Mitf-family transcription factor function is required within cranial neural crest cells to promote choroid fissure closure

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ABSTRACT

A crucial step in eye development is the closure of the choroid fissure (CF), a transient structure in the ventral optic cup through which vasculature enters the eye and ganglion cell axons exit. Although many factors have been identified that function during CF closure, the molecular and cellular mechanisms mediating this process remain poorly understood. Failure of CF closure results in colobomas. Recently, MITF was shown to be mutated in a subset of individuals with colobomas, but how MITF functions during CF closure is unknown. To address this issue, zebrafish with mutations in mitfa and tfec, two members of the Mitf family of transcription factors, were analyzed and their functions during CF closure determined. mitfa;tfec mutants possess severe colobomas and our data demonstrate that Mitf activity is required within cranial neural crest cells (cNCCs) during CF closure. In the absence of Mitf function, cNCC migration and localization in the optic cup are perturbed. These data shed light on the cellular mechanisms underlying colobomas in individuals with MITF mutations and identify a novel role for Mitf function in cNCCs during CF closure.

KEY WORDS: Coloboma, Choroid fissure, Mitf, Neural crest, Zebrafish

INTRODUCTION

The development of the vertebrate eye is a complex process, involving coordinated morphogenesis, migration and communication between the surface ectoderm, neural ectoderm and extraocular mesenchyme. During eye development, the optic primordia evaginate from the ventral forebrain and give rise to the optic vesicles (Bibbiovicz et al., 2011). Subsequent invagination of the vesicle gives rise to the optic cup, which eventually subdivides into the neural retina and retinal pigmented epithelium (RPE). During optic cup morphogenesis the optic stalk narrows ventrally and creates a transient opening, known as the choroid fissure (CF) (FitzPatrick, 2005). The CF is crucial for the entrance of the hyaloid vasculature between the surface ectoderm, neural ectoderm and extraocular mesenchyme (POM). A network of intrinsic factors and signaling and regulatory pathways influences CF closure (ALSomiry et al., 2019). The intersection of these major pathways mediates the transcription of downstream target genes within the CF but also in the surrounding POM, which subsequently influences optic cup morphogenesis. The POM plays a crucial role in this process, as mesenchymal cells physically interact with and signal to the CF and optic cup to facilitate optic cup morphogenesis and CF closure (Bryan et al., 2020; Fuhrmann et al., 2000; Gestri et al., 2018; James et al., 2016). A large proportion of POM cells are derived from cranial neural crest cells (cNCCs) (Williams and Bohnsack, 2015). Neural crest cells (NCCs) are born from the dorsal neural tube, after which they migrate ventrally and differentiate into mesenchymal components of the face (Bohnsack et al., 2011; Christiansen et al., 2000; Kaucka et al., 2016; Williams and Bohnsack, 2015). cNCCs additionally migrate anteriorly around the optic vesicle, during which they communicate with the optic stalk and retina (Groccott et al., 2011). Ultimately, morphogenetic and signaling events between the RPE, retina and cNCC-derived-POM facilitate the breakdown of basement membrane components within the fissure (Bryan et al., 2020; James et al., 2016; Lee and Gross, 2007), which enables fusion between the retina and RPE components of the CF. Despite these studies, the cellular and molecular underpinnings of CF closure remain unclear, with ECM interactions, signaling pathways and a variety of transcription factors implicated in the process. Confounding this further, closure involves three distinct cell types: retina, RPE and POM.

