Apoptotic and Proliferative Defects Characterize Ocular Development in a Microphthalmic BMP Model

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PURPOSE. Vision is critically dependent on ocular size, which is regulated by environmental and genetic factors. Mutation of human Growth and Differentiation Factor 6 (GDF6) or zebrafish gdf6a results in a spectrum of small eye phenotypes (microphthalmia, anophthalmia, and coloboma). However, current models do not explain their etiology fully. As such, analyses of apoptosis and cell cycle regulation were undertaken in a zebrafish gdf6a mutant.

METHODS. Microarray analysis was performed at 2 days after fertilization to uncover novel gdf6a-dependent cell cycle regulators. Altered expression of Gdf6a targets was confirmed by in situ hybridization, and resulting changes in cell proliferation were assessed by phosphohistone H3 immunohistochemistry. Analysis of apoptosis was evaluated through activated Caspase 3 immunohistochemistry and chemical inhibitors of cell death.

RESULTS. Reduced numbers of retinal progenitor cells are observed at 24 hours post fertilization (hpf), resulting in microphthalmic eyes in gdf6a+−/− embryos. At 28 hpf, a wave of apoptosis occurs; however, apoptosis inhibition does not rescue eye size, indicating a limited contribution. Mutants display altered proliferation and expression levels of cell cycle regulators, including members of the forkhead box transcription factor family expressed in the ciliary marginal zone. Notably, inhibition of foxi2 in gdf6a+−/− embryos further reduces eye size.

CONCLUSIONS. These data support a model whereby the gdf6a−−/induced microphthalmia is based on early regulation of retinal progenitor cell number, and later by regulation of proliferation in the ciliary marginal zone. Foxi genes represent downstream effectors of Gdf6a function in the CMZ required for eye size determination.

Keywords: bone morphogenetic protein, forkhead box, Gdf6, apoptosis, proliferation

Microphthalmia, the presence of a small eye, and anophthalmia, the complete lack of ocular tissue, represent part of a phenotypic spectrum of congenital anomalies present in up to 11% of blind children.12 Their complex etiology includes environmental and genetic factors.14 The importance of lens induction is illustrated by reduced levels of mouse Six3, Pax6, or Sox2 resulting in microphthalmia or anophthalmia.15,16 In mammals, Pax6, Sox2, and Otx2 contribute to the specification of the optic vesicle, whose inner layer becomes the neural retina, and requires Pax6 and Sox2 expression for accurate progenitor cell specification.13,14 Mouse and chick analyses similarly demonstrate the requirement of Mitf and Otx2 for RPE specification, which is essential for normal ocular growth.7,15,16

Central to the determination of eye size is the control of retinal progenitor cell proliferation and survival. Genes regulating both processes cause microphthalmia in patients and model organisms when mutated.17,18 Subsequent to cell cycle exit and differentiation of retinal neurons, the zebrafish eye continues to grow via the addition of new retinal neurons. A population of progenitor cells is maintained for this purpose and model organisms when mutated.17,18 Subsequent to cell cycle exit and differentiation of retinal neurons, the zebrafish eye continues to grow via the addition of new retinal neurons. A population of progenitor cells is maintained for this purpose and model organisms when mutated.17,18 Subsequent to cell cycle exit and differentiation of retinal neurons, the zebrafish eye continues to grow via the addition of new retinal neurons. A population of progenitor cells is maintained for this purpose and model organisms when mutated.17,18 Subsequent to cell cycle exit and differentiation of retinal neurons, the zebrafish eye continues to grow via the addition of new retinal neurons. A population of progenitor cells is maintained for this purpose and model organisms when mutated.17,18 Subsequent to cell cycle exit and differentiation of retinal neurons, the zebrafish eye continues to grow via the addition of new retinal neurons. A population of progenitor cells is maintained for this purpose and model organisms when mutated.17,18 Subsequent to cell cycle exit and differentiation of retinal neurons, the zebrafish eye continues to grow via the addition of new retinal neurons. A population of progenitor cells is maintained for this purpose and model organisms when mutated.17,18 Subsequent to cell cycle exit and differentiation of retinal neurons, the zebrafish eye continues to grow via the addition of new retinal neurons. A population of progenitor cells is maintained for this purpose and model organisms when mutated.
concerning the molecular regulation of cell proliferation in the CMZ.

