is associated with the cochlear tunnel developmental disorder in Cx26 knockout (KO) mice. These data reveal that Cx26-mediated intercellular communication is required for cochlear development and that deficiency of Cx26 can impair miRNA-mediated intercellular genetic communication in the cochlea, which may lead to cochlear developmental disorders and eventually congenital deafness as previously reported.

Tissue homeostasis and organ development rely on the well-orchestrated integration of intercellular communication and gene regulation to synchronize and coordinate cell proliferation and differentiation. Gap junctions are intercellular channels and represent the only selective intercellular conduit that possesses a large pore size (1.0–1.5 nm), allowing direct exchange of ions and small molecules between cells. It has been reported that small regulatory RNAs, such as siRNAs and miRNAs, can also pass through gap junctions, which provides a novel mechanism for intercellular genetic communication. In particular, miRNAs are single-stranded RNAs consisting of ~21 nucleotides and can broadly modulate gene expression by affecting the translation of mRNAs to proteins and mRNA target decay. To date, approximately 300 conserved miRNA families and thousands of additional poorly conserved miRNAs have been identified in mammals. Approximately two thirds of all human protein-coding genes are conserved targets of miRNAs. Thus, miRNAs provide a widespread mechanism for post-transcriptional control of gene expression and are important for the organ development.

Gap junctions have a crucial role in hearing. Connexin26 (Cx26, GJB2) mutations cause most cases of hereditary genetic deafness, responsible for >50% of nonsyndromic hearing loss. Recently, we found...
that Cx26 deficiency can cause cochlear developmental disorders leading to congenital deafness\textsuperscript{18,19}. However, the underlying mechanism for developmental disorders remains unclear. In this study, we found that miRNAs can pass through gap junctions in the cochlea. Cx26 and Cx30 are predominant connexin isoforms in the cochlea\textsuperscript{20,21}. Associated with Cx26 deficiency induced cochlear developmental disorders, Cx26 deficiency but not Cx30 deletion disrupted miRNA-mediated intercellular genetic communication in the cochlea.

Results

Gap junction and Cx26 and Cx30 expression in the cochlea. The organ of Corti has hair cells and supporting cells (Fig. 1a). The auditory sensory hair cells have no gap junctional coupling and connexin expression (Fig. 1f and also see ref. 20, 21). Gap junctions and connexin expression only existed in supporting cells (Fig. 1c–f). The organ of Corti contains four types of supporting cells, i.e., Deiters cells (DC), pillar cells (PC), Hensen cells (HC), and Claudius cells (CC) (see Supplementary Fig. S1). All of them had Cx26 and Cx30 expression and were well-coupled (Fig. 1 and also see ref. 20,21).

Intercellular transfer of miRNAs between cochlear supporting cells. MicroRNAs can pass through gap junctions between cochlear supporting cells (Figs 2, 5a,b and Supplementary Fig. S2). When fluorescence-tagged miRNA (miR-F) was injected into a cochlear supporting cell, the miR-F fluorescence could be detected in neighboring cells in the cochlear sensory epithelium (Fig. 5a,b) or in isolated cell preparation (Fig. 2). Out of a total of 20 injections (3 injections in DCs, 2 injections in PCs, 7 injections in HCs, and 8 injections in CCs), intercellular diffusion was observed in 18 injections. In two cases of no intercellular diffusion, the input capacitance ($C_{in}$) was at single cell level and demonstrated that the gap junctions between the recording cells were already uncoupled.

Blockage of miRNA intercellular transfer by gap junctional blockers. The intercellular transfer of miRNAs between cochlear supporting cells could be blocked by gap junctional blockers. Fig. 3 shows that application of 50 \textmu M 18\alpha-glycyrrhetinic acid (18-AGA) or 0.1 mM carbenoxolone (CBX) gap junctional blockers blocked miR-F diffusion between cells. The injected miR-F nucleotides were restricted within the injected cell and did not diffuse into the adjacent-contacts cells. Gap junction blocker blocked not only miR-F diffusion but also dye ethidium bromide (EB) diffusion between cells (Fig. 3d).

