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Separate Na,K-ATPase genes are required for otolith formation and semicircular canal development in zebrafish

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Abstract

We have investigated the role of Na,K-ATPase genes in zebrafish ear development. Six Na,K-ATPase genes are differentially expressed in the developing zebrafish inner ear. Antisense morpholino knockdown of Na,K-ATPase α1a.1 expression blocked formation of otoliths. This effect was phenocopied by treatment of embryos with ouabain, an inhibitor of Na,K-ATPase activity. The otolith defect produced by morpholinos was rescued by microinjection of zebrafish α1a.1 or rat α1 mRNA, while the ouabain-induced defect was rescued by expression of ouabain-resistant zebrafish α1a.1 or rat α1 mRNA. Knockdown of a second zebrafish α subunit, α1a.2, disrupted development of the semicircular canals. Knockdown of Na,K-ATPase β2b expression also caused an otolith defect, suggesting that the β2b subunit partners with the α1a.1 subunit to form a Na,K-ATPase required for otolith formation. These results reveal novel roles for Na,K-ATPase genes in vestibular system development and indicate that different isoforms play distinct functional roles in formation of inner ear structures. Our results highlight zebrafish gene knockdown-mRNA rescue as an approach that can be used to dissect the functional properties of zebrafish and mammalian Na,K-ATPase genes.

Keywords: Zebrafish; Na,K-ATPase; Sodium pump; Morpholino; Inner ear; Otolith; Semicircular canal; mRNA rescue

Introduction

Na,K-ATPase, also known as the sodium pump, is a key enzyme essential for maintaining cellular homeostasis. By transporting sodium and potassium ions, the sodium pump establishes and maintains electrochemical gradients that underlie electrical excitability of nerve and muscle and the transport of numerous solutes and water across epithelia (Thomas, 1972). The active enzyme is a major consumer of ATP, and is also the cellular receptor for cardiac glycoside drugs that have historically been used in the treatment of congestive heart failure and cardiac arrhythmias.

The sodium pump is composed of two functionally required subunits, α and β, which are present in equimolar amounts. A putative third subunit, termed γ, has been identified, although this subunit does not appear to be essential for Na,K-ATPase activity (Scheiner-Bobis and Farley, 1994). Multiple isoforms of each of the α and β subunits have been described in a variety of species. In mammals, four isoforms of the α subunit (Herrera et al., 1987; Shamraj and Lingrel, 1994; Shull et al., 1986) and three separate β subunit isoforms (Malik et al., 1996; Martin-Vasallo et al., 1989; Mercer et al., 1986) have been identified. Each α and β subunit is encoded by a distinct gene, and each gene is expressed in a unique tissue and developmentally regulated fashion. Most strikingly, in vitro experiments show

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that each α subunit is capable of interaction with any of the β subunits to form a functional enzyme (Crambert et al., 2000; Lemas et al., 1994; Schmalzing et al., 1997).

The potential for promiscuity in α/β subunit interactions raises the possibility for 12 different Na,K-ATPase isoenzymes in mammals. The situation in zebrafish is even more complex, with 9 α subunit and 6 β subunit genes having been identified (Blasiole et al., 2002; Rajarao et al., 2001, 2002). Thus, zebrafish have the potential for 54 possible α/β subunit combinations. A critical gap in our understanding of Na,K-ATPase diversity is whether the proteins encoded by each gene have unique or redundant physiological functions. One issue is that the different α/β subunit combinations produced in vitro all seem to have very similar biochemical properties (Crambert et al., 2000; Jewell and Lingrel, 1991). Attempts to address the question of Na,K-ATPase isoform diversity in knockout mice have been only partially successful, as homozygous null mutations at the α1 or α2 loci produce embryonic lethality (James et al., 1999), and homozyzous β2 mutant mice die shortly after birth (Magyar et al., 1994).

Several lines of evidence suggest that the question of Na,K-ATPase isoform diversity may be addressed in zebrafish. Experiments carried out by Shu et al. (2003) have shown that the zebrafish cardiac mutant *heart and mind* is caused by a defect in the Na,K-ATPase α1a.1 gene. The *heart and mind* mutant phenotype could be rescued by α1a.1 mRNA but not the zebrafish α2 subunit mRNA, indicating that these two Na,K-ATPase α subunits play distinct roles in zebrafish cardiac development and function.

In this report, we have investigated the role of several Na,K-ATPase genes in zebrafish inner ear development utilizing antisense morpholino gene knockdown. Knockdown of α1a.1, an α subunit gene previously shown to be required for normal myocardial (Shu et al., 2003) and brain ventricle (Lowery and Sive, 2005) development in zebrafish, resulted in otolith agenesis. Otoliths (or otoconia in mammals) are calcium carbonate deposits within the inner ear that facilitate the transmission of vibration and acceleration forces to macular hair cells, which is required for hearing and balance. Knockdown of a closely related α subunit, α1a.2, caused morphological defects in semicircular canals, which are inner ear structures responsible for sensing angular acceleration (Chang et al., 2004b).

The studies presented here indicate that different Na,K-ATPase genes are required for development of distinct components of the zebrafish inner ear. Our results highlight the utility of the zebrafish system for dissecting functional differences amongst zebrafish Na,K-ATPase isoforms. Rescue experiments of the type we describe show that zebrafish can be useful for exploring the functional significance of mammalian Na,K-ATPase gene diversity as well.

Materials and methods

Antisense morpholinos and phenotypic rescue

The antisense morpholinos (MOs; Gene Tools LLC; Philomath, OR) targeted against individual Na,K-ATPase α and β subunit genes are presented in Table 1. Two independent MOs were targeted against each gene. The MOs were resuspended in 1× Danieau buffer (58 mM NaCl, 0.7 mM KCl, 0.4 mM $MgSO_4$, 0.6 mM $Ca(NO_3)_2$, 5 mM HEPES, pH 7.6) and injected into the yolk of single-cell zebrafish embryos. MOs were 3′ labeled with either FITC or Lissamine to monitor for uniform oligonucleotide distribution in injected embryos.

The ability of a MO to specifically block translation of its cognate mRNA was analyzed using an in vitro translation assay. Full-length mRNAs were transcribed from each Na,K-ATPase gene (Table 1) using the mMESSAGE mMACHINE transcription kit (Ambion; Austin, TX). Protein synthesis was initiated with the addition of 0.5 μg of mRNA to a rabbit reticulocyte lysate translation mix (Ambion). Proteins were labeled by the addition of [^{35}S]-methionine to the translation reaction. The translation of each mRNA was tested in the presence or absence of MOs (4 μM). The entire in vitro reaction mixture (20 μl) was loaded onto a 10% SDS-polyacrylamide gel and fractionated by electrophoresis. The gels were dried, exposed to X-ray film, and the relative intensity of the bands quantified by densitometry.

For phenotypic rescue, full-length open reading frames (ORFs) corresponding to zebrafish α1a.1, α1a.2, and rat α1 Na,K-ATPase genes were synthesized by PCR from cDNA constructs (Rajarao et al., 2001). Rescue mRNA constructs were designed with a minimal Kozak consensus sequence adjacent to the initiating ATG so as not to match the native 5′-UTR MO target sequence. Each PCR product was verified by DNA sequence analysis, subcloned into the zebrafish expression vector pT3TS (Hyatt and Ekker, 1999), and used to synthesize full-length capped mRNA. Rescue mRNAs were injected into one-cell stage embryos either alone or in the presence of an antisense MO. For each rescue experiment, the amount of mRNA injected was titrated for the maximal dose that could be injected without causing toxicity to embryos.

