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Implications of long non-coding RNAs in the pathogenesis of diabetic retinopathy: a novel epigenetic paradigm.

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A thesis submitted in partial fulfillment of the requirements for the Doctor of Philosophy degree in Pathology and Laboratory Medicine

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Abstract

With the rising incidence of diabetic retinopathy (DR), there is an urgent need for novel therapies. Presently, several altered metabolic pathways have been implicated in the pathogenesis of DR. Recent advances in genomic technologies have identified considerable epigenetic alterations that also contribute to DR progression. Long non-coding RNAs (lncRNAs; >200 nucleotides), critical regulators of gene expression, are aberrantly expressed in DR and have not been comprehensively characterized. Our microarray analyses using human retinal endothelial cells (HRECs) revealed thousands of differentially expressed lncRNAs following high glucose (HG) exposure, with profound increases in the lncRNAs *MALAT1* and *HOTAIR*. Using multiple techniques, I sought to elucidate the roles of these two molecules in inflammation and angiogenesis during DR. My findings demonstrated that *MALAT1* is upregulated in HG and in diabetic animals, and regulates inflammatory transcripts (*IL-6* and *TNF-α*) through its association with polycomb repressive complex 2 (PRC2). Vitreous humors from diabetic patients revealed parallel findings. DNA methylation array analyses did not demonstrate significant alterations at CpG sites across the *MALAT1* gene, but inhibition of DNA methyltransferases significantly increased *MALAT1* and associated inflammatory transcripts. Furthermore, HG upregulated *HOTAIR* and angiogenic transcripts (*VEGF-A* and *ET-1*) in HRECs and promoted an association with RNA-binding proteins, P300 and EZH2. *HOTAIR* knockdown reduced the expressions of angiogenic cytokines, EZH2 and P300. HG did not induce significant hypomethylation in *HOTAIR* CpG regions, while inhibitors for histone methylation, DNA methylation and *HOTAIR* significantly impacted *VEGF-A* and *ET-1* expressions. *HOTAIR* expressions were elevated in the vitreous of DR patients and in the retinas of diabetic rodents. *HOTAIR* knockdown reduced HG-induced oxidative DNA and mitochondrial damage. The studies were further extended to delineate how these epigenetic mechanisms influence the regulation of a specific vasoactive factor, ET-1, in DR. DNA methylation array demonstrated hypomethylation in the *ET1* promoter in HG. Blocking DNA methylation or histone methylation significantly increased *ET-1* mRNA expressions in control and HG-treated HRECs; while, knocking down pathogenetic lncRNAs (*MALAT1* and *HOTAIR*) subsequently prevented glucose-

induced *ET-1* upregulation. Collectively, I uncovered a novel epigenetic paradigm that demonstrates a complex web of epigenetic mechanisms that regulate glucose-induced transcription of molecules in important pathological processes (inflammation and angiogenesis) during DR.

Keywords

Diabetic retinopathy; retinal microvascular abnormalities; metabolic pathway abnormalities; reactive oxygen species; epigenetics; DNA methylation; histone modifications; long non-coding RNAs; inflammation; angiogenesis; *MALAT1*; *HOTAIR*; endothelin-1.

Summary for Lay Audience

Background: Diabetic retinopathy (DR) is a serious eye complication that arises from both type 1 and 2 diabetes and is the leading cause of blindness among working-age adults in North America. To develop a better understanding of the processes involved in the advancement of DR, researchers are currently focusing on the 'epigenetic phenomena', which include any process that changes gene activity without making changes to the DNA sequence. In recent years, the field of epigenetics has shown that a special group of RNA molecules called long non-coding RNAs (lncRNAs) may be controlling many processes in cancer and heart disease. Although the expressions of several lncRNAs have been shown to increase in diabetes, the exact mechanisms of how certain lncRNAs contribute to the progression of DR are not known.

Hypothesis: In this study, we will test the hypothesis that two unique lncRNAs, known as *MALAT1* and *HOTAIR*, control inflammation and abnormal blood vessel growth that take place in diabetic retinopathy.

Methods: We will investigate the expressions of *MALAT1* and *HOTAIR* using the vitreous fluid from the eyes of diabetic patients (type 1 and type 2) and study the functions of these lncRNAs in different cell culture and animal models.

Expected Results and Significance: Results from this study will provide a better understanding on how lncRNAs can control inflammation and blood vessel growth in DR. We may use our findings to develop better therapies that can specifically target these disease-causing lncRNAs and ultimately reduce inflammation and abnormal blood vessel growth from happening, which are associated with severe diabetic eye damage.

Co-Authorship Statements

1. Chapter 2: "*MALAT1*: An Epigenetic Regulator of Inflammation in Diabetic Retinopathy". Saumik Biswas and Dr. Subrata Chakrabarti were involved in the conception and design of the study. Saumik Biswas performed a majority of the experiments, with assistance from Drs. Charlie (Shali) Chen, Anu Thomas, and Biao Feng. Drs. Erfan Aref-Eshghi and Bekim Sadikovic helped with DNA methylation experiments and analyses. Dr. John Gonder assisted with clinical sample collection and clinical data analyses. Dr. Subrata Chakrabarti contributed to the reagents/materials/analysis tools for the study. Saumik Biswas and Dr. Subrata Chakrabarti wrote the manuscript and performed all revisions.

2. Chapter 3: "The long non-coding RNA *HOTAIR* is a critical epigenetic mediator of angiogenesis in diabetic retinopathy". Saumik Biswas and Dr. Subrata Chakrabarti conceived, designed and directed the study. Saumik Biswas performed a majority of the experiments, with some assistance from Jieting Liu, Dr. Biao Feng, and Dr. Charlie Chen for the *in vitro* and *in vivo* experiments. David Malott assisted with electron microscopy experiments, and Linda Jackson-Boeters helped with immunohistochemical stains. Vy Ngo helped with RNA FISH experiments, while Drs. Erfan Aref-Eshgi and Bekim Sadikovic performed and analyzed the DNA methylation experiments. Dr. John Gonder assisted with clinical sample collection and clinical data analyses. Dr. Subrata Chakrabarti provided all reagents and materials used in the study. Saumik Biswas and Dr. Subrata Chakrabarti wrote the manuscript.

3. Chapter 4: "Endothelin-1 regulation is entangled in a complex web of epigenetic mechanisms in diabetes". Dr. Subrata Chakrabarti and Saumik Biswas conceived, designed and directed the study. Saumik Biswas performed a majority of the experiments, with some assistance from Drs. Biao Feng, Anu Thomas, Charlie Chen for the *in vitro* experiments. Drs. Erfan Aref-Eshghi and Bekim Sadikovic helped with the DNA methylation experiments. Dr. Subrata Chakrabarti provided all materials and reagents used in the study. Saumik Biswas and Dr. Subrata Chakrabarti wrote the manuscript.

Acknowledgments

We are all familiar with the traditional proverb, “it takes a village to raise a researcher”. If for some reason this proverb does not sound familiar, then your intuition is absolutely correct—I may have substituted a new word in there. Regardless, I would not have achieved what I have done today without the multitude of people that have supported me throughout this journey called “life”. Although my memory is completely capable, I will unfortunately not be able to mention every single individual who has helped me on here, as writing such a list may end up becoming longer than my actual dissertation. Just know that I still remember, and I am eternally grateful for your guidance and support over the years. I have learned a lot from all of my mentors and will continue learning and becoming the best version of myself. For the time being, I’ll thank the first few people that come to mind below.

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Being the first in my family to attend graduate school, one would expect several hurdles while navigating through graduate school. Luckily, this was not the case for me thanks to the wonderful folks in the Pathology Department. Dr. Chandan Chakraborty, Tracey, Cheryl, Susan, Lisa, Rachel, Angela, Linda, Mellonie: thank you so much for the great chats, answering all of my inquiries, continuously encouraging me and keeping up with my shenanigans over the years. You all do such an amazing job, and I know our Pathology students feel the same way. Thank you.

Thank you to all of my friends, who have been a great support network for me. Although there are too many individuals to list, I would like to specifically mention some of my friends who have supported me during the most critical stages of my life and professional development: Season C, Gaibrie S, Dylan C, Dylan J, Jonathan V, Nate M, Innes C, Thomas B, Ayan D, Marie S, Miso G, Mark V, and Dejan T. Thanks for everything.

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2D—Two-dimensional

2-DG—2-deoxy-D-glucose

3D—Three dimensional

3’ UTR— 3’ untranslated region

5-aza-dC—5-aza-2’-deoxycytidine

5’-UTR—5’ untranslated region

8-OH-dG—8-hydroxy-2′-deoxyguanosine

α-MSH— Alpha-melanocyte-stimulating hormone

β-value—Beta value

ΔΨM—Mitochondrial transmembrane potential

ACE— Angiotensin-converting enzyme

AGE— Advanced glycation end-product

ANRIL— Antisense non-coding RNA in the *INK4* locus

AP-1— Activator protein-1

Ang-2— Angiopoietin-2

ANGPTL4—Angiopoietin-like 4

ATP— Adenosine triphosphate

BRB— Blood-retinal barrier

BM— Basement membrane

cDNA—Complementary DNA

CGI—CpG island

-CH_3— Methyl group

CH_3CO— Acetyl group

ChIP-qPCR—Chromatin immunoprecipitation-qPCR

ChiRP-Seq—Chromatin Isolation by RNA purification

CNP— Capillary non-perfusion

COX-2— Cyclooxygenase-2

CTCF—CCCTC-binding factor

CytB—Cytochrome B

DACOR1—DNMT1-associated colon cancer repressed lncRNA 1

DAG— Diacylglycerol

DAPI-- 4′,6-diamidino-2-phenylindole

DCCT-EDIC— Diabetes Control and Complications-Epidemiology of Diabetes Interventions and Complications Trial

DGCR8— DiGeorge syndrome critical region 8

Dll4— Delta-like ligand 4

DM— Diabetes mellitus

DME— Diabetic macular edema

DMNTs-—DNA methyltransferases

DNA— Deoxyribonucleic acid

DR— Diabetic retinopathy

DZNep—3-Deazaneplanocin A

EBM-2—Endothelial basal media-2

EDN1—Gene for ET-1

EGM-2—Endothelial growth media-2

ECs—Endothelial cells

ELISA—Enzyme-linked immunosorbent assay

EndMT— Endothelial-to-mesenchymal transition

eNOS— Endothelial nitric oxide synthase

ER—Estrogen receptor

ET-1— Endothelin-1

ETC— Electron transport chain

EZH2— Enhancer of zeste homolog 2

$FADH_2$— Fully reduced form of flavin adenine dinucleotide

FasL— Fas Ligand

FN— Fibronectin

FOXO1— Forkhead box protein O1

GAPDH— Glyceraldehyde 3-phosphate dehydrogenase

GFAT— Glutamine:fructose-6-phosphate amidotransferase

H3K27me3—Trimethylation of lysine 27 on histone 3

HATs— Histone acetyltransferases

HDACs— Histone deacetylases

HDMCs— Histone demethylases

H&E—Hematoxylin and eosin

HG—High glucose

HIF-1α— Hypoxia-inducible factor-1α

HMGB1— High-mobility group box-1

HMTs— Histone methyltransferases

HOTAIR—Hox anti-sense intergenic RNA

HRECs—Human retinal microvascular endothelial cells

HSP— Hexosamine pathway

HUVECs—Human umbilical vein endothelial cells

ICAM-1—Intracellular adhesion molecule 1

IL-6— Interleukin-6

IL-1β— Interleukin-1β

iNOS— Inducible nitric oxide synthase

JAK-STAT— Janus kinase/signal transducers and activators of transcription

KLF2—Krüppel-like factor 2

KO—Knockout

LG—L-glucose, osmotic control

LSD1— Lysine-specific demethylase 1

MALAT1— Metastasis-associated lung adenocarcinoma transcript 1

mascRNA—MALAT1-associated small cytoplasmic RNA

MCP-1— Monocyte chemoattractant protein-1

MIAT—Myocardial infarction associated transcript

MIF— Macrophage migration inhibitory factor

MLECs—Mouse lung endothelial cells

MRECs—Mouse retinal microvascular endothelial cells

NF-κB—Nuclear factor kappa B

NG—Normal/basal glucose

NPDR— Non-proliferative diabetic retinopathy

PARP1—Poly (ADP-ribose) polymerase 1

PEDF— Pigment epithelium derived factor

PDR— Proliferative diabetic retinopathy

PDGF-BB-PDGFRß-—platelet-derived growth factor-BB-platelet-derived growth factor receptor subunit B pathway

PDGF-BB— platelet-derived growth factor-BB

PGF—Placental growth factor

PI3K— Phosphatidylinositol-3 kinase

PRC2—Polycomb Repressive Complex 2

RIP—RNA immunoprecipitation

RIP-Seq—RNA immunoprecipitation-sequencing

RNCR3—Retinal non-coding RNA 3

MAPK— Mitogen-activated protein kinase

MCP-1—Monocyte Chemotactic Protein 1

miRNAs— microRNAs

mM—mmol/L

MMP-9— Matrix metalloproteinase-9

mRNA— messenger RNA

NADH— Reduced form of nicotinamide adenine dinucleotide

NAPDH— Reduced form of nicotinamide adenine dinucleotide phosphate

NAD^+— Oxidized form of nicotinamide adenine dinucleotide

ncRNAs— Non-coding RNAs

nM—Nanomolar

sncRNAs— Small non-coding RNAs

lincRNAs—Long intergenic long non-coding RNAs

lncRNAs— Long non-coding RNAs

NF-κB— Nuclear factor-kappa B

NO— Nitric oxide

NSCLC—Non-small cell lung cancer

NV— Neovascularization

PAI-1— Plasminogen activator inhibitor-1

PARP— Poly (ADP-ribose) polymerase

PGE2— Prostaglandin E2

PKC— Protein kinase C

PLGF— Placental growth factor

PRC2— Polycomb repressive complex 2

RAGE— Receptor for AGEs

RAS— Renin-angiotensin system

RISC— RNA-induced silencing complex

RNA— Ribonucleic acid

RNA FISH—RNA fluorescence in situ hybridization

RNA pol II— RNA polymerase II

ROS— Reactive oxygen species

RT-qPCR—Quantitative real-time polymerase chain reaction

SAA3— Serum amyloid antigen three

SCR—Scrambled siRNA

SDF-1— Stromal derived growth factor

SHP-1— Src homology-2-domain-containing phosphatase-1

shRNA—Small hairpin RNA

SIRT1— Sirtuin (silent mating type information regulation 2 homolog) 1

siRNA—Small interfering RNA

SOD2— Manganese superoxide dismutase gene

STZ—Streptozotocin

TCA— Tricarboxylic acid

TET— Ten-eleven translocase

TGF-β1— Transforming growth factor-beta1

TNF-α— Tumor necrosis factor- alpha

UKPDS— United Kingdom Prospective Diabetes Study

VEGF— Vascular endothelial growth factor

VH—Vitreous humor

VHL— von Hippel-Lindau

VIP— Vasoactive intestinal peptide

WT—Wild-type

ZFAS1—ZNFX Antisense RNA 1

ZEB—Zebularine

Chapter 1 [i]

1 Diabetic retinopathy

With nearly 642 million people projected to live with diabetes in the year 2040, the risk for developing diabetes-related complications will drastically increase [1]. Diabetes mellitus (DM) is a chronic degenerative metabolic disease that is characterized by sustained hyperglycemia. Hyperglycemia correlates with a number of DM-related complications and is one of the preeminent factors for causing vascular damage in the human body [2, 3, 4]. The majority of diabetic complications can be viewed as either microvascular disease (small vascular injury) or macrovascular disease (large vessel injury) [4, 5]. Diabetic retinopathy (DR) remains the most prevalent chronic microvascular complication of DM [6, 7]. This debilitating ocular condition is also the leading cause of blindness in the working-age population in industrialized countries [7, 8]. The relationship between DR and diabetes has been reported in several studies with the majority of type 1 diabetic patients and over 60% of patients with type 2 DM developing evidence of DR within 20 years of diagnosis [9-14]. With the incidence of visual impairment due to DR strongly related to the duration of diabetes, DR remains asymptomatic to patients until the pathology significantly progresses [14, 15]. In this chapter, I will first highlight both clinical and

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[i] Content in Chapter 1 has been adapted and/or reproduced from:

1[*]. Biswas S, Chakrabarti S. Pathogenetic Mechanisms in Diabetic Retinopathy: From Molecules to Cells to Tissues. In: Mechanisms of Vascular Defects in Diabetes Mellitus. Kartha CC, Ramachandran S, Pillai RM (Eds). Springer International Publishing, 2017:209-247, doi:10.1007/978-3-319-60324-7_9.

2[*]. Biswas S, Chakrabarti S. The multifaceted roles of lncRNAs in diabetic complications: a promising, yet perplexing paradigm. In: The Chemical Biology of Long Noncoding RNAs. Jurga S, Barciszewski J (Eds). Cham: Springer Series, RNA Technologies (in press).

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pathological features of DR and then discuss our current understanding of the mechanisms involved in the pathogenesis of DR in diabetes.

1.1 Clinical features

To impede the progression of non-vision threatening DR to vision-threatening DR, distinct clinical features must be noted in the early stages of DR in order to implement appropriate treatments plans.

1.1.1 Non-proliferative diabetic retinopathy (NPDR)

The earliest stage of disease progression in DR is known as non-proliferative DR (NPDR). Although hyperglycemia-induced damage to endothelial cells and capillary pericytes in the retinal microvasculature are associated with the preclinical stages of DR, the loss of these cells underlies a number of clinical features in NPDR. These clinical features are characterized by microvascular abnormalities that consist of microaneurysms, intraretinal hemorrhages (dot and blot), increased retinal vascular permeability, nerve fiber layer infarcts (cotton wool spots), greater presence of intraretinal lipid deposits (hard exudates), and venous beading [16-19]. NPDR can be categorized into mild, moderate, severe, or very severe stages based upon the absence or presence of the aforementioned clinical features (**Figure 1.1**). In the natural course of DR, the severity of retinal vascular occlusion increases, which in turn leads to impaired perfusion and retinal ischemia [19, 20]. The sequelae of increasing ischemia include various venous abnormalities and considerable retinal vascular leakage that is markedly distinguished by the increased presence of hard lipid exudates and retinal hemorrhages [20]. Once the progression of these features surpasses clinically defined thresholds, severe NPDR is diagnosed. During this stage, the risk of progression to proliferative diabetic retinopathy heightens. Among the severe NPDR patients, nearly 50% will develop proliferative diabetic retinopathy (PDR) within one year and 15% will develop high-risk PDR [21, 22, 23]. Whereas, 75% of patients classified with very severe NPDR are at risk of developing PDR within one year and 45% will become high-risk PDR during this period [22, 23].

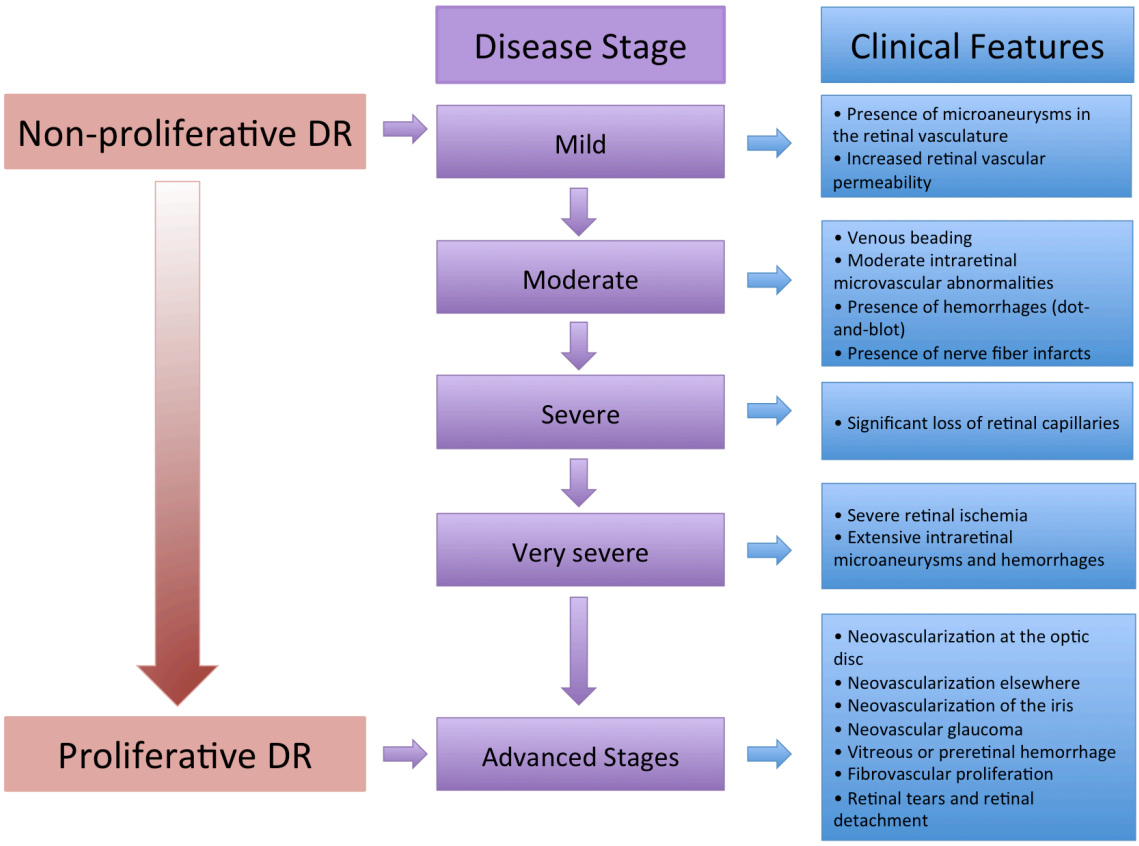

Figure 1.1. Respective clinical features of the various stages in diabetic retinopathy. Diabetic macular edema, not depicted, can occur at any point during DR progression, which is characterized by retinal thickening or hard intraretinal lipid exudates near the macula. Reproduced from *Pathogenetic Mechanisms in Diabetic Retinopathy: From Molecules to Cells to Tissues* (p. 213) by S. Biswas and S. Chakrabarti, 2017, Springer International Publishing.

1.1.2 Proliferative diabetic retinopathy (PDR)

Once diabetic retinopathy advances to the proliferative stage, visual loss becomes imminent if left untreated. In order to compensate for the sustained retinal ischemia, one of the distinguishing clinical hallmarks of PDR is the presence of neovascularization. The formation of abnormal vessels in the retinal circulation may occur through both endothelial cell migration and proliferation on or near the optic disc (neovascularization of the disk) or elsewhere in the retina (neovascularization elsewhere), on the iris (neovascularization of the iris), or into the vitreous cavity of the eye [24, 25]. Due to the fragility of the new vessels, the vessels become more

susceptible to bleeding, leakage, fibrosis, and contraction, which can result in vitreous hemorrhaging, retinal tears, and retinal detachment—crippling ocular complications that inevitably lead to vision loss [26-29]. Further, neovascularization of the iris, also known as rubeosis iridis, and neovascularization of the anterior chamber angle can lead to neovascular glaucoma, a painful ocular disease that may even necessitate enucleation of the affected eye [30].

1.1.3 Diabetic macular edema (DME)

Diabetic macular edema (DME) represents a common vision-threatening complication of DR that is defined as retinal thickening in the macular area [31-34]. Although DME has three severity levels, DME can occur at any point during DR progression and promotes the breakdown of the blood-retinal barrier via microaneurysms and hyperpermeability of capillaries—causing lipids and plasma to be leaked into the macula [31, 32, 33]. The increased presence of hard lipid exudates in close proximity or at the center of the macula is associated with clinically significant macular edema [34].

1.2 Pathological features of DR

There are five distinct vascular lesions underlying the DR response: dysfunctional pericytes and endothelial cells, basement membrane thickening, retinal capillary non-perfusion, retinal neovascularization, and breakdown of the blood retinal barrier. Each vascular disorder associated with DR is initiated by the microangiopathic properties of the diabetic process, which mainly occurs through numerous growth factors that are altered by the changing ocular environment [35]. In this section, we will discuss the pathological features of DR in detail as the presence of one or more of these vascular disorders will help us understand the pathogenetic mechanisms associated with DR.

1.2.1 Dysfunctional endothelial cells and pericytes

One of the earliest pathological features that occur in DR are alterations in the microvasculature, which consist of modifications in cellular structure [35, 36]. Two essential cell types in the microvasculature are pericytes and endothelial cells, and the

interaction between these cells is pivotal in the proper regulation of retinal hemodynamics and vascular function [36, 37]. With endothelial cells comprising the endothelium, which is the thin monolayer covering found in the interior surface of all blood vessels, retinal endothelial cells must ensure that proper nutritional requirements and protection of the ocular tissues, critical to vision are met [38, 39]. The general structure of the endothelium in the retinal microvasculature consists of adjoining endothelial cells that are linked by adherens junctions and tight junctions, which constitute much of the blood-retinal barrier (BRB) [40, 41, 42]. An essential prerequisite in the development of diabetic retinopathy is the loss of endothelium integrity caused by chronic hyperglycemic exposure. Following endothelial cell damage, the interendothelial junctions are unable to maintain the precise permeable properties that necessitate proper BRB function [43]. Therefore, the presence of dysregulated endothelial cell-to-cell junctions in the BRB allows for the extravasation of plasma constituents into the retina. Moreover, diabetic animal models have demonstrated that the apoptosis of retinal endothelial cells is enhanced by the activation of the Fas/Fas ligand (FasL) pathway upon leukocyte adhesion to the vascular endothelium [44].

In the context of maintaining vascular homeostasis, pericytes are important multifunctional cells that serve to stabilize blood vessels, form the BRB, regulate blood flow, and are involved in angiogenesis, endothelial proliferation, and leukocyte recruitment [45, 46]. Pericytes are situated on the abluminal surface of blood capillaries and are morphologically characterized as cells that possess finger-like projections, which extend along the capillary wall and wrap around endothelial cells [47, 48, 49]. While there are several intricate signalling pathways involved in the interaction between pericytes, astrocytes, and endothelial cells, the intercellular communication between endothelial cells and pericytes appears to determine the presence of pericytes on retinal microvessels [50]. One prominent signal transduction pathway utilized between pericytes and endothelial cells is the platelet-derived growth factor-BB-platelet-derived growth factor receptor subunit B pathway (PDGF-BB-PDGFRß) [50, 51]. During angiogenic or hypoxic stress, endothelial cells secrete PDGF-BB, which binds to the pericyte-specific PDGFRß with a strong affinity [52,

53]. Upon binding, the receptor is dimerized, autophosphorylated, and activated, which then further initiates the downstream cascade of PDGF-BB signalling, leading to pericyte survival, migration, proliferation, attachment, as well as leukocyte trafficking [54, 55]. In the case of diabetic retinopathy, both *in vitro* and *in vivo* studies have shown that hyperglycemic stress induces dysfunctional PDGF-BB-PDGFRß signalling, consequently leading to pericyte apoptosis and failure of proper pericyte recruitment [55, 56, 57]. The inability to replace damaged retinal pericytes will ultimately lead to aberrant retinal vascular morphologies, increased development of microaneurysms, endothelial cell hyperplasia, and blood-retinal barrier breakdown [58, 59]. Nevertheless, the loss of pericytes coupled with endothelial cell apoptosis contributes to the formation of acellular, nonperfused capillaries, which are tubes of basement membranes devoid of cell nuclei [44].

1.2.2 Basement membrane thickening

The vascular basement membrane (BM) is a thin extracellular sheet-like structure, comprised of numerous components (including types IV and V collagen, laminin, fibronectin (FN), nidogen, heparan and chondroitin sulfate proteoglycans), that exists between pericytes and endothelial cells [60]. The methodical arrangement of the BM components and molecular interactions between them manages cell survival and provides both a selective permeability barrier and physical support for cell attachment [61-64]. Early induction of hyperglycemia can provoke BM thickening in retinal capillaries through accelerated synthesis and decreased degradation of BM components, which can contribute to the occlusion of capillaries [64, 65]. More specifically, hyperglycemic conditions heighten the mRNA expression of FN, laminin (subunits beta-1 and gamma-1), and types IV (alpha-1 and alpha-2), and V collagen in the retinal BM of both diabetic animals and patients, which can be detected long before the onset of morphological lesions due to DR [65-68]. Further, any alterations in the vascular BM structure or its components may have detrimental effects on its ability to prevent vascular permeability, consequently leading to the development of macular edema [69, 70]. Since the careful balance between synthesis and degradation of BM components to sustain proper BM turnover is disrupted in DR, an

understanding of the mechanisms perpetuating BM thickening and accumulation of BM components is essential and will be later described in this chapter. The underlying mechanisms possibly include increases in protein kinase C (PKC) activity, polyol pathway flux, inflammation, advanced glycation end-product (AGE) accumulation, endothelin activity, and growth factor activity [71-78].

1.2.3 Breakdown of the blood-retinal barrier

The preservation of the blood-retinal barrier (BRB) is a mandatory requirement for proper vision. Compromised BRB integrity can result in numerous ocular pathologies that can have irreparable damage to one's visual perception; therefore, elucidation of the BRB structure is required. The BRB consists of both an inner and outer retinal barrier that serves to maintain a specialized environment for the neural retina [79]. In the inner BRB, retinal capillary endothelial cells form the inner lining of microvessels and are accompanied by pericytes, astrocytes, and glial cells (Müller cells) (shown in **Figure 1.2**) [80]. These endothelial cells are linked together via junctional complexes that facilitate the transport of highly selective molecules between the circulating blood and the neural retina through either transcellular or paracellular routes [81]. Retinal pericytes and astrocytes also interact with the endothelial cells to provide vascular integrity [82]. On the contrary, the outer BRB is comprised of retinal pigment epithelial cells that are connected by tight junctions; the primary role of the outer BRB is to sustain homeostasis in the outer retina [83]. During DR, however, hyperglycemic conditions result in both structural and functional alterations to the barrier, which subsequently leads to both inner and outer BRB breakdown (**Figure 1.3**). Following BRB damage, large amounts of plasma protein begin to extravasate into the neural interstitium, producing high oncotic pressures that will eventually contribute to macular edema [84]. As a result of chronic hyperglycemia, several known factors are implicated in BRB disruption: dysfunctional endothelial cells, pericytes, Müller cells, and astrocytes, increased levels of VEGF, hypoxia, oxygen-free radicals, inflammatory mediators, advanced glycated end products, and protein kinase C activity [85].

1.2.4 Retinal capillary non-perfusion

Satisfying the high metabolic demands of the retina requires the maintenance of adequate tissue perfusion, which ultimately preserves retinal function. The cessation of blood flow to certain areas of the retina is known as capillary non-perfusion (CNP) and this phenomenon is associated with occluded vessels, a consequence of glucose-induced retinal vascular damage [86]. Chronic retinal ischemia is manifested as large areas of CNP, which is the underlying cause of retinal neovascularization [87]. In the severe stages of non-proliferative DR (NPDR), the considerable presence of hypoxic regions resulting from retinal microvascular abnormalities can stimulate the retinal endothelial cells to release proinflammatory cytokines [88]. The subsequent release of cytokines perpetuates retinal hypoxia by recruiting and activating leukocytes, which adhere to the vascular endothelium—contributing to retinal capillary impedance [89, 90]. In the case of chronic retinal hypoxia, the heightened activation of several abnormal biochemical pathways induces the expression of numerous vasoactive factors [91]. These factors are instrumental in capillary dropout and the development of retinal neovascularization—a distinctive clinical feature of proliferative DR (PDR) [92]. Although the exact mechanisms of how retinal ischemia elevates the expression of vasoactive factors still require further elucidation, studies within the past decade have revealed that the activation of specific transcription factors increase a variety of vasoactive mediators implicated in the progression of DR [93-98].

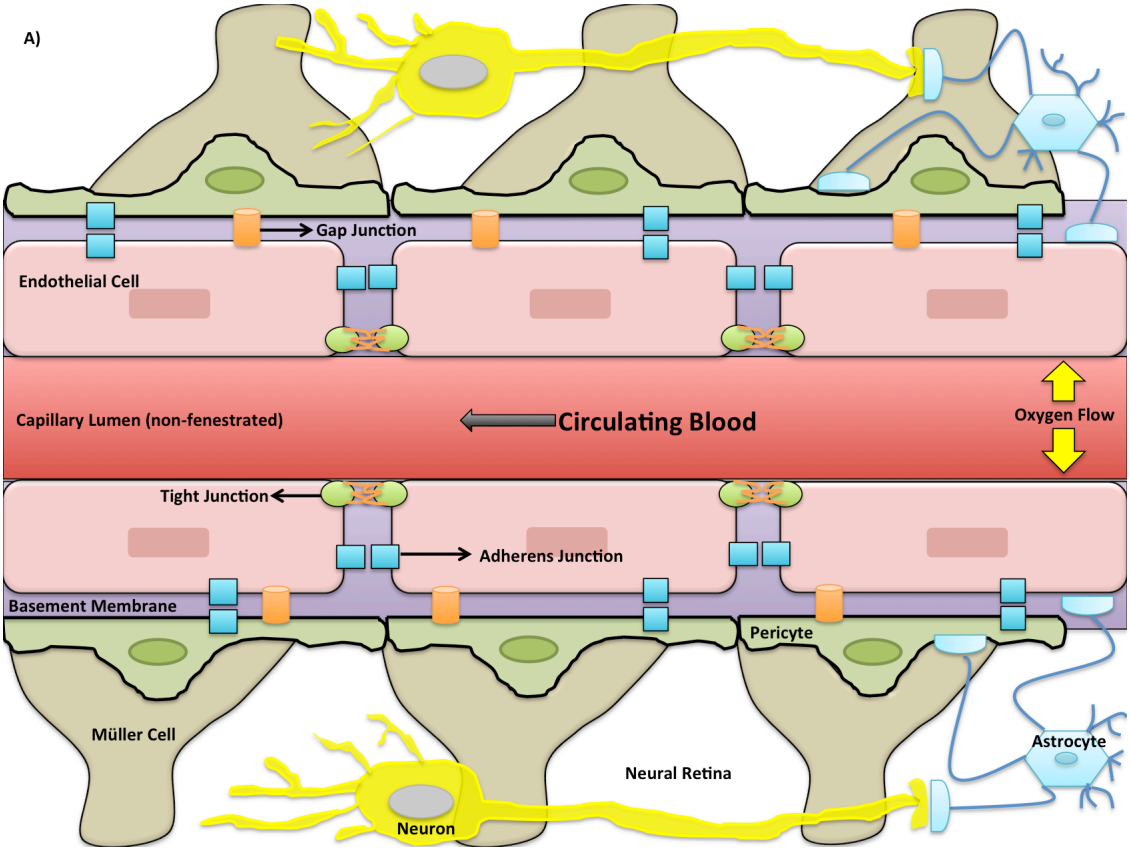

Figure 1.2. An illustration depicting a stable inner blood-retinal barrier in a healthy patient. The integrity of the endothelium is maintained by the presence of functional adherens junctions and tight junctions. Gap junctions authorize the passage of small molecules, and are predominantly located between pericytes and endothelial cells. Furthermore, Müller cells provide mechanistic support to the neural retina and also sustain balance of the extracellular environment in the retina. While, retinal astrocytes are involved in neuronal signaling and assist in managing the barrier properties of endothelial cells. Reproduced from *Pathogenetic Mechanisms in Diabetic Retinopathy: From Molecules to Cells to Tissues* (p. 217) by S. Biswas and S. Chakrabarti, 2017, Springer International Publishing.

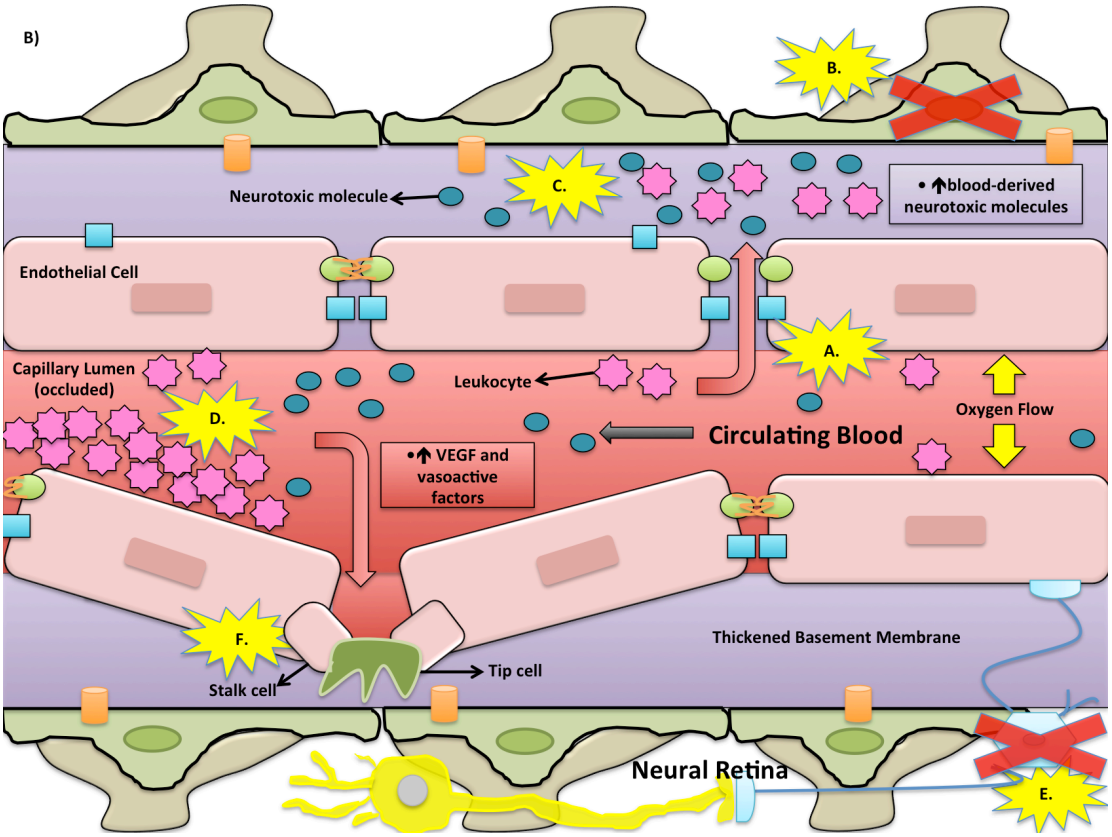

Figure 1.3. An illustration depicting an unstable inner blood-retinal barrier in a patient with advanced proliferative diabetic retinopathy. Chronic hyperglycemia compromises BRB integrity through numerous factors, which are depicted by letters in this figure: (A) Endothelial dysfunction, (B) Pericyte degeneration/apoptosis, (C) Basement membrane thickening, (D) Retinal capillary non-perfusion, (E) Neural inflammation and dysfunctional astrocytes, and (F) Retinal neovascularization. Reproduced from *Pathogenetic Mechanisms in Diabetic Retinopathy: From Molecules to Cells to Tissues* (p. 218) by S. Biswas and S. Chakrabarti, 2017, Springer International Publishing.

1.2.5 Retinal neovascularization

Angiogenesis is a critical physiological process in growth, development, and wound repair that induces the neogenesis of blood vessels from pre-existing vessels. However, in the case of DR, pathological retinal angiogenesis (retinal neovascularization) is a detrimental complication to vision. As observed in the mid-to late-1900s, retinal neovascularization (NV) transpires parallel to areas of CNP supporting the notion that vasoactive factors released from ischemic tissues are pivotal

in the development of pathological angiogenesis [99, 100, 101]. The discovery of hypoxia-related transcription factors and their role in mediating angiogenesis has shed more insight into the complicated pathogenesis of DR. The hypoxia-inducible factor (HIF)-1α protein is one such transcription factor that is significantly accumulated in the presence of low oxygen levels and subsequently upregulates numerous hypoxia-regulated gene products [102, 103]. Under normoxia, the tumor suppressor protein, von Hippel-Lindau (VHL) binds to HIF-1α, targeting it for degradation through the ubiquitin-proteasome pathway [104, 105]. In contrast, hypoxic conditions prevent HIF-1α and VHL interaction, which subsequently results in HIF-1α to cumulate, dimerise with HIF-1β, and translocate into the nucleus where it binds to the hypoxia-response elements in the promoters of vasoactive genes [106]. Following the activation of transcription, multiple pro-angiogenic factors are then upregulated including vascular endothelial growth factor (VEGF), placental growth factor (PLGF), stromal derived growth factor (SDF-1), platelet-derived growth factor (PDGF-B), and their receptors, and angiopoietin-2 (Ang-2) [107, 108]. In particular, VEGF not only stimulates the development of endothelial cells, but it also induces both the disassembly of endothelial cell-to-cell junctions, which drives vascular permeability, and the sprouting of new vessels in combination with Ang-2 [108, 109]. Before sprouting vessels develop, specific subsets of endothelial cells differentiate into either tip or stalk cells [110]. The sprouting process is controlled through the antagonistic actions of delta-like ligand 4 (Dll4) and Jagged1 ligands in the hypoxia-induced Notch signalling pathway [111-114]. As Notch-Dll4 and VEGF-induced signalling increases, the specialized endothelial tip cells direct the sprout vessel growth along a specific VEGF gradient, comprised of VEGF-A that is detected by VEGF receptor-2 expressed on the filopodia of these cells [115]. Although tip cells do not proliferate, the proliferative activity of stalk cells is driven by the availability of VEGF, Ang-2, and additional growth factors [116]. Together, the interaction between tip and stalk cells and the surrounding pro-angiogenic factors stimulate the growth of new blood vessels in the retina. It is important to note that within the retina, several cell types can produce VEGF: endothelial cells, pericytes, Müller cells, astrocytes, and retinal pigment epithelial cells [150]. To further emphasize the role of VEGF in DR, current

treatment of DME using intravitreal injections of anti-VEGF agents have met with success.

1.3 Biochemical and molecular mechanisms involved in the pathogenesis of DR

Hyperglycemic insult gives rise to a diverse number of biochemical pathways that are implicated in the pathogenesis of DR. As our knowledge over the years has significantly developed in regard to the molecular mechanisms attributed to DR, more theories begin to emerge and therefore expand and enrich our knowledge of preexisting DR mechanisms. Currently, as shown in **Figure 1.4**, several mechanisms/pathways that have an involvement in hyperglycemia-induced DR progression have been proposed: polyol pathway, protein kinase C pathway, hexosamine pathway, advanced glycation end-products formation, retinal renin-angiotensin system, and inflammatory mechanisms that include neural-and-immuno-inflammatory responses.

1.3.1 Polyol Pathway

Under normal glucose concentrations in non-diabetic patients, glucose metabolism utilizing the polyol pathway comprises only a small portion of total glucose use [117]. However, the elevation of intracellular glucose concentrations under diabetic conditions activates increased glucose flux through this pathway [118, 119]. Aldose reductase, the initial and NAPDH-dependent enzyme present in the polyol pathway, plays a critical role in the reduction of glucose to sorbitol [117, 118, 119]. Further metabolization of sorbitol is completed by sorbitol dehydrogenase, using NAD^+ as a cofactor, which allows for the formation of fructose [120]. Hyperglycemic conditions serve as a catalyst for enhancing aldose reductase activity, subsequently leading to sorbitol agglomeration [119, 120]. Although the polyol pathway and its exact mechanism in DR pathogenesis still remains inconclusive, several hypotheses have been reported that can ultimately commence and augment cellular damage mechanisms after the activation of the polyol pathway: changes in intracellular

tonicity (osmotic stress) via the accumulation of sorbitol and fructose, development of advanced glycation end-product precursors (methylglyoxal, fructose-3-phosphate, and 3-deoxyglucosone), decreased Na^+/K^+ ATPase activity, diminished cellular anti-oxidant defense mechanisms as a consequence of reduced glutathione levels, protein kinase C (PKC) activation by elevated diacylglycerol (DAG) formation, and increased generation of reactive oxidant species (ROS) through the hyperglycemic activations of poly (ADP-ribose) polymerase and NADH oxidase [121-127].

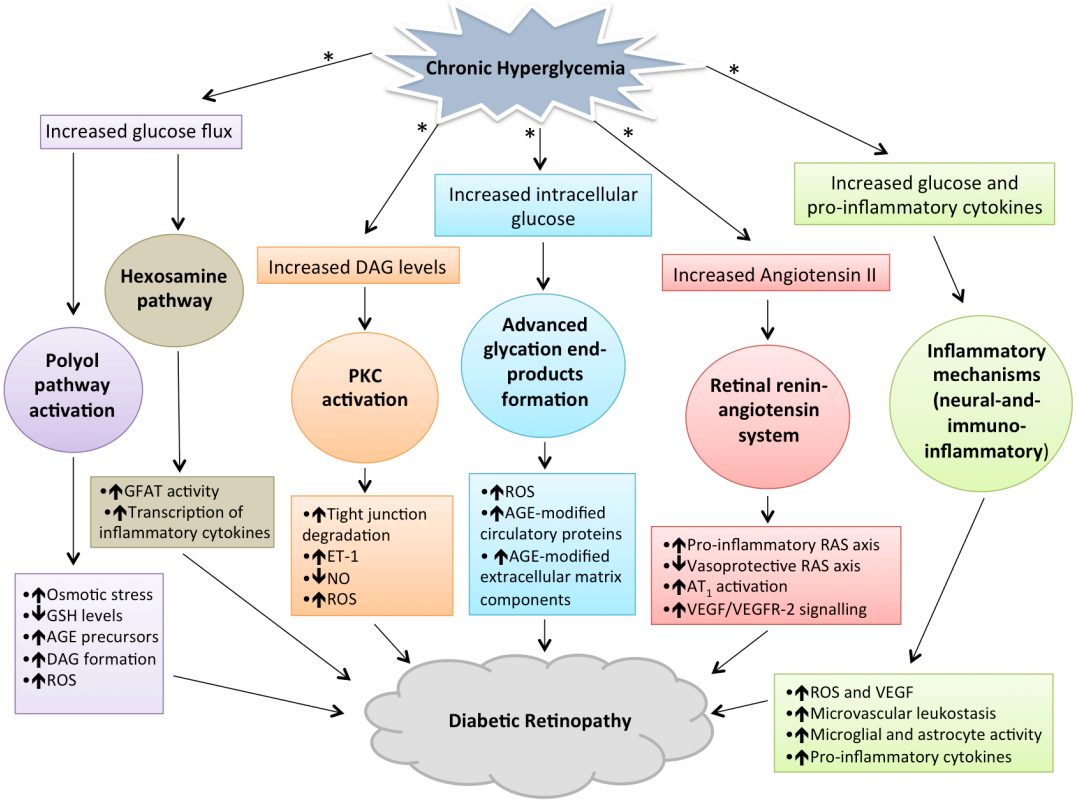

Figure 1.4. Chronic hyperglycemia in DR gives rise to abnormalities in diverse biochemical pathways, which ultimately contribute to and advance DR pathogenesis. The application of a large initial stimulus, such as increased glucose levels, can activate a chain of reactions that incorporate unique pathways: polyol, hexosamine, neural-and-immuno-inflammatory, retinal renin-angiotensin, advanced glycation end-products, and protein kinase C. The "*" in this figure signifies that reactive oxygen species (ROS) may regulate these pathways to some extent. Reproduced from *Pathogenetic Mechanisms in Diabetic Retinopathy: From Molecules to Cells to Tissues* (p. 221) by S. Biswas and S. Chakrabarti, 2017, Springer International Publishing.

1.3.2 Protein Kinase C (PKC) Pathway

As hyperglycemia elevates DAG levels via the *de novo* pathway, the subsequent elevation of this intracellular messenger activates several isoforms in the PKC family that consists of serine/threonine kinases. More specifically, both *in vitro* and *in vivo* experiments have shown that the hyperglycemic activation of PKC-β can mediate VEGF-A levels and increase vascular permeability by phosphorylating endothelial cell tight junction proteins. These tight junction proteins are then targeted for ubiquitin-mediated protein degradation—contributing to blood-retinal barrier breakdown [128, 129]. PKC-β is also involved in altering nitric oxide (NO) production, endothelial nitric oxide synthase (eNOS) expression, and endothelin-1 (ET-1) that consequently supports abnormal retinal hemodynamics [130, 131]. While, on the other hand, hyperglycemic activation of PKC-δ and Src homology-2-domain-containing phosphatase-1 (SHP-1) has been reported to induce retinal pericyte apoptosis through the nuclear factor-kappa B (NF-κB) signalling pathway [132]. The combined effects of hyperglycemic stimulus and PKC-δ activation can additionally provoke increased ROS generation, which will have detrimental consequences on retinal function [130-133]. Furthermore, the link between PKC activation and increased mitogen-activated protein kinase (MAPK) activity has been established—suggesting that the interplay between several PKC isoforms and MAPK activity can lead to subsequent phosphorylation of numerous transcription factors that heighten the expression of multiple stress-related genes associated with DR pathogenesis [134].

1.3.3 Hexosamine Pathway

During intracellular glucose metabolism, the redirection of fructose-6-phosphate from the glycolytic pathway to the hexosamine pathway (HSP) can ultimately induce increased transcription of pro-inflammatory cytokines, insulin desensitization, and oxidative stress—all of which are prominent features contributing to retinal neuronal apoptosis [135-138]. Glutamine:fructose-6-phosphate amidotransferase (GFAT) is the first and rate-limiting enzyme present in the HSP that catalyzes the conversion of fructose-6-phosphate and glutamine to glucosamine-6-phosphate and glutamate, respectively—preparing their entry into the HSP [139]. After a series of conversions,

the major HSP end product is uridine diphosphate-N-acetylglucosamine, which allosterically inhibits GFAT and serves as an important substrate for O-linked N-acetylglucosamine transferase to facilitate the process of O-linked glycosylation on the serine and threonine residues of nucleocytoplasmic proteins [139-142]. In the context of diabetes, hyperglycemia elevates both HSP flux and GFAT activity, which leads to post-translational over modifications and subsequently alters the expression of numerous genes implicated in DR pathogenesis. After all, O-linked glycosylation can target transcription factors, signalling molecules, cofactors, and even RNA polymerase II [143, 144, 145]. Although O-linked glycosylation may compete with phosphorylation for the specific serine and threonine sites on a protein, hyperglycemia-induced HSP activation has shown to elevate O-linked glycosylation and reduce serine and threonine phosphorylation of the transcription factor Sp1 [146]. The successive glycosylation of Sp1 additionally increases the transcription of transforming growth factor-beta1 (TGF-β1) and plasminogen activator inhibitor-1 (PAI-1)—assisting DR development [146, 147].

1.3.4 Formation of Advanced Glycation End-Products (AGEs)

The perpetual exposure of hyperglycemia to the retina can cause the formation and build-up of advanced glycation end-products (AGEs), which participate in endothelial dysfunction, chronic inflammation, BRB breakdown and retinal neuronal degeneration through a variety of mechanisms [148, 149]. The formation of AGEs is accomplished in a series of sequential chemical reactions that initially begins with the non-enzymatic interaction between carbonyl groups of intracellular glucose molecules (and other reducing sugars) and the free amino groups of intracellular proteins [150]. As a result of this interaction, an unstable compound known as a Schiff base is formed and then by molecular rearrangement, a more stable compound (an Amadori product) is constructed that later metabolizes to AGEs (an irreversible compound) [150, 151, 152]. Not only can the generation of AGEs modify and alter the function of intracellular proteins, but AGE precursors can also diffuse out of a cell and modify extracellular matrix components and their matrix receptors such as integrins, and

circulating plasma proteins that greatly contribute to retinal microvascular leukostasis [153-157]. The receptor for AGEs (RAGE) is ubiquitously expressed on multiple cell types and the activation of RAGE, upon the binding of AGE precursors, activates and enhances pro-inflammatory and pro-oxidant signal cascades involving MAPK, NF-κB, activator protein-1 (AP-1), Janus kinase/signal transducers and activators of transcription (JAK-STAT), phosphatidylinositol-3 kinase (PI3K), PKC, VEGF, and tumor necrosis factor- alpha (TNF-α) [158-163].

1.3.5 Retinal Renin-Angiotensin System

The systemic renin-angiotensin system (RAS) is responsible for regulating blood pressure and maintaining the balance of electrolytes. Within the last 30 years, research has revealed that local RAS exists in various tissues (including the retina) that are independent from the systemic RAS and play a role in sustaining local equilibrium [164]. In the eye, the localization of RAS components are found to be predominantly expressed on retinal microvessels (endothelial cells and pericytes), various glial cells (Müller cells, astrocytes, and microglia), neurons (ganglion cells), and in other structures, such as the choroid and ciliary epithelium [165]. In addition to their cellular localization, distinct local RAS components have been implicated in ocular pathogenesis. For example, studies have reported that DR patients have elevated plasma and intraocular concentrations of prorenin, renin, angiotensin II, and angiotensin-converting enzyme (ACE)—these levels additionally correlate with DR severity [166-169]. Furthermore, *in vivo* experiments have reported that DR may have an association with local RAS imbalances through the activation of the pro-inflammatory RAS axis (comprised of ACE, renin, the renin receptor, and angiotensin II receptor type I) that promotes vasoconstriction and a subsequent reduction in the vasoprotective axis (comprised of ACE2, angiotensin-(1-7), and Mas) of RAS [170]. Although the retinal RAS signalling mechanisms in DR still require further elucidation, several studies have demonstrated that the activation of angiotensin II receptor type I by angiotensin II facilitates the upregulation of VEGF/VEGFR-2 signalling [171, 172, 173]. This upregulation induces vascular permeability and retinal neovascularization that assists in BRB breakdown [171, 172, 173].

1.3.6 Inflammatory Mechanisms

Although acute inflammation is generally the body's natural way of protecting tissues from physiological stress or pathological stimuli, chronic inflammation, however, can mediate tissue destruction, enhance fibrosis, and drive angiogenesis. The persistence of low-grade inflammation is a prominent feature of DR that is mediated by several inflammatory mechanisms that can facilitate retinal vascular damage and neovascularization. Nevertheless, insight into the mechanisms regulating immune-and-neural-inflammation in DR pathogenesis is integral.

1.3.6.1 Immuno-Inflammatory Response

The immuno-inflammatory mechanisms involved in the ocular microenvironment remain ambiguous, as recent findings only allude to the complex interplay between the eye and the inflammatory system [174]. With the eye being an immune-privileged tissue, the presence of the inner and outer BRB allow the eye to create its own specialized microenvironment to suppress active inflammation and strictly regulate the activity of resident intraocular immune cells [174, 175]. The unique composition of the ocular immune microenvironment includes various immunosuppressive factors such as TGF-β2, alpha-melanocyte-stimulating hormone (α-MSH), neuropeptides, macrophage migration inhibitory factor (MIF), and vasoactive intestinal peptide (VIP), which restrict the actions of immune-competent cells (macrophages, microglial cells, dendritic cells, T-cells, and monocytes) [174-178]. During early NPDR stages, the chronic activation of pattern recognition receptors, such as RAGE and toll-like receptors expressed on immune-competent cells, can lead to the production of abnormal pro-inflammatory cytokines, upregulation of adhesion molecules, activation of other ocular resident immune cells, and increased retinal microvascular leukostasis [179]. More specifically, DR increases the production of several key ligands (includes high-mobility group box-1 (HMGB1) and AGEs) that greatly enhance RAGE activation in the retina [180]. Following activation, RAGE can stimulate MAPK and p38 signalling which in turn can trigger NF-κB transcription—contributing to pro-inflammatory cytokine production and ROS generation [181]. Heightened levels of inflammatory cytokines can then recruit additional leukocytes (i.e., monocyte

chemoattractant protein-1 (MCP-1)) and stimulate VEGF-A activation, which upregulates the expression of adhesion molecules [182, 183]. Intercellular adhesion molecule-1 is one of the upregulated adhesion molecules expressed on the surface of retinal endothelial cells that facilitates the binding of leukocytes to the vascular endothelium [183]. Upon binding, leukocytes generate ROS and additional inflammatory cytokines that promote retinal vascular permeability—consequently jeopardizing BRB integrity [184]. After all, maintenance of BRB integrity is critical for the preservation of the intraocular anti-inflammatory immune environment.

