Wnt signaling enhances FGF2-triggered lens fiber cell differentiation

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Summary

Wnt signaling is implicated in many developmental processes, including cell fate changes. Several members of the Wnt family, as well as other molecules involved in Wnt signaling, including Frizzled receptors, LDL-related protein co-receptors, members of the Dishevelled and Dickkopf families, are known to be expressed in the lens during embryonic or postembryonic development. However, the function of Wnt signaling in lens fiber differentiation remains unknown. Here, we show that GSK-3β kinase is inactivated and that β-catenin accumulates during the early stages of lens fiber cell differentiation. In an explant culture system, Wnt conditioned medium (CM) induced the accumulation of β-crystallin, a marker of fiber cell differentiation, without changing cell shape. In contrast, epithelial cells stimulated with Wnt after priming with FGF elongated, accumulated β-crystallin, aquaporin-0, p57kip2, and altered their expression of cadherins. Treatment with lithium, which stabilizes β-catenin, induced the accumulation of β-crystallin, but explants treated with lithium after FGF priming did not elongate as they did after Wnt application. These results show that Wnts promote the morphological aspects of fiber cell differentiation in a process that requires FGF signaling, but is independent of β-catenin. Wnt signaling may play an important role in lens epithelial-to-fiber differentiation.

Key words: Lens, Wnt signaling, Fibroblast growth factors, Lens fiber cell differentiation, Rat, β-crystallin, β-catenin

Introduction

The vertebrate ocular lens is composed of two distinctive cell types; differentiated fiber cells that make up the bulk of the lens, and a monolayer of epithelial cells that covers the anterior surface of the fiber cells. During growth of the lens, fiber cells are added to the bulk of the lens. This occurs through the proliferation of epithelial cells in a region just above the lens equator known as the germinative zone, and the subsequent differentiation of these cells into fiber cells in the transitional zone, just posterior to the equatorial region of the lens (McAvoy et al., 1999). Because the process of epithelial-to-fiber differentiation occurs continuously throughout the lifetime of the organism, the size of the lens increases with age (Harding et al., 1977). Epithelial-to-fiber differentiation is characterized by morphological and molecular changes, including exit from the cell cycle, cell elongation, the accumulation of fiber-specific proteins, including the β- and γ-crystallins, the intermediate filament proteins filensin and phakinin (CP49), and the intrinsic membrane protein MIP26 (also known as aquaporin-0), and the eventual loss of intracellular organelles and nuclei (Fromm and Overbeek, 1996; Krausz et al., 1996; McAvoy et al., 1999). The proliferation of epithelial cells and the differentiation of fiber cells have been thought to be regulated by factors that originate from surrounding tissues of the eye, such as the retina, iris and ciliary body. These factors, which are still not known with certainty, reach the lens by diffusion through the aqueous and vitreous humors (Lang, 1999).

Wnt proteins are known to control morphogenetic events during embryonic and postembryonic development (Moon et al., 1997). Wnts have been divided into functional classes, namely transforming and non-transforming Wnts, based on assays performed in mammalian cell lines (Wong et al., 1994). Ectopic expression of Wnt1, Wnt3a, Wnt7a and Wnt8 induces morphological transformation, whereas Wnt4 and Wnt5a lack transforming activity. Based on studies in various developmental systems, a mechanistic model of Wnt action has been proposed. Association of Wnt and its seven pass transmembrane receptor protein, Frizzled, leads to activation of Disheveled, resulting in the inhibition of GSK-3β, and in the subsequent accumulation and nuclear translocation of β-catenin. In the nucleus, β-catenin activates transcription factors of the T-cell factor (Tcf)/lymphoid enhancer factor (LEF) family, thereby affecting cell fate (Nusse and Varmus, 1992; Cadigan and Nusse, 1997). Wnts can also have a direct effect on the shape and fate of the cell through cytoskeletal reorganization (Shibamoto et al., 1998). For example, in vitro and in vivo studies show that the small GTPase, RhoA and Rac are directly activated by Frizzled-Disheveled signaling (Habas et al., 2003).

Wnts and proteins in the Wnt signaling pathway are expressed in the lens throughout its development. The Wnt receptors Frizzled 1, Frizzled 2 and Frizzled 7 were detected in the lens placode during lens induction (Stark et al., 2000). Transcripts encoding Wnt3, Wnt5a, Wnt5b, Wnt7a, Wnt7b,

Wnt8a, Wnt8b and Wnt13, and Frizzled receptors were identified in the postnatal lens epithelium and transitional zone at the lens equator (Jasoni et al., 1999; Stump et al., 2003; Liu et al., 2003). In addition, expression of Dishevelleds and Dickkopfs, which are involved in Wnt signaling, were detected in the lens (Stump et al., 2003). Although several Wnts and Frizzleds are expressed in lens cells and deletion of the Wnt co-receptor LRP5 leads to the death of central lens epithelial cells (Stump et al., 2003), Wnts have not been shown to direct lens formation or the differentiation of lens fiber cells.

In this study, we used three independent approaches, to show that Wnt signaling is involved in lens fiber cell differentiation: First, GSK-3β activity was decreased and nuclear β-catenin increased in the elongating fiber cells at the equatorial zone of the lens. Second, after FGF priming, Wnt induced cell elongation and the accumulation of β-crystallin. Finally, β-catenin activated the transcription of βB2-crystallin. These results suggest that Wnt acts as a regulatory factor of lens differentiation by regulating morphological change and the accumulation of lens proteins.

Materials and methods

Cell culture and preparation of conditioned medium

Epithelial explants were prepared from neonatal rat lenses according to a procedure reported previously (Lovicu and McAvoy, 2001). Eyes were removed from neonatal (P4-5) rats and lenses were dissected from the eyes. The lens capsule containing the adherent epithelial monolayer was peeled from the fiber cell mass and pinned out the edge of the explants, and then trimmed at the edge of explants to remove cells originating from equatorial regions of the lens. Explants were cultured in serum-free medium 199 (Sigma) supplemented with bovine serum albumin (BSA; Life Technologies, Grand Island, NY, USA) and antibiotic solution (100 units/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B; Life Technologies) in humidified 5% CO_2.

To activate the Wnt signal pathway, Wnt 3a-conditioned medium was prepared as described previously (Lyu et al., 2003), and the conditioned medium was diluted with normal culture medium (final concentration from 5× to 10×). Control treatment corresponds to the use of conditioned medium from the *lacZ* expressing L cells.

Bovine aqueous and vitreous humor were prepared from the eyes of freshly slaughtered animals as reported previously (Schulz et al., 1993), and then used to treat αTN4, B3 or HEK 293 cells at a final concentration of 20% aqueous- or vitreous humor.

For blocking experiments with sFRP-1, sFRP-1 CM was prepared from Myc-tagged sFRP1-expressing HEK 293 cells. Mouse vitreous humor was incubated with control medium and sFRP-1 CM for 30 minutes and then added to cells.

Immunochemical staining

For immunofluorescent labeling, explants were fixed with 4% paraformaldehyde and washed in phosphate-buffered saline (PBS), then permeabilized with 0.05% Triton X-100 in PBS. Fixed explants were incubated with blocking solution containing 1% normal goat serum (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) and 2% BSA (Sigma) for 1 hour and incubated overnight at 4°C with rabbit polyclonal anti-Ser9 phospho-GSK-3β (Cell Signaling Technology, Beverly, MA, USA), mouse monoclonal anti-GSK-3β (Transduction Laboratories, Lexington, KY, USA), rabbit polyclonal anti-Aquaporin-0 (Calbiochem, La Jolla, CA, USA), mouse monoclonal anti-β-catenin (Transduction Laboratories), or rabbit polyclonal anti-β-crystallin (kindly provided by John W. McAvoy, Sydney University). After a rinse with PBS, the explants were incubated with rhodamine, or FITC-conjugated (fluorescein-isothiocyanate; Jackson ImmunoResearch Laboratories) secondary antibodies at room temperature for 1 hour, and counterstained with Hoechst dye (Molecular Probes, Eugene, OR, USA). Labeled explants were rinsed with PBS, mounted and examined using a fluorescence microscope (Zeiss).

For immunofluorescent labeling of eye sections, dissected eyes from a neonatal (P4-P5) C57 mouse were immersed in Tissue Tek OCT compound and frozen in liquid nitrogen. Cryosections of eyes were fixed in 4% paraformaldehyde in PBS, and then incubated for 30 minutes in blocking solution. Antibodies to Ser9 phospho-GSK-3β and β-catenin were used on frozen sections to detect expression in the lens.

