Metalloproteinase \textit{Adamts16} Is Required for Proper Closure of the Optic Fissure

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Submitted: August 18, 2017
Accepted: January 25, 2018

\textbf{Purpose.} Coloboma is a sight-threatening congenital eye disease caused by a failure in optic fissure (OF) closure. The aim of this study was to investigate the role of \textit{Adamts16}, a metalloproteinase, in OF closure.

\textbf{Methods.} RNA in situ hybridization was used to examine the expression of \textit{Adamts16} in developing mouse and zebrafish eyes. Morpholino knockdowns were performed to study \textit{adamts16} function during zebrafish eye development. Additionally, immunofluorescent staining, RNA in situ hybridization, bromodeoxyuridine (BrdU) labeling, TUNEL assays, and high-throughput sequencing were used to examine altered cellular and molecular events in \textit{adamts16}-morphant optic cups (OCs).

\textbf{Results.} \textit{Adamts16} is expressed at the edges of the closing OF in both mice and zebrafish eyes. Zebrafish \textit{adamts16} knockdown resulted in coloboma formation. In \textit{adamts16}-morphant eyes, the basement membrane failed to disassemble at the closing OF edges. OC cells exhibited decreased proliferation and increased apoptosis, and fibroblast growth factor 8 (fgf8) was ectopically upregulated in the OC.

\textbf{Conclusions.} \textit{adamts16} is required for proper OF closure in zebrafish eyes. \textit{adamts16} controls OF closure possibly through the combined functions of degrading the basement membrane at the closing OF edges, promoting cell proliferation and survival, and restricting \textit{fgf8} expression. Our study linked a metalloproteinase to OF closure, which may facilitate future etiologic studies on human coloboma cases.

Keywords: optic fissure closure, coloboma, \textit{adamts16}, basement membrane

Ocular coloboma is a sight-threatening congenital eye disease, manifested as the absence of tissues (mainly retina) on the ventral side of the eye. Depending on the population under study, the incidence of coloboma ranges from 0.5 to 7.5 cases per 1000 births but accounts for 3.2% to 11.2% of childhood blindness worldwide.\textsuperscript{1} Coloboma formation is caused by a failure to close the optic fissure (OF)—a transient opening of the eye during morphogenesis. Vertebrate eye morphogenesis begins when the neural tube at the forebrain region protrudes bilaterally toward the surface ectoderm to form the optic vesicle (OV). When the evaginating OV reaches the surface ectoderm, its distal wall invaginates to form the bilayered optic cup (OC). At the same time, the ventral wall of the OV also invaginates to give rise to a groove extending distally to the end of the OC and proximally into the optic stalk (OS), which is termed the “optic fissure.”\textsuperscript{2,3} The OF provides the route for periocular mesenchymal (POM) cells to migrate into the eye, which then form blood vessels to supply nutrients for the developing OC. The OF also provides guidance for axons of newly generated retinal ganglion cells to exit the OC. After enough POM cells have migrated into the OC, the OF gradually fuses to seal the ventral wall, leaving only one central opening at the back of the retina—the optic disc.\textsuperscript{2,3} When the OF fails to close, the transient opening becomes permanent, and coloboma occurs.

Human genetic studies have linked many genes with coloboma, including \textit{ZEB2}, \textit{PITX2}, \textit{FBN2}, \textit{SHH}, \textit{PAX6}, \textit{PAX2}, \textit{CHD7}, \textit{ABC6}, and \textit{SALL2}. However, for more than half of coloboma cases, the genetic causes remain unclear, partially owing to the complexity of regulatory networks controlling OF\textsuperscript{1,4} Studies using animal models, such as mice and zebrafish, have further linked more genes and signaling pathways to coloboma formation and have revealed the cellular events that are essential for the development of the OF. \textit{Pax2} has been identified as an important regulator of OF development: at the OC stage before OF closure, \textit{Pax2} is strongly expressed in the ventral OC surrounding the OF and both mice and zebrafish \textit{Pax2} mutations have resulted in coloboma formation\textsuperscript{5,6}; humans carrying heterozygous \textit{PAX2} mutations develop optic nerve malformation, a structure closely related to OF in development.\textsuperscript{7} \textit{Pax2} maintains the cellular identity of the OF, as well as the OS, by repressing \textit{Pax6} expression in these regions.\textsuperscript{8} In addition, proximal OC fates, including the OF and OS, are determined by the body midline signal, Sonic hedgehog (SHH); therefore, mutations affecting the SHH signaling pathway may also lead to coloboma formation.\textsuperscript{9–11} Since the OF is a ventral OC-specific structure, mutations in genes affecting ventral OC fate determination, such as \textit{Vax2}, may cause coloboma.\textsuperscript{12} The OF provides the route for POM cells to migrate into the OC, and conversely, these cells also regulate OF development. Mutations in genes controlling POM devel-
adams16 regulates optic fissure closure

