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ORIGINAL ARTICLE

Single-cell RNA-sequencing of zebrafish hair cells reveals novel genes potentially involved in hearing loss

Fuping Qian[1] · Guanyun Wei[1] · Yajing Gao[2] · Xin Wang[2] · Jie Gong[1] · Chao Guo[1] · Xiaoning Wang[2] · Xu Zhang[2] · Jinxiang Zhao[2] · Cheng Wang[1] · Mengting Xu[2] · Yuebo Hu[2] · Guoli Yin[2] · Jiahui Kang[7] · Renjie Chai[2,3,4,5,6] · Gangcai Xie[7] · Dong Liu[1,2]

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Abstract

Hair cells play key roles in hearing and balance, and hair cell loss would result in hearing loss or vestibular dysfunction. Cellular and molecular research in hair cell biology provides us a better understanding of hearing and deafness. Zebrafish, owing to their hair cell-enriched organs, have been widely applied in hair cell-related research worldwide. Similar to mammals, zebrafish have inner ear hair cells. In addition, they also have lateral line neuromast hair cells. These different types of hair cells vary in morphology and function. However, systematic analysis of their molecular characteristics remains lacking. In this study, we analyzed the GFP+ cells isolated from *Tg(Brn3c:mGFP)* larvae with GFP expression in all hair cells using single-cell RNA-sequencing (scRNA-seq). Three subtypes of hair cells, namely macula hair cell (MHC), crista hair cell (CHC), and neuromast hair cell (NHC), were characterized and validated by whole-mount in situ hybridization analysis of marker genes. The hair cell scRNA-seq data revealed hair cell-specific genes, including hearing loss genes that have been identified in humans and novel genes potentially involved in hair cell formation and function. Two novel genes were discovered to specifically function in NHCs and MHCs, corresponding to their specific expression in NHCs and MHCs. This study allows us to understand the specific genes in hair cell subpopulations of zebrafish, which will shed light on the genetics of both human vestibular and cochlear hair cell function.

Keywords Hair cell · scRNA-seq · Zebrafish

Fuping Qian, Guanyun Wei, Yajing Gao and Xin Wang have contributed equally to this work.

✉ Renjie Chai
renjiec@seu.edu.cn

✉ Gangcai Xie
gangcai@ntu.edu.cn

✉ Dong Liu
liudongtom@gmail.com; tom@ntu.edu.cn

[1] School of Life Sciences, Nantong Laboratory of Development and Diseases, Nantong University, Nantong 226019, China

[2] Co-Innovation Center of Neuroregeneration, School of Life SciencesKey Laboratory of Neuroregeneration of Jiangsu and Ministry of Education, Nantong University, Nantong 226019, China

[3] State Key Laboratory of Bioelectronics, Co-Innovation Center of Neuroregeneration, Department of Otolaryngology Head and Neck Surgery, Zhongda Hospital, Jiangsu Province High-Tech Key Laboratory for Bio-Medical Research, School of Life Science and Technology, Southeast University, Nanjing 210096, China

[4] Institute for Stem Cell and Regeneration, Chinese Academy of Science, Beijing 100864, China

[5] Beijing Key Laboratory of Neural Regeneration and Repair, Capital Medical University, Beijing 100069, China

[6] Department of Otolaryngology Head and Neck Surgery, Sichuan Provincial People's Hospital, University of Electronic Science and Technology of China, Chengdu 610072, China

[7] Institute of Reproductive Medicine, Medical School, Nantong University, Nantong 226001, China

Introduction

Hair cells, getting their name from the hair-like structure on the surface of the cell body [1], detect mechanical vibrations through the cilia. Mechanoelectrical transduction (MET) channels on the tip of stereocilia open in response to stimuli, which cause the depolarization of the hair cells [2, 3] and the release of the neurotransmitters into the synaptic cleft between the hair cell and auditory neuron [4–6]. Inner ear mechanosensory hair cells, including cochlear hair cells and vestibular hair cells, play crucial roles in hearing and balance in mammals. The cochlear hair cells function as receptors of acoustic vibration, and they can be divided into outer hair cells (OHCs) and inner hair cells (IHCs) [7]. There are two types of vestibular sensory epithelia: maculae and cristae. The maculae are located in the utricle and saccule, and the cristae lie at the ends of the three semicircular canals. Both of the vestibular sensory epithelia are composed of hair cells and supporting cells. The vestibular hair cells can be divided into type I and type II hair cells [8]. The macula hair cells can sense linear acceleration and gravitational equivalent, and crista hair cells can sense angular acceleration and deceleration [8, 9]. In mammals, the cochlear hair cells get mechanical signals from the tectorial membrane, but the crista hair cells and macula hair cells sense the vibration from the endolymph and otolith, respectively.

As vertebrates, zebrafish have many organs similar to humans, which make them an excellent animal model for biological and medical research [10]. Zebrafish have an ear-like structure, otic vesicle; however, they do not have the cochlea. In the otic vesicle of zebrafish, there are five clusters of hair cells, three clusters of crista hair cells, and two clusters of macula hair cells. In the cristae, including anterior crista (AC), lateral crista (LC), and posterior crista (PC), hair cells put their kinocilia into the endolymph and detect the endolymph flow caused by head rotation. In the maculae, including the utricular macula (UM) and saccular macula (SM), the cilia of hair cells are in contact with the otoliths, which would produce movement if acceleration occurs. Therefore, the macula hair cells can sense linear acceleration and gravity. It has been demonstrated that the utricular macula is responsible for vestibular function [11] and the saccular macula is the hearing organ [12] in zebrafish. Unlike mammals, zebrafish have neuromast hair cells in their lateral line system. These hair cells are located on the surface of the skin, and are sensors of the surrounding water, which help them detect prey and avoid predators. Because they are easy to be administered to and imaged, the neuromast hair cells have been widely used in hair cell-related biomedical research, for example, in ototoxic drug screening.

The zebrafish model has been widely utilized in the hearing research field [13–18]. However, the systematic analysis of molecular and morphological differences among these hair cells is so far lacking. In this study, scRNA-seq was used to analyze zebrafish hair cells' gene expression, and uncover the molecular differences among the different subtypes of hair cells.

Results

The morphological characteristics of the three types of hair cells in zebrafish

Hair cells play crucial roles in hearing and balance, and they are variable in morphology and function. The typical feature of hair cells that differ from other cells is the cilia, including kinocilia and stereocilia, on the surface of the cell body. The cilia of hair cells can detect mechanical vibration by stereocilia bundle deflection, leading to the tension of the tip links, opening of the MET channels, and causing depolarization of hair cells. In zebrafish, except for the inner ear, there are hair cells in their lateral line system, and the hair cells can be divided into three types according to their morphology and location, namely crista hair cells, macula hair cells, and neuromast hair cells (Fig. 1A). The first two types are both located in the inner ear, and function importantly in hearing and balance, and the third type is key component of the lateral line system, which are distributed on the surface of the skin, and help the fish to sense the environmental water. Although the cilia are the common structure of all hair cells, there are also differences among the three types of hair cells. The crista hair cells and macula hair cells have straight kinocilia, which seem inflexible, and the kinocilia of neuromast hair cells can bend themselves to detect the water movement (Fig. 1B–D). For the length of kinocilia, taking the zebrafish larva of 3 dpf as an example, the crista hair cells have the longest kinocilia, which are almost 30 μm in length, and the kinocilia of macula hair cells and neuromast hair cells are approximately 10 μm and 20 μm, respectively (Fig. 1E). For the size of cell bodies, the macula hair cells are the biggest ones, and the cell bodies of the neuromast hair cells are the smallest among them (Fig. 1F).

