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The cellular bases of choroid fissure formation and closure

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ABSTRACT

Defects in choroid fissure (CF) formation and closure lead to coloboma, a major cause of childhood blindness. Despite genetic advances, the cellular defects underlying coloboma remain poorly elucidated due to our limited understanding of normal CF morphogenesis. We address this deficit by conducting high-resolution spatio-temporal analyses of CF formation and closure in the chick, mouse and fish. We show that a small ventral midline invagination initiates CF formation in the medial-proximal optic cup, subsequently extending it dorsally toward the lens, and proximally into the optic stalk. Unlike previously supposed, the optic disc does not form solely as a result of this invagination. Morphogenetic events that alter the shape of the proximal optic cup also direct clusters of outer layer and optic stalk cells to form dorsal optic disc. A cross-species comparison suggests that CF closure can be accomplished by breaking down basement membranes (BM) along the CF margins, and by establishing BM continuity along the dorsal and ventral surfaces of the CF. CF closure is subsequently accomplished via two distinct mechanisms: tissue fusion or the intercalation of various tissues into the inter-CF space. We identify several novel cell behaviors that underlie CF fusion, many of which involve remodeling of the retinal epithelium. In addition to BM disruption, these include NCAD downregulation along the $SOX2^+$ retinal CF margin, and the protrusion or movement of partially polarized retinal cells into the inter-CF space to mediate fusion. Proximally, the inter-CF space does not fuse or narrow and is instead loosely packed with migrating $SOX2^+/PAX2^+/Vimentin^+$ astrocytes until it is closed by the outgoing optic nerve. Taken together, our results highlight distinct proximal-distal differences in CF morphogenesis and closure and establish detailed cellular models that can be utilized for understanding the genetic bases of coloboma.

1. Introduction

During development, the vertebrate eye field evaginates from the forebrain to form the optic vesicle and stalk (O'Rahilly, 1975 and references therein). The optic vesicle subsequently invaginates to form a bilayered optic cup comprised of an inner retinal layer and an outer layer that gives rise to the retinal pigment epithelium (RPE) and other cell-types (Kwan et al., 2012; Li et al., 2000; Picker et al., 2009; Sidhaye and Norden, 2017; Venters et al., 2015). During this process, morphogenetic events create an opening along the ventral midline of the optic cup and stalk called the choroid fissure (CF), which allows embryonic vasculature to enter, and the optic nerve to exit the eye (Mann, 1921, 1922; O'Rahilly, 1975). The CF must subsequently close to form a ventrally continuous optic cup. A failure of CF closure results in disorders such as coloboma, with defects encompassing multiple tissues such as the lens, iris, ciliary body, retina, RPE, optic nerve, optic disc and the choroid (Chang et al., 2006; Fitzpatrick and van Heyningen, 2005; Graw, 2003; Gregory-Evans et al., 2004; Mann, 1921, 1922, 1964; Maumenee and Mitchell, 1990; Reis and Semina, 2015). CF defects account for up to 10% of cases of pediatric blindness and affect between 2.5 and 7.5/10,000 live births (Onwochei et al., 2000). Although causal mutations have been identified in ~ 20% of

Abbreviations: A, anterior; BM, basement membrane; CF, Choroid Fissure; D, dorsal; Di, distal; E, embryonic; Fib, fibronectin; HA, hyaloid artery; HH, Hamburger and Hamilton embryonic stages; hpf, hours post fertilization; ILM, inner limiting membrane; L, lens; LAM, laminin; M, medial; OC, optic cup; OD, optic disc; OL, outer layer of the optic cup; ON, optic nerve; OS, optic stalk; OV, optic vesicle; P, posterior; Pec, pecten; Phal, phalloidin; pHH3, phospho-histone H3; POM, periocular mesenchyme; Pr, Proximal; Pyr, Pyronine Y; R, retina; RPE, retinal pigment epithelium; Vim, Vimentin

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patients, precisely why they produce ocular defects remains unclear because a systematic, cellular analysis of CF formation and closure has not been conducted (Chang et al., 2006; Fitzpatrick and van Heyningen, 2005; Gregory-Evans et al., 2004).

The CF is formed in concert with lens and optic cup invagination. Physical contact with the lens is thought to invaginate the rapidly growing dorsal optic cup (Dudley et al., 1995; Hilfer, 1983; Luo et al., 1995; Wawersik et al., 1999). As the invagination progresses ventrally, the edges of the ventral optic cup grow towards each other and become juxtaposed to form the CF folds (Geeraets, 1976; Heermann et al., 2015; Hilfer, 1983; Li et al., 2000). Despite its simplicity, this model does not explain how CF formation occurs in medial-proximal optic cup and stalk, which do not directly contact the lens. Interestingly, the ventral midline invaginates in lens-less eyes, suggesting that lens-independent signals intrinsic to the optic vesicle (e.g., PAX2, VAX1, 2) may be sufficient to induce CF invagination (Barbieri et al., 2002; Hartsock et al., 2014; Hasegawa et al., 2016; Hyer et al., 2003; Lewis, 1907; Spemann, 1901; Take-uchi et al., 2003; Uemonsa et al., 2002). Notably, proximal, but not distal CF patterning is defective in *Pax2*$^{-/-}$ mice, supporting the idea that distinct patterning mechanisms regulate distal and proximal CF morphogenesis (Macdonald et al., 1997; Otteson et al., 1998; Torres et al., 1996). These observations suggest that gene mutations resulting in coloboma should be interpreted within the context of these proximal-distal differences. However, this is difficult due to the absence of a systematic spatial-temporal analysis of normal CF morphogenesis.

