METFORMIN INHIBITS EPITHELIAL-TO-MESENCHYMAL TRANSITION IN LENS EPITHELIAL CELLS

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ABSTRACT

Purpose: Posterior capsule opacification (PCO) is the most common complication after cataract surgery, affecting up to 50% of patients 2-5 years post-operatively. Epithelial-to-mesenchymal transition (EMT) is the main pathophysiology underlying PCO. Metformin, a drug with an excellent safety profile used primarily for diabetes, has been shown to suppress EMT. The objective of this study was to test the effectiveness of metformin in inhibiting EMT in an *in vitro* model of PCO.

Methods: The human lens epithelial cell (LEC) line HLE-B3 was exposed to transforming growth factor-beta (TGF-β) and fibroblast growth factor (FGF) to induce EMT. Subsequently, the effect of metformin on the following cellular parameters were determined: (1) survival, using a viability assay and drug concentrations ranging from 0-100 mM; (2) expression of epithelial (Pax6, E-cadherin) and mesenchymal (α-smooth muscle actin or α-SMA, fibronectin) markers via Western blot; (3) morphology, via microscopy and image analysis; (4) and migration, using the wound assay. The presence of the metformin uptake receptor SLC22A1 in the cell line and the ratio of active to inactive protein kinase B (pAkt/Akt) were assessed via Western blot. SLC22A1 expression was confirmed with immunohistochemistry on donor eyes. Statistical analysis of variance (ANOVA) with Tukey post-hoc test was done for analysis of cytotoxicity, morphology and migration data.

Results: Metformin is lethal to half (LC_{50}) and all cells at 30 and 80 mM, respectively. A decrease in viability ($P<0.05$) was noted at 5 mM. Compared to untreated cells, EMT-induced LECs treated

with 1 mM metformin showed increased Pax6 and E-cadherin and decreased α-SMA and fibronectin expression. LECs treated with metformin also maintained the roundness and circularity consistent with an epithelial phenotype. In addition, migration was significantly inhibited with 0.5 mM metformin ($P<0.05$). The HLE-B3 cell line and lens ocular epithelium in donor eyes were found to express SLC22A1, and treated HLE-B3 cells showed a decreased pAkt/Akt ratio.

Conclusion: Metformin inhibits EMT in LECs, decreasing survival and migration and maintaining the epithelial phenotype. These findings suggest that metformin entry into the cell is through the SLC22A1 receptor, and its action mediated via decreased Akt activation. Metformin thus has potential as an adjunct to treatment. Toxicity to proximal eye tissues and effectiveness *in vivo* must be tested to determine dose, route and timing of administration.

ABSTRACT (FRENCH)

Objectif: L'Opacification de la capsule postérieure (OCP) est la complication la plus courante suite à la chirurgie d'une cataracte et affecte environ la moitié des patients dans les 2-5 années suivant l'opération. Un phénomène appelé transition épithéliale-à-mésenchymale (TEM) est le principal mécanisme sous-jacent l'OCP. La metformin, un médicament principalement utilisé chez les diabétiques et ayant un excellent profil thérapeutique, est reconnue pour sa capacité d'inhibition de la TEM. L'objectif de cette étude est de vérifier et prouver l'efficacité de la metformin à inhiber la TEM dans un modèle *in vitro* d'OCP.

Méthodologie: Une lignée de cellules épithéliales du cristallin humain, HLE-B3, fut exposée à des facteurs de croissance bêta (TGF-β) ainsi que des facteurs de croissance de fibroblastes (FGF) afin d'induire la transition épithéliale-à-mésenchymale. Par la suite, les effets de la metformin ont été déterminés selon les paramètres suivants : (1) la survie, utilisant un test de viabilité et des concentrations de metformin allant de 0-100mM ; (2) l'expression de marqueurs épithéliaux (Pax6, E-cadhérine) et mésenchymaux (α-SMA, fibronectine) mesurée par Western-Blot ; (3) la morphologie, par microscopie et analyse d'image; (4) et la migration cellulaire, par le test de migration. La présence du récepteur de metformin SLC22A1 ainsi que le ratio de protéine kinase B active (pAkt/Akt) fut vérifiée dans la lignée cellulaire par Western-blot. L'expression de SLC22A1 fut aussi confirmée par immunohistochimie sur des yeux de donneurs *post-mortem*. Le test statistique d'analyse de la variance (ANOVA) ainsi que le test Tukey *post-hoc* ont été effectués sur les données d'analyse de cytotoxicité, de morphologie et de migration cellulaire.

Résultats: La metformin est létale pour la moitié (LC50) et la totalité des cellules à 30 et 80mM, respectivement. Un déclin de la viabilité ($p<0.05$) fut noté à 5mM. En comparaison aux cellules non-traitées, les cellules épithéliales de cristallin pour lesquelles la TEM a été induite et ensuite traitées avec 0.5mM de metformin, montrent une augmentation de l'expression de Pax-6 et E-cadhérine ainsi qu'une diminution de l'expression de α-SMA et fibronectine. Les cellules traitées avec la metformin maintiennent aussi leur rondeur et leur circularité, ce qui est consistant avec un phénotype de cellules épithéliales. De plus, la migration des cellules fut inhibée de façon significative avec 0.5mM de metformin ($p<0.05$). La lignée cellulaire HLE-B3 montre un déclin du ratio pAkt/Akt lorsque traités avec la metformin. Enfin, l'expression du récepteur à metformin SLC22A1 a été confirmée dans le modèle cellulaire *in vitro* ainsi que *in situ* avec des yeux de donneurs *post-mortem*.

Conclusion: La metformin a la capacité d'inhiber la TEM dans une lignée de cellules épithéliales de cristallin ; causant un déclin de la survie et de la migration de celles-ci, tout en maintenant leur phénotype original. En somme, ces résultats suggèrent que la metformin entre dans les cellules via le récepteur SLC22A1 et que son action est médiée par une baisse de l'activation de Akt. Ainsi, la metformin pourrait potentiellement être administrée en tant qu'adjuvant lors de chirurgies des cataractes. La toxicité au niveau oculaire et péri-oculaire ainsi que l'efficacité de la metformin restent à déterminer afin d'établir quelles doses, voies et fréquences d'administration seront à recommander.

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The Austrian philosopher Ludwig Wittgenstein wrote in 1971: *...And this is how it is: if only you do not try to utter what is inutterable then nothing gets lost. But the unutterable will be — unutterably — contained in what has been uttered!*

In that spirit, it is perhaps prudent to limit my acknowledgments to a few sentences and to generics, lest I forget someone. But woe on me if the depth and magnitude of my gratitude is not felt through a simple "thank you," and more so if I forget the people who have helped me!

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PREFACE

This is to certify that I have conducted all the experiments described in this thesis, under the supervision of Dr. Miguel Burnier and with the guidance of Dr. Denise Miyamoto, Dr. Pablo Zoroquiain and Dr. Patrick Logan. A manuscript originating from this thesis is under preparation for submission.

INTRODUCTION

A cataract is any opacity of the lens in the eye that, when it becomes clinically significant, causes blurred vision. It comes from the Greek word *katarraktes*, meaning waterfalls; the white opacities observed in the lens were named as such because the ancient Greeks originally thought that they resulted from humor cascading into the eye from the brain.[1]

Today, cataracts are the most common cause of reversible blindness worldwide, accounting for 51% of cases.[2] The World Health Organization (WHO) estimates that around 20 million individuals are affected globally,[2] and with the aging population worldwide, this number is expected to increase.

Fortunately, visual deterioration secondary to cataracts can be remedied through surgical intervention. There are a number of suitable operative techniques, but the most common method is phacoemulsification with subsequent insertion of an artificial intraocular lens (IOL). Briefly, phacoemulsification is performed by breaking the cataract into smaller pieces with ultrasonic energy followed by subsequent aspiration of these fragments. A new IOL is then inserted to replace the focusing power of the extracted lens, and the capsule is polished to remove residual lens epithelial cells (LECs) and other cell remnants.[3]

Cataract surgery is generally a safe and effective procedure that almost immediately restores vision. Within 2-5 years, however, visual decline recurs in 20%-40% of patients due to the development of posterior capsule opacification (PCO), also called a secondary cataract. PCO is the most common complication of cataract surgery[4] and manifests as deposits on the posterior capsule visible on lens retroillumination. The deposits affect light transmission and cause blurred vision.[3]

PCO treatment involves the use of a neodymium yttrium aluminum garnet (Nd-YAG) laser to perform a capsulotomy. With this procedure, short pulses of laser energy are delivered through the IOL to destroy the posterior capsule and create a clear path for light along the central visual axis. Nd-YAG capsulotomy is associated with infrequent but serious adverse events, which include retinal detachment and IOL dislocation, as well as corneal and macular edema.[3] This technology is also not readily available in undeveloped and developing countries. While available in developed economies it exerts a tremendous financial burden on the health care system.[5] Thus, strategies to prevent PCO are at the forefront of ophthalmology research.[4]

The pathophysiology underlying the development of PCO involves the residual LECs on the anterior capsule that, perceiving cataract surgery as an injury, attempt wound healing. The cells thus proliferate and migrate posteriorly to the intact capsule and secrete proteins to form an extracellular matrix. This collective response is called epidermal-to-mesenchymal transition (EMT) because the LECs lose their intrinsic properties and acquire those of mesenchymal cells.[6]

EMT is a biological process that is necessary for organogenesis and is a key mediator in cancer proliferation and metastasis.[7] Metformin, a biguanide drug used primarily in the treatment of diabetes, has been thrust into the spotlight for its observed activity against different cancers both *in vitro* and *in vivo.* Metformin has particularly been noted for its ability to inhibit proliferation and EMT[8] through a myriad of mechanisms, some of which have also been implicated in the development of PCO.[4]

With this synthesis of available knowledge, it is reasonable to ask: can metformin inhibit PCO?

This study aims to answer this question by utilizing an *in vitro* model of PCO, wherein LECs from the cell line HLE-B3 will be treated with growth factors to induce EMT.[9,10] The specific objectives of this study are as follows:

(1) The presence of the solute carrier family 22 member 1 (SLC22A1) protein in the HLE-B3 cell line will be determined. SLC22A1 is the cellular uptake receptor for metformin,[11] and its expression will suggest the ability of the cells to respond to the drug.

(2) The effect of metformin on cellular hallmarks of EMT will be evaluated. These parameters include the following: (a) survival as an index of proliferation; (b) a decrease in the expression of epithelial proteins in favor of mesenchymal markers; (c) a change towards spindle-shaped morphology; and (d) an increase in migratory potential.[12,13]

(3) Should metformin be demonstrated to impact cellular outcomes, the latter experiments in this study will seek to gain insight into its mechanism of action. This will be accomplished by identifying the effect of metformin on the expression of the SLC22A1 receptor and on the ratio of active to inactive protein kinase B (Akt). Akt is a key signaling protein through which metformin exerts its effects.[11]

(4) To verify the possible impact of this study's findings in a clinical setting, the presence of SLC22A1 in LECs will also be determined in anterior sections of donor eyes.

Current strategies to prevent PCO focus on IOL design and surgical techniques, but this approach has not produced significant results. Drugs tested to be successful against PCO, such as chemotherapeutic and anti-proliferative agents, are limited by expense, availability or narrow therapeutic indices.[4] In comparison, metformin is a promising candidate drug because of its established excellent safety profile and low cost.[11]

Metformin has never been tested on LECs in the setting of PCO prevention and treatment. Given the evidence in the literature demonstrating that metformin inhibits EMT in other cell types and with the knowledge that the EMT underlies PCO formation, it is imperative that experiments testing the effect of metformin on EMT in LECs be conducted. Should *in vitro* studies be satisfactory, metformin can thereafter be tested *in vivo* to determine the best route and timing of administration. This study is therefore essential in laying the groundwork for the possible clinical delivery of an effective and efficient intervention against PCO.

REVIEW OF RELATED LITERATURE

The objective of this study is to evaluate the effect of metformin on lens EMT in an *in vitro* model of posterior capsular opacification (PCO). To better frame this objective in the context of existing scientific evidence, this chapter will therefore review the following: (1) the anatomy and physiology of the lens, particularly the epithelium; (2) cataract development and surgical management; (3) the pathogenesis, experimental models, and prevention and treatment strategies for PCO; (4) EMT; (5) the pharmacokinetics and mechanisms of action of metformin; (6) studies performed on metformin and eye disease; (7) and the link between diabetes and PCO.

The Lens

Galen, the Greek philosopher (121-217 AD) who served as physician to the Roman emperors, wrote in detail about the anatomy and physiology of the eye. Although he wrongly espoused the theory of extramission, which stated that the eye directed light rays outward to enable man to see, he recognized the importance of the lens in vision.[14] He writes:

> "…the crystalline lens is the principal instrument of vision, a fact clearly proved by what physicians call cataracts, which lie between the crystalline humor and the cornea and interfere with vision until they are couched."[14]

Today, it is known that the lens is a crystalline, transparent, asymmetric oblate spheroid located in the anterior segment of the eye, posterior to the iris. It is suspended in place by zonular

fibers, which arise from the ciliary epithelium and insert into the lens capsule. The lens can be analyzed histologically as constituting of three parts: the outermost capsule, the lens epithelium and the lens substance. The lens capsule is acellular and is composed primarily of structural proteins and fibronectin. These proteins are secreted by the lens epithelium, a single layer of cuboidal cells on the anterior capsule. The lens epithelium divides throughout life, with greater proliferative capacity noted at the equator of the spheroid, and the newly-formed cells are pushed inward as they elongate, lose organelles and differentiate into what are now referred to as fiber cells. Thus, the lens substance has minimal extracellular space, being composed of the newest fibers at the outer cortex and the oldest fibers at the densely packed nucleus.[3]

Devoid of blood vessels, nerve and connective tissue, the lens relies on nutrition from both the aqueous and vitreous humor. The majority of glucose and amino acids come from the aqueous; these, along with electrolytes and proteins up to 70 kiloDaltons (kDa) can enter freely into the cells. Moreover, the cells also contain various channels and transporters for uptake of other nutrients. The energy requirement of the cells are met with adenosine triphosphate (ATP) produced from anaerobic metabolism of glucose via utilization of glucose-6-phosphate (G6P) through either the glycolytic or pentose phosphate pathway.[3]

The lens is primarily responsible for the transmission and refraction of light entering the eye for optimal focusing on the retina. Its refractive power corresponds to two-thirds of the total optical power of the eye, with one-third attributed to the cornea. To minimize spherical aberration, the defocus caused by the greater refraction of light in the periphery, the lens possesses the following features: the refractive index increases towards the center secondary to the compacted fibers; the curvature of both the anterior and posterior capsule increases towards the periphery, with the anterior capsule greater in curvature than the posterior surface; and the control of pupil

size prevents light from striking the periphery in non-mydriatic conditions. The zonular fibers that support the lens also enable it to change its shape to accommodate vision, maintaining focus even between near and distant objects.[3]

Because the lens is continuously subjected to radiation, particularly in the ultraviolet spectrum (295-400 nm), it is imperative that the lens has adequate defense against the generated free radicals and reactive oxygen species (ROS). To this end, the lens is equipped with an antioxidant system that uses enzymes such as superoxide dismutase (SOD) and glutathione peroxidase to protect itself.[3] The crystalline proteins, which make up 90% of the protein content of the lens, have also been hypothesized to enhance its ability to withstand stress through mediation of protein-protein interactions, cytoskeletal remodeling and inhibition of apoptosis.[15]

In view of the fact that the lens epithelium is the most metabolically active part of the lens, it is worthwhile to consider its molecular biology.

The Lens Epithelium

Resting on a thick basement membrane that is the anterior capsule, the lens epithelium is characterized by differences in proliferative potential. The anterior portion is quiescent, while the cells at the germinative zone of the lens equator have greatest proliferative capacity; putative stem cells also reside here, although no specific marker has been reported. Once the epithelial cells migrate to the posterior capsule, they differentiate into columnar cells. Inward migration, meanwhile, results in loss of organelle and nuclei.[16]

Lens epithelial cells (LECs) are cuboidal and are linked through apical cell-cell junction complexes, which include tight, gap and adherens junctions. These connections are responsible for maintaining epithelial integrity, as well as modulating gene expression and activities like cellular

proliferation and differentiation. Tight junctions express the usual protein markers of claudin, occludin and zonula occludens-1 (ZO1).[17] Other protein complexes, such as Par3/6, are crucial in establishing apical-basal polarity, while PDZ proteins that share the common structural PDZ domain (PDZ: Post-synaptic density protein, Drosophila disc large tumor suppressor, ZO-1) in the plasma membrane act as scaffolds for macromolecule assembly.[16] Gap junctions contain connexins for intercellular communication. Cx50, the connexin present in both LECs and fiber cells, is known to mediate cell proliferation through electrical coupling. Cx50 expression is upregulated in response to fibroblast growth factor (FGF) through the mitogen activated protein kinase (MAPK)-extracellular receptor kinase (ERK) pathway, as well as through the bone morphogenetic protein (BMP) pathway.[16] Adherens junctions are composed of intercellularly linked cadherins, which are calcium-dependent transmembrane proteins. There are different types of cadherins that predominate depending on the developmental stage of the lens. In the embryo, during morphogenesis, E- and P-cadherins are expressed in the lens placode; only E-cadherin remains as the vesicle is formed. Later in life, E-cadherin expression is limited to the epithelial cells, while N-cadherin can be found in both LECs and fiber cells. The loss of E-cadherin is associated with epithelial-to-mesenchymal transition (EMT), and gene mutation studies show impact in the later stages of this process.[16] The cytoplasmic domains of E-cadherin bind to β-catenin and through this interaction affect cytoskeletal reorganization.[7] Interestingly, LECs also highly express intermediate filament proteins such as vimentin, sometimes used as a marker of EMT; the intermediate filament proteins interact with the cadherins at the adherens junctions.[16]

Many transcription factors have been identified in lens induction and morphogenesis. Of primary importance is the highly conserved Pax6. It is highly expressed in the ectoderm during lens induction but is restricted to the epithelium later on, rapidly downregulated during fiber cell

differentiation. Two alternatively-spliced variants of Pax6 exist: Pax6 and Pax6(5a). While both promote fiber cell differentiation, each regulate different genes.[16] Pax6(5a) has been shown to activate expression of fibronectin in epithelial cells, driving EMT.[18] The relationship between Pax6 and transforming growth factor-beta (TGF-β) is less clear. In the developing lens, Pax6 and TGF-β exhibit similar expression patterns. Haploinsufficiency of Pax6 has been shown to decrease endogenous TGFβ, suggesting that the latter is required for the development of normal lens epithelium.[16] However, TGF-β-induced cataract models have demonstrated reduced Pax6 expression, in concordance with induction of EMT.[7]

Multiple growth factors and signaling pathways have also been demonstrated to regulate the lens epithelium. Intracellularly, these include the receptor tyrosine kinases (RTKs), TGF-β superfamily, Wnt, Notch and Salvador-Warts-Hippo pathways; matrix interactions are mediated through integrins and matrix metalloproteinases (MMPs). Mitogenic growth factors, such as FGF and insulin growth factor (IGF), have been shown to promote proliferation and differentiation of LECs *in vitro* via the MAPK and PI3/Akt (phopatidylinositol-3/protein kinase B) pathways. While growth factors are present in both the aqueous and vitreous humor, there exists an increasing concentration gradient from anterior to posterior. This explains the quiescent nature of LECs at the anterior pole; proliferation at the peripheral germinative zones; and differentiation into fiber cells at the posterior pole.[16] The Wnt/β-catenin and Notch pathways, meanwhile, are considered essential for maintaining the LECs, particularly the stem cells. The TGF-β pathways, whether Smad-dependent or independent downstream, regulate LEC development; however, as mentioned, these are also responsible for inducing EMT, leading to the development of anterior subcapsular cataracts (ASC) and posterior capsular opacification (PCO).[16]

The anterior capsule, which is also the LEC basement membrane, consists of collagen IV and matrix proteins such as laminin, fibronectin, tenascin and proteoglycans. Signaling in the extracellular matrix (ECM) rely on the integrins, which are transmembrane glycoproteins necessary for LEC survival. Integrins are upregulated in EMT and are thought to interact with α-smooth muscle actin (α-SMA) in the formation of stress fibers.[16] Integrin signaling also induces the formation of focal adhesion complexes, which aid cell mobility.[7] During EMT, MMPs have also been noted to be elevated and are thought to dissolve the ECM, thereby facilitating LEC migration.[16]

Understanding the molecular processes governing LEC development and behavior, especially of LECs, provide insight into the pathophysiology of cataracts and PCO. These will be considered in the next sections.

