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# Inhibition of hypoxia inducible factor-1α downregulates the expression of epithelial to mesenchymal transition early marker proteins without undermining cell survival in hypoxic lens epithelial cells

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**Purpose:** The purpose of this study was to identify potential therapeutic strategies to slow down or prevent the expression of early-onset epithelial to mesenchymal transition (EMT) marker proteins (fibronectin and alpha smooth muscle actin, α-SMA) without sacrificing the synthesis and accumulation of the prosurvival protein vascular endothelial growth factor (VEGF) in cultured virally transformed human lens epithelial (HLE) cells.

**Methods:** HLE-B3 cells, maintained in a continuous hypoxic environment (1% oxygen), were treated with SB216763, a specific inhibitor of glycogen synthase kinase-3β (GSK-3β) catalytic activity. Western blot analysis was employed to detect the cytoplasmic and nuclear levels of β-catenin, as well as the total lysate content of fibronectin and α-SMA. Enzyme-linked immunosorbent assay (ELISA) was used to measure the levels of VEGF in cell culture medium. A hypoxia-inducible factor-1α (HIF-1α) translation inhibitor and an HIF-2α translation inhibitor were independently employed to evaluate the effect of hypoxia inducible factor inhibition on EMT marker protein and VEGF expression. XAV932 was used to assess the suppression of nuclear β-catenin and its downstream effect on EMT marker proteins and VEGF expression.

**Results:** SB216763-treated HLE-B3 cells caused marked inhibition of GSK-3β activity prompting a significant increase in the translocation of cytoplasmic β-catenin to the nucleus. The enhancement of nuclear β-catenin looked as if it positively correlated with a significant increase in the basal expression of VEGF as well as increased expression of fibronectin and α-SMA. In conjunction with SB216763, coadministration of an HIF-1α translation inhibitor, but not an HIF-2α translation inhibitor, markedly suppressed the expression of fibronectin and α-SMA without affecting VEGF levels. Treatment with XAV932 significantly reduced the level of nuclear β-catenin, but the levels of neither the EMT marker proteins nor VEGF were changed.

**Conclusions:** Recently, we reported that nuclear β-catenin, but not HIF-2α, regulates the expression of fibronectin and α-SMA in atmospheric oxygen. In marked contrast, data from the hypoxic condition clearly establish that nuclear β-catenin plays little apparent role in the expression of EMT marker proteins. Instead, the loss of HIF-1α (but not HIF-2α) decreases the expression of the EMT marker proteins without sacrificing the levels of the prosurvival protein VEGF. These findings support the development of a potentially relevant therapeutic strategy to undermine the progression of normal cells to the mesenchymal phenotype in the naturally hypoxic lens without subverting cell viability.

The ocular lens and its complement of epithelial cells are adapted to exist under hypoxic conditions that would otherwise injure most types of cell. Human lens epithelial (HLE) cells survive under hypoxia through complex and interactive signal transduction pathways whose mechanisms of action are not well understood. A shift in the ratio of cytoplasmic β-catenin to activated, nuclear β-catenin increases vascular endothelial growth factor (VEGF) synthesis and epithelial to mesenchymal transition (EMT) protein expression under the atmospheric oxygen condition [1]. We have previously shown that the two pathways are independent of each other; that is, VEGF does not influence EMT progression, and EMT marker protein expression does not influence VEGF expression [1]. The two events, while occurring simultaneously but independently, likely provide a disadvantageous situation in which the newly emerging mesenchymal cell population is more likely to be resistant to apoptosis than the epithelial cell population from which the mesenchymal cell population stemmed.

During lens cataract surgery, atmospheric oxygen is unavoidably introduced to what would otherwise be the naturally hypoxic lens. The introduction of this brief oxidative insult has been linked to the initiation of a response that results in the activation of transforming growth factor beta (TGF-β); [2]. TGF-β promotes lens epithelial cell proliferation

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(and epithelial to mesenchymal transition) through the activation of the Wnt/β-catenin pathway [2]. Inhibition of glycogen synthase kinase-3β (GSK-3β) occurs by the activation of TGF-β/Wnt-β-catenin pathway [3]. Wnt3a activation leads to epithelial to mesenchymal transition and has been linked to breast carcinoma [4] and, in the lens, is a critical process in the progression of posterior capsular opacification (PCO) [5].

Lens epithelial cells likely experience acute high oxygen stress during cataract surgery [6], and once the insult is initiated, the ensuing damage likely carries over well after suture of the eye and the return to the naturally hypoxic state. In a previous study, we demonstrated that the inactivation of GSK-3β, under atmospheric conditions, is the initiating culprit that ultimately leads to the overexpression of early epithelial to mesenchymal markers and the prosurvival protein VEGF [1]. In this study, we addressed a similar question but extended it to its logical conclusion, "how does one explain the clinical situation insofar as the continued inactivation of GSK-3β in the presumed hypoxic condition post-surgery"? As was performed with studies in atmospheric oxygen [1], the experimental approach proposed here used direct GSK-3β catalytic inhibition with the specific GSK-3β inhibitor SB216763. We demonstrate that as with the atmospheric oxygen condition [1], inactivation of the catalytic site of GSK-3β, under the hypoxic condition, leads to translocation of cytoplasmic β-catenin to the nucleus. However, unlike what was previously reported under the atmospheric oxygen condition [1], under the hypoxic condition, hypoxia-inducible factor-1α (HIF-1α), and not nuclear β-catenin, which acts as the crucial transcription factor for the overexpression of the EMT marker proteins, fibronectin and α-smooth muscle actin (α-SMA), and the prosurvival protein VEGF.

We assert that these data represent a new and substantive shift from existing knowledge regarding the cause and progression of the overexpression of EMT marker proteins, eventually leading to PCO under hypoxic condition. We show that in the hypoxic condition, nuclear β-catenin plays a less prominent role in the regulation of the expression of EMT marker proteins and VEGF. Instead, HIF-1α controls the expression of EMT marker proteins, while at the same time (and in conjunction with HIF-2α) controlling the levels of the prosurvival protein VEGF [7]. Data presented here represent the beginnings of a feasible approach for suppressing HIF-1α in the hypoxic state, thus accomplishing two desired goals of the project: (i) lowering or suppressing the overexpression of EMT marker proteins and (ii) because HIF-2α compensates for the loss of HIF-1α, suppression of HIF-1α does not interfere with VEGF synthesis. The findings support the development of a potentially relevant therapeutic strategy to undermine the progression of normal cells to the mesenchymal phenotype in the naturally hypoxic lens without subverting cell viability. We are lens biologists interested in elucidating regulation pathways involved in early-onset EMT. The novelty of our preliminary work is that it suggests a means for devising a feasible approach to lower or suppress overexpression of EMT marker proteins while at the same time not interfering with the synthesis and accumulation of the prosurvival protein VEGF, under conditions where the cultured cells are maintained in low oxygen. Wnt/β-catenin pathways are universal, and the results presented here should have general widespread applicability for non-lens researchers.

