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Role of Caspase-8 on Human Retinal Microvasculature Endothelial Cells (HRMEC) Migration in Hyperglycemia

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ROLE OF CASPASE-8 ON HUMAN RETINAL MICROVASCULAR ENDOTHELIAL CELL (HRMEC) MIGRATION IN HYPERGLYCEMIA

A Thesis

by

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ABSTRACT

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Pathological angiogenesis is the hallmark of proliferative diabetic retinopathy that results from the advanced stage of Diabetic Retinopathy (DR). In response to prolonged tissue injury, retinal microvascular endothelial cells form new blood vessels to ensure the supply of oxygen and nutrients to the eye; however, progressive fibrovascular proliferation can eventually lead to retinal detachment and blindness. Caspase-8, the responsible protease for apoptosis has also been shown to modulate pathological and developmental angiogenesis but whether it exerts this role in a hyperglycemic environment has not yet been explored. Here we demonstrate that Caspase-8 decreased cell viability and was upregulated in hyperglycemic HRMEC. In addition, Caspase-8 K.D. resulted in inhibition of cell migration. Taken together, our data suggest that Caspase-8 plays a role that is involved in cell migration in HRMEC.

DEDICATION

The completion of my masters studies would not have been possible without the continuous support of my loved ones, especially my mother Cecilia Campano. Thank you for your never-ending love and encouragement.

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CHAPTER I

INTRODUCTION

Diabetic Retinopathy

Diabetic retinopathy (DR) is a condition affecting individuals with prolonged diabetes mellitus (DM) that is characterized by lesions in the retina. Not only is DR one of the most severe consequence arising from DM, but it is also the leading cause of blindness in the world (Bretts- Obregon et al., 2016). Type 2 diabetes (T2D) has reached an epidemic level globally while type 1 diabetes (T1D) rates are slowly rising (Duh et al., 2017). In the United States, the prevalence rate of diabetes in Hispanics is almost twice that of Caucasians. Moreover, 48% of the individuals aged ≥40 in the Hispanic community suffer from DR with 32% suffering from moderate to severe non-proliferative and proliferative retinopathy (West et al., 2001).

The retina is one of the most metabolically active tissues in the body, consuming high levels of oxygen and nutrients. The microvasculature in the retina responds to hyperglycemia through a number of biochemical changes that can be divided into an early and late stage. In the early non-proliferative stage, DR is denoted by pericyte dropout that leads to microaneurysms, thickening of the basement membrane, and increased permeability (Kharashi et al., 2018). These events are proceeded by ischemia of the retinal tissue which promotes upregulation of endothelial mitogens for secretion of growth factors. Extensive fibrovascular proliferations may lead to retinal detachment that is sometimes accompanied by vitreous hemorrhage.

At the end stage of DR, when the vitreous has been completely contracted, loss of vision is best explained by severe retinal ischemia (Danis et al., 2012). Because non-proliferative DR can progress to proliferative diabetic retinopathy (PDR), it is important to understand the biological processes that govern retinal vascular development. This has become an area of interest for researchers and clinicians to develop preventive and interventional therapeutics for vascular eye diseases that address promoters of abnormal vascular growth.

Caspase-8

The protein of interest in this project is caspase-8, a cysteine-aspartate specific protease that is well known for activation of the extrinsic cell death pathway (Kellar et al., 2018; Mandal et al., 2020; Van-Raam et al., 2012; Zhao et al., 2010). Apoptosis occurs when procaspase-8 is recruited by its N-terminal prodomain to the death-inducing signaling complex (DISC) following a death receptor ligation (Kellar et al, 2018 and Zhao et al, 2010). Caspase-8 aggregate and facilitate their dimerization of the C-terminal catalytic domain through the proximity-induced model which initiates autocatalytic processing (Kellar et al., 2018; Mendal et al., 2020; Zhao et al., 2010). Initially, cleavage occurs at Asp 374 and Asp 384 between the two subunits in the catalytic domain followed by cleavage of the catalytic domain from the prodomains. The released, active dimer cleaves a variety of downstream targets (Kellar et al., 2018). However, capsase-8 activity does not always result in cell death. In fact, low levels of caspase-8 activity promote the formation of the ripoptosome complex in which caspase-8 forms a heterodimer complex with FLIP. The FLICE Like Interleukin 1ß-Converting Enzyme (FLIP) is a catalytically inactive homologue of caspase-8 and is also recruited during the DISC formation. This heterodimer complex has catalytic activity but not enough to complete processing of caspase-8

thus, blocking apoptosis whilst promoting caspase-8 activity (Kellar et al., 2018, Mendal et al., 2020; Zhao, 2010).

The dimerization of c-FLIP with caspase-8 has less protease activity than that of the caspase-8 homodimer resulting in a translocation of free active caspase-8 into the cytosol promoting the activity to focus on local targets (Kellar et al., 2018). This complex also has the ability to prevent necroptosis, another type of cell death that is characterized by rupture of the cell membrane. Caspase-8 cleaves serine/threonine protein kinase (RIPK3) thus inhibiting activation of the ultimate effector of necroptosis, the pseudokinase mixed-lineage kinase domain-like (MLKL). However, RIPK3-dependent necroptosis can be activated by the absence of caspase-8 or signals that inhibit it (Kellar et al., 2018 and Mendal et al., 2020). Among the caspase family, caspase-8 is unique in that it acts as an environmental sensor, transduces a range of signals to cells, and modulates responses that extend beyond survival (Clauss and Breir, 2005). While it is well known for its role in cell-death signaling, caspase-8 has also been shown to take part in activities that promote cell survival independently of its cell-death processes which include cell migration and survial (Kellar et al., 2018; Kang et al., 2012; Chen et al., 2017).

Problem Statement

Prolonged hyperglycemia results in a series of events that if left untreated lead to neovascularization and potentially vision loss. Caspase-8 has been shown to be modulate pathological and developmental angiogenesis by being mechanistically involved in promoting endothelial cell proliferation, migration and sprouting (Tish et al., 2019). Whether inhibition of caspase-8 in a hyperglycemic environment can prevent the cell migration that leads to pathological angiogenesis has not yet been explored.

Research Objective

The aim of this project focuses on studying the role of caspase-8 in hyperglycemic induced human retinal microvascular endothelial cells (HRMEC). Here, we replicate the pathologic chain of events occurring Diabetic Retinopathy by inducing endothelial cells to a high glucose environment and identifying whether loss of caspase-8 can prevent cells from undergoing the cell migration that results in pathologic vascular formation.

CHAPTER II

REVIEW OF LITERATURE

Anatomy of Retinal Endothelial Cells

Retinal endothelial cells line the vasculature microvasculature that supplies and drains the neural retina. One distinguishing feature of endothelial cells in retinal circulation is the presence of intercellular junctions that form tight unions with neighboring cells (Aiello and Wong, 2000). This characteristic contributes to homeostasis that is maintained by the inner blood retinal barrier which is essential for proper vision. The blood retinal barrier helps protect the retina by excluding circulating molecular toxins, microorganisms, and pro-inflammatory leukocytes (Aiello and Wong, 2000). The interactions between endothelial cells with vascular mural cells, neurons, and glial cells are all critical for normal retinal endothelial cell functioning and also contribute to the development of retinal vascular diseases (Al-Kharashi et al., 2018; Bharadwaj et al., 2013).

