**Introduction**

Development of the mammalian eye begins with an evagination of forebrain neuroepithelium (the optic vesicle), which undergoes subsequent invagination to form a dual-layered optic cup. This invagination is asymmetric, such that a ventral opening (the optic fissure) forms around the fifth week of human gestation. To continue normal eye development, the two edges of the fissure must come into proximity and fuse. If the process of optic fissure closure is faulty, a uveal coloboma—a potentially blinding congenital ocular malformation—results [1,2]. This condition can affect the iris, retina, choroid, retinal pigment epithelium (RPE), and/or the optic nerve. Worldwide, uveal coloboma affects between 0.5 and 2.6 per 10,000 births [3–5]. Although patients display a wide range of visual acuities, uveal coloboma may account for as much as 10% of childhood blindness [6]. The genetics of coloboma are not fully understood. While most cases are sporadic, pedigrees displaying autosomal dominant, autosomal recessive, and X-linked inheritance have been reported [7–10]. Coloboma patients have displayed mutations in more than twenty developmentally-regulated genes (reviewed in [11]). These genes, however, account for a minority of patients, implying that other genes may be involved in the pathogenesis of coloboma and that uveal coloboma is a complex trait involving the action of many genes in concert [10,12,15].

Aldehyde dehydrogenase (ALDH) family members are important for eye development. In general, members of the aldehyde dehydrogenase superfamily catalyze the oxidation of aldehydes to their corresponding carboxylic acids [14]. ALDH class I (ALDH1) enzymes, for instance, synthesize the important morphogen, retinoic acid (RA) and are sometimes referred to as retinal dehydrogenases (RALDH). Knockout of *Aldh1a1* family members in mouse models causes congenital eye malformations such as uveal coloboma/optic fissure closure defects and biallelic mutations in *ALDH1A3* in humans cause microphthalmia/anophthalmia [15–18].

Because of our interest in understanding the molecular mechanisms and genes involved in optic fissure closure, we investigated the expression pattern of different *Aldh/aldh* family members in publically available databases, e.g. www.Zfin.org, focusing on relevant developmental time points. Aldehyde dehydrogenase 7 family, member A1 (*aldh7a1*) is expressed strongly in the developing zebrafish eye. It is a highly evolutionarily conserved gene and is the only member of the ALDH family noted to be active in several subcellular locations—namely, the cytosol, nucleus, and mitochondria [19]. Mutations of *ALDH7A1* have been linked to the human disorder, pyridoxine-dependent epilepsy; this patient population has a broad phenotypic spectrum of severity, including some ocular findings [20–24]. We therefore postulated that *aldh7a1*/*ALDH7A1* may play a role in ocular development.

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**aldh7a1 Regulates Eye and Limb Development in Zebrafish**

Holly E. Babcock¹, Sunit Dutta¹, Ramakrishna P. Alur¹, Chad Brocker², Vasilis Vasiliou², Susan Vitale³, Mones Abu-Asab⁴, Brian P. Brooks¹*

1 Unit on Pediatric, Developmental and Genetic Ophthalmology, Ophthalmic Genetics and Visual Function Branch, National Eye Institute, National Institutes of Health, Bethesda, Maryland, United States of America
2 Molecular Toxicology and Environmental Health Sciences Program, Department of Pharmaceutical Sciences, University of Colorado Denver, Aurora, Colorado, United States of America
3 Division of Biostatistic & Epidemiology, Clinical Trials Branch, National Eye Institute, National Institutes of Health, Bethesda, Maryland, United States of America
4 Immunopathology Section, National Eye Institute, National Institutes of Health, Bethesda, Maryland, United States of America

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Abstract

Uveal coloboma is a potentially blinding congenital ocular malformation caused by failure of the optic fissure to close during development. Although mutations in numerous genes have been described, these account for a minority of cases, complicating molecular diagnosis and genetic counseling. Here we describe a key role of *aldh7a1* as a gene necessary for normal eye development. We show that morpholino knockdown of *aldh7a1* in zebrafish causes uveal coloboma and misregulation of *nlz1*, another known contributor to the coloboma phenotype, as well as skeletal abnormalities. Knockdown of *aldh7a1* leads to reduced cell proliferation in the optic cup of zebrafish, delaying the approximation of the edges of the optic fissure. The *aldh7a1* morphant phenotype is partially rescued by co-injection of *nlz1* mRNA suggesting that *nlz1* is functionally downstream of *aldh7a1* in regulating cell proliferation in the optic cup. These results support a role of *aldh7a1* in ocular development and skeletal abnormalities in zebrafish.

