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Role of Long Noncoding RNAs in Diabetic Complications

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Abstract

LncRNAs, recently found to be pervasively transcribed in the genome, can play key roles in epigenetic modifications during gene transcription. Although, lncRNAs have no coding potential they can regulate various biological processes. Their role in diabetic complications are however not clear.

Using array analysis, we identified alterations of two lncRNAs in high glucose (25mM/L,HG) treated retinal endothelial cells (HRECs). We validated upregulations of lncRNA, ANRIL and downregulation of H19 in HRECs following incubation in HG. ANRIL expressions were further measured with or without siRNA knockdown in ECs. ANRIL knockout (KO) mice with or without streptozotocin (STZ)-induced diabetes were also investigated for mRNA and protein expression of VEGF and ECM proteins FN and Col1α4. ANRIL knockdown prevented glucose-induced increased VEGF, FN and Col1α4 levels. Comparable results were observed in the retina, heart and kidneys of diabetic ANRILKO mice compared to wildtype controls. Furthermore, ANRILKO had a protective effect on diabetes-induced renal functional changes. We further showed that these alterations under ANRIL's regulation are mediated by p300 and enhancer of zeste 2 (EZH2) of the PRC2 complex.

We also investigated the role of H19 in diabetes by silencing or overexpressing H19 in ECs exposed to various glucose levels. We extended our study to H19 knockout (KO) mice and vitreous samples from patients with proliferative DR. In both instances, diabetes caused downregulation of H19 expression. The cells and retinal tissues from animals were examined. H19 overexpression prevented glucose-induced endothelial-mesenchymal transition (EndMT) through TGF-β in a Smad-independent pathway. Additional experiments showed a regulatory relationship between ANRIL and H19.

These data suggest that glucose and diabetes cause alteration of specific lncRNAs in ECs and mouse tissues respectively. This led to increased production of vasoactive factors and ECM proteins; and alteration of cellular phenotypes to facilitate vascular remodeling and other changes in diabetic complications. Identification of such mechanisms help in a better understanding of the pathologies in diabetes and consequent development of RNA based therapies.

Keywords

lncRNA, ANRIL, H19, VEGF, FN, ECM, endothelial-mesenchymal transition.

Co-Authorship Statement

Chapter 2: Thomas AA, Feng B, Chakrabarti S. *ANRIL: a regulator of VEGF in diabetic retinopathy*. Invest Ophthalmol Vis Sci, 2017. 58: p. 470-480.

Dr. Biao Feng was involved in the conception and design of the study and assisted with animal experiments. Dr. Subrata Chakrabarti (Supervisor) conceived and designed the research study. He also edited and revised the manuscript. Anu Alice Thomas was involved in the study design, performed all experiments, data analysis and all aspects of manuscript preparation. All authors approved final version of the manuscript prior to its submission.

Chapter 3: Thomas AA, Feng B, Chakrabarti S. *ANRIL regulates production of extracellular matrix proteins and vasoactive factors in diabetic complications*. Am J Physiol Endocrinol Metab, 2018. 314: p. E191-E200.

Dr. Biao Feng was involved in the design of the study and performed all ultrasound experiments and ELISA for collagen protein expression. Dr. Subrata Chakrabarti (Supervisor) guided the project, assisted with feedback on experimental strategies and edited the manuscript. Anu Alice Thomas was involved in the study design, performed experiments, data analysis and all aspects of manuscript preparation. All authors approved final version of the manuscript prior to its submission.

Chapter 4: Thomas AA, Biswas S, Gonder J, Chakrabarti S. *LncRNA H19 reverses Endothelial-Mesenchymal Transition in Diabetic Retinopathy*. (*Manuscript Submitted)*.

Dr. John Gonder collected vitreous humor (VH) from PDR patients. Saumik Biswas extracted VH samples and converted to cDNA. Dr. Subrata Chakrabarti (Supervisor) conceived and guided the project and was involved in editing and finalizing the manuscript. Anu Alice Thomas was involved in the conception of the study, performed experiments, data analysis and all aspects of manuscript preparation. All authors approved final version of the manuscript prior to its submission.

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Anu
April 2018

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List of Abbreviations

AGE	Advanced glycation end-product
ATP	Adenosine triphosphate
ANOVA	Analysis of Variance
ANRILKO	ANRIL knockout
ANRIL	Antisense ncRNA in the *INK4* locus
Ago	Argonaute
BL	Basal lamina
BM	Basement membrane
BWS	Beckwith-Wiedemann Syndrome
BCA	Bicinchoninic acid assay
CHIP	Chromatin immunoprecipitation
CKD	Chronic Kidney Disease
CD-31	Cluster of differentiation 31
Col1α4	Type 4 Collagen 1α4
CBP	CREB binding protein
DAPK2	Death-associated protein kinase 2
DZNEP	3-Deazaneplanocin A
DCM	Diabetic Cardiomyopathy
DC	Diabetic Complications
DN	Diabetic Nephropathy
DR	Diabetic Retinopathy
DAG	Diacylglycerol
DMR	Differentially methylated region
DGCR8	DiGeorge syndrome critical region 8
DNMTs	DNA methyl transferases
DPP-4	Dipeptidyl Peptidase-4
DIRAS3	GTP-binding protein Di-Ras3
EED	Embryonic ectoderm development protein
EBM-2	Endothelial basal media-2

EC	Endothelial Cell
ET-1	Endothelin-1
EndMT	Endothelial-Mesenchymal transition
eNOS	Endothelial Nitric Oxide synthase
ESRD	End-Stage Renal Failure
EZH2	Enhancer of zeste homolog 2
ELISA	Enzyme-linked immunosorbent assay
EMT	Epithelial–mesenchymal transition
ECM	Extracellular matrix proteins
FA	Fatty acid
FSP1	Fibroblast-specific protein 1
FN	Fibronectin
FADH2	Flavin Adenine Dinucleotide
FISH	Fluorescence *in situ* hybridization
FFA	Free fatty acid
GWAS	Genome-wide association studies
GBM	Glomerular basement membrane
GFR	Glomerular Filtration Rate
GSH	Reduced Glutathione
GLP-1	Glucagon like Peptide-1
GAPDH	Glyceraldehyde-3 Phosphate Dehydrogenase
H19KO	$H19^{\Delta exI}$ mice
HF	Heart Failure
HGF	Hepatocyte growth factor
HATs	Histone acetyl transferases
HDACs	Histone de-acetyl transferases
HG	High glucose, 25mM D-glucose
H3K4, H3K36,	Histone 3 methylated at lysine 4/lysine 36/
H3K79, H3K9,	lysine 79/ lysine 9/
H3K27	lysine 27
H3K27me3	Tri-methylation at lysine27

H4K20	Histone 4 methylated at lysine 20
HRECs	Human retinal endothelial cells
ICR	Imprinting control region
IL-1β	Interleukin-1 β
Igf2	Insulin-like growth factor 2
JNK/SAPK	Jun Kinases/Stress Activated Protein Kinases
Keap 1	Kelch-like ECH associated protein 1
Kg	Kilogram
LVH	Left Ventricle Hypertrophy
lncRNA	Long noncoding RNA
LSD1	Lys-specific demethylase 1
MMP-9	Matrix metalloproteinase-9
MALAT-1	Metastasis-associated lung adenocarcinoma transcript-1
MBDs	Methyl-CpG binding proteins
MC	Mesangial cells
miRNA	microRNA
Mg	Milligram
mL	Millilitre
mmol/L	Millimoles per litre
miR200b	miRNA 200b
mtDNA	mitochondrial DNA
MAP	Mitogen-activated protein
NADPH	Reduced Nicotinamide adenine dinucleotide phosphate
NO	Nitric Oxide
ncRNA	Noncoding RNA
NG	Normal glucose, 5mM D-glucose
Nrf2	Nuclear factor (erythroid-derived 2)-like 2
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
p/CAF	p300/CBP- associated factor
PAS	Periodic acid-Schiff
PBS	Phosphate-buffered saline

PAI-1	Plasminogen Activator Inhibitor-1
PARP	Poly (ADP-ribose) polymerase
PRC1 and 2	Polycomb Repressor Complex 1 and 2
PTHMs	Post-translational histone modifications
PDR	Proliferative Diabetic Retinopathy
PKC	Protein kinase C
ROS	Reactive Oxygen Species
RAS	Renin–Angiotensin–System
RT-PCR	Reverse transcription polymerase chain reaction
RIP	RNA Immunoprecipitation
RISC	RNA-induced silencing complex
SAM	S-adenosylmethionine
SRS	Silver-Russell Syndrome
SIRT 1	Sirtuin 1
siRNA	Small (or short) interfering RNA
α-SMA	α-Smooth muscle actin
SM22α	Smooth muscle 22α
SGLT2	Sodium Glucose Transporter-2
STZ	Streptozotocin
SUZ12	Suppressor of zeste
TET	Ten-eleven translocation
TGF-β	Transforming growth factor β
TGFβRII	TGFβ receptor II
TNF-α	Tumor necrosis factor - α
T1D	Type 1 Diabetes
T2D	Type 2 Diabetes
UAE	Urine Albumin Excretion
VE-Cadherin	Vascular endothelial cadherin
VEGF	Vascular Endothelial Growth Factor
VH	Vitreous humor
XIST	X-inactive specific transcript

Chapter 1

1 General Introduction

1.1 Diabetes

Diabetes is a chronic disease caused by lack of sufficient insulin production by the pancreas or when the body cannot effectively utilize the produced insulin leading to elevated blood glucose known as hyperglycemia. The ancient Egyptians first recognized diabetes in 1500 B.C.E as a condition where a person excessively urinates and loses body weight. It's two types (later Type I and Type II diabetes) were described by ancient Indian physician, Sushruta, and the surgeon Charaka (400-500 C.E) but its association with elevated glucose wasn't discovered till the late 1700s (1). The prevalence of diabetes has increased drastically over the past three to four decades resulting in the elevation of diabetes to one of the most common and serious medical conditions faced by mankind today. According to the World Health Organization, the diabetic population has risen from 108 million in 1980 to 422 million in 2014, with numbers rising rapidly in middle and low-income countries (2). Overall diabetes caused 1.6 million deaths in 2015 and is a major cause of kidney failure, blindness, heart attacks, strokes and lower limb amputations (3).

Diabetes is comprised of various metabolic conditions involving cell's inability to transport and utilize glucose. The two major types are: Type 1 (T1D)- is due to insufficient insulin production caused by T-lymphocyte mediated autoimmune destruction of pancreatic β-cells, Type II (T2D)- is due to insulin resistance leading to compensatory β-cell hypertrophy. Other types could manifest during pregnancy, drug/chemical toxicity, genetic disorders, insulin receptor disorders and in association with pancreatic exocrine disease (4). Both T1D and T2D are characterized by chronic hyperglycemia, resulting in various secondary complications including kidney failure, neuropathy, peripheral vascular diseases, cardiac failure as well as retinopathy (5). Therefore, modern medical care lays emphasis on various interventions aimed at controlling the blood glucose levels, aimed at reducing the likelihood of chronic complications.

1.2 Diabetic complications

Pathophysiological changes in diabetes contribute to hyperglycemia resulting in disrupted cellular glycolysis and pyruvate oxidation (6). Chronic hyperglycemia and acute glucose fluctuations result in the onset and progression of several secondary complications including nephropathy, retinopathy, cardiomyopathy and neuropathy (7-11).

1.2.1 Diabetic Retinopathy

Diabetic retinopathy (DR) is the most common cause of blindness in individuals over 50 and affects 1 in 3 persons with diabetes (12). DR was first detected in the 19th century using an ophthalmoscope. It is a chronic vascular complication characterized by low-grade inflammation, serum leakage through microvasculature, loss of capillaries and increased vascular permeability ultimately leading to neovascularization (13,14).