Ouabain treatment

Zebrafish embryos were treated with ouabain essentially as described (Shu et al., 2003). Briefly, wild-type zebrafish embryos were raised in charcoal-filtered

Table 1
Na,K-ATPase antisense morpholinos

Morpholino	Targeted gene[a]	Target[b]	Morpholino sequence
α1a.1 MO-1	*atp1a1a.1* (α1a.1)	−7 to +18	5′-gccttctcctcgtcccattttgctg-3′
α1a.1 MO-2	*atp1a1a.1* (α1a.1)	−32 to −8	5′-cttttgattaaaatcgaccaactgg-3′
α1a.2 MO-1	*atp1a1a.2* (α1a.2)	−20 to +5	5′-tgccttgttgacttgtttggagaca-3′
α1a.2 MO-2	*atp1a1a.2* (α1a.2)	−52 to −28	5′-cccatttttccaagtttatttcaacc-3′
β1a MO-1	*atp1b1a* (β1a)	−1 to +24	5′-gtcaccatctttatttgcgggcatt-3′
β1a MO-2	*atp1b1a* (β1a)	−28 to −4	5′-cggtatttagttccctttttggtgg-3′
β2b MO-1	*atp1b2b* (β2b)	−25 to −1	5′-tttcctggactaaatgtcgctcac-3′
β2b MO-2	*atp1b2b* (β2b)	−78 to −54	5′-ctttgagagtaataaagagctattc-3′
Standard Control			5′-cctcttacctcagttacaatttata-3′

[a] The GenBank accession numbers for the genes are as follows: α1a.1, AF286372; α1a.2, AF286374; β1a, AF286375; β2b, AF373976.
[b] Target sequence of mRNA with $^{+}$1 corresponding to the adenosine of the initiating methionine.

H_2O up to 6 h post fertilization (hpf), then transferred into water containing a final concentration of 2 mM ouabain (Sigma; St. Louis, MO). Embryos were grown in the presence of ouabain until 24 hpf, washed three times in charcoal-filtered water, and grown for an additional 12 h in the absence of ouabain before scoring for the presence or absence of otoliths.

PCR mutagenesis and transfection

A ouabain-resistant zebrafish α1a.1 cDNA (α1a.1 OR) was generated by changing the nucleotides coding for the two amino acid residues located at the borders of the first extracellular domain (L121 → R and N132 → D) using PCR mutagenesis as described (Dahl et al., 2000). These mutations have previously been used to generate ouabain-resistant sheep α1 and rat α2 Na,K-ATPase α subunits (Canfield et al., 1990; Price et al., 1990). The mutated cDNA was verified by DNA sequencing, subcloned into the pCS2+ expression vector (Turner and Weintraub, 1994), and transfected into HEK293 cells using the Effectene transfection reagent (Qiagen; Valencia, CA). Cells were grown in Dulbecco's modified Eagle's medium (DMEM; Gibco; Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS; Gibco). Two days after transfection, cells were washed with phosphate buffered saline and cultured in DMEM + 10% FBS containing 0.5 μM ouabain. The appearance of ouabain-resistant colonies was scored 7 days after transfection.

RNA expression

Whole-mount in situ hybridization was performed as previously described (Thisse et al., 1994). Antisense RNA probes used in this study were *ncs-1a* (Blasiole et al., 2005), *dfna5* (Busch-Nentwich et al., 2004), and *ugdh* (Walsh and Stainier, 2001).

Histological analysis

Wild type and morphant embryos were fixed in 1.5% gluteraldehyde/0.5% paraformaldehyde in BT fix (Westerfield, 1993), and stored at 4°C. Microwave-accelerated processing was used for resin-embedding. Using a microwave setting of 182W for 40 s under vacuum, specimens were moved through 0.1 M Cacodylate buffer, pH 7.4 (2×), 1% osmium tetroxide/1.5% potassium ferricyanide (1 min without microwave; 40 s on; 3 min off), water (2×), graded ethanols (30%, 50%, 70%, 90%, 100%) and acetone. Using a microwave setting of 294W for 3 min under vacuum, specimens were immersed in 1:1 acetone: resin and 3 cycles of 100% resin (equal parts of Spurrs resin and Epon). The resin was then polymerized at 60°C for 2 days. One-micron sections were cut with a glass knife on an ultramicrotome and stained with 1% toluidine blue in water. Sections were analyzed using a Nikon Eclipse E800 microscope equipped with a 60× Plan Apo lens. Scanning electron microscopy was performed as previously described (Hughes et al., 2004).

Immunofluorescence

Embryos were anesthetized in 10 mg/ml Tricaine in E3 embryo medium (Nüsslein-Volhard and Dahm, 2002) and fixed in 4% paraformaldehyde in PBS for 8 h. They were washed in PBST (PBS with 0.1% Triton) for 2 days, placed in 1% Triton X-100 in PBS overnight to dissolve the otoliths, washed for several hours in PBST and immersed in blocking solution (2% goat serum, 0.1% DMSO, 1% bovine serum albumin in PBST) overnight before incubating for 1 day in primary antibody. All previous steps were at 4°C. Primary antibodies used were anti-HCS-1 (1:250; Gale et al., 2000), anti-acetylated tubulin monoclonal antibody (1:1000; Sigma), and anti-HuC (1:50; Molecular Probes; Carlsbad, CA; Raible and Kruse, 2000) diluted in blocking solution. Embryos were washed in PBST followed by overnight incubation at 4°C with secondary antibody diluted in blocking solution. HCS-1 immunoreactivity was visualized using goat anti-mouse IgG_{2a}-Alexa 568 secondary antibody (1:500; Molecular Probes), whereas tubulin immunoreactivity was visualized with goat anti-mouse IgG_{2b}-Alexa 488 secondary antibody (1:500; Molecular Probes). HuC immunoreactivity was visualized with donkey anti-mouse Ig-Alexa 488 (1:500; Molecular Probes). Specimens were then washed in PBST, mounted in 70% glycerol and examined using conventional fluorescence or confocal laser microscopy. Embryos used for tether cell differentiation analyses were collected at 18.75, 20, 22, 24, 26, 28, and 30 hpf.

Functional assay for hair cells

To assay functional channels in hair cells of the inner ear, 40 mM FM1-43 (Molecular Probes) diluted in extracellular solution (120 mM NaCl, 2 mM KCl, 10 mM HEPES, 2 mM $CaCl_2$, and 0.7 mM NaH_2PO_4, pH 7.30) was pressure-ejected directly into the fluid cavity of the otocyst of 58–64 hpf morphants. A 1-mm-diameter glass pipette was inserted into the otocyst under visual control using a 63× objective on a Zeiss Axioskop 2 FS microscope (Corey et al., 2004). Uptake of the dye was analyzed within 2 min of injection.

Results

Knockdown of Na,K-ATPase α1a.1 gene expression blocks otolith formation

Expression of Na,K-ATPase genes in zebrafish inner ear is complex, with transcripts encoding four α1-like and two β subunits exhibiting distinct spatiotemporal distribution patterns (Blasiole et al., 2003). To analyze the functional properties of these diverse Na,K-ATPase genes, we have utilized antisense morpholinos to knock down translation of Na,K-ATPase mRNAs in developing zebrafish. An advantage of the morpholino knockdown approach is that it is possible to generate hypomorphs at specific Na,K-ATPase alleles (Cheng et al., 2003). By titrating the dose of morpholinos, recognizable phenotypes can be produced without inducing lethality in early embryos. Two independent, non-overlapping MOs (α1a.1 MO-1 and α1a.1 MO-2) were targeted to the 5′ UTR and translational start site of α1a.1 (Table 1). The specificity of the α1a.1 MOs was analyzed by testing the ability of each MO to block α1a.1 mRNA translation using an in vitro translation assay. As shown in Fig. 1A, the translation of α1a.1 mRNA was reduced in the presence of either α1a.1 MO-1 or α1a.1 MO-2. Translation of α1a.1 mRNA was unaffected by MOs targeted against either the Na,K-ATPase α1a.2 (α1a.2 MO-1) or β2b (β2b MO-1) subunit genes (Fig. 1A). These in vitro results provide strong support for the idea that α1a.1 MO-1 and α1a.1 MO-2 should function relatively specifically in vivo to block α1a.1 mRNA translation in developing zebrafish.

Microinjection of α1a.1 MO-1 into one-cell stage zebrafish embryos produced a striking effect on inner ear development (Figs. 1B, C). By 24 hpf 100% of embryos injected with ≥0.25 ng of α1a.1 MO-1 failed to develop otoliths (Table 2). Microinjection of a second nonoverlapping morpholino (α1a.1 MO-2) targeted against α1a.1 mRNA also blocked otolith formation (Table 2). Injection of 8 ng of α1a.1 MO-2 blocked otolith formation in 69% of embryos (46/67). The otolith defect produced by the α1a.1 MO-1 morpholino was dose-dependent. Injection of 0.125 ng of α1a.1 MO-1 gave phenotypes ranging from the normal two otoliths per otocyst to one otolith per otocyst to one very small otolith paired with one large dysmorphic otolith (Fig. 5E). A small percentage (~6%) of morphant embryos that had completely failed to develop otoliths by 30 hpf exhibited rudimentary otoliths by 48 hpf.