1.3.6.2 Neural Inflammation

Neural inflammation plays a critical role in DR pathogenesis. The neural retina is separated from retinal blood supply by the inner and outer BRBs and is an extremely delicate nerve tissue that is mainly comprised of Müller cells, astrocytes, microglia, and retinal ganglion cells [185]. Macroglial cells, which include Müller cells and astrocytes, have critical functions in maintaining normal retinal physiology: ensuring appropriate neuronal functioning by contributing metabolic and physical support forming and maintaining BRB integrity, maintaining homeostasis in the extracellular environment, and regulating retinal blood flow, metabolic waste product removal, local immune responses, and retinal glucose metabolism [186]. In addition to macroglial cells providing a local immune response, microglial cells also play an important role in initiating and mediating appropriate intraocular immune responses. Microglial cells are mononuclear phagocytes that are derived from the bone marrow and represent the retinal innate immune cells as they use cytotoxic and phagocytic mechanisms to eliminate foreign materials in the eye and lack specificity and memory [187, 188]. On the other hand, retinal ganglion cells are involved in transmitting visual information to pertinent brain centers that constructs our vision [189]. DR ultimately alters the morphology and function of neural retinal components. For example, in the early stages of DR, Müller cells can become extremely responsive to the hyperglycemia-induced retinal changes and significantly contribute to the inflammatory environment by releasing VEGF and decreasing anti-angiogenic factors (such as pigment epithelium derived factor (PEDF)) to promote retinal vascular

permeability [190, 191]. Müller cells have also shown to induce RAGE activation, interleukin-1β (IL-1β), interleukin-6 (IL-6), nitric oxide (NO), prostaglandin E2 (PGE2), inducible nitric oxide synthase (iNOS), and cyclooxygenase-2 (COX-2) levels by numerous signalling pathways (including MAPK/NF-κB signalling) when exposed to high glucose *in vitro*—suggesting its roles in driving angiogenesis and furthering inflammation implicated in DR [190-194]. In regard to astrocytes, *in vivo* experiments have reported that astrocyte densities are significantly decreased in the retinas of diabetic rats and astrocytes can potentially excrete various inflammatory mediators (including TGF-α, COX-2, PGE2, and IL-1β) during hyperglycemic stress [195-199]. DR can also alter microglial morphology and induce microglial hyperactivity by RAGE activation—adding to the pro-inflammatory environment by releasing key inflammatory mediators [200, 201, 202]. Moreover, chronic hyperglycemia and accumulating levels of neurotoxic metabolites in the neuronal environment can induce apoptosis in retinal neurons by compromising the function of retinal ganglion cells [203].

1.4 Interconnection of the Pathways: Oxidative Stress

Although chronic hyperglycemia can provoke the involvement of multiple abnormal biochemical pathways, research has revealed that these pathways are interconnected by mitochondrial-derived ROS [153,154]. In aerobic cellular respiration, NADH and $FADH_2$ from the tricarboxylic acid (TCA) cycle are generated to donate electrons to specific complexes in the electron transport chain (ETC)—NADH provides electrons to complex I, while $FADH_2$ supplies electrons to complex II [204]. The electrons from both of these complexes are then shuttled through coenzyme Q, complex III, cytochrome-C, and finally complex IV, where oxygen is reduced to water [205]. The transfer of electrons through these complexes generates energy that is utilized to build an electrochemical proton gradient across the inner mitochondrial membrane [206]. Energy acquired from the electrochemical gradient regulates the generation of ATP through ATP synthase [206]. In the case of diabetes, however, elevated intracellular glucose levels lead to increased glucose oxidation in the TCA cycle; therefore, producing a substantial amount of electron donors that are then transported to the ETC

[154]. Following the increase of electron donors, the voltage across the proton gradient rises until a critical threshold is attained, which subsequently impedes the electron transfer properties of complex III [207, 208] As a result of the complex III blockade, superoxides are constructed at an elevated rate; since, coenzyme Q donates the accumulated electrons to molecular oxygen [209]. The build-up of mitochondrial-derived superoxides then creates breaks in the DNA strands that signals the activation for PARP [210, 211]. Consequently, activated PARP inhibits the activity of glyceraldehyde 3-phosphate dehydrogenase (GAPDH), a critical enzyme present in the cytosol and in the nucleus where it has a role in glycolysis and DNA repair, respectively [211, 212, 213]. The reduced capability of cytosolic GAPDH prevents glycolysis from completing, causing an aggregation of upstream glycolytic metabolites such as glucose, fructose-6 phosphate, fructose 1,6-biphosphate, and glyceraldehyde 3-phosphate [154]. Depending on the biochemical pathway and its respective glycolytic metabolite, the metabolites are subjected to additional modifications by pathway-specific enzymes that ultimately activate the major biochemical pathways previously mentioned in section 3 (shown in **Figure 1.5**). Nevertheless, although experimental data supports that hyperglycemia activates several downstream biochemical pathways and cellular aberrations that contribute to the pathogenesis of DR, accumulating research within the last three decades is beginning to demonstrate a strong role of epigenetics in such processes [214].

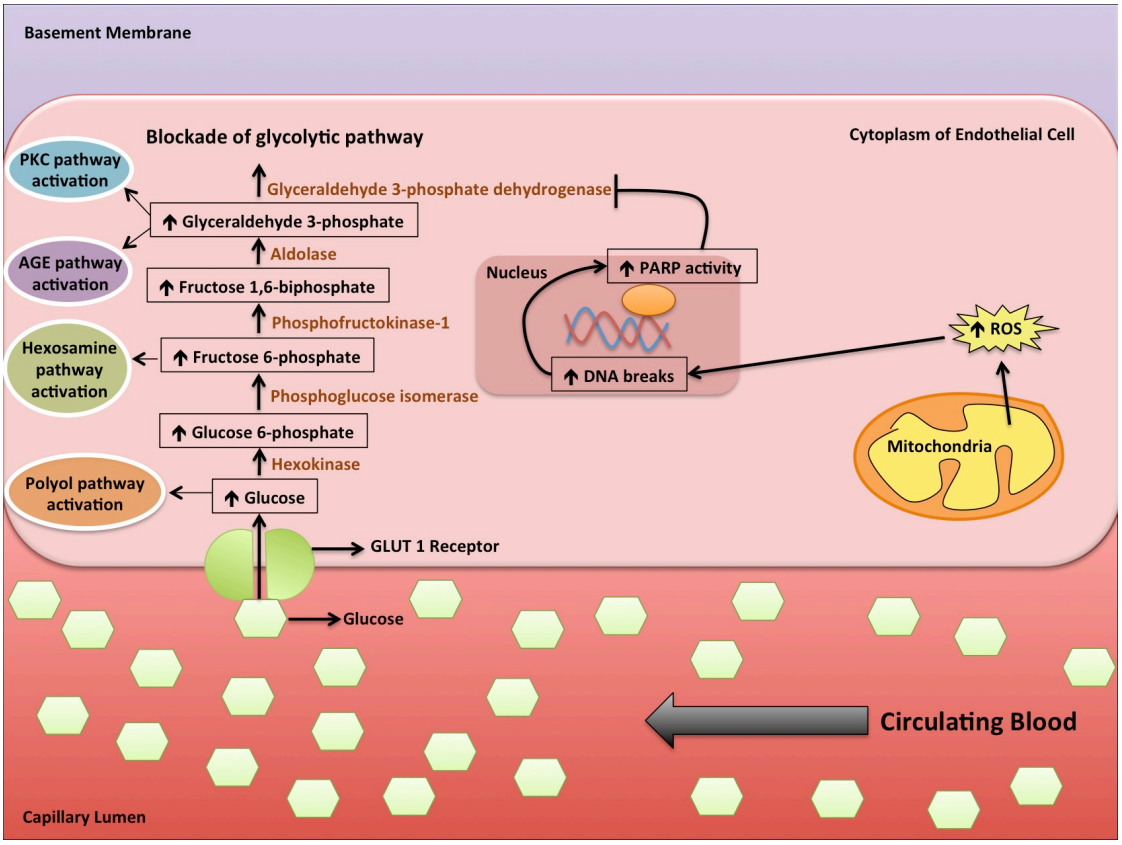

Figure 1.5. Effects of reactive oxygen species (ROS) on a retinal endothelial cell and the association of ROS with other biochemical pathways implicated in the pathogenesis of diabetic retinopathy. To summarize, the presence of high glucose stimulates the increased production of mitochondrial ROS, which induces breaks in DNA strands and activates poly (ADP-ribose) polymerase (PARP). As a consequence of PARP activation, glyceraldehyde 3-phosphate dehydrogenase activity is significantly reduced due to its interactions with PARP. Dysfunctional glyceraldehyde 3-phosphate dehydrogenase results in the accumulation of glycolytic metabolites upstream of this enzyme, which ultimately activates several biochemical pathways. Note, the endothelial junctional complexes, the retinal renin-angiotensin system, and the neural-and-immuno-inflammatory mechanisms are not shown in this figure. Furthermore, there are additional mechanisms of ROS generation that can subsequently contribute to cellular dysfunction. This figure only illustrates one of the ROS mechanisms in retinal endothelial cells. Reproduced from *Pathogenetic Mechanisms in Diabetic Retinopathy: From Molecules to Cells to Tissues* (p. 222) by S. Biswas and S. Chakrabarti, 2017, Springer International Publishing.

1.5 'Metabolic Memory' and the Rise of Epigenetics

Despite improving glycemic control, the early exposure of hyperglycemia can still be implicated in late complications and disease progression [215, 216, 217]. This phenomenon is known as "metabolic memory" or "legacy effect" and was documented in the Diabetes Control and Complications-Epidemiology of Diabetes Interventions and Complications Trial (DCCT-EDIC), and the United Kingdom Prospective Diabetes Study (UKPDS), respectively [215, 216, 217]. When diabetic patients are transiently exposed to hyperglycemia, metabolic abnormalities such as ROS generation, PKC activation, increased activation of the hexosamine pathway, AGEs formation, and RAGE activation can affect epigenetic mechanisms by altering the expression of target-specific genes in cells without modifying the DNA sequence [218, 219]. These heritable epigenetic alterations can still facilitate DR pathogenesis despite the achievement of normoglycemia as epigenetic modifications can sustain constant activation of pro-inflammatory genes [220, 221]. The involvement of epigenetic changes in diabetes, cancer, and heart disease stresses the importance of understanding how epigenetic modifications lead to the manifestation of these diseases [221, 222, 223]. Major epigenetic modifications that are being explored in depth include DNA methylation, histone modifications, and the activity of non-coding RNAs [224, 225].

1.5.1 DNA Methylation

Although the epigenetic modifications of DNA remain elusive, studies have reported that DNA methylation is typically associated with the silencing of gene transcription through the covalent addition of a methyl group on the fifth position of cytosine residues within CpG dinucleotide clusters (CpG Islands) [226, 227]. Moreover, the DNA methylation reaction involves the interaction between two enzymes that oppose each other: DNA methyltransferases (DMNTs) that create and sustain methylated DNA patterns, and DNA demethylases that remove methyl groups, such as the ten-eleven translocase (TET) enzyme [228]. In the context of diabetes, increases in DMNT have been reported with DMNT1, a maintenance enzyme among the three major types of DMNT, activity heightened in retinal capillary cells [229]. Further, new research

has shown that TET2 is activated in diabetes and its activation demonstrates increased binding at the promoter region of matrix metalloproteinase-9 (MMP-9)—establishing a hypomethylated state in the MMP-9 promoter [230]. Due to the novelty of DNA methylation, the exact mechanisms of how the DNA methylation machinery assists DR pathogenesis remains unclear.

1.5.2 Histone Modifications

Histone modifications are one of the better-characterized epigenetic mechanisms in DR. The nucleosome is the fundamental subunit that allows for the composition of chromatin, a tightly packaged structure that contributes to the final structure of eukaryotic chromosomes. Individual nucleosomes consist of an octamer of histones, two molecules of each histone protein (H2A, H2B, H3, and H4), encased by approximately 147 base pairs of DNA [231, 232]. Nucleosomes are prone to rapid adjustments from external stimuli [231, 232, 233]. More specifically, the core histones possess N-terminal tails where a large number of post-translational modifications can occur by targeting the amino acid residues in this area [233, 234]. Below, we will focus on the most common covalent histone modifications associated with gene expression and transcription in DR: histone acetylation and histone methylation [235].

1.5.2.1 Histone Methylation

In order for histone methylation to occur, histone methyltransferases (HMTs) are required to facilitate the transfer of methyl groups to amino acid residues including arginine and lysine residing in the N-terminal tails of histones [235, 236]. While, on the other hand, histone demethylases (HDMCs) possess the capacity to remove methyl groups from this area [237]. It is important to note that select amino acid residues (such as lysine) can be methylated using one, two, or three methyl groups [220, 237]. Depending on the type of stimulus present, the over-modifications performed by either HMTs or HDMCs on these amino acid residues will dictate chromatin accessibility to transcriptional factors that subsequently regulates the expression of specific genes and their respective translated products [231]. For example, studies have reported that the methylation of specific lysine residues, such as lysines 9 and 27 in histone H3 and

lysine 20 in histone H4 are associated with suppressed gene activity [236-239]. In contrast, methylation of lysines 4, 36, and 79 in histone H3 are associated with active gene regulation [236-239]. Moreover, epigenetic-related *in vitro* and *in vivo* experiments have demonstrated that diabetic conditions elevate the activity of lysine-specific demethylase 1 (LSD1), a type of HDMC, that hypomethylates lysine 9 in histone H3 at the promoter region of the MMP-9 gene [240]. Following hypomethylation of lysine 9, this amino acid residue is then subjected to increased acetylation that promotes NF-κB transcription—contributing to elevated MMP-9 activity that evokes retinal mitochondrial damage and apoptosis in retinal capillary cells [240]. Other hyperglycemic studies have also reported decreased activity of the manganese superoxide dismutase gene (SOD2) by increased methylation of lysine 20 in histone H4, which subsequently increases retinal oxidative stress [241, 242]. Furthermore, a multimeric complex known as polycomb repressive complex 2 (PRC2) plays a critical role in epigenetic regulation as it is associated with gene suppression including microRNAs (miRNAs) by tri-methylating lysine 27 of histone H3 [243, 244]. Previous work completed by our laboratory studied the role between PRC2 and miR-200b in DR, but we will discuss the detailed findings in the subsection discussing small non-coding RNAs.

1.5.2.2 Histone Acetylation

In addition to methylation, histones can also be acetylated. The acetylation process involves the interactions between histone acetyltransferases (HATs) and histone deacetylases (HDACs) that facilitates either the addition or removal of acetyl groups to lysine residues, respectively [245, 246]. More specifically, the acetylation of lysines 9, 14, 18, and 56 in histone H3 and lysines 5, 8, 13, and 16 in histone H4 are speculated to have a role in chromatin relaxation that augments transcription factor recruitment—subsequently contributing to gene activation [220, 247]. The direct modification of regulatory proteins and transcription factors can also occur by HATs and HDACs [247]. To further demonstrate the importance of HATs in DR, previous *in vitro* and *in vivo* work from our laboratory demonstrated that P300, a transcriptional coactivator that is also a HAT, was markedly expressed in a diabetic environment and

its increase in activity led to the overexpression of ET-1, VEGF, and FN—molecules that are upregulated in DR [248, 249]. On the other hand, sirtuin (silent mating type information regulation 2 homolog) 1 (SIRT1) is an important NAD-dependent deacetylase that is implicated in a dynamic range of cellular processes [250, 251, 252]. Due to its unique localization in both the nucleus and cytoplasm, SIRT1 is categorized as a type III HDAC [252]. *In vitro* and *in vivo* results from our laboratory demonstrated that chronic hyperglycemia reduces SIRT1 activity, which consequently drives ROS formation mediated by the acetylation of forkhead box protein O1 (FOXO1) through increased P300 activity [253].

1.5.3 The Emergence of Non-Coding RNAs

When the sequencing results materialized from the Human Genome Project, it become evident that the majority of our genome (>98%) is comprised of non-protein coding DNA, while less than 2% of the total genomic sequence is represented by protein-coding regions (amounts to ~20,000 protein-coding genes) [254, 255]. Furthermore, as demonstrated through genomic tiling arrays and large-scale cDNA cloning projects [255, 256], the process of transcription is pervasive throughout the mammalian genome and is not only restricted to protein-coding regions. In fact, more than 90% of our genome is transcribed and the resulting output of transcription produces a dynamic network of transcripts that includes thousands of non-protein-coding RNAs [255, 257]. Due to the breadth of information that can now be found on different classes of non-coding RNAs (ncRNAs), I will provide insight into some of the known functions of certain small and long ncRNAs, and their implications, in the pathogenesis of DR below.

1.5.3.1 Small Non-Coding RNAs

Although there are several classes of small non-coding RNAs (sncRNAs), microRNAs (miRNAs) are an extensively studied class in the context of disease, which will be the primary focus in this section. Being a class of small, endogenous, single-stranded, non-coding RNA molecules, active miRNAs typically range from 20 to 25 nucleotides in length and post-transcriptionally regulate gene expression [258, 259, 260]. The

initial synthesis of primary miRNA molecules transpires in the nucleus where they are synthesized by RNA polymerase II and are several kilobases long [260, 261]. Similarly, within the nucleus, RNA polymerase III enzymes, Drosha and DiGeorge syndrome critical region 8 (DGCR8), further processes these primary miRNA molecules into precursor miRNAs that are roughly 70 to 100 nucleotides in length and hairpin-shaped [260-265]. Following processing, exportin 5, a nuclear export factor, facilitates the transport of precursor miRNAs into the cytoplasm [264, 265]. Once reaching the cytoplasm, another RNA polymerase III enzyme, DICER, modifies the precursor miRNAs into their active miRNA forms, where they are then incorporated into the RNA-induced silencing complex (RISC) with the help of argonaute proteins [263, 264, 265]. The formation of RISC then binds to the targeted mRNA by interacting with its 3' untranslated regions (3'-UTR) [260-265]. As a result of this binding, the targeted mRNA is subjected to either repressed translation or degradation [260-265]. Earlier reports from our laboratory identified alterations of several miRNAs in hyperglycemia-induced endothelial cells and in numerous tissues affected by chronic diabetes: miR-1, miR-133a, miR-146a, miR-195, miR-200b, and miR-320 [39, 266-272]. Moreover, our recent findings report a novel regulation mechanism between PRC2 and miRNAs through histone methylation in diabetic complications. For instance, human retinal microvascular endothelial cells exposed to hyperglycemia and diabetic mice and rat retinas demonstrated heightened PRC2 activity, which in turn regulated the repression of miR-200b through tri-methylation of lysine 27 in histone H3 [270, 271]. Subsequently, reduced miR-200b levels promoted increased VEGF levels—contributing to vascular permeability and neovascularization [270, 271]. In addition to these findings, previous studies from our laboratory reported that miR-146a and miR-200b play an integral role in preventing glucose-induced endothelial-to-mesenchymal transition (EndMT), a pathogenetic mechanism implicated in diabetic complications that induces basement membrane thickening by accelerating the production and deposition of extracellular matrix proteins [39, 268].

1.5.3.2 Long Non-Coding RNAs

Long non-coding RNAs (lncRNAs) are a fundamental class of RNA molecules that are defined by their length (greater than 200 nucleotides) and limited protein-coding capacities. LncRNAs have putative roles in both biological (i.e. embryonic development [273] and pathological processes [274] and can take on unique regulatory capacities when governing the expression of genes (**Table 1.1**). Moreover, certain lncRNAs may execute more than one mechanism and present with specific functionalities depending on their subcellular localization. For instance, lncRNAs that are localized in the cytoplasm are generally involved in post-transcriptional modifications that govern the stability and translation potential of mRNAs [275]; whereas, lncRNAs primarily residing in the nucleus have implications in organizing nuclear architecture [276], alternative splicing [277], and transcriptional regulation [278]. Interestingly, certain lncRNAs can also be found in both the nucleus and cytoplasm [279], or other cellular compartments (such as the mitochondria [280]), where these RNA molecules have versatile roles in shaping the epigenome, influencing biological processes (such as transcription and translation), and regulating organelle formation and function [281]. Aside from their subcellular localization, the site of biogenesis can also classify lncRNAs (**Table 1.2**). Namely, lncRNAs can be broadly categorized as either intergenic (not overlapping with any protein-coding loci) or intragenic/genic (overlapping protein-coding genes), where intragenic lncRNAs are further classified as ‘antisense’, ‘bidirectional’, ‘intronic’, or ‘sense’ depending on their transcriptional orientation on the protein-coding loci. When compared to intragenic lncRNAs, long intergenic ncRNAs (lincRNAs), which arise from the intergenic regions of the human genome, oftentimes possess greater evolutionary conservation at both the sequence and RNA secondary structure level [282, 283]. Although particular differences in biogenesis exist between intragenic and intergenic lncRNAs, it is plausible that a majority of these lncRNAs share similar modes of action, through *cis* or *trans*-regulatory mechanisms, in order to govern fundamental biochemical and cellular processes.

Table 1.1. Regulatory capacities of lncRNAs. Reproduced from *The multifaceted roles of lncRNAs in diabetic complications: a promising, yet perplexing paradigm* by S. Biswas and S. Chakrabarti, 2020, Springer Nature.

Type of Mechanism	Description of Mechanism
Scaffold	LncRNAs can serve as adaptors that tether pertinent protein subunits into distinct complexes [284].
Guide	Once bound to a protein partner (i.e. chromatin-modifying enzymes), lncRNAs can direct enzymes to select regions of the genome [285].
Enhancer	Certain lncRNAs can directly enhance the activation of neighbouring genes [286].
Decoy/Sponge	LncRNAs can sequester proteins (i.e. transcription factors and alternative splicing factors) and small regulatory RNAs (i.e. miRNAs) in order to affect their regulation of target genes [287, 288].

Table 1.2. Classification of lncRNAs based on their site of biogenesis. Reproduced from *The multifaceted roles of lncRNAs in diabetic complications: a promising, yet perplexing paradigm* by S. Biswas and S. Chakrabarti, 2020, Springer Nature.

LncRNA classification	**Subcategory**	**Description of lncRNA subcategory**
Intergenic (does not intersect with protein-coding genes)	Long intervening/ intergenic lncRNA	Transcribed from the intergenic DNA regions (regardless of orientation) and generally possess greater evolutionary conserved regions compared to intragenic lncRNAs.
Intragenic/genic (overlaps with protein-coding genes)	Antisense	Transcribed from the antisense/opposite strand of a protein-coding gene and may overlap with coding exons.
	Bidirectional	Transcribed in the opposite direction from the promoter of a protein-coding gene (generally less than 1 kb away).
	Intronic	Transcribed entirely within the intronic regions of a protein-coding gene, and does not overlap with coding exons.
	Sense	Transcribed from the sense/coding strand of a protein-coding gene and may overlap coding exons.

In the context of DR, many aberrant lncRNAs have been identified that are expressed in the retina during diabetes; however, these lncRNAs have not been comprehensively characterized [289]. Therefore, defining the specific roles of each lncRNA becomes critical to understanding the interplay between lncRNAs and the pathogenesis of DR. Currently, a prominent lncRNA that is upregulated in DR and associated with increased inflammatory cytokine production is metastasis-associated lung

adenocarcinoma transcript 1 (*MALAT1*) [290, 291]. As a matter of fact, both *in vitro* and *in vivo* results from our laboratory identified that hyperglycemia induces an upregulation of *MALAT1* in endothelial cells, which in turn regulates increased expression of inflammatory mediators, IL-6 and TNF-α, through serum amyloid antigen three (SAA3) activation [291]. In addition to *MALAT1*, *in vitro* and *in vivo* experiments from our laboratory revealed another lncRNA, antisense non-coding RNA in the *INK4* locus (*ANRIL*), that is upregulated in human retinal endothelial cells under hyperglycemic conditions [292]. High glucose exposure elevated direct *ANRIL* binding to P300 and another component of PRC2, enhancer of zeste homolog 2 (EZH2). Furthermore, we demonstrated that VEGF regulation might involve *ANRIL*-mediated control of PRC2, P300 and miR-200b, since silencing of *ANRIL* simultaneously prevented the glucose-induced expressions of these epigenetic molecules and VEGF [292]. Moreover, a recent study from our laboratory has demonstrated the ability of *H19* (a lncRNA) to mediate the phenotypic switch of retinal endothelial cells in diabetic environments (this process is known as endothelial-to-mesenchymal transition, EndMT; where endothelial cells lose their endothelial markers and develop a more mesenchymal phenotype) [293]. More specifically, human retinal endothelial cells (HRECs) cultured in high glucose (HG) showed decreases in *H19* and endothelial cell markers (VE-cadherin and CD-31), while mesenchymal markers were significantly upregulated (SM22-α, α-SMA, and FSP-1). Remarkably, the overexpression of *H19* in HRECs drastically prevented the trends elicited by HG, which suggests that the lncRNA *H19* has a protective role in impeding EndMT in DR. In parallel with our *in vitro* findings, significant reductions of *H19* RNA levels were also observed in the vitreous humors of PDR patients. As well, retinal tissues from *H19* knockout non-diabetic mice revealed an EndMT phenotype (decreased endothelial markers and increased mesenchymal markers) that was comparable to retinal tissues from diabetic wild-type and diabetic *H19* knockout mice. Further expanding our findings, *H19* was found to suppress glucose-induced EndMT via the MAPK-ERK1/2 pathway of TGF-β signalling. In addition to *H19*, another lncRNA known as HOX anti-sense intergenic RNA (*HOTAIR*) has been shown to exert regulatory capabilities that promote oncogenesis [297], while other evidences

have suggested that aberrant *HOTAIR* expressions exist in diabetic kidney disease but its dysregulation may not necessarily contribute to disease pathogenesis [298]. Based on these reports, it may be possible that disease-specific phenotypes exist for *HOTAIR*, however, current work on this lncRNA in other diabetes models are limited and its implications in DR pathogenesis remain elusive.

Undoubtedly, long non-coding RNAs (lncRNAs) continue to evolve our understanding of the genomic landscape. The versatility of these non-protein-coding molecules warrants serious consideration for in-depth investigations of their roles in pathophysiological mechanisms, as novel information on these critical mediators will not only add to the existing molecular paradigms, but such knowledge will also contribute to the development of better-targeted diagnostics and therapies. As such, for my thesis, I sought to characterize the epigenetic mechanisms of two specific lncRNAs, *MALAT1* and *HOTAIR*, in the pathogenesis of DR.

1.6 Rationale

In our earlier studies, we performed microarray analyses that examined the global expression profiles of lncRNAs in HRECs cultured with 5 mM (mimicking euglycemia; NG) or 25 mM D-glucose (mimicking hyperglycemia; HG) for 48 hours (GEO: GSE122189; **Appendix B, Supplemental Figure 1**). Our findings identified thousands of differentially expressed long non-coding RNAs (lncRNAs) following 48 hours of HG stimulation. Among the transcripts, the lncRNAs *MALAT1* and *HOTAIR* were profoundly upregulated in HG environments. Based on our previous studies linking *MALAT1* to inflammation in diabetic complications [291, 294] and prior cancer-based studies demonstrating a relationship between *HOTAIR* and angiogenesis [295, 296], my research sought to elucidate for the first time the epigenetic mechanisms of *MALAT1* in inflammation and *HOTAIR* in angiogenesis during the pathogenesis of DR.

1.7 Central Hypothesis

I hypothesize that the lncRNAs *MALAT1* and *HOTAIR* are intertwined in a complex network of epigenetic mechanisms that regulate critical pathogenetic processes (such as inflammation and angiogenesis) in DR. To test this hypothesis, three specific aims were proposed.

1.7.1 Specific Aims

Aim 1: To investigate the implications of *MALAT1* in the epigenetic regulation of inflammation in DR (Chapter 2).

Synopsis: In this aim (Chapter 2), we investigated a prominent, cancer-related, intergenic lncRNA in DR known as *MALAT1*. More specifically, we established a novel epigenetic paradigm for *MALAT1* in DR by employing siRNA-mediated *MALAT1* knockdown in HRECs, a *Malat1* knockout animal model, vitreous humor from diabetic patients, pharmacological inhibitors for histone and DNA methylation, RNA immunoprecipitation, western blotting, and a unique DNA methylation array to determine glucose-related alterations in *MALAT1*. Our findings indicated that *MALAT1* is capable of impacting the expressions of inflammatory transcripts through its association with components of the PRC2 complex in diabetes. Furthermore, the vitreous humors from diabetic patients revealed increased expressions of *MALAT1*, TNF-α, and IL-6. Intriguingly, our DNA methylation array demonstrated that transient high glucose exposure in HRECs does not contribute to significant methylation alterations at CpG sites across the MALAT1 gene. However, global inhibition of DNA methyltransferases induced significant increases in MALAT1 and associated inflammatory transcripts in HRECs. Our collective findings demonstrated the importance of *MALAT1* in inflammation and epigenetic regulation in DR.

Aim 2: To examine whether *HOTAIR* is a critical epigenetic mediator of angiogenesis in the pathogenesis of DR (Chapter 3).

Synopsis: In Aim 2 (Chapter 3), we identified the lncRNA *HOTAIR* in HRECs through microarray screening and further examined its expression using several *in*

vitro and *in vivo* experimental models. As evident by our cellular findings, hyperglycemic environments can significantly augment *HOTAIR* expressions and the subsequent upregulation of *HOTAIR* can directly promote glucose-induced angiogenesis, oxidative damage, and mitochondrial aberrations. Similarly, vitreous fluid and serum samples from PDR patients and retinas from diabetic animals demonstrated significant *HOTAIR* expressions when compared to non-diabetic controls. Furthermore, *in vivo* knockdown of *Hotair* prevented early glucose-induced increases in several angiogenic markers and diabetes-associated molecules in the diabetic retina. Mechanistically, *HOTAIR* may be exerting its regulatory capabilities through its implications in a complex epigenetic axis involving histone methylation (PRC2), histone acetylation (P300), DNA methylation (DNMTs), and specific transcription factors (CTCF). Taken together, our findings suggest that the versatile lncRNA *HOTAIR* is a critical epigenetic mediator of angiogenesis in the pathogenesis of DR and *HOTAIR* may be a highly effective molecule for diagnostic and therapeutic targeting.

Aim 3: To examine the interrelationships between epigenetic mechanisms and specific molecular alterations in DR (Chapter 4).

Synopsis: Since endothelial cells (ECs) are primary targets of glucose-induced tissue damage, in Aim 3 (Chapter 4), we selected a prominent diabetes-related molecule, known as endothelin-1 (ET-1), for further follow-up. We carried out the investigation in HRECs using multiple approaches in order to identify novel transcriptional mechanisms that influence ET-1 regulation in diabetes. ECs were incubated with 5 mM glucose (NG) or 25 mM glucose (HG) and analyses for DNA methylation, histone methylation, or long non-coding RNA-mediated regulation of *ET-1* mRNA were then performed. DNA methylation array analyses demonstrated the presence of hypomethylation in the proximal promoter and 5' UTR/first exon regions of *EDN1* following HG culture. Further, globally blocking DNA methylation or histone methylation significantly increased *ET-1* mRNA expressions in both NG and HG-cultured HRECs, while knocking down pathogenetic lncRNAs (including *MALAT1* and *HOTAIR*) subsequently prevented the glucose-induced upregulation of *ET-1* transcripts. Based on our past and present findings, we present a novel paradigm that

reveals a complex web of epigenetic mechanisms regulating glucose-induced transcription of *ET-1*.

Following the discussion of these aims, in Chapter 5, I will examine the significance of my overall work and discuss potential follow-up experiments that could provide additional insights into this perplexing transcriptional paradigm.

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Chapter 2 [ii]

2 *MALAT1*: An Epigenetic Regulator of Inflammation in Diabetic Retinopathy

As the global prevalence of diabetes is projected to rise to 642 million by 2040, there is an urgent need for understanding the pathogenesis of diabetic complications to develop effective therapeutic agents [1, 2]. Diabetic retinopathy (DR), a debilitating ocular complication, is the leading cause of blindness among working-aged adults in industrialized nations [3]. The asymptomatic nature of DR, prior to the development of vision loss, is concerning, as nearly all type 1 diabetic patients and over 60% of type 2 diabetic patients will develop evidence of retinopathy within the first 20 years of diagnosis [4, 5]. Despite the presence of management strategies, the rate of DR is still expected to rise due to the increasing incidence of diabetes, which necessitates the need for exploration of new molecular aspects of DR to expand the current scope of therapy.

In the last two decades, the rapid advent of high-throughput genomic technology has made it evident that more than 97% of the human genome is comprised of non-protein-coding elements, such as non-coding RNAs (ncRNAs) [6]. Although significant research has been conducted in annotating the transcripts that arise from these genomic regions, a vast amount of information regarding the roles and functions of ncRNAs in DR remains elusive.

Long non-coding RNAs (lncRNAs) are a class of ncRNAs that are greater than 200 nucleotides in length and have diverse roles in a myriad of cellular processes including the ability to repress the expression of nearby protein-coding genes [7], X-chromosome inactivation [8], and the modulation of protein activity [9]. In DR, transcriptomic

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analyses have identified more than 300 lncRNAs that display aberrant expression profiles in the retina—with over 80 lncRNAs being overexpressed [10]. Among these upregulated lncRNAs, *MALAT1* (metastasis-associated lung adenocarcinoma transcript 1) is a prominent intergenic lncRNA that is known to be associated with metastasis in non-small cell lung cancer (NSCLC) [11]. Since it is accepted that endothelial cells (ECs) are main targets of diabetes-induced tissue damage, recent research has also revealed novel roles for *MALAT1* in diabetic complications. Results from our previous study indicate that *MALAT1* knockdown in human umbilical vein endothelial cells (HUVECs), under hyperglycemic conditions, down-regulates serum amyloid antigen 3 (SAA3) activation, subsequently reducing the RNA and protein expressions of key inflammatory mediators (IL-6 and TNF-α) implicated in diabetic complications [12]. Further, augmentation of *MALAT1* expression by hypoxia promotes a proliferative response in HUVECs [13].

In order to understand how lncRNAs, such as *MALAT1*, regulate the inflammatory processes underpinning these pathologies, the complex molecular interplay between lncRNAs and other epigenetic events must be examined in an integrated way. Several cancer-related studies have revealed that *MALAT1* is capable of binding to enhancer of zeste homolog 2 (EZH2), the main catalytic subunit of the histone methyltransferase polycomb repressive complex 2 (PRC2), and promotes oncogenesis by reprograming the chromatin state [14-17]. Furthermore, in the context of DNA methylation, it has been previously reported that lung cancer tissues exhibit reduced methylation in the *MALAT1* promoter, which subsequently enhances *MALAT1* expression [18]. However, in contrast, others have reported minimal methylation alterations at the CpG island in the *MALAT1* promoter of esophageal squamous cell carcinoma tissues and concluded that CpG island methylation status may not contribute to *MALAT1* dysregulation [19]. Nevertheless, despite the recent emergence of these epigenetic roles for *MALAT1* in cancer, the question of whether *MALAT1* influences other epigenetic mediator proteins to regulate inflammation in DR still remains unanswered.

Here, we first determined the expression level of *MALAT1* in human retinal microvascular endothelial cells (HRECs) cultured in high glucose (HG) and

subsequently analyzed the expressions of common inflammatory markers (IL-6, TNF-α, MCP-1, and IL-1β) along with components of PRC2 (EZH2, SUZ12, and EED) to represent histone methyltransferase activity. Following our initial findings, we employed *MALAT1* knockdown and *Malat1* knockout (KO) strategies in HRECs and in a mouse model, respectively, to determine the functional role and significance of *MALAT1* on inflammation and PRC2 activity in DR. Moreover, to substantiate the data from our *in vitro* and *in vivo* animal experiments, we examined *MALAT1* and its associated inflammatory markers in the vitreous humor (VH) of diabetic patients undergoing vitrectomy. We also examined *MALAT1* binding to EZH2 by RNA immunoprecipitation in HRECs. As well, we further investigated the effects of HG on CpG island methylation status in the *MALAT1* promoter of HRECs using a methylation array and then explored the impact of specific treatment(s) targeting *MALAT1*, histone methyltransferases, and DNA methyltransferases (DNMTs).

2.1 Research Design and Methods

2.1.1 Cell Culture

We utilized HRECs (Olaf Pharmaceuticals, Worcester, MA, USA) for the *in vitro* experiments. The experimental and culture conditions for HRECs were mentioned in our previous studies [44, 58]. Briefly, prior to experimentation, HRECs were plated at a density of $4.3x10^5$ cells/mL and used between passages 5 and 6 to reduce variability. Cells were grown to 80-90% confluency post-seeding and subjected to serum starvation for 24 hours, which was then followed by the administration of specific glucose levels (normal glucose [NG], 5 mM; high glucose [HG], 25 mM; and osmotic control [LG], 20 mM L-glucose + 5 mM D-glucose) at various time points. These glucose levels are based on a large volume of previous experiments [12, 32, 44, 58-61]. All cell culture reagents were purchased from Sigma (Oakville, Ontario, Canada) unless specified and *in vitro* experiments were performed with six replicates and independently repeated at least three times, unless specified.

2.1.2 Diabetic Mice Model

The Western University Council for Animal Care Committee approved all animals used in this study and the experiments were performed in accordance with *The Guide for the Care and Use of Laboratory Animals* (NIH Publication 85-23, revised in 1996). *Malat1* knockout (KO) mice, with a C57/BL6 background, were obtained through collaboration with Dr. Spector (Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, USA) [22] and only male mice were randomly organized into control and diabetic groups. Wild-type (WT) non-diabetic and WT diabetic mice were used as comparators to *Malat1* KO non-diabetic and *Malat1* KO diabetic mice. To generate a type 1 diabetic animal model, we used streptozotocin (STZ). STZ injection methods and monitoring have been previously described [59]. At two months following diabetes induction, animals (n= 6/group) were euthanized and the retinal tissues were either snap-frozen for future RNA or protein analyses or were placed in 10% formalin for paraffin embedding.

2.1.3 Clinical Sample Collection

The Western Research Ethics Board at the University of Western Ontario, London, Ontario, Canada, approved the clinical component of this study. Prior to the procurement of surgical specimens, patients provided informed consent and all of the samples were handled in accordance with the *Declaration of Helsinki*. VH was collected from patients undergoing pars plana vitrectomy by an experienced vitreoretinal surgeon. Specimens were then categorized into two groups: diabetic and non-diabetic. The diabetic group comprised of patients diagnosed with proliferative DR (PDR; n= 7; 3 males and 4 females; mean age ± SD= 61 ± 6.76 years); whereas, the non-diabetic group consisted of patients that had no previous history of diabetes mellitus and were diagnosed with idiopathic macular hole or a separate non-diabetic ocular condition (n= 6; 4 males and 2 females; mean age ± SD= 75 ± 3.52 years). As previously described [60], VH specimens were centrifuged (12,000g, 10 minutes, 4°C) and the pellet was used for RNA extraction using the TRIzol reagent (Invitrogen, Burlington, ON, Canada) and subsequently, real-time quantitative reverse transcription-PCR (RT-qPCR). In order to avoid contaminating RNA from blood cells, indications of vitreal haemorrhage in the

VH specimens were immediately excluded from this study.

2.1.4 Immunohistochemistry

To identify blood-retinal barrier (BRB) damage in the eye, paraffin-embedded mouse retinal sections were applied for immunohistochemical staining of immunoglobulin G (IgG) using anti-mouse IgG antibody (MP Biomedicals, OH, USA), as previously specified [44, 61]. Histological slides were evaluated for positive IgG immunoreactivity (arbitrarily scored 0-3, with 3 representing maximum IgG reaction) in a masked manner by an investigator.

2.1.5 Enzyme-Linked Immunosorbent Assay (ELISA)

In order to measure the cytokine levels from the cell supernatants, human IL-6 and TNF-α ELISA kits were purchased from ALPCO (Salem, NH, USA) and R&D Systems (Minneapolis, MN, USA), respectively. Concentrations for each cytokine were first quantified using the BCA protein assay kit (Pierce, Rockford, IL, USA) and equal protein concentrations were used for each ELISA (100 µg) according to the manufacturer's instructions. For the IL-6 chemiluminescence assay, the SpectraMax M5 (Molecular Devices, California, USA) was used to detect luminescence. Whereas, for the TNF-α Quantikine ELISA kit, the optical density for each well was determined at 450 nm and corrected at 568 nm using the Multiskan FC Microplate Photometer (Thermo Fisher Scientific, Massachusetts, USA).

2.1.6 SiRNA Transfection

HRECs were transfected with either pre-designed siRNAs targeting human *MALAT1* (ID numbers: n272231 [si1-MALAT1] and n272233 [si2-MALAT1], Life Technologies), *DNMT1* (ID number: s4216 [siDNMT1], Life Technologies) or scrambled siRNA (ID number: AM4635, Life Technologies) using Lipofectamine 2000 (Invitrogen, Burlington, ON, Canada) and Opti-MEM (Life Technologies). A lipofectamine-mediated transfection protocol has been indicated in our earlier studies [12, 58]. Briefly, 100 nM of siMALAT1, siDNMT1, or scrambled siRNA was used to

transfect the cells for 4 hours and subsequently recovered in full medium overnight. The following morning, cells were serum starved for 21 hours and were then incubated with specific glucose concentrations (5 mM or 25 mM) for 48 hours. *MALAT1* and *DNMT1* knockdown were confirmed using RT-qPCR. Following verification of knockdown, both siMALAT1s demonstrated similar knockdown activity (~75%) when compared to scrambled controls; therefore, we decided to select si2-MALAT1 (ID: n272233) for our subsequent experiments. Furthermore, when compared to scrambled HG controls, siDNMT1 (ID: s4216) demonstrated a ~72% reduction in *DNMT1* RNA expression in HG-treated HRECs.

2.1.7 3-Deazaneplanocin A (DZNep), 5-Aza-2'-deoxycytidine (5-aza-dC), and Zebularine

Based on previous literature, 5 μM of DZNep (Cayman Chemical, Ann Arbor, MI), 5-aza-dC (Sigma, St. Louis, USA), or zebularine (Cayman Chemical) pre-treatment was applied to HRECs for 1 hour prior to the addition of D-glucose [44, 58, 62, 67]. DZNep, 5-aza-dC, or zebularine-treated HRECs and their respective controls were collected at 48 hours for further analyses.

2.1.8 CpG Island Methylome Analysis

Whether a differential methylation pattern exists in a diabetic environment remains entirely elusive, therefore we investigated the CpG island methylation status in the promoter of the *MALAT1* gene in NG or HG-treated HRECs. At the 48-hour mark after glucose treatment, HRECs were collected and 1 μg of genomic DNA was used for bisulfite conversion using the EZ DNA Methylation Kit (Zymo Research, Irvine, California, USA). The bisulfite-converted DNA was then hybridized to the Illumina Infinium MethylationEPIC BeadChip array (Illumina, San Diego, California, USA) following the manufacturer's protocol. To achieve the readout from the array, we used the HiScan System (Illumina, San Diego, California, USA) and subsequently imported the methylated and unmethylated signal intensity data into R 3.4.0 for analyses. The methylation intensity was normalized using the Illumina normalization method with

background correction using the minfi package. Probes with a detection *P*-value > 0.01 were excluded from the downstream analyses. Further, probes known to contain SNPs at the single nucleotide extension, or the CpG interrogation, were removed. The beta value (β-value) was used to represent the methylation intensity for each CpG locus and was calculated from the ratio of unmethylated probe to methylated probe, ranging between 0 (no methylation) and 1 (full methylation). Three independent samples were used per group.

2.1.9 RNA Immunoprecipitation (RIP)

At the 48-hour mark, cell lysates from NG and HG-treated HRECs were collected for immunoprecipitation using the Magna RIP RNA-Binding Protein Immunoprecipitation Kit (Millipore, Etobicoke, ON, Canada) following the manufacturer's instruction [44]. Anti-IgG (control) and anti-EZH2 antibodies (Millipore) were used to co-precipitate the RNA-binding proteins of interest. The extracted RNAs were then analyzed by RT-qPCR.

2.1.10 Western Blotting

In order to evaluate the protein expression of EZH2 after siMALAT1 and HG treatments, western blotting was performed. As previously described [58], cell lysates were obtained from HRECs following 48 hours of NG or HG culture with scrambled or *MALAT1* siRNA. The Bicinconinic acid assay (Thermo Fisher Scientific, IL, USA) was used to determine protein concentration, in which 20 μg of protein was used for western blotting. Primary antibody incubation was performed overnight using monoclonal anti-EZH2 (1:500; Millipore) or for 1 hour using polyclonal anti-β-actin (1:10000; Abcam, Toronto, ON, Canada). While, secondary antibody incubation was conducted using anti-mouse IgG (1:5000; Santa Cruz Biotechnology, Santa Cruz, CA, USA) or anti-rabbit IgG (1:5000; Bio-Rad, Hercules, CA, USA) horseradish peroxidase conjugated secondary antibodies. Antigenic detection was performed using enhanced chemiluminescence following the manufacturer's instruction (GE Healthcare Life Sciences, QC, Canada).

2.1.11 RNA Isolation and Quantitative Real-Time Polymerase Chain Reaction (RT-qPCR)

Total RNA was extracted using TRIzol reagent (Invitrogen, Burlington, ON) as described [12, 44, 58-61]. After isolation, RNA concentrations were quantified using a spectrophotometer (260 nm; Gene Quant, Pharmacia Biotech, USA) and 1 μg of total RNA was reverse transcribed to complementary DNA (cDNA) using a high-capacity cDNA reverse-transcription kit (Applied Biosystems, Burlington, ON, Canada). In order to detect RNA expression, cDNA was amplified in the LightCycler 96 System (Roche Diagnostics, Laval, QC, Canada) using SYBR-green master mix (Clontech, Mountain View, CA, USA) and specific primers for the genes of interest (Sigma; **Appendix A, Table S1**). We analyzed results using the LightCycler 96 SW 1.1 software (Roche) and we calculated expression levels by the relative standard curve method using β-*actin* as an internal control for sample normalization.

2.1.12 Cell Viability Assay

As previously described [58], the cytotoxicity of glucose treatments (5 mM and 25 mM) in HRECs were determined using the WST-1 Cell Viability Assay (Roche). Viability was assessed at various durations of incubation (0, 24, and 48 hours). Absorbances were first measured at 450 nm, with the Multiskan FC Microplate Photometer (Thermo Fisher Scientific), and then corrected using 690 nm as the reference wavelength.

2.1.13 Statistical Analysis

Data are expressed as mean ± SEM, unless specified. To determine statistical significance, GraphPad Prism 7 was used to perform Student's *t* tests when comparing 2 conditions or 1-way ANOVA followed by Tukey's post hoc test for multiple comparisons. While, for the clinical samples, non-parametric statistical measures (Mann-Whitney U test) were applied. As well, two-sided Pearson Correlations were performed to determine a linear association between RNA expressions of *MALAT1* and PRC2 components, or EZH2 and inflammatory cytokines in HRECs cultured with HG

and siMALAT1. Differences below $P<0.05$ were considered statistically significant.

2.2 Results

2.2.1 *MALAT1* is upregulated in HRECs exposed to high glucose

Retinal ECs are a fundamental cell type in the retinal microvasculature [20]. Retinal ECs are also one of the earliest cells to undergo glucose-induced damage as a consequence of DR [21]. Hence, we used HRECs for our *in vitro* experiments. Of note, we have previously reported HG-induced increases in the expression of *MALAT1* in large vessel ECs [12]. In order to investigate the differential expression patterns of *MALAT1* and inflammatory markers, we cultured HRECs in HG and examined *MALAT1*, *IL-6*, *TNF-α*, *MCP-1* and *IL-1β* RNA expressions at 12, 24, 48, and 72 hours. RT-qPCR analyses demonstrated that expressions of *MALAT1* and inflammatory markers in HRECs peaked at 48 hours following HG incubation (see **Appendix A, Figures S1A-S1E**). Furthermore, no significant differences for *MALAT1*, *IL-6*, *TNF-α*, *MCP-1* and *IL-1β* expressions were observed osmotic controls (data not shown). Following our findings, we decided to use the 48-hour time point for our subsequent *in vitro* experiments.

2.2.2 *MALAT1* knockdown prevents augmented production of inflammatory cytokines and PRC2 components *in vitro*

After observing upregulation of *MALAT1* at 48 hours *in vitro*, we wanted to delineate the functional importance of the *MALAT1* transcript in inflammation and the role of PRC2 complex expression in HRECs. We, therefore, employed a siRNA-mediated knockdown approach targeting *MALAT1*. RT-qPCR analyses confirmed a ~75% reduction in total *MALAT1* RNA after siRNA treatments when compared to scrambled controls (**Fig. 2.1A**). *MALAT1* silencing in HG-treated HRECs dramatically reduced the

overall expression of tumor necrosis factor-alpha (*TNF-α*) and interleukin-6 (*IL-6*) by a ~74% and a ~93% reduction, respectively, when compared to scrambled controls (**Figs. 2.1B and 2.1C**). Similarly, *IL-1β* and *MCP-1* RNA expressions were also significantly reduced following siMALAT1 treatment (**Appendix A, Figs. S2A and S2B**). We further expanded our investigation to include the effects on inflammatory proteins after siMALAT1 treatment and findings from the ELISAs revealed that after knocking down *MALAT1* in a HG environment, IL-6 and TNF-α protein levels significantly decreased when compared to scrambled HG controls (**Figs. 2.1G and 2.1H**). The trends observed from these experiments are consistent with our previous findings of decreased expressions for IL-6 and TNF-α after siMALAT1 transfection in HUVECs [12].

To examine whether *MALAT1* knockdown can affect the components of PRC2, we analyzed *EZH2*, *SUZ12*, and *EED* RNA levels in HRECs following siMALAT1 transfection. As compared with scrambled controls, we found significantly reduced *EZH2*, *EED*, and *SUZ12* RNA levels in HG-treated HRECs transfected with *MALAT1* siRNA (**Figs. 2.1D-F**). Moreover, to demonstrate *MALAT1*'s ability to impact PRC2 at the protein level, we performed western blotting and examined EZH2 expression after *MALAT1* knockdown. Our western blot analyses confirmed that EZH2 protein expressions were reduced in HRECs transfected with siMALAT1 (**Appendix A, Fig. S2C**). In addition to our western blot data, we performed RNA immunoprecipitation and confirmed *MALAT1* binding with EZH2. In fact, *MALAT1* RNA was significantly enriched in the EZH2-antibody precipitated RNA fraction from HG-treated HRECs compared to controls (**Appendix A, Fig. S2D**).

Furthermore, extending our knockdown analyses, we observed significant positive correlations between *MALAT1* RNA expression and the RNA expressions of PRC2 components in HRECs subjected to HG and siMALAT1 treatments (two-sided Pearson correlation; **Appendix A, Figs. S3A-S3C**). As well, positive correlations existed between *EZH2* RNA expression and the RNA expressions of inflammatory markers, *IL-6* and *TNF-α*, in the siMALAT1+ HG group (**Appendix A, Figs. S3D** and **S3E**). Collectively, our findings from the knockdown experiment indicate that *MALAT1* RNA

levels play an important role in influencing glucose-induced upregulation of inflammatory cytokines and components of PRC2 in HRECs.

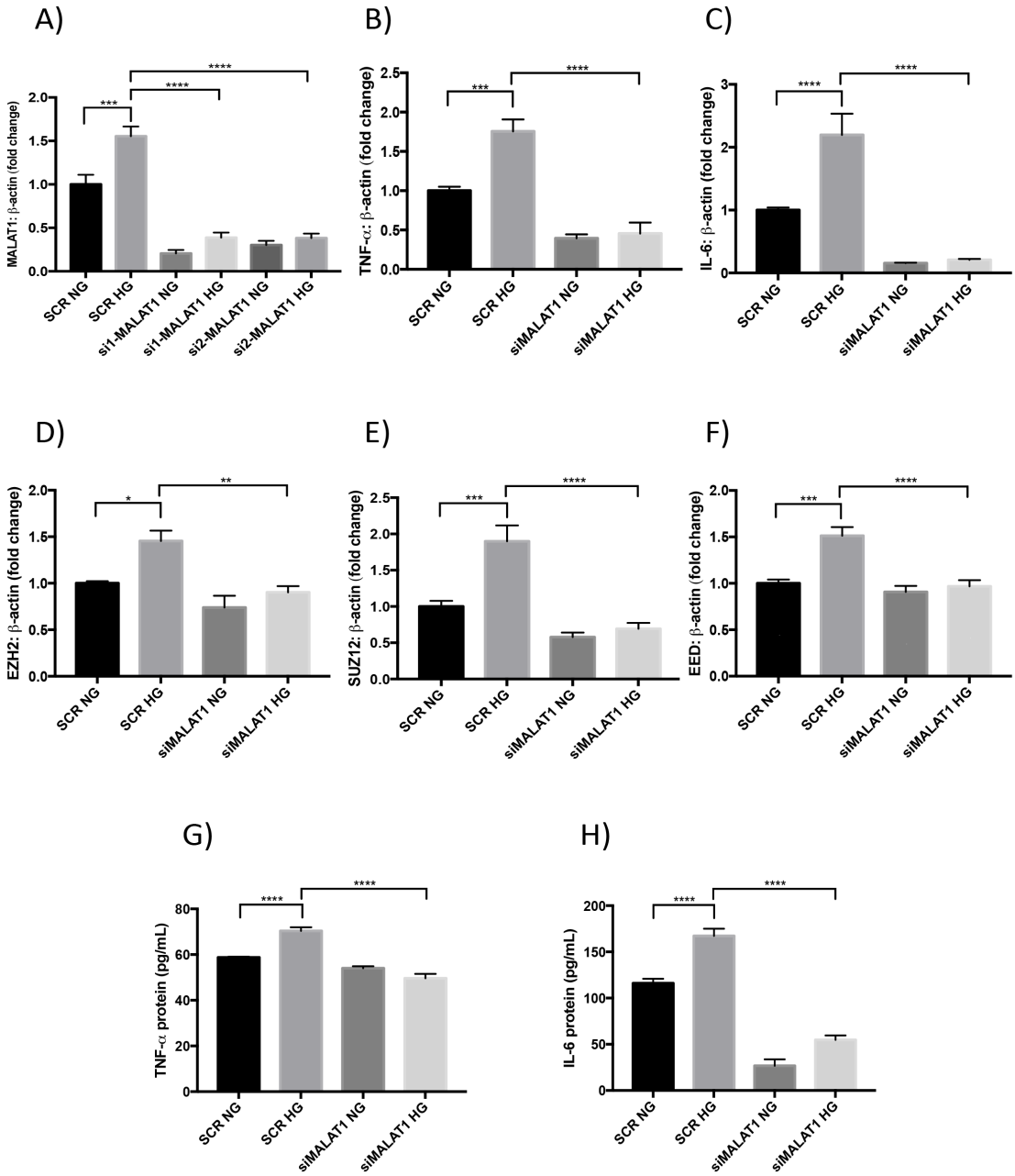

Figure 2.1. ***MALAT1*** **regulates glucose-induced production of inflammatory cytokines and PRC2 components** ***in vitro*****.** RT-qPCR analyses indicating HG-induced upregulation of A) *MALAT1* transcript, B, C) proinflammatory transcripts (*TNF-α, IL-6*), and D, E, F) PRC2 components (*EZH2, SUZ12*, and *EED*) in HRECs. SiMALAT1 transfections caused significant reductions in glucose-induced upregulation of these transcripts. G, H) ELISAs for protein levels of IL-6 and TNF-α (expressed as pg/mL) demonstrated prevention of glucose-induced increases

of these peptides following siRNA transfection (data expressed as a ratio to β-*actin* (mean ± SEM); normalized to SCR NG; *=*P*<0.05, **=*P*<0.01, ***=*P*<0.001, and ****=*P*<0.0001, compared to SCR NG or SCR HG; *n*= 6 from three independent experiments and performed in triplicates; SCR= scrambled siRNA; NG= 5 mM D-glucose; HG= 25 mM D-glucose; si1-MALAT1 (*MALAT1* siRNA 1, ID: n272231); and si2-MALAT1 (MALAT1 siRNA 2, ID: n272233)).

2.2.3 *Malat1* knockout alleviates diabetes-induced retinal inflammatory cytokines and elevated PRC2 expression

With the genetic ablation of the *Malat1* gene not contributing to noticeable developmental effects in mice under basal homeostatic conditions [22, 23, 24], we decided to use a diabetic *Malat1* KO mice model in order to evaluate the direct function of the *Malat1* gene on inflammation and PRC2 expression in retinal tissues. *Malat1* KO mice with STZ-induced diabetes and age-and sex-matched controls were monitored for 2 months. WT-diabetic (WT-D) mice showed hyperglycemia and reduced body weight gain (**Appendix A, Table S2**). Polyuria and glucosuria were also observed in diabetic mice (data not shown). Nevertheless, no effects on these parameters were seen following *Malat1* nullification.

Our analyses demonstrated that *MALAT1* RNA expression was significantly elevated (more than ~0.46-fold) in WT-D mice retinas (**Fig. 2.2A**) compared to WT non-diabetic control mice (WT-C). *MALAT1* RNA expressions were nearly non-existent in both *Malat1* KO animal groups (M1 KO-C and M1 KO-D), confirming that *MALAT1* transcripts are depleted in the retinal tissues of this global knockout model (**Fig. 2.2A**). Moreover, our initial findings of *MALAT1* upregulation in WT-D mice retinas are consistent with previous trends of increased *MALAT1* expression in retinas of diabetic rats [25, 26].

Intriguingly, upon *Malat1* gene inactivation, significant RNA expression changes were observed among *IL-6, TNF-α, IL-1β, MCP-1, EZH2, EED*, and *SUZ12* in retinal tissues of diabetic mice (**Figs. 2.2B-F**; **Appendix A, S4A** and **S4B**). When compared to WT-D

animals, all inflammatory transcripts were significantly downregulated (>50%) in *Malat1* KO diabetic animals (**Figs. 2.2B-C**; **Appendix A, S4A** and **S4B**). These changes were associated with downregulation of transcripts of PRC2 components in *Malat1* KO diabetic animals (**Figs. 2.2D-F**). Together, our data suggests that presence of the *Malat1* gene is important in regulating diabetes-induced inflammation and PRC2 components in retinal tissues.

