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A role for Wnt/β-catenin signaling in lens epithelial differentiation

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Abstract

The differentiation of epithelial cells and fiber cells from the anterior and posterior compartments of the lens vesicle, respectively, give the mammalian lens its distinctive polarity. While much progress has been made in understanding the molecular basis of fiber differentiation, little is known about factors that govern the differentiation of the epithelium. Members of the Wnt growth factor family appear to be key regulators of epithelial differentiation in various organ systems. Wnts are ligands for Frizzled receptors and can activate several signaling pathways, of which the best understood is the Wnt/β-catenin pathway. The presence of LDL-related protein coreceptors (LRPs) 5 or 6 has been shown to be a requirement for Wnt signaling through the β-catenin pathway. To access the role of this signaling pathway in the lens, we analyzed mice with a null mutation of *lrp6*. These mice had small eyes and aberrant lenses, characterized by an incompletely formed anterior epithelium resulting in extrusion of the lens fibers into the overlying corneal stroma. We also showed that multiple Wnts, including 5a, 5b, 7a, 7b, 8a, 8b, and Frizzled receptors 1, 2, 3, 4, and 6, were detected in the lens. Expression of these molecules was generally present throughout the lens epithelium and extended into the transitional zone, where early fiber elongation occurs. In addition to both LRP5 and LRP6, we also showed the expression of other molecules involved in Wnt signaling and its regulation, including Dishevelleds, Dickkopfs, and secreted Frizzled-related proteins. Taken together, these results indicate a role for Wnt signaling in regulating the differentiation and behavior of lens cells.

Keywords: Lens development; Wnt signaling; β-Catenin; LRP6; Lens epithelium

Introduction

How organs acquire their complex architectures and patterns of growth and maintenance has been an important area for research in developmental biology. The mammalian eye lens with its distinctive cellular architecture and growth patterns has been a useful model for studying mechanisms of morphogenesis and differentiation. Lens morphogenesis begins when ectoderm, situated next to the optic vesicle, grows and thickens to form the lens placode. Subsequent invagination of the placode forms the lens pit, which later closes to form the lens vesicle. Cells in the posterior segment of the lens vesicle, next to the optic cup, elongate to form the primary fibers, whereas cells in the anterior segment of the vesicle differentiate into the epithelium. The lens cells are contained within a capsule of extracellular matrix. From this stage onwards, the lens grows by continued proliferation of epithelial cells and differentiation of secondary fiber cells. Proliferation is restricted to the lens epithelial compartment. Progeny of epithelial divisions, that shift below the lens equator, elongate in the transitional

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zone to give rise to secondary fibers (McAvoy et al., 1999). These growth patterns ensure that lens polarity is maintained as secondary fibers are added to the fiber mass throughout life. This is important for the maintenance of the ordered cellular architecture that contributes to the transparency and optical properties of the lens (Trokel, 1962).

Throughout its morphogenesis and differentiation, the developing lens is closely associated with the optic vesicle/cup. Several early events in lens induction occur prior to association with the optic vesicle (Hirsch and Grainger, 2000); however, interaction with the optic vesicle appears to be important for localizing and promoting a lens-forming bias in the appropriate region of head ectoderm (Weaver and Hogan, 2001). Growth factors are important mediators of such inductive interactions and recent experiments indicate important roles for BMPs (Furuta and Hogan, 1998) and FGFs (Faber et al., 2001) in regulating some of these early inductive events.

Later stages of lens morphogenesis are characterized by the overt differentiation of the two forms of lens cells from the lens vesicle (McAvoy et al., 1999). Differentiation of epithelial and fiber cells involves acquisition of distinctive morphological and molecular characteristics that are required for the different structures and functions of cells in the two different lens compartments. The epithelial cells are predominantly cuboidal with strong intercellular adhesion and communication properties. They are firmly attached to the lens capsule and form an epithelial sheet that covers the anterior surface of the fiber mass. Lens fibers become highly elongated and, because of their hexagonal shape in cross-section, assume a highly ordered packing arrangement as they differentiate (see Taylor et al., 1996). How the two forms of lens cells differentiate in the two compartments of the lens to give it its distinctive polarity has been a major focus in lens developmental biology.

Studies over the last 20 years have concentrated on identifying factors that control fiber differentiation, and there is strong evidence that members of the FGF growth factor family (McAvoy et al., 2000; Govindarajan and Overbeek, 2001) and the TGFβ superfamily (De Iongh et al., 2001a; Faber et al., 2002; Belecky-Adams et al., 2002) are key regulators of this process. In contrast, little is known about the differentiation of the lens epithelium.

Members of the Wnt growth factor family have been shown to be involved in the regulation of many diverse functions during development in flies, frogs, and mice (Miller, 2002). In a number of organ systems, they appear to be key regulators of epithelial differentiation (see for example: Brisken et al., 2000; Tebar et al., 2001; Itaranta et al., 2002; Miller and Sassoon, 1998). Although extensively studied in other systems, little is known about the role of Wnts in lens development and growth. It is only recently that Wnts have been shown to be represented in the lens. The first Wnt to be detected in the lens was in the embryonic chicken, and this was identified as Wnt13 (Jasoni et al., 1999). Wnts function as ligands for members of the Frizzled (Fz) family of seven-pass receptors (Bhanot et al., 1996). So far, Fz1, Fz2, and Fz7 have been detected in the chick lens placode (Stark et al., 2000). Secreted frizzled-related proteins (sFRPs), which are thought to be involved in regulating the Wnt signaling pathway through interactions with the Fz receptors, have also been detected during mouse lens morphogenesis (Leimeister et al., 1998). In addition, the observation that Fz3 overexpression initiates ectopic eyes, including a morphologically distinct lens in *Xenopus,* indicates that Wnt signaling has an important role in eye development (Rasmussen et al., 2001).

The Fz receptors appear to be able to activate three main signaling pathways, including the Wnt/β-catenin pathway, the Wnt/planar cell polarity (PCP) pathway, and the Wnt/calcium pathway (Miller, 2002). Wnt/β-catenin is the best understood of the signaling pathways and is commonly referred to as the canonical pathway. The presence of LDL-related protein coreceptors (LRPs) 5 or 6, has been shown to be a requirement for Wnt signaling through this pathway (Tamai et al., 2000; Wehrli et al., 2000; Pinson et al., 2000; Zorn, 2001). Wnt signaling through this Fz/LRP complex leads to the accumulation of cytoplasmic β-catenin, a process that depends on the cell-autonomous activation of Dishevelled (Dvl), as well as the subsequent inactivation of the β-catenin destruction complex, which includes glycogen synthase kinase-3β, APC, and axin/conductin. This results in subsequent binding of β-catenin to members of the TCF/LEF transcription factor family. The second Wnt signaling pathway, the PCP pathway, does not require the LRP5/6 coreceptor and functions independently of β-catenin (McEwen and Peifer, 2001). Like β-catenin signaling, it depends on activation of Dvl; however, PCP signaling involves the DEP or DEP/PDZ domains as opposed to the DIX/PDZ domains used for β-catenin signaling (Moriguchi et al., 1999; Habas et al., 2003). Further details of this pathway in vertebrate tissues are uncertain, but there is evidence that PCP signaling may both affect the organization of the cytoskeleton directly (Schlesinger et al., 1999) and regulate the activity of the JNK signaling cascade (Boutros et al., 1998; Weston and Davis, 2002). Recent studies have shown that Dickkopf 1 (Dkk1) is an important regulator of Wnt signaling (Semenov et al., 2001). Dkk1 binding to LRP6 specifically blocks the β-catenin pathway but not PCP signaling. Moreover, recent evidence indicates that, when the canonical pathway is antagonized, the alternative JNK pathway is activated (Park and Moon, 2002). The third Wnt pathway, which also appears to be antagonistic to the canonical pathway (Torres et al., 1996), involves stimulation of intracellular calcium release and activation of two kinases, CamKII and PKC (Kuhl et al., 2000).

A growing list of gene targets for Wnt signaling includes E-cadherin, c-Myc, c-Jun, retinoic acid receptor, and connexin 43 (Hinck et al., 1994; He et al., 1998; Mann et al., 1999; McGrew et al., 1999; van der Heyden et al., 1998; and Ai et al., 2000); all of which are expressed in the lens and

play roles in cell adhesion, communication, and proliferation. Based on the potential for Wnt signaling to regulate expression of lens epithelial genes and the presence of Wnt and Wnt receptor expression in the embryonic chick lens, we hypothesized that Wnt signaling pathways play a role in governing the behavior of mammalian lens epithelial cells.