RNA isolation and RT-PCR analysis

Total RNA was isolated from the central epithelium (containing undifferentiated cells) and equatorial epithelium (containing both the proliferating epithelial cells and the elongating epithelial cells) of neonatal mouse lens, whole lenses, or explants using TRIzol Reagent (Life Technologies), according to the manufacturer's recommendations. Total RNA (2 μg) was reverse transcribed using the Superscript II kit (Invitrogen) and random hexamers. PCR amplification was performed using the following individual primer sets: for mWnt1, 5′-CAGTAGTGGCCGATGGTG-3′ and 5′-ATCG-ATGTTGTCACTGC-3′; for mWnt2b, 5′-GCCAAAGAGAAGAG-GCTTAA-3′ and 5′-TCAGTCCGGGTGGCGTGGCG-3′; for mWnt3, 5′-GCCGACTTCGGGGTGCTGGT-3′ and 5′-CTTGAAGAGCGC-GTACTTAG-3′; for mWnt3a, 5′-TAGTGCTCTGCAGCCTGAA-3′ and 5′-CCACAGATAGCAGCTGAT-3′; for mWnt4, 5′-ACAGTC-CTTTGTGGACGT-3′ and 5′-CGTCAATGGCTTTAGATG-3′; for mWnt5a, 5′-ATTGGAATATTAAGCCCG-3′ and 5′-GTGACCAT-AGTCGATGTT-3′; for mWnt5b, 5′-AGCTCTCATGAACCTACA-3′ and 5′-TGCAGTGGCAGCGTTCCA-3′; for mWnt7a, 5′-AACAT-GAAGCTGGAGTGT-3′ and 5′-TTACACTTGACGTAGCAGCA-3′; for mWnt7b, TCTGAGCAATTGTGGCTG; for rβB2-crystallin, 5′-CAGGCTCCGTCCTGGTGCAG-3′ and 5′-CGACGCACAGATTG-CACCTG-3′; for β-actin, 5′-AGGCCAACCGCGAAGATGACC-3′ and 5′-GAAGTCCAGGGCGACGTAGCAC-3′; for rGAPDH, 5′-CCATGGAGAAGGCTGGGG-3′ and 5′-CAAAGTTGTCATGGA-TGACC-3′. PCR was performed at 94°C for 30 seconds, 55°C (for mWnt1, mWnt4, mWnt5a, mWnt5b, mWnt7a, mWnt7b, rβB2-crystallin) or 58°C (for mWnt2b, mWnt3, mWnt3a, rGAPDH) or 60°C (for beta-actin) for 40 seconds, and 72°C for 1 minute for 30 cycles. The sequences of all PCR products were confirmed by automated sequencing.

Western blot analysis

Lenses of neonatal Sprague Dawley rats were dissected and the lens epithelium and the differentiating fiber cells of the zone of early fiber elongation at the equator were isolated under a dissecting microscope. Each tissue sample and explants was homogenized in lysis buffer containing 20 mM Tris-Cl (pH 7.5), 1% Triton X-100, 0.1% SDS, 0.5% deoxycholic acid, 10% glycerol, 100 mM sodium orthovanadate and protease inhibitor cocktail (1 mM EDTA, 1 mM PMSF, 5 μg/ml Aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin). Homogenates were clarified by centrifugation at 14,000 ***g*** for 15 minutes at 4°C and supernatant was collected as the total extract. Protein concentration was measured using Micro BCA Reagent kit (Pierce, IL, USA). Lysate was separated by SDS-PAGE (8-10%) and transferred onto nitrocellulose membranes. Blots were blocked with buffer containing 5% non-fat dry milk (Santa Cruz) and then reacted sequentially with the following antibodies: Mouse monoclonal antibodies for E-cadherin (Transduction Laboratories), N-cadherin (Transduction Laboratories), p27Kip1, α-SMA (Sigma), Actin (Sigma), α-tubulin (Sigma), and a rabbit polyclonal antibody for p57Kip2 (Santa Cruz Biotechnology) were used to detect the corresponding proteins. Peroxidase-conjugated goat anti-mouse and anti-rabbit secondary antibodies (Sigma) were then applied, and the proteins were detected

by using an enhanced chemiluminescence (ECL) reagent (Santa Cruz Biotechnology).

For western blotting assays of Ser9 phospho-GSK-3β, tissue samples were homogenized in lysis buffer containing 20 mM Tris-HCl, pH 7.5, 270 mM sucrose, 1 mM EDTA, 1 mM EGTA, 0.5% Triton X-100, 100 nM NaF, 1 mM sodium orthovanadate, 100 nM okadaic acid with protease inhibitors. Extracts were cleared by centrifugation at 13,000 ***g*** for 15 minutes at 4°C, and then the protein concentration was determined. Lysates were subjected to western blotting analysis with anti-Ser9 phospho-GSK-3β or a mouse monoclonal antibody specific to GSK-3β for normalization of GSK-3β. Band intensities were quantified using ImageMaster VDS (Pharmacia).

Nuclear extracts were prepared from lens epithelial cells and differentiating fiber cells in the zone of early fiber elongation at the equator, or from explants by the method described previously (Dignam et al., 1983). Protein concentration was determined using Bradford reagent (Bio-Rad). Western blotting was performed with anti-β-catenin antibody or a monoclonal anti-lamin A/C (Cell Signaling Technologies), for normalizing of nuclear protein loading. α-tubulin was not detected when blots were probed with anti-α-tubulin, suggesting that is was not due a contamination of cytoplasmic proteins.

In vitro GSK-3β kinase assay

Protein lysates were prepared from lens tissues or explants using lysis buffer. Total lysates were immunoprecipitated with antibody to GSK-3β. The immune complexes were washed three times in lysis buffer and three times in kinase assay buffer, and then were incubated for 20 minutes at 30°C with 62.5 μM glycogen synthase peptide-2 (Upstate Biotechnologies, Waltham, MA, USA), 100 μM [γ-^{32}P]ATP (0.25 μCi/ml), 10 mM $MgCl_2$. 32P-labeled reactions were spotted onto P81 phosphocellulose papers, and then were washed and then subjected to liquid scintillation counting. Kinase activity was normalized to background levels when 40 mM LiCl was included in the reaction.

Bromodeoxyuridine proliferation assay

Bromodeoxyuridine (BrdU) incorporation was determined by a BrdU labeling kit, according to the manufacturer's recommendations (Roche, Indianapolis, IN, USA). Nuclei were counterstained with Hoechst 33258. BrdU-reactive cells were captured by using an Axio camera and AxioVision 2.05 image analysis software (Zeiss) under a fluorescence microscope, and the number of BrdU-positive cells was determined.

Transient transfections and reporter gene assay

HEK 293 cells, B3 or αTN4 were plated into 5×10^5 per 35 mm tissue culture dish and transfected by Lipofectamine (Life Technologies) with reporter plasmid (TOPFLASH, FOPFLASH, pβB2-crystallin luciferase) and the internal control pRL-TK. Luciferase assays were performed 24 hours after transfection using the dual luciferase assay system (Promega).

Construction of plasmids

To construct reporter plasmids, a 624 bp βB2-crystallin promoter (–614/+10) was amplified from mouse genomic DNA using primers (Chen et al., 2002); for βB2-crystallin promoter, 5′-ATAGAA-CCCAGGACCACCAG-3′ and 5′-GAGTGCCGTGAAGCCAGGCT-3′. The PCR products were inserted in pGL2 basic-vector (Promega). For the pcDNA3-Myc/sFRP-1 and pcDNA3-Myc/ICAT constructs, a cDNA fragment for mouse sFRP-1 and ICAT was amplified by RT-PCR from mouse mRNA, as a template. Cloned cDNA was inserted into the pCS2-MT vector for Myc tagging of N-terminal, and subcloned into pcDNA3 vector. All constructs were confirmed by sequencing.