Cochlear development disorders and disruption of miRNA intercellular transfer in Cx26 KO mice. As previously reported\textsuperscript{18,22}, deletion of Cx26 could induce cochlear developmental disorders (Fig. 4). The tectorial membrane was attached to the inner sulcus cells and the cochlear tunnel was filled (Fig. 4e). Deletion of Cx26 also disrupted intercellular transfer of miRNAs in the cochlea (Fig. 5). The injected miR-F was restricted to the injected cell (Fig. 5c–f). In all 12 injections, no intercellular diffusion of miR-F was visible. However, deletion of Cx26 did not completely disrupt inner ear gap junctions, which still retained permeability to dye EB (Fig. 5g,h).

Figure 1. Cochlear structure and co-expression of Cx26 and Cx30 in the cochlea. (a) Schematic drawing of the cochlear structure in the cross-section. PC: Pillar cell, DC: Deiters cell, HC: Hensen cell, CC: Claudius cell. (b–e) Immunofluorescent staining for Cx26 (green) and Cx30 (red) in the cochlea. A white arrow in panel (b) indicates the cochlear tunnel. TM: tectorial membrane. (f) A high-magnitude image in the organ of Corti. Outer hair cells were visualized by prestin labeling (red). Scale bars: 25 \textmu m in (b–e), 10 \textmu m in (f).
that cochlear supporting cells in Cx26 KO mice still retained good gap junctional coupling. $C_{in}$ in the recording Hensen cells in Fig. 5e,f and Claudius cells in Fig. 5g,h was ~75 pF and 19.3 pF, respectively, showing that they were well-coupled.

**Normal cochlear development and miRNA intercellular transfer in Cx30 KO mice.** Cx30 is co-expressed with Cx26 in the cochlea (Fig. 1, and also see ref. 20, 21). However, deletion of co-expressed Cx30 displayed normal cochlear development (Fig. 6a–b). Intercellular transfer of miR-F also appeared normal in Cx30 KO mice and intercellular diffusion of miR-F among supporting cells was visible (Fig. 6c–f).

**Reduction of miRNA expression in Cx26 KO mice during cochlear postnatal development.** MicroRNA-96 is critical for cochlear development23. In mouse postnatal development, the cochlear tunnel starts to open at postnatal day 5 (P5) and fully opens at P10 (Fig. 7a). We found that prior to the cochlear tunnel opening, the expression of miR-96 in the cochlea was increased at P3 (Fig. 7b). Then, the expression decreased and reached a steady state at P10. However, the expression of miR-96 in Cx26 KO mice was not increased at P3 and remained at lower level during the postnatal period (Fig. 7b). On the other hand, the expression of miR-96 in the cochlea in Cx30 KO mice, which displayed normal cochlear development (Fig. 6a), was similar to WT mice, increasing at P3 and then reducing afterward.
during the postnatal development (Fig. 7b). There was no significant difference in miR-96 expression between Cx30 KO mice and WT mice (P = 0.43, one-way ANOVA).

Discussion
In this study, we found that miRNAs could pass through gap junctions between native cochlear supporting cells (Figs 2, 5a,b and Supplementary Fig. S2). Deletion of Cx26 disrupted cochlear development and miRNA intercellular transfer in the cochlea (Figs. 4 and 5). However, the inner ear gap junctions still remained permeable to cationic dye EB after Cx26 deletion (Fig 5g,h). Deletion of Cx26 also reduced...
miR-96 expression in the cochlea during postnatal development and the reduction is associated with over-development of the cochlear tunnel (Fig. 7). However, consistent with the normal cochlear development in Cx30 KO mice, deletion of Cx30 did not affect intercellular transfer of miRNA and miR-96 expression in the cochlea (Figs 6 and 7b). We previously reported that miRNAs can pass through gap junctional channels and regulate gene expression in neighboring cells to achieve intercellular genetic communication11. Our new data further demonstrate that this gap junction-mediated miRNA intercellular communication may have an important role in the cochlear development.