In this study, we analyzed mouse embryos homozygous for a mutation in the *lrp6* gene. These mutants show dysmorphogenesis of the lens, a notable feature being deficient differentiation of the epithelial sheet. We show that the lens expresses LRP6 and multiple Wnts and Fz receptors in the epithelium and in the transitional zone at the lens equator. We also show that key mediators and modulators of Wnt signaling, including Dvls, Dkks, and sFRPs, are expressed in, and near, the lens. These results indicate a role for Wnt/β-catenin signaling in lens development.

Materials and methods

Animal tissues

Eyes from 20–25 day postnatal (P20–P25) 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. Ciliary body and iris were also isolated from the sensory retina. The lens capsule and ciliary body/iris preparations were used for reverse polymerase chain reaction (RT-PCR) as described below. For in situ hybridization, eyes from P21 mice were fixed in 10% neutral buffered formalin (NBF) and embedded in paraffin wax. Knockout mice for *Wnt5a* (Yamaguchi et al., 1999) and *Wnt7a* (Parr and McMahon, 1998), and *lrp6* mutant mice (Pinson et al., 2000) were generated as described previously.

RT-PCR

Total RNA was extracted from dissected lens capsule (with adherent epithelial cells) or ciliary body and iris preparations by using Tri Reagent (Sigma, Sydney, Australia). First-strand cDNA synthesis was carried out by 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 1× reaction buffer (Promega, Sydney Australia). Samples were mixed at 4°C and heated to 94°C for 60 s, then amplified through 30–35 cycles (57–58°C for 30–60 s, 72°C for 45 s, and 94°C for 25 seconds), followed by a final extension at 72°C for 2 min. Specific primer sets were derived from the coding sequences for Wnts 1, 3, 4, 5a, 5b, 7a, 7b, 8a, 8b, 10a, and 11; Frizzleds (Fzs) 1–9; low-density Lipoprotein-Related Proteins (LRPs) 5 and 6; Dickkopfs (Dkks) 1, 2, and 3; secreted Frizzled-related proteins (sFRPs) 1, 2, 3, 4, and 5; Dishevelleds (Dvls) 1, 2, and 3 (see Table 1). PCR amplifications were optimized for each primer pair.

cDNAs

PCR products for Wnts 5a, 5b, 7a, 7b, 8a, and 8b; Fzs 1, 2, 3, 4, and 6; LRPs 5 and 6; Dkks 1, 2, and 3; sFRPs 1, 2, and 3; and Dvls 1, 2, and 3, were agarose gel purified and cloned into pGEM-T transcription vector (Promega). 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 by using SP6 and T3 RNA polymerases (Promega) and digoxigenin-labeled nucleotides (Roche, Mannheim, Germany).

In situ hybridization

In situ hybridization was performed on paraffin sections (6 μm) with digoxigenin-labeled probes for LRP6; Wnts 5a, 5b, 7a, 7b, 8a, 8b; Fzs 1, 2, 3, 4, and 6. For the LRP6 studies, sections of mice (FVB/N) at embryonic day 12.5 (E12.5), E14.5, and E18.5 were used. For the Wnt and Fz studies, sections of P21 mouse eyes were used. The in situ hybridization procedures were conducted as previously described for digoxigenin-labeled riboprobes (De Iongh et al., 2001b).

Histology and immunofluorescence

Formalin-fixed paraffin sections of *Wnt5a* and *Wnt7a* knockout mice and *lrp6* mutant mice were deparaffinized and rehydrated to PBS. Wild type littermate tissues were treated in parallel. For histological or histochemical analysis, 6-μm-thick sections were stained with either hematoxylin and eosin or Periodic-Acid-Schiff (PAS) reagent. Crystallin immunohistochemistry was carried out as described previously (De Iongh et al., 2001b).

Localization of the active form of β-catenin was carried out on wild type littermates of the LRP6 mutants by using a specific antibody generated against the nonphosphorylated Ser33 and Thr41, two of the N-terminal residues of β-catenin that are reported to be targets of Wnt signaling (see review at URL www.ana.ed.ac.uk/rnusse/pathway/bcatmut.html). Recent studies (van Noort et al., 2002) have used this antibody to visualize the active β-catenin from the canonical Wnt signaling pathway during mouse embryonic development. Sections were brought to PBS as described above. Prior to incubating sections with the antibody, an antigen-retrieval step was included (van Noort et al., 2002). Sections were then incubated in blocking solution as above, washed in PBS, and incubated with the hybridoma culture supernatant of anti-active β-catenin (α-ABC, clone 8E7). Reactivity was visualized with Alexa 488-conjugated goat

anti-mouse immunoglobulin as above. In controls, the α-ABC antibody was omitted or replaced by nonimmune IgG at an equivalent concentration.

Western blotting

To minimize animal numbers and maximize protein yield, the larger rat lenses were used to make lens cell lysate. Lens capsules with adherent epithelial cells were collected (according to the PCR protocol) in 500 μl of ice-cold PBS. Cells were lysed for 15 min in an equal volume of lysis buffer comprising 1.0% NP40, 0.2 M Tris base, 2 mM EDTA, 0.4 M NaCl, and one Complete Mini Protease Inhibitor Cocktail tablet (#1836153, Roche Basel, Switzerland) adjusted to pH 7.4 for 15 min on ice. Whole cell lysates were first centrifuged at 120*g* for 3 min to extract undissolved cellular and capsule material, and the resulting supernatant was recentrifuged at 35,000*g* for 5 min at 4°C to pellet noncytoplasmic debris. Protein concentrations were estimated by using a Bio-Rad protein assay (Bio-Rad Laboratories, Australia). Samples were mixed with dye loading buffer, denatured, and reduced (100°C), and samples (approximately 60 mg) were loaded and separated on 10% SDS–polyacrylamide gels with subsequent electrotransfer to PVDF membrane. The membranes were blocked in 5% nonfat milk powder, 5% FCS, in 0.1 M PBS overnight then rinsed two times in wash solution (1% FCS, 0.1% Tween 20, 0.1% nonfat skim milk powder in 0.1 M PBS). Membranes were incubated with primary antibodies (β-catenin; Transduction Laboratories, clone 14 and α-ABC) diluted in wash solution for 3 h at room temperature. Excess primary antibody was rinsed from membranes twice with wash solution and once with TPBS (0.01% Tween 20 and 0.1 M PBS). A goat anti-mouse IgG-HRP secondary antibody (1:3000; Bio-Rad) was applied for 1 h at room temperature. This was followed by three washes in TPBS and detection of HRP using the Vector NovaRed substrate kit (Vector, Burlingame, CA). The membranes were then dried and scanned.

Results

A functional role for Wnt signaling in the lens

LRP6 mutant

Recent studies have identified LRPs as key components of the receptor complex for Wnt ligands that signal through the β-catenin pathway. Here, we examined eye development in mice carrying a mutation for LRP6. This mouse model has an insertional mutation in the *lrp6* gene and no *lrp6* transcripts are detectable in homozygous mutant embryos (Pinson et al., 2000). At all stages, heterozygous mutant embryos could not be distinguished from wild type littermates. In the $lrp6^{-/-}$ embryos, considerable variation in the severity of the phenotype, at most embryonic stages, was noted. In the most severe cases, the eyes were substantially smaller and less advanced developmentally than their wild type and heterozygous littermates. Histological analysis showed that, at embryonic day 11.5 (E11.5), the wild type lens placode and the optic vesicle had grown and invaginated to form the lens vesicle and optic cup, respectively (Fig. 1B). An $lrp6^{-/-}$ embryo showed retarded lens development with only an incipient placode being evident (Fig. 1A). Pyknotic nuclei were also present in the lens and retinal primordia as well as the periocular mesenchyme. At E12.5, another $lrp6^{-/-}$ embryo with a severe phenotype had an eye that was distinctly smaller than the wild type. At this stage in the wild type lens, cells in the posterior hemisphere had elongated to form the primary fibers (Fig. 1D). However, in the mutant, there was relatively little elongation of the primary fibers (Fig. 1C). A quantitative analysis showed that mean length of fibers in $lrp6^{-/-}$ embryos (with a similar phenotype to that shown in Fig. 5D) was 60 ± SD 8.1 μms compared with 132 ± SD 8.9 μm in wild type mice at this stage. This difference was shown to be significant by the Mann–Whitney test ($U = 18$, $P = 0.03$, $n = 9$).