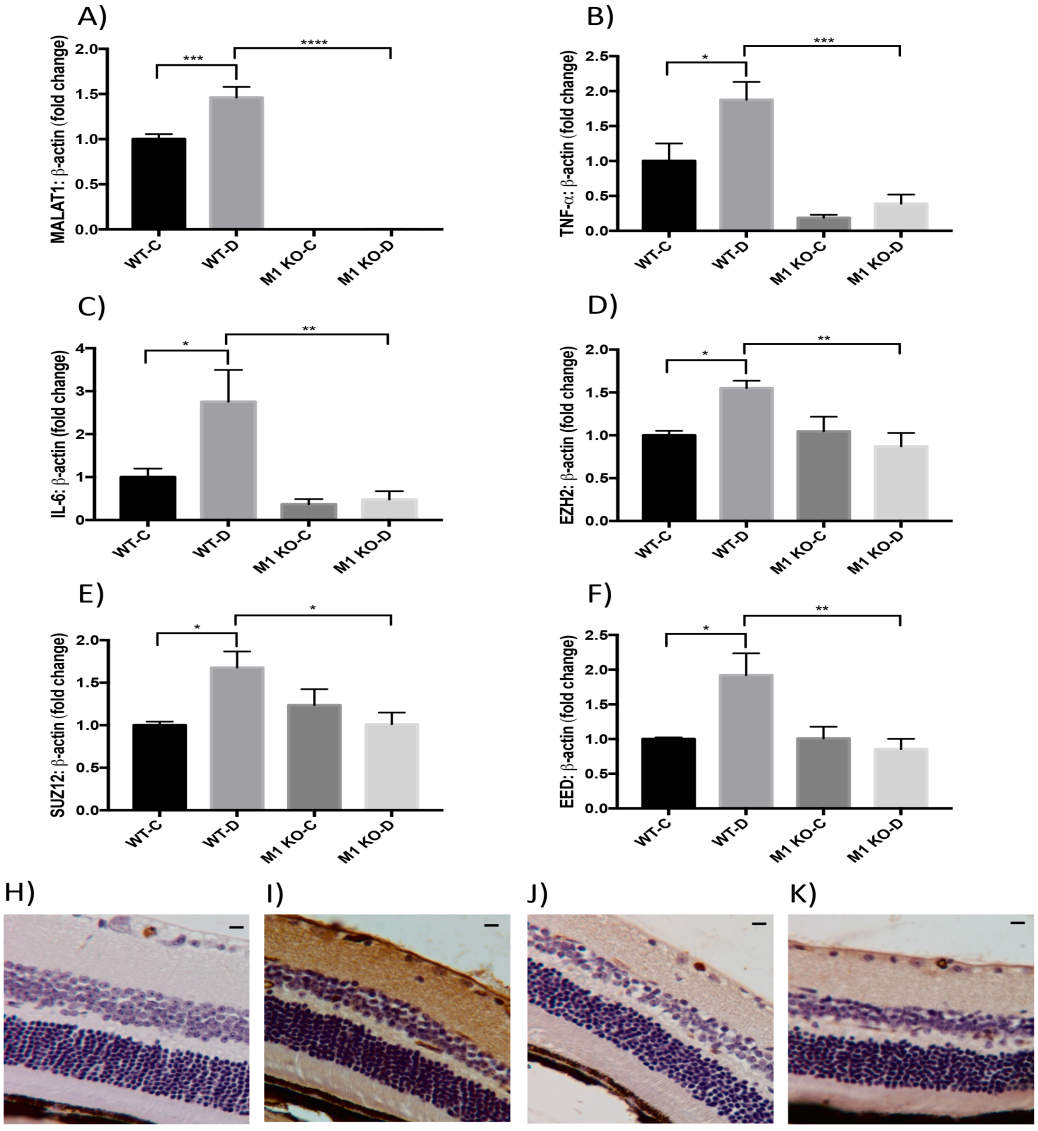

Figure 2.2. *Malat1* knockout alleviates diabetes-induced retinal inflammatory cytokines, elevated PRC2 expression, and IgG leakage *in vivo*. RT-qPCR analyses of the retinas from animals, following two months of poorly controlled diabetes showed increased expressions of A) *MALAT1*, B, C) inflammatory transcripts (*TNF-α, IL-6*), and D, E, F) PRC2 components

(*EZH2, SUZ12*, and *EED*) in WT-D retinas compared to WT-C retinas. *Malat1* KO prevented such increases in the M1 KO-D group (data expressed as a ratio to β-*actin* (mean ± SEM); normalized to WT-C; *=*P*<0.05, **=*P*<0.01, ***=*P*<0.001, and ****=*P*<0.0001, compared to WT-C or WT-D; *n*= 6/group). H, I, J, K) IgG staining shows elevated IgG leakage in I) WT-D retinas (score 3) and reduced leakage in the K) M1 KO-D retinas (score 1). No changes in IgG leakage were observed between H) WT-C (score 0) and J) M1 KO-C (score= 1) animals (scale bar= 10 μM). WT-C= Wild-type control; WT-D= Wild-type diabetic; M1 KO-C= *Malat1* KO control; and M1 KO-D= *Malat1* KO diabetic.

2.2.4 *Malat1* knockout diminishes vascular leakage in the diabetic retina

Damage to the blood-retinal barrier (BRB) becomes imminent during the severe stages of DR, which can consequently lead to irreparable vision damage [27]. In fact, risk for developing vision-threatening complications heightens when a chronic hyperglycemic environment allows for increased extravasation of large plasma proteins into the neural retina [27, 28]. Therefore, to examine functional alterations in our diabetic animal model, we used IgG staining on retinal tissues (**Figs. 2.2H-K**). Both in WT-C and M1 KO mice, IgG was mostly limited within the capillaries without any significant staining of retinal tissues (scores 0-1; **Figs. 2.2H** and **2.2J**). In contrast, diffuse staining of retinal tissues (score 3) were seen in WT-D animals (**Fig. 2.2I**). Such changes were prevented in M1 KO-D mice (score 1; **Fig. 2.2K**). Taken together, these findings support the theory that *MALAT1* is implicated in advancing BRB breakdown.

2.2.5 *MALAT1* is upregulated and associated with increased inflammatory markers in the vitreous of diabetic patients

Alteration in the VH composition ultimately reflects the retinal environment [29]. In individuals with late stage DR, there are increased concentrations of proinflammatory cytokines and soluble growth factors in the VH that mediate retinal neovascularization

within the eye [30, 31, 32]. Therefore, we decided to examine RNA levels of *MALAT1* and its potential downstream molecules, *IL-6* and *TNF-α* in the diabetic VH. RT-qPCR analyses revealed that *MALAT1* expression was significantly upregulated in the vitreous of PDR patients than that of non-diabetic patients (**Fig. 2.3A**). PDR patients also demonstrated significant upregulations of *TNF-α* and *IL-6* in the VH when compared to the vitreous of non-diabetic patients without retinopathy (**Figs. 2.3B and 2.3C**). In summary, our findings suggest that *MALAT1* upregulation in the diabetic vitreous is associated with a pathogenetic state.

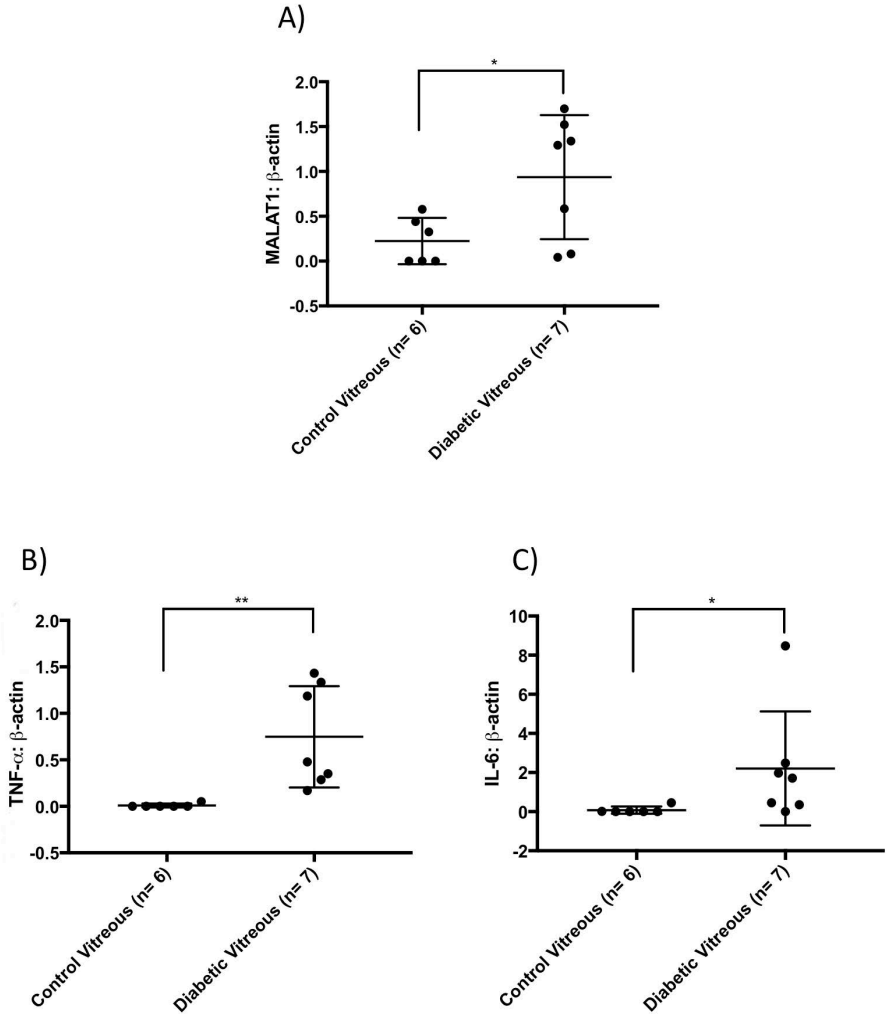

Figure 2.3. *MALAT1* upregulation is associated with increased inflammatory markers in the vitreous of diabetic patients. RT-qPCR analyses of the vitreous humors indicate that diabetic patients have elevated expressions of A) *MALAT1*, B) *TNF-α*, and C) *IL-6* transcripts (data expressed as a ratio to β-*actin* (mean± SD); *=P<0.05 and **=P<0.01, compared to control vitreous).

2.2.6 Histone methylation impacts *MALAT1* and some of its downstream targets

With epigenetic reprogramming by pharmacological intervention receiving critical recognition and showing considerable promise in cancer trials [33, 34], we decided to explore the function of DZNep on HRECs and elucidate the effects of inhibiting histone methyltransferases on *MALAT1* and inflammation in a diabetic environment. Following HG incubation, DZNep-treated HRECs demonstrated significant reductions in the RNA expressions of *EZH2*, *EED*, and *SUZ12*, compared to HG-treated HRECs (**Figs. 2.4D-F**). Accompanying the reduction of PRC2 components, HRECs in HG following DZNep treatment showed significant reductions in *MALAT1* and *TNF-α* RNA expressions when compared to control HG-treated HRECs (**Figs. 2.4A-B**). However, this is in stark contrast to the trends observed for *IL-6, IL-1β*, and *MCP-1*; in which, the RNA expressions of these transcripts were significantly upregulated after DZNep treatment in both NG-and HG-treated HRECs, as compared to control HG-treated HRECs (**Figs. 2.4C**; **Appendix A, S4C and S4D**).

In order to further verify this surprising observation of *IL-6*, *IL-1β*, and *MCP-1* upregulation and to determine whether the same trends observed in the RNA expressions exist at the protein level, we selected IL-6 and TNF-α for subsequent ELISA assays. Following the addition of both DZNep and HG treatments, HRECs demonstrated a significant induction of IL-6 compared to HG-treated HRECs (**Fig. 2.4G**), suggesting a positive correlation with the RNA expressions observed in **Figure 2.4C**. On the other hand, TNF-α protein levels were significantly decreased in the presence of DZNep + HG, as compared to HG-treated HRECs, which is in parallel to the trends observed with *TNF-α* RNA levels (**Fig. 2.4H**). Our DZNep findings demonstrate that PRC2 activity may have an important role in impacting *MALAT1*, TNF-α, IL-6, IL-1β, and MCP-1 expressions. However, of note, the effect of PRC2 depletion on the expression of IL-6, IL-1β and MCP-1 may also result from an indirect effect of decreased TNF-α levels, which needs further exploration.

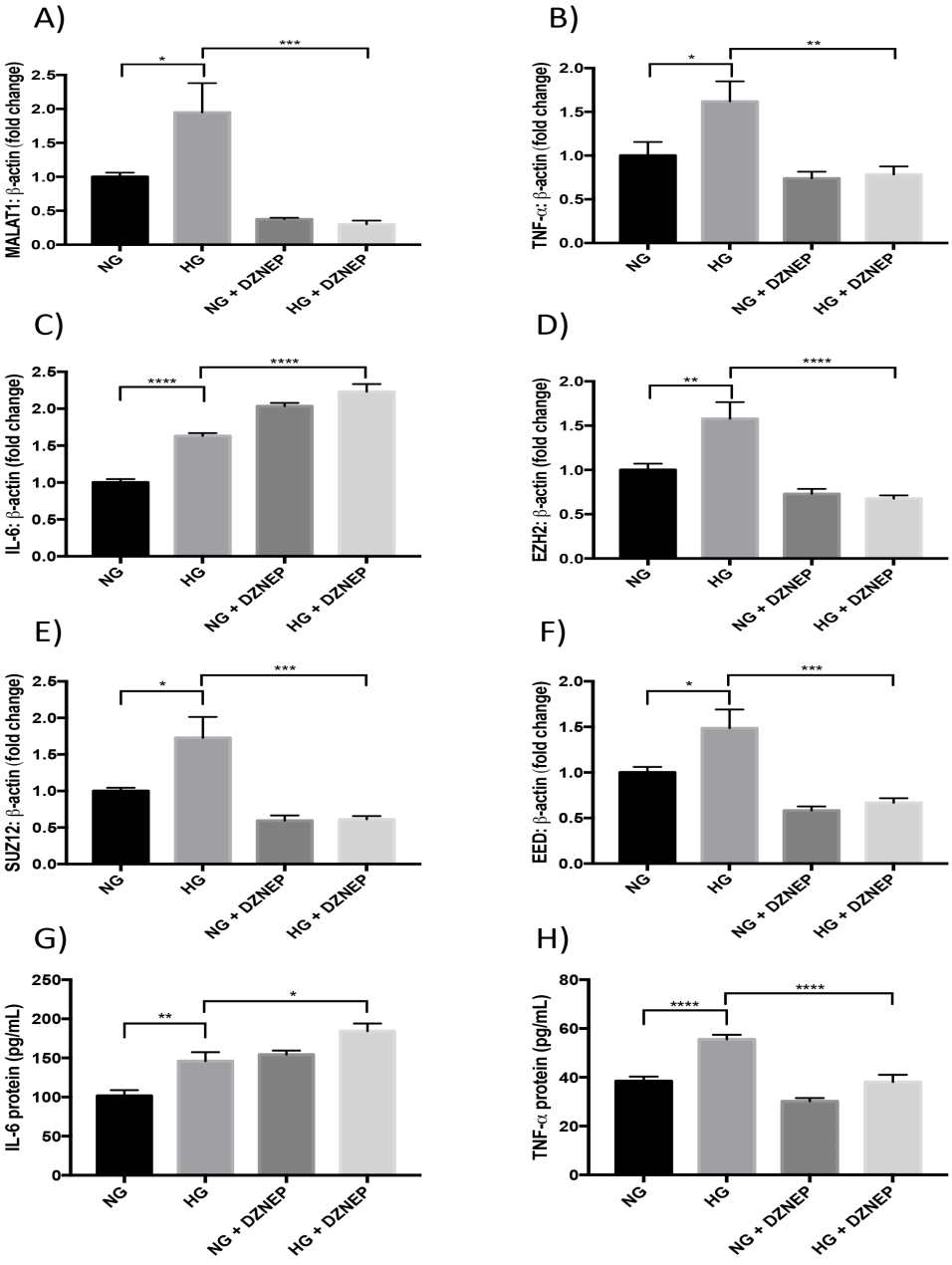

Figure 2.4. Global methylation inhibitor (DZNep) prevents glucose-induced upregulation of *MALAT1*, *TNF-α*, and PRC2 expressions. RT-qPCR findings indicate HG-induced elevations of A) *MALAT1*, B, C) inflammatory transcripts (*TNF-α, IL-6*), and D, E, F) PRC2 components (*EZH2, SUZ12*, and *EED*) compared to NG at 48 hours. Such upregulations were prevented (except *IL-6*) following DZNep treatment. Protein levels of G) IL-6 and H) TNF-α showed similar patterns (RNA data expressed as a ratio to β-*actin* (mean ± SEM); normalized to NG; *=P<0.05, **=P<0.01, ***=P<0.001, and ****=P<0.0001, compared to NG or HG; n= 6 from three independent experiments and performed in triplicates; and protein data are expressed as pg/mL).

2.2.7 Transient HG treatment does not alter methylation status of the CpG island in *MALAT1* promoter

To address whether differential methylation patterns exist in the *MALAT1* promoter in hyperglycemia, we cultured HRECs in NG and HG conditions and performed a genome-wide methylation analysis. Following detection of genome-wide methylation (over 830,000 methylation sites), we filtered to specifically examine the sites that spanned across the *MALAT1* gene, which amounted to 21 probes (**Data File S1,** doi: 10.1038/s41598-018-24907-w). We found that the average methylation intensity was generally lower across the 'Shore', 'Island', and 'Shelf" regions in both NG and HG-treated HRECs (β-values<0.2) compared to the 'Open Sea' region, **(**β-values= ~0.49, **Fig. 2.5C**). Furthermore, in both NG and HG conditions, HRECs demonstrated the lowest degree of methylation in the CpG islands compared to the other regions (β-values<0.074, **Fig. 2.5C**). Although eight probe sites indicated a slight reduction in methylation after HG treatment (**Fig. 2.5B**), no comparable differences in methylation were demonstrated overall between HRECs in NG and HG conditions (**Fig. 2.5C**).

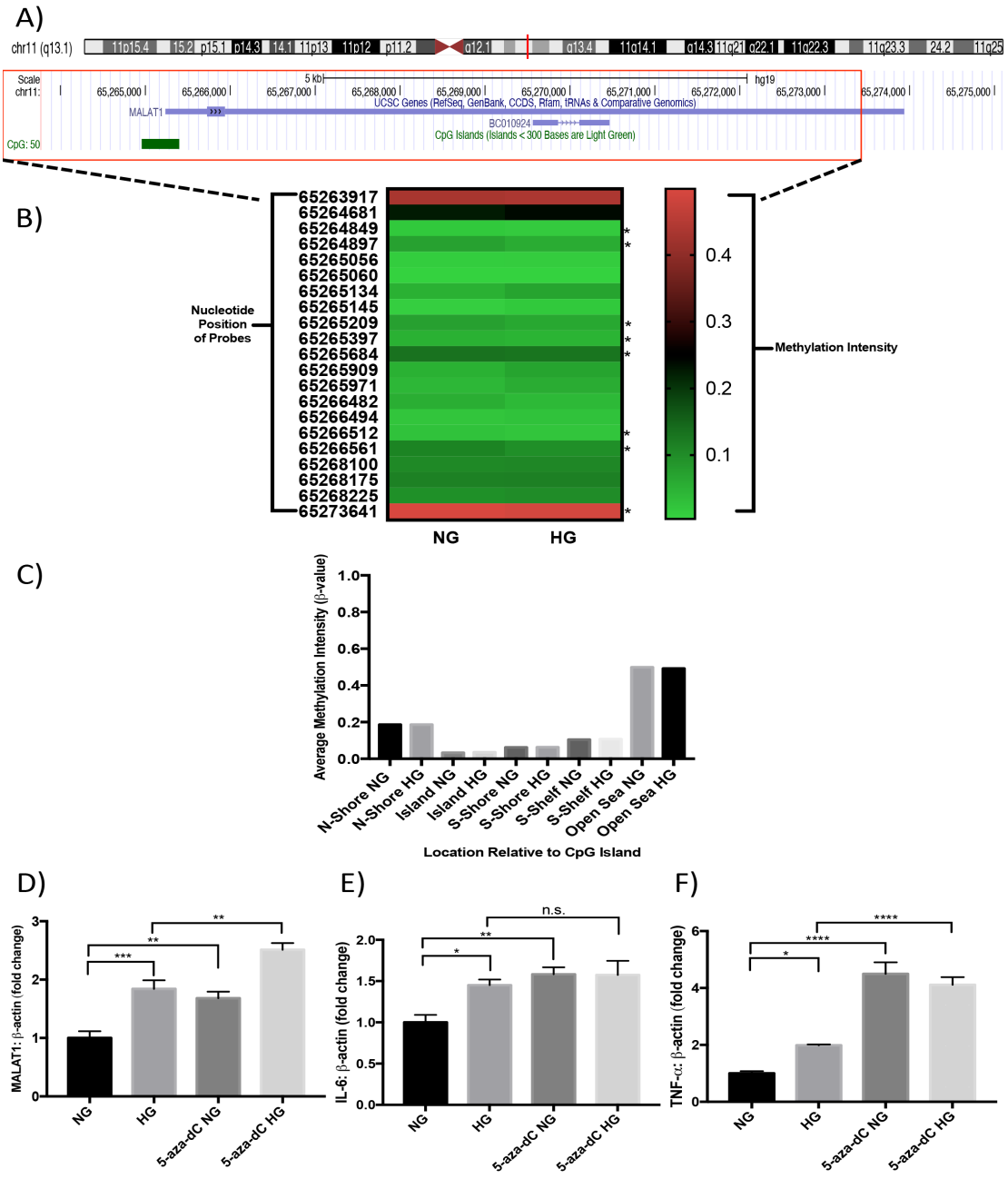

Figure 2.5. Transient HG treatment does not alter methylation status of CpG island in *MALAT1* promoter; however, DNA methylation inhibition augments glucose-induced upregulations of MALAT1, TNF-α, and IL-6 expressions. A) Information on the human *MALAT1* gene according to the UCSC database. The *MALAT1* gene is 8,707 nucleotides in length (nucleotide positions in chromosome 11.q13.1: 65,265,233 to 65,273,939) and contains a CpG island in its promoter region that is located from positions 65,264,958 to 65,265,398 (441 nucleotides in size)[63]. B) An intensity map depicting the β-values of CpG sites across the *MALAT1* gene, generated by the Illumina methylation array. A β-value of 0 indicates no

methylation, while a value of 1 indicates complete methylation at the interrogated site (the '*' demonstrates a reduction in methylation following HG treatment). C) A bar graph illustrating the *β*-values separated by regions relative to the CpG island in the *MALAT1* promoter. These regions are defined as 'Island' (an area of at least 500 base-pairs that contains an observed-to-expected CpG ratio greater than 60%), 'Shore' (areas 2 kilo-bases on either side of an island), 'Shelf' (areas 2 kilo-bases on either side of a shore), or 'Open Sea' (areas outside of the shelf). No significant differences were observed in methylation between NG and HG conditions in these regions (data expressed as average *β*-value per region; *n*=3 independent samples for each NG or HG group). Glucose-induced elevations of D) MALAT1, E) IL-6, and F) TNF-α transcripts were further increased following 5-aza-dC treatment (data expressed as a ratio to β-*actin* (mean ± SEM); normalized to NG; *=*P*<0.05, **=*P*<0.01, ***=*P*<0.001, ****=*P*<0.0001, and n.s.= not significant, compared to NG or HG; *n*= 6 from three independent experiments and performed in triplicates).

2.2.8 Inhibition of DNA methyltransferases (DNMTs) increases expression of *MALAT1* and inflammatory cytokines

We then examined the effects of inhibiting genome-wide DNA methylation on *MALAT1, TNF-α, IL-6, IL-1β*, and *MCP-1* RNA expressions. To produce such an environment *in vitro*, we administered the pan-DNMT inhibitors 5-aza-dC or zebularine to HRECs prior to glucose treatment and analyzed the effects of methylation loss on RNA expressions using RT-qPCR. Following these treatments, we confirmed the reductions of *DNMT1*, *DNMT3A*, and *DNMT3B* (**Appendix A, Figs. S6A-C** and **S6F-H**). Interestingly, inhibiting the activity of DNMTs in NG and HG conditions evoked further increases in *MALAT1, IL-6, TNF-α, IL-1β*, and *MCP-1* RNA expressions when compared to control NG and HG-treated HRECs (**Figs. 2.5D-F**; **Appendix A, S6D-E** and **S6I-M**). Among the markers analyzed, *TNF-α* and *IL-1β* demonstrated the greatest upregulations in 5-aza-dC or zebularine-treated HRECs (**Fig. 2.5F** and **Appendix A, S4E**). The findings implicate that although transient HG treatment on HRECs may not generate considerable DNA methylation alterations

across the *MALAT1* gene region, globally inhibiting the activity of DNMTs impacts the expression of *MALAT1* and inflammatory transcripts. Therefore, we speculate that DNMTs may have a potential role in regulating *MALAT1* and inflammatory RNA expressions, which needs further characterization.

To confirm the findings from our pan-DNMT inhibitors, we selected *DNMT1*, a constitutively expressed DNMT, for subsequent siRNA-mediated knockdown (**Appendix A, Fig. S7A**). After silencing *DNMT1*, we observed overall increases in *MALAT1, TNF-α, IL-6, IL-1β* and *MCP-1* RNA expressions in both NG and HG-treated HRECs (**Appendix A, Figs. S7B-F**).

Based on our findings, we propose a diagram for *MALAT1* in potentially regulating inflammation through independent and dependent pathways in DR (summarized in **Fig. 2.6**). In the independent pathways, DNA methylation may be capable of regulating the transcriptional status of *MALAT1* and if the actions of DNMTs are hindered, *MALAT1* RNA expressions could increase. Following upregulation, *MALAT1* may recruit PRC2 to the promoters of anti-inflammatory genes and epigenetically repress these targets, which might subsequently allow for heightened transcription of inflammatory genes. On the contrary, in the dependent pathway, the *MALAT1* transcript and *MALAT1* gene may directly interact with inflammatory transcripts and inflammatory genes to ultimately provoke a greater inflammatory response.

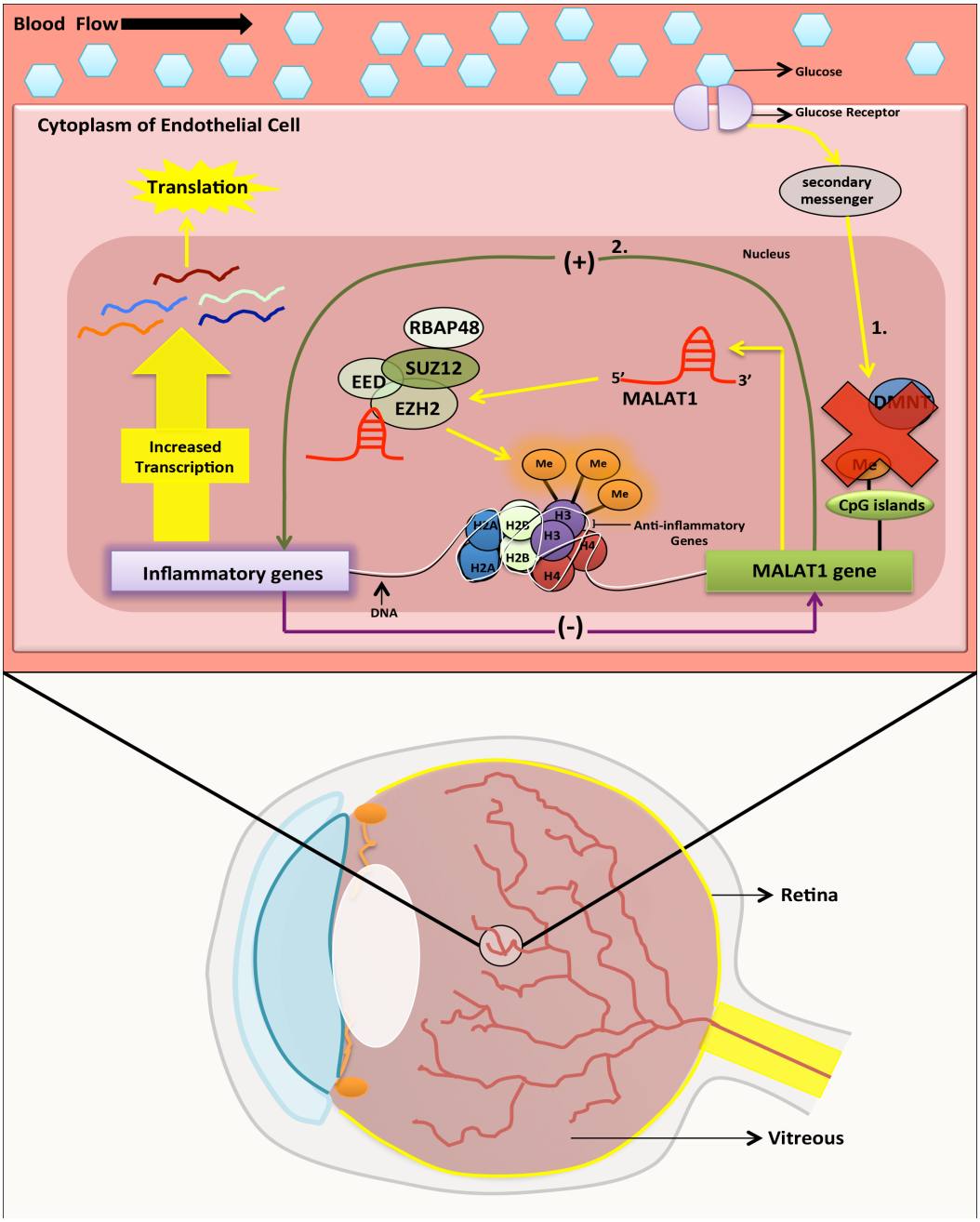

Figure 2.6. A schematic depicting the potential involvement of *MALAT1* in regulating inflammation through epigenetic mechanisms in diabetic retinopathy. Based on our findings, we present a diagram for *MALAT1* in potentially regulating inflammation by independent and dependent pathways in DR. In the independent pathways, indicated by '**1.**', DNA methylation regulates the transcriptional status of *MALAT1* and if the actions of DNA methyltransferases are hindered, *MALAT1* RNA expressions will increase. Following upregulation, *MALAT1* can recruit PRC2 to the promoters of anti-inflammatory genes and epigenetically repress these targets, which will subsequently allow for heightened transcription

of inflammatory genes. On the contrary, in the dependent pathway (indicated by '**2.**'), the *MALAT1* transcript and *MALAT1* gene may directly interact with inflammatory transcripts and inflammatory genes, respectively, to ultimately provoke a greater inflammatory response (Me= methylation; EZH2 + SUZ12 + EED + RBAP48= PRC2 complex components; (+) and (-)= feedback loop; 1. = Indirect effect of *MALAT1* on inflammation via DNA methylation and PRC2 pathways; and 2. = Direct effect of *MALAT1* gene activity on inflammation and vice versa). Reproduced from *MALAT1: An Epigenetic Regulator of Inflammation in Diabetic Retinopathy* (p. 9) by S. Biswas, A. Thomas, S. Chen, E. Aref-Eshghi, B. Feng, J. Gonder, B. Sadikovic, and S. Chakrabarti, 2018, Scientific Reports.

2.3 Discussion

When metabolic memory was first described [3, 4], it became clear that understanding the processes implicated in this phenomenon are of utmost importance. Epigenetic modifications, which can alter the expression of genes without altering the underlying DNA sequence, are critical players in metabolic memory [35] and characterizing these modifications will help us learn about the intricacies of epigenetic regulation in diseases. Recently, the advancements in experimental genome-wide approaches have allowed for the identification of lncRNAs, which play various roles in cellular physiology and are heavily involved in epigenetic regulation [35, 36]. Further insight into the functions of lncRNAs and their association with other epigenetic mechanisms in diseases will help with the development of better-targeted therapeutics. Here, not only do we show for the first time that the lncRNA *MALAT1* is present in the VH of diabetic patients, but we present through a series of well-designed *in vitro* and *in vivo* experiments, a novel epigenetic paradigm for *MALAT1* in the pathogenesis of DR.

MALAT1 was originally discovered in patients with non-small cell lung carcinoma and shortly after its discovery, *MALAT1* has also been reported in various pathologies such as heart disease and diabetes [10-13, 25, 26, 37]. Located on human Chr.11q13.1, the *MALAT1* gene produces a well-conserved non-coding transcript that is ~8000+ nucleotides in length and is further processed by RNAses P and Z, which ultimately results in the production of two transcripts: a mature *MALAT1* transcript and a ~61-

nucleotide *MALAT1*-associated small cytoplasmic RNA (mascRNA) [11, 22]. The mature *MALAT1* transcript is primarily localized to the nucleus, while the mascRNA is exported to the cytoplasm [22, 23, 24, 38].

The stability of the mature *MALAT1* transcript differs across cell-types, where *MALAT1* has been reported to have a half-life of ~9 hours in human cervical cells (HeLa Tet-off) [39], 16.5 hours in human B cells [40], and 3 hours in murine NIH 3T3 cells [40]. Of note, we have previously reported in HUVECs that *MALAT1* expression levels increase significantly at 12 hours [12]; whereas, other reports have indicated that *MALAT1* expression is highest at the 48-hour mark in RF/6A, primary retinal ganglion, and Müller cells [10, 26]. Our current results indicate that *MALAT1* expression peaks at the 48-hour mark following HG treatment in HRECs, which is in accordance with the findings from Liu et al. and Yao et al. [10, 26]. It is likely that *MALAT1* transcripts may exhibit similar half-life patterns among specific retinal cells, which may explain the variation in *MALAT1* expression between HUVECs and HRECs. As well, early increases of *MALAT1* by HG may regulate specific pre-mRNA splicing patterns and potentially activate a degradation mechanism that adjusts the *MALAT1* expression at later time points [64]. Nevertheless, it is important to note that although the initial increase of *MALAT1* at 48 hours may appear as a temporary event, *MALAT1* may be capable of activating persistent epigenetic changes involving its downstream targets—despite reductions in *MALAT1* expression later in the disease course. This phenomenon is best explained by the concept of metabolic memory, which has been previously demonstrated with epigenetic changes occurring in the NF-κB promoter following transient hyperglycemia [65].

Knocking down lncRNAs using siRNA-mediated approaches has allowed for critical discussion on the efficiency and specificity of these particular knockdowns. Despite the notion that siRNAs operate mainly in the cytoplasm, several studies have demonstrated that siRNA-programmed RNA-induced silencing complexes (RISCs) exist in the nucleus, where siRNAs can still be used to target nuclear lncRNAs [41, 42, 43]. In fact, previous studies have demonstrated efficient *MALAT1* knockdown using siRNA-mediated transfections [13-19, 25, 26, 38]. As indicated by our prior work, the

selective targeting of *MALAT1* via siRNAs resulted in a subsequent reduction of IL-6 and TNF-α mRNA and protein levels in HUVECs [12], which also resembles similar patterns observed in this study. A reduction in *MALAT1* with concomitant decreases in inflammatory transcripts suggests that *MALAT1* promotes an inflammatory phenotype in DR. Furthermore, in the context of PRC2, we recently demonstrated that knocking down lncRNA *ANRIL* downregulates the RNA expressions of PRC2 components in HG-treated HRECs [44]. Since we observed similar trends in PRC2 expressions after siMALAT1 treatment, our findings collectively support the notion that certain lncRNAs, such as *ANRIL* and *MALAT1*, may act as scaffolds to chromatin-associated complexes in order to further modulate the expression of genes in DR [45].

Under normal physiological conditions, the *Malat1* KO mice do not display a noticeable phenotype [22, 23, 24]. However, whether *Malat1* KO mice will reveal a disease-specific phenotype, when subjected to certain pathological conditions, remains of great interest. With respect to our *in vivo* diabetic animal model, our initial findings of *MALAT1* upregulation in the WT-D mice retinas at 2 months are consistent with previous demonstrations of increased *MALAT1* expression in the retinas of diabetic rats [25, 26]. However, of note, the relative *MALAT1* expressions documented from these studies were upregulated up to >3-fold at the aforementioned time points [25, 26]; whereas in our study, the relative *MALAT1* RNA expression significantly increased to ~1.46-fold in the WT-D retinas at 2 months. We believe the differences observed in *MALAT1* expressions may be accounted for potential species-specific or other unidentified variations [46, 47]. Since we also observed statistically significant reductions in the expressions of *IL-6, TNF-α, IL-1β, MCP-1, EZH2, SUZ12*, and *EED* in the diabetic *Malat1* KO retinas, we infer that *MALAT1* may be functionally capable of controlling the expressions of PRC2 components, through which it may regulate inflammation in DR. To further elaborate on the potential transcriptional capabilities of *MALAT1*, intergenic lncRNA transcripts have been previously shown to modulate the expression of protein-coding genes in *cis* by reducing the recruitment of transcription factors in the promoter regions of protein-coding genes and subsequently activating histone modification cascades to silence gene transcription- also known as transcriptional interference [48, 49, 50]. It is also likely possible that prolonged

diabetic conditions can enable *MALAT1* to act in *trans* by directly impacting RNA polymerase II activity, which could impact the transcription of nearby anti-inflammatory genes [51]. Moreover, confirming the immunohistochemistry findings from our *in vivo* animal model, previous work by Michalik et al. demonstrated that *MALAT1* is capable of regulating and enhancing angiogenesis in the neonatal mice retina, suggesting a proangiogenic role for *MALAT1* during retinal neovascularization [13]. Although the main focus of our manuscript has been on *MALAT1* and its epigenetic role in inflammation, future experiments should elucidate the underlying epigenetic mechanisms implicated in the complex crosstalk between inflammation and angiogenesis—as both of these processes converge and synergistically influence the progression of DR [68].

The close proximity of the VH to the lens and retina make it an ideal location to administer therapeutic agents to target ocular pathologies [29]. Yao et al. have previously indicated that *MALAT1* expression levels were significantly downregulated in the aqueous humor of patients with primary open-angle glaucoma [26]. However, in patients with Alzheimer's Disease or glioma, the cerebrospinal fluid and the tumor itself, respectively, showed *MALAT1* upregulation [26]. In contrast, Yan and colleagues have reported increased *MALAT1* expression in the fibrovascular membranes and aqueous humors of diabetic patients [10]. Previous studies have also shown that *MALAT1* upregulation contributes to epiretinal membrane formation in patients with proliferative vitreoretinopathy [52, 53]. In keeping with previous data, intraocular administration of *MALAT1* shRNA alleviates vascular leakage and dampens the inflammatory response in the diabetic rat retina through reduced protein expressions of TNF-α, intracellular adhesion molecule-1 (ICAM-1), and vascular endothelial growth factor (VEGF) [25]. To our knowledge, this is the first study to directly identify *MALAT1* RNA from the VH of PDR patients. Although much is unknown about the specific mechanisms of lncRNAs in diabetic complications, our novel finding suggests the possibility of therapeutically targeting *MALAT1* in PDR patients. Taken together, a prolonged diabetic environment can ultimately allow for increased *MALAT1* expression at various locations in the eye, which may associate with increased inflammation and subsequent cellular damage [10, 13, 25, 32].

Although interactions between lncRNAs and chromatin modification complexes have been studied in cancers [66], such studies have not been undertaken in the context of DR. *MALAT1* has been shown to directly bind to EZH2 in renal cell carcinoma [14], mantle cell lymphoma [17], prostate cancer [15], osteosarcoma [16], and gastric cancer [54], suggesting an important regulatory relationship between the two components. Of particular interest, Wang et al. revealed that *MALAT1* could facilitate the targeting capabilities of EZH2 and further augment histone 3 lysine 27 trimethylation (H3K27me3) levels at the target gene loci of EZH2 in prostate cancer cell lines [15]. These findings allude to the many pathogenetic capabilities of *MALAT1*. Moreover, our RIP findings demonstrate heightened *MALAT1* binding with EZH2 in HG-treated HRECs and we have also previously shown similar binding of EZH2 to *ANRIL* in HRECs cultured with HG [44]—suggesting a potential overlap in functional mechanism. Therefore, despite observing the expected reductions in *MALAT1*, TNF-α, EZH2, EED, and SUZ12 expression levels after DZNep treatment, it was surprising to see an increase in IL-6, IL-1β, and MCP-1 expressions in HRECs following DZNep treatment. These data are in keeping with Serresi and colleagues, who indicated previously that Polycomb-mediated repression exists on the *IL-6* gene in NSCLC by H3K27me3 [55]. Furthermore, a similar observation by Lee et al. demonstrated that the depletion of EZH2 or estrogen receptor (ER) in ER-positive breast cancer cell lines (T47D and MCF7) produced a significant upregulation of IL-6 and IL-8 expressions at basal levels [56]. In ER-negative breast cancer, EZH2 acts as a coactivator for RelA and RelB and this in turn activates a positive feedback loop through NF-κB signalling pathways to enhance the expressions of NF-κB targeted genes, such as *IL-6* [56]. Lee and colleagues concluded that EZH2 is capable of context-specific regulation on the expression of NF-κB target genes [56]. These findings are supportive of our data. However, since DZNep inhibits global histone methylation and is not completely selective [57], further research is warranted on elucidating the cellular-specific mechanisms of DZNep treatment in diabetic complications.

Although we did not observe significant methylation changes in CpG sites across the

MALAT1 gene after HG treatment, we did notice increased expressions of *MALAT1*, *IL-6, TNF-α, IL-1β*, and *MCP-1* after 5-aza-dC or zebularine treatments in both basal and HG conditions. Consistent with the findings from our methylation array, Hu et al. reported no effects of CpG island methylation status on *MALAT1* expression in esophageal squamous cell carcinoma (ESCC) cells [19]. On the other hand, Guo et al. analyzed the CpG island methylation patterns in the *MALAT1* promoter of lung cancer cells (A549) and observed a reduction of methylated sites in lung cancer cells [18]. They also demonstrated that a methyl donor could reduce *MALAT1* expression in these cells—implying that DNA methylation can regulate *MALAT1* expression [18]. Taken together, the diverse findings documented from previous DNA methylation reports, and from our study, imply that disease-and cell-specific responses may exist after inhibiting the activity of DNMTs. Despite our observations from this pharmacological experiment, it still remains to be determined whether the inhibition of genome-wide DNA methylation alters neighbouring (or distal) genes, which in turn may substantially impact the transcriptional activity of *MALAT1*. Future studies should include targeted genome editing techniques such as CRISPR-Cas9, to eliminate any potential off-targeting effects observed from our 5-aza-dC, zebularine, or siDNMT1 experiments.

Overall, our findings collectively demonstrate that *MALAT1* is capable of impacting the expressions of inflammatory transcripts through its association with epigenetic mediators, such as histone and DNMTs. It is important to note that the findings described in this manuscript are simply a starting point for future investigations to build and develop a more definitive model for lncRNAs in DR. Establishing such an all-encompassing model, which includes the interplay of miRNAs and additional epigenetic modifications, will enable the development of better-targeted treatment strategies.

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2.5 Appendix A: *MALAT1*: An Epigenetic Regulator of Inflammation in Diabetic Retinopathy

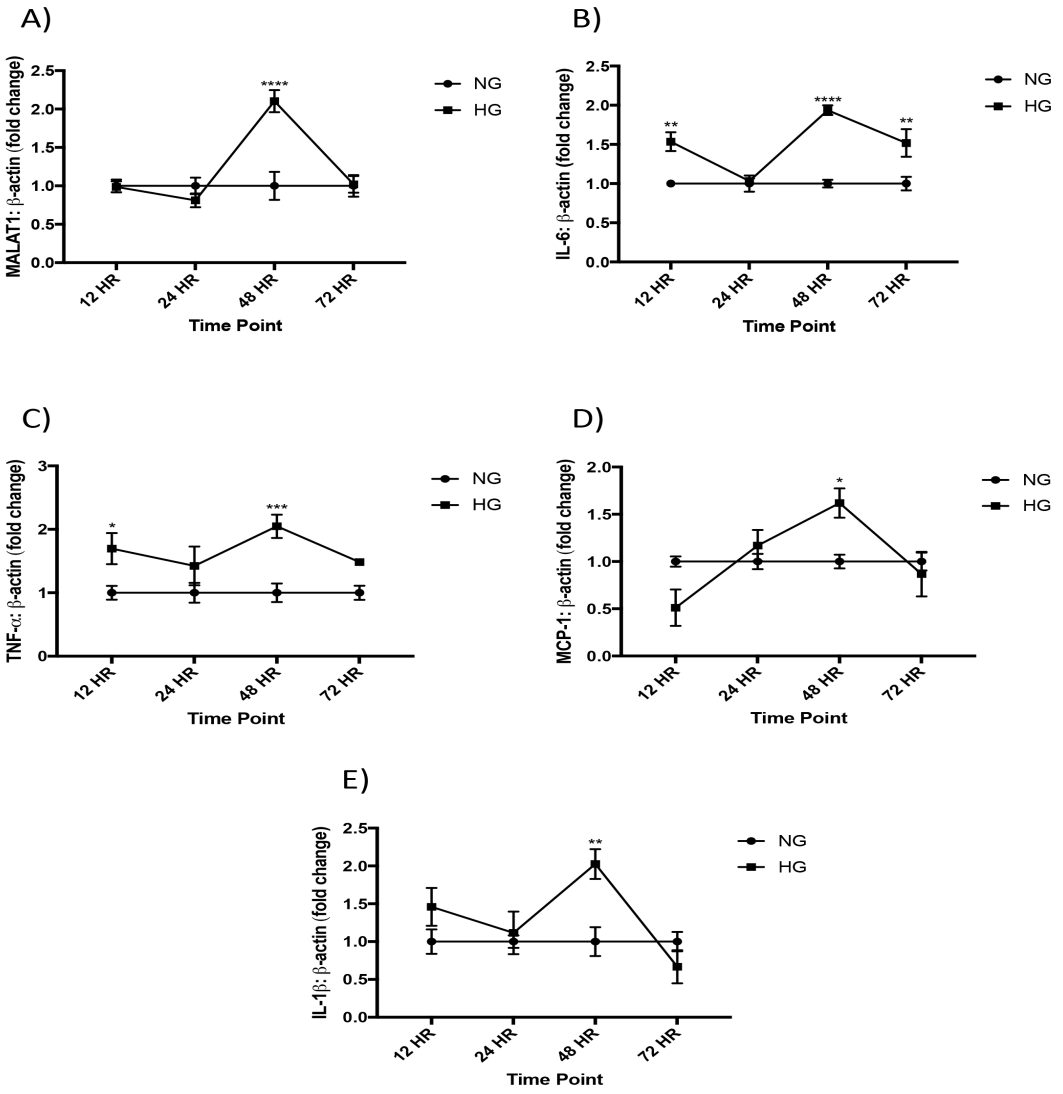

Figure S1: *MALAT1* and inflammatory transcripts are upregulated in HRECs following high glucose treatment. RT-qPCR analyses of A) *MALAT1*, B) *IL-6*, C) *TNF-α*, D) *MCP-1* and E) *IL-1β* expressions in HRECs exposed to 25mM (HG) or 5mM (NG) glucose over a time course of 72 hours. *MALAT1, IL-6, TNF-α, MCP-1* and *IL-1β* are upregulated in HG-treated HRECs at 48 hours compared to NG (*=P<0.05, **=P<0.01, ***=P<0.001, and ****=P<0.0001 compared to NG; data expressed as mean ± SEM; n=6/group; normalized to β-actin, and data represented as a fold change of NG).

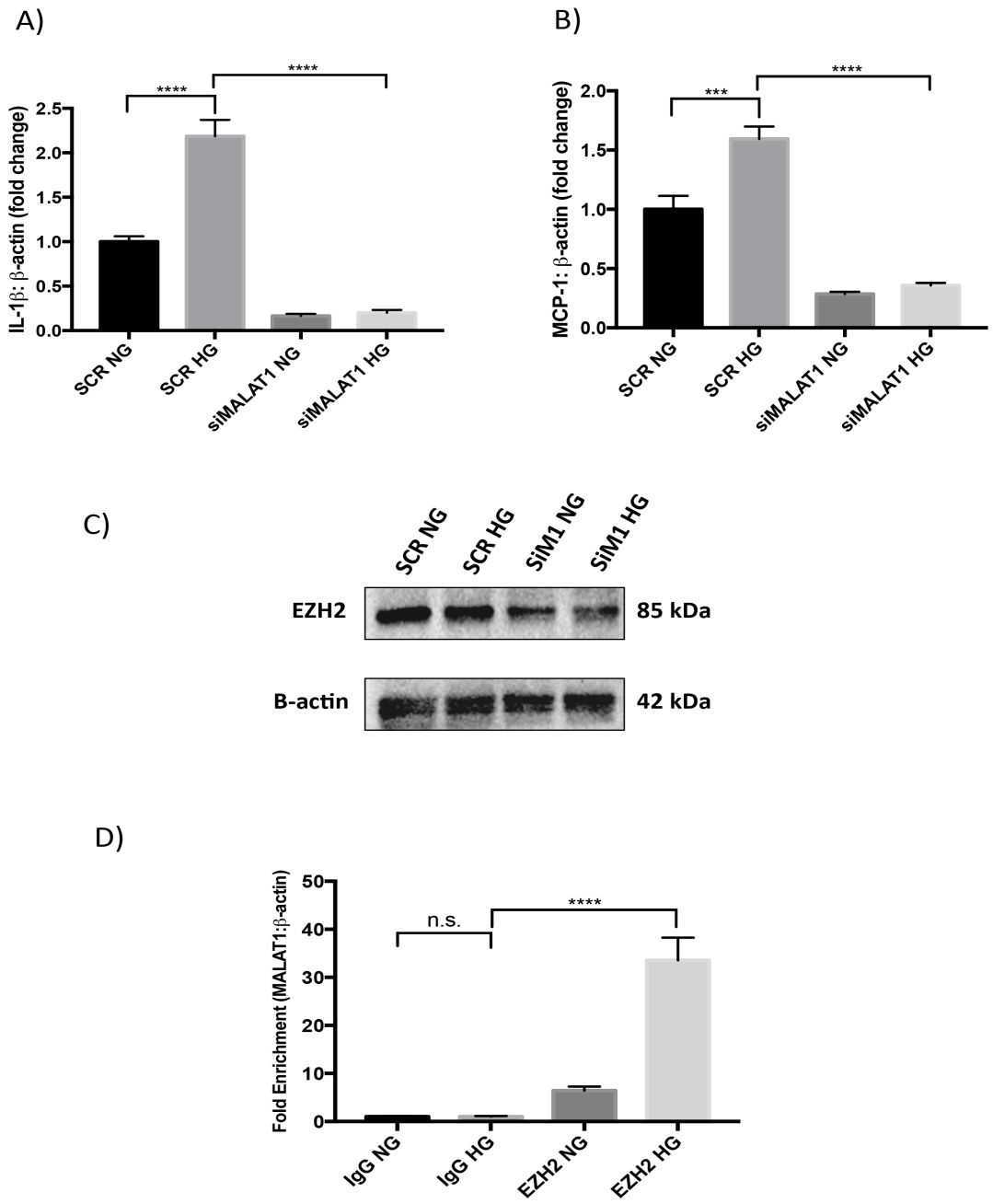

Figure S2: MALAT1 influences MCP-1, IL-1β, and EZH2 expressions and strongly binds with EZH2 in HG-treated HRECs. RT-qPCR analyses indicating reduced RNA expressions of A) IL-1β and B) MCP-1 following siMALAT1 treatment in HG-treated HRECs (data expressed as a ratio to β-*actin* (mean ± SEM); normalized to SCR NG; *=P<0.05, **=P<0.01, ***=P<0.001, and ****=P<0.0001, compared to SCR NG or SCR HG; n= 6 from three independent experiments and performed in triplicates). C) Representative Western blot image showing EZH2 expressions, with β-actin as a control, in NG or HG-treated HRECs transfected with scrambled controls or siMALAT1. Western blots presented (in C) were cropped from the

same membrane and were cropped to improve clarity. The bands within the range of the molecular marker were retained in this figure and the full-length blots are presented in **Supplementary Figure S5**. D) RNA immunoprecipitation, using an IgG control or EZH2 antibody, demonstrating elevated MALAT1 binding to EZH2 following HG treatment in HRECs. MALAT1 expression was determined by RT-qPCR (****P<0.0001 and n.s.= not significant compared to IgG controls; data expressed as mean ± SEM; n=3/group, and results are from one experiment that is representative of three independent experiments). SCR= scrambled siRNA; NG= 5 mM D-glucose; HG= 25 mM D-glucose; SiM1= siMALAT1 treatment.

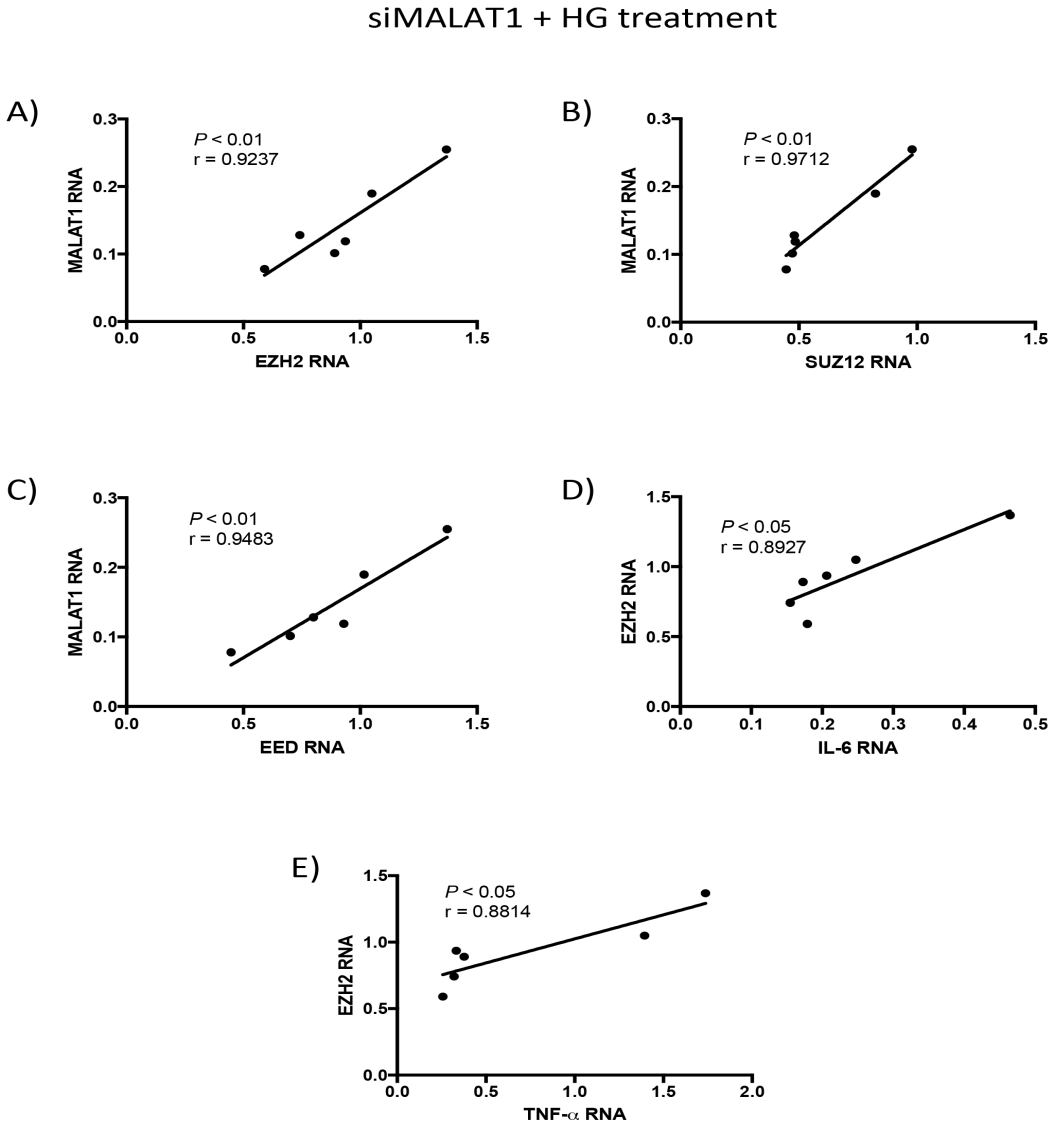

Figure S3: Positive correlations between PRC2 components and *MALAT1* expression, and between *EZH2* and inflammatory transcripts after *MALAT1* knockdown in HG-treated

HRECs. Pearson correlations between RNA expressions of A-C) *MALAT1* and the PRC2 components, and between D-E) *EZH2* and the inflammatory cytokines in HRECs following siMALAT1+HG treatments. RNA expressions were normalized to β-actin and *n*=6 in the HG+siMALAT group.