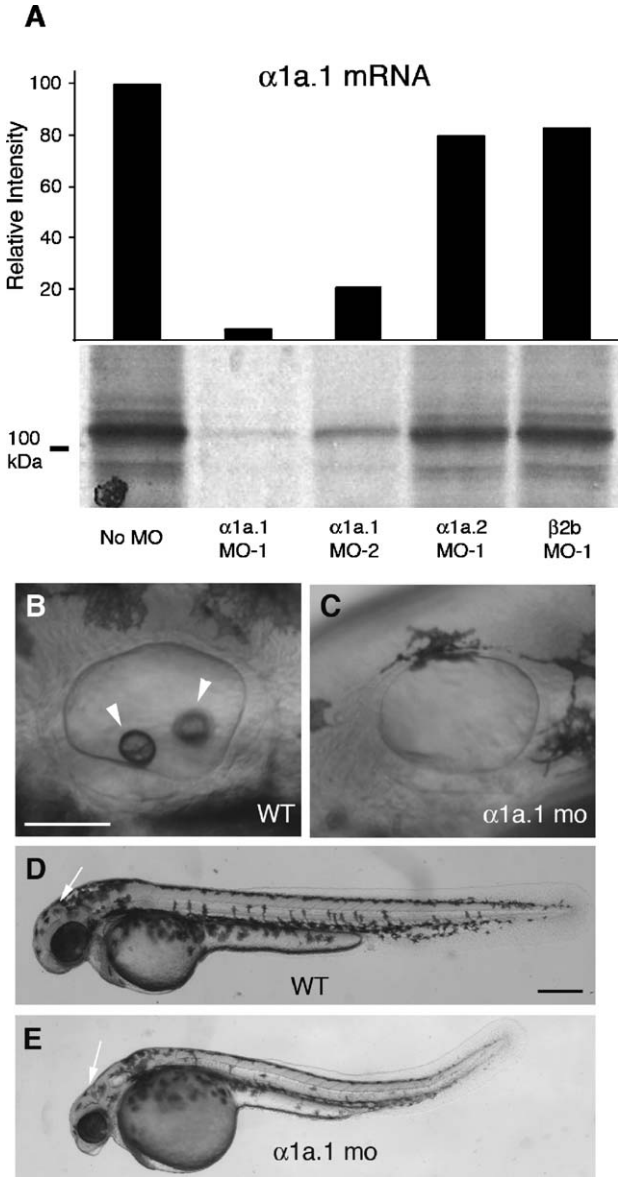

Fig. 1. Na,K-ATPase α1a.1 MOs block otolith formation. (A) Effect of antisense morpholinos on α1a.1 mRNA translation. α1a.1 mRNA was translated in vitro using a rabbit reticulocyte lysate system in the presence of 4 μM antisense MOs. [^{35}S]-methionine-labeled in vitro translation products were analyzed on a 10% SDS-polyacrylamide gel (lower panel). Molecular weight marker is shown at the left. Intensity of the bands relative to control was quantified by densitometry (upper panel). (B–E) Morphants were injected with 0.25 ng of α1a.1 MO-1. Lateral view with anterior to the left. (B) Otic vesicle (OV) of wild type (WT) embryo at 45 hpf. (C) OV of α1a.1 morphant at 45 hpf. (D) WT embryo at 45 hpf. (E) α1a.1 morphant at 45 hpf. Arrowheads indicate otoliths. Arrow indicates midbrain–hindbrain boundary. mo, morphant. Scale bars: A–B, 50 μM; C–D, 250 μM.

These results suggest that expression of α1a.1 is essential for formation of otoliths.

In addition to defective otolith development, α1a.1 morphants exhibited a decrease in the size of the otic vesicle and a delayed formation of semicircular canal pillars. At 45 hpf, the morphants exhibited a poorly defined midbrain–hindbrain boundary, and the brain and eyes appeared smaller compared to wild-type embryos (Figs. 1D, E). α1a.1 morphants exhibited a weak heartbeat, no evidence of circulation, and pericardial edema (data not shown). More than 65% of the morphants failed to survive past 4 days of development, at which point they were noticeably smaller than wild-type embryos, had abnormally curved tails, and exhibited brain necrosis and degeneration (data not shown). These defects are similar to those previously reported for zebrafish α1a.1 gene mutants (Lowery and Sive, 2005; Shu et al., 2003; Yuan and Joseph, 2004).

To further confirm that defective otolith development was caused by a reduction in Na,K-ATPase α1a.1 function, we treated wild-type zebrafish embryos with ouabain, a specific inhibitor of Na,K-ATPase. Ouabain treatment has previously been shown to phenocopy the cardiac defects caused by mutations in the α1a.1 gene in *small heart* (Yuan and Joseph, 2004) and *heart and mind* (Shu et al., 2003) mutant zebrafish. We confirmed that ouabain altered heart morphogenesis, and also observed that all embryos treated with 2 mM ouabain from 6 to 24 hpf failed to develop otoliths (87/87 embryos scored at 36 hpf). The effect of ouabain on otolith formation was found to be dose and time dependent (Blasiole, 2005). These results are consistent with the view that Na,K-ATPase activity is required for proper otolith development. Later effects of ouabain could not be assessed due to death of embryos at ~45 hpf.

mRNA rescue of Na,K-ATPase α1a.1 morphants

To provide additional confirmation that the Na,K-ATPase α1a.1 subunit plays an essential role in otolith biogenesis, we asked whether α1a.1 mRNA could rescue the otolith defect produced either by morpholino injection or ouabain treatment. As shown in Fig. 2 and Table 2, coinjection of embryos with α1a.1 rescue mRNA and α1a.1 MO-1 restored otolith formation in 48% of embryos (16/33). When used to program protein synthesis in vitro, α1a.1 rescue mRNA translation was not blocked by either α1a.1 MO-1 or α1a.1 MO-2 (Fig. 2E). We also tested the ability of rat Na,K-ATPase α1 mRNA to restore otolith formation in α1a.1 morphants. As shown in Table 2, the otolith defect was rescued in 44% (12/27) of embryos coinjected with α1a.1 MO-1 and rat α1 mRNA. In addition to correcting the otolith defect, injection of α1a.1 morphants with either zebrafish α1a.1 or rat α1 mRNAs appeared to rescue other aspects of the morphant phenotype and extended embryonic survival.

To test whether the ouabain-induced otolith defect could also be corrected by α1a.1 mRNA rescue, we converted the

Table 2
α1a.1 knockdown and mRNA rescue

α1a.1 MO-1 (0.25 ng)	α1a.1 MO-2 (8 ng)	α1a.1 rescue mRNA (125 pg)	rat α1 mRNA (0.25 ng)	Presence of otoliths	
				Number/total	Percent
+	−	−	−	0/86	0%
+	−	+	−	16/33	48%[a]
+	−	−	+	12/27	44%[b]
−	+	−	−	21/67	31%

[a] $P < 0.001$, Chi-squared = 48.17, degrees of freedom: 1.
[b] $P < 0.001$, Chi-squared = 42.76, degrees of freedom: 1.

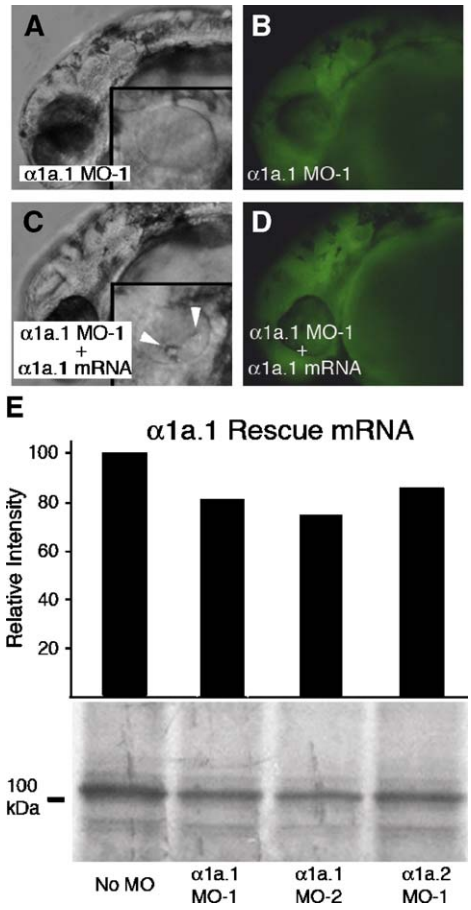

Fig. 2. mRNA rescue of α1a.1 morphant. Coinjection of 125 pg of α1a.1 rescue mRNA and 0.25 ng of α1a.1 MO-1 into one-cell stage embryos. All panels show a lateral view with anterior to the left. (A) α1a.1 morphant at 36 hpf injected with 0.25 ng of α1a.1 MO-1 alone. Inset is an enlarged view of the otic vesicle (OV) lacking otoliths. (B) Fluorescent image of panel A confirming presence of FITC-labeled morpholino in embryos. (C) 36 hpf embryo coinjected with α1a.1 MO-1 (0.25 ng MO-1) and α1a.1 rescue mRNA (125 pg). Inset is an enlarged view of the OV containing two otoliths. Arrowheads indicate otoliths. (D) Fluorescent image of panel C. (E) Effect of MOs on translation of α1a.1 rescue mRNA. The region adjacent to the initiating ATG of α1a.1 rescue mRNA was engineered to contain a minimal Kozak consensus sequence so as not to match the targeting MO. The α1a.1 rescue mRNA was translated in the presence of 4 μM antisense MOs and analyzed as described in Fig. 1A.

