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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.

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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.

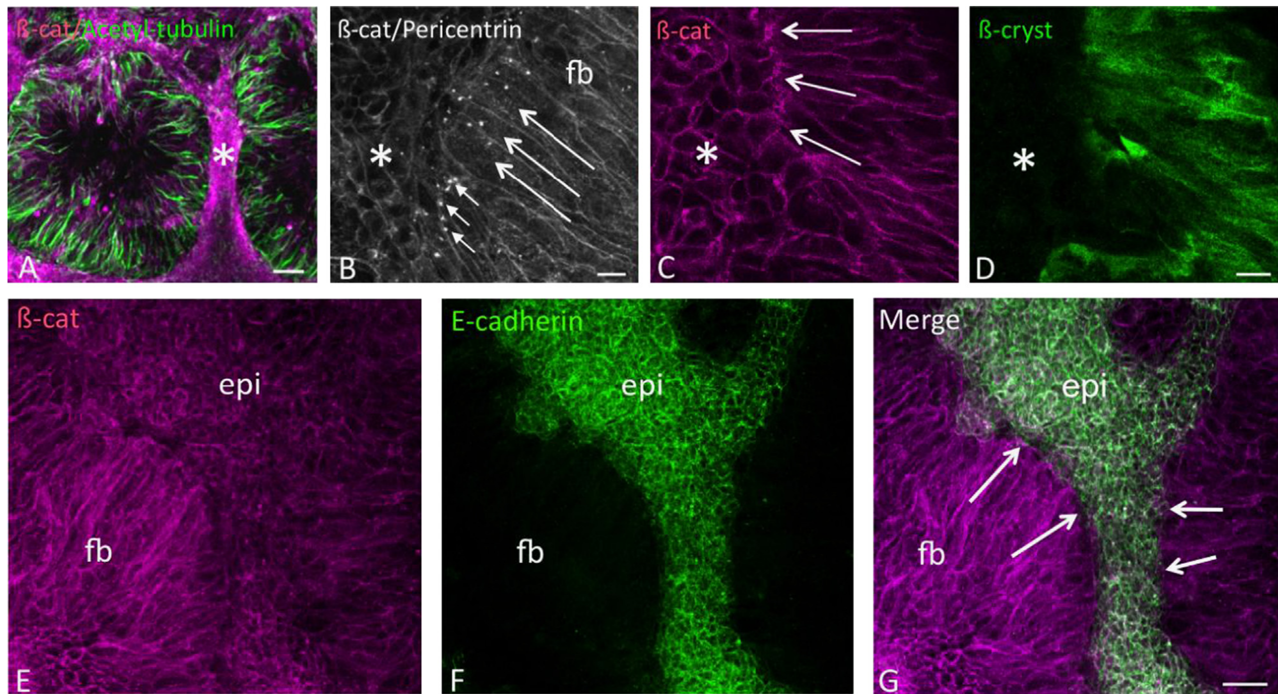

Fig. 1. Coordinated alignment/orientation of elongated fibers towards islands of epithelial cells. (A) An explant treated with 200 ng/ml FGF for 5 days shows elongated fibers indicated by acetylated-tubulin reactivity aligning perpendicular to islands of smaller cells (*) that have not elongated. (B) Elongated fibers (fb, long arrows) are shown at higher magnification. β-catenin localizes cell margins; bright spots (short arrows) localize pericentrin and the centrosomes. In most of the elongated fibers, the centrosome is localized to the same end of each cell and shows that these differentiating fibers are similarly polarized with their apical tips abutting onto the group of smaller cells. (C, D) The fiber-specific marker β-crystallin accumulates in elongated fibers (long arrows) that are aligned towards an island of smaller cells that display negative immunoreactivity for β-crystallin. (E–G) Localization of E-cadherin clearly demarcates the islands of small cells from the elongating fibers and identifies them as lens epithelial cells (F, epi). The merged image (G) clearly shows the structured relationship between the epithelial cells and the elongating fibers; the latter being highly aligned and oriented at right angles to the epithelial island (arrows). Scale bar: (A, E–G) 50 μm and (B–D) 10 μm.

Secondary antibodies conjugated to Alexa 488 and 594 dyes were used at a dilution of 1:1000 in 0.1% BSA in PBS and applied for 2 h in the dark at room temperature; this was followed by three washes in 0.1% BSA. Circular glass coverslips were mounted on top of lens explants using Aqua Poly/Mount Solution (Polysciences, Inc. PA, USA). Fluorescence was visualized and images were collected using a Zeiss LSM-5Pa confocal microscope (Zeiss, Jena, Germany) with LSM Image Browser 5 software (Zeiss).

Assessment of cell proliferation by EdU incorporation

To identify proliferating cells, 10 μM, 5-ethynyl-2′-deoxyuridine (EdU; EdU Click-iT® Imaging Kit, Molecular Probes, Invitrogen) was added to the lens explant culture medium after 72 h in experimental conditions and explants were incubated for a further 14 h. Inverted lens explants were fixed in 2% paraformaldehyde for 30 min at room temperature followed by four successive washes with 1 × PBS. Following fixation, explants were flipped over, as described above, and pinned to the base of the tissue culture dish. Lens explants were permeabilized in 0.5% Triton-X100 (VWR, IL, USA) in 1 × PBS for 20 min: this was followed by three successive washes in 1 × PBS and two washes in 3% BSA in 1 × PBS for 5 and 10 min respectively. Lens explants were then stained for EdU detection with AlexaFluor 488-azide using a Click-iT™ Kit for one hour according to the manufacturer's instructions (Invitrogen). Total nuclei were counterstained with propidium iodide (PI; 1 mg/ml; Molecular Probes, Invitrogen) at a dilution of 1:10,000 in 0.1% BSA in 1 × PBS and applied for 10 min in the dark at room temperature; this was followed by three washes in 0.1% BSA in PBS. Circular glass coverslips were mounted on top of lens explants using Aqua Poly/Mount Solution (Polysciences Inc). Fluorescence was visualized and images of × 10 and × 20 magnification were collected using a Zeiss LSM-5Pa confocal microscope.

Lens cell proliferation was determined by assessing the proportion of cells that were EdU-labeled. Three representative regions of 200 μm^2 from each explant were selected using LSM Image Browser 5 software (Zeiss). EdU-labeled cells were counted and expressed as a percentage of total cell count (PI labeled cells).

Co-culture of lens explants with Wnt5A over-expressing cells

The C17.2 cell line employed in this study is a Wnt5A over-expressing immortalized neural precursor line from the neonatal mouse cerebellum (Snyder et al., 1992; Blakely et al., 2011) and was a kind gift from Dr. C. Parish (Florey Institute, University of Melbourne, Australia). C17.2 cells were routinely cultured at 37 °C in 5% CO_2 in Dulbecco's Modified Eagle Medium (DMEM) (Gibco, VIC, Australia) supplemented with 10% Hyclone Fetal Bovine Serum (Thermo Scientific), 5% Horse Serum (Gibco), 50 IU/ml penicillin, 50 μg/ml streptomycin (Invitrogen), 0.2 mM L-glutamine (ThermoScientific) and 50 μg/ml Geneticin (Gibco). Tissue culture dishes of 35 mm were divided into four quadrants; C17.2 cells were seeded on one marked quadrant at 30,000 cells in 150 μl and left to adhere for 3 h before the addition of 1 ml DMEM supplemented medium. Cell cultures were maintained in conditions for 3 days after which culture medium was replaced with serum-free supplemented M199 medium with Earle's salts (as previously described) with culture for a further 24 h prior to the addition of lens explants. Inverted lens epithelial explants were positioned and pinned in a cell free area of the tissue culture dish at varying distances from the segment covered with C17.2 cells. Culture medium was replaced with 1 ml of fresh, equilibrated M199 with the addition of 200 ng/ml FGF or 0.2% BSA. To suppress the secretion of Wnt5A from C17.2 cells an Inhibitor of Wnt Production-2/Wnt Antagonist II (IWP-2) (Merck, Germany) was employed. IWP-2 inhibits cellular Wnt processing and secretion