A well-known relationship between pericytes and endothelial cells (EC) has also been established and is significant to the development of DR (Aiello and Wong, 2000). Pericytes are embedded in the EC basement membrane and pericyte death impacts EC function by diminishing their resistance to metabolic stress thus compromising blow flow in the retina leading to local hypoxia (Bharadwaj et al., 2013). In response to hypoxic conditions, angiogenic factors such as vascular endothelial factor (VEGF) are secreted which allow for the progression of neovascularization (Unait and Kazlauskas 2017).

Furthermore, retinal endothelial cell are particularly prone to oxidative stress caused by diabetes (Grammas and Riden, 2003). It has been shown that compared to brain derived endothelial cells, retinal endothelial cells release higher levels of superoxide, have less glutathione peroxidase activity as well as lower levels of junctional protein and superoxide dismutase when treated with high glucose (Grammas and Riden, 2003).

Hyperglycemia leads to VEGF secretion and angiogenesis

Of the many molecular mechanisms that underly vascular dysfunction, hyperglycemic induced hypoxia is one of the most important as it results in the secretion of vascular endothelial growth factor (VEGF) from retinal endothelial cells (Bretts-Obregon et al., 2016). Vascular endothelial growth factor (VEGF) is a key regulator of blood vessel growth and strongly increases vascular permeability. It functions as a survival factor for endothelial cells by stimulating proliferation, migration, and tube formation (Ferrara, N. 2013). In diabetic retinopathy, VEGF is excessively produced and results in endothelial cell proliferation, tissue damage, fluid leakage, new blood vessel formation and microaneurysms playing a major role in disease progression (Aiello and Wong, 2000; Bretts-Obregon et al., 2016; Ferrara, 2013).

Growth factors such as VEGF promote a neovascular response locally and by diffusing through the vitreous to other areas of the retina, to the optic disc, and into the anterior chamber (Danis and Davis 2012). In fact, studies have shown the accumulation of VEGF in the vitreous and fibrovascular tissue of patients with PDR (Danis and Davis, 2012; Bharadwaj et al., 2014; Sun., 2018; Gui et al., 2020). The main mechanism of hypoxia-induced angiogenesis involves the increase in hypoxia-inducible factor-1 (HIF-1) protein which in turn binds to regulatory regions of several hypoxia-sensitive genes like VEGF (Clauss and Breir, 2005). The expression of VEGF receptor type 1 mRNA can be induced by hypoxia and the endothelium can be

receptive to VEGF stimulation only if it senses hypoxic conditions. VEGF can then be secreted and bind to receptor tyrosine kinases (VEGFR-1 and VEGFR-2) located on the surface of vascular endothelial cells. The receptor ligation triggers a cascade of intracellular signaling pathways that initiate angiogenesis.

During angiogenesis endothelial cells migrate to form new capillaries from preexisting vessels. Migrating endothelial cells extend filipodia in association with astrocyte processes that are followed by "stalk cells" which proliferate to grow the endothelial sprout that initiates vessel formation (Aiello and Wong, 2000). VEGFR-1 and VEGFR-2 receptors direct endothelial tip cell migration and stalk cell proliferation (Bharadwaj et al., 2013).

Caspase-8 is involved in cell migration

Deletion of caspase-8 in mice results in death in utero, which is associated with cardiac deformations, neural tube defects, and hemopoietic progenitor deficiency implicating that it is required for the proper formation of a vascular system (Kang et al, 2004, Tish et al., 2019). Its deletion also results in reduced endothelial cell proliferation, sprouting and migration independently of its cell-death function whilst being catalically active (Tish et al., 2019).

In the extrinsic pathway which has survival and death inducing functions, CD95 recruits FADD and subsequently procaspase-8 which becomes activated by autocleavage at proteolytic sites to then create a catalytic dimer that cleaves downstream targets including the executioner caspases -3 and -7 (Kellar et al., 2004; Mandal et al., 2020; Zhao et al, 2020). Studies have shown that CD95 regulates vascular morphogenesis in the post-natal retina through PI3K and Erk signaling by promoting EC proliferation and branching, without affecting vessel regression (Chen et al, 2017). Furthermore, caspase-8 proteolytic activity becomes limited when associated with the binding of cFLIP which results in a proteolytic signaling complex that regulates

nonapoptotic caspase-8 function (Keller et al., 2018). This basal activity of caspase-8 is enough to prevent necroptosis and apoptosis (Keller et al., 2018; Hughes et al., 2016; Van Raam and Salvesen, 2011; Zhao et al., 2010).

Interestingly, caspase-8 has been shown to be required for proper angiogenesis by promoting VEGF induced sprouting, proliferation, and migration of human umbilical vein endothelial cells (HUVEC) (Tish et al., 2019). In the proposed signaling pathway, capase-8 inhibits RIPK3 phosphorylation which regulates phosphorylation of p38 resulting in proper cell migration whereas the loss of caspase-8 results in impaired angiogenesis of HUVEC. In a series of multiple experiments, Caspase-8 was initially silenced in HUVECs and underwent a tube formation, bead-sprout, and scratch assay, all failing to respond to VEGF stimulation. Loss of Casp-8 also affected the organization of adherens and tight junctions in endothelial cells (EC) via an increased basal phosphorylation of p38. Blocking caspase-8 activity resulted in increased basal p38 MAPK phosphorylation coinciding with previous studies showing that activation of p38 has been linked to the destabilization of VE-cadherin in the endothelium (Lee et al., 2009). The authors then tested the response of caspase-8^{KD} ECs to VEGF stimulation using the tube-formation assay which showed that caspase-8 in EC affects the proper formation of adherens and tight junctions in the postnatal retina of mice pups in addition to VEGF-induced remodeling of VE-cadherin in vitro (HUVECs). Furthermore, a tube formation and bead-sprout assay demonstrated that inhibition of p38 indeed rescue the ability of caspase-8^{KD} EC to respond to VEGF and to form tubes and sprouts of a length similar to that of caspase-8^{WT} EC (Tish et al., 2019). These results demonstrate that mechanistically, caspase-8 deletion in EC results in proliferation, sprouting, and VE-cadherin subcellular distribution defects.

CHAPTER III

METHODOLOGY AND FINDINGS

Methodology

Cell Culture and glucose exposure

Primary Human Retinal Microvascular Endothelial Cells (HRMEC) were purchased at passage 3 from Cell Systems, Kirkland, WA and propagated using Complete Classic Medium, Gibco™ Antibiotic-Antimycotic, and Culture Boost™. All cells used in the experiments were either passage 5 or 6 and were incubated at 37°C at 5% CO2. Cells used to measure glucose exposure were supplemented with physiological (5.5mM) or hyperglycemic (30mM) glucose levels using glucose-free DMEM prepared with 1% Gibco™ Antibiotic-Antimycotic and 10% FBS. Viable cells were counted using the Trypan blue dye exclusion method.