Adams16 Regulates Optic Fissure Closure

Adams16 regulates optic fissure closure (OF). This study aimed to uncover novel molecules governing OF closure, using microarrays to compare the transcriptome of the OC tissue at the OF region with that of the central OC tissue (manuscript in preparation). When inspecting genes specifically enriched in the OF transcriptome compared with the central OC transcriptome, we noticed *adams16*, a metalloproteinase that was the second most highly enriched gene in the OF transcriptome. *adams16* belongs to a large family of proteinases, called a disintegrin and metalloproteinase with thrombospondin type 1 motif 1 (ADAMTS). ADAMTSs are zinc-dependent secreted proteinases with multiple functions, including extracellular matrix degradation and protein post-translation processing and turnover. OF fusion involves dissolution of the basement membrane, yet the proteinases responsible for this process have not been investigated. *adams16* has been shown to be expressed in the lung, kidney, reproductive organs, and brain, and has been linked to fertility and blood pressure regulation. However, expression and function of *adams16* during eye development have not been examined. Here, we show that *adams16* is expressed in the OC cells lining the closing OF in both mice and zebrafish. Using zebrafish as the model organism, we determined that *adams16* is required for proper OF closure.

Further investigation indicated that *adams16* regulates zebrafish OF closure possibly through regulating basement membrane degradation, cell proliferation and survival, and fibrilalst growth factor8 (fgf8) expression.

**Methods**

**Animal Maintenance**

All animal studies were performed in compliance with the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the Institutional Animal Care and Use Committee of Zhongshan Ophthalmic Center. To obtain timed-matched embryos, wild-type C57BL/6j male and female mice were caged together in the afternoon and vaginal plugs were checked the next morning. Noon of the day when a plug was found was counted as embryonic day 0.5 (E0.5). Wild-type AB zebrafish embryos were obtained through spontaneous spawning or by microinjection male and female C57BL/6j mice were kept together overnight after fertilization. Fish embryos treated with 0.003% phenylthiourea (Sigma-Aldrich Corp., St. Louis, MO, USA) starting from 20 hpf to prevent pigmentation when post fertilization (hpf). Zebrafish embryos were treated with 4% formaldehyde at 4°C overnight. Fixed embryos were digested with 1 µg/ml proteinase K for 5 to 10 minutes, postfixed for 5 minutes in 4% formaldehyde, and hybridized with digoxigenin-labeled RNA probes at 56°C overnight. The next day, samples were washed and incubated with NB-TBICIP solution (Promega) for color development. Images were taken with the Leica stereomicroscope M205. The zebrafish ISH probe–synthesizing plasmids were constructed by T-cloning cDNAs into the pGEMT-easy vector (Promega). Mouse *adams16* cDNA was amplified by RT-PCR from E11.5 mouse embryonic head total RNA by using the following primer sequences: forward, 5'-CAACCTGTTGCTCTGAGATG-3' and reverse, 5'-TGATGTTTCCCCCAATTGTTCT-3'. Digoxigenin-labeled RNA probes were synthesized by using T7 RNA polymerase (Promega).

Zebrafish embryo whole-mount ISH was performed as follows: zebrafish embryos were fixed in 4% formaldehyde at 4°C overnight. Fixed embryos were digested with 1 µg/ml proteinase K for 5 to 10 minutes, postfixed for 5 minutes in 4% formaldehyde, and hybridized with digoxigenin-labeled RNA probes at 56°C to 65°C overnight. The next day, samples were washed and incubated with NB-TBICIP solution (Promega) for color development. Images were taken with the Leica stereomicroscope M205. The zebrafish ISH probe–synthesizing plasmids were constructed by T-cloning cDNAs into the pGEMT-easy vector. Zebrafish cDNAs were amplified by RT-PCR from zebrafish 36-hpf embryo total RNA by using the following primer sequences: *adams16*-forward: 5'-ACGGTATGCTGACTGTTG-3' and reverse: 5'-GTAATACGTGCTCGAGGTTG-3'; *fgf8*-forward: 5'-ATGGCCGAGACGTAAAGACCTG-3' and reverse: 5'-TCAGCTAGCTAGCTAGCTG-3'; vax2-forward: 5'-ATGGTTGATCAAGCCACAGGAG-3' and reverse: 5'-TTAGGAGACGGTCTCTTC-3'; aldh1a2-forward: 5'-ATGACCTCAGTGAAGTTG-3' and reverse: 5'-CATGTGCTGTGTTTAAAGAG-3'; pax6-forward: 5'-ATGGCAACAGGAGGACTGCTAGG-3' and reverse: 5'-CACAGCTGCTGACTGCTGCTAG-3'; foxC1a-forward: 5'-AAACAAATGTTGCTGAGTGC-3' and reverse: 5'-GTGAAAATGGGATCCAGTG-3'; pax6a-forward: 5'-CTGTAGACGGTCTCTTCGAGC-3' and reverse: 5'-CAATCCCTGCTGACATGG-3'; pax6b-forward: 5'-ATGAGACTCATACCTTCACG-3' and reverse: 5'-TTCAACGCTCTGCTGATTAC-3'.