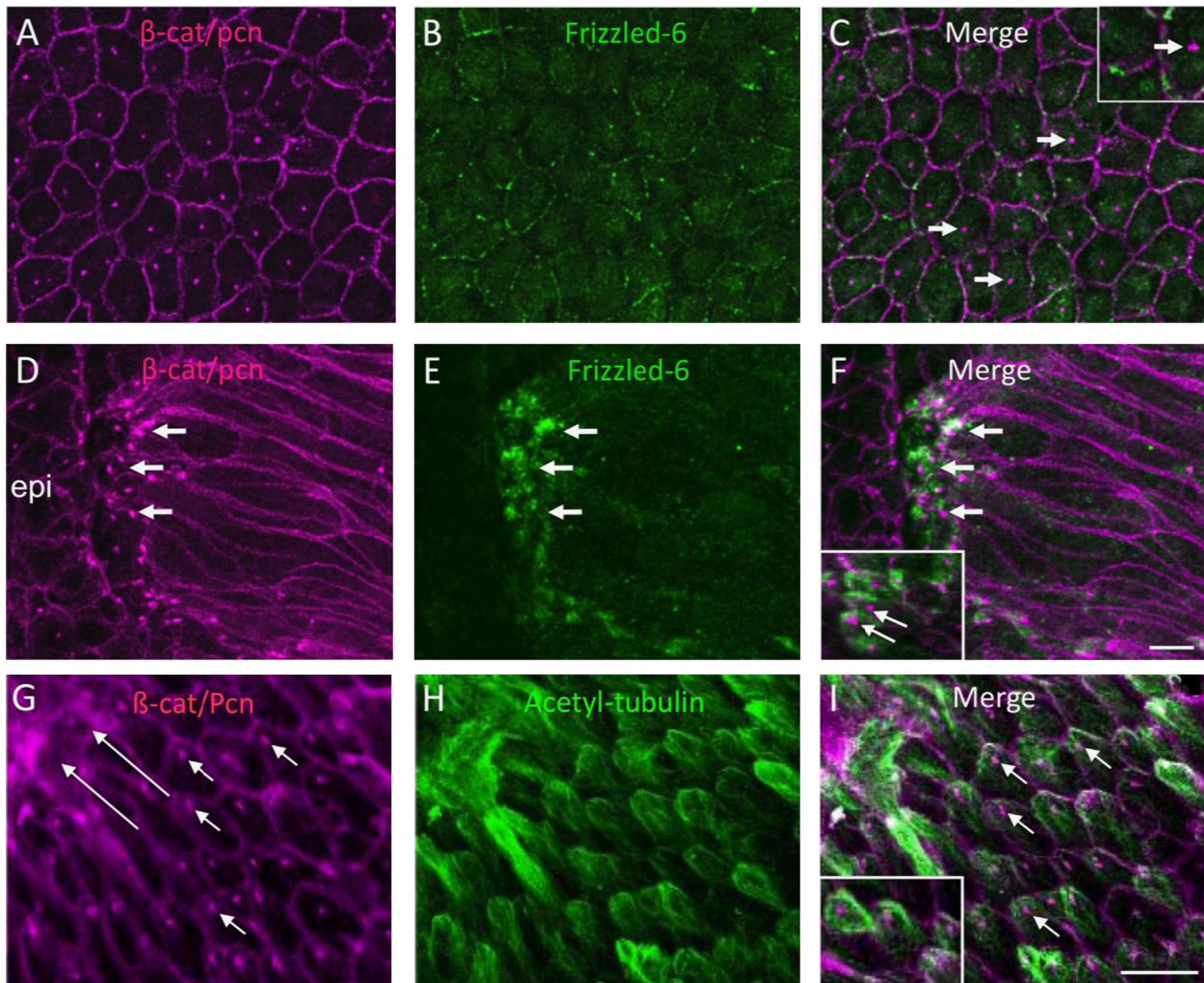

Fig. 2. FGF promotes translocation Fz6 to apical tips of elongating fibers which exhibit Planar Cell Polarity. In the absence of FGF treatment the lens epithelial cells retain their cobblestone-packed arrangement; β-catenin localizes cell margins and pericentrin immunoreactivity shows centrosome is mostly centrally placed in these epithelial cells (A). Fz6 localizes to discrete membrane domains in the cobblestone-packed cells (B) and does not associate with the centrosome (C, arrows). Explants treated with 200 ng/ml FGF for 4 days show elongated cells (D) that are aligned/oriented towards epithelial cells (epi). Elongated cells have Fz6 and their centrosome closely associated at their leading edge/apical tip (D–F, arrows); the Fz6 rich apical tips of elongating fiber cells are seen to abut onto the island of epithelial cells (F). Following 5 days of culture with 200 ng/ml FGF an oblique section through the apical region of a group of similarly oriented elongated cells shows that many of the cells have developed hexagonal profiles as indicated by β-catenin reactivity (G). The centrosome as indicated by pericentrin localization (G, small arrows), tends to be polarized to the same side of every cell (overall orientation is indicated by the two parallel long arrows). Strong fluorescence for acetlyated-tubulin shows that stabilized microtubules are prominent at the apical tips of most cells (H) and in many cases surround the centrosome (I, inset). Scale bar: (A–F) 15 μm and (G–I) 20 μm.

via selective blockage of the membrane bound actyltransferase, Porcupine (Chen et al., 2009). IWP-2 at 20 μM was applied to C17.2 cells 48 h after their initial seeding and was replenished upon the addition of the lens explants. Control dishes, lacking inhibitor, were supplemented with an equivalent volume of the vehicle, dimethylsulfoxide (DMSO). The explant-C17.2 cell co-cultures were maintained at 37 °C in 5% CO_2 for 3 days.

Assessment of cell outgrowth from lens epithelial explants

Measurements of cell outgrowth from the inverted lens explants were generated from digital images using phase contrast microscopy with DPC controller imaging software (IX71, Olympus, Japan). A complete profile of each explant was put together by combining several images using Office PowerPoint 2010 (Microsoft, USA). A circle divided into four quadrants labeled N, S, E and W was superimposed over the profile image of the explants such that the 'S' labeled quadrant was proximal to the segment of dish covered by C17.2 cells (see Fig. 6). Cell outgrowth from each quadrant was measured using Java-based image analysis software (ImageJ; available in the public domain by National Institutes of Health, Bethesda, MD, http://rsbweb.nih.gov/ij/). The distance between the explant and C17.2 cells was determined by analysis of fluorescent micrographs of the co-cultures immunostained for acetylated tubulin. Using a Zeiss LSM-700 confocal microscope (Zeiss) with ×10 objective a tiling function was performed to generate mosaic images of lens explants with proximal C17.2 cells. The distance between the edge of the explant and the leading edge of the C17.2 colony was measured using Image browser software (Zeiss LSM Image Browser 5); measurements were recorded in triplicate to ascertain a mean value of distance per co-culture.

Quantification of Wnt5 protein using ELISA

Following their dissection, 6–8 inverted lens epithelial explants were pinned to each tissue culture dish with the addition of 1 ml equilibrated M199 plus treatment conditions of 200 ng/ml FGF or 0.2% BSA for 3 days. For estimation of secreted Wnt5 from C17.2 cell line: cells were seeded onto 35 mm culture dishes at 30,000 cells in 150 μl and had identical treatment conditions and culture

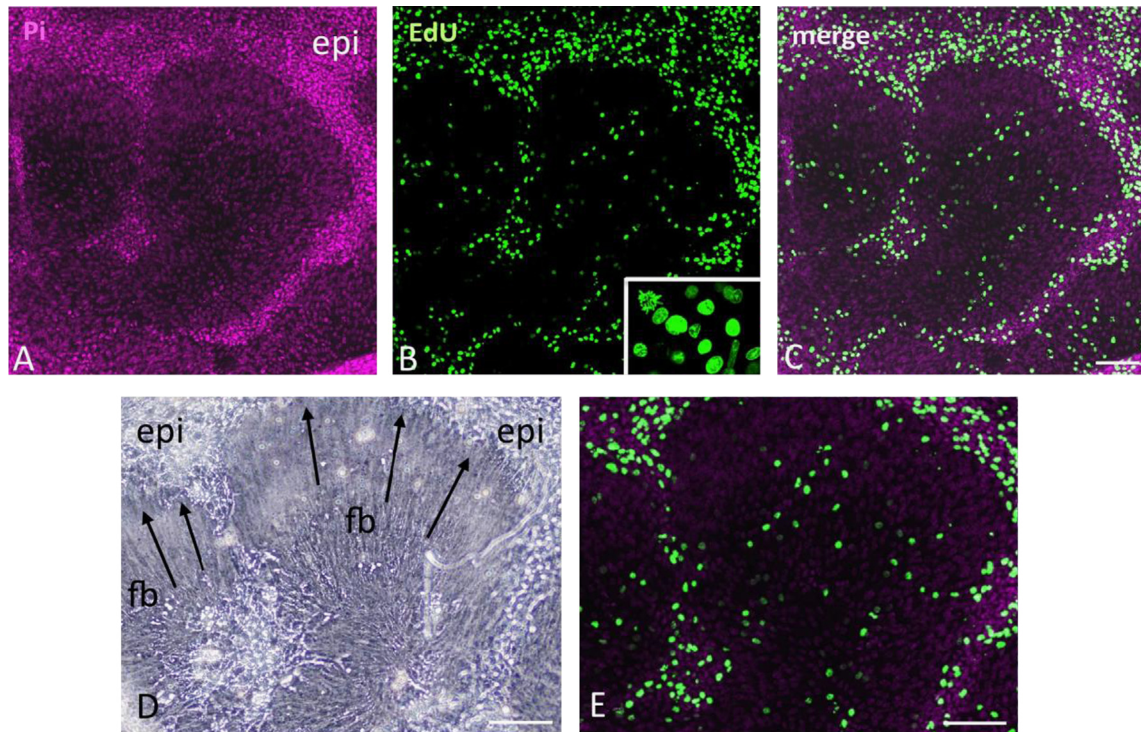

Fig. 3. Epithelial islands show high levels of DNA synthesis and mitosis. In explants treated with FGF for 4 days, propidium iodide (PI) staining for nuclei shows islands of densely packed cells indicating that epithelial islands (epi) have formed (A). Explants were incubated with EdU for 14 h prior to fixation. Cells incorporating EdU (green) are abundant in the epithelial islands but only occasionally an EdU positive cell is found outside these regions (B, C). Mitotic figures are also common in the epithelial islands (B, inset). Phase contrast images reveal more cellular detail and show elongated fibers (fb, arrows) aligned/oriented towards the epithelial islands (D). The EdU-labeled cells are seen to be concentrated in the regions occupied by the epithelial cells, whereas only a few labeled cells are evident in the regions of elongating cells (E), the percentage of EdU incorporation is reported in Table 1. Scale bar: 100 um

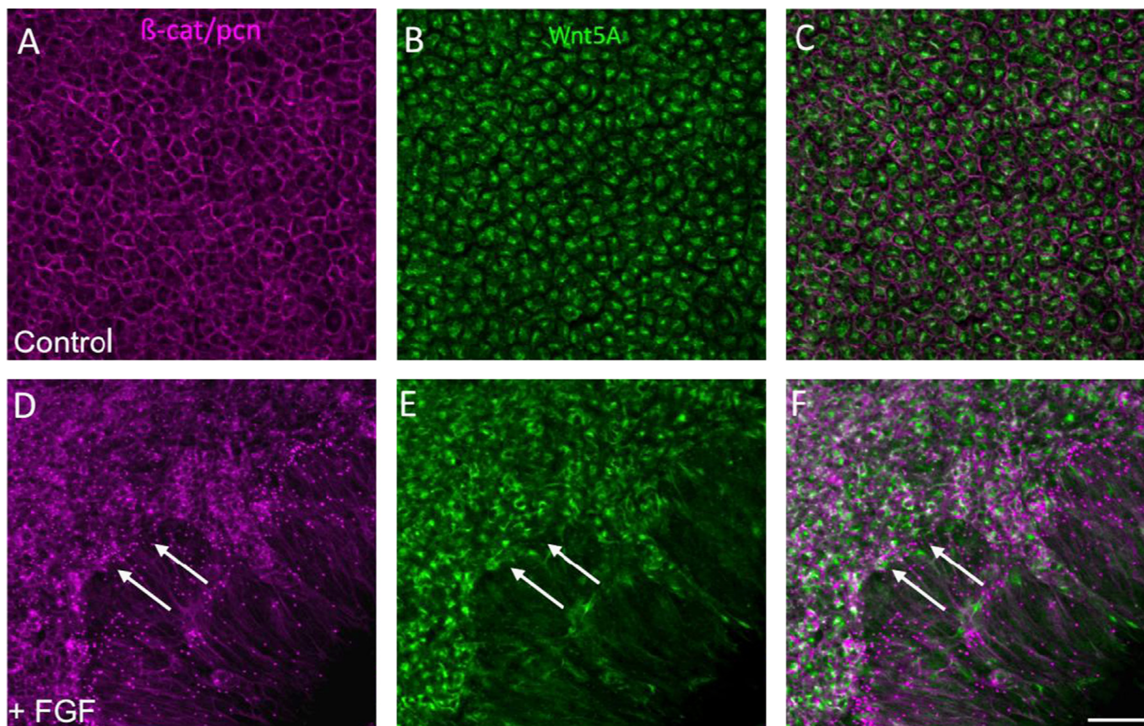

Fig. 4. Wnt5 is localized to epithelial islands in FGF-treated explants. Explants were cultured in control conditions (no FGF) (A–C) or in the presence of 200 ng/ml FGF (D–F) for 3 days. In the absence of FGF, β-catenin reactivity shows typical cobblestone packing of lens epithelial cells (A), which exhibit cytoplasmic immuno-reactivity for Wnt5 (B, C). In response to FGF, lens cells are elongated and oriented at right angles towards an island of epithelial cells (D, arrows). Pericentrin immuno-reactivity localizes the centrosome to the apical tip of the elongating fibers which abut the island of epithelial cells. Strong cytoplasmic immuno-reactivity for Wnt5 is evident in cells of the epithelial island (E, F). The elongating fibers that are polarized towards the epithelial island show weak and diffuse reactivity for Wnt5 (E, F). In this instance the fluorescent micrographs shown are representative images from repeat experiments using goat anti-Wnt5A (R&D systems) for immuno-detection, these images are also representative of results with rabbit anti-Wnt5A (Abcam). Scale bar: 50 μm.