Based upon our data, we speculate that the maintenance of normal cell growth as opposed to the mesenchymal transition is dependent on a shift in the ratio of HIF-1α/HIF-2α. We have shown that cultured human lens epithelial cells synthesize HIF-1α and HIF-2α [7]. Increased HIF-2α likely tends toward normal cell growth, and increased HIF-1α drives mesenchymal transition. Our data do not support a direct role for a complex of activated nuclear β-catenin and HIF-1α to drive the mesenchymal transition. However, the possibility should not be overlooked and, in our opinion, warrants further investigation.

## METHODS

*Materials:* The HIF-1α translation inhibitor (KC7F2) was purchased from Cayman Chemical (Ann Arbor, MI). The HIF-2α translation inhibitor (CAS882268–69–1) was purchased from EMD Chemicals (Billerica, MA). The GSK inhibitor 3-(2, 4-dichlorophenyl)-4-(1-methyl-1H-indol-3-yl)-1H-pyrrole-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-days]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, 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 with short tandem repeat (STR) profile analysis (American Type Culture Collection, Manassas, VA) and confirmed that the cell line was human and of female origin, as originally reported by Andley et al. [8]. A copy of the STR profile is available upon request (contact patrick.cammarata@unthsc.edu). All studies with HLE-B3 cells were performed with prefrozen 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 (FBS; 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% $CO_2$ to 95% $O_2$. Cells were sub-cultured 4 to 5 days before the experiment and placed in MEM containing 20% FBS. Twenty-four hours before the day of the experiment, the cells were switched to serum-free MEM. Unless otherwise specified, all experiments followed a common protocol; cells that had been maintained in atmospheric $O_2$ (approximately 21%) were then switched to hypoxic conditions (approximately 1% $O_2$) for 180 min. Each experiment was executed with control DMSO only cells (mock inhibitor treatment) and the cells treated with inhibitors. The DMSO concentration per experiment never exceeded 0.05%; DMSO levels higher than that have been proved to be cytotoxic to lens cell cultures.

*Western blot analysis:* Cytoplasmic and nuclear lysates were collected from HLE-B3 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 sodium dodecyl sulfate (SDS; Laemmli) buffer was added to the remaining lysates, which were subsequently incubated in boiling water for 5 min. The proteins were resolved with electrophoresis on 12% SDS-polyacrylamide gels (20 µg protein/lane). The proteins were then transferred to nitrocellulose membranes (Scheicher and Schuell, Keene, NH).

For the western blot analysis, nitrocellulose membranes were blocked with 1% bovine serum albumin (BSA) and 0.02% Tween-20 in Tris-buffered saline (TTBS) for 60 min. The membranes were incubated overnight at 4 °C with primary antibodies. The blots were then rinsed in TTBS (0.2% Tween-20, 500 mM NaCl, 20 mM Tris-HCl, pH 7.4–7.5, 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 (4 × 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-actin (Santa Cruz Biotechnology), rabbit anti-β-catenin, 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), and rabbit anti-fibronectin (Millipore, Billerica, MA). Western blot analysis was generally repeated in triplicate from three independent cell populations.

*ELISA:* Enzyme-linked immunosorbent assay (ELISA) was performed for the detection of VEGF using an Invitrogen VEGF ELISA kit (Grand Island, NY) using HLE-B3 cells. The HLE-B3 cells were cultured in 25 $cm^2$ tissue culture flasks in 20% FBS and transferred to serum-free media before the initiation of the experiment. The flasks were generally set up in triplicate for 3 h of incubation in hypoxia. At the end of 3 h, cell-free culture medium was collected and analyzed according to the manufacturer's instructions. The optical density at 450 nm was determined using a Molecular Devices SpectraMax 190 (Sunnyvale, CA).

*Statistical analysis:* Western blot densitometry was determined using ImageJ analysis. For ELISA, a Student *t* test was performed by collecting the cell culture medium from three individual cell cultures stemming from an initial single cell population using the software from GraphPad Prism, version 5.00 (La Jolla, CA). Statistical significance was determined based upon $p<0.05$. Error bars represent the standard error of the mean (SEM). For the bar graphs that represent the density of western blot bands, the Student *t* test was applied.

## RESULTS

*Inhibition of the enzymatic activity of GSK-3β leads to decreased pGS levels:* Our experimental approach involved the intentional bypass of the physiologic influence of activated TGF-β/Wnt by oxygen exposure (as might occur during cataract surgery) by going directly to inhibition of GSK-3β catalytic activity with pharmaceutical intervention. The direct inhibition of GSK-3β catalytic activity prevents phosphorylation of the downstream substrates, β-catenin and HIF-1α. 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 a useful indicator of the inactivation of GSK-3β activity. Treatment of HLE-B3 cells with SB216763 resulted in inhibition of phosphorylation of GS 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 the SB216763-treated cells (Figure 1). Many incorrectly use the autophosphorylation of GSK-3β as an indicator of GSK-3β activity. Note that the autophosphorylation of GSK-3β is unaffected by the treatment with SB216763, whereas inhibition of

the catalytic site prevented downstream phosphorylation of glycogen synthase. The data presented are reproduced from a previous publication [9]. Cultures of HLE-B3 cells were grown on 100 mm dishes until >85% confluence. Cells were treated with 12 µM SB216763 or mock-treated with DMSO (control). After 90 min in ambient oxygen, the cells were placed under hypoxic conditions (about 1% $O_2$) for 3 h and then switched back to atmospheric oxygen (about 21% $O_2$) for 3 h. Samples were collected from cells consistently maintained in atmospheric oxygen (control) immediately after hypoxic exposure and 1, 2, and 3 h of reexposure to atmospheric oxygen. The reader should note the effective blockage of phosphoglycogen synthase (pGS) with the SB216763 treatment irrespective of whether the cells were maintained in atmospheric oxygen (control) or taken through 3 h of hypoxic exposure or reintroduced to atmospheric oxygen subsequent to hypoxic exposure.