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Citation: Babcock HE, Dutta S, Alur RP, Brocker C, Vasiliou V, et al. (2014) *aldh7a1* Regulates Eye and Limb Development in Zebrafish. PLoS ONE 9(7): e101782. doi:10.1371/journal.pone.0101782

Editor: Bruce Riley, Texas A&M University, United States of America

Received February 27, 2014; Accepted June 11, 2014; Published July 8, 2014

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This work was funded by the Intramural Program of the National Eye Institute, National Institutes of Health, US Dept. Of Health and Human Services. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* Email: brooksbi@mail.nih.gov

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PLOS ONE | www.plosone.org 1 July 2014 | Volume 9 | Issue 7 | e101782
Here we show that aldh7a1 is dynamically expressed in ocular and skeletal tissue during zebrafish development. Knockdown of aldh7a1 in zebrafish leads to uveal coloboma and skeletal abnormalities. In addition, we provide mechanistic evidence that these phenotypes may result from abnormal cell proliferation.

Results

Expression of aldh7a1 in zebrafish during embryo development

Whole-mount in situ hybridization for aldh7a1 in zebrafish embryos was performed at approximately 9 hours post-fertilization (hpf), 18hpf, 24hpf, 36hpf, 48hpf, and 72hpf (Figure 1, and Figure S1 in File S1). aldh7a1 (GenBank: BC044367.1) was expressed ubiquitously at 18hpf (Figure S1B in File S1), but became more concentrated in the developing eye and brain by 24hpf (Figure 1A). At 36hpf the expression was restricted to the temporal and ventral eye, cerebellum, pharyngeal arch precursors and the fin bud (Figure S1C in File S1); by 48hpf, overall expression was decreased (Figure 1B). Pectoral fin expression persisted at around 48hpf (Figure 1B) and persisted through 72hpf (Figure S1 in File S1). We therefore inferred that aldh7a1 were likely important in the development of these structures.

To further investigate the role of aldh7a1 during embryonic development, we performed reduction-of-function analysis by microinjection of morpholino-oligonucleotides (MOs) targeted to translation start sites of the corresponding mRNA. In order to do semi-quantitative analysis of eye phenotypes, we used a four-point grading scale (Figure S2 in File S1), “0” being the control phenotype where the two edges of the optic fissure fused normally. Unjected and control-MO fish behaved identically in this and other measures used throughout this paper. Categories “I,” “II,” and “III” indicate increasing severity of coloboma phenotype where the two edges of the fissure remained increasingly further apart, similar to our previous report [25]. Knockdown of aldh7a1 by Aldh7a1 MO1 in zebrafish embryos resulted in bent tail (Figure S3A, D in File S1); coloboma of the eye evident at 28 hpf which persists until at least 5–6 days post-fertilization (dpf) [Figure 2B,D; Figure S3 E,F in File S1] compared to control embryos (Figure 2A; Figure S3B, C in File S1) where the optic fissure had nearly fused at 28 hpf (Figure 2A). Similar results were obtained by injecting Aldh7a1 MO2 in zebrafish embryos (data not shown). However, co-injection of Aldh7a1 MO1 and Aldh7a1 MO2 also resulted in coloboma (Figure S3 I, J in File S1), but with lower total concentrations of MO compared to controls (Figure S3 G, H in File S1). In this study most of the experiments were performed by using Aldh7a1 MO1, unless otherwise stated. Co-injection of aldh7a1 mRNA with the Aldh7a1 MO partially rescued the morphant phenotype, defined by a greater number of “0”/mildly affected embryos and fewer severely affected embryos based on our four-point scale of optic fissure closure (Figure 2E, p = 0.03 at 100 pg mRNA). At 48hpf, staining of axons using zn-5 antibody revealed that aldh7a1 morphant fish displayed smaller eyes and optic nerves compared to control fish (Figure 2F,G). At 5dpf, morphant fish showed shortened pectoral fins (Figure 5A,B, B’). We ascertained the cartilage structure of the control and morphant fish at 3dpf using Alcian blue staining. Fish exhibiting the more severe fin phenotypes (data not shown) also displayed abnormal cartilage structure in the head and jaw (Figure 3C, D). We could not show mRNA rescue of the fin and cartilage phenotypes because the mRNA did not persist until 5dpf.

