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Expression of Frizzleds and secreted frizzled-related proteins (Sfrps) during mammalian lens development

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ABSTRACT Recent studies indicate a role for Wnt signaling in regulating lens cell differentiation (Stump *et al.,* 2003). Here we investigated expression patterns of Wnt receptors, the Frizzleds (Fzs) and the Wnt signaling regulators, the secreted frizzled-related proteins (Sfrps), during rodent lens development. RT-PCR showed that Fz receptors, *Fz1-Fz8* are expressed in lens. *In situ* hybridization showed that all the *Fz* genes examined have similar expression patterns. *Fzs* are expressed throughout the early lens primordium. At embryonic day 14.5 (E14.5), *Fz* gene expression is predominantly localized to the epithelium and elongating cells at the lens equator. *Fz* expression is absent from lens fibers. This pattern of *Fz* gene expression continues throughout early postnatal development. Immunolocalization studies showed that Fz protein distribution closely follows that of the mRNAs. In addition, epithelial cells in FGF-treated explants show strongest Fz reactivity in cellular protrusions as they migrate and elongate. *Sfrp1- Sfrp5* are expressed and all, except *Sfrp2,* have similar patterns of expression to each other and to the *Fzs* during lens development. *Sfrp2* is strongly expressed in all lens pit cells but becomes restricted to the presumptive epithelial cells of the lens vesicle. By E14.5, *Sfrp2* is only present in a few cells above the lens equator. *Sfrp2* is not detected in the lens at E18.5 or at later stages. This study shows that multiple *Fz* and *Sfrp* genes are expressed during lens morphogenesis and differentiation. This is consistent with a role for Wnt-Fz signaling during both embryonic and postnatal lens development.

KEY WORDS: *frizzled, secreted frizzled-related protein, lens development, growth factor, Wnt*

Introduction

How the lens develops and acquires its distinctive morphology and growth patterns has been a major question in developmental biology. The lens originates from head ectoderm that overlies the optic vesicle. Shortly after these two tissues become associated, presumptive lens ectoderm grows and thickens to form the lens placode. By subsequent invagination, the placode forms the lens pit which later closes to form the lens vesicle. Cells in the posterior half of the lens vesicle, next to the optic cup, elongate to form the primary fibers whereas cells in the anterior half of the vesicle differentiate into epithelial cells (McAvoy *et al.,* 1999). These divergent fates of embryonic cells give the lens its distinctive anterior-posterior polarity. From this stage onwards the lens grows through the proliferation of epithelial cells and the differentiation of their progeny into secondary fiber cells. Only the cells that shift below the lens equator differentiate into fibers, hence lens polarity is maintained throughout life. These growth patterns are central to the maintenance of the ordered cellular architecture that contributes to the transparency and optical properties of the lens (Trokel, 1962).

Growth factors are known to play key roles in influencing cell fates during development. Some of the major growth factor families, including FGFs (McAvoy *et al.,* 1999; Faber *et al.,* 2001; Govindarajan and Overbeek, 2001) and TGFβ/BMPs (Furuta and Hogan, 1998; de Iongh *et al.,* 2001; Belecky-Adams *et al.,* 2002; Faber *et al.,* 2002) have been shown to be involved in regulating lens developmental processes, primarily fiber differentiation (Lang and McAvoy, 2004). In addition, recent studies in our laboratory have also indicated an important role for Wnt signaling in lens morphogenesis and differentiation (Stump *et al.,* 2003).

Wnts signal through Frizzled (Fz) receptors and can activate a number of pathways (Bhanot *et al.,* 1996; Pandur *et al.,* 2002).

Abbreviations used in this paper: bmc, basement membrane complex; BMP, bone morphogenic protein; E, embryonic day; FGF, fibroblast growth factor; Fzs, Frizzleds; LRP, LDL-related protein; P, postnatal day; Sfrps, secreted frizzled-related proteins; TGFβ, transforming growth factor-β.

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The presence of an LDL-related protein (LRP) co-receptor is required for signaling through the Wnt/β–catenin pathway (Pinson *et al.,* 2000). Our recent analysis of a mouse with a mutation in the Wnt co-receptor, LRP6, showed dysmorphogenesis of the lens, particularly in the epithelial layer, indicating a role for Wnt/β–catenin signaling in lens epithelial differentiation (Stump *et al.,* 2003). *Wnt* gene expression in the lens has been described in mice (Liu *et al.,* 2003; Stump *et al*., 2003) and expression profiles that include both early embryonic and postnatal developmental stages, have recently been reported (Ang *et al.,* 2004). Expression patterns of Fz receptors and key regulators of Wnt-Fz signaling, the secreted frizzled-related proteins (Sfrps), have also been described at several stages of embryonic development (Leimeister *et al.,* 1998; Wawersik *et al.,* 1999; Liu *et al.,* 2003). However, a full analysis of expression that includes early embryonic and postnatal developmental stages has not yet been conducted. Detailed knowledge of the expression patterns of Fzs, and the closely related Sfrps, will be required to identify the domains of Wnt-Fz signaling activity in the lens during its development. The current study describes patterns of Fz and Sfrp expression during embryonic and postnatal development of the murine eye. Expression of *Fz* genes, *Fz1-Fz8,* and all 5 *Sfrp* genes, *Sfrp1-Sfrp5,* were studied by RT-PCR and *in situ* hybridization. An immunohistochemical analysis of Fz receptor distribution was also conducted over similar developmental stages. In addition, changes in the subcellular distribution of Fz receptors were studied during FGF-induced fiber differentiation in rat lens epithelial explants.

Results

Frizzled expression in the lens

To investigate Fz expression in the lens RT-PCR was carried out on RNA extracted from P20-P25 rat lens epithelial cells. All reverse transcriptions were carried out using equivalent amounts of RNA, and equivalent volumes of reverse transcription reactions were used for PCR amplifications. RT-PCR using lens-derived RNA and primers for *Fz5, Fz7* and *Fz8* (note that *Fz1, Fz2, Fz3, Fz4* and *Fz6* have already been shown to be expressed in the lens by RT-PCR; Stump *et al.,* 2003) resulted in distinct amplification of products of expected sizes (Table 1). The specific PCR fragments for these *Fz* genes were cloned and sequenced to confirm sequence identity with those reported in GenBank.

To investigate the spatial expression patterns of *Fz* mRNAs in the murine lens, cDNAs were used as templates to prepare digoxigenin-labeled riboprobes for *in situ* hybridization analyses. As this study progressed it became clear that all the *Fzs* studied, (*Fz1* to *Fz8* inclusive) have similar, and generally indistinguishable, patterns of expression during ocular development. The results are summarized in Table 2 and for brevity only the expression pattern of one of these, *Fz6*, is presented (Fig. 1).

At embryonic day 9.5 (E9.5), *Fz6* is expressed weakly throughout the head region of the embryo, mostly in ectodermal and mesoderm cells (Fig. 1A). Expression is similar at E10.5; however, at this stage there is slightly stronger expression of *Fz6* in lens and retinal primordia (Fig. 1 B,B1). At E12.5 *Fz6* is expressed widely throughout the developing eye, including the lens, cornea, optic cup and hyaloid vasculature (Fig. 1 C,C1). In the lens, both the elongating primary fibers and the presumptive epithelial cells express *Fz6* transcripts. At E14.5 *Fz6* expression is absent from the most mature primary fibers in the center of the lens. The less mature elongating fibers at the lens equator and the epithelial cells show strong expression of *Fz6* transcripts (Fig. 1 D,D1). In the cornea, the epithelium and endothelium exhibit stronger expression than the stroma. In the optic cup the strongest expression is in the margin in the cells destined to form the ciliary and

TABLE 1

PRIMERS USED FOR RT-PCR

Gene	Accession No	Primer Sequences (5' to 3') Forward	*Reverse*	PCR product size	nucleotides
Fz5	AB052910	gacgccgaggttctgtgtat	tgcgcaccttgttgtagagt	**212**	**420-631**
Fz7	MMU43320	gccagacccacctttcact	cgaaccgtctctcctcttctt	**198**	**555-752**
Fz8	MMU43321	agtaccggcctgacgtgg	agacctgggacaatggca	**77**	**2168-2243**
Sfrp4	AF117709	gagtggcgttcaaggatga	gacttttgggggcacttct	**255**	**1051-1304**
Sfrp5	AF117759	gaccgaaagttgattggagc	gcagcagctgtccctctact	**184**	**881-1064**

