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Abstract: The human lens epithelium thrives in a naturally hypoxic environment resisting mitochondrial depolarization, a precursor of apoptosis. The ability of the lens to resist mitochondrial depolarization is referred to as lenticular mitoprotection and is rendered by a sustained synthesis of the pro-survival Vascular Endothelial Growth Factor (VEGF). Similar lenticular mitoprotective pathways come in to play when the hypoxic lens epithelium is exposed to atmospheric oxygen during ocular surgeries like the cataract surgery. Posterior capsular opacification (PCO) is a complication of cataract surgery resulting from the mesenchymal transition of the epithelial cells. The lenticular mitoprotective pathways involved in lens epithelial cell survival also initiate the mesenchymal transition of the epithelial cells, thus contributing to the pathogenesis of PCO. The progression of PCO involves the initiation phase in atmospheric oxygen (during the cataract surgery) and a persistence phase (persistence of the insult which occurred during cataract surgery) in hypoxia. The initial insult to epithelial cells occurs in atmospheric oxygen initiating the mesenchymal transition which persists when the lens epithelium is back in hypoxia after the surgery. Isolating the two events of lenticular mitoprotection and mesenchymal would be a beneficial therapeutic target. The data presented in this study supports a model whereby the onset of epithelial to mesenchymal transition may circuitously benefit from the enhanced synthesis of the pro-survival factor VEGF. As a result of which a mesenchymal cell resisting mitochondrial depolarization is generated, leading to the progression of PCO. The findings in this study support the possibility of generating a therapeutic target such that the mesenchymal transition of normal epithelial cells can be prevented without affecting the levels of pro-survival VEGF and compromising the lenticular mitoprotective pathways.

The Disadvantageous Interrelationship between Lenticular Mitoprotection and Epithelial-Mesenchymal transition via Nuclear Beta catenin

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By

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ABBREVIATIONS

VEGF- Vascular endothelial growth factor, **EPO** – Erythropoietin, **HIFs** – Hypoxia Inducible factors,**VEGFR2** – Vascular Endothelial Growth Factor Receptor 2, **TGF-β** – Transforming Growth **EMT** – Epithelial to Mesenchymal Transition, **α-SMA** – α Smooth Muscle Actin, **TCF-LEF** factors (T-cell and lymphoid enhancer factors), **GAPDH –** Glyceraldehyde -3 phosphate dehydrogenase, **LDH –** Lactate dehydrogenase, **APC**- Adenomatosis polyposis coli , **VHL-** Von Hippel–Lindau tumor suppressor,**HLE-B3**–Human Lens Epithelial cells, **MEM-** Minimum Essential Medium, **DMSO**- Dimethyl Sulphoxide, **SDS** – Sodium dodecyl sulphate, **ELISA-** Enzyme linked Immunosorbent Assay, **Bcl-2** – B cell lymphoma 2 and **BAX** – Bcl-2 associated X protein,

PHARMACOLOGICAL INHIBITORS USED IN THE STUDY: SB216763 – Selective Glycogen synthase kinase -3β inhibitor,Topotecan – HIF-1α topoisomerase inhibitor, KC7F2 - HIF-1α translation inhibitor,CAS882268-69-1- HIF-2α translation inhibitor,FM19G11- HIF-1/HIF-2α double translation inhibitor, AXITINIB(AG013736)-VEGF ~VEGF receptor inhibitor and XAV939- β-catenin inhibitor

LIST OF PUBLICATIONS:

1. **Neelam S,** Brooks MM, Cammarata PR. Lenticular cytoprotection. Part 1: the role of hypoxia inducible factors-1alpha and -2alpha and vascular endothelial growth factor in lens epithelial cell survival in hypoxia. Molecular vision. 2013;19:1-15.

2. Brooks MM, **Neelam S,** Fudala R, Gryczynski I, Cammarata PR. Lenticular mitoprotection. Part A: Monitoring mitochondrial depolarization with JC-1 and artifactual fluorescence by the glycogen synthase kinase-3beta inhibitor, SB216763. Molecular vision. 2013;19:1406-12.

3. Brooks MM, **Neelam S,** Cammarata PR. Lenticular mitoprotection. Part B: GSK-3beta and regulation of mitochondrial permeability transition for lens epithelial cells in atmospheric oxygen. Molecular vision. 2013;19:2451-67.

Introduction:

The human lens epithelium has the unique ability to survive in a naturally hypoxic environment and resist mitochondrial depolarization (lenticular mitoprotection), a precursor for apoptosis (1). The signal transduction pathways involved in the survival of hypoxic lens epithelium and their role in preventing mitochondrial depolarization are not fully understood. The hypoxic environment induces several transcription factors including the HIFs or the hypoxia inducible factors. These HIFs in turn regulate the transcription of various growth factors like VEGF (Vascular endothelial growth factor) or Erythropoietin (EPO) which promote cell survival and growth (2, 3).

Despite being an avascular tissue, the adult lens epithelium continues to synthesize VEGF under the influence of hypoxia and the HIF transcription factors. Atmospheric oxygen is introduced in to the hypoxic lens during ocular surgeries like the cataract surgery. The lens epithelium continues to synthesize VEGF in atmospheric oxygen, where the HIFs are degraded. The regulation and purpose of this sustained VEGF synthesis in hypoxia/atmospheric oxygen and whether VEGF plays a role in lenticular mitoprotection is yet to be determined.

The purpose of the lens is to focus light on to the retina and to accomplish this; the lens has to be transparent. During the life span of an individual, the lens epithelium is exposed to various insults

including oxidative damage. This damage accumulates over the years and eventually leads to loss of epithelial cells, a decrease in the cell density and impairs the ability of the lens epithelial cells in being mitotically active and maintaining the transparency of the lens. This leads to the development of a cataract (4, 5 6).

Cataract is a pathological condition in which the lens loses its transparency and becomes opacified (7). During the cataract surgery the opacified lens is replaced by placing an artificial intraocular lens in the lens capsular bag. The surgical procedure, brief introduction of atmospheric oxygen and the residual lens epithelial cells in the capsule contribute to the post-surgical complication known as PCO or posterior capsular opacification.

Introduction of atmospheric oxygen during ocular surgeries initiates the TGF-β/Wnt signaling pathways, which are known to play a role in the pathogenesis of PCO by inducing the mesenchymal transition of the residual lens epithelial cells through a process known as epithelial to mesenchymal transition or EMT. The initiation of EMT occurs in atmospheric oxygen and persists once the lens is back in its natural hypoxic environment after the surgery (8). The lenticular mitoprotective pathways that are involved in the survival of lens epithelial cells are likely to contribute to the mesenchymal transition of the epithelial cells.

The *central hypothesis* of the study is that the lenticular mitoprotective pathways in hypoxia and atmospheric oxygen contribute to the pathophysiology of PCO by inducing the mesenchymal transition of the epithelial cell and rendering resistance to the mesenchymal cell from mitochondrial depolarization via increased VEGF synthesis.

The rationale for this hypothesis is based on the following observations:

- The lens epithelium continues to synthesize VEGF under the influence of hypoxia and during exposure to atmospheric oxygen. The purpose of this sustained VEGF synthesis is not well established. Whether VEGF acts as a pro-survival factor, contributing to lenticular mitoprotection is yet to be determined.
- Exposure of the hypoxic lens to atmospheric oxygen leads to activation of Wnt/β-catenin signaling pathway and translocation of β-catenin to the nucleus. Once β-catenin is translocated to the nucleus, it induces the transcription of growth factors like VEGF and mesenchymal proteins like α-smooth muscle actin/ fibronectin initiating the process of EMT or epithelial to mesenchymal transition. This mesenchymal transition persists once the lens is back in hypoxia contributing to the pathophysiology of PCO (Posterior Capsular Opacificaiton).
- The signal transduction mechanisms which maintain the levels of VEGF either in hypoxia or atmospheric oxygen might also play a role in initiating the mesenchymal transition such that the net result will lead to generation of a mesenchymal cell resisting mitochondrial depolarization and onset of posterior capsular opacification or PCO.

The *long term goal* is to identify specific therapeutic targets in the mitoprotective pathways such that the mesenchymal transition of the cells can be prevented without affecting VEGF levels and compromising the lenticular mitoprotection.

BACKGROUND:

Structure of the Human Lens:

Human lens consist of the lens capsule, lens epithelium and the lens fiber cells. The lens capsule is a transparent sac like structure that surrounds the entire lens. The lens capsule is made up of collagen and acts like a basement membrane. It is secreted anteriorly by the epithelial cells and by the differentiating fiber cells in the posterior region. The lens epithelium is a single layer of cells which covers the entire lens. There are different cell regions or zones in the lens epithelium. The central zone consists of non-dividing cells. This layer of quiescent cells protects the underlying fiber cells from oxidative damage and injury and also provides nutrients to the fiber cells. This is surrounded by the germinal zone which consists of dividing cells and then the equatorial zone where the dividing cells differentiate in to the lens fiber cells. The newly formed fiber cells are added on top of the old cells such that the oldest fiber cells are buried deep inside the lens (9, 10).

The cells in the germinal zone are actively dividing and the newly formed cells elongate and form the fiber cells of the lens. The lens fiber cells are tightly packed with specialized organization and are composed of the crystallin proteins. The crystallin proteins help in maintaining the transparency of the lens (11, 12). The lens fiber cells undergo terminal differentiation by degradation of the cytoplasmic organelles including the golgi, nucleus, mitochondria and the endoplasmic reticulum and form the organ free zone, which again is important to maintain the lens transparency. Human lens epithelial cells are

unique because of their ability to be mitotically active, divide and differentiate throughout the life of an individual.

Hypoxic Lens Epithelium :

The lens exists in a natural state of hypoxia. The distribution of oxygen and the rate of oxygen consumption in isolated bovine lenses were studied by Mc nutly et al. (13) According to this study the partial pressure of oxygen in the lens core was less than 2 mm of Hg. This state of severe oxygen deprivation, an environment to which the lens is uniquely adapted, would be detrimental to most other cell types. The lens has developed several unique survival mechanisms enabling it to thrive in a chronically hypoxic environment and oppose oxidative injury (14, 15) Relatively little is known regarding these signaling mechanisms in the hypoxic lens and their role in preventing mitochondrial depolarization (referred to as Lenticular Mitoprotection in this study) a cellular event which under normal circumstances precludes the onset of apoptosis and cell death.

The predominant transcription factors activated in hypoxia are the HIFs- Hypoxia Inducible Factors, which promote cell survival via the transcription of genes like vascular endothelial growth factor (VEGF) and Erythropoietin (EPO).

Lens development is initiated in the ectoderm along with the formation of the lens placode. During development the mammalian fetal lens is surrounded by a network of capillary blood vessels arising from the posterior and anterior surfaces of the eye. These capillary networks form the hyaloid vascular system. VEGF is an important growth factor regulating the formation of vasculature during lens development. In

humans VEGF family consists of VEGF-A, -B, -C and –D. Alternative splicing of VEGF mRNA results in different isoforms of VEGF (16).

VEGF isoform 164 has been shown to play a significant role in the formation of the hyaloid vasculature and the fetal lens development. Studies in mice expressing only the VEGF 120 isoform showed abnormal ocular developments with significant defects in the lens development and differentiation, suggesting a significant role for VEGF 164 in lens development. As the fetal lens matures, these blood vessels are regressed and the adult lens is devoid of vascular supply. Previous studies in lens development suggest, VEGF-A may not play a significant role once the hyaloid vascular system is regressed (17, 18 19). However, the adult lens epithelium continues to synthesize VEGF under the influence of hypoxia.

It is generally acknowledged that hypoxia induces the expression of the hypoxia inducible factor, HIF-1α, which, in turn, plays a crucial role in the transcription of growth factors like vascular endothelial growth factor (VEGF). However, it has been previously shown that the hypoxic lens epithelium continues to synthesize VEGF in the absence of HIF-1α (17). Apart from HIF-1α another hypoxia inducible factor- HIF-2α is also known to induce the synthesis of VEGF (20). The functional expression of HIF-2α and whether HIF-2α compensates for the loss of HIF-1α in maintaining the VEGF levels in the lens are yet to be determined. These observations raise several important biological questions as to how the lens epithelium regulates VEGF synthesis and what is the purpose of this sustained VEGF synthesis in the adult lens epithelium.

Oxidative stress in the Lens and Cataract :

The naturally hypoxic lens is exposed to atmospheric oxygen during cataract surgery. Cataract is defined as a pathological condition in which the lens loses its transparency and becomes opacified (21, 22).

Depending on the area of the lens which is opacified, cataract can be divided into cortical which involves the outer region of the lens, nuclear which is the central core and posterior subcapsular opacification which is opacification below the lens capsule. Oxidative stress is known to play a major role in the development of cataract. Studies comparing the lipid, protein compositions and the levels of antioxidants and reactive oxygen species between cataract and normal lenses have shown that, an increase in the reactive oxygen species is a major contributing factor in the development of cataract (23).

The crystallin proteins play an important role in maintaining the transparency of the lens. The crystallin proteins act as molecular chaperones removing protein aggregates and must be maintained in a stable form (24). Oxidative damage to the lens proteins is a major risk factor leading to opacity and subsequent cataract formation. Crystallins are water soluble proteins and are divided in to alpha, beta and gamma crystallins. Alpha crystallins act as molecular chaperones by inhibiting the formation of insoluble protein aggregates and helps in maintaining the refractive index of the lens. This helps in the prevention of cataract. Beta and gamma crystallins are more of structural proteins. With age, the crystallin proteins lose their protective mechanisms and due to post translational changes they are accumulated in the lens forming protein aggregates eventually leading to opacification and cataract formation (25, 26).

The risk of cataract increases with age and the aging lens appears to be more susceptible to oxidative damage and cataract formation. Oxidative stress leading to the formation of reactive oxygen species and hydroxyl radicals is a major contributing factor in the development of cataract. The initial site of damage due to oxidative stress occurs in the epithelial cell layer and then progresses to the subcapsular region and eventually the entire lens becomes opaque.

Surgical Treatment of Cataract and PCO (Posterior Capsular Opacification):

The most effective treatment of cataract surgery is removal of the opacified lens and placing an intraocular lens near the capsular bag. During the cataract surgery, the lens capsular bag which separates the aqueous and the vitreous is used to place the artificial intra ocular lens. The anterior portion of the lens capsule is opened and the lens with cataract is removed carefully. The artificial intraocular lens is placed in the posterior portion of the lens capsule. Despite following a rigorous surgical protocol a significant number of the anterior and equatorial lens epithelial cells are left behind on the capsule. The remnants of the lens epithelial cells after the cataract surgery proliferate across the lens capsule, migrate towards the intraocular lens and undergo epithelial mesenchymal transition (EMT) in which the epithelial cells lose their polarity, E- cadherins, the cell–cell adhesion properties and transform in to mesenchymal cells. All these changes eventually lead to the development of posterior capsular opacification (PCO) (27, 28). PCO is the most common complication of cataract surgery and is seen in 20-40% of the patients after 2-3 years of cataract surgery (29).

Initiation and Persistence of Posterior Capsular Opacification:

The brief introduction of atmospheric oxygen during the surgery activates several signaling pathways chief among them being the TGF-β-Wnt signaling pathway. TGF-β is present in the aqueous and vitreous humor in an inactive state. Several studies in the lens have demonstrated that TGF-β is activated during exposure to atmospheric oxygen. Organ cultures of rat lens exposed to varying concentrations of TGF- β induced cataracts. Active TGF-β, Via the Wnt signaling pathway leads to inactivation of GSK-3β and translocation of β-catenin to the nucleus. Once β-catenin trans locates to the nucleus, it induces the

transcription of various transcriptions factors including the expression of mesenchymal proteins like α-smooth muscle actin and Fibronectin leading to the onset of EMT and PCO in atmospheric oxygen (30).

Once initiated in oxygen, PCO progresses in hypoxia under the influence of the HIFs. Previous studies by Flugel et.al.(31) have shown that the active Wnt/ β-catenin pathway influences HIF-1α to induce mesenchymal transition of epithelial cells in hypoxia. The active HIF-1α is also likely to influence the VEGF synthesis and promote the survival of the mesenchymal cell in hypoxia. Thus, the factors regulating mitoprotection through VEGF synthesis are also likely to promote the mesenchymal transition of the epithelial cells contributing to the pathophysiology of PCO. Whether the lenticular mitoprotective pathways regulating VEGF synthesis can be used to our advantage in isolating the two events of mesenchymal transition and VEGF such that, the mesenchymal transition can be inhibited without compromising the lenticular mitoprotection is yet to be determined.

SPECIFIC AIMS:

Specific Aim 1: Identify the signaling pathways promoting lenticular mitoprotection in the hypoxic lens epithelium. Cells respond to hypoxia by activating several signal transduction mechanisms which promote cell survival and proliferation. The hypoxia inducible factors (HIFs) activate the transcription of various hypoxia responsive genes, including vascular endothelial growth factor (VEGF). Previous studies in the lens epithelium demonstrated a continuous expression of VEGF in the absence of HIF-1α, suggesting that other regulatory factors are in play (17). The expression and role of another hypoxia inducible factor –HIF-2α is not well established in the lens epithelium. We propose that a compensatory interrelationship between two hypoxia inducible factors – HIF-1α and HIF-2α maintains the levels of VEGF, which in turn regulates the levels of anti- apoptotic Bcl-2 protein and provides resistance against mitochondrial depolarization. To demonstrate the reciprocity of HIFs, inhibition of HIF-1α, HIF-2α or simultaneous inhibition of both HIFs will be performed with specific pharmacological inhibitors and their effect on VEGF synthesis will be demonstrated by an ELISA. The pro-survival role of VEGF in mitoprotection will be demonstrated by suppressing the VEGF levels by inhibiting the expression of upstream HIFs, or by preventing the interaction of VEGF with its receptor using a VEGF~VEGFR2 inhibitor and their effect on anti-apoptotic Bcl-2/pBcl-2 levels and mitochondrial depolarization will be analyzed by western blot analysis and JC-1 assay.

Specific Aim 2: Identify the signaling pathways promoting lenticular mitoprotection during exposure of the hypoxic lens to atmospheric oxygen. The lens epithelium continues to synthesize VEGF during exposure to atmospheric oxygen, where HIF-1α is degraded. However, HIF-2α is stable at atmospheric

oxygen, therefore we hypothesize that HIF-2α may contribute to VEGF synthesis in atmospheric oxygen. Apart from the HIFs, exposure to atmospheric oxygen also activates TGF-β. Active TGF-β through the Wnt/β-catenin pathway leads to inactivation of GSK-3β and translocation of active β-catenin to the nucleus. Once β-catenin trans locates to the nucleus it activates several nuclear transcription factors promoting cell proliferation and survival including VEGF. Based on these studies we hypothesize that inactivation of GSK-3β either through TGF-β activation or by a specific pharmacological inhibitor will lead translocation of active β-catenin to the nucleus and VEGF synthesis. To demonstrate the association between increased nuclear β-catenin and VEGF levels, a specific pharmacological inhibitor will be used to inhibit the nuclear translocation of β-catenin and its effect on VEGF levels will be analyzed by ELISA. The pro-survival role of VEGF in atmospheric oxygen will be demonstrated by suppressing the VEGF levels by targeting upstream regulators like nuclear β-catenin/HIF-2 α and their effect on anti-apoptotic Bcl-2/pBcl-2 levels and mitochondrial depolarization will be analyzed by western blot analysis and JC-1 assay.

Specific Aim 3: Determine the association between lenticular mitoprotection (In hypoxia and atmospheric oxygen) and the initiation and progression of Posterior Capsular Opacification (PCO). Lenticular mitoprotective pathways either in hypoxia or atmospheric oxygen lead to increased VEGF synthesis. However, nuclear β-catenin which promotes increased VEGF synthesis in atmospheric oxygen is also known to induce mesenchymal transition of epithelial cells via the expression of mesenchymal proteins like α-smooth muscle actin and Fibronectin. We therefore hypothesize that nuclear β-catenin might play a dual role in promoting mesenchymal transition via EMT protein expression and increased resistance to mitochondrial depolarization via VEGF synthesis. A specific pharmacological GSK-3β inhibitor and β-catenin inhibitor will be used to demonstrate effects of nuclear translocation of β-catenin on mesenchymal protein expression. The pathophysiology of PCO is initiated in atmospheric oxygen and continues in hypoxia under the influence of the HIFs. The lenticular mitoprotective pathways in atmospheric oxygen

which involve inhibition of GSK-3β and increased translocation of nuclear β-catenin might lead to the formation of a mesenchymal cells resisting depolarizaiton via increased VEGF synthesis. When the lens returns to hypoxia (after the surgery) under the influence of GSK-3β inhibition (which is initiated in atmospheric Oxygen) the HIF-1α continues to regulate the expression of mesenchymal proteins and VEGF synthesis contributing to the progression of PCO. Taking advantage of the likely compensatory interrelationship between the two HIFs in hypoxia, we hypothesize that the mesenchymal transition can be prevented via inhibition of HIF-1α and HIF-2α compensates for loss of HIF-1α and maintains the VEGF levels without compromising the lenticular mitoprotective pathways.

CHAPTER 1

Hypothesis: Vascular endothelial growth factor (VEGF) synthesis in the hypoxic lens is regulated by a compensatory interrelationship between the two Hypoxia inducible factors – HIF-1α and HIF-2α.

Rationale: The hypoxic environment is known to induce the expression of the hypoxia inducible factor, HIF-1α, which in turn plays a crucial role in the transcription of growth factors like vascular endothelial growth factor (VEGF). However, it has been previously shown that the hypoxic lens epithelium continues to synthesize VEGF in the absence of HIF-1α (3,17). Apart from HIF-1α another hypoxia inducible factor- HIF-2α is also known to induce the synthesis of VEGF. HIF-1α is degraded in atmospheric oxygen by the enzyme prolyl hydroxylase-domain proteins (PHDs) which hydroxylate the α subunits on proline residues. The hydroxylated α subunit is recognized by VHL (Von –Hippel Lindau) tumor suppressor protein which degrades the HIF by proteasomal degradation (32,33). Lofstedt et al (34) have shown that HIF-2α is resistant to degradation in atmospheric oxygen by PHDs due to difference in the aminoacid sequences around the hydroxylation site. As a result HIF-2α is expressed in atmospheric oxygen and hypoxia (35-38). Although both HIF-1α and HIF-2α are similar in structure and are both activated by hypoxia, they are not redundant in function and have unique gene targets (39). Germane to the study presented herein, HIF-1α and HIF-2α have been shown to regulate VEGF expression under hypoxia. Using MCF-7 cells (a breast carcinoma cell line) in conjunction with siRNA knockdown, Carroll et. al. (20) has shown that both HIF-1α and HIF-2α regulates VEGF expression under hypoxia and inhibition of HIF-2α resulted in a reciprocal increase in the expression of HIF-1α, maintaining VEGF expression. Based on these studies we propose that the lack of HIF-1α in the lens epithelium is likely to

be compensated by HIF-2α and inhibition of both HIFs is essential to supress theVEGF expression in hypoxic lens epithelium.

<u>Hypothesis:</u> VEGF promotes lenticular mitoprotection in the hypoxic lens by maintaining the levels of the anti-apoptotic protein Bcl-2 and resisting mitochondrial depolarization.

Rationale: VEGF is one of the several factors that stimulate lens cell proliferation and fiber differentiation during the fetal lens development; however its role as a pro-survival factor in the adult hypoxic lens is not known (18,19). Beierle et. al. (40) has shown that an increase in VEGF levels in neuroblastoma cells increases the expression of pro-survival protein Bcl-2 and protects the cells from apoptotic stimuli. Cellular apoptosis is regulated by the anti-apoptotic Bcl-2 and the pro-apoptotic BAX protein levels. BAX, a member of the pro-apoptotic Bcl-2 family causes the release of cytochrome C from the mitochondria by opening the mitochondrial permeability transition pore (41). Before BAX can initiate this process it has to translocate from the cytoplasm to the mitochondria. Nomura et al (42) and others (43) have shown that anti-apoptotic Bcl-2 acts by partly inhibiting the translocation of BAX from the cytoplasm to mitochondria and thus prevents the release of cytochrome C and initiation of apoptosis. Based on these studies we hypothesize that the purpose of continous VEGF synthesis in the adult lens epithelium is to resisist mitochondrial depolarization by maintaining the levels of anti-apoptotic Bcl-2 proteins.

MATERIALS AND METHODS

Cell cultures: HLE-B3 (Human Lens Epithelial 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)(46). Authentication of the HLE-B3 cell line was verified by STR profile analysis (American Type Culture Collection, Manassas, VA.) and confirmed that the cells are human and of female origin, as originally reported by Andley et. al. (44). All studies with HLE-B3 cells were performed with pre-frozen stock cells (maintained in liquid nitrogen) between passages 14 to 17 and no experiments exceeded beyond 5 passages from 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), 2mM L-glutamine, nonessential amino acids, and 0.02g/L gentamycin solution (Sigma-Aldrich) and cultured at 37^0C and 5% CO_2-95% O_2. Cells were sub-cultured four to five days prior to the experiment and placed in MEM containing 20% FBS. On the day of the experiment, cells were switched to serum-free MEM.

The cells were maintained in hypoxia (1% oxygen) for 3 hours or 8 hours for the hypoxic exposure. Each experiment was executed with control DMSO only cells (mock inhibitor treatment) and cells treated with inhibitors. The DMSO concentration per experiment did not exceeded 0.05%.

Western blot analysis: Total cell lysates were collected from HLE-B3 cultures after treatments by rinsing adherent cells with ice-cold 1× PBS, pH 7.4, then adding hot lysis buffer (0.12M Tris HCl (pH 6.8), 4% SDS and 20% glycerol, 280 µl boiled to 100^0 C) directly to cell monolayers. Lysates were collected and sonicated for 5 sec, and a portion of the sample was removed for determination of protein

concentration. Protein concentration was determined using the EZQ protein quantification kit from Invitrogen (Carlsbad, CA); 3× SDS (Laemmli) buffer was added to the lysates, which were subsequently boiled for 5 min; and 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 Western blot analysis, nitrocellulose membranes were blocked with Tris-buffered saline (TTBS, 1% BSA and 0.02% Tween -20 in Tris–buffered saline) for 60 min. These membranes were probed overnight at 4°C with primary antibodies. The blots were then rinsed in TTBS (4× 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 pico chemiluminescent kit from Pierce (Rockford, IL).

Primary antibodies: The antibodies used in this specific aim were rabbit anti-BAX (Cell Signaling Technology, Danvers, MA), rabbit anti–Bcl-2 (Cell Signaling Technology, Danvers, MA), rabbit anti–Actin (Santa Cruz, CA), rabbit anti- HIF-1α (Bethyl Laboratories Inc., Montgomery, TX), rabbit anti-HIF-2α (Novus Biologicals, Littleton, CO).