Single-cell RNA-sequencing reveals different subpopulations of hair cells in zebrafish

To further distinguish the different types of hair cells at the molecular level, we used single-cell RNA-sequencing to analyze the gene expression patterns. Here, the transgenic zebrafish line *Tg(Brn3c:mGFP)* [19], in which the hair cells and retinal ganglion cells (RGCs) were labeled by the membrane-targeted green fluorescent protein (mGFP) (Fig. 2A),

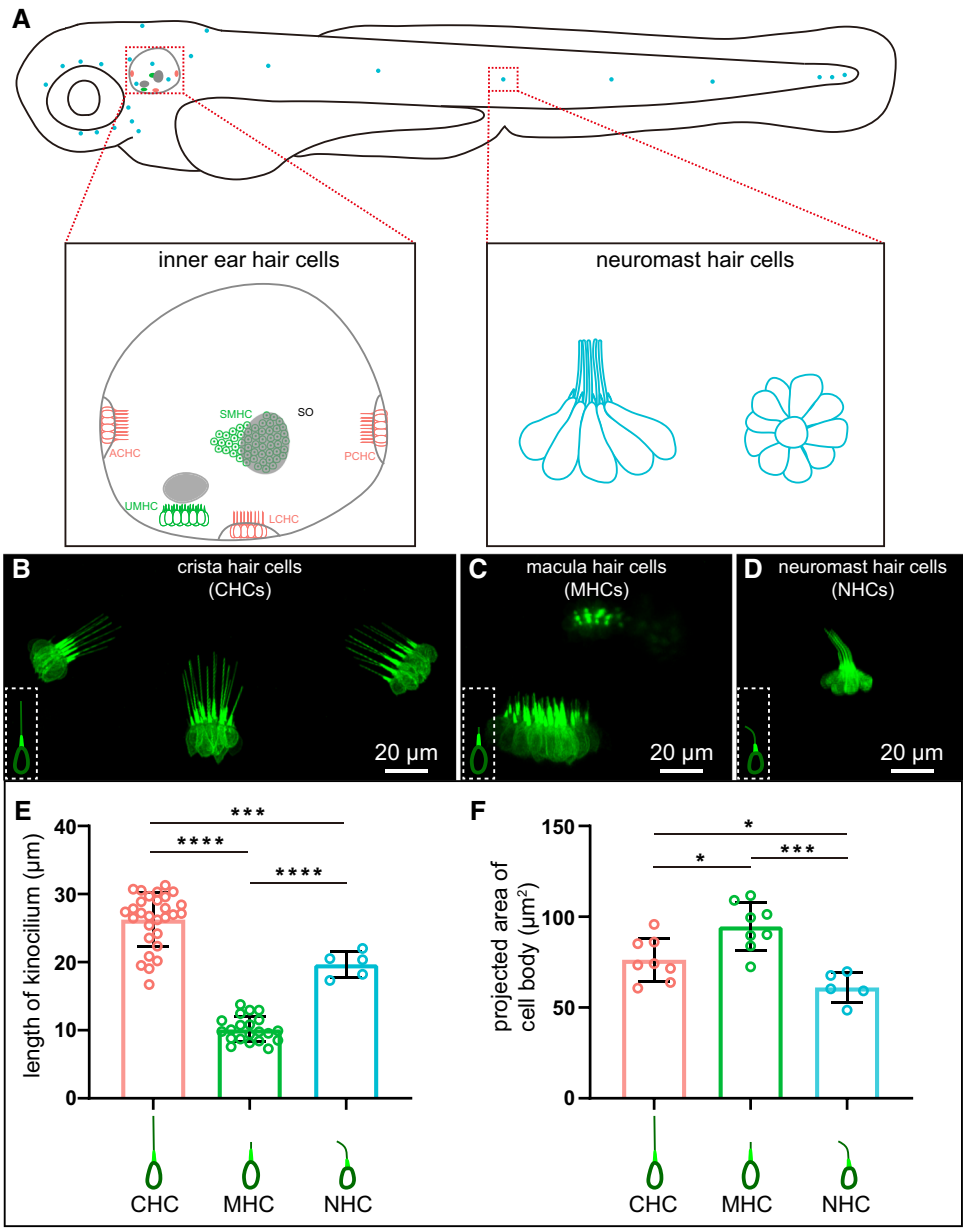


Fig. 1 The morphological feature of the three different types of hair cells in zebrafish. **A** The diagram of the zebrafish larva with hair cell labeling. UO, utricular otolith; SO, saccular otolith; UMHC, utricular macula hair cell; SMHC, saccular macula hair cell; ACHC, anterior crista hair cell; LCHC, lateral crista hair cell; PCHC, posterior crista hair cell. **B–D** The fluorescent image at high magnification of the three different types of hair cells. The diagrams of the different hair cells are presented in the boxes drawn with white dotted lines at the bottom left. **E–F** The comparison of the kinocilia and the cell bodies of the three different types of hair cells. $*P<0.05$, $***P<0.001$, $****P<0.0001$

was used as the animal model. The zebrafish larvae at 6 dpf were dissociated into the single cells by trypsin, and the GFP-positive cells were sorted using the fluorescence activated cell sorting (FACS) method (Figs. S1, S2). After high-throughput sequencing based on the 10 × Genomics system, the gene expression data were obtained. Based on Seurat [20] analysis, the cells were divided into 21 clusters. By analyzing the gene expression patterns of the top genes in each cluster, the four clusters of cells from the cluster 0, 5, 7, 12, were annotated as hair cells, and the cells from the cluster 2, 3, and 10 were identified as retinal ganglion cells. Both hair cells and retinal ganglion cells are GFP-expressing cells in the *Tg(Brn3c:mGFP)* zebrafish [19]. The rest were considered as other cells, which were GFP-negative and brought into the GFP-positive cells due to inevitable technical problems, including red blood cells, lymphocytes, muscle cells, and so on (Figs. 2B, S3). The number of cells and marker genes detected in each cluster was shown (Figs. 2 C, D). For each cluster, the top marker genes were listed, and the expression patterns of these genes were also presented (Fig. 2E).

Validation of the gene expression in the subtypes of hair cells using whole-mount in situ hybridization

Through our analysis, as shown above, the cells from cluster 0, 5, 7, 12 were considered as hair cells. In our subsequent study, we focused on these cells, and further analysis showed