By E13.5, it was evident that the variation in the severity of the phenotype also extended to individual embryos as commonly one eye was absent with an aberrant eye on the contralateral side. Absence of an eye may have resulted from widespread cell death as extensive pyknosis was detected in and around the underdeveloped ocular primordia of some embryos at earlier stages (see Fig. 1A). In other E13.5 $lrp6^{-/-}$ specimens, the eyes were comparable to wild type eyes both in size and in the level of differentiation. Fig. 1E shows that the optic cup of an $lrp6^{-/-}$ embryo had differentiated into pigmented epithelial and neural layers of the retina. In the lens, primary fiber cells had elongated to about 294 μms in length (Fig. 1E). Fibers in wild type lenses were a comparable 317 ± SD 52 μms in length at this stage. The strong expression of β-crystallin, a key fiber-specific marker, also indicated that molecular differentiation had occurred in the primary fibers (Fig. 1F). In contrast to the fibers, the epithelial layer was not well developed and did not form a complete monolayer (Fig. 1F). Epithelial cells, characterized by their cuboidal/columnar morphology and lack of β-crystallin expression, were present in the peripheral regions of the lens but were absent centrally, at the anterior pole. In place of the epithelium at the anterior pole were β-crystallin-immunoreactive cells (Fig. 1F). Furthermore, in the absence of a complete epithelial sheet, it appeared that some β-crystallin-expressing cells had been extruded from the lens. Attenuated β-crystallin fluorescing cells were present on the corneal side of the lens capsule (indicated by the small arrowheads in Fig. 1F). In another E13.5 embryo, a more severe phenotype was evident (Fig. 1G and H). Development in this specimen was clearly delayed. Although most of the cells expressed α-crystallin, indicating their commitment to lens differentiation (Fig. 1G), cells in the posterior hemisphere of the lens vesicle had elongated only slightly, and the expression of

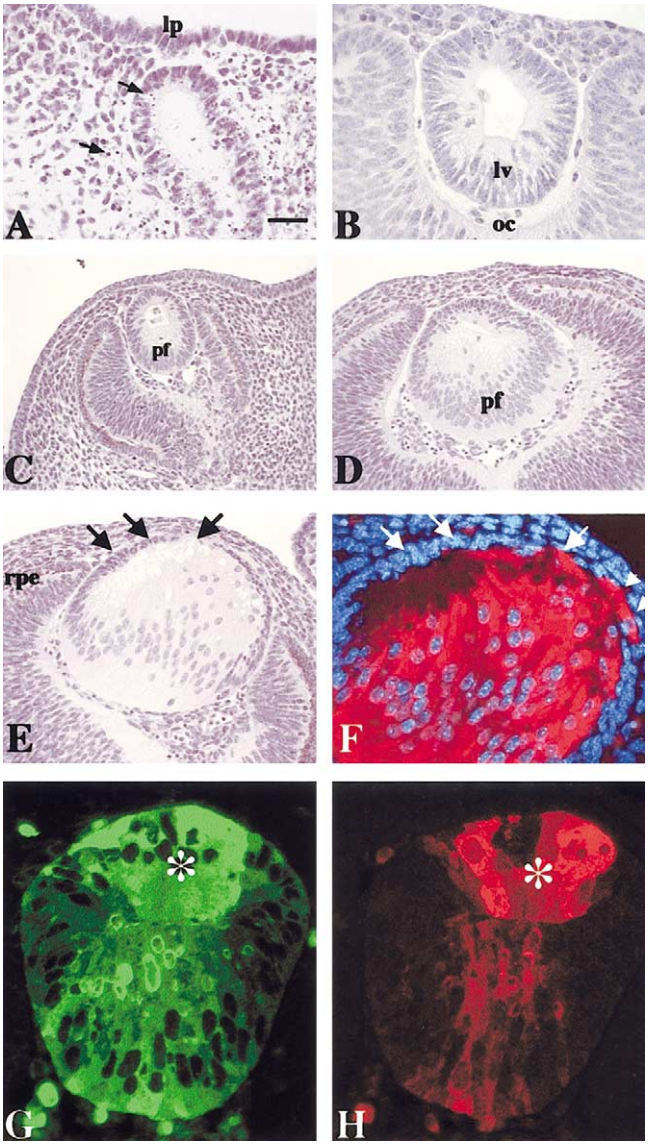

Fig. 1. *lrp6*$^{-/-}$ mutant mice: Sagittal sections of eyes from wild type (B, D) and *lrp6*$^{-/-}$ (A, C, E–H) embryos at E11.5 (A, B), E12.5 (C, D), and E13.5 (E–H) after staining with hematoxylin and phloxine (A–E), immunofluorescent localization of β-crystallin (F, H), or α-crystallin (G). Hoechst staining for nuclei was also included in (F). Sections through the developing eyes at E11.5 show that, in the wild type (B), the lens placode and the optic vesicle have grown and invaginated to form the lens vesicle (lv) and optic cup (oc), respectively. In contrast, lens morphogenesis at the same age in the *lrp6*$^{-/-}$ embryo is delayed and has only formed an incipient placode (lp, A). Pyknotic nuclei are present in the lens and retinal primordia and also in the periocular mesenchyme of the *lrp6*$^{-/-}$ embryo (arrows). At E12.5, the embryonic eye in the *lrp6*$^{-/-}$ mouse (C) is smaller than the wild type eye (D). At this stage, cells in the posterior hemisphere of the lens of the wild type have elongated to form the incipient primary fibers (pf, D). In the *lrp6*$^{-/-}$ lens, there is relatively little elongation of the primary fibers (C). At E13.5, in some *lrp6*$^{-/-}$ specimens, the eyes are comparable to wild types both in size and in the level of differentiation. The optic cup has differentiated into retinal pigmented epithelial (rpe) and neural layers (E). Lenses in some of the *lrp6*$^{-/-}$ mice show well-developed primary fibers (E) and express β-crystallin (F). In contrast, the epithelial layer in these embryos is not well developed and does not form a complete monolayer (E, F). In these embryos, epithelial cells with characteristic cuboidal/columnar morphology, and which do not express β-crystallin, are present in the peripheral regions of the lens (pair of arrows, E, F) but are absent centrally at the anterior pole of the lens, (single arrow, E, F). The cells at the anterior pole of the lens, are β-crystallin-immunoreactive cells (single arrow, F). In addition, elongated β-crystallin fluorescing cells are present on the corneal side of the lens capsule (arrowheads, F). In other E13.5 embryos, a more severe phenotype is evident (G, H). Most cells in the lens express α-crystallin (G), but cells in the posterior hemisphere of the lens vesicle have only elongated slightly and are weakly reactive for β-crystallin (H). Cells in the anterior hemisphere are also elongated and strongly express crystallins, including β-crystallin (asterisks, G, H). Scale bar: (A) and (B), 25 μm; (C) and (D), 60 μm; (E), 55 μm; (F), 35 μm; (G) and (H), 20 μm.

the fiber cell marker β-crystallin was weak (Fig. 1H). Cells in the anterior hemisphere remained large and strongly expressed β-crystallin (asterisk, Fig. 1H). Thus, these cells had acquired features of fiber cells and showed no evidence of epithelial differentiation, which is the normal developmental fate of cells in this anterior compartment.

At E14.5, the defect in the epithelium of the *lrp6*$^{-/-}$ embryos was more pronounced than seen earlier (Fig. 2A). At the anterior pole of the lens, epithelial cells appeared to be completely absent and in their place were clumps of cells (Fig. 2A) that were strongly reactive for β-crystallin (Fig. 2C and E). One of the most striking features of this lens at this stage was the presence of a large area of fiber-like lens material in the corneal stroma (Fig. 2A). Strong immunoreactivity for β-crystallin indicated that this material was lens-derived (Fig. 2C and E). Note that, in the wild type (Fig. 2B), the anterior layer of cuboidal epithelial cells was bounded by a continuous PAS-positive lens capsule (Fig. 2F), whereas in the *lrp6*$^{-/-}$ mutant, the PAS-positive lens capsule did not fully encapsulate the lens—gaps were present and this was where the fiber-like cells extruded from the lens (Fig. 2G). In the mutant, some epithelial-like cells were present in the peripheral regions of the lens. These cells did not extend very far above the lens equator and, because of the epithelial discontinuity at the anterior pole, did not form a continuous sheet.

