Lenticular cytoprotection, part 2: Link between glycogen synthase kinase-3β, epithelial to mesenchymal transition, and mitochondrial depolarization

Sudha Neelam, Morgan M. Brooks, Patrick R. Cammarata

Department of Cell Biology and Immunology, University of North Texas Health Science Center at Fort Worth, Fort Worth, TX

Purpose: The inhibition of GSK-3β blocks mitochondrial membrane permeability transition (mMPT) for HLE-B3 cells in atmospheric oxygen. GSK-3β, as part of a multifactorial complex, also regulates nuclear levels of β-catenin, a known coordinator of cell survival and adhesion. The purpose of these studies was to demonstrate a novel, but likely disadvantageous, link between β-catenin’s influence on the expression of the pro-survival protein, vascular endothelial growth factor (VEGF), resulting in enhanced lens epithelial cell mitochondrial protection against depolarization and nuclear β-catenin as an inducer of epithelial to mesenchymal transition (EMT).

Methods: Virally transformed human lens epithelial cells (HLE-B3) were treated with SB216763, a specific inhibitor of GSK-3β catalytic activity and XAV939, a specific β-catenin inhibitor that bars the translocation of β-catenin from cytoplasm to the nucleus. Western blot analysis was employed to detect the levels of cytoplasmic and nuclear β-catenin and phospho-β-catenin, pBcl-2 and the EMT proteins, α-smooth muscle actin (α-SMA), and fibronectin. ELISA was used to measure the levels of VEGF in cell culture supernatants. JC-1 analysis was performed to analyze the influence of either SB216763 or XAV939 on mitochondrial depolarization.

Results: Cultured lens epithelial cells maintained in hypoxia (1% oxygen) and subsequently reintroduced into atmospheric oxygen and treated with the GSK-3β inhibitor SB216763 illustrated a marked inhibition of phosphorylation of glycogen synthase (downstream substrate of GSK-3β) and significant increase in nuclear translocation of β-catenin. The augmented nuclear β-catenin levels positively correlated with increased expression of α-SMA and fibronectin, both marker proteins indicative of EMT. The enhanced nuclear β-catenin activity also elicited increased VEGF and pBcl-2 expression, resulting in increased resistance to mitochondrial depolarization. Treatment of the cells with the β-catenin inhibitor XAV939 resulted in decreased expression of nuclear β-catenin, VEGF levels, pBcl-2, and EMT proteins, as well as increased mitochondrial depolarization.

Conclusions: The data support a model whereby the onset of epithelial to mesenchymal transition may circuitously benefit from the enhanced synthesis of VEGF by setting up a potentially harmful situation whereby the resulting mesenchymal cell population may be more resistant to mitochondrial depolarization than the lens epithelial cell population from which it originated. These findings support the potential therapeutic relevance of developing strategies to undermine the progression of normal cells to mesenchymal transition without subverting cell viability.

The human lens thrives in a naturally hypoxic environment [1]. During ocular surgeries, oxygen may be introduced to the hypoxic lens. Upon introduction of atmospheric oxygen, there is the potential for the onset of posterior capsule opacification (PCO). PCO occurs as the residual lens epithelial cells that line the inside surface of the equatorial lens capsule proliferate and migrate along the capsule until they reach its posterior aspects. These cells undergo epithelial to mesenchymal transition (EMT), by which they become myofibroblast-like, express mesenchymal markers, and exhibit a contractile phenotype contributing to the wrinkling and fibrosis of the lens capsule [2,3]. Wallentin et al. [4] have shown that the aqueous humor, isolated from post-cataract surgery rabbit eyes, displayed proliferative effects on lens epithelial cells. Their studies demonstrated that growth factors, including basal fibroblast growth factor (bFGF) and transforming growth factor beta (TGF-β), contributed to the proliferation of the lens epithelial cells.

TGF-β induces morphological and molecular changes in lens epithelial cells, leading to the pathological PCO condition [4]. It has yet to be clarified whether elevated TGF-β levels are the consequence of the cataract or whether elevated TGF-β levels induce the cataract. Liu et al. [5] used rat epithelial cell explants to study the effect of TGF-β and bFGF on lens epithelial cell migration and proliferation. The authors demonstrated that TGF-β induced proliferation of lens epithelial cells and secretion of the extracellular matrix components. In a related study, Chong et al. [6] showed that Wnts
and their frizzled receptors are upregulated in lens epithelial cells in association with elevated TGF-β expression, which was linked to the formation of cataract. This study demonstrated that TGF-β promotes the expression of Wnts and frizzled receptors in lens epithelial cells, which leads to the activation and translocation of β-catenin from cell margins to the nucleus. Wnt/β-catenin signaling is initiated with the binding of Wnt ligands to a frizzled receptor and the formation of a complex with an LDL-related protein. The formation of this complex inactivates the enzyme glycogen synthase kinase-3β (GSK3-β). GSK3-β, when active, phosphorylates β-catenin and targets it for degradation through the ubiquitin pathway. In the absence of active GSK3-β, β-catenin is not phosphorylated and the unphosphorylated form of β-catenin translocates to the nucleus, subsequently activating several nuclear transcription factors, promoting cell proliferation and survival [7]. Cain et al. [8] further defined the role of β-catenin in the proliferation and differentiation of lens epithelial cells and fiber cells during development. Using a cre/lox-p system, β-catenin was deleted in the lens and the lens fibers, which resulted in abnormal morphology of epithelial cells. Collectively, these studies demonstrate that β-catenin plays an important role in the proliferation of lens epithelial cells, and that TGF-β contributes to the activation of β-catenin.

Skurk et al. [9] have previously shown that β-catenin is an important downstream target of GSK-3β and that inhibition of GSK-3β leads to increased nuclear β-catenin activity, which in turn promotes the synthesis of vascular endothelial growth factor (VEGF) in endothelial cells. Moreover, Easwaran et al. [10] have shown that, when colon cancer cells that exhibit elevated levels of β-catenin are treated with β-catenin antisense oligonucleotides, it leads to a significant decrease in the levels of VEGF, suggesting a link between β-catenin signaling and VEGF regulation. Studies of this nature have yet to be performed in an ocular system. Collectively, the work of Skurk et al. [9] and Easwaran et al. [10] support the data described herein, in that we demonstrate that the downstream effects of GSK-3β inhibition involves increased nuclear β-catenin and elevated VEGF synthesis. We have previously reported that increased VEGF synthesis is linked to maintaining enhanced levels of the anti-apoptotic protein pBcl-2, providing resistance to mitochondrial depolarization [11].