To examine the zebrafish *adams16* ISH signal on sections, after whole-mount ISH, fish embryos were briefly fixed in 4% formaldehyde for 2 hours at room temperature, soaked in 50% sucrose until sinking, and then embedded in OCT freezing medium and frozen. Fish embryos embedded in OCT blocks were cut and mounted onto glass slides by using a Cryostat Microtome, after which the slides were dehydrated with ethanol, covered with cover glass, and imaged by using the Zeiss Axio Observer Z1.

**Morpholino, mRNA, and Plasmid Injections**

The following morpholinos (MOs) were purchased from Gene Tools: *adams16*-MO1, AGGCTGTAAGACAGACGCTATCCTCCT (targeting translation initiation); *adams16*-MO2, GCTGTCATTACCTCTACTCACTTGTT (targeting splicing between exon2-intron2); Fgb8a-MO, 5'-AGCTCTCATGTATTAGCCCTAGTA-3' and pax2a-MO, 5'-ATATGGTGGTCTCCTGCTATAGTGT-3'.

The *adams16* full-length cDNA with 2-nt silent mutations in the M01 recognition sequence was cloned into the pCS2 vector. The translation initiation-mutant form of *adams16* cDNA-expressing plasmid was generated through site-directed mutagenesis by deleting the A from the ATG translation initiation codon. The catalytic site-mutant form of *adams16* cDNA-expressing plasmid was generated through site-directed...
mutagenesis to replace the three histidines in the HEXHXGXGXXH catalytic motif with prolines. Capped mRNAs were synthesized by using the mMESSAGE mMACHINE transcription kit (Thermo Fisher Scientific, Waltham, MA, USA). For the adamts16-MO1 efficacy test, an MO1 complementary sequence was inserted in-frame before the green fluorescent protein (GFP) coding sequence of the EF1α-GFP vector. For the adamts16-MO2 efficacy test, 18-hpf zebrafish embryos were collected, and then RNA was extracted, reverse transcribed, and amplified by using primers spanning the targeted exon2. MOs, capped mRNAs, or plasmids were injected into one-cell-stage embryos following the standard protocol.25

Western Blot
The 24-hpf zebrafish embryos (50 for each experimental group) were lysed on ice in RIPA buffer (50 mM Tris-HCl, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS) with protease inhibitor cocktails (Sigma-Aldrich Corp.). Equal volumes of lysate for each group were loaded onto 10% SDS-polyacrylamide gels. After the electrophoresis, proteins were transferred onto polyvinylidene fluoride membrane (Thermo Fisher Scientific). The membranes were blocked with 5% skim milk in TBST buffer (19 mM Tris base, 137 mM NaCl, 2.7 mM KCl, 0.1% Tween 20) for 1 hour, and incubated with anti-Adamts16 (ab45048, 1:1000; Abcam, Cambridge, UK) and anti-β-actin (ab3280 as loading control, 1:5000; Abcam) primary antibodies at 4°C overnight. The next day, membranes were washed with TBST and incubated with horseradish peroxidase-conjugated secondary antibodies (Cell Signaling, Danvers, MA, USA) for 2 hours at room temperature. The band signals were developed by using the enhanced chemiluminescence system (Roche).

Immunostaining
Zebrafish embryos were fixed in 4% formaldehyde at room temperature for 4 hours, washed, placed in 30% sucrose solution until embryos sank, embedded sagittally into OCT tissue freezing medium, and then frozen. Sample blocks were sectioned and mounted onto glass slides by using the tissue freezing medium, and then frozen. Sample blocks were transferred onto polyvinylidene fluoride membrane (Thermo Fisher Scientific) at room temperature for 2 hours. The membranes were blocked with 5% skim milk in TBST buffer (19 mM Tris base, 137 mM NaCl, 2.7 mM KCl, 0.1% Tween 20) for 1 hour, and incubated with anti-Adamts16 (ab45048, 1:100; Abcam, Cambridge, UK) and anti-β-actin (ab3280 as loading control, 1:5000; Abcam) primary antibodies at 4°C overnight. The next day, membranes were washed with TBST and incubated with horseradish peroxidase-conjugated secondary antibodies (Cell Signaling, Danvers, MA, USA) for 2 hours at room temperature. The band signals were developed by using the enhanced chemiluminescence system (Roche).

Bromodeoxyuridine (BrDU) Labeling and Immunostaining
Zebrafish embryos were dechorionated and mounted in 1.5% low-melting agarose gel (Sigma-Aldrich Corp.) with the ventral side up. Approximately 0.5 mL of 10 mM BrDU (Sigma-Aldrich Corp.) was injected into the yolk sac caudal to the heart by using a glass micropipette. Embryos were returned to fish water after injection. Two hours later, the embryos were harvested and treated for immunostaining as described above. An anti-BrDU antibody (RPN20, 1:50; GE Healthcare Bio-Sciences, Pittsburgh, PA, USA) was used.

