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Interactions between lens epithelial and fiber cells reveal an intrinsic self-assembly mechanism

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ABSTRACT

How tissues and organs develop and maintain their characteristic three-dimensional cellular architecture is often a poorly understood part of their developmental program; yet, as is clearly the case for the eye lens, precise regulation of these features can be critical for function. During lens morphogenesis cells become organized into a polarized, spheroidal structure with a monolayer of epithelial cells overlying the apical tips of elongated fiber cells. Epithelial cells proliferate and progeny that shift below the lens equator differentiate into new fibers that are progressively added to the fiber mass. It is now known that FGF induces epithelial to fiber differentiation; however, it is not fully understood how these two forms of cells assemble into their characteristic polarized arrangement. Here we show that in FGF-treated epithelial explants, elongating fibers become polarized/oriented towards islands of epithelial cells and mimic their polarized arrangement in vivo. Epithelial explants secrete Wnt5 into the culture medium and we show that Wnt5 can promote directed behavior of lens cells. We also show that these explants replicate aspects of the Notch/Jagged signaling activity that has been shown to regulate proliferation of epithelial cells in vivo. Thus, our in vitro study identifies a novel mechanism, intrinsic to the two forms of lens cells, that facilitates self-assembly into the polarized arrangement characteristic of the lens in vivo. In this way the lens, with its relatively simple cellular composition, serves as a useful model to highlight the importance of such intrinsic self-assembly mechanisms in tissue developmental and regenerative processes.

Introduction

How a particular tissue or organ develops its characteristic size and three-dimensional cellular architecture is often a poorly understood part of its developmental program; yet precise regulation of these features is often critical for function. The lens of the eye illustrates this well because it needs to develop very precise dimensions and curvature to do its job of focussing images onto the retina. In mammals, lens differentiates from ectoderm that overlies the optic vesicle. Presumptive lens ectoderm goes through placode, pit and vesicle stages. The lens develops its distinctive polarized structure because cells in the posterior half of the lens vesicle elongate and differentiate into primary fibers, whereas cells in the anterior half differentiate into epithelial cells that cover the anterior poles of the fiber cells (Lovicu and McAvoy, 2005). The lens maintains this polarity as it grows because it has highly ordered growth patterns. Proliferation is restricted to the epithelium, mostly in the germinative zone above the lens equator (McAvoy, 1978a, 1978b), and progeny migrate below the equator where they elongate and differentiate into secondary fiber cells that progressively become added to the primary fiber mass. Like primary fibers, secondary fibers are also highly polarized with their apical ends, at least initially, associated with the overlying epithelium.

As fiber differentiation is a major event in lens morphogenesis, much effort has been focussed on determining how this process is regulated. There is now compelling evidence that one, or several, members of the FGF growth factor family initiates and promotes the epithelial to fiber differentiation process (Lovicu and McAvoy, 2005; Robinson, 2006; Zhao et al., 2008; Qu et al., 2011). This information has been used to study the process of fiber differentiation in various in vivo and in vitro models. However, progress towards understanding lens morphogenesis depends, not only on knowing how fiber differentiation is triggered, but also how epithelial and fiber cells assemble into their characteristically ordered and polarized three-dimensional arrangement.

Studies in our laboratory have shown that as fibers undergo early stages of elongation, there are indications that their alignment and orientation depends on the Wnt-Frizzled/Planar Cell Polarity (Wnt-Fz/PCP) signaling pathway (Chen et al., 2008;

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Sugiyama et al., 2010, 2011). For example in mice overexpressing secreted frizzled-related protein 2 (Sfrp2), a well-known regulator of Wnt-Fz signaling, fiber orientation is severely disrupted and this is associated with reduced expression/activation of downstream components of the PCP pathway (Chen et al., 2008; Sugiyama et al., 2010). Moreover, in vitro explant studies show that FGF upregulates Wnt-Fz signaling and that this involves translocation of Fz and the centrosome to the leading edge (apical tip) of similarly polarized groups of elongating fiber cells (Dawes et al., 2013). How these processes are regulated has been a major research focus in our laboratory. Now we report, for the first time, that polarized/oriented behavior of elongating fibers in FGF-treated epithelial explants is coordinated by residual Wnt-expressing epithelial cells. We also show that these explants replicate aspects of the Notch/Jagged signaling activity that has been shown to regulate proliferation of epithelial cells in vivo (Jia et al., 2007; Rowan et al., 2008; Le et al., 2009; Saravanamuthu et al., 2009, 2012). This provides key insights into a self-regulatory mechanism intrinsic to the lens that involves reciprocal epithelial-fiber cell interactions and appears to be critical for the assembly and maintenance of the highly ordered three-dimensional architecture that is central to lens function.

Materials and methods

Animals

All animal procedures were performed in accordance with the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research and the animal care guidelines published by the Institute for Laboratory Animal Research (Guide for the Care and Use of Laboratory Animals). All studies were approved by the Institutional Ethics Committee of the University of Sydney.