Real-time polymerase chain reaction

HRMEC were seeded into a 6 well plate at a density of $8x10^4$ and treated with 5.5mM or 30mM glucose. Cell lysates were collected after 24 hours and stored at -80°C. One microgram total RNA was reversed transcribed with SuperScript™ IV VILO™ Master Mix with ezDNase™ Enzyme (Invitrogen, Carlsbad, CA). Primers were purchased from Integrated DNA Technologies, Inc and included: 5'-CTATCCTGTTCTCTTGGAGAGTCC-3'; Caspase-8: forward primer, 5'-CACTAGAAAGGAGGAGATGGAAAG-3'; reverse primer, and 18S which

was used as the housekeeping gene, forward primer; 5' CGGAGTCAAGGATTTGGTCGTAT-3', 5'-AGCCTTCTCCATGGTGGTAAGA-3'; reverse primer. Polymerase chain reactions were run on Gene Amp PCR system 9700 (Applied Biosystems, Foster City, CA) using a "hot start" method with the following conditions: 94 ºC for 5min, 94ºC for 1min, 60–65ºC for 1min, 72 ºC for 1min, 72ºC for 10min. RT-PCR was performed using PowerUp SYBR Green Master Mix (Thermo Scientific). After cell lysate storage from our primary run, an additional two trials were performed at different time points due to errors in value readings. After analyzing our experimental conditions, we chose the second trial that gave the least error in readings as well less mRNA degradation.

Human VEGF Immunoassay

Cells were supplemented with prepared media as indicated in cell culture and 'glucose exposure section'. Conditioned medium was harvested from cells seeded into 24 well plates at a density of $8x10^4$ and stored in -20^{o}C overnight for ELISA. Human VEGF ELISA (Quantikine ELISA Catalog number: DVE00) was performed per manufacturer instructions and analyzed using a Biotek plate reader equipped with Gen5 microplate reader with manager software). Data was quantified in comparison to VEGF standards.

Casp-8 Glo Assay

HRMEC were seeded at $3.5x10^4$ in an opaque bottom 96-well tissue culture plate (ref: 353296, Falcon™). After 24 hours, ECs were treated with 5.5mM or 30mM of glucose. The Casp-8 Glo assay was performed 24 hours following treatment according to the manufacturer's instructions (Promega) using a Thermo Scientific™ Flouroskan™ FL plate reader and Skan it 6.0.2 software.

siRNA Transfection

HRMEC were seeded in a 24 well plate at a density of $4.5x10^4$. At 80% confluent, cells were transfected drop-wise with validated Caspase-8 Silencer™ Select Pre-Designed siRNA® (6 RefSeqs; assay ID: s2427) and Silencer™ Negative Control No. 1 siRNA (Life Technologies) according to the manufacturer's instructions. Two days following transfection, cells were treated with 5.5mM or 30mM of glucose. The final concentration of siRNA solution was 5pmol per well.

Immunoblotting

HMREC used for western bot analysis were plated at $1x10^6$ in 100mm dishes and transfected with Caspase-8 Silencer™ Select Pre-Designed siRNA® or Silencer™ Negative Control No. 1. Final concertation of siRNA solution was 600pmol. Cells were lysed using RIPA lysis buffer mix. Proteins were separated by SDS-PAGE and immunoblotted according to standard protocols. 2% BSA was used for blocking p-p38. B-actin (1:10000, Lot:00923585, Invitrogen), p-p38 (1:1000, ref. 4511, Cell Signaling, and p38 (1:1000, ref. 9212, Cell Signaling).

Scratch Assay

After transfected cells were confluent, they were starved in 2% FBS DMEM medium overnight. A wound was induced by scraping the cell monolayer with a P200 pipette tip. A picture was acquired at time-point 0,12, and 24 hours after incubation at 37°C.

Analysis of wound closure

Images were captured using Nikon fluorescence microscope at 10x. Images for scratch assay of 0, 12, and 24 hours were analyzed using ImageJ software version 1.53a. Wound area for each image was outlined and determined using the measuring tool. 1 pixel represents 1 um^2.

Statistics

Error bars represent ± SEM. To calculate statistical significance, Students T-test was used.

Findings

Glucose concentration impacts HRMEC cell viability

There were 200,000 viable cells at time of treatment. After 24 hours, HRMEC in 5.5mM glucose the viable cell number increased to 232,500 while cells in 30mM showed a decrease of 42% to 119,000 (Figure 1). These findings are in line with previous studies showing that high glucose in endothelial cells may impact the integrity of the microvasculature in the diabetic retina leading to angiogenesis (Betts-Obregon et al., 2016).

Hyperglycemia affects Caspase-8 gene expression

Next, we investigated whether hyperglycemia would influence caspase-8 expression in HRMEC. RT-PCR analysis revealed that cells treated with high glucose increased in caspase-8 mRNA compared to the physiological group (Figure 2). It has been shown that high glucose mediates human pancreatic β cell apoptosis via up-regulation of the Fas receptor as well as in human coronary artery endothelial cells (Maedler et al., 2001; Kageyama et al., 2011). However, whether the increase in expression explains the cell death earlier observed in hyperglycemic HRMEC is unknown because caspase-8 is initially synthesized as a single-chain zymogen

(procaspase-8) (Zhao et al., 2010). Caspase-8 are recruited to the DISC as intact monomers and this recruitment subsequently leads to their dimerization and activation through induced proximity (Van Raam & Salvesen, 2011).

Hyperglycemia induces VEGF secretion

Figure 3 shows VEGF levels in cell medium after 24-hour glucose treatment. Compared to VEGF in cell media from the 5.5 mM glucose group, VEGF levels increased when treated with 30 mM of glucose from 16.296 to 22.407 pg/mL. This suggests that hyperglycemic cells were induced to release more VEGF. Results from a t-test show that the difference in VEGF secretion was significant ($P<.01$). The VEGF per cell (pg/cell) was calculated after 24 hours and each cell in the 5.5mM group secreted 0.000176 pg/cell whereas the 30mM group secreted 0.000341 pg/cell. The increase in glucose concentration is consistent with increased VEGF secretion in HRMEC suggesting a pro-angiogenic response as it occurs in the diabetic retina.

Caspase-8 Activity in Endothelial Cells is low but present

Because Caspase-8 activity is required to promote proper cell migration (Tish et al 2019), a Caspase-8 Glo assay was conducted. HRMEC in physiological glucose levels showed a slightly lower caspase-8 activity than hyperglycemic cells (Figure 3). The increase of caspase-8 activity is consistent with increase of VEGF protein previously shown by the ELISA. Both glucose groups showed between 1.5 and 2 luminescence units, falling at the lower end of the standard scale. Our Glo-Assay provides us with further insight that could explain what occurs at the DISC complex once procaspases are recruited. Studies imply that the Caspase-8/FLIP heterodimer with lower activity levels is likely to be responsible for cell migration (Tish et al., 2019) and is not the cause of the fully mature caspase-8 homodimer promoting the extrinsic cell death pathway (Tish el al 2019). Maedler et al., 2011 found that up-regulation of FLIP, by

incubation with transforming growth factor or by transfection with an expression vector coding for FLIP, protected β cells from glucose induced apoptosis, restored β cell proliferation, and improved β cell function.