TUNEL Assay
The TUNEL assay was performed on cryosections of zebrafish embryonic heads by using the ApopTag Fluorescein In Situ Apoptosis Detection kit (Millipore, Burlington, MA, USA). Sections were pretreated by boiling in Tris buffer in a microwave for 5 minutes, and then the assay was performed by following manufacturer’s instructions. Images were obtained by using the Zeiss confocal microscope.

High-Throughput Sequencing
The 36-hpf zebrafish eyes from control and morphant groups were collected and placed into TRIzol reagent (Thermo Fisher Scientific). Forty eyes from 20 embryos were pooled together as one sample. Triplicates were used for each experimental group. Samples were sent to BGI (Shenzhen, China) for RNA extraction, library construction, high-throughput sequencing, and data processing. The Fragments Per Kilobase of transcript per Million mapped reads method (FPKM) was used to calculate gene expression levels. NOIseq was used to compare gene expression between control and morphant groups; fold change ≥ 2 and divergence probability ≥ 0.8 were used to filter differentially expressed genes. KOBAS (http://kobas.cbi.pku.edu.cn/expression.php; in the public domain) was used to find enriched KEGG pathways.7

RESULTS
Adamts16 Is Expressed at the Edges of the Closing OF
To characterize the transcriptome of the mouse OF, we isolated the OC tissue surrounding the OF, as well as tissues in the center of temporal and nasal OCs of E11.5 mouse embryos, and then used microarrays to compare the transcriptomes of different OC regions (manuscript in preparation). Microarray analysis showed that there were five ADAMTS/ADAM family metalloproteinase genes (Adamts16, Adamts15, Adam23, Adamts6, and Adamts12) specifically enriched in the OF transcriptome when compared with the central OC transcriptome (Supplementary Fig. S1A). Since no studies have linked proteinases with OF closure, we decided to investigate the function of these genes during OF development. We used ISH to verify the OC expression patterns of these five metalloproteinases. We collected E11.5 mouse embryonic heads, the time point at which the mouse OF is closing, and made sagittal sections across the eye to better present the OF ISH of Adam23, Adamts6, and Adamts12 did not show clear signals in the OF region (Supplementary Figs. S1D-F) and were eliminated from further analysis. ISH of both Adamts15 and Adamts16 showed strong signals at the very edge of the OF (Fig. 1B, Supplementary Fig. S1B, arrows) and the signals became more prominent in the more proximal sections near the OS (Fig. 1C and data not shown); however, further examination of their expression patterns in zebrafish showed that adamts15 was not expressed in the developing zebrafish OC (Supplementary Fig. S1C), while adamts16 showed a conserved expression pattern at the very edge of the closing zebrafish OF (Figs. 1E-H); therefore, we decided to focus on Adamts16 for further functional analysis. It is notable that we also examined the expression of Adamts16 in the developing mouse OC before and after OF closure. In E10.5 embryos before OF closure, Adamts16 was expressed at the very edges of the OF, as well as the OS, very similar to its expression pattern in E11.5 embryos (Fig. 1A and data not shown). In E13.5 embryos after OF closure had completed, Adamts16 expression disappeared from the OC (Fig. 1D). In addition,
Adamts16 was also found to be expressed in the developing neural epithelium, liver, and urogenital ridge (Supplementary Fig. S2).

We then used zebrafish as a model organism to study Adamts16 function during OF development. For this purpose, we first used ISH to characterize adamts16 expression patterns in zebrafish embryonic eyes. ISH results showed that at 24 hpf, adamts16 was expressed in the ventral (future temporal, due to rotation of the eye during development) half of the OC (Figs. 1C, 1C') but was absent from the OF (arrow). At 36 hpf, when the zebrafish OF begins to close,29,30 adamts16 expression spread across the entire OC, but the signal was most intense in the inner portion of the OC close to the lens; the temporal side exhibited a stronger adamts16 signal than the nasal side (Figs. 1F, 1F'). Interestingly, at 36 hpf, adamts16 was expressed at the very edge of the closing zebrafish OF (Fig. 1F', red arrow), reminiscent of what was seen for mouse Adamts16 expression at the closing OF edges (Figs. 1H, 1H'). Moreover, adamts16 was also found in the periocular tissues between 24 and 48 hpf (Figs. 1E–H, 1E'–H') and in the developing brain, gut, otic vesicle, and horizontal myoseptum (Supplementary Fig. S3). Therefore, ISH demonstrated that Adamts16 is expressed at the edges of the closing OF in both mice and zebrafish.