Cataracts

A cataract is any opacity in the lens of the eye. Senile cataracts are the most common type, resulting from age-related decrease in lens transparency. There are different types of age-related cataracts depending on their location in the lens: nuclear, cortical and posterior subcapsular (PSC). Nuclear cataracts come from an increase in the optical density of the nucleus, followed by changes in color from clear to yellow to brown (brunescence). Cortical cataracts, meanwhile, result from the hydration of the cortex leading to the development of vacuoles; the formation of ray-like spaces that later become opaque; the lamellar separation of the cortex from linear opacities; and the formation of opacities from the periphery that grow towards the center. Posterior subcapsular cataracts, present singly or in association with other types, begins at the pole and move toward the periphery. These can present as either granules or vacuoles on the capsule.[3]

While genetics mainly influence the incidence of congenital cataracts, age is the major risk factor for senile cataracts. Other identified risk factors that may predispose to early cataracts include systemic diseases, such as diabetes and hypertension; trauma; sunlight and irradiation; smoking and alcohol use; toxic substances such as steroids and long-acting anticholinesterases; and concurrent ophthalmic conditions like myopia and uveitis.[3] Lens transparency depends on organization of the cells and the arrangement of proteins in the lens substance. The accepted pathophysiology underlying nuclear cataractogenesis is that as the cells divide and migrate inward, they lose their organelles, and proteins precipitate and form aggregates. The mechanisms governing these changes, however, as well as the other kinds of cataract, are still being elucidated.[19]

Several mechanisms have been implicated thus far; growth factors produced in the aqueous, failure of osmotic regulation, protein modifications and oxidative stress all play a role in cataract formation. Growth factors secreted by the ciliary epithelium, such as FGF and TGF-β, stimulate proliferation and differentiation of the LECs. Interestingly, it was also found that when there is increased intracellular calcium ions (Ca^{2+}) relative to its normal proportion to extracellular Ca^{2+}, LEC differentiation is altered and PSCs develop due to disturbances in endoplasmic reticulum signaling.[20] Lens transparency has been shown to rely heavily on the orientation and chemistry of the crystalline proteins: modifications such as methylation, acetylation, and glycation can cause discoloration, and subtractive alterations such as cleavage by calpains can cause denaturation and precipitation.[21] Finally, oxidative stress from light and from decreased antioxidant enzyme activity result in predominance of ROS in the aqueous, interacting with proteins and resulting in aggregate opacities, particularly in the cortex.[3]

The effect of cataracts on vision depend on their degree, morphology and location. The most common symptom associated with cataracts is blurring or cloudiness of vision, while other symptoms may include contrast sensitivity reduction, a myopic shift (nearsightedness), monocular diplopia (double vision), glares, shifts in color vision and, rarely, visual field loss.[22] Pharmacological strategies to prevent cataractogenesis have not yet been proven effective in a clinical setting. Drugs that have previously been tested include aldose reductase inhibitors, ibuprofen, antioxidant supplements such as ascorbate, beta-carotene and vitamin E, and estrogen replacement therapy.[3] Lanosterol, an amphipathic molecule produced in the lens and found to be absent in cases of congenital cataracts, has been shown to decrease opacities *in vivo* as tested in rabbits and dogs. Its effectiveness has yet to be tested in clinical trials.[23]

Cataracts require surgical intervention. It is generally agreed that the primary indication for surgery is functional visual impairment. Different surgical techniques exist subject to the available technology and to any co-existing eye pathology. Uncomplicated cataract cases, however, are generally managed with phacoemulsification followed by artificial intraocular lens (IOL) insertion. Phacoemulsification is performed with small incisions on the sclera and cornea, ranging from 1.9-3.2 mm; these are often self-sealing and sutureless. A continuous curvilinear capsulorrhexis is made on the anterior capsule, approximately 4.5-5.5 mm in diameter, which should be sufficient to cover the edges of the IOL haptics for proper placement “in the bag.” The cortex is then separated from the nucleus through hydrodissection, and the epinuclear shell is marked from the endonucleus through hydrodelineation. The phacoemulsification handpiece contains an ultrasonic transducer; the mechanical vibrations break the lens nucleus into smaller pieces or an emulsion, which are afterwards aspirated. The cortex is then irrigated, cleaned and aspirated, the IOL is inserted, and any external liquids injected — such as viscoelastics — are

removed completely. Any incisions made are sealed, and checks are performed to ensure there is no leakage.[3]

The most common complication of cataract surgery is the development of PCO. Other complications of cataract surgery can be categorized into intraoperative and postoperative. Potential intraoperative complications are numerous, but relatively infrequent. These include, in order of chronological incidence during surgery: detachment of the Descemet's membrane; thermal burns; tears during capsulorrhexis; iris prolapse; a trapped nucleus; subluxated lens, rupture of the posterior capsule, which may also lead to a dropped nucleus. Anterior segment hemorrhage may also occur at any point, blocking the surgeon's view. All these complications, however, can be solved with adjustments in surgical techniques. Post-operative complications, meanwhile, may affect any part of the eye and include the following: wound dehiscence and leakage; epithelial ingrowths; surgical astigmatism; corneal edema and bullous keratopathy; hyphema; increased intraocular pressure; lens decentration and dislocation; capsular block syndrome secondary to retained viscoelastics; cystoid macular edema; endophthalmitis; and retinal detachment.[3]

Despite this extensive list of potential complications, cataract surgery is considered to be very safe and effective. Vision improvement can occur as early as 24 hours after surgery, and patients are usually discharged on the same operative day. In an analysis of 90 studies looking at postoperative visual outcomes and complications, the percentage of eyes with visual acuity of 20/40 was 89.7% and 95.5% for all eyes and for eyes with co-existing morbidity, respectively. In addition, the incidence of sight-threatening complications was less than 2%.[24]

Posterior Capsule Opacification (PCO)

The most common post-operative complication after cataract surgery is the development of PCO. Also called a "second cataract," PCO results in decreased visual acuity and requires additional intervention.[4] Broadly speaking, PCO develops from the proliferation and migration of residual LECs after cataract surgery in response to surgical-induced trauma on the anterior capsule; the main mechanism governing this pathology is called EMT. Visual loss is due to two possible mechanisms: (1) the formation of swollen LECs called Elschnig's pearls that migrate onto the posterior capsule and block the visual axis; and (2) the transformation of LECs into myofibroblasts, which induce contraction and wrinkling of the posterior capsule.[3] The pearls and wrinkled streaks on the posterior capsule can easily be observed upon retroillumination on slit-lamp examination.[22]

The time lapse between surgery and PCO development varies from months to years. It is more common in younger individuals, in whom LECs have greater proliferative capacity. The incidence of PCO varies among studies, with values ranging from 3%-50% in the first 2-5 post-operative years in adults, and nearly 100% in children.[3,5] Other identified risk factors for the formation of PCO include uveitis and traumatic cataracts, while decreased risk has been noted for hydrophobic acrylic and squared IOLs. The risk of developing PCO after cataract surgery has not been found to be influenced by gender or axial length.[25]

Treatment of PCO necessitates the use of neodymium: yttrium-aluminum-garnet (Nd:YAG) laser capsulotomy. This technique utilizes a solid-state laser with a wavelength of 1064 nm that breaks down tissues via ionization with a high-energy pulse; this ionization results in shock waves that further disrupt tissue. In most cases, this is an uncomplicated procedure. However, some complications may arise, such as an acute increase in intraocular pressure, which can easily be managed with glaucoma medications if necessary. Other rare but infrequent adverse events

include retinal detachment, macular edema, corneal edema, iris hemorrhage and IOL subluxation.[3,5] In addition to the aforementioned side effects, this type of treatment exerts a financial burden on the public health care system. Also, in less developed countries, the technology is not readily available.[5]

Prevention of PCO is an active area of research, with the problem being approached through various strategies, such as improving surgical techniques, IOL materials and design and pharmacologic therapy.[5] There are general measures that the ophthalmic surgeon can employ to reduce the risk of developing PCO. These include the following: hydrodissection-enhanced cortical cleanup to ensure that there are no remnants of the lens cortex; ensuring that the IOL is fixed inside the capsular bag; and creating a surgical opening on the anterior capsule (capsulorrhexis) that is smaller than the IOL optic.[3] Research in IOL materials and design have brought a consensus that three factors must be maintained. First, the IOL material must be biocompatible to reduce induction of proliferation. Second, there must be maximum contact between the IOL optic and the posterior capsule, presumably to leave no space for LECs to migrate and proliferate.[5] Finally, an IOL with a square, truncated optic edge also discourages LEC activity through contact inhibition;[26] a meta-analysis of 23 randomized clinical trials confirmed that sharp-edged acrylic and silicone IOLs are superior in lowering incidence of PCO and Nd:YAG treatment.[27]

Numerous therapeutic agents have been tested *in vitro* and *in vivo* in attempts to discover a drug that can destroy residual LECs without causing damage to the surrounding ocular tissues. Different methods of administration have also been employed, such as direct injection to the anterior chamber, addition of the drug to the irrigating solution, or impregnating the IOL for slow release. Anti-metabolites such as methotrexate, mitomycin, daunomycin and 5-fluorouracil and

anti-inflammatories such as diclofenac and cyclooxygenase inhibitors, while effective *in vitro*, have shown to be damaging to the eye *in vivo*.[4,5,28,29] Compounds that specifically inhibit key signaling molecules implicated in the formation of PCO have also been tested both *in vitro* and *in vivo*. These include the following: the anti-fibrotic drug pirfenidone against TGF-β and Smads;[30] the anti-dyslipidemic drug lovastatin against Rho;[31] proteosome inhibition, which suppresses FGF;[32] the disintegrin salmosin to antagonize integrins;[33] and ethylenediaminetetraacetic acid, caffeic acid phenyl ester (CAPE) in rabbit lenses, nobiletin, and secreted protein acidic and rich in cystein (SPARC) inhibitors, which interfere with MMPs.[4,34-36] The disadvantage of inhibiting a single molecule is obvious: the development of PCO is a convergence of pathways. Integrins and MMPs as therapeutic targets are a more holistic approach but inherently increase the risk of affecting other ocular tissues, resulting in corneal edema *in vivo*, limiting clinical application.[4]

Newer strategies of PCO prevention involve interference with epigenetic mechanisms and non-pharmacological methods such as gene therapy, induction of osmotic changes to destroy residual LECs, nanomaterials and lens regeneration. Zebularine, a DNA methyltransferase inhibitor, has been shown to prevent LEC proliferation and migration *in vitro*.[37] Histone deacetylase inhibitors such as trichostatin A and vorinostat have also been shown to inhibit EMT in LECs.[38,39] The effect of manipulating epigenetic mechanisms, however, has yet to be observed in other ocular tissues.[4] Gene therapies that have been tested thus far and have shown promise include siRNAs for Snail, a molecule downstream of the TGF-β pathway, as well as for the TGF-β receptor.[40,41] Suicide gene therapy that code for apoptosis, using lentiviruses and adenoviruses, have also been found to inhibit PCO *in vitro* and in rabbits, respectively.[42,43] Despite these results, ethical and biological limitations arise. Gene therapy can induce an immune response, which is

already in full swing due to cataract surgery. The difficulty of the technique and the specificity it requires also render it impractical for a procedure as commonly performed as cataract surgery.[4]

Initiating cell death through osmotic pressure, whether through hyperosmotic or hypoosmotic agents, have also been tested with success *in vitro*, *ex vivo* and *in vivo* on LECs.[44,45] To prevent the effect of the drug on other ocular tissues, sealed-capsule irrigation of the capsular bag was performed with conflicting results as to prevention of PCO as a final outcome. A prospective clinical trial with 17 patients, each serving as their own control and having only one eye treated, showed no significant difference in reduction of PCO rates 24 months after surgery.[46] Another prospective clinical trial involving 60 patients, with separate treatment and control groups, found decreased PCO over a 24-month follow-up. This, however, was concomitant with increased endothelial cell loss.[47] This issue notwithstanding, whether hyperosmotic or hypoosmotic agents work better has also yet to be conclusively determined.[4]

Nanomaterials in themselves and as drug delivery systems have also been tested. Zinc oxide particles, in combination with UVB irradiation, for instance, have been shown to inhibit LEC proliferation.[48] A lipid nanoparticle delivery system using genistein, which inhibited LEC growth through tyrosine kinase block, was demonstrated to deliver effective sustained release.[49] Nanofibers designed to coat the inner capsular bag as well as IOL, whether or not impregnated with drugs, is another potential strategy to be tested.[4]

Despite the progress made in testing a variety of methods for PCO prevention, it is the consensus that meticulous surgical techniques and use of a square-edged IOL are most effective clinically. The ideal method to target LECs is unknown, and has it never been resolved whether eradication of the LECs are better than mere maintenance of an epithelial phenotype.[4] A study using an in vitro organ culture model has shown that total LEC destruction can lead to problems

with IOL fixation in the capsular bag. It has been suggested that fibrosis caused by LECs are essential for preventing IOL dislocation, and that there may be an appropriate time for PCO prevention strategies, between fibrosis and overt PCO formation.[50]

The plethora of pharmacotherapeutics tested against LECs have been shown to impact one or more pathways implicated in PCO development and have contributed to a better understanding of its pathophysiology. Essentially, during PCO, LECs proliferate, migrate to the posterior capsule and secrete an extracellular matrix. This collective response is called EMT, and available evidence points to this as the principal mechanism underlying PCO.[3]

EMT

EMT is the process by which epithelial cells lose their intrinsic properties and acquire those of mesenchymal cells. Characteristics of mesenchymal cells include increased proliferative and migratory capacity and the secretion of proteins into an extracellular matrix. It is a tightly regulated biological process necessary for normal embryogenesis, occurring during gastrulation and it allows the formation of mesoderm.[7] EMT can be pathologic, however, and is implicated in the initiation of cancer metastasis. In cancer, epithelial tumor cells acquire a mesenchymal phenotype, stray loose from the primary tumor and migrate to seed into other sites. The increase in TGF-β that favors EMT also favors angiogenesis, enabling cancer spread.[51] EMT also follows tissue injury and contributes to fibrosis and healing.[52]

The key pathways mediating EMT have been established after numerous *in vitro* and *in vivo* studies. Epithelial cells have apicobasal polarity and are linked together by tight junctions through zonula occludens and claudins, adherens junctions through E-cadherins, and desmosomes.[52] Epithelial cell markers, therefore, include E-cadherin, occludins and cytokeratins.

One of the earliest events in EMT is the disassembly of tight junctions alongside the disruption of cell polarity and cytoskeletal reorganization.[53] The loss of E-cadherin and the upregulation of N-cadherin is also considered a hallmark of EMT, although it alone is not sufficient.[54] Likewise, dissolution of desmosomes is a manifestation of EMT; its timing and role in the chronology of EMT, however, has yet to be terminated.

