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Evolution of Developmental Control Mechanisms

Wnt signaling is required for organization of the lens fiber cell cytoskeleton and development of lens three-dimensional architecture

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ABSTRACT

How an organ develops its characteristic shape is a major issue. This is particularly critical for the eye lens as its function depends on having appropriately ordered three-dimensional cellular architecture. Recent in vitro studies indicate that Wnt signaling plays key roles in regulating morphological events in FGF-induced fiber cell differentiation in the mammalian lens. To further investigate this the Wnt signaling antagonist, secreted frizzled-related protein 2 (Sfrp2), was overexpressed in lens fiber cells of transgenic mice. In these mice fiber cell elongation was attenuated and individual fibers exhibited irregular shapes and consequently did not align or pack regularly; microtubules, microfilaments and intermediate filaments were clearly disordered in these fibers. Furthermore, a striking feature of transgenic lenses was that fibers did not develop the convex curvature typically seen in normal lenses. This appears to be related to a lack of protrusive processes that are required for directed migratory activity at their apical and basal tips as well as for the formation of interlocking processes along their lateral margins. Components of the Wnt/Planar Cell Polarity (PCP) pathway were downregulated or inhibited. Taken together this supports a role for Wnt/PCP signaling in orchestrating the complex organization and dynamics of the fiber cell cytoskeleton.

Introduction

How the eye lens develops its characteristic shape and three-dimensional cellular architecture is a major developmental question. Lens tissue differentiates from ectoderm situated next to the optic vesicle. By thickening and invaginating, the ectoderm forms the lens vesicle. Subsequently, posterior vesicle cells elongate to form the primary fibers, whereas anterior vesicle cells differentiate into epithelial cells. The divergent fates of these embryonic cells give the lens its distinctive polarity. Once formed, the lens grows rapidly by cell division and differentiation. Cell proliferation occurs in the epithelial region just above the lens equator known as the germinative zone (McAvoy, 1978). Progeny of divisions migrate below the equator where they elongate and differentiate into secondary fiber cells. In this way, the lens grows throughout life and maintains its polarity.

Growth factors are key regulators of cell fates and behaviors and much attention has focused on identifying the factors that govern proliferation, migration and differentiation of lens cells. Initial studies with a mammalian lens epithelial explant system identified members of the FGF growth factor family as key regulators of lens fiber differentiation. Moreover, detailed analyses showed that FGFs could induce dose-dependent responses. Specifically, a low dose of FGF induced lens cell proliferation, whereas sequentially higher doses were required to induce epithelial cell migration and fiber cell differentiation (McAvoy and Chamberlain, 1989). This finding, together with the fact that FGF bioavailability differs throughout the eye (e.g. more FGF can be recovered from the vitreous than aqueous; Schulz et al., 1993), led to the hypothesis that the distinct polarity of the lens in the eye is determined by a gradient of FGF signaling. Subsequent genetic manipulations with the murine lens, where FGFs were overexpressed or FGF receptors were knocked out, have now provided compelling evidence for this hypothesis (Lovicu and McAvoy, 2005; Robinson, 2006; Zhao et al., 2008). However, it has become increasingly clear from both in vitro and in vivo studies that mammalian fiber differentiation also depends on a FGF-initiated cascade of growth factor signaling involving other growth factor-dependent pathways (de Iongh et al., 2001; Faber et al., 2002; Belecky-Adams et al., 2002; Lovicu and McAvoy, 2005; Boswell et al., 2008). Most recently, there is evidence that members of the Wnt family play key roles in regulating events in fiber differentiation in the mammalian lens (Lyu and Joo, 2004; Chen et al., 2006).

