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MicroRNA-200 family members are weakly expressed in the neurosensory epithelia of the developing zebrafish (*Danio rerio*) inner ear

J.T. Du[1,2], H. Cao[1,2], W. Zhou[1,2], K.T. Chen[1,2], D. Jiang[1,2], H.C. Tang[1,2], X.R. Wang[1,2], X.M. Zhang[1,2] and H.Y. Jiang[1,2]

[1]Department of Otorhinolaryngology, First Affiliated Hospital, Sun Yat-sen University, Guangzhou, China
[2]Institution for Otorhinolaryngology, Sun Yat-sen University, Guangzhou, China

Corresponding author: H.Y. Jiang
E-mail: jhongy@mail.sysu.edu.cn

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ABSTRACT. MicroRNA-200 family members are expressed in the developing mouse inner ear and in zebrafish (*Danio rerio*) olfactory epithelia, taste buds, and neuromasts, and have also been shown to be associated with differentiation of olfactory and taste buds. However, the role of the miR-200 family in the inner ear of zebrafish had not been studied. We investigated the expression and function of the miR-200 family in the zebrafish inner ear via *in situ* hybridization and loss-of-function methods. Expression of the miR-200 family was weak and dispersed throughout the developing zebrafish inner ear. After knockdown of miR-200 family members in the developing inner ear, no significant differences in development were observed compared to the controls. Otic vesicles, otoliths, and semicircular canals appeared normal. Compared with less differentiated olfactory filaments in olfactory epithelia, the development of hair cells and statoacoustic ganglion neurons were

normal. The kinocilia and stereocilia of hair cells, the innervation of hair cells, and the formation of ribbon synapses were also unaffected. Overall, we conclude that the miR-200 family has a negligible role in the development of zebrafish inner ear; the functions of the miR-200 family may be organ-specific.

Key words: miR-200; Inner ear; Zebrafish; Morpholino oligonucleotide

INTRODUCTION

The development of the inner ear is controlled by multiple signal pathways and genes, including Notch signaling (Pan et al., 2010), Wnt signaling (Freyer and Morrow, 2010), and atonal homolog 1 (Atoh1) (Raft et al., 2007). These have also been shown to be involved in the mechanisms that underlie the production of the hair cells of the inner ear (Kelley et al., 2009). Studies of non-coding microRNAs (miRNAs) revealed some novel mechanisms related to this developmental process, where these single-stranded RNAs post-transcriptionally repress complementary target gene expression and are involved in a variety of biological processes, including growth and differentiation (Ambros, 2004; Bartel, 2004; Kloosterman and Plasterk, 2006).

Although approximately one-third of known miRNAs have been detected in the inner ear (Weston et al., 2006), the miR-183 family members in particular (e.g., miR-96, miR-182, and miR-183) are specifically expressed in the inner ear and other sensory organs including the eye and nasal epithelium (Wienholds et al., 2005; Gessert et al., 2010; Wang et al., 2010; Weston et al., 2011). It has been reported that the mutation of non-coding genes, such as the loci in the seed region of miR-96, are associated with nonsyndromic deafness (Lewis et al., 2009; Solda et al., 2011). Publications concerning miRNA-183 family members suggest that the expression of miRNAs in the inner ear may be involved in the regulation of inner ear development in zebrafish (*Danio rerio*) (Li et al., 2010; Wang et al., 2012). For example, over-expression of miR-96 or miR-182 induces ectopic or expanded sensory patches and extra hair cells, accompanied by adversely affected morphogenesis of the statoacoustic ganglion (SAG) to varying degrees. In contrast, knockdown of miR-183, -96, and -182 results in a decrease in the numbers of hair cells and in smaller SAGs, although based on the average area occupied by $Sox2^+$ cells, the prosensory region of the posterior macula is not significantly impaired (Li et al., 2010). Interestingly, it has also been reported that inhibition of miR-96 in *Xenopus tropicalis* (the Western clawed frog) leads to defects of the eye (Gessert et al., 2010). These findings hint that the expression of specific miRNAs in these sensory organs may share a similar function. In other words, a miRNA expressed in a sensory organ may be expressed in other sensory organs and exert a similar function.

miR-200 family members include miR-200a, miR-200b, miR-200c, miR-141, miR-429, and miR-429b, whose expression in the mouse inner ear has been identified by microarray (Weston et al., 2006). Our previous study showed that miR-200 family members are expressed in the developing inner ear in the mouse (Wang et al., 2010). In the literature, the expression of miR-200 family members in the inner ear of neonatal mice and neuromasts of zebrafish [*Danio rerio* (Hamilton, 1822)] was determined by analysis using microarray or *in situ* hybridization (Wienholds et al., 2005; Weston et al., 2006). The expression of miR-200

family members in the olfactory epithelia, neuromasts, taste buds, and eye has been reported by several authors (Wienholds et al., 2005; Gessert et al., 2010). During olfactory placodal development in zebrafish, miR-200 family members are required for the regulation of proper differentiation of olfactory progenitor cells (Choi et al., 2008). Similarly, miR-200-mediated regulation is also required for taste bud formation and in particular for calbindin (Calb) 2b+ cell formation in zebrafish (Kapsimali et al., 2011). Gessert et al. (2010) reported that the depletion of miR-200b in *X. tropicalis* resulted in abnormal or absent eyes. These findings indicate that the miR-200 family may be sensory organ-specific and have regulatory roles in all sensory organs.

However, the expression of members of the miR-200 family and their role in the zebrafish inner ear remain unclear. In the current study, we investigated the expression and function of the miR-200 family in the zebrafish inner ear using *in situ* hybridization and imposing loss of function via morpholino oligonucleotide (MO) injection.