ELISA: An ELISA-based assay was used to detect secreted levels of vascular endothelial growth factor (VEGF). VEGF was determined using the Invitrogen VEGF ELISA kit (Grand Island, NY). HLE-B3 cells were cultured in 25 cm^2 tissue culture flasks in 20% FBS, transferred 24 h in serum-free media prior to the initiation of the experiment. Flasks were set up in triplicate for 3, 8, or 24 h of cell incubation in hypoxia (1% oxygen) or atmospheric oxygen (21% oxygen). Cell-free supernatants were collected and

analyzed according to manufacturer's instructions and optical density at 450 nm was determined using a Molecular Devices Spectramax 190 (Sunnyvale, CA).

JC-1 analysis to determine Mitochondrial depolarization: After treatment with the HIF-1/HIF-2α double translation inhibitor, the cells were stained with the cationic dye 5,5′,6,6′-tetrachloro1,1′,3,3′-tetraethyl-benzimidazolylcarbocyanine iodide (JC-1; Molecular Probes, Eugene, OR) as described previously (45) to visualize 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 fluorescence emission shift from red to green (45). That is, 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) 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.

Pharmacological inhibitors: Topotecan Hydrochloride, a recognized topoisomerase I inhibitor (46, 47) was purchased from AvaChem Scientific LLC, San Antonio, TX. HIF-1α translation inhibitor (KC7F2), HIF-2α translation inhibitor (CAS882268-69-1), HIF-1α and HIF-2α double translation inhibitors (FM19G11) were purchased from EMD Chemicals, Billerica, MA. Axitinib (N-methyl-[[3[(1E)-2-(2-pyridinyl) ethenyl]-1H-indazol-6-yl]thio]-benzamide) was purchased from Tocris Bioscience, Ellisville,

MO. All inhibitors were dissolved in dimethyl sulfoxide (DMSO). The final concentration of DMSO for all the inhibitor assays did not exceed 0.05%.

HIF Translation Inhibitors:

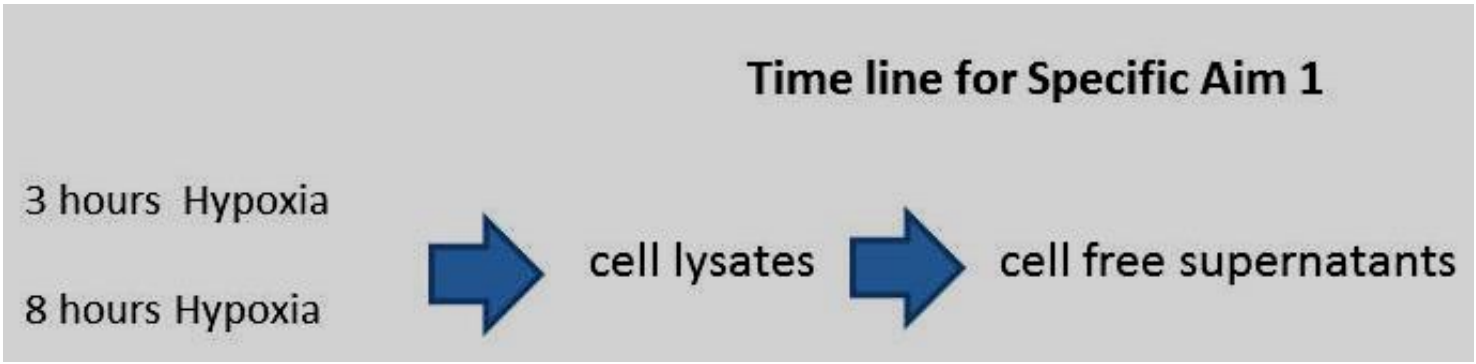
Inibition of HIF-1α by translation inhibitor: Studies have shown that mTOR signaling pathway increases the protein synthesis of HIF-1α by phosphorylation and supression of 4EBP-1 binding protein. HIF-1α translation inhibitors target 4EBP-1 or the mTOR signaling pathwys. KC7F2 is an HIF-1α translation inhibitor which inhibits HIF-1α protein synthesis and not the mRNA transcription. KC7F2 inhibits the phosphorylation of 4eBP-1, which inturn inhibits the translation of HIF-1α protein synthesis (48). In this study HLE-B3 cells were serum starved for 24hrs and incubated with three different concentratrions (0.5µm, 5µm and 50µm) of HIF-1α translation inhiitor KC7F2 dissolved in 0.05% DMSO for 3hrs of hypoxia. Control cells were incubated in serum free medium with 0.05% DMSO. Cell free supernatants and total cell lysates were collected at the end of 3hrs of hypoxia.

Inhibition of HIF-2α by translation inhibitor: Studies have shown that HIF-2α mRNA expresses the Iron responsive elements (IRE) at the 5' UTR region. Binding of iron regulatory proteins (IRP) to the IRE will inhibit the translation. Hypoxia is known to inhibit the binding of IRP to IRE and promote the translation of HIF-2α. In a study by Zimmer et.al, small molecules inhibitors of HIF-2α, were used to inhibit the translation of HIF-2α by promoting the binding of IRP to the iron responsive elements (IRE) (49,50). In the current study a small molecule HIF-2α inhibitor was used to test the effect of HIF-2α translation inhibition on VEGF levles in hypoxic lens epithelial cells. HLE-B3 cells were serum starved for 24hrs and incubated with three different concentratrions (0.5µm, 5µm and 50µm in 0.05% DMSO) of

HIF-2α translation inhibitor for 3hrs of hypoxia. Control cells were incubated in serum free media with 0.05% DMSO. Cell free supernatants and total lysates were collected at the end of 3hrs of hypoxia.

Inhibtion of both HIF-1α and HIF-2α using a double translation inhibitor: Hypoxia inducible factor (HIF) is a heterodimer. It consists of α subunit (HIF- 1α, HIF-2α) which is regulated by oxygen levels and a constitutively expressed β subunit. FM19G11 is an inhibitor of HIF proteins including both HIF-1α and HIF-2α. FM19G11 is a histone acetytransferase inhibitor. The compound was shown to cause an overall reduction in P300, which is a histone acetyltransferase (51). This enzyme is required as a co factor for HIF α transcription activation. FM19G11 was shown to inhibit HIF-1α and HIF-2α transcription activation in cancer cell lines. In this study we looked at the simultaneous inhibition of both HIF-1α and HIF-2 α proteins and its effect on VEGF expression. HLE-B3 cells were serum starved and incubated with three different concentrations (0.5µm, 5µm and 50µm in 0.05% DMSO) of the double translation inhibitor FM19G11 in hypoxia for 3hrs. Control cells were incubated in serum free media with 0.05% DMSO. Cell free supernatants and total cell lysates were collected at the end of hypoxic incubation.

Statistical Analysis: The images from JC-1 analysis were taken and separated into individual red and green channels using ImageJ software (Baltimore, MD). The background fluorescence was removed from each image. The fluorescence intensity signal from each image was quantified for the entire image and expressed as the ratio of green intensity over the red intensity. For ELISA, a student's t-test (Topotecan experiments) and a one-way ANOVA (HIF translation inhibitor experiments) were performed using the software from Graphpad Prism, version 5.00 (La Jolla, CA).



The cells were incubated with specific pharmacological inhibitors for either 3 or 8 hours of hypoxia and cell free supernatants and lysates were collected after the hypoxic incubation.

RESULTS

VEGF expression in HLE-B3 cells in hypoxia and atmospheric oxygen: The levels of VEGF synthesized by HLE-B3 cells maintained in either hypoxia or atmospheric oxygen were compared. HLE-B3 cells were serum-starved for 24 hours, followed by the introduction of fresh serum-free media and cell cultures switched to hypoxia (1% oxygen) or allowed to remain in atmospheric oxygen (~21% oxygen) for up to 72 hours. Cell free supernatants were collected in triplicates at 8, 24, 48 and 72 hours. The VEGF levels in the supernatants were analyzed by ELISA. VEGF steadily accumulated regardless of whether the cells were maintained in hypoxia or atmospheric oxygen throughout the duration of the time course. Significantly higher amounts of VEGF, at all collected time points beyond the initial 8 hour point, was evident in hypoxia-maintained cells relative to the corresponding collected time point from atmospheric oxygen (Figure 1).

Inibition of HIF-1α by Topotecan: Topotecan inhibits topoisomerase-I thereby preventing HIF-1α transcription and protein accumulation in hypoxia (46,47). HLE-B3 cells were serum-starved for 24 hours after which 500 nM Topotecan (dissolved in 0.01% DMSO) was added to the cells. Control cells were mock-treated by incubation in serum-free media with 0.01% DMSO. Cell lysates were collected after 8 hours of Topotecan treatment for Western blot analysis of HIF-1α and HIF-2α expression. At the same time, cell free supernantants were collected in triplicate and analyzed by ELISA to determine VEGF levels.

HIF-1α is a transcription factor, expressed and stabilized in hypoxia, which plays a major role in the regulation of VEGF synthesis. Western blot analysis of Topotecan-treated lysates displayed a marked decrease in protein levels of HIF-1α relative to control cells. The attenuation of HIF-1α did not affect VEGF synthesis relative to untreated cells (Figure 2D). HIF-2α has been shown to induce VEGF synthesis in other cell systems but a role for HIF-2α in the regulation of VEGF synthesis in human lens epithelial cells has never been reported. Topotecan-treated samples showed a marked increase in HIF-2α expression relative to control cells (Figure 2A). Thus, an elevated expression of HIF-2α was initiated as a result of the loss of HIF-1α by Topotecan treatment.

HIF-2α standard lysates: Western blot analysis of HIF-2α protein expression revealed a set of three bands of approximate molecular weight of 80 KDa. Foorogian et. al. (52) reported HIF-2α as a set of three bands of approximate molecular weight of 100 KDa in retinal pigment epithelial cells. In order to further confirm the identity of HIF-2α in HLE-B3 cells, a HIF-2α standard lysate (Novus Biologicals, Littleton, CO) was compared with lysate from HLE-B3 cells. Whereas the HIF-2α bands in HLE-B3 cells were of approximate molecular weight, 80 KDa, the standard lysate was of approximate molecular weight 97 KDa (Figure 2E). The three bands seen with HIF-2α could be due to the post translational

modifications of the protein or different isoforms of the protein being expressed in the lens epithelial cells. The difference in the molecular weights could be due to the nature of different cell lines used – the lens epithelial cells versus the Human embryonic kidney cell lines. The use of HIF-2α specific translational inhibitors inhibited the HIF-2α protein and the three bands in human lens epithelial cells, confirming the expression and biological function of HIF-2α protein in the lens epithelial cells.

Mitoprotection in HLE-B3 cells is not affected by the loss of HIF-1α: The effect of inhibition of HIF-1α on mitochondrial membrane permeability transition (i.e. lenticular mitoprotection) was evaluated with the JC-1 assay. Cells were incubated with 500 nM Topotecan for 3 hours in hypoxia and switched to atmospheric oxygen. The hypoxic media was replaced with fresh, oxygenated media without inhibitor and 5µg/ml of JC-1 dye introduced to the cell cultures and incubated at 37^0C for 30 min. The media was removed and fresh media without inhibitor or potentiometric dye was added to the cells. Control cells were incubated in serum-free media with DMSO but were otherwise treated identically. There was no significant difference in the green/red fluorescence ratio between the control cells and cells treated with the Topotecan (Figure 3). Inhibition of HIF-1α by Topotecan, did not influence the levels of pro and anti-apoptotic proteins – BAX and Bcl-2 (Figure 4)

Inhibition of hypoxia inducible factors by translation inhibitors: The role of hypoxia inducible factors in the regulation of VEGF expression was further evaluated by the use of specific translation inhibitors. KC7F2 is a specific HIF-1α translation inhibitor which suppresses HIF-1α protein synthesis but not the mRNA transcription (48). CAS882268-69-1 is a specific HIF-2α translation inhibitor which suppresses HIF-2α protein synthesis but not the mRNA transcription (49,50). FM19G11 is a histone acetylase inhibitor shown to simultaneously inhibit HIF-1α and HIF-2α transcription activation (51). Cells were maintained in serum-free media for 24 hours and treated in the presence and absence of one of

three conditions; 0.5µm, 5µm and 50µm of the HIF-1α translation inhibitor, KC7F2, the HIF-2α translation inhibitor, CAS882268-69-1 or the HIF-1α/HIF-2α translation inhibitor, FM19G11, dissolved in 0.05% DMSO for 3 hours in hypoxia. Control cells were incubated in serum-free media with 0.05% DMSO. Cell free supernatants were collected in triplicates at the completion of the 3 hour exposure period. The supernatants were analyzed for VEGF levels by ELISA. The inhibition of HIF-1α and Hif-2α by the translation inhibitors was confirmed by Western blot analysis (Figure 5A, E) . Inhibition of HIF-1α or HIF-2α by their respective specific translation inhibitors did not influence VEGF synthesis in hypoxia relative to their relevant controls (Figure 5D,H). In contrast, the simultaneous inhibition of HIF-1α and HIF-2α caused a significant decrease in VEGF synthesis (Figure 5L). In order to further confirm the sustained decrease in VEGF accumulation in the presence of the double translation inhibitor cells were treated for up to 8 hours in hypoxia. The prolonged exposure to FM19G11 continued to show a significant decrease in VEGF levels relative to control cells (Figure 5L).

Mitoprotection in HLE-B3 is affected by the combined loss HIF-1α and HIF-2α: HLE-B3 cells were treated with 50 µM of the HIF-1α/HIF-2α translation inhibitor, FM19G11, for 3 hours in hypoxia. After the hypoxic exposure the media was replaced with fresh, oxygenated serum-free media containing 5µg/ml JC-1 for 30min in atmospheric oxygen. The media was removed and fresh media without inhibitor was added to the cells. The control cells were incubated in serum-free medium with DMSO. A significant increase in the green/red ratios, indicating profound mitochondrial depolarization, was observed with the cells treated with the HIF-1α/HIF-2α translation inhibitor compared to control cells (Figure 6).

VEGF maintains Bcl-2 expression and promotes cell survival in hypoxia: Previous studies by Beierle et. al. (40) have shown that an increase in VEGF levels in neuroblastoma cells increases the expression of

pro-survival protein Bcl-2 and protects the cells from apoptotic stimuli. In our study, a significant decrease in VEGF levels coupled with a profound increase in mitochondrial depolarization was observed when the cells were treated with the HIF-1α/HIF-2α translation inhibitor (Figure 5L and Figure 6). To further evaluate the possible role of VEGF in lenticular cell survival we examined the association between VEGF levels and the expression of pro-apoptotic protein, BAX and the pro-survival protein, Bcl-2. Lysates were collected from cells treated with various inhibitors (Topotecan, HIF-2α (CAS882268-69-1), HIF-1α /HIF2α (FM19G11) as described above and analyzed by western blot analysis.

HIF translation inhibitors: The inhibition of HIF-1α by Topotecan or the inhibition of HIF-2α by the HIF-2α translation inhibitor did not alter the levels of VEGF (Figure 2D and Figure 5H) or the apoptotic proteins BAX and Bcl-2 (Figure 4 and 7). However, the simultaneous inhibition of HIF-1α and HIF-2α which resulted in a significant decrease in the levels of VEGF (Figure 5L) diminished the levels of the pro-survival protein Bcl-2, without alteration to the levels of the pro-apoptotic protein, BAX (Figure 8) and under these conditions profound mitochondrial depolarization was observed (Figure 6).

VEGF Receptor Tyrosine Kinase inhibitor (RTK): Axitinib is a selective RTK inhibitor which inhibits VEGFR1, 2 and 3 (53-55). The lens epithelial cells have been shown to express the VEGFR2. To test the effect of VEGF receptor inhibition on Bcl-2 levels, HLE-B cells were incubated with three different concentrations (0.05 µM, 0.5 µM and 5µM) of Axitinib for 3hrs of hypoxia. Control cells were treated with 0.05% DMSO. Cell lysates were collected for Western blot analysis of BAX and Bcl-2. Blocking the VEGF RTK activity significantly diminished the levels of anti-apoptotic Bcl-2 protein (Figure 9), confirming the role of VEGF as a pro-survival factor in the hypoxic adult lens epithelium.

FIGURES FOR CHAPTER 1

Figure 1

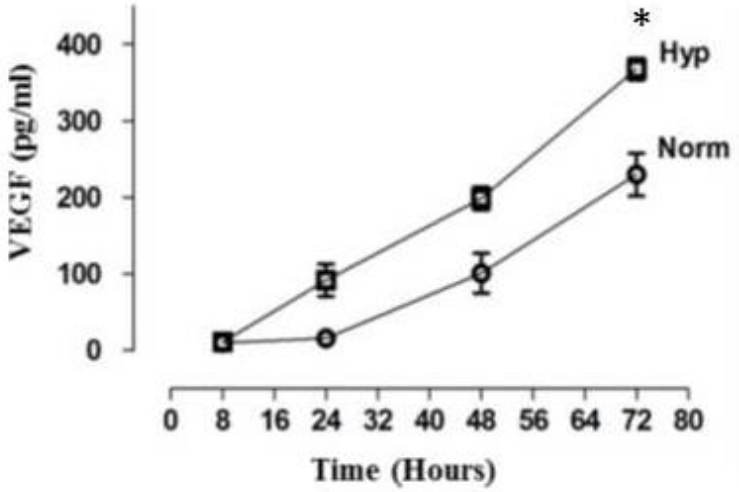


Figure 1: Sustained and cumulative expression of VEGF in hypoxia and atmospheric oxygen. HLE-B3 cells were cultured in 25 cm2 flasks with 20% FBS and switched to serum-free media 24 h before the experiment. The cells were incubated with 3 ml of serum-free media in hypoxia (1% oxygen) or remained in atmospheric oxygen (about 21% oxygen) for up to 72 h. Cell-free supernatants were collected in triplicate at 8, 24, 48, and 72 h and analyzed with ELISA to detect the VEGF levels. VEGF consistently accumulated throughout the 72 h incubation period regardless of whether the cells were maintained in hypoxia or atmospheric oxygen. A Student *t* test was performed to compare the VEGF levels between hypoxia (Hyp) and normoxia(Norm) (*$p<0.05$). Significantly higher levels of VEGF were detected at all-time points beyond the initial 8h point in hypoxia compared with atmospheric oxygen. Error bars are not shown at time points because the symbol is larger than the error bar.

Figure 2

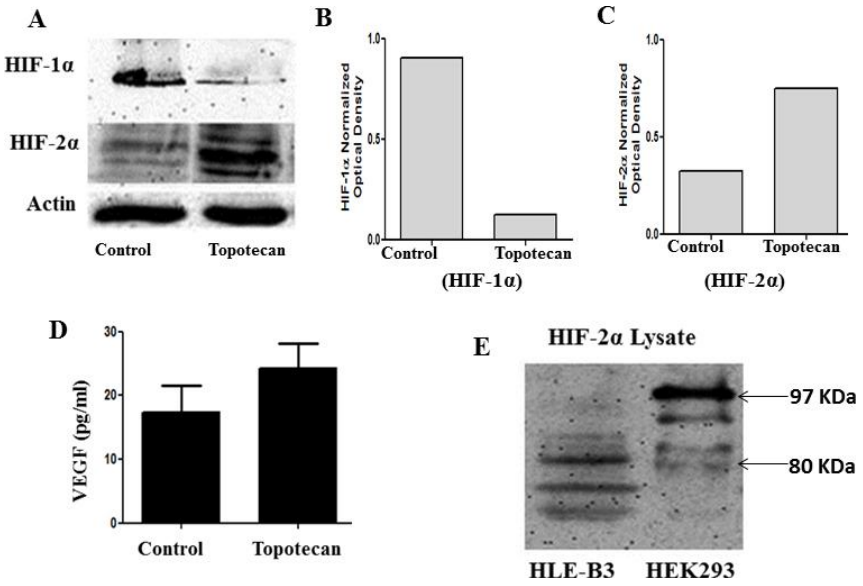


Figure 2. **A**: Western blot analysis of HIF-1α expression in HLE-B3 cells treated with Topotecan. Cell lysates were collected from cells treated with 500 nM Topotecan in 0.01% DMSO after 8 h of hypoxic incubation. Control cells were mock treated with 0.01% DMSO and maintained in hypoxia as the Topotecan-treated cells. Twenty μg protein/lane of cell lysates were analyzed with Western blot analysis and lane loading was normalized using a 1:1,000 dilution of rabbit anti-pan-actin antibody. Topotecan inhibited the expression of HIF-1α (1:1,000 dilution of rabbit anti- HIF-1α antibody) while a compensatory increase in HIF-2α was noted (1:1,000 dilution of rabbit anti- HIF-2α antibody). **B, C**: Densitometry analysis of HIF-1α and HIF-2α, respectively. **D**: Effect of HIF-1α inhibition on VEGF synthesis in hypoxia. HLE-B3 cells were cultured in 25 cm2 flasks with 20% FBS and switched to serum-free media 24 h before the experiment. The cells were incubated with 3 ml of serum-free media containing 500 nM Topotecan or 0.01% DMSO for 8 h of hypoxic exposure. Cell-free supernatants were collected in triplicate at the end of hypoxic incubation and analyzed for VEGF levels with ELISA. There was no significant difference in the VEGF levels between the Topotecan-treated cells and control cells. (A Student *t* test was performed to compare the VEGF levels between control and treated sample, and the p value was greater than 0.05 (not significant) **E**: HIF-2α protein from HLE-B3 control cells compared with a standard lysate prepared from Human embryonic kidney cells (HEK) 293 (Novus Biologicals, Litton, CO). The HIF-2α found in the standard lysate migrated at about 97 kDa. The HIF-2α from HLE-B3 cells was about 80 kDa).

Figure 3

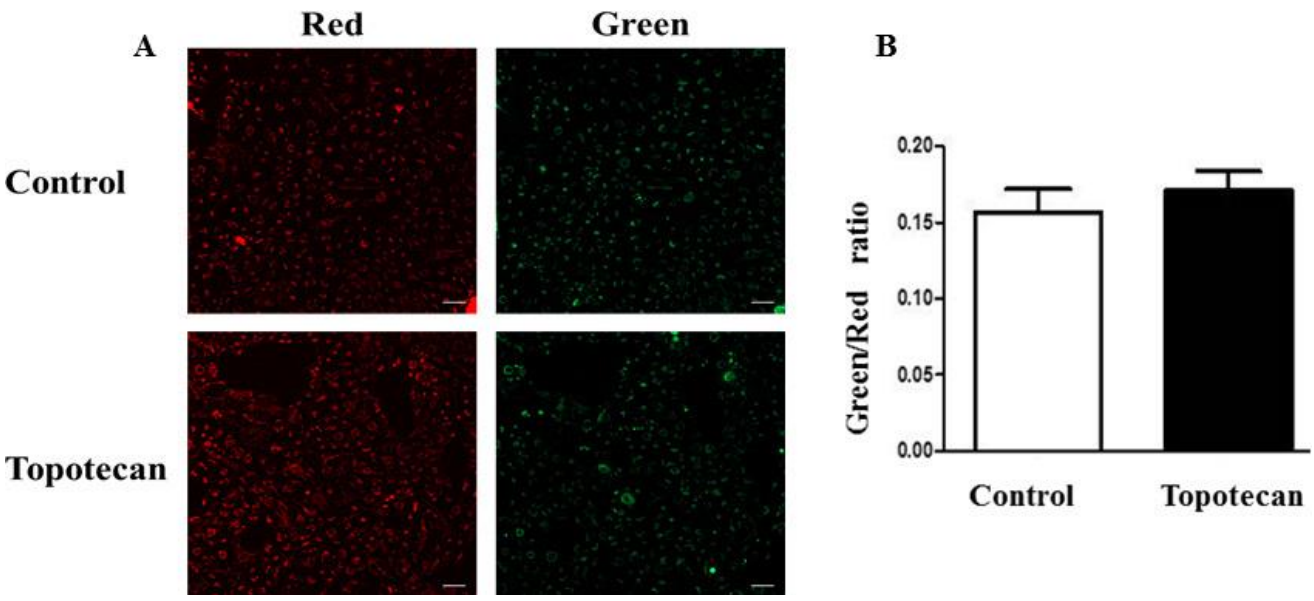


Figure 3: JC-1 analysis of HLE-B3 cells treated with Topotecan. Cells were incubated in serum-free media containing 500 nM of Topotecan in 0.01% DMSO for 3 h in hypoxia. Control cells were treated with 0.01% DMSO in serum-free media and likewise exposed for 3 h in hypoxia. After 3 h of hypoxic exposure, fresh, oxygenated media without the inhibitor but with the addition of 5 μg/ml of JC-1 dye were added and incubated at 37 °C for 30 min. JC-1 is a potentiometric dye that exhibits a membrane potential dependent loss as J-aggregates (polarized mitochondria) when transitioned to JC-1 monomers (depolarized mitochondria), as indicated by a fluorescence emission shift from red to green. Therefore, mitochondrial depolarization can be indicated by an increase in the green/red fluorescence intensity ratio. The media were removed, and fresh serum-free media without inhibitor or potentiometric dye were again added to the cells. **A**: Confocal imaging of mitochondrial membrane depolarization after inhibition of HIF-1α. Note the proportionally equivalent red and green fluorescence between Topotecan-treated and mock-treated cells, indicating that the membrane potential was not altered by inhibiting HIF-1α expression. These images were taken from a randomly chosen field. (The bar represents 20 μm.) **B**: There was no significant difference in the green/red fluorescence ratio between the control and Topotecan-treated cells. Student *t* test, $p>0.05$.

Figure 4

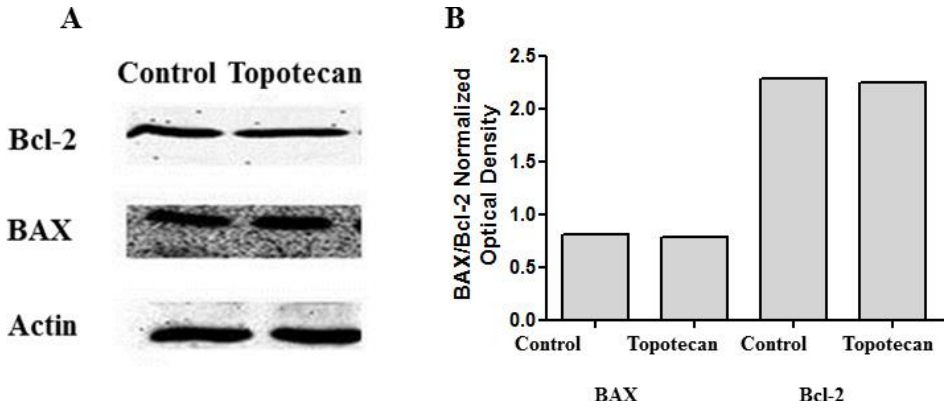


Figure 4: Inhibition of HIF-1α does not influence BAX or Bcl-2 levels. Cell lysates collected from cells treated with 500 nM Topotecan were analyzed with Western blot analysis for BAX and Bcl-2 levels. There was no change in the protein levels of BAX and Bcl-2 (A). (B) Represents the corresponding densitometry analysis.