Caspase-8 K.D. inhibits cell migration

After confirming caspase-8 activity in HRMEC, we questioned if silencing Caspase-8 would prevent cell migration in a hyperglycemic setting. Because VEGF had already been shown to be secreted in hyperglycemic cells, transfected cells were not stimulated with VEGF. Prior to treating cells with glucose, an rt-PCR was performed to ensure the knockdown was efficient (Figure 4). Following transfection, cells were treated with glucose for 24 hours and images were taken at 0,12, and 24 hours to track wound closure (Figure 5). At 12 hours, there appeared to be cell death and at 24 hours, cell death was more notable with a change in cell morphology in all groups. However, the wound area in the control group became smaller compared to the K.D. In addition, the 5.5 mM glucose treated Casp-8^{KD} EC group wound area decreased before reversing to almost its original size at 24 hours. Whether this was caused by cell dropout is unknown. Analyzation with imageJ software revealed a decreased surface area overall in the K.D. vs. the control group with no difference between the normal and high glucose group (Figure 6). Figure 5 shows a higher wound closure percentage in the control groups compared to the control. These findings suggest that K.D. of Caspase-8 in HRMEC prevents cell migration.

Caspase-8 K.D. resulted in decreased p38 MAPK activation

The p38 mitogen-activated protein kinase (MAPK) cascades are signaling pathways that regulate multiple functions of endothelial cells in response to exogenous and endogenous stimuli such as growth factors, stress and cytokines (Crcina, 2017). Loss of caspase-8 has been shown to increase basal p38 phosphorylation (Tish et al., 2019; Lee et al., 2009). The proposed pathway is

mediated by RIPK3 phosphorylation which results in increased downstream phosphorylation of p38 upon loss of caspase-8 (Tish et al., 2019). This p-p38 activity is the presumed downstream effector responsible for preventing the proper response to VEGF in EC resulting in impaired angiogenesis (Tish et al., 2019). Contrary to our expected results, western blot analysis revealed a decrease of p38 phosphorylated protein in the siCaspase-8 group compared to the control group (Figure 9). Tish et al 2019 also demonstrates that stimulation of Casp-8^{KD} ECs with VEGF further increases p-p38. It is possible that stimulation of HRMEC with VEGF to increase its protein concentration, could have resulted in increased p38 MAPK. However, we cannot ignore that p38 MAPK basal levels higher in the control group leading us to reject the proposed pathway in our cell line.

CHAPTER IV

DISCUSSION

Our studies have shown that hyperglycemia has a negative effect on endothelial cells by decreasing viability. Whether this is the result of Caspase-8 mediated apoptosis is unknown as cleaved Caspase-3 was not studied in this project. Because caspase-8 is initially synthesized as a single-chain zymogen (procaspase-8) (Zhao et al., 2010) we could not rule out that caspase-8 was further processed to become a mature caspase-8. However, Tish et al., 2019 showed that Casp-8– mediated apoptosis may have played a small but significant contribution to the physiological remodeling process in HUVEC, without affecting overall vessel regression. Furthermore, it is possible that TGFβ mediated apoptosis played a role in the cell death seen in the hyperglycemic group. A study by Betts-Obregon 2016 showed that TGFβ significantly induced apoptosis in retinal pericytes and endothelial cells which also accumulated in the inner retina arterioles.

It was important for us to identify VEGF secretion in HRMEC as this is an endothelial cell mitogen and key regulator of both physiological and pathological angiogenesis (Chavakis & Dimmeler, 2002). Here we also demonstrate that hyperglycemic HRMEC release higher levels of VEGF protein in comparison to the physiological glucose level group supporting previous results showing concentration-dependent increase of VEGF protein in human retinal endothelial cell and pericytes (Sun et al., 2010; Betts-Obregon et al., 2016). It has become clear that Caspase-8 possesses more function than its long-established role in triggering apoptosis. Here

we demonstrate that knockdown of caspase-8 inhibits cell migration and results in decreased p38 phosphorylation. In cancer cells, p38 regulates cell migration through Hypoxia-induced activation of HIF-1 that leads to the expression of VEGF which binds to tyrosine kinase receptor VEGFR2 present at the surface of blood vessel endothelial cells. Downstream of p38, MAPKAP kinase-2 (MK2), contributes to increase in the level of polymerized actin by phosphorylating HSP27 leading to actin polymerization, focal adhesion, turnover, and cell contraction lead to cell migration. This pathway has been identified in a study of endothelial cells where RIPK3 positively regulated the activation of p38 and HSP27 upon permeability factor treatment (VEGF- A, VEGF-B, FGF-b) and thus, promoting permeability in the tumor cells to metastasize (Hänggi et al., 2017). This potentially explains the loss of EC migration in HRMEC. In Tisch's study, in the absence of Casp-8, RIPK3 mediates increased phosphorylation of p38 in vitro and impaired developmental vascular growth in vivo. This RIPK3/p-p38 axis deregulates EC behavior, resulting in impaired angiogenesis. This makes us question why loss of caspase-8 resulted in decreased p38 phosphorylation in HRMEC rather than the increase phosphorylation observed in HUVEC.

In conclusion, further studies are needed to identify the molecular switches that govern caspase-8 behavior to stop from becoming active dimer in high glucose conditions to a caspase-8/FLIP heterodimer that influence cell migration. Repeated studies that test for Fas receptor activation and subsequently p38 phosphorylation in hyperglycemic Caspase-8^{KD} EC could provide us with further insight on how high glucose affects EC behavior.

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APPENDIX

APPENDIX

FIGURES

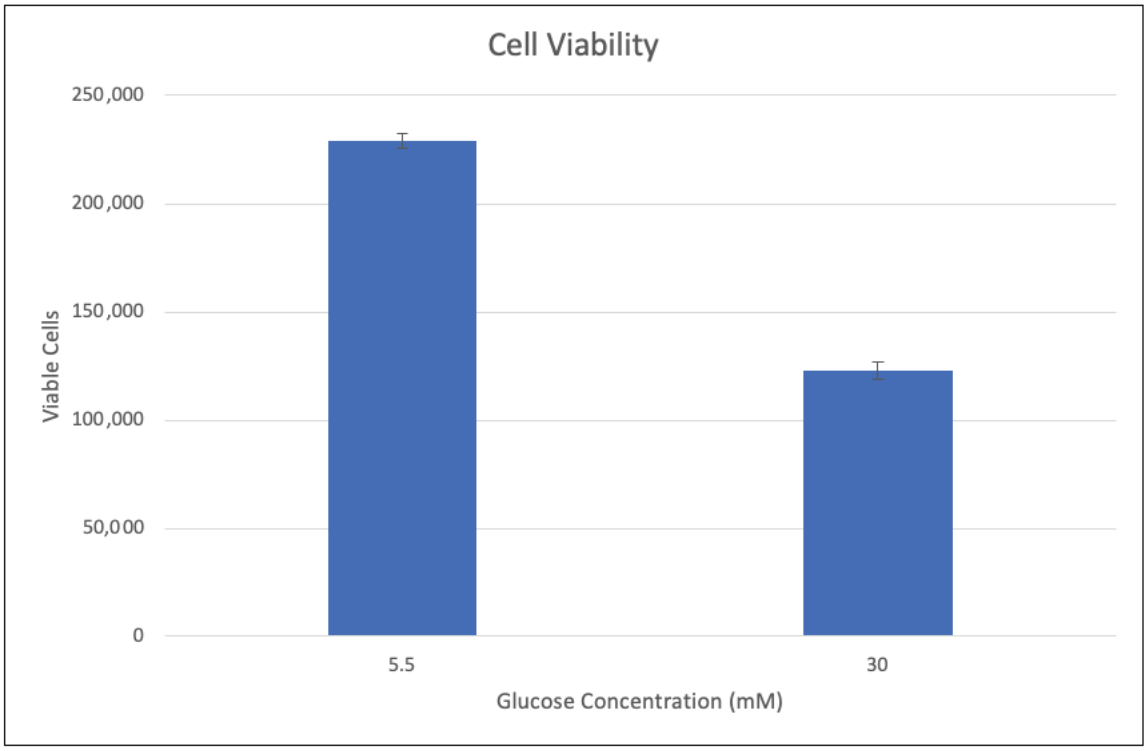

Figure 1. HRMEC were plated at 50,000 per ml in a 24 well plate. At confluency, cells were treated and viable cell number had increased to 200,000. At 24 hours of glucose treatment, there were 232,500 in 5.5mM and 119,000 in 30mM. Data is represented in ± SEM. *P*=.01, T-test.