MATERIAL AND METHODS

Zebrafish husbandry

Adult wild type (AB) zebrafish were maintained and bred, and their one-cell stage embryos were collected as previously described (Westerfield, 2000). Embryos were incubated at 28°C in E3 medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$, 0.33 mM $MgSO_4$) (zfin.org). The Sun Yat-sen University Animal Care and Use Committee approved all experimental procedures.

MO injection to initiate loss-of-function

MO injection was performed according to the protocol of Kloosterman et al. (2007). Pulled-glass capillary micropipettes with tips polished to a 30 to 45° angle were used to deliver 1 to 2 nL MO into the cytoplasm of one-cell zebrafish embryos. MOs, including standard control MOs (GeneTools, USA), were dissolved in water to make stock concentrations (3 mM) and diluted to working solution (3 ng/nL or 0.375 mM) in Danieau's buffer [58 mM NaCl, 0.7 mM KCl, 0.4 mM $MgSO_4$, 0.6 mM $Ca(NO_3)_2$, 5 mM HEPES, pH 7.2] (Ghiselli, 2006) before injection.

Triple MOs targeting all members of the miR-200 family were designed and synthesized by GeneTools. The sequences of the morpholino antisense oligos complementary to miRNAs were: miR-141-MO, GCATCGTTACCAGA CAGTGTTAGAA; miR-200C-MO, TGCATCATTACCAGGCAGTATTAGA; miR-429b-MO, GATGGCATTACCAG GCAGTATTAGA; and standard control, CCTCTTACCTCAGTTACAATTT ATA.

Whole-mount *in situ* hybridization to determine expressions of the miR-200 family

Whole-mount *in situ* hybridization was performed as previously described (Kloosterman et al., 2006; Thisse and Thisse, 2008). Phenylthiourea (0.03%) was used to inhibit pigmentation of embryos older than 22 h postfertilization (hpf). Embryos were fixed in 4% paraformaldehyde in phosphate buffered saline (PBS) at 4°C overnight, followed by dehydra-

tion in methanol at -20°C for at least 2 h. After rehydration through a graded methanol series and treatment with proteinase K (10 μg/mL), embryos were re-fixed and washed with 0.1% Tween-20 in PBS (PBST) for 5 min, 5X. Embryos were then pre-incubated in hybridization buffer for 2-3 h, followed by hybridization with locked nucleic acid-modified DNA probes for miRNA detection (10 nM; Exiqon) at 51°C overnight. After hybridization and high-stringency washing, embryos were blocked in 2% goat serum and 2 mg/mL bovine serum albumin in PBST at room temperature for 1 h and incubated with anti-digoxigenin conjugated to alkaline phosphatase antibody (Roche) at 4°C overnight. Embryos were incubated with BCIP (5-bromo-4-chloro-3-indolyl phosphate, toluidine salt; 188 μg/mL) and NBT (nitro-blue tetrazolium chloride; 373 μg/mL; Roche) at room temperature for 2 h or at 4°C overnight to yield a purple color reaction for visualization.

Whole-mount immunostaining to observe morphological change

At 24 to 72 hpf, zebrafish embryos were anesthetized with 0.02% tricaine (MS222, Sigma), fixed in 4% paraformaldehyde in PBS at 4°C overnight, and immersed in 0.1 or 1% Triton X-100 in PBS until otoliths were completely dissolved. Specimens were incubated in blocking solution (10% calf serum, 2% bovine serum albumin, and 0.1% Triton X-100 in PBS) at room temperature for 1 h and then in the above solution containing primary antibodies at 4°C overnight. After washing, samples were incubated with a fluorescein-coupled secondary antibody. Primary antibodies were: anti-myosin 6 (rabbit; 1:400; Proteus Biosciences), anti-human neuronal protein HuC/HuD (mouse; 1:400; Invitrogen), anti-acetylated tubulin (mouse; 1:400; Sigma), 3A10 (mouse, 1:50, Developmental Studies Hybridoma Bank), C-terminal-binding protein 2 (CTBP2; rabbit, 1:100, Proteintech), rhodamine-conjugated phalloidin (1:200; Invitrogen), and Hochest 33342 (1:500; Invitrogen). Secondary antibodies (1:400; Invitrogen) were: Alexa-488 or Alexa-594 donkey anti-mouse IgG and goat anti-rabbit IgG.

Imaging and data analyses

Live embryos and embryos subjected to whole-mount *in situ* hybridization were photographed at an Olympus X71 microscope equipped with differential interference contrast (DIC) optics. Immunostained embryos were visualized with an Olympus X71, Leica fluorescence microscope, or Olympus Fluoview confocal microscope. Only one side per embryo was used for analysis of quantitative data; cell counting was performed on reconstructed confocal stacks. The Student *t*-test was used to compare the differences between controlled and experimental groups. $P < 0.05$ was considered to be statistically significant.

RESULTS

MiR-200 family expression in the neurosensory epithelium of the inner ear

The expression of miR-200 family has been found in neurosensory epithelium, such as nose epithelium, taste buds, and neuromasts in zebrafish (Wienholds et al., 2005; Choi et al., 2008; Gessert et al., 2010; Kapsimali et al., 2011). In the present study, the results showed

that miR-141 (a member of the miR-200 family) was located in the inner ear from 24 to 72 hpf (Figure 1A). Also, we looked for other members of the miR-200 family and found that all members were expressed in the inner ear at least by 72 hpf (Figure 1B). However, all these miRNAs were weakly expressed in the inner ear neurosensory epithelium, as compared with their robust expression in other neurosensory epithelia such as the olfactory epithelia, taste buds, and neuromasts, and this was especially true for miR-141. miR-141 was expressed in the anterior macula (Figure 2A) and posterior macula (Figure 2B) of the inner ear, olfactory epithelium (Figure 2C), neuromast (Figure 2D), and taste bud (Figure 2E).