ouabain-sensitive zebrafish α1a.1 subunit into a ouabain-resistant isoform using site-directed mutagenesis (Canfield et al., 1990; Price et al., 1990). When transfected into ouabain-sensitive human HEK 293 cells, the mutated α1a.1 cDNA conferred ouabain-resistance to the cells. Ouabain resistant cells were capable of proliferating in the presence of 0.5 μM ouabain, a drug concentration that is normally cytotoxic to HEK 293 cells (Dahl et al., 2000). Injection of the cognate mRNA for the ouabain-resistant α1a.1 isoform rescued the otolith defect in 44% (21/46) of ouabain-treated zebrafish embryos. Our data shows that the zebrafish α1a.1 gene can form a functional Na,K-ATPase in mammalian cells, and that the rat α1 gene is functional in zebrafish. These results suggest that the essential function of Na,K-ATPase in otolith biogenesis may be conserved between fish and mammals.

Na,K-ATPase α1a.2 gene knockdown disrupts semicircular canal development

In addition to the α1a.1 gene, the Na,K-ATPase α1a.2 gene is also expressed in the developing zebrafish inner ear (Blasiole et al., 2003). Although these paralogous zebrafish genes show 81% amino acid sequence identity (Rajarao et al., 2001), they are differentially expressed during inner ear development (Blasiole et al., 2003). To analyze the role of the α1a.2 gene in otic morphogenesis, we used two independent non-overlapping α1a.2 MOs to knock down expression of α1a.2 mRNA in developing zebrafish embryos. By 45 hpf, the overall morphology and development of embryos injected with 2 ng of α1a.2 MO-1 appeared similar to that of uninjected controls (Figs. 3A, B), and all embryos analyzed (68/68) had developed otoliths. However, while semicircular canal epithelial protrusions were evident in uninjected embryos, they were absent in 83% (n = 86) of α1a.2 morphants (Figs. 3C, D). Microinjection of 6 ng of α1a.2 MO-2 phenocopied the semicircular canal defect in 35% of embryos (n = 73). In uninjected control embryos at 5 days post fertilization (dpf), the semicircular canal pillars were recognized by their characteristic cruciform appearance (Fig. 3E). In 5 dpf α1a.2 morphants, however, a ball-like mass of epithelial tissue was observed in the center of the otic vesicle (Fig. 3F).

We used an in vitro translation assay to confirm the targeting specificity of the α1a.2 MOs. As shown in Fig. 1A, α1a.2 MO-1 did not block translation of α1a.1 mRNA. When used to program protein synthesis in vitro, synthetic α1a.2 mRNA produced a polypeptide of the expected size (Fig. 3G). The translation of α1a.2 mRNA was reduced in the presence of either α1a.2 MO-1 or α1a.2 MO-2, and was unaffected by MOs targeted against either the Na,K-ATPase α1a.1 or β2b genes (Fig. 3G). These results are consistent with the view that the α1a.2 MOs specifically block α1a.2 mRNA translation.

We further analyzed the nature of the semicircular canal defect in α1a.2 morphants using *ncs-1a* (Blasiole et al., 2005), *dfna5* (Busch-Nentwich et al., 2004), and *ugdh* (Walsh and Stainier, 2001) as markers of epithelial pillars of semicircular canals. At 48 hpf, each of these markers was expressed in the otic epithelium and budding epithelial pillars of wild type embryos (Figs. 4A, E, and I), while at 72 hpf the markers were visible in the mature epithelial pillars (Figs. 4C, G, and K). In morphant embryos at 48 hpf, the markers were observed in the otic epithelium (Figs. 4B, F, and J), while at 72 hpf, the markers were present in the otic epithelium and in mislocalized semicircular canal protrusions (Figs. 4D, H, and L). These results suggest that α1a.2 knockdown does not affect *ncs-1a*- or *dfna5*/*ugdh*-mediated pathways of semicircular canal development.

The distinct inner ear defects that result from α1a.1 and α1a.2 gene knockdowns suggests that the α1a.1 and α1a.2 isoforms may play functionally unique roles in vestibular system development. To test this idea, we asked whether the α1a.1 and α1a.2 genes could compensate for each other using an mRNA knockdown-rescue protocol. Coinjection of 250 pg of α1a.2 mRNA with 0.25 ng α1a.1 MO-1 was unable to rescue

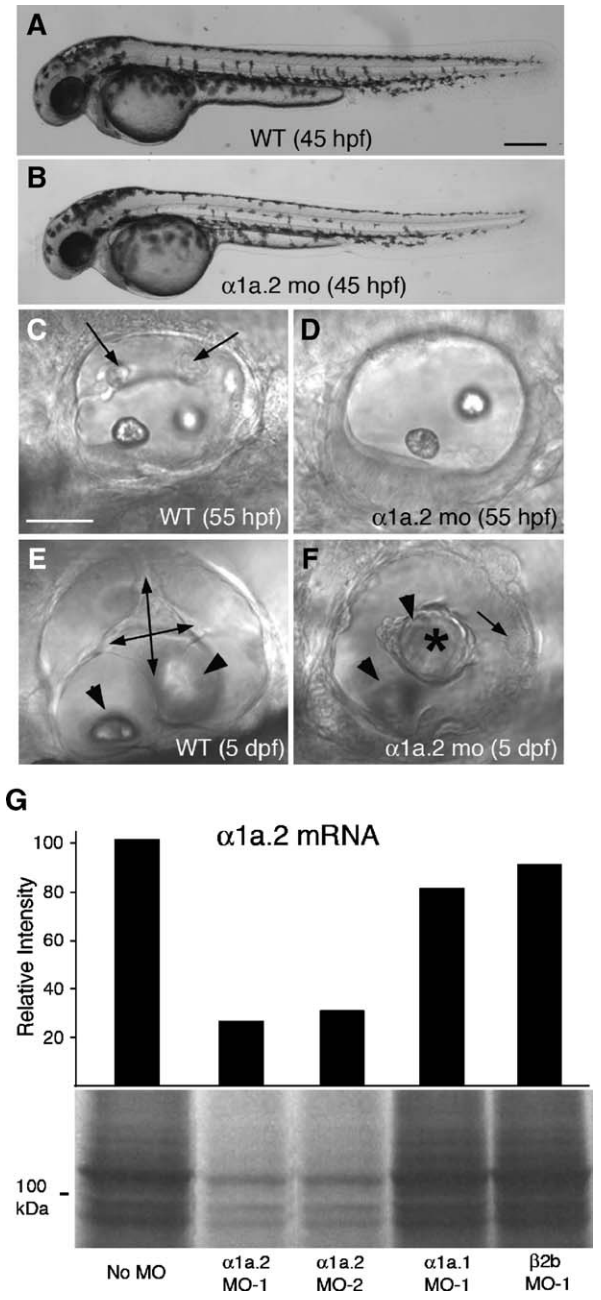

Fig. 3. Knockdown of Na,K-ATPase α1a.2 mRNA disrupts development of semicircular canals. All panels show a lateral view with anterior to the left. Morphants were injected with 2 ng of α1a.2 MO-1. (A) Wild type (WT) embryo at 45 hpf. (B) α1a.2 morphant at 45 hpf. (C) Otic vesicle (OV) of WT embryo at 55 hpf. Arrows indicate protrusions of semicircular canals. (D) OV of α1a.2 morphant at 55 hpf. (E) OV of WT embryo at 5 dpf. Double-headed arrows show hubs of the semicircular canals. Arrowheads indicate otoliths. (F) OV of α1a.2 morphant at 5 dpf. Asterisk shows epithelial mass in center of OV. Arrowheads indicate otoliths. Arrow indicates the posterior crista. (G) Effect of MOs on translation of α1a.2 mRNA. α1a.2 mRNA was translated in the presence of antisense MOs (4 μM) and analyzed as described in Fig. 1A. mo, morphant. Scale bars: A–B, 250 μM; C–F, 50 μM.