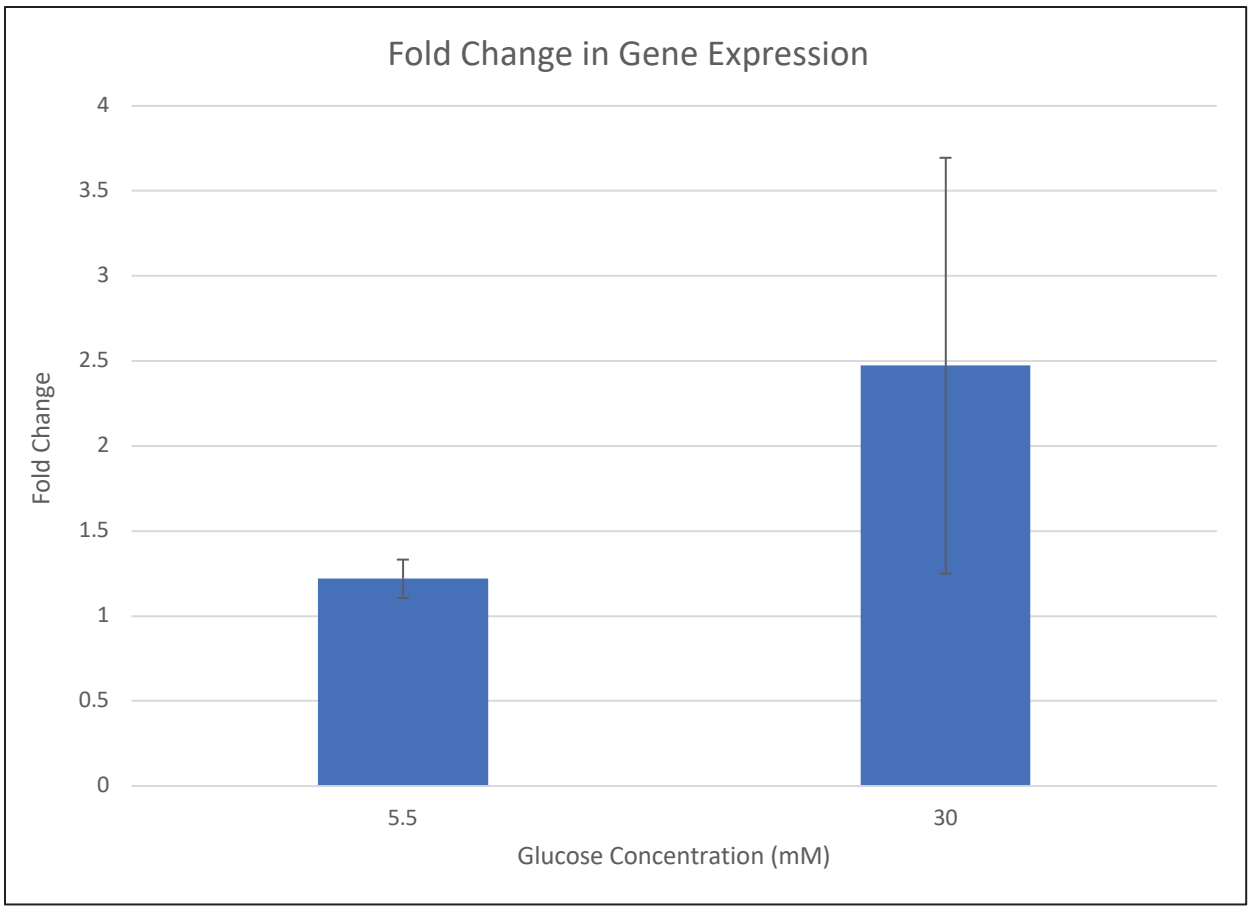

Figure 2. mRNA fold change measured by qRT-PCR for proaspase-8 after 24-hour treatment. Quantitative real time PCR of Procaspase-8 at indicated glucose concentrations were normalized according to housekeeping gene 18s as a control. Data is represented in ± SEM. *P= 0.36*, T-test.