Knockdown of aldh7a1 caused misregulation of genes necessary for proper eye development and was partially rescued by co-injection of nlz1 mRNA

Because of the strong expression of aldh7a1 in the developing zebrafish eye and the ventral nature of uveal coloboma, we examined expression of genetic markers known to be important in eye ventralization, including nlz1, vax2, pax2.1, nlz2, and vax1 in control (Figure 4A,C,E, data not shown) as well as in aldh7a1 morphant (Figure 4B,D,F; data not shown) embryos. Of these, only nlz1 (GenBank: AF222996.1) showed consistent down regulation in expression in aldh7a1 morphant eyes at 24hpf. The expression of nlz1 that was seen in the optic fissure and periocular mesenchyme in control embryos (Figure 4A) was lost in aldh7a1 morphant embryos (Figure 4B). This loss of expression led us to hypothesize that nlz1 functions downstream of aldh7a1. To test this hypothesis, we attempted to rescue the aldh7a1 morphant phenotype by co-injecting the Aldh7a1 MO with nlz1 mRNA. Because of the well-established role that vax2 plays in the ventralization of the eye during development, we performed the same rescue experiment with vax2 mRNA as a negative control to confirm specificity. We found that, as predicted, co-injection of vax2 mRNA did not rescue the morphant phenotype and may actually increase its severity (Figure 4G, p = 0.02); however, co-injection of nlz1 mRNA partially rescued the aldh7a1 morphants (p = 0.001). This, again, was demonstrated by a greater number of “0”/mildly affected embryos and fewer severely affected embryos. This partial rescue indicated that nlz1 functions downstream of aldh7a1.

Retinoic acid treatment does not rescues aldh7a1 morphant phenotype

It is well documented that RA plays an important role in eye morphogenesis. Members of the ALDH superfamily (specifically, the ALDH1A family) oxidize retinaldehyde to RA. Aldh1a3-/-, (Raldh3) knockout mice display coloboma, and although Aldh1a1-/-, (Raldh1) knockout mice did not have a recognizable ophthalmic phenotype, double knockout Aldh1a1-/-; Aldh1a3-/- mice displayed an even more severe eye phenotype [26]. In zebrafish, blocking RA during development resulted in coloboma [27]. Additionally, RA regulates nlz1 in zebrafish, demonstrated by a significant decrease in nlz1 expression in embryos where RA signaling is blocked [28]. More recently recessive mutation in ALDH1A3 have been associated with severe microphthalmia, anophthalmia, and hypoplasia of the optic tract [17,18]. These existing connections of RA to coloboma led us to pursue it as a chemical that is potentially involved in the aldh7a1 pathway. We attempted to rescue the morphant phenotype with one- or two-hour incubations in varying concentrations of RA at the 2 somite stage, the 10 somite stage and the 20-somite stage, when eye development is dynamic. We quantified the number of embryos displaying each of the four optic

Figure 1. Expression pattern of aldh7a1 in zebrafish. Whole-mount in situ hybridization of aldh7a1 at (A) 24 hpf and (B) 48 hpf. L, lens; OF, optic fissure; PF, pectoral fin. Scale bar: 65 μm in A; 60 μm in B. doi:10.1371/journal.pone.0101782.g001
fissure grades at 28 hpf and found that RA-treated morphants did not differ from control, untreated morphants (Figure S4 in File S1). RA concentrations beyond 3 \( \mu M \) lead to deformed and dead embryos at 28hpf (data not shown). Thus we conclude that exogenous RA is not able to rescue the \textit{aldh7a1} morphant eye phenotype and is less likely to be the endogenous substrate.