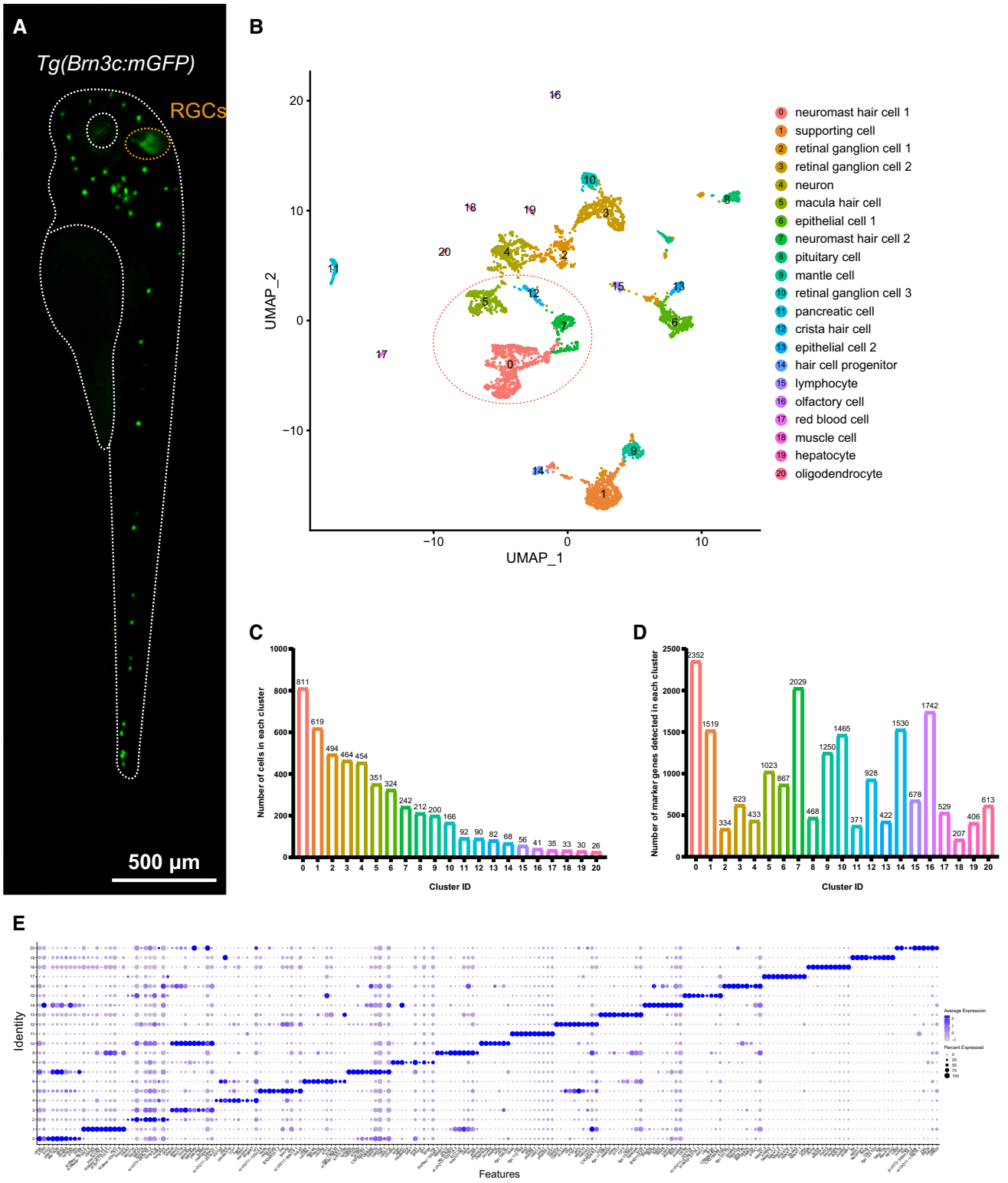


Fig. 2 Single-cell RNA-sequencing (scRNA-seq) of zebrafish hair cells. **A** The fluorescent image of the *Tg(Brn3c:mGFP)* transgenic zebrafish larva at 3 dpf. The GFP-expressing cells in the optic tectum, which were circled by the orange dotted line, are the retinal ganglion cells (RGCs), and the other GFP-expressing cells in the otic vesicle and lateral line system are hair cells. **B** The UMAP analysis of the zebrafish scRNA-seq data. The four clusters of cells circled by the red dotted line were annotated as hair cells. **C**, **D** The number of cells and marker genes detected in each cluster. **E** The dotplot of the top genes in each cluster

that these hair cells can be divided into three subpopulations, namely macula hair cells (cluster 5), crista hair cells (cluster 12), and neuromast hair cells (cluster 0, 7) (Fig. 3A). We further analyzed the top marker genes expressed in the four clusters, and found that some genes were mainly expressed in one of the clusters specifically, for example, *tectb* in cluster 5, *zpld1a* in cluster 12, *calm1b* in cluster 0 and 7 (Figs. 3B, S3). Functional enrichment analysis showed that many of the genes expressed in the four clusters of cells above have hair cell-related biological function (Fig. 3C–F), which indicated that these cells were hair cells. To further confirm the hair cell clustering and annotation, we performed the whole-mount in situ hybridization (WISH). As shown in Fig. 3G, the cells with high *tectb* gene expression were mainly gathering in the cluster 5, and the *tectb* gene was proved to be expressed specifically in the macula hair cells, including the utricular hair cells and saccular hair cells, by WISH. The *zpld1a* gene was mainly expressed in the crista hair cells, which were thought to be located in the cluster 12 according to our analysis (Fig. 3H), and this is consistent with the previous studies [21]. The cells from the cluster 0 and 7 were considered as neuromast hair cells because of the expression patterns of their marker genes, such as *calm1b* (Fig. 3I). As for cells from cluster 0 and 7, although they are both neuromast hair cells, they are different from each other. The cells from cluster 0 were classified to be mature neuromast hair cells, and the cluster 7 were classified to be young neuromast hair cells, according to the expression of the mature and young hair cell markers, *s100s* [22] and *prox1a* [23], respectively, in the two clusters (Fig. S4).

On the other hand, we analyzed the marker gene of supporting cells, *klf17* [24], and found that it was mainly expressed in the cells of cluster 1 (Fig. S5), which suggests that this cluster of cells are supporting cells. Likewise, we also analyzed the genes that were reported to be expressed in mantle cells, such as *tnfsf10*, *ponzr6*, *pkhd1l1*, *fat1b*, *crb3b*, *cts12*, *ovgp1*, and *cldne* [17], and found that cells expressing these genes with high level were clustering in cluster 9 (Fig. S6). For cluster 14, these cells are closer to the supporting cells (cluster 1) in UMAP clustering (Fig. 1B); however, they express some of the genes that have been proved to be expressed in hair cells specifically, such as *myo6b* [25], *myo7aa* [26] (Fig. S7), which raises a possibility that they are supporting cells that can differentiate into hair cells. Therefore, we concluded that the cells from cluster 1 and 9 were supporting cells and mantle cells, respectively, and the cells from the cluster 14 might be hair cell progenitors.

The molecular properties of the three types of hair cells

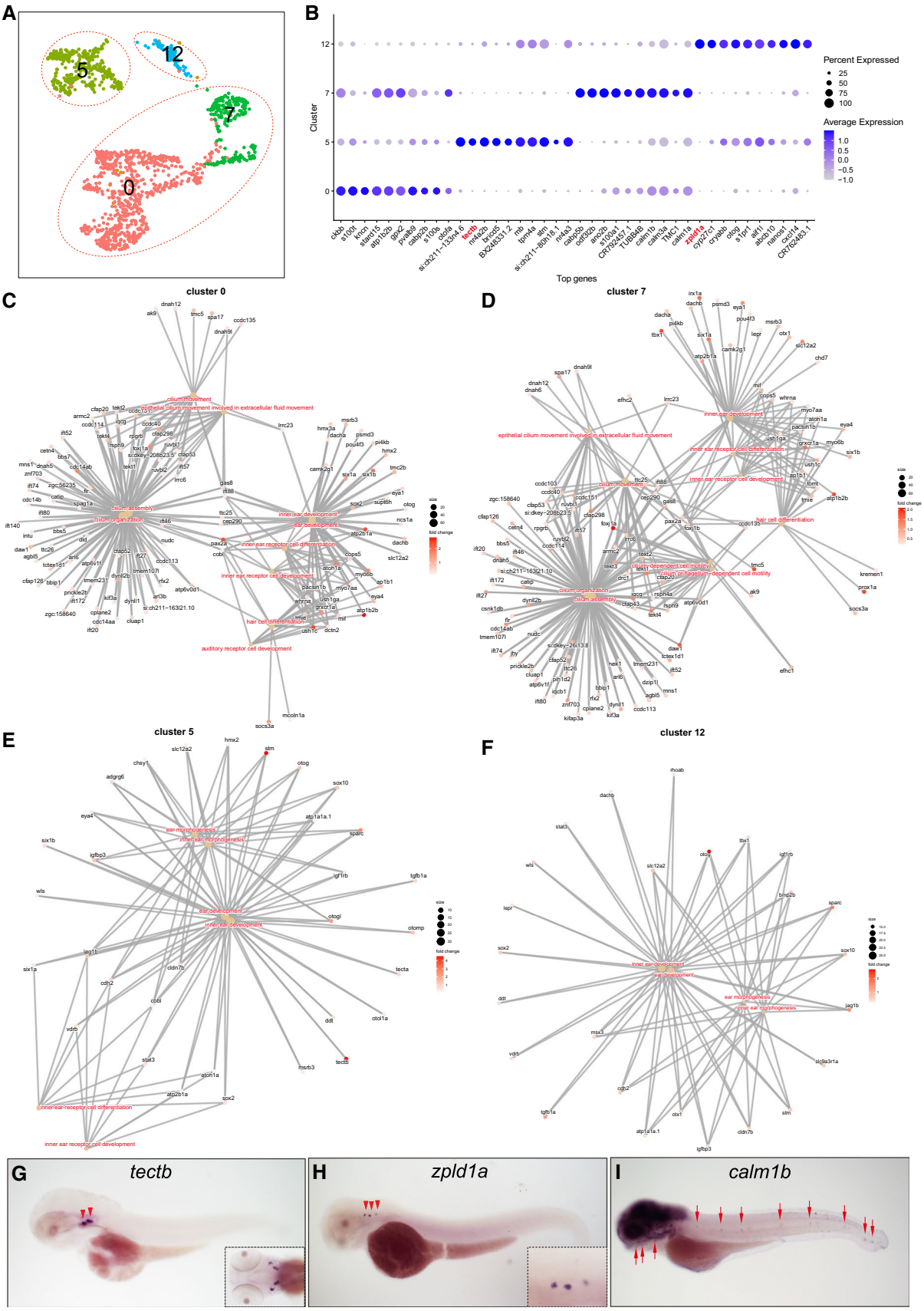
Given that different types of hair cells were clustered into different populations, we made a comparison among the distinguishable hair cells at the molecular level. As shown in Fig. 2D, the number of marker genes detected in the cluster 0, 5, 7, 12 was 2352, 1023, 2029, 928, respectively. The two types of inner ear hair cells, macula hair cells and crista hair cells (cluster 5 and 12), share 568 genes, and 1677 marker genes were expressed in both the mature and young neuromast hair cells (cluster 0 and 7). However, the neuromast hair cells and inner ear hair cells only share a small number of marker genes, which reveals their differences on the gene expression level (Fig. 4A). Furthermore, the top marker genes expressed in the different clusters were obviously different (Fig. 4B). We also performed gene enrichment analysis through gene ontology (GO) term, and found that these different types of hair cells vary dramatically in biological process, molecular function, and cellular component. For example, the neuromast hair cells have more energy metabolism-related activity, which raises the possibility that they require more energy to work compared to the inner ear hair cells (Fig. 4C–E).