The bone morphogenetic proteins, and growth and differentiation factors (BMPs/GDFs) have well-recognized ocular developmental roles. BMPs regulate apoptosis and proliferation in the eye, with mutation or copy number variation of 3 BMPs (GDF3, GDF6, and BMP4) associated with microphthalmia, anophthalmia, or coloboma (MAC) in patients. The contribution of this gene family is extended by the large number of paralogs implicated in eye development patients. The contribution of this gene family is extended by the large number of paralogs implicated in eye development through model organism analysis (e.g., bmp2, bmp7, gdf11). Likewise, perturbed function of multiple BMP antagonists, including gremlin, chordin-like 1, and Bambi, induce ocular anomalies in model organisms and for some (CHRD/1), comparable and severe patient anterior segment phenotypes have been defined. We demonstrated previously gdf6a−/− mutant zebrafish lack Smad 1/5/8 phosphorylation in the developing retina, and we and others demonstrated that gdf6a lies at the top of the hierarchy of genes controlling the patterning of the dorsal-ventral axis of the developing retina. In addition to this fundamental role in retinal patterning, gdf6a mutants display bilateral microphthalmia.

Since the cellular mechanisms that underlie this microphthalmic phenotype have yet to be elucidated, we hypothesized that aberrant regulation of cell cycle and apoptosis might contribute, and examined during gdf6a−/− ocular development in zebrafish. We demonstrated that gdf6a−/− eyes are microphthalmic at early developmental stages due to reduced retinal progenitor cell number. We showed that rescue of elevated ocular apoptosis during development does not restore normal eye size in gdf6a−/− larvae. Microarray analysis revealed aberrant expression of genes with roles in cellular proliferation in the CMZ, which included members of the forkbead box (foxi) family of transcription factors. Notably, several of these genes have fundamental roles in development and cell cycle control, and contribute to ocular disorders. We find a significant reduction in CMZ proliferation in gdf6a−/− larvae, with a requirement of foxi1 and foxi2 in mediating dorsal-ventral polarity of the CMZ, and in Gdf6a-mediated eye size determination.

**METHODS**

**Zebrafish Husbandry, Morpholino Injections, and In Situ Hybridization**

Zebrafish were cared for according to standard protocols, and embryos grown in embryo media at either 25.5°C, 28.5°C, or 32°C to be staged appropriately. Zebrafish embryos grown past 24 hours post fertilization (hpf) were treated with 0.003% 1-phenyl 2-thiourea (PTU; Sigma-Aldrich, St. Louis, MO) to prevent pigment formation. The AB strain of wild-type (WT) fish and the gdf6a homozygous line were used. The latter, hereafter described as gdf6a−/−, encodes a 555X truncation producing a 5 amino acid peptide lacking the mature domain. For inhibition of foxi2, 4 ng of a translation blocking morpholino (MO) (CCGATGTTGCTATCTCCGGTG) were injected into 1- to 2-cell stage embryos. In situ hybridization (ISH) was performed as described previously with embryos fixed overnight at 4°C in 4% paraformaldehyde (PFA) and permeabilized by incubation in 10 μg/ml Proteinase K for 20 minutes. Animal care protocols were approved by the University of Alberta Biosciences Animal Care Committee and comply with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

**Microarray Analysis**

Microarrays were performed using Agilent’s 4×44K whole genome chips (version 2; Agilent Technologies, Santa Clara, CA). Four biologic replicates were performed, with each array using RNA isolated from separate matings. RNA was isolated from 30 phenotypically WT and 30 gdf6a−/− eyes at 2 days post fertilization (dpf) using the RNasequeous kit (Ambion, Austin, TX), and then amplified and labeled (low input linear amplification kit; Agilent Technologies). Labeled RNA was hybridized for 17 hours at 65°C and then processed per the manufacturer’s protocol (Agilent Technologies), scanned on a GenePix 4000 scanner (Molecular Devices, Sunnyvale, CA) before data extraction using Agilent Technologies’ Feature Extraction Software.