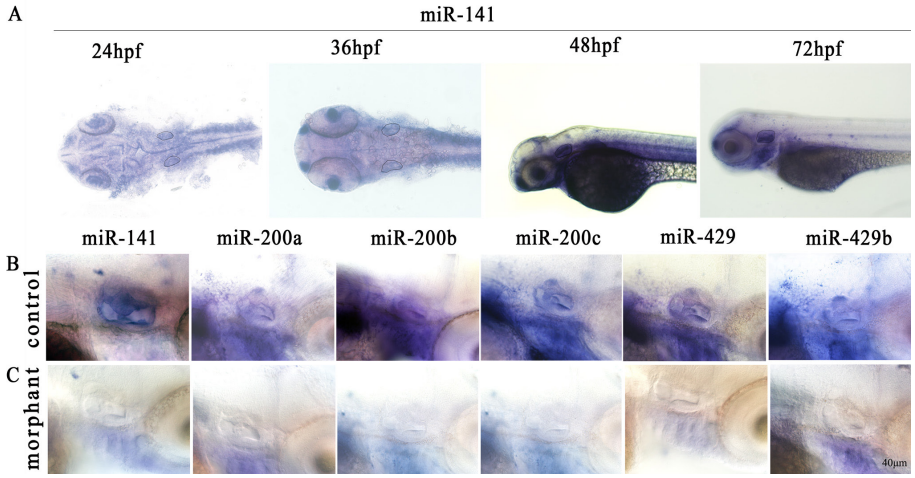

Figure 1. Expression of the miR-200 family in the inner ear of representative control and morphant embryos from 24 to 72 hpf. **A.** miR-141 was expressed in the developing inner ear (the black circle indicates the inner ear) from 24 to 72 hpf in wild type zebrafish. Lateral views: anterior to the right, dorsal to the top with focus on the plane of the anterior macula. **B.** All members of the miR-200 family were expressed in the inner ear at least by 72 hpf in control zebrafish. **C.** Downregulation of the expression of miR-200 family members in the inner ear by triple MO injection at the single-cell stage. Scale bar = 40 μm.

Knockdown of miR-200 family members by specific MOs

A single MO injection could block the expression of the relevant miRNAs. After miR-141 MO injection the expressions of miR-141 and miR-200a were effectively decreased to undetectable levels, as measured by *in situ* hybridization (Figure 3). At 72 hpf, the expression of miR-141 in gross morphology (Figure 3A, D), olfactory epithelia (Figure 3B, E), and the inner ear (Figure 3C, F) was almost depleted in morphants (Figure 3D to F), compared with that in the wild type (Figure 3A to C). Triple MOs (miR-141-MO, miR-200c-MO, and miR-429b-MO) which target all members of the miR-200 family were injected into the embryos at the single-cell stage. The dependence of otic development on the miR-200 family could be evaluated by this approach. The efficacy of MO-mediated knockdown of all members of the miR-200 family was confirmed by *in situ* hybridization, which showed a significant decrease (to undetectable levels) in miR-200 family expression, at 6 ng/embryo from 24 to 72 hpf (Figure 1C).

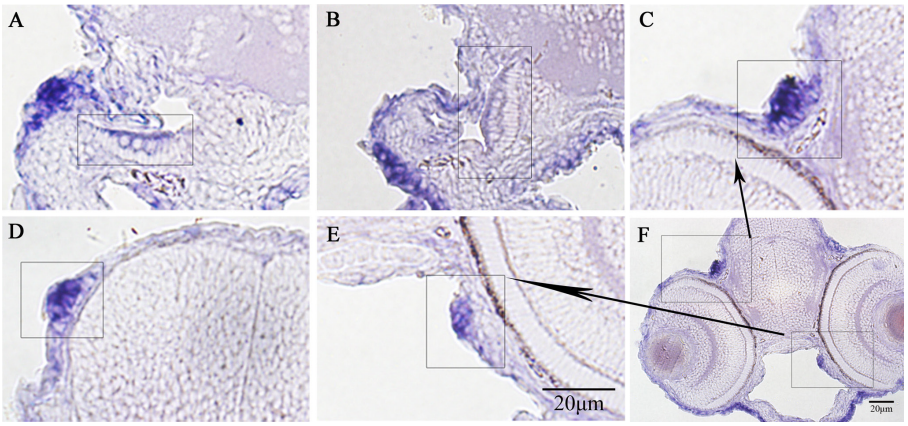

Figure 2. Expression of miR-141, a miR-200 family member in neurosensory organs. A to E: miR-141 is expressed in the anterior macula (**A**) and posterior macula (**B**) of the inner ear, olfactory epithelium (**C**), neuromast (**D**), taste bud (**E**); black rectangles indicate the location. (**F**) olfactory epithelium (**C**) and taste bud (**E**) from the same cryosection and their location. Compared with olfactory epithelium (**C**), taste bud (**E**), and neuromast (**D**), the expression of miR-141 in the inner ear (**A** and **B**) is relatively lower. Scale bar = 20 μm.