In embryos surviving to E18.5, further differentiation of ocular tissues was evident, but eyes tended to be smaller than those of wild type mice (Fig. 3). In the optic cup of the *lrp6*$^{-/-}$ embryo, the retinal pigmented epithelium was well developed and several layers could be distinguished in the neural retina. This was generally similar to the wild type, except that in some regions the ganglion cell layer in the *lrp6*$^{-/-}$ mutant was greatly expanded and extended into the neuroblast layer (Fig. 3A and B). This observation taken together with the detection of strong expression of Wnts and Fzs in ganglion cells (data not shown) indicates that Wnt/β-catenin signaling may have a role in ganglion cell differentiation. At the margin of the optic cup, the ciliary and iridial retina appeared to have differentiated to a level comparable with that of the wild type (Fig. 3A and B). In the lens, the fiber cells had differentiated further and contained abundant α-crystallin (Fig. 3C) and β-crystallin (data not shown). Fiber cells and their crystallin contents extruded

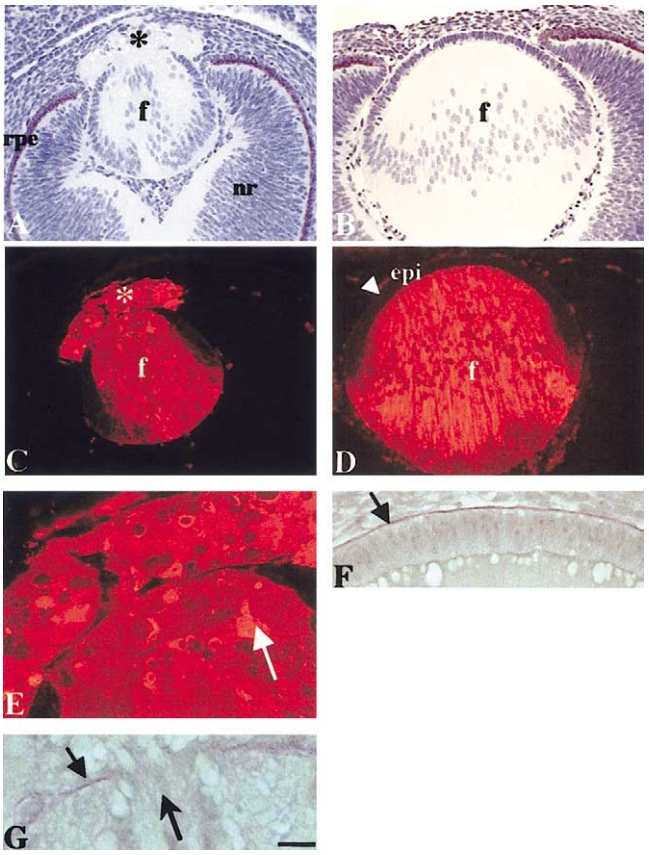

Fig. 2. *lrp6*$^{-/-}$ mutant mouse: Sagittal sections of an eye from an *lrp6*$^{-/-}$ mutant embryo (A, C, E, G) and a wild type embryo (B, D, F) at E14.5. Histological sections stained with hematoxylin and phloxine show that the embryonic eye in the *lrp6*$^{-/-}$ mutant (A) is smaller than the wild type (B). The optic cup in the *lrp6*$^{-/-}$ mutant has differentiated into neural retina (nr) and retinal pigmented epithelium (rpe). The lens epithelium is incompletely formed and elongating lens fibers (f) have extruded into the overlying cornea (asterisk). In the wild type (D), β-crystallin immunofluorescence shows the normal distribution of this protein: absent from the epithelium (arrowhead, epi) and present in the fibers (f). In the *lrp6*$^{-/-}$ mutant eye (C), immunofluorescence shows that lens fibers and/or their β-crystallin contents have extruded into the developing corneal stroma (asterisk). At higher magnification (E), the anterior pole of the lens shows the presence of large β-crystallin reactive cells (arrow) in place of the layer of cuboidal lens epithelial cells. PAS staining shows that the lens capsule forms a continuous layer along the basal surface of the lens epithelial cells in the wild type (arrow, F). In the *lrp6*$^{-/-}$ mutant lens, the PAS-positive capsule (small arrow, G) is discontinuous and at the anterior pole it does not form a barrier to the lens fiber mass. In this region, fiber cell material extrudes into the corneal stroma (large arrow, G). Scale bar: (A–D) 45 μm; (E), 15 μm; (F) and (G), 10 μm.

into the cornea from the anterior pole of the lens (large arrow, Fig. 3A, C, E, and G). Peripheral to the anterior pole, the epithelial cells formed a continuous layer but showed reduced reactivity for α-crystallin compared with the wild type lens (small arrows; cf. Fig. 3C and E with D and F, respectively). Also in the *lrp6*$^{-/-}$ mutant mice, the lens epithelial cells tended to be irregularly packed and more squamous, compared with the regularly packed cuboidal cells in the wild type lens (small arrows; cf. Fig. 3G and H).

β-Catenin signaling in the lens epithelium

The result with the *lrp6*$^{-/-}$ mutant mice indicates that functional LRP6, and hence Wnt/β-catenin signaling, is important for normal differentiation of the lens epithelium. To investigate whether this is likely to be due to a direct effect on the lens epithelium, we studied the expression of LRP6 in the embryonic lens from E12.5 to E18.5. At E14.5, LRP6 is strongly expressed throughout the developing epithelium. At the lens equator, the signal diminishes and is absent from the maturing fibers (Fig. 4A and B). This pattern of LRP6 expression is similar for E12.5 and E18.5 stages (data not shown).

To seek other evidence that Wnt/β-catenin signaling is a feature of normal lens cells, we conducted immunochemical studies using an antibody that specifically recognizes active β-catenin (α-ABC). At E14.5, strong reactivity for active β-catenin was detected in lens epithelial cells (Fig. 4D). Most of the reactivity was in the cytoplasm but some also appeared to be associated with some of the nuclei. The reactivity extended into the transitional zone at the lens equator but disappeared as the fibers elongated. The reactivity in the primary fiber cells was nonspecific as similar labeling was detected in controls where the primary antibody had been omitted (Fig. 4C). A similar pattern of reactivity was detected at E18.5 (data not shown). Western blotting was also carried out to confirm that active β-catenin was present in the lens epithelium. Material probed with antibodies against β-catenin and α-ABC showed bands at 92 kDa, the expected molecular weight for β-catenin (Fig. 4E).

Wnt7a and Wnt5a null mice

In another approach to investigate the role of Wnt signaling in the lens, we examined Wnt7a and Wnt5a knockout models. These Wnt family members are expressed strongly in the lens (see later) and represent different classes of ligands. According to some assays, Wnt7a is a representative of the Wnt1 class of ligands that mainly signal through the canonical β-catenin pathway, whereas the Wnt5a class of ligands signal through less well-characterized pathways (Moon et al., 1997).

In the *Wnt7a* homozygous null mouse, no gross morphological defects were observed in the lens in situ. Sections of postnatal lenses showed that the lens, and other ocular tissues, appeared normal (Fig. 5A). *Wnt5a* homozygous null mice die just before birth; however, the eyes were well developed and a careful histological analysis of an E18.5 embryo showed that the lens and other ocular tissues had apparently normal architecture (Fig. 5B).

Wnt expression in the lens

To investigate Wnt expression in the lens, RT-PCR was carried out on RNA extracted from P20–P25 rat lens capsules (with adherent epithelial cells). All reverse transcription reactions were carried out by 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 Wnts 5a, 5b, 7a, 7b, 8a, and 8b resulted in distinct amplification of products of expected sizes (Table 1; Fig. 6A). The specific PCR fragments for these Wnts were cloned and sequenced to confirm sequence identity with those reported in GenBank. RT-PCR with primers for other commonly expressed Wnts (including 1, 3, 4, 10a, and 11) resulted in weak amplification of product (data not shown).

To investigate the spatial expression patterns of the Wnts in the murine eye that were strongly amplified by PCR, cDNAs were used as templates to prepare digoxigenin-labeled riboprobes for in situ hybridization analyses. The probe for Wnt8a showed labeling in the cytoplasm of lens epithelial cells (Fig. 6B). Signal was present throughout the epithelium and extended to the equatorial region of the lens (Fig. 6C). This region includes (1) the region above the equator, at the epithelial periphery, where the cells change from cuboidal to columnar in shape, and (2) the transitional zone below the equator where the cells undergo major elongation and change from columnar to highly elongated cells. Expression in the lens tended to be strongest in the equatorial region. Outside the zone of early fiber elongation at the equator, expression was reduced and was absent from the fiber cells in the cortex and deeper regions of the lens. Transcripts were also prominent in the corneal epithelium and endothelium as well the ganglion cell layer of the neural retina (data not shown). In situ hybridization with probes for Wnts 5a, 5b, 7a, 7b, and 8b showed essentially similar patterns of expression (data not shown).

Frizzled expression in the lens

To investigate Fz expression in the lens, RT-PCR was carried out as for Wnts. Using primers for Fzs 1, 2, 3, 4, and 6 resulted in amplification of products of expected sizes (see Table 1; Fig. 7A); cloning and sequencing confirmed their identity. RT-PCR with primers for other Fzs (including 5, 7, 8, and 9) resulted in no detectable amplification of product.