**Immunohistochemistry**

For analysis of apoptosis, embryos fixed in 4% PFA were permeabilized with ice-cold acetone (7 minutes), washed in water (5 minutes), before four 5-minute washes in PBS with 0.5% Tween-20 (PBST). For analysis of cell proliferation, fixed embryos were permeabilized using Proteinase K (10 μg/ml for 20 minutes at 2 dpf or 45 minutes at 4 dpf), refixed (20 minutes in 4% PFA), washed four times in PBST (5 minutes each), and then treated with 95°C 10 mM citric acid buffer (10 minutes). Slides containing 15 μM sections were treated with 95°C citric acid buffer, but were not permeabilized. Embryos and slides were blocked for 1 hour at room temperature in 5% normal goat serum and 2% BSA. Primary antibodies used were rabbit anti-activated Caspase 3 (1/1000; BD Biosciences, San Jose, CA), and rabbit antiphosphohistone H3 (1/1000; BD Biosciences). Embryos and slides were incubated in secondary antibody (goat anti-rabbit Alexa Fluor 488, 1/1000; goat anti-rabbit Alexa Fluor 568; Molecular Probes, Eugene, OR) for 2 hours at room temperature. All embryos were washed for 5 minutes in PBST, then 4 times for 10 minutes in PBST after primary and secondary antibody incubations. Hoechst 33258 nuclear stain (1/1000; Molecular Probes) was added to the second 10-minute wash after secondary antibody incubation. Embryos and slides were mounted in Prolong Gold (Molecular Probes) for visualization.

**Genotyping**

The offspring of a gdf6a−/− heterozygous incross were genotyped by high resolution melt (HRM) analysis performed on genomic DNA, extracted in 10 μl of 50 mM NaOH (95°C, 10 minutes, neutralized with 1 μl Tris-HCl, pH 8.0). PCR was performed using primers optimized for HRM (GCGTTTGTGGCACAAGGTGC CCGGTGCTTAAAATCATCGC). MeltDoctor HRM Master Mix (Applied Biosystems, Foster City, CA) and an ABI 7500 HT Fast RT PCR machine (Applied Biosystems) or Qiagen Rotor Gene Q qPCR machine (1 cycle, 95°C for 10 minutes; 40 cycles, 95°C for 15 seconds and 60°C for 20 seconds; 1 cycle, 95°C for 15 seconds, 60°C for 1 minute, 95°C for 15 seconds, and 60°C for 15 seconds; 2 cycles, 95°C for 15 seconds and 60°C for 20 seconds; 1 cycle, 95°C for 15 seconds, 60°C for 1 minute, 95°C for 15 seconds, and 60°C for 15 seconds; Qiagen, Inc., Valencia, CA). Results were analyzed via HRM 2.0 Software (Applied Biosystems) or Qiagen software v2.02 (Qiagen, Inc.) and variants initially were confirmed by Sanger sequencing. Quantification of average eye size was performed using arbitrary units in ImageJ (National Institutes of Health [NIH], Bethesda, MD), with gdf6a−/− control-treated eyes quantified as 100% eye size.

**Pharmacologic Treatment**

Embryos were treated from 5 to 31 hpf with 0.1 μM PTC3 (Asinex, Moscow, Russia) or dimethyl sulfoxide (DMSO) as a
vehicle control. Then, 28 hpf embryos were fixed and whole-mount immunofluorescence was performed using anti-active Caspase 3 antibody (BD Pharmingen, San Diego, CA) with the nuclear counterstain Hoechst 33258. Dissected eyes were visualized using confocal microscopy (Zeiss LSM 700 on Axio Observer.Z1: Carl Zeiss Microscopy, LLC, Thornwood, NY). All photographed eyes were genotyped by PCR and sequencing. For quantification of eye size, embryos treated with P7C3 from 5 to 31 hpf were transferred into regular embryo media and grown to 3 dpf. Embryos were photographed and genotyped individually (as described above). Eye area was quantified using ImageJ (NIH). Student’s t-tests were performed with Bonferroni correction for multiple comparisons.

**Results**

**Loss of gdf6a Expression Results in Microphthalmia**

The zebrafish gdf6a^−/− line studied contains a c.164C → A mutation that creates a premature stop codon, resulting in premature truncation of the protein, with loss of the mature domain of the peptide.54 Homozygous mutant embryos display bilateral microphthalmia, and in comparison with WT or heterozygotes, the eye is noticeably smaller at 2 dpf (Figs. 1A, 1B), with microphthalmia more pronounced by 4 dpf (Figs. 1C, 1D). Previous studies demonstrated gdf6a's expression adjacent to the developing eye field at the 3 somite stage, and from the 10 somite stage onwards in the dorsal retina and dorsal epidermis,33,54 indicating that specification of retinal progenitor cells may influence eye size. From 2 to 4 dpf, gdf6a is expressed within the dorsal CMZ (Figs. 1E–H), highlighting a potential role for defective cell proliferation in the determination of gdf6a^−/− eye size.