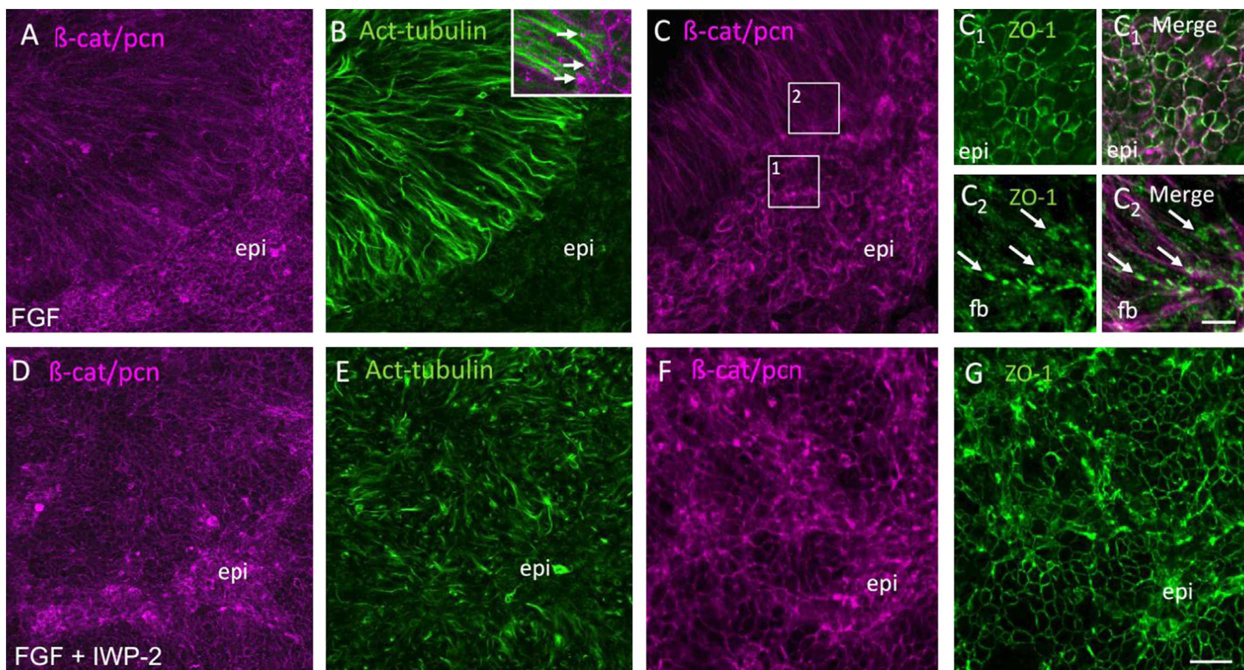

Fig. 5. Disruption of Wnt secretion abrogates FGF-promoted cell elongation and polarization. Explants were cultured with 200 ng/ml FGF (A–C) or in the presence of both FGF and Inhibitor of Wnt Production; IWP-2 (20 μM, D–G) for 3 days. β-catenin- and pericentrin-immunoreactivity (A, D) localizes cell margins and centrosomes, respectively. Acetylated-tubulin reactivity (B, E) localizes stabilized microtubules and helps visualize the extent of cell elongation. ZO-1 reactivity ($C_{1/2}$, G) localizes to tight junctions and helps assess cellular polarity. Explants treated with FGF (200 ng/ml) show substantial elongation of cells which are oriented/polarized towards a prominent island of epithelial cells (epi; A, B). Pericentrin-reactivity identifies centrosomes (arrows) at the apical tip of each of the elongated cells which are juxtaposed to the epithelial island (B, inset). In D, when IWP-2 is included with FGF, no prominent epithelial island is evident, instead small islands of epithelial cells are scattered throughout the explant (epi, D–G). Inclusion of IWP-2 reduces the extent of cell elongation (E) and cells do not exhibit polarized or oriented behavior toward groups of epithelial cells (D–G). The characteristic translocalization of ZO-1 to the apical tips of the elongating fibers (C_2) is also blocked by the inclusion of IWP-2; instead ZO-1 is maintained predominantly around cell margins (G) as in controls (C_1, no FGF or IWP-2). Scale bar: (A, B, D, E) 50 μm and (C, F, G) 20 μm.

duration to those previously described for explant-C17.2 cell co-cultures. At the end point of culture, for both lens explants and C17.2 cells a 500 μl sample of medium was removed for ELISA analysis and total proteins were extracted with supplemented RIPA buffer (as previously described). Mouse and rat Wnt5A ELISA kits (Cusabio, Wuhan Huamei Biotech, China) were employed to assess Wnt secretion from the C17.2 cell line and inverted lens explants respectively. Because it is present in a conjugated form we were unable to test the ELISA antibody for Wnt5A specificity. However, given the similarity between Wnt5A and Wnt5B as well results from Western blotting analysis of other Wnt5A antibodies (see Supplementary Fig. 2) that show they have equal ability to detect recombinant Wnt5A and Wnt5B proteins, we conclude that this ELISA kit likely detects both Wnt5 isoforms. Total proteins extracted from the C17.2 cells and lens explants were used to normalize Wnt5 levels.

Statistical analysis

A 2 tailed *t*-test analysis (Excel software; Microsoft, WA, USA) and one-way ANOVA (with Tukey's post-hoc analysis; IBM SPSS Statistics ver. 19 for Windows; SPSS Inc, IL, USA) were performed to determine statistical differences between experimental groups, set at $p \leq 0.05$.

Results

Epithelial islands promote polarized behavior during FGF-induced fiber differentiation

Previous studies in our laboratory with lens epithelial explants have shown that FGF induces a fiber differentiation response that recapitulates morphological and molecular processes characteristic of differentiation in vivo (Lovicu and McAvoy, 2005). One of the key features of differentiating fibers in the intact lens is that they exhibit a regular alignment and are polarized towards the overlying epithelial sheet. Now, using a modification of this explant system we show that after 4–5 days exposure to FGF, we can routinely generate groups of elongated cells that show similar orientation/alignment. Further analysis of these explants reveals that the similarly oriented/aligned cells are polarized towards groups of smaller cells; i.e. cells in the epithelial explant that failed to elongate and are present in compact clusters (Fig. 1A and B). We often find that when these groups or islands of cells are well developed, the elongated cells are highly ordered. Whilst the cellular arrangements vary considerably, one thing that is consistent is the alignment of the elongating cells at approximately right angles to a group, or island, of smaller cells. The elongated cells are readily visualized by immuno-localization of acetylated tubulin (Fig. 1A). We confirm that these cells are similarly polarized as the bright spot of pericentrin reactivity that localizes the centrosome and demarcates the apical tip of each cell is similarly localized in these groups of elongating cells and mostly this is juxtaposed to the clusters of smaller cells (Fig. 1B). Localization of the key fiber cell marker β-crystallin confirms that the elongating cells are undergoing molecular features of fiber differentiation. The absence of β-crystallin from the islands of smaller cells and the presence of the epithelial marker E-cadherin shows that these cells retain the epithelial phenotype (Fig. 1C–G).

Additional short-term explant cultures show that this cellular segregation process begins around 2 days. After 1 day culture in FGF, although there is some variation from the typical cobblestone packing arrangement typical of controls, all cells show strong immuno-reactivity for E-cadherin (Supplementary Fig. S1A and D). In contrast, by 2 days culture, distinct groups of cells have formed