Active nuclear β-catenin also initiates epithelial to mesenchymal transition [12]. Using human lung fibroblasts, Caraci et al. [13] have shown that TGF-β induces the expression of alpha smooth muscle actin (α-SMA) through GSK-3β inhibition and nuclear translocation of β-catenin. The study further demonstrated that inhibition of β-catenin by siRNA leads to decreased synthesis of α-SMA in TGF-β–treated fibroblasts. Kato et al. and others [14,15] have also shown that activation of β-catenin leads to EMT, which contributes to the pathogenesis of pterygium. In the lens, EMT plays an important role in the pathogenesis of posterior capsular opacification. PCO is a common complication of cataract surgery and is seen in as many as 25% of postoperative patients and may take up to five years post-cataract surgery before its onset is apparent [16].

Disruption of the Wnt/β-catenin pathway has been observed in many cancer cells, making this pathway an attractive target for anti-cancer therapies [17]. A study by Huang et al. analyzed a small molecule inhibitor, XAV939, which selectively mediates the destruction of β-catenin by stabilizing the AXIN protein in the β-catenin destruction complex [18]. A study by Tian et al. [19] using the β-catenin inhibitor XAV939 has demonstrated that inhibition of the Wnt/β-catenin signaling pathway in neuroblastoma cell lines resulted in apoptosis. Collectively, the studies cited above lend powerful support to the notion that either activation of the TGF-β/Wnt pathway or direct inhibition of GSK-3β catalytic activity results in increased nuclear β-catenin activity via its translocation from the cytosol. The resulting activated nuclear β-catenin promotes cell survival and proliferation, in part due to increased resistance to mitochondrial depolarization. On the other hand, a loss of nuclear β-catenin results in decreased resistance to mitochondrial depolarization, which, under appropriate conditions, may lead to apoptosis. The studies described herein reaffirm these observations and reveal a β-catenin–cell survival pathway in lens epithelial cells.

In the current study, we describe the regulatory pathways involved in preventing mitochondrial depolarization resulting from the inactivation of GSK-3β activity using the pharmacological inhibitor SB216763. At the same time, GSK-3β inactivation elicits epithelial to mesenchymal transition. As such, any ensuing occurrence of posterior capsular opacification brought on by the proliferative capacities of the mesenchymal cell population is likely to be accompanied by a situation whereby the newly generated cell type is more resistant to cell death (e.g., in our hands, mitochondrial depolarization) than the lens epithelial cell population from which it derives. We demonstrate that the link between mitochondrial protection and the ensuing EMT is via the translocation of cytoplasmic β-catenin to the nucleus, where the enzyme acts as the transcription factor for both events. Furthermore, we firmly establish the link between activated nuclear β-catenin, mitoprotection, and EMT using the β-catenin inhibitor XAV939.
Furthermore, we wish to clarify that, in an effort to not overstate our data, studies described herein were not intended to directly investigate the characterization of mesenchymal transition. Instead, we use an analysis of the EMT marker proteins, fibronectin, and α-SMA as predictors of mesenchymal transition. It is generally accepted that an increase in the expression of fibronectin and α-SMA is an early-onset indicator of the transition of epithelial cells to a mesenchymal phenotype.

**METHODS**

**Materials:** An HIF-2α translation inhibitor (CAS882268–69–1) was purchased from EMD Chemicals (Billericia, MA). The Glycogen Synthase Kinase inhibitor 3-(2,4-Dichlorophenyl)-4-(1-methyl-1H-indol-3-yl)-1H-pyrrrole-2,5-dione (SB216763) was purchased from Sigma-Aldrich (St. Louis, MO). The β-catenin inhibitor XAV939 (3,5,7,8-Tetrahydro-2-[4-(trifluoromethyl)phenyl]-4H-thiopyrano[4,3-d]pyrimidin-4-one) was procured from Sigma-Aldrich (Milwaukee, WI). All inhibitors were dissolved in dimethyl sulfoxide (DMSO).

**Cell cultures:** HLE-B3 cells, a human lens epithelial cell line immortalized by the SV-40 virus [13], were obtained from U. Andley (Washington University School of Medicine, Department of Ophthalmology, St. Louis, MO). Authentication of the HLE-B3 cell line was verified by STR profile analysis (American Type Culture Collection, Manassas, VA) and confirmed that the cell was human and of female origin, as originally reported by Andley et al. [20]. A copy of the STR profile is available upon request. All studies with HLE-B3 cells were performed with pre-frozen stock cells (maintained in liquid nitrogen) between passages 14 and 17, and no experiments exceeded five passages beyond the initial stock cell passage. The cells were maintained in minimal essential media (MEM) containing 5.5 mM glucose supplemented with 20% fetal bovine serum (Gemini Bio-Products, Sacramento, CA), 2 mM L-glutamine, nonessential amino acids, and 0.02 g/l gentamycin solution (Sigma-Aldrich) and cultured at 37 °C and 5% CO2 and 95% O2. Cells were sub-cultured four to five d before the experiment and placed in MEM containing 20% FBS. Twenty-four h before the day of the experiment, cells were switched to serum-free MEM. Unless otherwise specified, all experiments followed a common protocol; cells were maintained in atmospheric O2 (~21%) for 90 min, then switched to hypoxic conditions (~1% O2) for 180 min, followed by reintroduction to atmospheric O2. Each experiment was executed with control DMSO-only cells (mock inhibitor treatment) and cells treated with inhibitors. The DMSO concentration per experiment never exceeded 0.05%, and DMSO levels higher than that have proven to be cytotoxic to the lens cell cultures.

Bovine eyes obtained from a local abattoir were transported on ice to the laboratory, where the lenses were removed aseptically. Bovine lens epithelial cells (BLECs) were isolated and cultured in 20% bovine calf serum–supplemented Eagle’s minimal essential medium. All studies with BLECs were performed on cells of passage 2.