**Reduced Number of Retinal Progenitor Cells in gdf6a^−/− Embryos at 24 hpf**

As eye size is noticeably smaller in gdf6a^−/− embryos by 24 hpf, we conducted an analysis of the number of retinal progenitor cells and proliferation at this time point. Although the expression of a subset of genes that control proliferation is reduced in homozygous mutants (mycn, Figs. 2A, 2B and mycb, Figs. 2C, 2D), the number and percent of proliferating cells at this time point are unchanged (Figs. 2E–H). Thus, it is unlikely that early small eye phenotypes are due to a proliferation defect. However, the number of progenitor cells clearly is reduced in gdf6a^−/− eyes (111.7 ± 8.0) when compared to WT siblings (125 ± 7.7, P = 0.00017, t-test, Fig. 2G), indicating that processes occurring before 24 hpf influence eye size in gdf6a^−/− embryos.

**Inhibition of the Increased Rates of Apoptosis in the gdf6a^−/− Eye Does Not Rescue Eye Size**

Previous characterization of gdf6a^−/− embryos56 and our findings (Fig. 3) show high rates of apoptosis throughout the developing eye. To determine if this contributes to microphthalmia, as suggested previously,56 gdf6a^−/− embryos were treated with either P7C3, a pharmacologic agent that inhibits apoptosis,12 or DMSO control. Analysis of ocular activated Caspase-3 staining at 28 hpf indicated that heterozygous embryos have low levels of apoptosis when incubated with either P7C3 or DMSO (Figs. 3A, 3C). High levels of apoptosis are observed in control DMSO-treated gdf6a^−/− embryos (mean number of foci [μ] = 76, n = 34), which is decreased significantly when treated with P7C3 (μ = 16, n = 35, P < 0.00001, t-test; Figs. 3B, 3D). This rescue of apoptosis in gdf6a^−/− did not result in any significant rescue of eye size at 3 dpf, as P7C3-treated gdf6a^−/− eyes still were significantly smaller than their control-treated heterozygous siblings (50.3% of eye size, t-test P < 0.00000001) or P7C3-treated heterozygous siblings (62% of eye size, P < 0.001, t-test, Fig. 3E). These data demonstrated that gdf6a^−/−-induced apoptosis is not a major contributor to the microphthalmia phenotype, as partial rescue does not measurably affect ocular size. Incubation of embryos in P7C3 may affect other aspects of retinal development in addition to apoptosis, as evidenced by the subtle change in eye size of treated heterozygous eyes (Fig. 3E).

**Genes With Roles in Cell Cycle Progression Have Reduced Expression in gdf6a^−/− Eyes**

To investigate whether later developmental events contributed to microphthalmia in gdf6a mutants, we assessed transcriptome changes at 2 dpf via microarray analysis. Using cut-offs of ≥1.75-fold change in expression, 220 transcripts were down-regulated and 90 transcripts were upregulated in gdf6a^−/−/WT eyes (Supplementary Tables S1 and S2, respectively). Notably, the microarray results revealed significant alteration in expression of genes with roles in cell differentiation and patterning, including atonal homolog 7, t-box 2b, and t-box 4, forkhead box N4, and H6 family homeobox 1, with the findings validated by in situ hybridization (Fig. 4, Supplementary Figs. S1A-J). To identify gdf6a-dependent transcripts that have a role in proliferation, we focused on genes with ocular expression in the CMZ. Many genes responsible for regulation of cell cycle were down-regulated in gdf6a^−/− embryos (Fig. 5), suggesting that they contributed to the microphthalmia phenotype. Indeed, in situ hybridization demonstrated the expression of six genes reduced or eliminated specifically in the CMZ of gdf6a^−/− eyes, but minimally changed or unaffected in other areas of the embryo. This is compatible with a gdf6a specific role in control of ocular cell cycle. Altered genes include carbamoyl-phosphate synthetase 2, aspartate transcarbamylase, and dihydroyrotoase (cad, Figs. 5A–D), ubiquitin-like with PHD and ring finger domain containing 1 (ubhf1, Figs. 5E–H) and retinal homeobox1 (rx1, Figs. 5I–L). Expression of minichromosome maintenance complex component 3 (mcm3, Figs. 5M–P) and proliferating cell nuclear antigen (pcna, Figs. 5Q–T) is highly down-regulated in the outer retina and CMZ in gdf6a^−/− embryos, but minimally changed elsewhere. Similar results are seen for three zebrafish paralogs of the cell cycle regulator, C-Myc. The expression of myca, found at high levels in CMZ, was strongly reduced in gdf6a^−/− eyes (Figs. 5U–X). This also was the case for mycn (Figs. 5Y–B’) and mycb (Figs. 5C–F’), which displayed strongly reduced expression in the CMZ of gdf6a^−/− eyes, but were minimally affected in other areas of the embryo. Reduction in ocular expression of genes with roles in cell cycle progression suggested a role for gdf6a in cellular proliferation during eye development, and led to examination of possible anomalies in gdf6a^−/− larvae.