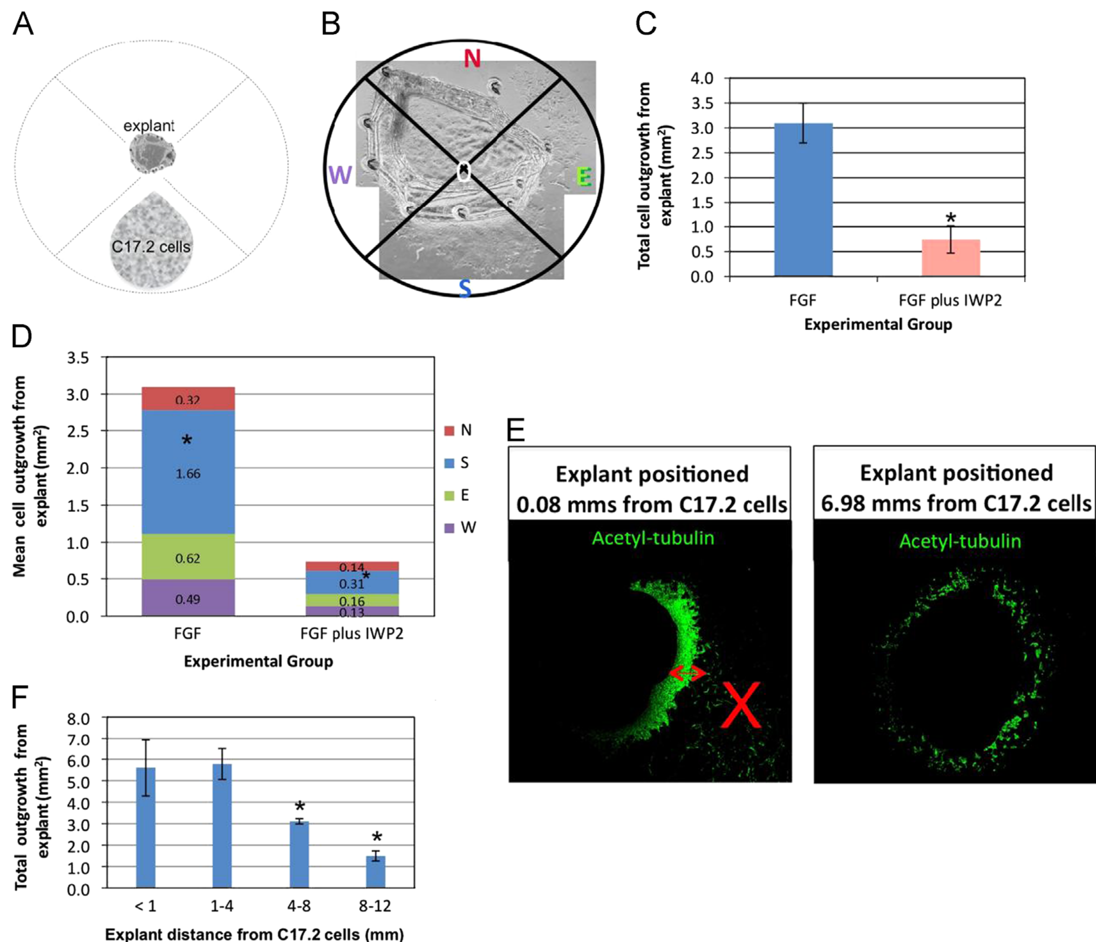

Fig. 6. Wnt5 producing cells promote directed lens cell migration. Lens epithelial explants were co-cultured with a Wnt5A expressing cell line (C17.2). Inverted lens epithelial explants were positioned in a cell-free area of the tissue culture dish opposite to the segment that had been previously seeded with C17.2 cells (diagrammatically represented in A). As depicted in (B), following 3 days with 200 ng/ml FGF treatment, cells were seen to grow out from the explant and onto the tissue culture dish. The histogram shows total area of cell outgrowth (square millimeter) from explants cultured with C17-2 cells (C). The explant-C17.2 cell co-cultures were maintained in the presence of FGF (200 ng/ml) alone or FGF plus 20 μM IWP-2 for 3 days. The inclusion of IWP-2 with FGF significantly reduced the total cell outgrowth from explants compared to FGF treatment alone (*, $p \leq 0.05$, 2-tailed *t*-test). To assess regional outgrowth, an image of each culture dish was superimposed by a circle representing the area of the culture dish. This was divided into four quadrants labeled N, S, E and W and was positioned so that the 'S' labeled quadrant was proximal to the segment of dish covered by C17.2 cells. The histogram in D shows the area of explant cell outgrowth from each of the four quadrants. There is a significant difference between cell outgrowth in quadrant 'S' compared to all other quadrants in FGF alone and FGF plus IWP-2 treatment conditions (*, $p \leq 0.05$, ANOVA with Tukey's test). To determine if the distance of lens explants from the Wnt5 producing cells influenced the results, explants were positioned at various distances from the area covered with C17.2 cells (E). Following 3 days with FGF, localization of acetylated tubulin (green) shows cellular outgrowth from the lens explants and X (E, left panel) demarcates the position of C17.2 cells. A prominent, confluent sheet of outgrowing cells is evident when the explant is only 0.08 mm away from the C17.2 cells (E, left panel, arrow). In contrast, an explant at a distance of 6.98 mm away from C17.2 cells, shows sparse cellular outgrowth that occurs all around the explant (E, right panel). The histogram in F, shows that explants positioned more than 4 mm from C17.2 cells exhibit significantly reduced cellular outgrowth activity compared to those explants in close proximity (*, $p \leq 0.05$, ANOVA with Tukey's test). Data for C, D and F represent the mean ± SEM ($n=4$).

that show little or no E-cadherin reactivity (Fig. S1B and E). Some of these E-cadherin depleted cells are slightly elongated and show evidence of polarization toward the epithelial cells as indicted by localization of pericentrin at their apical tips that abut the epithelial cells (Fig. S1B and C, inset). Although these aligned cells show little E-cadherin expression, N-cadherin is prominent and this is typical of cells undergoing early fiber differentiation (Fig. S1F).

We also show that during fiber differentiation, Frizzled 6 (Fz6) translocates from membrane domains in epithelial cells to the apical tips of the elongating fibers and during this process becomes closely associated with the centrosome (Fig. 2A–F). This is consistent with previous results that showed FGF-induced translocation of centrosome and Fz6 to the apical tip of each elongating cell (Dawes et al., 2013). Additionally, the current study now shows that the Fz-rich apical tips of elongating cells abut the cells of the epithelial islands. This is reminiscent of the situation in vivo where, during the epithelial to fiber transition, Fz and the centrosome/primary cilium become localized to the apical tip of each elongating fiber as it abuts the epithelium (see Sugiyama et al., 2010). Importantly, oblique sections that transect the apical ends of elongated cells show that many of them have developed hexagonal profiles having four long sides and two short sides (Fig. 2G–I). Note that whilst transverse sections along most of the length of a lens fiber in vivo show two long sides and four short sides (see for example, Maddala et al., 2011), at the apical tips this arrangement is reversed and the two long sides become short sides whilst the four short sides become long sides (see Fig. 3; Sugiyama et al., 2010). Also in the image presented most of the cells are oriented similarly as is shown by the polarization of the centrosome to the same short side of each cell. This shows that collectively this group of cells is polarized within the plane; i.e. they exhibit local PCP somewhat similar to the PCP that has been described in vivo where the centrosome is polarized to the same side of each cell (Sugiyama et al., 2010).

Table 1
EdU incorporation in cells of lens explants after 4 days exposure to 200 ng/ml FGF.

Cells	% EdU incorporation
Epithelial island	49.56 ± 3.05[a]
Aligned fibers	8.17 ± 0.65

Values in this table are derived from mean ± SEM ($n=4$).

[a] Significant difference between epithelial islands compared with aligned fibers in FGF treated explants ($p \le 0.05$, 2-tailed *t*-test).

Epithelial islands retain proliferative activity

To determine the proliferative status of the lens cells following FGF treatment we used EdU incorporation methods to detect cells undergoing DNA synthesis. After 48 h exposure to FGF, epithelial cells that form islands show positive EdU labeling (Fig. 3). In contrast, the areas occupied by elongated fibers show only sparse EdU labeling. Cell counts show that in epithelial islands 49.56% ± 3.05 of cells are labeled whereas only 8.17% ± 0.65 cells are labeled in the elongated fiber regions (see Table 1). Moreover, mitotic figures are observed within the islands of epithelial cells, providing further evidence of their proliferative activity. (Fig. 3B, inset). Thus, given their proliferative status these epithelial cells resemble the cells of the germinative zone of the lens epithelium.

Wnt5 promotes directed cell migration and promotes the polarized behavior of elongating fiber cells

A logical candidate for the putative polarizating/orienting cue from the epithelial cells is a member of the Wnt growth factor family. Given results from our previous study that shows FGF promotes Wnt-Fz signaling in explants (Dawes et al., 2013), one possibility is that an epithelial-derived Wnt acts as a ligand for the Fz that is prominently localized at the leading edge of the elongating fibers (see Fig. 2D–F). Previous studies have shown that a number of Wnt mRNAs, including non-canonical Wnt5A and Wnt5B, are expressed in the lens (Ang et al., 2004; Lyu and Joo, 2004). To investigate the possibility that Wnt5-expressing epithelial cells act as the polarizing/orienting cue, we first localized Wnt5 protein in explants. For this we used an antibody from R&D Systems. Initially to test for its specificity we conducted Western blotting on extracts of lens explants. This showed distinct bands corresponding to those previously reported for Wnt5A (Da Forno et al., 2008; Peters et al., 2004); however, the antibody also showed strong reactivity for both recombinant Wnt5A and Wnt5B (Supplementary Fig. S2). Taken together with the previous studies, the strong cytoplasmic immuno-reactivity shown in explants is likely to be in response to the presence of both Wnt5A and 5B (Fig. 4). After FGF treatment the epithelial islands that form after 3 days also show strong cytoplasmic reactivity for Wnt5, whereas the elongating fibers that are polarized/oriented towards the epithelial islands show weaker and more diffuse reactivity for Wnt5 (Fig. 4).

To determine if Wnt5 was secreted from the cells we analyzed the explant culture medium by ELISA. From this analysis we detected Wnt5 in the medium from FGF-treated and control cultures (eight explants were included/culture dish) at levels of 1.88 ng/ml and 0.36 ng/ml, respectively (Table 2). This shows that FGF promoted about a 5-fold increase in production/secretion of Wnt5 from epithelial cells into the culture medium and is consistent with previous studies that show FGF upregulates expression of other Wnt-Fz signaling components (Dawes et al., 2013). We also showed that the addition of the inhibitor of Wnt production (IWP-2) significantly reduced the level of Wnt in the culture medium of FGF-treated explants (Table 2), in line with its role in specifically inhibiting Wnt processing and secretion via selective blockage of the membrane bound acyltransferase, Porcupine (Chen et al., 2009).

Table 2
ELISA detection of Wnt5 concentration from culture medium extract following 3 days in experimental conditions.

Culture type	Experimental condition	Wnt5A concentration (ng/ml)
Lens epithelial explant	no FGF	0.36 ± 0.09
	200 ng/ml FGF	1.88 ± 0.40[a]
	200 ng/ml FGF plus 20 μM IWP-2	1.36 ± 0.42[b]
Lens epithelial explant +C17.2 cell line	200 ng/ml FGF	1.72 ± 0.34
	200 ng/ml FGF plus 20 μM IWP-2	0.84 ± 0.21[b]

Values in this table are derived from mean ± SEM($n=4$).

[a] Significant difference between FGF compared with control group ($p \le 0.05$, 2-tailed *t*-test).

[b] Significant difference between FGF plus IWP-2 compared with FGF alone for explant and cell line cultures ($p \le 0.05$, 2-tailed *t*-test).