Initially, hyperglycemia contributes to gradual biochemical and cellular alterations. During this period, vascular alterations surface, accompanied by elevated mitochondrial and extracellular reactive oxygen species (ROS) production injuring endothelial cells (ECs), pericytes and neuronal tissues. These events are accompanied by localized hypoxia due to blockage of retinal capillaries and release of angiogenic growth factors. As the disease progresses leaky blood vessels become prominent resulting in macular edema (non-proliferative). Progression of this process results in proliferative diabetic retinopathy (PDR), characterized by angiogenesis with tufts of highly permeable new weak blood vessels on the surface of retina. These vessels can rupture easily allowing blood to leak out into the vitreous and compromise vision (15).

Recent research has suggested that retinal dysfunction in diabetes is associated with changes in the retinal neurovascular unit (16). This unit is conferred with unique characteristics by the retinal architecture. The inner retinal layers are in close proximity with the neurovascular units, thereby aiding in oxygen and nutrient delivery, while the outer retinal layers support production of electrochemical impulses in response to light stimulation (16). In DR, the whole retinal neurovascular system is impaired causing loss of neurovascular coupling, neurodegeneration and neuroinflammation that can be detected before the development of vascular damage (17).

These insights suggest addressing both vascular dysfunction and neural degeneration for possible therapeutic interventions in DR.

Present day treatments for DR revolve around laser photocoagulation for macular edema and/or proliferative disease and vitreo-retinal surgery for serious complications (18). Other approaches that delay its progression include blood sugar management, reduction of LDL using statins, blockade of renin-angiotensin system and newer methods of inhibiting angiogenesis and edema using vascular endothelial growth factor (VEGF) and VEGFR inhibitors (15,19-21).

1.2.2 Diabetic Nephropathy

As the worldwide diabetic population continues to rise, advanced diabetic nephropathy (DN) continues to be leading cause of end-stage renal failure (ESRD) (22). Clinical development of DN is based on the level of urine albumin excretion (UAE) and decline in the glomerular filtration rate (GFR) (23). For unknown reasons 20% to 40% of patients with diabetes develop DN associated with microalbuminuria (>30 mg <300 mg albumin in the urine/day), macroalbuminuria (>300 mg albumin in the urine/day), and subsequent progression to ESRD (24,25).

The disease progression starts in the nephron where hyperfiltration and hyperperfusion leads to hypertrophy and subsequent thickening of the glomerular basement membrane (26). Glomerular hyperfiltration paves way to microalbuminuria, culminating in microalbuminuria and renal failure (27). Once the nephropathy is established multiple mechanisms contribute to its outcomes. These include hyperglycemia-induced hemodynamic changes such as activation of vasoactive factors like renin–angiotensin–system (RAS) as well as genetic predisposition (28). During the course of this process, the kidney undergoes functional derangement and structural remodeling that alters various cellular events and activates signaling pathways. Interaction of these pathways in a complex mechanism results in renal fibrosis, mesangial expansion, glomerular hypertrophy, oxidative stress and tubular inflammation (29). The body responds by releasing cytokines like transforming growth factor β1 (TGF-β1), further aggravating the hemodynamic changes (30-32).

Prevention and treatment strategies for DN involve targeting the risk factors including hyperglycemia, hypertension, smoking and dyslipidemia (33). Blood sugar control reduced the risk of disease development at young age, but its long-term effect on rate of progression of chronic kidney disease (CKD) is controversial (34, 35). Along with hypoglycemic effects, metformin, sodium glucose transporter-2 (SGLT2) inhibitors, thiazolidinediones, glucagon like peptide-1 (GLP-1) agonists and dipeptidyl peptidase-4 (DPP-4) inhibitors have positive effects on DN (36-40). RAS blockers and dietary salt restriction have been used to control blood pressure in DN patients with increased UAE. RAS blockers impact GFR in DN patients, decrease glomerular tuft pressure and inhibit cytokine overproduction (41). However, RAS blockade has failed to fully prevent renal injury progression in T1D and may cause accelerated disease progression in patients with advanced chronic kidney disease (42). Smoking plays a huge role in DN progression and quitting is mandatory for both T1D and T2D patients (43). Statins are important hypolipidemic treatment recommended for all DN patients as it reduces risk of atherosclerotic heart diseases in CKD patients but have minimal effect on CKD progression (41,44). However, these approaches are not effective for all diabetic patients or have varying side-effects. The accumulating scientific evidence call for a change in plan, raising the need for novel therapeutic strategies to prevent its development and progression of ESRD.

1.2.3 Diabetic Cardiomyopathy

Cardiovascular complications are responsible for majority of diabetes related morbidity and mortality (45). Such complications include atherosclerosis, autonomic neuropathy and diabetic cardiomyopathy (DCM). DCM causes structural and functional changes in the myocardium independent of coronary artery disease and hypertension (46). Rubler and colleagues first described ventricular dysfunction occurring in diabetic patients in 1972 (47). Alterations in myocardial structure, calcium signaling and metabolism are primary features that precede accelerated left ventricular hypertrophy and increase susceptibility to ischemic injury and heart failure (HF) (48). Myocardial tissue undergoes structural and functional modifications in diabetes induced by hyperglycemia, hyperlipidemia and insulin resistance (49). A histological trademark of DCM is interstitial and perivascular fibrosis, characterized by increased deposition of collagen accompanied by crosslinking of these collagen fibers contributing to reduced

ventricular compliance (47,50-51). Myocardial fibrosis is accompanied by left ventricle hypertrophy (LVH). LVH has been linked to elevated serum markers of systemic inflammation like fibrinogen, C-reactive protein and microalbuminuria (52). These structural changes are accompanied by functional alterations namely diastolic and systolic dysfunction. Diastolic dysfunction is defined as defective ventricular relaxation leading to pressure increase and impairment in blood filling during diastole (49). During systolic dysfunction, the myocardium fails to eject adequate blood volume, but is observed at later stages in DCM after subsequent diastolic dysfunction has been established in the patients (53,54). Early identification of these abnormalities is important to provide appropriate treatment and prevent advancement to HF.

Multiple mechanisms have been proposed to explain the pathogenesis of DCM. These include autonomic dysfunction, defects in lipid metabolism, abnormalities in ion homeostasis, alterations in structural proteins, increased oxidative stress, interstitial fibrosis and alterations in myocardial substrates and energy metabolism (55-60). As compensation for glucose assimilation, fatty acid transporters are increased to generate ATP through fatty acid (FA) degradation (59). However excess FAs accumulate in cytosol and causes lipotoxicity through generation of diacylglycerol and ROS. Hyperglycemia also triggers ROS and advanced glycation end-product (AGE) production culminating in cardiac gluco-toxicity. Lack of fuel and lipo/gluco-toxicity triggers cardiac inflammation, fibrosis and contractile dysfunction (61). In response RAS and TGF-β systems mediating cytokine/ chemokines responses are significantly enhanced (62).

Currently there is no single therapy for treating diabetic cardiomyopathy. Treatment options centers around dietary glycemic control, direct and indirect regulators of fatty acid metabolism, and inhibitors of factors triggering heart failure (53,63). Medical advancements and lifestyle interventions have contributed to reduction in cardiovascular mortality in diabetic patients. However epidemiological studies show higher incidence of diabetic cardiomyopathy despite adjustments for hypertension, microvascular diseases, hypercholesterolemia, body mass index and other factors (64-66).

1.3 Pathogenesis: Mechanism of diabetic complications

Hyperglycemia is the central event leading to these micro- and macrovascular pathologies in diabetes. Four distinct mechanisms have been proposed to explain the underlying effect of hyperglycemia. These are: increased polyol pathway flux, increased levels of AGE, activation of protein kinase C (PKC) isoforms as well as enhanced hexosamine pathway (67). Polyol pathway is a less frequent route for glucose metabolism under normal conditions. Hyperglycemia mediates increased conversion of glucose to polyalcohol sorbitol mediated by aldose reductase, the first enzyme in this pathway (68). This is accompanied by decrease in co-factor NADPH essential for regenerating reduced glutathione (GSH), resulting in intracellular oxidative stress (69). Intracellular hyperglycemia is a key accelerator of intracellular and extracellular AGE formation (70,71). The intracellular AGEs then functionally alter intracellular proteins, modify binding properties of extracellular matrix proteins (ECM) and facilitate binding of AGE precursor-modified plasma proteins to AGE receptors on endothelial and mesangial cells. These events then aid in activating the production of ROS (67). Majority of the PKC isoforms are activated by diacylglycerol (DAG) which are elevated during hyperglycemia (72). These isoforms are responsible for numerous effects such as elevated levels of vasoconstrictors like endothelin-1 (ET-1), permeability factors like VEGF, microvascular matrix proteins like, fibronectin and type IV collagen (Col1α4) as well as TGF-β1 (73). In addition, they also contribute to activation of nuclear factor-κB (NF-kB) and NAD(P)H oxidases whereas bioavailability of nitric oxide (NO) and endothelial nitric oxide synthase (eNOS) expression are reduced (73-76). Increased utilization of excess glucose by the hexosamine shunt pathway also contribute to pathogenesis of diabetic complications (DC) (77). This event mediates increased transcription of key genes such as TGF-β1 in arterial ECs and plasminogen activator inhibitor-1 (PAI-1) in vascular smooth muscles, through a 4-fold increase in *O*-GlcNAcylation of the transcription factor Sp1 (78-79). These mechanisms once thought to be independent of each other are currently known to reflect a single hyperglycemia-induced phenomenon; namely the over production of superoxide by mitochondrial electron transport chain (MTETC) (80). Elevated levels of glucose in ECs in diabetes increase the flux of electron donors (NADH and FADH2) into the MTETC and subsequently lead to voltage gradient across mitochondrial membrane rising above critical threshold (81). Crossing the threshold leads to increased superoxide formation and high levels of ROS. Mitochondrial superoxides produced

due to hyperglycemia further activate the above-mentioned pathways by inhibiting glyceraldehyde-3 phosphate dehydrogenase (GAPDH). Inhibition of GAPDH increases the intracellular levels of glucose by deregulating glycolysis. The accumulating GAPDH shuttles into the nucleus and gets modified by activated poly (ADP-ribose) polymerase (PARP). PARP usually resides in the nucleus in an inactive form and gets activated when increased ROS production in the mitochondria induces DNA strand breaks (82). PARP modified GAPDH is reduced in activity leading to intracellular AGE formation, activation of PKC and hexosamine pathway flux. The pathways then alter cell signaling by triggering various transcription factors, inflammatory markers, cytokines and growth factors leading to further cell damage in multiple ways (72,83-84). In the following sections I will describe specific mechanisms, which are of direct relevance to this research.