As with the Wnts, cloned Fz cDNAs were used to prepare digoxigenin-labeled riboprobes for in situ hybridization on sections of postnatal mouse eyes. The probe for Fz4 showed labeling in the cytoplasm of lens epithelial cells (Fig. 7B). Similar to the Wnts described above, the signal was present throughout the epithelium and extended to the lens equator (Fig. 7C). As for the Wnts, transcripts were reduced after the phase of early fiber elongation in the transitional zone and were absent from the fiber cells in the cortex and in the deeper regions of the lens. Transcripts were also prominent in the corneal epithelium and endothelium as well the ganglion cell layer of the neural retina (data not shown). The patterns of expression of Fzs 1, 3, and 6 were similar to those shown for Fz4 (data not shown).

For both Wnts and Fzs, sense controls showed no distinct labeling. In addition, because all Wnt and Frz genes examined were expressed primarily in the lens epithelium, we carried out a positive control to ensure that our in situ hybridization protocol was capable of detecting RNA in fiber cells. A riboprobe for β-crystallin (De Iongh et al., 2001a), which is known to be fiber-specific (McAvoy, 1978), showed strong β-crystallin expression in the fibers (Fig. 7D).

Wnt signaling in the lens

To further examine the potential for Wnt signaling in the lens, we looked for the expression of Dvl mRNA. RT-PCR using primers specific for Dvls 1, 2, and 3 resulted in distinct amplification of products of expected sizes for Dvl2 and Dvl3 (Table 1; Fig. 8A). A weak amplification product for Dvl1 was also detected. Cloning and sequencing of the PCR fragments confirmed their identities.

How the different Wnt signaling pathways are regulated is currently a major focus of research. There is compelling evidence that other factors, which are part of the ligand/receptor complex, are involved. For example, as described earlier, LRP5 and LRP6 act as coreceptors for Wnt/β-catenin signaling. Dkk1 is another important regulatory factor that binds to LRPs and specifically blocks the β-catenin signaling pathway. To investigate expression of these molecules in the lens epithelium, RT-PCR was carried out as for the Wnts. Using primers for both LRPs and Dkks 1, 2, and 3 resulted in strong amplification of products of expected sizes for LRP6, consistent with our in situ results, and LRP5 (Table 1; Fig. 8B) and Dkks 1 and 3 (Table 1; Fig. 8C). A weak amplification product for Dkk2 was also detected (not shown). Because the Dkks are secreted, we also examined expression in the extralenticular tissues such as the ciliary body and iris. RT-PCR was carried out on RNA extracted from P20-P25 mouse ciliary body/iris preparations. All three Dkks were detected in this preparation (Fig. 8C). Cloning and sequencing of PCR fragments for all these factors confirmed their identities.

Other important regulatory factors are the sFRPs. These generally interfere with ligand receptor interactions and can block all Wnt signaling pathways. To determine whether sFRPs are expressed in the postnatal lens epithelium, RT-PCR was carried out as for Wnts. Using primers for sFRPs 1, 2, 3, 4, and 5 resulted in amplification of products of expected sizes for sFRPs 1, 2, and 3 (Table 1; Fig. 8D); cloning and sequencing confirmed their identities. Weaker amplification of products of expected sizes for sFRPs 4 and 5 was also detected (not shown).

Discussion

In this study, we present evidence that Wnt/β-catenin signaling has a role in lens development. In one part of the study, we analyzed mouse embryos homozygous for a mutation in the *lrp6* gene. These mutants show dysmorphogen-

esis of the lens, a notable feature being deficient differentiation of the epithelial sheet. In another part of the study, we show that the lens expresses multiple Wnts and Fz receptors in the epithelium and in the transitional zone at the lens equator. We also show that key intracellular mediators of Wnt signaling, Dvls 1, 2, and 3, and modulators of Wnt signaling, including Dkks 1, 2, and 3 and sFRPs 1, 2, and 3 are expressed in, and near, the lens. These results indicate a role for Wnt/β-catenin signaling in the lens, particularly in relation to differentiation of the lens epithelium.

Although numerous studies have been carried out on the effects of Wnt signaling on many cell types, to date no method is available for reliably purifying Wnt proteins that retain biological activity (e.g., see URL www.stanford.edu/~rnusse/assays/wntproteins.html). In many cases, insights into the role of Wnt signaling during mammalian development have come from analysis of the phenotypes of mutant, transgenic, and knockout mouse models in which expression of Wnts or various signaling components is disturbed. To assess a role for Wnt signaling in the lens, we examined a mouse model that is deficient in a cofactor, which is required for signaling by several Wnts. Recent studies have identified LRP5 and LRP6 as key components of the receptor complex for Wnt ligands that signal through the β-catenin pathway (Tamai et al., 2000; Werhli et al., 2000; Zorn, 2001). A mouse that is homozygous for a mutation of the *lrp6* gene exhibits developmental defects that are a composite of those in mice carrying mutations of *Wnt1*, *Wnt3a*, and *Wnt7a*. This has been interpreted to indicate a requirement for *lrp6* for efficient signaling by several Wnt proteins that signal through the β-catenin pathway (Pinson et al., 2000). Recent studies have substantiated this and provided details of other key components of the LRP6-receptor complex that are involved in regulating canonical Wnt signaling (see Mao et al., 2002).

Our studies show that LRP6 is expressed, and active β-catenin is present, in the lens epithelium during development. Evidence for a functional role for Wnt/β-catenin signaling in the lens comes from our detailed analysis of the ocular phenotype in the $lrp^{-/-}$ mutant mouse. In these mutant embryos, although the eye is frequently smaller than the wild type, the optic vesicles differentiate into optic cups with retinal pigmented epithelial and neural retina layers. Differentiation of the lens vesicle in these mutants is characterized by deficiencies in the anterior segment. In most cases, the central epithelium appears to be absent or incomplete in the $lrp6^{-/-}$ mutant mice. This may be a result of degeneration of the cells of the anterior hemisphere of the lens vesicle. Alternatively, they may differentiate aberrantly into the short, swollen, β-crystallin reactive, fiber-like cells, that accumulate at the anterior pole of the developing lens. Primary fiber cells differentiate from the posterior hemisphere of the lens vesicle, and as they progressively grow and elongate they, and/or their cytoplasmic contents, extrude from the anterior pole of the lens into the corneal stroma. Thus, it appears that a major effect of the mutation is the disruption of the differentiation of the epithelial monolayer.

Close examination of the $lrp6^{-/-}$ mutant also indicates that the epithelial defect is primarily in the central region of the lens, whereas cells in the peripheral epithelium and transitional zone appear comparatively normal. Since Wnts and Fzs are expressed in the peripheral epithelium and transitional zone as well as the central epithelium, this may indicate that β-catenin signaling may not be so important, and/or that an alternative Wnt signaling pathway(s) may predominate, in regions peripheral to the central epithelium. In this context, it should be noted that, in the normal lens, there are structural and functional differences between cells in central and equatorial regions of the epithelium. In the central epithelium, the cells tend to be cuboidal, whereas toward the periphery, they tend to be low columnar. In the transitional zone below the lens equator, the columnar epithelial cells undergo major changes in cell shape and polarity as they begin the process of fiber differentiation. Coincident with the major elongation that occurs in this region, the cells take on a flattened hexagonal shape (exhibiting two long sides and four short sides in cross section) and form highly ordered arrays (Taylor et al., 1996). Changes in cell shape and polarity in other cellular systems are characteristically regulated by signaling through the Wnt-activated PCP pathway; eye and wing development in Drosophila and gastrulation movements in vertebrates are commonly used examples (Huelsken and Birchmeier, 2001). Therefore, it is plausible that alternative Wnt signaling pathways may be a feature of the peripheral lens epithelium and transitional zone, whereas cells in the central region may be more dependent on canonical Wnt signaling.