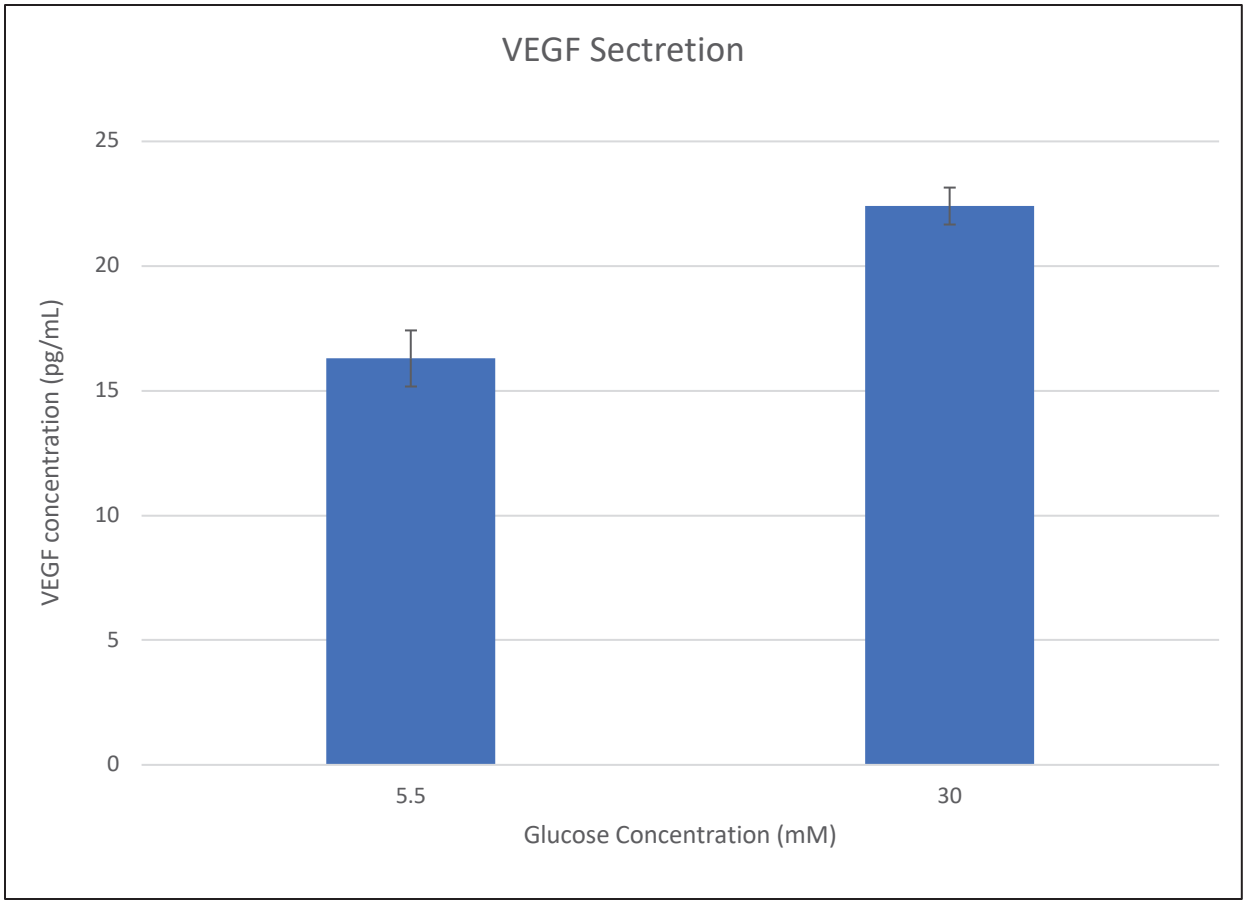

Figure 3. Concentration-dependent change in VEGF secretion after 24-hour glucose treatment. VEGF amounts secreted into conditioned medium was measured for by ELISA. Data is represented in ± SEM. Results from a t-test were significant. *P=0.01*, T-test.

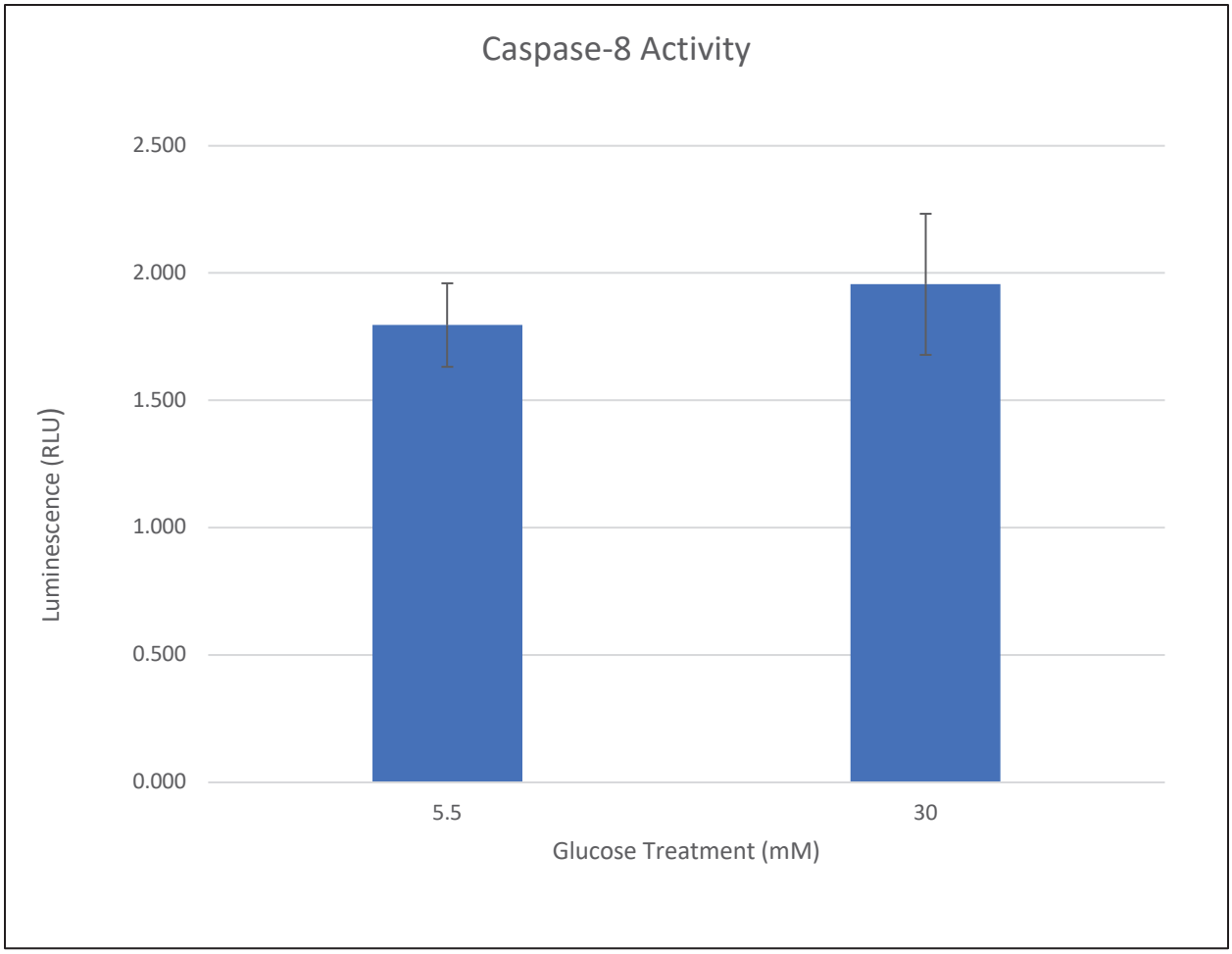

Figure 4. Caspase-8 Glo assay showing that Caspase-8 activity in the euglycemic HRMEC is lower after 24-hr. treatment. Normal and high glucose groups show low Caspase-8 activity. Activity of Caspase-8 is proportional to luminescent signal which is measured in relative light units. Data is represented in ± SEM. *P=0.66*, T-test.