Accompanying disintegration of epithelial connections is the expression of mesenchymal cytoskeletal proteins, such as vimentin α-SMA, and increased deposition of extracellular matrix proteins like collagens and fibronectin.[7] Extracellular matrix proteins in particular are essential for interacting with integrins and forming focal adhesion complexes that facilitate cell migration.[55] The elevation in matrix metalloproteinases also benefit cell mobility and migration to the other tissues.[56]

The alterations in gene expression are mediated by at least three families of transcription factors, including the Snail family, ZEB family and basic helix-loop-helix (bHLH) family.[57] Epigenetic changes include modification of histone proteins and DNA promoter regions with microRNAs.[58] Post-translational modification of proteins such as methylation, acetylation, phosphorylation, glycosylation, hydroxylation and SUMOylation enhance the diversity of the proteome expression profile and renders it difficult to target a native protein for therapeutics.[59]

In the midst of these challenges, there is a general consensus on the inducers and pathways involved. TGF-β has been identified as the primary inducer, interacting with the TGF-β and the bone morphogenic protein (BMP) receptors, activating both Smad-dependent and Smad-independent pathways.[7] Other growth factors, such as FGF, activate many non-SMAD pathways in cross-talk. Activation of the RAS-Erk-MAP kinase pathway propagates the activation of transcription factors towards downregulation of epithelial proteins and upregulation of

mesenchymal proteins.[60] The JNK MAP kinase has also been shown to be required for TGF-β induced EMT, by primarily affecting the cytoskeletal reorganization in EMT.[61] Rho-like GTPases, which hydrolyze guanosine triphosphate (GTP) and which are composed of the Rho, Rac and Cdc42 subfamilies, are also activated in EMT. The effector kinase is ROCK, the activity of which is cytoskeletal reorganization, particularly the formation of actin stress fibers.[62] Tyrosine kinase receptors are also stimulated, particularly the phospatidyl-inositol-3 kinase/protein kinase B (PI3/Akt) pathway.[63] Akt is a central regulator of downstream mechanisms involved in cell survival, cell size control via the molecule mechanistic target of rapamycin (mTOR), proliferation and migration.[64]

Cross-talks with the NF-κB, Notch and Wnt pathways also exist. NF-κB is increased in inflammation, highlighting its importance in fibrosis and cancer.[52] Notch signaling is highly localized, occurring between cells with Notch receptors and specific ligands of Delta or Delta-like (Serrate or Jagged) configurations. Activation of the Notch pathway has been associated with increase in Snail and in Smad3 expression, thereby indirectly promoting EMT.[65] The canonical Wnt pathway is arbitrated chiefly by β-catenin, which is also a component of the adherens junction and links E-cadherin to the cytoskeleton.[7] Activation of Wnt results in accumulation of β-catenin in the cytoplasm, allowing it to complex with transcription factors that also upregulate Smads 2 and 4.[66]

EMT in the Pathophysiology of PCO

An increase in inflammatory cytokines and growth factors has been observed following cataract surgery. Tumor necrosis factor-alpha (TNF-α) and prostaglandins, for example, modulate the tissue response to injury.[67] TGF-β, particularly isoform TGF-β2, which is already highly

expressed in the aqueous, becomes activated from its latent form and exists in elevated amounts post-operatively.[68] Interestingly, TGF-β2 alone has a suppressive effect on LEC proliferation.[69] In combination with FGF, however, which is also elevated in the aqueous after surgery, LECs proliferate.[70] Other growth factors that have been identified to promote EMT in LECs include epithelial growth factor (EGF), insulin growth factor (IGF-1), platelet-derived growth factor (PDGF) and connective tissue growth factor (CTGF).[71,72] Integrins likewise are essential in the development of PCO; they are regulated by the integrin-linked kinase (ILK), binding to the β-subunit of integrin and to another growth factor receptor.[73] Tenascin C, an extracellular matrix protein that interacts with fibronectin, is also elevated and is critical for injury-induced EMT in the lens epithelium.[74]

A multitude of pathways are activated in EMT to bring about the cellular outcomes seen in PCO: LEC proliferation, transdifferentiation into mesenchymal cells, formation of actin stress fibers, matrix remodelling and LEC migration (Figure 1). These pathways involve the Smad and non-Smad pathways; Wnt, Rho, Ras and Notch; and further downstream, PI3K/Akt, MAPK and ERK1/2.[4,6,12,13,75,76]

Epigenetic changes are also mediated through increases in histone deacetylases (HDACs), particularly from isoforms I and II.[39] The CD44/hyaluronan pathway is also activated in EMT; in an *ex vivo* experiment, a dose-dependent rise in LEC proliferation and subsequent PCO formation was noted alongside an increase of hyaluronan and CD44.[77] Viscoelastics used to maintain the anterior chamber during surgery contain hylauronan. One study compared the rates of PCO in cataract surgery patients in whom an air bubble was injected and in whom viscoelastics were used; a higher occurrence of PCO was noted in the latter.[78] As minimal amounts of viscoelastic may be

retained after surgery, the effect of the various kinds of viscoelastics and viscodispersives on the incidence of PCO may be worthwhile investigating.[4]

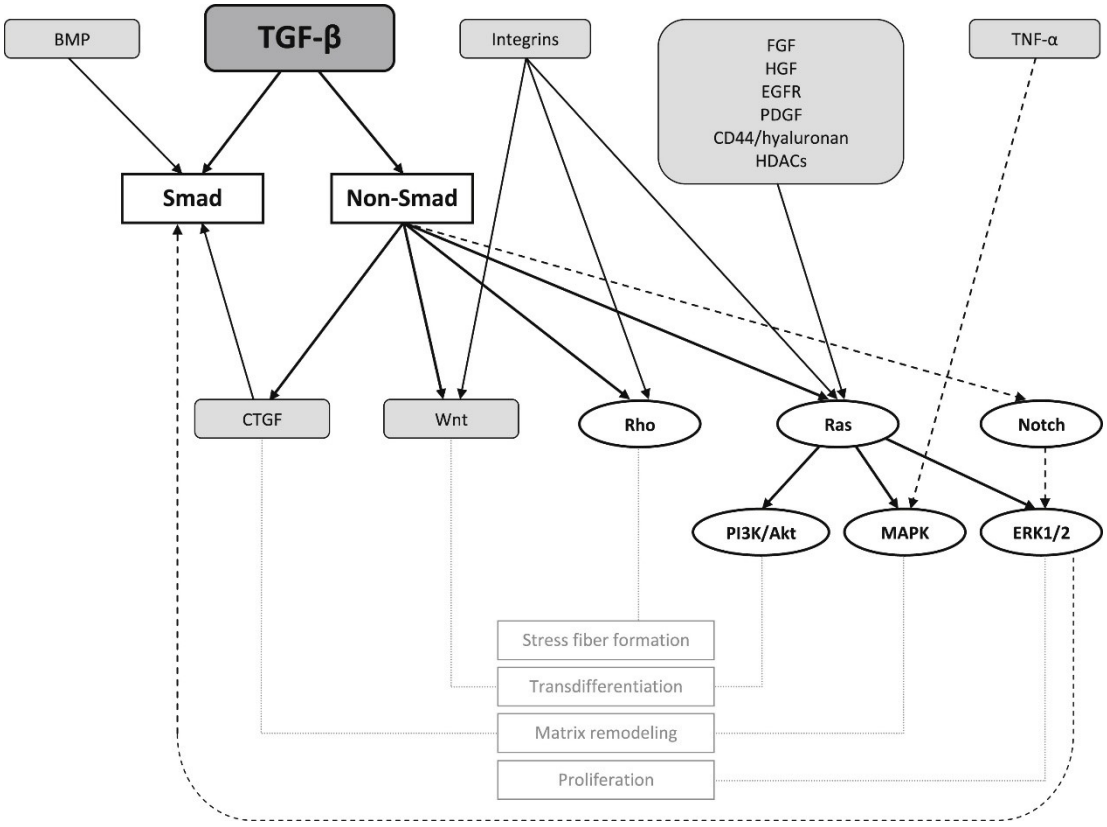

Figure 1. Schematic diagram of the key inducers and mechanisms involved in EMT during PCO development. TGF-β is the primary molecule, activating several pathways. All these converge to promote LEC proliferation; mesenchymal transdifferentiation; the formation of stress fibers, which are contractile actin filament bundles; and matrix remodeling.[4]

With identical pathways involved in the pathogenesis of EMT in cancer and tissue fibrosis and in PCO development, it is no surprise that drugs that have been used effectively in cancer have also been tested for the treatment and prevention of PCO.[4,5] It is both logical and timely to look at metformin, which is recently in the spotlight for its value in fighting EMT in cancer.[8]

Metformin

Metformin is a biguanide drug that belongs to the class of nitreones. It was originally extracted from the French lilac *(Galega officinalis)* plant and was historically used in the Middle Ages to treat polyuria. Today, it is the first-line treatment for diabetes mellitus; other clinical indications include management of polycystic ovarian syndrome (PCOS) and metabolic syndrome, and diabetes prevention in high-risk populations. Metformin has an excellent safety profile. The most common adverse side effects are diarrhea and dyspepsia.[8] Its most serious adverse effect, lactic acidosis, is very rare, with an incidence of 4.3 cases every 100,000 patient-years.[79]

Metformin exists in regular and extended-release tablets. It is primarily taken orally and is absorbed in the intestine through the plasma membrane monoamine transporter (PMAT) located on the luminal side of the enterocytes. Other receptors present on the brush border, specifically the solute carrier 22 (SLC22) family of receptors, which include organic cation receptors (OCT), may also mediate intestinal absorption. The hepatic uptake of metformin is through the OCT1/SLC22A1 and OCT3/SLC22A3 receptors, which are present on the basolateral membrane. Renal uptake, meanwhile, is through OCT2/SLC22A2, which is located mostly at the basolateral side of the distal tubules. Metformin is not metabolized and is renally excreted, with a half-life of 5 hours. Elimination is thought to be likewise through the PMAT receptors on the apical border of the renal epithelial cells.[80] Several polymorphisms of the gene coding for the SLC family of receptors have been identified with differences in response to metformin.[81]

Its effectiveness in diabetes is due mainly to its ability to acutely decrease hepatic glucose production, mostly through a mild and transient inhibition of its primary target: the mitochondrial respiratory-chain complex 1. This decrease in hepatic energy status activates the adenosine monophosphate-activated protein kinase (AMPK), which then suppresses gluconeogenesis during

cellular stress.[82] The metabolic action of metformin is mediated through the phosphorylation of tuberous sclerosis complex 2 (TSC2), a GTPase that hydrolyzes the Rheb protein. The Rheb/mTOR pathway is then blocked,[83] although metformin can act on mTOR independently of AMPK and TSC2.[84]

Traditionally, medicine has considered concomitant diabetes to be associated with a worse prognosis and outcome in cancer patients.[85] In 2005, however, an epidemiological study of more than 11,000 patients diagnosed with diabetes who were taking metformin showed an overall reduced rate of cancer. It thus theorized that this is because upstream of AMPK the tumor suppressor liver kinase B1 (LKB1) was activated by the drug.[86] The indirect effects of decreased systemic glucose and increased insulin sensitivity in vivo, contributing to the overall health, was also deemed advantageous to patients with cancer. This observation of reduced cancer incidence in diabetic patients taking metformin was confirmed in a prospective cohort study.[87] The decrease in cancer incidence ranged from 30%-50% in multiple cancers, such as of the breast, pancreas, kidney, prostate, malignant melanoma, endometrium, cervix, colorectum, stomach, thyroid, liver and lung.[11,88,89] The majority of subsequent studies have shown that long-term metformin use is necessary for this benefit.[8] However, a window-of-opportunity clinical trial in non-diabetic patients with breast cancer showed that short-term metformin may be of benefit as neoadjunct therapy.[90]

In vitro and *in vivo* studies on metformin and its effect on cancer cells have shed light on its possible mechanisms of action against tumor proliferation, migration and invasion. Metformin can exert its effect on the cell cycle through an AMPK-dependent manner via inhibition of mTOR activation.[8] It can stall cell replication by decreasing cyclin D1 and through AMPK-phosphorylation of p53, leading to apoptosis.[91,92] Metformin can also induce senescence in cancer

stem cells and can inhibit tyrosine kinase activity. Also through its activity on AMPK, metformin exerts pressure on the PI3/Akt/mTOR pathway, leading to decreased cell survival.[8] In addition, independent of AMPK, metformin also inhibits the mTOR pathway via inhibition of the RAG family GTPases.[93] Apoptosis may also be induced through upregulation of the growth arrest and DNA damage inducible gene 153 (GADD153) and through activation of the mitogen activated protein kinase (MAPK) pathway.[94] Upstream of the MAPK pathway are Ras and Raf, downstream effectors include the JNK/p38 and ERK1/2 kinases, which impact binding of transcription factors such as c-Jun, Fos, signal transducer and activator of transcription 3 (STAT3) and the cAMP response element binding (CREB) protein, to genes that control growth, proliferation and differentiation.[95,96]

Aside from its effects on cancer cell proliferation and survival, metformin has also been shown to specifically inhibit EMT. It does so by repressing production of transcriptional factors ZEB1, TWIST1 and SLUG, all of which are highly associated with EMT induction.[8] Moreover, metformin induces upregulation of DICER1, a ribonuclease enzyme involved in microRNA synthesis. This correlates with an increase in the tumor suppressive miRNA-let7a and miRNA-96 and a decrease in the oncogenic miRNA181a, thereby preventing the acquisition of mesenchymal characteristics by cancer stem cells.[97] Alterations in candidate miRNAs specific to various cell types have also been identified. In pancreatic cells, for instance, metformin has been shown to attenuate migration and invasion by deregulating miRNAs that interfere with the Notch signaling pathway necessary for mediating the inflammatory response to cytokines like TNF-α.[98,99] Metformin has also been shown to rescue expression of E-cadherin and inhibit vimentin expression in epithelial cells in TGF-β-induced EMT.[100] In addition, metformin decreases cellular migratory

ability *in vitro* via regulation of Rho kinase and matrix metalloproteinases and affects mesenchymal transdifferentiation with dysfunction of the canonical Wnt/β-catenin pathway.[101]

The majority of the preliminary experiments into metformin effectiveness and mechanisms of action, however, use supraphysiological doses that are 10-100 times greater than what can be observed in the blood. This is why caution is warranted in interpreting and extrapolating data for clinical trials; not all promising laboratory studies have thus translated into investigations in human subjects.[102] Presently, metformin is being tested in clinical trials for breast cancer, cutaneous melanoma, pancreas, endometrium, colorectum, blood, head and neck, brain and ovaries. An overview of the ongoing clinical trials that aim to test the efficacy of metformin across the multitude of cancers indicate this restraint: metformin is touted as either an adjuvant therapy in both primary and metastatic disease or as a preventive drug for high-risk populations. Dosages are well-within the established range, which is up to 3000mg/day in adults, and treatments may last anywhere from 1 week to several years. Primary endpoints include biochemical markers of disease progression, disease control, treatment response and recurrence-free and disease-free survival.[88]

Metformin and the Eye

The literature on metformin and the eye is sparse. While metformin is primarily used for diabetes and while good blood glucose control is essential to prevent ocular complications, it has always been presumed that the effect of metformin on the eye is secondary to its glucose-lowering effect. Studies on the effect of metformin on angiogenesis on the eye, however, have revealed mechanisms of action different from AMPK activation. Primary human retinal microvascular endothelial cells (RVECs) treated with metformin concentrations ranging from 5-50 mM had decreased cell viability, proliferation, migration, and capillary formation at 5mM.[103] In a study on

oxygen-induced retinopathy in mice, it was observed that intraperitoneal injection of metformin decreased angiogenesis not by decreasing production of the inducer vascular endothelial growth factor (VEGF), but by reducing expression of its receptor, FLK1.[104]

Metformin has also been shown to confer a protective effect on the lens in streptozocin-induced diabetes in rats, despite a mild increase in non-enzymatic glycosylation of proteins in the lens.[105] A case report associated metformin use with a sudden blood glucose decrease in a diabetic patient, which resulted in acute blurry vision and fluid-filled cortical cracks in the lens. It was hypothesized that this complication was due to osmotic regulation in response to the hypotonic extracellular fluid. The symptoms resolved with return of vision to normal and stabilization of glucose levels after 3 months of metformin use.[106]

An epidemiological retrospective cohort study considering more than 150,000 patients diagnosed with diabetes noted that 5893 (3.9%) developed open angle glaucoma (OAG).[107] Previous studies have associated increased risk of developing glaucoma in diabetic patients, although these did not consider levels of glycosylated hemoglobin (HbA1C) or class and quantity of medications being administered. The study found that after adjusting for confounding factors, including other diabetic medications being taken, patients who took the highest doses of metformin had a 25% reduced risk of OAG relative to those who took no metformin. Further analysis showed that every 1g increase in metformin hydrochloride use was associated with a 0.16% reduction in OAG risk. This benefit was present even when taking HbA1C levels into account, suggesting that metformin exerts its effects through mechanisms independent of its ability to lower glucose.[107]

Other ocular diseases in which metformin has been tested include uveitis and retinoblastoma. Endotoxin-induced uveitis was performed in rats through injection of lipopolysaccharide (LPS), after which metformin was injected intraperitoneally in the treatment

group. Analysis of the aqueous humor in the rats treated with metformin revealed a significantly decreased number of inflammatory cells and cytokines compared to the control group. Metformin was also found to prevent the expression of Cox-2 and to activate AMPK in the ciliary body and in the retina. These findings suggest that metformin may play a role in limiting inflammation in uveitis.[108] Studies on retinoblastoma, meanwhile, have not been as successful. *In vitro* experiments on the cell lines WERI and Y79 have revealed that metformin can inhibit growth and proliferation through the AMPK/mTOR pathway and activation of autophagy. However, this effect was not observed with xenografted Y79 tumors in nude mice when physiologic doses of metformin were used.[109]

That metformin can affect various processes in the eye is no surprise as metformin, administered orally and distributed systemically, can cross the blood-brain barrier.[110] An ophthalmic formulation of metformin also exists for pharmacokinetics research; it was found that a 7.85 mM preparation had a penetration efficiency of 0.058% across the cornea and into the anterior chamber. Thus, the maximal concentration of metformin was found in the aqueous 30 minutes after a single instillation, corresponding to a concentration of 0.108 nmol/mL.[111] The known distribution of the principal receptor for cellular uptake, SLC22A1/OCT1, also explains the effect of metformin on specific eye tissues. SLC22A1/OCT1 was demonstrated to be present in the cornea, in the iris and ciliary body, and in the blood-retina barrier.[81]

No studies have been performed to date to ascertain the presence of the SLC22A1/OCT1 receptor in the lens.