Figure 5

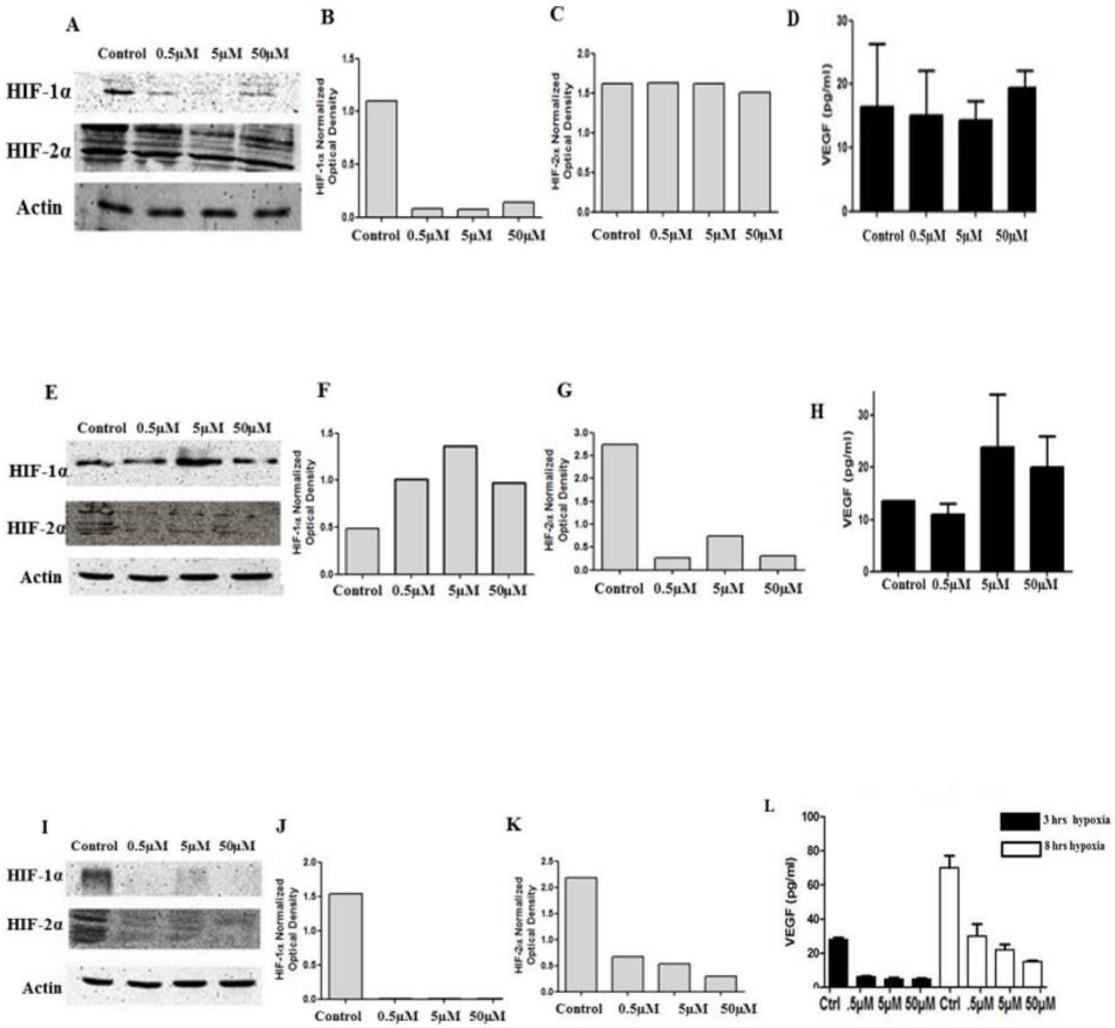

A
Control 0.5μM 5μM 50μM
HIF-1α
HIF-2α
Actin
B
HIF-1α Normalized Optical Density
Control 0.5μM 5μM 50μM
C
HIF-2α Normalized Optical Density
Control 0.5μM 5μM 50μM
D
VEGF (pg/ml)
Control 0.5μM 5μM 50μM
E
Control 0.5μM 5μM 50μM
HIF-1α
HIF-2α
Actin
F
HIF-1α Normalized Optical Density
Control 0.5μM 5μM 50μM
G
HIF-2α Normalized Optical Density
Control 0.5μM 5μM 50μM
H
VEGF (pg/ml)
Control 0.5μM 5μM 50μM
I
Control 0.5μM 5μM 50μM
HIF-1α
HIF-2α
Actin
J
HIF-1α Normalized Optical Density
Control 0.5μM 5μM 50μM
K
HIF-2α Normalized Optical Density
Control 0.5μM 5μM 50μM
L
3 hrs hypoxia
8 hrs hypoxia
VEGF (pg/ml)
Ctrl .5μM 5μM 50μM Ctrl .5μM 5μM 50μM

Figure 5: Inhibition of both HIF-1α and HIF-2α elicits the loss of VEGF expression. HLE-B3 cells were cultured in 25 cm2 flasks with 20% FBS and switched to serum-free media 24 h before the experiment. The cells were incubated with 3 ml of serum-free media containing 0.5 μm, 5 μm, and 50 μm HIF-1α inhibitor, HIF-2α inhibitor, and HIF-1α/HIF-2α double translation inhibitor for 3 or 8 h in hypoxia. The effect of the inhibitors on HIF-1α and HIF-2α protein expression was analyzed using Western blot analysis. Cell lysates (20 ug protein/lane) were identified using either anti-rabbit HIF-1α or HIF-2α at 1:1000 dilutions and lane loading was normalized using a 1:1000 dilution of rabbit anti- pan-actin antibody. HLE-B3 cells were cultured in 25 cm2 flasks with 20% FBS and switched to serum-free media 24 h before the experiment. The cells were incubated with 3 ml of serum-free media containing 0.5 μm, 5 μm and 50 μm HIF-1α inhibitor, HIF-2α inhibitor and HIF-1α/HIF-2α double translation inhibitor for 3 or 8 h in hypoxia. Cell free supernatants collected in triplicate were analyzed for VEGF levels by ELISA. The HIF-1α translation inhibitor at all concentrations inhibited HIF-1α without affecting the HIF-2α protein synthesis (**A**). Figure (**B**) and (**C**) represent the densitometry analysis for HIF-1α and HIF-2α protein expression. There was no significant difference in the VEGF levels between the control cells and cells treated with HIF-1α inhibitor (**D**). One-way ANOVA analysis was performed to compare the VEGF levels between the control and the three concentrations of HIF-1α inhibitor and the p value was >0.05. The HIF-2α translation inhibitor at all concentrations inhibited HIF-2α without affecting the HIF-1α protein synthesis (**E**). Figure (**F**) and (**G**) represent the densitometry analysis for HIF-1α and HIF-2α protein expression. There was no significant difference in the VEGF levels between the control cells and cells treated with HIF-2α inhibitor (**H**). One -way ANOVA analysis was performed to compare the VEGF levels between the control and the three concentrations of HIF-2α inhibitor and the p value was >0.05.The HIF-1α/HIF-2α double translation inhibitor at all concentrations inhibited HIF-2α and HIF-1α protein synthesis (**I**). Figure (**J**) and (**K**) represent the densitometry analysis for HIF-1α and HIF-2α protein expression. There was significant difference in the VEGF levels between the control cells and cells treated with HIF-1α/ HIF-2α double translation inhibitor at 3 h and 8 h of hypoxia. **L**: One-way ANOVA analysis was performed to compare the VEGF levels between the control and the three concentrations of HIF-1α/ HIF-2α double translation inhibitor and the p value was <0.05.

Figure 6

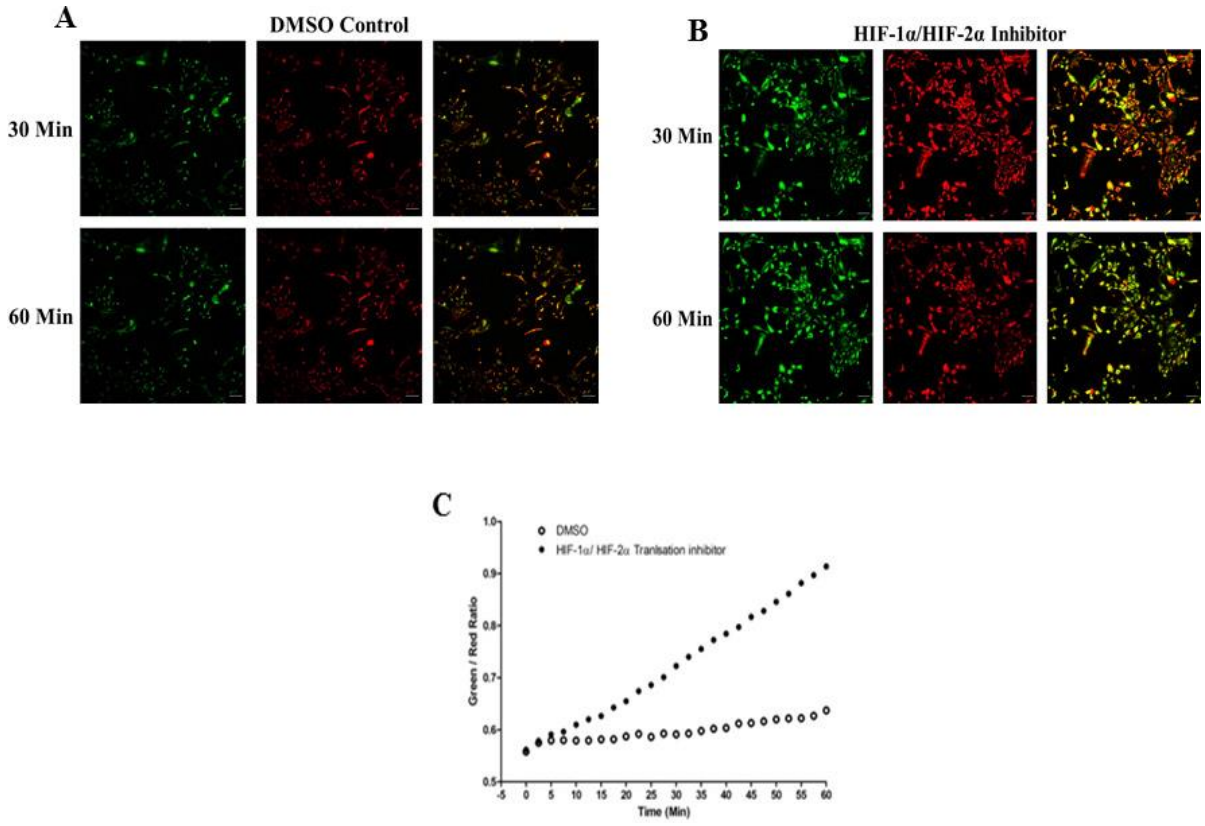


Figure 6. Inhibition of both HIF-1α and HIF-2α elicits mitochondrial membrane depolarization. Cells were treated with 50 μM of HIF-1α/HIF-2α translation inhibitor in 0.05% DMSO for 3 h in hypoxia. Control cells were treated with 0.05% DMSO. After the hypoxic exposure, the media was replaced with fresh oxygenated serum-free media containing 5 μg/ml JC-1 for 30 min in atmospheric oxygen. The media were removed, and fresh serum-free media were added to the cells. Control cells incubated with DMSO only were treated in a similar manner. We used serial confocal imaging to monitor mitochondrial membrane depolarization in HLE-B3 cells after treatment with the HIF-1α/HIF-2α double translation inhibitor. Sequential images of a random field of cells were taken every 150 s throughout the 60 min duration (Bar=20 μm). Confocal images of the HIF-1α/HIF-2α double translation inhibitor-treated cells indicated that there was a marked increase in green fluorescence intensity (indicative of depolarization) at both 30 and 60 min (**B**) compared with the control cells (**A**). **C**: HLE-B3 cells treated with the HIF-1α/HIF-2α double translation inhibitor exhibited a significantly increased green/red ratio compared with control, untreated cells.

Figure 7

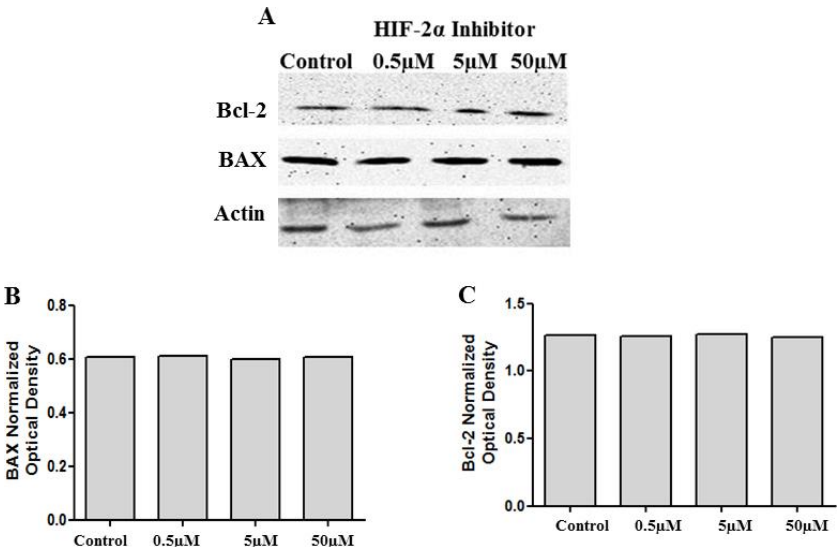


Figure7: Inhibition of HIF-2α does not influence BAX or Bcl-2 levels. Cell lysates collected from cells treated with 0.5 μM, 5 μM, and 50 μM of HIF-2α translation inhibitor were analyzed with Western blot analysis for BAX and Bcl-2 levels. There was no change in the protein levels of BAX and Bcl-2 (**A**). **B** and **C** represent the corresponding densitometry analysis.

Figure 8

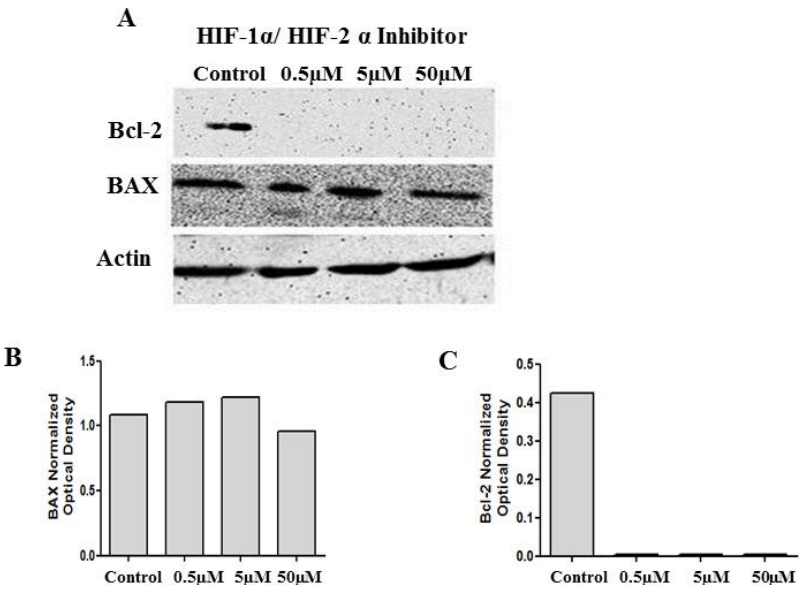


Figure8: Inhibition of both HIF1α and HIF-2α diminishes Bcl-2 levels without affecting BAX levels. Cell lysates collected from cells treated with 0.5 μM, 5 μM, and 50 μM of HIF-1α/HIF-2α translation inhibitors were analyzed with Western blot analysis for BAX and Bcl-2 levels. There was no change in the protein levels of BAX, and a significant decrease in the levels of Bcl-2 coupled with the loss of VEGF (Figure 4L) was observed with the double translation inhibitor (**A**). **B** and **C** represent the corresponding densitometry analysis.

Figure 9

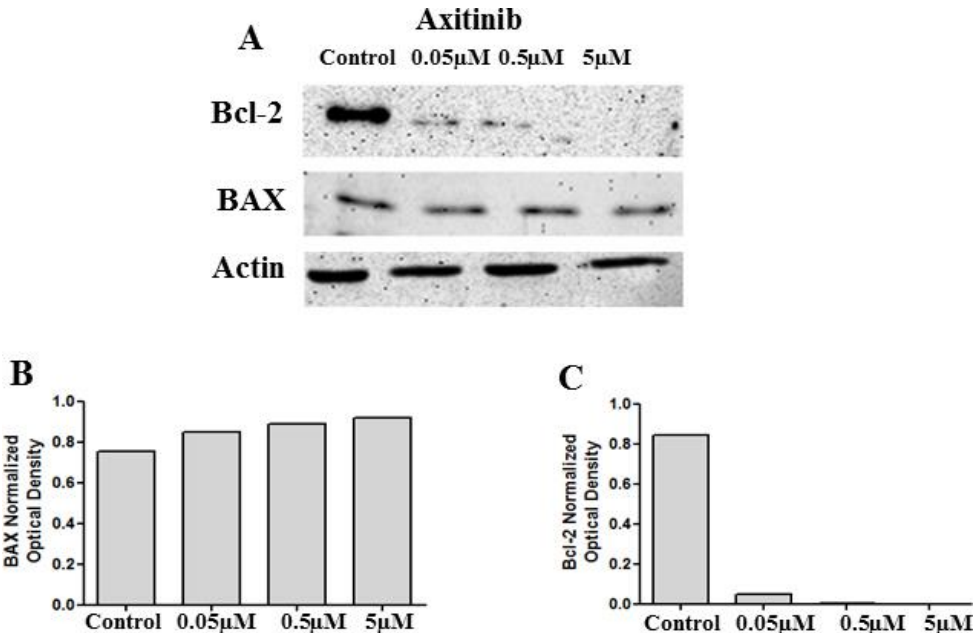


Figure 9: Axitinib blocks binding of VEGF to its receptor, VEGFR2 and prompts a loss of Bcl-2 levels. Cell lysates collected from cells treated with 0.05 µM, 0.5 µM, and 5 µM of axitinib (AG013736) were analyzed with Western blot analysis for the BAX and Bcl-2 levels. There was no change in the protein levels of BAX, and a significant decrease in the levels of Bcl-2 was observed with the VEGF receptor inhibitor (**A**). **B** and **C** represent the corresponding densitometry analysis.

DISCUSSION

Cells respond to hypoxia by undergoing various biological adaptations, chief among them is the transcription of hypoxia inducible factors (HIFs). The hypoxia inducible factors in turn activate the transcription of genes promoting cell survival and proliferation like vascular endothelial growth factor (VEGF) and Erythropoietin (EPO). Erythropoietin (EPO) is primarily a hematopoietic growth factor regulating the survival and proliferation of red blood cells. Patel et. al. (56) has studied the role of EPO in fetal eye. Their studies in the fetal retina show an increase in EPO mRNA and protein levels with increase in gestation age. Production of EPO is induced by the hypoxia inducible factors (HIFs). Radreau et. al. (3) has shown an increase in the mRNA levels of EPO in FHL-124 lens epithelial cells induced by HIF-1α. If this increase was translated to increased protein levels of EPO was not addressed in their study. Previous studies in our laboratory (data not shown) have shown a 10 fold increase in mRNA levels of EPO in hypoxia, but this increase in mRNA does not translate to an increase in protein synthesis. There were no detectable amounts of EPO protein in HLE-B3 cells in hypoxia. Based on these results we conclude that EPO message in HLE-B3 cells does not translate to protein synthesis and does not play a role in lenticular mitoprotection.

Saint-Geniez et. al. have shown the role of VEGF isoform 164 in lens development (18). Abnormalities in ocular development were observed in mice lacking this isoform suggesting a role for VEGF in ocular vasculature and lens development. The adult lens thriving in hypoxia, under the influence of hypoxia inducible factors (HIFs) continues to synthesize VEGF. Garcia et. al. (57) has shown sustained VEGF-A synthesis in HIF-1α^{CKO} mice suggesting the contribution of factors other than HIF-1α in regulating VEGF. In the current study, inhibition of HIF-1α with two different inhibitors (a topoisomerase inhibitor and a translation inhibitor) did not have an effect on the VEGF synthesis. Previous studies have

demonstrated an increased post transcriptional stabilization of VEGF mRNA in hypoxia compared to atmospheric oxygen. According to these studies an increased stability of VEGF mRNA in hypoxia contributes to sustained VEGF synthesis (58, 59). However, the HLE-B3 cells cultured in atmospheric oxygen, where there is no expression of HIF-1α and no post transcriptional stabilization of VEGF mRNA, continue to synthesize VEGF. This raises an important question as to what is driving VEGF synthesis in HLE-B3 cells in hypoxia in the absence of HIF-1α in atmospheric oxygen.

The role of another transcription factor–HIF-2α in regulating VEGF synthesis has not been investigated in the lens epithelium. In this study we have shown an increase in the protein expression of HIF-2α in the absence of HIF-1α. The HIF-2α protein appeared as a series of three bands around 80 KDa. To confirm the presence of HIF-2α in HLE-B3 cells, we used a HIF-2α standard lysate from Novus Bio chemicals. The standard lysate was prepared from HEK cells. The HIF-2α in HLE-B3 cells was around 80KDa and the standard was at 94kDa. Since the standard was prepared from a different cell lysate it did not match with the HIF-2α seen in the HLE-B3 cells. To further confirm the presence of HIF-2α in HLE-B3 cells we used a translation inhibitor specific for HIF-2α. The HIF-2α synthesis in HLE-B3 cells was inhibited in the presence of translation inhibitor. Based on these results we propose the role of another transcription factor- HIF-2α in regulating VEGF synthesis in HLE-B3 cells.

The HIF-1α protein expression in hypoxia was inhibited by Topotecan and HIF-1α translation. Under these conditions, there was a sustained VEGF synthesis and HIF-2α protein expression. Inhibition of either HIF-1α or HIF-2α translation did not have an effect on the VEGF synthesis. However, when both HIF-1α and HIF-2α were inhibited by a double translation inhibitor, there was a significant decrease in VEGF synthesis in hypoxia.

Based on these results, we propose the existence of a reciprocal relationship between HIF-1α and HIF-2α in regulating VEGF synthesis in the HLE-B3 cells in hypoxia. HIF-2α in HLE-B3 cells compensates for the loss of HIF-1α and vice-versa and continues to synthesize VEGF in hypoxia. Inhibition of both HIF-1α and HIF-2α is needed to suppress the VEGF expression in hypoxia.

What is the role of VEGF synthesis in an avascular cell like the adult lens epithelium? Studies in the RPE (retinal pigment epithelium) have shown that VEGF plays an important role in maintaining the RPE and nourishing the choriocapillaries. Loss of VEGF leads to atrophy and loss of RPE (60). Whether VEGF plays a similar role in lens epithelial cells and protects the cells from going towards apoptosis is not known. In this study we have shown the existance of a reciprocal relationship between HIF-1α and HIF-2α and inhibition of both the transcription factors caused a significant decrease in the VEGF levels. Under these conditions there was significant mitochondrial depolarization in HLE-BE cells. This indicates a significant correlation between maintaining sustained levels of VEGF in hypoxia and resisting mitochondrial depolarization (Mitoprotection).

The receptor for VEGF - VEGFR2 has been observed in the lens epithelium and differentiating fiber cells of the marginal zone of embryonic mouse (17,18). It is likely that within the natural lens the hypoxic response described in the current study is relegated, to some greater or lesser extent, to the immediate subjacent lens fiber cells in juxtaposition to the anterior epithelium and the differentiating marginal fiber cells, as well as the anterior epithelium. Our data with anterior lens epithelial cell cultures does not permit the distinction nor weigh the contribution of one zone (i.e., cell population) over the other with respect to the hypoxic response.

Axitinib is a potent inhibitor of VEGFR1, VEGFR2 and VEGFR3 (53-55). Our data with Axitinib reconfirms our observation with the HIF-1α/HIF-2α double translation inhibitor in that the blocking of

the autophosphorylation of VEGFR2 prevents the interaction of VEGF with its receptor, thereby functioning as a "loss" of VEGF from the cell system resulting in a loss of expression of Bcl-2. But more than that, it demonstrates the biological significance of VEGF signaling for lens epithelial cell survival and the role played by the VEGFR2~VEGF complex in the signaling pathway whereby VEGF regulates endogenous Bcl-2 levels. A potential procedure could be that the synthesized and secreted VEGF interacts with cell surface VEGFR2 in a cell autonomous, autocrine manner which then initiates the downstream cell survival mechanism. Our data supports the fact that these survival signals are triggered by the stress situation of hypoxia. We have yet to delve in to the precise mechanism by which VEGFR2~VEGF regulates downstream synthesis of Bcl-2, in the hypoxic lens epithelium, it is likely that Axitinib inhibits cellular autophosphorylation of VEGFR2 thereby blocking VEGF-mediated lens epithelial cell survival . Moreover, we must stress that our data cannot rule out the possibilty that the lens in its natural setting is influenced by paracrine activation mediating the activation of VEGFR2 receptor. As VEGF may also be secreted by the ciliary process we cannot exclude the possibility that paracrine VEGF signaling plays some role in hypoxic lens survival function.