**Knockdown of \textit{aldh7a1} alters cell proliferation in developing eye**

A prerequisite for optic fissure closure is the approximation of its two edges at the appropriate time during development. We noted that the edges of the optic fissure were widely spaced in our morphant fish, and hypothesized that this may be due to a decrease in the rate of cell division required to acquire a normal optic fissure configuration. We compared the number of dividing cells in the retina between uninjected control and morphant embryos at 24 hpf using an anti-phosphohistone H3 (PH3) antibody to label active histones in the M phase of the cell cycle. Qualitatively, there is a clear reduction in labeled cells in the morphant eye (Figure 5B) compared to control eye (Figure 5A). When quantified, the average number of labeled cells in morphant eyes compared to control eyes was statistically lower (Figure 5C). We also labeled dividing cells in the eyes of morphants rescued with \textit{nlz1} mRNA. The eyes of rescued morphants showed a statistically significant recovery in the number of PH3 labeled cells (Figure 5C).

To address the morpholino mediated nonspecific effects on apoptotic cell death in the eye, we performed immunostaining by using anti-active caspase 3 antibody in control MO and \textit{aldh7a1} morphant embryos; no significant difference in the staining was observed (Figure S5 in File S1). These findings offer plausible evidence that uveal coloboma involves a \textit{aldh7a1}-dependent mechanism that disrupts the cell proliferation.
In this study, our aim was to understand the function of \textit{aldh7a1} during embryo development. \textit{ALDH7A1} is part of a superfamily of aldehyde dehydrogenases involved in the NAD(P)\textsuperscript{+}-dependent oxidation of reactive aldehydes to carboxylic acids, as well as the metabolism of other important molecules such as tetrahydrofolate, \(\gamma\)-aminobutyric acid, RA, and betaine [14]. The gene is also suspected to play a role in regulation of hyperosmotic/oxidative stress and the cell cycle [19,29,30]. As previously noted, \textit{ALDH7A1} is unique amongst this family in that it is localized to the cytosol, nucleus, and mitochondria and is highly conserved through evolution, implying one or more essential roles in cell physiology [19]. In fact, \textit{ALDH7A1} was originally named "antiquitin" referring to its ancient origins and the high level of amino acid similarity of the homologues.

Biallelic mutations in \textit{ALDH7A1} have been associated with pyridoxine-dependent epilepsy (PDE) and folinic acid responsive seizures in humans [21,23]. Although we were not equipped to evaluate seizure activity in our morphants, many of PDE patients also have developmental abnormalities of the CNS, including optic nerve hypoplasia—a phenotype observed in our zebrafish model [20]. Although uveal coloboma and skeletal abnormalities have not been reported in PDE, it is unclear from the literature whether these phenotypes were systematically ascertained in the patients reported. An informative follow-up to this study would be the careful examination of patients with PDE (and, perhaps, their carrier parents) for subtle ocular and skeletal abnormalities consistent with the zebrafish phenotype. Since \textit{ALDH7A1} is a susceptibility gene for osteoporosis and is believed to play a role in bone formation and maintenance [31] as well as cancer metastasis[32], assessing these specific phenotypes in PDE patients (and, perhaps, their carrier parents) would be of particular interest. While published data do not support a clear link between ALDH7A1 mutations and optic fissure closure per se, they do not exclude the possibility that regulation of \textit{ALDH7A1} is an important modifier of ocular development.
Based on basic developmental principles, our lab and others have posited that in order for the optic fissure to close, the two edges of the optic cup must approximate in the correct anatomic location, at the correct developmental stage, and express the correct complement of genes; any event that disrupts one or more of these conditions results in uveal coloboma [1,2]. Abnormal regulation of the cell cycle would therefore be predicted to disrupt ocular morphogenesis by interfering with this developmental program. Other zebrafish models of coloboma, such as knockdown of chd7 and gdh6, in fact, result in reduced cell proliferation in the developing eye [33,34]. ALDH7A1 has been shown to localize, in part, to the nucleus and to be up-regulated using the G1-S phase transition [30]. Our observation that knockdown of aldh7a1 results in reduced cell proliferation is consistent with these findings. Furthermore, the observation that nlz1/NLZ1 which is important in optic fissure closure [23] acts functionally downstream of aldh7a1 is also consistent with the observation that NLZ1 is important in tumor cell division and metastasis [35]. The precise mechanism by which aldh7a1 levels affect nlz1 activity is an ongoing area of investigation in our laboratory. Our attempts to rescue the morphant phenotype with vax2 mRNA may make the morphant phenotype more pronounced, consistent with the rescue the morphant phenotype with ongoing area of investigation in our laboratory. Our attempts to rescue the morphant phenotype with nlz1/NLZ1 which is important in optic fissure closure [23] acts functionally downstream of aldh7a1 is also consistent with the observation that NLZ1 is important in tumor cell division and metastasis [35].