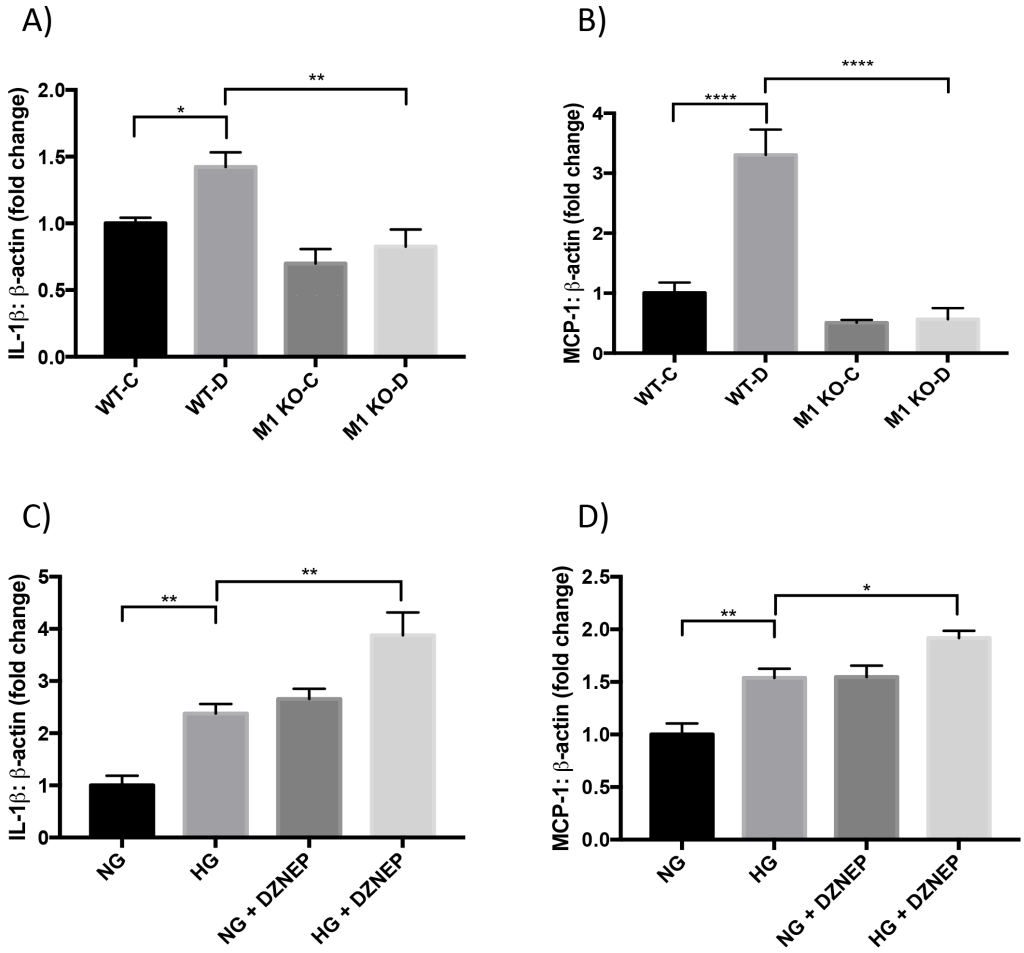

Figure S4: IL-1β and MCP-1 transcript expressions are impacted by *Malat1* knockout and histone methyltransferases. RT-qPCR analyses of the retinas from animals, following two months of poorly controlled diabetes showed increased expressions of A) IL-1β and B) MCP-1 inflammatory transcripts in WT-D retinas compared to WT-C retinas. *Malat1* KO prevented such increases in the M1 KO-D group (data expressed as a ratio to β-*actin* (mean ± SEM); normalized to WT-C; *=*P*<0.05, **=*P*<0.01, ***=*P*<0.001, and ****=*P*<0.0001, compared to WT-C or WT-D; *n*= 6/group). Following DZNep and HG treatments, RT-qPCR findings indicate elevations of C) IL-1β and D) MCP-1 RNA expressions compared to NG at 48 hours

(data expressed as a ratio to β-*actin* (mean ± SEM); normalized to NG; *=*P*<0.05 and **=*P*<0.01 compared to NG or HG; *n*= 6 from three independent experiments and performed in triplicates). WT-C= Wild-type control; WT-D= Wild-type diabetic; M1 KO-C= *Malat1* KO control; and M1 KO-D= *Malat1* KO diabetic.

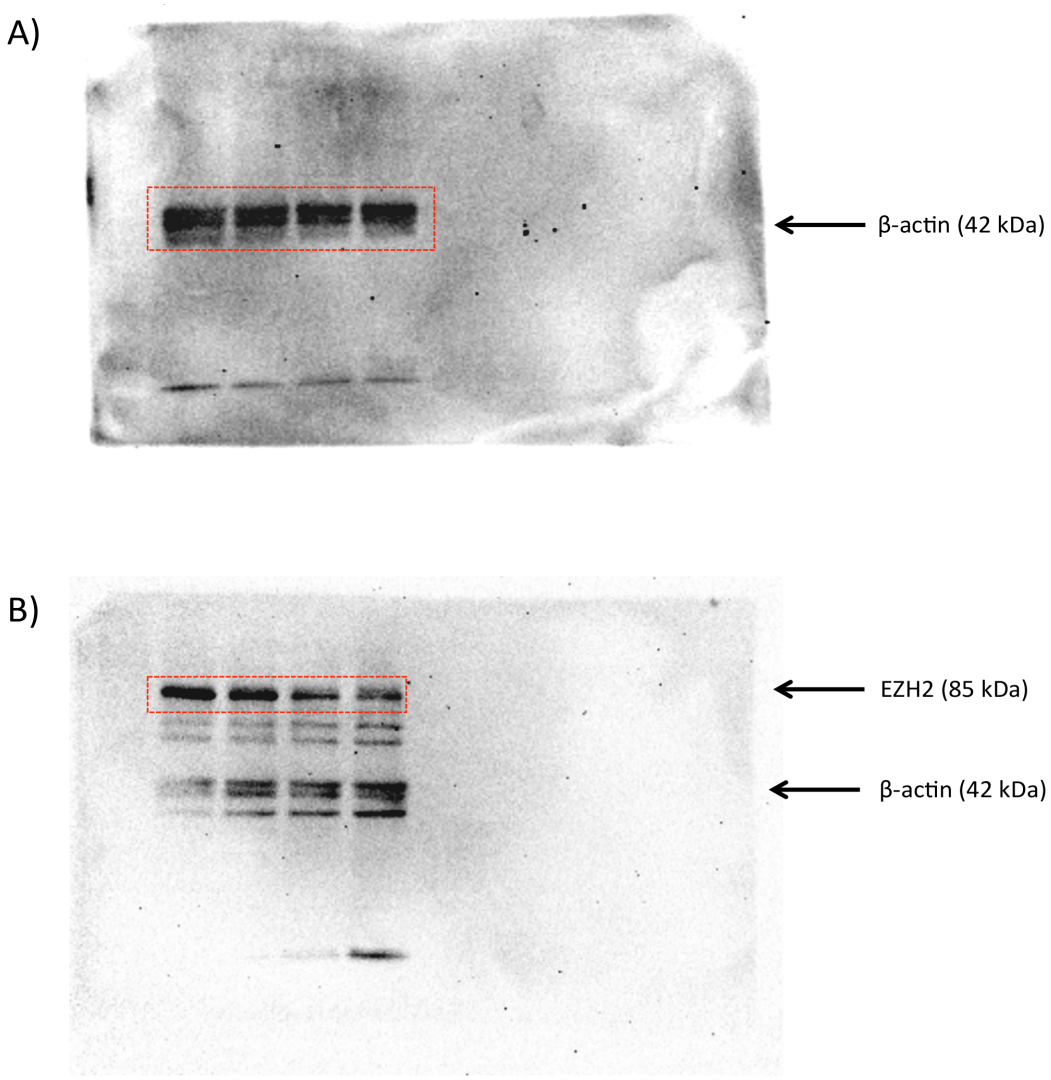

Figure S5: Full length blots of Figure S2C. Red dotted lines indicating the cropping locations. A) β-actin was detected first prior to measuring EZH2 expressions, which were detected the following day. B) Blot reveals EZH2 expressions, as well as residual β-actin expressions from the first imaging run.

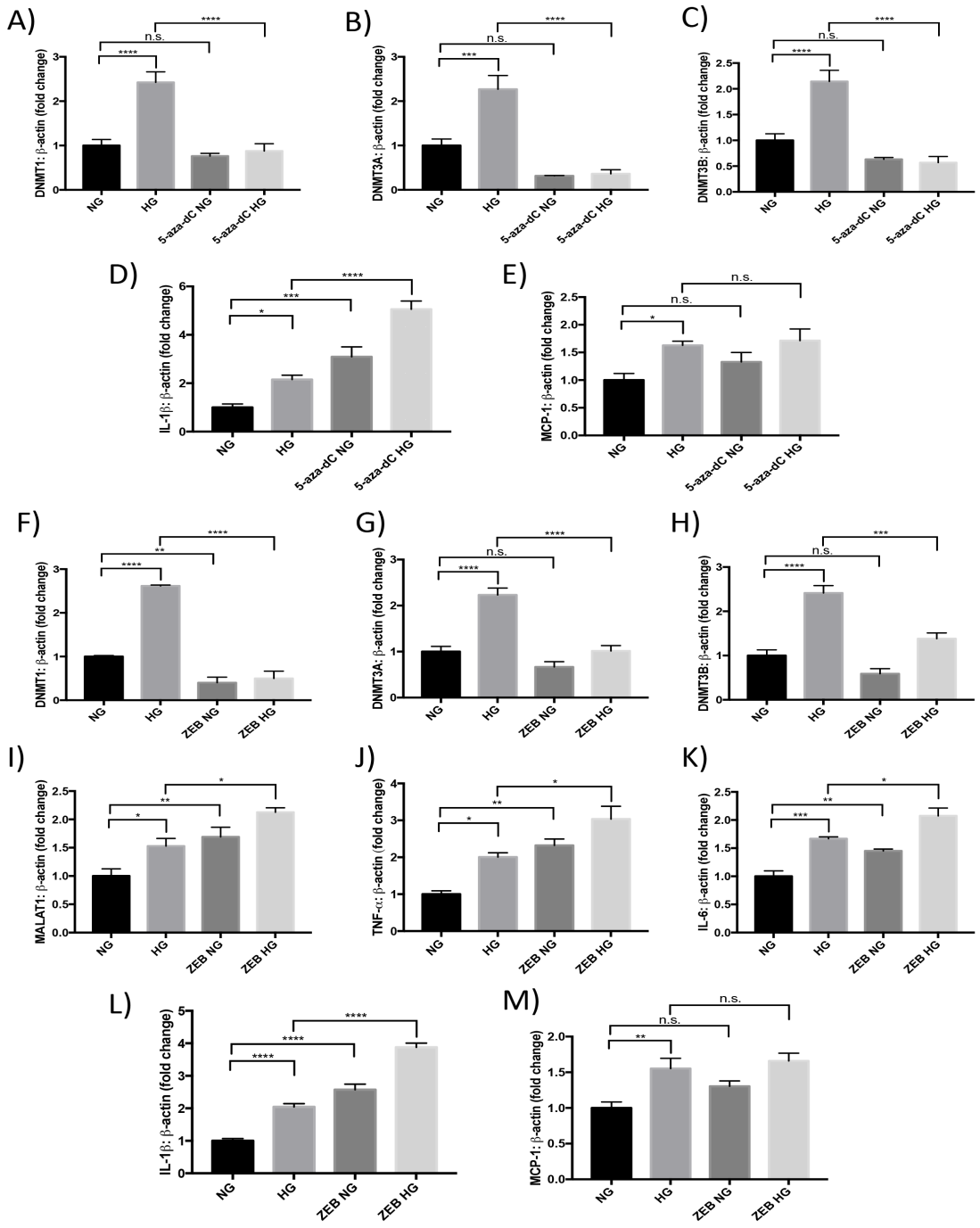

Figure S6: Global inhibition of DNMTs significantly elevates the RNA expressions of MALAT1, TNF-α, IL-6, MCP-1 and IL-1β. HRECs pre-treated with 5-aza-dC demonstrated overall reductions in A) DNMT1, B) DNMT3A, and C) DNMT3B transcripts. While, glucose-induced elevations of D) IL-1β and E) MCP-1 transcripts were further increased following 5-aza-dC treatment. HRECs were also incubated with zebularine (another pan-DNMT inhibitor) and demonstrated similar trends. Reductions in F) DNMT1, G) DNMT3A, and H) DNMT3B transcripts were observed; whereas, I) MALAT1, J) TNF-α, and K) IL-6, L) IL-1β, and M)

MCP-1 RNA expressions increased following zebularine treatment (*=P<0.05, **=P<0.01, ***=P<0.001, ****=P<0.0001, and n.s.= not significant compared to NG or HG controls; data expressed as mean ± SEM; n=6/group; normalized to β-actin, and data represented as a fold change of NG). ZEB= Zebularine.

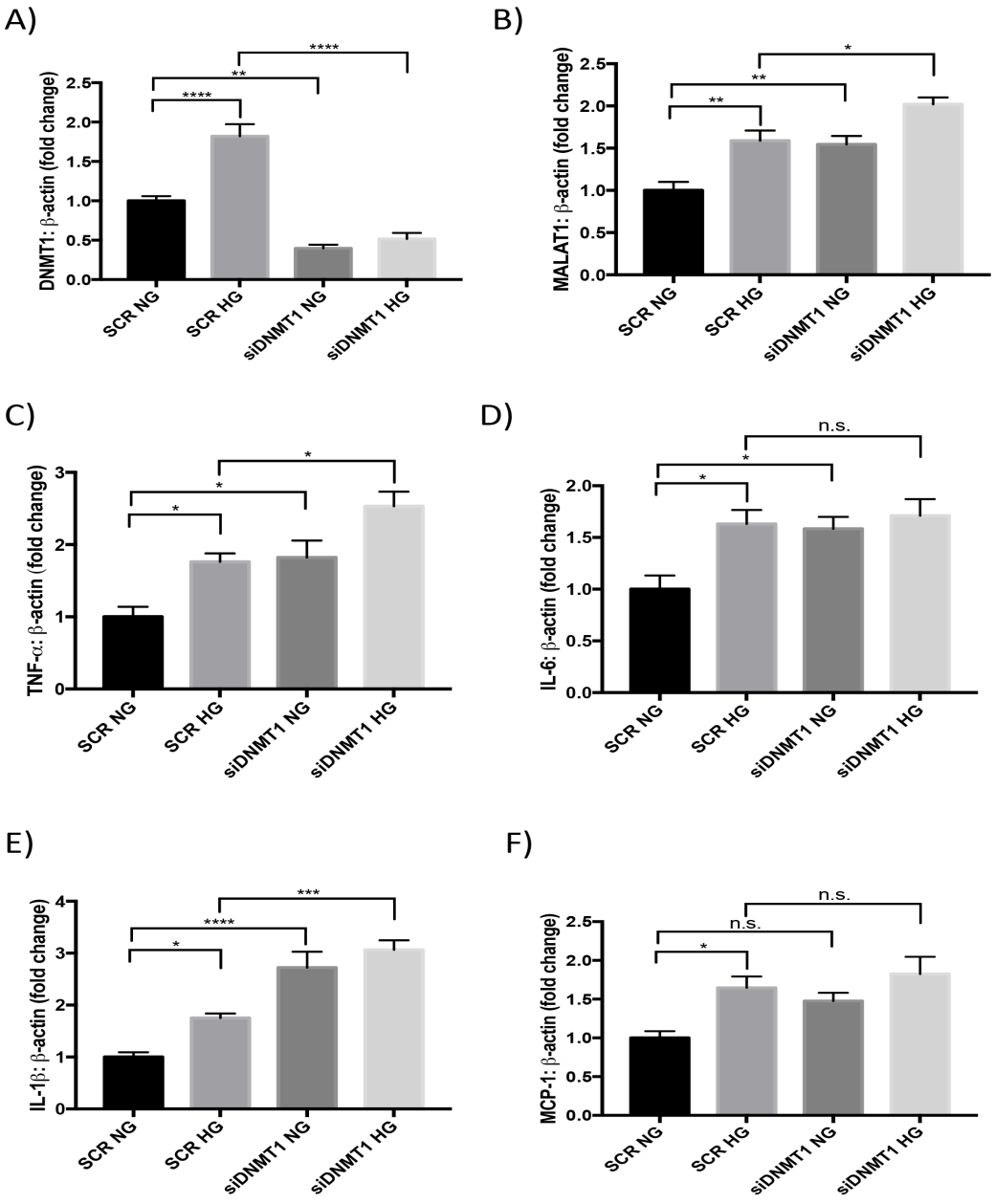

Figure S7: *DNMT1* knockdown impacts *MALAT1*, *TNF-α, IL-6, MCP-1* and *IL-1β* transcripts. HRECs transfected with siDNMT1 demonstrated significantly decreased A) *DNMT1* transcript expression and increased RNA expressions of B) *MALAT1*, C) *TNF-α*, D) *IL-*

6, E) *IL-1β*, and F) *MCP-1* compared to scrambled controls (*=*P*<0.05, **=*P*<0.01, ***=*P*<0.001, ****=*P*<0.0001, and n.s.= not significant compared to SCR controls; data expressed as mean ± SEM; *n*=6/group; normalized to β-actin, and data represented as a fold change of NG). HRECs were also incubated with 5 μM Zebularine (a DNA methylation inhibitor) and demonstrated similar increases in D) *MALAT1*, E) *TNF-α*, and F) *IL-6* RNA compared to controls (*=*P*<0.05, **=*P*<0.01, ***=*P*<0.001, and ****=*P*<0.0001 compared to SCR NG or SCR HG; data expressed as mean ± SEM; *n*=6/group; normalized to β-actin, and data represented as a fold change of NG). SiDNMT1= siDNMT1 treatment.

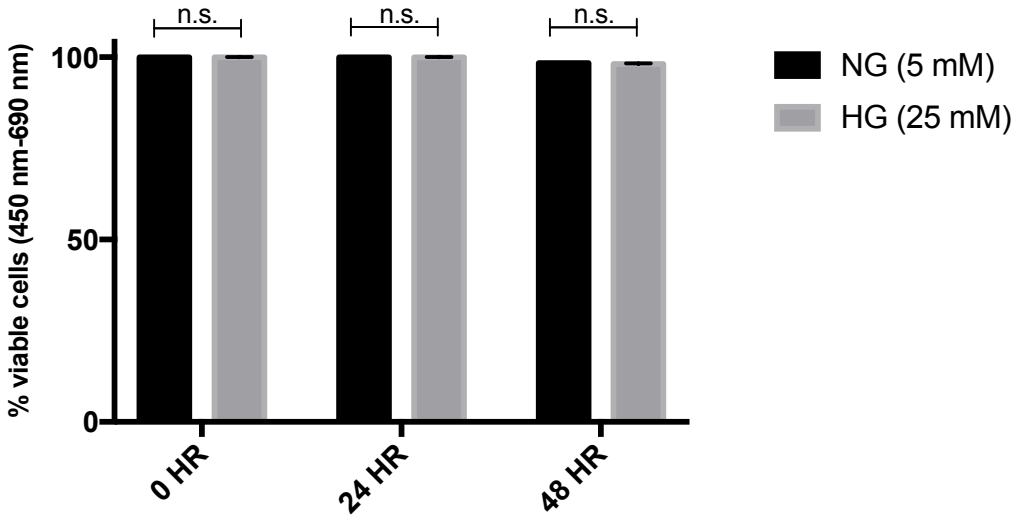

Figure S8: Cell viability of retinal endothelial cells following glucose treatments across various durations of incubation. WST assay showing no significant differences in human retinal endothelial cell viability between the two groups following various durations of incubation (data presented as mean± SD; n.s.= no significance compared to NG; and n=6/group). Legend: NG= normal glucose, HG= high glucose.

Table S1: Specific oligonucleotide sequences used for RT-qPCR.

Target Gene:	Oligonucleotide Sequence (5'→ 3'):
B-actin (*Homo* and *Mus*)	F: CCTCTATGCCAACACAGTGC R: CATCGTACTCCTGCTTGCTG
MALAT1 (*Homo*)	F: TCTTAGAGGGTGGGCTTTTGTT R: CTGCATCTAGGCCATCATACTG
Malat1 (*Mus*)	F: TGCAGTGTGCCAATGTTTCG R: GGCCAGCTGCAAACATTCAA
IL-6 (*Homo*)	F: GGGGCTGCTCCTGGTGTTG R: CTGAGATGCCGTCGAGGATGTA
Il-6 (*Mus*)	F: ACAACCACGGCCTTCCCTACTTT R: TCATTTCCACGATTTCCCAGAG
TNF-α (*Homo*)	F: AGGCGCTCCCCAAGAAGACAG R: AGCAGGCAGAAGAGAGCGTGGTG
Tnf-α (*Mus*)	F: AGGCACTCCCCCAAAAGAT R: CACCCCGAAGTTCAGTAGACAG
EZH2 (*Homo*)	F: CCACCATTAATGTGCTGGAA R: TTCCTTGGAGGAGTATCCACA
Ezh2 (*Mus*)	F: CGCGGGACTAGGGAGTGTTCAGT R: AGTACATTATAGGCACCGAGGCGA
EED (*Homo*)	F: GCAACTGTAGGAAGCAACAGA R: CATAGGTCCATGCACAAGTGT
Eed (*Mus*)	F: ATGCTGTCAGTATTGAGAGTGGC R: GAGGCTGTTCACACATTTGAAAG
SUZ12 (*Homo*)	F: TACGGCTCCTATTGCCAAAC R: TGCTTCAGTTTGTTGCCTTG
Suz12 (*Mus*)	F: AGCTCTGCCACAGCAGGTTCA R: TGCTTTTGTTCTTTTTGGCCTGCAA
IL-1β (*Homo*)	F: GCGGCATCCAGCTACGAATCT R: GGGCAGGGAACCAGCATCTT
Il-1β (*Mus*)	F: TTCAGGCAGGCAGTATCACTC R: GAAGGTCCACGGGAAAGACAC
MCP-1 (*Homo*)	F: TCGCCTCCAGCATGAAAGTC R: GGCATTGATTGCATCTGGC
Mcp-1 (*Mus*)	F: TTGTCACCAAGCTCAAGAGAGA R: GAGGTGGTTGTGGAAAAGGTAG
DNMT1 (*Homo*)	F: ACGGTGCTCATGCTTACAAC R: TTAGCCTCTCCATCGGACTT
DNMT3A (*Homo*)	F: GGCAAATTCTCAGTGGTGTG R: GTCACTCTCATCGCTGCTGT
DNMT3B (*Homo*)	F: TTGAATATGAAGCCCCCAAG R: TGATATTCCCCTCGTGCTTC

Table S2: Body weights and blood glucose levels for the mice at 2 months. Legend: WT-control=WT-C, WT-diabetic= WT-D, *Malat1* KO-control= *Malat1* KO-C, and *Malat1* KO-diabetic= *Malat1* KO-D [data expressed as mean ± SEM; *n*=6/group; and '*'=significantly different from WT-C].

	WT-C	WT-D	*Malat1* KO-C	*Malat1* KO-D
Average Body weight (grams)	29.33 ± 0.67	* 24.42 ± 0.86	27.25 ± 1.39	* 23.17 ± 0.99
Average Blood Glucose (mmol/L)	9.42 ± 0.50	* 30.00 ± 1.31	9.03 ± 0.57	* 26.15 ± 3.17

Chapter 3 [iii]

3 The long non-coding RNA *HOTAIR* is a critical epigenetic mediator of angiogenesis in diabetic retinopathy

With diabetes mellitus (DM) projected to impact over 600 million people globally in the next 20 years [1], the subsequent risk of acquiring micro- and macrovascular complications remain a serious concern. DM is a degenerative metabolic disease that is primarily characterized by chronic hyperglycemia, where sustained hyperglycemic insults can evoke alterations in biochemical and metabolic pathways that ultimately contribute to vascular damage and the pathogenesis of chronic diabetic complications [2-4]. Among these complications, diabetic retinopathy (DR) is a debilitating microvascular complication of DM and is also one of the leading causes of blindness worldwide [5]. Although there are different severity stages for DR, DR can be mainly categorized as non-proliferative DR (NPDR) and proliferative DR (PDR), where the latter may lead to imminent vision loss due to the presence of ocular pathological neovascularization [6].

As a result of long-term diabetes, pathological angiogenesis occurs and continuously activates various signal transduction cascades that promote the expressions of several pro-angiogenic genes, leading to increased concentrations of angiogenic factors than angiostatic factors [7-10]. Following upregulation, these angiogenic factors act synergistically to mediate the migration and proliferation of retinal endothelial cells in pre-existing blood vessels, which ultimately leads to the formation of new, abnormal blood vessels that are susceptible to bleeding, leakage, fibrosis and contraction [11,12]. Among the regulatory angiogenic molecules, vascular endothelial growth factor (VEGF) is a potent angiogenic factor expressed by ECs and non-ECs and has been studied extensively in DR. Several pathological processes, such as hypoxia [13], oxidative stress [14], advanced glycation end

[iii] Content in Chapter 3 contains data under publication review.

products [15], and inflammation [16], can stimulate VEGF expressions through a transcriptional regulation involving a complex milieu of transcription factors [17], mediator complexes [18] and non-coding RNAs [19,20]. Indeed, due to VEGF's critical role in DR, the standard first-line therapy for patients with diabetic macular edema and PDR consists of intravitreal injections of anti-VEGF and/or steroid compounds, which temporarily delay the progression of severe retinopathy. However, the use of such therapies comes at a cost to patients, where frequent intraocular injections are required, local or systemic adverse effects are associated with anti-VEGF compounds [21,22], and 40-50% of eyes with diabetic macular edema (another complication of diabetes) can not fully respond to anti-VEGF treatments [23]. Undoubtedly, in order to mitigate the effects of DR, a sense of urgency is warranted for diabetes research, as exploratory studies will provide novel insights into our understanding of ocular angiogenesis and open new avenues for better diagnosis and targeted therapies.

Within the last two decades, the rapid advent of genomic technologies has identified long non-coding RNAs (lncRNAs) as a fundamental class of RNA transcripts that are larger than 200 base-pairs and possess limited protein-coding capacities. LncRNAs are dynamically regulated and present with distinct functionalities that facilitate chromatin remodelling and/or help govern the expression of genes involved in a multitude of biological and pathological processes, including development [24], cancer [25], neurodegeneration [26] and even DR [27,28]. Despite the breadth of literature available regarding lncRNAs, limited studies have comprehensively characterized the functions of certain lncRNAs in mediating angiogenesis in the context of DR.

Here, we used microarray screening to identify glucose-induced expressions of the lncRNA *HOTAIR* (HOX transcript antisense RNA) in human retinal ECs and then investigated its functional implications in the pathogenesis of DR. Examination of retinal tissues from diabetic animals and vitreous and serum samples from patients with PDR showed that the lncRNA *HOTAIR* is specifically upregulated in hyperglycemic environments. Further mechanistic studies revealed that *HOTAIR*

directly mediates angiogenesis and other glucose-induced aberrations in ECs (such as mitochondrial and DNA damage) through its involvement in related epigenetic pathways, including histone methylation, histone acetylation, and DNA methylation. Collectively, our data highlights the complex regulatory nature of *HOTAIR* in the context of DR and identifies *HOTAIR* as a potential diagnostic marker and therapeutic target for angiogenesis in DR.

3.1 Materials and Methods

3.1.1 Cell culture

Human retinal microvascular endothelial cells (HRECs; Cell Systems, Kirkland, WA, USA; catalog number ACBRI 181), mouse retinal microvascular endothelial cells (MRECs; Applied Biological Materials Inc., Richmond, BC, CAN), and primary lung endothelial cells (MLECs) from C57BL/6J mice were cultured in endothelial basal media-2 (EBM-2, Lonza, Walkersville, MD, USA) containing endothelial growth media-2 (EGM-2) SingleQuots (Lonza). All cells were grown in 75 cm^2 culture flasks and maintained in a humidified incubator containing 5% CO_2 at 37°C. As described previously [19,27,31], in order to reduce variability for experimentation, cells were used between passages three and six and the cellular densities were determined accordingly based on the type of culture plates used for each experiment. Generally, once 80% confluence was obtained post-seeding, ECs were cultured in serum and growth factor-free medium overnight before exposure to different D-glucose levels (final glucose concentrations of 5 mmol/L, mimicking normoglycemia [NG], and 25 mmol/L, mimicking hyperglycemia [HG]) for various durations; the selected glucose levels are based on a large volume of previous experiments [9,19,20,27,28,31,38,85,86]. All *in vitro* or *ex vivo* experiments were independently repeated at least three times and performed with six replicates, unless specified.

3.1.2 siRNA transfections

HRECs were transfected using scrambled siRNAs (ID number: AM4635 [SCR],

Thermo Fisher Scientific) or pre-designed siRNAs targeting human *HOTAIR* (ID: n272221 [si1-HOTAIR], Thermo Fisher Scientific; n272222 [si2-HOTAIR], Thermo Fisher Scientific; R-187951-00-0005 [SMARTpool siHOTAIR], Horizon Discovery), *EZH2* (ID: M-004218-03-0005 [SMARTpool si-EZH2], Horizon Discovery), *CTCF* (ID: M-020165-02-0005 [SMARTpool siCTCF], Horizon Discovery), *DNMT1* (ID: s4216 [siDNMT1], Thermo Fisher Scientific) or mouse *Hotair* (ID: R-173526-00-0005 [SMARTpool siHOTAIR], Horizon Discovery) using Lipofectamine 2000 (Invitrogen, Burlington, ON, Canada) and Opti-MEM reduced serum media (Thermo Fisher Scientific). As documented previously by us [19,20,27,28,31,38], cells were transfected with 100 nM of each siRNA for 3-4 hours and subsequently recovered in complete EBM-2 overnight. Cells were then serum starved the following morning, between 18-24 hours, and then incubated with specific glucose concentrations (5 mmol/L or 25 mmol/L) for 48 hours. Knockdown of the target genes were then confirmed using RT-qPCR.

3.1.3 Enzyme-linked immunosorbent assay (ELISA)

Human VEGF-A (R&D Systems, Minnesota, USA) and mouse Vegf-a (Invitrogen) ELISA kits were used to measure the cytokine levels from HREC supernatants and mice retinal tissues, respectively. Cytokine concentrations were quantified using the BCA protein assay kit (Pierce, Rockford, IL, USA) and 100 μg protein concentrations were used for each ELISA kit according to the manufacturer's instructions. The optical density for each well was determined at 450 nm and corrected at 568 nm using the Multiskan FC Microplate Photometer (Thermo Fisher Scientific, Massachusetts, USA).

3.1.4 Endothelial tube formation assay

When performing the tube formation assay, ~$1.5x10^4$ HRECs (pre-treated with either SCR siRNA or siHOTAIR) were seeded into a 96-well plate containing 100 μL of BD Phenol red-free matrigel matrix (BD Biosciences, Bedford, MA, USA) per well. In the presence of growth medium, cells were allowed to attach for one hour in a humidified incubator with 5% CO_2 at 37°C. Following one-hour incubation, the

growth medium was replaced with serum-free medium containing appropriate glucose concentrations (5 mM or 25 mM) and/or exogenous VEGF-A protein concentrations (50 ng/mL). At the six-hour mark, images were taken at a 40x magnification using the Nikon Diaphot microscope (Nikon Canada, Mississauga, ON, CAN) with a PixeLINK camera (PixeLINK, Ottawa, ON, CAN) and images were captured from at least two field views per well (n= 8 independent samples/group). In order to assess the total number of tubules and branching points in the images, the WimTube Image analyzer software (Wimasis) was used and these results were plotted graphically.

3.1.5 RNA fluorescence *in situ* hybridization (RNA-FISH)

As previously described [20,27,86], HRECs were seeded at 75% confluency on glass cover slips in 12-well plates, serum starved over night, and treated with various glucose concentrations (NG or HG) for 48 hours. RNA fluorescence *in situ* hybridization (FISH) was performed according to the manufacturer's protocol for adherent cells (https://www.biosearchtech.com/support/resources/stellaris-protocols) and Stellaris FISH probes for Human *HOTAIR* with Quasar 570 dye (5 nmol; Biosearch Technologies, Petaluma, CA, USA) were used for hybridization. HRECs were also counterstained with 4′,6-diamidino-2-phenylindole (DAPI; Vector Laboratories, Burlingame, CA, USA) and mounted with Vectashield mounting medium (Vector Laboratories). Images were captured with the Cytation 5 Cell Imaging Multi-Mode Reader (BioTek, Winooski, VT, USA), at a magnification of 20X, by researchers blinded to the experimental groups. Yellow fluorescent protein (YFP), DAPI, and phase contrast filters were used and images were subsequently analyzed using ImageJ software (NIH, Bethesda, MD, USA).

3.1.6 RNA immunoprecipitation (RIP)

Cell lysates from HRECs cultured in NG or HG were collected for immunoprecipitation at the 48-hour mark using the Magna RIP RNA-binding protein immunoprecipitation kit (Millipore, Etobicoke, ON, CAN) [20,28], following the

manufacturer's instructions. Anti-IgG (control) and anti-EZH2 antibodies (Millipore) were used to co-precipitate the RNA-binding proteins of interest. The extracted RNAs were then reversed transcribed to cDNA, analyzed by RT-qPCR and normalized to the levels of *B-actin* mRNA (encoding a housekeeping protein).

3.1.7 3-Deazaneplanocin A (DZNep), 5-Aza-2'-deoxycytidine (5-aza-dC) and 2-deoxy-D-glucose (2-DG) treatments

Following the concentrations documented in previous studies, DZNep (Cayman Chemical, Ann Arbor, MI, USA; 5 μM), 5-aza-dC (Sigma, St. Louis, USA; 5 μM) or 2-DG (Sigma; 0.6 mM and 5 mM) pre-treatment was applied to HRECs for 1 hour prior to the addition of D-glucose [19,28,31,45]. DZNep, 5-aza-dC or 2-DG-treated HRECs and their respective controls were collected at 48 hours for further analyses.

3.1.8 JC-1 assay

In order to assess mitochondrial health and functional status, the JC-1 assay was employed [38]. Briefly, HRECs were treated with either SCR siRNA or siHOTAIR prior to glucose culture and at the 48-hour mark, cells were subsequently incubated for 10 minutes with 10 μM of the JC-1 dye (5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimi-dazolylcarbocyanine iodide; Abcam, Toronto, ON, CAN). Following the manufacturer's instructions, HRECs were then washed three times using the JC-1 dilution buffer. In order to stain the nuclear regions, DAPI (Vector Laboratories) was used following JC-1 staining. Fluorescence images were captured at 20X magnification using the Zeiss LSM 410 inverted laser-scanning microscope (Carl Zeiss Canada, North York, ON, CAN) and images were analyzed using ImageJ.

3.1.9 8-OH-dG staining

Following siRNA pre-treatment, HRECs were plated in eight-chamber tissue culture slides and incubated for 48 hours after glucose challenge (NG or HG) [38,85]. Following the manufacturer's instructions, cells were fixed with methanol and then

stained for 8-hydroxy-2′-deoxyguanosine (an oxidative DNA damage marker; 8-OHdG; 1:50, Santa Cruz Biotechnology, Dallas, TX, USA). Nuclear staining was also performed using DAPI (Vector Laboratories). Microscopy was performed by a blinded examiner, who was unaware of the identity of the samples, using a Zeiss LSM 410 inverted laser scan microscope (Carl Zeiss Canada) and the images were captured at a 20X magnification and subsequently analyzed using ImageJ.

3.1.10 Chromatin immunopreciation-qPCR (ChIP-qPCR)

ChIP assays (Milipore, Temecula, CA, USA) were carried out as previously described by us [19]. Briefly, HRECs were pre-treated with either SCR siRNA or siHOTAIR and subsequently cultured in NG or HG for 48 hours. Cells were then fixed with 1% formaldehyde, incubated for 10 minutes at 37°C, and then lysed and sonicated to shear DNA. ChIP assays were performed using anti-trimethyl-Histone H3 (Lys27; H3k27me3; Millipore), anti-RNA polymerase II (Millipore), anti-IgG (Millipore) and anti-acetyl-Histone H3 (K9, K14, K18, K23, K27; Abcam) antibodies. Anti-mouse IgG was used as a negative control. The immunoprecipitated DNA was detected by RT-qPCR using promoter-specific primers for *VEGF-A*: distal promoter region (forward: 5'-GTAGTCCCAGGGTGCAACAC-3', reverse: 5'-GACTGGCTAGAATGGGCATC-3', location relative to transcriptional start site [TSS]: -4896 bp) and proximal promoter region (forward: 5'-CGGTGCTGGAATTTGATATTCATTGAT-3', reverse: 5'-TTCAAGTGGGGAATGGCAAGC-3', location relative to TSS: -189 bp) [87].

3.1.11 WST-1 cell viability & proliferation assay

Following glucose and siHOTAIR treatments, the viability of HRECs was determined using the WST-1 Cell Viability Assay (Roche) at 48 hours. Using the Multiskan FC Microplate Photometer (Thermo Fisher Scientific), absorbances were first measured at 450 nm and then corrected using 690 nm as the reference wavelength.

3.1.12 Electron microscopy

Following the transfection of HRECs on coverslips, the inserts with attached cells were fixed in 2.5% glutaraldehyde in phosphate buffer and processed for EPON embedding as previously described [92]. Ultra-thin sections on 200 mesh nickel grids were stained with uranyl acetate and lead citrate and examined electronmicroscopically (Phillips EM-420 TEM).

3.1.13 Methylation analysis of CpG sites across *HOTAIR*

Differential methylation patterns of CpG sites across the *HOTAIR* gene were identified in HRECs, incubated in NG or HG environments for 2 days (48 hours) or 7 days (168 hours), using the Illumina Infinium MethylationEPIC BeadChip array (Illumina, CA, USA). At each respective time-point, genomic DNA was extracted from these cells and 1 μg of DNA was used for bisulfite conversion using the Blood & Cell Culture DNA Mini Kit (Qiagen, Toronto, ON, CAN). The HiScan System (Illumina, CA, USA) was used to obtain the array readout and the methylated and unmethylated signal intensity data were then imported into R 3.5.2 for analysis. Following our previously published protocols [55], normalization was performed using the Illumina normalization method with background correction using the minfi package. Probes with detection p-value >0.01 were excluded from the downstream analysis. In addition, probes known to contain single nucleotide polymorphisms (SNPs) at the CpG interrogation or the single nucleotide extension were removed. Methylation level for each probe was measured as a beta value (β-value), calculated from the ratio of the methylated signals versus the total sum of unmethylated and methylated signals, ranging between 0 (no methylation) and 1 (full methylation). Three independent samples were used per group.

3.1.14 Diabetic animal models

The Western University Council for Animal Care Committee approved all animal models used in this study and experiments were performed in accordance with *The Guide for the Care and Use of Laboratory Animals* (NIH Publication 85-23, revised in 1996). Beginning with our initial two-month *in vivo* model, male rats (Sprague-

Dawley; ~175 g, 6 weeks old) or male mice (C57/BL6 background; ~25 g, 8 weeks old) were obtained (Charles River, Wilmington, MA, USA) and randomly divided into control and diabetic groups. Streptozotocin (STZ) was used to generate a type 1 diabetic animal model and methods of diabetes induction and monitoring have been previously described [9,20,27,28,85,86,88]. At two months following diabetes induction, animals were euthanized (n= 8 for both mice groups; n= 5 for control rats and n=9 for diabetic rats) and retinal tissues were collected for RNA extraction and *Hotair* RNA levels were assessed using RT-qPCR.

For our short-term therapeutic *in vivo* model (4-week duration), wild-type mice were obtained (Charles River; C57BL/6J background; ~25 g, 8 weeks old) and randomly divided into four groups (n= 6/group): control mice administered intravitreal injections of SCR siRNA (negative control) or siHOTAIR, and diabetic mice administered intravitreal injections of SCR siRNA or siHOTAIR. Prior to administering intravitreal injections, STZ-induced diabetes was first confirmed in diabetic animals (>20 mmol/L blood glucose levels) using the above methodologies. After the onset of diabetes, a 1 µl solution (100 nmol/L) containing either SCR siRNA or siHOTAIR with Lipofectamine 2000 (Invitrogen) was injected into the vitreous chamber of the diabetic mice eye once every week for up to three weeks. Control mice were also injected similarly with the same volume of SCR siRNA or siHOTAIR with Lipofectamine. All mice were anaesthetized using isoflurane (2.25% mixed with 900 mL/min O_2) and intravitreal injections were performed with a 33-gauge needle attached to a 10 µl glass syringe (Hamilton, Reno, USA). Surgical positioning of the needle and the general duration of each intravitreal injection have been described previously [89]. No post-surgical ocular complications occurred throughout the 4-week study.

3.1.15 Toxicity and Histopathological Analyses

To determine potential adverse effects of siHOTAIR in mice, toxicity analyses were performed in addition to the regular monitoring of mice (C57/BL6 background) [85]. Age-matched mice were divided into four groups: SCR siRNA group (negative control; 100 nmol/L; n=3), siHOTAIR low-dose group (25 nmol/L; n=3), siHOTAIR

middle-dose group (50 nmol/L; n=3) and siHOTAIR high-dose group (100 nmol/L; n=3). SCR siRNA or siHOTAIR were intravitreally injected once, as a single-dose, and mice were followed-up for 7 consecutive days. Following this time-point, organs were excised, fixed in 10% buffered formalin solution and embedded, and sectioned into 5 μm thick sections. The tissue sections were then stained with hematoxylin and eosin (H&E) for routine histology. A blinded pathologist evaluated the histopathological damage using a light microscope and the images were captured (Nikon, Japan). Of note, in order to examine long-term toxicity of siHOTAIR, we had also performed H&E staining for the mice tissues obtained from our 4-week therapeutic model (n= 3/group).

3.1.16 Clinical sample collection

The Western Research Ethics Board and Lawson Health Research Institute at the University of Western Ontario (London, ON, CAN) approved the clinical component of this study. Patients provided informed consent prior to the procurement of specimens and all of the samples were handled in accordance with the *Declaration of Helsinki*. Both serum and undiluted vitreous humor (VH) were collected from patients undergoing a pars plana vitrectomy by an experienced vitreoretinal surgeon. Both specimens were categorized into two groups: control and diabetic retinopathy (DR). The DR group comprised of patients diagnosed with advanced stages of DR, including proliferative DR (PDR; n= 11; mean age ± SD= 60.9 ± 10.43 years; 10 males and 1 female), while the control group consisted of patients that had no previous history of diabetes mellitus and were diagnosed with idiopathic macular hole or a separate non-diabetic ocular condition (n= 10; mean age ± SD= 69.2 ± 8.87 years; 2 males and 8 females). PDR was defined as the presence of neovascularization or fibrous proliferation of the disc or elsewhere on the retina. As previously described [27,28], total RNA was extracted from 500 μL of VH samples and 200 μL of serum samples using the TRIzol reagent (Invitrogen) and a serum RNA extraction kit (Bio Basic Inc., Markham, ON, CAN) following the manufacturer's protocol. After conversion to cDNA, RT-qPCR was used to evaluate the expression of *HOTAIR* in these samples.

3.1.17 RNA isolation and quantitative real-time polymerase chain reaction (RT-qPCR)

As extensively described by us [9,19,20,27,28,31,38,85,86], total RNA was extracted using the TRIzol reagent (Invitrogen). Once total RNA was obtained, a spectrophotometer (260 nm; Gene Quant, Pharmacia Biotech, USA) was used to quantify RNA concentrations in which 1-2 μg of total RNA was reverse transcribed to complementary DNA (cDNA) using a high-capacity cDNA reverse-transcription kit (Applied Biosystems/Thermo Fisher Scientific). cDNA was then amplified in the LightCycler 96 System (Roche Diagnostics, Laval, QC, CAN) using the SYBR-green master mix (Takara Bio, Mountain View, CA, USA) and specific primers for the genes of interest (Sigma; **Appendix B, Supplementary Tables 1 and 2**). RT-qPCR results were analyzed using the LightCycler 96 SW 1.1 software (Roche) and expression levels were calculated by the relative standard curve method using β-actin as an internal control for sample normalization.

3.1.18 Statistical analyses

Statistical differences were evaluated between groups using GraphPad Prism 7 (La Jolla, CA, USA). Data were considered statistically significant if the *P* value was less than 0.05. All quantitative data for the *in vitro* experiments are presented as mean ± SEM, while all *in vivo* data are presented as mean ± SD. Experiments were performed in triplicate (n= 6 per group), unless specified. Statistical significance for samples with non-parametric distribution was identified using the Mann-Whitney *U* test, while two-tailed Student's *t*-test (when comparing two conditions) or one-way ANOVA (for multiple comparisons; followed by Tukey's post hoc test) was applied for parametric variables.

3.2 Results

3.2.1 *HOTAIR* RNA expressions are glucose-dependent and appear to follow oscillating patterns with significant

elevations at the 48-hour time-point

Using microarray analyses, we previously explored the global expression profiles of lncRNAs in HRECs cultured with NG or HG for 48 hours (Gene Expression Omnibus [GEO] ID: GSE122189). Interestingly, following stringent filtering criteria (fold change $\leq$ or $\geq$ 2 and an adjusted *p*-value <0.05), thousands of lncRNAs were differentially expressed after HG glucose culture; in particular, when examining between replicates, 2669-3518 lncRNAs were found to be upregulated and 890-1991 lncRNAs were found to be downregulated in HRECs challenged with HG (**Appendix B, Supplemental Figures 1A-C**). Among the upregulated lncRNAs, the lncRNA *HOTAIR* was increased by 2.67-fold in HG-treated HRECs compared to NG controls (**Appendix B, Supplemental Figure 1D**). Real-time quantitative reverse transcription-PCR (RT-qPCR) further confirmed the elevated expressions of *HOTAIR* following 48 hours of HG culture in HRECs (**Figure 3.1A**), which were also associated with augmented expressions of *VEGF-A* and *ET-1* transcripts (**Figure 3.1C-D**). Since lncRNAs have been reported to demonstrate differential expression patterns across various time-points [28-30], we investigated *HOTAIR* RNA expressions at 6, 12, 24, 48, and 72 hours. Intriguingly, when compared to their respective NG controls, *HOTAIR* demonstrated significant HG-induced elevations only at the 48-hour (**Figure 3.1C**; *p*=0.0014). Furthermore, to determine whether specific glucose concentrations can influence *HOTAIR* expressions, we cultured HRECs in the presence of 5, 10, 15, 20, and 25 mmol/L (mM) D-glucose for 48 hours. As demonstrated by RT-qPCR, *HOTAIR* RNA expressions peaked significantly following 25 mM glucose culture (mimicking hyperglycemia) compared to cells cultured with 5 mM glucose (mimicking euglycemia) (*p*=0.0077; **Figure 3.1D**). Therefore, based on the present findings and our previously published studies [19,20,27,28,31], the 48-hour time-point and 5 mM and 25 mM glucose concentrations were used for our subsequent *in vitro* experiments. Of note, for the above experiments, no significant differences in *HOTAIR* expressions were observed when using an osmotic control (25 mM L-glucose; data not shown).

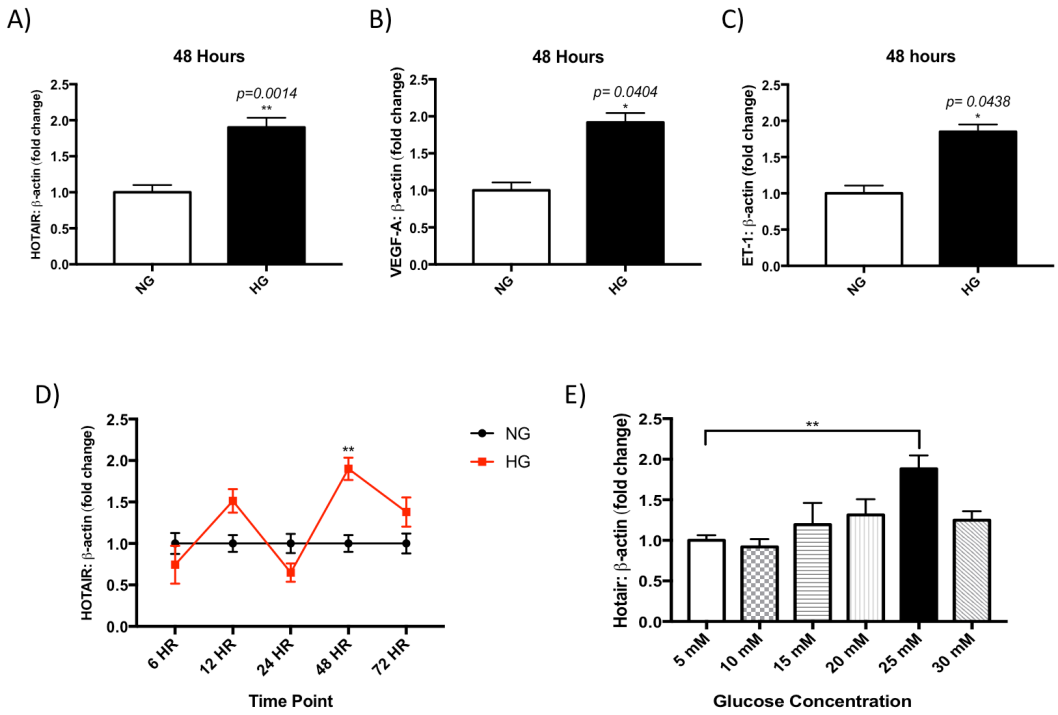

Figure 3.1. *HOTAIR* RNA expressions are associated with increased expressions of angiogenic markers in HRECs cultured with high glucose (HG) and appear to be glucose-dependent with significant elevations at 48 hours. RT-qPCR analyses demonstrating HG-induced increases of **(A)** *HOTAIR*, **(B)** *VEGF-A*, and **(C)** *ET-1* in HRECs compared to HRECs cultured in basal glucose levels at 48 hours (normal glucose; NG). **(D)** Expressions of *HOTAIR* across various time-points. **(E)** Relative *HOTAIR* RNA levels following different glucose concentrations following 48 hours of culture. β-actin was used as an internal control. Statistical significance was assessed using two-tailed Student's *t*-test when comparing two conditions or one-way ANOVA for multiple comparisons followed by Tukey's post hoc test (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, or n.s.= not significant). Data represents the mean ± SEM of 3 independent experiments (n=6/group).

To delineate the sub-cellular localization of *HOTAIR* in HRECs, we performed RNA fluorescence *in situ* hybridization (RNA FISH). RNA FISH showed that *HOTAIR* can be present in both nuclear and cytoplasmic compartments of HRECs, with a predominant localization in the perinuclear/cytosolic region (**Figure 3.2A**). Moreover,

further confirming our microarray and RT-qPCR findings, RNA FISH analyses demonstrated that HG significantly promotes elevated expressions of *HOTAIR* when compared to NG controls (**Figure 3.2B**; $p<0.0001$). Taken together, these data reveal that HG is an inducer of *HOTAIR* expressions and additionally imply an endothelial-specific role for *HOTAIR* during HG stress, where *HOTAIR* may be involved in the regulation of nuclear and cytoplasmic processes.

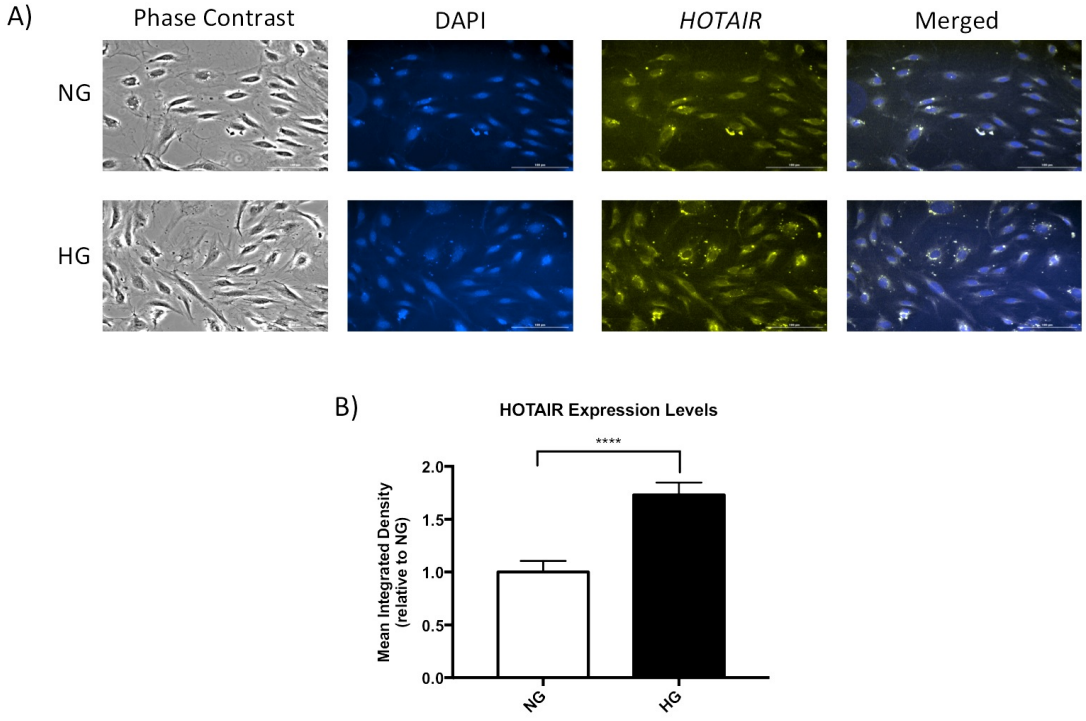

Figure 3.2. High glucose promotes *HOTAIR* expressions and *HOTAIR* can be localized in the nucleus and cytoplasm of retinal endothelial cells. (A) Visualization of *HOTAIR* localization in HRECs at 48 hours as indicated by RNA fluorescence *in situ* hybridization using Stellaris FISH probes for human *HOTAIR* with Qasar 570 dye. Cells were also counterstained with DAPI to visualize the nuclei. Original magnification, 20X; scale bars, 100 µm. **(B)** Mean integrated densities of *HOTAIR* expressions calculated using ImageJ. Statistical significance was assessed using two-tailed Student's *t*-test (****$p<0.0001$). Data represents the mean ± SEM of 50 cells captured per sample (n=4-5 independent samples/group).

3.2.2 *HOTAIR* directly mediates angiogenesis *in vitro*

With previous reports documenting the pro-angiogenic capabilities of *HOTAIR* in nasopharyngeal carcinoma [32] and glioma cells [33], we wanted to examine whether *HOTAIR* can mediate angiogenesis in hyperglycemic environments, since angiogenesis is a major pathological process in patients with proliferative diabetic retinopathy (PDR). Therefore, in order to determine *HOTAIR*'s angiogenic role, we used HRECs and performed an endothelial cell tube formation assay, which is a widely used *in vitro* assay that models the reorganization stage of angiogenesis and is a rapid method that can determine genes or pathways involved in angiogenesis [34]. As evident by the images in **Figure 3.3**, at the 6-hour mark, cells pre-treated with scrambled siRNAs (denoted as 'SCR') and cultured in the presence of HG have an elevated presence of capillary-like structures (tubules) compared to pre-treated SCR cells incubated in NG. However, when cells were treated with siHOTAIR, the degree of branching and total number of tubules significantly decreased in both NG and HG conditions at 6 hours (**Figure 3.3B** and **3.3C**; $p<0.0001$). Even more intriguing at the 6-hour mark, when HRECs were pre-treated with siHOTAIR, the presence of both exogenous VEGF proteins and HG were not able to completely recover the degree of branching and number of tubules compared to HG controls, which implies that the knockdown of *HOTAIR* may be further desensitizing ECs to other external angiogenesis-causing factors in HG. These findings encouraged us to explore other angiogenic factors [35], such as angiopoietin-like 4 (ANGPTL4), placental growth factor (PGF), hypoxia-inducible factor (HIF), interleukin-1 beta (IL-1β), and diabetes-related molecules including poly [ADP-ribose]-polymerase 1 (PARP1) [36], Cytochrome B [37,38], and several additional epigenetic mediators in the next set of experiments below.