**Western blot analysis:** Cytoplasmic and nuclear lysates were collected from HLE-B3 or normal bovine epithelial cell cultures after treatments using the NE-PER Nuclear and Cytoplasmic Extraction Kit (Thermo Scientific, Pittsburg, PA). A portion of the sample was used for protein quantification using the EZQ Protein Quantification Kit (Invitrogen, Carlsbad, CA) and 3X SDS (Laemmli) buffer was added to the remaining lysates, which were subsequently boiled for 5 min; the proteins were resolved by electrophoresis on 12% SDS-polyacrylamide gels (20 μg protein/lane). Proteins were then transferred to nitrocellulose membranes (Scheicher and Schuell, Keene, NH).

For the western blot analysis, nitrocellulose membranes were blocked with 1% BSA and 0.02% Tween-20 in Tris-buffered saline (TTBS) for 60 min. These membranes were incubated overnight at 4 °C with primary antibodies. The blots were then rinsed in TTBS (4X with 5-min washes) and incubated in either goat anti-rabbit horseradish peroxidase conjugate or goat anti-mouse horseradish peroxidase conjugate (Santa Cruz Biotechnology, Santa Cruz, CA) for 1 h at room temperature. Required concentrations of antibodies were determined according to the manufacturer’s suggested protocols. Blots were again rinsed in TTBS (four 5-min washes), and proteins were detected using a SuperSignal West Femto Chemiluminescent Kit (Pierce, Rockford, IL).

Primary antibodies used in this study were rabbit anti-BAX, rabbit anti-pBcl-2, rabbit anti–Bcl-2 (Cell Signaling Technology, Danvers, MA), rabbit anti–Actin (Santa Cruz, CA), rabbit anti-HIF-2α (Novus Biologicals, Littleton, CO), rabbit anti-β-catenin, rabbit anti-phospho-β-catenin antibody, rabbit anti-Glycogen Synthase, rabbit anti-phospho-Glycogen Synthase (Ser641), rabbit anti-phospho-GSK-3β (Ser9), rabbit anti-GSK-3β and rabbit anti-LaminA/C antibody (Cell Signaling Technology, Danvers, MA), mouse anti-α-SMA (Sigma-Aldrich, St. Louis, MO), rabbit anti-fibronectin (Millipore, Billerica, MA), and rabbit anti-E-cadherin (Bioss Inc., Woburn, MA). Western blot analysis was generally repeated in triplicate from three independent cell populations.

**ELISA:** ELISA was performed for the detection of VEGF using an Invitrogen VEGF ELISA kit (Grand Island, NY).
using HLE-B3 cells. Normal bovine lens epithelial cells were evaluated for VEGF levels using an ELISA kit from NeoBi- oLab (Cambridge, MA). HLE-B3 and normal bovine lens epithelial cells were cultured in 25 cm² tissue culture flasks in 20% FBS, then transferred to serum-free media before the initiation of the experiment. Flasks were generally set up in triplicate for 3 h of incubation in hypoxia. At the end of 3 h, fresh serum-free media was added and cells were incubated at atmospheric oxygen for 2, 4, and 24 h. Cell-free supernatants were collected at 2, 4, and 24 h of re-oxygenation and analyzed according to the manufacturer’s instructions. The optical density at 450 nm was determined using a Molecular Devices Spectramax 190 (Sunnyvale, CA).

**JC-1 analysis:** After treatment with specific inhibitors, cells were stained with the cationic dye 5, 5′, 6, 6′-tetraclorofluorocarbon (JC-1; Molecular Probes, Eugene, OR) as described previously [16] to demonstrate the state of mitochondrial membrane potential. JC-1 is a potentiometric dye that exhibits a membrane potential dependent loss as J-aggregates (polarized mitochondria) to accumulation of JC-1 monomers (depolarized mitochondria) as indicated by a fluorescence emission shift from red to green [21,22]. According to this assay, mitochondrial depolarization is indicated by an increase in the green-to-red fluorescence intensity ratio.

The cells were stained using the following procedure. Monolayers were rinsed one time with serum-free MEM. Cell monolayers were incubated with serum-free MEM and 5 μg/ml JC-1 at 37 °C for 30 min. After this incubation, cells were again rinsed two times with the serum-free MEM and multiple images were obtained using a 10X objective on a confocal microscope (Zeiss LSM410, Thornwood, NY) excited at 488 nm, set to simultaneously detect green emissions (510–525 nm) and red emissions (590 nm) channels using a dual band-pass filter.

**Statistical analysis:** Images from JC-1 confocal microscopy were analyzed as individual red and green channels using ImageJ (Baltimore, MD). The background fluorescence was removed from each image before the intensity was measured. The fluorescence intensity signal from each image was quantified for the entire image and expressed as the ratio of green fluorescent intensity over red fluorescent intensity. Western blot densitometry was determined using ImageJ analysis. For ELISA, a Student’s t-test was performed by collecting the supernatants from three individual cell cultures stemming from an initial single cell population using Graphpad Prism version 5.00 software (La Jolla, CA). Statistical significance was determined based upon a p value <0.05. Error bars represent SEM. For bar graphs representing the density of western blot bands, a Student’s t-test was likewise applied.

**RESULTS**

**Inhibition of the enzymatic activity of GSK-3β leads to decreased pGS levels:** Non-phosphorylated GSK-3β is the active form of the enzyme. The active form of the enzyme phosphorylates its downstream substrate, glycogen synthase (GS). Phosphorylation of GS is, therefore, a useful indicator of GSK-3β activity. Treatment of HLE-B3 cells with SB216763 resulted in inhibition of phosphorylation of GS as compared to the untreated controls (Figure 1). There was no significant change in the levels of GSK-3β and phosphoglycogen synthase kinase-3β (pGSK-3β) between the control and SB 216,763-treated cells (Figure 1). That is, the autophosphorylation of GSK-3β is unaffected by treatment with SB216763, whereas the inhibition of the catalytic site prevented downstream phosphorylation of glycogen synthase [23].