*Inhibition of the enzymatic activity of GSK-3β leads to increased nuclear β-catenin under hypoxic condition:* GSK-3β, as part of the multiprotein complex, is involved in the Wnt signaling pathway [10]. β-catenin is an important downstream target of GSK-3β activity [10]. In the absence of active GSK-3β, β-catenin is not phosphorylated, and in this active form, β-catenin translocates to the nucleus. We recently reproduced this result and showed that inhibition of GSK-3β leads to increased nuclear β-catenin under atmospheric conditions [1]. The studies described here sought to determine whether inhibition of GSK-3β catalytic activity likewise results in an increase in nuclear β-catenin under hypoxic conditions. To address this, HLE-B3 cells were treated with 12 µm SB216763 or SB216763 combined with either 0.5 µm HIF-1α translation inhibitor (KC7F2) or HIF-2α translation inhibitor (CAS882268–69–1) for 3 h in hypoxia (1% oxygen). At the end of the incubation period, the cytoplasmic and nuclear extracts were collected (refer to Methods) and analyzed with western blot for the β-catenin levels. Control cells mock-treated with DMSO were similarly analyzed. As previously reported, in atmospheric oxygen [1], inhibition of GSK-3β activity resulted in increased accumulation of nuclear β-catenin (Figure 2). The addition of neither an HIF-1α translation inhibitor nor an HIF-2α translation inhibitor with the GSK-3β inhibitor, SB216763, altered the observed results; in all cases, the nuclear β-catenin level was statistically higher than that of the control cells (Figure 2). The experiments shown in Figure 2, which followed non-phosphorylated β-catenin (treated with SB +/− HIF-1α of HIF-2α translation inhibitors) to the nucleus, show the cytoplasmic and nuclear lysate obtained from two independent cell populations. When cytoplasmic and nuclear extracts are separated, there is unavoidable cytoplasmic cross-contamination of the

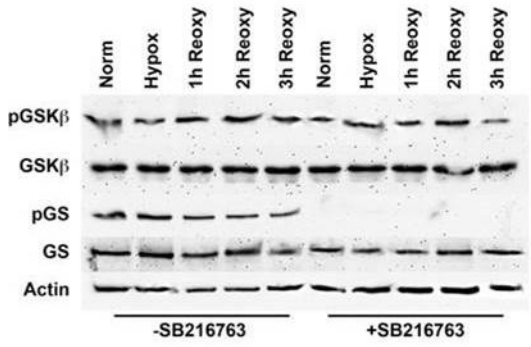


Figure 1. Western blot analysis of GSK-3β and GS phosphorylation in HLE-B3 cells in the presence or absence of SB216763. Total cell lysates were collected from >85% confluent HLE-B3 cell cultures that were incubated for 90 min in serum-free minimal essential media (MEM) containing either 12 µM SB216763 or 0.05% DMSO vehicle. Cells were then exposed to hypoxia for 3 h. At the end of the incubation period, the hypoxic medium was removed, and fresh, oxygenated serum-free MEM with SB216763 or dimethyl sulfoxide (DMSO) was added to the cultures. Cells were then placed in atmospheric oxygen for up to 3 h. Cultures were collected after continuous normoxic exposure (approximately 21% oxygen), hypoxic exposure (approximately 1% oxygen), or after reintroduction of atmospheric oxygen (approximately 21%) for 1, 2, or 3 h. Total cell lysates were analyzed by immunoblots using 25 µg of protein per lane. Antiactin was used to normalize the bands to ensure equivalent lane loading. Note: These data were taken from a prior publication [9] but are typical of SB216763 treatment. Note to reader: Look at the hypoxic exposure lanes to verify that glycogen synthase, a substrate of glycogen synthase kinase-3β (GSK-3β) treated with SB216763 fails to be phosphorylated. This is indicative of inactivation of GSK-3β catalytic activity that also prevents phosphorylation of β-catenin.

proteins in the nuclear fraction. We have demonstrated this phenomenon several times in the past (refer to reference 1) and shown that the amount of cytoplasmic extract GAPDH that spills into the nuclear extract is approximately equal irrespective of the experimental treatment over that of control. It is unlikely that the conclusions drawn from the increase in non-phosphorylated β-catenin translocating from the cytoplasm to the nucleus as shown in Figure 2 was misrepresented by cytoplasmic proteins spilling into the nuclear extract.

*Western blot analysis of fibronectin and α-SMA in HLE-B3 cells treated with SB216763 +/− HIF translation inhibitors under hypoxic condition:* We recently reported that under sustained atmospheric oxygen conditions, where HIF-1α was naturally degraded, suppression of HIF-2α did not affect the fibronectin or α-SMA levels [1]. Similar experiments were undertaken in hypoxia, where HIF-1α and HIF-2α have previously been shown to be expressed in virally transformed human lens epithelial cells [7]. The HLE-B3 cells were cultured in 25 $cm^2$ flasks with 20% FBS and switched to

serum-free media on the day of the experiment. The cells were incubated in triplicate in dishes with 3 ml of serum-free media containing 12 µm of SB216763, SB216763/KC7F2, or SB216763/CAS882268–69–1 for 3 h in hypoxia. At the end of 3 h, western blot analysis of fibronectin and α-SMA was performed on the total lysates. The experiment was repeated twice with independent cell cultures. Data were normalized to actin (10 µg) because the total lysates were compared. There was a significant increase in the expression of α-SMA and fibronectin in the SB216763-treated samples compared to the corresponding control samples treated with DMSO ($p<0.05$). The SB216763-treated samples with the added HIF-2α translation inhibitor showed a similar result. In marked contrast, the SB216763-treated samples with the added HIF-1α translation inhibitor revealed substantially suppressed expression of fibronectin and α-SMA (Figure 3).

*Detection of VEGF levels by ELISA in HLE-B3 cells treated with SB216763 +/− HIF translation inhibitors:* HLE-B3 cells were cultured in 25 $cm^2$ flasks with 20% FBS and switched to serum-free media for 24 h before the experiment. The cell culture medium was collected from the same cultures described above in Figure 3. At the end of 3 h of drug treatment, the cell culture medium was collected and analyzed for VEGF. The experiment was repeated twice using independent cell cultures stemming from a single cell passage. There was a significant increase in the VEGF level in the SB216763 treated samples compared to the corresponding control samples ($p<0.05$). The SB216763-treated samples with added HIF-1α translation inhibitor or HIF-2α translation inhibitor showed similar VEGF levels as those observed with the SB216763-treated cells (Figure 4).