1.4 Cell Signaling in diabetic complications

Signaling abnormalities in various cell types contribute to the pathogenesis of DC. As ECs are primary targets of these complications, we will focus on these cells. Alterations in both intra- and intercellular signaling evolve slowly during the years while the disease progresses. Hyperglycemia and possibly free fatty acid (FFA) levels are key factors implicated in the activation of biochemical pathways, including stress-activated signaling pathways of NF-κB, NH_2-terminal Jun kinases/stress activated protein kinases (JNK/SAPK), p38 mitogen-activated protein (MAP) kinase, ERK1/2 signaling, PKC signaling and others like PI3 kinase, Janus kinase (JAK/STAT) and mTOR pathway activation (85-91). One of the intracellular pathways most extensively studied as a target of hyperglycemia and oxidative stress, is transcription factor NF-κB (92). In the cytoplasm of unstimulated cells NF-κB exists as a latent dimer bound to IκB. On activation by factors like oxidative stress, NF-κB dissociates from IκB and translocate into nucleus where it binds to κB elements in the promoter region of certain genes and affects gene expression (93). NF-κB plays key roles in inflammatory responses ultimately leading to enhanced fibrosis especially in the kidneys; and regulates expression of large number of genes, including those involved in DC such as VEGF (94-96). Many of these factors, in turn, activate NF-κB, thereby initiating a vicious cycle. High glucose exposure on cultured ECs can activate number of protein kinase family members, especially PKC (97-98). Isoform βII of PKC

shows highest induction in the retina and heart of diabetic animals (99). PKC activation involves increased DAG levels which are observed to be elevated in high glucose treated vascular cells as well as retina of diabetic animals (99-100). Activation of PKC directly or in concert with other intracellular signaling proteins can cause number of effects such as mediating glucose-induced EC permeability, enhance ECM production, regulate cellular activity as well as involvement in the expression of various growth and vasoactive factors such as VEGF (97-98,101-104). Binding of VEGF to VEGF receptors causes release of DAG, and this increase in DAG in turn leads to selective activation of PKC isoforms which mediate vasoregulatory roles of VEGF such as permeability (105-106). VEGF also has critical effects on the physiology and pathophysiology of podocytes, which is the main source of renal VEGF (107). Secreted VEGF facilitates autocrine activation of podocytes, thereby inducing podocyte glomerular basement membrane matrix protein production; e.g. α3(IV) collagen synthesis via PI3K signaling (108). Further evidence suggests that accelerated glomerular angiogenesis may contribute to diabetic glomerular hypertrophy and ECM expansion (109). Although VEGF receptors are present on podocytes and mesangial cells, VEGF's effect of DN results largely from endothelial signaling leading to enhanced endothelial growth and proliferation as well as increase in permeability; possibly through subsequent activation of MAPK pathway (110-111).

One of the crucial steps in the progression of DR is thickening of basal lamina (BL) that surrounds ECs and pericytes of the retinal capillaries (112-114). Disturbances in the balance between synthesis and breakdown of ECM components induced by high intracellular glucose is considered the main cause of BL thickening (115-118). In addition, various growth factors like VEGF and transforming growth factor TGF-β, may play a more direct role in this balance disturbance (119-120). Excessive ECM deposition also occurs in the heart muscles accompanied by abnormal proliferation of cardiac fibroblasts (121). Cardiac fibrosis is characteristic of DCM and TGF-β is one of the most studied mediators of this phenomenon (122-123). Numerous studies have reported high-glucose mediated elevation in the transcription of TGF-β genes, thereby increasing the levels of the protein and its downstream signaling (124-125). In addition to its role in the canonical SMAD signaling pathway, TGF-β is also the key stimulator of three known MAP pathways namely; ERK, JNK and p38 pathways (126-128). ECM protein deposition leading to glomerulosclerosis and renal hypertrophy are characteristic

features of DN (129). Clinical studies suggest that responses triggered by TGF-β are involved in pathways leading to interstitial fibrosis and glomerulosclerosis observed in ESRD (130). Evidently, TGF-β is the chief cytokine in the regulation of ECM synthesis and is responsible for stimulating the production of its components including proteoglycans, fibronectin and collagen, while blocking matrix degradation (131).

1.4.1 Extracellular matrix proteins in diabetic complications

Chronic diabetes leads to structural and functional alterations in the vasculature. Modifications to ECM and basement membrane (BM) thickening are the structural hallmarks in the target organs of DC (132). First recorded by Siperstein and colleagues in 1968, these disturbances of ECM are directly linked to loss of function in the target organs (133). ECM encompasses an insoluble network of collagens, fibronectin, elastins, structural glycoproteins, proteoglycan hyaluronans and integrins that provide cells with mechanical support and mediate multifarious interactions between cells or cells and ECM of the vascular tissues (134). Excessive ECM deposition in the glomerular matrix and tubulointerstitium is associated with significant decline in the renal function during diabetes (135-136). As mentioned earlier ECM level depends on the balance between synthesis and degradation. Elevated glomerular basement membrane thickness in the glomeruli and the tubular system is seen in both T1D and T2D. Interstitial fibrosis and atrophy of the tubules follow these glomerular changes. The interstitium harbors inflammation and in the advanced stages of DN, glomerulosclerosis is prominent with a substantial accumulation of ECM in the mesangium accompanied by reduced capillary volume (137). In the retina, ECM provides a framework on which the cells reside and hence contributes to structural integrity and the vasculature. Here, it functions as a cell attachment substratum, promotes wound healing and enables intracellular communication among other functions (138-140). The cardiac ECM consists of fibrillar collagen localized within myocardial interstitium and non-fibrillary collagen as well as fibronectin and laminin in the myocyte basement membrane (141-142). With the progression of diabetes, left ventricle might hypertrophy with occurrence of hypertension-induced diastolic dysfunction (143-144). DCM is characterized as cardiac dysfunction and alterations to vascular ECM are accompanied by various cardiovascular complications. Regulating ECM composition by transforming growth factors and VEGF-

mediated angiogenic responses are compensatory responses to sustained EC damage by hyperglycemia (145). ECM breakdown and formation plays pivotal roles in angiogenesis. It can mobilize angiogenic growth factors to provide cues for directional growth of ECs.

1.4.2 Vascular endothelial growth factor in diabetic complications

VEGF is a vital part of tissue growth and organ repair segment of angiogenesis (new blood vessels sprouting from pre-existing vasculature) and vasculogenesis (new blood vessel formation) (146). Angiogenesis or neovascularization includes migration and proliferation of endothelial and smooth muscle cells, ECM breakdown, pericyte and macrophage accumulation and development of new vasculature (147). VEGF was discovered in 1983 by Senger, Dvorak and colleagues as a key mediator in angiogenesis (148). Subsequent isolation, cloning and clinical studies provided evidence for its critical role in occular neovascularization (149-151). Diabetes is closely linked to impaired neovascularization and anomalies associated with it includes increased angiogenesis in the retina and blood vessel walls, impaired wound healing, defective release and function of endothelial progenitor cells from bone marrow and increased production of angiogenesis inhibitor, angiostatin (152-154). Although VEGF plays a central role in the development of pathological microvascular complications such as DR, it is also essential for the survival of ECs, enhances microvascular permeability and is a potent vasodilator (155-157). In DR, VEGF is important for proliferative DR as well as diabetic macular edema (158). It alters retinal capillary permeability by enhancing protein phosphorylation and increases EC proliferation by activating MAP proteins (159-160). It also stimulates ECs to release matrix metalloproteinases leading to degradation of BM, in turn allowing for cell migration (161). The proliferation and migration of ECs are followed by synthesis of new BMs. These BMs have new capillaries which are stabilized by recruitment of pericytes and other growth factors to the site (162). Microvascular abnormalities in heart can cause ischemia in the absence of coronary atherosclerosis, leading to adverse cardiovascular events in diabetes. DCM is characterized by these microvascular abnormalities in the absence of coronary disease and leads to progressive heart failure (163-164). They are influenced by angiogenesis factors, especially VEGF, in response to hyperglycemia and ischemia (165). Impairments in VEGF expression and action have been observed to occur in diabetic patients

(166-168). Glucose plays a significant role in DN pathogenesis and evidence suggests its direct and indirect stimulation of VEGF expression in podocytes through TGF-β (169-170). Glucose also increases TGF-β expression in podocytes and mesangial cells, which thereby stimulates glomerular basement membrane (GBM) thickening and glomerulosclerosis through VEGF (169-170). VEGF in turn inhibits TGF-β expression through negative feedback mechanism. VEGF signaling affects GBM thickening, slit pore density and the quantity of nephrin, a protein required for proper functioning of the renal filtration barrier, which are all related to the extent of diabetic albuminuria (171). Obvious upregulation of VEGF in glomerular epithelial cells is a sign of early DN, while its increase in the proximal tubules mark a more progressed stage (172). VEGF in the glomeruli is important in DN development and the rise in glomerular VEGF speeds up DN progression to advanced stages (172).

1.4.3 Endothelial-mesenchymal transition in chronic diabetic complications

The entire circulatory system is lined by ECs, forming a boundary between circulating blood in the lumen and vessel walls from heart to small capillaries (173). They allow the blood to be pumped farther by reducing turbulence of flow and unambiguously play critical roles in maintaining overall homeostasis (173-174). The endothelium secretes several factors that regulate coagulation, fibrinolysis, platelet aggregation and vascular tone (174). Hyperglycemia causes the endothelium to be exposed to a range of negative intracellular occurrences that promote endothelial dysfunction, where the endothelium loses it physiological properties (174). Accumulation of fibrous connective tissue and excess ECM production in and around inflamed or damaged tissue, that culminates in organ failure or death is one of the consequences of endothelial dysfunction (175-178). Fibrosis involves proliferation of local fibroblasts and their differentiation into myofibroblasts (178). Myofibroblasts, in comparison to fibroblasts have elevated α-smooth muscle actin (α-SMA) and upregulated production of ECM proteins like type IV collagens (178-179). Initially the origin of these myofibroblasts was thought to be local proliferation of resident fibroblasts in response to factors like TGF- β, but subsequent research suggests other cellular sources such as ECs (180-181). ECs can adopt mesenchymal phenotype and express markers characteristic of myofibroblast differentiation such as α-SMA, smooth muscle 22α (SM22α), vimentin, fibroblast specific protein 1 (FSP1) and ECM proteins like

fibronectin and collagen and downregulate EC markers such as vascular endothelial cadherin (VE-Cadherin) and cluster of differentiation 31 (CD-31) (181-183). This phenomenon is known as endothelial-mesenchymal transition (EndMT) through which ECs gain an altered differentiated phenotype and obtain invasive and migratory abilities (173). At the cellular level, EndMT can be defined as loss of cell adhesion and actin reorganization to convert apical-basal polarity to front-end back end polarity resulting in change from compact, well-structured cobblestone-like shape to less organized spindle-shaped cells (183-184). The major regulatory cytokines that stimulate EndMT belong to TGF- β superfamily of proteins including TGF-β1 and TGF-β2 (181,184-186). Importance of this major regulator in the activation of EndMT has been described in several studies (187). TGF promotes EndMT through Smad-dependent and Smad-independent pathways like PKC (185,188). Several studies have shown that inhibition at TGF-signaling at various stages have resulted in decrease or partial reduction in EndMT and fibrosis in animal models (185,187,189). TGF-signaling plays important roles in myofibroblast differentiation and ECM alterations favor myofibroblast transdifferentiation through altered responses to mechanical stress or transduction of growth factor signals (181). TGF is also responsible for induction of cardiac fibroblast transdifferentiation to myofibroblasts and promotes cardiac fibrosis by inducing ECM protein synthesis and reducing collagenase expression (190-191). Renal fibrosis, caused by aberrant ECM accumulation, have also been linked to abnormal differentiation and proliferation of activated myofibroblasts (181,192-193). Therefore, TGF-signaling and EndMT are major contributors to generation of myofibroblasts that are key role players in the development of fibrotic diseases such as DCM or DN. Targeting these pathways and their specific components in myofibroblasts can possibly lead to effective anti-fibrotic therapies (181). There are several known inhibitors of EndMT including epigenetic regulators such as microRNAs (miRNA) and histone acetylators (194-195). Our lab has demonstrated that during DR and DCM, miRNA 200b (miR200b) modulates EndMT in concert with histone acetylator, p300 through regulation of TGF-β mediated Smad signaling pathways (196-197). Most miRNAs act through inhibition of TGF-β signaling although other ways of miRNA action in regulating EndMT have also been recorded in literature (198-203).