Consistent with our previous studies, (Dawes et al., 2013), the presence of IWP-2 reduced the fiber differentiation response as indicated by the presence of cells that were clearly shorter than cells in explants treated with FGF alone (Fig. 5). To expand upon this previous work, we now show cells treated with IWP-2 do not exhibit polarization or alignment towards occasional small islands of epithelial cells that have formed (Fig. 5, compare A and B with D and E). To further assess how Wnt/Fz signaling influences the polarity of lens cells we localized the tight junction protein ZO-1. Following FGF treatment, ZO-1 showed a distinctly polarized localization at the apical tips of lens fibers that abut a prominent epithelial island (Fig. 5C, inset C2). In contrast, cells of the epithelial island showed strong immuno-reactivity for ZO-1 around the cell margins (Fig. 5C, inset C1). Importantly, these localization patterns are consistent with the pattern of ZO-1 expression observed in the epithelium and underlying cortical fibers in vivo (Nielsen et al., 2003). The presence of the Wnt secretion inhibitor IWP2 abrogated such distinct patterns of ZO-1 expression. Notably, cells in direct contact with small epithelial islands maintained the characteristic epithelial membrane localization pattern for ZO-1 expression and showed no evidence of the polarized reorganization that ZO-1 characteristically undergoes during fiber elongation (Fig. 5G). This clearly indicates IWP-2 disturbed the polarized/oriented behavior of elongated cells towards the epithelial islands. Taken together with the ELISA result that shows less epithelial-derived Wnt5 is detected in the presence of IWP-2 (see Table 2), this appears to support our hypothesis that epithelial-derived Wnt acts as a polarizing/orienting cue for elongating fibers. However, the addition of IWP-2 also clearly reduced the presence and prominence of the epithelial islands and because of this we cannot exclude the alternative possibility that the lack of polarized/oriented behavior is due to the absence or reduced amount of another (e.g. a non-Wnt) epithelial-derived cue.

In another approach to assess the capability of Wnt ligands to promote directed behavior of lens cells, we conducted a series of experiments where epithelial explants were co-cultured with the C17.2 neuronal cell line that was engineered to express Wnt5A. For controls, we set up similar co-cultures but also included IWP-2 as this effectively reduces the level of Wnt secreted by these cells into the medium (Table 2). The C17.2 cells were grown up as previously described (Snyder et al., 1992; Blakely et al., 2011) and were plated so that they only covered a segment on one side of the dish. Lens epithelial explants were set up in the cell-free area and FGF was added to the medium to promote cell migration (Fig. 6A). Three days after setting up these co-cultures, cells had clearly grown out

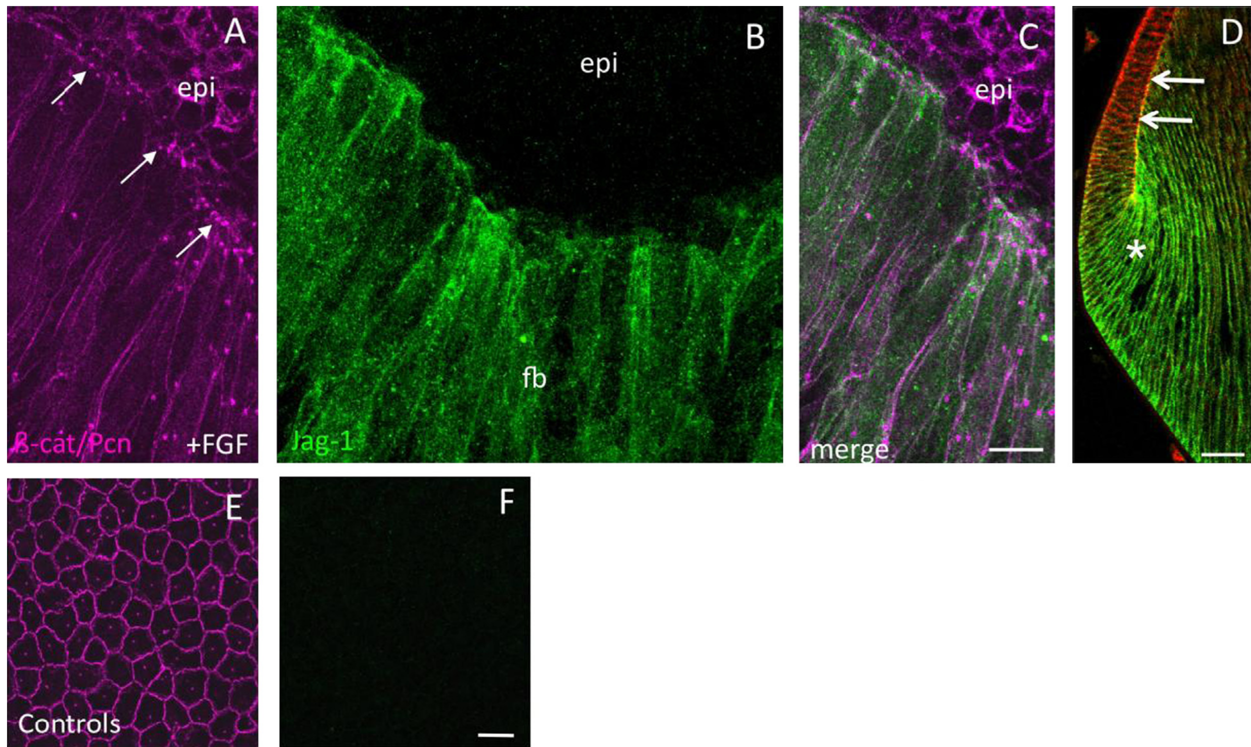

Fig. 7. Jag-1 is prominently localized in elongated fibers. In explants treated with FGF for 4 days, epithelial islands have formed and elongated fibers are clearly aligned with their apical tips polarized (as shown by pericentrin localization) towards them (arrows, A). Jag-1 is clearly localized in association with the membranes of elongating fibers and is absent from epithelial islands (A–C). To determine Jag-1 localization in vivo a paraffin section of a postnatal day 5 rat lens was immuno-stained for Jag-1 and β-catenin (D). Similar to explants cultured with FGF (B, C), Jag-1 shows strong localization along the membranes of early differentiating fibers (asterisk) and is absent from the adjacent epithelium (arrows). In control explants (no FGF) the epithelial cells have the same characteristic cobblestone packing seen in vivo and show no reactivity for Jag-1 (E, F). Scale bar: (A–C, E, F) 15 μm and (D) 100 μm.

from the lens explants (Fig. 6B) and showed significantly greater total cell outgrowth when compared to non-treated (no FGF) controls (Supplementary Fig S3). Quantitative analysis showed that in co-culture with the Wnt5 expressing cells, total outgrowth of lens cells was significantly greater than in the presence of IWP-2 (Fig. 6C). Moreover, the reduced outgrowth activity in the presence of IWP-2 coincided with a significant reduction in level of Wnt5 in the culture medium from 1.72 ± 0.34 ng/ml in the presence of FGF alone down to 0.84 ± 0.21 ng/ml when IWP-2 was included (Table 2). It was also clear that significantly more outgrowth activity occurred in the quadrant closest to the C17.2 cells, quadrant S (Fig. 6B and D). Whilst outgrowth activity was sometimes present in other quadrants (e.g. in Fig. 6B, some outgrowth is present in quadrants N and E), invariably the cells tended to be diffuse and dissociated unlike the prominent and contiguous sheet of cells consistently present in quadrant S. One interpretation of this result is that because lens cells in quadrant S are closer to the C17.2 cells than cells in the other quadrants, they are exposed to a higher concentration of Wnt5 and respond accordingly with more directed outgrowth activity. Support for the existence of such a Wnt5 gradient comes from experiments in which the explants were placed at different distances from the C17.2 cells. Consistently, the closer the explant to the C17.2 cells the more directed outgrowth behavior was observed (Fig. 6E and F). Taken together this is consistent with our hypothesis that in vivo, epithelial-derived Wnt provides a cue that promotes the directed behavior of lens cells.

FGF-treated explants recapitulate aspects of Notch/Jagged signaling in vivo

As described above, the prominence of cell proliferative activity in the epithelial islands likened them to epithelial cells of the germinative zone. Given that results from several recent studies have shown that in vivo Notch signaling is required for maintenance of a proliferating population of epithelial cells in the germinative zone and that this is activated by Jagged-1 (Jag-1)-expressing fiber cells in the outer lens cortex (Jia et al., 2007; Rowan et al., 2008; Le et al., 2009; Saravanamuthu et al., 2009, 2012), we wanted to determine if aspects of this regulatory mechanism operated in our explant system.

In FGF-treated explants Jag-1 is clearly localized to the elongating fibers (Fig. 7 A–C) but is absent from the epithelial islands as well as the epithelial cells of control explants (Fig. 7E, F). This mimics the situation in the intact rat lens at P5 as epithelial cells show no reactivity for Jag-1 but it becomes strongly expressed early in the fiber elongation process at the lens equator (Fig. 7D). Restriction of both Jag-1 mRNA and protein to early differentiating fibers has also been shown to be the case for other developmental stages (Jia et al., 2007; Le et al., 2009). When FGF-treated explants are cultured in the presence of the gamma secretase inhibitor, DAPT, which is widely used to inhibit Notch signaling, fiber elongation and Jag-1 expression is diminished (Fig. 8). This is consistent with the previously reported inhibition of Jag-1 and fiber differentiation marker expression that occurs following the application of DAPT to lens explant cultures (Saravanamuthu et al., 2012). Moreover, in relation to the role of Notch signaling in maintaining a proliferating epithelial phenotype, we also show that in the presence of the DAPT inhibitor the FGF-treated cells have an EdU incorporation rate of $9.35\% \pm 0.23$ that is significantly reduced from the $54.26\% \pm 1.83$ incorporation rate in the epithelial islands of explants treated with FGF alone ($p \leq 0.05$, 2-tailed t-test).