TABLE 2

EXPRESSION PATTERNS OF *FZS* AND *SFRPS* DURING EMBRYONIC AND POSTNATAL LENS DEVELOPMENT

Developmental Stage	Fzs/Sfrps	Levels of expression	
		lens primordia	
E 9.5	Fzs	+	
	Sfrps 1 & 5	+	
	Sfrp2	+/-	
	Sfrp3	-	
	Sfrp4	+	
E 10.5	Fzs	++	
	Sfrps 1 & 5	++	
	Sfrp2	+++	
	Sfrp3	-	
	Sfrp4	+	
		lens epithelium	**lens fibers**
E 12.5	Fzs	+++	+++
	Sfrps 1 & 5	+++	+++
	Sfrp2	+++	+/-
	Sfrp3	-	-
	Sfrp4	+++	+++
E 14.5	Fzs	+++	-
	Sfrps 1 & 5	+++	-
	Sfrp2	+++	-
	Sfrp3	++	-
	Sfrp4	+++	-
E 18.5	Fzs	+++	-
	Sfrps 1 & 5	+++	-
	Sfrp2	-	-
	Sfrp3	++	-
	Sfrp4	+++	-
P2	Fzs	+++	-
	Sfrps 1 & 5	+++	-
	Sfrp2	-	-
	Sfrp3	++	-
	Sfrp4	-	-
P8	Fzs	+++	-
	Sfrps 1 & 5	+++	-
	Sfrp2	-	-
	Sfrp3	++	-
	Sfrp4	-	-
P21	Fzs	+++	-
	Sfrps 1 & 5	+++	-
	Sfrp2	-	-
	Sfrp3	++	-
	Sfrp4	-	-

Key: Fzs refers to Fz1-Fz8 inclusive

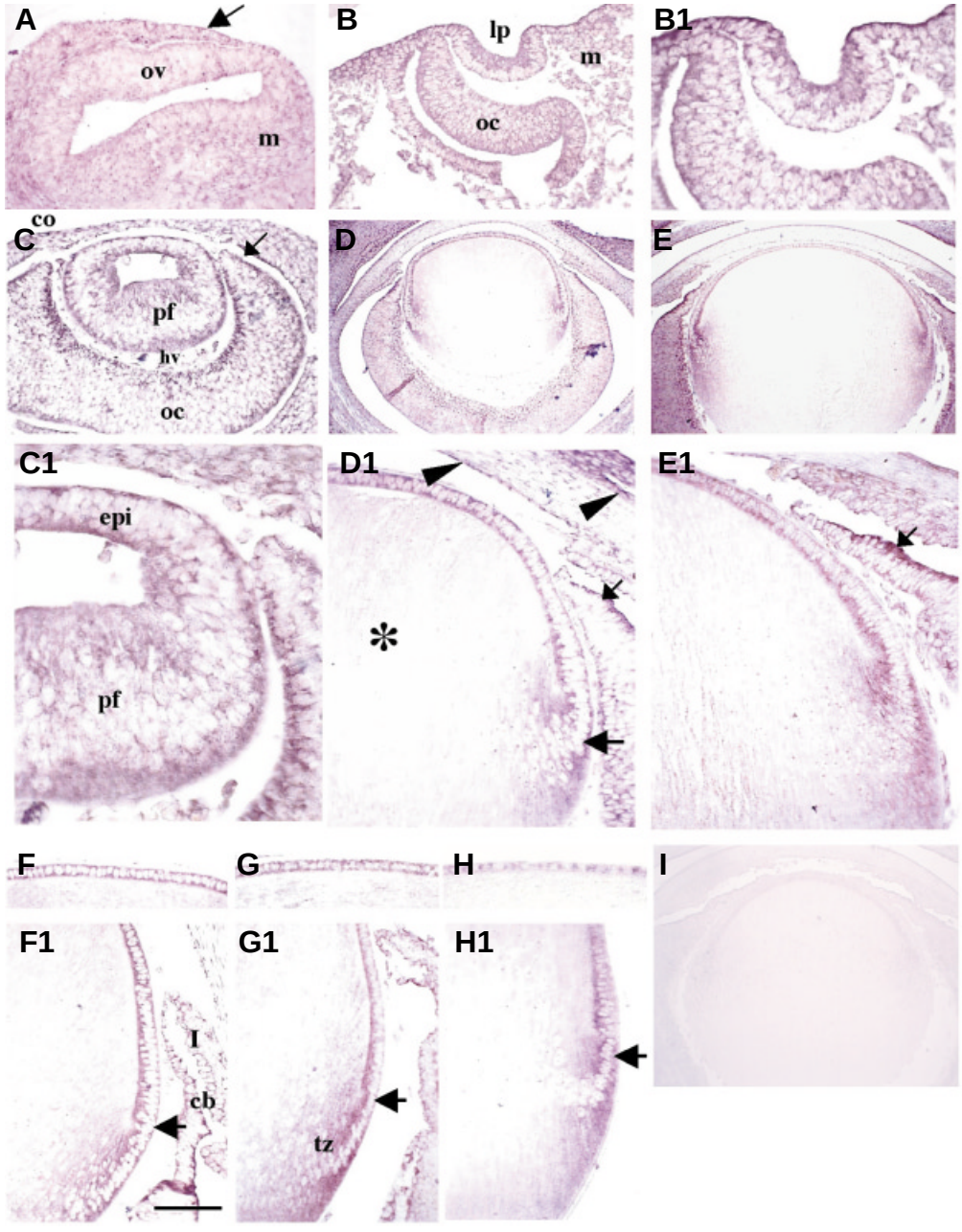

Fig. 1. *In situ* hybridization: expression patterns of *Fz6* during murine lens development. *Sections of embryonic stages E9.5 (A), E10.5 (B, B1), E12.5 (C, C1), E14.5 (D, D1), E18.5 (E, E1) and postnatal stages P1 (F, F1), P3 (G, G1) and P21 (H, H1) mouse eyes labeled for* Fz6 *mRNA expression and viewed by bright field microscopy. A control (sense probe) is included for E18.5 (I). At E9.5,* Fz6 *is expressed weakly throughout the lens placode region* **(A)**, *(arrow) and the underlying mesoderm. At E10.5,* Fz6 *expression is detected in the lens pit and surrounding ectoderm as well as the optic cup and mesoderm* **(B, B1)**. *At E12.5,* Fz6 *is expressed in all cells of the lens vesicle including the presumptive epithelium and the elongated primary fiber cells. The developing cornea and hyaloid vasculature also express* Fz6 **(C,C1)**. *By E14.5 the most mature primary lens fibers* **(D1)** *(asterisk) express little or no* Fz6. *At this stage* Fz6 *is expressed throughout the differentiating lens epithelium and also in cells undergoing early fiber elongation at the lens equator (D1, large arrow). In the cornea, the endothelial (D1, upper arrowhead) and epithelial (D1, lower arrowhead) layers show distinct expression. In the optic cup, the marginal region tends to show the strongest* Fz6 *expression (D1, small arrow). At E18.5, P1, P3 and P21 the pattern of* Fz6 *expression in the lens is similar to that described for E14.5. In the optic cup, expression of* Fz6 *tends to be strongest at its margin* **(E1)** *(arrow) where the iridial and ciliary epithelia form. At all postnatal stages* Fz6 *is expressed throughout the epithelium* **(F,G,H)** *and at the equator of the lens. See arrows in* **(F1,G1,H1)**. *Signal remains strong during early fiber elongation in the transitional zone but is lost in the cortical fibers (F1, G1 and H1). The sense control shows no signal in any ocular tissues* **(I)**. *Abbreviations: cb, ciliary body; co, cornea; epi, epithelium; hv, hyaloid vasculature; i, iris; lp, lens pit; m, mesoderm; oc, optic cup; ov, optic vesicle; pf, primary fibers; tz, transitional zone. Scale Bar: (D, E, I), 100 μm; (A, B, C, D1, E1, F, F1, G, G1, H, H1), 40 μm; (B1, C1), 20 μm.*

iridial epithelia. At E18.5 (Fig. 1 E,E1) the pattern of *Fz6* expression in the lens and other ocular tissues is similar to that seen at E14.5.

At postnatal stages P1 (Fig. 1F), P3 (Fig. 1G) and P21 (Fig. 1H), expression of *Fz6* in the lens epithelium remains strong. At all postnatal stages studied the signal for *Fz6* is also strong in the equatorial region of the lens (Fig. 1 F1,G1,H1). This includes the peripheral epithelium and the transitional zone just below the equator where epithelial cells commence elongation to form secondary fibers. During the fiber differentiation process the signal diminishes and is undetectable in the cortical fiber cells. At all postnatal stages examined, *Fz6* expression remains strong in the corneal epithelium and endothelium (not shown). Expression is also distinct in the ciliary body and iris. The sense control demonstrates no signal in any of the ocular tissues (Fig. 1I).