**adamts16** Is Required for Proper Closure of the OF in Zebrafish

To study adamts16 function during eye development, we synthesized two MOs against adamts16: adamts16-MO1 blocks translation initiation, while adamts16-MO2 disrupts splicing between exon2 and intron2, resulting in a frameshift mutation and premature translation termination (Fig. 2A). Before using these MOs to study adamts16 function, we first examined MO knockdown efficacy. For translation-blocking MO1, we used a modified GFP reporter that contains an MO1-targeting sequence. When the EF1α:MO1GFP plasmid was injected alone, the zebrafish body showed a strong GFP signal throughout; however, when EF1α:MO1GFP was coinjected with MO1, the GFP signal was barely detected (Fig. 2B, left), suggesting that adamts16 can be efficiently knocked down by MO1. We also used Western blot to examine the change in

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**FIGURE 1.** Adamts16 is expressed at the closing OF (A–D) Adamts16 mRNA in situ hybridization of mouse embryonic sagittal eye sections. (A) E10.5 OC. (B) E11.5 central OC. (C) E11.5 OC more proximal, close to the OS. (D) E13.5 OC. Arrows point to the specific Adamts16 signals in the OF region. (E–H) Lateral views of adamts16 mRNA whole-mount in situ hybridization of (E) 24-hpf, (F) 36-hpf, (G) 48-hpf, and (H) 72-hpf zebrafish embryos. (E', F') Section in situ hybridization images of the corresponding (E–H) stages. The red arrow in (F') points to the adamts16 signal at the edge of the closing OF. Scale bar in (C): 50 μm, applies to (A–C); in (D): 50 μm; in (H): 100 μm, applies to (E–H); and in (H'): 50 μm, applies to (E'–H').

Adamts16 was required for proper closure of the OF in zebrafish.
Adamts16 Regulates Optic Fissure Closure

**Figure 2.** *adams16* is required for zebrafish OF closure. Diagram illustrating a portion of the zebrafish *adams16* gene, MO1 and MO2 targeting sites, and primers used to examine splicing products before and after MO2 injection. (B) Left: MO1 knockdown efficacy reporter assay. Right: Western blot analysis showing *adams16* protein depletion from MO1-morphant fish bodies. (C) Testing MO2 knockdown efficacy by RT-PCR using primers indicated in (A); amplicons from control fish RNA were 401 bp, while MO2 disruption of splicing resulted in the appearance of smaller 319-bp amplicons (arrow). (D) Knockdown of *adams16* by either MO1 or MO2 resulted in coloboma and microphthalmia. (E) Eye surface area of MO1- and MO2-morphant zebrafish. Data were normalized as the percentage of average eye surface area to the standard control MO-injected zebrafish. (F) Quantification of coloboma prevalence shows that coloboma phenotype of MO1- or MO2-morphants can be rescued by *adams16* mRNA injection. (H) Quantification of coloboma prevalence shows that the coloboma phenotype in MO1- or MO2-morphants could not be rescued by the catalytic site–mutant form of *adams16* mRNA injection. Numbers under each column in (E), (G), and (H) denote the number of zebrafish injected in each group.
protein levels. Indeed, the adamts16 protein was almost completely depleted in 24-hpf MO1-morphant zebrafish (Fig. 2B, right), demonstrating the efficacy of adamts16 knockdown by MO1. We then performed RT-PCR by using primers flanking exon 2 (Fig. 2A) to detect the exon 2/intron 2-splicing-disrupted transcript. MO2 injections resulted in exon 2 deletion in some adamts16 transcripts (Fig. 2C, arrow), demonstrating that MO2 indeed disrupts splicing between exon 2 and intron 2.

We then used the two MOs to study adamts16 function in eye development. We injected MOs into one-cell-stage embryos and examined the eyes at 72 hpf when the OF is normally fully sealed (Figs. 2D–a). Interestingly, the OF of MO1- or MO2-injected fish remained open (Figs. 2D b–d, arrows), demonstrating that adamts16-morphant zebrafish developed coloboma. The appearance of the coloboma phenotype varied between morphants. From the extent of the opening of the unsealed OF, we grouped coloboma phenotypes into three categories: mild, where edges of retinal tissues on opposite sides of the unclosed OF approached each other but remained unfused (Figs. 2D–b); moderate, where the distance between edges of the unsealed OF was clearly visible (Figs. 2D–c); and severe, where a significant portion of the retina was missing (Figs. 2D–d). The penetrance of coloboma phenotypes in both MO1- and MO2-morphant fish depended on dosing dependence. At low dosages (4 ng MO1 and 0.5 ng MO2), approximately 15% of embryos exhibited the mild coloboma phenotype, whereas the incidence of coloboma gradually increased with increasing dosage to over 90% at high dosages (8 ng MO1 and 1.5 ng MO2). Coloboma severity also increased with increasing dosage (Fig. 2E). The consistent and dosage-dependent coloboma phenotype observed for both MOs suggests that adamts16 is required for OF closure. In addition to coloboma, adamts16-morphant eyes were also smaller than control MO-injected eyes (Fig. 2D). At a moderate dosage (6 ng MO1 and 1 ng MO2), the average surface area of MO1-morphant eyes was 92.2% of the standard control, while the average surface area of MO2-morphant eyes was only 71.7% of standard control MO-injected eye surface area (standard control group, n = 52 eyes; MO1 group, n = 54 eyes; MO2 group, n = 54 eyes; Fig. 2F).