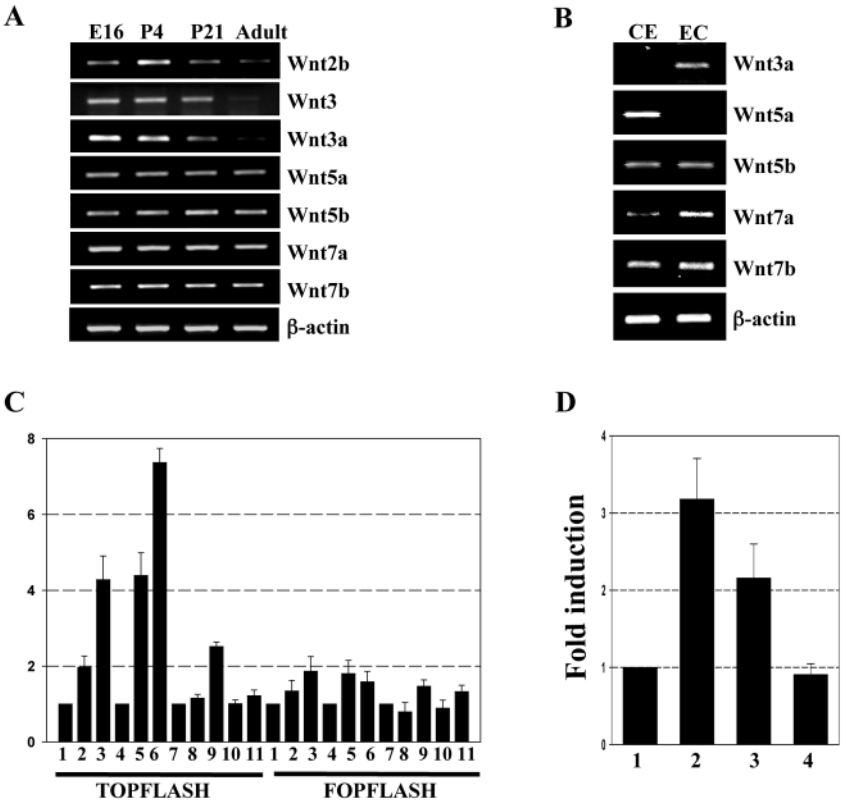


Fig. 1. Wnt gene expression in the mouse lens and activation of Wnt signaling by vitreous humor. Total RNA was isolated from E16, P4, P21 and adult rat lenses (A), or from rat (P4) lens central epithelium (CE) and equatorial epithelium, which contains both the proliferating cells and elongating cells (EC) (B). cDNA was prepared by reverse transcription and amplified with primers specific for each Wnt. Each reaction was normalized to β-actin. Analyses of at least three different RNA preparations from the same tissues provided similar results. (C) Mouse lens cells were transiently transfected with TOPFLASH or FOPFLASH reporter plasmid, and then stimulated with vehicle (lanes 1, 7), aqueous humor (lane 2), vitreous humor (lane 3), control medium (lane 4), 5×Wnt3a conditioned medium (lane 5), 10×Wnt3a CM (lane 6), 1 ng/ml EGF (lane 8), 50 ng/ml FGF2 (lane 9), 10 ng/ml PDGF (lane 10), or 5 ng/ml TGF-β (lane 11) for 16 hours. Cell lysates were assayed for luciferase activity. (D) Control medium (lane 1), vitreous humor pre-incubated with control medium (lane 2), vitreous humor pre-incubated with sFRP-1-conditioned medium (lane 3), or sFRP-1-conditioned medium alone (lane 4) were added to mouse lens cells transfected with the TOPFLASH reporter plasmid. Cell lysates were analyzed by luciferase assay.

Results

Expression of Wnts in the lens

The expression profile of Wnt genes is shown in Fig. 1A and Fig. 1B. RT-PCR assays were performed with RNA extracted from the whole lenses of 16-day-old mouse embryos, postnatal 4 days of age, 21 days and three-month-old adults. We found

that several Wnt genes (Wnt2b, Wnt3, Wnt3a, Wnt5a, Wnt5b, Wnt7a and Wnt7b) were expressed in the lens from the embryo through to adulthood. No positive result was obtained with Wnt1 and Wnt4. As a positive control, these products were amplified by RT-PCR from embryonic brain or kidney extracts (data not shown). The level of Wnt3a and Wnt7a mRNAs was significantly increased in the equatorial epithelium, including both the proliferating epithelial cells and the elongating epithelial cells, compared with that detected in central epithelial cells. In contrast, the level of Wnt5a mRNA was lower in cells of the equatorial epithelium compared with central cells (Fig. 1B).

As Wnt family proteins activate intracellular signaling pathways by both autocrine and paracrine mechanisms, the ocular environment may contribute to Wnt signaling in the lens. To test whether aqueous or vitreous humor could activate Wnt signaling, a luciferase reporter assay was performed. Vitreous humor and Wnt3a CM activated the TOPFLASH reporter gene, which regulates luciferase expression through Tcf/LEF binding sites in the TOP promoter (Brantjes et al., 2002). Neither aqueous humor nor control medium had a significant effect on reporter expression in this assay (Fig. 1C). When the Tcf/LEF binding sites were mutated (FOPFLASH), promoter activity was abolished. To test whether the activity in vitreous humor was because of Wnt proteins, we inhibited Wnt function using a Wnt antagonist, sFRP-1 (Finch et al., 1997; Hall et al., 2000). sFRP-1 CM from transfected cells had no effect on promoter activity when added alone. However, sFRP-1 inhibited the increase in TOP promoter activity caused by vitreous humor (Fig. 1D).

It is generally considered that canonical Wnt signaling has mitogenic activity. To determine the effects of Wnt on cellular proliferation in lens epithelial cells, we performed BrdU-incorporation as a marker of DNA synthesis. Medium harvested from confluent cultures of L cells expressing *lacZ* (control medium) or Wnt3a was applied to rat lens epithelial explants for 2 days. BrdU was administered 6 hours before collection of the explants. Significantly more BrdU-labeled cells were present in explants cultured in Wnt CM than in those cultured in control medium (Fig. 2).

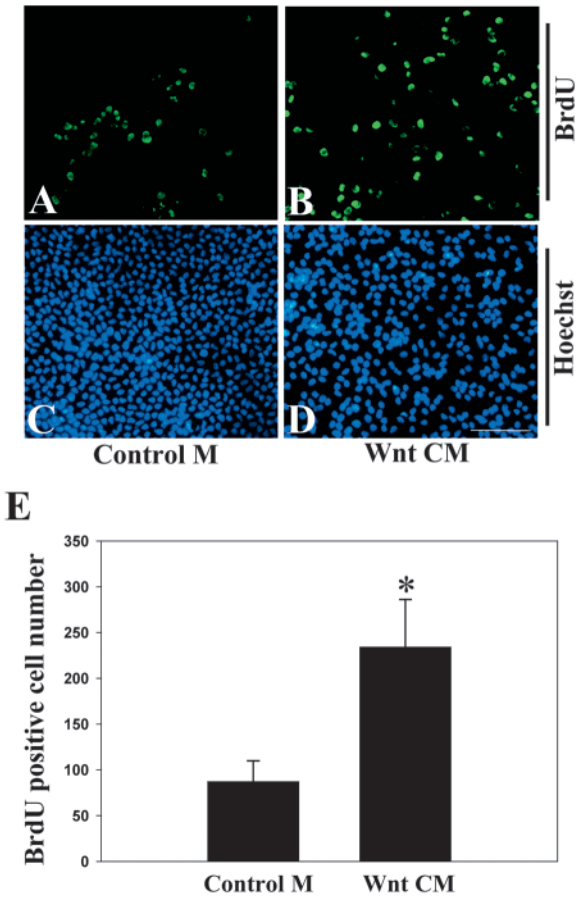


Fig. 2. Increased proliferation of epithelial cells in explants treated with Wnt CM. (A,B) BrdU incorporation assays demonstrated stimulation of the cell cycle in explants treated with Wnt 3a CM. (C,D) Nuclei of explants derived from rat lens capsule revealed by Hoechst 33258 staining. (E) Quantification of BrdU incorporation after a 6-hour labeling period. BrdU-positive nuclei from a total of six explants treated with each factor were counted. The mean values (±s.d.) are shown in the histogram. (n=5) [versus control (Cont), *P<0.005].

Inhibition of GSK-3β kinase and nuclear translocation of β-catenin in mouse lens fiber cells

If Wnt/GSK-3β signaling plays an important role in the lens cells, GSK-3β should be inhibited in these cells. An antibody to Ser9 phospho-GSK3β, which reacts with an inactivated form of the enzyme, strongly labeled elongating fiber cells (Fig. 3A, arrow). More mature fiber cells, located deeper in the secondary fibers, had a lower level of labeling, and labeling was not detected in the central fibers. Ser9 phospho-GSK-3β labeling was not detected in the epithelial cells (Fig. 3A, arrowhead). To further test the state of GSK-3β phosphorylation in fiber cells, we measured Ser9-phosphorylated GSK-3β levels by western blot analysis. As shown in Fig. 3C, the level of phosphorylated GSK-3β was higher in the extract from the differentiating cells in the zone of early fiber elongation at the equator compared with the extract from the lens epithelium (3.43±0.38-fold induction; n=3). To confirm the decreased activity of GSK-3β kinase in differentiating fiber cells, cell lysates were prepared from the lens epithelium and the differentiating fiber cells, and then subjected to immunoprecipitation with an anti-GSK-3β antibody, followed by assay with the glycogen synthase-2 peptide as substrate. GSK-3β kinase activity was 1.98±0.39-fold higher in epithelial cells compared with differentiating cells (n=3) (Fig. 3D). These results indicate that GSK-3β activity is decreased during lens fiber differentiation.