1.5 Epigenetic regulation in Diabetic Complications

The chronic nature of diabetes makes it a high-risk factor for development of many complications like cardiovascular disorders, kidney damage and retinal deterioration. Multifactorial causes can be ascribed to the development of these complications (204-206). There is considerable evidence indicating that the interaction between genes and the environment can influence an individual's susceptibility to develop a chronic complication (207-209). The environmental factors can alter signaling pathways and alter gene expression through epigenetic modifications. Epigenetics is defined as the study of heritable changes in gene expression that does not involve changes to underlying DNA sequence. This represents a change in phenotype without a change in genotype that affects how cells read genes (210).

The chromosomal DNA is tightly packaged into chromatin, and its status between transcriptionally "active" (euchromatin) and 'inactive'(hetero-chromatin) in response to extracellular signals are key aspects that regulate gene expression (211-213). Chromatin is composed of subunits known as nucleosomes. Each nucleosome has an octamer of histones, with two copies of histone proteins (H2A, H2B, H3 and H4), wrapped by 147 base pairs of chromosomal DNA. Post-translational modification of these histones (PTHMs) alter their interactions with DNA and represent one of the key epigenetic regulations (214-215). Epigenetic regulations can lead to non-heritable or heritable effects. During non-heritable changes, cells are able to respond fast to factors altered in the environment (216). Heritable or long-term epigenetic effects occur in response to long acting stimuli and can be transmitted to memory of the offspring cells (217,218). There are several components of epigenetic pathways. PTHMs along with methylation of DNA, short noncoding RNAs (ncRNAs; for example, micro RNAs (miRNAs) and long noncoding RNAs (lncRNAs) are all components of these pathways.

1.5.1 Histone modification

Histone methylation and acetylation are the most well characterized epigenetic marks implicated in diabetes and its complications (219-220).

Methylation*:* Histone methylation involves transfer of methyl groups from the methyl donor S-adenosylmethionine (SAM) to amino acid residues (lysine, arginine and histidine) by histone methyltransferases (221,222). Methylation at the arginine and lysine residues leads to either activation or repression of transcription. Arginine residue methylation results in activation while methylation at lysine can lead to activation or repression of transcription. Generally, H3K4 (histone 3 methylated at lysine 4), H3K36 and H3K79 are associated with transcriptional activation and H3K9, H3K27 and H4K20 are seen in repressed regions (223). To add to the complexity of this regulation, lysine residues can be mono-methylated (m1), di-methylated (m2) or tri- methylated (m3). Furthermore, the specific action of methylases and demethylases control chromatin accessibility to transcriptional enzymes and therefore influence protein expression (224).

A recent study has showed that human ECs transiently exposed to high glucose, stimulated transcriptional activation of NFκB-p65, followed by overexpression of inflammatory factors. These alterations were attributed to Set7-dependent mono-methylation of H3K4 (225-226). Reversal of oxidative stress through overexpression of ROS-scavenger enzymes prevented both NF-κB activation and vascular inflammation (225-226).

Evidence suggests involvement of important histone modifications in gene regulation associated with pathogenesis in DN. A gene expression study on DN-related genes in mouse and rat models, showed increase in transcriptional activating methylation H3K4me2, coupled with decreased levels of H3K27me3 levels (227). Silencing of histone methyltransferase SET7 in mesangial cells (MC) attenuated TGF-β1-induced gene expression (228). TGF-β is implicated in fibrosis and expression of these genes and others like cell cycle inhibitor p21 in renal cells, which contribute to DN pathogenesis (229). High glucose treatments in rat MC led to similar histone modifications at fibrotic and cell cycle gene p21 promoters (228). Recent results from Finnish DN study reported linkage between the polymorphism in *SUV39H2*, gene encoding for a histone methyltransferase and various diabetic microvascular complications, including retinopathy.

Recently our lab described regulatory roles of tri-methylation at lysine27 (H3K27me3) catalyzed by H3K27me3 transferase EZH2 (PRC2 component) in DC (230). EZH2 is part of PRC2 multimeric complex known to negatively regulate expression of genes including miRNAs. Other components of PRC2 complex are EED, SUZ12 and RpAp46/48 and this complex has been widely studied in tumor biology where increased EZH2 promoted VEGF stimulation and subsequent angiogenesis (231-232).

Acetylation: Histone acetylation involves interplay between two families of enzymes to transfer acetyl group to lysine residues (233). Histone acetyl transferases (HATs) utilize acetyl CoA and catalyze transfer of acetyl groups to lysine. Histone de-acetyl transferases (HDACs) oppose HATs and reverse lysine acetylation (234). HATs are grouped, based on sequence homology, structural motifs and function (235). Over activity of HATs results in hyper acetylated histones with neutralized ε-amine positive charge on H3K and H4K N-terminal tails, that obliterates interactions with negatively charged DNA backbone (236). Hyperacetylation therefore favors euchromatin formation; allowing better access to DNA templates by RNA polymerase and transcription factors. HDAC on the other hand removes acetyl groups from e-amine groups, resulting in creation of heterochromatin and transcriptional silencing (237).

Enhanced ROS production has vital roles to play in the pathogenies of diabetic complication especially in the development of endothelial dysfunction (77). Epigenetic regulation of ROS generating genes can have important implications in understanding the mechanisms underlying these complications. Recent research suggests regulatory roles of both HATs and HDACs on several genes associated with endothelial dysfunction and inflammation in diabetes. Activation of sirtuin 1 (SIRT 1) from the sirtuin family of HDACs reduces ROS formation, thereby suppressing NF-κB activation and PARP cleavage (238). Similarly, HATs can also regulate NF-κB transcription, resulting in alterations in the gene expression of inflammatory pathway genes. High glucose treatment on monocytes resulted in increased transcriptional activity of HATs CREB binding protein (CBP) and p300/CBP- associated factor (p/CAF) leading to enhanced acetylation of histone lysine at the promoter regions of inflammatory genes and TNF-α and rise in their expression (239). HATs-mediated increase in pro-inflammatory cytokines were prevented by p300 blocker, curcumin treatment on human monocytes exposed to high

glucose by reducing HAT activity and acetylation of CBP/p300 complex (240). Increased promoter histone lysine acetylation of inflammatory genes has also been described in monocytes of T1D and T2D patients (241).

Li and colleagues recently reviewed the roles of HATs in DN and have reported that high glucose-mediated elevation in HATs such as p300, CBP and p/CAF are accompanied by activation of pro-inflammatory cytokines, fibrotic processes, ECM proteins and endothelial function in DN through acetylation of histone and non-histone proteins like Smads, p53, SP1 and NF-κB (242). Available literature on DN also suggests that HDAC1, HDAC2 and HDAC5 modulate genes induced by TGF-β1 (243). TGF-β1, a key signaling molecule implicated in fibrosis, increases acetylation at H3K by recruiting the HATs like transcriptional co-activator p300 and CBP to the PAI-1 and p21 promoters (243). A chromatin immunoprecipitation (CHIP) assay to study histone modifications at promoters for AGEs and PAI-1 showed increased active marks (mostly acetylation) in the glomeruli of diabetic db/db mice versus non-diabetics (244). Increased H3K9 acetylation at promoters of inflammatory genes is tightly linked to elevated HbA1c levels and induction of DC (245). In our work with DC, we have previously shown miR200b dependent increased production of p300 (246,247). Furthermore, glucose induced upregulation of vasoactive factors like VEGF and endothelin 1 (ET-1) were prevented by p300siRNA (247). Moreover, p300 and SIRT1 (HDAC) were shown to regulate each other directly and indirectly in endothelial cells exposed to high glucose (248).

In DR, increased histone acetylation has been partly ascribed to lowering of HDAC activity as a result of high glucose treatment on retinal cells. HDAC initiators and inhibitors, mitigated or aggravated diabetes-induced histone acetylation and pro-inflammatory protein expression in Müller glial cells, thereby confirming role of histone acetylation in the pathophysiology of DR (249). Recent research has shown that HDAC1 and HDAC2 modulate expression of cardiac hypertrophy genes (250). In DCM, HDAC3 exerts a pro-hypertrophic effect in diabetic mice by reducing expression of a MAP kinase phosphatase, DUSP5 (251). However, additional work is warranted to identify specific types altered and their molecular targets in chronic diabetic complications.

1.5.2 DNA methylation

Methylation of DNA occurs at position C5 of cytosine in CpG dinucelotides, mostly in the promoter regions by DNA methyl transferases (DNMTs). The presence of symmetrical CpG methylation marks on both DNA strands facilitates post-replicative maintenance of the DNA methylation patterns (252). There are two key components in the DNA methylation machinery of mammals, DNMTs which establish and maintain DNA methylation patterns, and methyl-CpG binding proteins (MBDs), involved in identifying the methylation marks (253). These modifications usually repress gene expression by disrupting the binding of transcriptional machinery to promoter or by recruiting co-repressors to the promoter site. The methylation patterns on the DNA are established by DNMTs, DNMT3A and DNMT3B53 along with DNMT3L54 (254). These patterns are maintained through cell division by the methyl-transferase, DNMT1 (254). DNMTs are wide-spread and involve similar catalytic mechanisms where the enzyme and the substrate base react to form a covalent reaction intermediate (255). All DNMTs use SAM as the methyl donor and transfers the methyl group to positon 5 on the cytosine ring or amino group at position 6 of adenine (256). However, DNA methylation is not an irreversible process. A family of protein known as Ten-eleven translocation (TET) are actively catalyzing demethylation, primarily at the transcription factor binding sites (254). Regulation of DNA methylation is associated with various human diseases including diabetes.

Alterations in DNA methylation of inflammatory genes, lipid and glucose metabolism genes as well as those involved in oxidative stress have been reported in diabetes (257). Short-term high glucose exposure to aortic endothelial cells led to promoter DNA methylation of NFκB-p65 (225). In streptozotocin (STZ)-induced diabetic rats, demethylation of liver X receptor α (LXR α) a key regulator of macrophage function increased myocardial ventricular expression (258). Another study in diabetic rats reported cell death of cardiomyocytes and DCM as a result of altered methylation of p53-inducible *p21WAF1/CIP1* promoter (259). In T2D patients with DCM, demethylation of CpG islands in the Kelch-like ECH associated protein 1 (Keap1) promoter increased Keap1 expression and targeted nuclear factor (erythroid-derived 2)-like 2 (Nrf2), a regulator of antioxidant proteins to proteosomal degradation (260). These studies indicate that gene regulation by DNA methylation under high glucose environments may have important roles in pathophysiology of DCM.

Hyperglycemia mediated alterations of DNA methylation have also been reported in DN (261). DNA methylation profiling of DN patients showed altered methylation of 1,061 genes (262). Fibrotic and non-fibrotic kidneys isolated from animal models of kidney disease showed aberrant hypermethylation at RASALI, induced by TGF-β1. It leads to increased Ras activation in fibroblasts, leading to proliferation and fibrosis (263). ECs exposed to high glucose showed altered DNA methylation of several genes involved in endothelial function (264). DNA methylation profiling of genomic DNA from diabetic patients with or without DN, also showed differential methylation of various genes, including *UNC13B,* that is known to mediate apoptosis in glomerular cells exposed to hyperglycemia, indicating its association with initiation and pathogenesis of DN (265).

Altered DNA methylation of several genes dysregulated in DR, have been observed in diabetic patients and *in vitro* studies. A genome-wide analysis of DNA methylation in T1D patients identified differential methylation of close to 233 unique genes in cases of PDR versus controls, suggesting the prospects of this distinct methylation pattern as a marker for PDR (266). Matrix metalloproteinase-9 (MMP-9) is known to be elevated in retina and capillary cells during diabetes (267). Kowluru and colleagues showed that prevention of hypomethylation at the MMP-9 promoter prevents mitochondrial damage in retinal ECs and tissues from diabetic mouse retina (268). Persistent hypermethylation at the regulatory region of the catalytic subunit of mitochondrial DNA (mtDNA) replication enzyme, affected its binding to mtDNA which was associated with continued progression of DR in STZ-induced diabetic rats (269). These aforementioned studies in ECs, animals and patients highlight the significant role of DNA methylation in the pathophysiology of diabetes and its complications like DCM, DN and DR.