**Ocular Proliferation During Development Is Reduced in gdf6a^−/− Larvae**

The decrease in ocular expression of multiple genes with roles in the cell cycle in gdf6a^−/− implicated altered levels of cellular proliferation in the microphthalmia phenotype. Accordingly, the number of dividing cells was assayed using antiphospho-histone H3 (PH3) antibodies at two time points: 2 dpf, when proliferation occurs primarily in the inner neural layer, and 4 dpf, when postmitotic retinal layers are present in gdf6a mutants, and there is a greater contribution from the CMZ. To
address potential confounding effects from reduced ocular size, the percentage of proliferating cells was quantified in addition to the total number of cells per section. While there is a difference in the number of proliferating cells per section at 2 dpf (\(gdf6a^{-/-}\)/WT: number of cells \(\mu = 28, n = 7\); WT: \(\mu = 47.5, n = 6\), \(P = 0.0016, t\)-test), there is no significant difference in the proportion of PH3-positive cells per section (\(gdf6a^{-/-}\)/WT: percent of cells \(\mu = 10.0\%, n = 7\); WT: \(\mu = 11.7\%, n = 6\); \(P = 0.262, t\)-test; Figs. 6A, 6B, 6E, 6F, 6I). By 4 dpf there is a statistically significant difference in the total number of PH3-positive cells in the CMZ (\(gdf6a^{-/-}\)/WT: \(\mu = 1.1, n = 9\); WT: \(\mu = 8, n = 6\); \(P = 0.00004, t\)-test). These findings are validated by the proportion of positive cells (\(gdf6a^{-/-}\)/WT: mean = 0.7%, \(n = 6\); WT: mean = 3.4%, \(n = 6\); \(P = 0.0017, t\)-test, Figs. 6C, 6D, 6G, 6H, 6I). The strongly reduced proportion of proliferating cells at 4 dpf suggested that loss of \(gdf6a\) during ocular development results in decreased proliferation in the CMZ, and, taken together with previous results, suggested that \(gdf6a\) has a role in regulation of proliferation and apoptosis in the eye during development.

**Figure 1.** Analysis of \(gdf6a\) expression and ocular size. At 2 dpf \(gdf6a^{-/-}\) embryos exhibit microphthalmia (A, B) that is more evident by 4 dpf (C, D). At 2 dpf, \(gdf6a\)'s ocular expression is confined to a small area of the dorsal retina (E), and in cross-section this domain includes the dorsal CMZ (between black and white arrows) and ganglion cell layer (F). Expression remains in the dorsal retina (G), with low levels of expression detected throughout the ganglion cell layer and CMZ at 4 dpf (H). Live pictures taken at \(\times 90\), in situ pictures taken at \(\times 200\).

**Figure 2.** Reduced number of retinal progenitors contribute to microphthalmia in \(gdf6a^{-/-}\) embryos. While a difference in the expression of cell cycle regulators, such as myca (A, B) and mych (C, D), is observed in \(gdf6a\) mutants at 24 hpf, no defects in the number or percentage of proliferating cells is observed (E, F). These data are quantified in (H). However, by this time there is a clear reduction in the number of retinal progenitor cells in \(gdf6a\) mutant eyes (\(P = 0.00017, t\)-test, [G]), indicating that reduced cell number contributes to the observed microphthalmia at early stages of development. Graphic data are presented as mean ± SE. In situ pictures taken at \(\times 200\).
FIGURE 3. Inhibition of apoptosis in gdf6a<sup>−/−</sup> eyes does not rescue microphthalmia. In contrast to the low levels of ocular apoptosis observed in gdf6a<sup>+/−</sup> embryos treated with DMSO (A) or the apoptosis inhibitor P7C3 (C), gdf6a<sup>−/−</sup> embryos exhibit high levels of retinal apoptosis (B). Treatment of gdf6a<sup>−/−</sup> embryos with P7C3 results in significant reduction in the number of cells undergoing apoptosis in the eye at 28 hpf (P < 0.00001, t-test, [D]). At 3 dpf, no rescue of eye size is observed, as P7C3 treated gdf6a<sup>−/−</sup> eyes remain significantly smaller than DMSO-treated (P < 0.00000001, t-test) or P7C3-treated (P < 0.001, t-test) heterozygous siblings. Graphic data are presented as mean ± SE. Confocal pictures taken at ×400.
Expression of the Forkhead Box Transcription Factors foxi1 and foxi2 Are Altered in gdf6a<sup>−/−</sup> Eyes