Previous studies have shown that the Notch effector Herp2/Hey1 is expressed only in the lens epithelium (Jia et al., 2007). Here we also show that Herp2/Hey1 expression is clearly localized

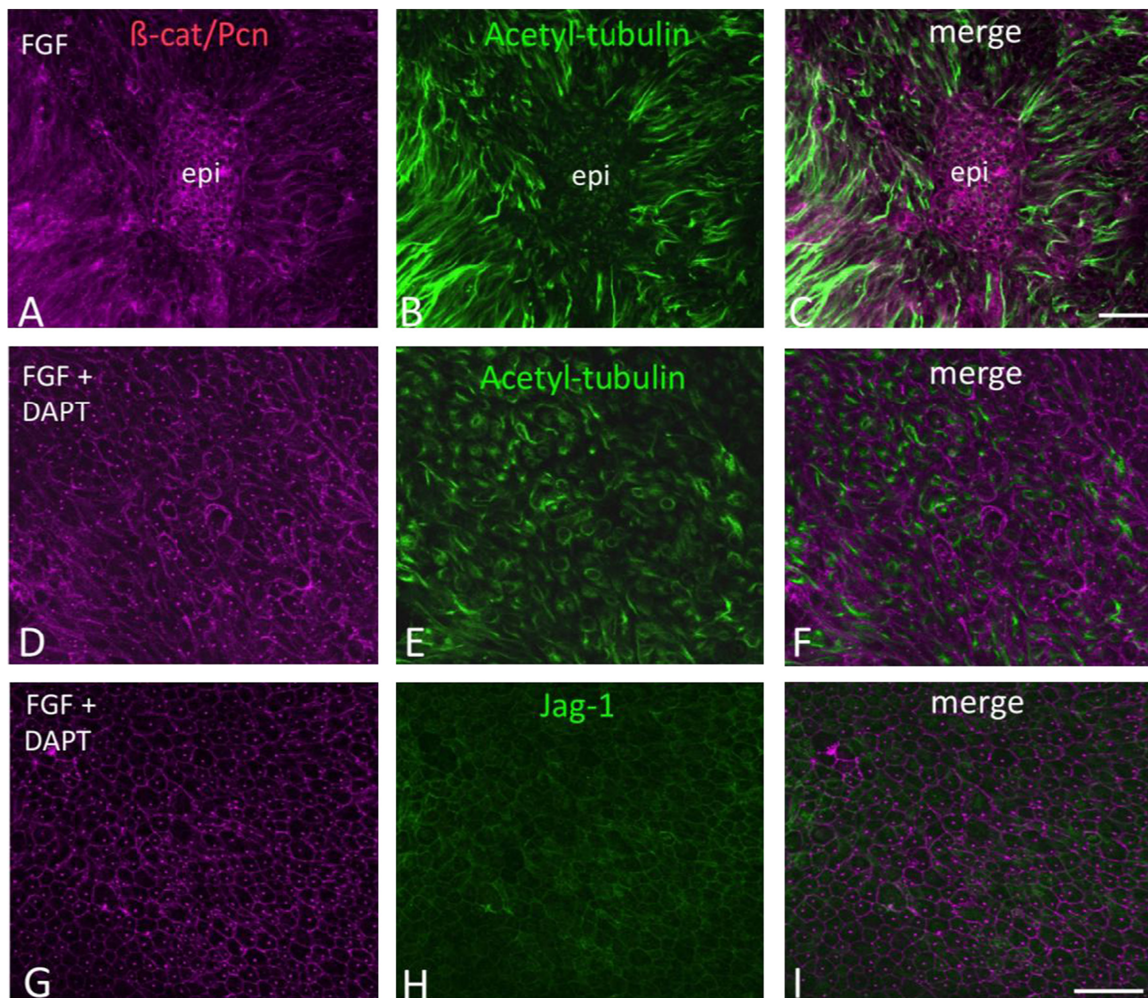

Fig. 8. The Notch inhibitor, DAPT, inhibits fiber elongation and Jag-1 expression. Explants were cultured in FGF alone (A–C) or in the presence of both FGF and 75 μM DAPT (D–F and G–I) for 4 days. B-catenin/pericentrin (A, D, G) and acetylated tubulin (B, E) localization shows that in response to FGF, cells elongate and align/orient around a centrally situated island of epithelial cells (epi, A–C). When DAPT is included with FGF, cell elongation is inhibited (D–F) and Jag-1 is barely detectable (H, compare with Fig. 7B). Abbreviation; epi, epithelium. Scale bar: 40 μm.

to the proliferating epithelial islands and is absent from the elongating fibers (Fig. 9A–C). Also in the presence of the DAPT inhibitor, Herp2/Hey1 shows weak expression and cell proliferative activity is diminished (Fig. 9D–F). Taken together this shows that our epithelial explant system recapitulates key features of the fiber/epithelial interaction that previous studies have shown to be mediated by Notch/Jagged signaling.

Discussion

This study shows that, when provided with the appropriate stimuli and conditions, an intrinsic mechanism is activated that controls the self-assembly of the two forms of lens cells, epithelial and fiber cells, into arrangements that replicate important features of three-dimensional lens cellular organization. Whilst it is well known that FGF provides the stimulus for epithelial cells to elongate and differentiate into fibers, the current study has shown that when explants are set up in an inverted configuration (see Section 2) and exposed to FGF, rows of similarly oriented fiber cells become prominent. A key finding is that these fibers are invariably oriented towards foci of epithelial cells that are clearly distinguished from the elongating fibers by their compact appearance and strong expression of E-cadherin (Fig. 1). The polarization of the fibers is shown by the observation that in virtually every one of these aligned fiber cells the centrosome that defines the apical tip of each cell is juxtaposed to the epithelium (Fig. 1B). This mimics the alignment and polarized behavior that fibers exhibit in vivo (see Sugiyama et al., 2010).

This raises the question of the identity of the polarizing cue (s) from the epithelium. Given the role of Wnts as ligands for Fz receptors, an obvious candidate for the polarizing cue is epithelial-derived Wnt. Support for a Wnt ligand acting as a guidance cue for fiber cells comes from analysis of mice that were generated to overexpress the Wnt-Fz regulator, Sfrp2, in the lens (Chen et al., 2008; Sugiyama et al., 2010). In these mice fiber cells were severely disorientated and lacked their characteristic global alignment/orientation toward the lens poles (Sugiyama et al., 2010). Several members of the Wnt family have been reported to be expressed in the lens (Stump et al., 2003, Lyu and Joo, 2004). Array data (e.g. http://biogps.gnf.org/#goto=genereport&id=22419) also shows that these Wnts are expressed at relatively high levels in lens compared with other tissues. Here, consistent with the in vivo data, we show that Wnt5 is prominent in the epithelial islands. We then set out to assess the possibility that an epithelial-derived Wnt could act as a polarizing cue and promote directed behavior of lens cells.

In our first approach to address this question, we used the inhibitor of Wnt production and secretion, IWP-2, to reduce Wnt bioavailability in the explant cultures. This had the desired

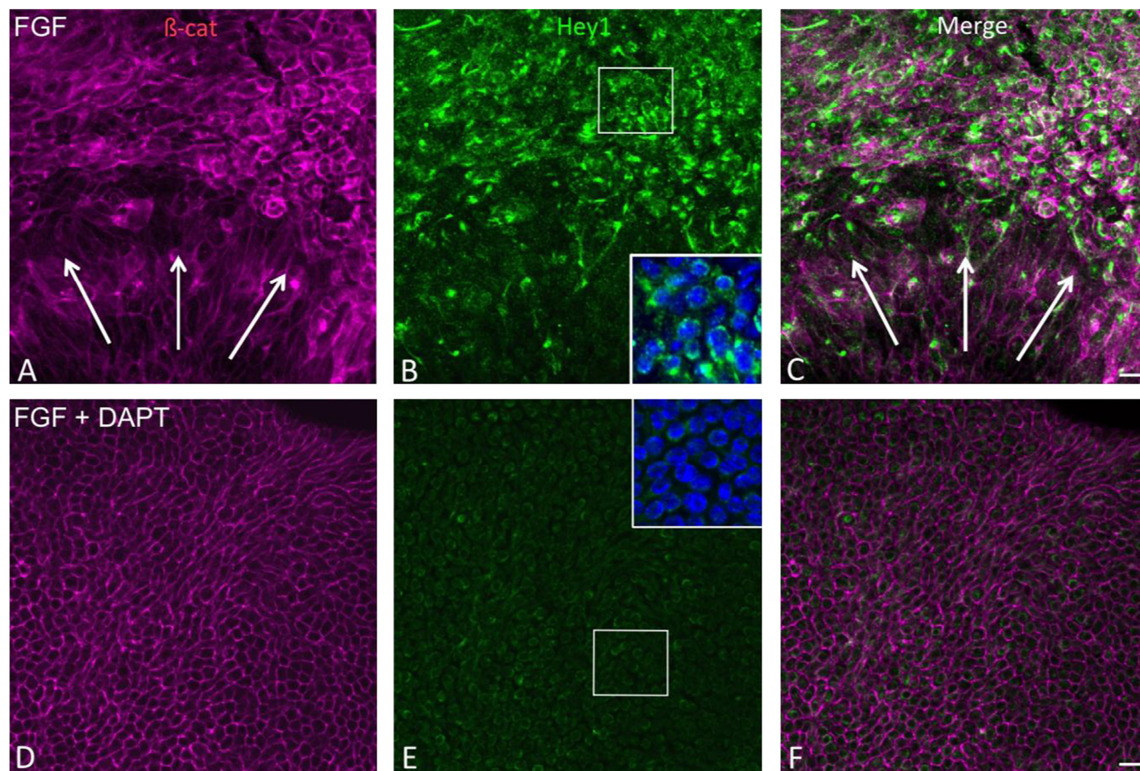

Fig. 9. The Notch effector Hey1 is expressed in epithelial islands and is blocked by DAPT. Explants were cultured in FGF alone (A–C) or in the presence of both FGF and DAPT (D–F) for 4 days. β-catenin (A) localization shows that in response to FGF alone, cells appear elongated and aligned/oriented towards an island of epithelial cells (A and C, arrows). Immuno-reactivity for Hey1 localizes to the epithelial cells that form the prominent island (B). Hoechst nuclear staining shows Hey1 is particularly prominent at the nuclear periphery of cells; however, in some cases cytoplasmic localization of Hey1 cannot be excluded (inset of boxed region in B). In the presence of DAPT, cells did not elongate (D and F) and Hey-1 can only be weakly detected (E and inset of boxed region with Hoechst staining). Scale bar: 20 μm.

effect and reduced the amount of Wnt5 detected in the culture medium. However, because reduced Wnt bioavailability inhibits both FGF-induced fiber elongation and the formation of the epithelial islands, this approach led to inconclusive results. The alternative approach was to set up a Wnt gradient in the culture dish and determine if this promoted directed behavior. To achieve this we co-cultured explants next to a Wnt5A expressing cell line. After 3 days this resulted in prominent cellular outgrowth from the explants that was predominantly directed towards the Wnt-producing cells; an activity that was significantly inhibited by IWP-2. Quantification of Wnt5 in the medium of these cultures also showed that the presence of IWP-2, in keeping with its role in inhibiting Wnt production/secretion, significantly reduced the level of Wnt5 in the culture medium. The observation that cells in the explant which were closer to the Wnt-producing cells (quadrant S in Fig. 6) showed significantly more directed outgrowth than cells further away, indicated the presence of a gradient of Wnt bio-availability. Strong support for this also comes from the observation that explants placed further away from the C17.2 cells showed less directed outgrowth activity than explants closer to the source of the Wnt. Taken together this identifies a role for Wnts, and in this particular case Wnt5, in providing a directional cue for lens cell migration. We could not assess if the Wnt gradient promoted oriented/polarized behavior of fibers because in these explant culture conditions the predominant response of cells was to migrate rather than to differentiate. Nevertheless, the result clearly shows that lens cells respond to a Wnt gradient and exhibit polarized behavior in the form of directed migration to the source of the Wnt.