Several proteins in the ALDH superfamily have the primary function of converting retinal to RA (e.g., ALDH1A1, ALDH1A2, ALDH1A3) [14]. Coloboma results when zebrafish embryos were treated with citral, a compound that decreases the production of RA in the ventral retina. This phenotype was partially rescued by incubation in RA [27]. When retinoic acid receptor (RAR) signaling was inhibited by treating zebrafish embryos with AGN194310 (AGN), coloboma was present in 75% of embryos quantified at 60 hpf [28]. Our experience with aldh7a1 revealed no relationship with RA, in that incubation of morphants in various RA concentrations showed no rescue of phenotype. Consistent with this observation, Tang et al. found that Aldh7a1 from the seabream fish—which shares 94% identity with ALDH7A1—could not utilize retinal as a substrate [37]. Lastly, in silico analysis demonstrates that ALDH7A1 has low sequence identity with other, known RA-metabolizing members of the superfamily: ALDH1A1 (25.7%), ALDH1A3 (20%), and ALDH1A3 (25.7%). Taken together, these observations make it unlikely that Aldh7a1 plays a major role in RA production in our model system.

In summary, we demonstrate that aldh7a1 has a critical role in eye and skeletal development in zebrafish. The optic fissure closure defects in our morphant fish occurred at least in part because of reduced nlz1 expression and reduced cell proliferation. Regulation of ALDH7A1/ALDH7A1 expression and/or activity may therefore play an important role in human optic fissure closure, optic nerve formation and skeletal development.

Materials and Methods

Ethics Statement

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. All animal experiments were conducted under protocols approved by the National Eye Institute’s Animal Care and Use Committee (NEI-ACUC) at the National Institutes of Health (ASP # NEI-648/). Fish Husbandry

AB×TL strains of zebrafish were raised and maintained according to standard protocols [38]. Embryos were collected after natural spawning and maintained at 28.5°C.

RNA Probe Synthesis and Whole-Mount In Situ Hybridization

Antisense RNA probes were synthesized from full-length cDNA IMAGE clones (Open Biosystems, Huntsville, AL) using digoxigenin RNA-labelling kit (Roche, Indianapolis, IN). Whole-mount in situ hybridization was carried out as described [39] at 65°C using probes for aldh7a1, nlz1 [25], nlz2 [25], vax1 [40], vax2 [40], and pax2.1 [41].

Morpholino Gene Knockdown and mRNA Rescue in Zebrafish

Translation-blocking morpholino oligonucleotides were obtained from Gene Tools, LLC (Philomath, OR), and diluted in 0.1 M KCl. Zebrafish embryos were injected with 7.5 ng Aldh7a1 MO1, (5’TGGGACACTCGGGCAGTGTTATGCG3’), 10 ng of Aldh7a1 MO2 (5’AGTGCCGCAAGTC TCAGCGTCAGCA3’), and 10 ng of control MO (5’ATCCAGGAGGCAGTTCGCT- CATGCTG3’) at the 1–2 cell stage. In some experiments, the embryos were co-injected with 2 ng Aldh7a1 MO1 and 2 ng Aldh7a1 MO2 (4 ng, a lower total concentration of MO). The ORFs for aldh7a1, nlz1, and vax2 were amplified by PCR from IMAGE clones obtained from Open Biosystems (Table S1). PCR products were purified and sub-cloned into pcS2* [42]. Recombinant clones were linearized with NotI and capped mRNA were synthesized in vitro using mMESSAGE mMACHINE kit (Ambion, Grand Island, NY). Zebrafish embryos were co-injected with 50–100 pg of synthetic mRNA and 7.5 ng of Aldh7a1 MO at the 1–2 cell stage. In rescue experiments, datasets were compared using Chi-squared analysis. Because some severity scores contained a low number of embryos, affected phenotypes were sometimes grouped together for analysis (e.g., grades II and III were grouped in the analysis of nlz1 and vax2 rescue of aldh7a1 morphants.)