When CF closure is perturbed, colobomas result. Colobomas are estimated to occur in 1 in 10,000 live births and severe cases account for up to 10% of childhood blindness (Bermejo and Martinez-Frias, 1998; Onwochei et al., 2000; Stoll et al., 1997). Depending on where along the proximal-distal axis of the CF closure fails, one or several parts of the eye can be involved (Gregory-Evans et al., 2004). Although causative mutations have been identified in some coloboma patients, these loci only account for ~20% of reported cases (Chang et al., 2006; FitzPatrick, 2005). Recently, mutations were identified in MITF, a member of the microphthalmia-associated transcription factor/TFE family of transcription factors (TFs), that result in colobomas (Georgie et al., 2016). Individuals with compound heterozygous mutations in MITF display a phenotype termed COMMAD syndrome (coloboma, osteoporosis, microphthalmia, macrocephaly, albinism and deafness). MITF TFs are well known for their roles in pigmentation, melanocyte development and RPE specification (Hsiao and Fisher, 2014; Lister, 1999; Martina et al., 2014). MITF mutations are also associated with melanomas and with diseases like Waardenburg syndrome type II, which presents with pigmented abnormalities and congenital sensorineural hearing loss (Kawakami and Fisher, 2017). Dominant-negative mouse Mitf mutations (Mitfmut) result in colobomatous microphthalmia (Hero, 1989;
Takebayashi et al., 1996), and loss of MITF in human embryonic stem cells impairs proliferation in optic vesicles differentiated from these cells (Capowski et al., 2014), which also highlights the crucial role of MITF in the gene regulatory network regulating retina/RPE development. MITF mutations in COMMAD patients result in decreased MITF nuclear localization and DNA-binding ability (George et al., 2016). However, despite these biochemical results, little is known about how MITF contributes to CF closure.

The Mitf family of TFs includes four members: MITF, TFEC, TFEB and TFE3 (Martina et al., 2014; Steingrimsson et al., 2004; Zhao et al., 1993). MITF TFs homodimerize or heterodimerize with one another and bind to E-box and M-box DNA regions to regulate transcription of downstream targets (Hemesath et al., 1994; Pogenberg et al., 2012). MITF is the most well-studied member of the family, with functions identified in cell cycle regulation, motility, metabolism, cell survival and pigmentation (Cavodeassi and Bovolenta, 2014; Hsiao and Fisher, 2014). In addition to paralogueous genes in zebrafish (mitfa and mitfb), there are at least nine isoforms of Mitf/MITF, each maintaining a specific expression pattern in developing mouse and zebrafish embryos (Bharti et al., 2008; Lister et al., 2001). Many Mitf isoforms have overlapping expression in zebrafish, but mitfa is expressed specifically in cNCCs, neural crest-derived melanocytes and the developing RPE (Lister et al., 2001). Tfec has a similar expression pattern to mitfa in the zebrafish eye (Lister et al., 2011). Although expressed in the RPE and cNCCs during early eye development, tfec is also upregulated in the CF during closure (Cao et al., 2018). Owing to the convergence of RPE and cNCC-derived POM in the CF, it remains unknown in which cell type(s) mitf TFs function to promote closure.

Ocular development and morphogenesis are highly conserved between zebrafish and humans, making this an ideal system with which to study CF closure. Using a novel mitfa::GFP mutant line, we demonstrate that the loss of these Mitf-family TFs phenocopies colobomas observed in MITF individuals. Both RPE and cNCC development are perturbed in mitfa::GFP mutants. Through a series of embryological manipulations and rescue experiments, we demonstrate that Mitf-family function is required within the cNCCs to facilitate CF closure. Furthermore, our data suggest that Mitf TFs act within cNCCs to promote their localization in around the eye and their survival. These data identify potential cellular underpinnings of colobomas in human COMMAD patients with mutations in MITF, and provide a platform through which cNCC-specific functions during CF closure can be further elucidated.

RESULTS AND DISCUSSION

Previous studies have shown that mitfa−/− mutants possess pigmentation defects but have normal ocular development (Lister, 1999). Mitf-family members are co-expressed in many tissues and, in zebrafish, mitfa and tfec have similar expression patterns within the RPE and cNCCs (Lister et al., 2001, 2011). Therefore, using CRISPR/Cas9, we generated a tfec mutant (Fig. S1). Tfec:60 contains a frameshifting indel in exon 7, which encodes the second helix of the dimerization domain, resulting in a complete loss of function (Petratou et al., 2019 preprint). Tfec−/− mutants did not live beyond larval stages as their swim bladders failed to inflate. Tfec−/− mutants also possessed pigmentation defects and were mildly microphthalmic, but optic cup formation was normal and they displayed no obvious signs of colobomas (Fig. S1).