The inhibition of GSK-3β leads to the stabilization of β-catenin and its subsequent translocation into the nucleus (Nusse, 1999). Thus, we analyzed the localization of β-catenin in lens tissue. Staining for β-catenin was detected in the nuclei of elongated fiber cells, but was weaker in the nuclei of the earliest stages of fiber cell differentiation (Fig. 4C,D). In contrast, nuclear β-catenin was barely detectable in the lens epithelium (Fig. 4B,E). To confirm the translocation of β-catenin into nuclei, we measured the level of nuclear β-catenin in the lens epithelium and differentiating cells at the equator using immunoblot analysis. Nuclear extracts were prepared from central epithelial cells and the outer fiber cells near the epithelium of the

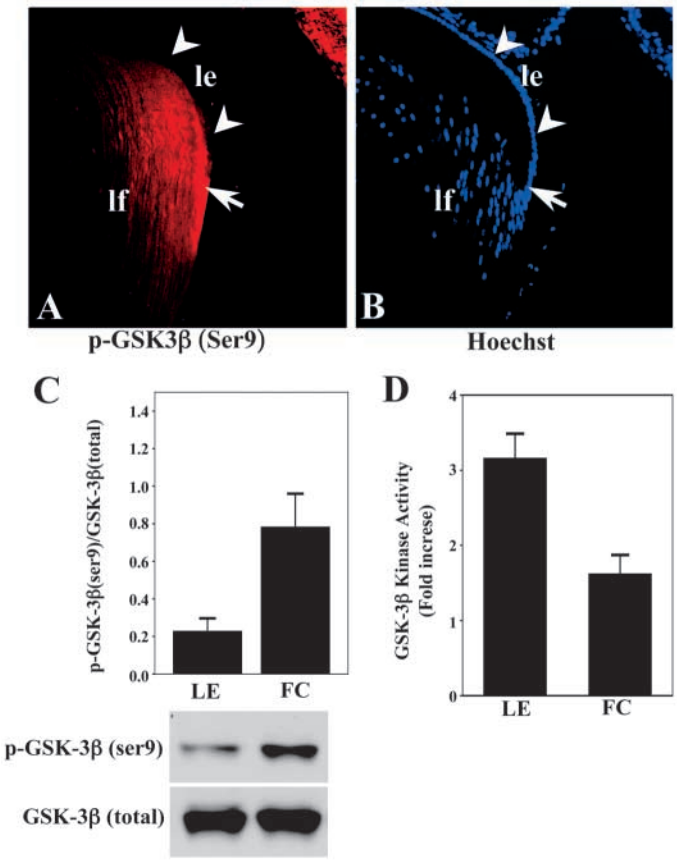
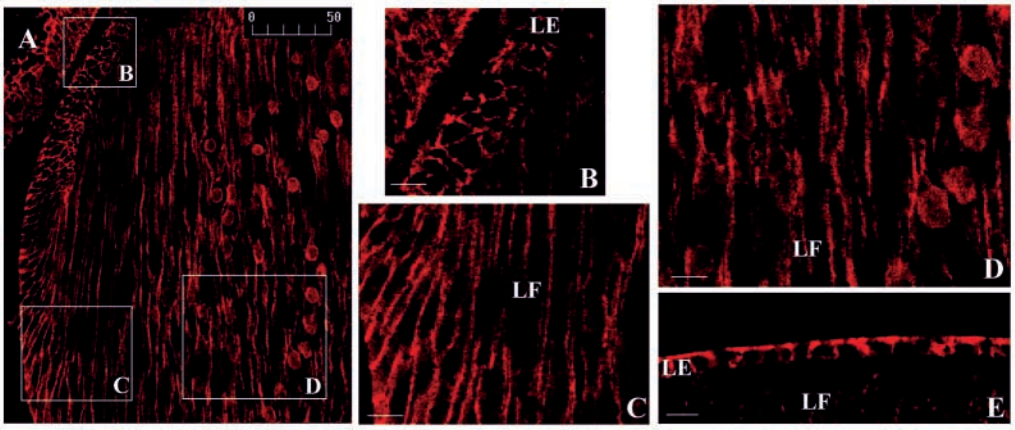


Fig. 3. Representative immunolabeling of Ser9 phospho-GSK-3β in the bow region of cryosections from mouse lenses (A), counterstained with Hoechst dye (B). Strong reactivity for phospho-GSK-3β in fiber cells near the lens equator (arrow), but not in lens epithelial cells (arrowhead). (C) Western blotting of cell extracts was performed with anti-Ser9 phospo-GSK-3β and normalized with anti-total GSK-3β. Ser9 phosphorylation of GSK-3β was increased in differentiating lens cells. Normalized quantification is shown in the graph. (D) Protein extracts from undifferentiated lens epithelium (LE) and differentiating lens fiber cells of the equatorial zone (FC) were assayed by GSK-3β immune complex kinase assay. GSK-3β kinase activity was decreased in the rat lens fiber cells. Each error bar represents the mean±s.d. of three independent experiments; each assay was performed in duplicate. le, lens epithelium; lf, lens fiber.

equatorial zone. Nuclear β-catenin was 4.7±0.83-fold higher in differentiating cells than in undifferentiated epithelial cells (*n*=4) (Fig. 4F). Taken together, these data suggest that GSK-3β and β-catenin contribute to lens fiber cell formation.

Wnts stimulate cultured lens epithelial cells to form lens fiber cells

Our results suggest that Wnts contribute to lens fiber formation. To further test this possibility, we determined whether Wnt protein could stimulate cell elongation, a measure of in vitro fiber cell differentiation. Treatment of lens epithelial explants with Wnt CM alone was not sufficient to induce cell elongation (Fig. 5B). Because treatment with FGFs can initiate fiber cell differentiation (Leenders et al., 1997), we tested whether Wnt CM could induce lens epithelial cell elongation after priming by FGF2 (50 ng/ml). Explants were exposed to FGF2 for 1 hour, then washed with phosphate-buffered 2 M NaCl to remove the FGF2, and cultured further in the presence of Wnt CM or control medium for 5 days. Explants that were first incubated with FGF2 for 1 hour and then cultured in control medium for 5 days showed no morphological changes (Fig. 5E). In contrast, when the explants were incubated with FGF2 for 1 hour and further cultured with Wnt CM, a significant increase in lens epithelial cell elongation was observed (Fig. 5F). These morphological changes were similar to those seen in FGF2-induced fiber differentiation (Fig. 5D). FGF/Wnt CM-treated explants cultured in the presence of DMSO showed cell



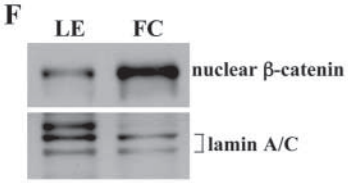


Fig. 4. Localization of β-catenin on mouse lenses. β-catenin immunostained cryosections of the bow region of mouse lenses. (A) β-catenin antibodies localized to the membrane of lens epithelial and fiber cells. However, nuclear β-catenin was detected only in differentiating fiber cells near the lens equator (C,D). β-catenin antibodies did not label the nucleus of central (B) and equatorial (E) epithelial cells. Scale bars: A, 50 μm; B-E, 10 μm. (F) Lysates prepared from the nuclei of undifferentiated lens epithelial cells (lane LE) and differentiating lens fiber cells (lane FC) were separated by 10% SDS-PAGE and analyzed with antibodies against β-catenin. Western blots showed that nuclear β-catenin was higher in differentiating lens cells compared with undifferentiated epithelial cells. Antibodies against lamin A/C were used to confirm the purity of the nuclear fraction.

elongation (Fig. 5G), whereas FGF/Wnt CM-treated explants treated with U0126 (an inhibitor of ERK signaling) during the FGF pre-treatment did not elongate (Fig. 5H), indicating that FGF-induced ERK signaling is required to prime the cells to respond to Wnt CM.

In similar experiments, we tested the activity of GSK-3β in explants where cell elongation was observed using antibodies against Ser9 phospho-GSK-3β and by measuring the activity of GSK-3β by immune complex kinase assay using a GSK-3 substrate. Explants exposed to FGF2 for 1 hour were washed, and cultured further in the presence of Wnt CM or control medium for 5 days. At the end of the culture period, explants were fixed and immunolabeled. FGF/Control medium-treated explants did not contain detectable Ser9 phospho-GSK-3β. However, FGF/Wnt CM-treated explants accumulated Ser9 phospho-GSK-3β (Fig. 6A). When explants were incubated with FGF2/Wnt CM, GSK-3β kinase activity decreased to