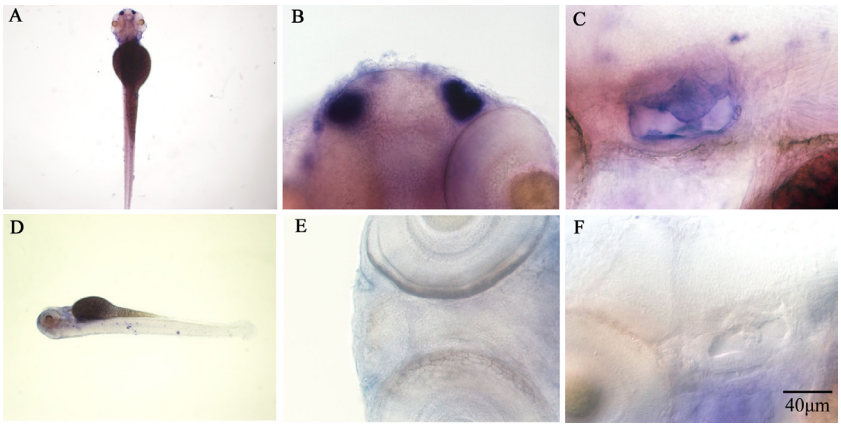

Figure 3. Expression of miR-141, a miR-200 family member in neurosensory organs, and its depletion in representative triple MO zebrafish morphants at 72 hpf. Triple MO injection mediated depletion of miR-141 in all neurosensory organs at 72 hpf. **A. D.** Expression of miR-141 in gross morphology in wild type (**A**) and morphant (**D**). B. E. Expression of miR-141 in olfactory epithelium in wild type (**B**) and morphant (**E**). **C. F.** Expression of miR-141 in inner ear in wild type (**C**) and morphant (**F**). Compared with olfactory epithelia (**B**), the expression of miR-141 in the inner ear (**C**) is relatively lower. Scale bar = 40 μm.

Normal development of inner ear morphology after triple MO injection

Beginning at 18 hpf, a slit-shaped lumen from the placodal mass of cells developed and rapidly enlarged to form the otic vesicle. The otoliths appeared at opposite ends of the lumen by 21 hpf, and hair cells differentiated just beneath them at 24 hpf (Figure 4A). From 24 to 36 hpf (Figure 4B), the otoliths were on the same plane. Up to 48 and 72 hpf (Figure 4C and D), the

anterior otolith lay on the anterior macula, and the posterior otolith lay on the posterior macula, which appeared at the posteromedial of the otic vesicle (Haddon and Lewis, 1996). When triple MOs targeting all members of the miR-200 family were injected into the single-cell stage embryos, the otic vesicle developed normal gross morphology without significant change (Figure 4E to H). In the dorsal regions of the vesicle, protrusions grew out from the walls of the vesicle into its lumen (48 hpf, Figure 4C = control; Figure 4G = morphant). These protrusions met and fused (60-72 h) to form pillars of tissue spanning the lumen, which became axial hubs of the three semicircular canals (Figure 4D and H). Compared to the control (Figure 4A to D), no observable changes occurred in the embryos after triple MO injection (Figure 4E to H).

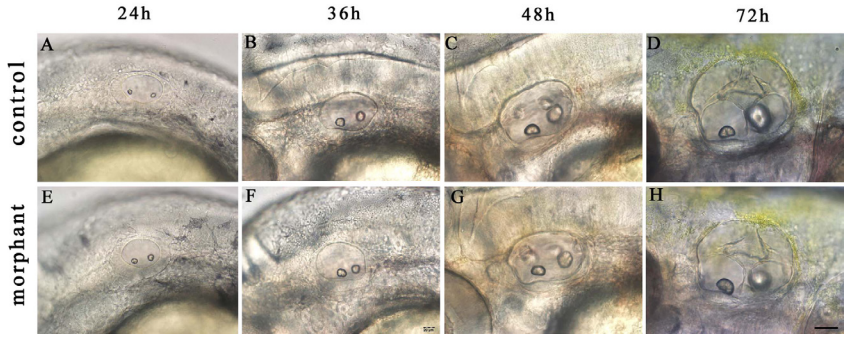

Figure 4. Developing ear in live embryos. After injection of control or triple MOs, the gross development of the inner ear showed no significant change. **A. E.** By 24 hpf, two otoliths appeared at opposite ends of otic vesicle with obvious tether cells (the first hair cells differentiated) beneath them. **B. F.** Up to 36 hpf, two otoliths were still located in the same focus plane. C. G. In the dorsal regions of the vesicle, protrusions grew out from the walls of the vesicle into its lumen. **D. H.** These protrusions met and fused (60-72 h) to form pillars of tissue spanning the lumen-the axial hubs of the three semicircular canals. Meanwhile the three cristae developed (48 h onwards), ventral to the horizontal canal hubs. Lateral views: anterior to the left, dorsal to the top with focus in the plane of the anterior otolith. Scale bar = 40 μm.

Although the general morphology of the otic vesicle was normal after triple MO injection, the development status of hair cells after treatment was still not clear. Via staining with anti-myosin 6, we found that the earliest tether cells developed normally in morphants compared to controls at 24 hpf, and four hair cells were separated into two groups at the anterior and posterior otic vesicle. With the development of the otic vesicle, the numbers of hair cells in the anterior macula and posterior macula and their location in the macula of morphants were similar to that of the controls (Figure 5A to D = controls; Figure 5E to H = morphants). By 72 hpf, all three cristae formed and hair cells were similar to those of the controls (Figure 5D = control; Figure 5H = morphant). Alternatively, we counted the number of cells positive for myosin 6 in the anterior macula and posterior macula and found no statistical difference between morphants and controls from 24 to 72 hpf (Figure 5I and J, $N \geq 10$, $P > 0.05$). Similar results were obtained when the hair cells of cristae were counted (Figure 5 K, $N \geq 10$, $P > 0.05$). These results suggest that it may be impossible to influence the specification, differentiation, or maintenance of hair cells in maculae and the sensory patches of cristae.