Diabetes and PCO

With metformin as the initial treatment of choice in diabetes, it is reasonable to examine the incidence of PCO in diabetic versus non-diabetic patients. Multiple studies have been performed to this end, but to date, results have been inconclusive. The difficulty in performing such analyses lay in the following: the differences in surgical techniques and IOLs used; the lack of standardization in quantitating PCO, including wrinkling of the anterior capsule and time to follow-up; the failure to consider factors, such as diabetes control and medications being taken.[112,113]

A previous study has looked at the rates of Nd: YAG-laser capsulotomy as a surrogate outcome for the incidence of PCO. A greater incidence of capsulotomy being performed in diabetic patients when compared to healthy controls was found,[114] although this result was later criticized, as the procedure is more commonly performed in diabetic patients who require more frequent ophthalmic screening for retinopathy.[115] Other related risk factors have also been analyzed between diabetic and non-diabetic patients, including the incidence of anterior capsule wrinkling and the degree of post-operative inflammation. Studies have reported an increased risk of anterior capsule contraction in diabetic patients.[116,117] While a subsequent study found no correlation between anterior capsule contraction and PCO,[118] the methods employed for evaluating PCO, particularly in the periphery, was appraised to be inaccurate.[113] Furthermore, because diabetes is considered to be a microvascular disease and to manifest increased capillary permeability, it was theorized that diabetic patients would have greater post-operative inflammation. A prospective study on diabetic patients without retinopathy who had undergone cataract surgery found that, compared to controls, there was no mean difference between cells and flares in the anterior chamber up to day 28 post-operatively.[119]

Later studies evaluated PCO using Scheimpflug videophotography, which is a slit-lamp with modified optics to enable visualization of the entire anterior segment.[120] Different scoring systems were also utilized, alone or in combination with videophotography. One prospective study comparing 26 diabetic and non-diabetic patients who received heparin-surface modified polymethylmethacrylate IOL implants concluded that at 2 years post-operatively, the rate of PCO after phacoemulsification was lower in diabetic patients. This finding was thought to be secondary to the inherent low proliferative capacity of LECs in diabetic patients due to accumulation of sorbitol and other advanced glycosylation products.[115] Culture of primary LECs obtained from patients after cataract surgery corroborated that LECs from diabetic patients have a lower proliferative potential.[121]

Another prospective case-control study that had 42 patients in each group and that secured follow-up until 1 year after surgery showed that over this short period, patients with diabetes had a greater chance of developing PCO.[122] The results of this study were later contested due to the noted patient dropout rate and the high standard deviation in the data for the diabetic group.[112] The results of this study, however, were confirmed in another prospective case-control study with 100 patients per group. Each subject had the same IOL inserted: 6.0 mm in diameter, with a soft acrylic optic and modified C polymethylmethacrylate loops. After 1.5 years of follow-up, PCO was found to be increased in patients with diabetes, although HbA1C status and degree of retinopathy did not correlate with the severity of the PCO in diabetic patients.[123]

Finally, a fourth prospective case-control study, this time with 75 patients for each group, concluded that at 4 years, there was no difference in the incidence rate of PCO between groups. The length of time since diagnosis of diabetes, however, correlated with an increased risk, although the degree of retinopathy did not.[124]

In all these studies, no stratified analysis was done to evaluate the effect of any medication—whether insulin, metformin or the other anti-hyperglycemic agents—on PCO development.

This review of available literature can be summarized as follows: (1) EMT is the main pathophysiologic mechanism underlying PCO; (2) metformin has been shown to inhibit EMT in cancer; (3) there exists a gap in knowledge on the effect of metformin on the lens. With this rationale established, it is useful to restate the primary objective of this research: to determine the effectiveness of metformin in an *in vitro* model of PCO. The methodology that follows seeks to address this question.

MATERIALS and METHODS

The development of PCO necessitates the proliferation, differentiation and migration of the residual LECs as they acquire the properties of mesenchymal cells. This collective response is called EMT. Other characteristics observed are the loss of phenotypic epithelial markers and the increased expression of mesenchymal proteins, both of which are concomitant with the acquisition of a spindle cell shape.

This study utilized an *in vitro* model of PCO, in which immortalized LECs from the HLE-B3 cell line were exposed to transforming growth factor-beta (TGF-β) and fibroblast growth factor (FGF) for EMT induction.[125] The expression of solute carrier family 22 member 1 (SLC22A1), also known as organic cation transporter 1 (OCT1), was determined in cell line extracts. SLC22A1 is the receptor that is most commonly associated with the cellular uptake of metformin. Afterwards, the effect of a range of metformin concentrations on cell viability, migration, morphology, and expression of epithelial and mesenchymal protein markers was determined in a series of experiments detailed below. Insights into how metformin might exert its effects were also obtained in additional experiments. The effect of increasing metformin on SLC22A1 expression was demonstrated. The relative proportions of Akt and phosphorylated Akt, proteins in the signaling pathway known in literature to be primarily impacted by metformin in its action against cancer cells, were also evaluated. Lastly, to confirm future applicability of the results of this study in possible subsequent *in vivo* experiments, the presence of the SLC22A1 receptor in the anterior segments of donor eyes was demonstrated.

Cell Culture of Human Lens Epithelial Cells

This cell line was isolated from fetal human lens epithelium and was immortalized with transformation with the adenovirus 12-SV40.[126] Cells were maintained on Minimum Essential Media (MEM, Gibco®, Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 20% fetal bovine serum (FBS) and 50 ug/mL gentamicin (Thermo Fisher Scientific). Cells were kept at a humidified incubator (37ºC, 5% CO_2). Media was changed every 2-3 days, and cells were grown to 70%-80% confluency before passaging. Cells were monitored daily under an EVOS XL (Thermo Fisher Scientific) phase-contrast and brightfield microscope.

For each passage, adherent cells were washed with warm phosphate buffered saline (PBS) and treated with 0.05% trypsin with ethylenediaminetetraacetic acid (EDTA; Gibco®, Thermo Fisher Scientific). Trypsin was neutralized with culture media containing 20% FBS, and cell suspensions were centrifuged at 1500*g* for 5 minutes. The supernatant was decanted, and the cell pellets were resuspended in fresh, pre-warmed media with 20% FBS. Cell counts were performed with trypan blue exclusion and using an automated cell counter, and suspensions were diluted to the appropriate cell concentration needed for the purpose of the experiment. For cryopreservation of cells, a concentration of $1x10^6$ cells/mL was used, and cells were stored in 5% dimethylsulfoxide (DMSO) prior to storage in liquid nitrogen.

A previous study compared proteomes of HLE-B3 and freshly isolated lens epithelium and saw that in the former there was a significant amount of crystalline proteins expressed. It was also observed that αA-crystallin was not produced after passage 11.[127] Thus, only cells passaged up to 11 times were used in the succeeding experiments.

Epithelial-to-Mesenchymal Transition (EMT) Induction

LECs were exposed to a combination of TGF-β and FGF to induce EMT and also because both growth factors have been directly implicated in the formation of PCO.[128] Post-operatively, increased levels of both growth factors have been observed in the aqueous humor. The increase of FGF occurs earlier and has been shown to promote proliferation and differentiation of the lens epithelium, while TGF-β levels increase later after surgery and induce cellular migration and fibrosis.[9,10,68]

TGF-β and FGF were obtained from Thermo Fisher Scientific (MA, USA). Serum-free (SF) MEM was prepared with the addition of 50 ug/mL gentamicin. No anti-fungal agent was used, as was done in previous studies,[12,13] as this decreases LEC viability.[129] TFG-β and FGF were then dissolved in serum-free (SF) MEM at a concentration of 10 ng/mL and 40 ng/mL, respectively. These concentrations have been previously used in *in vitro* PCO models.[12] These components constituted the EMT induction media used for the rest of the experiments.

Metformin Concentrations

Metformin hydrochloride (Cayman Chemical, MI, USA) was dissolved in PBS to a concentration of 1440 mM for storage. Fresh working solutions of metformin were made by preparing 200 mM in EMT medium. The range of concentrations of metformin were based on the minimum (0.1 mM)[130] and maximum (100 mM)[131] used against cancer cells as reported in literature.

The following concentrations of metformin were initially used for the cytotoxicity assay: 0.1 mM, 0.5 mM, 1 mM, 5 mM, 10 mM, 20 mM, 30 mM, 50 mM, 80 mM and 100 mM. Metformin used in subsequent experiments was limited to 10 mM, as a significant decrease in the number of viable cells, compared to SF, was noted in higher concentrations (≥20 mM). It was imperative that

the cell counts across treatments be kept consistent in studies on migration, morphology and protein expression.

Data Analysis

Data processing and statistical analysis was done using Microsoft Excel (Microsoft Corporation, Redmond, WA, USA) and GraphPad Prism (La Jolla, CA, USA).

SLC22A1 Expression in the HLE-B3 Cell Line

To check if the HLE-B3 cells have the potential to respond to metformin, the presence of the SLC22A1 receptor was determined using Western blot.

Cell Culture and Protein Extraction

For this study, $1x10^6$ cells were seeded in 10-millimeter culture plates (Corning®) and grown to 80% confluency. Cells were starved in SF media prior to treatment with either SF or EMT. After incubation for 48 hours, the media was aspirated, and the cells were washed twice with cold PBS. To lyse the cells, 300 uL radioimmunoprecipitation (RIPA) buffer (Sigma-Aldrich, St. Louis, MO, USA) with anti-phosphatase and anti-protease tablets (Roche, Basel, Switzerland) was added to each plate. The plates were incubated on ice for 10 minutes, after which the cells were detached with cell scrapers. Stringy precipitates were presumed to be of nucleic acid origin and were carefully removed to prevent interference in the protein quantification assay. The cell lysis extracts were then vortexed once briefly for 10 seconds (8 watts, Sonic Dismembrator Model 100, Quebec, CA) and then incubated on ice for an additional 20 minutes. Extracts were centrifuged at 13,000*g* (relative centrifugal force, RCF) for 10 minutes at 4ºC (IEC Micromax RF,

Thermo Electron Corporation, Milford, MA, USA). The supernatant was aspirated without disturbing the cellular debris pelleted. Protein extracts were stored at -80ºC until further analysis.

Protein Quantification

To quantitate the amount of protein in the extracts, the bicinchoninic acid (BCA) assay was performed using a commercial kit (Pierce BCA Protein Assay Kit, Thermo Fisher Scientific). Briefly, the process is as follows: fresh standards of bovine serum albumin (BSA), ranging from 0-2000 ug/mL were prepared. Next, 2 uL of samples and standards were loaded in triplicates on a 96-well plate, and 5 uL of the RIPA buffer and water were also added, after which the working reagent (190 uL) was added. The working reagent was a mix of Reagents A and B in a ratio of 50:1; Reagents A and B contained bicinchoninic acid and cupric sulfate, respectively. The plate was incubated at 60ºC for 30 minutes (Boekel Scientific, PA, USA) and was read at 562 nm using a spectrophotometric reader. The standard curve obtained from the dilutions of BSA was used ($R^2 \geq 0.99$) to quantitate the amount of protein in the extracts. The BCA assay was performed immediately prior to sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE).

Western Blot

Gel Preparation. Bio-Rad TGX Stain-Free™ FastCast™ in 7.5%, 10% and 12% (Bio-Rad, Irvine, CA, USA) were used in this study, depending on the molecular weight of the protein of interest. The resolving and stacking gels were prepared on 1.5 mm glass plates as per manufacturer's recommendations, with freshly made 10% ammonium persulfate (APS) each time.

Sample Preparation. Protein samples were prepared by adding 4x Laemmli buffer (Bio-Rad) with β-mercaptoethanol (Sigma Aldrich) in a 3:1 ratio. These were afterwards placed in a

digital dry bath (Accublock™, Labnet International Inc, Edison, NJ, USA) set to 95ºC for 10 minutes. Two molecular weight ladders were used: the Bio-Rad Precision Plus Protein™ All Blue Prestained Standard to track subsequent transfer onto membrane, and the Unstained Standard (Bio-Rad) for visualization on stain-free gels.

SDS-PAGE. The same amount of protein for each treatment was loaded in each well and in each gel run. The amount of sample loaded ranged from 10-20 ug. The electrophoresis run was set at a maximum of 70 volts (V) until the proteins were properly stacked, after which the voltage was increased to a maximum of 100V for adequate separation. The gels were afterwards activated for 1 minute using the Chemidoc™ MP System and Image Lab software (Bio-Rad).

Membrane Transfer. Proteins were transferred from the gel to the Trans-Blot Turbo™ nitrocellulose blots using the Trans-Blot Turbo™ Transfer System (Bio-Rad). The settings for efficient transfer were as follows: 7 minutes of transfer, with 1.3 amperes (A) of current and voltage ranging from 21-25V. Transfer efficiency was documented and images for subsequent total protein standardization were afterwards obtained using the Chemidoc™ system.

Membrane Blotting. The membranes were thrice rinsed with 1X Tris buffered saline (8.77g sodium chloride (NaCl), 2.42 g Tris in 1L distilled deionized water (ddH_2O)) with 0.1% Tween-20 (TBST) for 5 minutes, after which they were blocked with 5% non-fat dry milk in TBST for 1 hour and with gentle agitation (Twist Shaker TW3, FinePCR Finemould Precision Ind Co, Seoul, Korea). The membranes were then incubated overnight at 8ºC with the primary antibody and with gentle agitation (VWR Scientific, Radnor, PA USA). Afterwards, the membranes were rinsed thrice in 1x TBST for 5 minutes each time and were incubated with the corresponding secondary antibody at room temperature for 1 hour and with gentle mixing. Blots were again rinsed thrice in 1x TBST for 5 minutes each time before the addition of the luminol-peroxide solution for

chemiluminescent substrate detection (ECL Prime Western Blotting System, Amerhsam, GE Healthcare, Chicago, IL, USA). The blots were visualized using the Chemidoc™ system with auto-exposure arranged.

Antibodies. The mouse monoclonal anti-SLC22A1 (2C5, Novus Biologicals, Littleton, CO, USA) was used in a 1:1000 dilution to probe the blot, after which it was targeted with the goat-anti-mouse HRP-linked IgG (sc-2005, Santa Cruz, Dallas, TX, USA, 1:1000 dilution). Protein extracts from the hepatocellular cancer cell line HepG2 and the uveal melanoma cell line OCM-1 were used as weak and strong positive controls, respectively.

Image Analysis. No loading control was used in the Western blots performed, as the Stain-Free™ gels allowed for total protein normalization with the use of the Image Lab software. With this software, a multi-channel image of both the membrane after transfer and the blot after chemiluminescent detection was created. The molecular weight of the bands of interest were determined using the same overlay of images.[132] For this part of the experiment, the presence of the band specific to SLC22A1 was sufficient, and relative quantification of protein expression was unnecessary.

Effect of Metformin on Cell Viability (Cytotoxicity Assay)

To assess the effect of metformin on cell survival, the Cell Counting Kit-8 (CCK-8) viability assay was used (Dojindo Molecular Technologies, Kumamoto, Japan). The CCK-8 assay is a colorimetric test that utilizes the ability of live cells to metabolize, through dehydrogenases, a water-soluble tetrazolium salt (WST-8).[133]

A standard curve for the absorbance of different concentrations of HLE-B3 cells, when incubated with the tetrazolium salt, was first performed. Different cell counts were incubated in

duplicates in a 96-well plate (Falcon®) as follows: $2.5x10^4$, $2.25x10^4$, $2x10^4$, $1.75x10^4$, $1.5x10^4$, $1.25x10^4$, $1x10^4$, $7.5x10^3$, $5x10^3$, $2.5x10^3$ and 0. After 24 hours of incubation to minimize cell division, the cells were starved for 4 hours in SF media. Next, 10 uL of the WST-8 reagent was added to each well. The plate was incubated at 37ºC and 5% CO_2 for 2 hours. Spectrophotometric reading was done at 450 nm using an infinite M200 Pro plate reader and the Tecan i-Control software (Tecan, Männedorf, Switzerland), and a standard curve was calculated.

Initial optimization experiments were first performed to determine the number of cells to inoculate for the cytotoxicity assay. The number of cells that resulted in 80% confluence after allowing attachment for 24 hours was selected. As such, to test the effect of metformin on cell viability, $1.5x10^4$ cells (100 uL of $1.5x10^5$ cells/mL suspension) were inoculated into each well in a 96-well plate. After 24 hours, the cells were starved in SF media for 4 hours. This was afterwards replaced by various treatment media. These included SF; EMT; and the following concentrations of metformin dissolved in EMT: 0.1-100 mM. After 24 hours of incubation, 10 uL of the WST-8 reagent was added to all wells. The plate was incubated at 37ºC and 5% CO_2 for 2 hours. Spectrophotometric reading was done at 450 nm using the same plate reader.

Three replicates were used for each treatment, and three independent experiments were performed. Wells with only SF media were used as negative controls. Cell counts were calculated using the standard curve previously obtained. Analysis of variance (ANOVA) with the Tukey post-hoc test was done to determine at which metformin concentration was there a significant decline in cell viability.

Effect of Metformin on Cell Death (Acridine Orange-Ethidium Bromide Assay Apoptosis)

Metformin has been shown to induce cell death in tumor cells primarily through apoptosis.[134] To confirm this, a fluorescence assay was performed on cells in a 96-well microplate; it enables differentiation between apoptosis and non-apoptotic cell death based on nuclear staining, is easy to perform, and requires no additional steps that may inadvertently cause cell death.[135] The same amount of cells in each independent experiment ($1.5x10^4$ cells per well) was plated and allowed to attach for 24 hours. Treatments of SF, EMT, and representative concentrations of metformin treatment (0.5 mM, 5 mM and 50 mM) were administered after a 4-hour starvation period. Treatment with a high concentration of glucose (25 mM) was used as a positive control for apoptosis.[136]

After 24 hours of treatment, the acridine orange/ethidium bromide (AO/EB) assay was performed as previously described. This protocol was chosen because its *in situ* method is designed to minimize unnecessary induction of cell death and to include in its analysis cells floating in the culture media.[135] The 96-well plate was centrifuged in the Sorvall™ RT6000B Refrigerated Centrifuge (ThermoFisher Scientific) with 96-well plate inserts (5 minutes, 1000*g*, 10ºC) to spin down dead cells. Next, 6 uL of the AO/EB mixture (100 ug/mL AO and EB) was added to each well in batches, prior to imaging with an Olympus IX81 fluorescence microscope (Tokyo, Japan) and using TRITC and FITC filters. The wells were imaged within 20 minutes of adding the dye mixture, as recommended in the protocol.[135]

Acridine orange stains both live and dead cells, but ethidium bromide stains only cells that have lost membrane integrity. As such, live and dead cells were stained green and red-orange, respectively. The distinction between apoptotic and non-apoptotic cell death was made by analyzing nuclear morphology. With non-apoptotic cell death, the cell nuclei were rounded,

swollen and with indistinct borders and did not have condensed chromatin. With apoptosis, the nuclei were either pyknotic or fragmented.[135] The total number of cells was counted automatically using particle analysis in ImageJ (Figure 2).