Cellular apoptosis is regulated, in part, by maintaining a balance between the levels of the pro-survival protein, Bcl-2 and the pro-apoptotic protein, BAX. BAX is a pro-apoptotic member of the Bcl-2 family. Activation of BAX causes its translocation to the mitochondrial outer membrane and induces mitochondrial depolarization but before BAX can initiate this process it must first translocate from the cytoplasm to the mitochondria. Using lenses isolated from E10 chick embryo fractions, Weber et. al. (41) has demonstrated that an increase in Bcl-2 expression during lens differentiation binds to BAX and "helps in the controlled release of cytochrome C from mitochondria without tipping the balance toward apoptosis." Indeed, Nomura et. al. (42) have shown that Bcl-2 inhibits the translocation of BAX from the cytoplasm to mitochondria and thus prevents initiation of apoptosis. Others have shown that VEGF

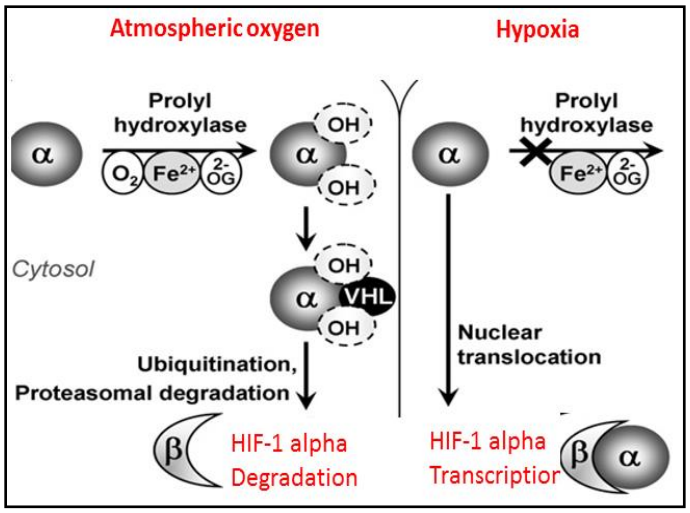
protects endothelial cells from apoptotic stimuli by increasing Bcl-2 expression (43). Using a specific HIF-1α/HIF-2α translation inhibitor, we have demonstrated that a decrease in VEGF levels resulted in markedly diminished Bcl-2 levels. The loss of Bcl-2 was associated with a significant increase in mitochondrial depolarization (Figure 6).

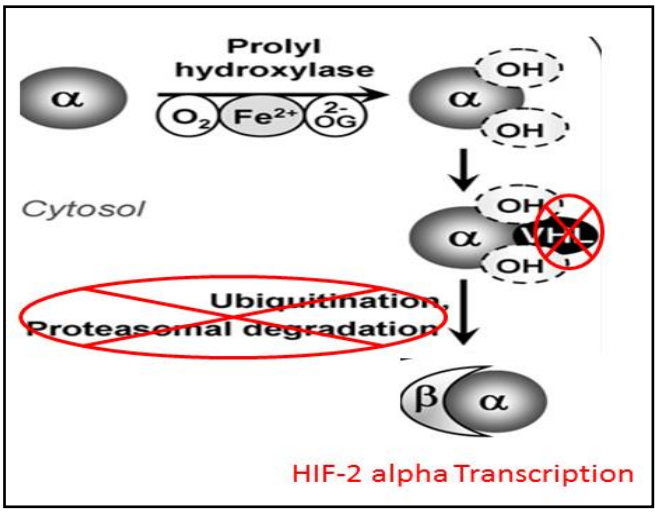
Our data suggests a potential mechanism by which the naturally hypoxic lens epithelium survives in low oxygen concentrations and resist cell death. That mechanism entails sustained VEGF synthesis in hypoxia, which is tightly regulated by the hypoxia inducible factors, HIF-1α and HIF-2α. Sustained VEGF expression maintains a consistent level of Bcl-2. Any reduction in the levels of intracellular VEGF, in association with the loss of Bcl-2, is likely to permit the transolocation of BAX from cytosol to mitochondria and induce mitochondrial depolarization. The existance of reciprocal relationship between transcription factors HIF-1α and HIF-2α has not been reported in HLE-B3 lens epithelial cells.

In this study, we have shown the expression of HIF-2α in HLE-B3 cells and how loss of HIF-1α is compensated by an increase in HIF-2α protein expression. VEGF synthesis in the adult lens epithelium is tightly regulated by both the transcription factors. Inhibition of both HIF-1α and HIF-2α is essential to suppress the VEGF expression in lens epithelial cells. The role of VEGF synthesis in fetal lens development and formation of hyaloid vascular system is well studied, however the role of VEGF in the adult lens epithelium is not well documented. In conclusion, our study demonstrates the synergistic effect of HIF-1α and HIF-2α in regulating VEGF synthesis and the role of VEGF as a prosurvivial factor contributing to lenticular cytoprotection (61).

SUMMARY OF CHAPTER 1

Degradation of HIF-1 and HIF-2α

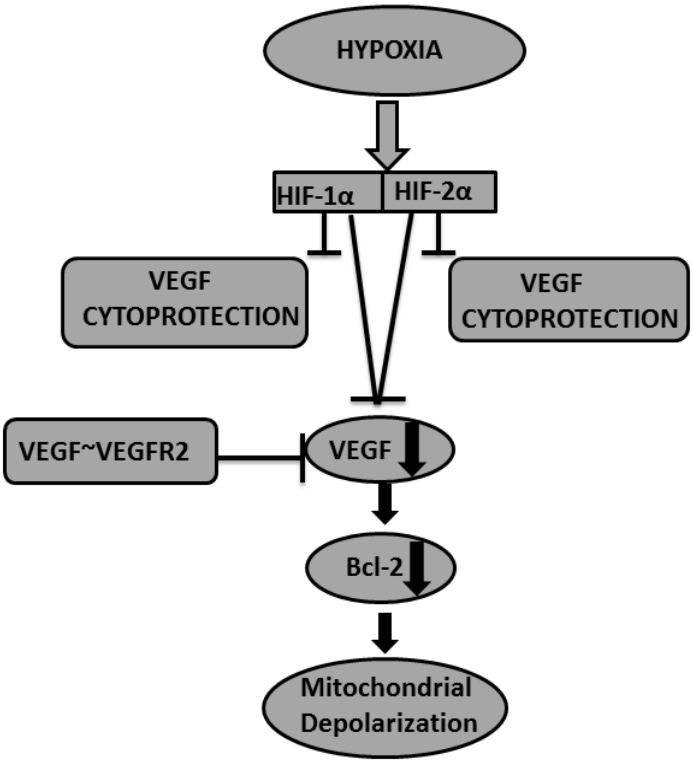




Both HIF-1 and HIF-2α are similar in structure, except for the differences in the amino acid sequences of the alpha subunit. The Hypoxia inducible factors are made of alpha and beta subunits. The beta subunit is constitutively expressed; however it's the hydroxylation of the alpha subunit by the enzyme prolyl hydroxylase (which is active only in the presence of oxygen) that determines the stability of the HIFs. In the presence of atmospheric oxygen, the enzyme prolyl hydroxylase adds hydroxyl groups to the alpha subunit. This hydroxylated alpha subunit is recognized by the VHL protein and degraded through the ubiquitin proteasome pathway. Thus, HIF-1α is degraded in atmospheric oxygen. The alpha subunit of HIF-2α has an altered amino acid sequence which is not recognized by the prolyl hydroxylase enzyme, as a result the alpha subunit is not degraded by the VHL protein and HIF-2α is constitutively expressed in both hypoxia and atmospheric oxygen.

Figure HIF degradation: Reference: Radreau P, Rhodes JD, Mithen RF, Kroon PA, Sanderson J. Hypoxia-inducible factor-1 (HIF-1) pathway activation by quercetin in human lens epithelial cells. Experimental Eye Research. 2009

SUMMARY OF CHAPTER 1



VEGF levels in the hypoxic lens are maintained by a compensatory interrelationship between two Hypoxia inducible factors –HIF-1 and HIF-2α. The loss of either Hypoxia inducible factor is compensated by the other, such that there is a sustained VEGF synthesis, which in turn acts as a pro-survival factor resisting mitochondrial depolarization by maintaining the levels of anti-apoptotic Bcl-2 proteins. Inhibition of both HIF-1/HIF-2α is essential to inhibit the VEGF synthesis leading to loss of lenticular mitoprotection. To further elucidate the role of VEGF as a pro-survival factor in the hypoxic lens, a VEGF receptor inhibitor (VEGF~VEGFR2) which prevents the binding of VEGF to the VEGFR2 and essentially removes VEGF from the system was used. The prevention of interaction between VEGF and its receptor resulted in loss of Bcl-2 proteins, confirming the role of VEGF as a pro-survival factor in the hypoxic lens epithelium.

CHAPTER 2

Hypothesis: Lack of HIF-1α in atmospheric oxygen is compensated by HIF-2α. HIF-2α maintains the VEGF levels when the hypoxic lens is exposed to atmospheric oxygen.

Rationale : In the hypoxic lens, VEGF synthesis is regulated by both the hypoxia inducible factors (HIFs) HIF-1α and HIF-2α (61). The lens epithelium continues to synthesize VEGF in atmospheric oxygen where HIF-1α is degraded. The HIF-1α is degraded in atmospheric oxygen, by the enzyme prolyl hydroxylase-domain proteins (PHDs). In atmospheric oxygen the PHDs hydroxylate the HIFα subunits on the proline residues and hydroxylated HIF is recognized by VHL (Von –Hippel Lindau) tumor suppressor protein which polyubiquitinates and degrades the HIF. HIF-2α is resistant to degradation in atmospheric oxygen by PHDs due to difference in the amino acid sequences around the hydroxylation site (34). As a result, HIF-2α is constitutively expressed in atmospheric oxygen. There fore, we investigated whether the presence of HIF-2α in atmospheric oxygen could contribute to the VEGF synthesis in the lens epithelial cells.

Hypothesis: Inhibition of GSK-3β during exposure to atmospheric oxygen leads to increased nuclear β-catenin and VEGF expression in the lens epithelial cells

Rationale: Previous studies in the lens have demonstrated that exposure to atmospheric oxygen during cataract surgery activates TGF-β which in turn activates the Wnt pathway leading to inactivation of GSK-3β and translocation of β-catenin to the nucleus (62). Although a role for β-catenin has been established in the lens epithelial cell development and fiber cell differentiation, its role in regulating VEGF levels is not known in the lens (63-65). Wnt/β-catenin signaling is initiated when the Wnt ligand binds to the frizzled receptor and forms a complex with the LDL related protein. The formation of this complex will inactivate GSK3-β. GSK3-β when active as a part of a multiprotein destruction complex consisting of the

scaffolding protein, Axin and APC (Adenomatous Polyposis Coli) leads to phosphorylation and degradation of β-catenin by the ubiquitin/proteasome pathway. In the absence of active GSK3-β, β-catenin is not phosphorylated and translocates to the nucleus. Active β-catenin once translocated to the nucleus is known to initiate the transcription of various genes promoting cell proliferation and survival including VEGF (66, 67). Previous studies in cancer literature have demonstrated that VEGF-A mRNA has seven consensus binding sites for the β-catenin/TCF complex, thus making it a potential target gene for β-catenin. The TCF/LEF (T cell factor and the Lymphoid enhancing factor) family of transcription factors are involved in the Wnt signaling pathway. They promote the interactions between β-catenin and the enhancer elements of the genes they target (68, 69). Previous studies from our laboratory have demonstrated that inhibition of GSK-3β in atmospheric oxygen provides resistance against mitochondrial depolarization (70, 71). Based on these observations we propose that inhibition of GSK-3β in atmospheric oxygen using a specific pharmacological inhibitor like SB216763 leads to increased nuclear β-catenin and VEGF synthesis (72). To further prove the association between increased nuclear β-catenin and VEGF levels, a specific pharmacological inhibitor, XAV939 which 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 will be utilized (73,74). To mimic the *in-vivo* conditions during cataract surgery, the lens epithelial cells will be exposed to hypoxia followed by atmospheric oxygen with the inhibitor treatments.

Hypothesis: **The lens epithelium resists mitochondrial depolarization in atmospheric oxygen by maintaining a sustained VEGF synthesis.**

Rationale: Despite being an avascular tissue, the adult lens epithelium continues to synthesize VEGF either in hypoxia or atmospheric oxygen. Increased VEGF synthesis has been shown to act as a pro-

survival factor by maintaining the levels of anti-apoptotic protein Bcl-2 in the hypoxic lens (61). In this study we propose that VEGF synthesis in the lens epithelium during exposure to atmospheric oxygen promotes survival by resisting mitochondrial depolarization and maintaining the levels of anti-apoptotic proteins Bcl-2. VEGF acts through a tyrosine kinase receptor–VEGFR2 in the lens epithelial cells. Axitinib is a selective tyrosine kinase receptor inhibitor which inhibits VEGFR1, 2 and 3.To determine whether VEGF, acts through the VEGF~VEGFR2 complex to regulate endogenous Bcl-2/pBcl-2 levels in atmospheric oxygen, the VEGF receptor inhibitor, Axitinib, will be used. To further support and reaffirm the inter-relationship between inhibition of GSK-3β-β-catenin axis and its effect on VEGF and Bcl-2/pBcl-2 levels, we will employ a strategy which involves the use of pharmacological inhibitors to either inhibit the enzymatic activity of GSK-3β (using SB216763) or deplete the nuclear β-catenin (using XAV939) in lens epithelial cells exposed to atmospheric oxygen and determine the downstream effects of their inhibition on VEGF and Bcl-2/pBcl-2 levels.

MATERIALS AND METHODS

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 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. (44). All studies with HLE-B3 cells were performed with pre-frozen stock cells (maintained in liquid nitrogen) between passages 14 to 17 and no experiments exceeded 5 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), 2mM L-glutamine, nonessential amino acids, and 0.02g/L gentamycin solution (Sigma-Aldrich) and cultured at 37^0C and 5% CO_2-95% O_2. Cells were sub-cultured four to five days prior to the experiment and placed in MEM containing 20% FBS. Twenty four hours prior to 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 O_2 (~21%) for 90 minutes, then switched to hypoxic conditions (~1% O_2) for 180 minutes, followed by reintroduction to atmospheric oxygen. 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%.

Primary Bovine Lens Epithelial 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.

Cytoplasmic and Nuclear Lysates: To demonstrate the translocation of β-catenin to the nucleus, instead of total cell lysates the cytoplasmic and nuclear lysates were collected. To rule out the cross contamination between the two lysate fractions cytoplasmic markers – GAPDH (glyceraldehyde -3 phosphate dehydrogenase) and LDH (lactate dehydrogenas) were used. 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 3× SDS (Laemmli) buffer was added to the remaining lysates, which were subsequently boiled for 5 min; and the proteins resolved by electrophoresis on 12% SDS-polyacrylamide gels (20 μg protein/lane). Proteins were then transferred to nitrocellulose membranes (Scheicher and Schuell, Keene, NH).

Western blot analysis: For the Western blot analysis, nitrocellulose membranes were blocked with 1% BSA and 0.02% Tween-20 in Tris–buffered saline (TTBS) for 60 minutes. These membranes were incubated overnight at 4°C with primary antibodies. The blots were then rinsed in TTBS (4× 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 hour 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).

ELISA: ELISA was performed for the detection of VEGF using an Invitrogen VEGF ELISA kit (Grand Island, NY) for HLE-B3 cells and Bovine VEGF ELISA kit form NeoBioLab (Cambridge, MA) for normal bovine cells. HLE-B3 and normal bovine lens epithelial cells were cultured in 25 cm^2 tissue culture flasks in 20% FBS, transferred to serum-free media prior to the initiation of the experiment.

Flasks were generally set up in triplicate with the inhibitor treatement for 3 hours of incubation in hypoxia. At the end of 3 hours, fresh serum-free media with the inhibitor was added and cells were incubated at atmospheric oxygen for 3 and 8 hours. Cell-free supernatants were collected at 3 and 8 hours of re-oxygenation and analyzed according to manufacturer's instructions. The optical density at 450 nm was determined using a Molecular Devices Spectramax 190 (Sunnyvale, CA).

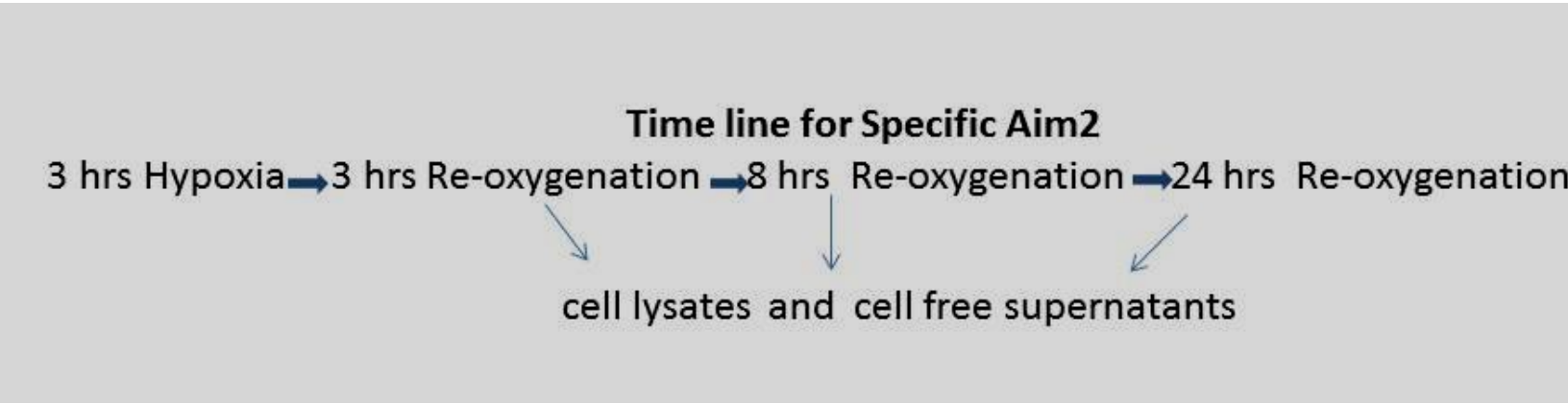
Primary antibodies used in this study were rabbit anti-BAX, rabbit anti- pBcl-2, rabbit anti–Bcl-2 (Cell Signaling Technology, Danvers, MA), rabbit anti–Actin (Santa Cruz, CA), rabbit anti- HIF-2α (Novus Biologicals, Littleton, CO), rabbit anti- β-catenin, rabbit anti-phospho-β-catenin antibody, rabbit 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). Western blot analysis was generally repeated in triplicate from three independent cell populations.

JC-1 analysis to determine Mitochondrial Depolarization: After treatments with the specific inhibitors cells were stained with the cationic dye 5, 5′, 6, 6′-tetrachloro1, 1′, 3, 3′-tetraethyl-benzimidazolylcarbocyanine iodide (JC-1; Molecular Probes, Eugene, OR) as described previously (45) 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 fluorescence emission shift from red to green. 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 minutes. After this incubation, cells were again rinsed two times with the serum-free MEM and multiple images

were obtained using a ×10 objective on a confocal microscope (Zeiss LSM410) 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 Image J (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 Image J 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 the software from Graphpad Prism, version 5.00 (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.



The cells were incubated with specific pharmacological inhibitors for 3 of hypoxia and reintroduced to atmospheric oxygen for a period of 3, 8 or 24 hours along with the inhibitors. The cell free supernatants and lysates were collected after the incubation in atmospheric oxygen.

RESULTS

Regulation of VEGF by HIFs (hypoxia inducible factors) in atmospheric oxygen: We have previously demonstrated the functional expression of 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 condition, is regulated by a compensatory inter-relationship between HIF-1α and HIF-2α (61). HIF-1α is not expressed in atmospheric oxygen, whereas HIF-2α is resistant to degradation in atmospheric oxygen by the enzyme prolyl hydroxylase. Since we observed a consistent accumulation of VEGF in the HLE-B3 cells switched from hypoxia to atmospheric oxygen (61), the burden of proof was upon us to demonstrate whether or not HIF-2α inhibition affected VEGF synthesis.

HLE-B3 cells cultured in 25 cm^2 tissue culture flasks were treated with CAS882268-69-1, a specific HIF-2α translation inhibitor which suppresses HIF-2α protein synthesis but not the mRNA transcription. 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 inhibitor for 3 hours in hypoxia, and after 3 hours, the media was replaced with fresh media containing the same concentrations of inhibitor and incubated in atmospheric oxygen for an additional 3, 8 and 24 hours. Control (mock-treated) cells were incubated with DMSO and jointly collected under the same conditions. Inhibition of HIF-2α did not influence VEGF synthesis in atmospheric oxygen relative to the corresponding controls (Figure 1C). At all concentrations of inhibitor utilized, HIF-2α was markedly suppressed albeit not completely eradicated (Figure 1A).

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 (61). As inhibition of HIF-2α did not influence VEGF levels (Figure 1C), it followed that HIF-2α inhibition should not influence the levels of the anti-apoptotic protein, pBcl-2, thereby supporting a conclusion that HIF-2α neither plays a role in VEGF regulation in atmospheric oxygen nor affect 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 hours in hypoxia, after 3 hours fresh serum-free media containing similar concentrations of the inhibitor was added and incubated at atmospheric oxygen for 3 hours. Control cells incubated with DMSO were treated similarly. After 3 hours, 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.

Inhibition of the enzymatic activity of GSK-3β leads to decreased pGS levels: GSK-3β in its non-phosphorylated form is the active form of the enzyme. The active form of the enzyme phosphorylates the downstream substrate glycogen synthase (GS). Phosphorylation of GS is a useful indicator of GSK-3β activity. Treatment of HLE-B3 cells with 12 μm of GSK-3β inhibitor SB216763 resulted in inhibition of phosphorylation of GS as compared to the untreated controls (Figure 2). There was no significant change in the levels of GSK-3β and phosphoglycogen synthase kinase-3β (pGSK-3β) between the control and SB 216763 treated cells (Figure 2). This could be explained by the fact that autophosphorylation of GSK-3β is unaffected by the treatment with SB216763, whereas the inhibition of the catalytic site prevented downstream phosphorylation of glycogen synthase (71).

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 hours, either

in hypoxia or atmospheric oxygen. At the end of 3 hours of hypoxic exposure, cell free supernatants were collected in triplicates 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 hours of incubation post atmospheric oxygen-exposure. Similarly, at the end of 3 hours of incubation (from the control cells maintained in atmospheric oxygen and never exposed to hypoxia) cell free supernatants were collected in triplicates and fresh serum-free media was added to the cells. As above, supernatants were collected in triplicates after 2, 4 and 24 hours of further incubation in atmospheric oxygen. The VEGF expression in the supernatants from the set of hypoxic-exposed and later switched to atmospheric oxygen, as well as the cells maintained continuously in atmospheric oxygen, were 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). We have previously shown that cells consistently maintained in hypoxia also demonstrate a significantly higher expression of VEGF over similar time course when compared to cells continuously kept in atmospheric oxygen (61), suggesting that the act of reintroducing atmospheric oxygen to cells held in hypoxia is not, in and of itself, 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 the hypoxic lens epithelial cells and prevents mitochondrial depolarization by maintaining the levels of the anti-apoptotic protein, pBcl-2 (61). Brooks et al. (70) 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 whether said increase is linked to pBcl-2 levels thereby establishing the link between inhibition of GSK-3β and inhibition of mitochondrial depolarization in lens epithelial cells

exposed to atmospheric oxygen is not currently known and constitutes one of the fundamental questions raised in this study. To test this, HLE-B3 cells cultured in 25 mm^2 tissue culture flasks were treated with 12 μm of the specific GSK-3β inhibitor, SB216763, in serum-free media for 3 hours in hypoxia. At the end of 3 hours, fresh serum-free media with the same concentration of SB216763 was added and incubated at atmospheric oxygen. Cell free supernatants were collected in triplicates at the end of 3, 8 and 24 hours of re-oxygenation and analyzed by ELISA to determine the VEGF levels. Control cells, mock-treated with DMSO, was analyzed in a similar manner. Inhibition of the enzymatic activity of GSK-3β resulted in a significant increase in VEGF levels after 24 hours of incubation in atmospheric oxygen (Figure 3B).

Inhibition of the enzymatic activity of GSK-3β leads to increased nuclear β-catenin: GSK-3β, as a part of multi-protein complex, is involved in the Wnt signaling pathway (62). β-catenin is an important downstream target of GSK-3β activity (62). In the absence of active GSK-3β, β-catenin is not phosphorylated and in this active form, β-catenin translocates to the nucleus and initiates the nuclear transcription of growth factors like VEGF (66). Whether the inhibition of GSK-3β likewise leads to increased nuclear β-catenin and stimulation of VEGF synthesis and accumulation in lens epithelial cells had not yet been demonstrated. To address this, HLE-B3 cells were treated with 12μm SB216763 for 3 hours in hypoxia, followed by exposure to atmospheric oxygen with the inhibitor for 3 and 24 hours. At the end of the incubation periods, cytoplasmic and nuclear extracts were collected (refer to Methods) and analyzed by Western blot for the 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 4).

Effect of GSK-3β inhibition on the levels of the anti-apoptotic proteins Bcl-2/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 (61) 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 mm^2 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 SB2167632-treated cells as compared to the corresponding nuclear extracts of the control cells was noted. However, this increase in the pBcl-2 levels was not statistically significant (Figure 5).

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 2-4). 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 which involved the use of a pharmacological inhibitor in order 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 protein, Axin and APC (Adenomatous Polyposis Coli) 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 (73,74). In order to further establish the association between nuclear β-catenin and VEGF/pBcl-2 levels, we utilized the pharmacological inhibitor, XAV939, to deplete the nuclear levels of β-catenin.

HLE-B3 cells were cultured in 100 mm^2 culture dishes and incubated with 1μm XAV939 for 3 hours in hypoxia, at the end of which time, fresh serum-free media with the inhibitor was added and the cells incubated in atmospheric oxygen for an additional 3 hours. At the end of 3 hours, cytoplasmic and nuclear extracts were collected and analyzed by Western blot. Treatment of HLE-B3 cells, with the pharmacological inhibitor, XAV939, led to the depletion of nuclear β-catenin without any significant changes in the cytoplasmic levels of β-catenin (Figure 6).

Loss of nuclear β-catenin leads to decreased VEGF levels in HLE-B3 cells: A link between total β-catenin levels and VEGF expression has previously been established in colon cancer cells (67). In the lens epithelial cells, inhibition of GSK-3β leads to increased nuclear β-catenin (Figure 4) and increased VEGF levels (Figure 3B) thereby demonstrating, but not definitely proving, a direct association between β-catenin and VEGF. To reaffirm and firmly establish an inter-relationship between β-catenin in VEGF expression we felt it necessary to suppress nuclear β-catenin. 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 triplicates 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-2levels in HLE-B3 cells: In order 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 (Fig. 6) were also analyzed by Western blot

forBcl-2/ pBcl-2 levels (Figure 8). The pharmacological depletion of nuclear β-catenin not only resulted 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 lab have demonstrated that VEGF acts as a pro-survival factor in the hypoxic lens epithelium by maintaining the levels of the anti- apoptotic protein, Bcl-2 (61). Brooks et. al. (71) has 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 1µM of the inhibitor in serum-free media for 3 hours in hypoxia. At the end of the hypoxic exposure, the hypoxic media was poured off, and 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 but no JC-1 dye) was added.

