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Epha2 and *Efna5* participate in lens cell pattern-formation

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ABSTRACT

Ephrin type-A receptor 2 (EPHA2) and one of its ligands, ephrin-A5 (EFNA5), have been associated with loss of eye lens transparency, or cataract, - an important cause of visual impairment. Here we show that mice functionally lacking EPHA2 (*Epha2*-null), EFNA5 (*Efna5*-null), or both receptor and ligand (*Epha2/Efna5*-null) consistently develop mostly transparent lenses with an internal refractive disturbance and a grossly disturbed cellular architecture. *In situ* hybridization localized *Epha2* and *Efna5* transcripts to lens epithelial cells and nascent fiber cells at the lens equator. *In vivo* labeling of *Epha2*-null lenses with a thymidine analog detected a significant decrease in lens epithelial cell proliferation within the germinative zone resulting in impaired early lens growth. *Ex vivo* imaging of *Epha2*-null, *Efna5*-null, and *Epha2/Efna5*-null lenses labelled in vivo with a membrane-targeted red fluorescent protein revealed misalignment of elongating fiber cells at the lens equator and loss of Y-suture pattern formation near the anterior and posterior poles of the lens. Immunofluorescent labeling of lens major intrinsic protein or aquaporin-0 (MIP/AQP0) showed that the precise, radial column patterning of hexagonal fiber cells throughout the cortex region was disrupted in *Epha2*-null, *Efna5*-null and *Epha2/Efna5*-null lenses. Collectively, these data suggest that *Epha2* and *Efna5* participate in the complex, global patterning of lens fiber cells that is necessary for maximal optical quality.

1. Introduction

Originally discovered in a human erythropoietin-producing hepatoma cell-line, EPH-receptors constitute the largest sub-family (14/58) of mammalian receptor tyrosine kinases (RTKs) that, along with their eph-receptor interacting ligands or ephrins, elicit diverse signaling pathways in embryonic development, adult tissue homeostasis, and various diseases (Lisabeth et al., 2013; Barquilla and Pasquale, 2015; Kania and Klein, 2016). First identified as epithelial cell kinase (ECK), EPH-receptor A2 (EPHA2) belongs to the type-A, EPH-receptor subfamily (EPHA1–8, EPHA10) and like other RTKs, shares a single-pass transmembrane glyco-protein topology with multiple functional domains including a cytoplasmic (C-terminal) tyrosine-kinase signaling domain and an extracellular (N-terminal) ligand-binding domain. EPHA2 preferentially binds glycosyl-phosphatidyl-inositol (GPI)-anchored or type-A ephrin ligands (EFNA1–5) and is also referred to as ephrin type-A receptor 2. Canonical EPH-receptor signaling requires direct interaction between EPHA2 and an ephrin-A ligand in neighboring cells (i.e. contact dependent) in order to elicit 'forward' signaling in the receptor-expressing cell and 'reverse' signaling in the ligand-expressing cell. Such bi-directional signaling often results in opposite cellular effects (e.g. adhesion versus repulsion) depending on the specific cellular context (Lisabeth et al., 2013; Barquilla and Pasquale, 2015; Kania and Klein, 2016).

The crystalline lens is a transparent, ellipsoidal, structure located toward the front of the vertebrate eye that plays a central role in anterior eye development and the establishment of normal refractive vision (emmetropia) by facilitating the variable fine-focusing of images onto the photosensitive retina (Beebe and Coats, 2000; Iribarren, 2015; Donaldson et al., 2017). In mammals, the lens develops from a placode of head ectoderm under the influence of a paired box 6 (PAX6)-dependent gene regulatory network and several extracellular signaling pathways to form an exquisitely patterned, cellular structure composed of two cell types enclosed in a collagenous basement membrane or capsule. (Bassnett et al., 2011; Cheng et al., 2017b; Cvekl and Zhang, 2017). The anterior lens surface comprises a monolayer of mitotically

Abbreviations: EPH, erythropoietin producing hepatocellular-carcinoma; EPHA2, EPH receptor A2 or ephrin type-A receptor 2 protein symbol; EFNA5, ephrin-A5 protein symbol; *Epha2*, mouse EPHA2 gene symbol; *Efna5*, mouse ephrin-A5 gene symbol; EdU, 5-ethynyl-2'deoxyuridine; ISH, *in situ* hybridization; tdT, tandem-dimer Tomato; DAPI, 4,6-Diamidino-2-phenylindole; MIP/AQP0, major intrinsic protein/aquaporin-0