1.5.3 Non-coding RNAs

Genomics studies over the years have influenced the identification of potential drug targets for validation. However, target validation does not promise a drug candidate. Regulation of gene expression by controlling post-transcriptional modification is an expensive mechanism for the protein-coding genes. Hence novel approaches that target the process at the transcriptional level would save energy involved in RNA modification and subsequent protein production. Majority

of the human genome sequences studied to date are protein-coding which comprises only 1.5% of the entire genome (270). Recently focus has been drawn onto the remaining non-coding area of the genome and its importance in normal development and relevance in diseases (271). These ncRNAs when functional, exert regulatory activities independent of the protein encoding route. Hence on manipulation, they constitute potential drug targets capable of phenotypic changes in the organism (272). The potential roles of these ncRNAs are still baffling, although they are known to play regulatory roles in various biological processes. For instance, miRNAs are a category of short ncRNAs (< 22-nts) involved in multiple physiological and pathological functions in the living system (273-275). On the other hand, lncRNAs are of larger size (>200nts) which works through a variety of mechanisms.

1.5.3.1 Short non-coding RNA (miRNAs)

miRNAs (~20-25 nucleotide) are short RNA molecules that do not code for proteins. miRNAs as synthesized through RNA polymerase II, processed to precursors in the nucleus by RNAse III Dorsha and DiGeorge syndrome critical region 8 (DGCR8) and exported to cytoplasm by exportin 5. They are further processed in the cytoplasm by Dicer into functionally active~22 nt double-stranded RNA molecules (miRNA duplex). Recent evidence show that the thermodynamic stability of the duplex determines which strand is to be selected to be loaded onto RISC (RNA-induced silencing complex) by Ago (Argonaute) proteins (276). Regardless of the strand chosen, once loaded into RISC the miRNAs direct the complex to the target, based on complementarity between the miRNA seed sequence and target transcript, culminating in transcriptional repression of the target. Mechanisms of repression by miRNAs are diverse, where a single miRNA can target multiple mRNAs or a single mRNA can by targeted by multiple miRNAs (277). Presently, more than 2000 human miRNAs are listed in the miRBase, and this huge number combined with the vast range of their targets make them candidates for numerous functions (278). miRNAs play important roles in controlling histone modification (277,279-280), in diverse cellular processes (281) and regulate lncRNAs (282). miRNAs are implicated in number of diseases and have emerged has key regulators of specific aspects of disease pathology in DC (283).

Nephropathy is an important research area where most miRNA dysregulations have been identified. Podocyte-specific deletion of Dicer was attributed to kidney failure and early death (284,285). Abnormal expression of several miRNAs in cell lines and kidney of diabetic mice emphasis importance of these molecules in renal fibrosis and DN. TGF-β1-treated murine mesangial cells and mouse models of diabetes (T1D and T2D) showed elevation of several miRNAs (miR192, miR200b/c, miR216a and miR217) in their renal glomeruli compared to non-diabetics (286-289). However, another miRNA, miR-200a was decreased in TGF-β1-treated proximal tubular epithelial cells (290). miR21 upregulated in T1D mice, could also target PTEN and promote mTOR signaling; important components in DN. Other miRNAs alterations were found in glomeruli of db/db mice as well as podocytes and renal endothelial cells exposed to high glucose. These included miR93, miR29c, miR192 and miR200b (291,292). To decipher their role in DR, an in-depth miRNA profiling of the retina and retinal ECs in STZ-induced rats were conducted. The results showed upregulation of miRNAs responsive to NF-κB (such as miR146, miR155, miR132 and miR21) and VEGF in the diabetic samples versus controls (293). Our lab has also shown regulatory roles on miRNAs, miR146a and miR200b in ECs treated with high glucose and retinas from diabetic mouse models. These miRNAs were themselves reduced in a high glucose environment and regulated expression of fibronectin and VEGF respectively, both mediated by increase in the co-activator p300 (294,246). Another miRNA, miR29b was upregulated in the early stage of diabetes in cells of retinal ganglion cells and inner nuclear layer of retinas of STZ-induced rats versus normal animals (295). The results indicate a protective role of miR29b against apoptosis of retinal neurons in DR and can be considered with other miRNAs for intravitreal injections as therapy for DR. Several studies have also focused on identifying miRNAs as therapeutic and diagnostic tools for diabetic cardiovascular complications (296). DCM is the result of dysfunction in a number of cells types in the heart such as vascular ECs, cardiomyocytes and myoblasts (297). Along with these, there is an increased recruitment of M1 macrophage leading to elevated cytokine levels in diabetic hearts (298). These cytokines along with transcriptional factors mediate effects that result in cardiac hypertrophy and fibrosis (297). Many miRNAs have been recognized in these processes including miR133, miR320, miR29, miR30, miR1, miR206 and miR21 (299). We have demonstrated pathogenic roles of miR146a, miR200b, miR133a, miR 195, miR1, miR9 in glucose exposed ECs and in various chronic DC (300).

1.5.3.2 Long non-coding RNA (lncRNA)

The vast span of non-coding regions brings us to question of its role in the cellular system, especially the poorly understood lncRNAs. The idea of lncRNAs first surfaced some two decades ago with the description of X-inactive specific transcript (*XIST*), the gene that is responsible for X-chromosome inactivation in eutherian lineage and lacks an open reading frame (301,302). They are defined as transcripts >200 nucleotides that may be transcribed by RNA polymerase II or III, subjected to splicing or contain a single exon (303). On the basis of their location and orientation relative to nearby genes they can be classified as sense/antisense, divergent/convergent or intronic/intergenic (304). These tissue-specific RNAs tend to form thermodynamically stable secondary and higher-order structures. Their myriads of roles include modulation of alternate splicing, chromatin remodeling, *cis/trans*-acting regulators of gene expression and RNA metabolism (305-309). *Cis*-acting lncRNAs mediate local genes while *trans*-acting lncRNAs are involved with multiple targets (309). Dysregulation of target genes leads to abnormal lncRNA expression responsible for cellular defects and disease progression including carcinogenesis and neurodegeneration (271,310). *MALAT1,* lincRNA-p21, *HOTAIR, LSINCT5, PTCSC3 and H19* are some of the lncRNAs associated with various kinds of cancers (311). Another lncRNA, *CDKN2B*-AS1 (ANRIL) has been linked to cancer, diabetes and cardiovascular diseases (312). Along with cancer, *GAS5* lncRNA has been linked to autoimmune diseases (313).

Recent publications since 2012, has shown a significant increase in the focus of lncRNAs in diabetes mellitus and its complications. In retinal samples from patients with early DR, 303 lncRNAs were found to be perversely expressed, of which 214 were downregulated and remaining 89 were upregulated (314). Additionally, metastasis-associated lung adenocarcinoma transcript-1 (MALAT1) that interacts with NF-κB, was significantly upregulated in retinas of STZ-induced diabetic rats and db/db mice as well as in the aqueous humor of DR patients (314,315). We have also reported MALAT1 levels to be elevated in high glucose-treated ECs and diabetic kidneys, which regulated glucose-induced inflammatory cytokine production (316). In DN, TGF-β activated Akt by stimulating the expression of miR-216a and miR-217 along

with their host lncRNA, RP23, as well as miR192 and its host lncRNA, CJ241444 resulting in p300 activation (288,317). These alterations resulted in mesangial cell proliferation and hypertrophy. Another lncRNA, plasmacytoma variant translocation 1 (PVT1), was classified as a candidate gene for ESRD in T2D and later found to be associated with ESRD in T1D as well (318,319). Upregulated under high glucose conditions, PVT1 was revealed to be a regulator of ECM, and targeted TGF-β1 and PAI-1 (320). DCM is a prominent cardiovascular complication of diabetes and a few lncRNAs have been associated with DCM also. In a rat model of DCM, Zhou and colleagues reported upregulation of lncRNA myocardial infarction–associated transcript (MIAT) (321). Their recent work showed MIAT to function as competing endogenous RNA to increase death-associated protein kinase 2 (DAPK2) through sponging of miR22-3p, facilitating cardiomyocyte apoptosis in DCM (322). MALAT1 was shown to be upregulated in cardiac tissues of diabetic rats and patients with myocardial infarction (323-325). MALAT1 knockdown was able to reduce myocardial inflammation as a result of diabetes and led to improved left ventricular function in the diabetic rats (323, 324). LncRNA H19 is reported to have multiple mode of actions on cardiomyocytes exposed to high glucose. It can bind and recruit EZH2 at the promoter of GTP-binding protein Di-Ras3 (DIRAS3), causing inhibition of DIRAS3-mediated autophagy and imparting a protective effect of the cardiomyocytes exposed to high glucose (326). H19 can also serve as a precursor to miR675 and post-transcriptionally regulate numerous target genes important for cell proliferation and differentiation (327,328).

Implications of lncRNAs in diseases broaden the avenues for potential drug targets such as antisense therapies. Although ncRNAs do not give any room for protein product manipulations for therapy, use of techniques like siRNA has shown regulation of sense transcript expression levels. Taking into account the long road ahead, these approaches could be useful in overcoming a key problem in traditional therapies: development of drug resistance.

1.5.3.2.1 H19 lncRNA

Over the years several lncRNAs have been identified in mammals, but many of their biological roles remain unknown. The *H19* gene (also known as ASM; BWS; WT2; ASM1; D11S813E;

LINC00008 and NCRNA00008) produces a 2.3kb spliced, capped and polyadenylated lncRNA which is abundantly expressed in developing embryos and down-regulated after birth (329). *H19* was isolated by Tilghman and colleagues in 1984 while screening for genes upregulated by α-fetoprotein in mice fetal liver (330). The paternally imprinted gene is conserved between humans (*Ch 11p15*) and mice (*Ch 7*) but lacks a common open reading frame, ruling out existence of proteins coded by this gene (329). Predominantly cytoplasmic, H19 is co-regulated by Igf2 (Insulin-like growth factor 2); a maternally imprinted gene located on the same locus (331). In the *H19-Igf2* locus, a primary imprint is set on *H19*-ICR (imprinting control region) during gametogenesis and secondary imprinting is established on *Igf2*-DMR (differentially methylated regions) after fertilization (332). The two genes share endodermal and mesodermal enhancers that regulate the promoter region activation.

H19 was one of the first lncRNAs to be well characterized since its discovery 20 years ago. However most of its biological roles remain undeciphered. Speculations revolve around potential functions as post-transcriptional regulator binding to certain proteins like Igf2 mRNA-binding proteins, or interaction with molecules like miRNAs through DNA methylation or histone modification of DMRs (333,334).

H19 is known to harbor protumorigenic properties as ectopic H19 expression enhanced tumorigenic property of carcinoma cells in vivo (335). Its carcinogenic properties have been recently described in various cancers like bladder carcinoma (336), hepatocellular carcinoma (337) and gliomas (338) although some research described tumor suppressor roles of H19 in the initial stages of colorectal cancer (339) and other cancers (332). This gene has also been implicated in genetic disorders like Beckwith-Wiedemann Syndrome (BWS) (340) and Silver-Russell Syndrome (SRS) (341) as well as during gestational diabetes in mouse models (342,343).