Control cells were maintained as described but without inhibitor being administered. A random field of cells was imaged every 2.5 min for 90 min using an x10 objective on a confocal microscope (Ziess LSM410, LSM410, Thornwood, NY). 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 bandpass filter. Inhibition of β-catenin in atmospheric oxygen resulted in profound mitochondrial depolarization as compared to the control cells treated with DMSO (Figure 9).

VEGF maintains the levels of pBcl-2 in lens epithelial cells exposed to atmospheric oxygen: We observed a significant increase in VEGF when the enzymatic activity of GSK-3 β was inhibited. To further evaluate the link between increased VEGF synthesis and pBcl-2 levels, a VEGF receptor inhibitor Axitinib was used. VEGF acts through a tyrosine kinase receptor. Axitinib is a selective tyrosine kinase receptor inhibitor which inhibits VEGFR1, 2 and 3 (53, 54). To determine if extracellular VEGF acts through VEGF~VEGFR2 complex to regulate endogenous pBcl-2 levels, HLE-B3 cells were treated with 0.05μm, 0.5 μm and 5μm of the receptor inhibitor Axitinib. The cells were treated with the inhibitor in serum free media for 3 hours in hypoxia at the end of which fresh serum free media with the inhibitor was added to the cells and incubated in atmospheric oxygen. Total cell lysates were collected at the end of 3 hours of incubation in atmospheric oxygen and analyzed by western blot for pBcl-2 and BAX levels. Inhibiting the binding of VEGF to VEGFR through the inhibitor Axitinib resulted in a significant loss of pBcl-2 levels without affecting the levels of pro- apoptotic protein BAX (Figure 10).

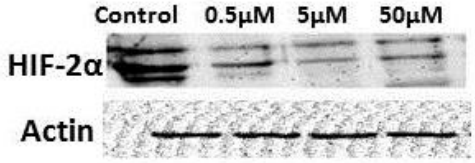
Bovine lens epithelium demonstrates a similar response to inhibition of enzymatic activity of GSK-3β as compared to HLE-B3 cells: We are aware that the HLE-B3 cell line is T-antigen transformed and thus may not be accurately representing normal cell proliferation and apoptosis responses, calling into question whether these cells have biological or clinical relevance. 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. Preliminary findings demonstrated that, similar to HLE-B3 cells, inhibition of the enzymatic activity of GSK-3β resulted in increased nuclear β-catenin, and concomitant decrease in the phospho-β-catenin as compared to the control cells (Figure 11A). Inhibition of GSK-3β, as a result of elevated nuclear β-

catenin, also led to stimulation of VEGF synthesis and accumulation in the HLE-B3 cells (Figure 3B). To determine the effect of increased nuclear β-catenin on VEGF in normal bovine lens epithelial cells, the cells were cultured in 25 mm^2 tissue culture flasks and treated with SB21763 as described previously. The cell free supernatants were collected after 3 and 8 hours 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 hours of incubation in atmospheric oxygen (Figure 11B). Based on these results, we conclude, that HLE-B3 cells recapitulate the same signaling pathways functional in normal bovine lens epithelial cells.

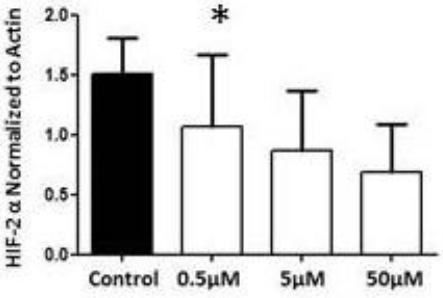
FIGURES FOR CHAPTER 2

Figure 1

A

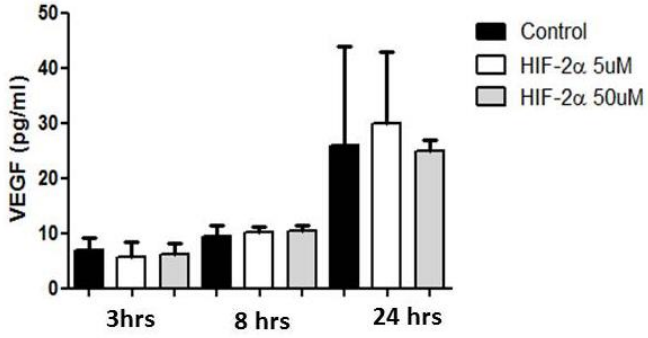


B



HIF-2 α Inhibitor

C



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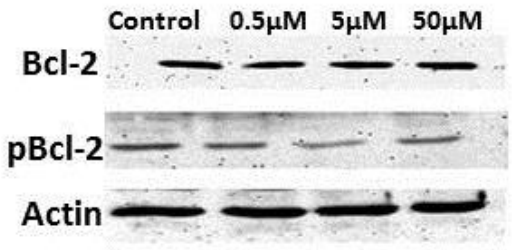

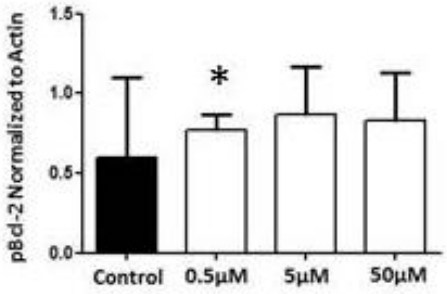

Figure 1A/B: Cells were cultured in 100 mm^2 dishes with 20% FBS and switched to serum-free media on the day of the experiment. The cells were incubated with 3 ml of serum-free media containing 0.5µm, 5µm and 50µm of HIF-2α inhibitor for 3 hours in hypoxia. At the end of the 3 hour hypoxic exposure, cells were switched to atmospheric oxygen and replaced with fresh serum-free media containing the same concentrations of the inhibitor. Total cell lysates were collected at the end of 3 hours incubation in atmospheric oxygen and analyzed for HIF-2α levels. Control cells were incubated with DMSO and analyzed in a similar manner .The experiment was repeated three times using independent cell populations. The normalized lysates were analyzed for levels of HIF-2α using image J analysis Treatment of the cells with HIF-2α inhibitor resulted in significant suppression of the HIF-2α protein levels (* $P < 0.05$).**C:** 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 with 3 ml of serum-free media containing 5µm and 50µm HIF-2α inhibitor for 3 hours in hypoxia. At the end of 3 hours, the cells were switched to atmospheric oxygen and replaced with fresh serum-free media containing the same concentrations of the inhibitor. Control cells treated with DMSO were analyzed in a similar manner. Cell-free supernatants were collected in triplicate from three independent cell populations after 3, 8 and 24 hours of incubation in atmospheric oxygen. The supernatants were analyzed for VEGF levels by ELISA. Inhibition of HIF-2α in atmospheric oxygen did not influence the levels of VEGF as compared to control cells (* $P > 0.05$). **D/E:** Cells were cultured in 100 mm^2 dishes with 20% FBS as described above and total cell lysates were collected at the end of 3 hours of incubation in atmospheric oxygen. Inhibition of HIF-2α did not alter the levels of Bcl-2/pBcl-2 levels as compared to the control cells. The experiment was repeated three times using three independent cell populations and there was no statistically significant difference in the Bcl-2/pBcl-2 levels (* $P > 0.05$).

Figure 2

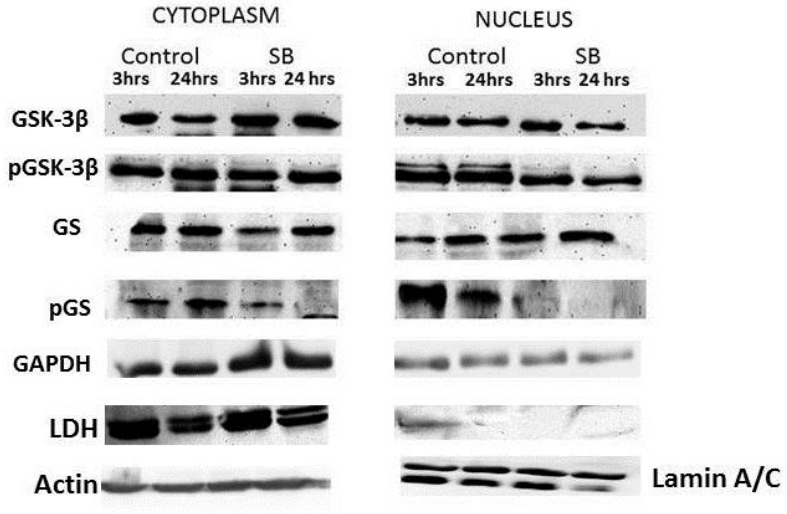

Figure 2: Western blot analysis of pGSK-3β, GSK-3β, pGS and GS in HLE-B3 cells treated with GSK-3β inhibitor (SB216763). The cells were treated with 12µm of the inhibitor SB216763 for 3 hours in hypoxia, at the end of which cells were switched to atmospheric oxygen and fresh serum-free media with the inhibitor was added. At the end of 3 and 24 hours of incubation in atmospheric oxygen cytoplasmic and nuclear lysates were collected using the NE-PER Nuclear and Cytoplasmic Extraction kit(refer to methods)and analyzed by Western blot for pGSK-3β, GSK-3β, pGS and GS. Control cells mock treated with DMSO were analyzed in a similar manner. Treatment of HLE-B3 cells with SB216763 resulted in inhibition of phosphorylation of glycogen synthase as compared to the untreated controls (* $P < 0.05$). There was no significant change in the levels of GSK-3β and phosphoglycogen synthase kinase -3β (pGSK-3β) between the control and SB 216763 treated cells in both the cytoplasmic and nuclear lysates.

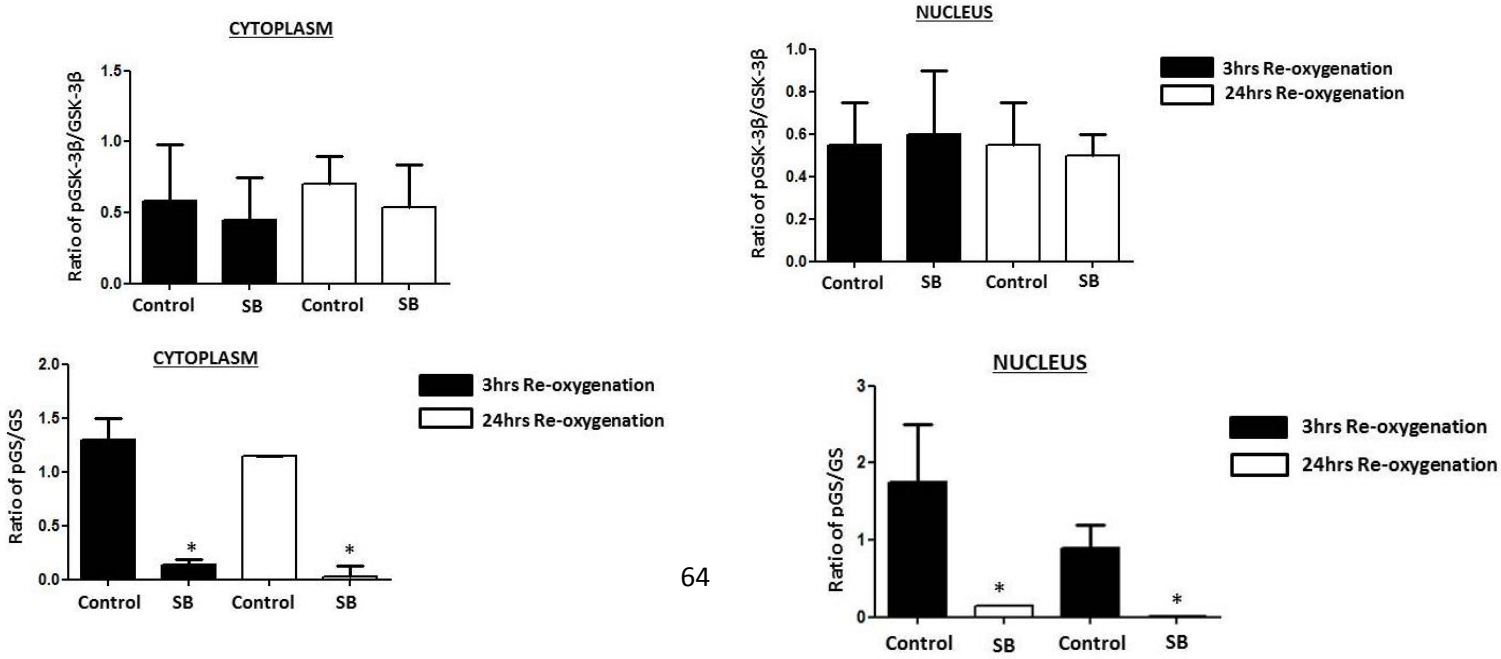

Figure 3

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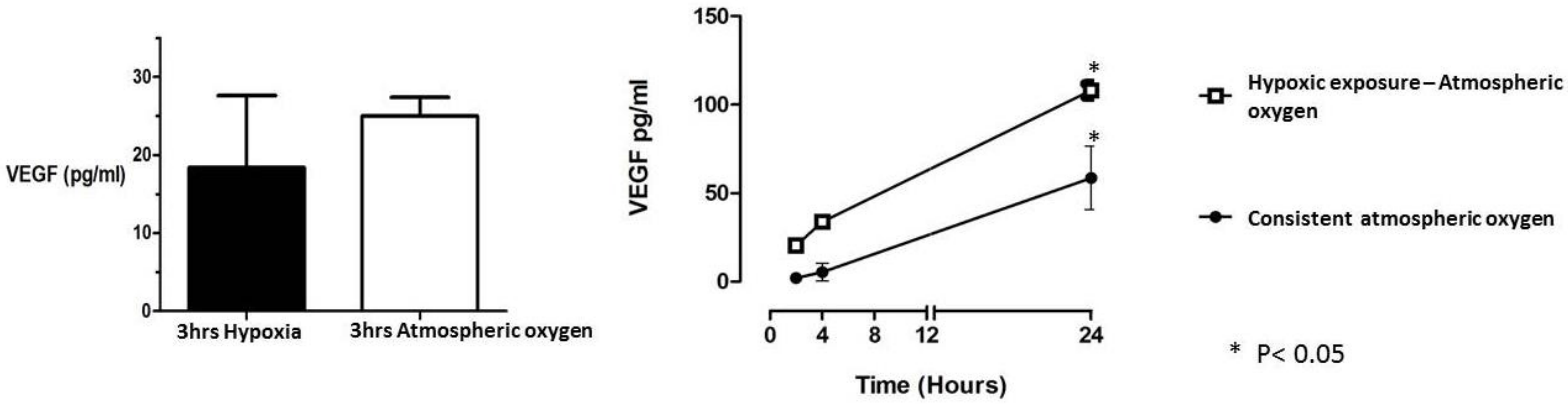

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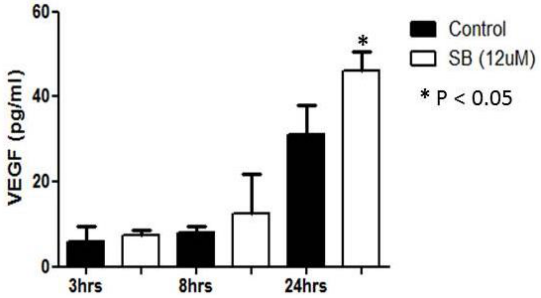


Figure 3: HLE-B3 cells were cultured in 25 cm^2 flasks with 20% FBS and switched to serum-free media on the day of the experiment. The cells were incubated with 3ml of serum-free media in hypoxia (1% oxygen) or remained in atmospheric oxygen (~21% oxygen) for 3 hours. At the end of 3 hours cell-free supernatants were collected in triplicates from the cells exposed to hypoxia as well as cells that remained in atmospheric oxygen. Fresh serum-free media was added to the hypoxic exposed cells and incubated at atmospheric oxygen for 2, 4 and 24 hours. Cell-free supernatants were collected after 2, 4 and 24 hours of incubation in atmospheric oxygen. The control cells which remained in atmospheric oxygen throughout were treated in a similar manner and cell-free supernatants were likewise collected after 2, 4 and 24 hours of incubation in atmospheric oxygen. 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 atmospheric oxygen (*P <0.05). **B:** The 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 triplicates with 3ml of serum-free media containing 12µm of GSK-3β inhibitor SB216763 for 3 hours in hypoxia. At the end of 3 hours, fresh serum-media with the same concentration of the inhibitor was added and further incubated in atmospheric oxygen for an additional 3, 8 and 24 hours. At the end of the incubation , cell- free supernatants were collected in triplicate and analyzed by ELISA . The control cells were treated with DMSO in 3ml of serum-free media and analyzed in a similar manner. There was a statistically significant (*P <0.05) increase in the VEGF levels at 24 hours in the SB216763 treated cells as compared to the control cells at the same time point.

Figure 4

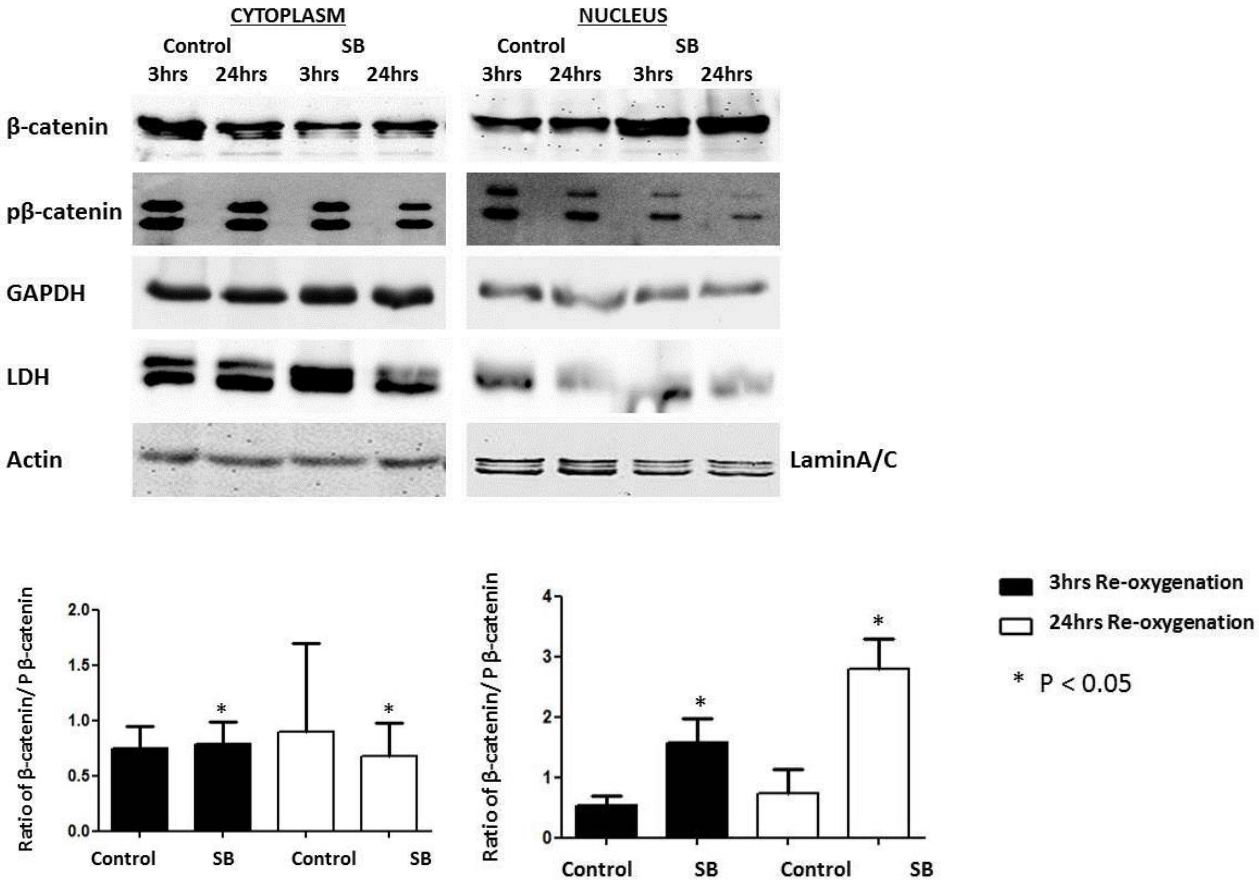


Figure 4: The cells were treated with 12µm of SB216763 for 3 hours in hypoxia. At the end of 3 hours, the cells were switched to atmospheric oxygen and fresh serum-free media with the same concentration of the inhibitor was re-added. At the end of 3 and 24 hours of incubation in atmospheric oxygen, cytoplasmic and nuclear lysates were collected. Control cells were incubated with DMSO and analyzed in a similar manner. The normalized lysates were analyzed for levels of β-catenin and phospho-β-catenin. To rule out the cross contamination between the cytoplasmic and nuclear fractions, the cytoplasmic markers GAPDH and LDH were utilized. The experiment was repeated three times with independent cell populations and the ratio between β-catenin and phospho- β-catenin was quantified using Image J analysis. The levels of β-catenin and phospho- β-catenin in the cytoplasmic extracts were unchanged(* p>0.05), 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 (*P< 0.05). There was no significant carryover of the cytoplasmic markers in the nuclear fractions.

Figure 5

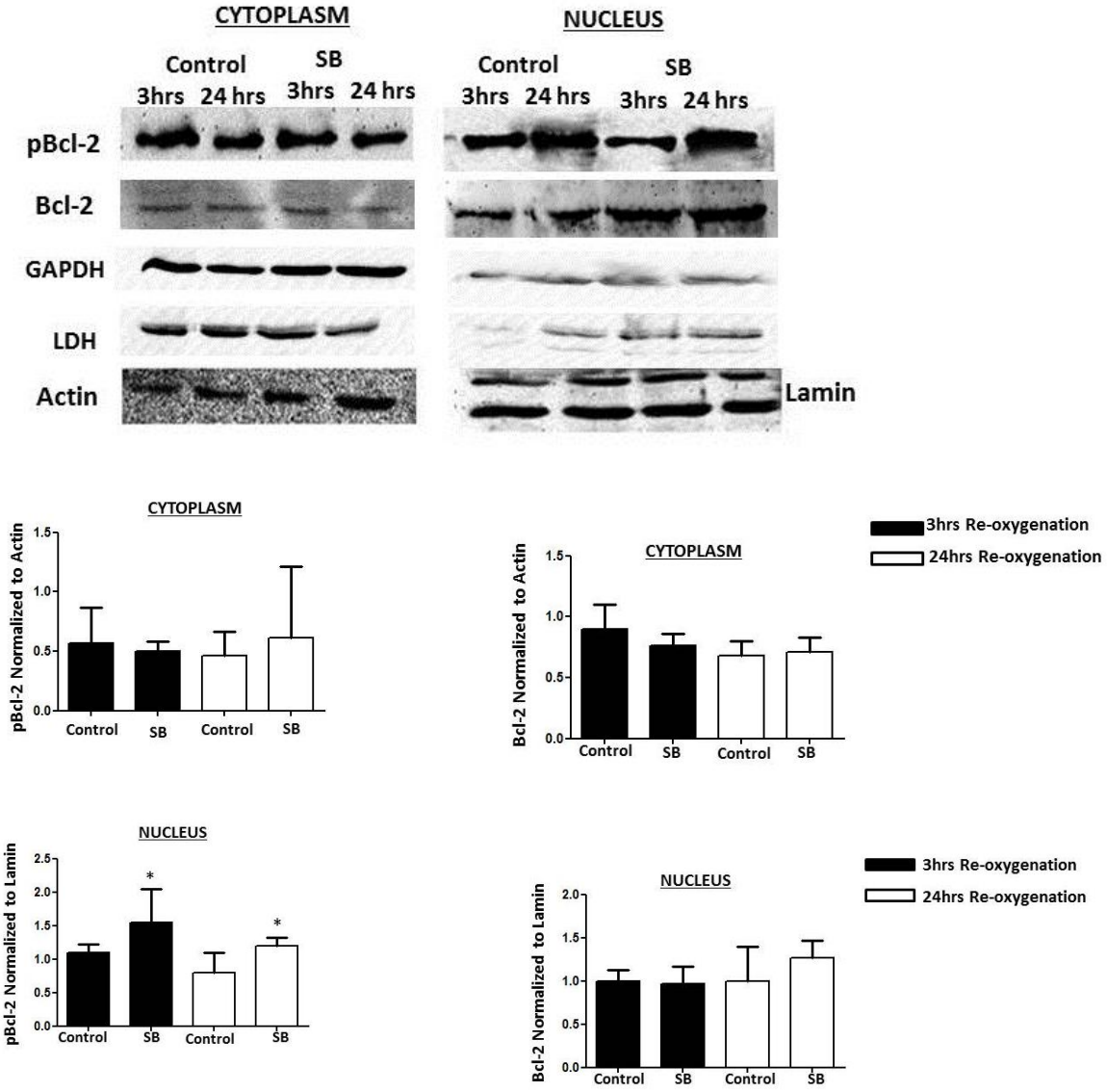


Figure 5: The cells were treated with 12µm of SB216763 for 3 hours in hypoxia. At the end of 3 hours, the cells were switched to atmospheric oxygen and fresh serum-free media with the same concentration of the inhibitor was re-added. At the end of 3 and 24 hours of incubation in atmospheric oxygen, cytoplasmic and nuclear lysates were collected. Control cells were incubated with DMSO and analyzed in a similar manner. The normalized lysates were analyzed for levels of pBcl-2 and Bcl-2. To rule out the cross contamination between the cytoplasmic and nuclear fractions, the cytoplasmic markers GAPDH and LDH were utilized. The experiment was repeated three times with independent cell populations and the levels of pBcl-2 and Bcl-2 in the cytoplasmic and nuclear fractions were quantified using Image J analysis. The increase in the pBcl-2 levels in the nuclear fractions of SB216763 treated cells as compared to the control cells was not statistically significant (*P >0.05). There was no change in the levels of Bcl-2 or pBcl-2 in the cytoplasmic fractions. There was no significant carryover of the cytoplasmic markers in the nuclear fractions.