In addition to identifying altered expression of genes with roles in the cell cycle, microarray analysis also revealed genes with uncharacterized roles in ocular development and deregulated expression in gdf6a<sup>−/−</sup> embryos. The forkhead genes foxi1 and foxi2 showed decreased (2.20-fold) and increased (1.97-fold) expression, respectively (Fig. 4). As these genes are members of the forkhead box family of transcription factors that have well documented roles in ocular development,<sup>35-46</sup> we conducted additional studies to elucidate potential roles in determining ocular size. In situ analysis confirmed the microarray findings, and demonstrates that the two transcription factors are expressed in converse patterns in the dorsal (foxi1) and ventral (foxi2) CMZ (Figs. 7A, 7B, 7E, 7F). In WT embryos, foxi1 is expressed in the dorsal retina in a pattern similar to gdf6a (Figs. 1E, 1F, 7A, 7B), with loss of gdf6a resulting in complete loss of foxi1 expression in the dorsal CMZ (Figs. 7C, 7D). The expression domain of foxi2 is the mirror image of foxi1 (Figs. 7E, 7F), and with loss of gdf6a, this ventral retinal expression of foxi2 expands to encompass the dorsal CMZ where foxi1 normally would reside (Figs. 7G, 7H). These findings indicated that foxi1 and foxi2 lie downstream of gdf6a. Their specific dorsal or ventral CMZ expression patterns are compatible with a role regulating proliferation in the CMZ, consistent with previously characterized roles of foxi1 transcription factors in regulation of the cell cycle. (Figs. 4).

Injection of foxi2 Morpholino Into gdf6a Mutants Further Reduces Eye Size

Expression of foxi1 miRNAs delineates dorsal and ventral domains of the ciliary marginal zone. We assessed whether foxi2 and gdf6a function in the same genetic pathway by morpholino inhibition of foxi2 in gdf6a<sup>−/−</sup> and gdf6a<sup>+/−</sup> embryos. Normally, the ocular size of gdf6a<sup>+/−</sup> embryos closely resembles that of gdf6a<sup>−/−</sup> (Figs. 8A and 1A, respectively), and genetic differences are discernible only upon genotyping. While injection of 4 ng of foxi2 morpholino into WT embryos resulted in no discernible phenotype (data not shown), injection into gdf6a<sup>−/−</sup> embryos resulted in a small eye phenotype by 2 dpf (μ = 76.6% of eye size, n = 15, P = 0.004; t-test, Figs. 8C, 8E), similar to that of gdf6a<sup>−/−</sup> ocular size (Fig. 8B). Moreover, injection of gdf6a<sup>−/−</sup> embryos with foxi2 morpholino resulted in a further reduction in eye size relative to uninjected gdf6a<sup>−/−</sup> embryos (Figs. 8D, 8E, μ = 77% of gdf6a<sup>−/−</sup> eye size, n = 10, gdf6a<sup>−/−</sup>/n = 15 gdf6a<sup>−/−</sup>/Foxi2<sup>MO</sup>, P = 0.00014; t-test). On the basis of these findings, we observed a synergistic phenotype when we injected foxi2 morpholino into gdf6a<sup>−/−</sup> embryos, consistent with these genes functioning on the same or converging genetic pathways.