Another possible explanation for the central location of the epithelial deficiency in the $lrp6^{-/-}$ mutant is that the other LDL homologue, LRP5, is able to compensate for the absence of LRP6 in the more peripheral regions of the epithelium. Investigation of LRP5 expression in both wild type and $lrp6^{-/-}$ lenses may provide some insight into such putative compensatory mechanisms. However, LRP5 and LRP6 may not be fully interchangeable as some differences in their properties have been reported. For example, overexpression of LRP6, but not LRP5, induced dorsal axis duplication in Xenopus (Tamai et al., 2000). Also, recent studies of mice (Kato et al., 2002) and human patients (Gong et al., 2001) with mutations of *LRP5* show different phenotypes to those reported for $lrp6^{-/-}$ (Pinson et al., 2000). Both murine and human *LRP5* mutations result in bone and eye phenotypes. While eye phenotypes are present in both $lrp6^{-/-}$ and *LRP5* mutants, the former is evident during embryogenesis, whereas the latter is evident postnatally. The eye phenotype of the *LRP5* mutants does not appear to involve the lens but appears to be largely due to a failure of programmed regression of capillaries and, at least in the mouse, results in retention of the hyaloid vasculature throughout life (Gong et al., 2001; Kato et al., 2002). The $lrp6^{-/-}$ mutant is embryonic lethal so no information is

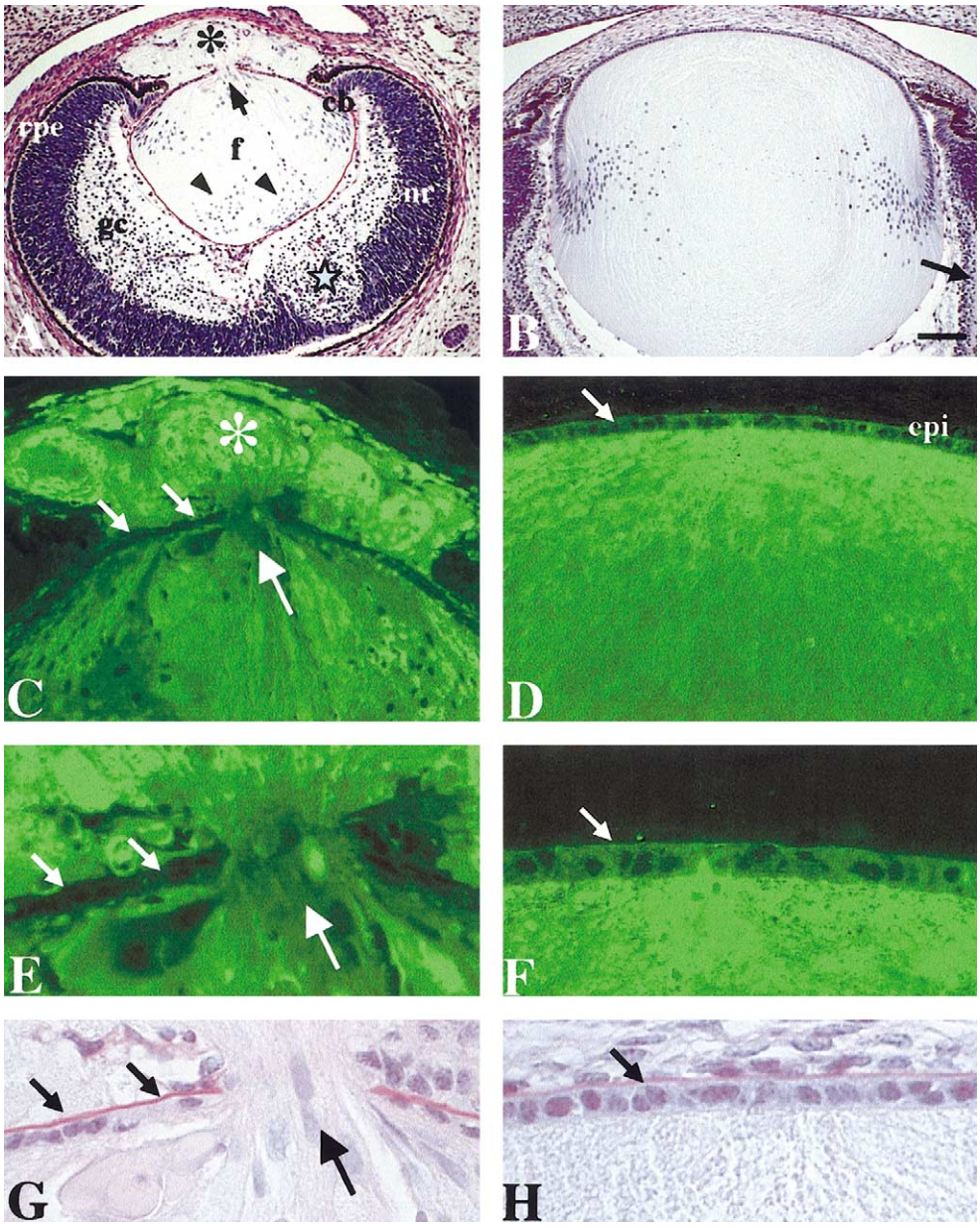

Fig. 3. *lrp6*$^{-/-}$ mutant mouse: Sagittal sections of eyes from *lrp6*$^{-/-}$ (A, C, E, G) and wild type littermate (B, D, F, H) embryos at E18.5. Histological sections stained with hematoxylin and phloxine show that the embryonic eye in the *lrp6*$^{-/-}$ mutant (A) is smaller than the eye of the wild type littermate (B). The optic cup has differentiated into neural retina (nr) and retinal pigmented epithelial (rpe) layers. The neural retina in the *lrp6*$^{-/-}$ mutant is generally similar to the wild type, except that the ganglion cell (gc) layer in the *lrp6*$^{-/-}$ mutant appears to be much more extensively layered compared with the wild type (B, arrow), and in one region, the ganglion cells have extended into the neuroblast layer (A, star). At the margin of the optic cup, the presumptive ciliary body (cb) and associated iris appear to have differentiated to a level comparable with that of the wild type. In the lens, fiber (f) cells have elongated, and immunofluorescence shows that they have accumulated α-crystallin (C, E). Abnormal distribution of nuclei in the posterior hemisphere of the lens indicates that the denucleation process of the fibers is disturbed (A, arrowheads). The lens epithelium is incompletely formed and through a gap at the anterior pole (A, C, E, G, large arrow) elongating lens fibers (f) have extruded into the overlying cornea (A, C, asterisk). Immunofluorescence of the *lrp6*$^{-/-}$ mutant shows cells reactive for α-crystallin present in the corneal stroma (C, E). In the wild type littermate, the epithelial (epi) cells form a continuous layer over the fiber mass and are reactive for α-crystallin (D, F, arrow). In the *lrp6*$^{-/-}$ mutant, α-crystallin is barely detectable in the epithelial cells (C, E, pair of small arrows). In the *lrp6*$^{-/-}$ mutant, the PAS-positive capsule is discontinuous in the region where the fibers are extruded and the epithelial cells that are present tend to be more squamous and irregularly packed compared to the cuboidal regularly packed cells in the wild type (cf. G and H, small arrows). Scale bar: (A) and (B), 110 μm; (C) and (D), 45 μm; (E–H), 20 μm.

available on hyaloid vasculature regression. Thus, available evidence indicates that the two Wnt coreceptors may recognise different classes of Wnts. Further evaluation of the relative roles of LRP5 and LRP6 in the lens awaits further experimentation.

Consistent with an important role for Wnt signaling in

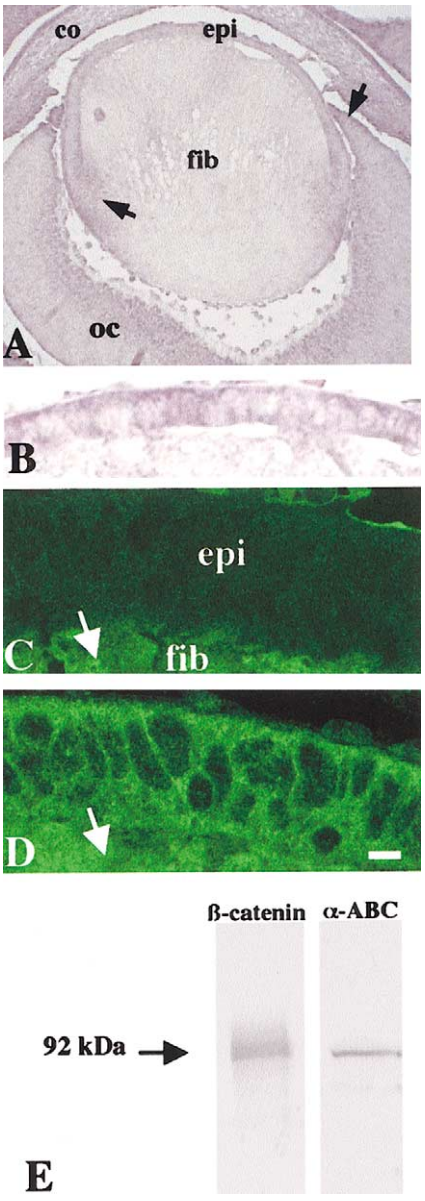

Fig. 4. β-Catenin signalling in the lens epithelium: (A, B) Sagittal sections of an E14.5 lens hybridised with a riboprobe for LRP6. Transcripts were detected in the cytoplasm of epithelial (epi) cells. Signal intensity diminished sharply just after the onset of fiber elongation below the lens equator (A, lens arrow) and was absent from the primary fibers (fib). Signal for LRP6 was also distinct in the cornea (co), particularly at its surface, and at the optic cup (oc) margin (A, arrow), (C, D) Immunohistochemistry for nonphosphorylated (active) β-catenin using the α-ABC antibody. Confocal microscopy shows reactivity in the lens epithelial cells (D). Reactivity is mainly cytoplasmic although punctate reactivity is detectable in some nuclei. Underlying fibers (arrows) show reactivity but this is nonspecific as it is also present in the control (C) where the primary antibody was omitted. (E) Western blotting for β-catenin: Protein (60 mg/lane) from P21 rat epithelial cell lysates was blotted with an antibody to β-catenin and an antibody specific for nonphosphorylated β-catenin (α-ABC). A strong 92-kDa band for total β-catenin was detected and a weaker, but distinct, band was detected for nonphosphorylated β-catenin. Scale bar: (A), 65 μm; (B), 15 μm; (C, D), 5 μm.