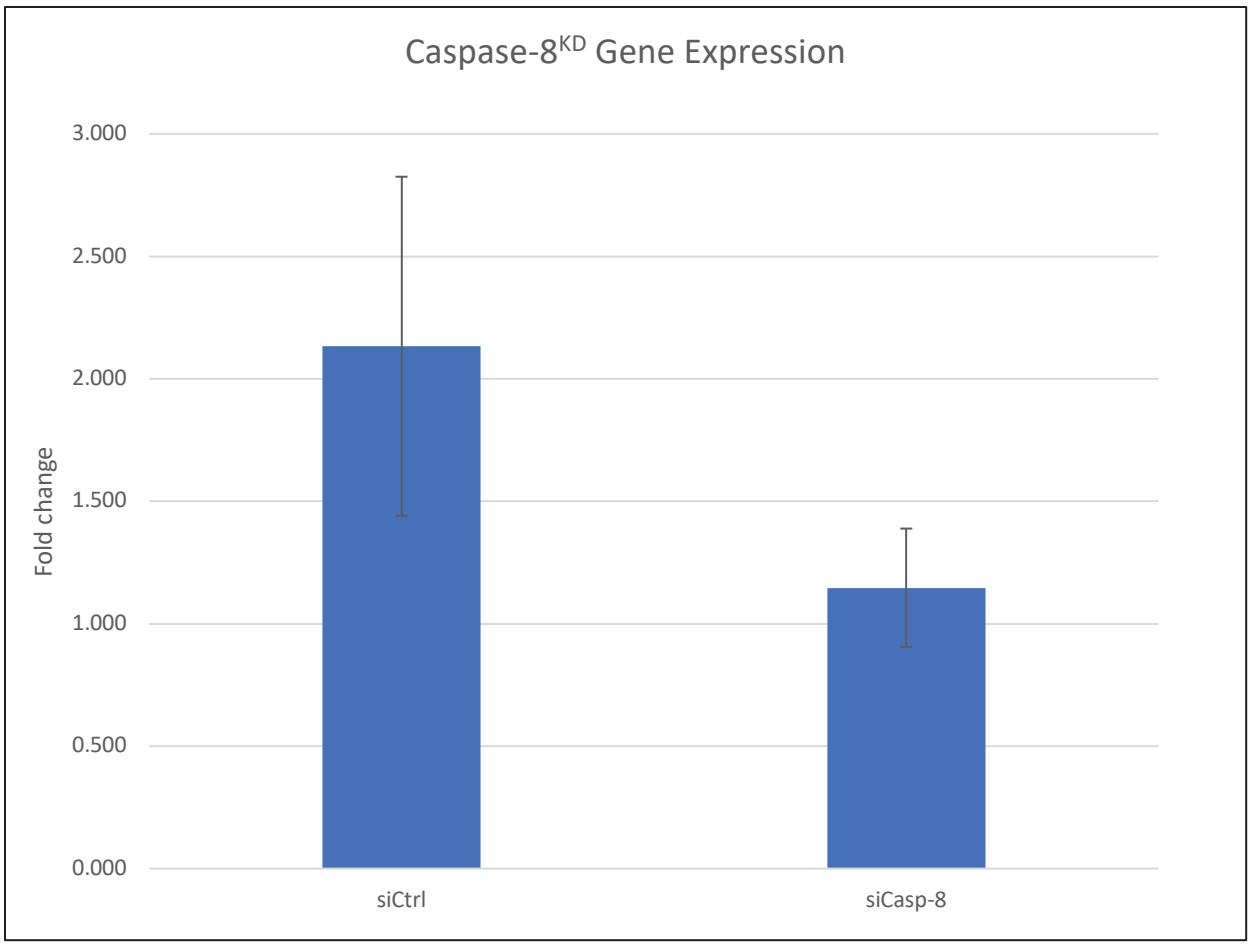

Figure 5. mRNA fold change measured by RT-PCR for caspase-8 after transfection. Quantitative real time PCR showed a lower expression in siCasp-8 HRMEC vs the control. Data is represented in ± SEM. *P=0.24*, T-test.

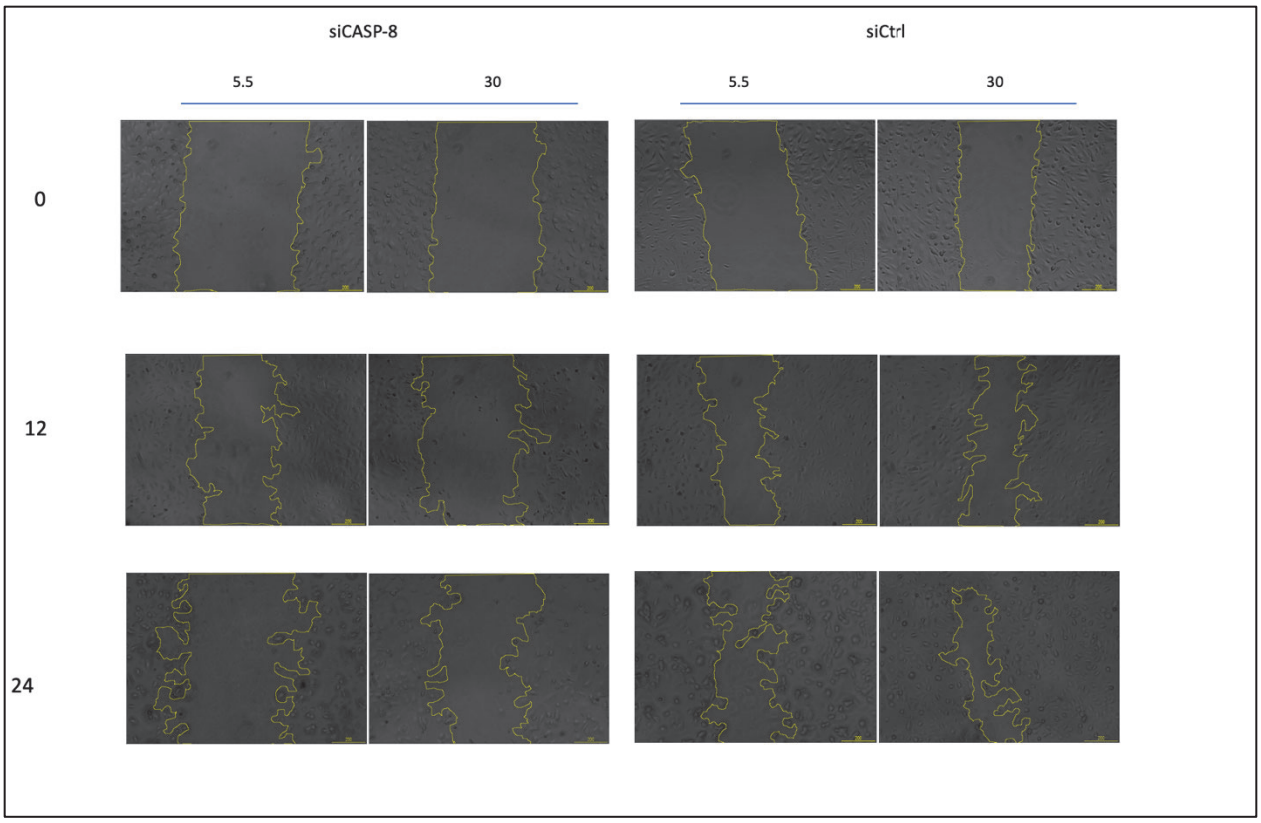

Figure 6. Representative bright field images of the scratch wound assay of HMREC transfected with control siRNA or Casp-8 and 5.5 or 30 mM of glucose. Wound closure is reduced in the siCtrl compared to the Casp-8^{KD} EC after 24 hrs.