*Effect of XAV939 exposure on nuclear β-catenin levels in HLE-B3 cells:* Our results thus far with the GSK-3β inhibitor, SB216763, have resulted in a demonstration of increased nuclear β-catenin (Figure 2) and a corresponding increase in EMT marker proteins, fibronectin and α-SMA (Figure 3), and VEGF levels (Figure 4). These results suggest, but do not definitely prove, a hypoxic model in which there is an association between nuclear β-catenin, EMT marker protein expression, and VEGF synthesis and accumulation. To further support or

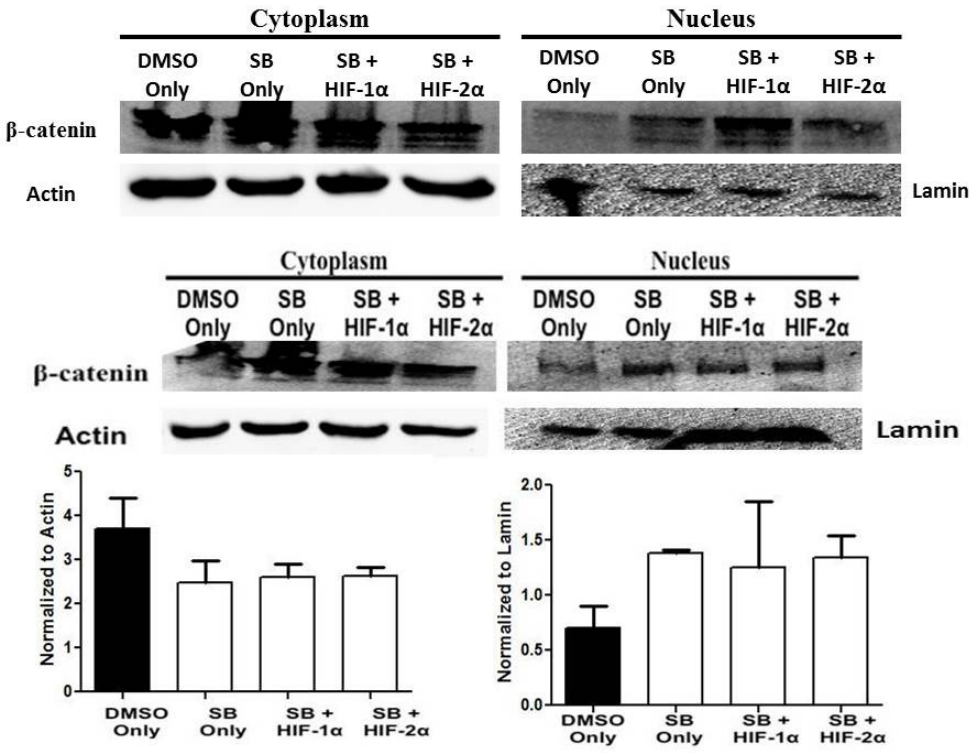


Figure 2. Western blot analysis of β-catenin in HLE-B3 cells treated with SB216763. HLE-B3 cells were cultured in 25 $cm^2$ flasks with 20% fetal bovine serum (FBS) and switched to serum-free media for 24 h before the experiment. The cells were incubated with 3 ml of serum-free media containing 12 µm SB216763 or SB216763 combined with either 0.5 µm hypoxia-inducible factor-1α (HIF-1α) translation inhibitor (KC7F2) or HIF-2α translation inhibitor (CAS882268–69–1) for 3 h in hypoxia (1% oxygen). Cytoplasmic and nuclear lysates were collected from HLE-B3 cell cultures after treatments using the NE-PER Nuclear and Cytoplasmic Extraction Kit. A portion of the sample was used for protein quantification using the EZQ Protein Quantification Kit, and 3X sodium dodecyl sulfate (SDS) 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). The proteins were then transferred to nitrocellulose membranes. The experiment was repeated twice with independent cell populations and the image density of β-catenin was quantified using ImageJ analysis. The β-catenin levels in the cytoplasmic extracts were essentially unchanged, while in the nuclear extracts there was a significant increase in β-catenin in the SB216763-treated cells, as well as SB treatment with the HIF translation inhibitors, compared with the controls. SB=SB216763.

disprove 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 sustained hypoxia and to determine the downstream effects of nuclear β-catenin inhibition on EMT marker protein expression and VEGF levels.

XAV939 has been reported to stimulate β-catenin degradation by stabilizing Axin, the concentration-limiting component of the destruction complex. XAV939 stabilizes Axin by inhibiting the poly-ADP-ribosylating enzymes tankyrase-1 and tankyrase-2, leading to degradation of β-catenin [3]. To further establish the association between nuclear β-catenin, EMT marker proteins, and VEGF levels, we used the pharmacological inhibitor XAV939 to deplete the nuclear levels of β-catenin. The HLE-B3 cells were cultured in 100 $mm^2$ culture dishes and incubated with 1 µm XAV939 for 3 h in hypoxia. At the end of 3 h, cytoplasmic and nuclear extracts were collected and analyzed with western blot. Treatment of the HLE-B3 cells with the pharmacological inhibitor XAV939 led to a statistically significant decrease in nuclear β-catenin without any significant changes in the cytoplasmic levels of β-catenin (Figure 5). We previously reported a similar result under atmospheric oxygen conditions [1].

*Inhibition of the nuclear β-catenin and its effect on expression of EMT proteins:* To further confirm whether there is an association between nuclear β-catenin and mesenchymal marker protein expression with hypoxic lens epithelial cells, HLE-B3 cells were cultured in 100 $mm^2$ culture dishes and incubated with 1 µm XAV939 for 3 h in hypoxia. The total cell lysates were collected and used to detect the fibronectin and α-SMA levels with western blot analysis. The decrease in nuclear β-catenin with the XAV939 treatment (Figure 5) correlated with a small but statistically significant increase in the expression of the EMT proteins, fibronectin and α-SMA, compared to the control cells (Figure 6). The experiment was