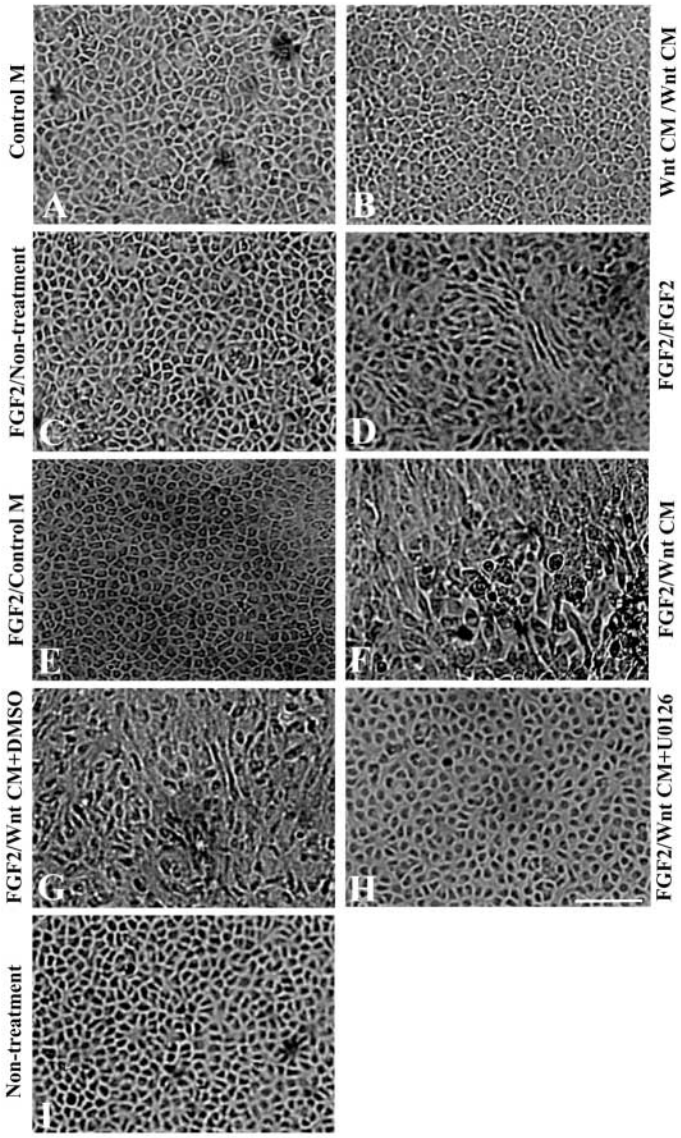


Fig. 5. Wnt CM induces elongation of lens epithelial cells. Explants were pre-stimulated with 50 ng/ml FGF2 or Wnt3a CM for 1 hour, followed by stimulation with control medium, Wnt3a CM, 50 ng/ml FGF for 5 days, or in the presence of 50 μM U0126 or DMSO. Explants were also cultured with vehicle or control medium in the absence of stimulation. Untreated explants or explants cultured in control medium after pre-stimulation with FGF2 showed normal epithelial cell morphology (A,C,E,I). Explants pre-stimulated by Wnt3a and cultured in Wnt3a did not undergo morphological change (B). However, explants that were pre-stimulated by FGF2 and cultured for 5 days in Wnt3a elongated (F) similar to explants cultured continuously with FGF2 (D). In the presence of DMSO (as a control for U0126), explants cultured with FGF2/Wnt CM showed a cell elongation (G). However, cell elongation did not occur in the presence of U0126 (H). Scale bar: 50 μm.

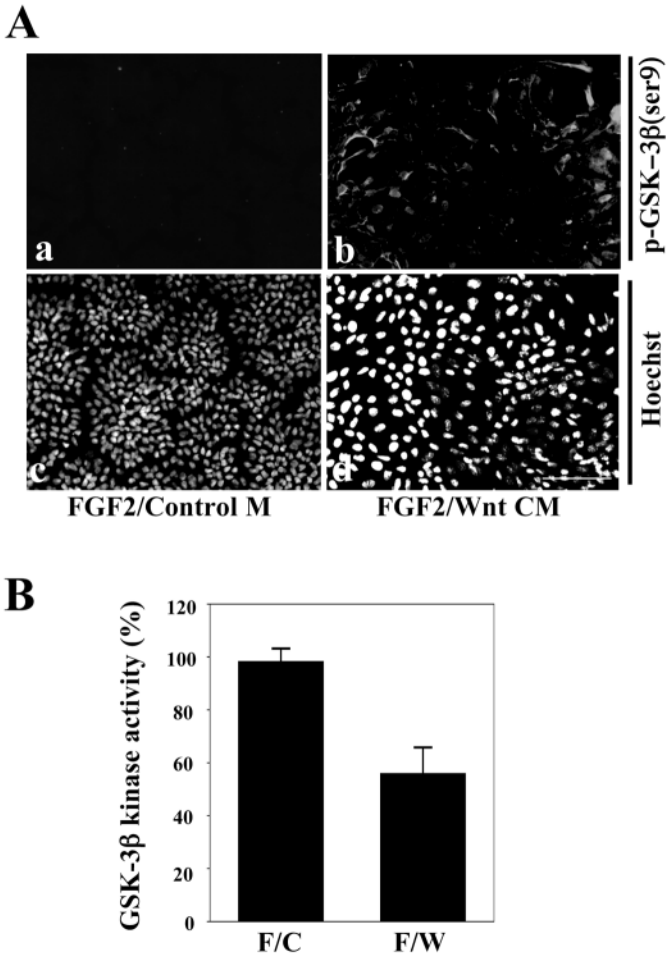


Fig. 6. GSK-3β is inactivated in lens cells treated with Wnt CM. (A) Explants exposed to FGF2 for 1 hour were washed with 2 M NaCl, and cultured in control medium or Wnt3a CM for 5 days. Explants were immunolabeled by using anti-Ser9 phospo-GSK-3β (a,b), or Hoechst 33258 to stain nuclei. Ser9 phospho-GSK-3β accumulated in explants cultured with FGF2/Wnt CM (b), but not in explants cultured with FGF2/Control medium (a). (B) In vitro GSK-3b kinase assays were performed using a GSK-3 substrate and [γ-^{32}P]. Lysates containing 150 μg of protein prepared from explants cultured with FGF2/Control medium (F/C) or FGF2/Wnt CM (F/W) were immunoprecipitated with anti-GSK-3β and analyzed using a GSK-3β immune complex kinase assay. The activity of GSK-3β was decreased in explants cultured with FGF2/Wnt CM compared with explants cultured with FGF2/Control medium. Values represent the mean±s.d. of three independent experiments and are expressed as a percentage of the activity in FGF2/Control explants.

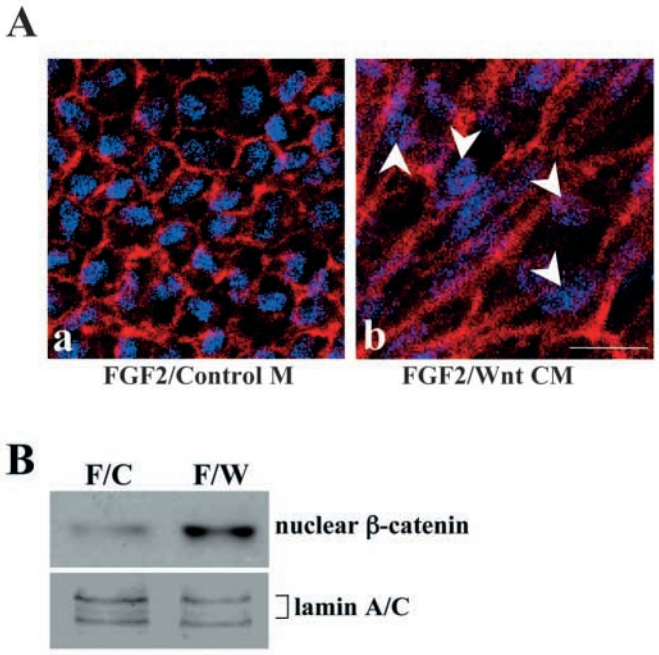


Fig. 7. Wnt CM induces the accumulation of β-catenin in nuclei. (A) Explants were pre-stimulated by 50 ng/ml FGF2, followed by stimulation with control medium or Wnt3a CM for 5 days. Explants were immunolabeled with anti-β-catenin or Hoechst 33258 to stain nuclei. β-catenin accumulated in the nuclei of cells that elongated after treatment with FGF2/Wnt CM (b; arrow), but not in the nuclei of morphologically normal cells cultured with control medium or FGF2/control medium (a). Scale bar: 10 μm. (B) Nuclear extracts were prepared from explants cultured with FGF2/control medium (F/C) or FGF2/Wnt CM (F/W). Western blotting was performed with anti-β-catenin. Explants displayed an increased level of nuclear β-catenin in response to FGF2/Wnt CM. Blots probed with an anti-lamin A/C antibody demonstrated equal loading of nuclear proteins.

approximately 42% of that of explants cultured with FGF2/Control medium (Fig. 6B).

We next examined the localization of β-catenin using immunolabeling. Explants were exposed to FGF2 for 1 hour, and cultured further in the presence of Wnt CM or control medium for 5 days. At the end of the culture period, explants were fixed and immunolabeled. Although β-catenin staining was weak, the elongated cells in FGF2/Wnt CM-treated explants showed β-catenin in their nuclei (Fig. 7A). To confirm that β-catenin translocates into the nuclei of FGF/Wnt-treated cells, we performed western blot analysis of nuclear extracts prepared from FGF2/Control medium- or FGF2/Wnt CM-treated explants. In these experiments, the levels of nuclear β-catenin increased in FGF2/Wnt CM-treated explants compared with FGF2/Control medium-treated explants (Fig. 7B).