the otolith defect (0%, n = 33), while coinjection of 150 pg of α1a.1 rescue mRNA with 2 ng of α1a.2 MO-1 failed to rescue the semicircular canal defect (0%, n = 24). Although these results suggest that α1a.1 cannot compensate for loss of α1a.2 activity and vice versa, it is important to note that neither α1a.1 nor α1a.2 mRNAs can rescue the semicircular canal defect in α1a.2 morphants. The failure of either mRNA to rescue the semicircular canal defect may reflect inability of the injected mRNAs to remain intact until the time at which semicircular canals begin to form.

Na,K-ATPase β2b gene is required for otolith formation

To investigate the role of the Na,K-ATPase β subunit in zebrafish ear development, we used antisense MOs to knock down β subunit mRNA expression in the developing zebrafish ear. Two Na,K-ATPase β subunit genes, β1a and β2b, are differentially expressed in the developing inner ear (Blasiole et al., 2003). Two independent non-overlapping MOs were targeted against the 5′ UTR of the β1a gene and tested for specificity in the in vitro translation assay. Each of the β1a MOs blocked translation of β1a but not β2b mRNA (data not shown). When microinjected into one-cell stage zebrafish embryos, neither 2 ng of β1a MO-1 (0/83) or 0.5 ng β1a MO-2 (0/47) alone or in combination, was found capable of blocking otolith biogenesis (data not shown).

We also generated two independent MOs to target the β2b gene. Each of these β2b-specific MOs blocked translation of β2b but not β1a mRNA in the in vitro translation assay (data not shown). Microinjection of β2b MO-1 (6 ng) in zebrafish produced a noticeable effect on otolith biogenesis (Fig. 5). Abnormal otolith phenotypes occurred in 94% (n = 53) of β2b morphant embryos. Various phenotypes were observed ranging from one very small otolith paired with one large dysmorphic otolith, to a third ectopic otolith, to one otolith per otocyst (Fig. 5D). At 72 hpf, it was noted that β2b morphants also exhibited a delay in semicircular canal formation. Microinjection of β2b MO-2 (8 ng) phenocopied the otolith defects seen with β2b MO-1 (data not shown), suggesting that the β2b isoform plays a role in zebrafish otolith development.

Neither β2b MO-1 or β2b MO-2, injected alone or in combination, completely blocked formation of otoliths. However, the various otolith defects produced by knockdown of β2b mRNA translation were strikingly similar to some of the abnormal otolith phenotypes produced by low dose injection of α1a.1 MO-1 (<0.25 ng; Fig. 5E), suggesting a possible functional relationship between the α1a.1 and β2b subunits. To test this idea, we coinjected sub-effective doses of α1a.1 MO-1 (0.125 ng) and β2b MO-1 (2 ng) and analyzed the effect on inner ear development. Injection of 0.125 ng of α1a.1 MO-1 alone produced abnormal otoliths in only 2/22 embryos (9%), while no loss of otoliths was detected in embryos injected with 2 ng of β2b MO-1 alone (n = 20) (Fig. 6, Table 3). However, coinjection of α1a.1 MO-1 (0.125 ng) and β2b MO-1 (2 ng) caused complete inhibition of otolith development in 93% of embryos (n = 30; Fig. 6C, Table 3). In contrast, coinjection of sub-effective doses of α1a.1 MO-1 (0.125 ng) and β1a MO-2 (0.5 ng) did not cause any apparent defects in otolith development in 100% of embryos tested (n = 13; Table 3). These results indicate that the Na,K-ATPase required for otolith

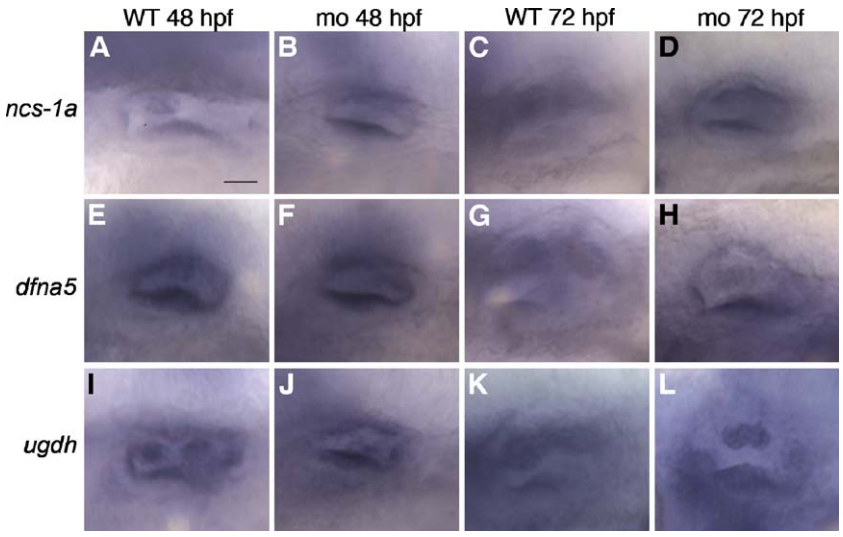

Fig. 4. Expression of semicircular canal markers in α1a.2 morphant ears. All panels are lateral views with anterior to the left. Embryos were injected with 2 ng of α1a.2 MO-1 at the one-cell stage. (A–D) *ncs-1a* staining in the otic vesicle (OV) of (A) 48 hpf wild type (WT) embryo, (B) 48 hpf morphant, (C) 72 hpf WT embryo, and (D) 72 hpf morphant. (E–H) *dfna5* staining in OV of (E) 48 hpf WT embryo, (F) 48 hpf morphant, (G) 72 hpf WT embryo, and (H) 72 hpf morphant. (I–L) *ugdh* staining in OV of (I) 48 hpf WT embryo, (J) 48 hpf morphant, (K) 72 hpf WT embryo, and (L) 72 hpf morphant. mo, morphant. Scale bar: 25 μm.

formation is most likely composed of a combination of α1a.1/β2b subunits.

Tether cells are present in α1a.1 morphant embryos

Tether cells are precocious hair cells that are essential for otolith seeding (Riley et al., 1997). To determine whether knockdown of α1a.1 expression caused defects in tether cell development, we visualized tether cells by immunostaining with antibody to acetylated tubulin. Tether cell kinocilia (tethers) were compared between control and morphant ears, and the results summarized in Table 4. In control embryos, formation of tethers appeared to be virtually complete by 18.75 hpf, with 94% of embryos showing a pair of tethers at each of the otocyst poles. We observed that tethers also formed in embryos injected with α1a.1 MO-1, although their appearance was typically delayed by 2–3 h compared to controls (Table 4). This delay is consistent with the general delay in size and morphogenesis of α1a.1 morphants. Tethers were visible in 16% of morphant otocyst poles at 18.5 hpf and 31% at 20 hpf. By 22 hpf, tethers were present in 100% of otocyst poles in morphant embryos. In contrast, no otoliths were observed in sister α1a.1 morphants at 30 hpf, and only one of 8 morphant ears had otoliths at 48 hpf. Taken together, these results suggest that the failure of α1a.1 morphants to form otoliths is not due to the absence of tether cell kinocilia.