the mammalian lens, this study also shows the expression of multiple Wnts, their cell surface receptors, and Wnt signaling molecules. By RT-PCR, we detected strong expression of the ligands Wnt 5a, 5b, 7a, 7b, 8a, and 8b, and by in situ hybridization studies in postnatal mice, showed that they all have essentially similar patterns of expression. They are expressed throughout the lens epithelium and in the transitional zone at the lens equator, where the epithelial cells undergo early fiber elongation. Similarly, RT-PCR and in situ hybridization analyses showed that the receptors Fz 1, 2, 3, 4, and 6 are expressed in the lens in very similar patterns to each other (and to the Wnt ligands), being detected throughout the epithelium and transitional zone. Co-receptors, LRP5/6, which are required for Wnt signaling through the β-catenin pathway were also expressed in the lens. In addition, expression of other molecules that are known to be involved in Wnt signaling and its regulation, including Dvls, Dkks, and sFRPs, were detected in the lens.

Considerable attention is currently being directed at understanding how different Wnt signaling pathways are regulated. There is growing evidence that other factors associated with the ligand/receptor complex are involved. As mentioned above, LRP5/6 is required only for β-catenin signaling. Recent studies indicate that LRP5/6, when part of the Wnt ligand-receptor complex, removes axin from the β-catenin destruction complex, resulting in stabilization of β-catenin and its translocation to the nucleus (Mao et al., 2001; Zorn, 2001). In the absence of LRP5/6, the β-catenin pathway is inactive, but Wnt signaling can proceed through the PCP pathway, for example (Semenov et al., 2001). Dkks have been shown to be important regulators of β-catenin signaling (Semenov et al., 2001). For example, Dkk1 binds to LRP6 and specifically blocks the β-catenin signaling pathway. This does not inhibit PCP signaling, and in fact, recent evidence indicates that when the canonical pathway is antagonized, the alternative JNK pathway is activated (Park and Moon, 2002). Dkks are expressed in the lens and therefore may have a role in regulating Wnt/β-catenin signaling so that cells in different functional domains can alternate between different Wnt signaling pathways.

Wnts and Fzs are expressed in the central lens epithelium

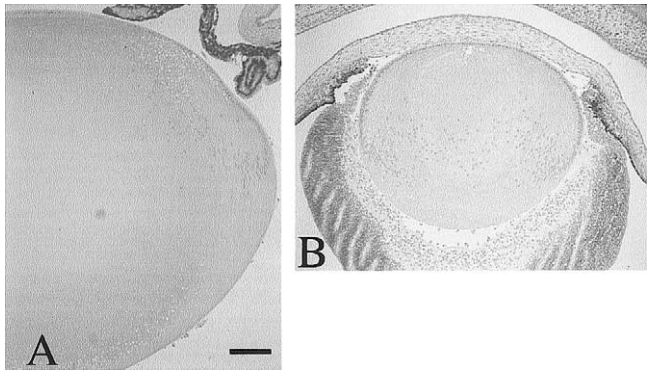

Fig. 5. Histology of *Wnt* null mice eyes: Hematoxylin and phloxine-stained sections of *Wnt7a* (A) and *Wnt5a* (B) null mice eyes. At P21, eyes from *Wnt7a* null mice showed normal morphology and were similar in size to wild type eyes. At E18.5, eyes from *Wnt5a* null mice showed normal morphology and were similar in size to wild type eyes. Scale bar, 110 μm.

Table 1
Primers used for RT-PCR

Gene	Accession No.	Primer sequences (5′ to 3′)		PCR product size	Nucleotides
		Forward	Reverse		
Wnt5a	M89798	aatattaagcccaggagtgg	tggcagagtttcttctgtcct	234	568–801
Wnt5b	M89799	cattgggatgggttgagg	caggaagttggcagcacac	181	16–196
Wnt7a	M89801	cctgggccacctctttct	tgggagccaggcctgggat	116	24–139
Wnt7b	M89802	tcgaaagtggatcttttacgt	tggggccaggccaggaatca	124	15–138
Wnt8a	Z68889	ctgactactgcaaccgcaac	tgacagtgcaacaccactga	201	823–1023
Wnt8b	AF130349	ccagagttccgggaggtag	gagatggagcggaaggtgt	131	726–856
Fz1	AF054623	cagttcacttccgacaaagg	aggtaggaaggcaccctgag	170	673–842
Fz2	NM02051	ggaactcctgcgctactcac	gcgctcacccagaaacttat	183	568–750
Fz3	U43205	ttttccatgggcgtagga	taacacggttcatgctggtg	447	1841–2287
Fz4	U43317	acaaccacatgtgcatggaa	tccttagctgagcggctgta	190	788–977
Fz6	U43319	ccctcgtaagaggacacagc	ttgcaagatgcagaaagtgc	156	186–341
Dv11	U10115	gggggtagtggcagtgaa	acctgtaagttctggagggaca	237	2051–2287
Dv12	U24160	gcagtggcagtgagtcagaa	tcatggggttataggggagag	192	2071–2262
Dv13	U41285	caaggagaaggacccaaaag	atcgggggaccatagagag	240	1971–2210
LRP5	AF064984	gatgtgcggctagtggatg	gcccgagatgacaatgttct	195	233–427
LRP6	AF074265	gggccgatgcaaaacttaat	cctctgttggctgaaagcat	242	678–919
Dkk1	AF030433	gaggggaaattgaggaaagc	gcaggtgtggagcctagaag	356	488–843
Dkk2	AJ243963	ccaaacccaactccatcaa	gcaacacatcccatctctgt	254	239–492
Dkk3	AF177400	ctcctatgccagccacaca	aatctcctcctctccgccta	237	913–1149
sFRP1	U88566	tgctcaaatgtgacaagttcc	atgagaaagttgtggctgaggt	349	717–1065
sFRP2	U88567	agctcccaaggtgtgtgaag	accagatacggagcgttgat	276	736–1011
sFRP3	BCO16884	caagggacaccgtcaatctt	catatcccagcgcttgactt	182	1056–1237

and in the transitional zone at the lens equator (see Figs. 6 and 7). This expression pattern indicates a role for Wnt signaling in promoting the epithelial phenotype and possibly in mediating major changes in cell shape and polarity in the transitional zone. In contrast, the cessation of Wnt/Fz expression during fiber differentiation at about the time that the fiber-specific marker, β-crystallin appears (compare Fig. 7C with D) indicates that Wnt signaling is not required for, and may even inhibit, aspects of the fiber differentiation process. This is consistent with the lens phenotype in

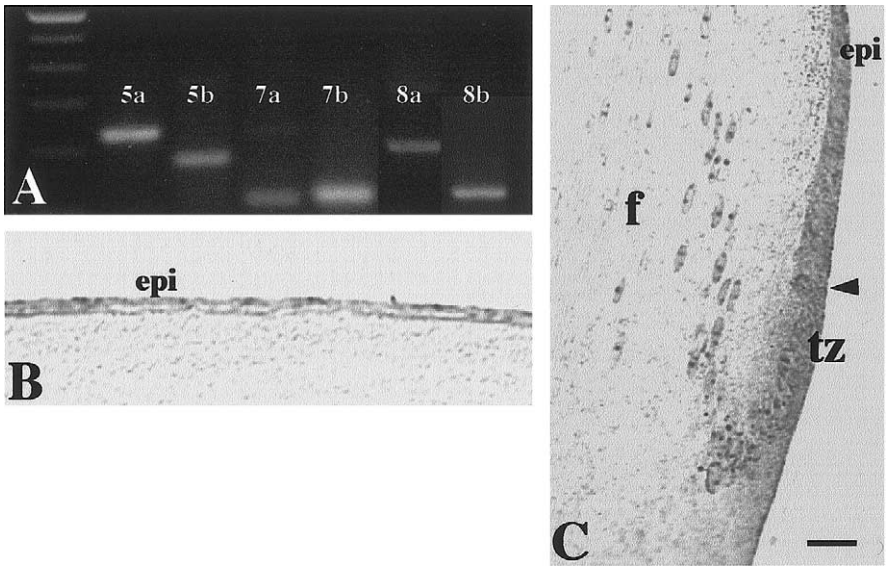

Fig. 6. Expression of Wnts in the postnatal lens. RT-PCR products were amplified from P20–P25 rat lens epithelial-derived cDNA (A). Specific amplification products for Wnts 5a, 5b, 7a, 7b, 8a, and 8b were detected. (B, C) Sagittal section of a P21 mouse lens hybridized with a riboprobe for Wnt8a. Transcripts were detected in the cytoplasm of lens epithelial cells (epi) in both the central (B) and peripheral (C) regions. Signal intensity tended to be stronger in the cuboidal/columnar cells at the epithelial periphery just above the lens equator (arrowhead). Below the equator, in the transitional zone (tz), the elongating cells expressed Wnt8a. Signal was reduced or absent from the cytoplasm of fiber (f) cells in the cortex and deeper regions of the lens, although it was commonly detected in their nucloli. Scale bar, 35 μm.