According to a previous study, the neurons that innervate the hair cells in the inner ear derive from neuroblasts that originate in the otic epithelium, but delaminate from it to form a

ganglion close beneath it from 22 hpf onward (Haddon and Lewis, 1996). Delaminated precursors finally develop into SAGs in the anterolateral and medial posterior floor of the otic vesicle, appearing comma-shaped (Haddon and Lewis, 1996) (Figure 6A to D). As in the control, the SAG in embryos injected with triple MOs developed into a comma-like shape (Figure 6E to H).

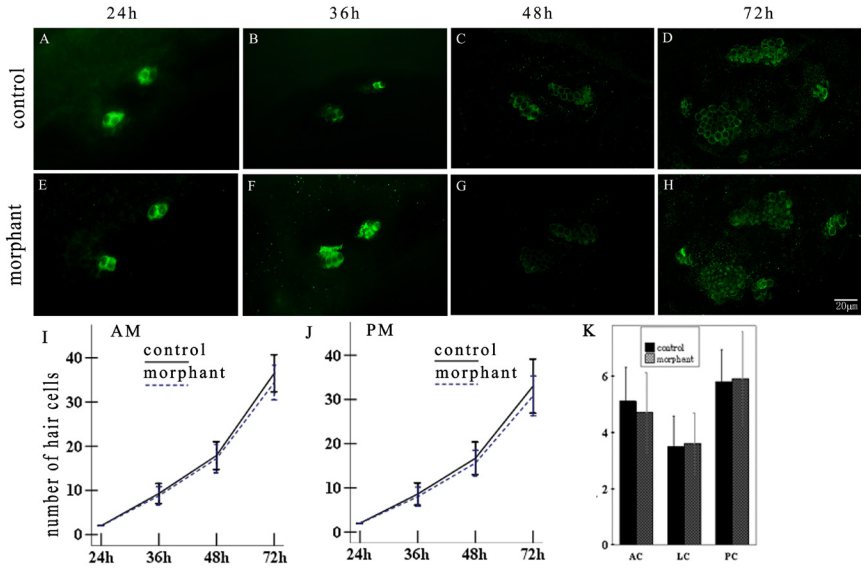

Figure 5. Development of hair cells after triple MO injection. Lateral views: anterior to the left, dorsal to the top, merging with focus in the plane of the anterior and posterior macula. Hair cells were stained by anti-myosin 6 coupled with green fluorescein. **A.** to **D.** Development of hair cells from 24 to 72 hpf in the inner ear of the control. **E.** to **H.** Development of hair cells from 24 to 72 hpf in the morphant inner ear. The development of hair cells in morphants (**E** to **H**) appeared no different from that of the control (**A** to **D**). **I.** and **J.** Number of hair cells in the anterior macula (**I**) and posterior macula (**J**) of morphants was similar to those of the control during inner ear development. K. Three cristae developed the same as the control by 72 hpf. AM = anterior macula; PM = posterior macula; AC = anterior cristae; LC = lateral cristae; PC = posterior cristae. Scale bar = 20 μm.

Abnormal development of olfactory epithelia after triple MO injection

According to these observations, miR-200 family members were robustly expressed in olfactory epithelium, consistent with a previous report (Choi et al., 2008). This suggests that olfactory epithelium would develop abnormally after the triple MO injection. Therefore, knockdown of all members of the miR-200 family could induce defects in olfactory development, and this would be a positive control for triple MO injection. We subsequently stained the olfactory filament with anti-acetylated tubulin, and found a remarkable reduction in filaments after knocking down all members of the miR-200 family with the triple MO injection (Figure 7). At 36 and 48 hpf, olfactory filaments formed a circle at the nose in the controls (Figure 7A, B and D). However, after knockdown of the miR-200 family olfactory filaments formed sporadic structures at 36 hpf (Figure 7E) and were greatly missing at 48 hpf (Figure 7F and H). Meanwhile, the nose epithelia at 48 hpf developed abnormally in the morphants (Figure 7G) compared with the controls (Figure 7C).

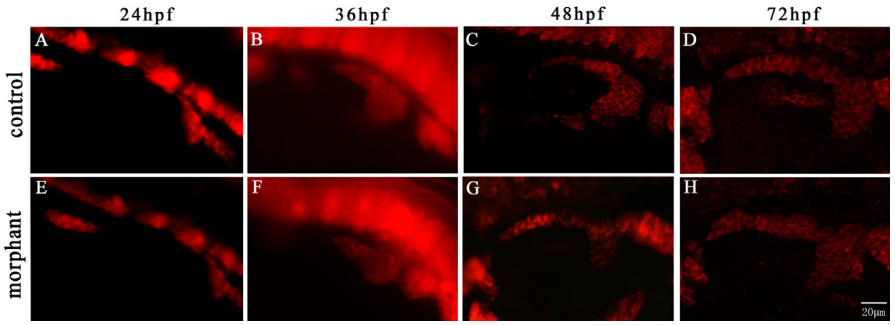

Figure 6. Development of SAG neurons in both the morphant and control groups. Lateral views: anterior to the right, dorsal to the top. The development of SAGs in morphants (**A** to **D**) was no different from that of the controls (**E** to **H**). **A. E.** By 24 hpf, the neurons of SAGs stained with anti-HuC (red) developed normally in the morphants and controls (**E** and **A**), like a tadpole. **B. F.** Increasingly differentiating SAG neurons gradually formed a comma-shaped structure with no significant difference between the morphants (**F**) and controls (**B**) by 36 hpf. **C.** to **D.** and **G.** to **H.** Increasingly, SAG neurons expanded forward to anterolateral and posteromedial ventral at 48 and 72 hpf with no difference between the morphants (**G** and **H**) and the controls (**C** and **D**). Scale bar = 20 μm.