Considering that there may be developmental compensation and/or functional redundancy between these genes, we next generated mitfa−/−;tfec−/− mutants and assessed eye development. At 4 days postfertilization (dpf), mitfa−/−;tfec−/− mutants displayed prominent bilateral colobomas (Fig. 1A). Only mitfa−/−;tfec−/− mutants possessed colobomas; CF closure was normal in mitfa−/−;tfec+/− (Fig. 1A) and mitfa−/−;tfec+/+ embryos. mitfa−/−;tfec−/− mutants were viable and therefore for the remainder of the experiments, mitfa−/−;tfec−/− incrosses were used to generate mitfa−/−;tfec−/− mutants. Through this breeding scheme, expected Mendelian ratios of mutant embryos were recovered and colobomas appeared, on average, in 21% of progeny (Fig. S2). Colobomas vary in severity in most models of the disease, as well as in human patients, and we likewise observed a range of severities in mitfa−/−;tfec−/− mutants, as assessed by the angle of CF opening (Fig. 1B).

CF closure is a multistep process that relies on the breakdown of the basement membrane (BM) lining the tightly apposed sides of the CF (Bernstein et al., 2018; Carrara et al., 2019; James et al., 2016). A hallmark of colobomas in many models of the disease is BM retention within the CF (Barbieri et al., 2002; Geeraets, 1976; Hero, 1990; Hero et al., 1991; James et al., 2016; See and Clagett-Dame, 2009; Tsuji et al., 2012). Using laminin as a marker of the BM, we assayed BM degradation in mitfa−/−;tfec−/− mutants. mitfa−/−;tfec−/− mutants retained strong laminin expression in the CF, whereas mitfa−/−;tfec+/− controls have degraded the intervening BM by 48 h postfertilization (hpf) (Fig. 1C).

mitfa and tfec are expressed in two distinct cellular populations within the developing eye: the RPE and cNCCs (Lister et al., 1999, 2011). Activity in one or both of these tissues could mediate CF closure events that are disrupted in COMMAD patients. With this in mind, we examined the development of these two cell types in mitfa−/−;tfec−/− mutants. At 48 hpf, there was a dose-dependent effect on RPE pigmentation: the RPE of mitfa−/−;tfec+/− controls have degraded the intervening BM by 48 h postfertilization (hpf) (Fig. 1C).
lapse data, some cNCCs in mitfa<sup>−/−</sup>;tfec<sup>−/−</sup> mutants appeared to burst (see t=8:08-10:08 in Movie 2), consistent with cell death during their migration. To directly assess whether elevated apoptosis contributed to the lack of cNCCs around the eye of mitfa<sup>−/−</sup>;tfec<sup>−/−</sup> mutants, we performed TUNEL staining at 24 hpf. Although the number of TUNEL<sup>+</sup> cNCCs was elevated in mitfa<sup>−/−</sup>;tfec<sup>−/−</sup> mutants, the difference was not significant when compared with wild-type controls, suggesting that lack of cNCCs within the POM could be driven by a defect in cell migration (Fig. S3B). Combined, these data demonstrate a transient delay in RPE pigmentation in the absence of mitfa/tfec function and a significant defect in cNCC localization to the POM in mitfa<sup>−/−</sup>;tfec<sup>−/−</sup> mutants.

Ocular hypopigmentation or albinism is a common feature of a number of human disorders but they do not commonly correlate with colobomas, and most defects in RPE formation/function do not result in colobomas (e.g. Ma et al., 2019; Fuhrmann 2010; Reissmann and Ludwig, 2013; Jeffery 1998). On the other hand, there are many studies demonstrating functional requirements for cNCCs and POM in regulating early eye development and CF closure (Akula et al., 2019; Bryan et al., 2020; Dee et al., 2013; Fuhrmann et al., 2000; Gestri et al., 2018; James et al., 2016; Lupo et al., 2011; McMahon et al., 2009; Sedykh et al., 2017). Although our imaging data demonstrate abnormalities in the cNCC population of mitfa<sup>−/−</sup>;tfec<sup>−/−</sup> mutants, given that Mitf-family genes are expressed in both RPE and cNCCs, it remains possible that defects within the RPE could also contribute to colobomas. Thus, we wanted to determine in which cell population mitfa and tfec function was required to close the CF. Zebrafish are highly amenable to embryonic transplantation experiments, in which cells can be transplanted between embryos to create genetic mosaics (Carmany-Rampey and Moens, 2006), with cells targeted to specific tissues or cell types based on established fate maps (Woo and Fraser, 1995) to determine in which cell type(s) a gene product functions.