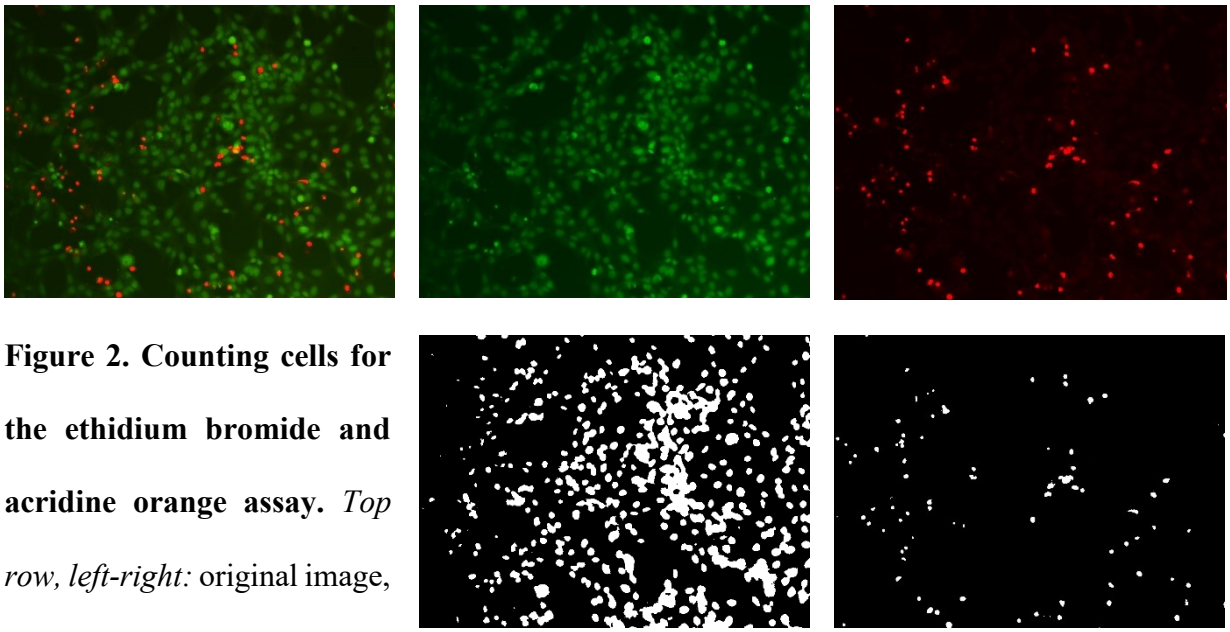

Figure 2. Counting cells for the ethidium bromide and acridine orange assay. *Top row, left-right:* original image, green filter, red filter. *Bottom row: (left)* green filtered image and *(right)* red filtered image made binary, with edges outlined and the interior filled. Total number of particles were counted using particle analysis inclusive of areas that measured only up to 1500 square pixels; this size was optimized to constitute the maximal cell surface area. Apoptotic and non-apoptotic cells were counted manually and differentiated by nuclear size and border appearance. Only dead (red-orange) cells were counted; live cells in early apoptosis were excluded. Images were taken at 10X magnification.

Manual cell counting of apoptotic and non-apoptotic cells was done on the digital images captured for two reasons: Image J cannot discriminate border appearances, and fragmented nuclei

in proximity may be counted as two cells. Numbers of both apoptotic and non-apoptotic cells were recorded as a percentage of the total number of dead cells visualized in the frame.

At least two independent experiments were performed, with three replicates per experiment. Analysis of variance (ANOVA) with the Tukey post-hoc test was used to determine at which concentration of metformin were apoptotic and non-apoptotic mechanisms significant.

Effect of Metformin on Cellular Shape and Symmetry (Microscopy)

EMT induction through TGF-β and FGF has been reported to cause a change in the cellular shape of lens epithelial cells, from cuboidal to spindle-shaped; this is theorized to be secondary to a reorganization of actin and intermediate filaments of the cytoskeleton.[7] The effect of different concentrations of metformin on changes in cellular shape and symmetry was therefore evaluated with microscopy and image analysis.

HLE-B3 cells ($0.5x10^5$ cells/well) were seeded on a 96-well plate (Falcon®, Franklin Lakes, NJ, USA) and grown to 70% confluence. Eight replicates were used for each treatment. Cells were starved for 4 hours before the following treatments were initiated: SF, EMT and 0.1-10 mM metformin in EMT. Incubation was maintained for 48 hours to allow changes in cell morphology to be evident.

After 48 hours, cells were washed with cold PBS twice and were fixed in ice-cold methanol (Fisher Scientific, Nepean, ON, Canada) for 10 minutes. Methanol was aspirated, and the wells were coated with 0.5% crystal violet (Fisher Scientific) in 25% methanol. The plate was kept at room temperature for 10 minutes. The wells were rinsed with distilled water until the dye was completely washed off, and the plate was left to dry overnight.

One image of each well was taken under brightfield microscopy, 20x magnification, using the EVOS XL (Thermo Fisher Scientific) microscope.

Images were saved and processed with Image J (National Institutes of Health, Bethesda, MD). Digital processing was as follows: (1) images were made binary; (2) borders were dilated and then (3) eroded; (4) holes inside cells were filled; (5) cells were measured as particles, and only particles with sizes from 500-15000 pixels were included to exclude dirt particles and overlapping cells. Circularity and roundness were selected for measurement and were averaged for all particles measured per image (Figure 3).

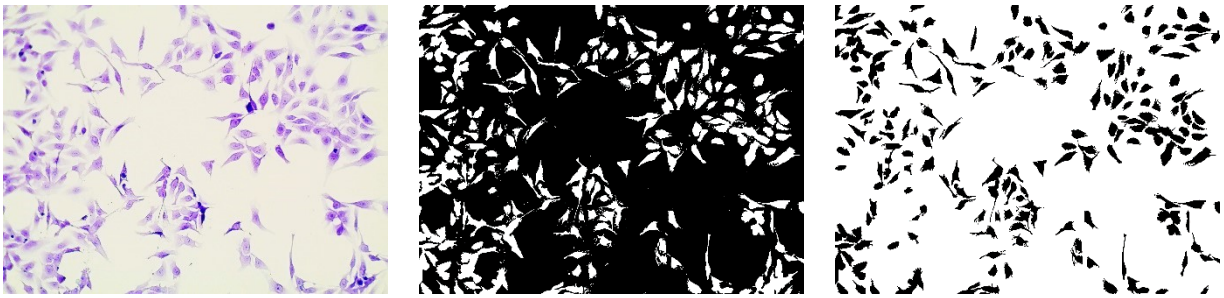

Figure 3. Digital processing of images captured from EVOS XL (20X). *(Left)* Original image of HLE-B3 cells in SF, fixed in methanol and stained with crystal violet. Image was taken under brightfield microscopy. *(Middle)* Image after binary conversion, dilation, erosion and after filling holes. *(Right)* Overlay of some of the cells measured for morphology using particle analysis, with cell size limited to a range of 500-15000 pixels. Counts excluded cells at the edges.

The formula for circularity (C), as used by Image J, is as follows:

$$C = \frac{4\ x\ \pi\ x\ area\ (pixels)}{perimeter\ (pixels)}$$

It thus takes into consideration the form and roughness of the perimeter. If the shape is more circular, the value of *C* approaches 1. The value of *C*, however, does not reach 0.1 until the shape is thread-like.[137] Another method of describing the shape of a particle entails the detection of an ellipse that best approximates the particle. The maximum and minimum axes of this ellipse are then measured; this is called the aspect ratio (AR). The reciprocal of AR is referred to, in Image J, as roundness (*R*). The value of *R* approaches 1 as the object is more symmetric, as in a circle, and moves further from 1 as it assumes the shape of an ellipse.[137]

Both parameters were considered in this study to better describe the general shape and symmetry of the cells. The mean values of *C* and *R* for each group was calculated. ANOVA with the Tukey post-hoc test was done to compare values across treatments. Three independent experiments with 6-8 replicates were performed.

Effect of Metformin on Cell Migration (Wound Assay)

The effect of metformin on chemokinetic cell migration was studied using the wound assay. In this technique, a scratch is introduced into a confluent layer of cells; the natural response is for cells to heal this scratch through migration. The extent of migration can therefore be determined by measuring the cell-free space after wound induction and after a set time period.[138]

For this assay, $2x10^4$ cells were seeded per well in two 48-well plates (Costar®, Corning, NY, USA). Cultures were grown to 95%-100% confluence. Care was taken to ensure that only a single monolayer of cells was maintained, as sheets of cells could easily be dislodged. Cells were starved for 4 hours in SF media prior to treatment.

Media was aspirated from the sides of each well. A 200 uL pipette tip, angled 30^o to the wells, and a ruler were used to draw a vertical line down the monolayer of cells. Afterwards, the

wells were carefully washed with pre-warmed PBS and then aspirated to be free of liquids. Each well was documented with three images, spanning the top to bottom, using the EVOS XL microscope. The condenser was set to phase contrast and images were taken at 4x magnification.

After imaging at 0 hours (wound induction), different media treatments were then given as follows: SF, EMT and 0.1-10 mM metformin in EMT.

The plates were incubated for 24 hours, after which the media was removed. The cells were once again imaged as previously described and were fixed and stained with crystal violet for later documentation with the Nikon Eclipse Ts2 (Tokyo, Japan) and using brightfield microscopy. Phase contrast images were processed using Image J (National Institutes of Health). An Image J macro, the MRI Wound Healing Tool (MRI Redmine),[139] was used for calculating the area of the cell-free gap at 0 and 24 hours after wound induction. This tool has previously been validated in literature.[140] Settings included using variance to calculate the cell-free area; a variance filter radius of 10; a threshold of 50; a radius open of 4; and a minimum size of 5000 pixels (Figure 4).

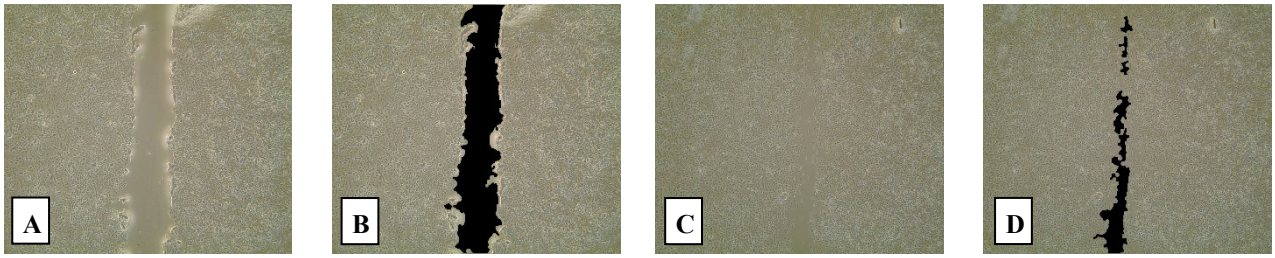

Figure 4. Wound assay images after processing with the MRI Wound Healing Tool (4X). *(A, B)* 0 hours, upon wound induction. *(A)* Raw image, phase contrast. *(B)* After application of the MRI plugin on Image J. Areas in black are the cell-free area. *(C, D)* After 24 hours. *(C)* Raw image, phase contrast. *(D)* Note the decrease in cell-free area, indicating that the HLE-B3 cells have migrated. Cells were treated only with serum-free media.

The percent cellular migration was calculated using the following formula:

$$\%\ cellular\ migration = \frac{area_{0hr} - area_{24h}}{area_{0hr}}\ x\ 100$$

ANOVA with the Tukey post-hoc test was done to compare values across treatments. Three independent experiments with 12 replicates each were performed.

Effect of Metformin on Epithelial and Mesenchymal Markers (Western Blot)

Western blot was used to assess the effect of metformin on protein expression. Proteins of interest in this study included the following: the lens epithelial marker Pax6, which is a transcription factor that influences differentiation of the epithelium to a lens fiber cell;[16] E-cadherin, a transmembrane protein part of forming adherens junctions between cells and a marker of epithelial cells; α-smooth muscle actin (α-SMA), an actin isoform that promotes contractility and migration of mesenchymal cells; and fibronectin, a high-molecular weight glycoprotein that functions as an extracellular matrix scaffold and is associated with EMT *in vitro*.[141] Vimentin was not used in this study as an analysis of the proteome in freshly cultured LEC and in the HLE-B3 cell line showed high expression of this intermediate filament protein.[127]

Cell Culture, Protein Extraction, and Protein Quantification

For this study, $1x10^6$ cells were seeded in 10-millimeter culture plates (Corning®) and grown to 80% confluency. Cells were starved in SF media prior to treatment with either SF, EMT, or 0.1-20 mM metformin in EMT. Proteins were extracted and quantified as previously described.

Western Blotting

SDS-PAGE and Membrane Transfer. Gels and protein samples were prepared as described. SDS-PAGE and membrane transfer were performed as earlier detailed.

Primary Antibodies. Membrane blotting was performed as described previously. The following primary antibodies were used in the following optimized concentrations: a mouse monoclonal anti-Pax6 (1:500, clone 1C8, ThermoFisher Scientific); a mouse monoclonal anti-E-cadherin (1:500, clone 4A2, Cell Signaling Technology, Danvers, MA, USA); a mouse monoclonal anti-α-SMA (1:500, clone 1A4, Dako, Santa Clara, CA, USA); and a rabbit monoclonal anti-fibronectin (1:500, ab32419, Abcam, Cambridge, UKs).

Secondary Antibodies. For mouse anti-Pax6 and anti-αSMA antibodies, the goat anti-mouse IgG-HRP linked secondary antibody (sc-2005, Santa Cruz) was used in a 1:1000 dilution. Meanwhile, the anti-E-cadherin primary antibody was probed with the horse anti-mouse IgG HRP-linked antibody (#7076, Cell Signaling) with a 1:2000 dilution. The rabbit anti-fibronectin was targeted with the goat anti-rabbit HRP-linked IgG (#7074, Cell Signaling) using a 1:1000 dilution.

Image Analysis. Image analysis was conducted using Image Lab as described previously. The multi-channel image was created and total protein normalization was automatically performed by the software. Relative quantitation of the amount of target protein in each treatment, in comparison to the first lane (SF), was then obtained using the measured densitometric volumes.

Reproducibility. Two independent experiments using SF, EMT, and EMT treated with 0-20 mM of metformin was performed on cells plated on 10-mm culture dishes. Western blots probing for a specific protein were performed at least twice on samples in each independent experiment.

Effect of Metformin on SLC22A1 Expression (Western Blot)

Following the observed outcome in LECs treated with metformin alongside EMT induction, the effect of metformin on the expression of the SLC22A1 uptake receptor was evaluated. This was done through Western blot, utilizing the identical protein extracts analyzed in earlier experiments and with the same protocol as previously described.

Effect of Metformin on the Phosphorylation of Akt (Western Blot)

Metformin is known to exert its anti-tumor action through many mechanisms. The main mechanism is primarily associated with the inhibition of the mammalian target of rapamycin complex (mTORC), which is responsible for the proliferation of cancer cells. This pathway is indirectly activated by phosphorylated Akt (protein kinase B). As such, the relative ratio of phosphorylated Akt (pAkt) to Akt was determined among different treatment concentrations.[11]

Western blots were performed according to the method described above. The primary antibodies used were the following: a polyclonal rabbit anti-Akt (#9272, Cell Signaling, 1:500 dilution) and rabbit anti-pAkt (#9271, Cell Signaling, 1:500 dilution), the latter specific for pAkt phosphorylated at the Ser473 residue. The secondary antibody used in both instances was the goat anti-rabbit HRP-linked IgG (#7074, Cell Signaling, 1:1000 dilution).

The membranes were initially probed with pAkt and then Akt. A mild stripping procedure was performed as follows.[142] Blots were rinsed for 5 minutes four times with TBST and were afterwards incubated in stripping buffer twice for 20 minutes per time and with gentle agitation. The stripping buffer was prepared with 7.5g glycine, 0.5 g SDS and 5 mL Tween 20 dissolved in 500 mL ddH_2O, with pH adjusted to 2.2. Stripping of the primary antibody was confirmed adequate with ECL detection after reprobing with the secondary antibody. Ponceau red (Sigma Aldrich) was

also used to confirm minimal stripping of the proteins on the membrane. After each checkpoint, the membranes were washed for 5 minutes four times with TBST, blocked with 5% non-fat milk in TBST for 1 hour, and then re-probed with the primary antibody.

Expression of SLC22A1 in the Lens Tissue (Immunostaining)

To substantiate the possibility that the results of the previous experiments will be applicable in vivo, it was necessary to verify the expression of SLC22A1 in fixed lens tissue. This was accomplished through immunostaining.

Four anterior segment sections of normal human eyes, routinely processed and embedded in paraffin, were obtained from the Henry C. Witelson Laboratory Archive (Montreal, Quebec, Canada). These were baked overnight at 60ºC (Boekel Scientific, Feasterville, PA, USA) and then subsequently for 1 hour at 37ºC to ensure adherence of the tissues to the slide. Automated immunohistochemistry was done using the Ventana Benchmark machine per the manufacturer's recommended protocol (Ventana Medical Systems, Tucson, AZ, USA). Processing of the barcoded slides included baking, solvent-free deparaffinization and CCl (Tris-EDTA buffer pH 8.0)-based antigen retrieval. The slides were incubated afterwards with the mouse monoclonal IgG primary antibody against SLC22A1 (2C5, Novus Biologicals) at a dilution of 1:400 for 30 minutes at 37ºC, followed by the addition of an avidin/streptavidin enzyme conjugate complex. The ultraView Universal Detection Kit (Ventana Medical Systems) was utilized as a chromogenic substrate; slides were then counterstained with hematoxylin. Uterus sections were used as negative controls, and the cornea present in the same slides were used as positive controls. The slides were evaluated by two pathologists in the laboratory and were afterwards scanned for documentation using the Aperio AT2 (Leica Biosystems, Wetzlar, Germany).

RESULTS

Expression of the SLC22A1 receptor in HLE-B3

Since this was the first study conducted on how metformin can possibly affect LECs, it was essential to determine if the HLE-B3 cell line could in fact respond to the drug. Western blotting with revealed a 40-kDa band in HLE-B3 extracts treated with either SF or EMT media (Figure 5). This band corresponded to the SLC22A1 receptor, reported in literature to be responsible for the cellular uptake of metformin.[80] This suggested that the HLE-B3 cells were capable of metformin uptake, and that metformin may exert its effects through intracellular mechanisms.