Figure 6

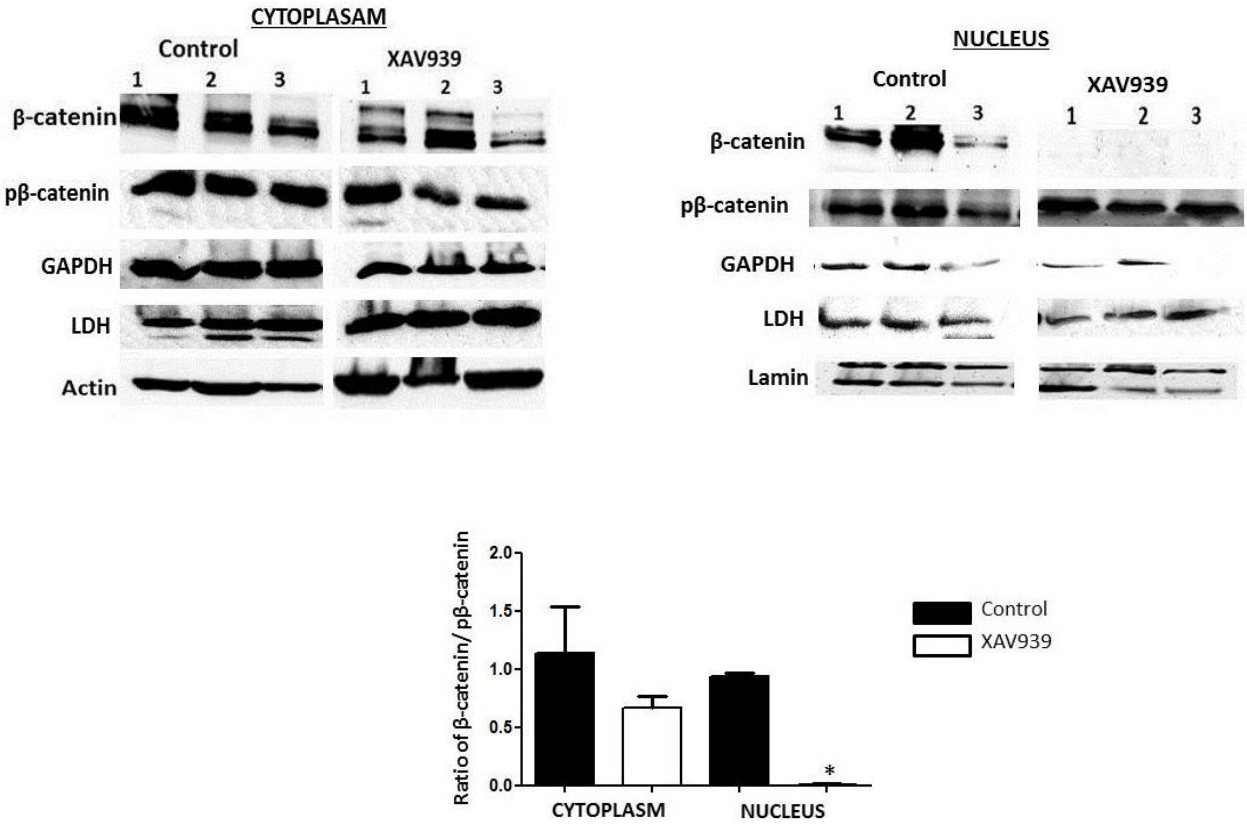


Figure 6: The cells were treated with 1µm XAV939(The drug acts by stabilizing the Axin protein in the destruction complex, leading to destruction of β-catenin) for 3 hours in hypoxia. At the end of the hypoxic exposure, fresh serum-free media with the same concentration of inhibitor was re-added and the cells were further incubated in atmospheric oxygen for additional 3 hours. Control cells were mock-treated with DMSO in a similar manner. Cytoplasmic and nuclear lysates were collected after the 3 hours of incubation and analyzed by Western blot. The normalized lysates were analyzed for levels of β-catenin and phospho-β-catenin using image J analysis. To rule out the cross contamination between the cytoplasmic and nuclear fractions, the cytoplasmic markers GAPDH and LDH were utilized. The experiment was repeated three times with independent cell populations and the ratio between β-catenin and phospho- β-catenin was quantified using Image J 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 decrease in β-catenin in the SB216763-treated cells as compared with the controls (*P< 0.05). There was no significant carryover of the cytoplasmic markers in the nuclear fractions.

Figure 7

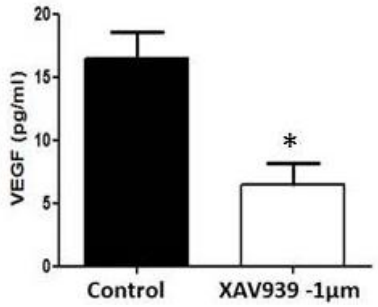


Figure 7: The cells were cultured in 25 cm^2 flasks with 20% FBS and switched to serum-free media on the day of the experiment. Cells were incubated in triplicate using three independent cell populations with 3ml of serum-free media containing 1µm XAV939 for 3 hours in hypoxia. At the end of the 3 hour hypoxic incubation, fresh serum-media with the same concentration of inhibitor was re-added and incubated in atmospheric oxygen for an additional 3 hours. Control cells treated with DMSO were incubated in a similar manner. Cell-free supernatants were collected at the end of 3 hours of incubation and analyzed by ELISA. There was a statistically significant decrease in the levels of VEGF (* $P < 0.05$) in cells treated with XAV939 as compared to the control cells at the end of 3 hours of incubation in atmospheric oxygen.

Figure 8

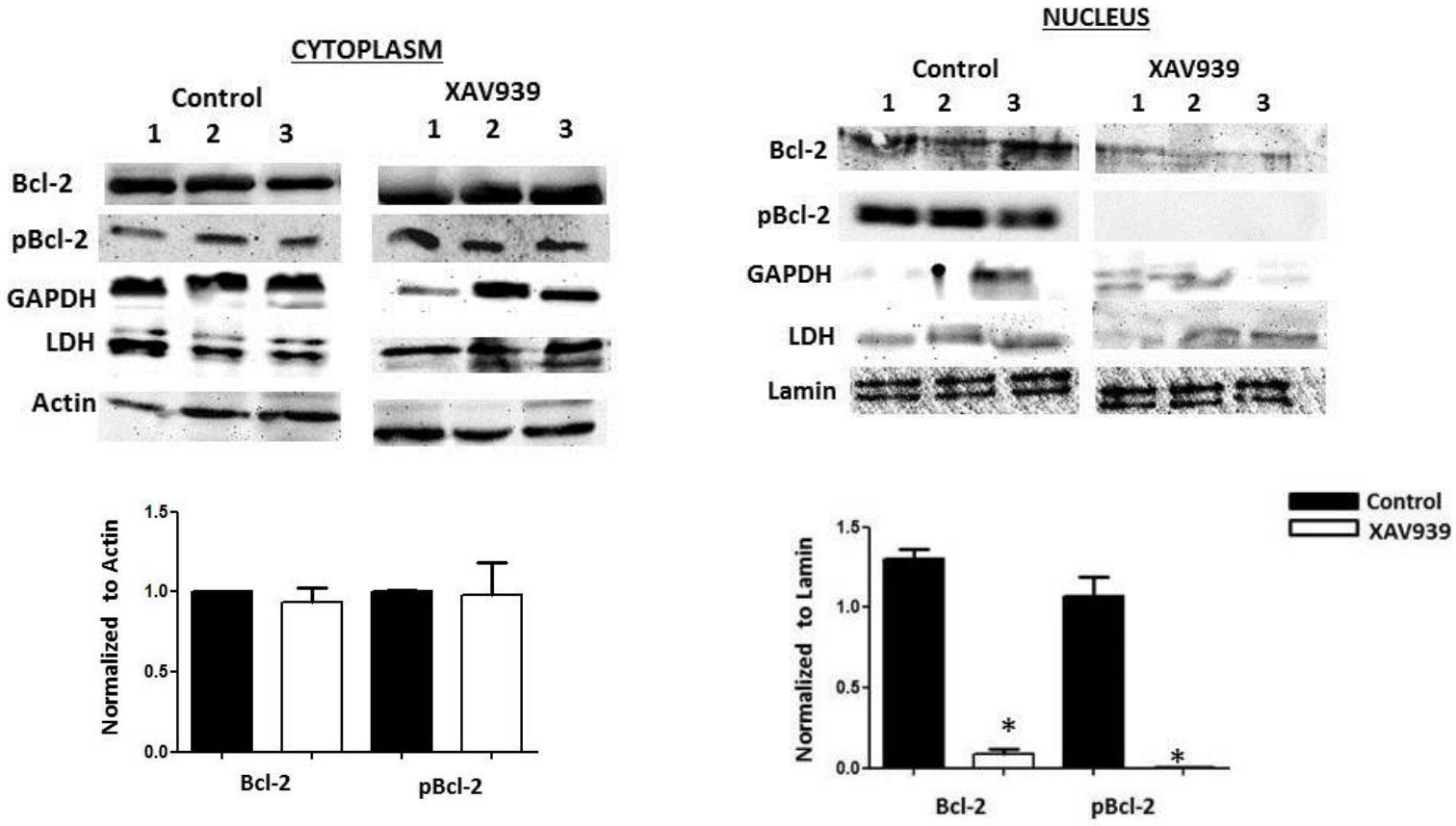


Figure 8: The cells were treated with 1µm XAV939 for 3 hours in hypoxia. At the end of the hypoxic exposure, fresh serum-free media with the same concentration of inhibitor was re-added and the cells were further incubated in atmospheric oxygen for additional 3 hours. At the end of 3 hours incubation in atmospheric oxygen, cytoplasmic and nuclear isolates were collected. Control cells were mock-treated with DMSO in a similar manner. The normalized lysates were analyzed for levels of Bcl-2/pBcl-2 using image J analysis. To rule out the cross contamination between the cytoplasmic and nuclear fractions, the cytoplasmic markers GAPDH and LDH were utilized. The experiment was repeated three times with independent cell populations.There was a significant decrease (* $P < 0.05$) in the levels of nuclear Bcl-2 and pBcl-2 levels as compared to the control cells. The cytoplasmic levels of Bcl-2 and pBcl-2 remained unchanged in both the control and XAV939 treated cells.

Figure 9

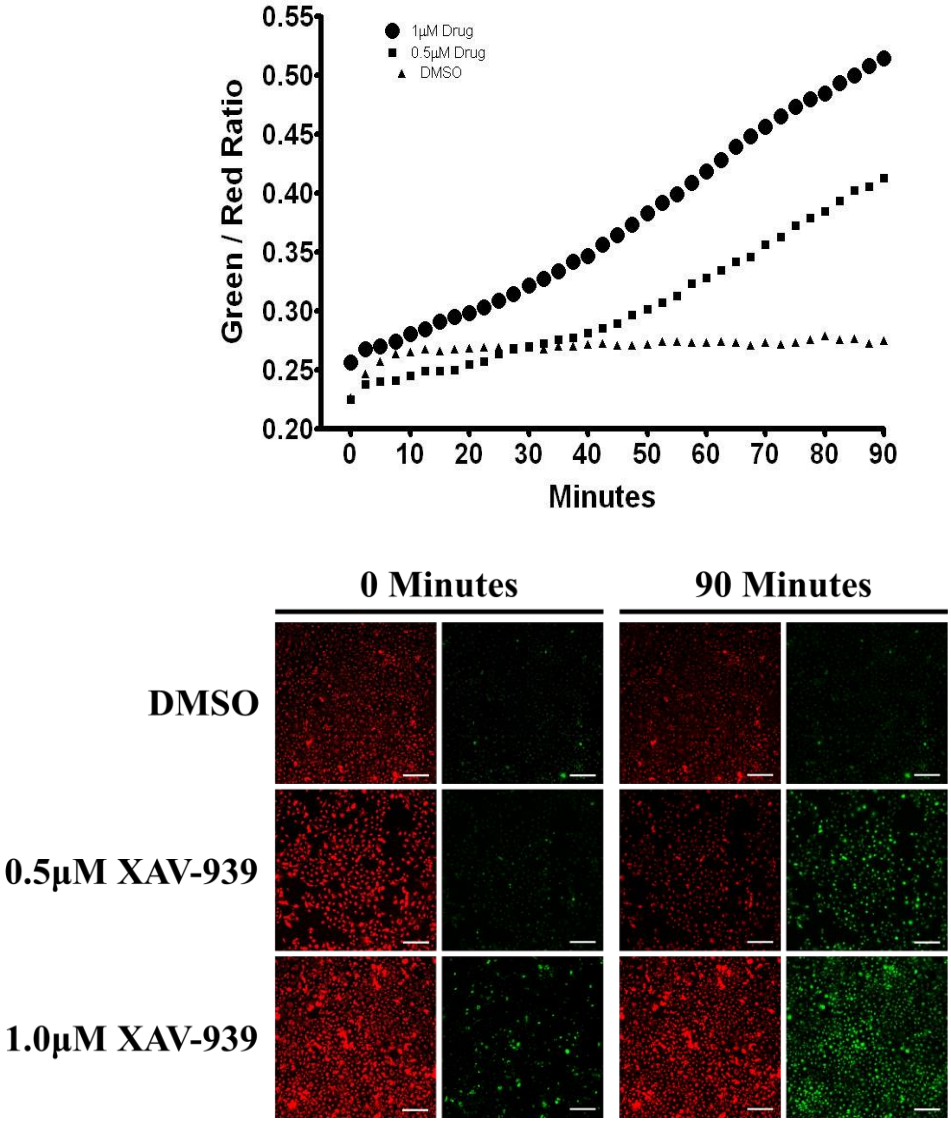


Figure 9: (Top panel) Serial confocal imaging was used to determine the extent of mitochondrial depolarization in cells treated with XAV939. Sequential images of the same random field of cells were taken every 150 seconds for a 60 minute duration (Bar=20 μm). Confocal images indicated that the cells treated with either 0.5μm or 1μm of XAV939 showed a marked increase in the green/red fluorescence ratio, indicative of depolarization as compared to control cells. (Bottom panel) Confocal images of the XAV939-treated cells indicated that there was a marked increase in green fluorescence intensity (indicative of depolarization) at 0.5 μM, and to an even greater degree at 1 μM compared with the control cells.

Figure 10

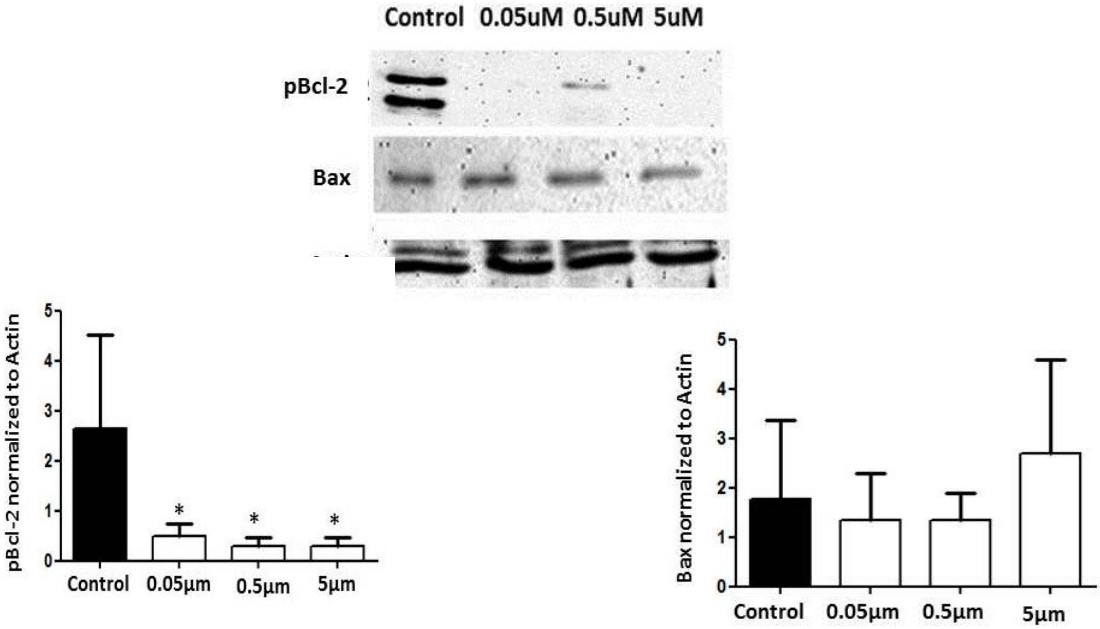
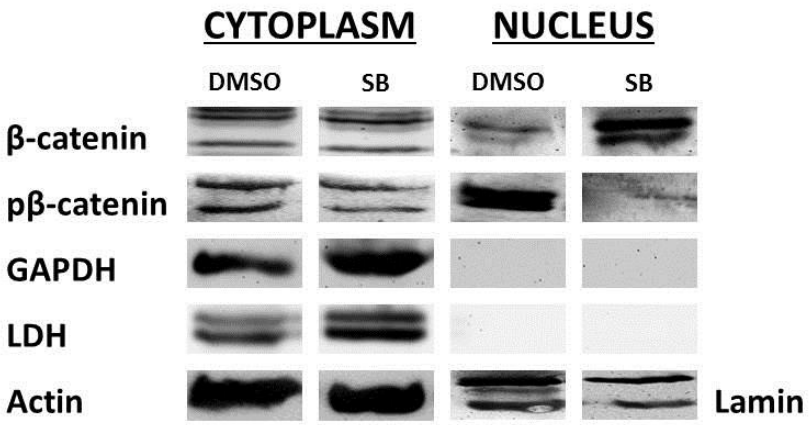


Figure 10: Western blot analysis of pBcl-2 and Bax in HLE-B3 cells treated with the VEGF~VEGFR inhibitor Axitinib. The cells were treated with 0.05 µM, 0.5 µM and 5µM of Axitinib in serum-free media and incubated in hypoxia for 3 hours. At the end of 3 hours, fresh serum-free media with the same concentrations of the inhibitor were added and incubated at atmopsheric oxygen. Control cells treated with DMSO were treated similarly.Total cell lysates were collected at the end of 3 hours of incubation at atmopsheric oxygen using ice-cold 1× PBS, pH 7.4, then adding hot lysis buffer (0.12M Tris HCl (pH 6.8), 4% SDS and 20% glycerol, 280 µl boiled to 100^0 C) directly to cell monolayers. The lysates were normalized with actin and analyzed for pBcl-2 and Bax levels. The experiment was repeated three times using three independent cell populations and the levels of pBcl-2 and Bax were quantified using Image J analysis. There was a significant decrease in the levels of pBcl-2 in cells treated with Axitinib relative to the control cells (* P< 0.05). There was no significant difference in the levels of Bax between the Axitinib treated and control cells.

Figure 11

A



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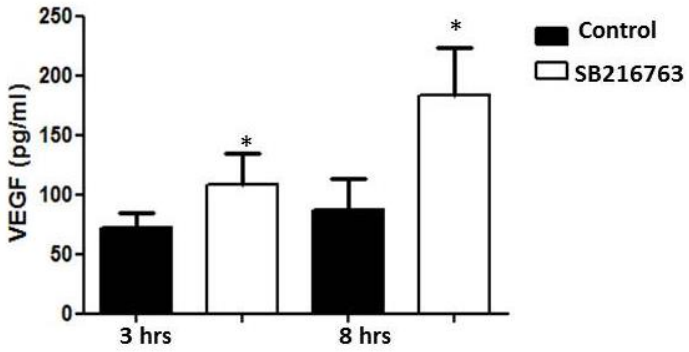


Figure 11A: Normal bovine lens epithelial cells at passage 2 were utilized for this experiment. To rule out the cross contamination between the cytoplasmic and nuclear fractions, the cytoplasmic markers GAPDH and LDH were utilized. The cells were incubated with GSK-3β inhibitor SB216763 for 3 hrs in hypoxia followed by re-oxygenation and supernatants and lysates were collected after 3 hrs of incubation in atmospheric oxygen. 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 .There was no significant carryover of the cytoplasmic markers in the nuclear fractions. **B:** The asterisk (*) indicates a statistically significant (* $P<0.05$) increase in the levels of VEGF in SB216763 treated cells at both 3 and 8 hour time points as compared to the control cells. SB = SB216763.

DISCUSSION:

Previous data from specific aim 1 supports a model in which the sustained synthesis of VEGF in human lens epithelial cells in hypoxia 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 the levels of anti-apoptotic protein Bcl-2, which prevents the translocation of cytosolic BAX to the outer mitochondrial membrane, thus providing resistance against mitochondrial depolarization (61). VEGF is also consistently synthesized and accumulated in lens epithelial cells exposed to atmospheric oxygen. Whereas the HIF's regulate VEGF levels in hypoxia, it is primarily glycogen synthase kinase-3β (GSK-3β) which regulates VEGF levels in atmospheric oxygen (Figure 3). We recently reported that inhibition of the enzymatic activity of GSK-3β prevented mitochondrial depolarization (70, 71) but in that study we did not specifically identify the downstream mechanism(s) involved in the protection pathway. Here, 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 which subsequently increased VEGF synthesis thereby preventing mitochondrial depolarization (Figures 3 and 9).

To monitor the effect of SB216763 on the catalytic site of the enzyme GSK-3β, we scrutinized the phosphorylation of a downstream substrate of GSK-3β, glycogen synthase (GS). In order to possibly 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) prior to Western blot analysis. GSK-3β, pGSK-3β, GS and pGS were detected in both the cytoplasmic and nuclear fractions of control cell lysates. 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.

However, the pGS levels were markedly reduced in the cytosolic fraction and completely eradicated from the nuclear fraction of the SB216763-treated samples. 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. 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. 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. These results were repeated with three independent cytosolic and nuclear samples from three independent cell populations. The presence of glycogen synthase kinase and glycogen synthase in the nucleus and its effect on the downstream signaling pathways, via a nuclear location, is not obvious at this time.

The data presented in this study indicates that the catalytic activity of GSK-3β resulted in consistent accumulation of the pro-survival factor, VEGF over a time period of 3–24 hours in atmospheric oxygen. 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 (70) 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 (71), 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 (71), 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 separated cytoplasmic and nuclear fractions in an attempt to gain greater insight into the relevance of pBcl-2 levels between control and SB216367-treated cells. Doing so revealed consistent levels of cytoplasmic and nuclear Bcl-2 and pBcl-2 between control and SB216763 treated sample (Figure.4). Portier and Taglialatela (75) have shown that, "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. (76) 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 Bcl-2 and pBcl-2 levels between control and SB216763 treated sample, the data as shown in figure 5 neither supports nor refutes the possibility that nuclear Bcl-2 or pBcl-2 influences a trend towards apoptosis. However, additional data discussed below (Figure 8) strongly suggests 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 (71). 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, pBcl-2 acts as a pro-survival factor in lens epithelial cells.

Thus far, we have inferred an association between elevated levels of VEGF and the enhanced levels of nuclear pBcl-2 resulting from SB216763 treatment, but have yet to show a definitive link between the two phenomena. In prior studies, we utilized, Axitinib, a potent inhibitor of VEGFR1, VEGFR2, and VEGFR3, which prevents association of VEGF receptor with VEGF, to demonstrate the link between VEGF and Bcl-2 in hypoxia (61). Repeating this experiment in atmospheric oxygen (Figure 10) demonstrated the biologic significance of VEGF signaling for lens epithelial cell survival and the role played by the VEGF-VEGFR2 complex in the signaling pathway in which VEGF regulates endogenous Bcl-2 levels. The disassociation of the VEGF-VEGFR2 complex led to decreased levels of pBcl-2. In atmospheric oxygen conditions, as with hypoxic conditions, the VEGF positively influences and regulates the level of pBcl-2, thereby establishing the VEGF~pBcl-2 pathway.

Activation of GSK-3β initiates apoptosis, while its inhibition is involved in anti-apoptotic signaling via the Wnt/β-catenin pathway (72). Activation of β-catenin is an important downstream effect of GSK-3β inhibition. 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 (67). In this study, we demonstrated a significant

association between inhibition of GSK-3β and increased translocation of β-catenin from cytoplasm to nucleus with increased VEGF and nuclear pBcl-2 levels.

The data with the GSK-3β inhibitor demonstrates a strong association between resistance to mitochondrial depoplarization and increased nuclear β-catenin levels. To further prove the direct association between nuclear β-catenin and resistance to mitochondrial depolarization, a pharmacological inhibitor was utilized. Degradation of cytoplasmic β-catenin is mediated by the protein destruction complex consisting of GSK-3β and Axin proteins. The pharmacological inhibitor XAV939 binds to the Tankyrase enzyme which leads to the stabilization and increased expression of the Axin protein in the destruction complex, leading to the degradation of β-catenin. In this study we utilized the inhibitor XAV939 to inhibit β-catenin translocation to the nucleus and demonstrate the association between β-catenin, mitochondrial depolarization and the VEGF expression.

The use of the pharmacological inhibitor, XAV939, with isolation of the nuclear and cytoplasmic fractions, was used to determine whether the loss of nuclear β-catenin negatively influenced the levels of VEGF and pBcl-2. The inhibitor increases the expression of the APC protein in the destruction complex resulting in depletion of nuclear β-catenin, but had no effect on the levels of cytoplasmic β-catenin (Figure 6). In turn, the loss of nuclear β-catenin resulted in decreased VEGF content diminished levels of pBcl-2 and markedly increased mitochondrial depolarization (Figures 7,8 and 9). Thus, we conclude that nuclear β-catenin participates in cell survival by inducing the pro-survival proteins, VEGF.