**Inhibition of the enzymatic activity of GSK-3β leads to increased nuclear β-catenin:** GSK-3β, as part of a multiprotein complex, is involved in the Wnt signaling pathway [7]. β-catenin is an important downstream target of GSK-3β activity [7]. In the absence of active GSK-3β, β-catenin is not phosphorylated, and in this active form, β-catenin translocates to the nucleus, where it initiates the nuclear transcription of growth factors like VEGF [9]. Whether the inhibition of GSK-3β likewise leads to increased nuclear β-catenin and stimulation of VEGF synthesis and accumulation in lens epithelial cells has not previously been demonstrated. To address this, HLE-B3 cells were treated with 12 µm SB216763 for 3 h in hypoxia, followed by exposure to atmospheric oxygen with the inhibitor for 3 and 24 h. At the end of the incubation periods, cytoplasmic and nuclear extracts were collected (refer to Methods) and analyzed by western blot for levels of β-catenin and phospho-β-catenin. Control cells, mock-treated with DMSO, were similarly analyzed. Inhibition of GSK-3β activity resulted in increased accumulation of nuclear β-catenin and concomitant decrease in nuclear phospho-β-catenin (Figure 2).

**VEGF expression in HLE-B3 cells exposed to hypoxia (1% oxygen) followed by re-introduction of atmospheric oxygen (21% oxygen):** HLE-B3 cells were cultured in 20% serum and switched to serum-free media on the day of the experiment. The cells were incubated in serum-free media for 3 h, either in hypoxia or atmospheric oxygen. At the end of 3 h of hypoxic exposure, cell-free supernatants were collected in triplicate from the hypoxia-exposed set of cells. Fresh serum-free media was added to the hypoxia-exposed cells and then exposed to atmospheric oxygen. Cell-free supernatants were
then collected in triplicate at 2, 4, and 24 h of incubation post-atmospheric-oxygen-exposure. Similarly, at the end of 3 h of incubation (from the control cells maintained in atmospheric oxygen and never exposed to hypoxia), cell-free supernatants were collected in triplicate and fresh serum-free media was added to the cells. As above, supernatants were collected in triplicate after 2, 4, and 24 h of further incubation in atmospheric oxygen. The VEGF expression in the supernatants from the set of hypoxic-exposed cells later switched to atmospheric oxygen, as well as the cells maintained continuously in atmospheric oxygen, was analyzed by ELISA. There was a statistically significant increase in the VEGF levels, at all time points, when the cells were switched from hypoxic exposure to atmospheric oxygen as compared to the control cells incubated in continuous atmospheric oxygen (Figure 3A). This suggests that the act of reintroducing atmospheric oxygen to cells held in hypoxia is sufficient impetus to stimulate VEGF synthesis and accumulation.

Inhibition of the enzymatic activity of GSK-3β leads to increased VEGF levels: We recently reported that VEGF acts as a pro-survival factor in hypoxic lens epithelial cells and prevents mitochondrial depolarization by maintaining the levels of the anti-apoptotic protein pBcl-2 [11]. Brooks et al. [23] have shown that inhibition of GSK-3β catalytic activity prevents mitochondrial depolarization in lens epithelial cells exposed to atmospheric oxygen. Whether inhibition of GSK-3β catalytic activity leads to increased VEGF synthesis and accumulation in atmospheric oxygen has not previously been shown with cultured lens epithelial cells. To test this, HLE-B3 cells cultured in 25 mm² tissue culture flasks were treated with 12 µm of the specific GSK-3β inhibitor SB216763 in serum-free media for 3 h in hypoxia. At the end of 3 h, fresh serum-free media with the same concentration of SB216763 was added and incubated at atmospheric oxygen. Cell-free supernatants were collected in triplicate at the end of 3, 8, and 24 h of re-oxygenation and analyzed by ELISA to determine the VEGF levels. Control cells, mock-treated with DMSO, were analyzed in a similar manner. Inhibition of the enzymatic activity of GSK-3β resulted in a significant increase in VEGF levels after 24 h of incubation in atmospheric oxygen (Figure 3B).

Figure 1. Analysis of GSK-3β enzymatic activity. The experiment was repeated three times using independent cell populations, and the ratio between pGSK-β/GSK-3β and pGS/GS was quantified using ImageJ analysis. Treatment of HLE-B3 cells with SB216763 resulted in inhibition of phosphorylation of glycogen synthase as compared to the untreated controls in both the cytoplasmic and nuclear fractions. The asterisk (*) indicates a significant decrease of pGS (p<0.05). There was no significant change in the levels of GSK-3β and phosphoglycogen synthase kinase-3β (pGSK-3β) between the control and SB 216,763 treated cells in both the cytoplasmic and nuclear lysates. There was no significant carryover of the cytoplasmic markers in the nuclear fractions. SB=SB216763.
Figure 2. Western blot analysis of β-catenin and phospho-β-catenin in HLE-B3 cells treated with SB216763. The experiment was repeated three times with independent cell populations, and the ratio between β-catenin and phospho-β-catenin was quantified using ImageJ analysis. The levels of β-catenin and phospho-β-catenin in the cytoplasmic extracts were unchanged, whereas in the nuclear extracts there was a significant increase in β-catenin and decrease in phospho-β-catenin in the SB216763-treated cells as compared with the controls. The asterisk (*) indicates a significant increase in nuclear β-catenin (p<0.05). There was no significant carryover of the cytoplasmic markers in the nuclear fractions. SB=SB216763.

Figure 3. Detection of VEGF levels in atmospheric oxygen by ELISA. A: The supernatants from the cells incubated in hypoxia and later switched to atmospheric oxygen were compared with the supernatants collected from cells in consistent atmospheric oxygen for VEGF levels by ELISA. Samples were derived from three independent cell populations. A student’s t test was performed and significantly higher levels of VEGF were detected in cells switched from hypoxia to atmospheric oxygen as compared to cells maintained consistently in atmospheric oxygen (p<0.05). B: Detection of VEGF levels in cells treated with SB216763. The cells were incubated in triplicate from a single cell population with 3 ml of serum-free media containing 12 µm of SB216763 for 3 h in hypoxia. The asterisk (*) indicates a statistically significant (p<0.05) increase in the VEGF levels at 24 h in the SB216763-treated cells as compared to the control cells. SB=SB216763.
Effect of GSK-3β inhibition on the levels of the anti-apoptotic protein pBcl-2: VEGF acts as a pro-survival factor in the hypoxic lens epithelium by maintaining the levels of the anti-apoptotic protein Bcl-2 [11], and it is likely that a similar protective scheme is at play with cells in atmospheric oxygen. Since there was an increase in the levels of VEGF with the inhibition of GSK-3β enzymatic activity (Figure 3B), we ran a subsequent experiment, using HLE-B3 cells cultured in 100 mm² culture dishes. The cells were treated with 12 µm of the specific GSK-3β inhibitor SB216763, as described above. Cytoplasmic and nuclear lysates were collected (refer to Methods) and analyzed by western blot for pBcl-2 levels. There was no significant difference in the pBcl-2 levels in the cytoplasmic extract; there was a slight increase in the pBcl-2 levels in the nuclear extracts of SB216763-treated cells as compared to the corresponding nuclear extracts of the control cells. However, this increase in the pBcl-2 levels was not statistically significant (Figure 4).