To further demonstrate the specificity of MO1 and MO2 against adamts16, we performed mRNA coinjection rescue experiments. We synthesized adamts16 mRNA with a 2-nt mutation in the MO1-targeting sequence and coinjected this mRNA with MO1 or MO2. Indeed, coinjection of adamts16 mRNA (440 pg) with MO1 (8 ng) or MO2 (1.5 ng) rescued the coloboma phenotype by decreasing formation incidence from 77.2% to 22.4% and 92.2% to 8.8%, respectively (Fig. 2F), whereas the mutant form of adamts16 mRNA (440 pg), in which the translation initiation codon was mutated, failed to rescue the phenotype (Fig. 2G); this suggests that coloboma formation in MO1- and MO2-morphant fish was specifically caused by adamts16 knockdown. To examine if proteinase activity of adamts16 is necessary for proper OF closure, we synthesized a catalytically inactive form of adamts16 mRNA, in which the essential histidines in the HEXHXXXGXXH catalytic motif were mutated to prolines. Rescue experiments showed that the catalytic site–mutant form of adamts16 mRNA (400 ng) failed to rescue the coloboma phenotype of both MO1 (6 ng)- and MO2 (1 ng)-morphant fish (Fig. 2H), suggesting that proteinase activity is necessary for adamts16 function in OF closure.

Altogether, MO knockdown and rescue experiments indicate that adamts16 is required for proper OF closure in zebrafish eyes.

**adamts16-Morphant OCs Proliferate Slowly**

In addition to coloboma, adamts16-morphant zebrafish also developed microphthalmia (Figs. 2D, 2F). In addition, finely tuned cell proliferation is critical for proper OF closure. Therefore, we examined whether cell proliferation was altered in adamts16-morphant OCs. We labeled live 3–4-hpf zebrafish embryos with BrdU for 2 hours and harvested fish at 36 hpf when the proliferation wave that sweeps across the OC is halfway complete, and when OF fusion is initiated. We counted all BrdU+ cells in the middle four sections of each eye and analyzed four to six eyes with moderate coloboma phenotypes for each MO group. In standard control MO (6 ng)-injected fish, approximately 55% of retinal progenitor cells in each OC were BrdU+, which decreased to 36% and 41% for MO1- and MO2-morphant groups, respectively (Figs. 4A–C). There was no observable change in the distribution pattern of BrdU+ cells in adamts16-morphant OCs (Figs. 4A, 4B). Therefore, adamts16-morphant OCs proliferate slower than normal, which may be a significant factor in microphthalmia phenotype development and may contribute to coloboma formation.

**adamts16 Knockdown Affects Survival of OC Cells**

Since increased cell death may also result in small eyes, we next analyzed apoptosis in 56-hpf adamts16-morphant OCs. We used the TUNEL assay to reveal apoptotic events and selected one middle sagittal section representative of each eye for cell counting. In zebrafish developing OCs, apoptosis is a rare event and only 1.2 apoptotic cells per section, on average, were observed in standard control MO (6 ng)-injected embryos (Figs. 4D, 4F). However, in adamts16-morphant OCs, the number of apoptotic cells increased dramatically to 3.8 and 8.4 cells per section in MO1 and MO2 groups, respectively (Figs. 4E, 4F). This increased rate of cell death may contribute to the microphthalmia phenotype of adamts16-morphant zebrafish, as well as failure in OF closure.

It has been reported that MO injection can elicit a sequence-independent p53-dependent cell death pathway. To test if
**FIGURE 3.** *adams16*-morphant OCs fail to disassemble the basement membrane at the closing OF. The 60-hpf zebrafish embryonic eye sagittal sections were stained with anti-laminin (A–C) or anti-fibronectin (D–F) antibodies. (A, D) Control eyes. Arrows point to the location of the previous OF. (B, E) *adams16*-morphant eyes with mild coloboma. Arrows point to the unfused OF with intact basement membrane. (C, F) *adams16*-morphant eyes with moderate coloboma. Arrows point to the unclosed OF. Scale bar: 50 μm.
p53-mediated cell death was responsible for coloboma formation in *adamts16*-morphant fish, we coinjected MO1 or MO2 with p53 MO. Knocking down p53 did not rescue the coloboma phenotype of both MO1- and MO2-morphant fish (Fig. 4G), suggesting that coloboma was not caused by the nonspecific toxicity or off-target effects of MOs.