Preparation of inverted lens epithelial explants

P5 Wistar rats were sacrificed by decapitation; eyes were removed and placed in pre-warmed 37 °C M199 medium with Earle's salts (Gibco, Invitrogen, CA, USA), supplemented with 50 IU/ml penicillin, 50 μg/ml streptomycin (Invitrogen), 0.2 mM L-glutamine and 2.5 μg/ml Amphotericin B (ThermoScientific MA, USA). With the use of a dissecting microscope and jeweller's forceps, the eyes were torn open at the optic nerve to release the lens. Lens epithelial explants were prepared by gently tearing the posterior lens capsule adjacent to the posterior suture and slowly removing the lens fiber mass. The anterior lens capsules were flattened and pinned in an inverted position to the base of the tissue culture dish such that epithelial cells were in direct contact with the base of the tissue culture dish with their lens capsule facing and exposed directly to the media. These are known as 'inverted' explants (see Fig. 3B in Lovicu and McAvoy, 2008) After explantation, culture medium was replaced with 1 ml of fresh, equilibrated M199 with the addition of 200 ng/ml FGF2 (R&D systems, MN, USA). Control dishes for FGF treatments were supplemented with 0.2% BSA respectively. Explants were maintained at 37 °C in 5% CO_2 for 4–5 days unless otherwise indicated.

Antibodies

Primary antibodies used in this study were as follows: mouse antibodies against E-cadherin (clone 36, BD Transduction Labs, CA, USA), β-catenin (clone 14, BD Transduction Labs), GAPDH (HyTest Ltd., Finland) and α-acetylated tubulin (T-6793, Sigma-Aldrich, MO, USA); rabbit antibodies against β-catenin (H102, Santa Cruz, TX, USA), pericentrin (ab4448, Abcam, MA, USA), Hey1 (ab22614, Abcam) and Wnt5A (ab72583, Abcam); and goat antibodies against Fz-6 (M-19, Santa Cruz), Jagged-1 (sc6011, Santa Cruz) and Wnt5A (AF645, R&D systems). For Western blot analysis the following horseradish peroxidase (HRP) conjugated secondary antibodies were employed: goat anti-mouse IgG (Upstate, PA, USA); goat, anti-rabbit IgG (Millipore, PA, USA) and rabbit anti-goat IgG (Invitrogen, VIC, Australia). For immunocytochemistry negative controls of mouse, rabbit and goat whole molecule IgGs were used (Jackson Immuno Research Laboratories, PA, USA); secondary antibodies employed were Alexa Fluor 488 or 594-conjugated donkey anti-rabbit, mouse or goat IgG (Invitrogen).

Application of Notch inhibitor

To determine the role of Notch signaling in FGF-induced fiber differentiation, lens explants were exposed to the γ-secretase inhibitor N-[N-(3,5-Difluorophenacetyl-L-alanyl]-S-phenylglycine t-butyl ester (DAPT) (Sigma-Aldrich) (Abello et al., 2007). DAPT inhibits the activity of β-secretase which is specifically required for internal cleavage of the intracellular domain of Notch (NICD) that leads to gene activation (Abello et al., 2007; Micchelli et al., 2003; Saravanamuthu et al., 2009). Lens epithelial explants were treated with 75 μM DAPT for 4 h before the addition of 200 ng/ml FGF or 0.2% BSA; explants remained in these culture conditions for 4 days. Control dishes, lacking inhibitor, were supplemented with an equivalent volume of the vehicle, dimethylsulfoxide (DMSO).

Western blot analysis

Lens explants for each western blotting experiment were obtained from littermates and extracts prepared from pools of 6–9 explants. Following 4 days in experimental conditions explants were rinsed in cold PBS and lens proteins extracted in RIPA buffer (50 mM Tris–HCl pH 8.0, 150 mM NaCl, 1% Igepal, 0.5% sodium deoxycholate, 0.1% SDS) (0.5%), SDS (0.1%) containing complete Mini-protease inhibitor cocktail tablet (Roche Diagnostics, Germany) and 10 mM sodium fluoride. Lysates were pre-cleared by centrifuging at 13,000 rpm at 4 °C for 15 min, and the protein content of the soluble fraction was determined using the QuantiPro™ BCA Assay Kit (Sigma-Aldich) according to manufacturer's instructions. Equal amounts of protein per sample along with 50 ng/ml recombinant Wnt5A and Wnt5B (R&D systems) were loaded onto 8% SDS-PAGE gels for electrophoresis and transferred onto an Invitrolon™ polyvinylidene fluoride (PVDF) membrane (Invitrogen); with a Mini Trans-Blot® Electrophoretic Transfer Cell (Bio-Rad, CA, USA). Western blotting was carried out as previously described (Stump et al., 2003). Proteins were detected using SuperSignal West Dura Extended Duration ECL Substrate (Thermo Scientific) and visualized using a G:Box with imaging software, GeneSnap v.6.08 (Syngene, UK).

Immunocytochemistry

Inverted lens epithelial explants were fixed in 100% methanol for 45 s at room temperature followed by four successive washes with PBS. Following fixation, explants were flipped over and pinned to the base of the same tissue culture dish such that lens cells faced uppermost towards the bathing medium with their capsule closest to the base of the dish. Non-specific cellular sites were blocked with the addition of normal donkey serum (1:10) in 0.1% BSA in PBS with incubation for 1 h at room temperature. Primary antibodies (1:200–1:1000 dilution) were diluted in 0.1% BSA in PBS with normal donkey serum (1.5:100) and applied overnight at 4 °C. To remove unbound antibody, explants were washed in 0.1% BSA in PBS three times for 5, 10 and 15 min.

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