Inhibition of the enzymatic activity of GSK-3β and expression of EMT/E-cadherin proteins: Nuclear β-catenin is associated with epithelial-to-mesenchymal transition. The expression of EMT proteins like α-SMA and fibronectin is generally taken as a strong early-onset indicator of progression toward EMT. To test whether increased nuclear β-catenin positively correlates with an increase in EMT proteins in the lens epithelial cells, total cell lysates from HLE-B3 cells treated with SB216763 in atmospheric oxygen, as described above, were used to detect the levels of α-SMA and fibronectin by western blot analysis. The increase in nuclear β-catenin with SB216763 treatment decisively correlated with an increase in the expression of the EMT proteins, α-SMA, and fibronectin as compared to the control cells (Figure 5, top panel). At the same time, the decreased expression of E-cadherin is also generally taken as a strong indicator of EMT. The increase in nuclear β-catenin with SB216763 treatment also positively correlated with a decrease in the expression of E-cadherin as compared to the control cells (Figure 5, bottom panel).

Demonstration of the association between nuclear β-catenin with VEGF and pBcl-2 levels using a pharmacological inhibitor: Our studies with the GSK-3β inhibitor SB216763 resulted in increased nuclear β-catenin, VEGF, and pBcl-2 levels (Figures 2B,C, and Figure 3). These results reinforce a model whereby there is an association between nuclear β-catenin and VEGF/pBcl-2 levels. To further support and reaffirm this inter-relationship, we employed a strategy that involved the use of a pharmacological inhibitor to deplete
nuclear β-catenin in lens epithelial cells exposed to atmospheric oxygen and determine the downstream effects of nuclear β-catenin inhibition on VEGF/pBcl-2 levels.

*Treatment of HLE-B3 cells with XAV939 leads to decreased nuclear β-catenin levels:* GSK-3β, as a part of the destruction complex consisting of the scaffolding proteins Axin and adenosomatous polyposis coli (APC), leads to phosphorylation and degradation of β-catenin by the ubiquitin/proteasome pathway. The pharmacological inhibitor XAV939 binds to the Tankyrase enzyme (TNKS) domain and leads to increased stabilization of the Axin protein in the destruction complex, leading to degradation of β-catenin [18,19]. To further establish the association between nuclear β-catenin and VEGF, we suppressed nuclear β-catenin levels. HLE-B3 cells were treated with 1 µm of the β-catenin inhibitor XAV939 in atmospheric oxygen as described previously. Cell-free supernatants were collected in triplicate and analyzed by ELISA for VEGF levels. There was a statistically significant decrease in VEGF levels in the cells treated with XAV939 as compared to control cells (Figure 7).

*Loss of nuclear β-catenin leads to decreased Bcl-2/pBcl-2 levels in HLE-B3 cells:* To establish the downstream link between decreased VEGF levels (refer to Figure 7) and Bcl-2/pBcl-2 content, lysates collected for cytoplasmic and nuclear β-catenin (Figure 6) were also analyzed by western blot for Bcl-2/pBcl-2 levels (Figure 8). The pharmacological depletion of nuclear β-catenin not only resulted in the attenuation of VEGF levels (Figure 7) but also in the significant lessening of Bcl-2/pBcl-2 levels in the nuclear extracts of the cells treated with XAV939(Figure 8). These data reaffirm that VEGF influences Bcl-2 levels in the adult lens epithelium maintained in atmospheric oxygen.

*Depletion of nuclear β-catenin leads to mitochondrial depolarization in HLE-B3 cells exposed to atmospheric oxygen:*
Previous studies from our laboratory have demonstrated that VEGF acts as a pro-survival factor in the hypoxic lens epithelium by maintaining the levels of the anti-apoptotic protein pBcl-2 [11]. Brooks et al. [24] have also reported that loss of pBcl-2 in the lens epithelium leads to mitochondrial depolarization in atmospheric oxygen. To demonstrate the role of β-catenin in regulating the pBcl-2 levels in atmospheric oxygen, a JC-1 assay was performed on lens epithelial cells cultured with the β-catenin inhibitor XAV939 with either 0.5 µM or 1 µM of the inhibitor in serum-free media for 3 h in hypoxia. At the end of the hypoxic exposure, the hypoxic media was poured off and a fresh serum-free medium (with inhibitor) containing 5 µg/ml JC-1 was added for 30 min and incubated at atmospheric oxygen. The stained cells were then rinsed twice using serum-free medium, and fresh oxygenated serum-free medium (with no JC-1 dye) was added.

Control cells were maintained as described but without the inhibitor being administered. A random field of cells was imaged every 2.5 min for 90 min using an 10X objective on a confocal microscope (Ziess LSM410). The excitation wavelength was 488 nm, and the microscope was set to simultaneously detect green emission (540 nm) and red emission (595 nm) channels using a dual band-pass filter [22]. Inhibition of β-catenin in atmospheric oxygen resulted in profound mitochondrial depolarization as compared to the control cells treated with DMSO (Figure 9).

Control cells were maintained as described but without the inhibitor being administered. A random field of cells was imaged every 2.5 min for 90 min using an 10X objective on a confocal microscope (Ziess LSM410). The excitation wavelength was 488 nm, and the microscope was set to simultaneously detect green emission (540 nm) and red emission (595 nm) channels using a dual band-pass filter [22]. Inhibition of β-catenin in atmospheric oxygen resulted in profound mitochondrial depolarization as compared to the control cells treated with DMSO (Figure 9).