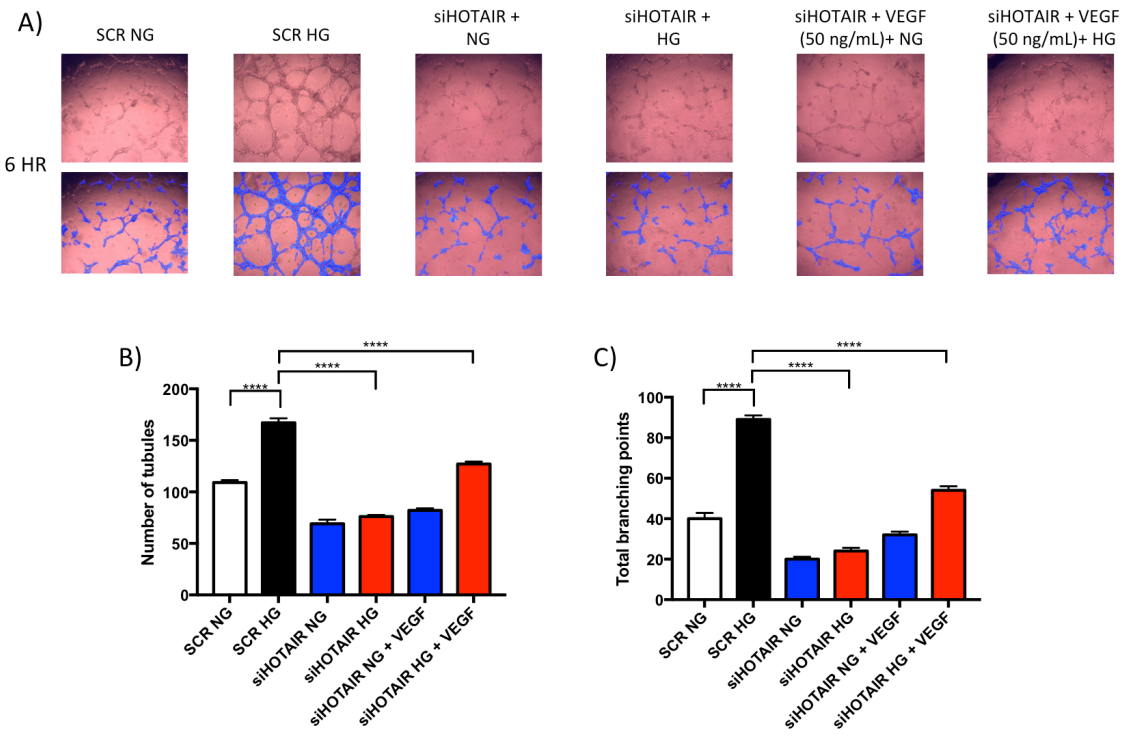

Figure 3.3. *HOTAIR* directly mediates angiogenesis *in vitro*. (A) Images captured from the endothelial tube formation assay at the 6-hour mark for HRECs treated with scrambled siRNAs (SCR), siHOTAIR or exogenous VEGF proteins and cultured in NG or HG conditions. The WimTube Image analyzer software was used to calculate **(B)** the number of tubules and **(C)** the total branching points in each group. Statistical significance was assessed using one-way ANOVA for multiple comparisons, followed by Tukey's post hoc test (****p<0.0001). Data represents the mean ± SEM of 3 independent experiments (n=8/group) and images were captured from at least two field views per well. Original magnification, 40X.

3.2.3 *HOTAIR* knockdown can prevent the induction of several angiogenic factors and diabetes-related molecules *in vitro*

To determine the direct regulatory capabilities of *HOTAIR* on the aforesaid molecules of pathogenetic significance in DR *in vitro*, we carried out a loss-of-function experiment that involved siRNA-mediated knockdown of *HOTAIR* in HRECs. Amongst the three siRNAs tested and albeit significant decreased *HOTAIR*

expressions were observed across all siRNA treatments, the 'SMARTpool siHOTAIR' evoked the largest reduction of *HOTAIR* RNA levels (by ~91%, $p<0.0001$) in HG-cultured HRECs compared to HG SCR controls (**Appendix B, Supplemental Figure 2**). Therefore, as such, we selected the 'SMARTpool' siRNA for our subsequent downstream analyses.

Accompanying the reduced *HOTAIR* levels in siHOTAIR-treated HRECs challenged with HG, significantly decreased expressions of various RNA transcripts implicated in angiogenesis (*VEGF-A*, *ET-1*, *ANGPTL4*, *PGF*, *HIF-1α*; **Figure 3.4**), DNA and oxidative damage (*PARP-1* and *Cytochrome B*; **Figure 3.4**), and epigenetic regulation (*EZH2*, *SUZ12*, *DNMT1*, *DNMT3A*, *DNMT3B*, *CTCF*, and *P300*; **Appendix B, Supplemental Figure 3**) were also evident when compared to HG controls. These findings indicate that the lncRNA *HOTAIR* is directly implicated in the transcriptional regulation of several DR-related molecules. To determine whether these molecular changes are also reflected at the protein level, we selected one of the angiogenic markers (VEGF-A) for further follow-up via ELISA. In parallel to our RNA results, the knockdown of *HOTAIR* can significantly prevent glucose-induced upregulations of VEGF-A proteins in HRECs (**Appendix B, Supplemental Figure 4A**; $p<0.0001$). Extending our findings, we had additionally examined the expressions of *HOXD3* and *HOXD10*, since *HOTAIR* has been implicated in the transcriptional repression of *HOXD* loci [39]. Indeed, the knockdown of *HOTAIR* in HRECs cultured with HG can induce significant upregulations of *HOXD3* ($p=0.0473$) and *HOXD10* ($p=0.0001$) compared to SCR HG controls (**Appendix B, Supplementary Figures 4B** and **4C**). We had also investigated the viability of HRECs following siHOTAIR treatment and as evidenced by our WST-1 findings, siRNA-mediated knockdown of *HOTAIR* can significantly improve cellular viability compared to SCR controls (**Appendix B, Supplemental Figure 4D**; $p<0.0001$). Collectively, these results suggest that *HOTAIR* is a critical regulator of glucose-induced EC dysfunction *in vitro*.

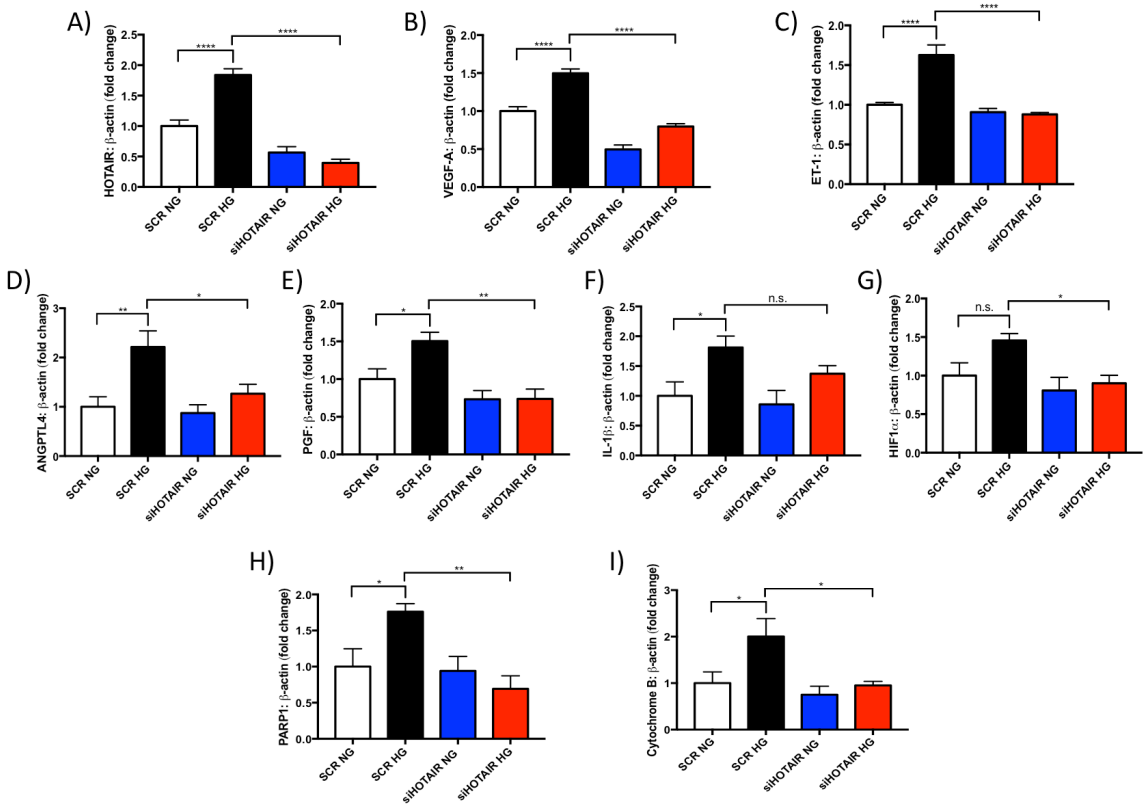

Figure 3.4. *HOTAIR* knockdown can prevent the induction of several angiogenic factors and diabetes-related molecules *in vitro*. RT-qPCR analyses of **(A)** *HOTAIR*, **(B)** *VEGF-A*, **(C)** *ET-1*, **(D)** *ANGPTL4*, **(E)** *PGF*, **(F)** *IL-1β*, **(G)** *HIF-1α*, **(H)** *PARP1*, and **(I)** *Cytochrome b* expressions following the administration of SCR siRNA or siHOTAIR in HRECs subjected to 48 hours of NG or HG culture. β-actin was used as an internal control. Statistical significance was assessed using one-way ANOVA for multiple comparisons, followed by Tukey's post hoc test (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, or n.s.= not significant). Data represents the mean ± SEM of 3 independent experiments (n=6/group).

3.2.4 *Hotair* is significantly elevated in the retinas of diabetic mice and rats at 2 months

Following our *in vitro* findings, we wanted to confirm *in vivo* whether *HOTAIR* had a similar pathogenetic phenotype in the retina in diabetes. As such, we employed a streptozotocin (STZ)-induced diabetic animal model involving both C57/BL6 mice and Sprague-Dawley rats and subsequently extracted retinal tissues after 2 months of

diabetes. Diabetic animals showed hyperglycemia and glucosuria (data not shown), as well as reduced body weight gain and hyperglycemia (**Appendix B, Supplemental Figures 8C** and **8D**). In parallel to the trends observed from our *in vitro* experiments, distinct patterns of *Hotair* RNA expressions were evident between the retinas of control and diabetic animals, with significant upregulations of *Hotair* in the retinas of both diabetic mice (p=0.0281; **Appendix B, Supplemental Figure 5A**) and rats (p=0.0420; **Appendix B, Supplemental Figure 5B**) at 2 months, suggesting that retinal *Hotair* expressions share a positive association with diabetes.

After confirming the significance of *Hotair* in the retina in diabetes, we sought to evaluate the therapeutic potential for siRNA-mediated modulation of *HOTAIR* as a new approach to treat DR. In order to determine this, we had first acquired a SMARTpool siHOTAIR that specifically targeted mouse *Hotair,* tested this siRNA compound on two EC-specific mouse cell lines (mouse retinal microvascular ECs [MRECs] and primary mouse lung ECs [MLECs] (C57/BL6)), and then elucidated the therapeutic significance of siHOTAIR using a short-term, one-month, diabetic animal model. Beginning with our *in vitro* and *ex vivo* experiments, we found that 50 nM and 100 nM concentrations of siHOTAIR can evoke significant reductions in *Hotair* RNA levels across both EC-lines cultured with HG, when compared to SCR HG controls (**Appendix B, Supplemental Figure 6**). In fact, using a 50 nM concentration, ~79% and ~53% reductions were observed in MRECs and MLECs challenged with HG, respectively, when compared to SCR HG controls; whereas, at a 100 nM concentration, ~80% and ~43% reductions were noted in HG-cultured MRECs and MLECs, respectively (**Appendix B, Supplemental Figure 6A** and **6D**). Similarly, when compared to controls, statistically significant reductions for both *Vegf-a* and *Angptl4* transcripts were also found after *Hotair* knockdown in MRECs (at a 100 nM concentration; **Appendix B, Supplemental Figure 6B** and **6C**). While, conversely, significant reductions in these angiogenic transcripts were not observed in MLECs (**Appendix B, Supplemental Figure 6E** and **6F**), which suggests that transfection efficiencies may differ between EC subtypes. Nevertheless, based on the findings from MRECs, we selected the mouse-specific siHOTAIR for our subsequent animal experiments.

3.2.5 Intravitreal administration of siHOTAIR is non-toxic and prevents early DR-related retinal changes

We initially performed a toxicology study involving siHOTAIR. Wild-type C57BL/6 mice were subjected to a one-time intravitreal injection that consisted of either scrambled siRNA control (50 nM; SCR) or siHOTAIR at varying concentrations (25 nM, 50 nM, and 100 nM) and were monitored for seven days and then euthanized for tissue collection. No behavioral changes or ocular complications were observed in the mice throughout the duration of the experiments. As evidenced by hematoxylin and eosin (H&E) staining, no structural abnormalities were observed across retinal, heart, lung, liver, and kidney tissues following intravitreal siHOTAIR injection at 25, 50, or 100 nM concentrations (**Appendix B, Supplemental Figure 7**). Furthermore, at the 7-day mark, retinal *HOTAIR* expressions appeared to be the lowest following a 100 nM dose of siHOTAIR (~50% reduction) when compared to SCR controls and other siHOTAIR concentrations (**Appendix B, Supplemental Figure 8A**). Using this information, we opted to select 100 nM as the optimal concentration of siHOTAIR for our therapeutic animal model.

To understand the therapeutic effects of siHOTAIR, diabetes was induced in C57BL/6 mice using STZ injections. All diabetic mice showed significant hyperglycemia and a progressive loss of body weight (**Appendix B, Supplemental Figures 8B** and **8C**), as well as polyuria and glucosuria (data not shown). Compared to SCR diabetic controls, *Hotair* knockdown did not further affect body weight and blood glucose levels (**Appendix B, Supplemental Figures 8B** and **8C**). When examining the pathogenetic molecules implicated in DR-related microvascular dysfunction, we found elevated RNA expressions of *Hotair*, *Vegf-a*, *Ctcf*, *Et-1*, *Angptl4*, *Mcp-1*, *Il-1β*, *Pgf*, *Hif-1α*, *p300*, polycomb repressive complex 2 [Prc2] components (*Ezh2*, *Suz12*, and *Eed*) and *Parp1* in the retinal tissues of diabetic mice administered SCR siRNAs (**Figure 3.5**). Whereas, the knockdown of *Hotair* (a ~58% reduction) could significantly reduce diabetes-induced upregulations of *Hotair*, *Vegf-a*, *Et-1*, *Angptl4*, Mcp-*1*, *Ctcf*, *Hif-1α*, *p300*, Prc2 components (*Ezh2*, *Suz12*, and *Eed*), and *Parp1*—suggesting that *HOTAIR* knockdown can alleviate early molecular aberrations induced by a diabetic milieu

within the retina (**Figure 3.5**). Of note, we did not observe statistically significant changes in retinal expressions of *Hoxd3* between SCR and siHOTAIR-treated diabetic animals (**Appendix B, Supplemental Figure 8D**). Furthermore, as indicated by H&E stains and in comparison to SCR controls, we did not find any observable cellular anomalies or toxic effects in retinal, cardiac, lung, liver, kidney, and brain tissues following 1 month of siHOTAIR injections (**Appendix B, Supplemental Figure 9**).

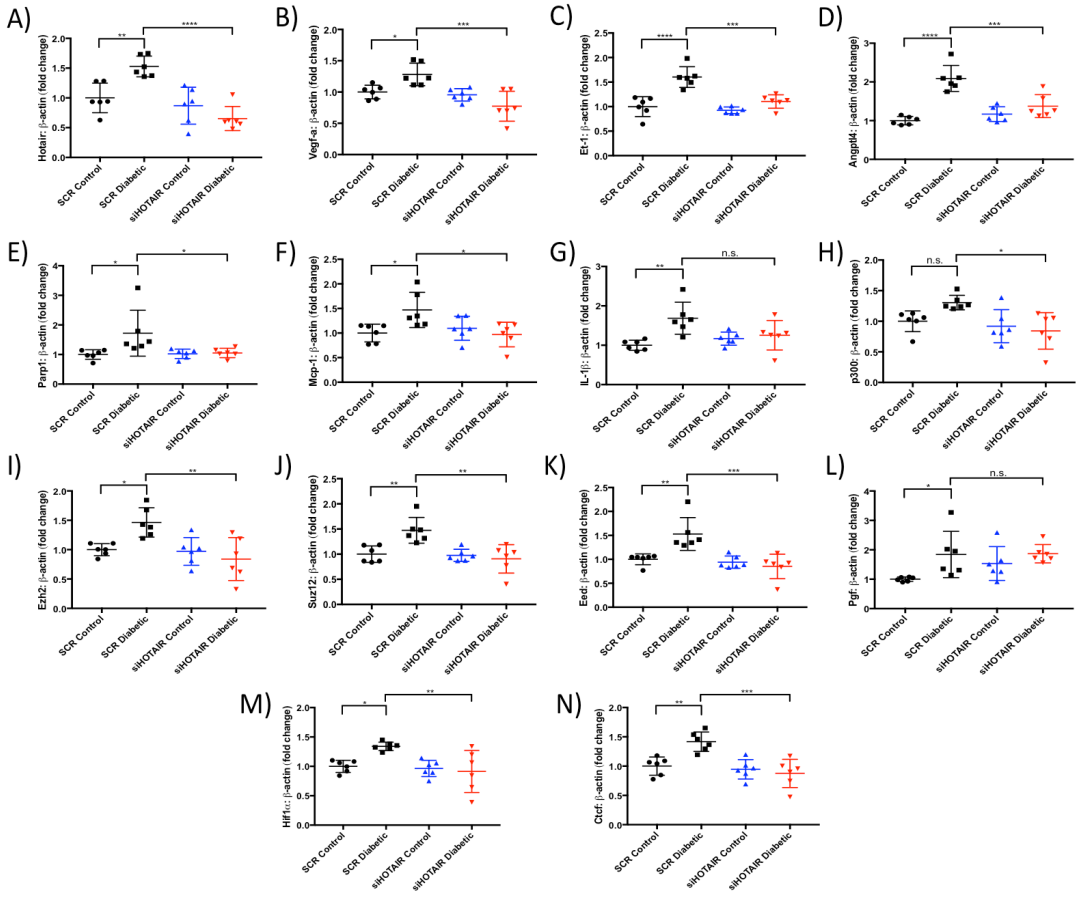

Figure 3.5. *In vivo* knockdown of *HOTAIR* can significantly prevent early glucose-induced elevations of angiogenic and diabetes-associated molecules in the diabetic retina. Non-diabetic and diabetic C57BL/6J mice were administered intravitreal injections of scrambled siRNAs (SCR) or siHOTAIR once every week for up to 3 weeks. Animals were then euthanized at 4 weeks (1 month) and retinal tissues were isolated and extracted for RNA. RT-qPCR was employed to analyze **(A)** *Hotair*, **(B)** *Vegf-a*, **(C)** *Et-1*, **(D)** *Angptl4*, **(E)** *Parp1*, **(F)** *Mcp-1*, **(G)** *Il-1β*, **(H)** *p300*, **(I)** *Ezh2*, **(J)** *Suz12*, **(K)** *Eed*, **(L)** *Pgf*, **(M)** *Hif-1α*, and **(N)** *Ctcf* expressions. β-actin was used as an internal control. Statistical significance was assessed

using one-way ANOVA for multiple comparisons, followed by Tukey's post hoc test (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, or n.s.= not significant). Data represents the mean ± SD (n=6/group).

3.2.6 *HOTAIR is upregulated in the vitreous and serum of diabetic patients*

After establishing *HOTAIR*'s biological importance in diabetic animals, we wanted to determine whether *HOTAIR* expressions have similar clinical importance from a potential biomarker angle. To this extent, we examined *HOTAIR* expressions in the serum and vitreous humor (VH) of patients with DR. Based on our RT-qPCR analyses, *HOTAIR* expressions were distinct and significantly upregulated in the vitreous (p<0.0001; **Figure 3.6A**) and serum (p=0.0021; **Figure 3.6B**) of patients with PDR than that of non-diabetic patients without retinopathy. Moreover, we performed two-sided Pearson correlations to determine whether a linear association for *HOTAIR* expressions existed between the two sample types. Interestingly, statistically significant correlations for *HOTAIR* were found between serum and vitreous samples, where increased serum *HOTAIR* expressions positively correlated with increased vitreous *HOTAIR* expressions (p=0.0005, R^2=0.482; **Figure 3.6C**). Taken together, our clinical findings suggest that *HOTAIR* expressions in the vitreous and serum are associated with DR and may have potential to be used as a prognostic and diagnostic biomarker for DR.

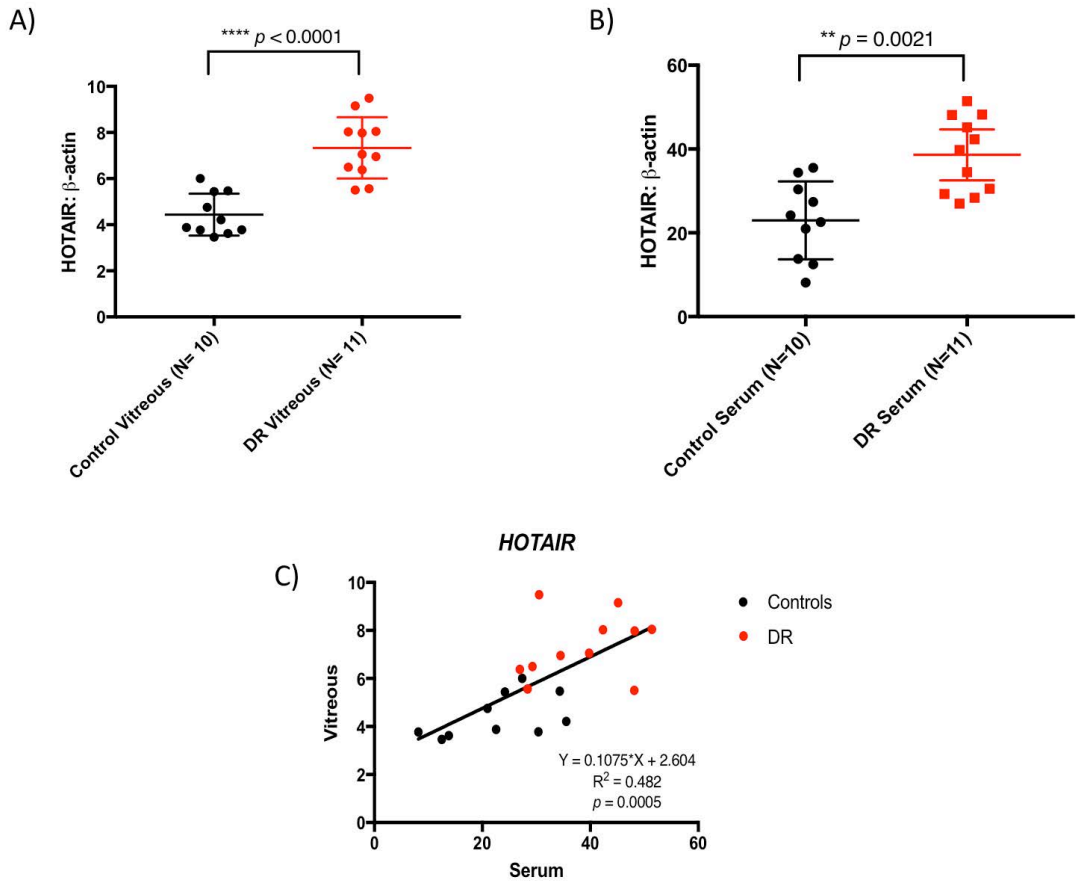

Figure 3.6. *HOTAIR* is upregulated in the serum and vitreous of patients with proliferative diabetic retinopathy (PDR). RT-qPCR analyses were used to examine *HOTAIR* expressions in the **(A)** vitreous and **(B)** serum from non-PDR (control) and PDR patients. β-actin was used as an internal control. Statistical significance was assessed using the Mann-Whitney U test. Data represents the mean ± SD (n=10 per control group or n=11 per DR group; **p<0.01 or ****p<0.0001). **(C)** Two-sided Pearson correlations determined that a linear (positive) association for *HOTAIR* expressions existed between the two sample types (***p<0.001). Legend: DR= PDR.

3.2.7 *HOTAIR* knockdown can partially prevent glucose-induced DNA and mitochondrial damage, as well as disruptions of endothelial cell junctions *in vitro*

We then wanted to further explore some of the molecular mechanisms for *HOTAIR in vitro*. With a previous report documenting *HOTAIR*'s implications in mitochondrial dysfunction in HeLa cells [40] and based on the localization of *HOTAIR* from our

RNA FISH experiments and the impact of siHOTAIR on *Cytochrome B* RNA levels, we first assessed the mitochondrial transmembrane potential (ΔΨM) in HRECs after *HOTAIR* knockdown through the detection of JC-1 signals. As shown in **Figure 3.7**, HG significantly evoked mitochondrial depolarization (indicated by more green and less red fluorescence; low ΔΨM) compared to scrambled NG controls ($p<0.0001$), suggesting that HG induces mitochondrial depolarization/dysfunction in HRECs. Conversely, when compared to SCR NG controls, the knockdown of *HOTAIR* in cells cultured with NG can markedly increase mitochondrial activity (indicated by more red and less green fluorescence; normal to high ΔΨM; $p<0.0001$). As expected, *HOTAIR* knockdown partially reduces HG-induced mitochondrial dysfunction/depolarization when compared to SCR HG controls ($p=0.0459$). Collectively, the JC-1 results indicate that *HOTAIR* contributes to mitochondrial aberrations in hyperglycemic environments.

Since it is well documented that HG can induce abnormalities in several glucose metabolic pathways that subsequently lead to intracellular oxidative stress and DNA damage [3,4,41], we examined the relationship between HG, *HOTAIR*, and 8-hydroxy-2'-deoxyguanosine (8-OHdG) levels, a biomarker for oxidative DNA damage [42]. Indeed, in comparison to SCR NG cells, HRECs in the presence of SCR siRNAs and HG demonstrated significant expressions of 8-OHdG (increase in green fluorescence; $p<0.0001$, **Figure 3.8**). In contrast, however, *HOTAIR* knockdown significantly reduced glucose-induced increases in 8-OHdG expressions compared to SCR HG cells ($p=0.0264$), which suggests that *HOTAIR* may be implicated in HG-induced oxidative damage. Moreover, an essential prerequisite in the development of DR is the loss of endothelium, which is caused by chronic hyperglycemic exposure and demonstrated by dysregulated endothelial cell-to-cell junctions [4]. To investigate this in our cell culture model, we examined SCR or siHOTAIR-treated HRECs in HG using electron microscopy. As expected, HG induced disruptions of cell junctions in HRECs treated with SCR siRNAs (**Appendix B, Supplemental Figure 17A**). However, conversely, the knockdown of *HOTAIR* preserved EC junctional integrity following HG culture (**Appendix B, Supplemental Figure 17B**). These results further suggest that *HOTAIR* contributes to DR-related EC dysfunction.

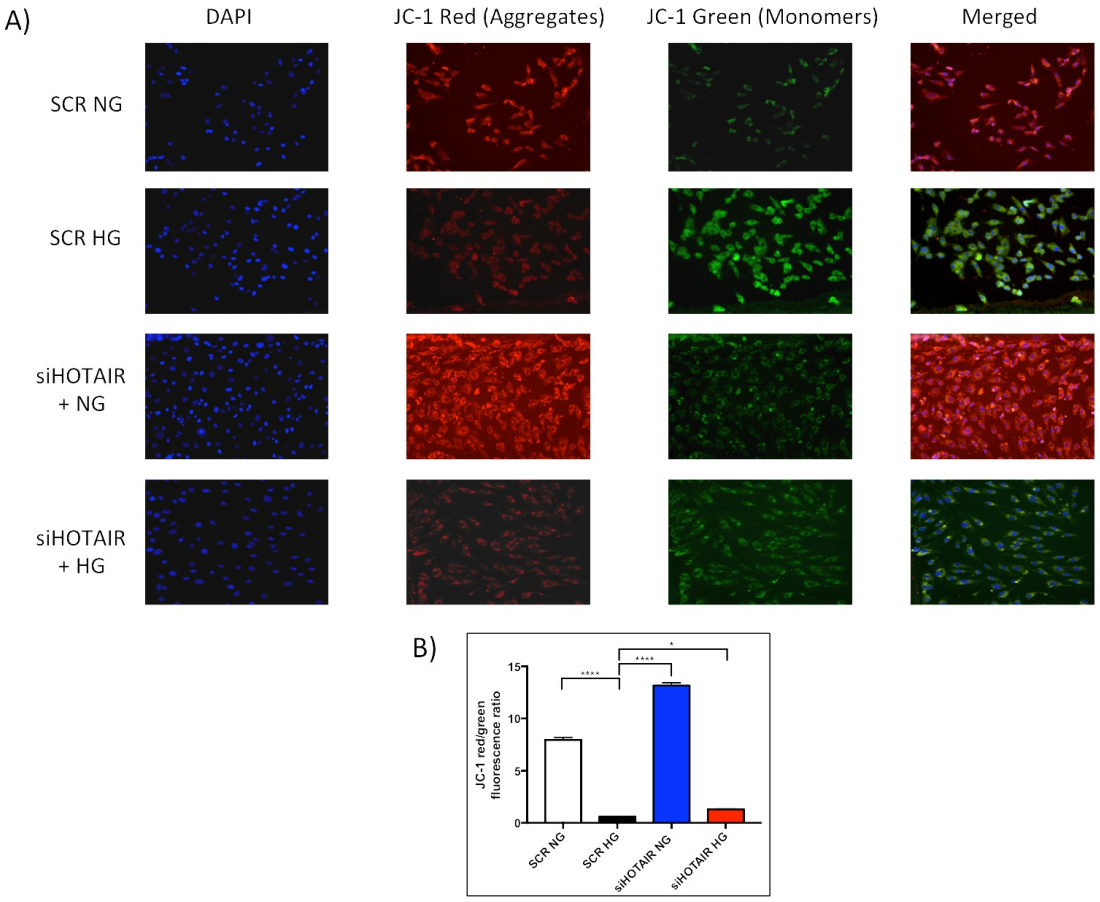

Figure 3.7. *HOTAIR* knockdown can partially prevent glucose-induced mitochondrial depolarization/dysfunction. (A) Images captured from the JC-1 assay, where HRECs were pre-treated with scrambled (SCR) siRNAs or siHOTAIR and subsequently cultured in NG or HG for 48 hours. Mitochondrial depolarization is indicated by more green and less red fluorescence (low ΔΨM, suggesting unhealthy/dysfunctional mitochondrial), while a polarized mitochondrial state is indicated by more red and less green fluorescence (normal to high ΔΨM, suggesting healthy/functional mitochondria). Cells were also counterstained with DAPI to visualize the nuclei. Original magnification, 20X; scale bars, 100 μm. **(B)** JC-1 red/green fluorescence ratio was calculated using ImageJ. Statistical significance was assessed using one-way ANOVA for multiple comparisons, followed by Tukey's post hoc test (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, or n.s.= not significant). Data represents the mean ± SEM of 20 cells captured per sample (n=8 independent samples/group).

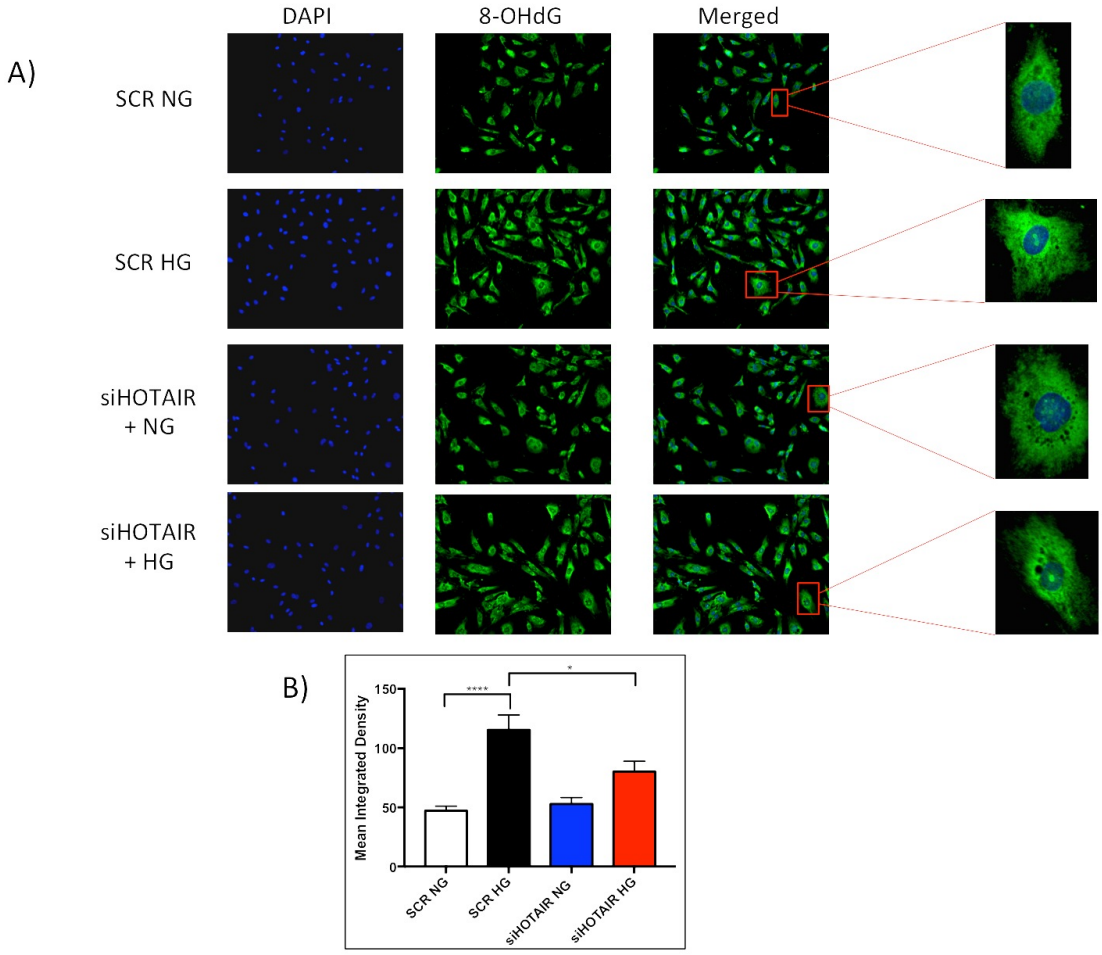

Figure 3.8. Knockdown of *HOTAIR* can significantly prevent glucose-induced oxidative damage. (A) Images captured from the 8-OHdG assay, where HRECs were pre-treated with scrambled (SCR) siRNAs or siHOTAIR and subsequently cultured in NG or HG for 48 hours. 8-OHdG is a biomarker for nuclear and mitochondrial oxidative DNA damage, where heightened oxidative damage is indicated by strong green fluorescence. Cells were also counterstained with DAPI to visualize the nuclei. Original magnification, 20X; scale bars, 100 µm. **(B)** Mean integrated densities of 8-OHdG expressions calculated using ImageJ. Statistical significance was assessed using one-way ANOVA for multiple comparisons, followed by Tukey's post hoc test (*p<0.05 or ****p<0.0001). Data represents the mean ± SEM of 20 cells captured per sample (n=8 independent samples/group).

3.2.8 *HOTAIR*-induced production of DR-related molecules depends on glycolytic metabolism

To have a better understanding of the regulatory mechanisms, we sought to examine the upstream role of glucose on *HOTAIR* and the expression of its target molecules. We employed 2-deoxy-D-glucose (an inhibitor of the glycolytic pathway [43]; 2-DG) and investigated the effects of this glucose analogue on HRECs *in vitro*. Accordingly, albeit the apoptotic nature of 2-DG (~45-55% viability indicated by trypan blue exclusion assay [data not shown]), 2-DG treatment significantly blocked HG-induced expressions of *HOTAIR*, *VEGF-A*, *ET-1*, *ANGPTL4*, *MCP-1*, *IL-1β*, *CTCF,* and *Cytochrome B* (**Figure 3.9**), which further emphasized the upstream regulatory roles played by glucose. Furthermore, inhibiting effective glucose metabolism (at 5 mM of 2-DG) evoked significant reductions in epigenetic molecules including *EZH2*, *SUZ12*, *EED*, and *DNMT1*, but no differences were observed for *DNMT3A* and *DNMT3B* (**Appendix B, Supplemental Figure 10**). Interestingly, even at 5 mM concentrations, 2-DG treatments also did not induce significant reductions in *PARP1* and *P300* expressions and did not augment *Cytochrome B* expressions, which may suggest that the blockade of glycolysis may continue to produce direct oxidative stress through nuclear transport mechanisms involving PARP1 and P300 rather than contributions of oxidative damage from the mitochondria [44]. Moreover, at 5 mM concentrations, *HOXD3* and *HOXD10* expressions were significantly upregulated in 2-DG-treated HRECs cultured with HG (**Appendix B, Supplemental Figures 10H** and **10I**) compared to HG controls, further highlighting the inverse relationship shared between *HOTAIR* and *HOXD* expressions. Taken together and in keeping with previous reports that confirm the anti-angiogenic and apoptotic effects of 2-DG on ECs [45], our data indicates that glucose works upstream of *HOTAIR* and inhibiting glucose uptake can ultimately prevent the upregulation of *HOTAIR* and most of its downstream targets.

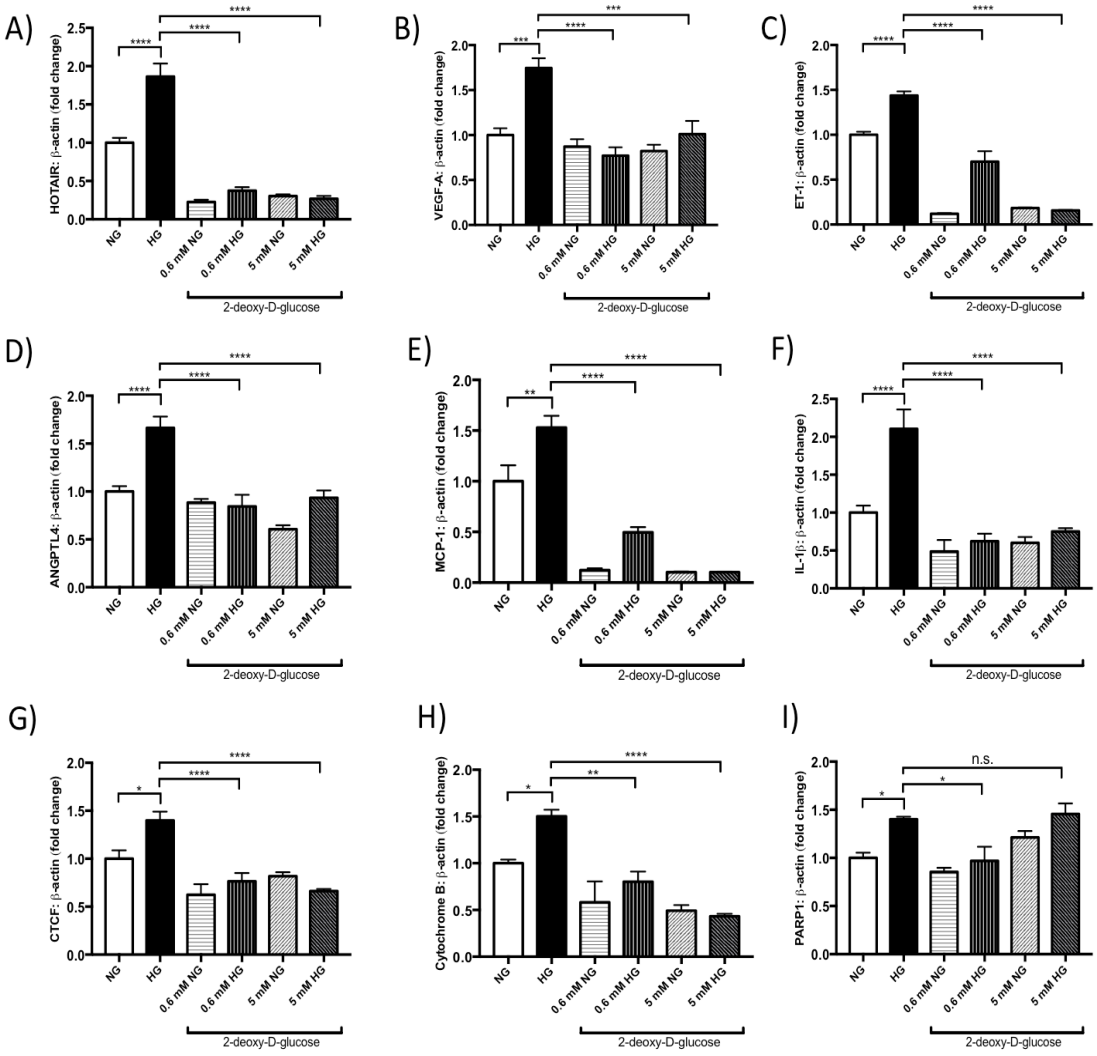

Figure 3.9. Glucose metabolism regulates *HOTAIR* and most of its downstream targets *in vitro*. RT-qPCR analyses of **(A)** *HOTAIR*, **(B)** *VEGF-A*, **(C)** *ET-1*, **(D)** *ANGPTL4*, **(E)** *MCP-1*, **(F)** *IL-1β*, **(G)** *CTCF*, **(H)** *Cytochrome B*, and **(I)** *PARP1* expressions following 2-deoxy-D-glucose treatment (0.6 or 5 mM) in HRECs subjected to 48 hours of NG (5mM D-glucose) or HG (25 mM D-glucose) culture. 2-deoxy-D-glucose is a potent inhibitor of glycolysis. β-actin was used as an internal control. Statistical significance was assessed using one-way ANOVA for multiple comparisons, followed by Tukey's post hoc test (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, or n.s.= not significant). Data represents the mean ± SEM of 3 independent experiments (n=6/group).

3.2.9 Histone methylation epigenetically regulates *HOTAIR* and its downstream targets

As *HOTAIR* and PRC2 (a critical histone methyltransferase) have shown a relationship in cancer [46,47], we decided to investigate the role of histone methylation in the context of diabetes. Beginning with the administration of a global histone methylation inhibitor known as 3-deazaneplanocin A (DZNep) [48], we confirmed that HRECs in the presence of HG plus DZNep had significantly reduced expressions of PRC2 components; in particular, *EZH2*, *SUZ12*, and *EED* transcripts were reduced by ~72% (p<0.0001), ~48% (p=0.0005), and ~61% (p<0.0001), respectively, when compared to SCR HG cells (**Appendix B, Supplemental Figure 11A-C**). Accompanying the reduced expressions of PRC2 components in HRECs treated with DZNep and HG, statistically significant reductions were also evident for *HOTAIR*, *VEGF-A*, *ANGPTL4*, *CTCF*, PARP1, *P300*, and *Cytochrome B* transcripts when compared to SCR HG controls (**Figure 3.10**). On the contrary, opposite trends were observed for *ET-1*, *MCP-1*, *IL-1β*, *HOXD3* and *HOXD10* transcripts, where DZNep pre-treatment plus HG culture of HRECs significantly augmented the expressions of the aforementioned molecules (**Figure 3.10** and **Appendix B, Supplemental Figures 11D** and **11E**). These dynamic observations are in keeping with our previous studies [20,28] and could suggest that DZNep is not completely selective and as such, may be disrupting a number of different cellular crosstalks in ECs within a hyperglycemic environment.

To confirm and expand our experimental findings using DZNep, we selected *EZH2* (the catalytic subunit of PRC2 [49]) and *CTCF* (a critical transcription factor that can maintain chromosome organization and is possibly implicated in the direct regulation of *HOTAIR* [50,51]) for subsequent siRNA-mediated knockdown. Following siRNA treatments and when compared to SCR HG controls, we confirmed significant reductions for *EZH2* (~77% knockdown; **Figure 3.11E**) and *CTCF* (~55% knockdown; **Figure 3.11F**). Interestingly, inhibiting the expressions of *EZH2* in HG conditions also evoked significant reductions in *HOTAIR*, *VEGF-A*, *ET-1*, *ANGPTL4*, *CTCF, SUZ12, PARP1, MCP-1*, *IL-1β*, *Cytochrome B,* and *DNMT1* RNA expressions,

while significant increases were seen for *P300*, *HOXD3* ad *HOXD10* transcript levels, compared to SCR HG controls (**Figure 3.11** and **Appendix B, Supplemental Figure 12D-F**). No significant differences in expressions were observed for *DNMT3A* and *DNMT3B* following siEZH2 treatment. Taken together, these findings imply that *EZH2* (the critical component of PRC2) is also directly involved in the transcriptional regulation of *HOTAIR* and several other downstream genes in a hyperglycemic environment. Of note, the differences observed between *ET-1*, *MCP-1*, and *IL-1β* RNA expressions for DZNep and siEZH2 treatments may have been due to the particular selectivity profile for each compound (i.e. siRNAs are generally more specific in gene knockdown versus global inhibitors for histone methylation).

On the other hand, the knockdown of *CTCF* in HRECs cultured with HG produced differential expressions of several genes, including significant increases in *HOTAIR*, *ANGPTL4*, *EED*, *IL-1β*, *Cytochrome B*, *HOXD3*, and *HOXD10* and significant decreases in *ET-1*, *EZH2*, *PARP1*, *MCP-1*, *DNMT1*, and *P300* transcripts when compared to their respective SCR HG controls. As well, no significant differences were observed for *VEGF-A*, *SUZ12*, *DNMT3A*, and *DNMT3B* transcripts after the silencing of *CTCF* in HRECs cultured with HG (**Figure 3.11** and **Appendix B, Supplemental Figure 12**). Based on these results, our collective findings allude to the diverse roles of *CTCF* in gene regulation, where siRNA-mediated depletion of *CTCF* can either augment glucose-induced expressions of certain genes (possibly through the inability of CTCF to block the interaction between enhancers and promoters, leading to subsequent gene induction) or repress the expressions of select genes, which may occur due to changes in chromatin architecture that prevent gene induction [51,52].

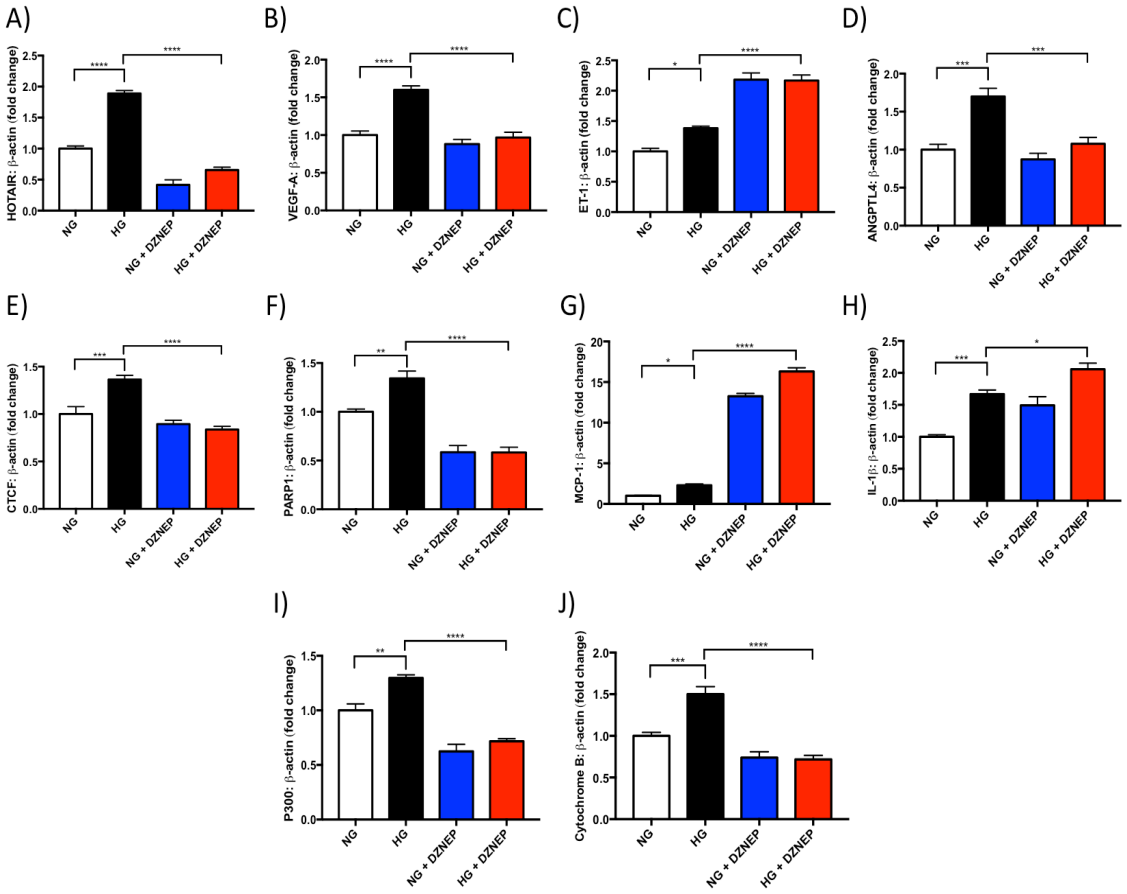

Figure 3.10. Histone methylation differentially regulates *HOTAIR* and its downstream targets. HRECs were pre-treated with DZNep (a global histone methylation inhibitor) prior to NG or HG culture for 48 hours. RT-qPCR was then used to analyze the expressions of **(A)** *HOTAIR*, **(B)** *VEGF-A*, **(C)** *ET-1*, **(D)** *ANGPTL4*, **(E)** *CTCF*, **(F)** *PARP1*, **(G)** *MCP-1*, **(H)** *IL-1β*, **(I)** *P300*, and **(J)** *Cytochrome B*. β-actin was used as an internal control. Statistical significance was assessed using one-way ANOVA for multiple comparisons, followed by Tukey's post hoc test (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, or n.s.= not significant). Data represents the mean ± SEM of 3 independent experiments (n=6/group).

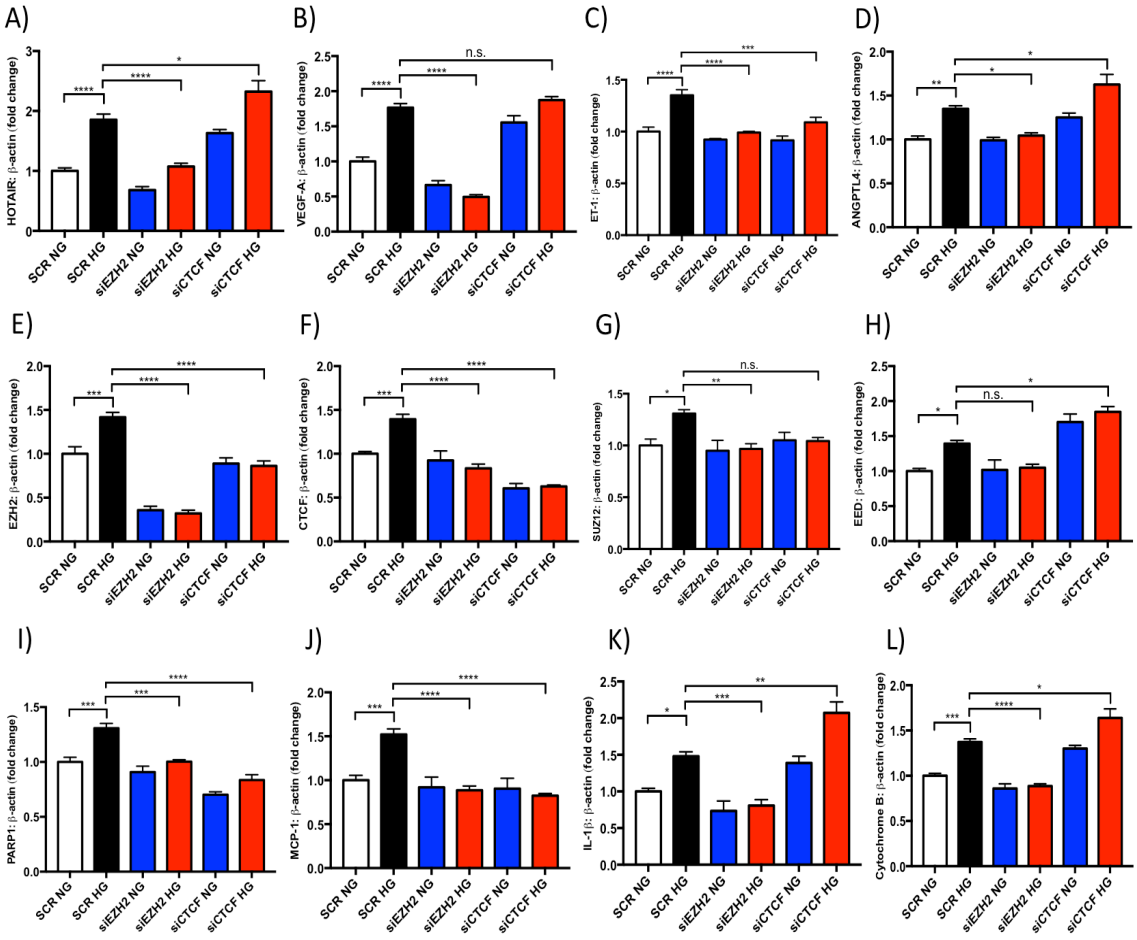

Figure 3.11. EZH2 and CTCF are directly involved in the transcriptional regulation of *HOTAIR* and several other downstream genes. RT-qPCR analyses of **(A)** *HOTAIR*, **(B)** *VEGF-A*, **(C)** *ET-1*, **(D)** *ANGPTL4*, **(E)** *EZH2*, **(F)** *CTCF*, **(G)** *SUZ12*, **(H)** *EED*, **(I)** *PARP1*, **(J)** *MCP-1*, **(K)** *IL-1β*, and **(L)** *Cytochrome B* expressions following the administration of scrambled (SCR) siRNAs, siEZH2, or siCTCF in HRECs subjected to 48 hours of NG or HG culture. EZH2 is the catalytic subunit of PRC2 (a critical histone methyltransferase) and CTCF is an important epigenetic transcription factor involved in the direct regulation of genes. β-actin was used as an internal control. Statistical significance was assessed using one-way ANOVA for multiple comparisons, followed by Tukey's post hoc test (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, or n.s.= not significant). Data represents the mean ± SEM of 3 independent experiments (n=6/group).

3.2.10 *HOTAIR* binds with histone modifying enzymes and regulates VEGF transcription

We then examined for possible direct relationships shared between *HOTAIR* and critical histone modifying enzymes in HRECs and thus, we performed a RNA immunoprecipitation (RIP). In comparison to IgG controls, our results demonstrated that *HOTAIR* RNA levels were distinctly enriched in the precipitated anti-EZH2 and P300-antibody fractions obtained from HRECs cultured in HG (p<0.0001; **Appendix B, Supplemental Figure 13**), suggesting that HG promotes strong *HOTAIR* binding associations to EZH2 and P300. Our findings are in agreement with previous reports that have documented similar relationships with *HOTAIR* and these epigenetic mediators [53,54].

Next, to demonstrate the involvement of histone modifications at the genomic level, we performed chromatin immunoprecipitation (ChIP)-qPCR using antibodies for IgG (negative control), RNA polymerase II (indicative of transcriptional activity; Pol II), H3K27me3 (indicative of transcriptional repression), and pan-H3K9/14/18/23/27 acetylation (indicative of transcriptional activation). We treated HRECs with siHOTAIR and employed primers that specifically spanned across the proximal and distal promoter regions of *VEGF-A* for subsequent ChIP-qPCR analyses. Accordingly, compared to NG controls, RNA Pol II levels were significantly enriched in both the distal (p<0.0001; **Figure 3.12A**) and proximal promoter (p<0.0001; **Figure 3.12D**) regions of *VEGF-A* in HRECs cultured with HG; whereas, the knockdown of *HOTAIR* can markedly reduce Pol II enrichment in these regions compared to HG controls. Conversely, under HG stimulation, significant reductions of H3K27me3 enrichment were observed in both *VEGF-A* distal (p<0.0001; **Figure 3.12B**) and proximal promoter (p<0.0001; **Figure 3.12E**) regions and siHOTAIR treatment significantly reversed glucose-induced reductions of H3K27me3 in the *VEGF-A* promoter. Moreover, when compared to NG controls, HG conditions significantly augmented the enrichment of H3K9/14/18/23/27 acetylation in both *VEGF-A* promoter regions, while the knockdown of *HOTAIR* significantly prevented glucose-induced increases in pan-acetylation levels of H3K9/14/18/23/27 across the *VEGF-A* distal (p<0.0001; **Figure**

3.12C) and proximal promoter (p<0.0001; **Figure 3.12F**) regions, compared to HG groups. Hence, we concluded that a dynamic interplay exists between *HOTAIR*, histone–modifying enzymes, and RNA Pol II in the transcriptional regulation of genes, such that *HOTAIR* may have an active role in modulating the epigenome during hyperglycemic stress. Of note, no significant differences were observed between IgG NG and HG groups, confirming the specificity of the antibodies.