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competent epithelial cells that terminally differentiate at the lens equator into concentric layers (growth-shells) of tightly packed, highly elongated, secondary fiber cells that form the refractive mass of the lens. Lens fiber cell formation is characterized by several unique remodeling processes including accumulation of crystallins in the cytoplasm, remodeling of the cytoskeleton, specialization of the plasma-membrane, programmed loss of organelles, and formation of a core syncytium (Bassnett et al., 2011; Cheng et al., 2017b; Cvekl and Zhang, 2017). Collectively, these cellular processes are designed to establish and maintain lens transparency, minimize light scattering, and generate a high refractive index.

EPHA2 is a surprisingly abundant component of the lens cell-membrane proteome, where it accounts for approximately 10% of cell signaling molecules (Bassnett et al., 2009). However, the precise role(s) of EPHA2 signaling in lens cell biology remains unclear. Genetic variants in the human EPHA2 gene (*EPHA2*) have been widely associated with clinically heterogeneous forms of inherited pediatric cataract and with acquired or age-related forms of cataract (Shiels et al., 2008; Jun et al., 2009; Bennett et al., 2017; Chen et al., 2017) (https://sites.wustl.edu/catmap). In addition, variants in the human gene for ephrin-A5 (*EFNA5*) have been tentatively associated with age-related cataract (Lin et al., 2014). Lenses of knock-out mice that are functionally null for EPHA2 and/or EFNA5 have also been reported to develop a highly variable cataract phenotype with respect to morphology, severity, progression, and penetrance (Cooper et al., 2008; Jun et al., 2009; Cheng and Gong, 2011; Shi et al., 2012; Son et al., 2013; Cheng et al., 2013, 2017a; Biswas et al., 2016). Here we uncover a consistently abnormal lens cell patterning phenotype in predominantly transparent lenses of mice lacking EPHA2 and/or EFNA5.

2. Materials and methods

2.1. Mice and lenses

Epha2-null mice (Stock no. 006028) (Brantley-Sieders et al., 2004), transgenic tandem-dimer (td)-Tomato (tdT) reporter mice (Stock no. 007576) (Muzumdar et al., 2007), and C57BL/6 J (B6J) mice (Stock no. 000664) were obtained from The Jackson Laboratory (Bar Harbor, ME). *Efna5*-null mice (Frisen et al., 1998) were generously provided by Dr. David Feldheim (University of California, Santa Cruz). Absence of EFNA5 protein in lenses of these mice has been confirmed by others (Cooper et al., 2008; Cheng and Gong, 2011; Cheng et al., 2017a). Null mice were genotyped by PCR-amplification as described (Frisen et al., 1998; Shi et al., 2012) and maintained on a predominantly B6J background that lacks a deletion mutation in the gene for lens beaded-filament-structural-protein-2 (CP49) carried by some inbred strains (Simirskii et al., 2006). *Epha2* -null and *Efna5*-null mice were crossed to generate double null (*Epha2/Efna5*-null) mice. Null mice were crossed with tdT-reporter mice (B6J background) to generate null and wild-type littermates that constitutively express membrane-targeted tdT. Expression of tdT was detected in vivo by means of a Dual Fluorescent Protein Flashlight (Nightsea, Lexington, MA) and confirmed by PCR-genotyping as described (Muzumdar et al., 2007). Mice were humanely killed by CO_2 asphyxiation followed by cervical dislocation or decapitation. Eyes were removed from age and sex matched littermates and lenses dissected in pre-warmed (37 °C) phosphate buffered saline (PBS, #P4417-100TAB, Sigma-Aldrich, St. Louis, MO) then photographed using a dissecting microscope fitted with a digital camera (Stemi 2000; Zeiss, Thornwood, NY). Images were processed with Photoshop Creative Suite 6 (CS6) software (Adobe Systems, San Jose, CA). All mouse procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at Washington University in compliance with the Institute for Laboratory Animal Research (ILAR) guidlines.