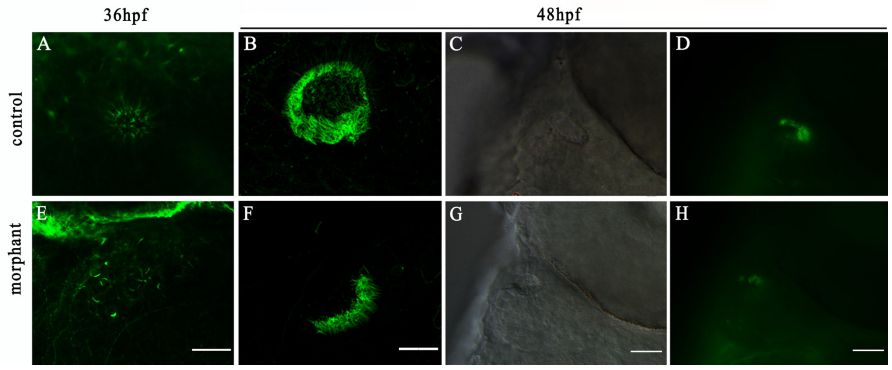

Figure 7. Olfactory epithelium developed abnormally after triple MO injection. Olfactory filaments were stained with anti-acetylated tubulin (green). **A.** to **D.** Olfactory of the control embryo. **E.** to **H.** Olfactory epithelium of the morphant embryo. **A. E.** Embryos at 36 hpf; **B.** to **D.** and **F**. to **H**. Embryos at 48 hpf. After injection of triple MOs, olfactory filaments remarkably decreased, as compared with the control at 36 hpf (**A.** control; **E.** morphant) and 48 hpf (**B.** control; **F.** morphant). **C.** (48 hpf, control) and **G.** (48 hpf, morphants) showed the development of nose epithelia under the DIC microscope. Nose epithelia developed abnormally after triple MO injection. **D. H.** Olfactory filaments stained with anti-acetylated tubulin, relative to **C.** and **G.** respectively. Scale bar = 20 μm.

Morphology and innervation of hair cells develop normally after triple MO injection

The functional hair cell in the inner ear of the zebrafish is characterized by stereocilia that form after the kinocilium. This could be observed by immunofluorescent staining with tubulin (anti-acetylated tubulin) for kinocilia and with F-actin (rhodamine phalloidin) for stereocilia. At 24 hpf, compared with the control inner ear (Figure 8A), the kinocilia of hair cells in the anterior and posterior macula in the morphant inner ear (Figure 8B) were similar. At 48 hpf, compared with that of the control inner ear (Figure 8C to E), the stereocilia of hair cells in the morphant inner ear (Figure 8F to H, hair cells were stained by anti-myosin 6 coupled with

green fluorescence) were normal. Therefore, the planar cell polarity of hair cells in the inner ear after triple MO injection was the same as that of the control.

Besides planar cell polarity, the innervations of hair cells from SAG neurons are also an important structure of inner ear function. At 48 hpf, the dendrites from the differentiated neurons (stained red with 3A10 antibody) (Yamada et al., 1991; Haddon and Lewis, 1996) projected into the ear epithelium and contacted the bottom of the control hair cells (Figure 9A to D). In morphants, the dendrites also reached the bottom of hair cells (Figure 9E to H). As above, the innervations of hair cells of the morphants and wild-type zebrafish were comparable.

Furthermore, the formation of synapses is also crucial to the transmission of impulses from hair cells to the central nervous system. C-terminal binding protein 2 (CTBP2, syn. RIBEYE), a protein component of the presynaptic ribbon, is exclusively localized in the basolateral portion of hair cells in a large puncta-like pattern (Zanazzi and Matthews, 2010; West and McDermott, 2011) (Figure 10 A` to D`). Acetylated tubulin is tubulin-associated protein that is expressed in both hair cells and neurons and can be detected with anti-acetylated tubulin antibody. With the section taken at the proper position, positive staining for acetylated tubulin could be observed in both hair cells and nerves that were in contact with each other. At 48 hpf, morphants and controls