The neuromast hair cells were enriched with MET gene expression

The MET channels are required for functional hair cells and they are complex comprising of several components, such as TMC1, TMC2, TMIE, LHFPL5, and CIB2 [2, 27–31]. Hair cells with functional MET channels are crucial for normal hearing and balance. In addition, the tip links play important roles in the stereocilia deflection and MET channel-gating, and it was proved to be composed of two cadherins, CDH23 and PCDH15 [32–34]. To determine whether these key molecules are also expressed in zebrafish hair cells and what differences there are among these different hair cells, we analyzed the expression patterns of the genes encoding these important proteins. As illustrated in Fig. 5A–F, the orthologs of mammalian MET complex components were expressed in most of the neuromast hair cells; however, only a small proportion of inner ear hair cells expressed these genes. The genes encoding the tip link components, *cdh23* and *pcdh15a*, were expressed in a few hair cells (Fig. 5G–H).

The zebrafish hair cell scRNA-seq reveals potential hearing loss genes

Hearing is one of the most important sensory functions, and it relies on functional hair cells. Healthy hair cells are the receptors of acoustic signals; therefore, genes responsible for hair cell development, survival, and function are also important for normal hearing. 119 genes have been identified as non-syndromic hearing loss (NSHL) genes in humans (https://hereditaryhearingloss.org/). Among these genes, 96 human genes have orthologous genes in zebrafish (Fig. 6A), which demonstrates that zebrafish are quite similar to humans in terms of NSHL genes. Furthermore, 51


A
5
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7
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Average Expression
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−0.5
−1.0
ckb
s100l
vnn1
stard13
atp1b2b
gpx2
pvalb9
cabp2b
s100s
dio1a
si:ch211-153h4.6
tectb
nkx2b
prox1
BX248333.2
ntn
tpm4a
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TMC1
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zpld1a
cyp27c1
crybb
otog
s1pr1
alif1l
abcb10
nanos1
cxcl14
CR762483.1
Top genes
C
cluster 0
cilium movement
epithelial cilium movement involved in extracellular fluid movement
cilium assembly
cilium organization
inner ear development
ear development
inner ear receptor cell differentiation
inner ear receptor cell development
hair cell differentiation
auditory receptor cell development
D
cluster 7
epithelial cilium movement involved in extracellular fluid movement
inner ear development
inner ear receptor cell differentiation
cilium movement
inner ear receptor cell development
hair cell differentiation
cilium-dependent cell motility
cilium or flagellum-dependent cell motility
cilium organization
cilium assembly
E
cluster 5
ear morphogenesis
inner ear morphogenesis
ear development
inner ear development
inner ear receptor cell differentiation
inner ear receptor cell development
F
cluster 12
inner ear development
ear development
ear morphogenesis
inner ear morphogenesis
G
tectb
H
zpld1a
I
calm1b

◂**Fig. 3** The hair cell classification in zebrafish. **A** The four clusters of hair cells can be further divided into three types, namely macula hair cells (cluster 5), crista hair cells (cluster 12), and neuromast hair cells (cluster 0 and 7). **B** The dotplot of top genes in the four clusters of hair cells. **C–F** The cnetplot of the genes expressed in the four clusters of hair cells. **G-I** The results of the WISH. The genes presented here are expressed in the macula hair cells (**G**), crista hair cells (**H**), and neuromast hair cells (**I**), respectively. The images at the bottom right in **G** and **H** show the dorsal and enlarged view of the gene expression. The red arrowheads and arrows indicate the inner ear hair cells and neuromast hair cells, respectively

human NSHL genes have 57 orthologous genes which are expressed in zebrafish hair cells (cluster 0, 5, 7, and 12), and the detailed information was shown in Fig. 6B. Except for the orthologs of identified human NSHL genes, there are still more than 3000 genes being specifically expressed in the zebrafish hair cells (Fig. 6C). Some of these genes are thought to be crucial for hair cell function, and it is quite possible that they are potential hearing loss genes. In other words, these data may provide us clear direction for our scientific research in hearing loss gene identification. Under the guidance of this idea, we randomly selected some genes for further analysis, and found that these genes were specifically expressed in either or both of the zebrafish inner ear and neuromast hair cells (Fig. 6D), indicating that these genes function in hair cell-related biological processes, and may even be essential for hearing function.

Functional analysis of the candidate genes involved in hair cell development

Our scRNA-seq analysis revealed genes that are specifically expressed in zebrafish hair cells, some of which are orthologs of human NSHL genes; however, many genes have not been reported to play roles in hair cell development or function. To investigate the function of hair cell-enriched genes, we took the *capgb* and *mb* gene as examples, which were mainly expressed in neuromast hair cells and macula hair cells, respectively (Figs. 6D, S8). Here, we used morpholino-mediated gene knockdown to down-regulate the gene expression (Fig. S9). As illustrated in Fig. 7A, B, the *capgb*-morphants exhibited decreased hair cells in their lateral line neuromasts compared to the littermate control, and this abnormality can be rescued by supplying with wild-type *capgb*-mRNA. Furthermore, the *capgb*-morphants had less response to the acoustic stimuli in the startle response test (Fig. 7C, D), which indicated that their hair cells had lost their function to some extent. Likewise, in another experiment, the *mb* gene knockdown resulted in reduced macula hair cells, which can be rescued by coinjecting with *mb*-mRNA (Fig. 7E, F), and the *mb*-morphants showed severely abnormal balance ability in the vestibulo-ocular reflex (VOR) test (Fig. 7G, H).

Discussion

In mammals, inner ear hair cells can detect the mechanical signals and transform them into physiological signals, which were then transmitted to the brain through the auditory neurons [35]. Inner ear hair cells can be divided into cochlear hair cells and vestibular hair cells, which play important roles in hearing and balance, respectively. The two types of hair cells differ from each other in both structure and function [7].

As an excellent animal model, zebrafish have hundreds of hair cells, making it a good model to study hair cell function. Similar to mammals, zebrafish have inner ears, consisting of semicircular canals and otolith; however, zebrafish have no cochlea in their inner ear. Therefore, inner ear hair cells can be divided into macula hair cells and crista hair cells in zebrafish. Except for inner ear hair cells, zebrafish have a third type of hair cells, neuromast hair cells, in their lateral line system. The three different hair cells differ from each other in morphology; however, the molecular difference among them is unknown.