Deletion of Cx26 can result in filling of the cochlear tunnel and attachment of the tectorial membrane to inner sulcus cells leading to loss of the under-tectorial-membrane space (Figs 4e, 7a, and 7b).
junctions extensively exist in almost all cell types and organs. Recently, it has been found that miRNAs intercellular-exchangeable through gap junctions except small non-coding RNAs such as miRNA. Gap transcription factors, and mRNA polyA polymerization. However, none of these regulatory factors is Gene expression can be regulated by many factors at many stages, such as enhancer and promoter, in Cx26 deficiency.

sharpened needle. The isolated sensory epithelium was dissociated by trypsin (1 mg/ml) for 3–5 min32–35. TT- 3

′ GTG GCA GAT GGC GCG GCA ACA CCA

and CreR: 5′ TGC ATT ACC GGT CGA TGC AAC GA- 3′

was selected. Intracellular injection was performed by patch clamp recording under the whole-cell...
configuration. The patch pipette was 1.5–2 μm in tip diameter and filled with the normal intracellular solution (KCl 140, EGTA 5, and HEPES 10 in mM, pH 7.2 and 300 mM) with 100 μM miR-F. The holding voltage was set at −40 mV. Gap junctional coupling between cells was continuously monitored by input capacitance \( (C_{in}) \), which was recorded online at 1–3 Hz and calculated from the transient charge elicited by small (−10 mV) test pulses at the holding potential. The diffusion was captured with a CCD camera under a fluorescence microscope (Nikon, TE300) as we previously reported.

In some cases, cationic dye ethidium bromide (EB, 0.1 mM) was also used and mixed with miR-F for injection. EB can distinctly identify the transjunctional-diffused cells and clearly demonstrate transjunctinal transport, because it can bind to DNAs labeling cell nuclei showing bright fluorescence.

**Immunofluorescent staining.** The immunofluorescent staining was performed as previously reported. The cochlear section or culture cells were fixed with 4% paraformaldehyde for 30 min and washed out with PBS. After 30 min of incubation in a blocking solution (10% goat serum and 1% BSA in PBS) with 0.1% Triton X-100, the cochlear section or culture cells were incubated with monoclonal mouse anti-Cx26 (1: 400, Cat#33–5800, Invitrogen) in the blocking solution at 4°C overnight. For double immunofluorescent staining for Cx26 and Cx30, polyclonal rabbit anti-Cx30 (1:400, Cat#71–2200, Invitrogen), or polyclonal goat anti-prestin (1:50, Cat# sc-22694, Santa Cruz Biotech Inc, CA) was used. After being washed with PBS, the section or cells were incubated with corresponding Alexa Fluor 488- or 568-conjugated goat anti-mouse IgG and Alexa Fluor 568-conjugated goat anti-rabbit IgG (1:500, Molecular Probes) in the blocking solution at room temperature (23°C) for 1 hr. In some cases, following the 2nd antibody incubation, the section or cells were stained by 4’, 6-diamido-2-phenylindole (DAPI, 0.1 mg/ml, D1306, Molecular Probes) for ~15–20 min to visualize cell nuclei. After completely washing out with PBS, the sections or cells were mounted with a fluorescence mounting medium (H-1000, Vector Lab, CA) and observed under a fluorescence microscope (Nikon, T2000) or a confocal microscope (Leica TCS SP2). The fluorescent image was saved in the TIFF format and assembled in Photoshop (Adobe Systems, CA) for presentation.

**miRNA extraction and quantitative PCR measurement.** The cochlear sensory epithelia were freshly isolated as described above and miRNAs were extracted by mirVana miRNA Isolation Kit (AM1560, Ambion, USA) following manufacturer’s instructions. The purity and quantity of miRNA was determined by a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Inc., Rockland, DE). Then, miRNAs were converted to cDNA using TaqMan® MicroRNA Reverse Transcription Kit (#4366596, Applied Biosystems, CA, USA) with corresponding mouse-specific miRNA reverse transcription templates according to manufacturer’s instructions and measured by use of MyiQ real-time PCR detection system (Bio-Rad Laboratories) with TaqMan® MicroRNA Assay (Applied Biosystems, CA, USA). An internal standard U6 snRNA (#001973, Applied Biosystems, CA) was used as an internal control. The relative quantity of miRNA expression was calculated from the standard curve and normalized to the amount of the internal standard U6 snRNA.

**Data analysis.** Data were expressed as mean ± s.e.m. and plotted by SigmaPlot (SPSS Inc. Chicago, IL). The statistical analyses were performed by SPSS v18.0 (SPSS Inc. Chicago, IL) using one-way ANOVA with a Bonferroni correction.

**References**

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Author Contributions
Y.Z., L.Z., L.M. and H.B.Z. designed and performed experiments and analyzed data. H.B.Z. wrote paper.

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