Switching the focus to the groups/islands of epithelial cells, it was shown that they were still proliferating as evidenced by the high level of DNA synthesis and abundance of mitotic figures. This is in contrast to the juxtaposed elongating cells, which mostly had exited the cell cycle and only a small number were shown to be positive for EdU. This observation is similar to previous reports of occasional BrdU-positive nuclei in the fiber cell compartment of wild-type mouse lenses (Garcia et al., 2005; Rajagopal et al., 2008). Altogether, our observations of elongated cells juxtaposed to groups of proliferating epithelial cells is similar to the situation in vivo, where post-mitotic fibers of the outer lens cortex underlie the proliferating epithelial cells in the germinative zone. Recent studies have shown that Notch signaling is required for maintenance of this population of proliferating cells (Jia et al., 2007) and that this is mediated by Jag-1-expressing fiber cells (Rowan et al., 2008; Le et al., 2009; Saravanamuthu et al., 2009). Consistent with this we showed that, in our explants, Jag-1 was strongly expressed in the elongated fibers that were juxtaposed to the proliferating epithelial cells. We also showed that when we applied the Notch signaling inhibitor DAPT, Jag-1 was reduced in the FGF-treated explants. The fact that Jag-1 was still detected, although much reduced, is consistent with previous studies that showed the initial induction of Jag-1 is Notch independent but robust Jag-1 expression is dependent on lateral Notch/Jagged signaling between elongating fibers (Jia et al., 2007; Rowan et al., 2008; Saravanamuthu et al., 2009). Also in line with the role for Notch/Jagged maintaining proliferative activity, the presence of the Notch inhibitor led to a reduction of the level of EdU incorporation in the epithelial cells. In addition, previous studies showed that the Notch signaling effector, HERP2/Hey1 is restricted to the epithelium and that its suppression of expression of the cell cycle inhibitor $p57^{Kip2}$ provides a mechanism whereby Notch signaling maintains a proliferating pool of lens fiber precursors (Jia et al., 2007). Similarly we show in FGF-treated explants that prominent HERP2/Hey1 localization is restricted to the epithelial

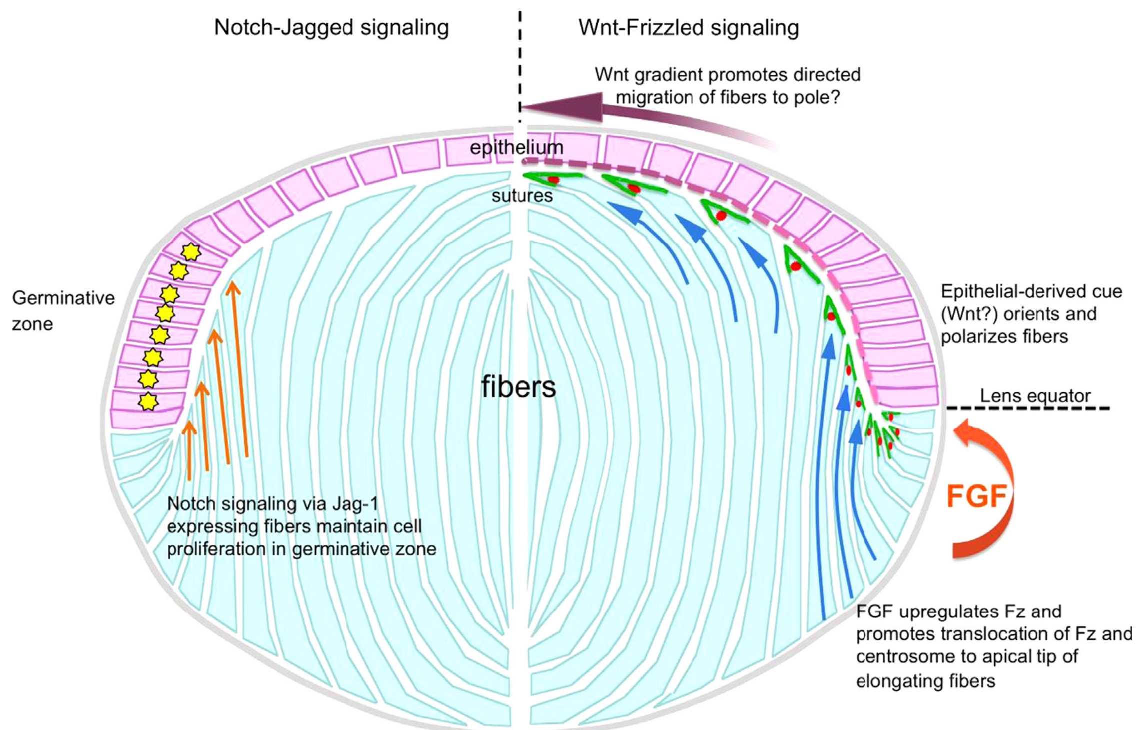

Fig. 10. Proposed model of interactions between fibers and epithelium. Right hand side of the diagram (Wnt-Frizzled signaling) shows that when epithelial cells shift below the lens equator they initiate fiber differentiation in response to FGF in the posterior segment. This includes upregulation of Wnt-Fz signaling components and pathway activation that plays a key role in fiber elongation and differentiation (Dawes et al., 2013). This response also involves translocation of Fz (green) and centrosome (red spot) to the apical tip of elongating fibers and we propose this acts as the key organizer center for cytoskeletal assembly and dynamics. Early in the elongation process the fibers are concave (long blue arrows) as they orient towards the overlying epithelium in response to a polarizing cue, possibly a Wnt (purple). As elongation progresses the fibers take on convex curvature (short blue arrows) as their apical tips migrate towards the pole, possibly in response to a Wnt concentration gradient. Left hand side (Notch-Jagged signaling) shows that where the Jag-1 expressing cortical fibers (orange arrows) abut the epithelium this determines the germinative zone (yellow star).

islands and that this is diminished in the presence of DAPT. The suppression of FGF-promoted proliferation and Jag-1 expression due to loss of Notch signaling as detailed in the current study is consistent with previous reports (Jia et al., 2007; Saravanamuthu et al., 2009). However, it is unclear if loss of Notch signaling impacts upon FGF signaling. Interestingly, FGFR and Notch pathways have been reported to play reciprocal roles in regulating cell growth (Ikeya and Hayashi, 1999; Small et al., 2003) suggesting potential feedback mechanisms between pathways. In particular, Notch signaling has been implicated in refining FGF signaling via regulation of MAPK activation in the Drosophila trachea (Ikeya and Hayashi, 1999). Therefore, a role for Notch signaling in regulating FGFR signaling pathways that promote lens cell proliferation and differentiation remains an intriguing possibility.

Through utilizing an in vitro lens explant culture system we can recapitulate key elements of the previously reported fiber to epithelial interaction that is mediated by Jag-1/Notch signaling and is clearly important for development and continued viability of the lens. In addition, and for the first time, we have identified a reciprocal interaction wherein the epithelium promotes polarized behavior of the elongating fibers and ensures their correct alignment/orientation towards the epithelium. Experiments with Wnt producing cells indicate that this polarizing influence, may be due, at least in part, to epithelial-derived Wnt5. Thus it appears that interactions between the two main forms of lens cells, play important roles, not only for maintaining a proliferating progenitor population of cells, but also ensuring that elongating fiber cells assemble into their characteristically polarized alignment up against the epithelium and possibly their directed migration towards the pole to form sutures (summarized in Fig. 10). Such mutually dependent processes are clearly important for the development and maintenance of lens three-dimensional cellular architecture.

Although the present work investigates Wnt and Notch signaling pathways in isolation it is important to consider their potential interaction in relation to regulation of lens cell self-assembly. Recent studies have implicated Wnt and Notch signaling crosstalk in regulating various cellular processes (Ann et al., 2012; Hayward et al., 2008; Hing et al., 1994). In the current context, it is interesting that a role for Notch in promoting Wnt5A expression has been suggested (Katoh and Katoh, 2009; Koyanagi et al., 2007). Specifically, Wnt5A expression in human endothelial progenitor cells was promoted by Notch via immoblilzed Jag-1 and was blocked by gamma secretase inhibition (Koyanagi et al., 2007). Wnt5A has also been suggested to regulate Notch signaling by promoting Hes-1 expression (Duncan et al., 2005) and Wnt-Fz/PCP regulation of Notch has been observed in photoreceptor fate choice in the *Drosophila* eye (Cooper and Bray, 1999). In the current study, we can only speculate if Notch signaling directly promotes Wnt5A secretion from lens cells as blockade of Notch prevents formation of islands of proliferative cells that are the likely source of secreted Wnt5A. Given these and other limitations of our explant system, further studies using other models will be required to tease out further details of the interplay between these signaling pathways.

Thus, if we provide appropriate stimuli we can generate conditions that promote mutually dependant interactions between the two forms of lens cells and their subsequent self-assembly into structural and functional arrangements like those in vivo. This is in line with recent in vitro studies that show embryonic stem cell-derived retinal epithelial layers, when given appropriate conditions, can interact and recapitulate intrinsic self-

organizing programs to differentiate into optic cup replete with appropriately organized neural and pigmented layers (Eiraku et al., 2011). Indeed, in previous studies with paired rat lens epithelial explants we have shown that, given appropriate stimuli and conditions, these epithelial layers can differentiate and self-assemble into polarized lentoid structures with a monolayer of epithelial cells overlying aligned fibers. These lentoids were also shown to be transparent and capable of focussing light (O'Connor and McAvoy, 2007). The current study provides insights into mechanisms that underlie this phenomenon of lens cell self-assembly and serves to emphasize the importance of understanding the interactions between the different cell types that normally occur within a tissue/organ. This is fundamental to defining the specific conditions and stimuli needed to promote the inherent capacity of particular cell types to recapitulate developmental programs in vitro and self-assemble into three-dimensional structures with functional capabilities.

Acknowledgments

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ydbio.2013.10.030.