To study Fz protein expression in the lens during embryonic and postnatal development, we carried out an immunohistochemical analysis using an antibody that recognizes all Fz family members. At E9.5, Fz reactivity is detected in the lens placode region but also shows patchy expression throughout the head region of the embryo, mostly in ectodermal and mesoderm cells (Fig. 2A). Expression is similar at E10.5; however, at this stage Fz

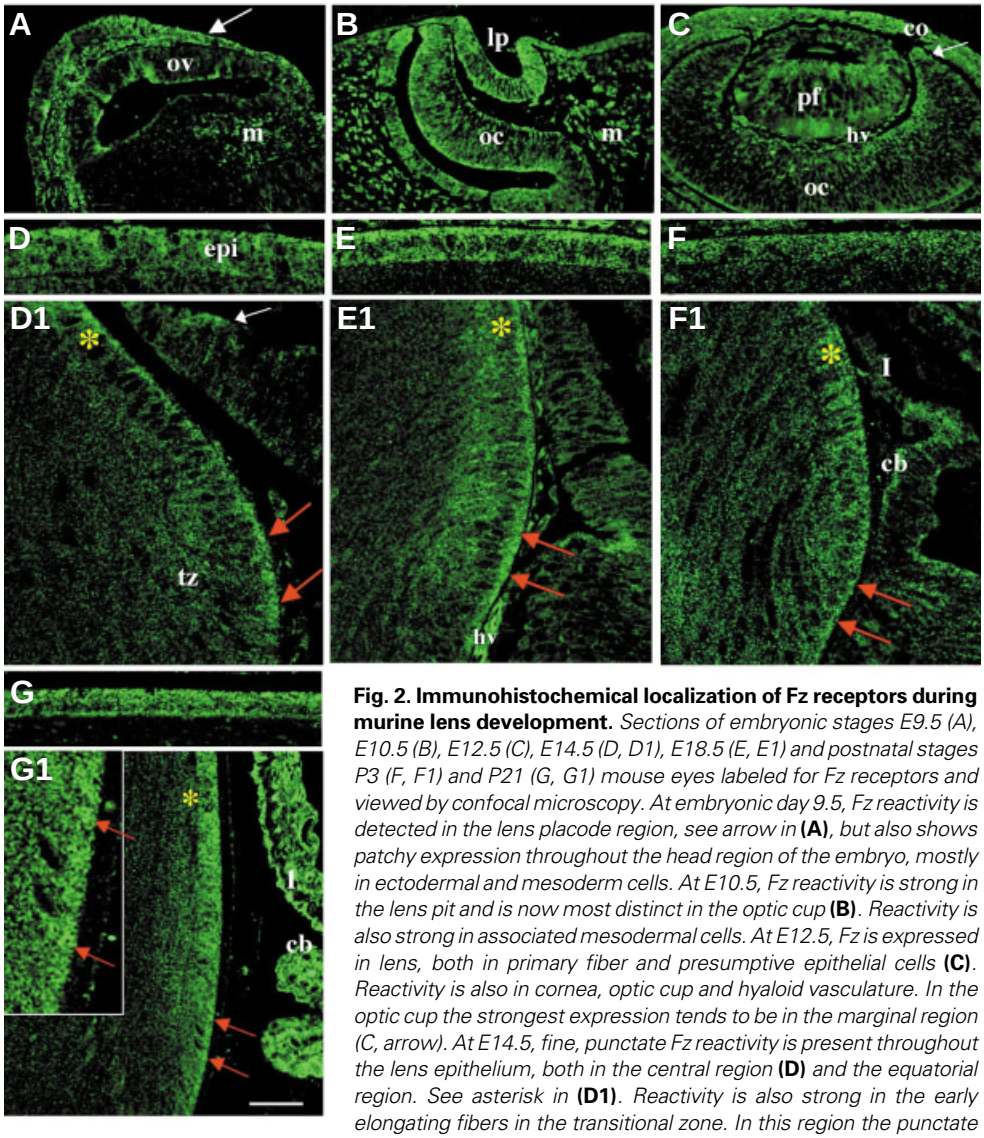

Fig. 2. Immunohistochemical localization of Fz receptors during murine lens development. *Sections of embryonic stages E9.5 (A), E10.5 (B), E12.5 (C), E14.5 (D, D1), E18.5 (E, E1) and postnatal stages P3 (F, F1) and P21 (G, G1) mouse eyes labeled for Fz receptors and viewed by confocal microscopy. At embryonic day 9.5, Fz reactivity is detected in the lens placode region, see arrow in* **(A)**, *but also shows patchy expression throughout the head region of the embryo, mostly in ectodermal and mesoderm cells. At E10.5, Fz reactivity is strong in the lens pit and is now most distinct in the optic cup* **(B)**. *Reactivity is also strong in associated mesodermal cells. At E12.5, Fz is expressed in lens, both in primary fiber and presumptive epithelial cells* **(C)**. *Reactivity is also in cornea, optic cup and hyaloid vasculature. In the optic cup the strongest expression tends to be in the marginal region (C, arrow). At E14.5, fine, punctate Fz reactivity is present throughout the lens epithelium, both in the central region* **(D)** *and the equatorial region. See asterisk in* **(D1)**. *Reactivity is also strong in the early elongating fibers in the transitional zone. In this region the punctate reactivity is strongest at their basal pole (D1, red arrows). In the optic cup the strongest expression is in the margin in the cells destined to form the ciliary and iridial epithelia (D1, white arrow) At E18.5* **(E, E1)** *the pattern of Fz expression in the lens and other ocular tissues is similar to that seen at E14.5. Hyaloid vessels also show strong reactivity at this stage (E1). At postnatal stages P3 and P21, reactivity for Fz is detected in the epithelium and fibers. Reactivity is strong in the central epithelium* **(F,G)**. *Fz protein is also strongly expressed in the peripheral epithelial cells. See asterisk in* **(F1,G1)**. *In the elongating fibers, the strongest reactivity is often detected at the basal ends of the elongating fibers (F1, G1, arrows). Fine punctate reactivity remains weakly detectable in the cortical fibers (F1, G1). Abbreviations: cb, ciliary body; co, cornea; epi, epithelium; hv, hyaloid vasculature; i, iris; lp, lens pit; m, mesoderm; oc, optic cup; ov, optic vesicle; pf, primary fibers; tz, transitional zone. Scale Bar: (A, B, C), 100 μm; (D1, E1, F1, G1) 50 μm; (D, E, F, G), 40 μm; inset 10 μm.*

expression is stronger in lens and retinal primordia (Fig. 2B). At E12.5, Fz is expressed widely throughout the developing eye, including the lens, cornea, optic cup and hyaloid vasculature (Fig. 2C). In the optic cup the strongest expression tends to be in the marginal region. In the lens, both the elongating primary fibers and the presumptive epithelial cells express Fz protein. Reactivity is present throughout the cytoplasm of the cells. At E14.5, fine, punctate Fz reactivity is present throughout the lens but tends to be stronger in the epithelium and in the transitional zone at the lens equator, than in the primary fibers (Fig. 2 D,D1). In the optic cup the strongest expression is in the margin in the cells destined to form the ciliary and iridial epithelia. At E18.5 (Fig. 2 E,E1), the pattern of Fz expression in the lens and other ocular tissues is similar to that seen at E14.5. At this stage strong reactivity is detected in the corneal epithelial and endothelial layers (not shown)

Postnatal stages are similar to E18.5. In the lens at P3 and P21, fine, punctate reactivity for Fz is detected in the epithelium and fibers. Reactivity is strong in the central epithelium (Fig. 2 F,G). Fz protein is also strongly expressed and in the equatorial region of the lens (Fig. 2 F1,G1). This includes the peripheral epithelium and the transitional zone just below the equator where epithelial cells commence elongation to form secondary fibers. In this region the strongest reactivity is often detected at the basal ends of the elongating fibers. During the fiber differentiation process reactivity for Fz diminishes but is still detectable in the cortical fibers.