**Gene Expression Changes in *adamts16*-Morphant Eyes**

Human genetic and animal model studies have revealed several key cellular events involved in successful OF closure. To test if *adamts16* regulates OF closure through regulating these cellular events, we performed ISH to analyze expression of the key genes involved in these events. Since the OF is a ventral-specific structure of the OC, mutations affecting retinal ventral fate establishment may cause coloboma; therefore, *tbx5* and *vax2* were selected to represent dorsal and ventral retinas, respectively. The results showed that *tbx5* and *vax2* were expressed normally in *adamts16*-morphant OCs (Figs. 5A–D), suggesting unchanged dorsal-ventral fates. RA signaling is an important regulatory pathway for eye morphogenesis, including OF closure. The two major RA synthesizing enzymes, *aldh1a2* and *aldh1a3*, are also reliable markers of dorsal and ventral retinas, respectively. We therefore performed ISH with these two genes to test for RA production and dorsal-ventral polarity. The results showed that *aldh1a2* and *aldh1a3* were expressed normally in dorsal and ventral OCs of *adamts16*-morphants, respectively (Figs. 5E–H), suggesting unchanged RA signaling and dorsal-ventral fates. The OF provides the pathway for POM cells to migrate into developing OCs, and conversely, POM cells also provide regulatory signals that promote proper OF closure. Therefore, we examined the expression of a POM regulator—*foxc1*—to examine POM cell distribution. POM cells were found to be normally distributed around the OF in *adamts16*-morphant OCs (Figs. 5I, 5J). Moreover, we examined the expression of *pax2*, a marker and regulator of the OF, and found that *pax2* was expressed normally in the OF region in *adamts16*-morphant OCs (Figs. 5K, 5L), suggesting that OF identity was properly established and maintained in *adamts16*-morphants.

Finally, to systematically examine how *adamts16* knockdown affected OC gene expression, we used high-throughput sequencing to compare the transcriptomes of 36-hpf *adamts16* MO1- and MO2-morphant eyes with those of control eyes. Data analysis showed that *adamts16* knockdown did not alter OC gene expression drastically; only 54 genes were found to be downregulated, and 47 genes were upregulated in MO1- or MO2-morphant eyes (fold change ≥ 2, probability ≥ 80% FDR). KEGG pathway analysis showed that differentially expressed genes (DEGs) in *adamts16*-morphant eyes compared with wild-type control eyes were enriched in the following pathways: p53, apoptosis, ribosome biogenesis, FoxO, and Toll-like receptor signaling. Ribosome biogenesis pathway enrichment might reflect on the slightly different metabolism
status between *adamts16*-morphant and wild-type eyes. DEGs contributing to FoxO and Toll-like receptor pathways all overlapped with the p53 or apoptosis pathways (see Supplementary Table S2 column J for the gene names); this may merely reflect the involvement of these two pathways in cell survival and stress response regulation. Even though p53 pathway components, including p53, were enriched in the DEG list, MO knockdown of p53 did not rescue the coloboma phenotype of *adamts16*-morphant fish (Fig. 4G); this suggests that upregulation of the p53 pathway may be due to nonspecific off-target effects of MO1/MO2 injection. In addition to p53, caspase8—a core component of the apoptosis cascade pathway—was significantly upregulated in *adamts16*-morphant eyes (Fig. 5M, right), and this upregulation may be responsible for the increased apoptosis activity observed in morphant eyes. Among the significantly downregulated genes, there were several retinal neurogenesis regulatory transcription factors, such as atoh7, crx, rorb, sox12, and foxn4, and several crystallin genes, such as crygmd2d and cryaa (Fig. 5M, left), which may reflect the slightly delayed development of *adamts16*-morphant eyes. The cell cycle regulator cdkn1a (p21) was among the significantly upregulated genes (Fig. 5M, right), possibly leading to the decreased proliferation activity of *adamts16*-morphant eyes. Interestingly, *fgf8* was also significantly upregulated in *adamts16*-morphant eyes (Fig. 5M, right, red underline). It has been shown that increased expression of *fgf8* in *aussicht* (*aus*) mutants disrupts ventral OC development and leads to coloboma. Therefore, we performed ISH to examine changes in *fgf8* expression. In the developing zebrafish head at 36 hpf, *fgf8* is strongly expressed at the midbrain-hindbrain boundary (Fig. 5N, red arrowhead) and in the OS (Fig. 5N, red arrow) but can barely be seen in the OC (Fig. 5N, dashed circle). However, in *adamts16*-morphants, *fgf8* was clearly upregulated in the OC (Fig. 5O, dashed circle). To investigate whether upregulated *fgf8* was responsible for...
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coloboma formation in adams16-morphant fish, we co-injected fgf8 MOs and adams16 MOs. Indeed, knocking down fgf8 partially rescued the coloboma phenotype of both MO1- and MO2-morphant fish (Fig. 5P), suggesting that coloboma formation in adams16-morphants was partially mediated by ectopic fgf8 upregulation. Overall, gene expression analysis and rescue experiments suggest that adams16 regulates OF closure through regulation of cell proliferation, survival, and restriction of fgf8 expression.