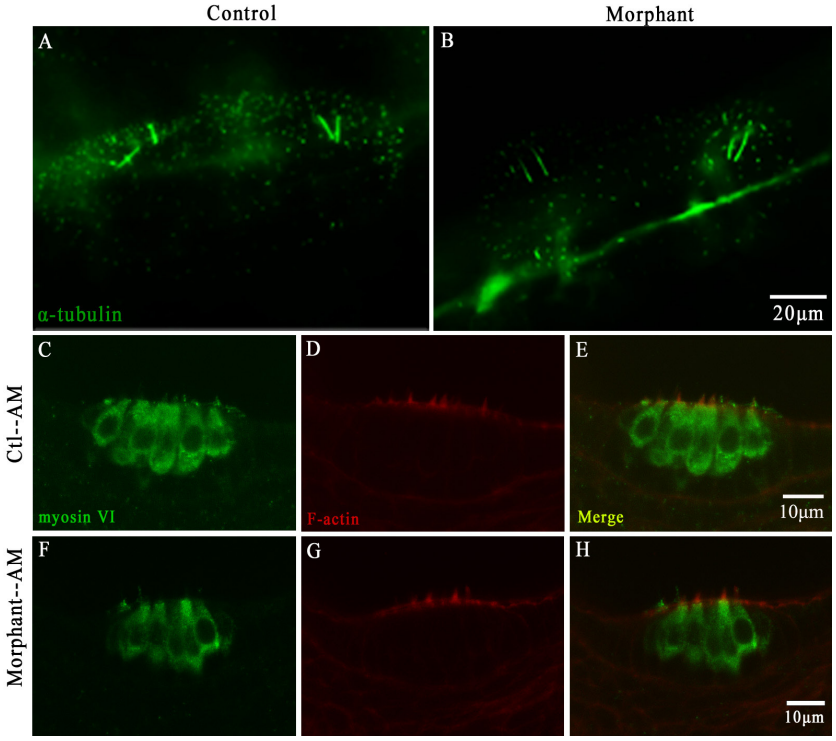

Figure 8. Plane cell polarity of hair cells in the inner ear developed normally in morphants and controls. **A.** and **B.** Kinocilia of hair cells (stained with anti-acetylated tubulin coupled with green fluorescein) developed normally in the morphant (**B**) and the control (**A**) by 24 hpf. **C.** to **E.** The stereocilia (marked by phalliodin with red fluorescein) developed upon the hair cells (stained by anti-myosin 6 coupled with green fluorescein) at the anterior macula of the control by 48 hpf. **F.** to **H.** Stereocilia (red) developed upon the hair cells (green) at the anterior macula of the morphant by 48 hpf, identical to that of the control (**C.** to **E.**). AM = anterior macula. Scale bar = 20 μm for **A.** and **B.** 10 μm for **C.** to **H**.

showed similar innervation of hair cells, stained with antibody for acetylated tubulin (Figure 10 A to D), and similar presynaptic components were also observed (Figure 10 A``` to D```).

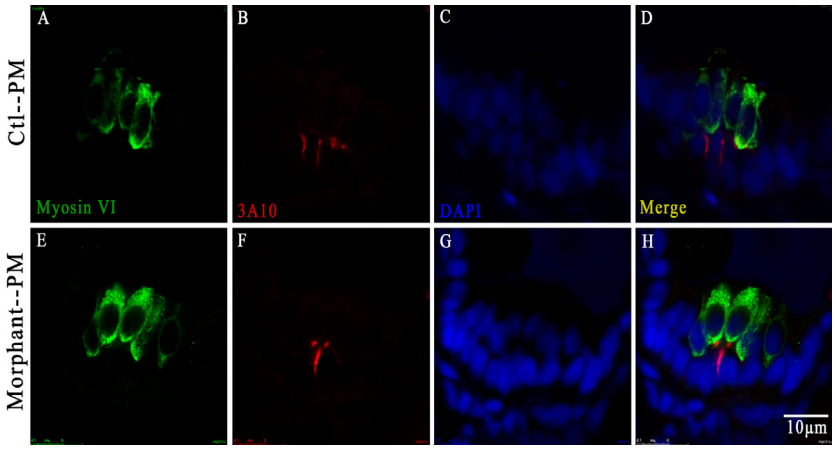

Figure 9. Innervation of hair cells from the SAG. At 48 hpf, the nerves innervating the hair cells were stained with 3A10 antibody (recognizing a phosphorylated neurofilament-associated antigen). **A.** to **D.** Cryosection (7 μm) of control zebrafish was stained with antibody 3A10 (red) for dendrities from SAG and anti-myosin 6 (green) for hair cells. **E.** to **H.** Cryosection (7 μm) of morphant zebrafish was stained with antibody 3A10 and anti-myosin 6 for dendrities and hair cells separately. PM = posterior macula. Scale bar = 10 μm.

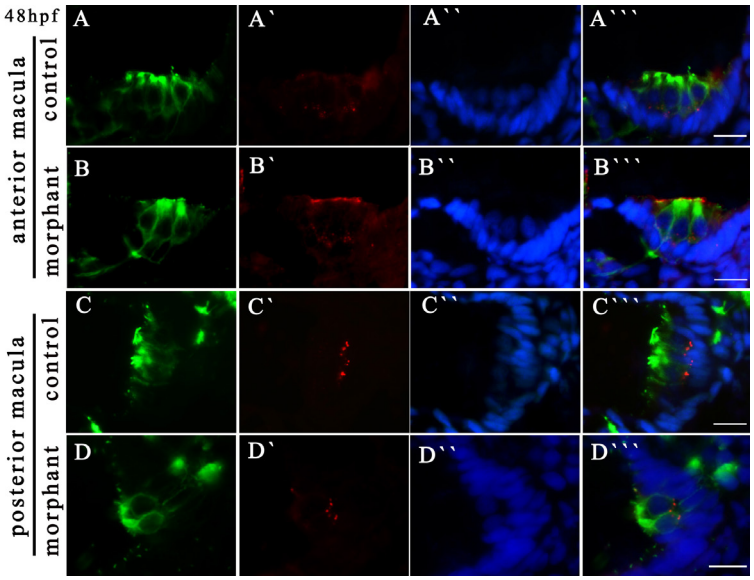

Figure 10. Development of auditory ribbon synapses of hair cells in morphants was similar to that of the controls at 48 hpf. **A.** to **D.** Staining for the acetylated-tubulin of hair cells and nerve fibers (green). **A.`** to **D.`** Dot-like pattern expression of CTBP2 to form the presynaptic ribbon component. **A.``** to **D.``** Nucleus stained by Hochest 33342 (blue). **A.```** to **D.```** Merging of hair cells, nerve fibers, and presynaptic ribbon component to display the exclusively basal pole expression of CTBP2. Scale bar = 10 μm.

DISCUSSION

In the present study, we investigated the expression patterns of miR-200 family members in the inner ears of zebrafish, as well in the olfactory epithelia, neuromasts, and taste buds. We found that miR-200 family members were weakly expressed and dispersed in the inner ears, but were abundant in the olfactory epithelia, taste buds, and neuromasts. After knockdown of miR-200 family members in developing inner ears, no significant changes in development were observed between these and normal (wild-type) ears. Otic vesicles, otoliths, and semicircular canals appeared normal. Compared with less differentiated filaments in olfactory epithelia, the development of hair cells and statoacoustic ganglion neurons were normal. The kinocilia and stereocilia of hair cells, the innervation of hair cells, and the formation of ribbon synapses were also unaffected. In brief, we did not observe pronounced variation of otoliths or related sensory epithelium and ganglion defects associated with knockdown of miRNAs.