We also tracked tether cell differentiation by staining embryos with HCS-1, an antibody that labels *H*air *C*ell *S*oma in lower vertebrates (Gale et al., 2000). HCS-1 staining was first detected at 22 hpf in both control embryos (n = 4 ears) and α1a.1 morphants (n = 10 ears). Representative images of HCS-1 staining at 24 and 28 hpf are shown in Fig 7. At 24 hpf, HCS-1-positive cells averaged 3.36 in control ears (n = 11 ears) and

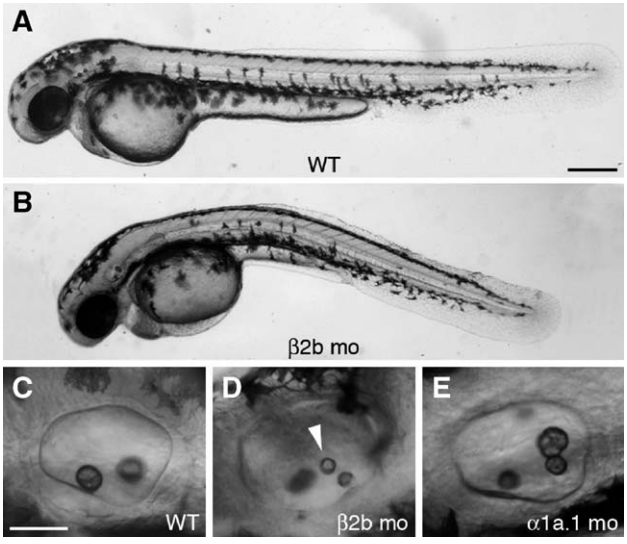

Fig. 5. Knockdown of Na,K-ATPase β2b expression. All panels show a lateral view with anterior to the left. (A) Wild type (WT) embryo at 45 hpf. (B) Embryo injected with 6 ng of β2b MO-1 at 45 hpf. (C) Otic vesicle (OV) of WT embryo at 45 hpf. (D) OV of embryo injected with 6 ng of β2b MO-1 at 45 hpf. Arrowhead indicates ectopic otolith. (E) OV of embryo injected with 0.125 ng of α1a.1 MO-1 at 45 hpf. mo, morphant. Scale bars: A–B, 250 μm; C–H, 50 μm.

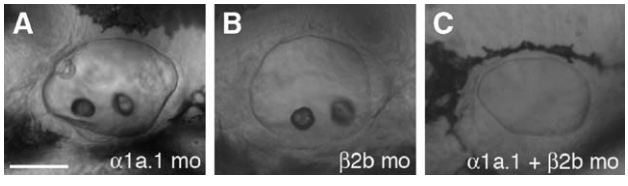

Fig. 6. Coinjection of Na,K-ATPase α1a.1 and β2b MOs. Embryos were injected with sub-effective doses of α1a.1 and β2b MOs. Lateral views of otic vesicle (OV) at 45 hpf, anterior to the left. (A) OV of embryo injected with 0.125 ng of α1a.1 MO-1. No effect on otolith formation was observed. (B) OV of embryo injected with 2 ng of β2b MO-1 showing normal otolith formation. (C) Embryo co-injected with 0.125 ng of α1a.1 MO-1 and 2 ng of β2b MO-1 failed to form otoliths. mo, morphant. Scale bar: A–C, 50 μm.

Table 3
Synergistic effect of α1a.1 and β2b MOs on otolith formation

α1a.1 MO-1 (0.125 ng)	β1a MO-2 (0.5 ng)	β2b MO-1 (2 ng)	Absence of otoliths	
			Number/total	Percent
+	−	−	2/22 [a]	9%
−	+	−	0/17	0%
+	+	−	0/13	0%
−	−	+	0/20	0%
+	−	+	28/30	93% [b]

[a] Number represents abnormal, dysmorphic otoliths.
[b] $P \leq 0.001$, Chi-squared = 56.84, degrees of freedom: 2.

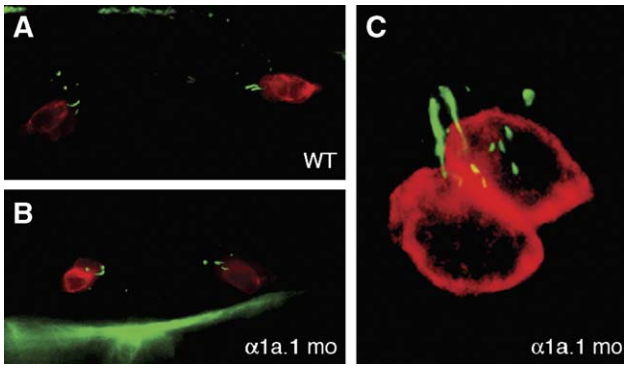

Fig. 7. Tether cells are present in α1a.1 morphant ears. Immunofluorescence was used to detect tether cell somae by labeling with HCS-1 (red) and tether kinocilia by labeling with anti-acetylated tubulin (green). (A) Lateral view of 24 hpf wild type (WT) otocyst. A pair of tether cells, each with a single kinocilium, is present at the anterior and posterior pole. (B) Dorsal view of α1a.1 morphant at 24 hpf. Two pairs of tether cells with kinocilia are visible. The tether cell pairs are closer together than in WT embryos due to the smaller size of the morphant otocyst. (C) Confocal image of α1a.1 morphant at 28 hpf. A pair of tether cells with kinocilia are located at the anterior pole of the otocyst. mo, morphant.

3.22 in morphants (n = 9 ears). The full complement of four HCS-1-positive tether cells was reached in 100% of control ears at 26 hpf (n = 6 ears), 67% of morphant ears by 26 hpf (n = 6 ears) and 100% of morphant ears at 28–30 hpf (n = 8 ears). These results are consistent with our observation that tether cells are present in α1a.1 morphant embryos, and that there is a 2–3 h delay in the appearance of the full set of tether cells compared with control embryos.

Histopathological and functional analysis of α1a.1 morphant sensory cells

Immunofluorescent staining with HCS-1 confirmed the presence of hair cells in two distinct maculae in the inner ears of all morphants at 3 dpf (Fig. 8). The anterior maculae of wild type embryos contained a compact clusters of hair cells (Fig. 8D), whereas this sensory patch in α1a.1 morphants contained fewer numbers of more loosely packed hair cells (Figs. 8E, F). An extreme example is shown in Fig. 8F, where 12 relatively dispersed hair cells were observed compared to more than 40 hair cells present in the wild type ear (Fig. 8D). The mean number (±SD) of hair cells per anterior macula was 38.3 ± 2.1 in controls (n = 3 embryos) as compared to a mean of 19.4 ± 4.0 (n = 12) in α1a.1 morphants ($P < 0.001$). The reduction in mature hair cell numbers, as well as the increase in hair cell dispersion, could result from a perturbation in sensory organ morphogenesis and/or an increase in hair cell death within developing sensory patches.

In order to determine whether α1a.1 morphants contained cranial ganglion neurons, we stained embryos with antibodies against the early neuronal marker HuC. Immunostaining revealed the presence of otic ganglia in all morphants examined, an observation confirmed by examining 1 μm plastic sections (data not shown). These results confirm that otic neurogenesis was present in α1a.1 morphants.

Histological analysis of the inner ear morphology of α1a.1 and β2b morphant embryos is shown in Fig. 9. In the wild type zebrafish inner ear at 72 hpf, the anterior macula lies on the ventral floor and the posterior macula lies on the medial wall. The maculae are composed of sensory hair cells and supporting cells organized in a pseudostratified epithelium. An otolith overlies each of the maculae and is attached to the ends of hair cell stereociliary bundles (Figs. 9A, B). At 72 hpf, α1a.1 morphants contain maculae with recognizable hair cells and supporting cells. However, no otoliths were present in embryos injected with 0.25 ng of α1a.1 MO-1 (Figs. 9C, D). Hair cell stereociliary bundles were evident in wild type and α1a.1 morphant embryos using either light (Figs. 9A–D) or scanning electron microscopy (Figs. 9I, J). Light microscopic analysis of 72 hpf β2b morphants (6 ng β2b MO-1) revealed the presence of dysmorphic otoliths overlying the anterior and posterior maculae. Sensory patches were present and appeared morphologically normal (Figs. 9E, F). We also examined sensory patch morphology of embryos coinjected with sub-effective doses of α1a.1 and β2b MOs. Coinjection of the two MOs (Figs. 9G, H) phenocopied the otolith defect produced by injection of 0.25 ng of α1a.1 MO-1 alone (Figs. 9C, D), providing further support for the idea that the Na,K-ATPase required for otolith formation is composed of α1a.1 and β2b subunits.