Wnt signaling is initiated when a Wnt ligand complexes with a frizzled (Fz) receptor and a LDL-related protein (Lrp) co-receptor. Once this complex is formed, dishevelled (Dvl) is activated and axin is

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recruited to the cell membrane. In the absence of Wnt, axin is part of a module containing glycogen synthase kinase-3β (GSK3β) that phosphorylates β-catenin and targets it for destruction (Cong et al., 2004; Logan and Nusse, 2004). Thus ligand/receptor interaction leads to an increase in stabilized (unphosphorylated) β-catenin. In this form, β-catenin can then associate with the DNA binding transcription factors TCF or Lef, and in the nucleus regulate expression of target genes. Other, non-canonical Wnt signaling pathways have also been identified, and these include the Wnt/Ca^{+} and the Wnt/planar cell polarity (PCP) pathways (Karner et al., 2006; Jones and Chen, 2007). Although not as well understood as canonical Wnt signaling, there is a growing awareness that non-canonical signaling, particularly through the Wnt/PCP pathway, has a critical role in regulating many key developmental processes that involve intricate remodeling of the cell cytoskeleton. For example, the elongation and narrowing of tissues, or convergent extension movements, that are fundamental to critical processes such as gastrulation and neurulation involve highly coordinated cellular shape changes and rearrangements that are driven by Wnt/PCP signaling (Green and Davidson, 2007). Wnt signaling through the PCP pathway involves activation of Dvl, although through a different domain(s) than for canonical signaling, and generally involves activation of the small Rho GTPases and results in the activation of JNK (Logan and Nusse, 2004; Wallingford and Habas, 2005; Karner et al., 2006; Jones and Chen, 2007).

Wnts, their Fz receptors and Lrp5/6 co-receptors are expressed in the mammalian lens during development (Stump et al., 2003; Liu et al., 2003; Chen et al., 2004 Ang et al., 2004). In previous studies we showed that mouse embryos homozygous for a mutation in the *lrp6* gene that have impaired Wnt/β-catenin signaling did not form a normal lens (Stump et al., 2003). This defect was evident at E13.5 when cells of the anterior region of the lens vesicle did not normally differentiate into a polarized sheet of adherent epithelial cells, but instead accumulated the fiber-specific marker, β-crystallin, and extruded into the corneal stroma. Results from TCF/Lef-LacZ reporter mice also show a period of Wnt/β-catenin reporter activity in the lens epithelium at this time (between E11.5-14.5; Liu et al., 2003, 2006, 2007). Taken together this indicates a requirement for Wnt/β-catenin signaling between E11.5–14.5 for the lens epithelium to differentiate from the anterior cells of the lens vesicle.

For lens fibers, explant studies indicate a role for Wnt signaling in mediating FGF-triggered fiber differentiation (Lyu and Joo, 2004). Fiber differentiation in vivo is unlikely to involve canonical Wnt signaling as no TCF/Lef-LacZ reporter activity is detected in any part of the lens beyond E14.5 (Liu et al., 2003, 2006, 2007). However, given that Wnt/PCP core signaling components Prickle and Van Gogh-like (Bekman and Henrique, 2002; Tissir and Goffinet, 2006) as well as Fzs and Dvls (Stump et al., 2003; Liu et al., 2003; Chen et al., 2004; Ang et al., 2004; Lyu and Joo, 2004) are prominent in elongating mammalian fiber cells, this effect may be mediated through the PCP pathway. In particular, Wnt/PCP signaling may have a role in mediating the effects of FGF in promoting the complex cytoskeletal reorganization required for the transition of epithelial cells into elongated, highly ordered fiber cells (Chen et al., 2006). A role for Wnt/PCP signaling in this process is consistent with studies that show impairment of the fiber cell cytoskeleton is a consequence of inhibiting the small Rho GTPases (Maddala et al., 2001, 2003, 2004; Rao et al., 2002). However, whilst these studies have identified roles for these GTPases in fiber cell elongation, it is not clear in this context if their activity is related to Wnt/PCP signaling.