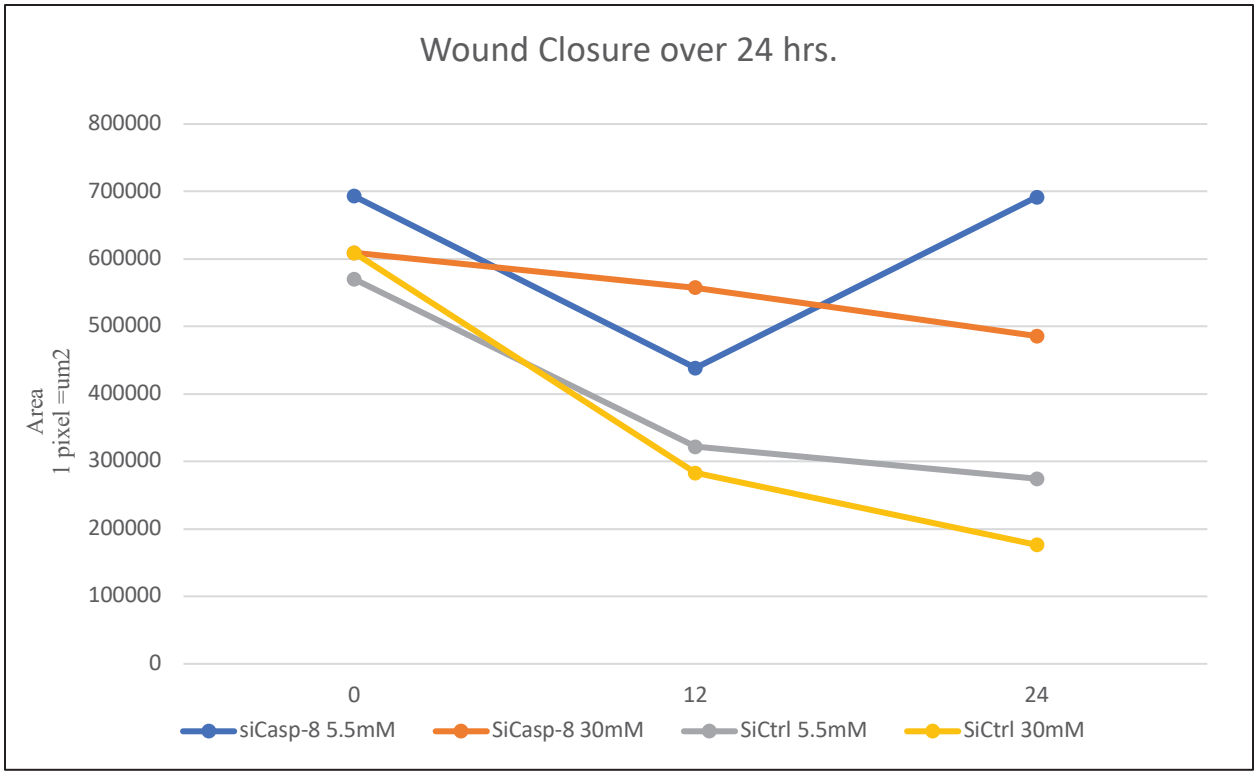

Figure 7. Wound area measured in mm^2 of the different HRMEC groups at 0,12, and 24 hours. siCtrl groups show the greatest decrease in surface area at 12 hours.

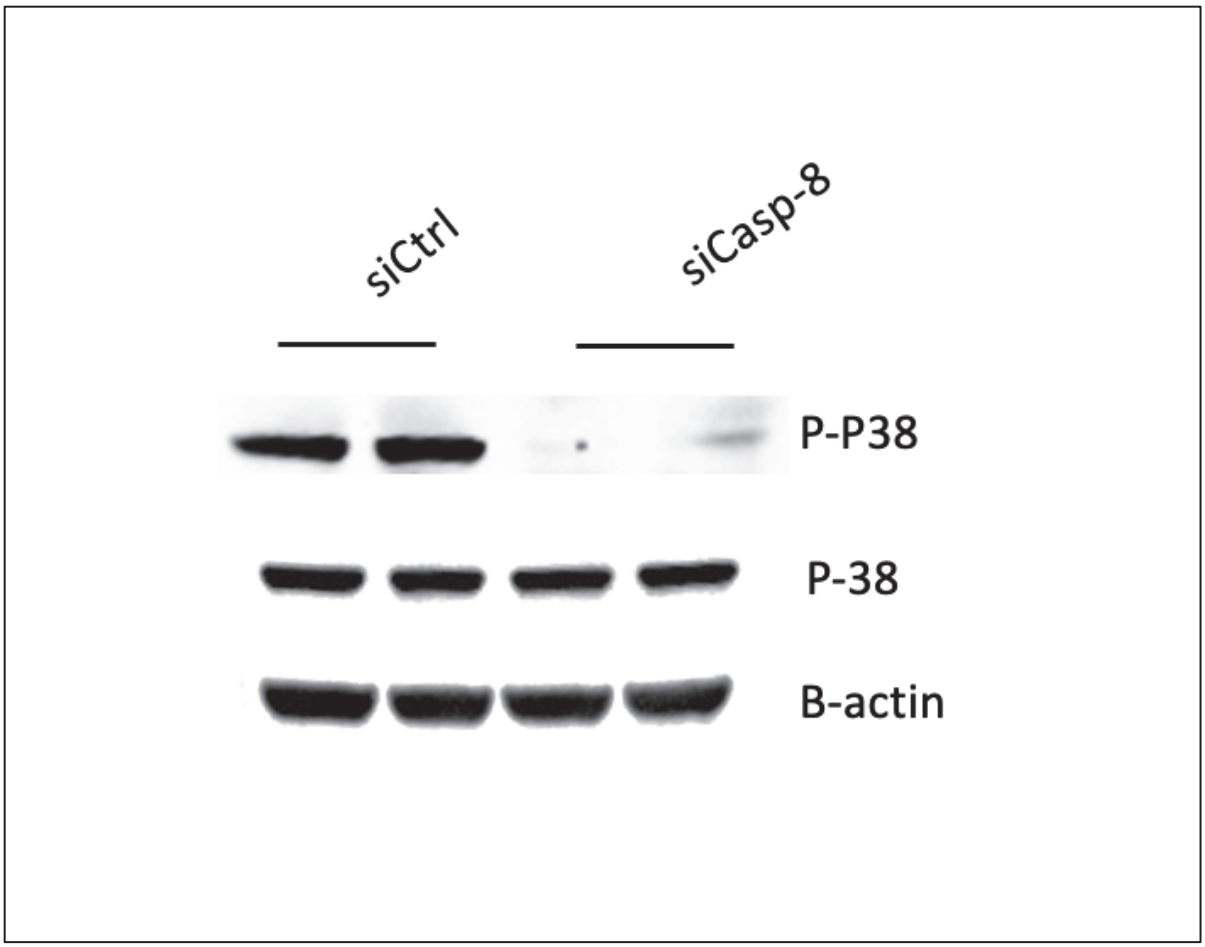

Figure 8. Western blot analysis showing that p38 signaling pathways are normally activated in basal conditions and is decreased in Casp-8^{KD} ECs.

BIOGRAPHICAL SKETCH

The author, Paola Alejandra Campano was born on December 27, 1994 in the state of Tamaulipas, Mexico and is now living in Texas as the youngest of three children. Paola attended The University of Texas at San Antonio and later returned home where she earned her Associates degree in Biology from South Texas College. Paola continued her bachelor's at The University of Texas Rio Grande Valley and graduated with a degree in Biology with focus on Biological Sciences. During her junior and senior years at UTRGV, Paola became greatly determined to enter the Physician Assistant program and volunteered at Rio Grande Regional hospital while working as a scribe. She also became a member of the UTRGV Pre-P.A. chapter and the Texas Academy of Physician Assistants. Immediately after receiving her Bachelor's, the author pursued her Master of Biology at UTRGV to become a more competitive candidate for the P.A. program. In an attempt to apply her knowledge and learn about clinical science, Paola joined Dr. Tsin's Lab and focused her project on Diabetic Retinopathy which would provide an appreciation for research and its role in medicine. Paola earned her Master of Science in Biology at The University of Texas Rio Grande Valley in August 2021 and is a member of the P.A. Enrichment Program at UTRGV. She can be reached at campanopaola@gmail.com.