Lens whole-mounts were also used to study Fz expression in lens cells. These preparations show patterns of Fz reactivity similar to that reported for histological sections. In the epithelial whole-mount, reactivity is present throughout the cytoplasm of the cells (Fig. 3A). In the incipient fibers included at the periphery of these preparations (the meridional rows), fine, punctate reactivity is present throughout these cells, although it tends to be stronger at their basal ends where the nuclei are located (Fig. 3B). Fibers deeper in the cortex also show punctate reactivity, although some filamentous reactivity is also detected (Fig. 3C). To investigate if Fz receptors are associated with cytoskeletal components we co-localized F-actin and Fz. Here we used posterior capsule whole-mounts. These preparations retain some attached fibers and also the broken ends of the fibers that maintain their attachment to the posterior capsule. This latter region is known as the basal membrane complex (bmc; Bassnett *et al.*, 1999). In a transverse optical section of cortical fibers we show that Fzs and F-actin have similar localization patterns (Fig. 3 D-F). F-actin and Fz is predominantly found at the cell margins, although fine granular reactivity is also obvious throughout the cytoplasm. In the bmcs that are retained on the posterior capsule, the F-actin clearly delineates the fiber cell margins (Fig. 3E). Fz also

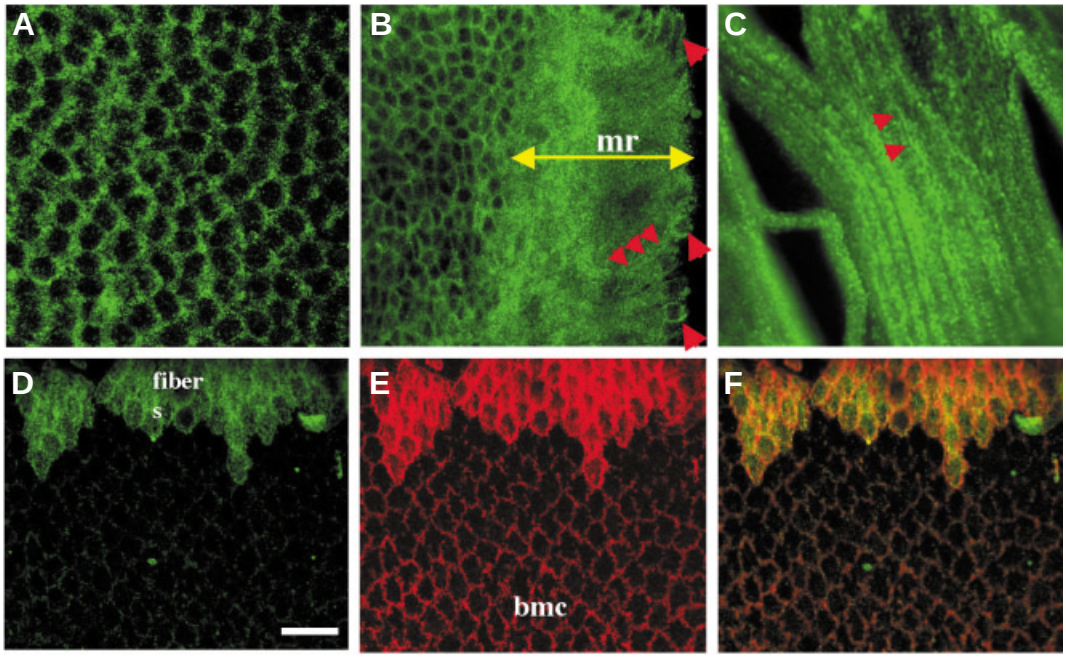

Fig. 3. Immunocytochemical localization of Fz receptors and F-actin in P21 rat lenses. *Whole-mount preparations of lens epithelial cells and fibers labeled for Fz (A-D, F) and F-actin (E, F). Throughout the epithelial sheet, Fz reactivity is present in the cell cytoplasm* **(A)**. *At the lens equator, in the meridional rows where fiber elongation commences, punctate Fz reactivity is detected. This can be seen to extend throughout the length of the incipient fibers* **(B)** *(set of small arrows), but tends to be more prominent at the basal ends of the cells in the cytoplasm around the cell nuclei (B, large arrows). In cortical fibers deeper in the lens, punctate Fz reactivity is present throughout the fiber length* **(C)**. *In some regions, filamentous arrangements are discerned (C, arrows). To investigate if Fz receptors are associated with cytoskeletal components we co-localized F-actin and Fz. Here we used posterior capsule whole-mounts. In these preparations the basal membrane complex (bmc) and some fibers remain attached to the posterior capsule. In a transverse optical section of cortical fibers, Fz receptors* **(D)** *and F-actin* **(E)** *have a similar localization pattern. Both are predominantly found at the cell margins, although fine granular Fz reactivity is also obvious throughout the cytoplasm. In the bmcs, the F-actin clearly delineates the fiber cell margins (E). Fz also appears to be present in the bmcs but is only very weakly reactive (D). When images A and B are merged, the yellow color indicates that there is considerable overlap of Fz with F-actin, particularly at the cell margins* **(F)**. *Some fine granular reactivity, mostly for Fz, is present throughout the fiber cell cytoplasm. The bmcs clearly label for F-actin but by comparison, Fz reactivity is very weak. Abbreviations: bmc, basal membrane complexes; mr, meridional rows. Scale bar: (A,D,E,F), 50 μm; (B), 75 μm; (C), 75 μm.*

appears to be present along these margins but is only very weakly reactive (Fig. 3D).

Frizzled localization in FGF-treated epithelial explants

It was noted that Fz reactivity was consistently strongest at the basal ends of the early elongating fibers in the transitional zone at the lens equator (Fig. 2 D1,E1,F1,G1). To study the localization of Fz during early stages of fiber differentiation we employed a lens epithelial explant culture system. Lens epithelial explants were treated with a fiber differentiating dose of FGF2, and Fz localization was carried out after 1 and 5 days of culture. In these explants, it was noted that after 24 hours, cells took on migratory morphologies and produced numerous lamellipodia and filopodia (Fig. 4B). These were generally randomly directed and strongly reactive for Fz receptors. Cells in untreated explants also showed some of these features but

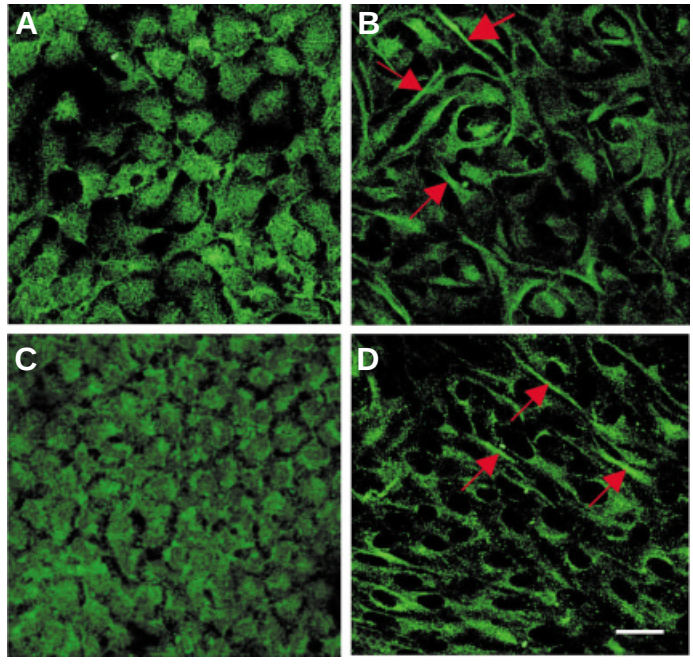

Fig. 4. Fz receptor localization in FGF-treated epithelial explants from P10 rat lenses. *Lens epithelial explants treated with a fiber differentiating dose of FGF2, show Fz localization after 1* **(B)** *and 5* **(D)** *days of culture. Controls explants (unsupplemented with FGF) also show Fz localization after 1* **(A)** *and 5* **(C)** *days. In FGF-treated explants, after 1 day, cells have migratory morphologies and exhibit numerous randomly directed lamellipodia and filopodia that are strongly reactive for Fz receptors (B, arrows). After a similar period, cells in controls also show some migratory features but these are less pronounced and the Fz reactivity is not as strong as in the presence of FGF (A). After 5 days of culture with FGF, cells have begun to multilayer and elongate. Fz reactivity is strong in these cells, particularly cell processes. Many of the processes still resemble the filopodia seen earlier but at this stage there is evidence of alignment of these processes, at least within some groups of cells (D, arrows). In control explants, after 5 days of culture, cells are present in a monolayer and show cobblestone-like packing, Weak reactivity for Fz is present in the cytoplasm of these cells. Scale bar: (A,B,C,D), 25 μm.*