Here, we used the single-cell RNA-sequencing method to analyze the gene expression patterns in zebrafish hair cells, and uncovered the molecular difference among the different types of hair cells. In brief, after quality control, the scRNA-seq data received dimensional reduction processing using the UMAP method, and all of the cells sequenced were classified as 21 clusters. After annotating according to the known marker genes and gene expression patterns in the database (http://zfin.org/), we identified four clusters of cells as macula hair cells, crista hair cells, and neuromast hair cells. Further validation using WISH confirmed our cell clustering and annotation in our following analysis. As for other types of cells, such as supporting cells, mantle cells, and epithelial cells, they are GFP-negative (Fig. S3), but they cannot be filtered out in our FACS sorting. The possible mechanism involved is that they interact with the GFP-positive cells, namely hair cells and retinal ganglion cells, and they were also sorted, coupling with GFP-positive cells.

Furthermore, we performed a comparison among these different subtypes of hair cells, and found that the neuromast hair cells have stronger expression of MET components and energy metabolism-related activity compared to the inner ear hair cells. Moreover, we analyzed the relationship between the known human NSHL genes and zebrafish hair cell marker genes revealed by the scRNA-seq, and found that 42.86% of human NSHL genes have orthologous genes expressed in zebrafish hair cells, which further reflects the reliability of our data. On the other hand, there are plenty of genes in our hair cell scRNA-seq gene pool that have not been reported to function in

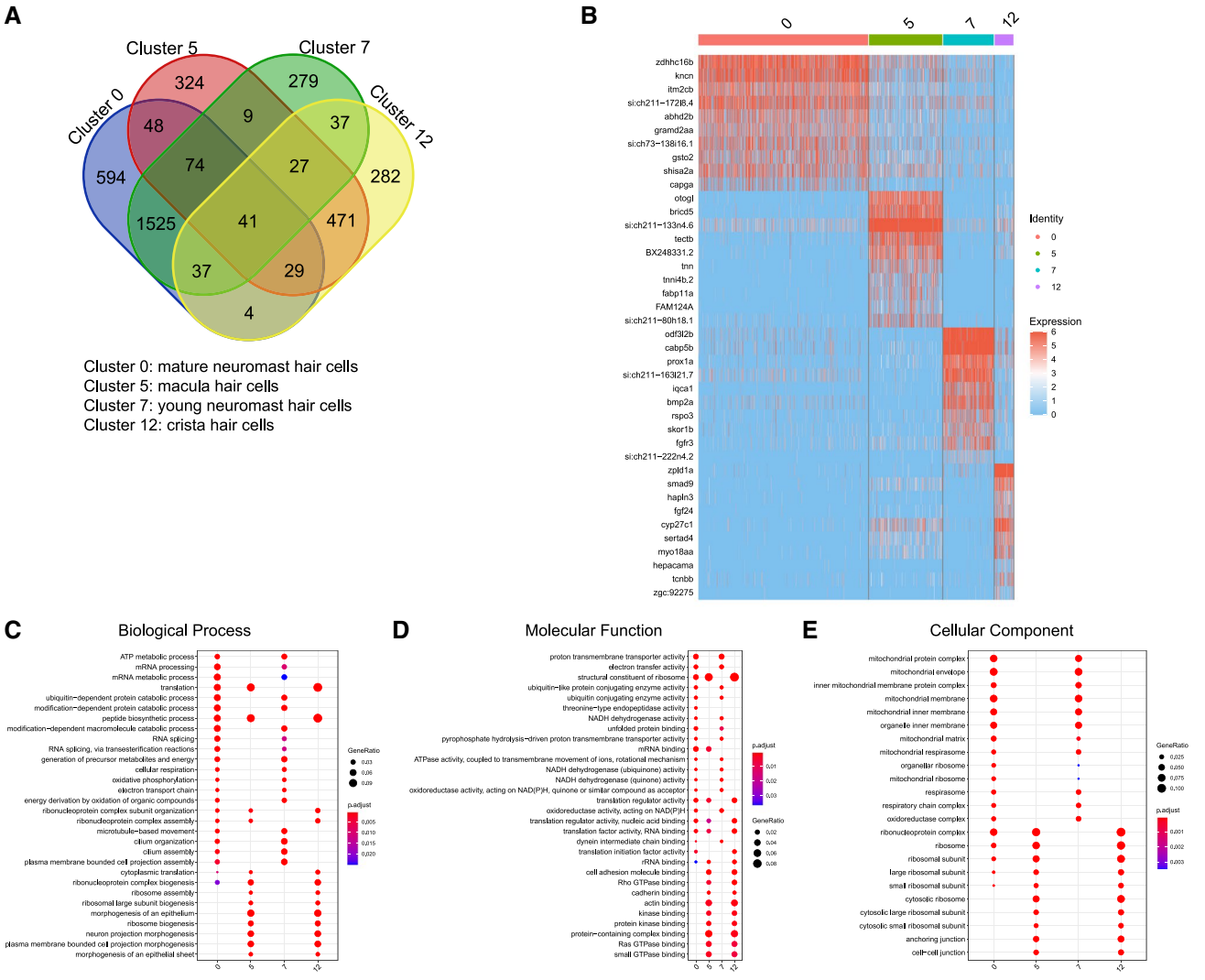


Fig. 4 The molecular difference among the different hair cells. **A** The marker genes that are expressed in different hair cells and the relationship among them. **B** The heatmap of the top marker genes in each cluster of hair cells. **C–E** The GO analysis reveals the different properties among the different hair cells in biological process (**C**), molecular function (**D**), and cellular component (**E**)

hair cells, which raises the possibility that these genes have potential value in hair cell function and even hearing loss gene identification. We randomly picked some of the marker genes for further analysis, and found that *mb* and *capgb* gene were specifically expressed in zebrafish inner ear macula hair cells and lateral line neuromast hair cells, respectively. Gene functional analysis also demonstrated that these two genes were required for hair cell development or function; for that knockdown of these two genes can lead to hair cell loss and hair cell dysfunction.

As far as we know, myoglobin, encoded by the *Mb* gene, is a single-chain heme protein containing 153 amino acids. It mainly exists in cardiac and skeletal muscle, and recent studies showed that it also exists in a variety of non-muscle tissues, such as the brain, kidney, gill, and liver [36]. Myoglobin functions in transporting and storing oxygen in muscle cells, and it can also promote the removal of reactive oxygen species (ROS) and NO in cancer cells [37]. However, the role of myoglobin in hair cells was not reported before, and it is the first time that the *mb* gene was proved to be expressed in zebrafish inner ear hair cells and important for hair cell development and function in our current study. Further investigation was needed to uncover the molecular mechanisms involved in hair cell function.

CAPG, encoding a gelsolin-like capping protein, as a proto-oncogene [38], is involved in the migration and invasion of various cancer cells [39–42]. Recently, a rare homozygous deletion of the chromosome 2p11.2 region was found in a Tunisian patient with autism, intellectual disability, and hearing impairment, and the affected genes