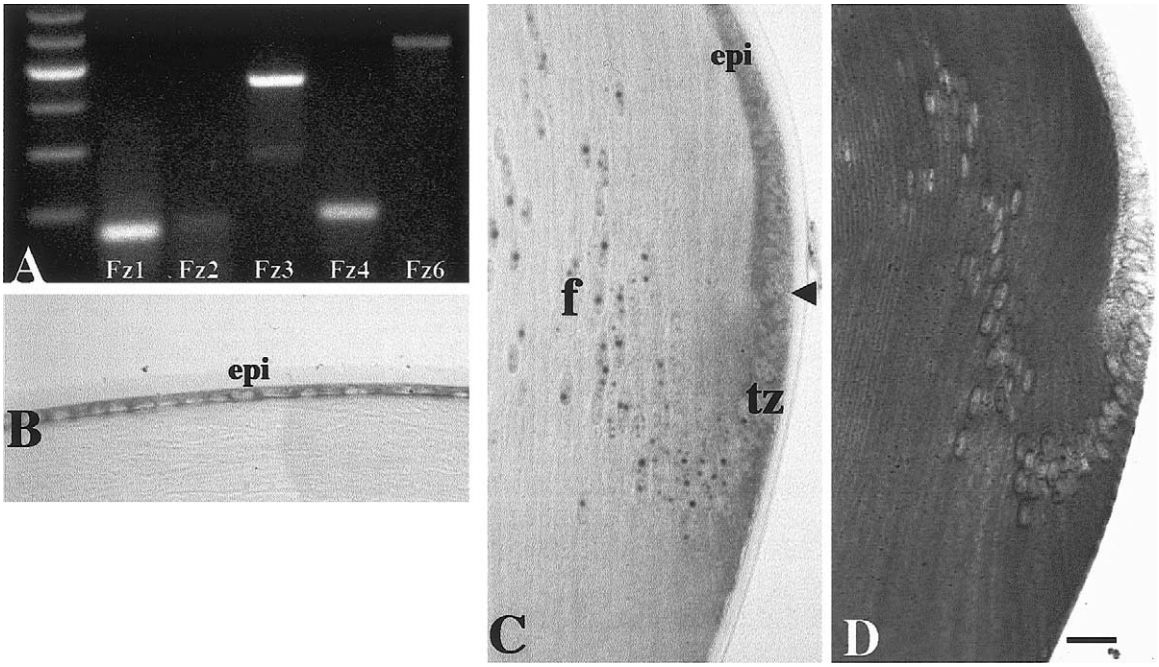

Fig. 7. Expression of Fzs in the postnatal lens. RT-PCR products were amplified from P20–P25 mouse (except Fz1 which was from rat) lens epithelial-derived cDNA (A). Specific amplification products for Fzs 1, 2, 3, 4, and 6 were detected. (B, C) Sagittal section of a P21 mouse lens hybridized with a probe for Fz4. Transcripts were detected throughout the cytoplasm of lens epithelial cells (epi) in both the central (B) and peripheral (C) regions. Below the lens equator (arrowhead), the elongating cells in the transitional zone (tz) expressed Fz4. Signal was reduced or absent from the cytoplasm of the fiber (f) cells in the cortex and deeper regions of the lens, although it was commonly detected in their nucleoli. Another sagittal section was hybridized with a probe for fiber-specific β-crystallin (D). This showed a distinctly different pattern of expression, with abundant expression detected in the fibers but not in the epithelial cells. Scale bar, 35 μm.

lrp6$^{-/-}$ mutant mice; no central lens epithelial layer forms, instead there is a layer of swollen β-crystallin reactive cells. These may have arisen due to the absence of Wnt signaling in this region. Thus, Wnt signaling in the anterior segment of the lens may have a role in inhibiting aspects of fiber differentiation. Such inhibitory molecules have been proposed to play important roles in maintenance of lens polarity (Hyatt and Beebe, 1993).

In this study, we also examined knockout mouse models for *Wnt7a* (Parr and McMahon, 1998) and *Wnt5a* (Yamaguchi et al., 1999). These purportedly represent different classes of Wnts that appear to signal via different pathways. By some assays, Wnt7a belongs to the Wnt1 class that act through the canonical signaling pathway that involves stabilization of β-catenin and its translocation to the nucleus, where it forms transcriptional complexes with members of the LEF/TCF family of DNA binding factors to control expression of Wnt target genes. On the other hand, Wnt5a belongs to another class of Wnts that act through less well-defined pathways that mediate cell proliferation, cell polarity, and cell movements (Moon et al., 1997). Neither knockout mouse model displayed any abnormal lens phenotype at either the gross morphological or histological level, indicating that neither of these Wnts, on their own, is essential for the development and growth of the lens. Given that multiple Wnts with similar expression patterns are present in the lens, the lack of a lens phenotype in these two

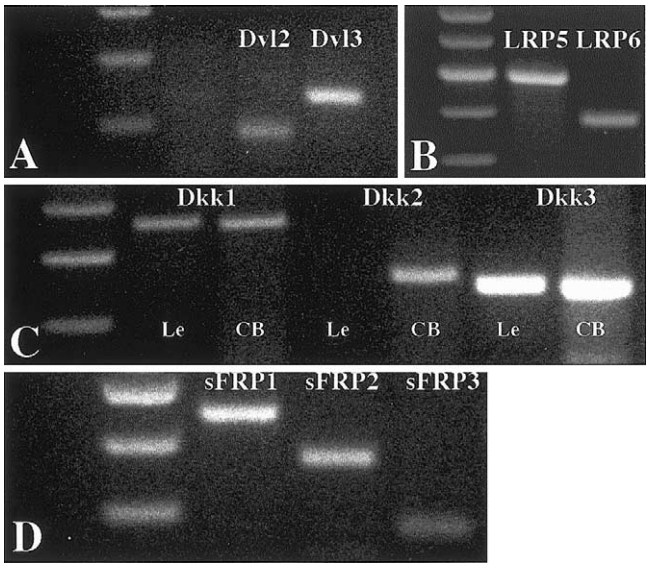

Fig. 8. Expression of Wnt regulatory and signaling molecules: RT-PCR products were amplified from mouse (Dkks and sFRPs) or rat (Dvls and LRPs) lens epithelial- or ciliary body-derived cDNA (A–D). Specific amplification products for Dvls 2 and 3 (A), LRPs 5 and 6 (B), were detected in the lens epithelium. Dkks 1 and 3 (C) were detected in the lens epithelium (Le) and ciliary body (CB), and Dkk2 was also detected in the ciliary body. Specific amplification products for sFRP1, sFRP2, and sFRP3 were detected in the lens epithelium (D).

mutant mice suggests there may be some functional redundancy for the Wnt ligands.

The Wnt family of secretory glycoproteins is one of the major families of developmentally important signaling molecules that play important roles in embryonic induction, generation of cell polarity, and specification of cell fate. Up to now, little is known about Wnt signaling in the mammalian eye. This study has reported on the expression of Wnts and Wnt signaling molecules in the lens. It has also presented evidence from analysis of the phenotype of the *lrp6*$^{-/-}$ mutant mouse that indicates an important role for Wnt signaling in lens development. Specifically, it appears to be important for the formation of the lens epithelium. This study also raises questions about the roles of the different putative signaling pathways: the Wnt/β-catenin, Wnt/PCP, and Wnt/calcium pathways. Detailed knowledge of the Wnts, their receptors, the Fzs and LRP5/6, and key Wnt signaling regulators will be required to elucidate the domains of Wnt signaling activity in the lens and their roles in influencing cell behavior in the different cellular compartments.

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