**DISCUSSION**

In the present study, we showed that adams16—a metalloproteinase—is specifically expressed at the edges of the closing OF in both mice and zebrafish eyes. Using zebrafish as a model organism, we demonstrated that adams16 is required for proper OF closure. Key cellular events, gene expression analysis, and rescue experiments indicate that adams16 regulates OF closure through mediating basement membrane disassembly, promoting cell proliferation and survival, and restricting fgf8 expression. It has been shown that OF closure involves the two sides of the retina moving close and apposing tightly against each other, disassembly of the basement membrane between the two sides of the OF, and finally, establishment and rearrangement of cell-cell interactions between cells from both sides of the OF. Although previous studies have elucidated the possible mechanisms contributing to the forces that draw together retina tissues and the cell-cell contact rearrangement during OF closure, the questions of how basement membrane disassembly is triggered and which proteinases are involved remained unanswerable. For this reason, we were particularly interested in Adams16 from the list of genes that were significantly enriched in the OF transcriptome, since it belongs to a large family of metalloproteinases that function in degrading extracellular matrices. Further supporting the possibility that Adams16 may be involved in the disassembly of the basement membrane for OF closure is the overlapping expression pattern of Adams16 with that of the OF fusion process. We found that Adams16 is specifically expressed at the very edge of retinal tissues at the OF region during the initial stage of OF closure, in both mice and zebrafish. Our gene knockdown and rescue experiments showed that disruption of adams16 expression resulted in coloboma, demonstrating the importance of adams16 for proper OF closure. Furthermore, we found that adams16-morphant eyes with mild coloboma phenotypes, where the two sides of retinal tissues had come close and were apposed tightly, exhibited intact basement membrane, indicating that adams16 is associated with basement membrane degradation. Whether or not adams16 directly degrades basement membrane to drive OF fusion requires further investigation.

Unlike in mice where Adams16 expression is restricted to the OF region, zebrafish adams16 is also expressed inside the OC. In addition, adams16-morphants developed a microphthalmia phenotype, and cell proliferation and apoptosis were affected by adams16 knockdown. How adams16 regulates cell proliferation and survival is unclear. Adams16 is a secreted proteinase and other members of the ADAMTS family and its related ADAM family have been shown to process many different molecules, such as thrombospondin-1, TGF-β, and Notch, for their release or maturation, thereby regulating a variety of biological processes, such as angiogenesis, cell differentiation, and cell proliferation. It is feasible that adams16 regulates retinal progenitor cell proliferation and survival through non-cell-autonomous mechanisms, possibly by mediating the release or maturation of extracellular growth factors and survival factors.

Interestingly, high-throughput sequencing and ISH showed that fgf8 was ectopically upregulated inside the OC, a phenomenon that was also seen in zebrafish aus mutants that also develop coloboma. It is unclear which gene is mutated in aus mutants, but it has been demonstrated that fgf8 signaling is upregulated at many sites in aus mutant embryos, including the OC. fgf8 expression around the developing zebrafish eye appears to require tight regulation for proper OF closure, since loss of fgf8 in acerebellar (ace) mutants also results in mild coloboma (Fig. 5P). It appears that adams16 is involved in restricting the expression pattern of fgf8 in the developing OC to ensure proper OF morphogenesis. In agreement with this notion, our experiments showed that co-knockdown of adams16 and fgf8 partially rescued the coloboma phenotype observed in adams16-morphants. How adams16 regulates fgf8 expression in the OC is unclear, but the regulation is probably indirect, since adams16 is not a transcription factor and does not regulate gene transcription directly. Several regulatory pathways implicated in OF closure were not altered in adams16-morphant OCs, but it is possible that adams16 functions downstream of some of these pathways. Indeed, we found that adams16 protein expression was dramatically downregulated in pax2a-morphant eyes (Supplementary Fig. S4), suggesting that adams16 expression is controlled by pax2a. It has been reported that fgf8 regulates pax2a expression during eye development. Our findings showed that pax2a promotes adams16 expression, while adams16 restricts fgf8 expression; this suggests that fgf8, pax2a, and adams16 form a regulatory feedback loop that controls OF morphogenesis.

Ophthalmic genetic studies have revealed that human coloboma exhibits significant genetic heterogeneity, with the causative genes of many cases unknown, reflecting the complexity of cellular and molecular events involved in OF development regulation. To the best of our knowledge, this study marks the first time a metalloproteinase—Adams16—is linked to the disassembly of basement membrane during OF closure, a key step that clears the way for cells from both sides of the OF to directly contact each other and form connections to complete the fusion process. Adams16 may not be the only ADAMTS family member involved in OF closure, since several other family members, especially Adams15, are also enriched in the OF region (Supplementary Fig. S1). In the future, it would be interesting to examine metalloproteinase expression patterns in human OCs and their potential involvement in human coloboma cases.

**Acknowledgments**

Supported by the National Natural Science Foundation of China (81371057), the Guangzhou Science Technology and Innovation Commission (201504010030), the Guangdong Provincial Department of Science and Technology (2015B020225005), and the Ministry of Science and Technology of China 973 Program (2015CB964400).

Disclosure: M. Cao, None; J. Ouyang, None; J. Guo, None; S. Lin, None; S. Chen, None

**References**