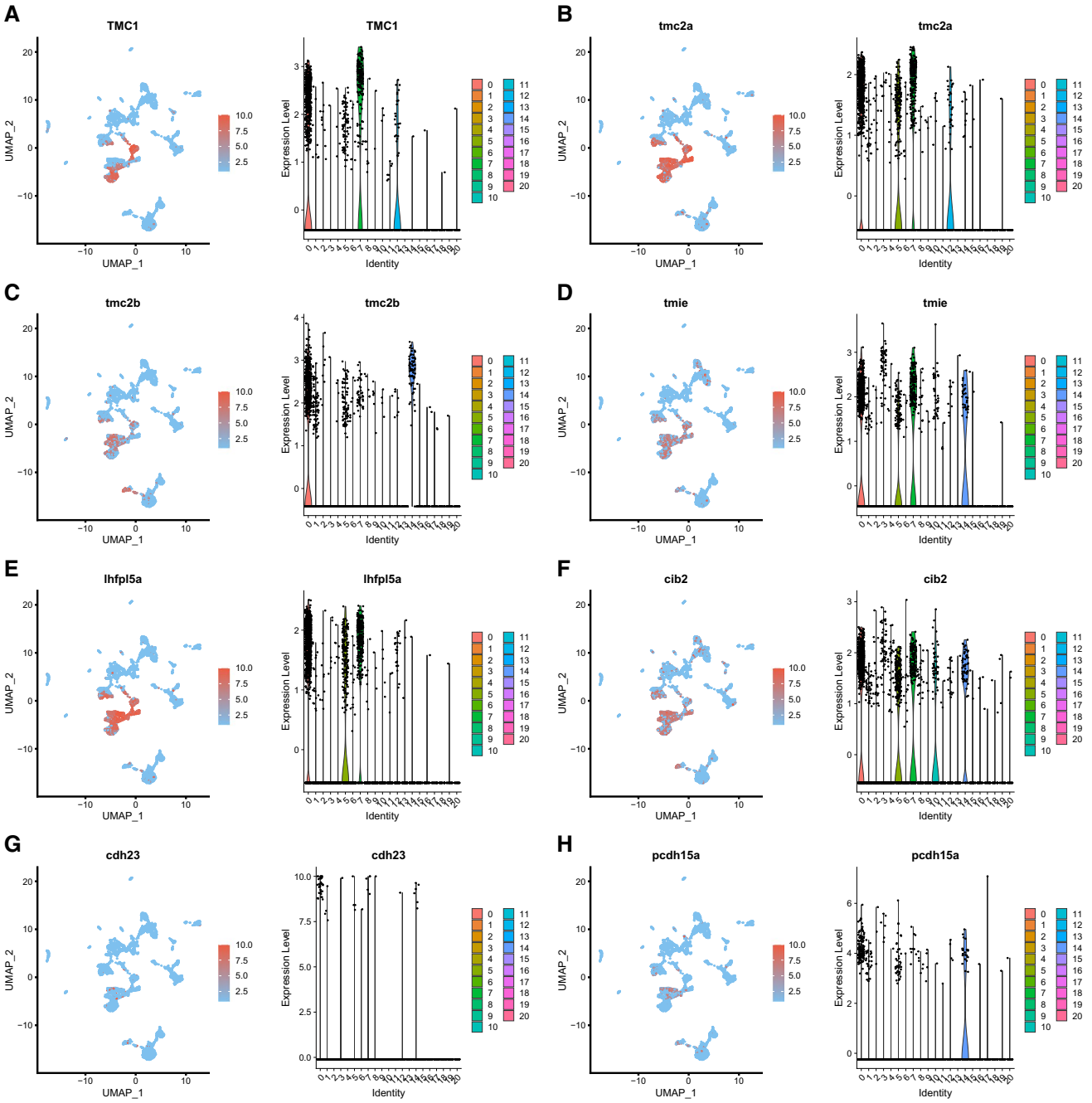


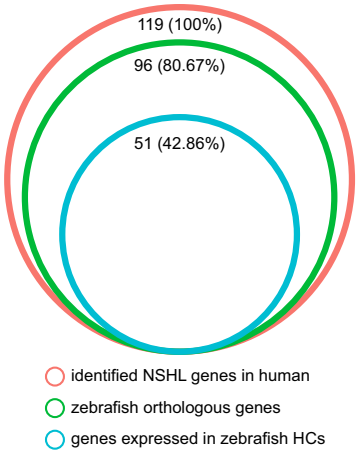
Fig. 5 The distribution of the cells expressing the genes encoding the MET and tip link components. The feature plot and violin plot are shown for each gene

mainly included *ELMOD3*, *CAPG*, and *SH2D6* [43]. In our study, we found that the *capgb* gene was mainly expressed in zebrafish neuromast hair cells, and essential for hearing, indicating its crucial role in auditory function. Nevertheless, how *CAPG* contributes to hair cell development, as well as hearing, merits further investigation.

To sum up, in this study, we identified three subpopulations of hair cells, which corresponded with macula hair cells, crista hair cells, and neuromast hair cells in zebrafish, through scRNA-seq. In addition, it also uncovered thousands of genes in zebrafish hair cells, which would be helpful for our research in hair cell biology in the future.

Based on differential gene expression, Lush., et al. [17] found that hair cells can be subdivided into young hair cells and mature hair cells, and these two different hair cells have distinct distribution and gene expression that the young hair