Inhibition of the nuclear β-catenin and expression of EMT proteins: Increased nuclear β-catenin positively correlates with an increase in EMT proteins in the lens epithelial cells (Figure 5). To further confirm the association between nuclear β-catenin and mesenchymal marker proteins, total cell lysates from HLE-B3 cells treated with XAV939 in atmospheric oxygen, as described above, were used to detect the levels of α-SMA and fibronectin by western blot analysis. The decrease in nuclear β-catenin with XAV939 (Figure 6) treatment decisively correlated with a decrease in the expression of the EMT proteins α-SMA and fibronectin as compared to the control cells (Figure 10).

Treatment of HLE-B3 cells with HIF-2α translation inhibitor: We have previously demonstrated the functional expression of the hypoxia inducible factors HIF-1α and HIF-2α in hypoxic human lens epithelial cells. Our data supported a model in which the sustained synthesis of VEGF in human lens epithelial cells, maintained under hypoxic conditions, is regulated by a compensatory inter-relationship between HIF-1α and HIF-2α [18]. HIF-1α is not expressed in atmospheric oxygen, whereas HIF-2α is resistant to degradation in atmospheric oxygen by the enzyme prolyl hydroxylase (refer to reference 11). Since we observed a consistent accumulation of VEGF in the HLE-B3 cells switched from hypoxia to atmospheric oxygen (Figure 3A), we examined whether HIF-2α inhibition affected VEGF synthesis.

HLE-B3 cells cultured in 25 cm² tissue culture flasks were treated with CAS882268–69–1, a specific HIF-2α translation inhibitor that suppresses HIF-2α protein synthesis but not mRNA transcription (refer to reference 11). The HIF-2α translation inhibitor was prepared to final concentrations of 0.5 µm, 5 µm, and 50 µm in serum-free media using DMSO.
that did not exceed 0.05% under any treatment concentration. Cells were incubated with the inhibitor for 3 h in hypoxia, and after 3 h, the media were replaced with fresh media containing the same concentrations of inhibitor and incubated in atmospheric oxygen for an additional 3, 8, and 24 h. Control (mock-treated) cells were incubated with DMSO and jointly collected under the same conditions. At all concentrations of the inhibitor used, HIF-2α was markedly suppressed but not completely eradicated (Figure 11A).

HLE-B3 cells were treated with 5 µm and 50 µm of HIF-2α translation inhibitor in atmospheric oxygen as described above. Cell-free supernatants were collected in triplicate at the end of 3, 8, and 24 h of incubation in atmospheric oxygen and analyzed by ELISA to determine the VEGF levels. Inhibition of HIF-2α did not influence VEGF synthesis in atmospheric oxygen relative to the corresponding controls (Figure 11B).

VEGF has been shown to prevent mitochondrial depolarization and promote cell survival in hypoxic HLE-B3 cells by maintaining the levels of the anti-apoptotic protein Bcl-2 [11]. As inhibition of HIF-2α did not influence VEGF levels (Figure 11B), it followed that HIF-2α inhibition should not influence the levels of the anti-apoptotic proteins Bcl-2/ pBcl-2, thereby supporting a conclusion that HIF-2α neither plays a role in VEGF regulation in atmospheric oxygen nor affects downstream pBcl-2 levels. The cells were treated with 0.5 µm, 5 µm, and 50 µm of HIF-2α translation inhibitor in serum-free media for 3 h in hypoxia; after 3 h, fresh serum-free media containing similar concentrations of the inhibitor were added and incubated at atmospheric oxygen for 3 h. Control cells incubated with DMSO were treated similarly. After 3 h, cell lysates were collected and analyzed by western blot to determine the levels of anti-apoptotic proteins Bcl-2 and pBcl-2. Inhibition of HIF-2α had no effect on the levels of pBcl-2 (Figure 11C).

**Bovine lens epithelium demonstrates a similar response to inhibition of enzymatic activity of GSK-3β as compared to HLE-B3 cells:** The HLE-B3 cell line is T-antigen-transformed and thus may not be accurately representing normal cell proliferation and apoptosis responses, calling into question whether these cells have biologic or clinical relevance. That is, transformed cells are often dismissed as a poor model for studying early-onset epithelial-to-mesenchymal transition since specific molecules exert their effects through cell cycle-specific mechanisms and the transformed cells have abnormal levels of cell cycle control that may falsely lead to misinterpretation of metabolic and biologic function. We are keenly receptive to the fact that the use of virally-transformed cells may not necessarily be representative of survival mechanisms operating in normal cells. We resolutely affirm that conclusions based upon virally transformed cells must be carefully replicated under the same experimental conditions using early-passage non-transformed cells. To rule out the possibility of viral transformation influencing our results and interpretations, we repeated the SB216763 treatment with HLE-B3 cells as previously described, in normal secondary cultures of bovine lens epithelial cells. Our findings demonstrate that, similar to HLE-B3 cells, inhibition of the enzymatic activity of GSK-3β results in increased nuclear β-catenin and concomitant decreases in the phospho-β-catenin as compared to the control cells (Figure 12A). Inhibition of GSK-3β also led to stimulation of VEGF synthesis and accumulation in the HLE-B3 cells due to elevated nuclear β-catenin activity (Figures 2 and 3). To reconfirm this effect of increased nuclear β-catenin on VEGF in normal bovine lens epithelial cells, the cells were cultured in 25 mm² tissue culture flasks and treated with SB21763 as described above. The cell-free supernatants were collected after 3 and 8 h of incubation at atmospheric oxygen and analyzed for VEGF levels by ELISA. Inhibition of the enzymatic activity of GSK-3β resulted in a significant increase in VEGF levels after 3 and 8 h of incubation in atmospheric oxygen (Figure 12B). Finally, we show that increased nuclear β-catenin (Figure 12A) positively correlated with increased fibronectin (Figure 12C) in the lens epithelial cell lysates from bovine
cells. For the purposes of this study, we conclude that normal, early secondary-passage bovine lens epithelial cells display the same functional pathways as observed in HLE-B3 cells.