To test the hypothesis that Mitf-family function was required in cNCCs during CF closure, we transplanted wild-type cells into the cNCC domain (Fig. 3A) or the retina/RPE domain (Fig. 3C) of mitfa<sup>−/−</sup>;tfec<sup>−/−</sup> mutant host embryos, and quantified CF closure at 4 dpf. Donor embryos were injected with fluorescein isothiocyanate-dextran at the one- or two-cell stage to track their progeny in hosts and validate correct targeting. Wild-type cells transplanted into the cNCC-fated domain in mitfa<sup>−/−</sup>;tfec<sup>−/−</sup> mutants appeared to burst (Fig. 3A), supporting a cell-autonomous role for mitfa/tfec within cNCCs in mediating their migration to the eye.

We next tested the alternative hypothesis, that Mitf-family function was required in the retina/RPE to enable CF closure. Wild-type cells transplanted into the retina/RPE-fated domain of mitfa<sup>−/−</sup>;tfec<sup>−/−</sup> mutants resulted in a strongly labeled optic cup at 24 hpf (Fig. 3C). Only embryos in which at least 20% of the optic cup was
composed of transplanted cells were used for subsequent analyses. Despite robust transplantation, wild-type retina/RPE cells failed to rescue colobomas in \( \text{mitfa}^{-/-};\text{tfec}^{-/-} \) mutants, even in cases in which they were largely restricted to the ventral optic cup (Fig. 3D, Fig. S4C). The maximum angle of CF opening in non-transplanted mutant eyes was 26.9°±3.04°, whereas in \( \text{mitfa}^{-/-};\text{tfec}^{-/-} \) mutants containing wild-type retina/RPE it was 39.82°±4.87° (P=0.037; Fig. 3E). Transplantation of wild-type cells into the retina/RPE of \( \text{mitfa}^{-/-};\text{tfec}^{-/-} \) embryos had no effect on CF closure (Fig. 3E). Control embryos indicated that the localization of cNCCs can be rescued by the expression of wild-type \( \text{mitfa}^{-/-};\text{tfec}^{-/-} \) transplanted with wild-type \( \text{mitfa}^{-/-};\text{tfec}^{-/-} \) mutants, even in cases where the eye of \( \text{mitfa}^{-/-};\text{tfec}^{-/-} \) embryos in which transplanted wild-type \( \text{mitfa}^{-/-};\text{tfec}^{-/-} \) mutants reduced the CF opening from 20.58°±4.31° to 9.66°±2.49° (P=0.023; Fig. 3F). These data indicate that Mitf-family function in cNCCs is sufficient to mediate CF closure, and also that cNCCs outside of the POM surrounding the eyes. Dorsal is upwards in the images in B,C. Data are mean±s.e.m. Scale bars: 100 μm in A; 50 μm in B,C.