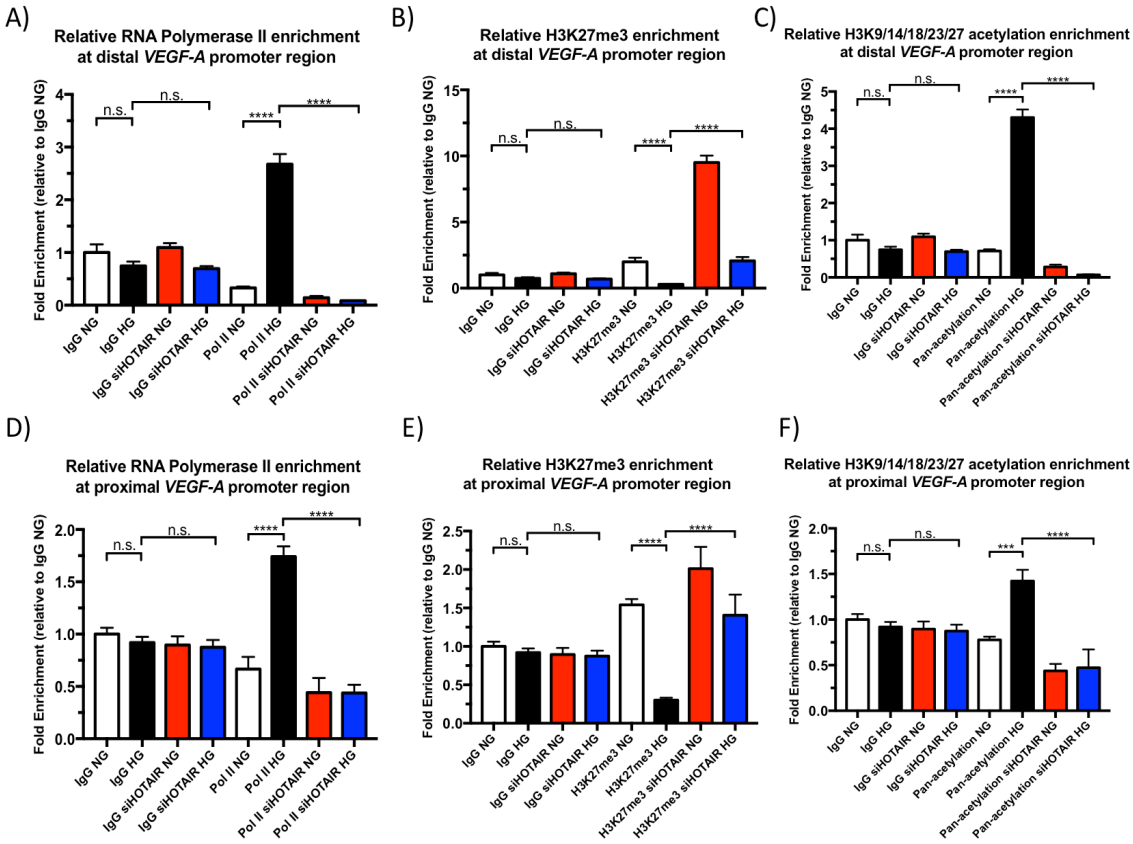

Figure 3.12. *HOTAIR* can govern the transcriptional status of *VEGF-A* in hyperglycemic environments. ChIP-qPCR analyses examining the enrichment of **(A,D)** RNA polymerase II (Pol II), **(B,E)** tri-methylation of lysine 27 in histone 3 (H3K27me3; a repressive histone mark), and **(C,F)** acetylation of lysines 9, 14, 18, 23, and 27 in histone 3 (H3K9/14/18/23/27; an active histone mark) in the distal (top panel) and proximal (bottom panel) regions of *VEGF-A*. In order to determine the role of *HOTAIR* in transcriptional regulation, HRECs were pre-treated with scrambled (SCR) siRNAs or siHOTAIR and subsequently cultured in NG or HG for 48 hours prior to ChIP-qPCR experimentation. IgG antibodies were used as a negative control and β-actin was used as an internal control. Statistical significance was assessed using one-way ANOVA for multiple comparisons, followed by Tukey's post hoc test

(****p<0.0001 or n.s.= not significant). Data represents the mean ± SEM of 3 independent experiments (n=3/group).

3.2.11 Duration-dependent and glucose-induced alterations of CpG methylation patterns across the *HOTAIR* gene were not observed in HRECs

In order to investigate glucose-induced implications of DNA methylation on *HOTAIR* regulation, we incubated HRECs in NG and HG conditions for durations of 2 and 7 days and then performed a genome-wide DNA methylation experiment using Infinium EPIC arrays and quality controls. Following the detection of >860,000 CpG sites (probes), we exclusively selected CpG sites that spanned across the *HOTAIR* gene (5kb upstream to 1kb downstream of the gene), which corresponded to 59 probes (**Figure 3.13**). We found that the average methylation intensity was generally lower for a majority of the probes (β-values < 0.3), except for 7 probes where slightly greater methylation intensities were observed (0.2 < β-values < 0.5; these sites mainly corresponded to North/South Shelf and North/South Shore regions; **Figure 3.13**). Furthermore, when examining the methylation patterns between the various groups across the *HOTAIR* genomic region (chromosome 12: 54,351,994 to 54,373,040; **Appendix B, Supplemental Figure 14A**), it was interesting to observe that a stable DNA methylation pattern persisted across all groups despite different culture durations (2 and 7 days) and glucose concentrations (NG and HG). However, of note, although not statistically significant, HRECs stimulated with HG at both 2 and 7 days displayed a slight trend towards the reduction of DNA methylation intensities in the *HOTAIR* promoter, compared to their respective NG controls (**Appendix B, Supplemental Figure 14B**). Nevertheless, these findings may allude to the stable epigenetic nature of DNA methylation marks in HRECs during hyperglycemic stress [55].

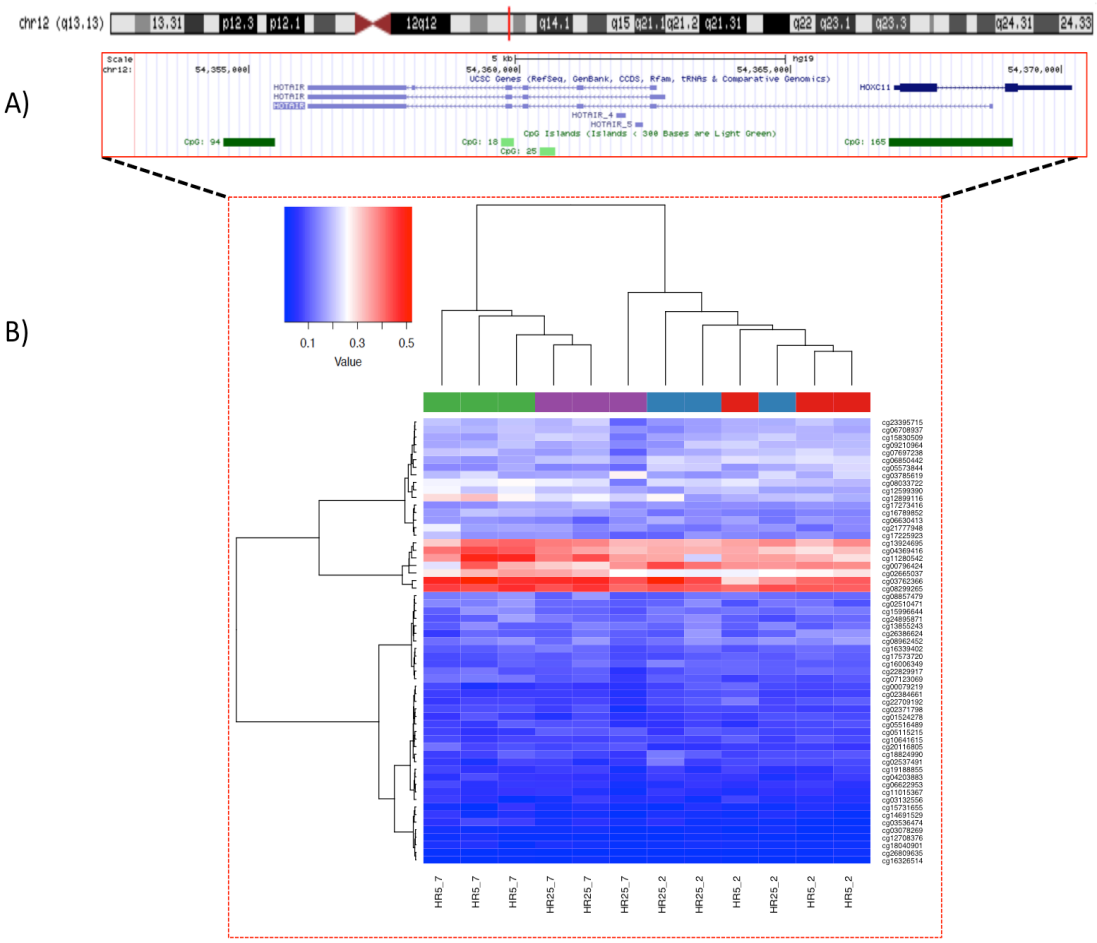

Figure 3.13: DNA methylation profiling of HRECs. (A) Information on the human *HOTAIR* gene according to the UCSC database, where the position of *HOTAIR* is located on chromosome 12: 54,356,092-54,368,740 (hg19) and its approximate size (for transcript variant 1) is 12,649 nucleotides containing a total of 6 exons (90). **(B)** Unsupervised hierarchical clustering with heatmap using all the CpGs in HRECs that span across HOTAIR, which amounted to 59 probes. Interestingly, there are no distinctions between cells treated with different concentrations of glucose (5 mM versus 25 mM) and the duration of culture (2 versus 7 days)—alluding to the stable epigenetic nature of DNA methylation in these cells following glucose treatment for different durations. Rows indicated CpGs and columns show the samples; the color scale from blue to red indicates the level of methylation from zero to one (with zero indicating no methylation and one indicating maximum methylation; n= 3 independent samples per group indicated by the top panel colors; HR5_2= HRECs cultured in 5 mM glucose for 2 days, HR25_2= HRECs cultured in 25 mM glucose for 2 days, HR5_7= HRECs cultured in 5 mM glucose for 7 days, and HR25_7= HRECs cultured in 25 mM glucose for 7 days).

3.2.12 Blockade of DNA methyltransferases differentially regulates the expressions of *HOTAIR* and some of its targets

We then wanted to examine the cause-effect relationship of genome-wide DNA methylation on the expressions of *HOTAIR* and its downstream targets. Accordingly, we pre-treated HRECs with the DNA de-methylating agent, 5-Aza-2'-deoxycytidine (5-aza-dC) prior to NG or HG culture. Following 5-aza-dC administration and compared to HG controls, *DNMT1*, *DNMT3A*, and *DNMT3B* RNA levels were reduced by ~69%, ~58%, and ~69%, respectively (p<0.0001; **Appendix B, Supplemental Figures 15A-C**). Accompanying the significantly reduced expressions of *DNMTs* in 5-aza-dC-treated HRECs, we also observed significant elevations in *HOTAIR*, *ET-1*, *CTCF*, *Cytochrome B*, *MCP-1*, *IL-1β*, *HOXD3*, and *HOXD10* transcripts, while no significant differences were observed for *ANGPTL4*, *P300*, and *PARP1* expressions (**Figure 3.14** and **Appendix B, Supplemental Figure 15**). Intriguingly, however, globally inhibiting the expressions of DNMTs significantly prevented glucose-induced increases in *VEGF-A* RNA expressions (p<0.0001; **Figure 3.14B**), which is in keeping with previous observations documented by others [56,57].

To further confirm the findings from our 5-aza-dC experiments, we specifically silenced *DNMT1* (a constitutively expressed DNMT) using a siRNA-mediated approach. With a ~71% knockdown in *DNMT1* RNA levels following the administration of siDNMT1 and HG (p<0.0001; **Appendix B, Supplemental Figure 16A**), *DNMT3A* and *DNMT3B* also exhibited significant reductions in transcript expressions by ~43% (p=0.0005; **Appendix B, Supplemental Figure 16B**) and ~51% (p<0.0001; **Appendix B, Supplemental Figure 16C**), respectively. In parallel, significant increases in RNA expressions were observed for *HOTAIR*, *ET-1*, *CTCF*, *Cytochrome B*, *PARP1*, *IL-1β*, *HOXD3*, and *HOXD10* after the knockdown of *DNMT1* in HG-cultured cells, relative to SCR HG controls (**Figure 3.15** and **Appendix B, Supplemental Figure 16**). Although no significant differences were observed for *ANGPTL4*, *P300*, and *MCP-1* transcripts after knockdown in HG conditions, significant reductions in *VEGF-A* transcripts still remained in siDNMT1-treated

HRECs cultured with HG (p<0.0001; **Figure 3.15B**), confirming the observations from our 5-aza-dC experiments. Indeed, it may be possible that depending on the genomic location, the inhibition of DNA methylation can have varying methylating effects on distal or intragenic regulatory elements with different degrees of CpG density, which subsequently dictate the regulation of gene expression [58]. Nevertheless, our findings suggest that DNA methylation is critically implicated in the regulation of *HOTAIR* and its target molecules in hyperglycemic environments.

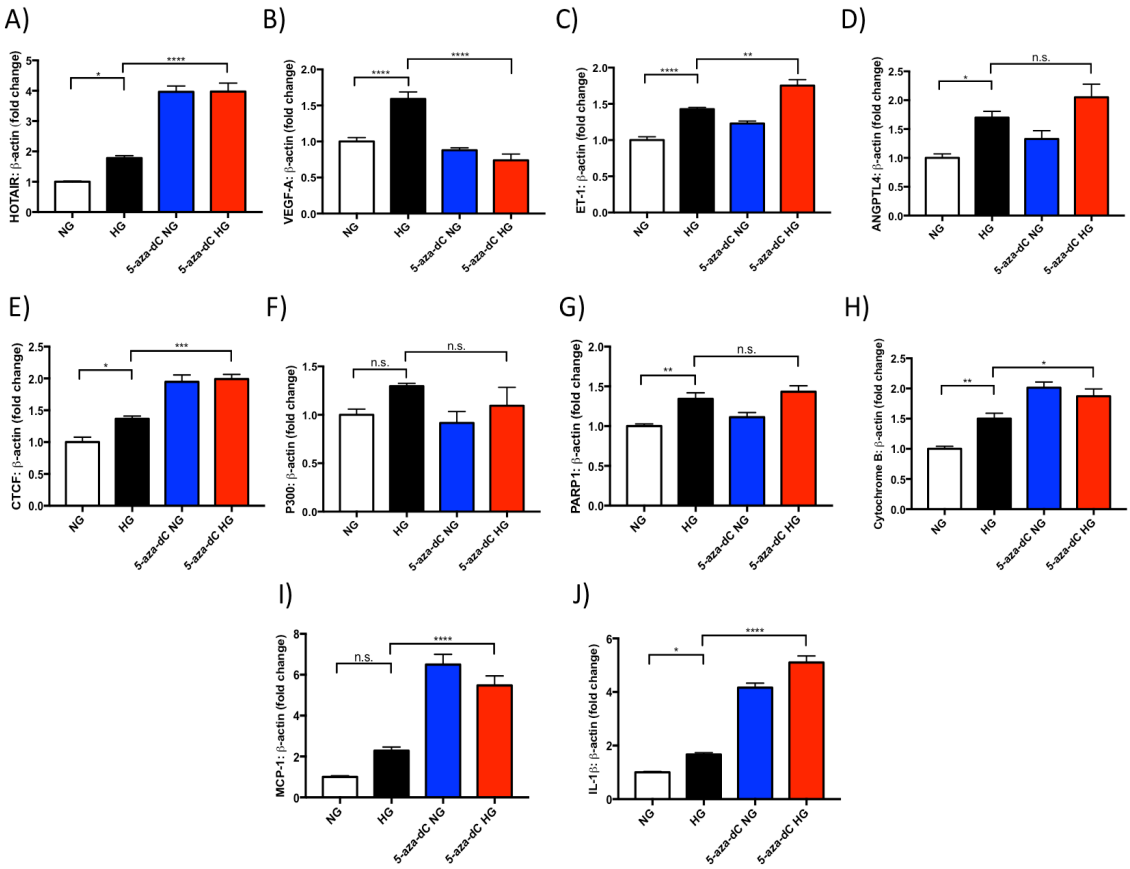

Figure 3.14. Global inhibition of DNA methyltransferases (DNMTs) can differentially regulate the expressions of *HOTAIR* and its targets. HRECs were pre-treated with 5-aza-dC (a pan-DNMT inhibitor) prior to NG or HG culture for 48 hours. RT-qPCR was then used to analyze the expressions of **(A)** *HOTAIR*, **(B)** *VEGF-A*, **(C)** *ET-1*, **(D)** *ANGPTL4*, **(E)** *CTCF*, **(F)** *P300*, **(G)** *PARP1*, **(H)** *Cytochrome B*, **(I)** *MCP-1*, and **(J)** *IL-1β*. β-actin was used as an internal control. Statistical significance was assessed using one-way ANOVA for multiple comparisons, followed by Tukey's post hoc test (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, or n.s.= not significant). Data represents the mean ± SEM of 3 independent experiments (n=6/group).

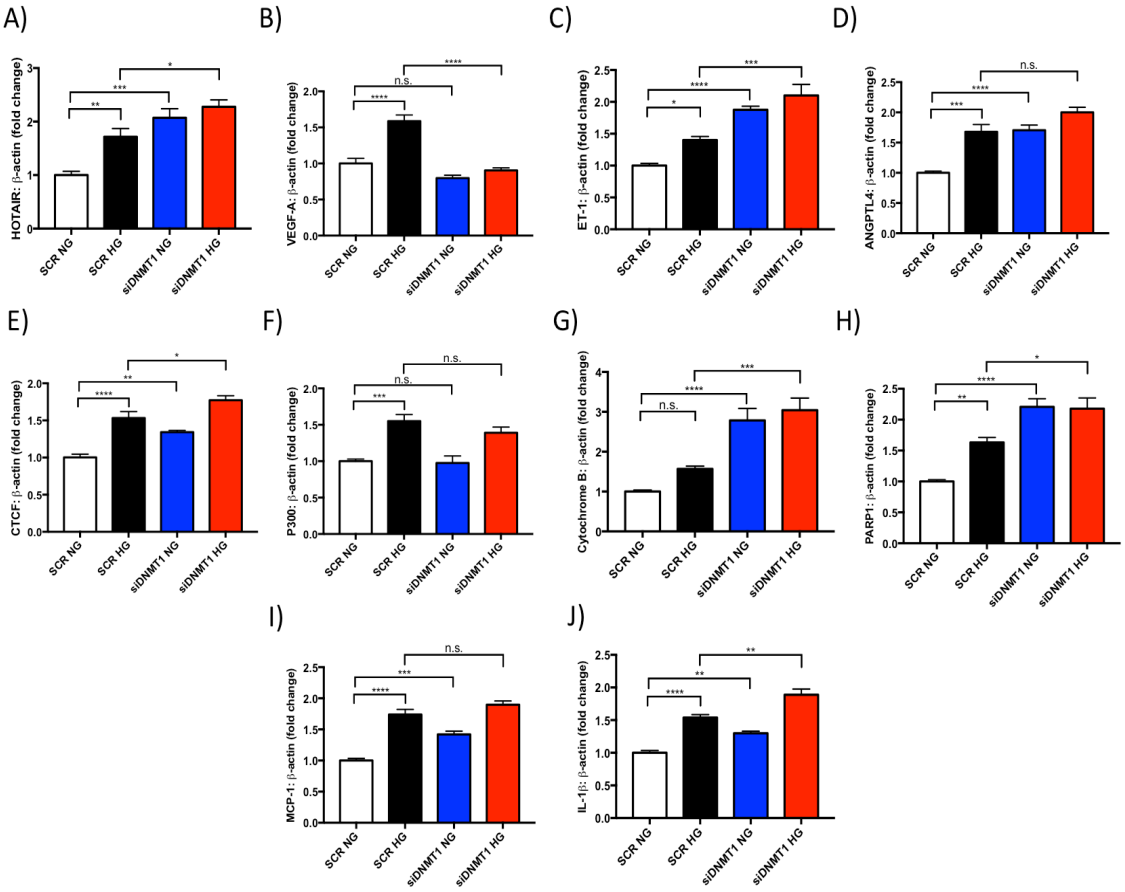

Figure 3.15. Selective knockdown of *DNMT1* can impact the expressions of *HOTAIR* and some of its downstream targets *in vitro*. RT-qPCR analyses of **(A)** *HOTAIR*, **(B)** *VEGF-A*, **(C)** *ET-1*, **(D)** *ANGPTL4*, **(E)** *CTCF*, **(F)** *P300*, **(G)** *Cytochrome B*, **(H)** *PARP1*, **(I)** *MCP-1*, and **(J)** *IL-1β* expressions following the administration of scrambled (SCR) siRNAs or siDNMT1 in HRECs subjected to 48 hours of NG or HG culture. DNMT1 is a constitutively expressed DNA methyltransferase. β-actin was used as an internal control. Statistical significance was assessed using one-way ANOVA for multiple comparisons, followed by Tukey's post hoc test (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, or n.s.= not significant). Data represents the mean ± SEM of 3 independent experiments (n=6/group).

3.3 Discussion

The process of physiological angiogenesis is critical for vascular homeostasis, since the formation of new blood vessels from pre-existing vasculature provides sufficient blood flow, oxygen, and nutrients to metabolically active tissues [59]. A delicate

balance between angiogenic and angiostatic factors regulates angiogenesis and any shifts in this balance will ultimately dictate the activity of the vascular network. However, in disease states such as DR, chronic hyperglycemia drastically alters the spatial and temporal kinetics of numerous biochemical and metabolic pathways that promote active pathological angiogenesis in the eye, subsequently leading to vision-threatening complications [4,7-10]. Although it is well-established that pathological angiogenesis, as well as macular edema early in the disease progression, is primarily mediated by VEGF-A in DR [60], limitations in current anti-VEGF therapies suggest that our understanding of the molecular networks underlying the pathobiology of ocular angiogenesis remains far from complete. As such, deciphering and exploring novel molecular mediators in this network may allow for the development of better-targeted therapies and diagnostic biomarkers. In recent years, lncRNAs have garnered considerable attention due to their critical regulatory capabilities in a number of diseases, particularly in cancer. In the context of DR however, several lncRNAs have been identified in the diabetic retina but very few lncRNAs have been comprehensively characterized.

In the present study, we provide for the first time direct evidence that the lncRNA *HOTAIR* mediates angiogenesis in DR through a complex web of epigenetic mechanisms involving histone methylation, histone acetylation, DNA methylation and the CTCF transcription factor. Using cell culture and animal models of diabetes, the targeted knockdown of *HOTAIR* via siRNAs made it evident that the inhibition of this lncRNA significantly prevented glucose-induced increases of several angiogenic factors, diabetes-associated molecules, and critical epigenetic mediators. Mechanistically, *HOTAIR* can contribute to glucose-induced mitochondrial and DNA damage and this lncRNA can also facilitate the epigenetic activation of VEGF-A by recruiting RNA polymerase II and acetylation writers (such as P300) to the promoter regions of *VEGF-A*, resulting in heightened VEGF-A expressions and subsequent angiogenesis. As well, *HOTAIR* and its target molecules can be differentially regulated through histone and DNA methyltransferases and CTCF. Collectively, our findings provide in-depth mechanistic insights into the epigenetic paradigm behind *HOTAIR*'s regulatory capabilities in DR and based on its clinical implications,

HOTAIR may also serve as a novel diagnostic biomarker and therapeutic target for DR.

LncRNAs can exhibit a wide range of stability profiles that are comparable to protein-coding transcripts. In fact, using genome-wide microarrays, lncRNA half-lives (ranging from $t^{1/2}$< 2 hours to $t^{1/2}$> 16 hours) were shown to correlate with several lncRNA features, including genomic location, splicing, GC percentage, and subcellular localization [30]. LncRNAs that were either spliced, localized in the cytoplasm, classified as intergenic or cis-antisense, or contained high GC percentage were considered more stable than those lncRNAs that were unspliced, classified as intronic, nuclear-retained, or contained low GC percentage. Interestingly, the degree of stability is critical for regulating lncRNA function, where lncRNAs (particularly those residing in the nucleus) with short half-lives have demonstrated rapid turnover in order to contribute to the dynamic processes that they regulate [30,61]. Published studies have made it evident that cell-specific variations exist for the half-life of *HOTAIR*, in which trophoblasts can exhibit a $t^{1/2}$ of ~7.7 hours [62], while HeLA cells demonstrate a $t^{1/2}$ of ~1.3 hours [63]. In the present study, although we did not examine *HOTAIR* stability in HRECs, we instead investigated *HOTAIR* expression levels across various time-points and observed oscillating *HOTAIR* patterns, with significant glucose-induced elevations at the 48-hour mark. Intriguingly, our results are in keeping with similar expression patterns observed for another lncRNA, *MALAT1*, in HRECs [28], which suggests that HREC-derived lncRNAs may have distinct and overlapping expression profiles compared to other cell types. Furthermore, based on a previous report documenting the long-lasting, activating, epigenetic changes that occur in the NF-KB-*p65* promoter region following transient hyperglycemia [64], it may be plausible that initial glucose-induced spikes of *HOTAIR* expressions could similarly induce persisting epigenetic changes that involve altered gene expressions of its downstream targets, despite reduced *HOTAIR* expressions at later time-points. Nevertheless, future research is warranted to further validate *HOTAIR*'s role in metabolic memory.

The subcellular localization of lncRNAs can provide insights into their potential functions. For example, lncRNAs localized in the cytoplasm are typically implicated in post-transcriptional modifications that govern mRNA stability and translation [65], while nuclear-retained lncRNAs can be involved in transcriptional regulation [66], organization of nuclear architecture [67], and alternative splicing [68]. On the other hand, certain lncRNAs can also be found in both the nucleus and cytoplasm [69], where these versatile RNA molecules shape the epigenome, regulate organelle formation and function, and influence transcription and translational processes [70]. In order to elucidate the subcellular localization of *HOTAIR* in ECs and develop potential insights into its regulatory capabilities, we performed RNA FISH and found that *HOTAIR* is present in both nuclear and cytoplasmic compartments. Our findings are in keeping with previous studies that depict the dual localization patterns for *HOTAIR* in other cells, such as cutaneous squamous cell carcinoma [71] and fibroblasts [72]. Extending our RNA FISH findings and on the basis of our mechanistic-based *in vitro* experiments, it quickly became evident that *HOTAIR* can regulate the expressions of several angiogenic and diabetes-associated genes, while simultaneously targeting enzymes involved in histone modification and DNA methylation processes. Lending further credence to our observations, earlier reports have demonstrated that *HOTAIR* is directly involved in PRC2-mediated silencing of the chromatin, where *HOTAIR* depletion in fibroblasts can lead to a loss of *HOXD* silencing and H3K27me3 by PRC2 [53]; while, *HOTAIR* overexpression in breast cancer cells can promote cellular invasiveness through selective, genome-wide re-targeting of PRC2 and H3K27me3, which ultimately enables the expression of genes that are conducive to cancer invasion [47]. Indeed, additional reports have suggested that *HOTAIR* may function as a molecular scaffold in order to regulate gene expressions through its direct interactions with epigenetic mediators, including PRC2 [73] and histone demethylases (LSD1) [39]. Based on these findings and the reciprocal relationships documented between this lncRNA and other epigenetic mechanisms in cancer (such as DNA methylation [74] and histone acetylation [75]), our findings imply that *HOTAIR* also possesses a similar regulatory profile as a critical epigenomic modulator in the pathogenesis of DR.

In addition to its role as a transcriptional regulator, we demonstrated that *HOTAIR* could also pathogenetically contribute to glucose-induced mitochondrial and oxidative DNA damage in ECs, which further highlight the dynamism of *HOTAIR* in diabetes. Although these roles of *HOTAIR* have not been previously investigated in the context of DR, other studies have shown that *HOTAIR* can mediate DNA damage response in ovarian cancer cells, through the regulation of NF-kB activation [76], and maintain mitochondrial function in HeLa cells [40]. Intriguingly, the findings by Zheng et al further demonstrate that *HOTAIR* knockdown in HeLA cells is accompanied with several mitochondrial aberrations, including dysregulations of 32 proteins associated with mitochondrial function, decreased Ubiquinol-Cytochrome C Reductase Complex III Subunit VII expressions, elevated mitochondrial swelling, reduced glucose uptake, and decreased $\Delta\Psi M$ [40]. In contrast to these findings, we observed that *HOTAIR* knockdown in HRECs could protect the mitochondria in NG and HG environments as evidenced by higher $\Delta\Psi Ms$ and decreased *Cytochrome b* expressions (a fundamental component for the assembly and function of complex III [77]), compared to SCR HG controls. Although it is likely that cell-specific regulations may exist for *HOTAIR*, another possible explanation for our results could be attributed to certain stress-induced mechanisms involving mitophagy and autophagy. More precisely, prolonged hyperglycemia has been reported to evoke significant mitochondrial oxidative stress and membrane depolarization in Müller cells, consequently leading to mitochondrial fragmentation [78]. Following the induction of mitochondrial dysfunction and fragmentation, increased levels of reduced Cytochrome b, along with specific mitophagy factors (including autophagy-related genes, *ATG11* and *ATG32*) have been shown to trigger non-specific autophagy [79]. Interestingly, a recent study has also demonstrated that *HOTAIR* can promote autophagy by upregulating the expressions of *ATG7* in pancreatic cancer cells [80], which warrants further research into the mechanisms revolving around *HOTAIR* and mitophagy in diabetes.

Aberrations in the microvasculature represent one of the earliest pathological manifestations of DR. In fact, chronic hyperglycemic exposure can compromise the function of ECs and the integrity of the endothelium in the retinal microvasculature, which consequently leads to the extravasation of plasma constituents into the retina

due to blood-retinal barrier damage [4]. Coupled with chronic ischemic regions in the retina (known as capillary non-perfusion), endothelium damage and the heightened activation of glucose-induced biochemical pathways further stimulate retinal cells to release inflammatory cytokines and vasoactive factors that cause increased vascular permeability and lay the foundation for pathological angiogenesis in DR [4,81]. For these reasons, we specifically examined retinal ECs in our experiments. Accordingly, glucose can directly induce the expressions of *HOTAIR* and numerous angiogenic factors in ECs and diabetic retinas, while the subsequent knockdown of *HOTAIR* can preserve endothelial cell junctions in HG environments and significantly alleviate early glucose-induced upregulations of angiogenic factors in both *in vitro* and *in vivo* models. Our observations on the pro-angiogenic capabilities of *HOTAIR* are consistent with previous reports in cancer, where *HOTAIR* has been shown to enhance angiogenesis by directly targeting the *VEGF-A* promoter in nasopharyngeal carcinoma cells [32] or through transmission of glioma cell-derived extracellular vesicles into ECs [33]. Hence, based on these findings, it is no surprise that *HOTAIR* is involved in the pathogenesis of DR, possibly through its role in EC dysfunction.

With *HOTAIR* exhibiting pro-oncogenic functions [32,33,46,47,54,71,74-76,80] and potential involvement in diabetic kidney disease [91], several emerging studies are beginning to address the clinical utility of *HOTAIR* as a lncRNA biomarker [82]. We wanted to delineate the clinical significance of this lncRNA in the context of DR. As such, we decided to examine the presence of *HOTAIR* in VH and serum samples because the vitreous fluid can be used to indirectly assess the pathophysiological events that take place in the diabetic retina [83] and circulating serum non-coding RNAs have been previously associated with the pathogenesis of DR [84]. When compared to controls, we found that *HOTAIR* expression levels are significantly upregulated in the VH and serum samples of PDR patients, implying a pathogenetic association between *HOTAIR* and DR. These results are in keeping with our previously published findings for another pathogenetic lncRNA, *MALAT1*, in DR [28]—alluding to the potential overlapping patterns shared between pathogenetic lncRNAs in certain disease states. Furthermore, to the best of our knowledge, this is the first study to identify positive correlations in *HOTAIR* RNA levels between serum

and VH of PDR patients, which raise the possibility of using *HOTAIR* as a potential serum-based biomarker for DR. Although the complete clinical validation of *HOTAIR* as a potential prognostic or diagnostic marker for DR will require larger sample sizes with appropriate power calculations, stringent cut-off criteria and determination of clinically meaningful endpoints, our results simply provide a proof of principle for the pathological significance of *HOTAIR* in DR and further large-scale investigations are undoubtedly warranted.

In summary, this report establishes for the first time that the lncRNA *HOTAIR* is a critical epigenetic regulator of angiogenesis in DR. Mechanistic experiments confirmed *HOTAIR*'s ability to directly regulate the transcriptional expressions of multiple angiogenic factors and DR-associated molecules *in vitro* and *in vivo*, possibly through a complex epigenetic axis involving PRC2/P300/DNMTs/CTCF. In addition to transcriptional regulation, *HOTAIR* may also have implications in other organelle functions, since the knockdown of *HOTAIR* can partially prevent glucose-induced oxidative mitochondrial and DNA damage. We further highlight the clinical significance of *HOTAIR*, where *HOTAIR* RNA expressions in the vitreous fluid and serum strongly correlate with PDR. Taken together, these findings allude to *HOTAIR*'s potential as a possible diagnostic and therapeutic target in DR.

3.4 References

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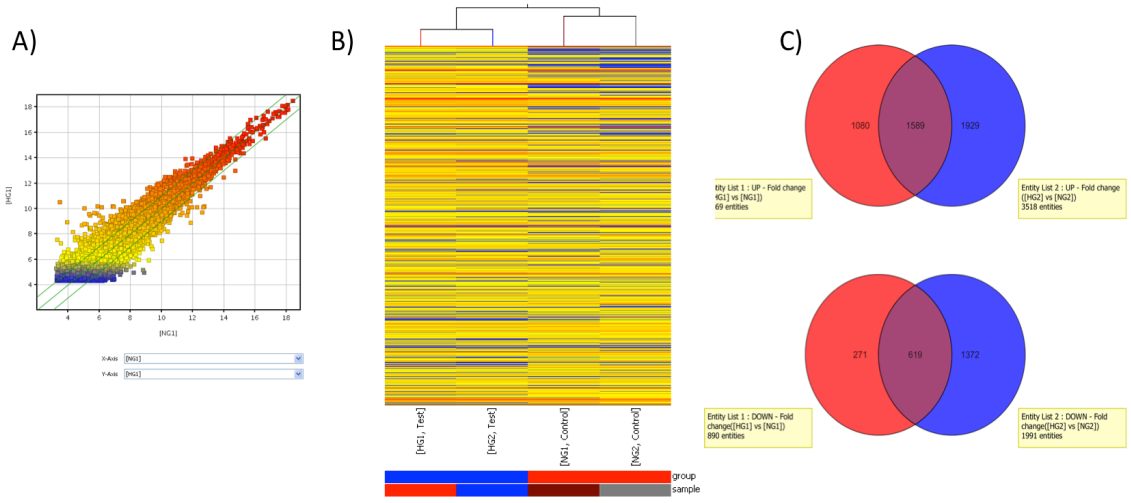
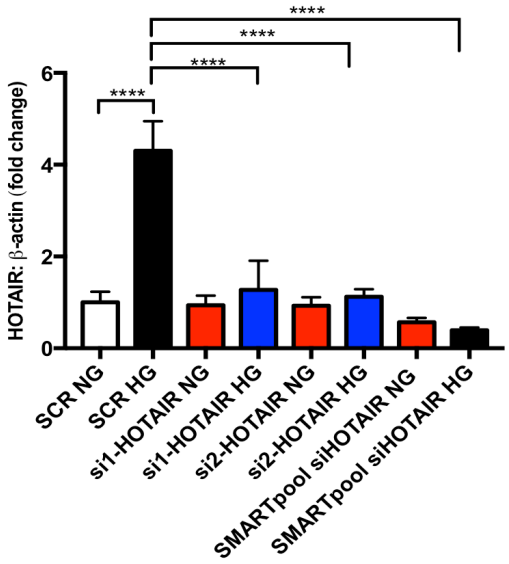
3.5 Appendix B: The long non-coding RNA *HOTAIR* is a critical epigenetic mediator of angiogenesis in diabetic retinopathy



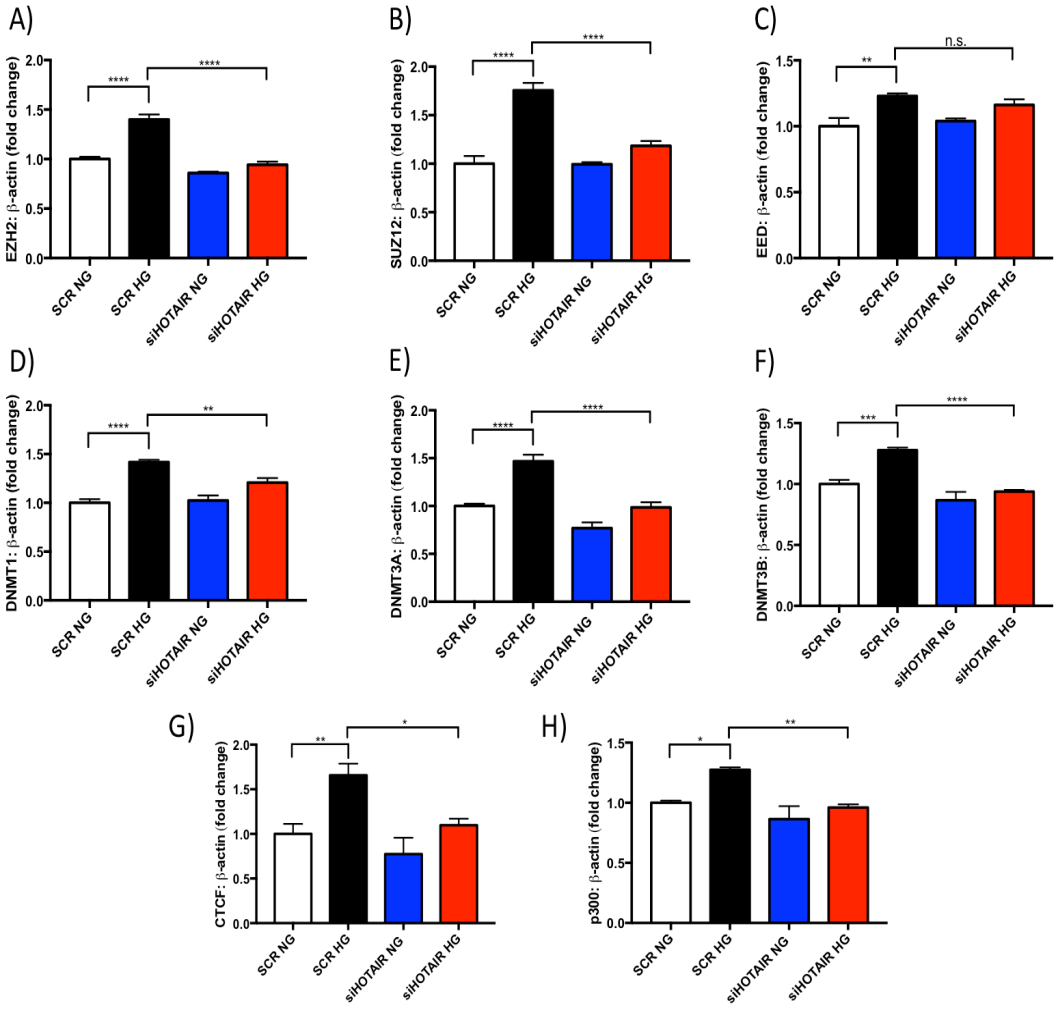
D)

ProbeName	**ASHGA5P015895**
Absolute Fold change ([HG1] vs [NG1])	2.6724913
Regulation and Type	Up; non-coding
Seqname	NR_047517
Gene Symbol	HOTAIR
RNA length	2370
chrom	12
Sequence	GAAGGAAAGCCCTCCAGCCTCCAGGCCCTGCCTTCTGCCTGCACATTCTGCCCTGATTTC

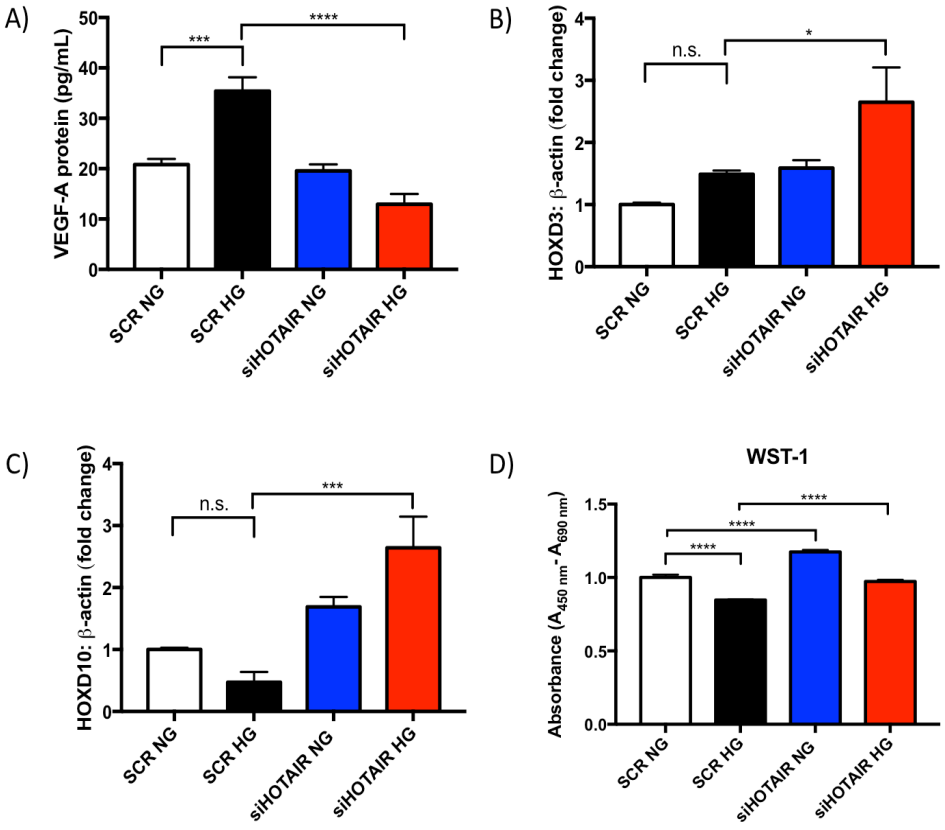
Supplemental Figure 1. LncRNA microarray findings from HRECs cultured in NG or HG for 48 hours (GEO: GSE122189). (A) Scatter plot demonstrates lncRNA expressions between normal glucose (NG) and high glucose (HG) replicates. In general, the scatter plot is a visualization method used for assessing the lncRNA expression variation (or reproducibility) between the two compared samples (or groups). The values of X and Y axes in the scatter plot are the normalized signal values (log2 scaled) of two samples or the averaged normalized signal values (log2 scaled) for two groups. The green lines are fold change lines (the default fold change value given is 2.0). The lncRNAs above the top green line and below the bottom green line indicates more than 2.0 fold change of lncRNAs between the two compared groups or samples. **(B)** Hierarchical clustering for the lncRNAs in all sample groups. "Red" indicates high relative expression, and "blue" indicates low relative expression. **(C)** Venn diagrams depicting the total number of lncRNAs that were upregulated (top) or downregulated (bottom) between NG and HG replicates. **(D)** Specific microarray readout for *HOTAIR*.



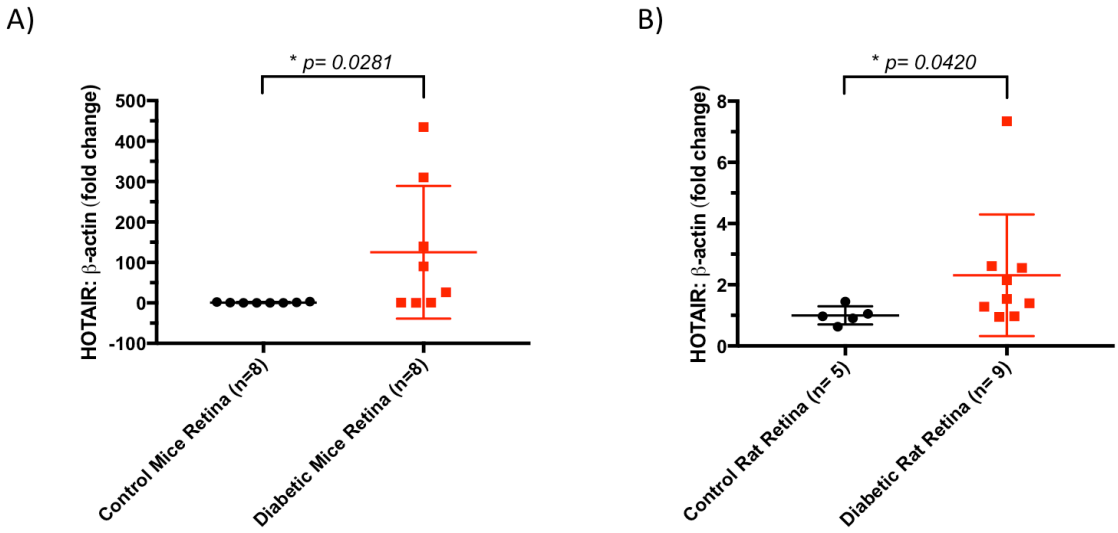
Supplemental Figure 2. *HOTAIR* knockdown using three different siRNAs. HRECs were pre-treated with scrambled (SCR) siRNAs or specific siRNAs targeting *HOTAIR* prior to NG or HG culture for 48 hours. RT-qPCR was then used to analyze the expressions of *HOTAIR*. β-actin was used as an internal control. Statistical significance was assessed using one-way ANOVA for multiple comparisons, followed by Tukey's post hoc test (****p<0.0001). Data represents the mean ± SEM of 3 independent experiments (n=6/group).



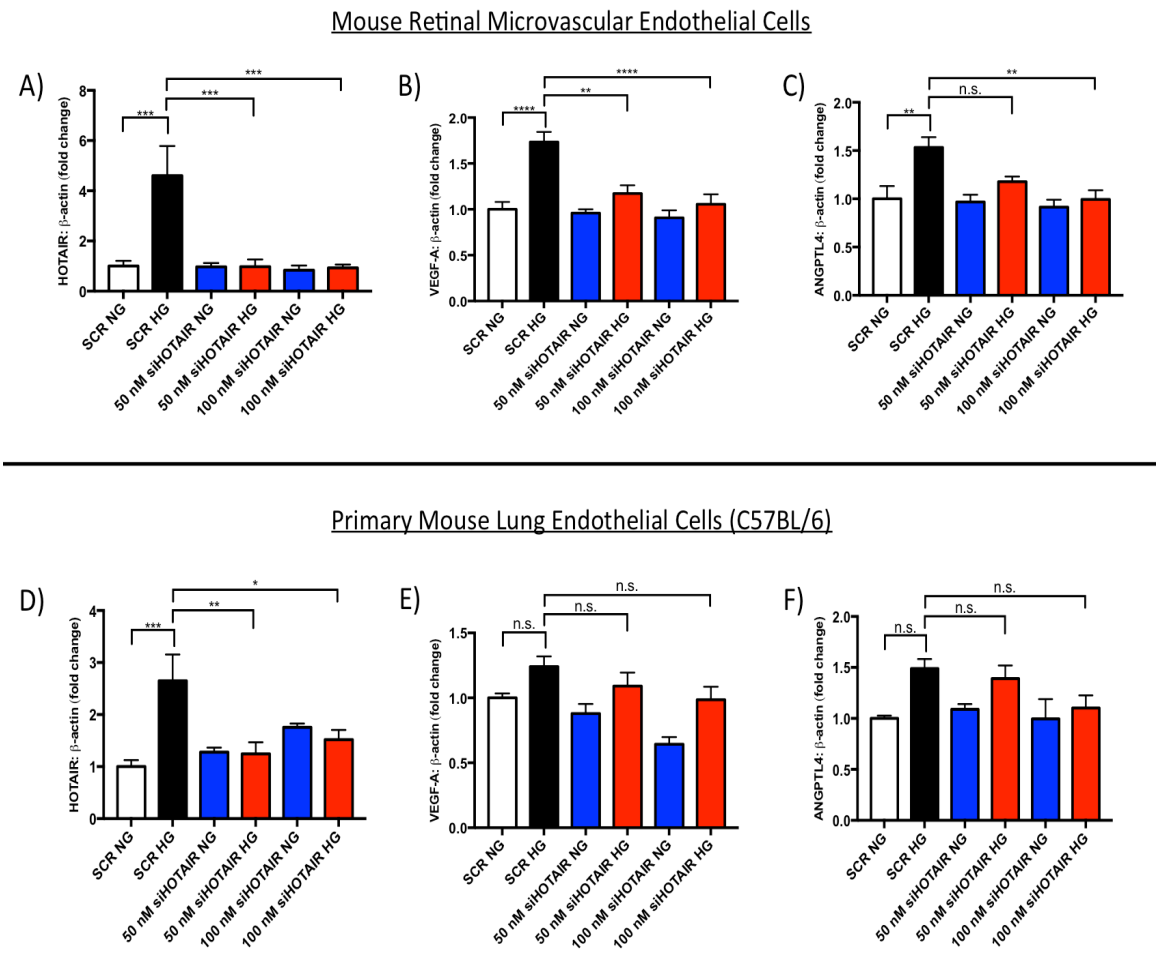
Supplemental Figure 3. *HOTAIR* knockdown can prevent the induction of several epigenetic mediators in hyperglycemic environments. RT-qPCR analyses of **(A)** *EZH2*, **(B)** *SUZ12*, **(C)** *EED*, **(D)** *DNMT1*, **(E)** *DNMT3A*, **(F)** *DNMT3B*, **(G)** *CTCF*, and **(H)** *P300* expressions following the administration of SCR siRNA or siHOTAIR in HRECs subjected to 48 hours of NG or HG culture. β-actin was used as an internal control. Statistical significance was assessed using one-way ANOVA for multiple comparisons, followed by Tukey's post hoc test (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, or n.s.= not significant). Data represents the mean ± SEM of 3 independent experiments (n=6/group).



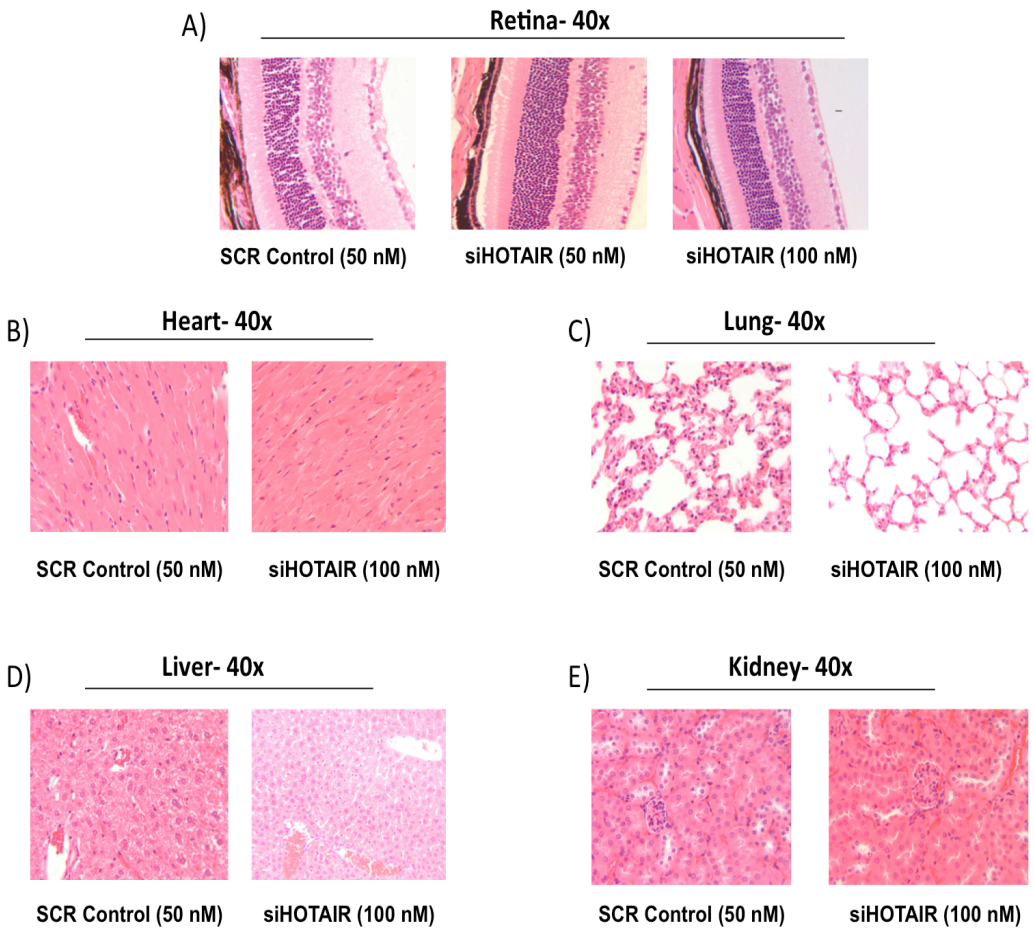
Supplemental Figure 4. *HOTAIR* knockdown can reduce VEGF-A proteins, improve cellular viability, and prevent glucose-induced decreases of *HOXD* loci in HRECs. (A) VEGF-A ELISA results (expressed as pg/mL) from HRECs that were pre-treated with SCR siRNA or siHOTAIR and subjected to NG or HG culture for 48 hours. **(B,C)** RT-qPCR analyses of *HOXD3* and *HOXD10* expressions following *HOTAIR* knockdown. β-actin was used as an internal control. **(D)** WST-1 findings for SCR or siHOTAIR-treated HRECs. Statistical significance was assessed using one-way ANOVA for multiple comparisons, followed by Tukey's post hoc test (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, or n.s.= not significant). Data represents the mean ± SEM of 3 independent experiments (n=6/group).



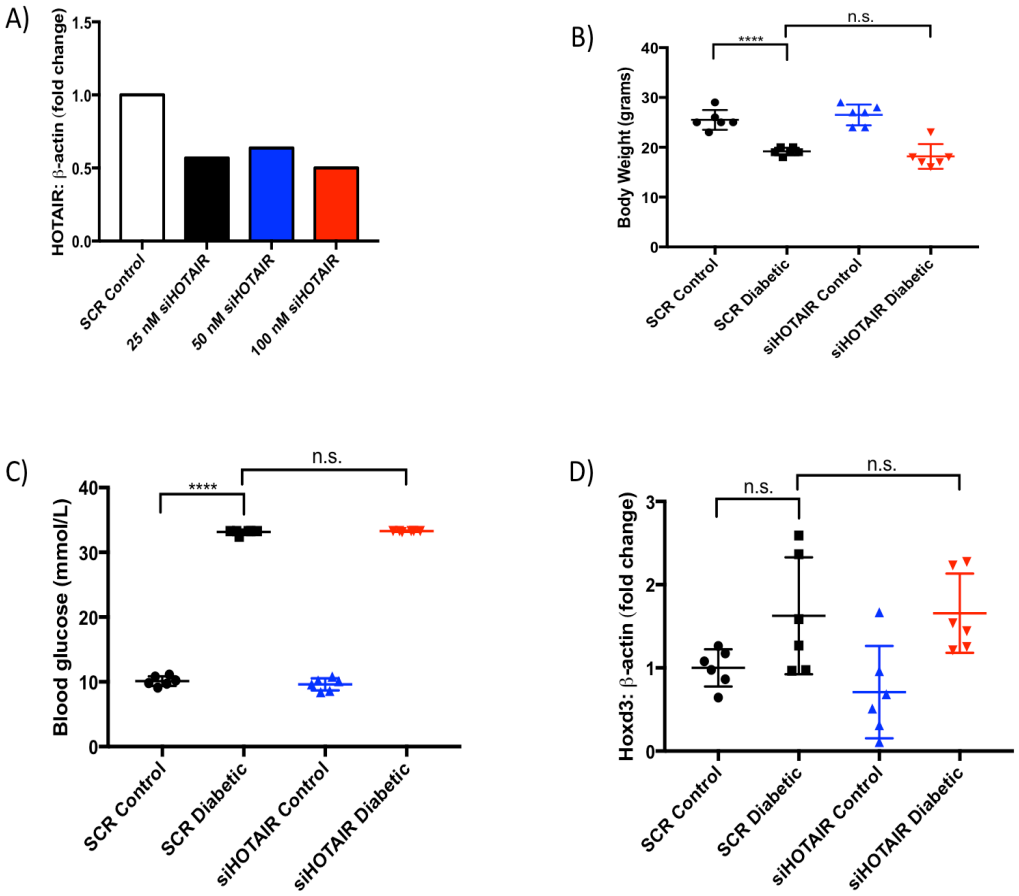
Supplemental Figure 5. *HOTAIR* is significantly elevated in the retinas of diabetic animals at 2 months. Non-diabetic (control) and streptozotocin-induced diabetic C57BL/6J mice or Sprague-Dawley rats were followed for 2 months. Retinal tissues were isolated and extracted for RNA. RT-qPCR was employed to analyze retinal *Hotair* expressions in **(A)** mice and **(B)** rats. β-actin was used as an internal control. Statistical significance was assessed using the Mann-Whitney U test. Data represents the mean ± SD (n=8 per control or diabetic mice group, n=5 per control rat group or n=9 per diabetic rat group; **p*<0.05).