DISCUSSION

During zebrafish ocular development, the neural retina is formed as progenitor cells exit the cell cycle and differentiate into populations of retinal neurons. Throughout the life of the fish, a population of self-renewing cells remains at the ciliary margin and adds new retinal neurons as the eye grows. We demonstrated that gdf6a, already known to
In situ hybridization validation of Gdf6a responsive genes with known roles in cell cycle. Gdf6a−/− embryos have ocular specific reduction in expression of genes with roles in the cell cycle. The expression of cad is lost almost completely in gdf6a−/− (A–D), while expression of uhfr1 (E–H) and rx1 (I–L) are down-regulated. The cell cycle regulators mcm3 (M–P) and pcna (Q–T) are expressed at high levels throughout the eye, including the CMZ at 2 dpf, and are down-regulated in gdf6a−/− embryos. The expression of three zebrafish C-Myc paralogs is found in the CMZ at 2 dpf, and is highly down-regulated (myca [U–X], mycn [Y–B']) or eliminated (mych [C–F']) in gdf6a−/− eyes. Whole embryo pictures are taken at ×90, and cross-section pictures are taken at ×200.
initiate dorsal retina patterning and having a key role in lens development, also regulates the cell cycle during retinal development. Parallels existed between the requirement of \( gdf6a \) for initiation of dorsal retina identity, and \( gdf6a \)’s comparable role in specifying the dorsal identity of the CMZ. Notably, the CMZ has a polarized axis specified by \( foxi1 \) and \( foxi2 \), with a requirement of \( foxi2 \) in maintenance of ocular size in \( gdf6a^{+/+}/C0 \) fish. Mutations in \( GDF6 \) resulted in microphthalmia, anophthalmia, and coloboma, phenotypes that are recapitulated in mouse, frog, and zebrafish model systems. While the early role of \( gdf6a \) for dorsal retinal identity is well documented, few studies have addressed the mechanism underlying the small eye phenotype. Microphthalmia is observed as early as 24 hpf, and becomes more pronounced at later developmental stages. By 24 hpf, \( gdf6a^{+/+}/C0 \) eyes contain less progenitor cells than their WT or heterozygous counterparts, indicating a potential role for \( gdf6a \) in retinal precursor cell specification, or optic cup evagination as one facet of the microphthalmic phenotype. Consistent with this hypothesis, it has been shown that other BMP ligands, such as Bmp2b, have a role in selecting the eye field from surrounding forebrain tissue. Given \( gdf6a \)’s expression adjacent to the early eye fields, it also may have a role in such processes. The microphthalmia observed in \( gdf6a \)-deficient zebrafish and \( Xenopus \) embryos had been attributed to increased levels of retinal apoptosis. Despite these reports, we found that inhibition of this apoptosis with P7C3, and anti-apoptotic compound thought to protect mitochondrial membrane integrity, does not rescue microphthalmia. Although incubation with this agent may have other effects on retinal cell development that were not tested, it clearly is able to inhibit
retinal apoptosis with no associated increase in eye size. This lack of eye size rescue via incubation with P7C3 also has been observed up to 7 dpf. Although this result does not exclude an apoptotic contribution to the \textit{gdf6a/C0/C0} small eye phenotype, additional factors clearly are involved.

Increasing evidence supports a distinct role for \textit{gdf6a} in regulating targets that control ocular proliferation during development. Indeed, there are significant alterations in the transcriptome after loss of \textit{gdf6a} that are involved in cell cycle progression, such as \textit{mcm3}, \textit{pcna}, \textit{myca}, \textit{mycn}, and \textit{mych}. MYC genes have well-documented roles in the regulation of proliferation in normal and tumorigenic cells, and the \textit{MYC} proto-oncogene is a known downstream target of the TGF-β signaling pathway. Myc family members previously have implicated roles in ocular development, and are responsible for microphthalmic phenotypes seen in \textit{meis1} mutant zebrafish, as \textit{myca} expression is lost from the developing eye, and coinjection of \textit{c-myc} mRNA with \textit{meis1} MO rescues cell cycle defects and eye size. It recently has been demonstrated that knock-down of \textit{bmx1} results in microphthalmia in part due to the failure of retinal progenitors to exit the cell cycle. Since \textit{bmx1} is one of the most down-regulated genes on the microarray, it is likely that this also may contribute to \textit{gdf6a/C0/C0}-induced microphthalmia. Similarly, \textit{cad} expression is down-regulated in \textit{gdf6a} mutants, consistent with \textit{cad} mutant phenotypes that involve small eyes and reduced retinal proliferation.