To further test the hypothesis that Mitf-family function in cNCCs is sufficient to mediate CF closure, we expressed wild-type \( \text{tfec} \) within cNCCs of \( \text{mitfa}^{-/-};\text{tfec}^{-/-} \) mutants and assessed CF closure. \( \text{tfec} \) is enriched at the CF during CF closure (Cao et al., 2018) and is sufficient for CF closure in \( \text{mitfa}^{-/-} \) mutants (Fig. 1, Fig. S1). Using the Tol2 system (Kwan et al., 2007), we generated pDestTol2pA2\text{-}\text{sox10}\text{-}m\text{Cherry}\text{-nostop}\text{-}t\text{2A}\text{-}\text{FLAG}\text{-}\text{tfec}\text{-}P\text{A} and injected embryos derived from \( \text{mitfa}^{-/-};\text{tfec}^{-/-};\text{sox10}\text{-}\text{eGFP} \) incrosses. Embryos that possessed detectable mCherry+ cNCCs were assessed at 4 dpf for colobomas, sectioned serially and the maximum angle of CF opening quantified. Expression of wild-type \( \text{tfec} \) in \( \text{mitfa}^{-/-};\text{tfec}^{-/-} \) mutants reduced the CF opening from 20.58°±4.31° to 9.66°±2.49° (P=0.033; Fig. 3E). Control embryos retained no mCherry+ expression, which correlated with a lack of CF closure (Fig. 3F). Quantification of the number of \( \text{sox10}\text{-}\text{eGFP} \) cNCCs within the cranial field (P=0.023; Fig. 3F). These data indicate that the localization of cNCCs can be rescued by the presence of wild-type \( \text{tfec} \), and also that cNCCs outside of the \( \text{mitfa} \) lineage are affected by the loss of \( \text{mitfa} \) and \( \text{tfec} \).
In summary, these data identify defects in cNCC localization in mitfa<sup>−/−</sup>:tfec<sup>−/−</sup> mutants and defects in BM breakdown in the CF, a function attributed to the POM (Gestri et al., 2018; James et al., 2016). Data from cell-transplantation and cDNA rescue experiments demonstrate that Mitf-function in cNCCs is sufficient to rescue CF closure in mitfa<sup>−/−</sup>:tfec<sup>−/−</sup> mutants. Taken together, these data support a model in which Mitf-family function is required in cNCCs to facilitate CF closure. Without sufficient cNCC contribution/function within the POM, colobomas result. These data are consistent with those from other studies implicating cNCCs as crucial regulators of optic cup morphogenesis (Bryan et al., 2020; Fuhrmann et al., 2000). Additionally, they highlight the expanding role of Mitf-family TFs in a variety of developmental processes beyond their well-known roles in regulating pigmentation. Finally, these data identify a potential cellular mechanism underlying colobomas in NCC-deficient individuals with MITF mutations (George et al., 2011). Several human congenital disorders/syndromes that include colobomas also have phenotypes consistent with NCC defects (e.g. Akula et al., 2019; Asad et al., 2016). Further studies can now focus on the NCC origin of ocular diseases and how cNCCs modulate optic cup morphogenesis and CF closure. Future studies addressing these topics will be essential for understanding the link between NCC functions and congenital disorders of eye formation.

**MATERIALS AND METHODS**

**Zebrafish husbandry**

Zebrafish (Danio rerio) were maintained on a 14 h/10 h light-dark cycle at 28.5°C. For the mitfa and tfec lines, fish were maintained in a mitfa<sup>−/−</sup> mutant background and double mutant embryos were obtained by mating pairwise heterozygous tfec<sup>−/−</sup> crosses. The mutant alleles mitfaw2<sup>−/−</sup> (Lister et al., 1999) and tfecw64<sup>−/−</sup> (Petratou et al., 2019 preprint) were used for all experiments. Wild-type AB animals were used as donors for rescue transplantation experiments. Fish genotyping was performed using high resolution melt analysis with the following primers for tfec: forward, GTGATATGCGCTGGAACAAAGGGA; reverse, GCTTTTCTGCAGCCACTTAATGTAT. sox10:egFP<sup>−/−</sup> fish were generated previously (Hoffman et al., 2007) and obtained from Dr. Ann Morris (University of Kentucky, Lexington, USA). All fish were housed and maintained in accordance with the University of Pittsburgh School of Medicine Institutional Animal Care and Use Committee.

**Tissue preparation and cryosectioning**

Embryos were collected and fixed in 4% paraformaldehyde (PFA) in PBS for 1 h at room temperature. Embryos were subsequently washed with 25% and 35% sucrose in PBS, and embedded in Tissue Plus optimal cutting
temperature compound (Fisher Scientific). Tissue blocks were frozen and maintained at −80°C. Tissue sections (12 μm) were cut on polylysine-coated FrostPlus slides (Fisher Scientific). Slides were maintained at 4°C until analyzed.