CF invagination extends from the ventral midline of the optic cup into the stalk, establishing continuity between the distal and proximal segments of the CF (Otteson et al., 1998; Silver and Robb, 1979). During this process, PAX2^{+} cells that line the ventral edges of proximal CF are thought to invaginate and surround the optic nerve and hyaloid artery, thereby forming the optic disc (Morcillo et al., 2006; Otteson et al., 1998; Petros et al., 2006; Soukkarieh et al., 2007; Take-uchi et al., 2003). Optic disc formation and proximal CF morphogenesis are thus tightly associated and a common set of genes (BMP7, SHH, PAX2, FGFs), regulate the formation of both (Cai et al., 2013; Dakubo et al., 2003; Morcillo et al., 2006; Otteson et al., 1998). As a result, optic disc defects lead to optic nerve hypoplasia and axon guidance defects, phenotypes also associated with coloboma (Cai et al., 2013; Dakubo et al., 2003; Morcillo et al., 2006; Otteson et al., 1998; Silver and Robb, 1979). Despite their tight association, very little is known about how CF and optic disc morphogenesis are integrated during development.

Following its juxtaposition, the CF must close to form a ventrally continuous optic cup. Cell proliferation, movement and death have all been implicated in CF closure, although their involvement in CF formation and growth, makes it difficult to identify cell behaviors specifically involved in CF closure (Heermann et al., 2015; Laemle et al., 1999; Lee et al., 2013; Morcillo et al., 2006; Ozeki et al., 2000). Thus the sequence of events and cell behaviors that achieve CF closure remain poorly understood. In fact, there is little agreement on a cellular definition of CF closure that is applicable across animals.

The CF folds must be apposed to ensure CF closure, followed by the breakdown of the BM lining the CF folds. Mutations that prevent BM breakdown frequently cause coloboma, although the cellular bases of this is poorly understood (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). Even less is known about how BMs lining the retina [the inner limiting membrane (ILM)] and the RPE (Bruch's membrane) become continuous along dorsal and ventral CF even though this continuity is integral to CF closure.

It is clear from the above discussion that a systematic cellular examination of normal CF morphogenesis is necessary and currently not available, particularly at light microscopic levels. We address this deficit by comparing CF development and closure in the mouse, zebrafish and the chick. Our study identifies several novel features of CF formation and closure. We show that CF closure occurs via two distinct morphogenetic processes in all species, which we term “fusion” and “intercalation”. During fusion, the leading edges (folds/margins/lips) of the CF become continuous by eliminating the intervening basement membranes, a process which employs a surprising degree of retinal epithelial remodeling. By contrast, proximal CF folds do not fuse during tissue intercalation. Rather, the inter-CF space is retained and subsequently closed via the intercalation of incoming astrocytes and the outgoing optic nerve. Our study thus identifies unique and conserved features of CF morphogenesis and closure across species and establishes detailed cellular models that can be utilized to understand the genetic bases of coloboma.

2. Materials and methods

2.1. Animals

All protocols used in this study were approved by the Institutional Animal Care and Use Committee of the University of Texas at Austin and the University of Pittsburgh School of Medicine, and conform to the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

2.1.1. Chick

Fertilized Leghorn eggs (Texas A & M, Bryan, TX) were incubated at 38 °C in a humidified forced-draft incubator. Embryos were staged according to Hamburger and Hamilton (1951).

2.1.2. Mouse

Swiss-Webster mouse embryos were used in the current study. The morning of vaginal plug detection was designated E0.5. Embryos were staged by counting somites.

2.1.3. Zebrafish

Zebrafish were maintained at 28.5 °C on a 14-h-light/10-h-dark cycle. Embryos were obtained from the natural spawning of wild-type parents and collected according to established protocols (Kimmel et al., 1995; Westerfield, 1995).

2.2. Sectioning and orientation

In this study, we have oriented optic cup sections with reference to the eye of the animal. This permits us to refer to identical planes of sections by the same name, regardless of whether the animal has frontal (humans, owls) or lateral (chick, mouse, zebrafish) eyes. For most figures in the paper, we use the term “tangential” to refer to sections cut parallel to the external surface of the eye in which the dorsoventral and nasal-temporal (anterior-posterior) axes can be seen in lateral-eyed animals (e.g., cartoon in Fig. 1A; Fig. 1). Serial tangential sections are grouped arithmetically into three groups designated distal (Di), medial (M) and proximal (Pr) based on their proximity to the lens (distal) or the diencephalon (proximal). When using the head as a reference point, this axis is referred to as sagittal in animals with lateral eyes and as coronal in animals with frontal eyes.

Horizontal sections are cut along the antero-posterior axis of the eye, orthogonal to the dorsoventral axis of the eye. These sections display the distal-proximal and nasal-temporal (antero-posterior) axes in animals with lateral eyes (cartoon, Fig. 2; Fig. 2A–F). In humans, these sections are referred to as axial and display the distal-proximal (antero-posterior) and nasal-temporal axes.

Sagittal sections are cut orthogonal to the nasal-temporal axis of the eye and display the distal-proximal and dorsoventral axes in animals with lateral eyes (cartoons, Fig. 2H; Fig. 2I–L’). When using the head as a reference, these are referred to as coronal in animals with lateral eyes, and as sagittal in animals with frontal eyes. Cartoons are provided at the top left of every figure to avoid the confusion generated by the different terminologies.