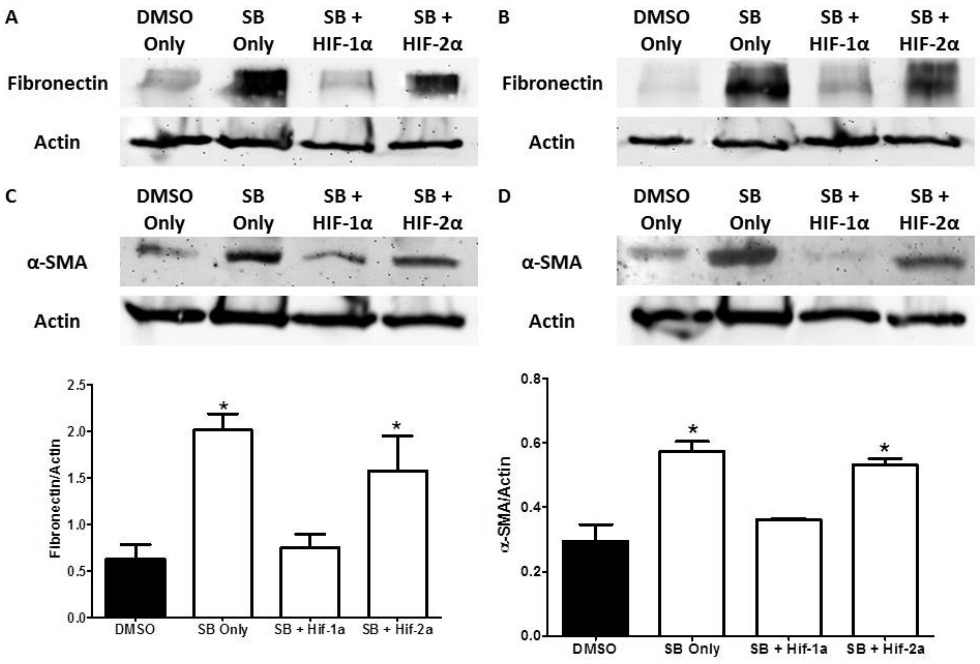


Figure 3. Western blot analysis of fibronectin and α-SMA in HLE-B3 cells treated with SB216763 +/− hypoxia inducible factor translation inhibitors. HLE-B3 cells were cultured in 25 $cm^2$ flasks with 20% fetal bovine serum (FBS) and switched to serum-free media for 24 h before the experiment. The cells were incubated with 3 ml of serum-free media containing 12 µm SB216763 or SB216763 combined with either 0.5 µm hypoxia-inducible factor-1α (HIF-1α) translation inhibitor (KC7F2) or HIF-2α translation inhibitor (CAS882268–69–1). Total cell lysates were collected from the HLE-B3 cell cultures. A portion of the sample was used for protein quantification using the EZQ Protein Quantification Kit and 3X sodium dodecyl sulfate (SDS) 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. The experiment was repeated twice with independent cell cultures. **A** and **C** show the lysate from one cell sample, while **B** and **D** show the lysate from the second, independent cell sample. The normalized lysates were analyzed for alpha smooth muscle actin (α-SMA) and fibronectin using ImageJ analysis. There was a significant increase in the in the SB216763-treated samples compared to the corresponding expression of both epithelial to mesenchymal transition (EMT) marker proteins, fibronectin and α-SMA compared to corresponding control samples treated with dimethyl sulfoxide (DMSO) ($p<0.05$). SB216763-treated samples with added HIF-2α translation inhibitor showed similar results. The asterisk (*) signifies there was a statistically significant increase in the levels of fibronectin and α-SMA in the SB only treated and SB+HIF-2α treated samples relative to the control (DMSO) sample ($p<0.05$). In marked contrast, the SB216763-treated samples with the added HIF-1α translation inhibitor revealed substantially suppressed expression of α-SMA and fibronectin. SB=SB216763.

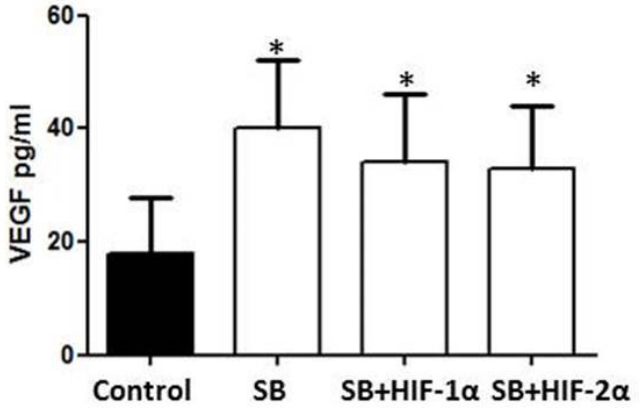


Figure 4. Detection of VEGF levels by ELISA in HLE-B3 cells treated with SB216763 +/− hypoxia inducible factor translation inhibitors. HLE-B3 cells were cultured in 25 $cm^2$ flasks with 20% fetal bovine serum (FBS) and switched to serum-free media for 24 h before the experiment. We used the cell culture medium from the cell populations described in Figure 3. At the end of 3 h of drug exposure, the cell culture medium was analyzed for vascular endothelial growth factor (VEGF). The experiment was repeated twice using independent cell populations stemming from a single cell passage. The cell culture medium was evaluated for vascular endothelial growth factor (VEGF) levels using six samples from two independent cell populations. The asterisk (*) signifies there was a statistically significant increase in the VEGF levels. Resulting data indicated that the level of VEGF in the SB216763-treated samples was substantially higher than that of the non-treated control samples ($p<0.05$). In both cases, SB216763-treated samples with supplemented hypoxia-inducible factor-1α (HIF-1α) translation inhibitor or HIF-2α translation inhibitor showed the same propensity to be elevated above the control levels. SB=SB216763. HIF-1α=hypoxia inducible factor-1 translation inhibitor. HIF-2α=hypoxia inducible factor-2 translation inhibitor.

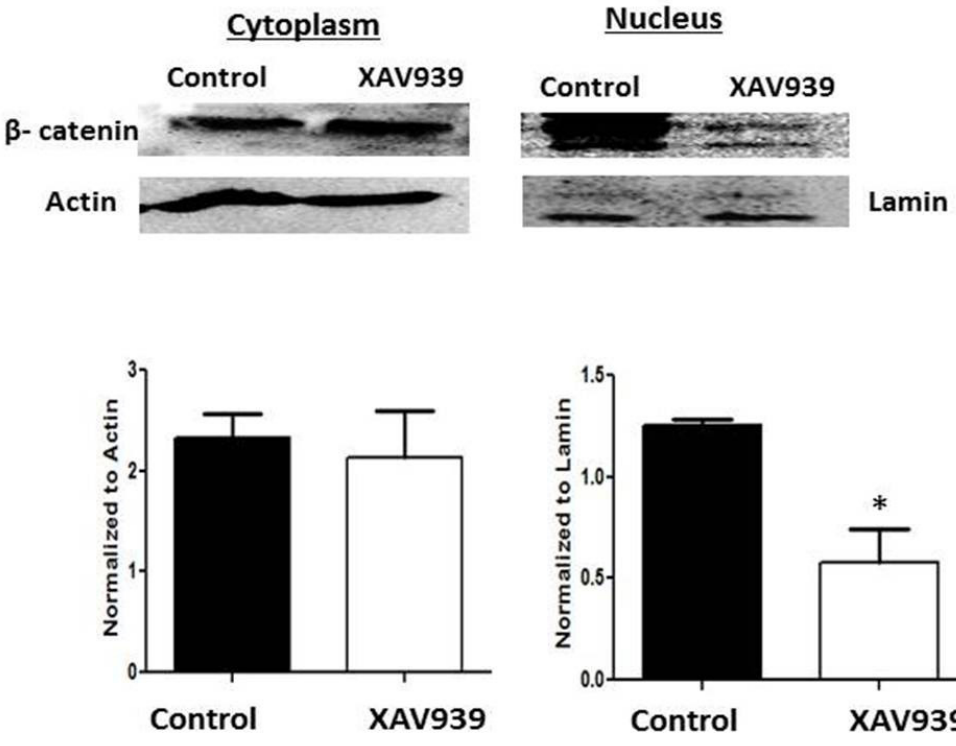


Figure 5. Effect of XAV939 on nuclear β-catenin levels in the HLE-B3 cells. To further establish the association between nuclear β-catenin, epithelial to mesenchymal transition (EMT) marker proteins, and vascular endothelial growth factor (VEGF) levels, we used the pharmacological inhibitor, XAV939, to decrease the nuclear levels of β-catenin. HLE-B3 cells were cultured in 100 $mm^2$ culture dishes and incubated with 1 µm XAV939 for 3 h in hypoxia. At the end of 3 h, cytoplasmic and nuclear extracts were collected and analyzed with western blot. The experiment was repeated twice with independent cell populations and was quantified using ImageJ analysis. The asterisk (*) signifies there was a significant decrease in nuclear β-catenin in the XAV939-treated cells compared with the controls ($p<0.05$). There was no significant change in the cytoplasmic level of β-catenin with XAV939 relative to controls.