β-crystallin and aquaporin-0 (also known as Major Intrinsic Protein or MIP26) are expressed and accumulate in fiber cells exclusively and therefore serve as useful markers for fiber differentiation (Yancey et al., 1988). Lens explants were incubated in unsupplemented medium, FGF/control medium or FGF/Wnt CM for 5 days. At the end of the culture period, explants were fixed and labeled with antibodies against lens fiber proteins. In response to treatment with FGF/Wnt CM, the elongated epithelial cells accumulated β-crystallin (Fig. 8A) and aquaporin-0 (Fig. 8B). In contrast, FGF2/control-treated explants did not accumulate detectable levels of β-crystallin and aquaporin-0.

E-cadherin, an epithelial cadherin, is expressed in the mouse lens epithelium. E-cadherin expression ceases at the onset of fiber cell differentiation (Xu et al., 2002; Wigle et al., 1999). In contrast, N-cadherin is strongly expressed in elongating fiber cells (Ferreira-Cornwell et al., 2000; Xu et al., 2002). As lens cells exit the cell cycle at the lens equator, $p27^{kip1}$ and $p57^{kip2}$ are highly expressed (Zhang et al., 1998). We were therefore interested in measuring the expression of these proteins in vitro and in vivo. When explants were stimulated

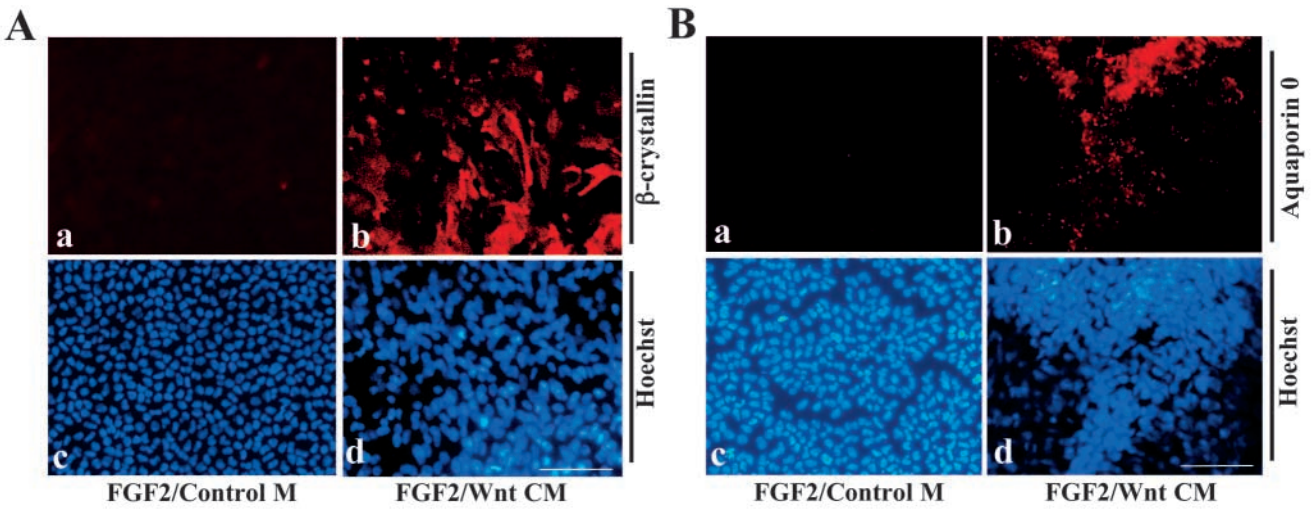


Fig. 8. Wnt CM induces the accumulation of lens fiber proteins in FGF2-stimulated epithelial cells. Explants pre-stimulated by FGF2 for 1 hour were washed and cultured in control medium or medium supplemented with Wnt3a CM and incubated for 5 days. Explants were analyzed by immunolabeling with antibodies against β-crystallin (A), aquaporin 0 (B) or were stained with Hoechst 33258. Explants cultured in control medium did not accumulate β-crystallin or aquaporin 0, but these fiber-specific markers accumulated in cells exposed to Wnt CM. Scale bar: 50 μm.

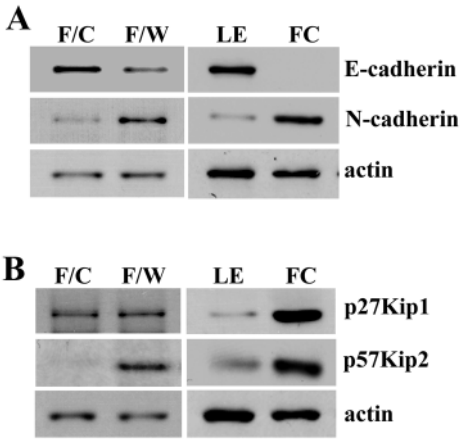


Fig. 9. Wnt CM induces alteration in the protein levels of cadherins and $p57^{Kip2}$ in epithelial cells pre-treated with FGF2. Explants were cultured for 5 days with control medium (lane F/C) or Wnt3a CM (lane F/W), after pre-stimulation with FGF2 for 1 hour. Lens epithelium (lane LE) and differentiating lens cells of the zone of early elongation at equator (lane FC) were isolated from neonatal rat lens. Cell lysates were separated by 10% SDS-PAGE and probed with antibodies against E-cadherin, N-cadherin, $p27^{Kip1}$ or $p57^{Kip2}$. E-cadherin levels were decreased and N-cadherin levels increased after treatment with FGF2/Wnt CM, consistent with the changes seen during fiber cell differentiation (A). $p57^{Kip2}$ levels increased in explants treated with FGF2/Wnt CM, similar to the increase in differentiating fiber cells. However, $p27^{Kip1}$ levels were not affected by FGF2/Wnt CM, unlike the expression pattern seen in differentiating fiber cells (B). Actin was used as a loading control.

by FGF2/Wnt CM, levels of N-cadherin increased compared with explants treated with FGF2/Control medium. In contrast, E-cadherin levels decreased in FGF2/Wnt CM-treated explants. The increased N-cadherin and decreased E-cadherin accumulation in explants treated with FGF2/Wnt CM was similar to the expression of these proteins in differentiating fiber cells (Fig. 9A). When explants were treated with FGF/Wnt CM, $p57^{kip2}$ also increased. However, the levels of $p27^{kip1}$ did not change in explants, although $p27^{kip1}$ levels were higher in lens fiber cells than in epithelial cells (Fig. 9B).

In addition, we excluded the possibility of epithelial-mesenchymal transition (EMT) by Wnt signaling (Muller et al., 2002) by showing that α-SMA did not accumulate in explants cultured with Wnt CM or FGF/Wnt CM (data not shown).

Induction of β-crystallin expression by the Wnt/β-catenin-dependent pathway

Lithium inhibits GSK-β and mimics Wnt signaling through β-catenin (Stambolic et al., 1996). We therefore determined whether lithium could induce fiber differentiation in explants. Cell elongation was not observed when epithelial explants were treated with FGF for 1 hour, followed by continuous exposure to 10 mM LiCl (Fig. 10A). To test whether GSK-3β responds to lithium in lens epithelial explants, extracts were analyzed by Western blot assay using antibodies against Ser9 phospho-GSK-3β. Treatment with FGF2/LiCl resulted in an increase in Ser9 phospho-GSK-3β, suggesting that GSK-3β was inactivated (Fig. 10B). We next examined the accumulation of β-crystallin in explants. Explants cultured with FGF2/LiCl or LiCl accumulated β-crystallin without cell elongation, whereas explants cultured with FGF/NaCl or NaCl did not accumulate β-crystallin (Fig. 10C). As shown earlier, explants cultured with FGF2/Wnt CM accumulated β-crystallin and elongated. We noted that cell elongation was inhibited by U0126, and explants cultured with Wnt CM accumulated β-crystallin without cell elongation (Fig. 10C). These results confirm that β-crystallin expression and cell elongation can be uncoupled, as shown previously (Lovicu and McAvoy, 2001).

If Wnt-induced cell elongation and β-crystallin accumulation can occur independently, Wnt/β-catenin signaling might regulate the expression of β-crystallin in the absence of FGF2. Therefore, we continuously exposed explants to control medium, Wnt CM, NaCl or LiCl and analyzed the accumulation of a specific β-crystallin transcript using RT-PCR. Even in the absence of FGF2, lithium and Wnt CM were sufficient to induce expression of the βB2-crystallin gene (Fig. 11A). To further test whether β-catenin-dependent signaling regulates β-crystallin expression, we performed reporter assays in αTN4 cells, a transformed lens cell line (Fig. 11B). Overexpression of β-catenin increased the activation of the βB2-crystallin promoter, and overexpression of ICAT, an inhibitor of the interaction between β-catenin and TCF, repressed the transcriptional activation of βB2-crystallin by Wnt CM, lithium, β-catenin or stabilized β-catenin (S37A). Similar results were obtained using the B3 human lens epithelial cell line (data not shown). Co-expression of GSK-3β with stabilized β-catenin did not prevent the increased accumulation of βB2-crystallin transcripts, indicating that βB2-crystallin is expressed by a β-catenin-dependent pathway.