Alcian Blue Staining

Cartilage was stained with Alcian Blue according to a protocol modified from Neuhauss et al. [96] [43]. Embryos at 5dpf were fixed overnight in 4% PFA and then washed in PBST. The embryos were bleached of pigments in 1 mL of solution containing 100 μL 30% H2O2, 100 μL 1% KOH, 800 μL ddH2O. After rinsing again with PBST, they were stained overnight in 0.1% Alcian Blue dissolved in acidic ethanol (70% EtOH, 5% concentrated HCl). Post-staining, embryos were washed extensively in acidic ethanol and then dehydrated and transferred to 80% glycerol for storage and visualization.

Retinoic Acid treatment

Developing control and morphant zebrafish embryos were incubated in various concentrations of retinoic acid prepared according to Hyatt et al. [92] [44] for 1 hour at the 20-somite stage, and 2 hour at the 2-somite stage, and 10-somite stage. The embryos were washed several times in embryo media after RA treatment, and incubated in embryo media until 28hpf.

Immunohistochemistry

Embryos were fixed overnight in 4% PFA and washed with PBST0.2 (1X PBS with 0.2% Triton X-100). Blocking was completed for 30 minutes at RT in solution of PBST0.2 with 2% goat serum. Proliferating cells were labeled with rabbit anti-
phospholipid-H3 (H3P, Millipore, Billerica, MA) primary antibody at 1:250 and incubated overnight. Embryos were rinsed thoroughly with PBST0.2 and then incubated for 2 hours at RT with secondary antibody Alexa Fluor 488 anti-mouse IgG (Molecular Probes, Grand Island, NY) at 1:400 for visualization by fluorescence microscopy. The same procedure was used to analyze optic nerve with zn-5 primary antibody to label axons. Mouse-conjugated zn-5 (ZIRC 021009) was diluted at 1:200 and embryos were incubated overnight. Alexa Fluor 488 goat anti-mouse IgG (Molecular Probes, Grand Island, NY) at 1:400 was used as secondary antibody. Apoptotic cells were labeled with rabbit anti-active-caspase3 antibody (BD Pharmingen), and Alexa Fluor 568 secondary antibody. Apoptotic cells were labeled with rabbit anti-active-caspase3 antibody (BD Pharmingen), and Alexa Fluor 568 secondary antibody. Apoptotic and proliferative defects characterize ocular development in a model of CHARGE syndrome. Developmental Biology 382: 57–69.

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Acknowledgments

We would like to sincerely thank Dr. Igor Davíd, The Eunice Kennedy Shriver National Institute of Child Health and Development, for use of his zebrafish facility and for a critical reading of the manuscript draft.

Author Contributions

Conceived and designed the experiments: HEB SD RPA VV BPB. Performed the experiments: HEB SD CB MAA. Analyzed the data: HEB SD RPA BPB. Contributed reagents/materials/analysis tools: HEB SD RPA VV BPB. Wrote the paper: HEB SD BPB.

Supporting Information

File S1 Supporting figures and table. Figure S1. aldh7a1 expression. Figure S2. Grades of severity for eye development. Figure S3. aldh7a1 loss-of-function phenotype. Figure S4. aldh7a1 morphant phenotype is not rescued by retinoic acid. Figure S5. Coloboma in aldh7a1/morphant fish is not due to apoptosis. Table S1. List of Image clones and Oligonucleotides used in this study. (PDF)
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