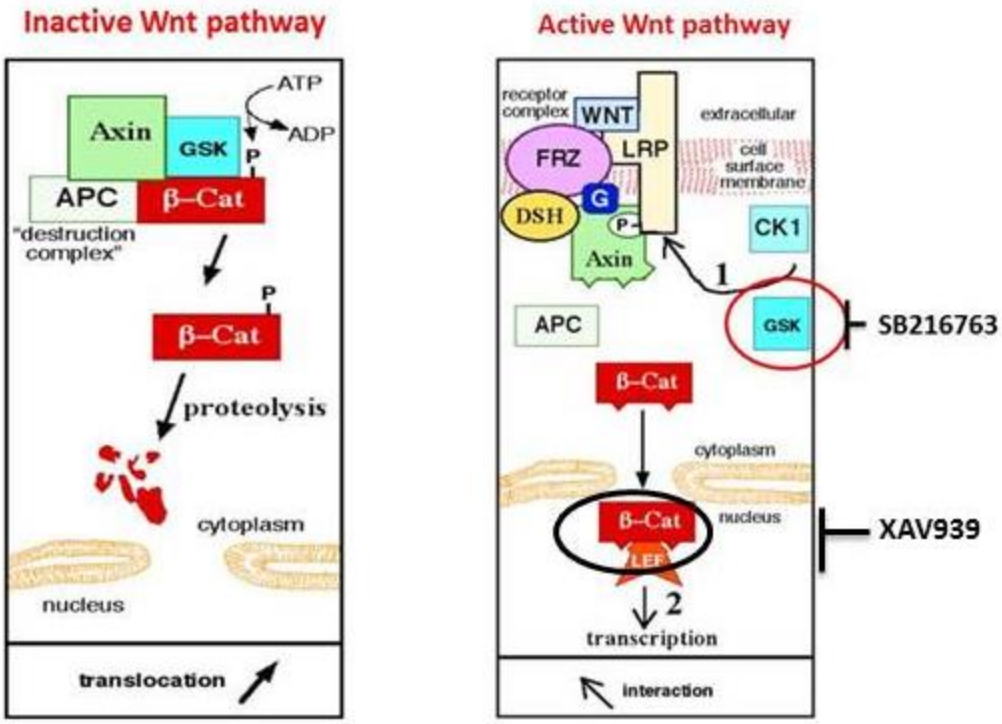
In the hypoxic lens, VEGF synthesis is regulated by both the hypoxia inducible factors (HIFs) HIF-1α and HIF-2α (61). The HIF-1α is degraded in atmospheric oxygen, but HIF-2α, is resistant to degradation

in atmospheric oxygen. HIF-1α is degraded in atmospheric oxygen by the enzyme prolyl hydroxylase-domain proteins (PHDs). In atmospheric oxygen the PHDs hydroxylate the HIFα subunits on the proline residues and hydroxylated HIF is recognized by VHL (Von –Hippel Lindau) tumor suppressor protein which polyubiquitinates and degrades the HIF. HIF-2α is resistant to degradation in atmospheric oxygen by PHDs due to difference in the amino acid sequences around the hydroxylation site. As a result, HIF-2α is constitutively expressed in atmospheric oxygen (refer to reference 61). There fore, we investigated whether the presence of HIF-2α in atmospheric oxygen could contribute to the VEGF synthesis in the lens epithelial cells.We have established the finding that the survival (i.e. mitochondrial depolarization) of human lens epithelial cells in atmospheric oxygen depends on the continuous synthesis and accumulation of VEGF However, with a specific HIF-2α translation inhibitor (CAS882268–69–1), HIF-2α decreased without affecting the levels of VEGF or pBcl-2. Our data demonstrated that it is not HIF-2α which is involved in maintaining continuous VEGF expression but rather, β-catenin.

To summarize, the data 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, in turn prompting elevated VEGF expression, a forerunner to increased resistance to mitochondrial depolarization.

Figure source http://en.wikipedia.org/wiki/Wnt_signaling_pathway

SUMMARY OF CHAPTER 2



Exposure of the hypoxic lens epithelium to atmospheric oxygen during cataract surgeries activates the Wnt signaling pathway. Wnt/β-catenin signaling starts with binding of Wnt ligand to the frizzled receptor which leads to inactivation of GSK3-β - part of a destruction complex consisting of Axin and APC proteins. When the Wnt pathway is inactive, GKS-3β is not phosphorylated. In its active form GSK-3β phosphorylates β-catenin which is degraded through the ubiquitin proteasome pathway. With the activation of Wnt, GKS-3β is inactivated by phosphorylation. In the absence of active GSK3-β, β-catenin is not phosphorylated. Active β-catenin translocates to the nucleus and promotes the transcription of pro-survival factors like VEGF. In this study, we bypassed the upstream Wnt signaling and directly inhibited GSK-3β activity (SB216763), which lead to increased nuclear β-catenin and VEGF expression. We further confirmed the association between nuclear β-catenin and VEGF by using a specific pharmacological inhibitor for β-catenin-XAV939.

CHAPTER 3

Hypothesis: Inhibition of GSK-3β in atmospheric oxygen using a pharmacological inhibitor (SB216763) leads to active nuclear β-Catenin which promotes the mesenchymal transition of the epithelial cells leading to initiation of Posterior Capsular Opacification or PCO.

Rationale: Posterior Capsular Opacification (PCO) is a complication of cataract surgery in which the residual lens epithelial cells that line the inside surface of the equatorial lens capsule proliferate; lose the characteristics of a epithelial cell and transition towards a mesenchymal cell, a phenomenon which is referred to as epithelial to mesenchymal transition or EMT. The mesenchymal cells are more motile than the epithelial cell population from which they were generated, as a result of which they migrate along the lens capsule and reach its posterior aspects (77, 78).

The initiation of the mesenchymal transition occurs during the brief exposure of the hypoxic lens epithelium to atmospheric oxygen during the cataract surgery. The Wnt/β-catenin pathway is activated during exposure to atmospheric oxygen and has been shown to play a role in the mesenchymal transition of the epithelial cells. Activation of the Wnt signaling pathway leads to translocation of β-catenin to the nucleus. Once β-catenin translocates to the nucleus, it binds to the TCF-LEF factors (T-cell and lymphoid enhancer factors) and induces the transcription of target genes like mesenchymal protein markers including α-Smooth muscle actin and Fibronectin (79). In a normal lens epithelial cell β-catenin is associated with E-cadherins at the cell membrane and any residual cytoplasmic β-catenin is degraded by the protein destruction complex consisting of GSK-3β, Axin and APC (adenomatosis polyposis coli) proteins. Once the Wnt signaling pathway is activated, it inactivates the GSK-3β (a component of the protein destruction complex) as a result the cytoplasmic β-catenin translocates to the nucleus binds to

TCF-LEF factors and induces the expression of mesenchymal proteins and thus initiates the mesenchymal transition of the epithelial cells contributing to the pathogenesis of PCO (80).

<u>Hypothesis:</u> The mesenchymal transition of epithelial cells initiated in atmospheric oxygen persists in hypoxia under the influence of hypoxia inducible factor – HIF-1α.

The pathogenesis of PCO involves two stages; the initiation phase which occurs during exposure to atmospheric oxygen and the persistence phase which occurs once the lens epithelial cells are back in hypoxia following the cataract surgery. The initiation of the mesenchymal transition of the epithelial cells which occurs in atmospheric oxygen persists in hypoxia under the influence of the HIFs. The rationale for initiation and persistence phase of PCO is based on the fact that clinical manifestation of PCO occurs 2- 6 years following the cataract surgery, and the insult to the epithelial cells in atmospheric oxygen persists and continues when the lens is back in hypoxia (81).

The hypoxia inducible factors or HIFs are regulated by the hydroxylation of the proline residues on the α-sub unit and degraded by the ubiquitin proteasome pathway in a VHL (Von Hippel–Lindau tumor suppressor) dependent manner. A study Fugel et. al. (31) has demonstrated that GSK-3 can negatively regulate and degrade HIF-1α in a VHL independent manner and bypass the hydroxylation of the proline residues. Furthermore, it has been previously demonstrated that HIF-1α competes with the TCF-4 binding site for β-catenin and promotes the transcription of mesenchymal proteins like α-smooth muscle actin and fibronectin.

Based on these observations, we propose that inactivation of GKS-3β, which occurs via the TGF-β/Wnt signaling pathway, during exposure to atmospheric oxygen persists when the lens is back in hypoxia (82). Under the influence of GSK-3β inhibition, HIF-1α will be activated in hypoxia and drives the expression of mesenchymal proteins leading to persistence of PCO in the hypoxia. In this study, we propose that the

HIFs in hypoxia under the influence of nuclear β-catenin or other transcription factors might be regulating the expression of mesenchymal proteins in hypoxia. Depending on if its HIF-1α or HIF-2α driving the mesenchymal protein expression in hypoxia, the presence of two HIFs – HIF-1α/HIF-2α could be used to our advantage to separate the two events of mitoprotection (via VEGF synthesis) and mesenchymal transition (Via the expression of mesenchymal proteins).

MATERIALS AND METHODS

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 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 (44). All studies with HLE-B3 cells were performed with pre-frozen stock cells (maintained in liquid nitrogen) between passages 14 to 17 and no experiments exceeded 5 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), 2mM L-glutamine, nonessential amino acids, and 0.02g/L gentamycin solution (Sigma-Aldrich) and cultured at 37^0C and 5% CO_2-95% O_2. Cells were sub-cultured four to five days prior to the experiment and placed in MEM containing 20% FBS. Twenty four hours prior to the day of the experiment, cells were switched to serum-free MEM. Unless otherwise specified, all experiments followed a common protocol; cells were either maintained in atmospheric O_2 (~21%) for 90 minutes, then switched to hypoxic conditions (~1% O_2) for 180 minutes, followed by reintroduction to atmospheric O_2 or maintained in hypoxia (1% oxygen) for 3 hours. 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%.

Western blot analysis: Total cell lysates were collected from HLE-B3 cultures after treatments by rinsing adherent cells with ice-cold 1× PBS, pH 7.4, then adding hot lysis buffer (0.12M Tris HCl (pH 6.8), 4% SDS and 20% glycerol, 280 µl boiled to 100^0 C) directly to cell monolayers. Lysates were collected and sonicated for 5 sec, and a portion of the sample was removed for determination of protein concentration. Protein concentration was determined using the EZQ protein quantification kit from Invitrogen (Carlsbad, CA); 3× SDS (Laemmli) buffer was added to the lysates, which were subsequently boiled for 5 min; and the proteins were resolved by electrophoresis on 12% SDS-polyacrylamide gels (20 µg protein/lane). Proteins were then transferred to nitrocellulose (Scheicher and Schuell, Keene, NH).

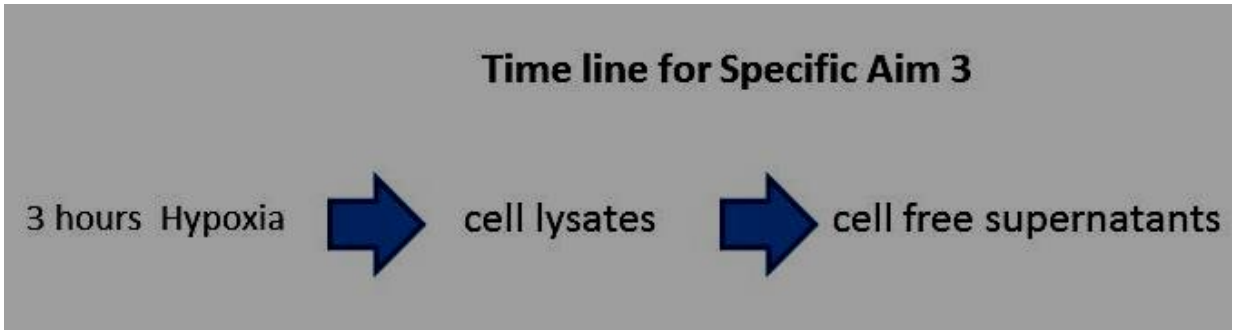
For Western blot analysis, nitrocellulose membranes were blocked with Tris-buffered saline (TTBS, 1% BSA and 0.02% Tween -20 in Tris–buffered saline for 60 min. These membranes were probed overnight at 4°C with primary antibodies. The blots were then rinsed in TTBS (4× with 5-min washes) and incubated 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 pico chemiluminescent kit from Pierce (Rockford, IL).

ELISA: ELISA was performed for the detection of VEGF using an Invitrogen VEGF ELISA kit (Grand Island, NY) for HLE-B3 cells. HLE-B3 were cultured in 25 cm^2 tissue culture flasks in 20% FBS, transferred to serum-free media prior to the initiation of the experiment. Flasks were generally set up in triplicate for 3 hours of incubation in hypoxia. At the end of 3 hours, fresh serum-free media was added

and cells were incubated at atmospheric oxygen for 3 hours. Cell-free supernatants were collected at 3 hours of re-oxygenation and analyzed according to manufacturer's instructions. The optical density at 450 nm was determined using a Molecular Devices Spectramax 190 (Sunnyvale, CA).

Primary antibodies used in this study were; mouse anti-α smooth muscle actin (Sigma) and rabbit anti-Fibronectin antibodies (Millipore).

Statistical analysis: Images from JC-1 confocal microscopy were analyzed as individual red and green channels using Image J (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 Image J 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 the software from Graphpad Prism, version 5.00 (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.



The cells were incubated with specific pharmacological inhibitors for 3 of hypoxia. The cell free supernatants and lysates were collected after the hypoxic incubation.

RESULTS

Inhibition of the enzymatic activity of GSK-3β and expression of EMT/E-cadherin proteins: Nuclear β-catenin is associated with EMT (Epithelial to Mesenchymal transition). The expression of EMT proteins like alpha smooth muscle actin (α-SMA) and fibronectin is generally taken as a strong indicator of progression towards 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 1, 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 1, bottom panel).

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 1). 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 previously 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 – specific aim 2) treatment decisively correlated with a decrease in the expression of the EMT proteins, α-SMA and fibronectin as compared to the control cells (Figure 2).

Effect of GSK-3β/ HIF-1α inhibition on the expression of Mesenchymal proteins in hypoxia: HLE-B3 cells were cultured using either 12µM of the specific GSK-3β inhibitor, SB216763 alone or SB216763 with 50 µM of either HIF-1α or HIF-2α inhibitor for 3 hours in hypoxia. At the end of 3 hours of hypoxic incubation, total cell lysates were collected and analyzed by Western blot to determine the expression of the mesenchymal proteins.

Inhibition on GSK-3β in hypoxia resulted in increased expression of the mesenchymal proteins α-smooth muscle actin and Fibronectin. Inhibition of both GSK-3β and HIF-1α resulted in loss of mesenchymal proteins as compared to the control and inhibition of both GSK-3β and HIF-2α did not have an effect on the expression of mesenchymal proteins (Figure 3).

Effect of GSK-3β/ HIF-1α inhibition on the levels VEGF of in hypoxia: HLE-B3 cells were cultured using either 12µm of the specific GSK-3β inhibitor, SB216763 alone or SB216763 with 50 µm of either HIF-1α or HIF-2α inhibitor for 3 hours in hypoxia. At the end of 3 hours of hypoxic incubation, cell free supernatants were analyzed by ELISA to determine the levels of VEGF. Inhibition of GSK-3β resulted in increased VEGF levels as compared to the control. Inhibition of both GSK-3β and HIF-1α resulted in increased VEGF synthesis – the lack of HIF-1α is compensated by HIF-2α. Similarly inhibition of both GSK-3β and HIF-2α, resulted in increased VEGF synthesis- the lack of HIF-2α is compensated by HIF-1α (Figure 4).

FIGURES FOR CHAPETER 3

Figure 1

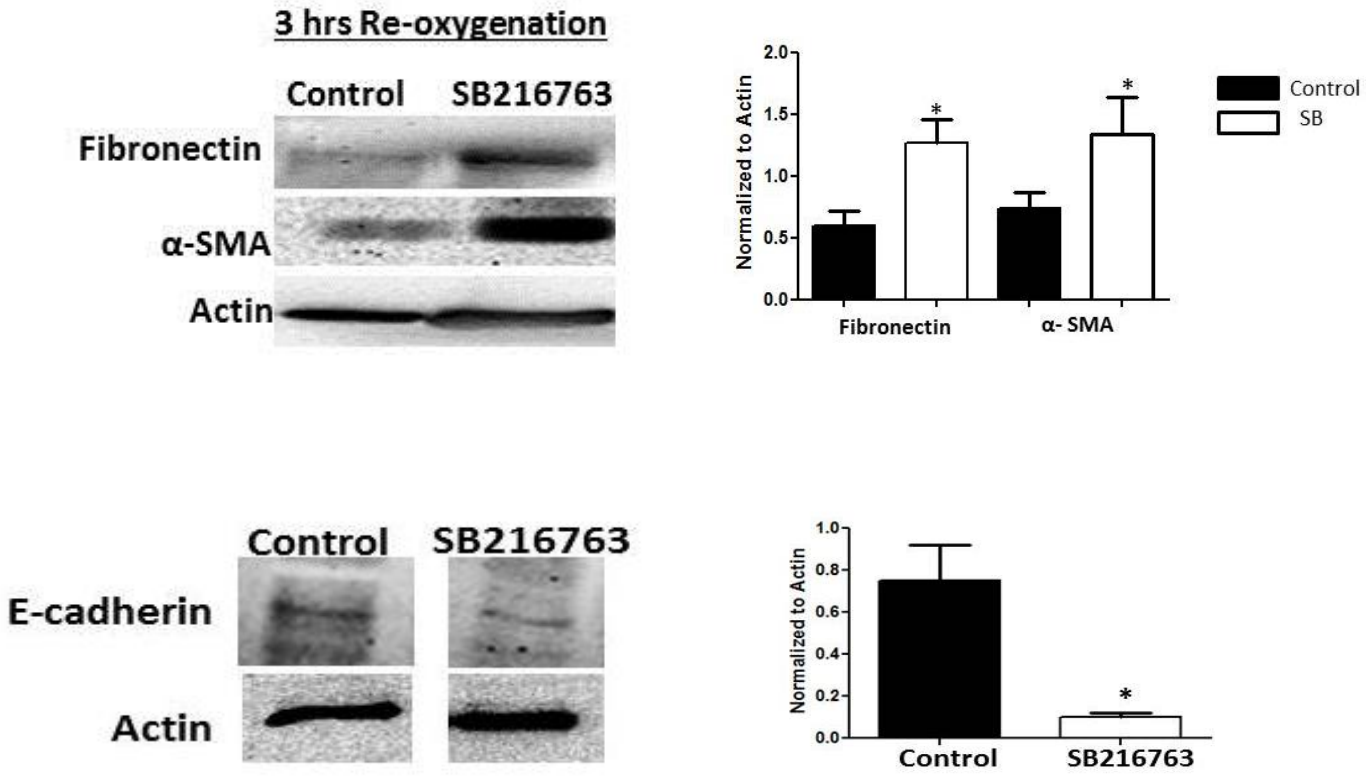


Figure 1: (Top panel) The experiment was repeated three times using independent cell populations stemming from a single cell passage. The normalized lysates were analyzed for levels of α-SMA and fibronectin using ImageJ analysis. There was a significant increase in the expression of both α-SMA and fibronectin in the SB216763 treated samples as compared to the corresponding control samples treated with DMSO (*P<0.05). (Bottom panel) The normalized lysates were analyzed for levels of E-cadherin using ImageJ analysis. The experiment was repeated three times using independent cell populations stemming from a single cell passage. There was a significant decrease in the expression of E-cadherin in the SB216763 treated samples as compared to the corresponding control samples treated with DMSO (*P<0.05). SB = SB216763.

Figure 2

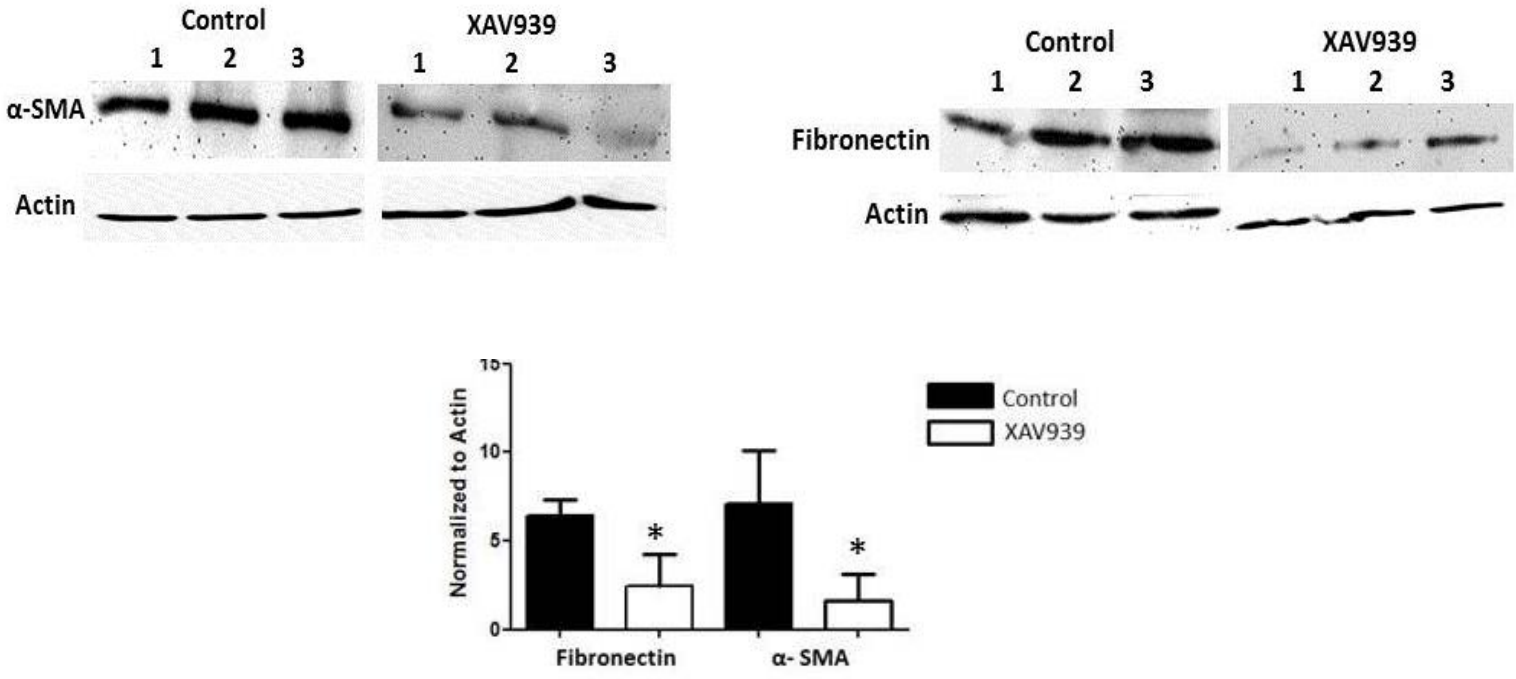


Figure 2: The experiment was repeated three times using independent cell populations (represented as 1 , 2 3 in the figure).The cells were treated with XAV939 (the drug acts by stabilizing the expression of Axin protein in the destruction complex and degrading nuclear β-catenin) for 3 hours in hypoxia followed by re-oxygenation and lysates were collected after 3 hours of incubation in atmospheric oxygen. The normalized lysates were analyzed for levels of α-SMA and fibronectin using ImageJ analysis. There was a significant decrease in the expression of both α-SMA and fibronectin in the XAV939 treated samples as compared to the corresponding control samples treated with DMSO (*P<0.05).

Figure 3

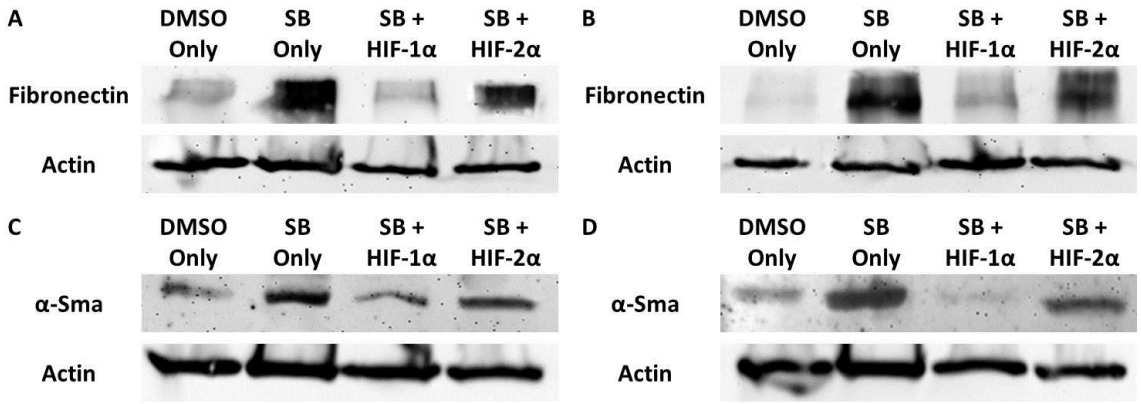


Figure 3: HLE-B3 cells were cultured in 100 mm^2 culture dishes with 20% FBS and switched to serum-free media on the day of the experiment. Two independent cell populations were tested with 12µm SB216763 alone or 12µm of SB216763 with 50 µm of either HIF-1α or HIF-2α inhibitor for 3 hours in hypoxia. At the end of the 3 hour hypoxic incubation, total cell lysates were collected and analyzed by western blot for the levels of mesenchymal proteins α- Smooth muscle actin (α-SMA) and Fibronectin. Control cells treated with DMSO were incubated and analyzed in a similar manner. In both the cell populations compared to the control there was a significant increase in the levels of fibronectin (A and B) and α-SMA(C and D) in the samples treated with either SB216763 alone or SB216763 with HIF-2 α. There was a significant decrease in the expression of mesenchymal proteins in the samples treated with SB216763 and HIF-1α.

Figure 4

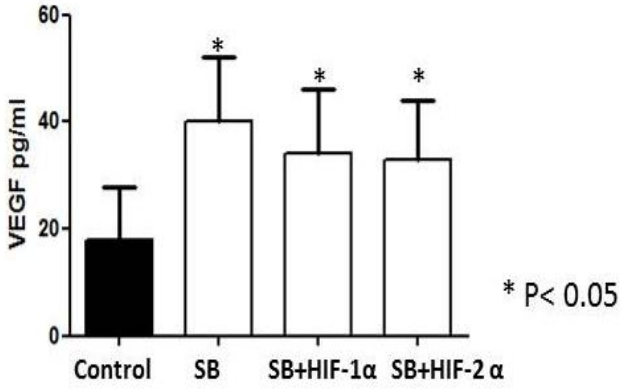


Figure 4: HLE-B3 cells were cultured in 25 cm^2 flasks with 20% FBS and switched to serum-free media on the day of the experiment. Two independent cell populations were tested with 12µm SB216763 alone or 12µm of SB216763 with 50 µm of either HIF-1α or HIF-2α inhibitor for 3 hours in hypoxia. Control cells treated with DMSO were analyzed in a similar manner. At the end of the 3 hour hypoxic incubation cell free supernatants were collected and analyzed by ELISA to detect the levels of vascular endothelial growth factor (VEGF). Compared to the controls there was a significant increase in the levels of VEGF in samples treated with either SB216763 alone or SB216763 with either HIF-1α or HIF-2α inhibitors (*P< 0.05).

DISCUSSION:

Initiation of PCO in Atmospheric oxygen:

Posterior capsular opacification (PCO) is a complication of cataract surgery. The pathophysiology of PCO involves mesenchymal transition of the epithelial cells lining the lens capsule. Introduction of atmospheric oxygen during cataract surgery activates the TGF-β/Wnt signaling pathway. TGF-β acts through the type I and type II receptors coupled to the Smad proteins. Under normal physiological conditions TGF-β exists in its latent form. Biologically active form of TGF-β is detected in patients with PCO (83, 84).

TGF-β is abundant in the aqueous humor and the lens cells express different isoforms of TGF-β and its receptors. A study by Gordon–Thomson et al. (85) compared the ability of three isoforms of TGF-β in inducing cataract. The three isoforms studied were TGF-β1, β2 and β3. Rat lens tissues were used to determine the protein and mRNA expression of different isoforms of TGF-β. All three isoforms of TGF-β were identified in the rat lens and were capable of inducing cataract in the lenses. Activation of TGF-β predominantly occurs through the Smads. Smad proteins are intracellular proteins which carry the signals from the receptors to the specific target genes in the nucleus. The TGF-β signaling pathway is initiated by binding of the TGF-β ligand to the TGF-β specific type II receptors (86).