If knockdown of Na,K-ATPase activity alters the ionic composition within the endolymph of larval zebrafish, this

Table 4
Effect of α1a.1 MOs on tether kinocilia formation

	No. of tethers/ no. of otocysts poles [a]	Percent of otocyst poles with:		
		0 tether	1 tether	2 tethers
Controls [b]				
18.75 hpf	30/16	0%	6%	94%
20 hpf	40/22	3%	5%	92%
22 hpf	38/20	5%	0%	95%
24 hpf	29/16	6%	6%	88%
α1a.1 morphants				
18.75 hpf	3/12	83%	8%	8%
20 hpf	5/16	69%	31%	0%
22 hpf	23/12	0%	8%	92%
24 hpf	28/14	0%	0%	100%

[a] Refers to the anterior and posterior pole of each otolith; there are 4 otocyst poles per embryo.
[b] Includes approximately equal numbers of WT and embryos injected with standard morpholinos.

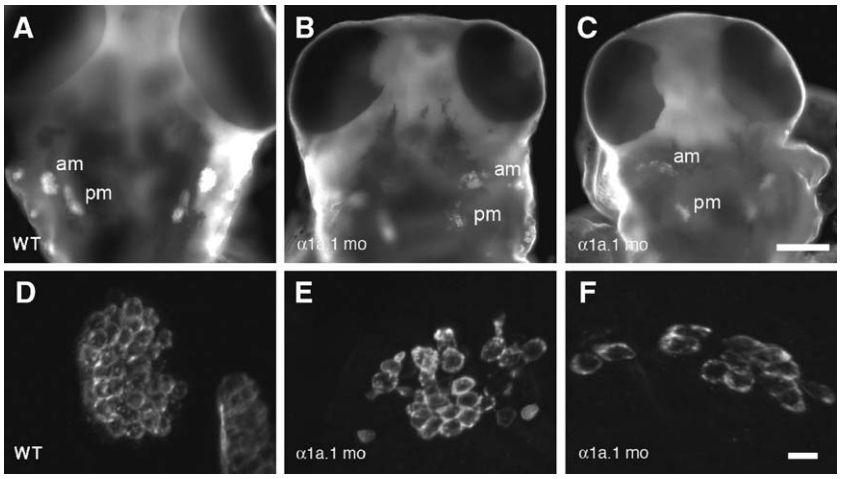

Fig. 8. Macular sensory organs develop in α1a.1 morphants. Hair cells were labeled with HCS-1 antibody in wild type (WT) and α1a.1 morphant embryos at 75 hpf to visualize the anterior (am) and posterior (pm) maculae. (A–C) Low power images of embryos, dorsal views. Scale bar: 100 μm. (D–F) High power views of embryos showing labeled anterior maculae. Scale bar: 10 μm. (A, D) WT embryo. (B, C, E, F) α1a.1 morphants. Both pairs of maculae are present, but they have fewer hair cells and are less tightly clustered than WT. mo, morphant.

might non-specifically disrupt the physiological homeostasis of otic epithelial cells. We reasoned that hair cell transduction activity, which can be monitored with the fluorescent dye, FM1–43, should be a sensitive assay for the physiological status of the inner ear sensory epithelium. The steryl dye FM1–43 is known to permeate through open hair cell transduction channels (Gale et al., 2001; Meyers et al., 2003). Embryos injected at the 1–2 cell stages with standard control morpholino (n = 3), α1a.1 MO-1 (n = 2), β2b MO-1 (n = 3), or a combination of sub-effective doses of α1a.1 MO-1 and β2b MO-1 (n = 3) were analyzed at 58–64 hpf by pressure ejection of FM1–43 into the otic cavity. Small patches of fluorescently-labeled macular hair cells were evident within two min of dye injection in all embryos. Labeled hair cells could also be observed in the cristae of at least some embryos from each morphant group. These data indicate that the ionic milieu of the endolymph in morphant ears is conducive to hair cell development, differentiation and transduction, despite the absence of otoliths. Together, the histological analysis we performed indicates that all the sensory elements of the inner ear are present in morphant embryos, and that the defects in otolith biogenesis do not seem to occur via complete loss of specific cell types within the sensory regions.

Discussion

Previous studies have hinted at important roles for Na,K-ATPase in inner ear function. It is been proposed that a key function of the enzyme is to establish and maintain endolymph homeostasis (Peters et al., 2001). This view is supported by the observation that the sodium pump inhibitor ouabain causes a reduction in endolymph potassium levels (Kuijpers and Bonting, 1970; Kuijpers and Wilberts, 1976). Additionally, the auditory dysfunction associated with mice homozygous for mutations at the *viable dominant spotting* and *Steel-dickie* loci results from failure to develop and maintain a high endocochlear potential. This defect appears to be correlated with aberrant Na, K-ATPase gene expression in cells of the stria vascularis (Schulte and Steel, 1994). Together, these studies raise the possibility that alterations in Na,K-ATPase expression may contribute to deafness. Our studies now point to an earlier role for Na,K-ATPase genes in the development of the inner ear structures involved in gravity sensing and balance. By using antisense morpholinos, we created hypomorphs at three distinct Na,K-ATPase alleles in zebrafish, and discovered that the α1a.1 and β2b genes are required for formation of otoliths, whereas the α1a.2 gene is essential for development of the semicircular canal system.

Na,K-ATPase genes are required for otolith biogenesis

A variety of cell types contribute the organic and inorganic substrates required for formation of otoliths (Thalmann et al., 2001). In fish, this biomaterial is secreted into the otocyst and is then captured by the cilia of two pairs of specialized ‘tether cells’ located at the anterior and posterior poles of the otocyst. Micro-otoliths are initially formed and appear to serve as the seeds for further growth and maturation of the biomatrix of the anterior and posterior otoliths (Riley et al., 1997). Mature otoliths (and otoconia in mammals) are anchored above macular sensory patches that are comprised of hair cells and supporting cells. Knockdown of α1a.1 gene expression could alter otolith formation by affecting tether cells, hair cells whose mature stereocilia eventually replace the otolith-anchoring function of the tether cells, support cells, non-sensory cells that secrete the otolith matrix proteins (otoconins), or ionocytes (functional equivalent of mammalian dark cells) that contribute to the endolymph ionic environment in which otoliths form (Shiao et al., 2005; Sterkers et al., 1988). The ability of α1a.1 mRNA to rescue the otolith defect in α1a.1 morphants and ouabain-treated embryos provides compelling evidence that the failure to form otoliths is due to absence of the Na,K-ATPase α1a.1 subunit, and not a nonspecific consequence of morpholino treatment.

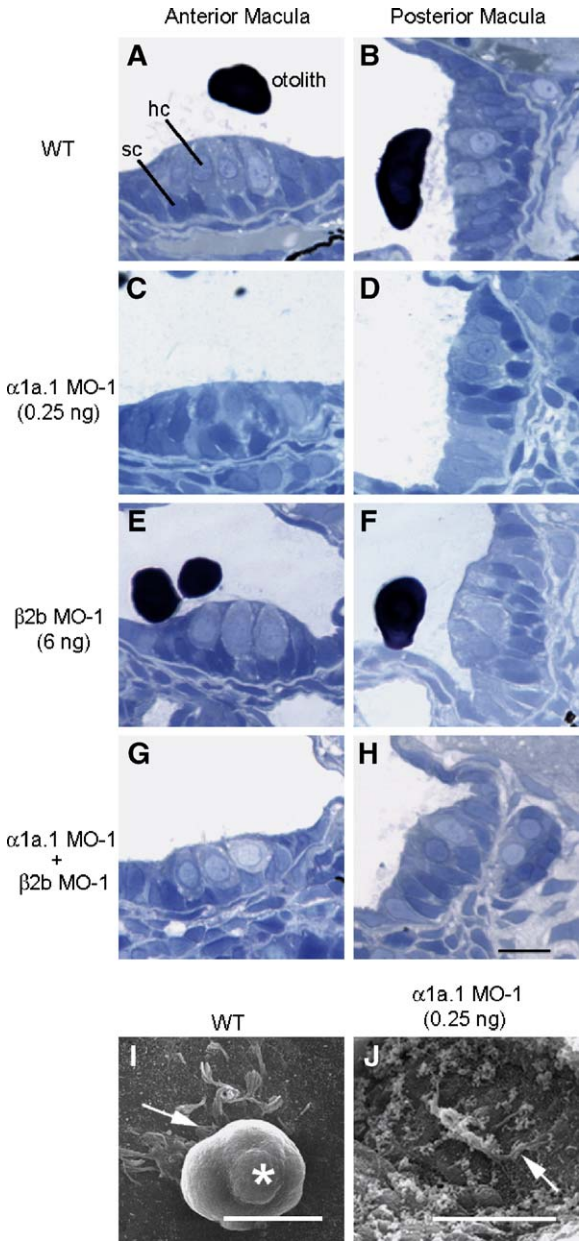

Fig. 9. Histological analysis of α1a.1 and β2b morphant ears. (A–H) 72 hpf wild type (WT) and morphant embryos were fixed, embedded in resin, sectioned at 1 μm, and stained with Toluidine blue. (A, C, E, and G) Sections through the anterior macula. (B, D, F, and H) Sections through posterior macula. Embryos injected with 0.25 ng of α1a.1 MO-1 are shown in panels C and D. Embryos injected with 6 ng of β2b MO-1 are shown in panels E and F, while embryos coinjected with 0.125 ng of α1a.1 MO-1 and 2 ng of β2b MO-1 are shown in panels G and H. hc, hair cell; sc, supporting cell. (I and J) Scanning electron micrographs of posterior macular otolithic membrane of WT embryo (I) and α1a.1 morphant (J) (injected with 0.25 ng α1a.1 MO-1) at 72 hpf. Arrows indicate hair cell ciliary bundles. Asterisk indicates otolith. Scale bars: 10 μm.