2.2. Ex vivo imaging of membrane-localized tdT

Lenses labelled with tdT were positioned in agarose-coated petri-dishes in which a triangular wedge-shape chamber had been cut out then overlaid with pre-warmed cell-culture medium (DMEM/F12 without phenol red, ThermoFisher, Waltham, MA) and imaged using a water-immersion objective lens attached to a confocal, fluorescence microscope (FluoView FV1000, Olympus, Center Valley, PA) as described (Bassnett and Shi, 2010). Fluorescent images were acquired at various depths (20–400 μm) from the lens surface with the 'multi-area time-lapse' function and reassembled into a complete lens image using system-integrated FluoView software.

2.3. In vivo 5-ethynyl-2'deoxyuridine (EdU) labeling

Lens epithelial cell proliferation was measured by labeling S-phase nuclei with the thymidine analog EdU (Invitrogen/ThermoFisher) as described (Bassnett and Shi, 2010; Wiley et al., 2010). Briefly, mice were given an intra-peritoneal (IP) injection of EdU (10 μg/g) one hour before death then dissected lenses were fixed, permeabilized, and labelled using the Click-iT EdU Alexa Fluor 488 Imaging Kit (C10337, ThermoFisher) according to the manufacturers' instructions. EdU labelled lenses were immobilized in agarose, overlaid with PBS, and imaged as above (2.2). Fluorescent image stacks (500–800 μm) of lens anterior and equatorial quadrants or sectors were acquired (Z-plane) and projected (Y-plane) using system-integrated FluoView software (Olympus). EdU-positive nuclei were counted manually using the count-tool in Photoshop CS6. Hoechst-stained nuclei were counted using MetaMorph software (Molecular Devices, San Jose, CA).

2.4. In situ hybridization (ISH)

Lens RNA transcripts were localized using the RNAscope 2.0 HD Detection Kit (RED) (P/N 310034) and custom-synthesized oligonucleotide probes to *Epha2* (NM_010139.3, target region 214–1758 bp) and *Efna5* (NM_207654.2, target region 328–987 bp) according to the manufacturers' instructions (Advanced Cell Diagnostics, Inc, Hayward, CA). Briefly, mouse eyes were fixed (24 h, 20 °C) in 10% neutral buffered formalin (Fisher Scientific) and processed using standard formalin-fixed-paraffin-embedded (FFPE) section techniques. Microtome sections (5 μm, RM2255, Leica Microsystems, Buffalo Grove, IL) on glass slides (SuperFrost Plus, ThermoFisher) were baked (1 h, 60 °C), de-waxed in xylene, dehydrated in ethanol, boiled in citrate buffer, then protease treated (10 μg/ml, 40 °C, 30 min) in a HybEZ Oven (ACD). Pre-treated sections were hybridized with target probes (2 h, 40 °C), followed by signal amplification oligonucleotides (15–30 min, 40 °C). For chromogenic labeling, hybridized sections were treated with alkaline phosphatase (AP)-conjugated Fast-Red label probe (15–30 min, 20 °C) and Fast-Red substrate (10 min, 20 °C), then counterstained (Gill's Hematoxylin-1/0.01% ammonia-H_2O), mounted (Acrymount, StatLab, McKinney, TX), and imaged under a bright-field microscope fitted with a digital camera (BX61, Olympus, Center Valley, PA).

2.5. Immuno-fluorescence microscopy

Eyes were processed using standard cryo-section or FFPE-section techniques and immuno-localization performed as described (Shi et al., 2012; Zhou et al., 2016). The following primary antibodies were used, anti-EPHA2 (AF639, R & D Systems, Minneapolis, MN), anti-ephrin A5 (38–0400, ThermoFisher), anti-aquaporin 0 (AB3071, EMD Millipore, Billerica, MA). Briefly, for anti-EPHA2 and anti-EFNA5, eyes were fixed (1 h., 4 °C) in 4% paraformaldehyde (16% aqueous solution, #15710, Electron Microscopy Sciences, EMS, Hatfield, PA) diluted in PBS, then cryo-protected by serial incubation in 15% and 30% sucrose/PBS, embedded in Tissue-Tek (EMS) and cryo-sectioned (15 μm) using

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