**Immunohistochemistry**

Slides were rehydrated with three 20-min washes in 1× PBS. For laminin staining, antigen retrieval was performed by incubation in 0.5% SDS at 37°C for 20 min. Slides were washed in PBS and then blocked using 10% normal goat serum in PBS for 1 h. Staining was performed overnight at 4°C in blocking solution. The antibodies used in this study were as follows: anti-laminin (1:100; Sigma-Aldrich, L9393); 1:100 and goat anti-rabbit Alexa 488 secondary (1:500; Jackson ImmunoResearch, 111-545-144) 1:500. DNA was counterstained with DAPI (1:100; Life Technologies).

**cNCC quantification and TUNEL assay at 24 hpf**

mitfa-GFP embryos were collected at 24 hpf and fixed in 4% PFA in PBS for 1 h at room temperature. Genotyping and tissue preparation of embryos were performed as outlined above. Tissue was sectioned (12 μm) and an In Situ Cell Death Detection Kit, TMR red (Roche) was used to assess cell death. Cell death and quantification of total NCCs was ascertained by counting the total number of cranial mitfa:GFP cells and mitfa:GFP+ TUNEL+ cells in each half of the embryo. Numbers were normalized to the area of the eye in the respective side of the head and results were displayed as the number of NCCs per 1000 μ² area of the eye.

**Imaging**

Slides were imaged using an Olympus FV1200 confocal microscope and Olympus software. Ten to 12 μm optical sections were acquired and then stacked using ImageJ software (https://imagej.nih.gov/ij/).

Whole-mount imaging was performed using an Olympus FV1200 confocal microscope. Embryos were mounted in 0.5% low melt agarose and then immersed in Danieau’s embryo media. Embryos were imaged in vivo for 15 h starting at 25 hpf and analyzed using Olympus software.

**Transplantation assays**

Fish lines were bred as outlined above. Donor embryos were injected with 3.5% Alexa Fluor Dextran 488 (Thermo Fisher Scientific, D22910). Embryos were maintained in Danieau’s embryo media and dechorionated at 4 hpf. At 6 hpf, embryos were embedded in 1.5% methylcellulose and two drops of Ringer’s high calcium solution. For transplant rescue experiments, 10-15 dexteran+ cells of a wild-type embryo were transferred into the region of neural crest or retinal/RPE origin in a mitfa−/−:tfec−/− embryo. Embryos were then maintained in sterile filtered Danieau’s embryo media with 1× pen/strep for 24 h. At 24 h, embryos were assessed for successful transplantation using a Zeiss Axio Zoom V16 Dissecting Microscope and Z3 Zeiss software. For retinal/RPE transplants, we required that at least 20% of the optic cup (primarily ventral optic cup) was composed of dextran+ cells to be counted.

**Quantification of CF opening**

To quantify the CF opening, ImageJ was used to define a line between the dorsal and ventral inner plexiform boundaries of the retina from the central point on this line, the angle of opening of the CF was calculated from each section. Mild mutants were defined as possessing CF openings smaller than 20°; moderate openings were defined as 20°-35°; and severe openings were larger than 35°.

**cDNA injections**

The plasmid pDestTol2pA2-sox10:mCherry-nostop-t2A-FLAG-tfec-pA used for cDNA microinjection was generated by multi site Gateway cloning (Invitropic/Thermo Fisher Scientific) from the plasmids pDestTol2pA2 (Kwan et al., 2007; Tol2Kit, 394), p5E-MCS-sox10-4.8 (Pendergast et al., 2012), pME-mCherry-no stop (Kwan et al., 2007; Tol2Kit, 456) and p3E-2A-FLAG-tfec-pA (Petratou et al., 2019). Control cDNA embryos were microinjected but retained no mCherry+ cells within the cranial region, therefore the construct was not successfully incorporated.

**Statistics**

Student’s t-test (unpaired and two tailed) was used to assess significance and data are mean±s.e.m.

**Competing interests**

The authors declare no competing or financial interests.

**Author contributions**


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**Supplementary information**

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