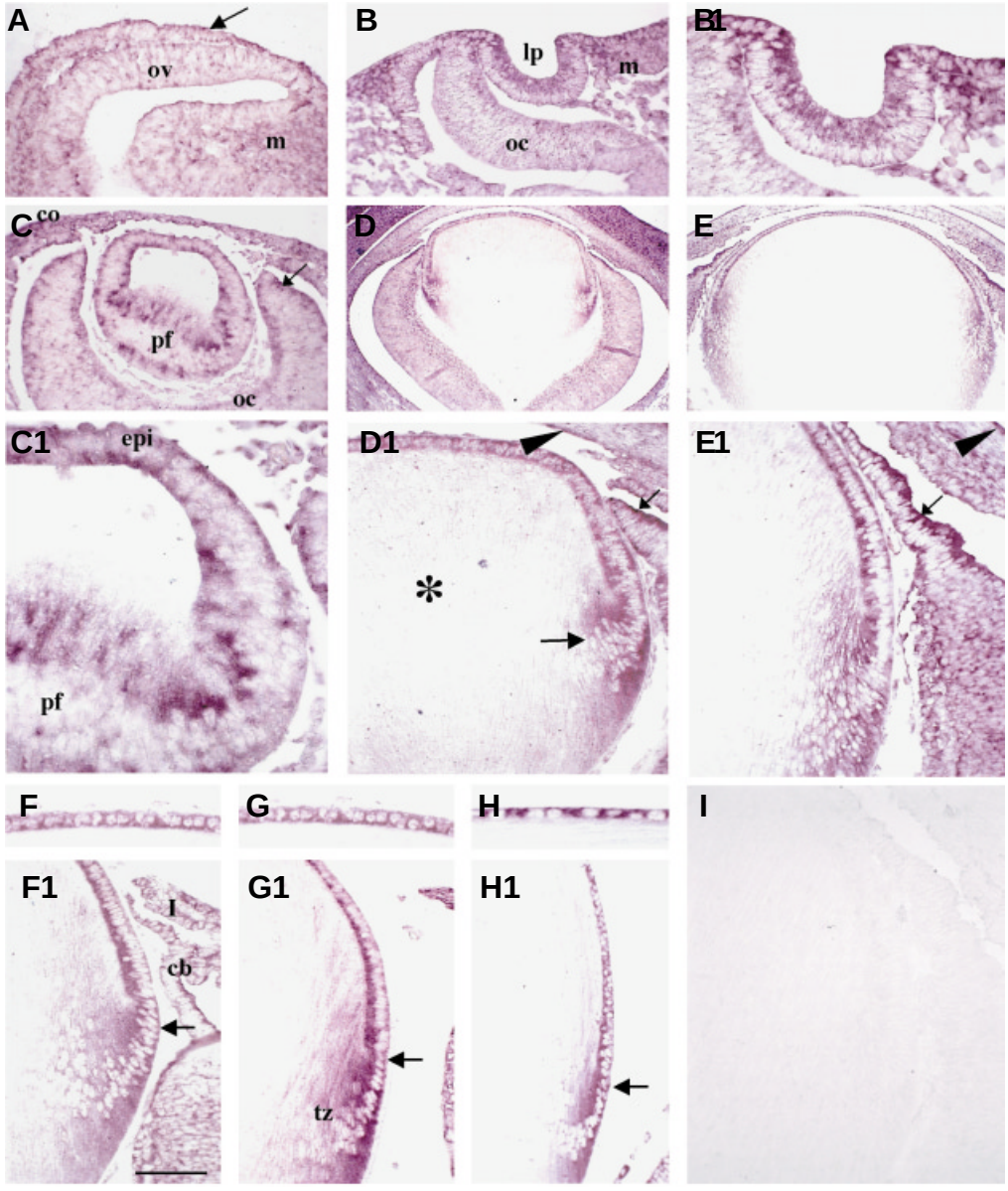

Fig. 5. *In situ* hybridization: expression patterns of *Sfrp1* during murine lens development. *Sections of embryonic stages E9.5 (A,), E10.5 (B, B1), E12.5 (C, C1), E14.5 (D, D1), E18.5 (E, E1) and postnatal stages P1 (F, F1), P3 (G, G1) and P21 (H, H1,) mouse eyes labeled for* Sfrp1 *mRNA expression and viewed by bright field microscopy. A control (sense probe) is included for E18.5 (I). At E9.5,* Sfrp1 *is expressed weakly throughout the lens placode region, see arrow in* **(A)** *and the underlying mesoderm. At E10.5,* Sfrp1 *expression is detected mostly in the lens pit and surrounding ectoderm as well as the underlying mesoderm* **(B, B1)**. *At E12.5,* Fz6 *is expressed in all cells of the lens vesicle including the presumptive epithelium and the elongated primary fiber cells. The developing cornea and the optic cup, particularly cells in the marginal region, see arrow in* **(C)**, *also express* Sfrp1. *By E14.5 the most mature primary lens fibers, see asterisk in* **(D1)** *express little or no* Sfrp1. *At this stage* Sfrp1 *is expressed throughout the differentiating lens epithelium and also in cells undergoing early fiber elongation at the lens equator (D1, large arrow). In the cornea, the endothelial (D1, arrowhead) layer shows distinct expression. In the optic cup, the marginal region tends to show the strongest* Sfrp1 *expression (D1, small arrow). At E18.5, P1 P3 and P21, the pattern of* Sfrp1 *expression in the lens is similar to that described for E14.5. In the optic cup, expression of* Sfrp1 *tends to be strongest at its margin , see arrow in* **(E1)**, *where the iridial and ciliary epithelia form. In the cornea, the epithelium (E1, arrowhead) shows distinct expression. At all postnatal stages* Sfrp1 *is expressed throughout the epithelium* **(F,G,H)** *and at the lens equator; see arrows in* **(F1, G1, H1)**. *Signal remains strong during early fiber elongation in the transitional zone but is lost in the cortical fibers (F1, G1, H1). The sense control shows no signal in any ocular tissues* **(I)**. *Abbreviations: cb, ciliary body; co, cornea; epi, epithelium; i, iris; lp, lens pit; m, mesoderm; oc, optic cup; ov, optic vesicle; pf, primary fibers; tz, transitional zone. Scale Bar: (D,E), 100 μm; (A,B,C,D1,E1,F,F1,G,G1,H,H1,I), 40 μm; (B1, C1), 20 μm.*

they were less pronounced (Fig. 4A), This is consistent with previous studies in our laboratory that showed cell migration occurs in epithelial explants and that it is significantly stimulated by FGF early in the culture period (McAvoy and Chamberlain, 1989). At this stage, Fz reactivity is strongest at the leading edges of migrating cells (Fig. 4B). By 5 days of culture, differentiation had begun and the cells were beginning to multilayer and become elongated. In these cells filopodia-like processes were still evident and, within groups of cells, were often aligned in one direction. Strong Fz reactivity was detected within these cellular processes (Fig. 4D). Note that as previous studies have shown FGF-induced cell migration ceases in epithelial explants after 2 days (McAvoy and Chamberlain, 1989), the filopodia-like processes may represent the elongating ends of differentiating fibers.

Secreted frizzled-related protein expression in the lens

To investigate *Sfrp* expression in the lens RT-PCR was carried out on RNA extracted from E15.5 mouse embryos or P20-P25 rat lens epithelial cells. All reverse transcriptions were carried out using equivalent amounts of RNA, and equivalent volumes of reverse transcription reactions were used for PCR amplifications. RT-PCR using RNA and primers for *Sfrp4* and *Sfrp5* (note that *Sfrp1*, *Sfrp2* and *Sfrp3* have already been shown to be expressed in the lens by RT-PCR; Stump *et al.,* 2003) resulted in distinct amplification of products of expected sizes (Table 1). The specific