Dysregulation of H19 and other ncRNAs present pathological consequences through versatile functional roles. Different ncRNAs including lncRNA and miRNAs may have close relationships depending on their location and sequence similarity. This has become increasingly evident in current research on functional roles of H19 where it is observed to interact with

several miRNAs. These include; modulation of miR-let-7 in skeletal muscles (344), interaction with its direct precursor miR-675 in gliomas (338) and regulation of miR-200 family in hepatocellular carcinomas (337). Our lab has previously shown a novel miR200b mediated regulation of histone acetylator, p300 in diabetic retinopathy (246). Hence, modulating H19 may potentially lead to correction of abnormalities in several molecules altered during different DC, directly or through p300. In this study, we plan to elucidate the role of H19 in DR and its interactions with members of miR200 family in DC. Such alterations will be studied using *in vitro* and *in vivo* models of diabetes.

1.5.3.2.2 ANRIL lncRNA

The *INK4/ARF* locus on chromosome 9p21 was highlighted as the strongest genetic susceptibility locus for cardiovascular disease (CVD) (345,346). Later it was associated with other conditions such as type 2 diabetes (346, 347), Alzheimer disease (348), glaucoma (349,350), endometriosis (351) and periodontitis (352). The CVD and diabetes-associated hot-spot lies in a region overlapping a newly discovered lncRNA, ANRIL (antisense ncRNA in the *INK4* locus). However, the CVD-risk alleles have been associated with both, increased (353) and reduced expression of ANRIL (354,355). ANRIL is also known as CDKN2B-AS1; CDKN2B-AS; CDKN2BAS; NCRNA00089 and p15AS. ANRIL consists of 19 exons, spanning 126.3kb. It produces a 3834bp RNA. It is situated in the p15/CDKN2B-p16/CDKN2A-p14/ARF gene cluster, in the antisense direction (312).

ANRIL's transcription is initiated from *INK4A-ARF-INK4B* gene cluster and it represses *INK4A, ARF* and/or*INK4B* (355). ANRIL may have a direct transcriptional effect or an indirect effect in association with a histone modifier and methylating agent, the member of the multiprotein Polycomb Repressor Complex 1 and 2 (PRC1, PRC2). The PRC2 system consists of many subunits (eg. EZH2, EED, SUZ12 and RpAp46/48), each having its own role in maintaining and stabilizing PRC2 activity. These complexes are critical in the epigenetic regulation of the CDKN2A/B locus (356). Moreover, histone modifications caused by Polycomb proteins occur in coordination with other epigenetic mechanisms. Polycomb proteins CBX7 and EZH2 interact with DNA methyltransferase DNMT3B and the activity of chromatin

remodelers have shown to influence PRCs occupancy in the CDKN2A/B locus (356). Furthermore, EZH2 bound and directly methylated transcription factor GATA4 is a key dose sensitive regulator of heart development in mice and human (357,358). GATA4 methylation by PRC2 proteins impaired its acetylation by histone acetylator, p300 resulting in reduced transcriptional potency (359). Conversely, loss of PRC2 activity in embryonic stem cells resulted in histone H3K27 acetylation catalyzed by acetyltransferases p300 and CBP (360). Recently over expression of ANRIL has been shown to regulate p300 and hence might be a key player PRC complex-p300/CBP mediated epigenetic modifications (361). Past research by us and others have reported that VEGF, ECM proteins and other factors characteristically altered in DC are regulated by p300 and specific miRNAs (196,197, 230,246,247,283,294). We have also shown that miR200b regulates VEGF production by altering p300 levels and works through PRC2 complex (230). We predict that ANRIL too may regulate important factors elevated in DC through PRC2 complex with / without mediation by miR200 and p300. We hope to decipher this regulatory role of ANRIL in DC.

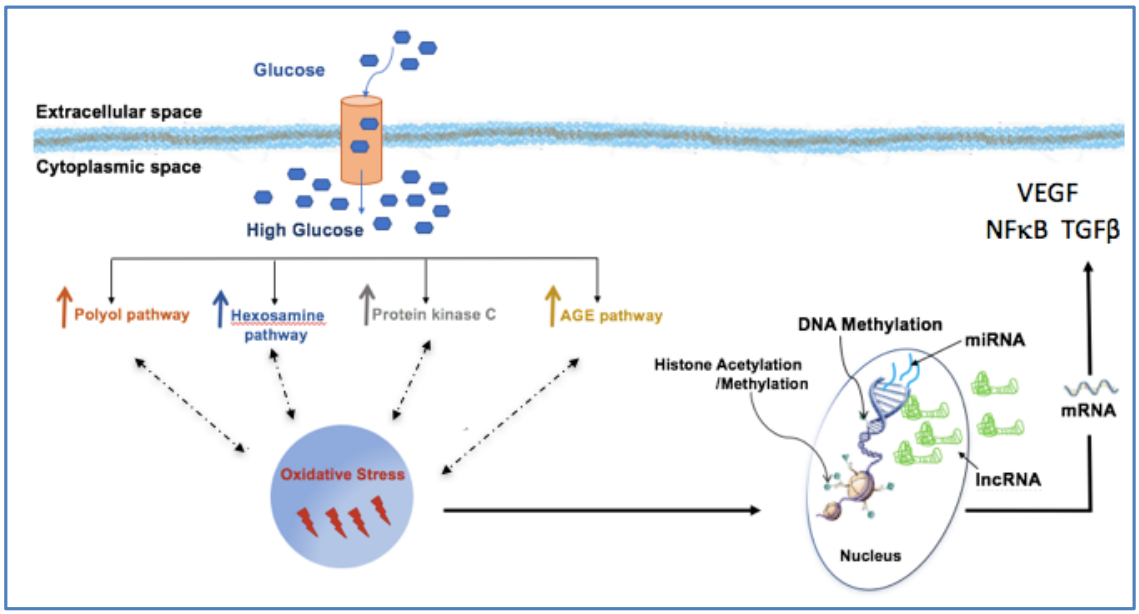

Figure 1.1: An overview of the various processes at the cellular and molecular level during h hyperglycemia. Glucose enters endothelial cells from blood. High levels of intracellular glucose activate several pathways such as polyol pathway, hexosamine pathway, protein kinase C and advanced glycation (AGE) pathways. These pathways increase superoxide production by the mitochondrial electron transport chain, leading to oxidative stress. Oxidative stress thorough

epigenetic regulators, activate expression of transcription factors like NF-κB as well as multiple pathogenic molecules such as vascular endothelial growth factors (VEGF) and transformation growth factors TGF-β. Downstream signaling from these molecules causes cellular damage in diabetic complications.

1.6 Rationale

Microarray analysis of the long non-coding RNAs from microvascular ECs exposed to high glucose (25mM) showed alterations of multiple lncRNAs (Fig 1.2). We specifically examined two lncRNAs, ANRIL and H19 in this study due to their known interactions with key epigenetic regulators of interest to us, namely the PRC2 complex, p300 and miR200b (337,356,361). We have previously shown that these epigenetic regulators control diabetes-induced upregulation of VEGF and ECM proteins as well as cellular phenotypic alterations such as EndMT. Furthermore, these lncRNAs are also known to be of importance in cardiovascular diseases and diabetes (345-347, 326). In this thesis, we hope to decipher key regulatory roles of ANRIL and H19 on these pathogenic changes through mediation by PRC2 complex, p300 and miR200b. We identified alterations of ANRIL and H19 in the array analysis and validated such changes by RT-PCR. As discussed above, lncRNAs may mediate their action directly or indirectly through p300, miR200b or changing actions of PRC complex. Hence modulating these lncRNAs may potentially lead to correction of abnormalities in several molecules altered. In this study, we plan to elucidate the roles of ANRIL and H19 in diabetic complications, possibly directly or through interactions with miR-200b and p300. We have previously shown alterations of p300, miR200b and PRC methylation in multiple chronic diabetic complications. Hence, based on our previous discussion and these preliminary findings we hypothesized that **in chronic diabetic complications lncRNAs ANRIL and H19 regulate production of key pathogenic molecules and cellular phenotype changes through specific pathways.**

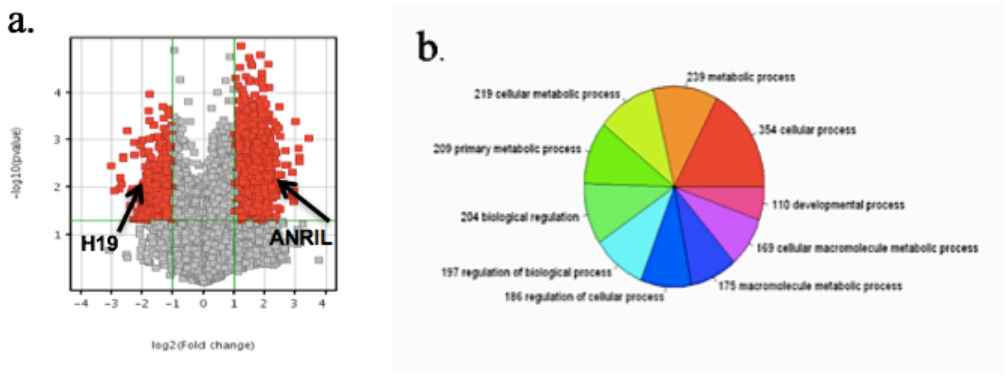

Figure 1.2: a) Volcano plot of differentially expressed lncRNAs in hyperglycemic versus normoglycemic condition treated endothelial cells. Vertical lines correspond to 1.2-fold change up and down, respectively, and the horizontal line represents a p-value of 0.05. Red points differentially expressed genes with statistical significance. b) The pie chart shows potential function of the lncRNAs altered in diabetes as detected by the array.

1.7 Central Hypothesis

In chronic diabetic complications, lncRNAs- ANRIL and H19 regulate production of key pathogenic molecules and cellular phenotype changes through specific pathways.

1.8 Specific Aims

Aim 1: To elucidate the role of ANRIL in regulating VEGF in diabetic retinopathy (Investigated in Chapters 2 of the thesis).

In this aim we examined the changes in the expression of ANRIL in human retinal endothelial cells exposed to high glucose and retinal tissue from diabetic mice. Then we moved onto unravelling the regulatory role of ANRIL on the expression of VEGF in a high glucose environment. The last part of the study explores the mechanism of ANRIL's action whether it occurs through its interaction with miR200b, p300 and PRC2 complex.

Aim 2: To investigate the regulatory role of ANRIL in upregulation of extracellular matrix protein and VEGF in diabetic nephropathy and diabetic cardiomyopathy (Investigated in Chapter 3).

This study explores the role of ANRIL, extending it to other diabetic complications namely the kidneys and heart. Expression of ANRIL were measured in the kidneys and cardiac tissues. The study further explores alterations of extracellular matrix proteins and VEGF under the influence of ANRIL and examines whether these are mediated by p300 and PRC2 complex.

Aim 3: To examine the role of H19 in regulating endothelial-mesenchymal transition during diabetic retinopathy. (Investigated in Chapter 4).

The expression of H19 in diabetic retinopathy is explored here. The study utilizes human retinal endothelial cells exposed to high glucose and retinal tissue from diabetic mice and samples from diabetic patients. The study aims to elucidate the role of H19 in regulating endothelial-mesenchymal transition and the specific mediator pathways.

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Chapter 2

2 ANRIL: A Regulator of VEGF in Diabetic Retinopathy[a]

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2.1 Introduction

Diabetic retinopathy is the most common cause of blindness in individuals over 50 years of age, affecting 1 in 3 persons with diabetes (1). It is a chronic vascular complication characterized by low-grade inflammation, serum leakage through microvasculature, loss of capillaries, increased vascular permeability and eventually neovascularization (2,3). Last decade has seen scientific and medical progress on early diagnosis and prevention in order to combat this condition. However, in this complication a variety of defects lead to functional and structural alterations in the retina that remains a significant challenge (4).