In lens epithelial cells active TGF-β in response to injury is known to induce the transformation of lens epithelial cells in to fibroblasts. Active TGF-β promotes the binding of the Wnt to its frizzled receptor proteins. Wnt signals through the Frizzled, which in turn activates intracellular Dishevelled, leading to activation and nuclear translocation of β-catenin. Wnt/β-catenin signaling starts with binding of Wnt ligand to the frizzled receptor and forms a complex with the LDL related protein. The formation of this complex will inactivate GSK3-β. GSK3-β when active will phosphorylate β-catenin which is then

degraded through the ubiquitin proteasome degradation pathway. In the absence of active GSK3-β, β-catenin is not phosphorylated and the unphosphorylated form of β-catenin translocates to nucleus and activates its downstream nuclear transcription factors (87-89).

Chong et al. (62) have shown that Wnts and their frizzled receptors are up regulated in lens epithelial cells in association with TGF-β leading to EMT and cataract development. This study demonstrated that TGF-β promotes the expression of Wnts and frizzled in lens epithelial cells which lead to the activation and translocation of β-catenin from the cell margins to the nucleus. Using lens epithelial cell explants, the study by Chong et.al., demonstrated that β-catenin is associated with cell margins in controls and in explants treated with TGF-β, there was an increase in the translocation of unphosphorylated β-catenin to the lens nucleus. In conclusion, the study suggests that TGF-β may play a role in activation and translocation of β-catenin to the nucleus eventually leading to EMT(epithelial to mesenchymal transition) and initiation of PCO (posterior capsular opacification) in the lens epithelial cells.

Cain et. al. (65) studied the role of β-catenin in the proliferation and differentiation of lens epithelial cells and fiber cells during development. Using a cre/loxp system they specifically deleted β-catenin in the whole lens and the lens fibers. Loss of β-catenin resulted in abnormal epithelial cells with loss of cadherins and adhesion junctions. The ability of the epithelial cells to transition to a mesenchymal phenotype represents the inherent plasticity of the epithelial cells. The transition towards a mesenchymal phenotype involves loss of the apical – basal polarity of the epithelial cells, reorganization of the actin cytoskeleton, and increased migration. Specialized protein complexes like the cadherins form the cell-cell junctions which maintain the polarity of the epithelial cells. In the lens epithelial cells during the transition to a mesenchymal phenotype there is a significant loss of the epithelial cadherins or E-cadherin proteins. E-cadherins are cell adhesion molecules whose cytoplasmic domain binds to β-catenin. Under

normal circumstances any unbound cytoplasmic β-catenin is degraded by the ubiquitin proteasome pathway. With the activation of Wnt signaling pathway, β-catenin is translocated to the nucleus and dissociates from E-cadherins leading to loss of cadherins and cell adhesion properties (90). Using human lung fibroblasts, Caraci et. al. (91) has shown that TGF-β induces nuclear translocation of β-catenin. and the expression of alpha smooth muscle actin (α-SMA) through GSK-3β inhibition The study further demonstrated that inhibition of β-catenin by siRNA leads to decreased synthesis of α-SMA in TGF-β treated fibroblasts. In another study with human corneal cells, Kato et. al. (92) has also shown that activation of β-catenin leads to EMT which contributes to the pathogenesis of pterygium. Collectively, these studies demonstrate that β-catenin plays an important role in the transition of the epithelial cells towards a mesenchymal phenotype.

In this study we bypassed the upstream signaling factors – TGF-β/Wnt and directly targeted GSK-3β inactivation (using the pharmacological inhibitor SB216763) which leads to increased translocation of β-catenin to the nucleus. We propose that increased nuclear β-catenin activity elicits lenticular mitoprotection through increased VEGF levels, but at the same time via an independent pathway educes a marked increase in the expression of the EMT proteins, α-smooth muscle actin and fibronectin. These data, therefore, uncovered a unique observation in the lens literature in that elevated nuclear β-catenin is coupled to two distinct parallel phenomena. One pathway leads to the increase in the pro-survival protein, VEGF (which is linked to the pro-survival protein, pBcl-2). The second and independent pathway is the initiation of epithelial to mesenchymal transition (as indicated by the increase in the EMT proteins, α-SMA and fibronectin and loss of E-cadherins). Other reports in the literature lend support to our conclusion. In a study by Martinez et al. (93) 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. This study further

elucidated the role of Wnt/ β-catenin pathway as an initiator of EMT. In another study, Bao et. al. (94) examined the effect of Wnt 3a on β-catenin levels and progression of PCO. In that study, Wnt 3a was over-expressed in the HLE-B3 lens epithelial cell, resulting in increased total β-catenin, decreased E-Cadherins, and increased expression of EMT proteins like fibronectin. The HLE-B3 cells, in that study, also displayed an irregular morphological pattern, characteristic of a mesenchymal-like cell. The association between E-cadherins and β-catenin is important in maintaining the integrity of the epithelial cells. Disruption of this association and activation of the Wnt signaling pathways leads to not only loss of cell adhesive properties, but also initiation of the mesenchymal transition.

In this study, data shown with the GSK-3β inhibitor, SB216763, suggested an association between elevated nuclear β-catenin activity, increased VEGF and an increase of α-SMA and fibronectin of the SB216763-treated cells. Such data implies, but does not necessarily prove, that the two phenomena (enhanced lens mitochondrial protection and EMT) are the result of increased nuclear β-catenin expression. In order to firmly establish the inter-relationship we re-approached this suggested association by suppressing the expression of β-catenin. We utilized a 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 degradation of β-catenin (73,74).

The use of the pharmacological inhibitor, XAV939, with subsequent isolation of the nuclear and cytoplasmic fractions, was used to determine whether the loss of nuclear β-catenin negatively influenced the levels of the pro-survival factors, VEGF and pBcl-2 and the EMT proteins, α-SMA and fibronectin. The inhibitor results in the depletion of nuclear β-catenin, but had no effect on the levels of cytoplasmic β-catenin. In turn, the loss of nuclear β-catenin resulted in decreased VEGF content, diminished levels of pBcl-2, and a failure to detect α-SMA and fibronectin. As anticipated, XAV939 treatment also markedly

increased mitochondrial depolarization (refer to specific aim 2). Thus, we conclude that nuclear β-catenin participates in cell survival by inducing the transcription of pro-survival proteins, VEGF and pBcl-2 but at the same time triggers induction of α-SMA and fibronectin - early marker proteins of epithelial to mesenchymal transition. The translocation of nuclear β-catenin to the nucleus and the expression of mesenchymal proteins drive the initiation of PCO in atmospheric oxygen. In an effort to not overstate our data, studies described herein were not intended to directly investigate the measure of mesenchymal transition. Instead, we use an analysis of the EMT marker proteins, fibronectin and α-SMA, as early-onset predictors of mesenchymal transition.

Persistence of PCO in Hypoxia:

A compensatory interrelationship between the two HIFs in hypoxia regulates the VEGF levels providing resistance against mitochondrial depolarization (61). The exposure of the lens to atmospheric oxygen (during the cataract surgery) leads to inhibition of GSK-3β, which persists, when the lens is back in hypoxia (after the cataract surgery). Whether the HIFs under the influence of GSK-3β inhibition promote the expression of mesenchymal proteins is not well established in the lens. Previous studies in the cancer literature have demonstrated that inhibition of GSK-3β in hypoxia leads to accumulation of active HIF-1α, which in turn induces the mesenchymal transition of the epithelial cells (95-98).

The HIFs are made up of α and β subunits. The β subunits are constitutively expressed, and it's the stability of the subunit which determines the transcriptional response initiated by the HIFs. The α subunit is degraded in oxygen by hydroxylation of the proline residues via the enzyme PHD- proline hydroxylase and degraded by the ubiquitin proteasome pathway in a VHL dependent manner. The proline hydroxylase

enzyme is inactive in hypoxia; as a result the subunit is constitutively expressed leading to activation of HIFs and the downstream transcription factors.

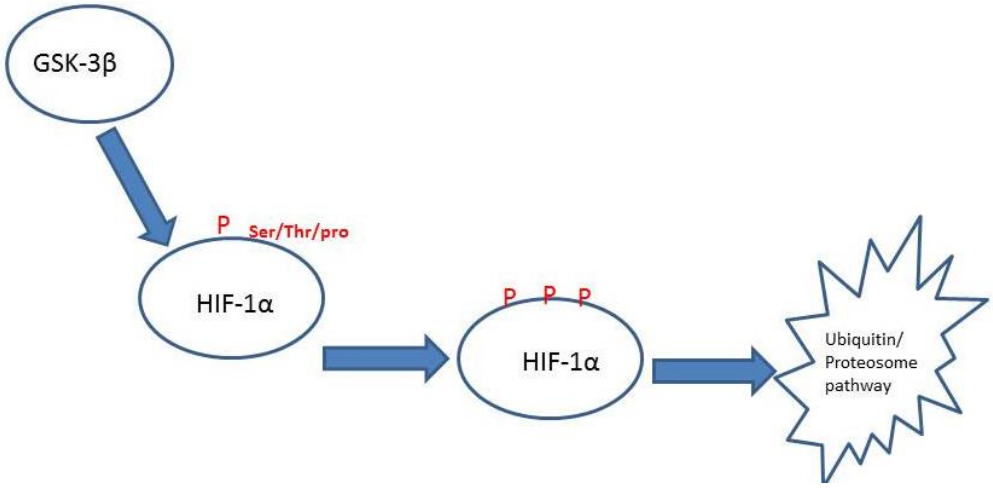
The enzyme GSK-3β is involved in various signaling pathways. The activity of GSK-3β is influenced by various cellular responses, including hypoxia. Studies in prostate cancer have demonstrated that inhibition of GSK-3β lead to activation of HIF-1α and over expression of GSK-3β suppressed HIF-1α synthesis in hypoxia. According to these studies, GSK-3β phosphorylates HIF-1α at specific residues, which are recognized by the E3 ubiquitin ligase pathway and are degraded in a VHL independent manner (99,100).

Based on these studies we proposed that following the exposure to atmospheric oxygen and hypoxia, HIF-1α is activated under the influence of GSK-3β inhibition. Inhibition of HIF-1α (which drives the expression of the mesenchymal proteins) will be compensated by HIF-2α, such that the mesenchymal transition will be prevented and HIF-2α maintains the VEGF levels without altering the mitoprotective pathways. Our preliminary data with HLE-B3 cells indicates that inhibition of HIF-1α in hypoxia leads to loss of EMT proteins without affecting the VEGF levels (which are maintained by the HIF-2α).

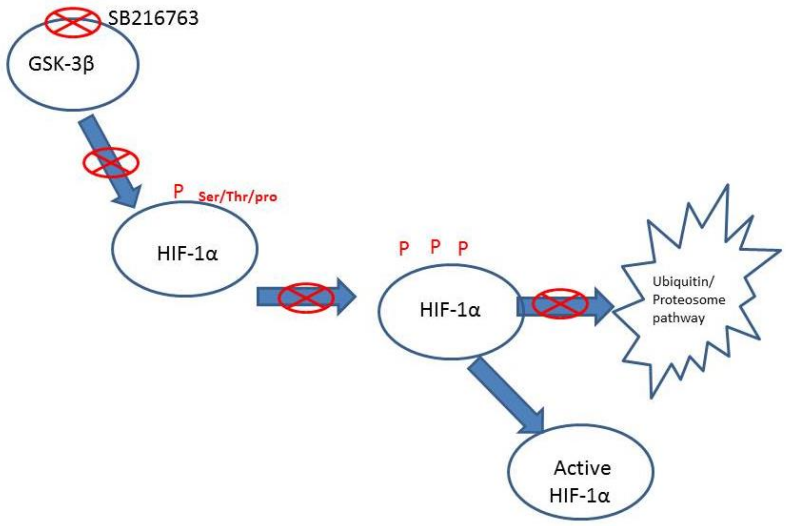
Future studies in the laboratory are geared towards understanding the interactions between the Hypoxia inducible factors, β-catenin and the downstream transcription factors regulating the expression of mesenchymal proteins in hypoxia. Understanding these interactions will help in designing therapeutic targets which can selectively inhibit the expression of mesenchymal proteins without altering the levels of pro survival VEGF and the resistance to mitochondrial depolarization.

SUMMARY OF CHAPTER 3

Active GSK-3β inactivates HIF-1α by phosphorylation



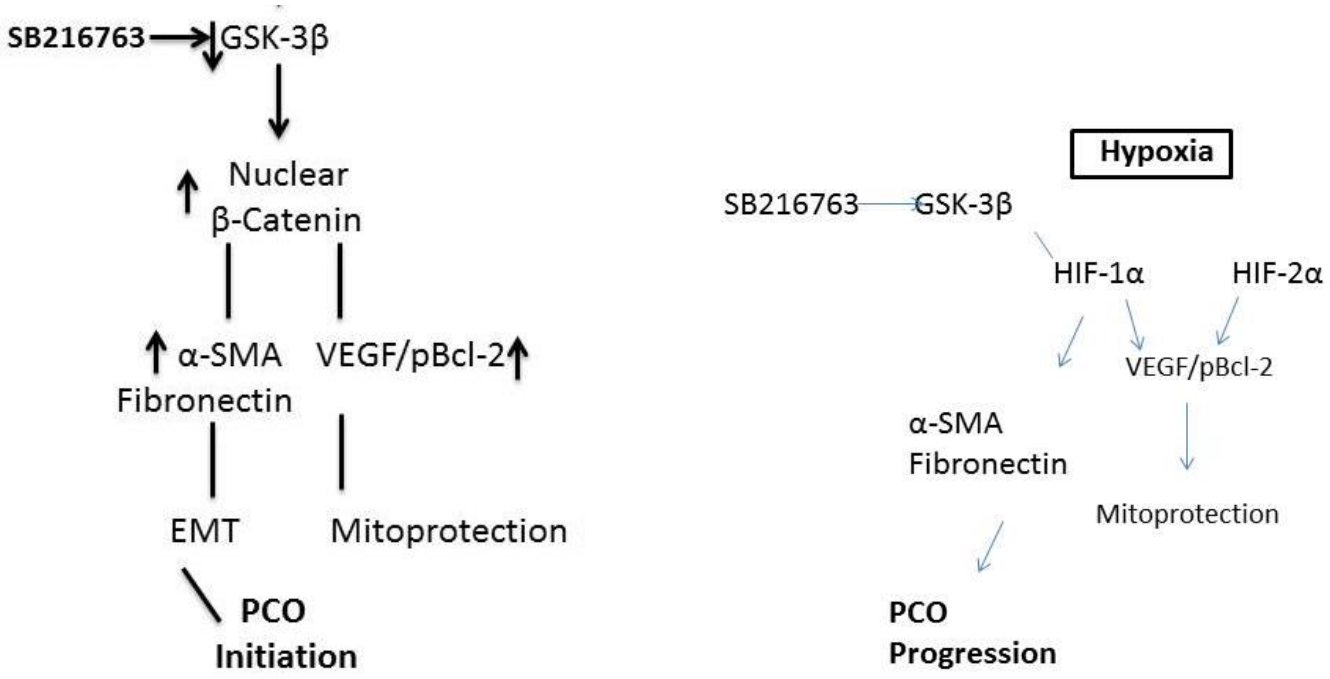
Inactivation of GSK-3β in hypoxia activates HIF-1α



Active GSK-3β phosphorylates HIF-1α at the serine/threonine/proline residues. The phosphorylated HIF is degraded by ubiquitin proteasome pathway in a VHL independent manner in hypoxia. Inactivation of GSK-3β in hypoxia prevents the phosphorylation of HIF-1α in hypoxia. Active HIF-1α induces the transcription of downstream targets like VEGF/mesenchymal protein markers.

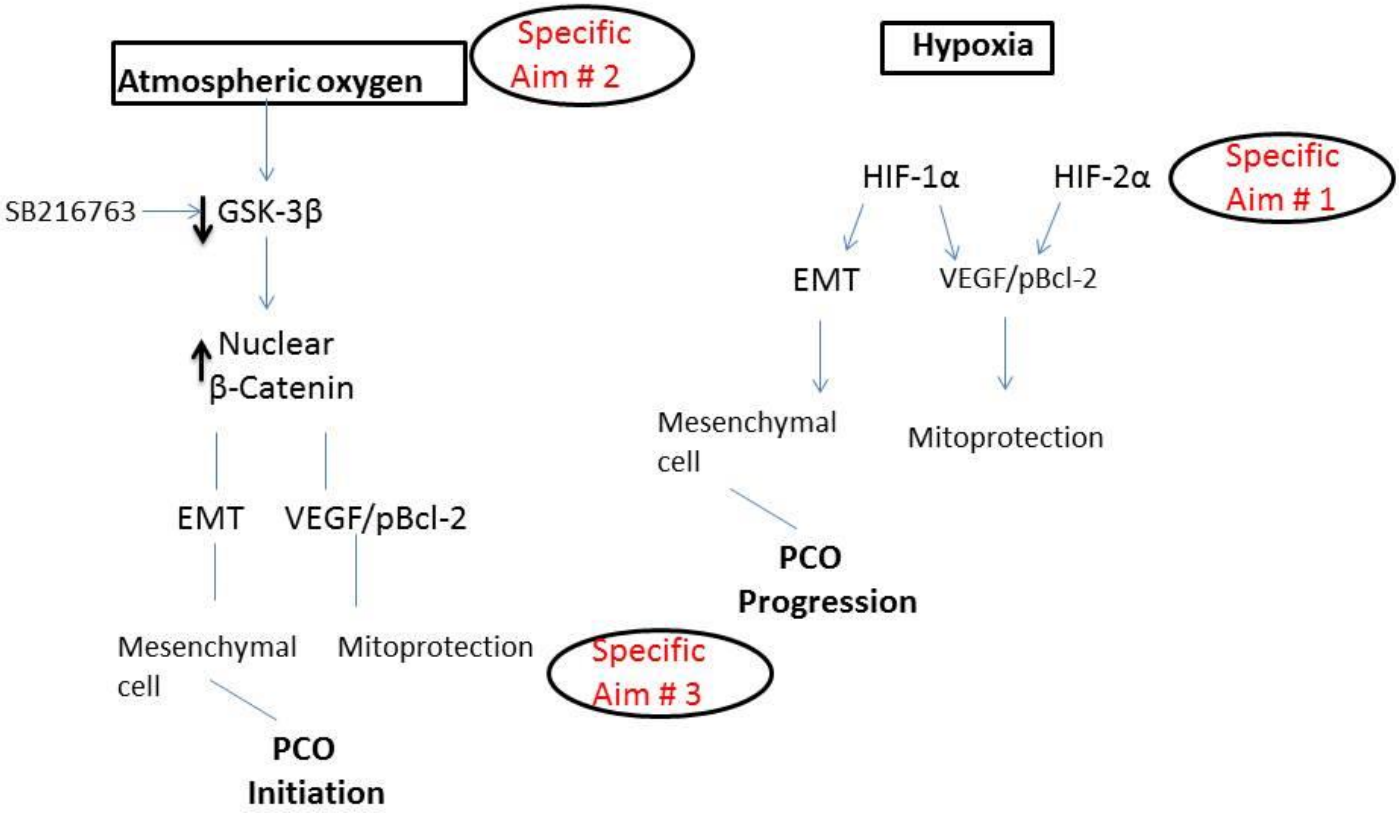
SUMMARY OF CHAPTER 3

INITIATION PROGRESSION



Exposure to atmospheric oxygen leads to inactivation of GSK-3β and activation of β-catenin. Active β-catenin through two parallel pathways leads to the expression of pro-survival VEGF and mesenchymal proteins. As a result, a mesenchymal cell resisting mitochondrial depolarization is generated leading to initiation of posterior capsular opacification in atmospheric oxygen. Once the lens is back in hypoxia under the influence of GKS-3β inhibition, the HIFs especially the HIF-1α continues the mesenchymal protein synthesis contributing to the progression of PCO in hypoxia. Utilizing the compensatory nature of two HIFs in hypoxia, the mesenchymal transition by HIF-1α can be inhibited by a HIF-1α translation inhibitor and HIF-2α will compensate for loss of HIF-1α in maintaining the VEGF levels providing resistance to mitochondrial depolarization.

OVERALL SUMMARY



A compensatory interrelationship between the two HIFs- HIF-1α and HIF-2α in the hypoxic lens epithelium maintains a sustained VEGF synthesis. VEGF acts a pro-survival factor by regulating the levels of anti-apoptotic pBcl-2 protein levels and resisting mitochondrial depolarization. Exposure of the hypoxic lens epithelium to atmospheric oxygen during cataract surgery activates a series of events which lead to inhibition of GSK-3β (SB216763) and increased translocation of β-catenin to the nucleus. Once β-catenin translocates to the nucleus through two independent parallel pathways it promotes the expression of mesenchymal proteins and VEGF synthesis. While the VEGF synthesis protects the lens epithelium

from mitochondrial depolarization, the mesenchymal proteins induce the epithelial to mesenchymal transition. The net result is the generation of a mesenchymal cell resisting depolarization leading to the initiation of Posterior Capsular Opacification (PCO). After the surgery, the lens epithelial cells are back in hypoxia and the HIF-1α under the influence of GSK-3β inhibition continues the expression of the mesenchymal protein, thus contributing to the progression of PCO in hypoxia. Based on our date we conclude that HIF-2α does not play a role in the expression of mesenchymal proteins in hypoxia. The presence of the hypoxia inducible factors – HIF-1α and HIF-2α in hypoxia can be utilized to our advantage in separating the two events of mesenchymal transition and mitoprotection. Inhibition of HIF-1α in the hypoxic lens could be a potential therapeutic target for PCO such that the mesenchymal transition can be prevented and HIF-2α will compensate for loss of HIF-1α and maintain the levels of VEGF without compromising the mitoprotection pathways.

SIGNIFICANCE:

The lens epithelium has developed several unique mechanisms which promote survival by resisting mitochondrial depolarization in the natural hypoxic environment and during exposure to atmospheric oxygen. The signal transduction mechanisms involved in the lenticular mitoprotective pathways provide resistance against mitochondrial depolarization by maintaining the levels of the pro-survival factor – VEGF. From a Clinical perspective, these pathways while providing resistance against mitochondrial depolarization also initiate the mesenchymal transition of the epithelial cells contributing to the pathophysiology of posterior capsular opacification (PCO). The initiation of PCO occurs in atmospheric oxygen under the influence of nuclear β-catenin which through two independent parallel pathways regulates both the mesenchymal transition and VEGF synthesis. This results in a mesenchymal cell resisting mitochondrial depolarization contributing to the initiation of PCO. Inhibition of nuclear β-catenin in atmospheric oxygen is not a beneficial therapeutic target, because while it can eliminate the expression of mesenchymal proteins, β-catenin inhibition also leads to loss of VEGF making the lens epithelial cells susceptible to mitochondrial depolarization.

The mesenchymal transition initiated in atmospheric oxygen is sustained once the lens epithelial cells are back in hypoxia under the influence of the hypoxia inducible factor- HIF-1α. The hypoxic lens epithelium resists mitochondrial depolarization by maintaining the levels of the pro-survival protein VEGF. The VEGF levels in hypoxia are regulated by a compensatory relationship between two HIFs – HIF-1α and HIF-2α. Since HIF-2α does not contribute to the mesenchymal transition, the compensatory relationship between two HIFs in hypoxia can be used to our advantage in separating the two events of mesenchymal transition and mitoprotection.

Inhibition of HIF-1α in hypoxia would prevent the mesenchymal transition of the epithelial cells and HIF-2α will compensate for the loss of HIF-1α and maintain the VEGF levels providing resistance against mitochondrial depolarization.

Exposure to atmospheric oxygen during ocular surgeries like vitrectomy is also known to induce the formation of cataracts. These findings suggest that not only during cataract surgery, but introduction of oxygen during ocular surgeries can lead to the activation of the TGF-β/Wnt β- catenin pathways. Despite rigorous surgical techniques PCO continues to be a serious post-surgical complication and inhibition of HIF-1α could be a potential therapeutic target which can prevent the mesenchymal transition and progression of PCO without affecting the mitoprotective pathways.

FUTURE DIRECTIONS

There is a remaining but very relevant aspect to this project that has not been addressed in the present study. During lens cataract surgery, atmospheric oxygen is briefly introduced to what would otherwise be the naturally hypoxic lens. The introduction of what may be interpreted as oxidative stress has been linked to the activation of growth factors like TGF-β (62). TGF-β promotes lens epithelial cell proliferation through the activation of Wnt/β-catenin pathway. Inhibition of GSK-3β, either by the activation of TGF-β/Wnt-β-catenin pathway or, in the case of this study, by the use of GSK-3β catalytic inhibition, leads to increased nuclear β-catenin. A significant role for β-catenin in lens epithelial and fiber differentiation has been established (65). In this study, we have demonstrated a role for β-catenin in lens epithelial cell survival via the induction of VEGF synthesis and downstream elevation in the pro-survival protein pBcl-2, thereby providing resistance to mitochondrial depolarization. Paradoxically, increased nuclear β-catenin through another independent pathway is also associated with the onset of posterior capsular opacification by initiating the mesenchymal transition. Of particular interest to the pathophysiology of PCO, however, is that the onset of posterior capsular opacification may occur within a few months to 5 years post-surgery (79). Put in perspective, this suggests that the brief introduction of oxygen during cataract surgery is the causative factor for the residual cells to migrate and proliferate under what would be a naturally hypoxic state. Studies in lens epithelial cells involving the mechanisms of PCO and expression of mesenchymal proteins were always conducted in atmospheric oxygen. The expression of mesenchymal proteins in the hypoxic lens and the role of HIFs in pathogenesis of PCO are not well understood.

Future studies will be geared towards understanding two important questions

(i) why the insult to lens epithelial cells during exposure to atmospheric oxygen is carried forward when the lens is back in its natural hypoxic state and

(ii) Understand how the VEGF levels are regulated in the normal lens without initiating the expression of EMT marker proteins.

To address these questions experiments will be conducted with normal cultured bovine lens epithelial cells passed through hypoxia to atmospheric oxygen and back again into hypoxia. We will then endeavor to determine whether and why the elevated nuclear β-catenin~epithelial to mesenchymal transition~elevated VEGF/Bcl-2 axis is maintained once the cells are returned to their naturally hypoxic environment, as well as why the pathological condition may be delayed by as much as five years post-cataract surgery.

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