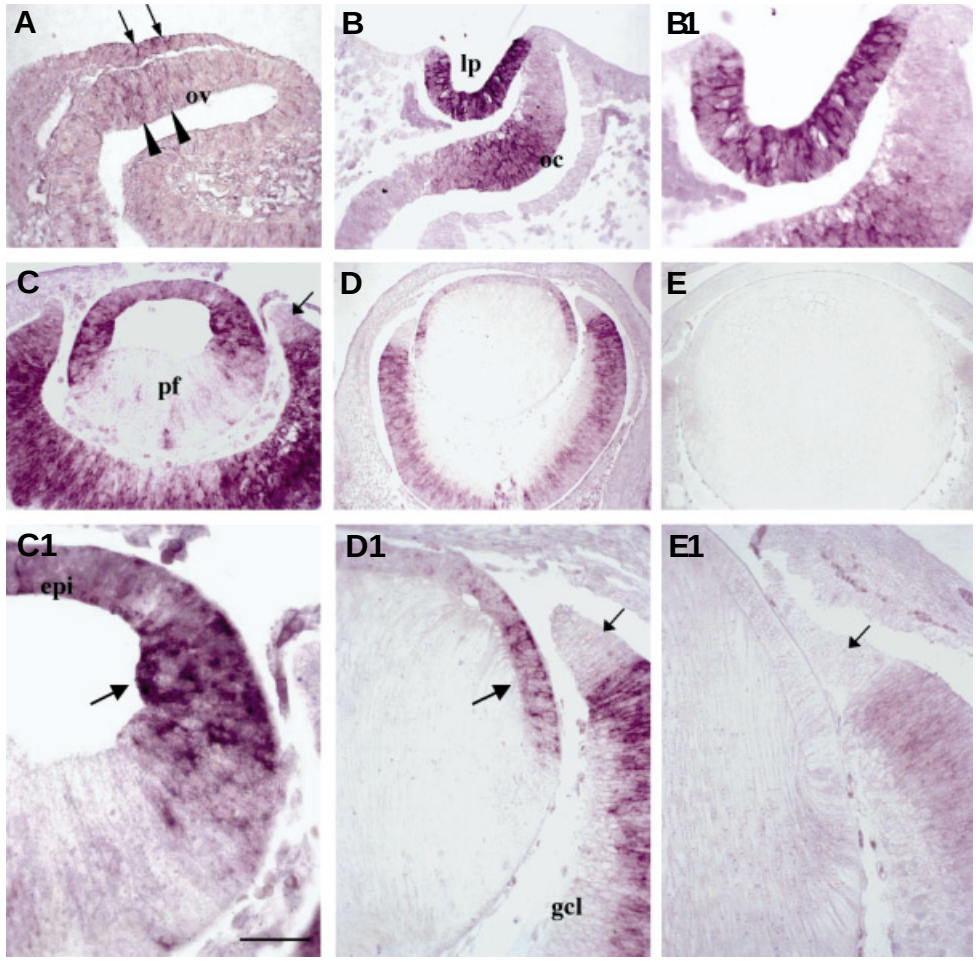

Fig. 6. *In situ* hybridization: expression patterns of *Sfrp2* during murine lens development. *Sections of embryonic stages E9.5 (A), E10.5 (B, B1), E12.5 (C, C1), E14.5 (D, D1), E18.5 (E, E1) mouse eyes labeled for Sfrp2 mRNA expression and viewed by bright field microscopy. At E9.5,* Sfrp2 *is expressed weakly in a group of cells in the center of the early lens placode but not in neighboring ectodermal cells; see pair of arrows* **(A)**. *At this stage, there is also weak expression in some cells in the central region of the inner layer of the optic vesicle (A, pair of arrowheads). At E10.5,* Sfrp2 *is strongly expressed throughout the lens placode but not in the surrounding ectoderm* **(B, B1)**. *Strong* Sfrp2 *expression is also detected in the central region of the inner layer of the optic cup (B, B1). At E12.5,* Sfrp2 *is restricted to cells in the presumptive lens epithelium, particularly in the peripheral region, see arrow, (C1). The elongating primary fibers in the posterior hemisphere of the lens vesicle show no, or patchy, expression of* Sfrp2 **(C, C1)**. *At this stage expression of* Sfrp2 is *strong throughout the optic cup, except at the marginal tip where expression is weak or absent (C, arrow). By E14.5,* Sfrp2 *is only weakly expressed in the central epithelium but is still strongly expressed in groups of epithelial cells just above the lens equator; see large arrow in* **(D1)**. *Expression of* Sfrp2 *is evident throughout the optic cup but is notably absent from its margin (D1, small arrow). Expression is also absent from the innermost region of the optic cup where the ganglion cells arise (D1).* Sfrp2 is *not detected in the lens at E18.5* **(E, E1)**. *At this stage the expression in the optic cup is similar to that at E14.5, although it is weaker* **(E, E1)**. *No* Sfrp2 *expression is detected at the margin of the optic cup (E, E1, arrow). Abbreviations: epi, epithelium; gcl, ganglion cell layer; lp, lens pit; oc, optic cup; ov, optic vesicle; pf, primary fibers. Scale Bar: (D, E), 100 μm; (A, B, C, D1, E1), 40 μm; (B1, C1), 20 μm.*

PCR fragments for these *Sfrp* genes were cloned and sequenced to confirm sequence identity with those reported in GenBank.

To investigate the spatial expression patterns of *Sfrp* mRNAs in the murine lens, cDNAs were used as templates to prepare digoxigenin-labeled riboprobes for *in situ* hybridization analyses. This included cDNAs for *Sfrp1*, *Sfrp2* and *Sfrp3* from our previous study (Stump *et al.,* 2003). As this study progressed it became clear that, except for *Sfrp2*, the expression patterns of *Sfrp1, Sfrp3, Sfrp4* and *Sfrp5* were very similar. As summarized in Table 2, the only differences were that *Sfrp3* was not detected until E14.5 and *Sfrp4* was not detected postnatally. At other developmental stages these *Sfrps*, although weaker, showed a pattern of expression that was similar to *Sfrp1* and *Sfrp5*. Except for these differences, the results presented for *Sfrp1* in Fig. 5 are representative for *Sfrp3, Sfrp4 and Sfrp5* genes.

At E9.5, *Sfrp1* is expressed weakly throughout the head region of the embryo, mostly in ectodermal and mesoderm cells (Fig. 5A). Expression is similar at E10.5; however, at this stage expression of *Sfrp1* is slightly stronger (Fig. 5 B,B1). At E12.5 *Sfrp1* is expressed widely throughout the developing eye, including the cornea, optic cup and lens (Fig. 5 C,C1). In the lens, both the elongating primary fibers and the presumptive epithelial cells express *Sfrp1* transcripts. In the optic cup, the strongest signal is at its margin (Fig. 5C). At E14.5 *Sfrp1* expression is absent from the most mature primary fibers in the center of the lens. The less mature elongating fibers at the lens equator and the epithelial cells show strong expression of *Sfrp1* transcripts (Fig. 5 D,D1). In the cornea, the epithelium and endothelium exhibit stronger expression than the stroma. In the optic cup the strongest expression is in the margin in the cells destined to form the ciliary and iridial epithelia (Fig. 5 D,D1). At E18.5 (Fig. 5 E,E1), the pattern of *Sfrp1* expression in the lens and other ocular tissues is similar to that seen at E14.5.

At postnatal stages P1 (Fig. 5 F,F1), P3 (Fig. 5 G,G1) and P21 (Fig. 5 H,H1), the expression of *Sfrp1* is strong throughout the epithelium and in the equatorial region. At all postnatal stages the signal diminishes during fiber differentiation and is undetectable in the cortical fiber cells. At all postnatal stages examined, *Sfrp1* expression remains strong in the corneal epithelium and endothelium (not shown). Expression is also distinct in the ciliary and iridial epithelium (Fig. 5 F1). The sense control demonstrates no signal in any ocular tissues (Fig. 5I).

In contrast to the other *Sfrps*, *Sfrp2* has a very distinctive pattern of expression (Fig. 6). At E9.5, the *Sfrp2* gene is ex-

pressed weakly in a group of cells in the center of the early lens placode but not in the neighboring head ectoderm (Fig. 6A). At this stage, there is also weak expression in some cells in the central region of the optic vesicle. At E10.5, *Sfrp2* is strongly expressed throughout the lens placode but not in the surrounding ectoderm (Fig. 6 B,B1). Moreover, the only other site of expression in this region of the embryonic head is in a group of cells in the center of the optic cup (Fig. 6 B,B1). At E12.5, *Sfrp2* is restricted to cells in the presumptive lens epithelium, particularly in the peripheral region (Fig. 6 C,C1). The elongating primary fibers in the posterior hemisphere of the lens vesicle show little to no expression of *Sfrp2* (Fig. 6 C,C1). At this stage expression of *Sfrp2 is* strong throughout the optic cup; however, at the marginal tip expression is weak or absent (Fig. 6C). By E14.5, as epithelial differentiation progresses, *Sfrp2* is weakly expressed in the central epithelium and is only strongly expressed in groups of epithelial cells just above the lens equator (Fig. 6 D,D1). Expression of *Sfrp2* is evident throughout the optic cup but is notably absent from its margin. Expression is also absent from the inner region of the optic cup where the ganglion cells arise (Fig. 6 D,D1). *Sfrp2* is not detected in the lens at E18.5 (Fig. 6 E,E1), or at later stages. At this stage the expression in the optic cup is similar to that at E14.5, although it is weaker (Fig. 6 E,E1).

Discussion

In this study multiple members of the Fz and Sfrp receptor families are shown to be expressed in the lens during development. The *in situ* hybridization analysis shows that the *Fzs* and most of the *Sfrps* are expressed throughout the epithelium and the transitional zone during both embryonic (E9.5-E18.5) and postnatal (P1-P21) development; the most notable exception being *Sfrp2* which is restricted to the presumptive epithelium and is only detectable during early stages of lens morphogenesis. A pan-specific antibody for the Fzs also shows that the protein is expressed in the epithelium and in the differentiating fibers during embryonic and postnatal development.