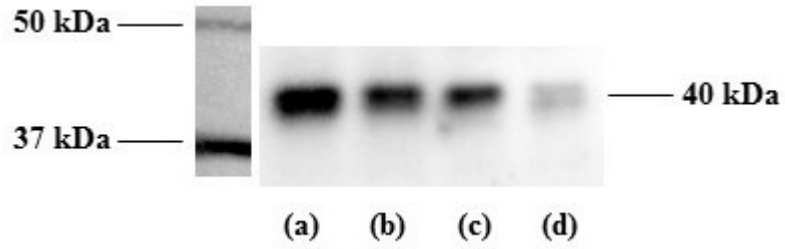

Figure 5. SLC22A1 is expressed in the HLE-B3 cell line.
(a) SF; *(b)* EMT; *(c)* the uveal melanoma cell line OCM-1; *(d)* the hepatocellular cancer cell line HepG2. HepG2 is known to weakly express the SLC22A1 receptor.

Effect of Metformin on Lens Epithelial Cell Viability

The results of the CCK-8 viability assay showed that metformin, at increasing concentrations, could be cytotoxic to LECs (Figure 6). EMT treatment resulted in a calculated survival rate greater than 100%, signifying that there were more cells after 24 hours of treatment than what was originally plated. This suggested not only improved viability but also proliferation, as was expected from exposure to TGF-β and FGF.

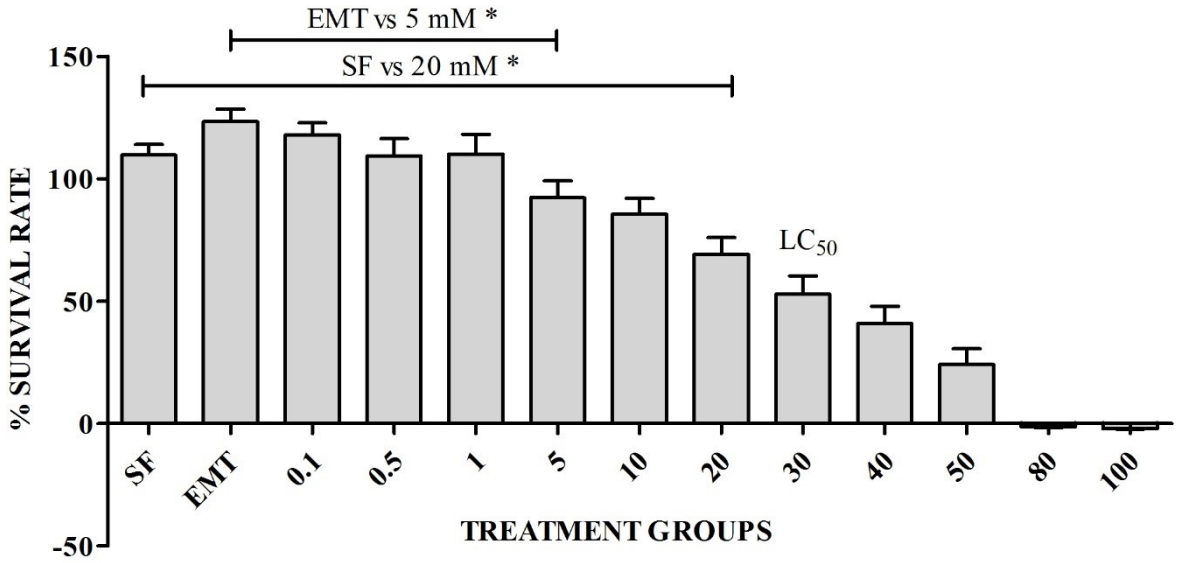

Figure 6. Metformin was cytotoxic to LECs. The lethal concentration that decreased cell counts by 50% (LC_{50}) was 30 mM metformin. The drug was completely cytotoxic at 80 mM. There was an increase in survival rate in cells grown in EMT media (SF vs. EMT). Compared to SF and EMT, there was a significant decrease ($P<0.05$) in the survival rate of cells treated with 20 mM and 5 mM metformin, respectively. As such, all subsequent experiments determining the effect of metformin on other cellular parameters were designed to limit treatment to 10 mM.

Effect of Metformin on Lens Epithelial Cell Death

To confirm that metformin reduced viability by inducing apoptotic cell death in LECs, as has been reported in literature for other cell types,[134] the ethidium bromide-acridine orange fluorescence assay was utilized. This assay could differentiate, *in situ* on a 96-well culture plate, between apoptotic and non-apoptotic cell death through features of the stained nuclei. Cells treated with EMT medium had lower rates of cell death when compared to SF (Figure 7). In all representative concentrations of metformin used, there was a greater percentage of nuclei bearing features of apoptosis (Figure 7), such as distinct borders, fragmentation and pyknosis (Figure 8).

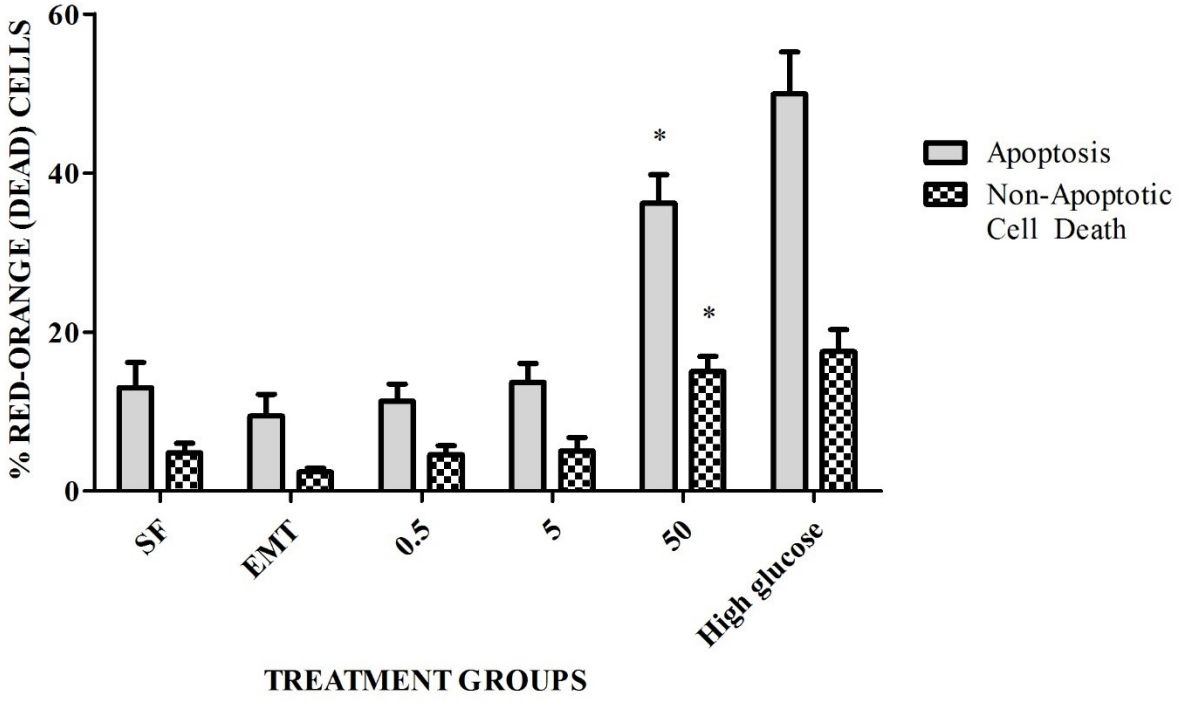

Figure 7. Metformin induced cell death primarily by apoptosis. At 50 mM metformin, majority of the dead cells (40%) had pyknotic or fragmented nuclei. Metformin also induced non-apoptotic cell death at this concentration, although to a lesser degree. Compared to SF, there was decreased cell death in the cells treated with the EMT medium.

Nuclei of other cells undergoing necrosis, meanwhile, were larger, rounder and had indistinct borders (Figure 8), as described in the literature.[135] Percentages of apoptotic and non-apoptotic cell death were only significant at 50 mM metformin ($P<0.05$); these were comparable to LECs treated with high glucose as a positive control. While the same number of cells were initially inoculated into each well, there is an obvious abundance of cell-free space with increasing concentrations of metformin. This is most likely due to the dissolution of nuclei from cells that had earlier perished and were not captured at the observational timepoint.

With this technique, cells in early apoptosis could also be identified on the basis of bright green nuclear staining and condensed chromatin. The major limitation of this method was that it did not discriminate among other mechanisms of non-apoptotic cell death, such as necroptosis and pyroptosis.[143] Regardless, this technique proved useful in confirming that metformin decreased viability of LECs through induction of cell death primarily through apoptosis.

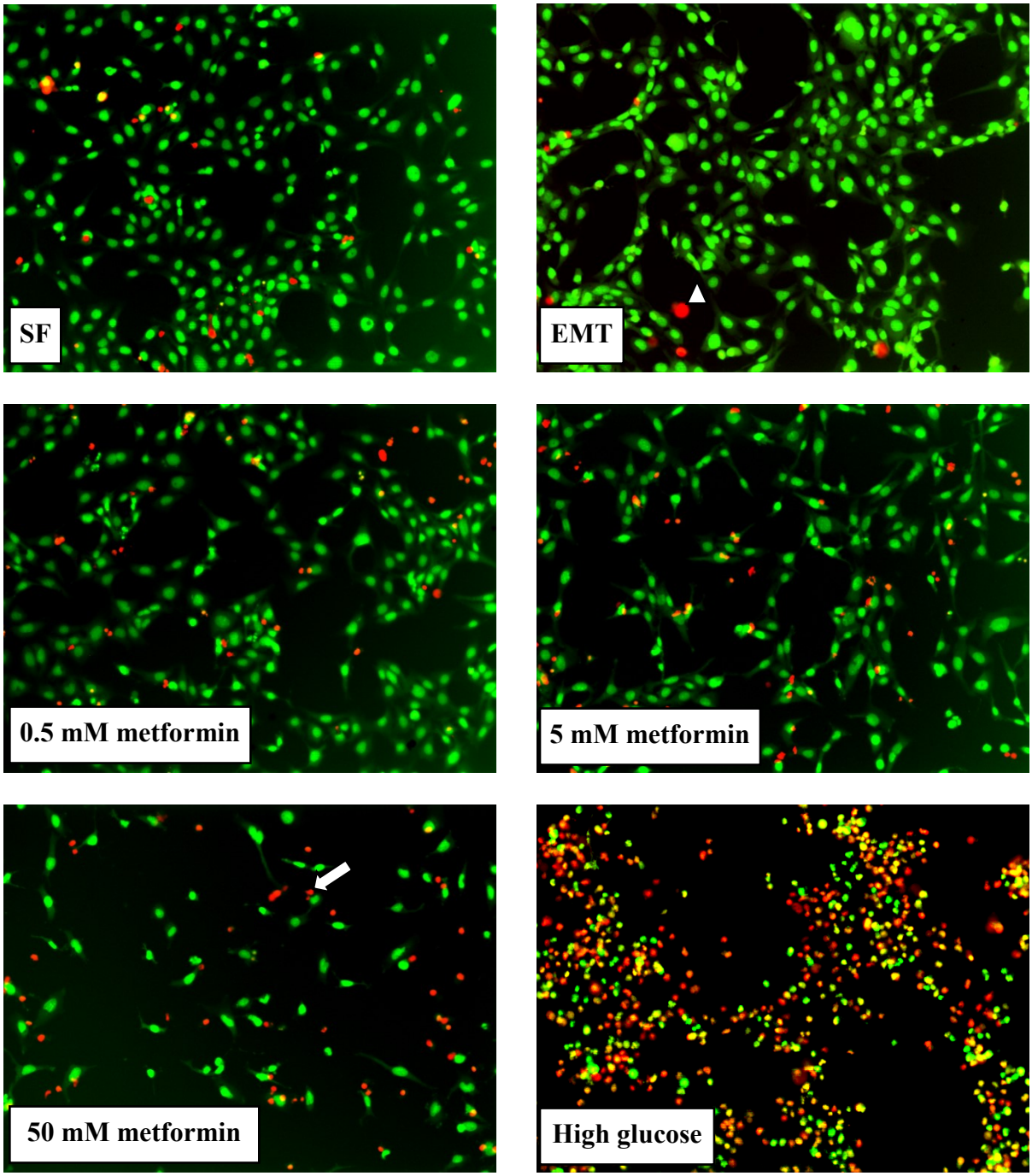

Figure 8. AO/EB Assay (10x). Green and red nuclei represent viable and non-viable cells, respectively. There are more fragmented nuclei with increasing metformin concentration. Note the appearance of the nucleus in a necrotic cell *(white arrowhead, EMT)* and the fragmented nucleus in an apoptotic cell *(white arrow, 50 mM)*.

Effect of Metformin on Cellular Shape and Symmetry

Metformin was also noted to maintain the morphology of LECs amid exposure to TGF-β and FGF. Upon EMT induction, LECs were expected to lose symmetry and acquire a spindlier shape, as was observed during experimentation (Figures 9-11). With the addition of metformin, however, circularity was observed to be greater than EMT-treated cells at a concentration of 1 mM (Figure 9). Roundness was also greater in LECs treated from 0.1-1 mM metformin (Figure 10).

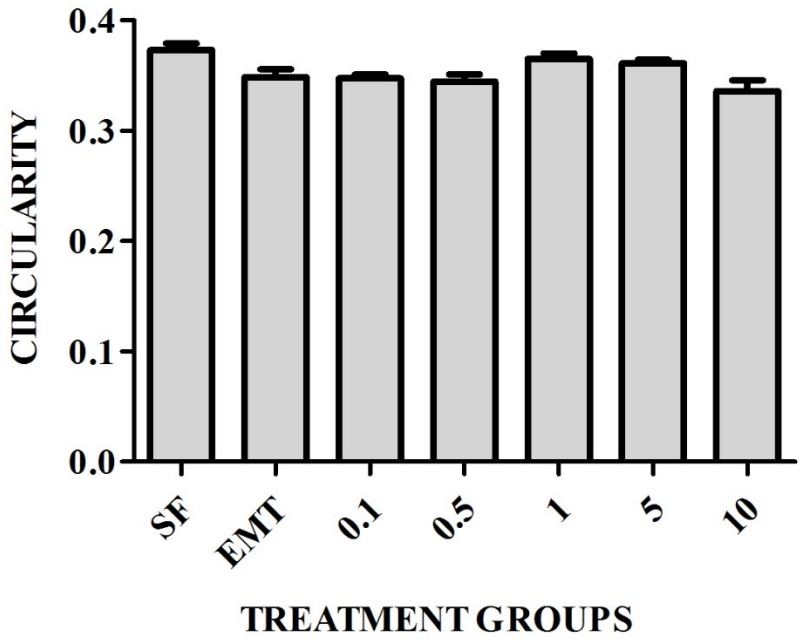

Figure 9. Metformin maintained LEC circularity. Circularity was decreased with EMT treatment. This alteration was not affected with lower concentrations of metformin. At 1 mM, however, the circularity of the cells approximated that of treatment with SF.

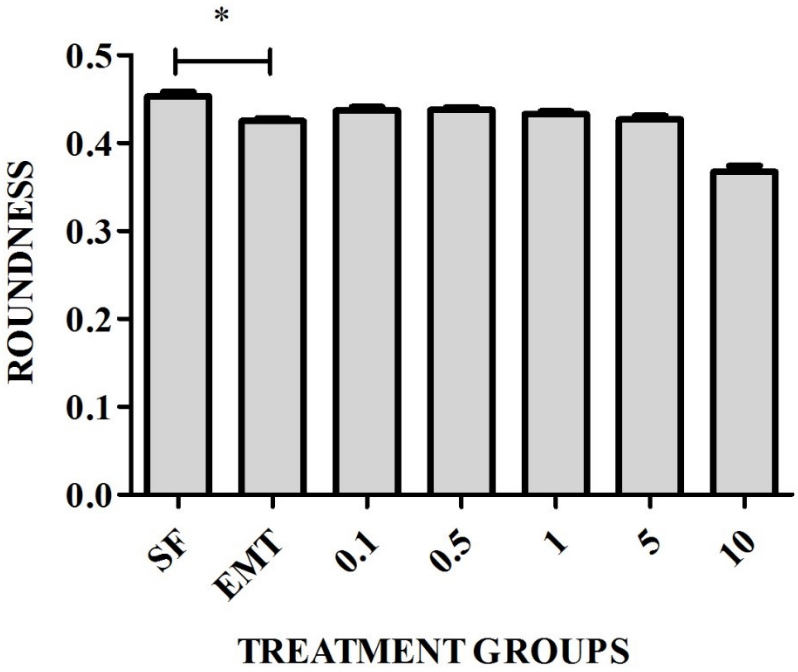

Figure 10. Metformin maintained LEC roundness. Roundness was significantly decreased ($P<0.05$) with EMT medium. Cell roundness was maintained with the lower concentrations of metformin used (0.1-1 mM) in the study, although the values never attained those of untreated cells (SF).

However, measurements of both circularity and roundness—which corresponded to shape and symmetry, respectively—never reached that of cells treated only with SF. Interestingly, cells treated with the highest concentration of metformin used (10 mM) had significantly decreased roundness and circularity ($P<0.05$). Brightfield microscopy showed that these cells appear more spindle-like and elongated, resembling EMT-treated cells (Figure 11).