Na,K-ATPase α1a.1 mRNA is ubiquitously expressed in the developing zebrafish inner ear (Blasiole et al., 2003), a feature shared by other genes involved in otolith biosynthesis such as *starmaker* (Sollner et al., 2003) and GP96 (Sumanas et al., 2003). The ubiquitous expression of α1a.1 in early otic epithelium, however, fails to implicate a particular cell type whose function may be compromised in α1a.1 morphants. In addition, histological analysis revealed the presence of tether, hair and supporting cells in morphant ears, while the uptake of FM1-43 by morphant hair cells suggests that the ciliary hair bundles contain functional transduction channels. Our data indicate that β2b is the β subunit most likely to partner with α1a.1 to form the Na,K-ATPase required for otolith formation. Since β2b mRNA expression is limited to the sensory regions in the developing ear (Blasiole et al., 2003), the sensory patches may be the only regions in the ear where functional α1a.1/β2b-containing isoenzymes are found. It is therefore possible that reduced expression of α1a.1/β2b-containing isoenzymes in sensory patch epithelial cells may disturb the physiological functions required for otolith seeding and/or maturation.

We found no evidence of hair cell extrusion into the underlying mesenchyme in α1a.1 and α1a.1/β2b morphants, such as that seen at 2–3 dpf in *mind bomb* mutants, which completely lack sensory organ supporting cells (Haddon et al., 1998). We therefore conclude that supporting cells must be at least partially functional in morphant embryos. A consideration of the *mind bomb* phenotype is also of interest with respect to otolith biogenesis in the absence of supporting cells: tiny otoliths arise and seed in the mutants although they subsequently fail to enlarge (Haddon et al., 1998, 1999). This indicates that the total absence of otoliths seen when Na,K-ATPase subunits are knocked down is unlikely to result from an incomplete differentiation or function of sensory organ supporting cells.

A distinct Na,K-ATPase isoform is required for semicircular canal morphogenesis

The mechanism by which knockdown of α1a.2 expression causes a defect in semicircular canal formation in zebrafish is still unclear. Key events in the development of the semicircular canal system include outgrowth of epithelial protrusions at ~48 hpf and fusion at ~68 hpf to form the mature pillars of the semicircular canals (Haddon and Lewis, 1996; Waterman and Bell, 1984). Directed outgrowth of the protrusions depends on an increase in the volume of extracellular matrix secreted into the lumen of the epithelial pillars (Haddon and Lewis, 1991). We have previously shown that Na,K-ATPase α1a.2 mRNA is expressed in semicircular canal protrusions and in the epithelial pillars of the semicircular canals (Blasiole et al., 2003). Given the importance of Na,K-ATPase activity for cell survival, reduced expression of α1a.2 could potentially compromise the function or survival of epithelial cells that form the protrusions and pillars. However, the fact that we detect expression of several markers of semicircular canal development in α1a.2 morphant ears suggests that widespread loss of epithelial cells does not account for the failure of semicircular canals to form.

A second potential mechanism by which knockdown of α1a.2 could affect semicircular canal development is by damaging the cristae, sensory regions which have recently been shown to specify formation of the non-sensory epithelial pillars of the semicircular canals in mice (Chang et al., 2004a).

However, all three cristae were identified in α1a.2 morphant embryos by differential interference contrast imaging (Blasiole et al., unpublished observations).

Na,K-ATPase activity creates an osmotic gradient that regulates the transport of solutes and water across epithelial cell membranes. This activity of the sodium pump has been proposed as the mechanism responsible for inflation of developing brain ventricles in the zebrafish *snakehead* mutant (Lowery and Sive, 2005). It is tempting to speculate that a similar mechanism might underlie the failure of the semicircular canal protrusions to form in α1a.2 morphants, where decreased Na,K-ATPase α1a.2 isoform expression inhibits establishment of the transepithelial ion potential in the developing otic cysts. The resulting decrease in intra-otic pressure and/or volume could adversely affect protrusion and morphogenesis of the semicircular canal epithelial pillars.

Functional diversity of Na,K-ATPase isoforms can be effectively analyzed in zebrafish

A fundamental unresolved issue regarding the sodium pump is whether the multiple α and β subunit isoforms possess unique or redundant functional properties. Studies in transgenic mice provided the first genetic evidence that the α1 and α2 subunits serve different physiological roles in cardiac (James et al., 1999) and skeletal muscle (He et al., 2001) contractility. Recent work using zebrafish has shown that the Na,K-ATPase α1a.1 and α2 isoforms regulate distinct aspects of cardiac development (Shu et al., 2003), and that the α1a.1 gene is required for proper formation of the brain ventricles (Lowery and Sive, 2005). The work described here shows for the first time that two closely related zebrafish Na,K-ATPase α1 subunit genes, α1a.1 and α1a.2, play differing roles in inner ear development; α1a.1 being necessary for otolith formation while α1a.2 is essential for proper development of the semicircular canal system. It will clearly be of interest to determine whether the two additional α1-like genes (α1a.4 and α1a.5) expressed in the developing zebrafish ear (Blasiole et al., 2003) have unique or redundant functions in inner ear development. The morpholino-based gene knockdown method in zebrafish now provides a powerful new in vivo approach for deciphering which Na,K-ATPase isoforms perform unique versus redundant functions.

The gene knockdown approach also provides a novel strategy for identifying which Na,K-ATPase α and β subunit combinations are likely to form functional isoenzymes in vivo. Based on the synergism obtained by coinjection of subeffective doses of morpholinos, we were able to deduce with a high degree of certainty that the sodium pump enzyme responsible for otolith formation is composed of α1a.1/β2b subunit pairs. It will clearly be of interest to determine which of the two β subunits expressed in ear (β1a or β2b) partners with the α1a.2 subunit to form the isoenzyme necessary for semicircular canal formation. Interestingly, while knockdown of β1a expression did not affect formation of otoliths, β1a morphants and embryos co-injected with sub-effective doses of α1a.1 and β1a MOs exhibited severe cardiac morphological abnormalities that phenocopied those described in *heart and mind* and *small heart* α1a.1 zebrafish mutants (Shu et al., 2003; Yuan and Joseph, 2004). Since β1a is the predominant β subunit expressed in the developing zebrafish heart (Canfield et al., 2002), it seems likely that α1a.1 and β1a subunits combine to form an isoenzyme that plays an important role in cardiac morphogenesis.

It is of note that the gene knockdown/mRNA rescue approach in zebrafish can also be used to test the functional properties of mammalian Na,K-ATPase genes. The ability of the rat α1 subunit to rescue the otolith defect in α1a.1 morphants provides clear evidence that these two genes are functionally conserved. This type of approach will now make it feasible to analyze the functional properties of additional mammalian α subunit isoforms using zebrafish as an in vivo model system. Further, the ability of the mutated zebrafish α1a.1 gene to rescue human cells from ouabain cytotoxicity raises an additional point of interest. These results indicate that in human cells, the transfected zebrafish α1a.1 subunit can substitute for the endogenous α1 subunit and form, together with the mammalian β subunit, a biologically active Na,K-ATPase. These results provide the first evidence that the essential function of Na,K-ATPase in maintaining cellular viability has been preserved between fish and mammals.

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