References

Abello, G., Khatri, S., Giraldez, F., Alsina, B., 2007. Early regionalization of the optic placode and its regulation by the Notch signaling pathway. Mech. Dev. 124, 631–634.

Ang, S.J., Stump, R.J., Lovicu, F.J., McAvoy, J.W., 2004. Spatial and temporal expression of Wnt and Dickkopf genes during murine lens development. Gene Expr. Patterns 4, 289–295.

Ann, E.J., Kim, H.Y., Seo, M.S., Mo, J.S., Kim, M.Y., Yoon, J.H., Park, H.S., 2012. Wnt5a controls Notch1 signaling through CaMKII mediated degradation of the SMRT corepressor protein. J. Biol. Chem. 287, 36814–36829.

Blakely, B.D., Bye, C.R., Fernando, C.V., Horne, M.K., Macheda, M.L., Stacker, S.A., Arenas, E., Parish, C.L., 2011. Wnt5a regulates midbrain dopaminergic axon growth and guidance. PLoS One 6, e18373.

Chen, B., Dodge, M.E., Tang, W., Lu, J., Ma, Z., Fan, C.W., Wei, S., Hao, W., Kilgore, J., Williams, N.S., et al., 2009. Small molecule-mediated disruption of Wnt-dependent signaling in tissue regeneration and cancer. Nat. Chem. Biol. 5, 100–107.

Chen, Y., Stump, R.J., Lovicu, F.J., Shimono, A., McAvoy, J.W., 2008. Wnt signaling is required for organization of the lens fiber cell cytoskeleton and development of lens three-dimensional architecture. Dev. Biol. 324, 161–176.

Cooper, M.T.D., Bray, S.J., 1999. Frizzled regulation of Notch signalling polarizes cell fate in the *Drosophila* eye. Nature 397, 526–530.

Da Forno, P.D., Pringle, H., Hutchinson, P., Osborn, J., Huang, Q., Potter, L., Hancox, R. A., Feltcher, A., Saldanha, G.S., 2008. WNT5A expression increases during melanoma progression and correlates with outcome. Clin. Cancer Res. 14, 5825–5832.

Dawes, L.J., Sugiyama, Y., Tanedo, A.S., Lovicu, F.J., McAvoy, J.W., 2013. Wnt-frizzled signaling is part of an FGF-induced cascade that promotes lens fiber differentiation. Invest. Ophthalmol. Vis. Sci. 54, 1582–1590.

Duncan, A.W., Rattis, F.M., DiMascio, L.N., Congdon, K.L., Pazianos, G., Zhao, C., Yoon, K., Cook, J.M., Willert, K., Gaiano, N., Reya, T., 2005. Integration of Notch and Wnt signaling in hematopoietic stem cell maintenance. Nat. Immunol. 6, 314–322.

Eiraku, M., Takata, N., Ishibashi, H., Kawada, M., Sakakura, E., Okuda, S., Sekiguchi, K., Adachi, T., Sasai, Y., 2011. Self-organizing optic-cup morphogenesis in three-dimensional culture. Nature 472, 51–56.

Garcia, C.M., Yu, K., Zhao, H., Ashery-Padan, R., Ornitz, D.M., Robinson, M.L., Beebe, D.C., 2005. Signaling through FGF receptor-2 is required for lens cell survival and for withdrawal from the cell cycle during lens fiber cell differentiation. Dev. Dyn. 233, 516–527.

Hayward, P., Kalmar, T., Arias, A.M., 2008. Wnt/Notch signalling and information processing during development. Development 135, 411–424.

Hing, H.K., Sun, X., Artavanis-Tsakonas, S., 1994. Modulation of wingless signaling by Notch in *Drosophila*. Mech. Dev. 47, 261–268.

Ikeya, T., Hayashi, S., 1999. Interplay of Notch and FGF signaling restricts cell fate and MAPK activation in the *Drosophila* trachea. Development 126, 4455–4463.

Jia, J., Lin, M., Zhang, L., York, J.P., Zhang, P., 2007. The Notch signaling pathway controls the size of the ocular lens by directly suppressing p57Kip2 expression. Mol. Cell Biol. 27, 7236–7247.

Katoh, M., Katoh, M., 2009. Transcriptional mechanisms of WNT5A based on NF-kappaB, Hedgehog, TGFbeta, and Notch signaling cascades. Int. J. Mol. Med. 23, 763–769.

Koyanagi, M., Bushoven, P., Iwasaki, M., Urbich, C., Zeiher, A.M., Dimmeler, S., 2007. Notch signaling contributes to the expression of cardiac markers in human circulating progenitor cells. Circ. Res. 101, 1139–1145.

Le, T.T., Conley, K.W., Brown, N.L., 2009. Jagged 1 is necessary for normal mouse lens formation. Dev. Biol. 328, 118–126.

Lovicu, F.J., McAvoy, J.W., 2005. Growth factor regulation of lens development. Dev. Biol. 280, 1–14.

Lovicu, F.J., McAvoy, J.W., 2008. Epithelial Explants and their Application to Study Developmental Processes in the Lens. In: Tsonis, P.A. (Ed.), Animal Models in Eye Research. Academic Press, Burlington, pp. 134–136.

Lyu, J., Joo, C.K., 2004. Wnt signaling enhances FGF2-triggered lens fiber cell differentiation. Development 131, 1813–1824.

Maddala, R., Skiba, N.P., Lalane III, R., Sherman, D.L., Brophy, P.J., Rao, P.V., 2011. Periaxin is required for hexagonal geometry and membrane organization of mature lens fibers. Dev. Biol. 357, 179–190.

McAvoy, J.W., 1978a. Cell division, cell elongation and distribution of α-, β- and γ-crystallins in the rat lens. J. Embryol. Exp. Morphol. 44, 149–165.

McAvoy, J.W., 1978b. Cell division, cell elongation and the co-ordination of crystallin gene expression during lens morphogenesis in the rat. J. Embryol. Exp. Morphol. 45, 271–281.

Micchelli, C.A., Esler, W.P., Kimberly, W.T., Jack, C., Berezovska, O., Kornilova, A., Hyman, B.T., Perrimon, N., Wolfe, M.S., 2003. Gamma-secretase/presenilin inhibitors for Alzheimer's disease phenocopy Notch mutations in *Drosophila*. FASEB J. 17, 79–81.

Nielsen, P.A., Baruch, A., Shestopalov, V.I., Giepmans, B.N., Dunia, I., Benedetti, E.L., Kumar, N.M., 2003. Lens connexins alpha3C $\times$ 46 and alpha8C $\times$ 50 interact with zonula occludens protein-1 (ZO-1). Mol. Biol. Cell 14, 2470–2481.

O'Connor, M.D., McAvoy, J.W., 2007. In vitro generation of functional lens-like structures with relevance to age-related nuclear cataract. Invest. Ophthalmol. Vis. Sci. 48, 1245–1252.

Peters, S., Mix, E., Bauer, P., Weinelt, S., Schubert, B., Knoblich, R., Bottcher, T., Strauss, U., Pahnke, J., Cattaneo., E., et al., 2004. Wnt-5a expression in the rat neuronal progenitor cell line ST14A. Exp. Brain Res. 158, 189–195.

Qu, X., Hertzler, K., Pan, Y., Grobe, K., Robinson, M.L., Zhang, X., 2011. Genetic epistasis between heparan sulfate and FGF-Ras signaling controls lens development. Dev. Biol. 355, 12–20.

Rajagopal, R., Dattilo, L.K., Kaartinen, V., Deng, C.X., Umans, L., Zwijsen, A., Roberts, A.B., Bottinger, E.P., Beebe, D.C., 2008. Functions of the type 1 BMP receptor Acvr1 (Alk2) in lens development: cell proliferation, terminal differentiation, and survival. Invest. Ophthalmol. Vis. Sci. 49, 4953–4960.

Robinson, M.L., 2006. An essential role for FGF receptor signaling in lens development. Semin Cell Dev. Biol. 17, 726–740.

Rowan, S., Conley, K.W., Le, T.T., Donner, A.L., Maas, R.L., Brown, N.L., 2008. Notch signaling regulates growth and differentiation in the mammalian lens. Dev. Biol. 321, 111–122.

Saravanamuthu, S.S., Gao, C.Y., Zelenka, P.S., 2009. Notch signaling is required for lateral induction of Jagged1 during FGF-induced lens fiber differentiation. Dev. Biol. 332, 166–176.

Saravanamuthu, S.S., Le, T.T., Gao, C.Y., Cojocaru, R.I., Pandiyan, P., Liu, C., Zhang, J., Zelenka, P.S., Brown, N.L., 2012. Conditional ablation of the Notch2 receptor in the ocular lens. Dev. Biol. 362, 219–229.

Small, D., Kovalenko, D., Soldi, R., Mandinova, A., Kolev, V., Trifonova, R., Bagala, C., Kacer, D., Battelli, C., Liaw, L., Prudovsky, I., Maciag, T., 2003. Notch activation suppresses fibroblast growth factor-dependent cellular transformation. J. Biol. Chem. 278, 16405–16413.

Snyder, E.Y., Deitcher, D.L., Walsh, C., Arnold-Aldea, S., Hartwieg, E.A., Cepko, C.L., 1992. Multipotent neural cell lines can engraft and participate in development of mouse cerebellum. Cell 68, 33–51.

Stump, R.J., Ang, S., Chen, Y., von Bahr, T., Lovicu, F.J., Pinson, K., de Iongh, R.U., Yamaguchi, T.P., Sassoon, D.A., McAvoy, J.W., 2003. A role for Wnt/beta-catenin signaling in lens epithelial differentiation. Dev. Biol. 259, 48–61.

Sugiyama, Y., Stump, R.J., Nguyen, A., Wen, L., Chen, Y., Wang, Y., Murdoch, J.N., Lovicu, F.J., McAvoy, J.W., 2010. Secreted frizzled-related protein disrupts PCP in eye lens fiber cells that have polarised primary cilia. Dev. Biol. 338, 193–201.

Sugiyama, Y., Lovicu, F.J., McAvoy, J.W., 2011. Planar cell polarity in the mammalian eye lens. Organogenesis 7, 191–201.

Zhao, H., Yang., T., Madakashira, B.P., Thiels, C.A., Bechtle, C.A., Garcia, C.M., Zhang, H., Yu, K., Ornitz, D.M., Beebe, D.C., Robinson, M.L., 2008. Fibroblast growth factor receptor signaling is essential for lens fiber cell differentiation. Dev. Biol. 318, 276–288.