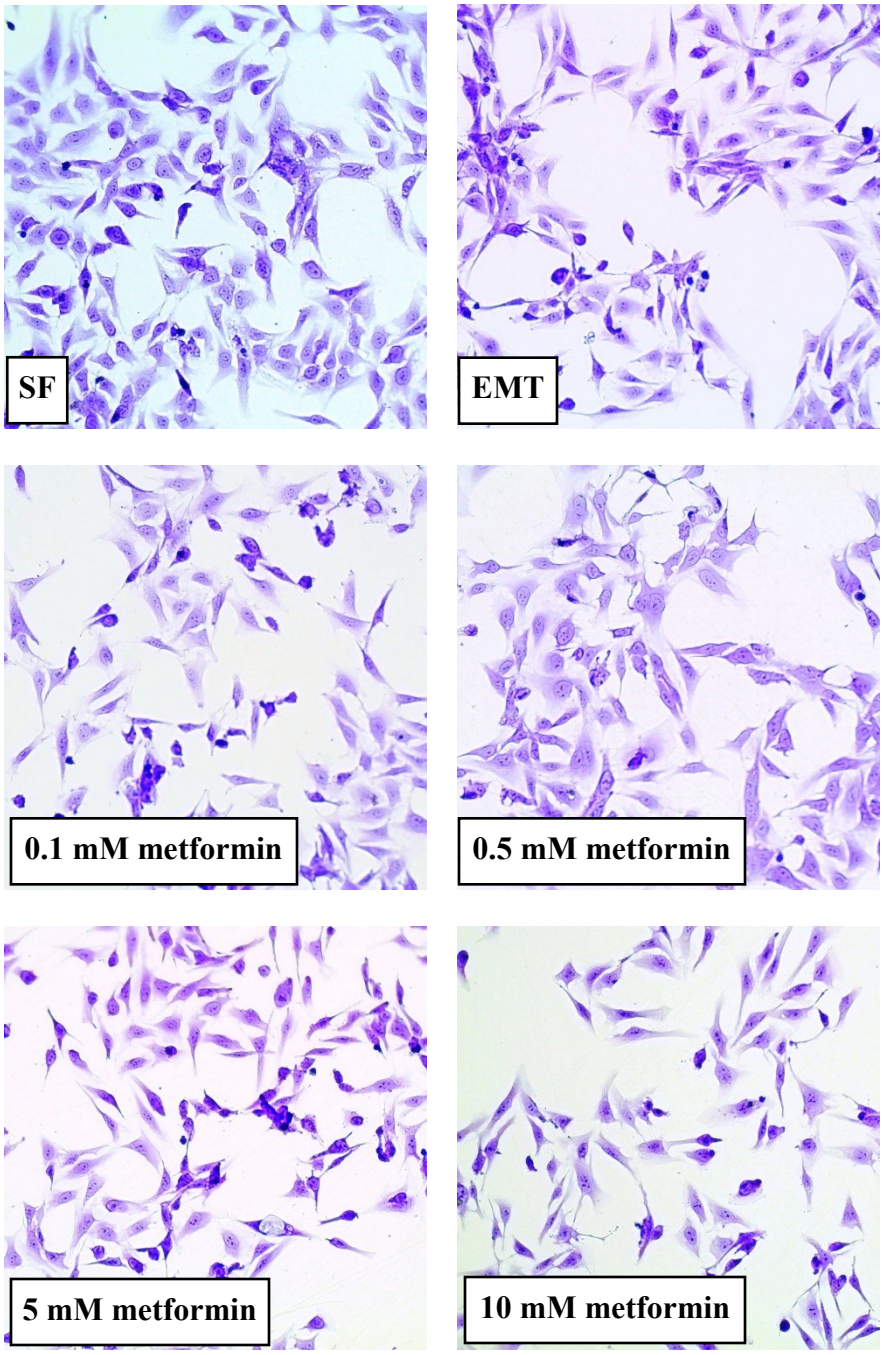

Figure 11. Low concentrations of metformin (0.5 mM) maintained the lens epithelial phenotype. Cells in EMT media became more elongated and spindly. Higher concentrations (5-10 mM) induced morphological changes similar to treatment with EMT.

Effect of Metformin on Lens Epithelial Cell Migration

The cellular parameter most easily affected by metformin was migratory ability (Figure 12). EMT treatment significantly increased migration, as expected. The lowest concentration of metformin (0.1 mM) was able to markedly decrease the ability of cells to migrate, with the migration rate approximating cells grown in SF. Inhibition of migration was significant at 0.5 mM and at higher doses, so that the gap induced in the wound assay was maintained (Figure 13).

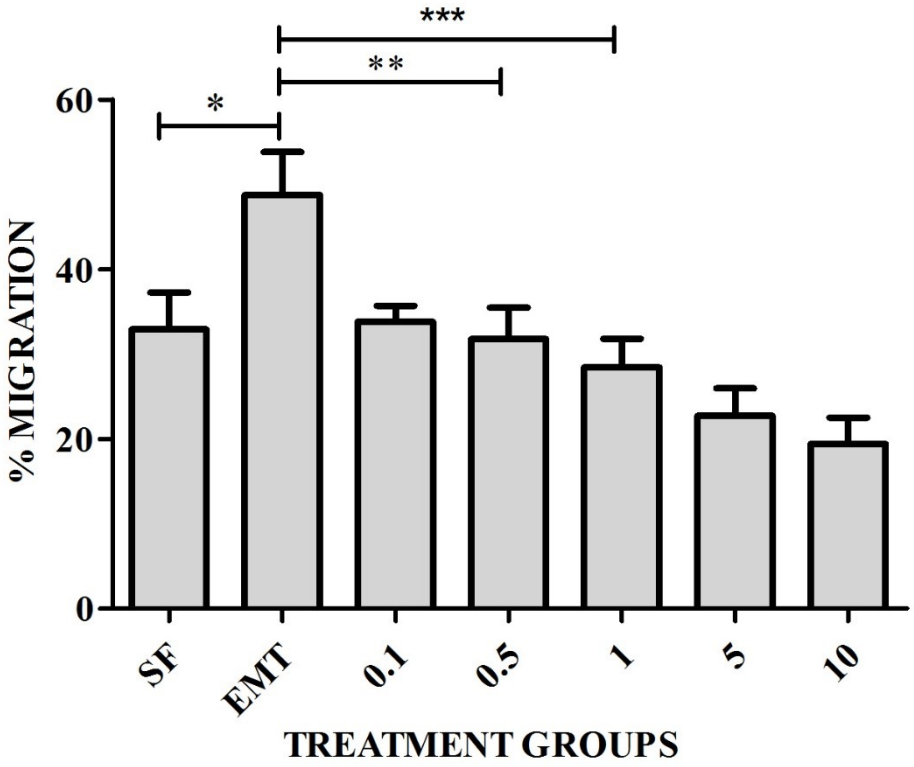

Figure 12. Metformin decreased LEC migration. EMT induction significantly increased the ability of LECs to migrate ($P<0.05$). This decrease was marked at 0.5 mM metformin ($P<0.05$) and was observed with higher concentrations ($P<0.05$).

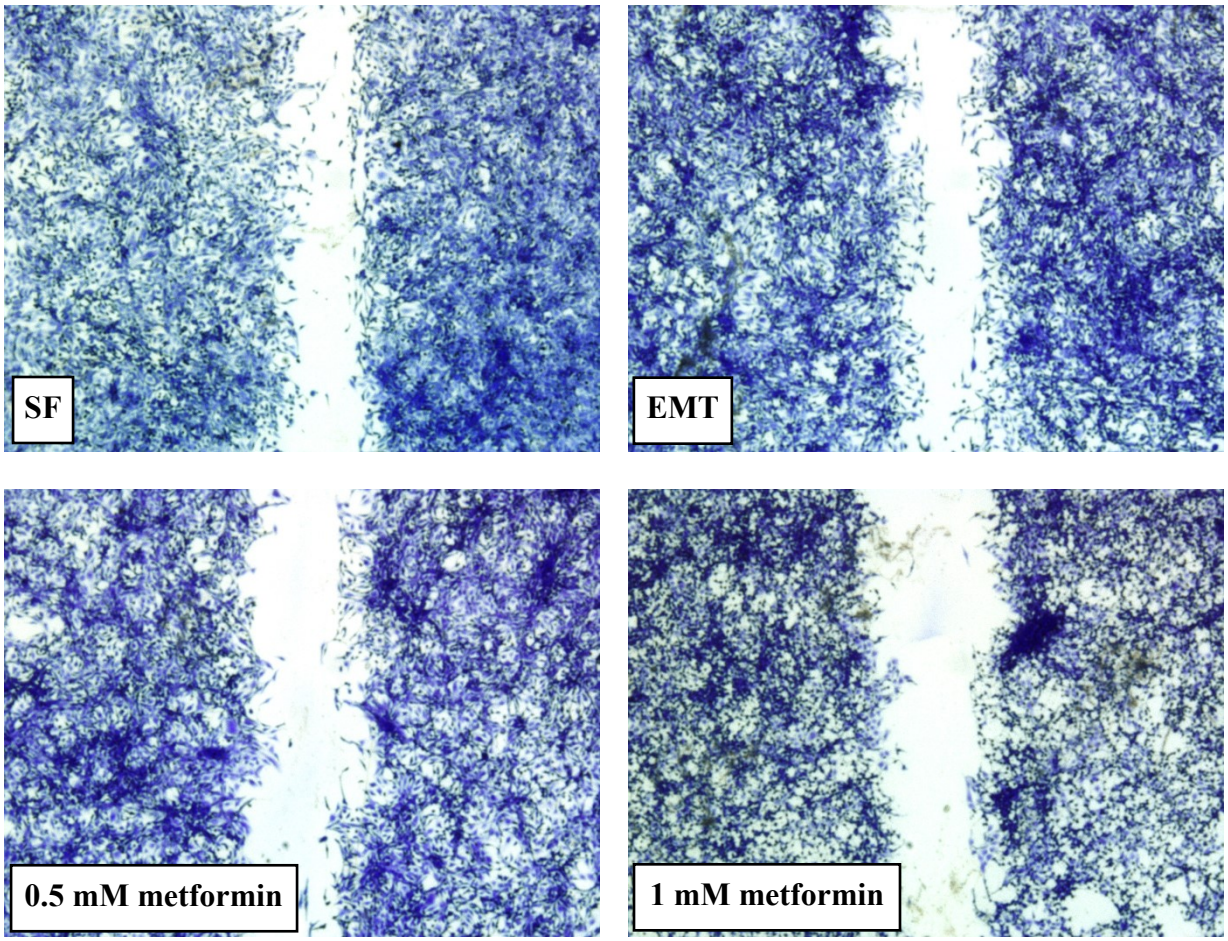

Figure 13. Wound assay (4X magnification). Cells treated with TGF-β and FGF possessed greater capacity to migrate and close the wound gap. Metformin inhibited the ability of LECs to migrate so that the gap was maintained.

Effect of Metformin on Lens Epithelial and Mesenchymal Markers

Western blot analysis of the expression of epithelial and mesenchymal markers validate these outcomes observed at the cellular level. The lens epithelial markers Pax6 and E-cadherin were expected to decrease with EMT treatment, as reported in literature.[7,54] This decrease was observed in EMT-induced LECs in this study (Figure 14). An increase in both markers was noted with treatment of 1 mM metformin, although at levels less than observed in cells cultured in SF. However, with concentrations of metformin at 5 and 10 mM, a stark decrease in pax6 and E-cadherin expression was noted. This was consistent with loss of the epithelial phenotype.

The mesenchymal markers α-SMA and fibronectin were increased after EMT induction and decreased with metformin treatment (Figure 15). For fibronectin, this decrease was proportional to increasing concentrations of metformin. For α-SMA, this decrease was only observed from 0.1-1 mM metformin; higher concentrations (5 and 10 mM) showed an increase in α-SMA.

This increase in α-SMA most likely resulted in the reorganization of the cytoskeleton and the excessive formation of actin stress fibers, resulting in the spindle-like appearance observed earlier. That the cells treated with higher concentrations of metformin had the same morphology as EMT-induced cells begged the question of why there was decreased migration in the former. Contrary to the prevalent belief that any increase in stress fibers would translate to improved cell motility, it has been noted that this process might actually instead prohibit movement due to temporal limitations.[144] Different types of actin stress fibers have also been characterized, with some that are less dynamic than others.[62] It is possible that LECs developed less dynamic stress fibers in response to high concentrations of metformin. Finally, it must be noted that motility and contractility involves proteins other than α-SMA and stress fibers.[62]

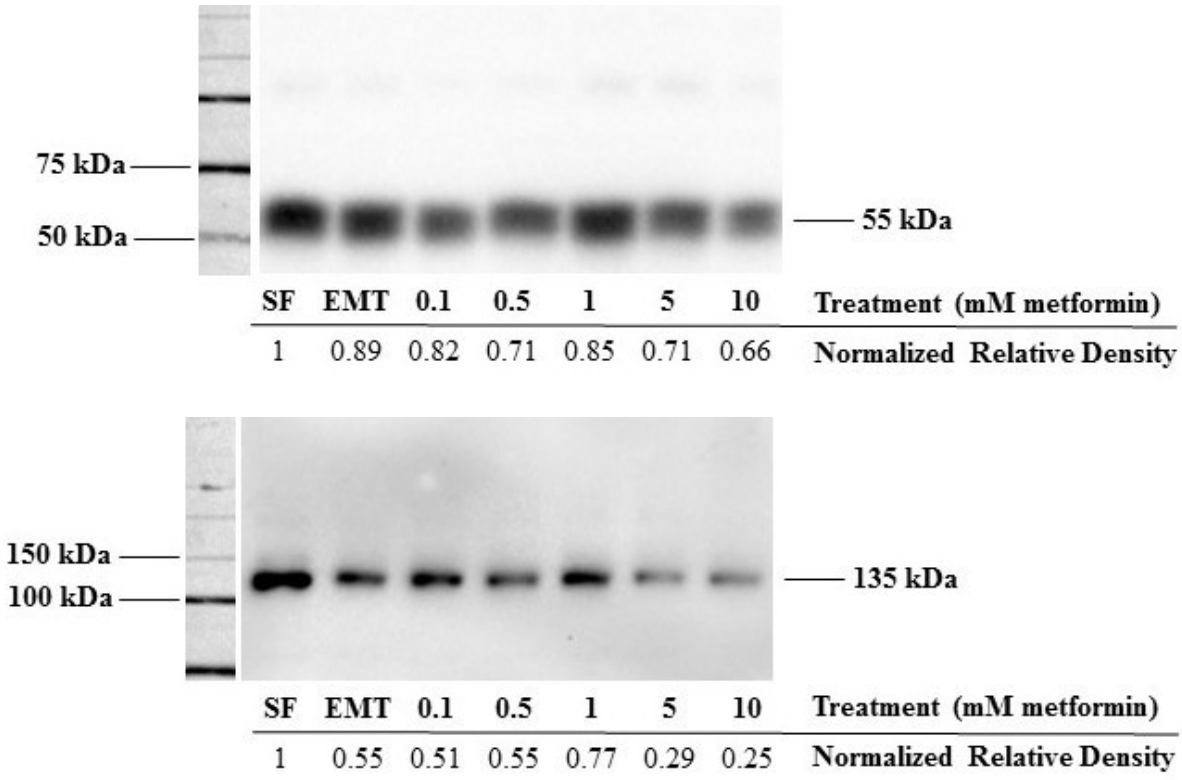

Figure 14. Metformin maintains expression of lens epithelial markers. Pax6 *(top)* and E-cadherin *(bottom)* expression. Pax6 and E-cadherin decreased with EMT treatment. Metformin had minimal effect on the expression of pax6 and E-cadherin; increased expression of both markers was noted only at 1 mM metformin and at levels less than observed with cells cultured in SF. It should be noted that higher concentrations of metformin (5 and 10 mM) resulted in decreased expression of lens epithelial markers.

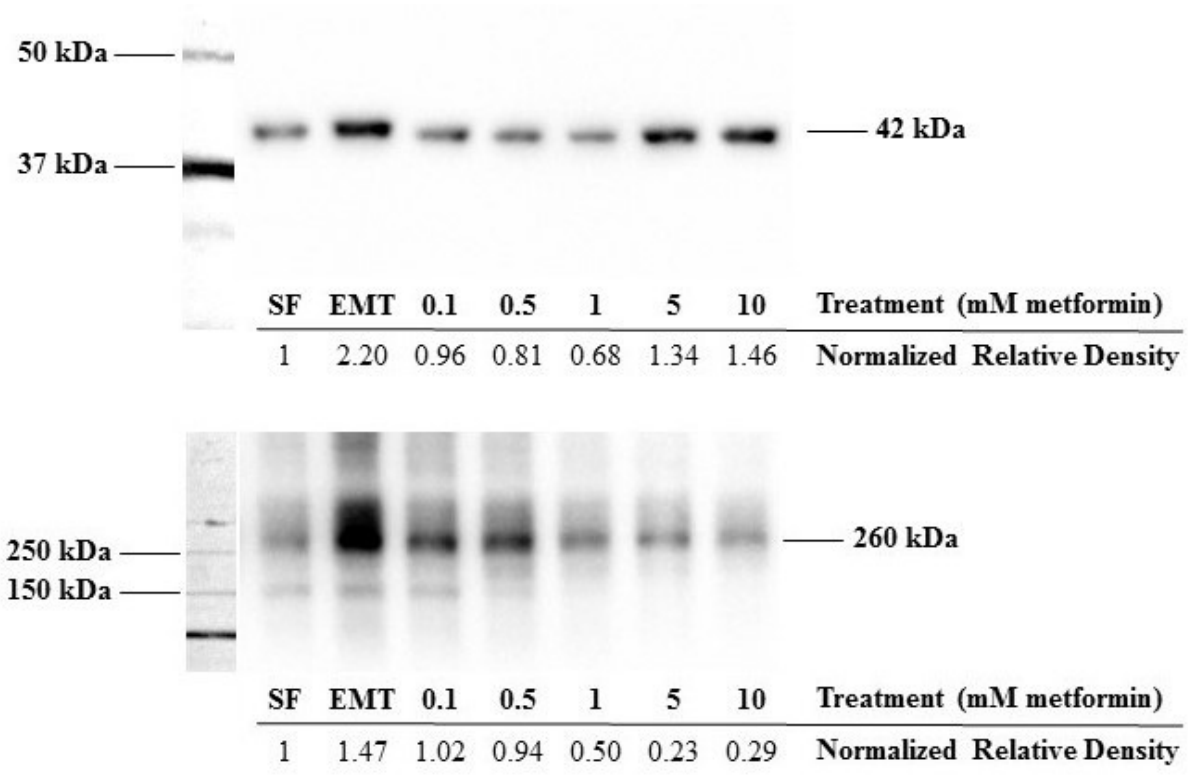

Figure 15. Metformin inhibits expression of mesenchymal markers. Expression of α-SMA 9 *(top)* and fibronectin *(bottom)* in LECs. Culture in EMT induction media resulted in increased expression of both mesenchymal markers. Treatment with metformin decreased expression of both α-SMA and fibronectin. For fibronectin, this decrease was sustained and inversely related to increasing concentrations of metformin. On the other hand, an increase in α-SMA was observed at concentrations of 5 and 10 mM metformin.

Effect of Metformin on SLC22A1 Expression in HLE-B3

With the HLE-B3 cell line demonstrated to express SLC22A1, the effect of various levels of metformin on the expression of its uptake receptor was determined. Western blotting revealed upregulated SLC22A1 expression in response to increasing concentrations of metformin (Figure 16). This increase is most likely due to a positive feedback mechanism.