Discussion

FGFs, IGF-1 and BMPs have been implicated as factors that stimulate fiber cell differentiation in cultured explants in vitro (Beebe et al., 1987; Chamberlain and McAvoy, 1987; Peek et al., 1992; Belecky-Adams et al., 2002). Although FGFs, IGF-1 and BMPs play important roles in the lens developmental processes, these factors may not be sufficient for the full differentiation of lens fiber cells. For example, FGF1 and FGF2-deficient mice exhibited no defects in lens development (Dono et al., 1998; Miller et al., 2000). Overexpression of IGF-1 in the lens cells of transgenic mice caused an increase in epithelial cell proliferation, but did not stimulate the premature differentiation of lens epithelial cells (Shirke et al., 2001). Furthermore, expression of a dominant-negative BMP family receptor in the lens of transgenic mice inhibited primary fiber differentiation on the nasal, but not the temporal side of the lens (Faber et al., 2002). Together, these reports indicate that other factors may be involved in fiber differentiation. Here, we suggest that Wnt signaling plays a role in epithelial-to-fiber differentiation, by showing that the lens expresses various Wnt genes in vivo, and that Wnt CM regulates the components of fiber cell differentiation, including the accumulation of fiber-specific proteins, and cell elongation.

In lens development, diffusible factors in the ocular environment are an important part of cellular development. The aqueous humor from the anterior chamber (adjacent to the lens epithelium) favors proliferation, and the vitreous body (adjacent to the fiber cells) promotes epithelial-to-fiber

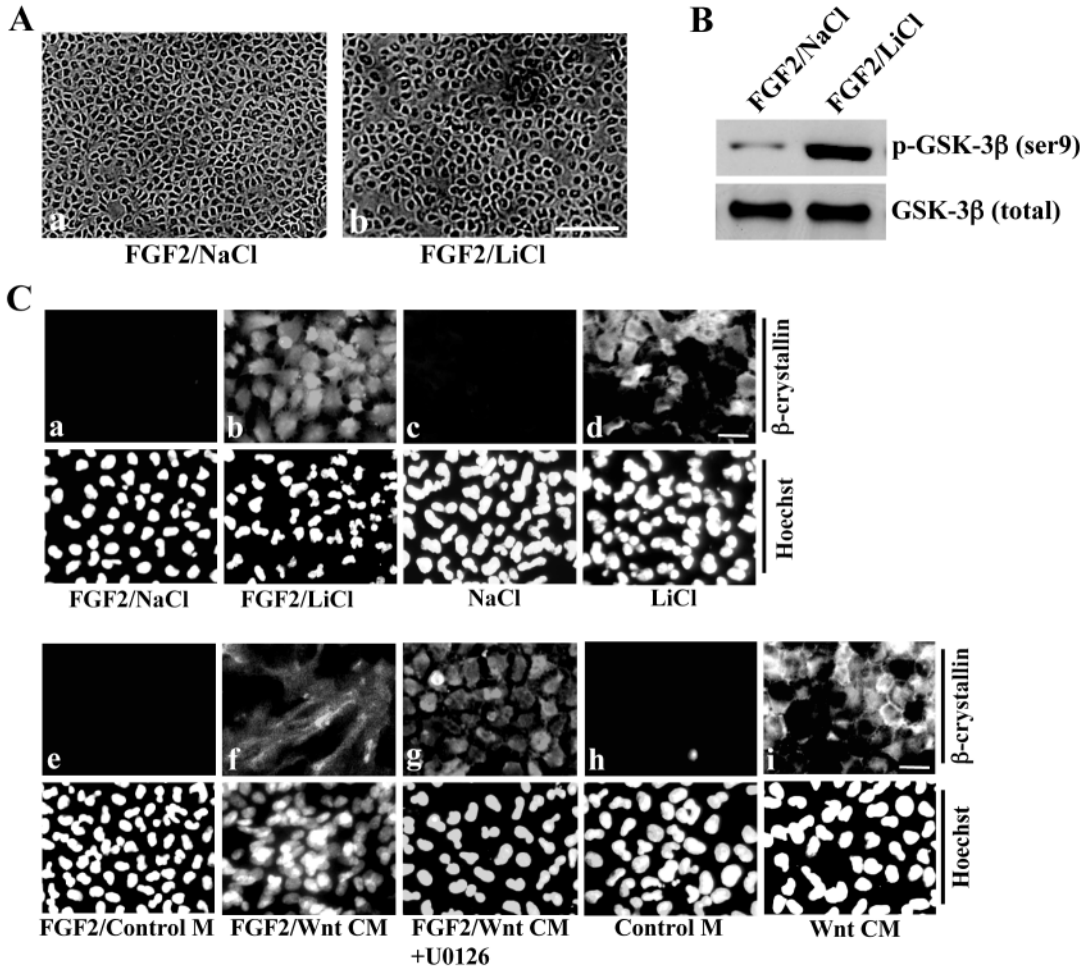


Fig. 10. Treatment with lithium or Wnt CM leads to the accumulation of β-crystallin protein without FGF2 pre-stimulation. (A) After treatment with 50 ng/ml FGF2, explants were cultured for 5 days in the presence of 10 mM NaCl (as a control) or 10 mM LiCl and observed by microscopy. Note that cell elongation did not occur in response to FGF2/LiCl. Scale bar: 50 μm. (B) Western blot analysis of explants cultured for 5 days in the presence of FGF2/NaCl and FGF2/LiCl showed that lithium induced a significant induction of Ser9 phospho-GSK-3β. (C) Explants were pre-stimulated with or without 50 ng/ml FGF2 for 1 hour, and then cultured with 10 mM NaCl, 10 mM LiCl, control medium, or Wnt3a CM for 5 days, or in the presence of 50 μM U0126 or DMSO. Explants were then fixed and labeled with antibodies against β-crystallin or Hoechst 33258 for nuclei staining. Immunolabeling for β-crystallin in FGF2/LiCl-treated explants showed that β-crystallin was accumulated without cell elongation (b). Lithium also induced the accumulation of β-crystallin in explants cultured without FGF2 pre-stimulation (d). In the presence of U0126, FGF2/Wnt CM-treated explants revealed an accumulation of β-crystallin without cell elongation (g), whereas the accumulation of β-crystallin and cell elongation are induced in FGF2/Wnt CM-treated explants (f). In the presence of Wnt CM without FGF2 pre-stimulation, β-crystallin was also accumulated (i). Scale bar: 10 μm.

differentiation (Schulz et al., 1993). For example, the BMPs FGFs that are required for lens fiber cell differentiation are produced by the retina during development and are secreted into the vitreous humor (Belecky-Adams et al., 2002; Nakashima et al., 1999). We showed that vitreous humor induced the activation of the TOP promoter and that the activity in vitreous humor was inhibited by the Wnt antagonist sFRP-1 (Fig. 1). In other experiments, CyclinD1 and fla-1 levels were significantly increased by treatment of HEK293 cells with vitreous humor (data not shown), consistent with induction by β-catenin (Shtutman et al., 1999; Mann et al., 1999). These results suggest that vitreous humor, the fluid that bathes differentiating fiber cells, contains Wnt proteins or factors that can activate the Wnt/β-catenin pathway.

Recent studies reported that several different Wnt genes are expressed in lens cells. Wnt5a, 5b, 7a, 7b, 8a and 8b are expressed in the lens epithelium of postnatal mice, including the transitional zone, and they are reduced outside the zone of early fiber elongation at the equator (Stump et al., 2003). In the embryonic mouse lens, Wnt3 is expressed in the lens epithelium, Wnt5b is expressed at the lens equator, and Wnt7a is expressed in the lens fibers (Liu et al., 2003). Frizzled receptors, which have at least 10 orthologs in mammals, were detected in the lens. Frizzled 1, 2 and 7 are expressed in the chick lens placode (Stark et al., 2000). Also, Frizzled 1, 2, 3, 4, 6 and 7 are expressed in the embryonic and postnatal lens (Stump et al., 2003; Liu et al., 2003). Consistent with these studies, we also showed the expression of Wnt2b, Wnt3, Wnt3a, Wnt5a, Wnt5b, Wnt7a and Wnt7b in lens. Levels of Wnt3a and Wnt7a at the equator of the neonatal rat lens, where