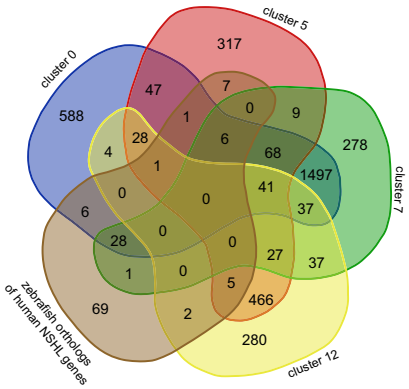
A



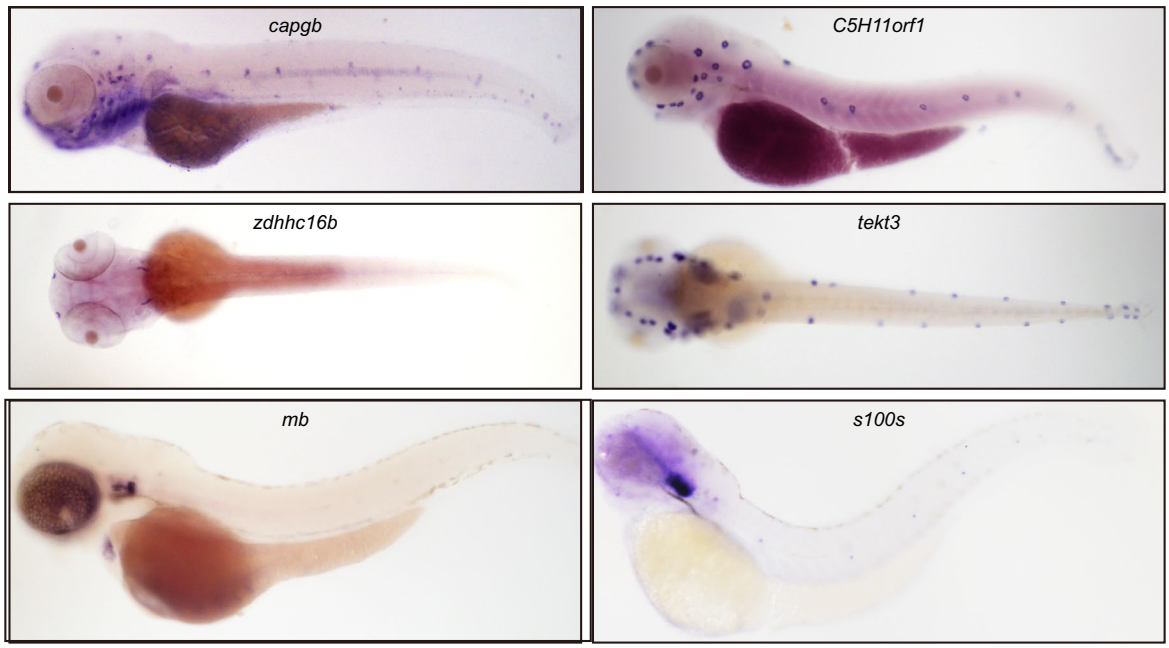
B

Human NSHL Genes	Zebrafish Orthologous Genes	UMAP-Cluster
AIFM1	*aifm1*	0,7
CDC14A	*cdc14aa; cdc14ab*	0,14; 0,7,14
CIB2	*cib2*	0,7,14
CLIC5	*clic5a; clic5b*	0,7; 0,7,14
COCH	*coch*	5,12
COL11A1	*col11a1a*	5,12
COL11A2	*col11a2*	5
COL4A6	*col4a6*	5,12
DMXL2	*dmxl2*	0,7,14
EPS8L2	*eps8l2*	0,14
ESPN	*espn*	0,5,7,14
EYA4	*eya4*	0,5,7,4
GAB1	*gab1*	5,12
GIPC3	*gipc3*	0,7,14
GJB2	*cx30.3*	5
GJB6	*cx30.3*	5
GPSM2	*gpsm2*	0,1,14
GRXCR1	*grxcr1a; grxcr1b*	0,7,14
KARS	*kars1*	0
KITLG	*kitlga; kitlgb*	1,5,12; 1,12
LHFPL5	*lhfpl5a*	0,5,7
LOXHD1	*loxhd1a*	0,7
LRTOMT	*tomt*	7,14
MARVELD2	*marveld2a*	5,9
MET	*met*	1,12
MSRB3	*msrb3*	0,5,7,14
MYH14	*myh14*	0,7,14
MYO15A	*myo15aa*	0,7,14
MYO6	*myo6b*	0,7,14
MYO7A	*myo7aa*	0,7,14
OSBPL2	*osbpl2b*	0,7
OTOF	*otofa; otofb*	0,7,14
OTOG	*otog*	0,5,12
OTOGL	*otogl*	5
PLS1	*pls1*	0
POU4F3	*pou4f3*	0,7,14
SIX1	*six1a; six1b*	0,5,7; 0,5,7,14
SLC17A8	*slc17a8*	0,7
SLC26A5	*slc26a5*	5
SMPX	*smpx*	0,7,14
SPNS2	*spns2*	5
STRC	*strc*	0,7
TECTA	*tecta*	5
TMC1	*TMC1*	0,7
TMIE	*tmie*	0,7,14
TMPRSS3	*tmprss3a*	0,7,14
TNC	*tnc*	0,5
TRIOBP	*triobpa; triobpb*	0,7,14; 0,14
USH1C	*ush1c*	0,7
WBP2	*wbp2*	0,7,14
WHRN	*whrna*	0,7,14

C



D



◂**Fig. 6** The zebrafish hair cell scRNA-seq reveals potential hearing loss genes. **A** The relationship between the known human NSHL genes and zebrafish hair cell-enriched genes. **B** The list of the human NSHL genes, which have orthologs expressed in zebrafish hair cells. **C** Venn diagram of the zebrafish orthologs of human NSHL genes and marker genes expressed in each cluster of hair cells in zebrafish. The numbers indicate the number of the genes. **D** The expression patterns of the hair cell-enriched genes in zebrafish embryos

cells form a ring and express *atoh1b*. Differently, our current work revealed the molecular difference among three different types of hair cells and specific marker genes for the first time, which could provide further insights into understanding the mechanisms underlying hearing and balance.

Materials and methods

Zebrafish embryos

Zebrafish were maintained at 28.5 °C. Two zebrafish lines were used in the study, including the wild-type AB and the transgenic line *Tg(Brn3c:mGFP)*, which was described in the previous work [19]. All animal procedures were performed according to protocols approved by the Animal Care and Use Committee of Nantong University and were consistent with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Single-cell RNA-sequencing

The *Tg(Brn3c:mGFP)* transgenic zebrafish larvae at 6 days post-fertilization (dpf) were anesthetized and then treated with 0.25% trypsin to dissociate into single cells, which were divided into GFP-positive and GFP-negative cells through the fluorescent activated cell sorter, and the RNA expressed in GFP-positive cells were then obtained and sequenced using the 10 × Genomics platform.

Single-cell sequencing analysis

The basic procedure for single-cell sequencing analysis was carried out as previously described [44]. Briefly, Seurat V4.0.1 [20] was used for the integrated analysis of the single-cell sequencing data, including data filtering, data normalization, cell clustering, and cluster-level marker gene identification. First, the Seurat object was created by "CreateSeuratObject" function and the raw data were primarily filtered through the parameter settings "min.cells = 5,min.features = 200", which required that only the genes expressed in at least five cells were considered and only the cells with minimal 200 genes detected were kept. Then, the data were further filtered through the "subset" function in Seurat with the parameter settings "nFeature_RNA < 5000 & percent.mt < 10", which required the cells containing no more than 10% reads from mitochondrial genes and up to 5000 detected genes. Second, "SCTransform" function in Seurat was applied to data normalization, and the data were regressed by several factors(percentage of mitochondrial expression, total number of UMIs, and total number of genes detected) through a second non-regularized linear regression (variable.features.n = 1000,vars.to.regress = c("nFeature_RNA","nCount_RNA","percent.mt")). For cell clustering, the principal component analysis (PCA) was first applied for extracting the top principal components (top 50 PCs), and then, the clusters were identified through shared k-nearest neighbor graph construction and cluster modularity function optimization (k.param = 20, resolution = 0.25). To identify marker genes for each cell type, "FindAllMarkers" function in Seurat was used with the parameter settings "only.pos = TRUE, min.pct = 0.25,logfc.threshold = 0.25". The default Wilcoxon rank-sum test in "FindAllMarkers" function was selected for differential expressed gene detection between clusters, where the *p* values were further adjusted by Bonferroni correction.

For GO enrichment analysis, the R package clusterProfiler [45] was used, and the marker genes identified by Seurat were used as input. The three GO categories: BP (Biological Process), CC (Cellular Component), and MF (Molecular Function) were analyzed respectively against the marker genes for each cell cluster, and the p values were corrected by Benjamini & Hochberg (BH) method, where the significantly enriched GO terms were defined as the ones with BH adjusted p value less than 0.05. The GO enrichment results were further visualized through the enrichplot method integrated in clusterProfiler.

Whole-mount in situ hybridization

The whole-mount in situ hybridization procedure was similar to our previous description [46, 47]. In brief, the gene-specific primers targeting the coding sequence of interest were designed and synthesized. After amplification using the PCR method, the fragments were subcloned into the pGEM-T Easy vector (Promega). The DIG-labeling RNA probe was transcribed in vitro using the linearized recombinant plasmid as a template. For the hybridization step, the pretreated zebrafish embryos were incubated with the digoxigenin-labeling RNA probe overnight first, and then, alkaline phosphatase-conjugated primary antibody against digoxigenin was used to detect the RNA probe following three washes. Subsequently, for color reaction, the nonspecific binding was washed out and the substrate of alkaline phosphatase, NBT/BCIP solution, was added to the reaction system. Finally, the samples were imaged and the gene-specific mRNA expression was visualized through the microscope. The primers used for probe synthesis are as follows:

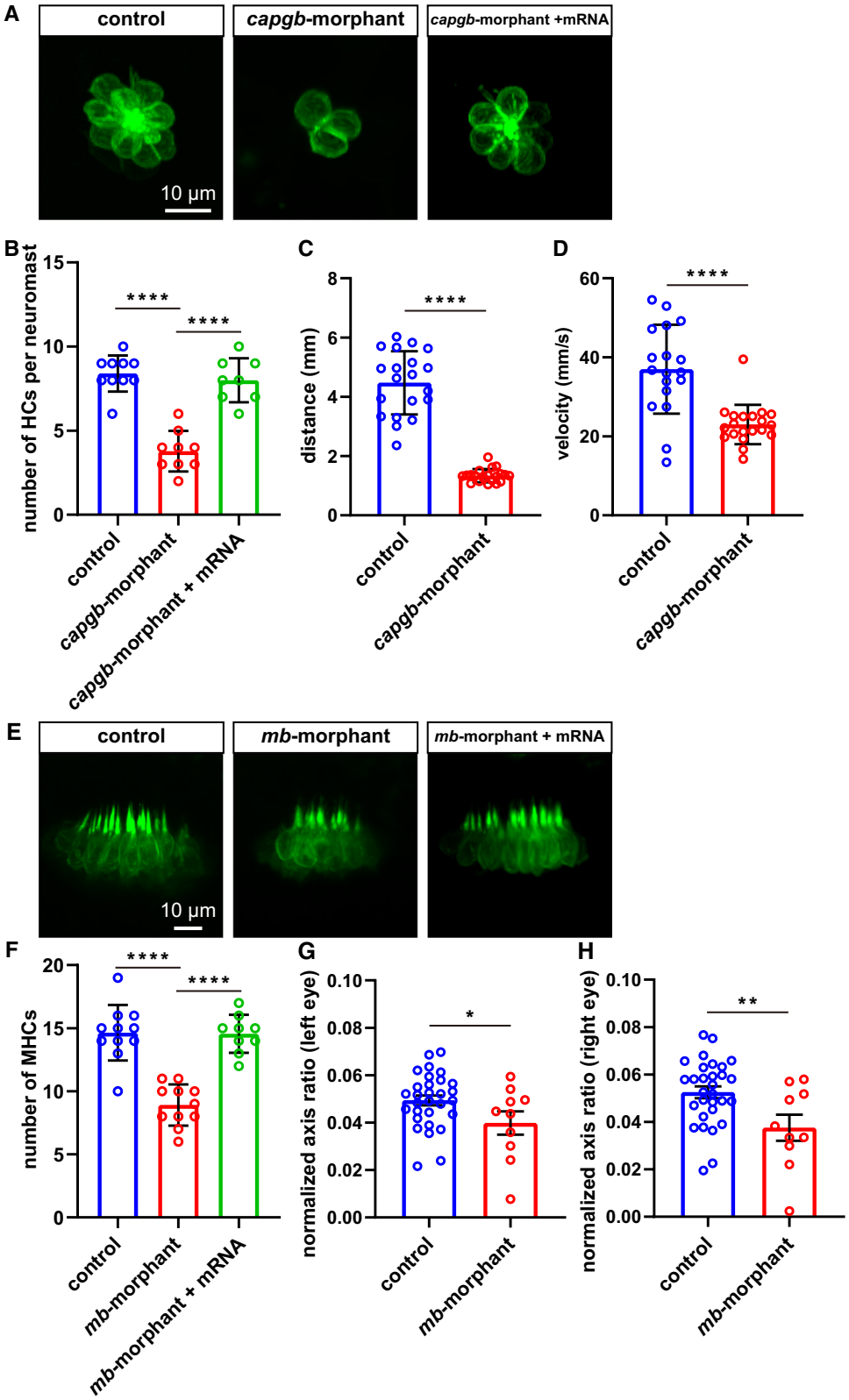


Fig. 7 Functional analysis of the candidate genes involved in hair cell development. **A**, **B** The *capgb* gene knockdown resulted in decreased neuromast hair cells in zebrafish. **C**, **D** The *capgb*-morphants showed less response to the acoustic stimuli. **E**, **F** The *mb* gene knockdown resulted in reduced macula hair cells in zebrafish. **G**, **H** The *mb*-morphants showed damaged balance ability. $*P<0.05$, $**P<0.01$, $****P<0.0001$

tectb-F: GCCGTGTTTAGCCAAAGAGT;
tectb-R: ACAACTTCTCCCCGTCACAT;
zpld1a-F: CCACGGTTGTGCTGTTTAGC;
zpld1a-R: GTTGGTGGCTTTTACAGCGG;
calm1b-F: AAGTGTTGTGTCGTGGGGTT;
calm1b-R: GCAGGGGAGATTTCACTGGT;

capgb-F: ACCTGGTGCTGGATAACAGG;
capgb-R: ATCTGAGCTTTGCCGTGTCT;
C5H11orf1-F: TGATGTCCAATAAAAGCCAGGT;
C5H11orf1-R: CACACATTGAGGCTCTGAAGT;
zdhhc16b-F: CCTGTGGAATTATGGGATGG;
zdhhc16b-R: ATGCTGCAGTGATGAGTTCG;
tekt3-F: AGATTTCAGCGCTGTCCGAT;
tekt3-R: AAGCAGCACGTTCACTCTGA;
mb-F: TGATCTGGTTCTGAAGTGCTG;
mb-R: GGCAAATCCGATCTCCTTGT;
s100s-F: CCAAGATGCCACGCTCAAAG;
s100s-R: CCCGCTAACACTTCTCTCGG.

Morpholino-mediated gene knockdown

The gene-specific morpholinos (MOs) were synthesized by the Gene Tools, LLC. For gene knockdown, the 2–3 nL of 0.3 mM of gene-specific morpholinos were microinjected into the 1–2-cell-stage zebrafish embryos. Here, the morpholinos were used to block the splicing of the pre-mRNA, and therefore down-regulate the target gene expression. The sequences of the morpholinos used in this study were as follows:

capgb-MO: TCTGGAGGAACAAAGATGAGATGGT;
mb-MO: ATCAGAGAGTCCTGCTTTACCCTGA.

Reverse transcription-polymerase chain reaction (RT-PCR)

The RT-PCR was performed following the standard procedure. Briefly, the total RNA was extracted from the zebrafish embryos and then reverse-transcribed into cDNA. The PCR experiment was performed to detect the gene expression using the cDNA as a template. Herein, the primers used for PCR were listed as follows:

capgb-F: TCTGACAGCATGCCGGAGC;
capgb-R: TAACATTGGTGATCTGAGCTTTGC;
mb-F: GACTTTTCCAAAGCCACAGGC;
mb-R: TCCTGAGACCCTAACGAACCA.

mRNA rescue experiment

The rescue experiment was performed by coinjecting the specific mRNA with the morpholino. The mRNA was transcribed in vitro using the linearized recombinant plasmid as a template, which contains the coding sequence of specific gene. The primers used in the PCR to amplify the specific genes were as follows:

capgb-mRNA-F: ACCTAGGTGCAGGAACAGG;
capgb-mRNA-R: ACAACTGGTTGAGTGCAGTTTA;
mb-mRNA-F: GCCCCGATATTGAAGACAGGT;
mb-mRNA-R: TGACTCCCATTTGAGATCTGGT.

Startle response test

The startle response test in zebrafish was carried out following the procedure [48, 49]. In this test, 20 zebrafish larvae at 5 dpf were put into the culture dish for free swimming. When acoustic stimuli occurred, the behavior of the zebrafish was recorded by a high-speed camera (500 fps). In response to the stimuli, the zebrafish with normal auditory function would have a characteristic C-bend motion lasting less than 10 ms; however, the zebrafish with damaged auditory function would not. Herein, both the distance moved and the number of the C-bend motions were used to quantify the startle response.

VOR test

As previously described [49, 50], the VOR test was performed according to standard procedure. Briefly, the zebrafish larva at 5 dpf was fixed in the chamber in a head-up position with 5% methylcellulose. The rotary platform, with the chamber unit fixed, rotated back and forth at a speed of 30 rpm. The eye movements of the zebrafish were recorded by an infrared camera, and the periodical changes of the projection area of the eyes were used to evaluate the VOR.

Imaging and statistical analysis

For the confocal fluorescence microscopic analysis, the zebrafish were embedded in the 0.8% low melt agarose following anesthetization with MS-222, and the imaging was carried out by the Nikon A1 microscope. The Olympus MVX10 microscope was used for bright-field imaging in the WISH experiments. All data were presented as mean with SD, and the unpaired Student's t tests were used to determine statistical significance. A value of $P < 0.05$ was considered statistically significant.

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Author contributions FQ, DL, GX, and RC conceived and designed the experiments, and wrote the manuscript. FQ, YG, XW, JG, CG, XW, XZ, JZ, CW, MX, YH, and GY performed the experiments. FQ, GW, JK, GX, DL, and RC analyzed the data. All authors read and approved the final manuscript.

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Availability of data and materials All the high-throughput sequencing data generated in this study have been deposited in the Gene Expression Omnibus database under accession number GSE221471 and are available at the following URL: https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE85337. All the experimental materials generated in this study are available from the corresponding authors upon reasonable request.

Declarations

Conflict of interest The authors declare that they have no conflicts of interest.

Ethical approval and consent to participate All zebrafish experimentation was carried out in accordance with the NIH Guidelines for the care and use of laboratory animals (http://oacu.od.nih.gov/regs/index.htm) and ethically approved by the Administration Committee of Experimental Animals, Jiangsu Province, China (Approval ID: 20180405-039).

Consent for publication The authors declare the consent for publication.

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