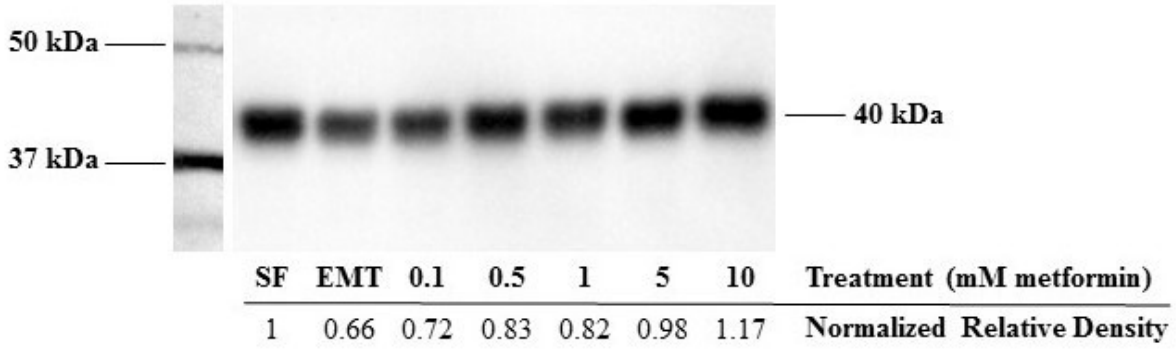

Figure 16. SLC22A1 increased in response to metformin. Expression of the cellular uptake receptor for metformin decreased with EMT treatment and increased with greater concentrations of metformin.

Effect of metformin on the phosphorylation of Akt (protein kinase B)

Metformin was also found to exert its effects via the key signaling protein Akt (protein kinase B), a central regulator of downstream pathways involved in cell survival, proliferation and migration.[64] EMT induction resulted in the increased activation of Akt (Figure 17), thereby promoting the changes in LECs previously observed.

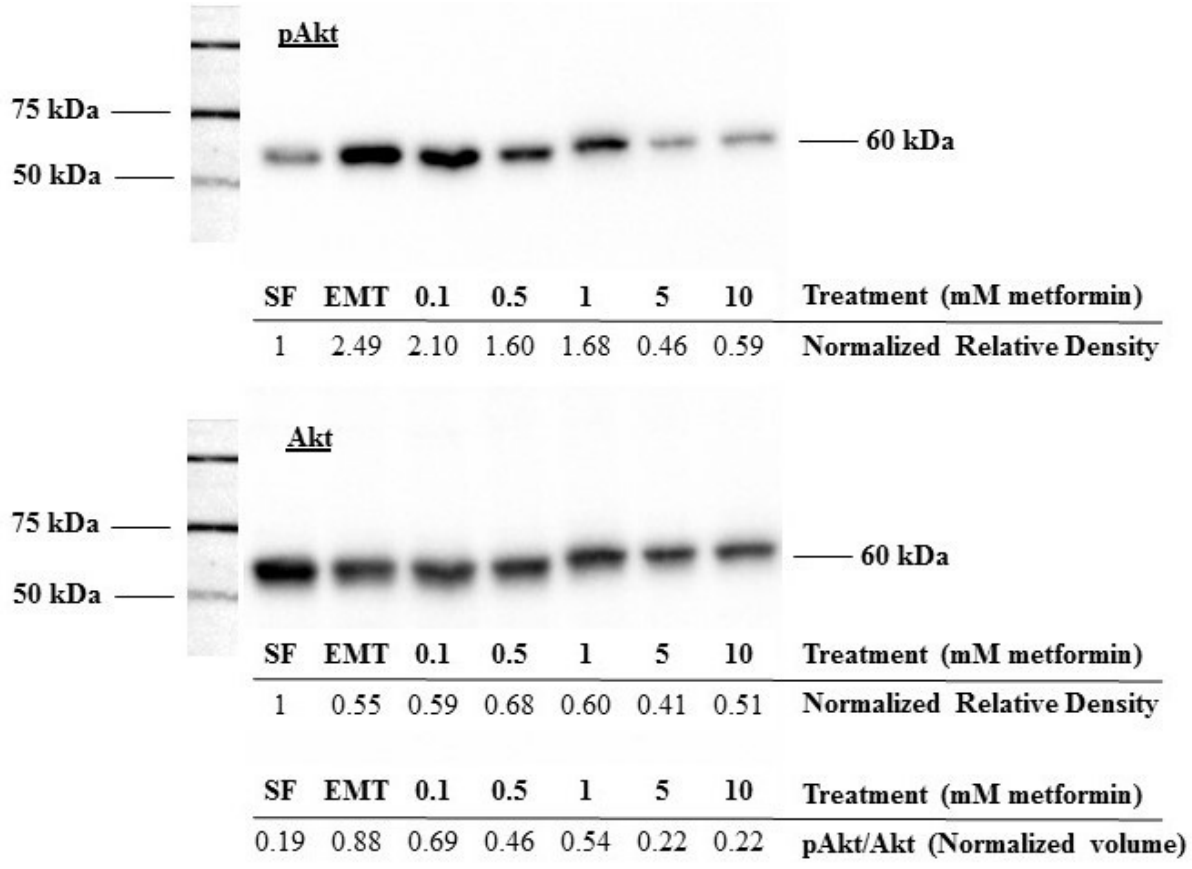

Figure 17. Metformin inhibited EMT in LECs through decreased activation of Akt. EMT induction increased phosphorylation of Akt (pAkt); note the decreased amount of inactive Akt. Metformin concentrations from 0.1-1 mM decreased the pAkt/Akt ratio by decreasing activation of Akt. Higher metformin concentrations decreased the pAkt/Akt ratio by reducing both Akt production and phosphorylation.

Treatment with metformin at concentrations ranging from 0.1-1 mM, however, led to a decrease in phosphorylated Akt (pAkt). The pAkt/Akt ratios were consequently lower with metformin treatment. The decrease in total and phosphorylated Akt observed at higher concentrations of metformin (5 and 10 mM) could be attributed to the effect of metformin on cellular stress, particularly on the endoplasmic reticulum.[145,146]

Expression of the SLC22A1 Receptor in Lens Tissue

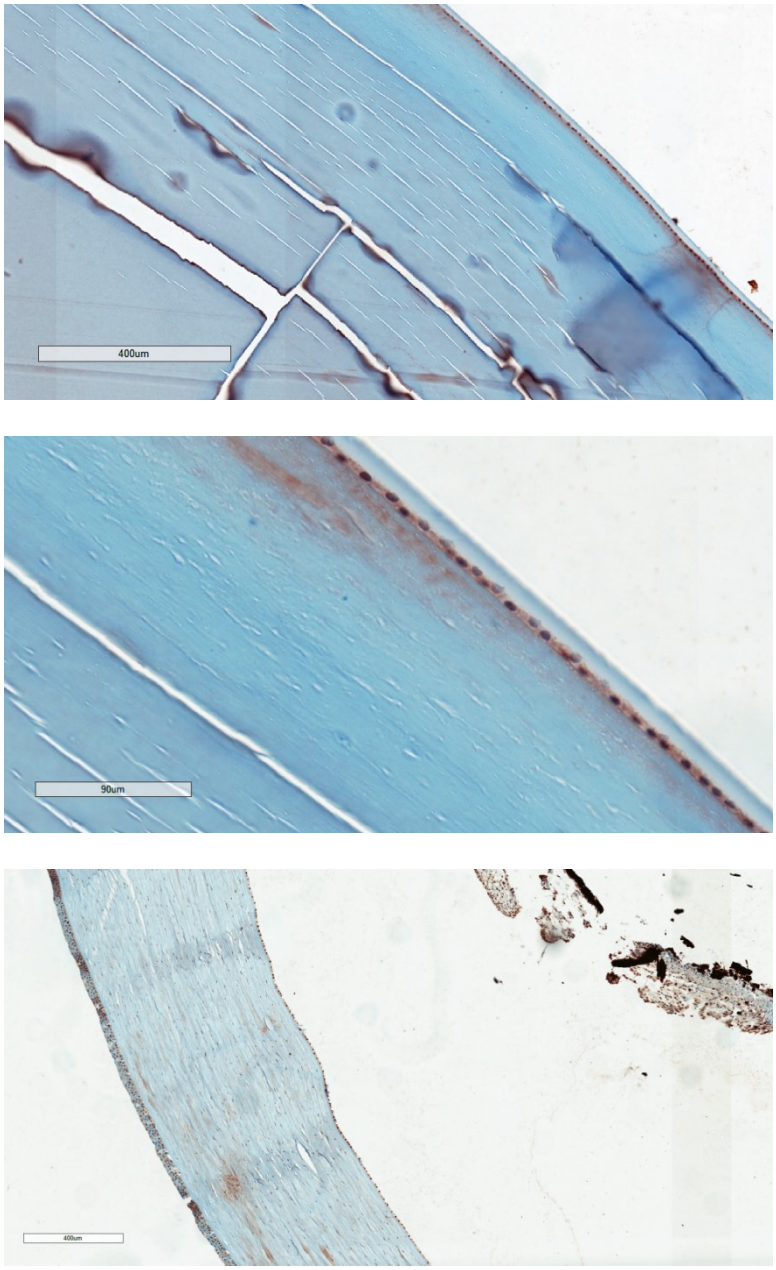

Figure 18. SLC22A1 was expressed in the human lens epithelium. *(a)* The lens epithelium. *(b)* Higher magnification of the lens epithelium. Note cytoplasmic expression of the receptor. *(c)* SLC22A1 is known to be expressed in the corneal epithelium and endothelium; these served as internal positive controls.

DISCUSSION

Metformin has been shown to prevent EMT in different types of cancer cells. Because EMT is also the key mechanism underlying the development of posterior capsule opacification (PCO), this study has sought to determine the effect of metformin on lens epithelial cells (LECs) through utilization of an *in vitro* model of PCO. The data obtained from these series of experiments demonstrate that metformin inhibits EMT in LECs. This encompassed reduced survival and induction of apoptosis; preservation of the LEC phenotype, as seen in the maintenance of epithelial marker expression and cell morphology; and reduced mesenchymal marker expression and cell migration. Such effects are evident with effective concentrations of metformin ranging from 0.1 mM to 1 mM. These outcomes are also most likely mediated through the uptake of metformin through the SLC22A1 receptor and the decreased phosphorylation of Akt.

This is the first study to utilize quantitative measures of cell morphology in LECs exposed to EMT induction. Previous studies have noted the acquisition of a spindly shape after exposure to TGF-β, both *in vitro* and in histological sections of the eye, but the data documented was descriptive and qualitative.[147-150] With this study, the objective parameters of circularity and roundness, as automated and standardized by the image analysis software, could more definitively characterize the changes in LEC morphology during EMT. This method of measurement and analysis can be used in future studies evaluating cell morphology in PCO.

More importantly, this is the first study investigating the effect of metformin on LECs and the expression of SLC22A1 in the ocular lens epithelium. The demonstrated efficacy of metformin against EMT in LECs *in vitro* is encouraging for three reasons. First, it is highly probable that LECs *in vivo* can respond to metformin, as it has been shown, through immunostaining of anterior

segment sections obtained from human donor eyes, that the ocular lens epithelium do express the SLC22A1 receptor.

Second, EMT is the main pathophysiology underlying PCO formation, the most common complication after cataract surgery. It is logical to suppose that inhibition of EMT will translate into PCO prevention. Lastly, metformin has an excellent pharmacologic profile: it is inexpensive, readily available, and safe. Current practice utilizes metformin in diabetes and metabolic diseases, wherein the drug is taken orally up to a maximum of 2.55 grams per day.[151] Even then, because metformin is not metabolized and it is rapidly excreted, systemic levels remain sufficiently low so that adverse events are rare.[151]

The quick elimination of metformin, while advantageous for its safety, poses a problem for its dosing, route and timing of administration. The effects of metformin against EMT in LECs, as revealed in this study, manifest with concentrations ranging from 0.1-1 mM metformin. Oral intake of the maximum dose of metformin in humans results in a peak blood concentration of only 5 ug/mL (38.7 uM);[151] even if metformin can cross the blood-brain barrier and reach the aqueous humor with perfect efficiency, this concentration is likely to be too small to induce its desired effects. No clinical trial studying the effect of systemic metformin on PCO formation has ever been done, however. Considering the widespread influence of metformin on metabolism and inflammation, so much so that systemic intake of metformin has been associated with a 25% risk reduction of open-angle glaucoma,[107] such a clinical trial will definitely be worthwhile.

Topical administration of metformin is another option that should be evaluated. An ophthalmic preparation of metformin dissolved in PBS, as was used in this study, has been utilized to study the ocular pharmacokinetics of organic cation transporters like SLC22A1.[111] In this experiment, an ophthalmic drop solution containing 7.85 mM was prepared and instilled into the

lacrimal sac of rabbits. Liquid chromatography mass spectrometry of the aqueous humor revealed that it took 30 minutes for the concentration of metformin to peak. However, this peak concentration was only at 0.108 nm/mL, translating to a penetration efficiency of 0.058%.[111] It is important to note that there are certainly differences between the ocular anatomy and physiology of rabbits and humans that can result in differences in pharmacokinetics. For instance, rabbits blink less than humans, resulting in higher surface concentrations and lower rates of drainage. Rabbits also have a nictitating membrane, which act as a reservoir and are not present in humans. Even the lack of pigmentation in albino rabbits used in pharmacology may alter drug absorption and metabolism.[152]

Ignoring these qualifications and extrapolating the ocular kinetics of metformin, it means that to attain 0.1 mM in the aqueous humor, an ophthalmic preparation of 200 mM (25.8 mg/mL) needs to be prepared and administered. As this is far beyond the solubility of metformin in PBS, which is 1mg/mL, a suspension might be more suitable.[153] The use of other solvents may resolve this difficulty. Likewise, there are other factors in drug design that may be manipulated to improve absorption and decrease elimination. For instance, the addition of preservatives such as benzalkonium chloride, polymers or basic amino acids, as well as increasing lipophilicity and osmolarity, may improve absorption. Other drug delivery platforms to maximize the limits of physiologic doses also exist; these include biodegradable inserts, liposomes and nanoparticles.[152]

Because the objective of metformin use is to prevent PCO formation, it is practical to visualize the intraocular lens (IOL) implant as a drug delivery device. The IOL can be coated with the drug, or the drug can be loaded into a separate reservoir and linked to either the haptic or the optic.[154] Using this strategy, two drugs have thus far been tested against PCO. One employed polymethylmethacrylate (PMMA) lenses coated with thapsigargin, a hydrophobic inhibitor of the

endoplasmic reticulum adenosine triphosphatase (ATPase), in a capsular bag explant culture model. This technique resulted in death of residual LECs.[155] Another used the chemotherapeutic agent daunorubicin, linked with a carrier and to the IOL, in a PCO model in rabbits. A significant reduction in PCO was found with this approach, although there was some loss of corneal endothelial cells.[156] In this regard, metformin can be used in conjunction with IOLs, whether through soaking, supercritical fluid impregnation, layer-by-layer coating, or chemical grafting.[157]

Aside from route, the timing of drug administration is also a question that needs to be addressed: does metformin need to be given pre-, intra-, or post-operatively, or in multiple settings? Dosing is also another issue that must be resolved. Metformin was not completely cytotoxic until a concentration of 80 mM was reached. Nevertheless, this is not the ideal amount of metformin to use *in vivo,* primarily because it will be difficult to administer this supraphysiologic concentration without causing damage to proximal tissues. There is also the debatable issue of the risk of lens dislocation with early elimination of LECs.[50] In addition, while the findings of this study report noticeable effects against EMT even at 0.1 mM metformin, particularly with respect to cellular migration, EMT is not significantly inhibited across all parameters until levels reach 1 mM. Considering the differences in pharmacokinetics between cell culture systems and living organisms, however, a range of doses, schedules and routes must be tested to determine the optimum conditions for metformin to produce the desired outcome, whether it be the elimination of any residual LECs, the inhibition of EMT, or the prevention of PCO formation.

Whereas the safety profile of metformin with systemic administration is established, its direct toxicity on eye tissue has yet to be determined. SLC22A1 receptors have been identified in the cornea, in the iris and ciliary body, and in the blood-retina barrier.[81] It is thus likely that these

portions of the eye will be most receptive to metformin. Since metformin is intended for use in the anterior segment and its target distribution site is the aqueous humor, assessing its toxicity on the cornea is of utmost priority. No corneal edema or endothelial cell loss were reported with the use of the aforementioned ophthalmic formulation (7.85 mM metformin in PBS) in rabbits, although these results have yet to be confirmed in human eyes.

The efficacy of metformin against EMT in this *in vitro* model of PCO, alongside the pharmacologic characteristics of the drug and the myriad of strategies for its delivery, is promising. The results of this investigation usher in questions of safety, and of the dose, route and timing of metformin administration that will be best answered in subsequent *in vivo* experimentation and clinical trials. While numerous *in vivo* models of PCO exist, the use of continuous curvilinear capsulorrhexis with phacoemulsification in rabbits is the most applicable. This technique replicates the surgery done as standard of care, and rabbit eyes are sufficiently similar in size and structure to represent human eyes. With this model, there is rapid PCO development due to the magnitude of inflammation after surgery. IOLs may also be implanted in rabbits, so that the efficacy of metformin utilizing the IOL as a delivery platform can be examined. Finally, even if rabbit LECs respond differently to pharmaceutical compounds, a review of literature has proven the use of rabbits to be reliable for predicting positive outcomes in humans.[158] These attributes of the rabbit designate it as the ideal organism for *in vivo* PCO models, particularly for the translation of the findings of this study from the bench to the clinic.

CONCLUSION

Upon utilization of an *in vitro* model of PCO, metformin effectively prevents EMT in LECs through inhibition of cell survival via apoptosis induction; maintenance of epithelial morphology by suppression of mesenchymal marker expression and retention of epithelial markers; and reduction of cell motility. These effects are significantly evident with a concentration of 1 mM metformin. These cellular outcomes are also most likely mediated through the uptake of metformin through the SLC22A1 receptor and the decreased phosphorylation of Akt.

The efficacy of metformin *in vitro* has potential to be extended *in vivo* and translated into clinical application due to three key reasons: the safety, accessibility and low cost of metformin; the variety of drug platforms and parameters available for optimized ophthalmic formulation and delivery; and the demonstrated presence of the SLC22A1 receptor in human lens epithelial cells.

Further investigation using the rabbit as an animal model are necessary to confirm these findings *in vivo,* as well as to determine the optimum dose, route and timing of metformin administration.

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