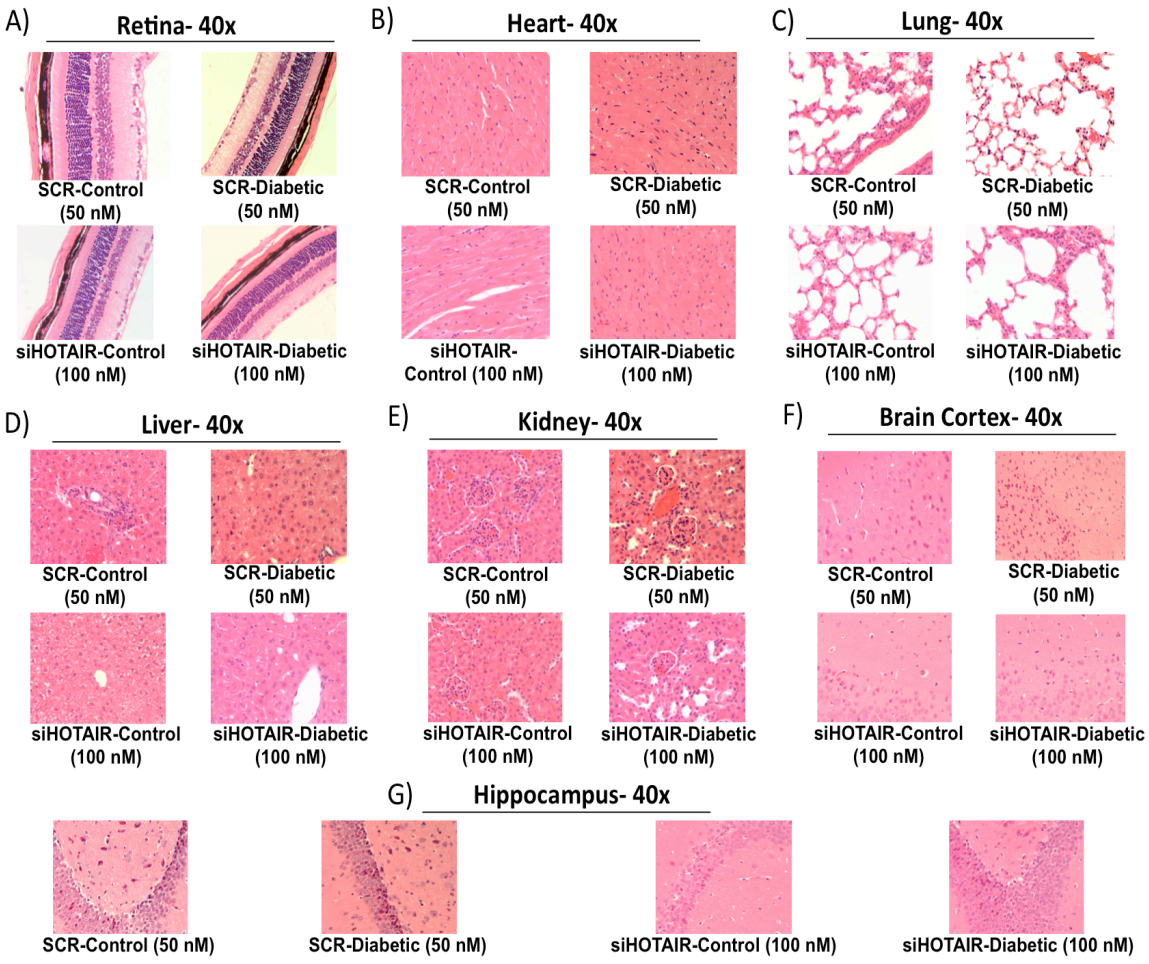
Supplemental Figure 6. SiRNA-mediated knockdown of *mus Hotair* and its impact on angiogenic markers in mouse retinal and lung endothelial cells. RT-qPCR analyses of **(A,D)** *Hotair*, **(B,E)** *Vegf-a*, and **(C,F)** *Angptl4* expressions following the administration of SCR siRNA or siHOTAIR in mouse retinal endothelial cells (top panel) and primary mouse lung endothelial cells (bottom panel) subjected to 48 hours of NG or HG culture. β-actin was used as an internal control. Statistical significance was assessed using one-way ANOVA for multiple comparisons, followed by Tukey's post hoc test (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, or n.s.= not significant). Data represents the mean ± SEM of 3 independent experiments (n=6/group).



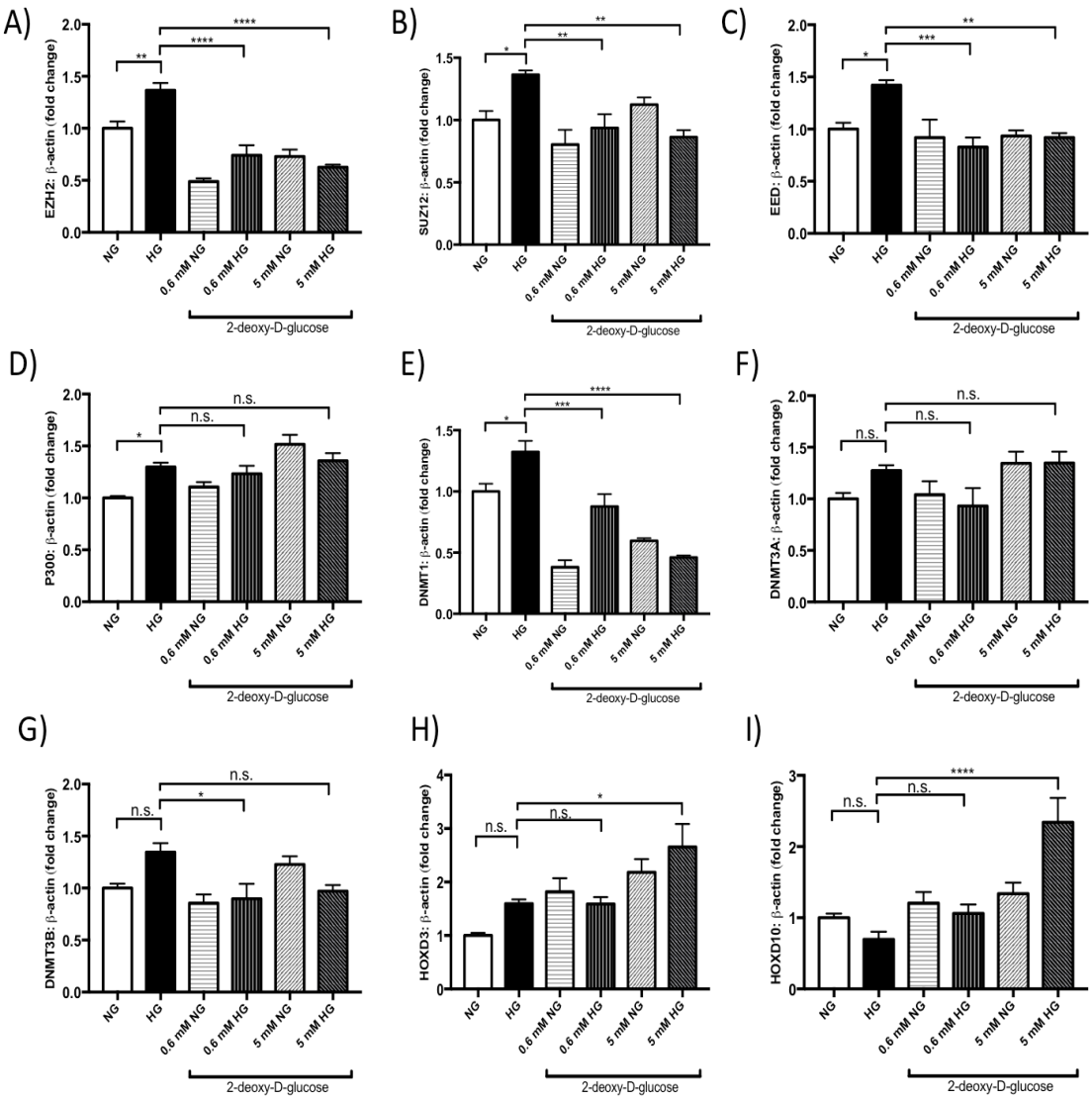
Supplemental Figure 7. Hematoxylin and eosin (H&E) staining of various mouse tissues following siHOTAIR toxicology experiments. Wild-type C57BL/6 mice were subjected to a one-time intravitreal injection that consisted of either scrambled siRNA control (50 nM; SCR) or siHOTAIR at varying concentrations (25 nM, 50 nM, and 100 nM) and were monitored for seven days and then euthanized for tissue collection (n=3 per group). No behavioural changes or ocular complications were observed in the mice throughout the duration of the experiment and as evidenced by H&E staining, no cellular abnormalities were also observed across **(A)** retinal, **(B)** heart, **(C)** lung, **(D)** liver, and **(E)** kidney tissues following the one-time intravitreal siHOTAIR injection at 25, 50 or 100 nM concentrations (images not shown for 25 nM). Original magnification, 40X; scale bar= 5 microns.



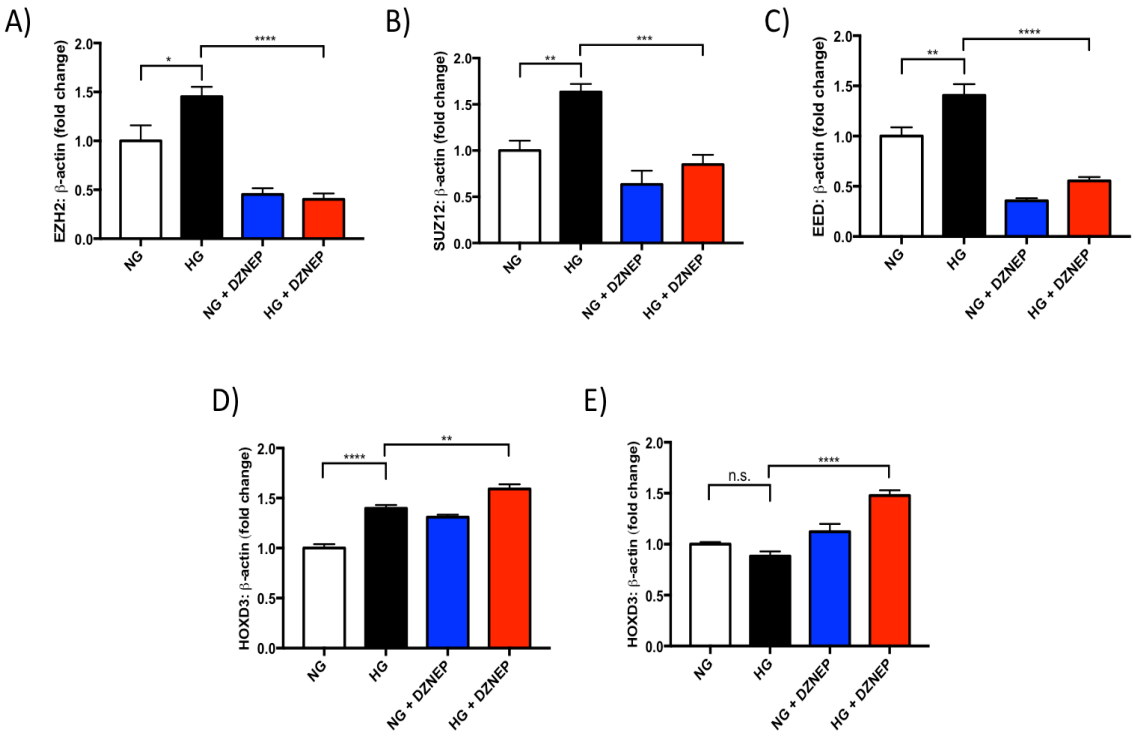
Supplemental Figure 8. *In vivo* results following the knockdown of *Hotair*. (A) Relative *Hotair* knockdown expressions, as indicated by RT-qPCR, in the retinal tissues of C57BL/6J mice from our toxicology experiments involving different siHOTAIR concentrations (n=3 per group). β-actin was used as an internal control. **(B,C)** Body weights and blood glucose levels of all C57BL/6J mice involved in our short-term, one-month therapeutic model, where intravitreal injections of scrambled (SCR; 50 nM) siRNAs or siHOTAIR (100 nM) were administered to non-diabetic and diabetic mice eyes once every week for up to three weeks. **(D)** RT-qPCR analysis of retinal *Hoxd3* expressions at 1 month following siHOTAIR treatment. β-actin was used as an internal control. Statistical significance was assessed using one-way ANOVA for multiple comparisons, followed by Tukey's post hoc test (****p<0.0001 or n.s.= not significant). Data represents the mean ± SD (n=6/group).



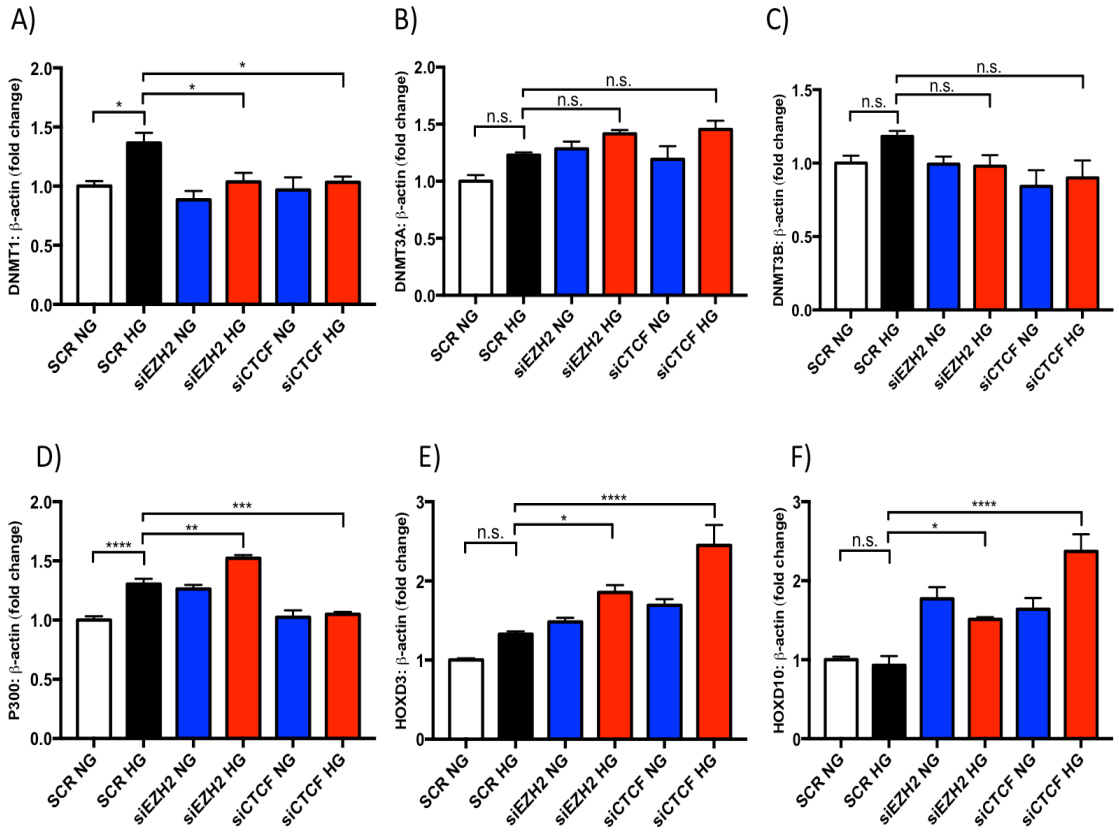
Supplemental Figure 9. Hematoxylin and eosin (H&E) staining of various mouse tissues following our short-term, 1-month therapeutic animal model involving siHOTAIR. Non-diabetic (control) and diabetic C57BL/6 mice were subjected to intravitreal injections of scrambled (SCR; 50 nM) siRNAs or siHOTAIR (100 nM) once every week for up to three weeks. Mice were monitored throughout the duration of the experiment and subsequently euthanized for tissue collection at 4 weeks (n=3 per group). Similar to our initial toxicology experiments, no behavioural changes or ocular complications were observed in the mice and as evidenced by H&E staining, no cellular abnormalities were also observed across **(A)** retinal, **(B)** heart, **(C)** lung, **(D)** liver, **(E)** kidney, **(F)** cortical, and **(G)** hippocampal tissues following multiple intravitreal siHOTAIR injections at 1 month. Original magnification, 40X; scale bar= 5 microns.



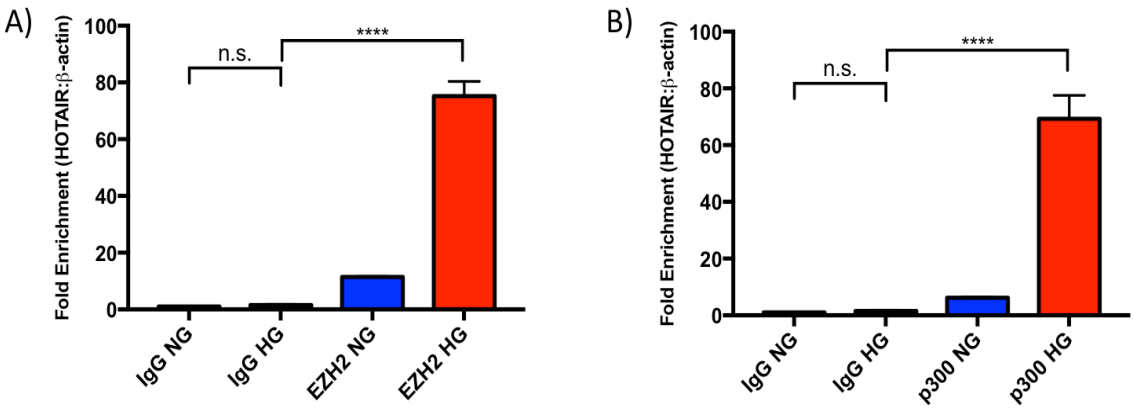
Supplemental Figure 10. Glycolytic inhibition can impact certain epigenetic molecules and may also influence nuclear transport molecules involved in oxidative stress, independent of the mitochondria. RT-qPCR analyses of **(A)** *EZH2*, **(B)** *SUZ12*, **(C)** *EED*, **(D)** *P300*, **(E)** *DNMT1*, **(F)** *DNMT3A*, **(G)** *DNMT3B*, **(H)** *HOXD3*, and **(I)** *HOXD10* expressions following 2-deoxy-D-glucose treatment (0.6 or 5 mM) in HRECs subjected to 48 hours of NG (5mM D-glucose) or HG (25 mM D-glucose) culture. 2-deoxy-D-glucose is a potent inhibitor of glycolysis. β-actin was used as an internal control. Statistical significance was assessed using one-way ANOVA for multiple comparisons, followed by Tukey's post hoc test (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, or n.s.= not significant). Data represents the mean ± SEM of 3 independent experiments (n=6/group).



Supplemental Figure 11. DZNep pre-treatment reduces the expression of PRC2 components and stimulates the transcription of *HOXD* loci. HRECs were pre-treated with DZNep (a global histone methylation inhibitor) prior to NG or HG culture for 48 hours. RT-qPCR was then used to analyze the expressions of **(A)** *EZH2*, **(B)** *SUZ12*, **(C)** *EED*, **(D)** *HOXD3*, and **(E)** *HOXD10*. β-actin was used as an internal control. Statistical significance was assessed using one-way ANOVA for multiple comparisons, followed by Tukey's post hoc test (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, or n.s.= not significant). Data represents the mean ± SEM of 3 independent experiments (n=6/group).

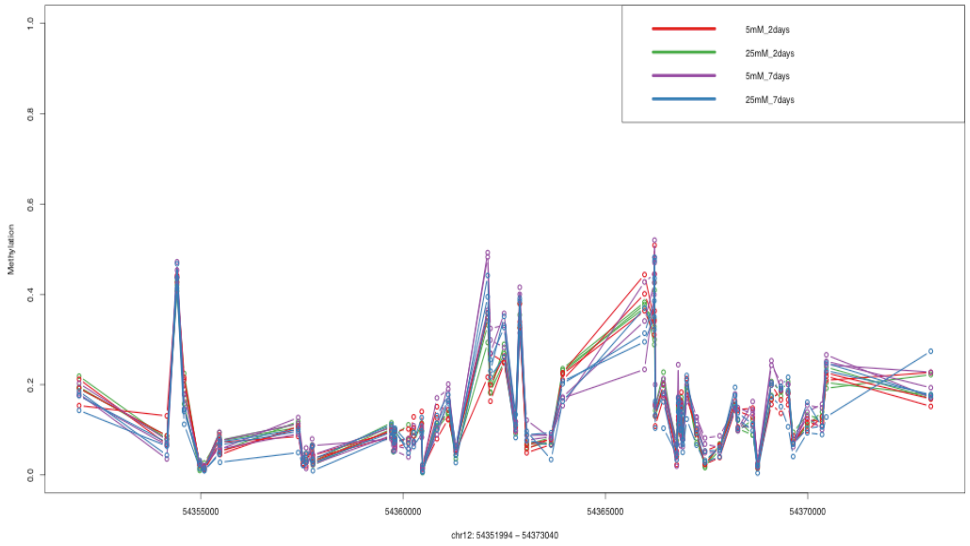


Supplemental Figure 12. The knockdown of *EZH2* and *CTCF* can alter glucose-induced expressions of certain epigenetic molecules. RT-qPCR analyses of **(A)** *DNMT1*, **(B)** *DNMT3A*, **(C)** *DNMT3B*, **(D)** *P300*, **(E)** *HOXD3*, and **(F)** *HOXD10* expressions following the administration of scrambled (SCR) siRNAs, siEZH2, or siCTCF in HRECs subjected to 48 hours of NG or HG culture. EZH2 is the catalytic subunit of PRC2 (a critical histone methyltransferase) and CTCF is an important epigenetic transcription factor involved in the direct regulation of genes. β-actin was used as an internal control. Statistical significance was assessed using one-way ANOVA for multiple comparisons, followed by Tukey's post hoc test (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, or n.s.= not significant). Data represents the mean ± SEM of 3 independent experiments (n=6/group).

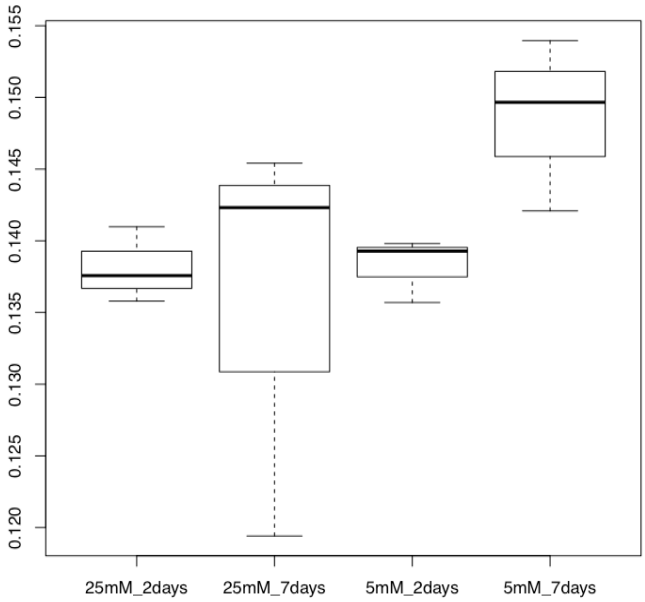


Supplemental Figure 13. High glucose promotes strong binding associations between *HOTAIR* and epigenetic enzymes. RNA immunoprecipitation (RIP) experiments were conducted using anti-IgG, anti-EZH2 (catalytic subunit of the histone methyltransferase, PRC2), or anti-P300 (a histone acetyltransferase) antibodies on HRECs cultured with NG or HG for 48 hours. RT-qPCR was then used to determine the fold enrichment of *HOTAIR* following IgG, **(A)** EZH2 and **(B)** P300 pulldown. IgG antibodies were used as a negative control and β-actin was used as an internal control. Statistical significance was assessed using one-way ANOVA for multiple comparisons, followed by Tukey's post hoc test (****p<0.0001 or n.s.= not significant). Data represents the mean ± SEM of 3 independent experiments (n=3/group).

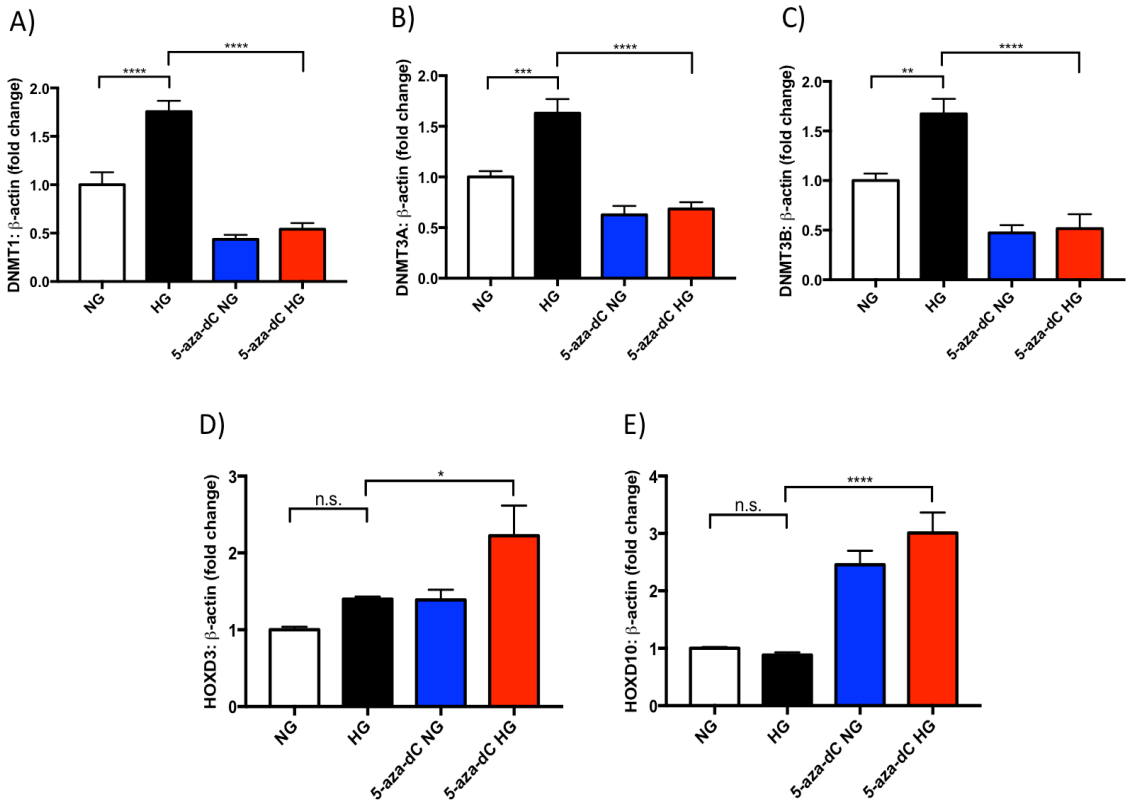
A)



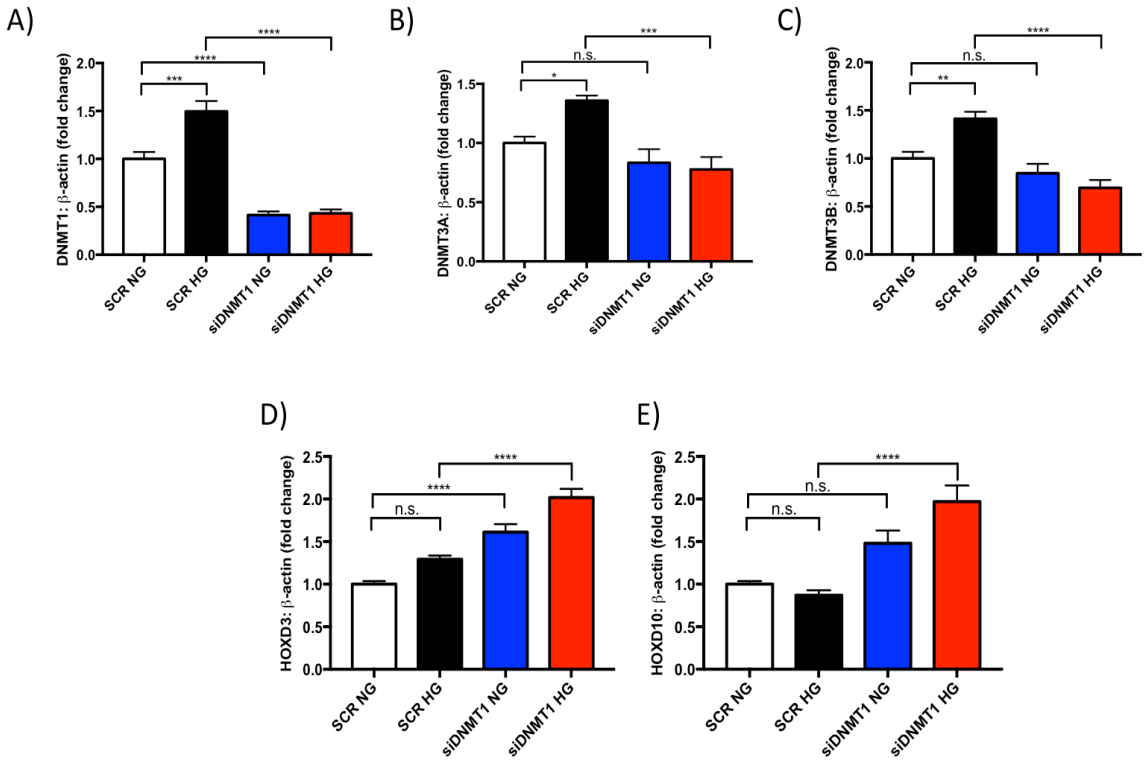
B)



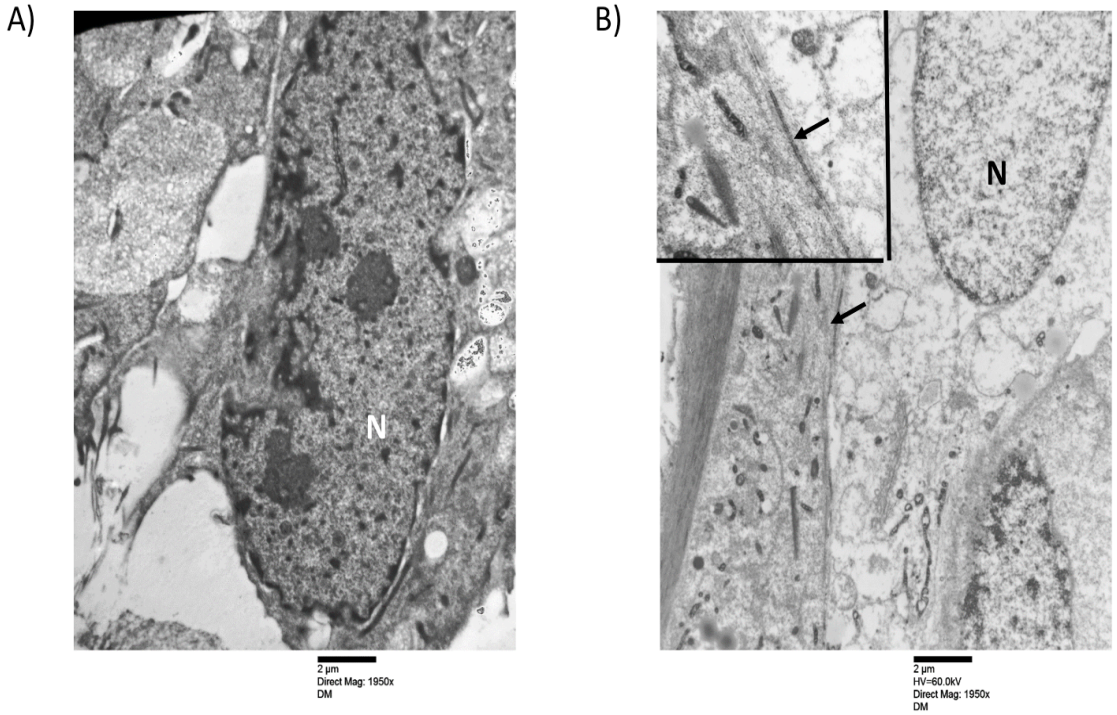
Supplemental Figure 14. DNA methylation profiling of HRECs. (A) Panel that depicts the stable DNA methylation patterns across the *HOTAIR* genomic regions shared between HRECs cultured with NG or HG for various durations (2 or 7 days). **(B)** Differential methylation patterns in the *HOTAIR* promoter region. The box plots represent the distribution of median methylation values across all of the probes mapping to this region as stratified by glucose concentration and culture duration. Center line: median of regional methylation levels across samples; lower and upper bounds: first and third quartiles; whiskers: interquartile ranges (n= 3 independent samples per group).



Supplemental Figure 15. 5-aza-dC can decrease the expressions of *DNMT*s, while promoting *HOXD3* and *HOXD10* gene expressions. HRECs were pre-treated with 5-aza-dC (a pan-DNMT inhibitor) prior to NG or HG culture for 48 hours. RT-qPCR was then used to analyze the expressions of **(A)** *DNMT1*, **(B)** *DNMT3A*, **(C)** *DNMT3B*, **(D)** *HOXD3*, and **(E)** *HOXD10*. β-actin was used as an internal control. Statistical significance was assessed using one-way ANOVA for multiple comparisons, followed by Tukey's post hoc test (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, or n.s.= not significant). Data represents the mean ± SEM of 3 independent experiments (n=6/group).



Supplemental Figure 16. SiRNA-mediated knockdown of *DNMT1* can influence glucose-induced expressions of *DNMT*s and *HOXD* loci. RT-qPCR analyses of **(A)** *DNMT1*, **(B)** *DNMT3A*, **(C)** *DNMT3B*, **(D)** *HOXD3*, and **(E)** *HOXD10* expressions following the administration of scrambled (SCR) siRNAs or siDNMT1 in HRECs subjected to 48 hours of NG or HG culture. DNMT1 is a constitutively expressed DNA methyltransferase. β-actin was used as an internal control. Statistical significance was assessed using one-way ANOVA for multiple comparisons, followed by Tukey's post hoc test (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, or n.s.= not significant). Data represents the mean ± SEM of 3 independent experiments (n=6/group).



Supplemental Figure 17. HOTAIR knockdown can prevent glucose-induced disruptions of endothelial cell junctions *in vitro*. Representative images, by electron microscopy, of **(A)** scrambled siRNA (SCR) or **(B)** siHOTAIR-transfected HRECs after high glucose culture (n=3 samples per group). Disruptions of endothelial cell junctions can be visualized in SCR plus HG cells compared to preservation of junctions in siHOTAIR plus HG cells (a higher magnification was inserted in **B** showing the intact junctions [indicated by the black arrows]). Direct magnification, 1950X; scale bar= 2 microns; 'N'= nucleus.

Supplemental Table 1. qPCR primers for human-specific genes.

Target Gene (Human):	Oligonucleotide Sequence (5'→3'):
ACTB	F: CCTCTATGCCAACACAGTGC R: CATCGTACTCCTGCTTGCTG
HOTAIR	F: GGTAGAAAAAGCAACCACGAAGC R: ACATAAACCTCTGTCTGTGAGTGCC
VEGFA	F: GAACTTTCTGCTGTCTTGGG R: CTTCGTGATGATTCTGCCCT
EDN1	F: AAGCCCTCCAGAGAGCGTTAT R: CCGAAGGTCTGTCTGTCACCAATGT
ANGPTL4	F: GGACACGGCCTATAGCCTG R: CTCTTGGCGCAGTTCTTGTC
PGF	F: CCGGCTCGTGTATTTATTACCG R: GGCAACCACTGTTCTCCAGAGC
IL1β	F: GCGGCATCCAGCTACGAATCT R: GGGCAGGGAACCAGCATCTT
HIF1α	F: CATAAAGTCTGCAACATGGAAGGT R: ATTTGATGGGTGAGGAATGGGTT
PARP1	F: CCACACACAATGCGTATGAC R: CCACAGCAATCTTCGGTTATG
CYTB	F: TCACCAGACGCCTCAACCGC R: GCCTCGCCCGATGTGTAGGA

Target Gene (Human):	Oligonucleotide Sequence (5'→3'):
MCP1	F: TCGCCTCCAGCATGAAAGTC R: GGCATTGATTGCATCTGGC
CTCF	F: GACCCCACCCTTCTTCAGATG R: CCACAGCAGCCTCTGCTTCT
P300	F: GGGACTAACCAATGGTGGTG R: ATTGGGAGAAGTCAAGCCTG
EZH2	F: CCACCATTAATGTGCTGGAA R: TTCCTTGGAGGAGTATCCACA
SUZ12	F: TACGGCTCCTATTGCCAAAC R: TGCTTCAGTTTGTTGCCTTG
EED	F: GCAACTGTAGGAAGCAACAGA R: CATAGGTCCATGCACAAGTGT
DNMT1	F: ACGGTGCTCATGCTTACAAC R: TTAGCCTCTCCATCGGACTT
DNMT3A	F: GGCAAATTCTCAGTGGTGTG R: GTCACTCTCATCGCTGCTGT
DNMT3B	F: TTGAATATGAAGCCCCCAAG R: TGATATTCCCCTCGTGCTTC
HOXD3	F: CAGCCTCCTGGTCTGAACTC R: ATCCAGGGGAAGATCTGCTT
HOXD10	F: ATGTACATGCCACCACCTAGC R: TTGCTGTGTAACAGGTTGCTC

Supplemental Table 2. qPCR primers for mouse-specific genes.

Target Gene (Mouse):	Oligonucleotide Sequence (5'→3'):
Actb	F: CCTCTATGCCAACACAGTGC R: CATCGTACTCCTGCTTGCTG
Hotair	F: GCGCCAACGTAGACCAAAAG R: TCTACCGATGTTGGGGACCT
Vegfa	F: ATGCGGATCAAACCTCACCA R: CTTTCTTTGGTCTGCATTCAC
Edn1	F: TTAGCAAGACCATCTGTGTG R: GAGTTTCTCCCTGAAATGTG
Angptl4	F: TTGGTACCTGTAGCCATTCC R: GAGGCTAAGAGGCTGCTGTA
Pgf	F: TGCTGGGAACAACTCAACAG R: CCTCATCAGGGTATTCATCCA
Il1β	F: TTCAGGCAGGCAGTATCACTC R: GAAGGTCCACGGGAAAGACAC
Hif1α	F: TCAAGTCAGCAACGTGGAAG R: TATCGAGGCTGTGTCGACTG
Parp1	F: GGAAAGGGATCTACTTTGCCG R: TCGGGTCTCCCTGAGATGTG
Cytb	F: TCCTTCATGTCGGACGAGGC R: AATGCTGTGGCTATGACTGCG

Target Gene (Mouse):	Oligonucleotide Sequence (5'→3'):
Mcp1	F: TTGTCACCAAGCTCAAGAGAGA R: GAGGTGGTTGTGGAAAAGGTAG
Ctcf	F: TGGTCCAGATGGCGTAGAGG R: GTCATCGAGATCCGGCTCAG
p300	F: AGGCAGAGTAGGACAGTGAA R: CTCAGTCTGGGTCACTCAAT
Ezh2	F: CGCGGGACTAGGGAGTGTTCAGT R: AGTACATTATAGGCACCGAGGCGA
Suz12	F: AGCTCTGCCACAGCAGGTTCA R: TGCTTTTGTTCTTTTTGGCCTGCAA
Eed	F: ATGCTGTCAGTATTGAGAGTGGC R: GAGGCTGTTCACACATTTGAAAG
Hoxd3	F: GAGACCTGGCACTGGGAATA R: TCCAGGGGAAGATCTGTTTG
Hoxd10	F: ATAAGCGCAACAAACTCATTTCG R: ATATCGAGGGACGGGAACCT

Chapter 4 [iv]

4 Endothelin-1 regulation is entangled in a complex web of epigenetic mechanisms in diabetes

With the continuous global rise in diabetes [21], the risk for developing glucose-induced injuries to the microvasculature also remains high. As a consequence of hyperglycemia, microvascular damage gives rise to debilitating complications affecting the eyes, kidneys, and peripheral nerves [15]. To minimize the risk of subsequent diabetes-induced vessel damage, current therapeutic modalities are comprised of lifestyle changes and pharmaceutical intervention [5, 15]. Although these therapies may be effective in impeding some progression of late stage diabetic complications, the presence of 'metabolic memory' contributes to unique molecular alterations that may alter an individual's response to treatments [36]. Epigenetic mechanisms comprise the 'metabolic memory' phenomenon and identification of these mechanisms would expand current therapeutic modalities, allowing for the development of targeted treatment strategies.

Modifications to the epigenome influence gene expression without changing the underlying nucleotide sequence [45]. DNA methylation, histone modifications, and the activity of non-coding RNAs are examples of such epigenetic mechanisms that are involved in metabolic memory [2]. The identification of these processes have been made evident by high throughput genomic technologies, however new questions arise with respect to our current understanding of the documented mechanistic properties for pro-inflammatory markers.

Endothelin-1 (ET-1) is a prominent peptide in diabetes that has been well documented within the last three decades. As previously demonstrated by our laboratory and

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[iv] Content in Chapter 4 has been adapted from Biswas S, Feng B, Thomas AA, Chen S, Aref-Eshghi E, Sadikovic B, Chakrabarti S. Endothelin-1 regulation is entangled in a complex web of epigenetic mechanisms in diabetes. Physiological Research: 2018;67(Suppl 1): S115-S125, doi: 10.33549/physiolres.933836. The work was published under a Creative Commons Attribution License.

others, aberrations in metabolic pathways can heighten the production of ET-1 and subsequently allow for an increased pathologic state in various diabetic complications [4, 6, 42]. Using such knowledge and information from ligand-binding studies, drugs were developed to target the ET-1 receptors, in order to mitigate cellular dysfunction and other effects in diabetic complications [25, 38]. Despite the promising results for endothelin receptor antagonists in animal models, investigators discovered that there were adverse, undesirable and heterogeneous responses present among numerous patients using these drugs—suggesting that there are alternative mechanisms implicated in ET-1 regulation [34]. As the mechanistic impact of epigenetic machinery on ET-1 production still remains unclear, further characterization is warranted to understand such a process.

In this study, we examined the roles of DNA methylation, histone methylation, and long non-coding RNAs (lncRNAs) on ET-1 regulation in retinal microvascular endothelial cells (HRECs). We performed a DNA methylation array to identify whether unique methylation patterns exist across the ET-1 gene (*EDN1)* after high glucose (HG) incubation. To further follow-up our findings from the methylation array, we employed a global DNA methylation inhibitor in HRECs to determine the impact of DNA methyltransferases on ET-1 regulation. Next, we subjected HRECs to a histone methylation inhibitor to understand the influence of histone methyltransferases on ET-1 mRNA production. Moreover, based on our previous lncRNA microarray analysis [43], we selected three prominent HG-induced upregulated lncRNAs and silenced these lncRNAs to determine the effects on ET-1. We then collated our previous and current findings to present an all-inclusive epigenetic paradigm for ET-1 regulation.

4.1 Research Design and Methods

4.1.1 Cell culture

Endothelial dysfunction is one of the earliest pathological features during chronic hyperglycemia [1]. Hence, we used HRECs (Olaf Pharmaceuticals, Worchester, MA) for our experiments. Such experimental conditions have been previously described

[24, 32, 35, 43] and all cell culture reagents were purchased from Sigma (Oakville, Ontario, Canada). Based on our previous findings, the 48-hour time point was selected for our *in vitro* experiments. All experiments were independently repeated at least three times and performed with six replicates, unless specified.

4.1.2 *EDN1* CpG DNA methylation analysis

The Illumina Infinium MethylationEPIC BeadChip array (Illumina, CA, USA) was used to identify differential methylation patterns of CpG sites across the *EDN1* gene in HRECs incubated in 5 mM glucose (NG, mimicking euglycemia) and in 25 mM glucose (HG, mimicking hyperglycemia). Genomic DNA was extracted from HRECs after 48 hours of glucose culture and 1 μg of DNA was used for bisulfite conversion using the EZ DNA Methylation Kit (Zymo Research, CA, USA). The HiScan System (Illumina, CA, USA) was used to obtain the array readout and the methylated and unmethylated signal intensity data were then imported into R 3.4.0 for analysis. Normalization was performed using Illumina normalization method with background correction using the minfi package. Probes with detection *P*-value > 0.01 were excluded from the downstream analyses. In addition, probes known to contain SNPs at the CpG interrogation or the single nucleotide extension were removed. Methylation level for each probe was measured as a beta value (β-value), calculated from the ratio of the methylated signals versus the total sum of unmethylated and methylated signals, ranging between 0 (no methylation) and 1 (full methylation). Three independent samples were used per group.

4.1.3 3-Deazaneplanocin A (DZNep) and 5-Aza-2'-deoxycytidine (5-aza-dC)

In order to understand the impact of histone and DNA methylation inhibition on ET-1, we used pharmacological inhibitors that globally blocked the methylation process. Therefore, based on previous literature, 1 hour pre-treatment of either 5 μM of DZNep (Cayman Chemical, Ann Arbor, MI) or 5-aza-dC (Sigma, St. Louis, USA) was applied to HRECs prior to addition of D-glucose [35, 43, 46]. DZNep or 5-aza-dC-treated

HRECs, and their respective controls, were collected at 48 hours for further analyses.

4.1.4 SiRNA-mediated transfection

The lncRNA array analysis from our earlier study identified several upregulated lncRNAs in HG-treated HRECs [43]. Therefore, in this study, we selected the three most upregulated lncRNAs, which potentially target ET-1, and silenced each of these lncRNAs to determine the impact on ET-1 regulation. HRECs were initially transfected with either two pre-designed siRNAs targeting human *ANRIL* (antisense RNA to *INK4* locus), *MALAT1* (metastasis-associated lung adenocarcinoma transcript 1), or *ZFAS1* (zinc finger antisense 1), or scrambled siRNA (catalog number: AM4635, Life Technologies, CA, USA) using Lipofectamine 2000 (Invitrogen, ON, CA) and Opti-MEM (Life Technologies, CA, USA). Details regarding the lipofectamine-mediated transfection protocol can be found in our previous studies [36, 44]. Following confirmation of knockdown by RT-qPCR, the siRNA sequences with the best knockdown were selected for this study: *ANRIL* (Lincode CDKN2B-AS1; catalog number: R-188105-00-0005, Dharmacon, IL, USA), *MALAT1* (catalog number: n272233, Life Technologies, CA, USA), and *ZFAS1* (catalog number: n271357, Life Technologies, CA, USA).

4.1.5 RNA Isolation and Quantitative Real-Time Polymerase Chain Reaction (RT-qPCR)

As previously described, the TRIzol reagent (Invitrogen, Burlington, ON) was used to extract total RNA and a spectrophotometer (260 nm; Gene Quant, Pharmacia Biotech, USA) was utilized to quantify RNA concentrations [24, 32, 35, 43]. A high-capacity complementary DNA (cDNA) kit (Applied Biosystems, Burlington, ON) was then employed to reverse transcribe 1 μg of total RNA to cDNA. Next, in combination with SYBR-green master mix (Clontech, Mountain View, CA, USA) and specific target gene primers (Sigma; **Appendix C, Table S1**), cDNA was amplified in the LightCycler 96 System (Roche Diagnostics, QC, CA) to detect RNA expression. Expression levels were further calculated by the relative standard curve method using

β-actin as internal control for normalization.

4.1.6 Statistical analysis

All statistical tests were performed using GraphPad Prism 7 (GraphPad, CA, USA). Data are expressed as mean ± SEM. Student's *t*-test or 1-way ANOVA, followed by Tukey's post hoc test, were used as appropriate. Differences with a *P* value below 0.05 were considered significant.

4.2 Results

4.2.1 Transient HG treatment results in hypomethylation of CpG sites in the proximal promoter regions of *EDN1*

Technological advancements in next-generation sequencing have allowed for the identification of genome-wide DNA methylation patterns, which have critical implications in disease pathogenesis. Following our methylation profiling of NG and HG-treated HRECs, we exclusively selected the CpG sites that spanned across *EDN1*, which corresponded to 12 probes (**File S1, doi: 10.33549/physiolres.933836**). All of the probes were placed in the 'Open Sea' category, since the array did not detect a CpG island (defined as regions that have a GC content greater than 50% and are greater than 200 base pairs in length [16]) in the *EDN1* region—which was also confirmed by the UCSC Human Genome Browser (University of California, Santa Cruz, CA, USA; **Figure 4.1A**). Using annotations derived by Illumina, we further categorized the probes based on functional location relative to the gene region: TSS1500 (region that is -200 to -1500 nucleotides upstream from the transcription start site), TSS200 (the region from -200 nucleotides upstream to the transcription start site itself), 5' UTR (can include the region through the first exon), gene body, and 3' UTR. Analyzing the *EDN1* regions, there were four probes for TSS1500, five probes for TSS200, one probe for 5' UTR, one probe for gene body, and one probe for 3' UTR. According to our array readout, nearly 58% of CpG sites were hypomethylated following 48 hours of HG treatment (**Figure 4.1B**). In addition, a substantial number

of these hypomethylated CpG sites were predominantly located in the TSS200 region of *EDN1*, while a hypomethylated CpG site was also detected in both TSS1500 and 5' UTR regions (**Figure 4.1C**). Despite the overall methylation intensities between the NG and HG groups being low for each gene region (0≤ β-value≥0.119; a value of 0 indicates no methylation, while a value of 1 indicates maximal methylation), the percentage of methylation further reduced at CpG sites in the proximal promoter and promoter regions of *EDN1* following high glucose treatment.

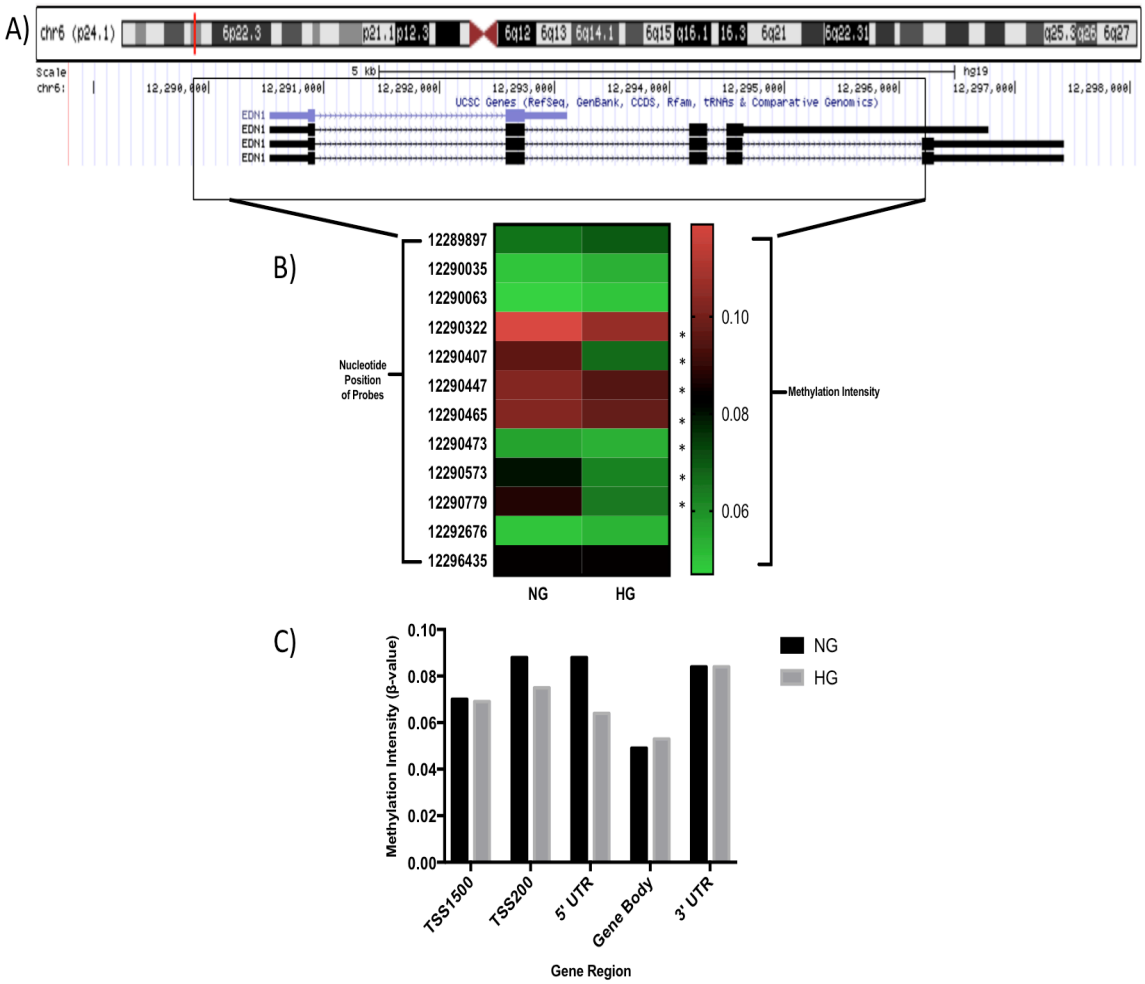

Figure 4.1. CpG sites in specific regions of *EDN1* are hypomethylated following high glucose treatment *in vitro*. A) Information on the human *EDN1* gene annotated from the UCSC database. *EDN1* is located on chromosome 6 and spans from the nucleotide positions 12, 289, 311 to 12, 296, 209 (6899 nucleotides in size [23]). B) A heat map depicting the methylation intensity (in ß-values) of specific CpG dinucleotides across *EDN1* in HRECs following 48 hours of NG or HG culture. The Illumina methylation array indicates that a ß-

value of 1 suggests complete methylation at the interrogated site, while a value of 0 indicates no methylation. C) A bar graph categorizing the methylation intensities of probes based on gene region. The proximal promoter and 5' UTR/first exon regions demonstrated CpG hypomethylation following HG treatment (data expressed as average ß-value per region; *N*= 3 independent samples for each NG or HG group). Legend: '*' demonstrates a significant reduction in methylation following HG treatment.

4.2.2 Global inhibition of DNA methyltransferases increases *ET-1* expression

To further investigate the impact of DNA methylation on *EDN1* regulation, we administered a DNA demethylating agent in HRECs prior to glucose treatment. Following the global inhibition of DNA methyltransferases, we subsequently analyzed *ET-1* mRNA expression (the main product of *EDN1* transcription) using RT-qPCR. Interestingly, 5'-aza-dC administration significantly augmented *ET-1* expressions in both NG and HG-treated HRECs compared to controls (**Figure 4.2A**). Among the groups analyzed, HRECs that were cultured with NG and 5'-aza-dC demonstrated comparable *ET-1* expression levels to HRECs cultured solely in HG—suggesting the importance of DNA methyltransferases in governing the transcriptional activity of *EDN1*. Collectively, both of our DNA methylation experiments indicate that hyperglycemic environments can greatly impact DNA methylation patterns in *EDN1* and its transcriptional products.

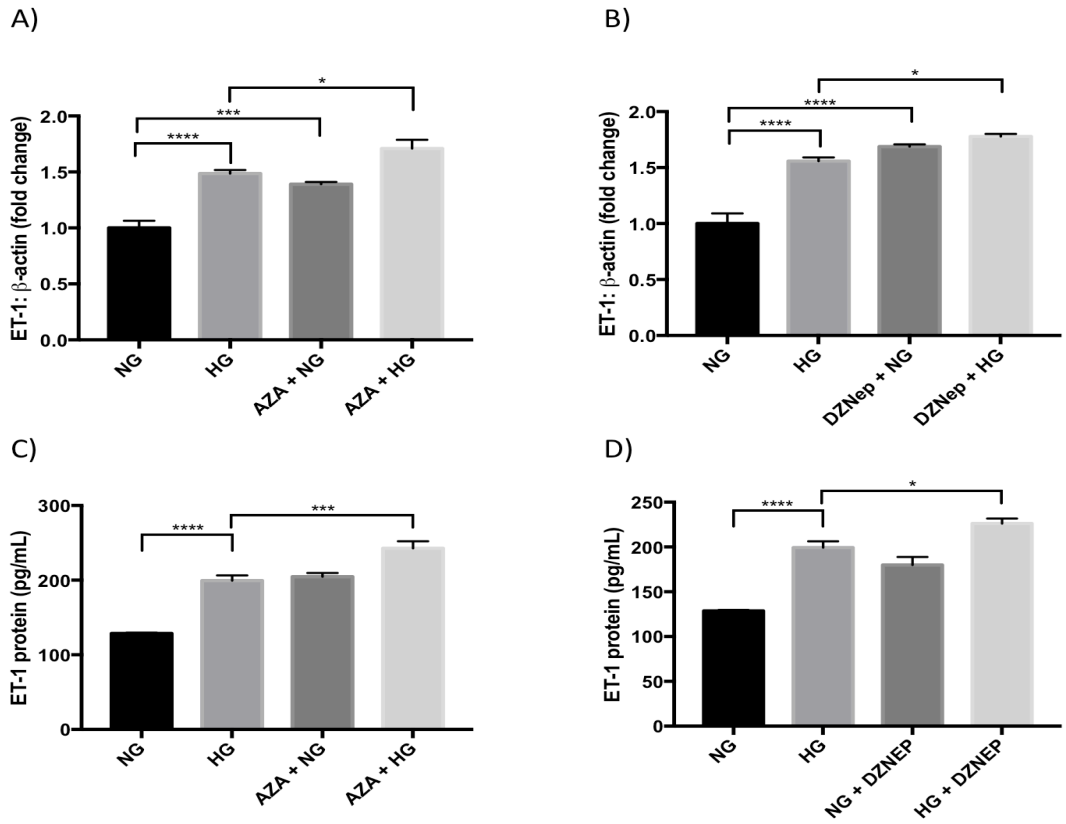

Figure 4.2. Global inhibition of DNA or histone methylation significantly increases *ET-1* mRNA expressions in HRECs. RT-qPCR findings indicate that A) 5-aza-dC treatment increases *ET-1* expressions in NG or HG-treated HRECs, B) DZNep treatments also showed similar patterns of ET-1 upregulation (data expressed as a ratio to β-actin (mean ± SEM); normalized to NG; *=P<0.05, ***=P<0.001, and ****=P<0.0001 compared to NG or HG; and *N*= 6 from three independent experiments and performed in triplicates).