Endothelial cells (ECs), the main target of hyperglycemic damage undergoes growth, increased permeability, remodeling and phenotypic alterations in diabetes (5). Various vasoactive factors are involved in angiogenesis as seen in diabetic retinopathy. However, vascular endothelial growth factor (VEGF) signaling in ECs, represent a major rate-limiting step in this process (6). Induced in response to increased oxidative stress, VEGF binds to and activates tyrosine kinase receptors VEGFR-1 and VEGFR-2. Production of VEGF may however be regulated at transcriptional and post-transcriptional levels (7-10).

Transcriptional regulation in mammalian systems is critically characterized by dynamic switching between “active” and “inactive” states of chromatin in response to extracellular and intrinsic signals. Such signals include histone and DNA modifications as major epigenetic mediators, recently joined by several RNA molecules including microRNAs (miRNAs) and long non-coding RNAs (lncRNAs) (11,12). Advances in DNA/RNA sequencing techniques have highlighted the existence of numerous noncoding RNA (ncRNA) molecules with significant regulatory roles, impacting both physiology and diseases (13-16). In particular, lncRNAs have been shown to be of importance in various biological process such as transcription, translation, splicing, intracellular/extracellular trafficking, etc (17). Recent studies have identified large and small-scale mutations affecting noncoding genomes. Mutations in lncRNAs have been linked to diseases such as cancers, neurodegenerative diseases as well as genetic disorders (18-23).

A recent genome-wide study revealed existence of lncRNA ANRIL (Antisense RNA to *INK4* locus). ANRIL has been noted to be significant in cardiovascular diseases, type 2 diabetes, glaucoma, intracranial aneurysm and cancers (24-28). ANRIL consists of 19 exons, spanning 126.3kb and producing a 3.8bp RNA. It is situated in the p15/CDKN2B-p16/CDKN2A-p14/ARF gene cluster (29). ANRIL may have direct transcriptional effects or an indirect role as a recruiter of chromatin remodeling complexes like polycomb repressor complex 2 (PRC2) to specific genomic loci (30,31). Overexpression of ANRIL has also been shown to regulate histone acetylator, p300 (32). Its role in epigenetic silencing of specific miRNAs have also been reported (33).

We and others have previously shown that VEGF and other vasoactive factors are regulated at the transcriptional level through p300 and specific miRNAs (34-36). We have also shown that in diabetic retinopathy, microRNA 200b (miR200b) regulates production of VEGF directly or through regulation of p300 and/or through PRC2 complex (8,9). ANRIL may work though PRC2 complex, and in other systems has been shown to have a regulatory role on p300 (31,32). Here, we examined the role of ANRIL in regulating VEGF production, its molecular mechanism and action in diabetic retinopathy. These phenomena were studied in human retinal endothelial cells (HRECs) exposed to various levels of glucose as well as in retinal tissues of diabetic mice. We further investigated whether ANRIL facilitates such effects by regulating miR200b in concert with PRC2 complex and p300.

2.2 Methods

All reagents were obtained from Sigma (Oakville, ON, Canada) unless otherwise specified.

2.2.1 Cells

We used human retinal endothelial cells (HRECs, Olaf Pharmaceuticals, Worcester, MA, USA), grown in endothelial basal media-2 (EBM-2), supplemented with 10% fetal bovine serum. The cells were plated at a density of $4.3x10^5$ cells/mL. Following 24h incubation in serum-free media (EBM-2), cells were incubated with various levels of D- glucose [5mM/L,normal glucose (NG); 25mM/L,high glucose (HG)] or 25mM/L L-glucose, osmotic control (LG)] at 75% cell

confluency as previously described (37). 3-Deazaneplanocin A (DZNEP) pre-treatment was also carried out where needed (9). Each experiment was performed atleast in triplicates.

2.2.2 siRNA transfection

HRECs were transfected with Lincode^{TM} Human CDKN2B-AS1 siRNA (5nmol/L,Dharmacon,Chicago,IL) or silencer siRNA CDKN2B-AS (5nmol/L, Ambion,Austin,TX) using Lipofectamine2000 (Invitrogen, Burlington, ON, Canada). Scrambled controls were used. The cells were transfected for 3h and recovered in full media overnight. Cells were then incubated in media with various glucose levels as previously described by us (9). Gene knockdown was verified by real time RT-PCR, which showed ≈70% reduction of ANRIL expression (compared to scrambled control).

2.2.3 Animals

All procedures were conducted in accordance with the ARVO statement for the Use of Animals in Ophthalmic and Vision Research. All animals were cared for according to the guiding principles in the care and use of animals. Western University and Animal Care and Veterinary Services approved the experiments. All experiments conform to the guide for care and use of laboratory animals published by the NIH (NIH publication no. 85-23, revised in 1996).

ANRIL knockout (KO) mice with a 70kb deletion on Chr 4 [129S6/SvEvTac-Del(4C4-C5)1Lap/Mmcd] corresponding to 58kb interval on chromosome 9p21 in humans, were used (38). The ANRILKO mice were obtained from Mutant Mouse Resource & Research Centre (MMRC, Davis, CA); and administered five doses of STZ intraperitoneally (50mg/kg in citrate buffer, pH5.6) on alternate days. Age and sex-matched littermate controls received identical volume of citrate buffer. Diabetes was confirmed by measuring blood glucose (>20mmol/L) from tail vein using a glucometer. Animals were monitored for changes in body weight and blood glucose. After 8 weeks of diabetes, mice were sacrificed and tissues collected.

2.2.4 Microarray

For lncRNA microarray analysis, HRECs were incubated with various levels of glucose. Cellular RNA was extracted using RNA isolation kit (Ambion, Carlsbad, CA). Custom analysis

of lncRNA expression profiling was performed by Arraystar Inc. (Rockville, MD).

2.2.5 RNA analysis

RNA was extracted with TRIzol reagent (Invitrogen) as previously described (37). cDNA for PCR was synthesized using high-capacity cDNA reverse-transcription kit (Applied Biosystems, Burlington, ON, Canada). Real time RT-PCR to detect mRNA expression was done in the LightCycler (Roche Diagnostics). Housekeeping gene β-actin mRNA was used to normalize the data.

2.2.6 miRNA analysis

MiRNA was isolated using mirVANA miRNA isolation kit (Ambion, Austin, TX) (8,34,37). cDNA for PCR was synthesized with high-capacity cDNA reverse-transcription kit (Applied Biosystems, Burlington, ON, Canada). Real time RT-PCR was performed with TaqMan miRNA Assays (Applied Biosystems) in a LightCycler (Roche Diagnostics). miR-200b expression levels were normalized to housekeeping gene U6.

2.2.7 ELISA

ELISA was performed to measure the expression of VEGF protein using a commercially available kit (ALPCO, Salem, NH; R&D Systems, Minneapolis, MN) according to the manufacturer's instructions (34).

2.2.8 Tube formation assay

HRECs ($1.5x10^4$ per well) were seeded on BD Phenol Red-Free Matrigel matrix (100µL per well; BD Biosciences, Bedford, MA) into a 96-well plate. After 1h incubation at 37°C, growth medium was replaced with serum-free medium containing appropriate amounts of glucose (9). Recovery experiments were performed by incubating treated cells with VEGF (20ng/ml). Pictures were taken at 40X magnification using a Nikon Diaphot microscope (Nikon Canada, Mississauga, ON) with PixeLINK camera (PixeLINK, Ottawa, ON). Images were captured

from at least two field views/plate. Branch points were counted for each treatment and plotted graphically.

2.2.9 Cell proliferation assay

Cell proliferation was analyzed colorimetrically by quantifying cleavage of WST-1 (Roche, USA) by mitochondrial enzymes. Transfected HRECs were seeded at density of $2x10^4$ cells/well in 96-well plates and cultured overnight. Cells were serum starved and treated with various glucose levels for 48h. At the end of the timepoint, 20µl of WST-1 was added to each well and incubated for 1h at 37 °C. Absorbance was measured at 490nm (39).

2.2.10 Fluorescence in situ hybridization

Cells were seeded at 75% confluency in 12-well plates and treated with various glucose levels for 48h. FISH was performed according to the manufacturer's protocol (biosearchtech.com/ stellaris protocols) (40). The probes were custom designed using the Stellaris FISH probe designer and tagged with Cal Fluor Red 610 Dye (Biosearch technologies, Petaluma,CA). Following hybridization of probes, the cells were counter-stained with Hoechst and mounted with Vectashield mounting medium (Vector Labs, Burligame, CA). Positive stains were detected with a fluorescent microscope (Olympus BX51; Olympus, Richmond Hill, ON, Canada) and analyzed with ImageJ software.

2.2.11 RIP

RNA Immunoprecipitation (RIP) assay was conducted using Magna RIP RNA-Binding Protein Immunoprecipitation Kit (Millipore, USA) following the manufacturer's instruction (41). Anti-EZH2 or anti-p300/CBP antibodies and IgG (control) were used for RIP (Millipore, USA). Co-precipitated RNAs were detected with real time RT-PCR.

2.2.12 Immunohistochemistry

Mouse retinal sections were immunocytochemically stained for IgG using anti-mouse IgG antibody (MP Biomedicals, OH,USA,) as previously described (42). The stains were arbitrarily scored (0-3) in a masked fashion.

2.2.13 Statistical analysis

Data are expressed as mean ± SEM. To determine statistical significance, one-way ANOVA followed by Tukey's post-hoc test was performed as appropriate, using fixed-level testing with level of significance set at 5%. GraphPad Prism 5 (GraphPad, San Diego, CA, USA) software was used for statistical analysis.

2.3 Results

2.3.1 Glucose regulates ANRIL expression

Primary aim of this study was to identify the roles of glucose-induced alterations in lncRNA, to mediate gene transcription in the pathogenesis of diabetic retinopathy. As endothelial cells are primary targets of glucose-induced damage, we have used these cells to perform our in vitro studies. VEGF is reported here as a downstream molecule, that is regulated by ANRIL. Hence, HRECs were used to investigate alterations in ANRIL expression and its effect on VEGF expression and function. HRECs were exposed to 25mM D-glucose (HG) environments for various time points, using 5mM D-glucose (NG) glucose levels as controls. We observed HG induced elevation in VEGF expression in a time dependent manner, with highest expression at 48h (data not shown). Hence for subsequent expression analysis 48h glucose incubation was used. Microarray analysis conducted on HRECs post 48h HG incubation showed a 2.5fold elevation in ANRIL (Fig 2.1A). The results were validated by real time RT-PCR (Fig 2.1B). A significant, (≈1.8 fold) increase was seen with HG. Such relatively smaller increase may possibly be explained by relatively small sample size. However, no significant ANRIL alteration was seen following 25mM L-glucose (osmotic control) incubation (Fig 2.1B). We performed RNA-FISH for ANRIL to examine its cellular distribution and to detect its subcellular localization. We used custom designed RNA FISH probes consisting of 34

complementary oligonucleotides, each 20 bases long and labeled with 3'end flurophore tag. This approach has been shown to be highly specific for RNA detection (43). Glucose induced upregulation of ANRIL expression was confirmed by this technique. No change in subcellular distribution was noted following incubation with HG (Fig 2.1C and D). Interestingly, the cells showed both cytoplasmic and nuclear distribution of ANRIL, although predominately localized in an around the cytoplasmic side of the nuclear envelope (Fig 2.1C).