Our results on Fz expression show some agreement with an earlier *in situ* study of *Fz3, Fz4, Fz6* and *Fz7* expression in mouse embryos at E12.5, E14.5 and E18.5 (Liu *et al.,* 2003). This study also reported that the overall pattern of *Fz* expression was in the lens epithelium and equatorial region. However, they also reported that *Fz6* was not expressed in the anterior epithelium and that *Fz4* and *Fz7* were not expressed in the equatorial region. In our study the Fzs we examined, *Fz1 to Fz8* inclusive, all showed a similar pattern of localization in both the anterior epithelium and equatorial region. Although occasionally there were slight differences in intensity of expression between the anterior and equatorial regions, we did not find these variations to be consistent or reproducible. It is not clear if the differences between our results and those reported by Liu and colleagues (2003) relate to the different probes used in these studies or to the different tissue processing methodologies.

The localization of the Fzs in the lens epithelium during development is consistent with a role for Wnt-Fz signaling in the formation and maintenance of the lens epithelium. In recent studies, we showed that mouse embryos homozygous for a mutation in the *lrp6* gene did not form a normal lens epithelium (Stump *et al.,* 2003). A common feature of these mice was the absence of the anterior epithelium and extrusion of the lens fibers into the corneal stroma. As LRPs are required for Wnt-Fz signaling through the β–catenin pathway it was concluded that the normal formation of the epithelium requires β–catenin signaling. Independent evidence for a role for β–catenin signaling during early stages of lens epithelial differentiation comes from a recent study of reporter gene expression in the eyes of *TCF/Lef-LacZ* transgenic mice (Liu *et al.,* 2003). Expression of this reporter indicates that signaling through the canonical Wnt/β–catenin pathway is restricted to an early stage of differentiation of the lens epithelium, namely E13.5. Thus Wnt/β–catenin signaling may have a key role in the specification of the lens epithelium from the lens vesicle. However, as Fzs and Wnts continue to be expressed in the mouse lens during subsequent embryonic and postnatal development (Liu *et al.,* 2003; Stump *et al.,* 2003; Ang *et al.,* 2004), this raises the possibility that non-canonical Wnt-Fz signaling pathways may also have roles in regulating lens cell behavior. For example, Wnt-Fz signaling can also activate the planar cell polarity (PCP) pathway. This pathway does not require LRP co-receptors and appears to function independently of β–catenin (McEwen and Peifer, 2001). Changes in cell shape and polarity in other cellular systems are characteristically regulated through the PCP pathway (Huelsken and Birchmeier, 2001). Therefore, it is possible that this pathway may have a role in regulating cell polarity across the epithelium as well as the major cellular rearrangements that occur at the lens equator.

In relation to a role for Wnt-Fz signaling at the lens equator, it was noted that in elongating fibers at the equatorial region, Fz receptor reactivity tends to be strongest at their basal ends. It has been reported that the basal ends of the fibers migrate along the posterior capsule towards the sutures (Bassnett *et al.,* 1999; Al-Ghoul *et al.,* 2003). In another approach to study the localization of Fz during fiber differentiation, we employed a rat lens epithelial explant culture system. Lens epithelial explants can be induced to differentiate by the addition of FGF to the culture medium (Chamberlain and McAvoy, 1989). This is characterized by a period of FGF-induced random cell migration followed by cell elongation (McAvoy and Chamberlain, 1989). Consistent with this, after 24hrs of FGF treatment, most cells exhibit filopodia and randomly directed protrusive activity. Strong reactivity for Fzs is present at leading edges and filopodia. As differentiation progresses and the cells begin to multilayer and elongate, polarization of Fz reactivity is still evident in the filopodia-like extensions, which also show some alignment within some groups of cells (Fig. 4D). Thus Wnt-Fz signaling may be involved in cytoskeletal reorganization that is associated with the cellular migration and/or fiber elongation processes that occur below the lens equator. This is consistent with a role for Wnt/PCP signaling in regulating the cytoskeleton during major cellular reorganizations in various developmental systems (Mlodzik, 2002). A frequently used example is convergent cell extension which occurs during gastrulation (Mlodzik, 2002; Copp *et al.,* 2003). This process involves initial random protrusive activity that is followed by stabilization of filopodia and aligned bipolar protrusive activity. Such polarized cell behavior bears some similarity with the early stages of FGF-induced fiber differentiation in lens epithelial explants. Other studies show that polarized cell behaviors involve the small GTPases, CDC42, Rac and Rho (Fukata *et al.,* 2003). Recently it has been shown that growth factor-activated Rac and Rho appear to mediate cytoskel-

etal reorganization in cultured lens cells (Maddala *et al.,* 2003). In addition, inhibition of Rho activation in the lens leads to defects in fiber differentiation (Rao *et al.,* 2002). In future studies it will be important to examine the relationship between the small GTPases in the lens and Wnt-Fz signaling.

In this context it is also interesting that in the lens fibers there is some co-localization of Fz and F-actin (Fig. 3 D-F). In a culture system that mimics convergent extension, Fz becomes localized to the actin cytoskeleton during cellular rearrangements, migration and cytoskeletal reorganization (Wiggan and Hamel, 2002). In this study it was hypothesized that Wnt/PCP signaling may be involved in regulating the turnover of actin structures that give rise to lamelliform and filiform protrusions. Such a role for Wnt-Fz signaling could be envisaged during the migration/major cellular rearrangements that occur at the lens equator. However, how an interaction between Fz and actin may be involved in regulating the more stable cytoskeleton of cortical fibers is harder to envisage. Clearly further studies are needed to unravel the details of the relationship between these molecules in lens cells.

The expression patterns of *Sfrp* mRNA*s* are also reported in this study. This family of molecules is important for regulation of Wnt-Fz signaling (Jones and Jomary, 2002). The distribution of most of the *Sfrp* mRNAs in the developing lens is largely similar to the *Fzs*, being expressed in the epithelium and the equatorial region. Expression of *Sfrp 2* between E9.5 and E10.5 (Wawersik *et al.,* 1999), *Sfrp1, Sfrp 2* and *Sfrp4* between E10.5 and E15.5 (Leimeister *et al.,* 1998) and *Sfrp1*, *Sfrp2*, and *Sfrp3* between E12.5 and E18.5 (Liu *et al.,* 2003) in the developing mouse eye have been reported previously. Whilst there were similarities with these studies, the main difference was that in our study at E18.5, unlike Liu and colleagues (2003), no *Sfrp1* was detected in the most mature fibers. Also, they did not detect *Sfrp3* in the lens; however, we did show it to be present at this stage. Other studies on *Sfrp1* have shown it to be expressed in the adult mouse epithelium but not in the fibers (Rattner *et al.,* 1997). This is in agreement with our reported *Sfrp1* expression pattern during postnatal development. Currently it is not clear if the differences between our results and those reported by Liu and colleagues (2003) relate to the different probes used in these studies or to the different tissue processing methodologies. Additional information from the current study shows that *Sfrp3* and *Sfrp5* are also expressed postnatally and have similar patterns of expression to each other and to the *Fzs*, being present in the epithelium and transitional zone and absent from the fibers. The only exception is *Sfrp4*, which is not detected in the lens postnatally.

The most distinctive expression pattern is exhibited by *Sfrp2*. This family member is clearly different from the other *Sfrps* (and the *Fzs*). Expression of *Sfrp2* is restricted to the lens placode, pit and the presumptive epithelium. At E9.5 it is only weakly expressed in a few cells in the center of the lens placode and the apposing central region of the optic vesicle. This is intriguing because these regions are thought to be the most developmentally advanced cells of the lens and neural retina, respectively. Cells in the central region of the lens primordium are the first to withdraw from the cell cycle and initiate primary fiber elongation (McAvoy, 1978) and cells in the central region of the retinal primordium are the first to form laminations (Rapaport and Stone, 1982). *Sfrp2* is only present for a short period during early embryonic development and is not detected in the postnatal lens. As it is not detected in any of the neighboring ectodermal cells, this restricted pattern of expression is suggestive of a specific requirement for tight regulation of Wnt-Fz signaling in the early lens primordia. In this context it is also interesting that studies on *Pax6*-deficient (*Sey/Sey*) mice indicate that, at least at early stages of lens morphogenesis, *Sfrp2* expression appears to be dependent on *Pax6* expression (Wawersik *et al.,* 1999).

How Sfrps influence Wnt-Fz signaling in the lens is uncertain. Whilst Sfrps are most commonly viewed as Fz antagonists; they have also been reported to have the capacity to act as agonists in some contexts (Jones and Jomary, 2002). In addition, members of the Sfrp family can have different effects in the same system; for example, Sfrp1 and Sfrp2 can have opposing effects on human breast cells (Melkonyan *et al.,* 2002). Other regulatory molecules, such as the Dickkopfs, which are expressed in the lens epithelium and the equatorial region (Ang *et al.,* 2004), may also comprise a regulatory network that influences Wnt-Fz signaling at different stages of ocular development. Clearly, gaining an understanding of how individual components of this network influence the behavior of lens cells is an important area for further study.