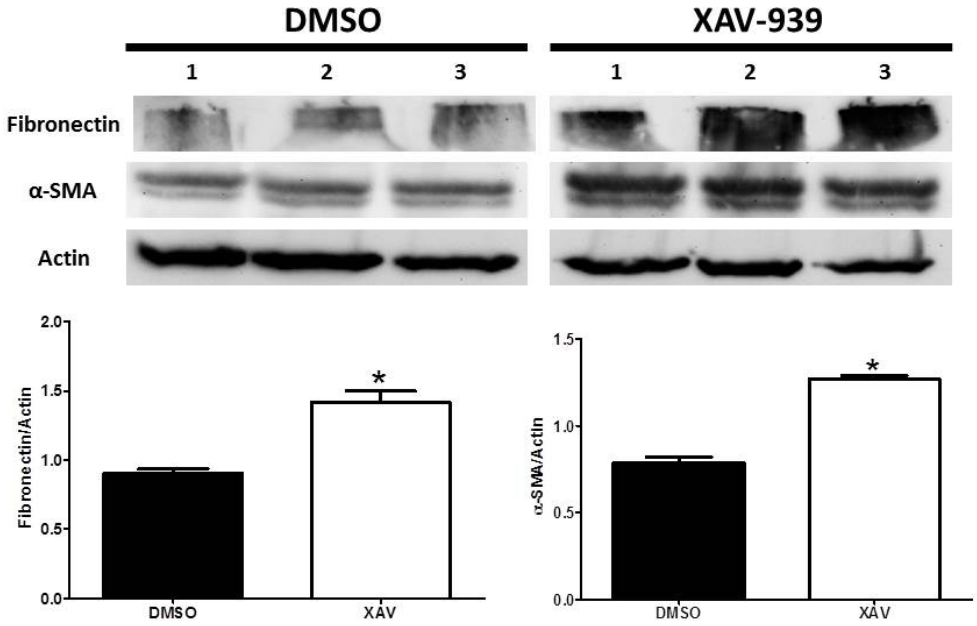


Figure 6. Western blot analysis of fibronectin and α-SMA in HLE-B3 cells treated with XAV939. HLE-B3 cells were cultured in 100 mm$^2$ culture dishes and incubated with 1 µm XAV939 for 3 h in hypoxia. At the end of 3 h the total cell lysate was collected and analyzed with western blot. The experiment was repeated three times with independent cell populations and was quantified using ImageJ analysis. The asterisk (*) signifies there was a small but significant increase in fibronectin and alpha smooth muscle actin (α-SMA) in the XAV939-treated cells compared with the controls ($p<0.05$).

repeated three times, each time with independent cell populations of HLE-B3 cells.

*Effect of XAV939 exposure on VEGF levels in HLE-B3 cells:* To firmly establish whether there is an inter-relationship between β-catenin and VEGF expression, we suppressed nuclear β-catenin levels as described above (Figure 5). HLE-B3 cells were cultured in 100 mm$^2$ culture dishes and incubated with 1 µm XAV939 for 3 h in hypoxia using the same cell populations as employed in Figure 6. At the end of 3 h, the cell-free cell medium was collected before the cell lysates were harvested and analyzed with ELISA for VEGF levels. There was a minor but statistically positive increase in the VEGF levels of the XAV939-treated cells compared with the control cells (Figure 7).

## DISCUSSION

Inactivation of GSK-3β's active catalytic site was monitored by following the phosphorylation of a downstream substrate of GSK-3β, GS, under the hypoxic oxygen condition. The GSK-3β, pGSK-3β, and GS levels were essentially unaffected in the SB216763-treated cells (Figure 1). However, the pGS levels were markedly decreased in the SB216763-treated samples indicating that the catalytic site of GSK-3β was inactive as a result of treatment with the GSK-3β inhibitor SB216763 (Figure 1).

Inhibition of the GSK-3β catalytic site also prevents phosphorylation of β-catenin (data not shown); the resulting cytoplasmic non-phosphorylated β-catenin translocates and accumulates in the nucleus (Figure 2). The simultaneous addition of hypoxia inducible factor inhibitors for either HIF-1α or HIF-2α did not impede the translocation of cytoplasmic β-catenin to the nucleus.

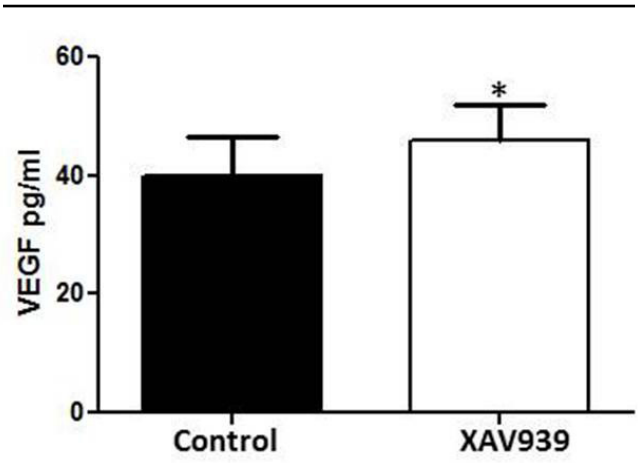


Figure 7. Detection of VEGF levels by ELISA in HLE-B3 cells treated with XAV939. HLE-B3 cells were cultured in 100 mm$^2$ culture dishes and incubated with 1 µm XAV939 for 3 h in hypoxia as described in Figure 6. At the end of 3 h, the cell culture medium was collected. The experiment was repeated three times with independent cell populations, and the cell culture medium was evaluated for vascular endothelial growth factor (VEGF) levels using nine samples from three independent cell populations. The asterisk (*) signifies there was a slight but statistically sound increase in the VEGF levels of XAV939-treated cells compared with the controls ($p<0.05$).