**DISCUSSION**

Previous data from our laboratory have supported a model in which the sustained synthesis of VEGF in human lens epithelial cells, maintained under hypoxic condition, is regulated by a compensatory inter-relationship between HIF-1α and HIF-2α. VEGF acts as a pro-survival factor in hypoxic lens epithelial cells by maintaining consistent expression of the anti-apoptotic protein Bcl-2, which likely prevents the translocation of cytosolic BAX to the outer mitochondrial membrane, thus preventing the initiation of mitochondrial depolarization [11]. In the current study, we demonstrate that VEGF is also consistently synthesized and accumulated in lens epithelial cells maintained in atmospheric oxygen (Figure 3A). Whereas HIFs regulate VEGF levels in hypoxia, the present study demonstrates that it is primarily glycogen synthase kinase-3β (GSK-3β) that regulates VEGF levels in atmospheric oxygen. We recently reported that inhibition of the enzymatic activity of GSK-3β prevents mitochondrial depolarization [23], but in that study we did not specifically identify the downstream mechanism(s) involved in the protection pathway. In this study, we define the downstream pathways influenced by GSK-3β inhibition. Collectively, these pathways involve the increase of activated nuclear β-catenin via translocation from cytoplasm to nucleus (Figure 2), which in turn subsequently increases VEGF synthesis and accumulation (Figure 3B), thereby preventing mitochondrial depolarization [24].

First, we sought to reconfirm our previous results in which treatment with SB216763 resulted in inactivating GSK-3β’s catalytic site as opposed to blocking GSK-3β’s autophosphorylation site, which led to the prevention of mitochondrial depolarization [23]. To monitor the enzyme’s active catalytic site, we scrutinized the phosphorylation of a downstream substrate of GSK-3β, glycogen synthase (GS). In the previous study [23], enzymatic activity was illustrated using whole-cell lysates with western blot analysis. To gain a better perspective on the biochemistry involved, cell lysates in this study, upon treatment with SB216763, were first separated into cytoplasmic and nuclear components (refer to Methods) before western blot analysis. GSK-3β, pGSK-3β, GS, and pGS were detected in both the cytoplasmic and nuclear fractions of control cell lysates (Figure 1). Our data showed that the levels of GSK-3β, pGSK-3β, and GS were essentially unaffected in both the cytosolic and nuclear fractions of SB216763-treated cells (Figure 1). However, the pGS levels were markedly reduced in the cytosolic fraction and completely eradicated from the nuclear fraction of the SB216763-treated samples (Figure 1). Therefore, as expected, SB216763 did not block the autophosphorylation of GSK-3β relative to control cells, but successfully eliminated the phosphorylation of GS, indicating that the catalytic site of GSK-3β was inactivated (Figure 1). Stated another way, measuring GS phosphorylation and not GSK-3β autophosphorylation appears to be a better predictor of whether inhibiting GSK-3β’s enzymatic
activity positively correlates with the downstream signaling pathways. We can state with confidence that the action of SB216763 was effective, as demonstrated by the failure to phosphorylate GS, which in turn agrees with the inability to phosphorylate another downstream substrate of GSK-3β, namely β-catenin. We should add, however, that the separation of the cytoplasmic and nuclear lysates revealed the unanticipated observation that GSK-3β, pGSK-3β, GS, and pGS were present in the nuclear fraction (Figure 1). These results were repeated with three independent cytosolic and nuclear samples from three independent cell populations, and the cytoplasmic markers GAPDH and LDH were used to rule out cross-contamination between the cytosolic and nuclear fractions. The presence of glycogen synthase kinase-3β and glycogen synthase in the nucleus and what effect this might have on downstream signaling pathways remains to be determined.

Our data indicated that inhibition of the catalytic activity of GSK-3β resulted in consistent accumulation of the pro-survival factor VEGF over a time period of 3–24 h in atmospheric oxygen (Figure 3B). These results suggest, but do not definitely prove, a link between GSK-3β catalytic activity and prevention of mitochondrial depolarization via an elevation of VEGF accumulation. Data discussed below further defined the VEGF protection pathway in atmospheric oxygen.

We recently reported [23] that “inhibition of GSK-3β activity by SB216763 blocked mitochondrial membrane permeability transition relative to a slow but consistent depolarization observed with the control cells.” We concluded
that “inhibition of GSK-3β activity by the GSK-3β inhibitor, SB216763, provides positive protection against mitochondrial depolarization.” In a successive study [24], we further reported that “lenticular mitoprotection normally afforded by the inactivation of GSK-3β activity may be bypassed by a loss of pBcl-2, an anti-apoptotic member of the Bcl-2 family. Bcl-2 prevents the translocation of BAX to the mitochondrial outer membrane inhibiting depolarization by disrupting the normal electrochemical gradient leading to mitochondrial membrane permeability transition.” In that study [24], inhibition of GSK-3β activity using SB216763 did not show any significant difference in either Bax, Bcl-2, or pBcl-2 levels using total cell lysates. In the present study, we refined our approach by separating cytoplasmic and nuclear fractions in an attempt to gain greater insight into the relevance of pBcl-2 levels between control and SB216367-treated cells. To the best of our knowledge, this is the first time that the cellular distribution of cytoplasmic and nuclear Bcl-2 and pBcl-2 levels has been analyzed in the lens epithelium. Doing so revealed consistent levels of cytoplasmic and nuclear Bcl-2 and pBcl-2 between control and SB216763-treated samples (Figure 4). Portier and Taglialatela [25] have shown that “nuclear compartment-associated Bcl-2 functions as a pro-apoptotic protein and that localization of Bcl-2 at the nucleus results from failure of FKBP38-mediated delivery of Bcl-2 to the mitochondria.” In another study, with breast cancer cells, Hoetelmans et al. [26] demonstrated that the Bcl-2 proteins are not only associated with the cytoplasm, but are also seen in the interphase nuclei. In that study, the authors suggested that the role of Bcl-2 proteins can also be extended to nuclear compartments. Based on the lack of statistical significance relating to Bcl-2 and pBcl-2 levels between control and SB216763-treated samples (Figure 4), the data as shown in Figure 4 neither support nor refute the possibility that nuclear Bcl-2 or pBcl-2 influence a trend toward apoptosis. However, additional data discussed below (Figure 8) strongly suggest that the loss of either Bcl-2 or pBcl-2 from the nucleus does, indeed, lead to mitochondrial depolarization, suggesting that one of these proteins acts as a pro-survival factor. We have previously shown that virally transformed HLE-B3 cells, as well as normal bovine lens epithelial cells, could be made to depolarize with UO126 treatment [24]. In that study, we took advantage of the observation that UO126-treatment with HLE-B3 cells instigated a loss of Bcl-2, whereas UO126 treatment with normal bovine lens epithelial cells did not diminish the levels of Bcl-2. However, with both the human virally transformed lens epithelial cell and normal bovine lens epithelial cell, a profound loss of pBcl-2 was apparent with UO126 treatment. Therefore, since the bovine cells depolarized without the loss of Bcl-2, we concluded that pBcl-2 confers pro-survival resistance in lens epithelial cells. In the context of the current study, we suggest that it is the loss of nuclear pBcl-2 that leads to mitochondrial depolarization (see below). In other words, it is pBcl-2 that acts as a pro-survival factor in lens epithelial cells.