The aim of the current study was to assess the role of the Wnt/PCP pathway in lens fiber differentiation by blocking Wnt signaling in the lens. To achieve this we overexpressed (using a lens crystallin promoter) the Wnt signaling antagonist, secreted frizzled-related protein 2 (Sfrp2), in the lenses of transgenic mice. Sfrps antagonize Wnt cascades by binding to Wnts or Fz receptors (Kawano and Kypta, 2003), and have the potential to modulate Wnt signaling activity in cells through all Wnt signaling pathways (Jones and Jomary, 2002). Although all five *Sfrp* genes are expressed during lens development (Chen et al., 2004), *Sfrp2* was selected for this transgenic study because, unlike the other *Sfrp* genes, it has a distinctive expression pattern early in lens morphogenesis that coincides with regions where inactivation of Wnt signaling is required for normal lens development (Smith et al., 2005). Evidence that Sfrp2 can inhibit Wnt signaling also comes from studies in various other systems (Lescher et al., 1998; Ladher et al., 2000; Deb et al., 2007) and recently a plethora of studies have linked Sfrp2 inhibition of Wnt signaling with an important tumor suppressor function (Nojima et al., 2007).

Results from the current study have shown that overexpression of Sfrp2 in fiber cells of transgenic mice led to the development of severe cataracts. Lens fiber elongation was attenuated and at their anterior and posterior tips they did not migrate towards the lens poles to form sutures. As a result fibers did not develop the characteristic convex curvature that is central to the development of normal three-dimensional lens architecture. Microtubules, microfilaments and intermediate filaments were disorganized in the incipient fiber cells of these transgenic mice. In addition, components of the PCP pathway, including Dvl, Cdc42, Rac1, RhoA and JNK were disturbed and, in the case of Dvl and the small GTPases, were downregulated/inhibited. Taken together this supports a role for Wnt/PCP signaling in regulating the complex organization of the cytoskeleton that is required for normal lens fiber differentiation and maturation.

Materials and methods

Animals

The use of animals in this study conformed to the Association for Research in Vision and Ophthalmology (ARVO) Statement for the use of animals in Ophthalmic and Vision Research. Mutant mice deficient for Sfrp2 were generated as previously described (Satoh et al., 2006).

Construction of the plasmid with the Sfrp2 full-length gene

Full length *Sfrp2* cDNA (U88567 in GenBank) was sub-cloned into the δ1αA-crystallin promoter plasmid (provided by Dr. Reneker, University of Missouri, USA) that consisted of a modified mouse αA-crystallin promoter, the chick δ1-crystallin enhancer element and the human growth hormone polyadenylation (polyA) region (Fig. 1H; Reneker et al., 2004).

Generation of transgenic mice and genotyping

The Sfrp2 transgene was injected into pronuclei of one-cell-stage BCBF1 (C57/BL6xCBA) mouse embryos by the Transgenic Animal Service of Queensland (Queensland, Australia). Transgenic mice were genotyped by PCR using primers specific for the *Sfrp2* transgene (forward: 5′tcgctagtccacgatgcc3′; reverse: 5′actgggaaactagcattgca3′) or the δ1-crystallin enhancer (forward: 5′gctgacgcaaaaatccctaatg3′; reverse: 5′caggaaataccaacttctgccatc3′). Total RNA was extracted from 5-day-old postnatal lenses (P5) of Sfrp2 overexpressing transgenic mice or WT littermates as described previously (Chen et al., 2004). Briefly, 2 μg of RNA treated with DNAase I was reverse transcribed, with or without reverse transcriptase, for PCR amplification using specific primer pairs to amplify Sfrp2. To genotype littermates from heterozygotic Sfrp2 matings, PCR amplification using genomic DNA from crude tail digests was performed using DirectPCR (Viagen Biotech CA, USA) as described above.

Tissue collection and histology

Embryos at embryonic day 12.5 (E12.5), E14.5, E16.5 or eyeballs at P1 and P21 of Sfrp2 transgenic mice and WT littermates were fixed in 10% neutral buffered formalin (NBF) overnight, or with HistoChoice