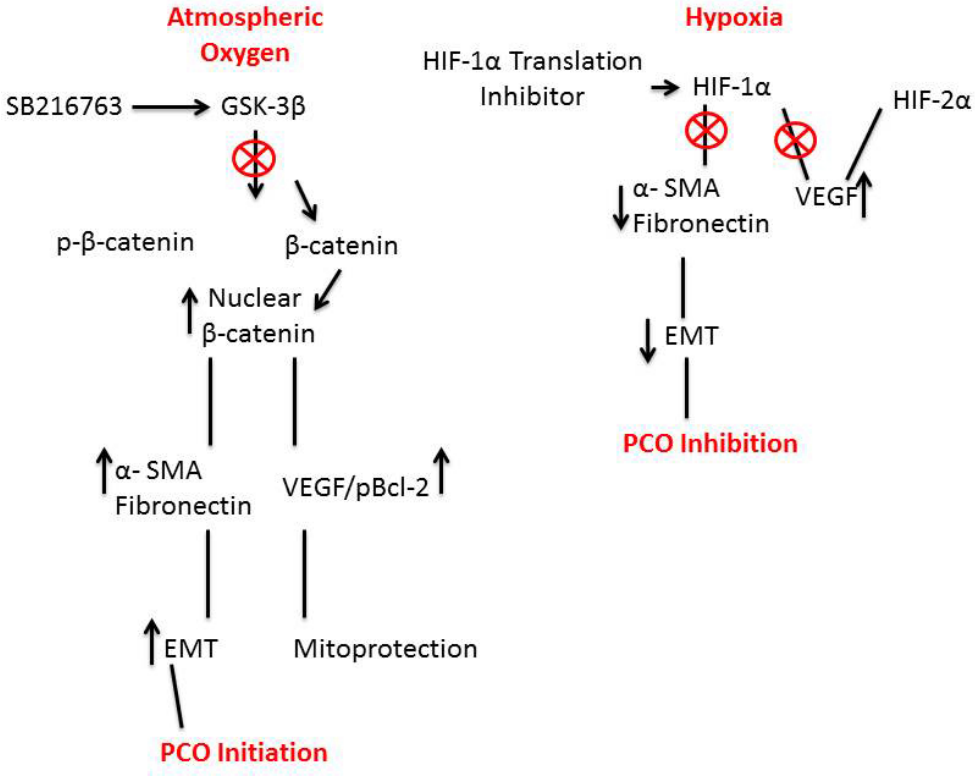


Figure 8. Schematic representation of the role of hypoxia inducible factor-1α or β-catenin on the regulation of expression of epithelial to mesenchymal transition early marker proteins and VEGF synthesis. Atmospheric oxygen: Catalytic activity of glycogen synthase kinase-3β (GSK-3β) is blocked using the specific GSK-3β inhibitor, SB216763. Inhibition of GSK-3β activity prevents the phosphorylation of β-catenin. Non-phosphorylated β-catenin translocates to the nucleus. Nuclear β-catenin acts as transcription factor in two independent pathways leading to [1]; elevated synthesis and accumulation of fibronectin and alpha smooth muscle actin (α-SMA) and [2] elevated synthesis of the prosurvival protein, vascular endothelial growth factor (VEGF). Increased accumulation of fibronectin and α-SMA is an early marker indicator for epithelial to mesenchymal transition, which in the lens is a contributing factor to posterior capsular opacification. At the same time, increased accumulation of VEGF promotes cell survival via the prevention of mitochondrial depolarization and cell death. Although present in the cell, hypoxia-inducible factor-2α (HIF-2α) plays no discernible role in these pathways under atmospheric oxygen conditions. Hypoxia: HIF-1α intracellular levels are decreased using the specific HIF-1α translation inhibitor KC7F2. The resulting inhibition of the transcription factor HIF-1α suppresses the synthesis of fibronectin and α-SMA, thus minimizing the trend toward epithelial to mesenchymal transition. Because of the cooperation between HIF-1α and HIF-2α under hypoxic conditions, the suppression of HIF-1α is compensated for by HIF-2α resulting in sustained VEGF synthesis and accumulation. Unlike the situation found with atmospheric oxygen, β-catenin appears to play little to no role in these processes.

We have previously reported that under atmospheric oxygen conditions, inhibition of the catalytic activity of GSK-3β similarly leads to increased nuclear β-catenin activity. In turn, the increase in nuclear β-catenin was positively correlated with overexpression of the early onset mesenchymal transition markers, fibronectin and α-SMA, as well as elevated VEGF expression [1]. These studies involved cultured HLE-B3 cells maintained under hypoxic conditions. The use of the GSK-3β inhibitor SB216763 also led to increased α-SMA and fibronectin (Figure 3). The addition of an HIF-1α translation inhibitor, but not an HIF-2α translation inhibitor, with SB216763 markedly reduced α-SMA and fibronectin expression (Figure 3). Unlike the situation found with atmospheric oxygen (where HIF-1α is naturally degraded), HIF-1α clearly plays a crucial role in mesenchymal marker protein synthesis under the hypoxic oxygen condition. The issue of whether nuclear β-catenin plays a role in mesenchymal marker protein synthesis under hypoxia is further discussed below.

Akin to the situation previously shown with atmospheric oxygen [1], inhibition of the catalytic activity of GSK-3β resulted in accumulation of the prosurvival factor VEGF in hypoxia (Figure 4). The addition of an HIF-1α translation inhibitor or an HIF-2α translation inhibitor with SB216763 did not diminish VEGF accumulation. We previously reported that "survival of human lens epithelial cells in hypoxia depends on the uninterrupted and sustained synthesis of VEGF levels controlled by the expression of either of the hypoxia inducible factors, HIF-1α or HIF-2α" [7]. The importance of that prior statement relative to this study is that a decrease in the manifestation of HIF-1α may be compensated for by HIF-2α accumulation and vice versa. That is, the naturally hypoxic lens epithelium regulates VEGF levels via the coordinated adjustment of two hypoxia

inducible factors, HIF-1α and HIF-2α [7]. The suppression of one hypoxia inducible factor or the other is not sufficient to depress VEGF synthesis and accumulation.

To firmly establish whether, in hypoxia, there is an inter-relationship between nuclear β-catenin, overexpression of the early onset mesenchymal transition markers, α-SMA and fibronectin, and elevated VEGF expression, further experiments were performed that involved the degradation of nuclear β-catenin. We used the pharmacological inhibitor XAV939, known to bind to the tankyrase enzyme (TNKS) domain, which leads to increased stabilization of the Axin protein in the destruction complex leading to the general degradation of β-catenin [3]. Treatment of the cells with the β-catenin inhibitor XAV939 in atmospheric oxygen has previously been reported to decrease the expression of nuclear β-catenin, VEGF levels, and EMT proteins [1]. Under hypoxic conditions, XAV939 treatment similarly resulted in the marked diminution of nuclear β-catenin (Figure 5). XAV939 did not appear to cause the general degradation of β-catenin. The cytoplasmic β-catenin levels were similar to those of the control, whereas the levels of nuclear β-catenin decreased by greater than half that of the control (Figure 5). Notwithstanding the difference in the action of XAV939 (i.e., only nuclear β-catenin was degraded instead of total cellular β-catenin), the loss of nuclear β-catenin did not positively correlate with the concurrent loss of α-SMA and fibronectin (Figure 6) or VEGF (Figure 7) in hypoxia.