\begin{figure}
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\caption{Altered \textit{foxi} expression in the ciliary marginal zone of \textit{gdf6a/C0/C0} eyes. At 48 hpf \textit{foxi1} is expressed in the dorsal retina, CMZ, lens, otic vesicle, and pharyngeal arches of WT fish (A, B), while in \textit{gdf6a/C0/C0} mutants \textit{foxi1} expression is absent in the dorsal retina, reduced in the pharyngeal arches, and unchanged in the lens and otic vesicle (C, D). Expression of \textit{foxi2} at 48 hpf in WT embryos is in the ventral retinal and surrounding the choroid fissure (E, F), while in \textit{gdf6a/C0/C0} mutants it expands to the CMZ surrounding the lens with reduced expression also noted in the pharyngeal arches (G, H). In situ pictures taken at ×90.}
\end{figure}

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\includegraphics[width=\textwidth]{figure8.png}
\caption{Loss of \textit{foxi2} in \textit{gdf6a/C0/C0} and \textit{gdf6a/C0/C0} fish reduces eye size. Heterozygous (\textit{gdf6a/C0/C0}) larvae have normal ocular size (A), in contrast to the microphthalmia evident in homozygous mutant (\textit{gdf6a/C0/C0}) at 48 hpf (B). Foxi2 morpholino injection into \textit{gdf6a/C0/C0} embryos results in \textit{gdf6a/C0/C0} larvae developing microphthalmic eyes (C), n = 15 almost indistinguishable from those of \textit{gdf6a/C0/C0} (B). Quantification of average eye size demonstrates that \textit{gdf6a/C0/C0} larvae injected with 4 ng of \textit{foxi2MO} have significantly smaller eyes than un.injected \textit{gdf6a/C0/C0} larvae (E, n = 15, P = 0.004, t-test). Similarly, a reduction in the eye size of \textit{gdf6a/C0/C0} embryos is observed when compared to un injected (D). Quantification of this change reveals significantly smaller eyes of \textit{gdf6a/C0/C0} larvae injected with \textit{foxi2MO} when compared to un injected \textit{gdf6a/C0/C0} larvae (E, n = 10 \textit{gdf6a/C0/C0}; n = 15 \textit{gdf6a/C0/C0} + \textit{Foxi2MO}, P = 0.00014, t-test). Graphic data are presented as mean ± SE. Live pictures taken are at ×90.}
\end{figure}
ways, similar to studies in mice where loss of Bmp receptors (Bmpr1a and Bmpr1b) affect eye size through multiple mechanisms.55

Although our data at 2 dpf were in accord with previous studies demonstrating no significant alteration in proliferation rates,56 we observed a profound reduction in the number and proportion of proliferating cells at 4 dpf in the CMZ. From such findings, we concluded that the microphthalmia in gdf6a−/− larvae is due in part to significant decreased ocular proliferation. Although Gdf6a has been shown to regulate processes specifically in the dorsal retina,55.54 the decrease in proliferation is uniform across the dorsal ventral axis. In addition, gdf6a−/− eyes clearly are smaller than their WT siblings at earlier stages, such as 24 hpf, and contain less progenitor cells, suggesting that specification of progenitors, or migration within the optic cup may influence early eye size in these animals. Apoptosis does not appear to have a major role in the induction of microphthalmia in gdf6a mutants, as rescuing apoptosis does not improve eye size significantly. However, the possibility that cells that fail to progress through the cell cycle undergo apoptosis (at stages not tested), remains to be explored. We also cannot rule out other processes, such as eye patterning and neural differentiation as contributing to gdf6a−/−-induced microphthalmia, as Gdf6a clearly is involved in such processes.

The forkhead box transcription factors, foxi1 and foxi2, are present at 28 hpf in WT embryos in multiple sites, including the dorsal and ventral CMZ, respectively, and the otic vesicle and pharyngeal arches. Zebrafish foxi1 has a key role in otic placode formation and jaw development through maintenance of survival of neural crest cell populations.56–60 Full elucidation of the developmental roles of foxi2 has yet to occur; expression in the mouse neural retina layer has been described, and Xenopus Foxi2 was shown recently to be required for activation of Foxie1, critical for consequent ectodermal gene expression.61,62 gdf6a−/− embryos have significant alterations in ocular expression of foxi1 and foxi2, as foxi1 is lost from the dorsal CMZ, and foxi2 expands to encompass the ventral and dorsal CMZ. The highly specific pattern expression of these transcription factors, coupled with loss of foxi1 when dorsal retinal identity is not initiated, suggests that the CMZ has dorsal-ventral identity specified by foxi1 and foxi2, with foxi2 having a role in the control of ocular size.

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