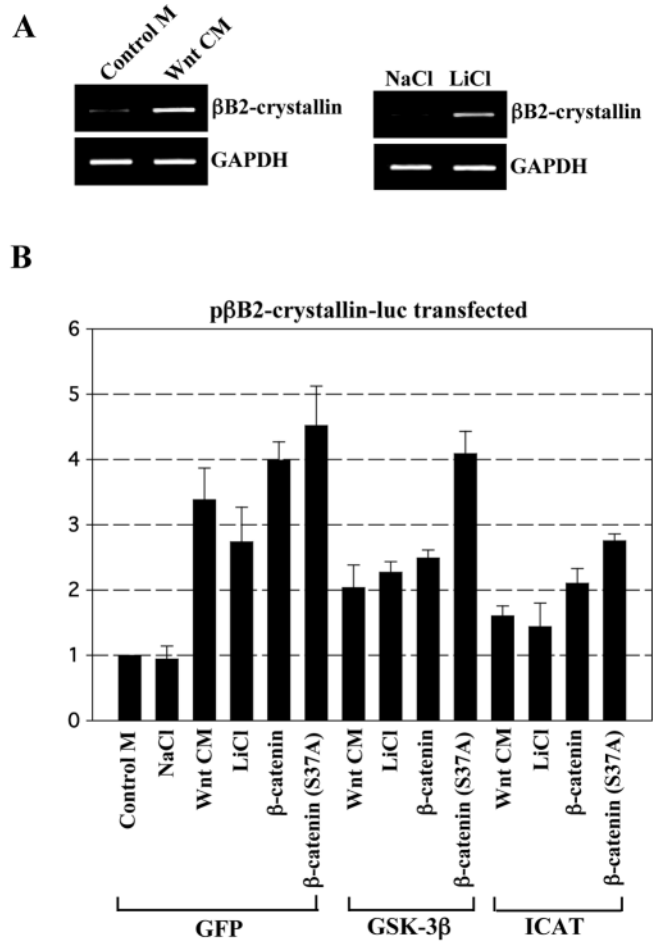


Fig. 11. Wnt/β-catenin activates the transcription of β-crystallin. (A) Explants were cultured for 5 days with control medium, Wnt3a CM, 10 mM NaCl, or 10 mM LiCl in the absence of stimulation by FGF2. Total RNA was prepared from each explant and βB2-crystallin expression was assayed by RT-PCR. (B) To analyze the transcriptional activity of β-crystallin, mouse lens cells were transiently transfected with pβB2-crystallin-luciferase plasmid, and co-transfected with an expression plasmid of GSK-3β, ICAT or GFP (as a control). Cells were stimulated by Control medium, Wnt CM, 10 mM NaCl, 10 mM LiCl or a co-transfection of β-catenin and stabilized β-catenin (S37A). The activity was measured by dual luciferase analysis. Values present the mean±s.d. (n=5) and are expressed as an n-fold increase relative to the control medium.

the epithelial cells undergo early fiber differentiation. Previous studies have shown that Wnt3a and 7a lead to β-catenin nuclear translocation (Shimizu et al., 1997). Consistent with this, we found that β-catenin accumulated in the nuclei of differentiating cells at the lens equator. These findings, together with the pattern of Wnt expression, indicate that Wnt signaling in the transitional zone at the lens equator may have a role in promoting aspects of fiber differentiation, including changes in cell shape and the expression of genes that are involved in fiber cell differentiation.

In the rat lens epithelial explants system that we used, treatment with Wnt alone lead to the accumulation of β-crystallin without cell elongation. Although lens fiber differentiation usually involves cell elongation and crystallin expression, these events are not necessarily linked (Lovicu and McAvoy, 2001). We also showed nuclear translocation of β-catenin, along with decreased GSK-3β kinase activity, in Wnt-treated explants. β-catenin has been implicated as a transcriptional component in Wnt signal transduction. We therefore examined the ability of β-catenin to activate the β-crystallin promoter; as expected, β-catenin increased the activity of the β-crystallin promoter. This effect of β-catenin is further supported by the demonstration that overexpression of ICAT and GSK-3β, negative regulators of Wnt/β-catenin signaling, inhibited the increase in activity induced by β-catenin. In the canonical mechanism of Wnt signaling, β-catenin activates transcription via its interactions with the Tcf/LEF family of transcription factors, which bind to the sequence 5′-A/T A/T CAAAG-3′ (van de et al., 1997). However, the promoter of β-crystallin does not contain the core Tcf/LEF binding site. It is possible that β-catenin activates β-crystallin expression indirectly, through the activation of another factor that binds to this promoter.

Cell elongation is a well-recognized component of lens fiber differentiation (Fromm and Overbeek, 1996). FGF induces cell elongation in rat lens explants. This event requires a continuous signal from FGF (Lovicu and McAvoy, 2001). We showed that cell elongation is induced by a longer exposure to FGF alone, but not by exposure to Wnt alone. Interestingly, after priming by FGF, Wnt induced cell elongation accompanied with β-crystallin expression. Previous studies have shown that although a brief FGF signal is not sufficient to induce cell elongation, the process of lens fiber differentiation can be initiated by a short pulse of high-dose FGF if that pulse is followed by the longer-term application of a second factor, such as insulin or IGF-1 (Leenders et al., 1997; Klok et al., 1998). Thus, these findings indicate the necessity of the initial signal of FGF for morphological differentiation. What mechanism might account for the Wnt-induced cell elongation after FGF priming? FGF is essential to lens differentiation but the molecular mechanism is not clear (Schulz et al., 1993). FGF2 induces Lef/Tcf-dependent transcription of cyclin D1, a known target gene of the β-catenin/Lef pathway, and this growth factor enhances the nuclear translocation of β-catenin and reduces GSK-3 activity in human umbilical vein endothelial cells (Holnthoner et al., 2002). In primary rat neuronal cells, FGF1 promotes GSK-3β inactivation through ERK-independent Akt phosphorylation and stimulates the translocation of β-catenin into the nucleus (Hashimoto et al., 2002). This suggests the possibility that an FGF/GSK-3β-mediated increase of nuclear levels of β-catenin is enhanced further by Wnt, thereby promoting the expression of genes required for morphological differentiation, such as those involved in cell adhesion and exit from the cell cycle. However, we did not observe any inactivation of GSK-3β by FGF2 in lens explants (data not shown), and showed that cell elongation is dependent on ERK activity (Fig. 5H). In addition, TOP promoter activity was not increased significantly by FGF2 in a lens epithelial cell line (Fig. 1C). Therefore, FGF signaling is unlikely to cause the inhibition of GSK-3β kinase activity. Another possibility is that β-catenin-independent Wnt pathways cooperate with an initial FGF signal to induce cytoskeletal reorganization, an event shown previously to be critical for the requisite change in cell shape that occurs during morphogenetic differentiation (Ferreira-Cornwell et al., 2000). Wnt3a is able to induce

cytoskeletal reorganization (Shibamoto et al., 1998). Recent evidence has shown that Wnt signaling can activate both the β-catenin pathway and the small GTPases Rho and Rac separately or together (Pandur and Kuhl, 2000; Ziemer et al., 2001; Habas et al., 2003). Interestingly, recent studies in lens cells showed that Rho and Rac GTPase are activated by FGF signaling within 1 hour and that cytoskeletal reorganization in lens cells may require the function of Rho and Rac GTPase (Maddala et al., 2003). Thus, FGF-induced Rho or Rac activation may be enhanced by a β-catenin-independent Wnt pathway to induce cell elongation. In this context, further study will be needed to analyze direct cross-talk between the initial FGF signal and the β-catenin-independent Wnt pathway.

It is generally considered that Wnt/β-catenin signaling promotes cell proliferation, rather than triggering differentiation. In our studies, Wnt induced mitogenic activity and fiber cell differentiation in lens epithelial cells. These results raise the question of how the same Wnt protein directs two opposite functions, namely mitogenic activity and fiber cell differentiation. Previous studies have shown that lower doses of FGFs stimulate the proliferation of lens epithelial cells, whereas higher doses promote fiber differentiation (Chamberlain and McAvoy, 1987). It is possible that the effects of Wnt signaling are also dependent on dose or that different Wnt proteins preferentially mediate different responses in lens cells. These issues will have to be addressed by studies that define the function of the Wnt signaling pathway in the lens and the roles of each of the different Wnts in lens cell proliferation and differentiation.

In conclusion, our results strongly suggest that Wnt signaling has the capacity to induce fiber differentiation in cultured rat lens epithelial explants via the regulation of β-crystallin expression and cell elongation. Our findings also imply that at least two different Wnt pathways are involved in lens fiber differentiation: one is a β-catenin-dependent pathway that promotes β-crystallin expression, and the other is a β-catenin-independent pathway that promotes morphological differentiation. Furthermore, given the fact that lithium mimics some but not all of the activities of Wnt in explants pre-stimulated by FGF, Wnt signaling will probably function in a second yet-to-be-defined pathway, cooperating with the initial signal of FGF to induce cell elongation, but not involving GSK-3β/β-catenin. Our continuing studies will focus on the direct contribution of the Wnt/Frizzled pathway to FGF-triggered fiber differentiation and the molecules involved in the convergence of the FGF and Wnt pathway that mediate the morphological aspects of fiber cell formation.

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