Our standalone SB216763 data, had we not further examined the data, would have incorrectly suggested and supported a role for nuclear β-catenin in the regulation of the expression of early-onset mesenchymal marker proteins with hypoxic cell cultures. SB216763 treatment led to the enhancement of nuclear β-catenin, and simultaneous addition of either an HIF-1α or HIF-2α translation inhibitor did not impede that enhancement (Figure 2). However, subsequent experiments with XAV939 demonstrated that the loss of nuclear β-catenin (Figure 5) did not diminish the expression of fibronectin or α-SMA below that of control values (Figure 6), which shows that nuclear β-catenin does not play a direct regulatory role in mesenchymal marker protein expression in hypoxia. Our data suggest that HIF-1α plays a significant role in the regulation of expression of these early-onset mesenchymal marker proteins. Suppression of HIF-1α resulted in the marked decrease in fibronectin and α-SMA relative to SB216763 treatment alone or supplemented with an HIF-2α translation inhibitor (Figure 3). Our data are, in part, explained by the work of Flugel et al. [11], who demonstrated that the inhibition of GSK-3β catalytic activity prevents phosphorylation of HIF-1α, thus preventing its degradation and increasing HIF-1α intracellular levels. Others have confirmed that GSK-3β inhibition in hypoxia raises HIF-1α levels [12]. Inactivation of GSK-3β catalytic activity leads to enhanced nuclear β-catenin because β-catenin is a downstream substrate for phosphorylation by GSK-3β. However, with the inactivation of GSK-3β catalytic activity, there is also a failure to phosphorylate HIF-1α (a second downstream substrate of GSK-3β). Phosphorylation of HIF-1α represents a crucial degradation pathway that helps downregulate cytoplasmic HIF-1α. The inability to phosphorylate HIF-1α results in a marked shift in the HIF-1α/HIF-2α ratio such that HIF-1α is significantly increased. Any increase in HIF-1α availability would favor increased expression of fibronectin and α-SMA.

Other explanations, although we believe they are less likely, have been suggested in the literature. We have yet to definitely rule out the possibility that activated nuclear β-catenin is coupled with the hypoxia inducible factor HIF-1α and that this functional complex regulates EMT marker protein expression and VEGF expression. There is (cancer) literature to support this notion. Choi et al. [13], using renal carcinoma cells, suggested, "actions of HIF-2α oppose those of HIF-1α on β-catenin." Furthermore, Jiang et al. [14], using a human prostate cancer cell line, demonstrated that overexpression of HIF-1α induced an EMT response via Wnt/β-catenin pathway activation. Luo et al. [15] showed that the enhanced binding of HIF-1α to nuclear β-catenin induces an EMT response.

The studies described here sought to determine pathways that could be important for lens epithelial cell differentiation. These pathways point to potentially important targets for the prevention of posterior capsular opacification and other types of cataract formation. Our preliminary efforts provide important information and point to a unique direction, which merits further intensive investigation. Figure 8 is a summation of the work presented in this study, as well as information summarized from our previous four manuscripts related to this topic [1,7,9,16]. We have reported a compensatory inter-relationship between the two hypoxia inducible factors, HIF-1α and HIF-2α, in the hypoxic lens epithelium insofar as they cooperatively maintain VEGF synthesis. VEGF acts as a prosurvival factor by regulating the levels of the anti-apoptotic protein, pBcl-2, thus resisting mitochondrial depolarization or, as we refer to the process, mitoprotection [7]. Exposure of the normally hypoxic lens epithelium to atmospheric oxygen during cataract surgery is the potential insult that activates a series of events leading to the inhibition of GSK-3β resulting in the translocation of non-phosphorylated β-catenin to the nucleus. Once the transition of cytoplasmic β-catenin to nuclear β-catenin is accomplished, two parallel

pathways independently promote the expression of early EMT marker proteins and VEGF synthesis. Although VEGF protects the lens epithelium from mitochondrial depolarization, the onset of elaboration of mesenchymal proteins indicates epithelial to mesenchymal transition. We hypothesize that the net result is an environment more supportive of a population of mesenchymal cells better capable of resisting mitochondrial depolarization and, at the same time, favoring the onset of PCO in the lens (or, metaplasia, should the reader wish to equate the model to other tissue types). After cataract surgery, and upon reestablishment of a hypoxic environment, HIF-1α (not nuclear β-catenin) is primarily involved in the regulation of the mesenchymal marker proteins fibronectin and α-SMA, thus playing a key role in the progression of PCO in hypoxia. We have established that HIF-2α does not play a role in the expression of mesenchymal marker proteins either in hypoxia [1] or atmospheric oxygen [9].

Our data support a new and substantive shift from existing knowledge regarding the cause and progression of the overexpression of epithelial to mesenchymal transition marker proteins and VEGF synthesis under the hypoxic condition. By focusing on HIF-1α (and its influence on, as yet, unidentified downstream pathways) and leaving β-catenin and HIF-2α activity unperturbed, we enhance the well-being of the lens epithelium but at the same time achieve the desired goal of suppressing overexpression of the two EMT marker proteins. These preliminary findings support the development of a potentially relevant therapeutic strategy to undermine the progression of normal cells to the mesenchymal phenotype in the naturally hypoxic lens without subverting cell viability.

Future directions for our preliminary studies must involve the demonstration of reproducibility using normal lens epithelial cell cultures. Moreover, it is essential that greater depth of detail be explored in identifying the pathways influenced by HIF-1α insofar as why HIF-1α suppression blocks fibronectin and α-SMA synthesis, as well as the association between HIF-1α and matrix metalloproteinase-9 [17–19]. New Wnt target genes and their potential function in the epithelial to mesenchymal transition and cell motility also merits further detailed examination. To that end, the Wnt/β-catenin target genes Nuclear factor interleukin-3-regulated protein (*NFIL3*; OMIM 605327), Block of proliferation 1 protein (*BOP1*; OMIM 610596) and CDC28 Protein Kinase Regulatory Subunit 2 (*CKS2;* OMIM 116901) have recently been shown to induce EMT, cell migration, and experimental metastasis in colorectal cancer cells [20].

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