Activation of GSK-3β initiates apoptosis, while its inhibition is involved in anti-apoptotic signaling via the Wnt/β-catenin pathway [7]. Activation of β-catenin is an important downstream effect of GSK-3β inhibition [9]. Studies in colon cancer have previously demonstrated that VEGF-A is an important downstream target of β-catenin activation, as demonstrated by the fact that active β-catenin induced VEGF mRNA and protein expression in these cell lines [10]. Herein we demonstrate a significant association between inhibition of GSK-3β and increased translocation of β-catenin from the cytoplasm to the nucleus (Figure 2), with increased VEGF expression (Figure 3B). But in addition to inducing the
transcription of cell survival factors like VEGF, β-catenin is also involved in the pathophysiology of EMT [12]. During EMT, the epithelial cells lose their polarity, with loss of E-cadherins and cell–cell adhesion properties, and transform into mesenchymal cells. One of the important characteristic features of PCO, a complication of the cataract surgery, is the mesenchymal transition of the lens epithelial cells. The remnants of the lens epithelial cells after the cataract surgery proliferate across the lens capsule, migrate toward the intraocular lens, and undergo EMT, eventually leading to the development of PCO [3]. We propose that increased nuclear β-catenin activity elicits lenticular mitoprotection through increased VEGF levels, (Figure 3B) but at the same time, via an independent nuclear β-catenin-controlled pathway, a marked increase in the expression of the EMT proteins, α-SMA, and fibronectin is simultaneously occurring (Figure 5). These data, therefore, have uncovered a heretofore unappreciated observation in the lens literature in that elevated nuclear β-catenin activity is coupled to two distinct parallel phenomena. One pathway leads to the increase in the pro-survival protein VEGF (Figure 3B), which is linked to the pro-survival protein pBcl-2 (Figure 4). The second and independent pathway leads to the initiation of epithelial-to-mesenchymal transition (as indicated in the increase in the EMT marker proteins, α-SMA and fibronectin) (Figure 5). Other reports in the literature lend support to our conclusion. In a study by Martinez et al. [27], Wnt/β-catenin signaling was activated by conditional knockout of the APC genes in the whole lens. The mutant APC protein inactivated the degradation complex of β-catenin, resulting in constitutively high expression of β-catenin. The Martinez et al. study [27] further elucidated the role of the Wnt/β-catenin pathway as an initiator of EMT. Bao et al. [15] have examined the effect of Wnt 3α on β-catenin levels and progression of PCO. Wnt 3α was overexpressed in the HLE-B3 lens epithelial cell, resulting in increased total β-catenin, decreased E-cadherins, and
increased expression of EMT proteins, such as fibronectin. The HLE-B3 cells reportedly also displayed an irregular morphological pattern, characteristic of a mesenchymal-like cell.

Our data have shown that the use of the GSK-3β inhibitor SB216763 suggested an association between elevated nuclear β-catenin activity (Figure 2), increased VEGF (Figure 3B), increased α-SMA and fibronectin, and a concomitant decrease in E-cadherin with virally transformed B3 cells (Figure 5). The elevation of nuclear β-catenin activity, increased VEGF expression, and accompanying increase in fibronectin expression were reproduced with normal bovine lens epithelial cells (Figures 12). These data support and confirm previous observations from other laboratories that have shown that a loss of E-cadherin with accompanying elevation in EMT marker proteins (i.e., fibronectin and α-SMA) represent early-onset indicators of mesenchymal transition [28,29]. Such data imply, but do not necessarily prove, that the two phenomena (enhanced lens epithelial cell mitochondrial protection and early expression of EMT markers with concomitant loss of E-cadherin) are the result of increased nuclear β-catenin activity. To firmly establish the inter-relationship between nuclear β-catenin activity, lens mitochondrial protection, and early-onset EMT, we refined our experimental approach to include suppression of the expression of β-catenin. Doing so permitted a more...
polarized lens epithelial phenotype and inhibits proliferation, migration and epithelial mesenchymal transition. However, we acknowledge that epithelial-to-mesenchymal transition is not necessarily accepted by all lens researchers as a PCO-associated event and that the lens may not be a mesenchymal tissue. While we could find no direct evidence to support this statement in the lens literature, we cannot, at this time, rule out the possibility that a non-mesenchymal event may drive the development of posterior capsular opacification.

In summary, the work reported herein unites myriad observations in multiple tissue preparations, and, for the first time in an ocular system, demonstrates a heretofore unappreciated inter-relationship stemming from the inhibition of the enzymatic activity of GSK-3β, which leads to increased nuclear β-catenin activity, in turn prompting both early-onset epithelial-to-mesenchymal transition and elevated VEGF expression, a forerunner to increased resistance to mitochondrial depolarization. Given that both cellular events occur concurrently, our data strongly suggest a previously unknown association describing a potentially deleterious situation whereby the newly created “mesenchymal-like” cell population is likely to be more resistant to mitochondrial depolarization.

ACKNOWLEDGEMENTS

The corresponding author dedicates this manuscript to, Robert G. Spiro, whom more than anyone else in his past, he attributes a profound influence on his pursuit of a career as a research scientist.
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Articles are provided courtesy of Emory University and the Zhongshan Ophthalmic Center, Sun Yat-sen University, P.R. China. The print version of this article was created on 31 December 2014. This reflects all typographical corrections and errata to the article through that date. Details of any changes may be found in the online version of the article.