4.2.3 Histone methylation is important in *ET-1* regulation

Following demonstration of DNA methylation's impact on ET-1, we investigated the role of histone methylation on *ET-1* mRNA expression. Therefore, prior to glucose treatment, we subjected HRECs to a global histone methylation inhibitor and subsequently analyzed *ET-1* mRNA. Similar to the trends observed in our 5'-aza-dC experiment, DZNep pre-treatment significantly upregulated *ET-1* in both NG and HG-treated HRECs (**Figure 4.2B**). Following pharmacologic inhibition of histone methylation, the sharp induction of *ET-1* expression in both NG and HG-treated HRECs alludes to the regulatory capabilities of histone methyltransferases on ET-1 transcription.

4.2.4 lncRNAs regulate *ET-1* expression in hyperglycemia

With lncRNAs gaining widespread recognition as important epigenetic mediators, we examined the impact of lncRNAs on ET-1 regulation. Based on the lncRNA array analysis from our previous study [43], we selected the top three upregulated lncRNAs in HG-treated HRECs for silencing in this investigation: *ANRIL*, *MALAT1*, and *ZFAS1*. Compared to scrambled controls, approximately 70%, 75%, and 90% knockdown activity was observed in HRECs following siANRIL, siMALAT1, and siZFAS1 treatments, respectively (data not shown). Interestingly, the siRNA-directed inhibition of *ANRIL*, *MALAT1*, or *ZFAS1* also significantly reduced *ET-1* mRNA in HG-treated HRECs (**Figure 4.3**). More specifically, when compared to scrambled controls, *ANRIL* knockdown contributed to the greatest reduction of *ET-1* expression in HG-treated HRECs (~75%; **Figure 4.3A**); while, siMALAT1 (**Figure 4.3B**) and siZFAS1 (**Figure 4.3C**) demonstrated comparable levels of *ET-1* mRNA reduction (~37-42%) in HG-treated HRECs. Taken together, the findings observed from our knockdown experiments suggest that pathogenetic lncRNAs can also influence glucose-induced upregulation of *ET-1* in microvascular endothelial cells (ECs).

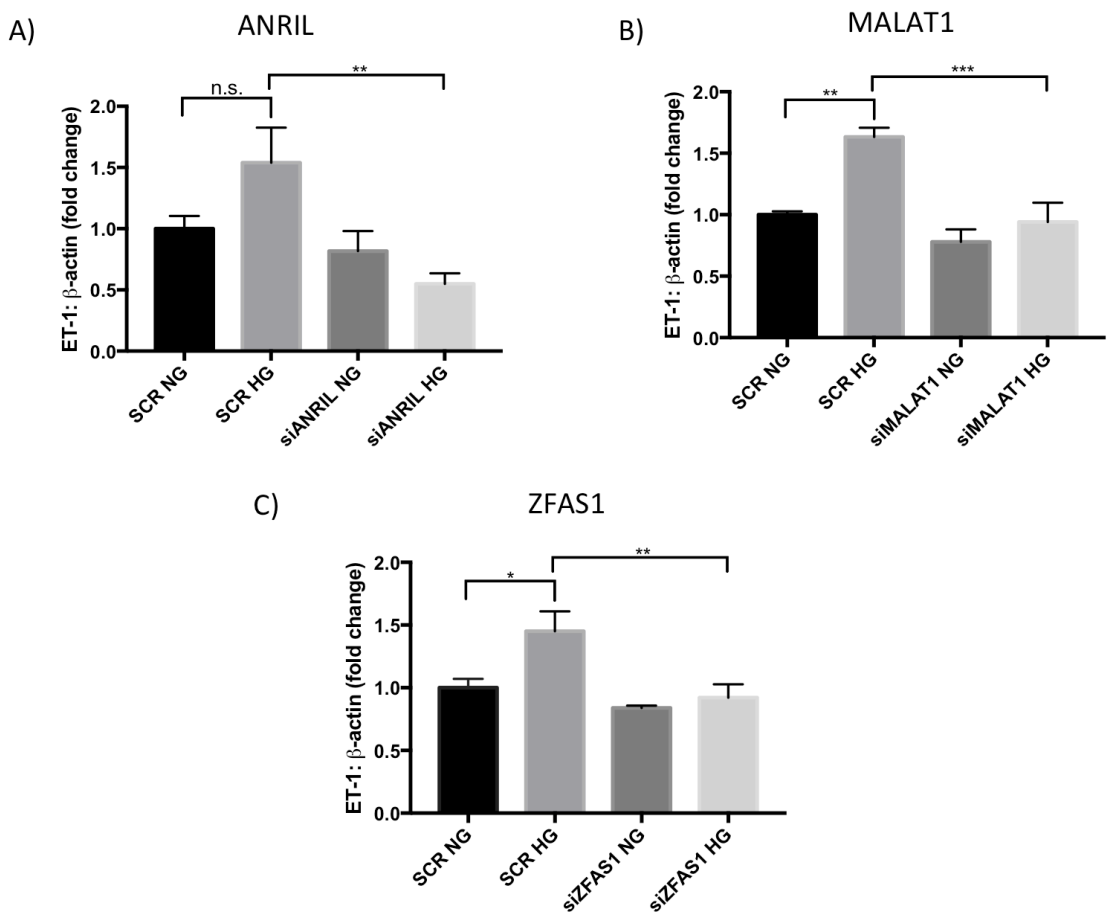

Figure 4.3. *ANRIL*, *MALAT1*, and *ZFAS1* regulates glucose-induced production of *ET-1* mRNA *in vitro*. RT-qPCR analyses indicating that A) siANRIL, B) siMALAT1, and C) siZFAS1 caused significant reductions in the glucose-induced upregulation of *ET-1* transcripts (data expressed as a ratio to β-actin (mean ± SEM); normalized to SCR NG; *=P<0.05, **=P<0.01, ***=P<0.001, and n.s.= not significant compared to SCR NG or SCR HG; and *N*= 6 from three independent experiments and performed in triplicates).

4.3 Discussion

When a chronic hyperglycemic environment jeopardizes the integrity of the endothelium, the risk of advancement to severe diabetic microvascular complications dramatically heightens. ECs are one of the earliest cells to undergo dysfunction in diabetes, contributing to disease progression [17]. During the development of endothelial dysfunction, one well-documented characteristic is the heightened production and activity of ET-1 [20, 39].

With the biologically active form being only 21 amino acids in length, the mature ET-1 peptide can elicit a potent proinflammatory and vasoactive response [31]. ET-1, belonging to the group of endothelin peptides, mediates its response through G-protein-coupled receptor subtypes, ET_A and ET_B [37]. In the vasculature, ECs predominantly express the ET_B receptors, while both receptor subtypes can be found on vascular smooth muscle cells [31]. Furthermore, the vascular response is characterized by the fine balance between the concentrations of ET-1 and its receptor subtypes. When this balance becomes disrupted in various pathologies such as atherosclerosis and diabetes, enhanced activities of vasoconstriction, inflammation, and mitogenesis can subsequently follow [37]. In order to truly understand the conundrum behind aberrant activation of the ET-1 axis, the transcriptional regulatory features behind ET-1 production must be thoroughly examined.

Located on chromosome 6, the human *EDN1* generates a 2.8-kb transcript that encodes for the pre-pro-ET-1 protein [48]. Once pre-pro-ET-1 is generated, the 212-amino-acid protein then undergoes a series of proteolytic modifications to ultimately produce the mature ET-1 peptide [48]. Although post-translational mechanisms are moderating ET-1 processing, large bodies of scientific reports indicate that ET-1 bioavailability is mainly directed by transcriptional regulation [4, 7, 19, 30, 41]. In fact, the 6.8-kb spanning mammalian *EDN1* can be transcriptionally governed by a plethora of transcription factors, which can include hypoxia inducible factor-1, NF-κB, activator protein-1, GATA-2, and c-Myc [22, 33, 40, 47]. Despite the well-documented literature on the various stimuli driving *EDN1* transcription, recognizing and incorporating novel regulatory mechanisms, such as epigenetic regulation, into the current ET-1 signalling paradigm will allow for improved understanding and exploration of innovative therapeutics.

Based on our DNA methylation array, we were able to develop significant insights into the glucose-induced methylation patterns present in the regulatory regions of *EDN1*. Specifically, in HRECs, transient high glucose exposure sufficiently evoked hypomethylation in the proximal promoter and 5' UTR/first exon regions of *EDN1*. In association, HG-treated HRECs also demonstrated a significant upregulation of *ET-1*

mRNA at the 48-hour mark. Our findings, together with previous reports by Han et al [18] and Feinberg and Vogelstein [11], highlight the importance of reduced methylation at CpG dinucleotides in the regulatory regions of actively transcribed genes. In fact, hypomethylated CpG sites in the promoter regions of an active gene provide a greater degree of accessibility for transcription factors, which will then facilitate transcription [9, 10]. Moreover, Vallender and Lahn [44] have previously indicated that hypermethylation of CpG regions near intron 1 in *Edn1* blocks the binding site for the Sp1 transcription factor, which may contribute to *Edn1* silencing in mice fibroblasts [44].

Despite the presence of differential CpG methylation patterns across *EDN1* between NG and HG-treated HRECs, the administration of a global DNA demethylating agent resulted in a significant induction of *ET-1* mRNA in both HREC groups. This data is in keeping with past reports in which similar trends for ET-1 mRNA were observed in fibroblasts [44] and 1833-bone metastatic cells [27] following the complete inhibition of DNA methylation. These findings, along with our study, suggest that DNA methyltransferases have a critical role in regulating the transcriptional status of *EDN1* and that pathological stimuli may be capable of altering the DNA methylating properties of these enzymes in the *EDN1* region.

In the context of histone modifications, globally inhibiting histone methylation provoked a significant elevation of *ET-1* mRNA in HRECs cultured with NG and HG. The profound influence of histone methylation on *EDN1* transcriptional activity has been previously documented in renal epithelial cells, where aldosterone treatment induced specific histone conformation changes that maintained active transcription in the *EDN1* promoter [41]. Although chronic hyperglycemic environments can facilitate chromatin remodeling by histone methylation, histone acetylation can also significantly contribute to chromatin state dynamics. Former studies from our laboratory have indicated that the upregulation of histone acetyltransferase P300 can cause increased *EDN1* promoter activity [7], reduced expression of the class II histone deacetylase SIRT1 [29], and increased expressions of *ET-1* transcripts, extracellular matrix (ECM) proteins and vasoactive factors [7, 14]. Collectively, our past and

current findings demonstrate that pathologies like diabetes can cause unique alterations in histone modification patterns, which may ultimately enhance *EDN1* transcriptional activity and contribute to elevated ET-1 protein levels.

With lncRNAs greatly influencing the epigenetic landscape, we knocked down three pathogenetic glucose-induced lncRNAs in HRECs in order to determine the impact of lncRNAs on *ET-1* transcript expression. Following siRNA-mediated knockdown of *ANRIL*, *MALAT1*, and *ZFAS1*, significant reductions in *ET-1* mRNA levels were observed. These findings are consistent with the notion that lncRNAs can alter mRNA transcription by controlling chromatin structure, transcription factors, nuclear organization, or specific protein complexes [26]. To further support these notions, we have previously shown that *ANRIL* can regulate VEGF (vascular endothelial growth factor) through its direct interactions with P300 and PRC2 (polycomb repressive complex 2; a histone methyltransferase) in HRECs [43]. Such a mechanistic study is warranted for ET-1, so that the direct relationship between epigenetic mediator proteins and *EDN1* can be confirmed in a diabetic context. Moreover, we have previously demonstrated the importance of microRNAs (miRNAs) 1 and 320 in ET-1 regulation [12, 13], miR-200b in mediating PRC2 and P300 activities [28, 35], and miR-146a impacting ECM proteins [14], inflammatory cytokines [8], and endothelial-to-mesenchymal transitioning [3]. Therefore, based on our past and current findings, we collated the pertinent information and developed an epigenetic paradigm for ET-1 regulation (**Figure 4.4**).

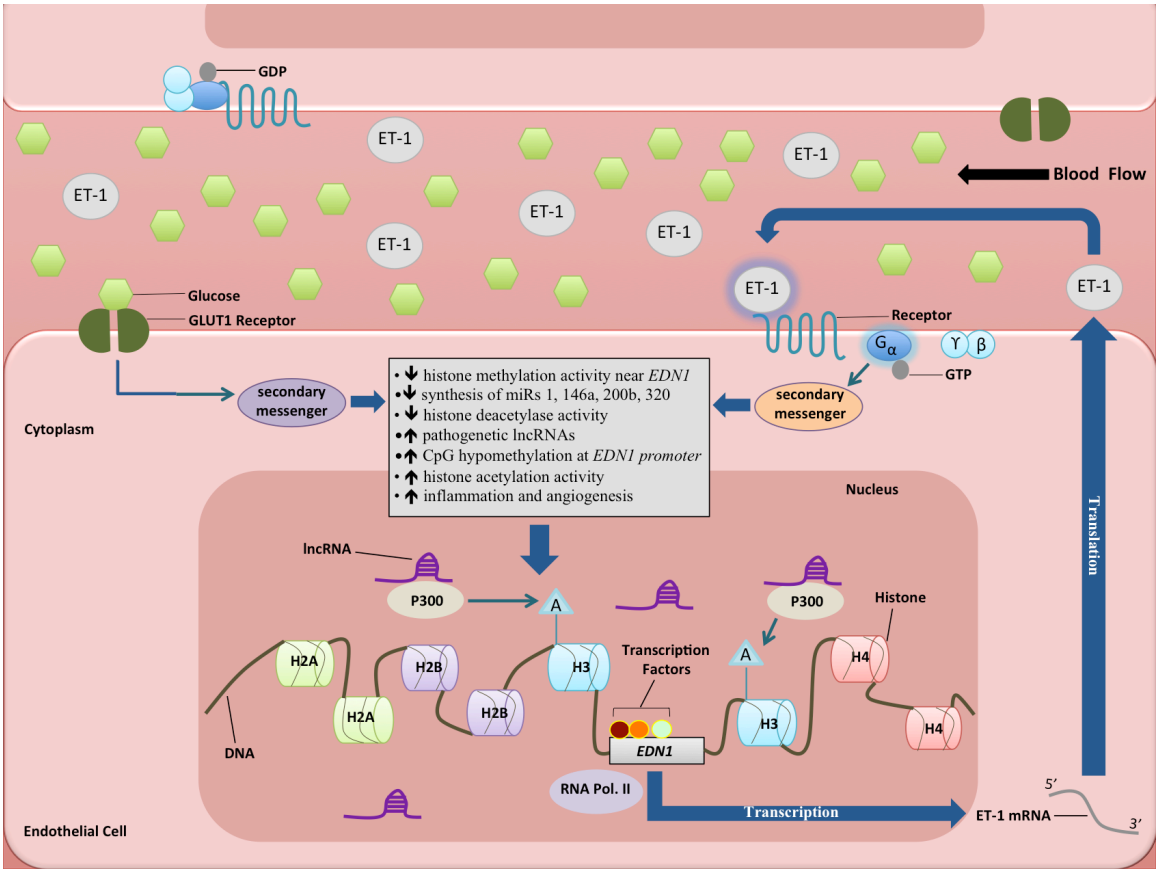

Figure 4.4. An illustration depicting the proposed epigenetic paradigm underlying ET-1 regulation in microvascular endothelial cells in diabetes. Based on our previous and current findings, we propose this epigenetic model in which histone modifications, DNA methylation, miRNAs, and lncRNAs can possibly influence *EDN1* transcriptional activity and ultimately ET-1 RNA and protein levels. Legend: GDP= guanosine diphosphate; GTP= guanosine triphosphate; lncRNA= long non-coding RNA; miRs= microRNAs; P300= histone acetyltransferase, H2A-H4= histones; RNA Pol. II= RNA polymerase II; and A= acetylation. Reproduced from *Endothelin-1 Regulation Is Entangled in a Complex Web of Epigenetic Mechanisms in Diabetes* (p. 8) by S. Biswas, B. Feng, A. Thomas, S. Chen, E. Aref-Eshghi, B. Sadikovic, and S. Chakrabarti, 2018, Physiological Research.

Incorporation of the emerging regulatory mechanisms into the previously understood paradigm of ET-1 signalling becomes imperative in the development of novel ET-1-based therapeutics. Although the findings from our study shed novel insights into the epigenetic influence on ET-1 regulation, further mechanistic studies are necessary to

elucidate the direct relationship between epigenetic mediator proteins and ET-1 in the diabetes context.

4.4 References

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4.5 Appendix C: Endothelin-1 regulation is entangled in a complex web of epigenetic mechanisms in diabetes

Supplemental Table 1. qPCR primers for human *ET-1*.

Target Gene:	Oligonucleotide Sequence (5' → 3')
B-actin (Homo)	Forward: CCTCTATGCCAACACAGTGC Reverse: CATCGTACTCCTGCTTGCTG
Endothelin-1 (Homo)	Forward: AAGCCCTCCAGAGAGCGTTAT Reverse: CCGAAGGTCTGTCTGTCACCAATGT

Chapter 5 [v]

5 General Discussion

5.1 Thesis Summary

The body of work presented in this thesis describes studies of lncRNAs and their molecular mechanisms in the pathogenesis of DR. Namely, through early microarray analyses (GEO: GSE122189) involving NG and HG-cultured retinal ECs, I selected two of the most prominent glucose-induced lncRNAs for further follow-up: *MALAT1* and *HOTAIR*. In Chapter 2, I specifically examined the epigenetic implications of *MALAT1* in inflammation in DR using various cell culture and diabetic animal models. I showed that the lncRNA *MALAT1* is capable of impacting the expressions of inflammatory transcripts (including *TNF-α* and *IL-6*) through its association with histone methylation and DNA methylation processes. Similarly, I demonstrated *in vivo* that the *Malat1* gene is directly involved in DR pathogenesis, as the genetic ablation of *Malat1* in diabetic animals significantly alleviated diabetes-induced upregulations of retinal inflammatory transcripts and prevented vascular leakage, compared to the retinas from diabetic animals with a wild-type background. Furthermore, I also alluded to the potential clinical significance of *MALAT1* in human DR, with distinct differences for *MALAT1* existing in the vitreous fluid between diabetic patients with PDR and non-diabetic patients without diabetes.

After establishing the relationship between *MALAT1* and inflammation, I wanted to investigate whether lncRNAs are linked to other critical pathogenetic processes in DR, such as angiogenesis. Therefore, based on our microarray findings and previous literature in cancer [1, 2], I wanted to delineate whether the lncRNA *HOTAIR* can similarly mediate angiogenic processes in DR, which was the focus of Chapter 3. Indeed, *in vitro* and *in vivo* experiments involving siRNA-mediated knockdown of

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[v] Some content in Chapter 5 has been adapted from Biswas S, Sarabusky M, Chakrabarti S. Diabetic Retinopathy, lncRNAs, and Inflammation: A Dynamic, Interconnected Network. Journal of Clinical Medicine: 2019;8(7): 1033, doi: 10.3390/jcm8071033. This work was published under a Creative Commons Attribution License.

HOTAIR demonstrated that this lncRNA could directly impact the expressions of several angiogenic cytokines, as well as critical epigenetic and DR-related molecules. *HOTAIR* was also shown to be involved in glucose-induced mitochondrial and oxidative DNA damage, while mechanistic-based experiments further illustrated that *HOTAIR* may be regulating the transcriptional status of certain angiogenic genes through its association with epigenetic RNA-binding proteins, including histone methyltransferases, histone acetyltransferases and RNA polymerase II. Moreover, I established that DNA methylation mechanisms are also implicated in the regulation of *HOTAIR* and its downstream targets. Extending my *in vitro* findings, I discovered that *HOTAIR* RNA levels are specifically elevated in the vitreous fluid and serum of diabetic patients with PDR, which further allude to the biological significance of lncRNAs in human DR.

In Chapter 4, I examined the interrelationships shared between epigenetic mechanisms and specific molecular alterations in DR. As such, using HRECs, I explored the implications of DNA methylation, histone methylation, and lncRNAs in the regulation of endothelin-1 (ET-1), a well-documented molecule that is dysregulated in diabetic complications. My findings indicated that HG environments could induce hypomethylation patterns in the promoter regions of *ET-1* (*EDN1*), while global blockade of DNA or histone methyltransferases could further augment glucose-induced elevations of ET-1. In addition to our findings, siRNA-mediated knockdown of certain pathogenetic lncRNAs also altered the expressions of *ET-1* transcripts, which ultimately confirmed the presence of an epigenetic axis in the regulation of *ET-1*.

Taken together, this work has uncovered a novel epigenetic paradigm involving an intricate web of epigenetic mechanisms that regulate glucose-induced transcription of DR-associated molecules in important pathological processes (inflammation and angiogenesis).

5.2 LncRNAs: Missing Pieces of the Epigenetic Puzzle in DR?

There is no doubt that the existing molecular network is complex. We know that under homeostatic conditions, cellular mechanisms are carried out in a very coordinated manner. However, in the event of chronic hyperglycemia, the activities of several genes go awry, which significantly alter the spatial and temporal kinetics of various signalling pathways and consequently promote damaging cellular processes. Interestingly, several studies have made it evident that damaging cellular events (such as the generation of oxidative stress) can still mediate the persistence of diabetic vascular complications even after glucose normalization—this is known as 'metabolic memory' [3-8]. Epigenetics, which refer to mechanisms that modify the expression of genes without changing the underlying nucleotide composition, is an attractive field of study underlying metabolic memory, since these mechanisms have been critically implicated in diabetes [9-11]. Epigenetic mechanisms include histone modifications, DNA methylation, and the activity of non-coding RNAs and as discussed before, several lncRNAs can exert their functionalities through these epigenetic mechanisms in various pathologies. Considering the diverse types of epigenetic mechanisms present, very few studies exist that take into consideration the complex crosstalk between lncRNAs and other epigenetic mechanisms in the pathogenesis of DR. Therefore, in my work, several experimental approaches were carried out in order to provide some insights into this dynamic epigenetic network and these findings, along with observations from current literature, will be discussed below.

5.2.1 Crosstalk between lncRNAs and DNA methylation

Considered one of the earliest discovered epigenetic mechanisms [12], DNA methylation involves the interactions between two opposing enzymes that facilitate the methylation status of cytosine residues in CpG dinucleotides: either through the addition (via DNA methyltransferases; DNMTs) or removal (via DNA demethylases) of methyl groups [13]. Furthermore, genomic regions that contain a high frequency of CpG dinucleotides are known as CpG islands (CGIs), which reside in the

regulatory/promoter regions of genes, and the CGIs can ultimately determine the transcriptional activity of a gene based on its degree of methylation [13, 14]. For instance, promoter CGIs that are hypermethylated are associated with gene silencing, while conversely hypomethylation is associated with gene activation [13, 14]. Indeed, in recent years, the impact of DNA methylation has been documented in DR, where previous reports suggest that hyperglycemia can evoke distinct methylation patterns in the promoters of miRNAs [15] and several DR-related genes (such as *TNF* and *MMP-9*) [16-18], which further the progression of DR. Adding to these results, findings from my *MALAT1* and *HOTAIR* studies demonstrate for the first time that reciprocal relationships exist between lncRNAs and DNMTs in the regulation of specific inflammatory and angiogenic mediators during DR pathogenesis (**Chapters 2** [19] and **3**). In fact, the knockdown of *MALAT1* or *HOTAIR* prevented glucose-induced elevations of DNMTs in HRECs, while blocking the actions of DNMTs (through siDNMT1 or pan-DNMT inhibitors) exacerbated glucose-induced lncRNA expressions and also evoked differential expressions of various downstream targets of *HOTAIR* and *MALAT1*. These results suggest that both DNMTs and lncRNAs actively participate in the transcriptional regulation of several genes under hyperglycemic environments. In support of our notion, previous reports in colon cancer [20], myogenesis [21], and neural differentiation [22] have revealed that certain lncRNAs can regulate gene expressions and DNA methylation in biological or pathological processes through their potential association with DNMTs. For example, using ChiRP-Seq (Chromatin Isolation by RNA purification), Merry et al [20] demonstrated that the induction of the lncRNA *DACOR1* (DNMT1-associated Colon Cancer Repressed lncRNA 1) could enhance DNA methylation at multiple loci without affecting DNMT1 protein levels in colon cancer cells [20]. Following additional mechanistic-based experiments, the authors concluded that *DACOR1* could indirectly regulate the methylome through its influence on DNMT1 genomic occupancy and/or its ability to govern the levels of certain substrates (Cystathionine β-synthase) that are required for the generation of key methyl donors utilized by DNMTs. Nevertheless, similar mechanistic studies are warranted to further solidify the role(s) of DNMT-associated lncRNAs in the context of DR. As well, it still remains to be determined whether the

inhibition of DNA methylation through siDNMT1 or pan-DNMT inhibitors alters neighbouring (or distal) genes, which in turn may substantially impact the transcriptional activity of *MALAT1* and *HOTAIR*.

We further interrogated the methylation status of individual CpG sites across the *MALAT1* and *HOTAIR* genes in both NG and HG-cultured HRECs using a DNA methylation array. Interestingly, we observed that transient glucose treatments (48 hours) did not significantly alter the methylation status of several CpG sites across the *MALAT1* and *HOTAIR* gene. One possible explanation for this result may be attributed to the stable epigenetic nature of DNA methylation marks during hyperglycemic stress. For example, a previous report has documented that active global DNA methylation changes can take place prior to the physiological elevation of glucose levels in humans [23]. Based on this, although we conducted our DNA methylation experiment at one particular time-point, it would be intriguing to see whether initial hyperglycemic treatments can provoke persistent, long-lasting changes in the methylation status of CpG sites across the *MALAT1* and *HOTAIR* genes. Constructing such an *in vitro* cell culture model involving multiple time-points and alternating glucose treatments will provide unique insights behind metabolic memory and the regulatory nature of DNA methylation on the biogenesis of lncRNAs during the progression of DR.

5.2.2 The Interplay between lncRNAs and Histone Modifications

Another fundamental epigenetic mechanism involved in the coordination of gene expression is histone modifications. Histone-modifying enzymes, including histone demethylases, histone methyltransferases, histone deacetylases and histone acetyltransferases, coordinate their actions by chemically modifying particular amino acid residues within histone proteins (H2A, H2B, H3, and H4), which govern the overall conformation of the chromatin and its accessibility to transcription factors for gene transcription at that modified region [24-28]. For example, an open configuration (euchromatin) can be induced by histone acetyltransferases through the acetylation of

lysine residues, which generally leads to active gene transcription [24-26]. Conversely, depending on the degree of methylation and the specific residue, histone methyltransferases facilitate the methylation of lysine residues that can drive gene silencing (a heterochromatin state) or activation [27, 28]. Changes in histone modifications have been extensively reported in multiple cancers [29] and in recent years, several studies are also documenting the presence of aberrant histone modifications in diabetic environments [30-36]. Despite the breadth of information available, few studies have addressed the involvement of histone modifications on lncRNA-mediated mechanisms in DR. In fact, presently, only studies from our laboratory have addressed the influence of histone methylation and acetylation processes on lncRNAs in DR, which will be the topic of discussion in the paragraphs below.

Polycomb repressive complex 2 (PRC2) is a multimeric histone methyltransferase complex that catalyzes the tri-methylation of lysine 27 on histone 3 (H3K27me3), a distinct chromatin mark linked with gene repression [37]. A previous report from our laboratory demonstrated that the core components of PRC2 (EZH2, SUZ12, and EED) were significantly augmented in HG-cultured HRECs and retinal tissues of diabetic rats and mice, which were also accompanied by increased VEGF expressions and reduced miR-200b levels (a negative regulator of VEGF) [38]. Interestingly, using ChIP-qPCR analyses, HG-cultured HRECs exhibited increased H3K27me3 and decreased RNA polymerase 2 associations in the promoter region of *miR-200b* when compared to NG controls. Furthermore, administration of DZNep significantly prevented HG-induced reductions of miR-200b, while VEGF RNA and protein levels were dramatically decreased in parallel—suggesting that PRC2 can negatively regulate miR-200b, while indirectly promoting the expressions of VEGF, in hyperglycemic environments.

Another study from our laboratory indicated for the first time that lncRNAs are closely connected with PRC2 functions in DR. Notably, the lncRNA *ANRIL* (antisense RNA to *INK4* locus) was shown to exhibit a pathogenetic phenotype in DR through its augmented expressions in HRECs and retinal tissues following hyperglycemia [39].

Moreover, retinal tissues from *Anril* knockout diabetic mice revealed depressed expressions of *Ezh2* and *Eed* RNA levels when compared to retinas from wild-type diabetic animals. Similar observations were also reported in HG-cultured HRECs following *ANRIL* silencing—confirming *ANRIL*'s direct impact on the EZH2 and EED subunits of PRC2. On the other hand, administration of DZNep in HG-cultured HRECs dramatically reduced both *ANRIL* and *VEGF* RNA expressions, which suggests that a highly interactive network may exist between these molecules. Potential binding interactions between *ANRIL* and PRC2 were further assessed by RNA immunoprecipitation analyses, where it was demonstrated that HG could promote strong binding associations between EZH2 and *ANRIL*. Of note, *ANRIL* also shared a similar relationship with P300, which is a prominent histone acetyltransferase involved in the regulation of several glucose-related genes [40-42]. Generally, HG-induced upregulations of P300 were corrected following *ANRIL* silencing in HRECs and *Anril* knockout diabetic mice exhibited reduced retinal *p300* levels compared to retinas from wild-type mice. Interestingly, transfection with siP300 in HG-treated HRECs did not alter *ANRIL* expressions [39].

Extending our *ANRIL* study, I demonstrated in my thesis that two other lncRNAs (*MALAT1* and *HOTAIR*) can also significantly influence PRC2 and P300 regulation of target gene expressions in DR (Chapters 2 [19] and 3). Indeed, it was evident that siRNA-mediated silencing of *MALAT1* or *HOTAIR* is capable of significantly preventing diabetes-induced increases of PRC2 components and P300, while the inhibition of PRC2 (through DZNep or siEZH2) can evoke differential expressions of these lncRNAs and their target downstream molecules. Moreover, strong binding associations were apparent between histone modifying enzymes (i.e. EZH2 and P300) and these two lncRNAs in HG environments. Interestingly, the knockdown of *MALAT1* was also shown to directly reduce protein expressions of EZH2 (**Appendix A, Figure S2C**) in HG, while *HOTAIR* knockdown was capable of governing methylation and acetylation statuses in the *VEGF-A* promoters following HG culture (**Figure 3.12**). These results suggest that lncRNAs may be able to govern the expressions of certain histone modifying enzymes in addition to influencing their functional capabilities. Supporting our observations, previous reports have shown that

MALAT1 and *HOTAIR* can share direct regulatory relationships with histone modifying enzymes [43, 44]. For instance, *MALAT1* has been shown to detach EZH2 from binding with the HIV1-long terminal repeat promoter, subsequently removing the PRC2-mediated H3K27me3 mark in this region and relieving epigenetic silencing of HIV-1 transcription [43]. Similarly, *HOTAIR* can tether distinct RNA-binding complexes and coordinate their targeting to chromatin for coupled histone modifications on target genes. For example, PRC2 has been shown to directly bind to the 5' domain of *HOTAIR*, while the 3' domain of *HOTAIR* binds to a demethylase that mediates enzymatic demethylation of H3K4me2 (lysine-specific demethylase 1; LSD1) [44]. Intriguingly, the knockdown of *HOTAIR* in primary foreskin fibroblasts was shown to decrease the occupancy of SUZ12 and LSD1 in the proximal promoters of *HOXD* genes [44]. Taken together, these findings allude to the potential abilities of lncRNAs to form scaffolds or act as guides with certain chromatin-modifying enzymes in diabetic environments. Further mechanistic-based studies are undoubtedly warranted that closely examine the relationship between lncRNAs (such as *HOTAIR* and *MALAT1*) and other key RNA-binding proteins in mediating the pathogenesis of DR.

5.2.3 The Relationship Between lncRNAs and miRNAs in DR: Friend or Foe?

Emerging as critical post-transcriptional regulators of gene expression [45, 46], miRNAs are small ncRNAs (~22 nucleotides in length) that can bind to the 3'-UTR of their target mRNAs, subsequently leading to mRNA degradation and/or inhibition of protein translation [47]. Within the last decade, the implications of miRNAs have been well identified in cardiovascular disease [48], cancer [49], neurodegenerative diseases [50], and even DR [42, 51-54]. Although I did not examine the role of miRNAs in my experimental work, emerging studies in DR are beginning to reveal a unique relationship shared between miRNAs and lncRNAs, which will be briefly discussed below.

Beginning with the lncRNA *MIAT* (myocardial infarction associated transcript), Yan et al. have shown that this lncRNA can function as a molecular sponge/decoy that sequesters miR-150-5p, which subsequently promotes the expression of its target mRNA (*VEGF*) in DR [55]. Using bioinformatics, the authors first identified predicted binding sites for miR-150-5p on its targets, *VEGF* and *MIAT*. These predictions were then confirmed *in vitro* through luciferase assays, which demonstrated that miR-150-5p directly targeted *VEGF* and *MIAT* transcripts. Additional mechanistic-based experiments involving miR-150-5p mimics and *MIAT* overexpressing ECs suggested that a dynamic interplay exists between *MIAT*, miR-150-5p, and VEGF in the critical functions of ECs during HG stress. Specifically, VEGF expressions were significantly upregulated in *MIAT*-overexpressing cells, while VEGF expressions were markedly reduced with increasing levels of miR-150-5p in *MIAT*-overexpressing cells. In a similar manner, gradual increases in *MIAT* were capable of restoring miR-150-5p-induced downregulations of VEGF in miR-150-5p-overexpressing ECs, further alluding to the regulatory crosstalk between these molecules. Other studies have also alluded to miRNAs as potential negative regulators of lncRNAs and mRNAs. For instance, miR-185-5p was shown to directly regulate the lncRNA *RNCR3* (retinal non-coding RNA 3) and the mRNA Krüppel-like Factor 2 (*KLF2*) [56]. In fact, miR-185-5p mimics decreased the expressions of *RNCR3* and *KLF2* and subsequently contributed to reduced viability and proliferation in chorioretinal ECs. Moreover, inverse regulatory relationships have also been documented for the lncRNA *MEG3* and miR-34a in retinal epithelial cells [57]. Accordingly, HG environments were shown to promote the upregulation of miR-34a and downregulations of *MEG3* and a histone deacetylase (SIRT1), while the overexpression of *MEG3* in these cells dramatically reversed HG-induced effects. Interestingly, using luciferase assays and mimics and inhibitors for miR-34a, the researchers confirmed that *MEG3* could positively regulate SIRT1 by directly sponging its negative regulator, miR-34a. With the consequent downregulation of miR-34a, *MEG3* overexpression was shown to reduce HG-induced apoptosis and inflammation. Based on these reports, future studies should continue investigating the dynamic crosstalks shared between lncRNAs,

miRNAs and their target mRNAs, as these findings will provide significant insights into perturbed regulatory networks that are implicated in the pathogenesis of DR.

5.3 Limitations and Future Directions

Although the findings generated from my experiments highlight the critical regulatory capabilities of lncRNAs in DR, one must recognize that inherent limitations oftentimes exist in experimental models. Beginning with my *in vitro* experiments, HRECs were grown in monolayers using culture flasks and plates, which constitute a two-dimensional (2D) cell culture model. As such, it is likely that ECs organized in a 2D culture system may not accurately recapitulate the bioactivities of ECs in an *in vivo* environment, since differences in motion and migration, extracellular matrix composition, and mechanical responses exist between the structure of ECs in 2D culture and *in vivo* environments [58]. These differences may also contribute to variations in gene expressions. Therefore, due to these reasons, it would be interesting to implement novel 3D (three-dimensional) cell culture models that could accurately model the composition of blood vessels *in vivo*.

Furthermore, ECs were the primary cell-type used in my experiments because these cells are the earliest (and primary) targets of diabetes-induced tissue damage. Considering that the retina is comprised of multiple layers of cells, simply focusing on ECs may not provide a complete picture of the molecular events transpiring across the retina during DR. Thus, future experiments should examine the expressions of lncRNAs in other retinal cell types. For example, in a study by Liu et al., the knockdown of *MALAT1* was shown to prevent capillary degeneration, microvascular leakage, retinal inflammation, as well as diabetes-induced retinal pericyte loss [59]—alluding to potential molecular crosstalks shared between various cell types. Interestingly, recent evidences have also demonstrated that inflammatory environments can induce cerebrovascular ECs to shed endothelial-derived exosomes, containing bioactive molecules (i.e. miRNAs), which can subsequently modulate pericyte responses [60]. With exosomal lncRNAs being reported in various pathologies [61], it would be logical to explore the potential regulatory functions and

transport pathways of these extracellular vesicles in cell-to-cell communication (i.e. between retinal ECs and pericytes) during DR pathogenesis.

Although we examined specific subsets of diabetes-related molecules across our studies, there is a possibility that we may have missed out on other critical molecules regulated by these lncRNAs. As such, future experiments should continue to examine the global transcriptome and methylome, using high-throughput sequencing methods, after the administration of epigenetic inhibitors (from DZNep to 5-aza-dC) and/or lncRNA-specific siRNAs in HRECs. Such findings, through ChIP-Seq, RIP-Seq or even DNA methylation arrays, can enable researchers to further elucidate the effects of these compounds on the epigenome in its entirety. Similarly, with the optimization of next-generation sequencing technologies in the last few years, it would be fruitful to perform single-cell RNA-seq using diabetic retinal tissues, as this information will help us understand whether discrete and abundant expressions for lncRNAs exist among individual cells.

Furthermore, in our studies, we examined very specific time-points across our cell culture and animal models, which impose its own limitations, as we are unable to examine the effects of metabolic memory on transcriptional regulation and the long-term consequences of inhibiting pathogenetic lncRNAs in the retina of diabetic animals. For instance, in our diabetic animal models, the one and two-month end-points were selected because early retinal aberrations can be detected during this time. Of course, if graduate degrees did not possess certain time constraints, later end-points for diabetes could have definitely been examined using the animal models presented in Chapters 2 and 3. Regardless, future work should definitely investigate the long-term implications of *Malat1* knockout or *Hotair* knockdown in the retinas of diabetic animals. Moreover, unlike *Malat1*, we were unable to use a global *Hotair* knockout mice model because the targeted deletion of *Hotair* was shown to cause homeotic transformation of the spine and malformation of metacarpal-carpal bones in mice [62]. An alternative solution to this limitation could be through the use of a Cre/loxP system to generate a retinal tissue-specific or inducible knockout of *HOTAIR* [63].

Other limitations in my studies include not examining histone and DNA demethylases, histone deacetylases, histone phosphorylation, histone ubiquitination and other histone modifications. Future studies should definitely investigate these mechanisms, as potential crosstalks could exist between different histone modifications and may uncover novel interactions shared between lncRNAs and other chromatin-modifying complexes. Furthermore, in addition to the double-stranded siRNAs used in our *MALAT1* and *HOTAIR* knockdown experiments, future investigations should also consider alternative approaches that ensure complete gene knockout or stronger knockdowns. For example, CRISPR/Cas9 knockout strategies could be used in HRECs to ablate the lncRNAs of interest, while other novel antisense molecules, such as locked nucleic acids (i.e. GapmeRs), or short hairpin RNAs (shRNAs) can also be used to target the lncRNAs of interest and obtain strong knockdowns in retinal ECs.

Lastly, in regard to our clinical experiments, further large-scale investigations are warranted to determine the complete clinical validation of *MALAT1* and *HOTAIR* as potential prognostic or diagnostic markers for DR. As such, larger sample sizes with appropriate power calculations, stringent cut-off criteria and determination of clinically meaningful endpoints will be required to truly understand the clinical significance of these lncRNAs in DR.

5.4 Concluding Remarks

In recent years, the rapid advent of genomic technologies has drastically improved our capacity to survey the intricate complexities of the genomic landscape. What were once considered ‘junk DNA’ or ‘dark matter’, lncRNAs (and other ncRNAs) are now proving to be dynamic regulators of gene expression, as these versatile RNA molecules can interact with different layers of regulation: epigenetic, transcriptional, and post-transcriptional. In this thesis, I have discussed two lncRNAs and their regulatory implications in the pathogenesis of DR.

With new, unannotated lncRNAs frequently being discovered, added efforts must be taken to functionally characterize these lncRNAs in the field of diabetes, especially DR. Additional mechanistic understanding of lncRNAs will allow us to appreciate the

dynamic regulatory networks behind these RNA molecules in pathophysiological processes, which will further create novel avenues for research and drug discovery in DR. As well, from the lncRNAs I have examined in my thesis, it is quite evident that these lncRNAs co-exist in a highly coordinated molecular network. Therefore, going forward, future studies should consider implementing integrated experimental approaches. Such approaches, and with the help of appropriate computational and database-driven tools, will help pinpoint potential lncRNA regions in disease-specific contexts. Once the target lncRNA is identified, subsequent gain-of-function and loss-of-function experiments will further provide in-depth understanding of the lncRNA functionalities. Nevertheless, given the pervasive nature of transcription throughout the mammalian genome, it is not surprising that other new ncRNA groups (circular RNAs, snoRNAs, and piwi-interacting RNAs) are also beginning to emerge as critical players in diabetes [64-66], which truly adds new dimensions to this perplexing transcriptional paradigm and require further consideration as well.

5.5 References

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Curriculum Vitae

Name: Saumik Biswas

EDUCATION

Ph.D. Candidate in Pathology and Laboratory Medicine *2015–Expected 2020*
Supervisor: Dr. Subrata Chakrabarti
Schulich School of Medicine and Dentistry
University of Western Ontario, London, Ontario

Bachelor of Science in Medical Sciences (Hon., First Class Standings) *2011–2015*
Brock University, St. Catharines, Ontario

RESEARCH AND WORK EXPERIENCE

Graduate Research Student, Western University *2015–2020*

- Involved in a translational research project that both identifies and improves knowledge of lncRNAs in diabetic complications

Summer Research Intern, The University of Hong Kong *2019*

- Developed a novel epigenetics-based project on non-alcoholic fatty liver disease using similar research principles from my PhD projects

Graduate Teaching Assistant, Western University *2017–2019*

- Assisted in the development of active learning exercises for two fourth year Medical Sciences' courses: Medical Sciences 4200G (Inflammation in Diseases) and Medical Sciences 4300F (Addressing Healthcare Misconceptions Using Scientific Inquiry)

PEER-REVIEWED PUBLICATIONS

1. **Biswas S,** Chakrabarti S. Pathogenetic Mechanisms in Diabetic Retinopathy: From Molecules to Cells to Tissues. In: Kartha CC, Ramachandran S, Pillai RM, eds. Mechanisms of Vascular Defects in Diabetes Mellitus. Cham: *Springer International Publishing*; 2017:209-247. doi:10.1007/978-3-319-60324-7_9. **Book chapter**.

2. Gordon A, **Biswas S**, Feng B, Chen S, Chakrabarti S. MALAT1: A Regulator of Inflammatory Cytokines in Diabetic Complications. *Endocrinology, Diabetes & Metabolism*; 2018. doi: 10.1002/edm2.10. **Research article**.

3. **Biswas S**, Thomas AA, Chen S, Aref-Eshghi E, Feng B, Gonder J, Sadikovic B, Chakrabarti S. MALAT1: An Epigenetic Regulator of Inflammation in Diabetic Retinopathy. *Scientific Reports:* 2018;8(1). doi:10.1038/s41598-018-24907-w. **Research article**.

4. **Biswas S**, Feng B, Thomas AA, Chen S, Aref-Eshghi E, Sadikovic B, Chakrabarti S. Endothelin-1 regulation is entangled in a complex web of epigenetic mechanisms in diabetes. *Physiological Research*: 2018;67(Supplementum 1): S115-S125. **Research article**.

5. **Biswas S**, Thomas AA, Chakrabarti S. LncRNAs: Proverbial Genomic "Junk" or Key Epigenetic Regulators During Cardiac Fibrosis in Diabetes?. *Frontiers in Cardiovascular Medicine*. 2018; 5:28. doi:10.3389/fcvm.2018.00028. **Review article**.

6. Thomas AA, **Biswas S**, Feng B, Chen S, Gonder J, Chakrabarti S. lncRNA H19 prevents endothelial--mesenchymal transition in diabetic retinopathy. *Diabetologia*. January 2019. doi:10.1007/s00125-018-4797-6. **Research article**.

7. **Biswas S**, Chakrabarti S. Increased Extracellular Matrix Protein Production in Chronic Diabetic Complications: Implications of Non-Coding RNAs. *Non-Coding RNA*. 2019;5(1). doi:10.3390/ncrna5010030. **Review article**.

8. **Biswas S**, Sarabusky M, Chakrabarti S. Diabetic Retinopathy, lncRNAs, and Inflammation: A Dynamic, Interconnected Network. *J. Clin. Med.* 2019; 8, 1033. https://doi.org/10.3390/jcm8071033. **Review article**.

9. **Biswas S**, Chen S, Liang G, Feng B, Cai L, Khan ZA, Chakrabarti S. Curcumin Analogs Reduce Stress and Inflammation Indices in Experimental Models of Diabetes. *Frontiers in Endocrinology*, 10, 887. https://doi.org/10.3389/fendo.2019.00887. **Research article.**

10. Liu J, Chen S, **Biswas S**, Nagrani N, Chu Y, Chakrabarti S. Glucose-induced oxidative stress and accelerated aging in endothelial cells are mediated by the depletion of mitochondrial SIRTs. *Physiological Reports*. 2020; 8(3). p.e14331. **Research article**.

11. **Biswas S**, Chakrabarti S. The multifaceted roles of lncRNAs in diabetic complications: a promising, yet perplexing paradigm. In: Jurga S, Barciszewski J, eds. The Chemical Biology of Long Noncoding RNAs. Cham: *Springer Series, RNA Technologies* (in press, 2020). **Book chapter**.

12. Aref-Eshghi E, **Biswas S**, Chen C, Sadikovic B, Chakrabarti S. Glucose-induced duration-dependent genome-wide DNA methylation changes in human endothelial

cells. *American Journal of Physiology-Cell physiology*. 2020, Advance online publication. https://doi.org/10.1152/ajpcell.00011.2020. **Research article**.

13. Zheng H, Baranova K, Song J, Yan L, **Biswas S**, Chakrabarti S, Zhang Q. Overexpression of Long Noncoding RNA HOTAIR is a Unique Epigenetic Characteristic of Myxopapillary Ependymoma (in preparation, 2020). **Research article.**

14. **Biswas S**, Feng B, Chen S, Liu J, Aref-Eshghi E, Gonder J, Ngo V, Sadikovic B, Chakrabarti S. The long non-coding RNA *HOTAIR* is a critical epigenetic mediator of angiogenesis in diabetic retinopathy (submitted, 2020). **Research article.**

E. CONFERENCE PRESENTATIONS

1. **Biswas S,** Feng B, Chen S, Aref-Eshghi E, Gonder J, Sadikovic B, Chakrabarti S. *The Long Non-Coding RNA HOTAIR is an Important Angiogenic Mediator in Diabetic Retinopathy.* **Oral presentation** at the 55th European Association for the Study of Diabetes Annual Meeting, Barcelona, Spain, September 16-20, 2019.

2. **Biswas S,** Chen S, Feng B, Aref-Eshghi E, Ngo V, Duennwald M, Gonder J, Sadikovic B, Chakrabarti S. *The Long Non-Coding RNA HOTAIR is an Important Angiogenic Mediator in Diabetic Retinopathy.* **Poster presentation** at the London Health Research Day Conference, London, ON, April 30, 2019.

3. **Biswas S,** Feng B, Chen S, Aref-Eshghi E, Gonder J, Sadikovic B, Chakrabarti S. *The Long Non-Coding RNA HOTAIR is an Important Angiogenic Mediator in Diabetic Retinopathy.* **Oral presentation** at the Annual Pathology and Laboratory Medicine Research Day, London, ON, March 28, 2019.

4. **Biswas S**, Thomas AA, Feng B, Chen S, Aref-Eshghi E, Gonder J, Sadikovic B, Chakrabarti S. *MALAT1 and HOTAIR: Key Epigenetic Regulators in Diabetic Retinopathy*. **Poster presentation** at the TREnD Conference, Toronto, ON. July 31, 2018.

5. **Biswas S**, Thomas AA, Feng B, Chen S, Aref-Eshghi E, Gonder J, Sadikovic B, Chakrabarti S. *MALAT1 and HOTAIR: Key Epigenetic Regulators in Diabetic Retinopathy*. **Oral presentation** at the 78th American Diabetes Association Conference, Orlando, Florida, US. June 25, 2018.

6. **Biswas S**, Thomas AA, Feng B, Chen S, Gonder J, Chakrabarti S. *Role of long non-coding RNA MALAT1 in the pathogenesis of diabetic retinopathy*. **Oral presentation** at the 20th Annual Canadian Diabetes Association Conference, Edmonton, Alberta, November 1-4, 2017.

7. **Biswas S**, Thomas AA, Chen S, Aref-Eshghi E, Feng B, Gonder J, Sadikovic B, Chakrabarti S. *Role of long non-coding RNA MALAT1 in the pathogenesis of diabetic retinopathy*. Presented a **poster** at TREnD conference, Toronto, ON, August 2, 2017.

8. **Biswas S**, Gordon A, Thomas AA, Feng B, Chen S, Gonder J, Chakrabarti S. *Role of long non-coding RNA MALAT1 in the pathogenesis of diabetic retinopathy*. Presented **poster** at the Annual Pathology and Laboratory Medicine Research Day, London, ON, March 30, 2017.

9. **Biswas S**, Gordon A, Thomas AA, Feng B, Chen S, Gonder J, Chakrabarti S. *Role of long non-coding RNA MALAT1 in the pathogenesis of diabetic retinopathy*. **Oral presentation** at the London Health Research Day Conference, London, ON, March 28, 2017.

F. SCHOLARSHIPS AND AWARDS

The Cameron Wallace Graduate Student Award in Pathology ($1400) *Apr. 2020*

- An award that recognizes a graduate student's high achievement in research, course work, and social involvement

WORLDiscoveries Vanguard Award (Pin) *Nov. 2019*

- An award that recognizes an inventor's first report of invention (ROI; **Tech ID W-20-010**) to WORLDiscoveries

EASD Annual Meeting Travel Grant Application ($1,200.00) *Sept. 2019*

- A limited number of travel grants were awarded to presenting authors of accepted abstracts at the 55th Annual Meeting of the European Association for the Study of Diabetes (EASD) in Barcelona, Spain

Dutkevitch Award ($1000.00) *Sept. 2019*

- Awarded to graduate students who are presenting their research work at a national or international conference

Nellie Farthing Fellowship in Medical Sciences Recipient ($3,000.00) *Jul. 2019*

- Established in 1960 through a generous legacy donation from Nellie Farthing, the Fellowship recognizes excellence in research to a full-time doctoral student in Medical Sciences. Candidate's record of research is of primary importance and this award is provided every year to only three graduate students out of the entire Schulich School of Medicine and Dentistry faculty

Ontario Graduate Research Scholarship Recipient ($15,000.00) *Sept. 2019–2020*

- Merit-based scholarship that is awarded to Ontario's best graduate students in all disciplines of academic study. The scholarship program at Western University (UWO) is jointly funded by the Province of Ontario and UWO. Specific number of awards varies between departments.

Mitacs Globalink Research Award ($6,000.00) *May-Aug. 2019*

- Merit-based funding that is awarded to undergraduate or graduate students who are planning to partake in an international research internship. Applicants must develop a unique project proposal and submit a detailed application package, which is later scored by Mitacs

Top Downloaded Article for *Endocrinology, Diabetes & Metabolism* *Jun. 2019*

- Amongst the articles published between January 2017 and December 2018 by *Endocrinology, Diabetes & Metabolism*, my research article entitled "MALAT1: A regulator of inflammatory cytokines in diabetic complications" was recognized as the top 20 most read paper

Top 100 Read Cell and Molecular Biology Papers for *Scientific Reports* *Apr. 2019*

- Out of 664 cell and molecular biology papers published by *Scientific Reports* in 2018, my research article entitled "MALAT1: An Epigenetic Regulator of Inflammation in Diabetic Retinopathy" placed in the top 100 most highly read articles

Dr. P.C. Raju & Jyoti Shah Graduate Education Prize ($1,000.00) *Mar. 2019*

- This award recognizes the Pathology and Laboratory Medicine graduate student with the best first-authored original or research article published

Ontario Graduate Research Scholarship Recipient ($15,000.00) *Sept. 2018–2019*

- Merit-based scholarship that is awarded to Ontario's best graduate students in all disciplines of academic study. The scholarship program at Western University (UWO) is jointly funded by the Province of Ontario and UWO. Specific number of awards varies between departments

Lawson Studentship Internal Research Fund ($15,000.00) *Dec. 2017–2018*

- Award provided to the project with the highest ranked proposal/application per competition. As determined by the Lawson IRF Review Committee, my research proposal/application received a score of 4.4 out of 5, which was considered in the "Excellent" category

Best Basic/Clinical Science Collaborative Poster Presentation ($100.00) *Apr. 2018*

- This award recognizes excellence in research design and performance, as well as the presentation of a research poster. This award is given to a Graduate Student or a Pathology Resident to recognize collaborative basic and clinical science research

Diabetes Canada Travel Award Recipient ($500.00) *Nov. 2017*

- Top 10 abstracts were selected out of 235 abstracts for this award. The purpose of this award is to encourage outstanding research in Canada by scientific trainees in the field of diabetes

Dutkevitch Award ($500.00) *Nov. 2017*

- Awarded to graduate students who are presenting their research work at a national or international conference

Proteus Innovation Competition Finalist *Mar. 2017*

- Advanced to 'Live Pitch Finale' stage of this four-month competition, where only six teams were selected out of 32 teams. We developed a viable commercialization strategy for an efficient snow removal system in Canada using cloud-based data collection technology

Western Graduate Research Scholarship (*$4500.00/year)* *2015–2018*

- Offer is based on undergraduate average (80%+), which must also be maintained throughout the duration of the graduate program to renew

Board of Trustees Spirit of Brock Medal (*Gold Medal)* *2015*

- A prestigious award made to one graduating student, selected by the Faculty, who exemplifies leadership, courage, innovation, inspiration, and community involvement

Brock University, Dean's Honour List *2011–2015*

- Honored for maintaining an average that is 80% or higher

Brock University, Brock Scholars Award (*$14,000.00)* *2011–2015*

- Offer is based on admission average (93%-100%) for which must be maintained throughout the duration of the undergraduate program to renew

Volunteer Recognition Award/Honour, City of St. Catharines *2013*

- Awarded to the top volunteers in the City of St. Catharines and recognized by the Mayor of St. Catharines

Brock University, Brock Academic Excellence Award *($1000.00)* *2011*

- Awarded to students entering first year at Brock with a 90% average

Queen Elizabeth Aiming for the Top Scholarship (*$7000.00)* *2011–2012*

- Awarded to the top 10% high school students that demonstrated academic excellence and financial need

G. VOLUNTEER EXPERIENCE AND COMMUNITY INVOLEMENT

Pathology Graduate Student Mentor, Western University *2017–2020*

- Became a mentor for new graduate students in the Pathology program

Retiring With Strong Minds (RWSM) Volunteer, Western University *2016–2019*

- Participated in graduate-related research talks at retirement homes around London, Ontario

Pathology Departmental SOGS Councillor, Western University *2016–2017*

- Attended all meetings of Council to advocate issues concerning the activities, programs, and decisions of the Graduate Society

Research Mentor for High School Students, Western University *2015–2020*

- Trained 5 high school students throughout the years and helped them become familiar with laboratory experiments and equipment

H. PEER-REVIEW ACTIVITIES

Journal Reviewer

- Reviewer (ad hoc), *Acta Ophthalmologica*
- Reviewer (ad hoc), *Biomedicine & Pharmacotherapy*
- Reviewer (ad hoc), *Cellular Physiology and Biochemistry*
- Reviewer (ad hoc), *Scientific Reports*
- Reviewer (ad hoc), *Ageing*
- Reviewer (ad hoc), *Acta Diabetologica*
- Reviewer (ad hoc), *Frontiers in Pharmacology*

I. PATENT(S)

1. Report of Innovation submitted to WORLDiscoveries (**Tech ID: W-20-010**): “A long non-coding RNA as a diagnostic marker and therapeutic target for ocular diseases and diabetic vasculopathies”.

2. Report of Innovation submitted to WORLDiscoveries (**Tech ID: W-21-001**): “A novel lncRNA-based diagnostic panel for monitoring chronic diabetic complications”.