Sfrp2 also has a highly restricted expression pattern in the developing retina and other parts of the neuroectoderm (see for example, Assimacopoulos *et al.,* 2003). In the retinal primordium, *Sfrp2* is expressed weakly in the center of the optic vesicle. It is strongly expressed throughout the optic cup but is notably absent at its margins. The region devoid of *Sfrp2* expression demarcates the region that will form the ciliary and iridial retina (Coulombre, 1965). This pattern of *Sfrp2* expression in the mouse optic cup has been noted previously by Liu and colleagues (2003). This observation, taken together with studies that show this marginal region strongly expresses *Fz* receptor and *Wnt* mRNAs (Liu *et al.,* 2003) as well as the strong reporter gene expression in this region in the eyes of *TCF/Lef-LacZ* transgenic mice (see above), has led to the suggestion that Wnt-Fz signaling may have an important role in promoting patterning in this region of the retina (Liu *et al.,* 2003). Support for this hypothesis comes from recent studies on chick eyes that show Wnt2b is necessary for the formation of ciliary body and iris (Kubo *et al.,* 2003; Nakagawa *et al.,* 2003).

In summary, the current study is consistent with a role for Wnt/Fz/β–catenin signaling in the early stages of differentiation of the epithelium from the anterior cells of the lens vesicle. Furthermore, expression of Wnts, Fzs and the Wnt-Fz signaling regulators, Sfrps and Dkks in the older embryo and at all postnatal stages so far examined, raise the possibility that Wnt signaling is also important for maintenance as well as development of the lens. The Wnt/PCP signaling pathway is involved in regulating cell polarity and cytoskeletal changes during major cellular rearrangements. Epithelial cells exhibit distinct apical-basal polarity and at the lens equator there are major cellular rearrangements as epithelial cells differentiate into fibers. To explore the putative role of Wnt-Fz PCP signaling in these developmental processes, it will be important to examine the expression and distribution of the PCP signaling components, such as Dishevelled and the Rho-family GTPases, and how they are related to Fzs and Wnts in the lens. In addition, genetic manipulation of Wnt, Fz and key PCP signaling molecules in lens explants and the lenses of transgenic mice should help elucidate functional roles for this complex family.

Methods

Animal Tissues

Eyes from 20-25 day postnatal (P20-25) mice (FVB/N) or rats (Wistars) were dissected to isolate the lens from other eye tissues. Lens capsules (with adherent epithelial cells) were then separated from the fiber mass. These preparations were used for reverse polymerase chain reaction (RT-PCR) as described below. For *in situ* hybridization, embryonic day 9.5 (E9.5), E10.5, E12.5, E14.5, E18.5 mice and eyes from postnatal day 1 (P1), P3 and P21 mice (FVB/N) were fixed in 10% neutral buffered formalin (NBF) and embedded in paraffin wax. P10 and P21 rats (Wistars) were used to prepare lens epithelial explants and lens epithelial whole-mounts, respectively.

RT-PCR

Total RNA was extracted from dissected lens capsules (with adherent epithelial cells) or E15.5 embryos using Tri Reagent© (Sigma, Sydney, Australia). First strand cDNA synthesis was carried out using 2 μg of RNA with a reverse transcription system (Promega, Sydney, Australia) according to the manufacturer's instructions. In control reactions AMV reverse transcriptase was omitted. For PCR amplification, 2 μl of template cDNA was combined with 0.5 μM primers, 400 μM dNTPs, (Astral Scientific, Sydney Australia), 2-4 mM $MgCl_2$, 1.25 U Taq DNA polymerase and 1x reaction buffer (Promega, Sydney Australia). Samples were mixed at 4ºC and heated to 94ºC for 60 seconds then amplified through 30-35 cycles (57-58ºC for 30-60 seconds and 72ºC for 45 seconds and 94ºC for 25 seconds), followed by a final extension at 72ºC for 2 minutes. Specific primer sets were derived from the coding sequences for *Fz5, Fz7, Fz8*, *Sfrp4 and Sfrp5* (see Table 1). PCR amplifications were optimized for each primer pair. RT-PCR studies had previously detected *Fz1, Fz2, Fz3, Fz4* and *Fz6*, as well as *Sfrp1, Sfrp2* and *Sfrp3* in the lens (Stump *et al.,* 2003).

cDNAs

PCR products for *Fz5, Fz7, Fz8, Sfrp4* and *Sfrp5* were agarose gel purified and cloned into pGEM-T transcription vector (Promega, Australia). The identity of resulting clones was confirmed by restriction enzyme digests and by direct sequencing. Digoxigenin-labeled complementary RNA probes were transcribed from linearized plasmid templates using SP6 and T7 RNA polymerases (Promega, Australia) and digoxigenin-labeled nucleotides (Roche Diagnostics Australia).

In situ *hybridization*

The expression patterns of *Fz1-Fz8* inclusive and *Sfrp1-Sfrp5* inclusive in the murine eye were examined by *in situ* hybridization using antisense and sense probes, prepared as described previously with digoxigenin as a label (Stump *et al.,* 2003). Paraffin sections of mice from embryonic day E9.5 to E18.5 and postnatal days P1, P3 and P21 were analyzed for *Fz* and *Sfrp* gene expression as described previously (de Iongh *et al.,* 2001).

Immunohistochemistry

Immunohistochemistry was carried out as described previously (de Iongh *et al.,* 2001). In brief, formalin-fixed, 5μm paraffin sections of mice embryos and whole eyes were deparaffinized and hydrated to PBS. Prior to incubating sections with the primary antibody, sections were incubated with 3% normal goat serum (blocking solution). Localization of Fz was carried out using a rabbit polyclonal antibody raised against a recombinant protein corresponding to amino acids 301-400 of human Fz2 (Santa Cruz Biotechnology, CA). This antibody reacts with mouse and rat Fz1-Fz10. Sections were then incubated with the primary antibody and washed in PBS/0.1% BSA. Following washes in PBS, reactivity was visualized with Alexa-conjugated goat anti-rabbit immunoglobulin. For controls the Fz antibody was omitted or replaced by non-immune IgG at an equivalent concentration.

The method for preparing whole-mounts was adapted from the method of Howard (1952). Briefly, the lens was dissected from the eye and fixed in HistoChoice (Amresco, Solon, USA) fixative for 2 mins in the case of epithelial whole-mounts, or 2% paraformaldehyde in PBS for posterior capsule whole-mounts. The capsule was torn at the posterior (epithelial whole-mounts) or anterior (posterior capsule whole-mounts) pole and the torn ends were pulled away from the fiber mass. The capsule was laid out flat on a SuperFrost Plus (Menzel-Glaser, Braunschweig, Germany) glass slide. After washing in PBS, and incubating in PBS/0.1% Tween 20 for 20 mins, immunocytochemistry was conducted as described above. Posterior capsule whole-mounts were also prepared for the immunmocytochemical analysis of attached fibers. These were made according to the procedure described by Al-Ghoul and colleagues (2003). To label F-actin, the capsule whole-mounts were incubated in phalloidin-FITC (Sigma Chemical Company, St Louis, MO) as previously described (Al-Ghoul *et al.,* 2003). Material was viewed and photographed using both fluorescence (Leitz Diaplan; Leica Microsystems, N. Ryde, NSW), and confocal (Zeiss LSM 5 PASCAL; Carl Zeiss, Camperdown, NSW) microscopy.

Preparation of Lens Epithelial Explants

All tissue culture was performed in Medium 199 with Earle's salts (ThermoElectron Corp., Victoria, Australia), supplemented with 0.1% bovine serum albumin (BSA), 0.5 mM L-glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin and 2.5 μg/ml Amphostat (all from ThermoElectron Corp., Victoria, Australia). Lens epithelial explants were prepared as follows: under sterile conditions, for each culture dish, two eyes were removed from P10 rats and placed in culture medium. Using a dissecting microscope, lenses were dissected from eyes and transferred to fresh culture medium. With fine forceps, the posterior lens capsule was torn and peeled from the fiber cell mass which was discarded. The remaining lens capsule (containing the adherent epithelial monolayer) was gently pinned out flat by pressing the forceps around the edge of the explant. Culture medium was then removed and replaced with 1 ml of fresh, equilibrated (37ºC, 5% CO_2) medium. To induce fiber differentiation, culture dishes were supplemented with 150 ng/ml of FGF-2 (PeproTech, Rocky Hill, USA) and cultured for either 24 hours, or 5 days. At the end of the culture period, immunocytochemistry for Fz receptors was carried out as described for the whole-mounts.

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