Earlier studies have indicated that in zebrafish, the miR-200 family is expressed in many sensory epithelia. Advanced investigation has shown that miR-200 family members are involved in the development and differentiation of olfactory epithelia, taste buds, and eyes (Choi et al., 2008; Gessert et al., 2010; Kapsimali et al., 2011). Consistent with previous reports (Weston et al., 2006; Wang et al., 2010), our data also showed that miR-200 family members were expressed in the developing inner ear, as well in olfactory epithelia, taste buds, and neuromasts in zebrafish. However, all these miRNAs were weakly expressed in the inner ear neurosensory epithelia, compared with their robust expression in other neurosensory epithelia. Using embryonic injection of MOs, the present study showed that the expression of all the miR-200 family members was knocked down to an undetectable level via *in situ* hybridization. No significant changes in the developing inner ear were observed compared with the controls. However, consistent with a previous report (Choi et al., 2008), the olfactory epithelium (where miR-200 family members were robustly expressed) appeared with olfactory filaments that were less differentiated. Our results imply that miR-200 family members may not be required for the maturation of the inner ear but are required for the development of olfactory and gustatory systems (Choi et al., 2008; Kapsimali et al., 2011).

Although research on genes related to the gross development of the inner ear and the differentiation of hair cells have been remarkably comprehensive, some fine regulatory mechanisms controlled by these genes are still not completely clear. microRNAs are involved in such fine regulation through post-transcriptional inhibition. Therefore, investigations of the expression and function of microRNAs in the developing inner ear should give us some insight into the finely-tuned function of genes in inner ear development and hair cell regeneration. Our study and earlier reports showed that the miR-200 family could be sensory organ-specific. Unlike other sensory organ-specific miRNAs such as the miR-183 family which is involved in the development of all sensory organs, we found no evidence that the miR-200 family is required for the development of the inner ear in zebrafish. This suggests that the miR-200 family functions differently in different organs.

It was reported that the differentiation of olfactory neurons was promoted by miR-200, which may be mediated by lunatic fringe (*lfng*) and zinc-finger homeobox 1 (*zfhx1*), two key factors associated with the Notch and bone morphogenetic protein (BMP) pathways, respectively (Choi et al., 2008). The classical Notch pathway, a functional evolutionarily conserved signaling system, is important for the diversification of olfactory and auditory systems

(Kelley, 2006; Endo et al., 2012). In the early stage of the developing inner ear, the Notch signal promotes the proliferation of sensory epithelial precursors, but at a later stage, it regulates the differentiation of hair cells through lateral inhibition (Kelley, 2006; Jayasena et al., 2008; Kelley et al., 2009). In our study, the depletion of all members of the miR-200 family possibly led to a loss of *lfng* translation inhibition that might have enhanced the Notch signal transduction pathway (Johnston et al., 1997). However, there are other regulatory mechanisms related to the Notch signaling pathway, such as miR-34a mediated-inhibition of cell proliferation through targeting the molecules downstream of the Notch signaling pathway (Pang et al., 2010). Similarly, the BMP signal pathway is also required for the development of the inner ear. Previous studies observed that different doses of exogenous BMP4 had contrary effects on the development of hair cells in the explants of chicken embryos (Li et al., 2005; Pujades et al., 2006). There may be both autoregulatory loops and neighboring inhibition of BMP in the development of the inner ear (Pujades et al., 2006). After knockdown of miR-200, the autoregulatory loops of BMP signaling could balance the variation of zfhx1.

Moreover, the miR-200 family could also promote the differentiation of taste bud cells, in particular for Calb2b+ cell formation by targeting SRY (sex determining region Y)-box 2 (Sox2) (Kapsimali et al., 2011). Similar to its function in the development of taste buds, Sox2 is also important for the development of the inner ear (Dabdoub et al., 2008). The expression of Sox2 may be enhanced after depletion of miR-200 family. However, it was also reported that Sox2 is required for the maintenance and regeneration of hair cells in the zebrafish inner ear, rather than for initial development (Millimaki et al., 2010). Additionally, miR-200 family members are strong inducers of epithelial differentiation through epithelial-mesenchymal transition by targeting zeb1 and zeb2 (Mongroo and Rustgi, 2010; Brabletz et al., 2011; Hill et al., 2013). Although the miR-200 family is weakly expressed in the inner ear and may promote the differentiation of neurosensory epithelium, this weak expression also indicates that the genes regulated by the miR-200 family have no more than a subtle influence, as shown after knockdown of the miR-200 family. Especially, one gene could be targeted by many miRNAs, so a subtle change in the miR-200 family would not produce a significant effect at the level of post-transcription. Accordingly, no matter what the miR-200 family targets (for instance, the Notch signal, BMP signal, sox2, or zeb) with autoregulation, network dynamic regulation, and low expression of miR-200 family members, knockdown of the miR-200 family would not affect the development of the inner ear in zebrafish.

Altogether, our results and other recent reports show that the miR-200 family is expressed in many sensory organs including the olfactory epithelia and taste buds. We also found that miR-200 was weakly expressed in the zebrafish inner ear, and that depletion of the miR-200 family in the inner ear did not significantly change development. However, the olfactory filaments were significantly reduced after knocking down the miR-200 family. These results lead us to conclude that the miR-200 family appears to be dispensable for maturation of the zebrafish inner ear during the early stages, and that the functions of the miR-200 family may be organ-specific.

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