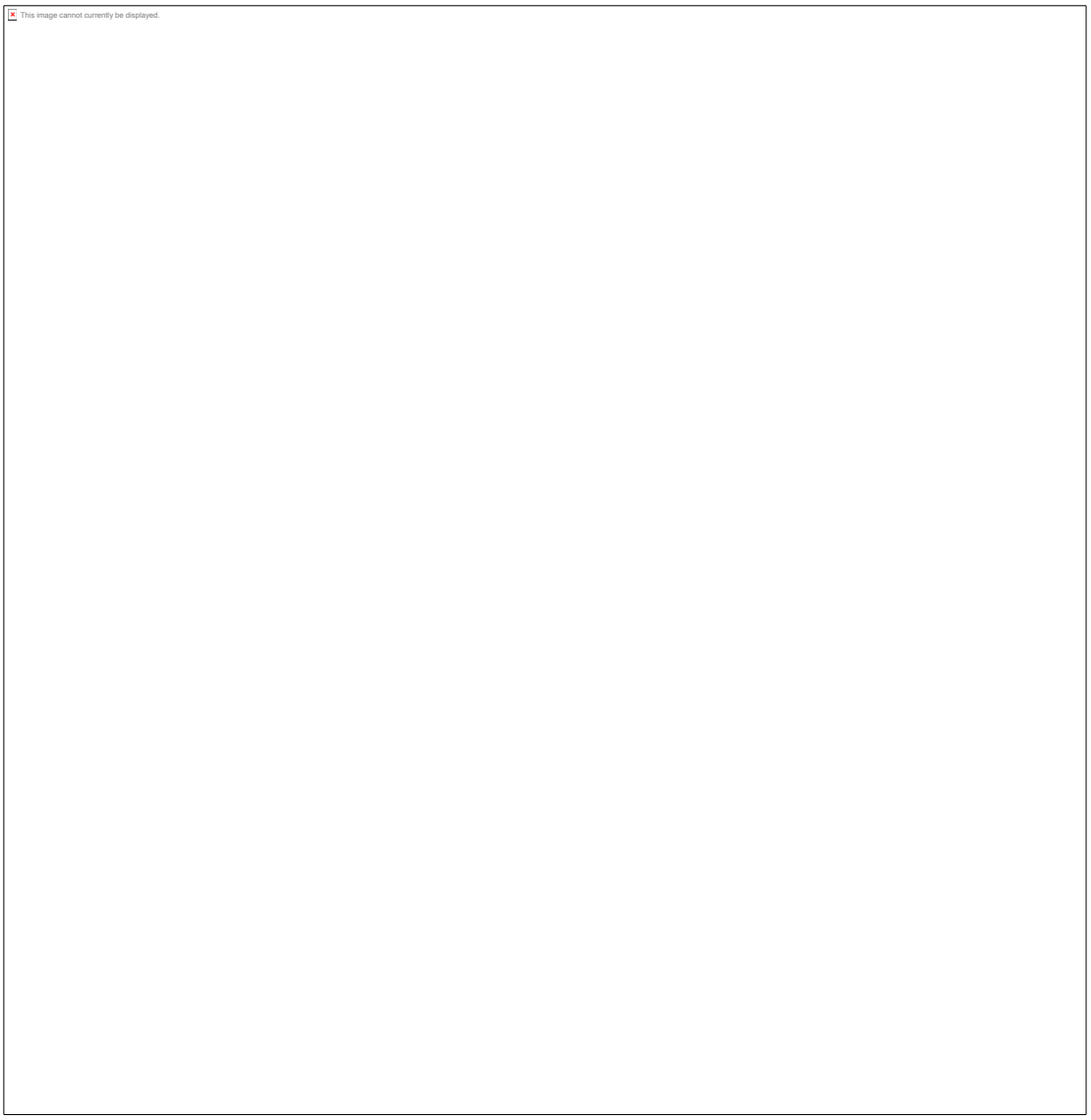

Figure 2.1: Alteration of lncRNA ANRIL in HG (25 mM) versus control (NG; 5 mM). (A) Volcano plot of differentially expressed lncRNAs in NG versus HG in HRECs. Vertical lines correspond to 1.2-fold changes up and down, respectively, and the horizontal line represents a P

value of 0.05. Red points indicate differentially expressed genes with statistical significance. Arrow represents location of ANRIL. (B) Array data were validated by real-time RT-PCR analysis of ANRIL, which confirmed its increased expression on exposure to HG (48 hours). No alterations were seen following incubation with 25 mM L-glucose (osmotic control, Osm). (RNA expressions are presented as a ratio of b-actin, normalized to NG.) (C) Fluorescence in situ hybridization using probes against human ANRIL showed increased expression (nuclear and perinuclear cytoplasmic) in HRECs exposed to HG versus NG (48 hours). (D) Quantitative data following analysis using ImageJ confirmed glucose-induced ANRIL upregulation (MFI, mean fluorescence intensity). Images quantified by ImageJ (*P < 0.05 versus NG. n=4 or more/group).

2.3.2 ANRIL regulates glucose-induced expression and function of VEGF

We then proceeded to investigate mechanisms and significance of glucose-induced ANRIL upregulation. ANRIL has been shown to interact with PRC2 complex, that regulates VEGF expression (9,31). The role of ANRIL in VEGF regulation was explored by knocking down ANRIL levels using siRNAs in HRECs. We used two siRNAs. As both gave similar results, data from one are shown. VEGF upregulations at mRNA and protein levels are characteristic of exposure of ECs to high levels of glucose. Such upregulations were prevented following ANRIL siRNA transfection, showing a direct regulatory relationship (Fig 2.2A and B). As a functional parameter, we examined VEGF-mediated angiogenesis using tube formation assays on HRECs. Glucose-induced increased tube formation was prevented by ANRIL silencing. In addition, such reduction in siANRIL transfected cells were rescued on further incubation with VEGF (Fig 2.2C and D). WST-1 assay further revealed that cell proliferation was inhibited in siANRIL transfected cells compared to scrambled controls. Meanwhile, rescue experiments showed that VEGF incubation increased the cell proliferative ability in ANRIL transfected cells both in low and high glucose (Fig 2.2E).

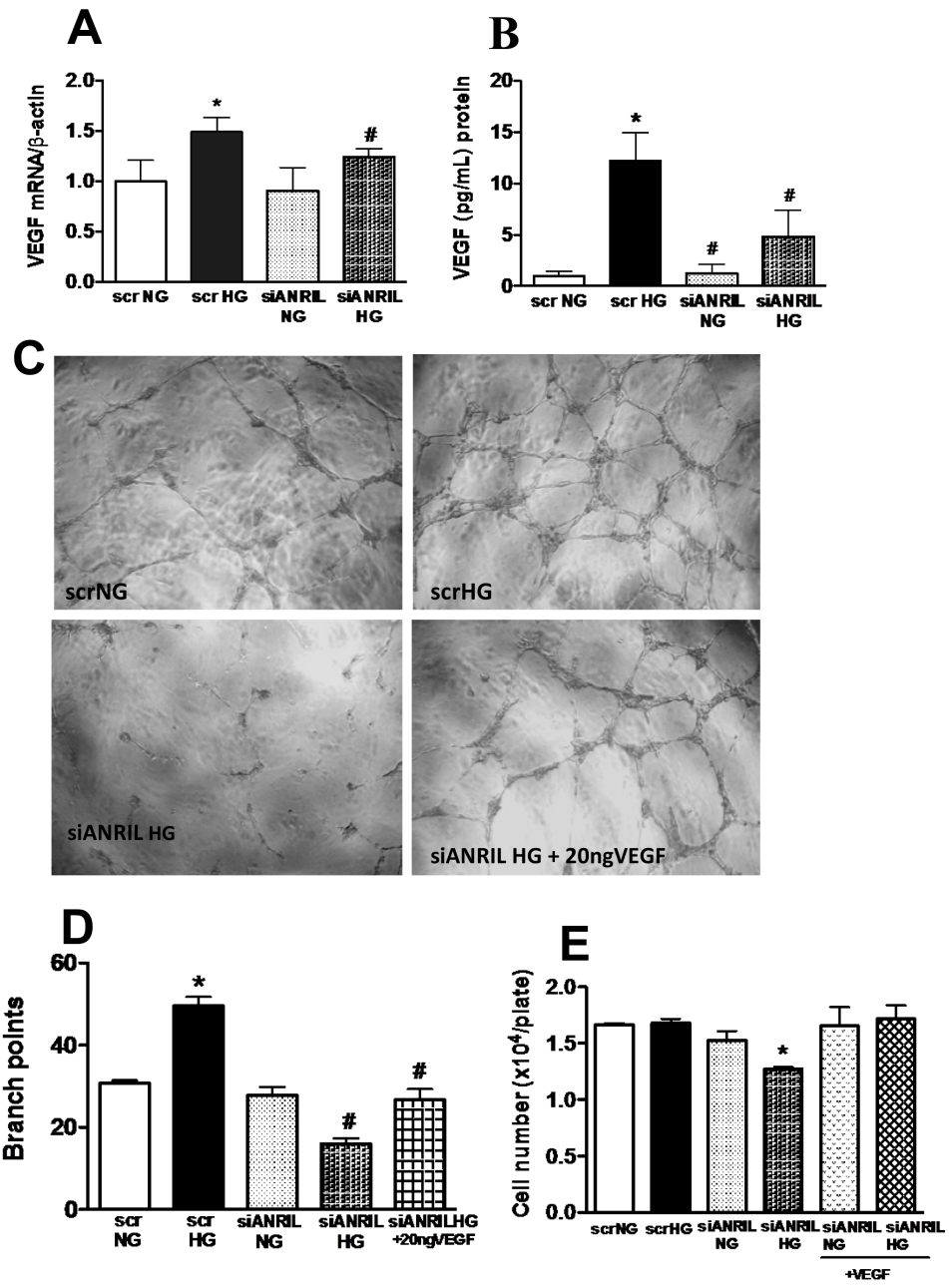

Figure 2.2: ANRIL regulates glucose-induced VEGF expression in HRECs (48 hours). Transfection of HRECs with ANRIL siRNA reduced (A) HG (25mM glucose)-induced upregulation of VEGF mRNA and (B) protein expression compared with scrambled siRNA (scr). Endothelial tube formation assay shows (C) representative micrograph and (D) quantification using branch point assay. High glucose–induced increased endothelial tube formation was prevented by ANRIL siRNA, which was subsequently rescued by incubation with VEGF. (E) In parallel, ANRIL siRNA reduced endothelial cell proliferation (WST-1

assay), which was also reversed following VEGF incubation (NG.5 mM glucose). Messenger RNA expressions are presented as a ratio of b-actin, normalized to NG. Protein levels are normalized to NG. *,#P < 0.05 versus scrNG or scrHG, n=4 or more per group).

2.3.3 Diabetes-induced retinal VEGF upregulation and function are prevented in the ANRILKO mice

With goals to translate our findings and establish the observation in diabetic conditions, we employed ANRILKO mouse models. Due to uncharacterized ANRIL mouse RNA sequence, as per standard practice, its expression in mice was measured using p15, as a surrogate marker for ANRIL (Fig 2.3B) (38). The wild type and KO animals following STZ-induction were monitored for a period of 2 months. Diabetic mice demonstrated hyperglycemia (Fig 2.3A), polyuria, glycosuria and reduced body weight gain, distinctive of poorly controlled diabetes (data not shown). ANRILKO had no effect on these parameters except for urine volume. Urine findings are further being investigated and will be reported separately.

We examined retinal tissues from the diabetic and control animals and analyzed for ANRIL and VEGF expression. ANRIL upregulation was observed in the retina of wild type diabetic animals. In addition, VEGF mRNA and protein levels were elevated in diabetes. All such changes were prevented in the ANRILKO diabetic animals (Fig 2.3C and D).

We stained the retinal tissues for IgG. Extravasated IgG is a marker for increased permeability, a functional effect of VEGF (45). Diabetes caused increased retinal microvascular permeability in wild type diabetic animals (score 3, compared to score 0 in wild type controls), which was prevented in the retina of diabetic ANRILKO mice (score1) (Fig 2.3E). These observations suggested that ANRIL regulates VEGF expression and its functional consequences in diabetic retinopathy.

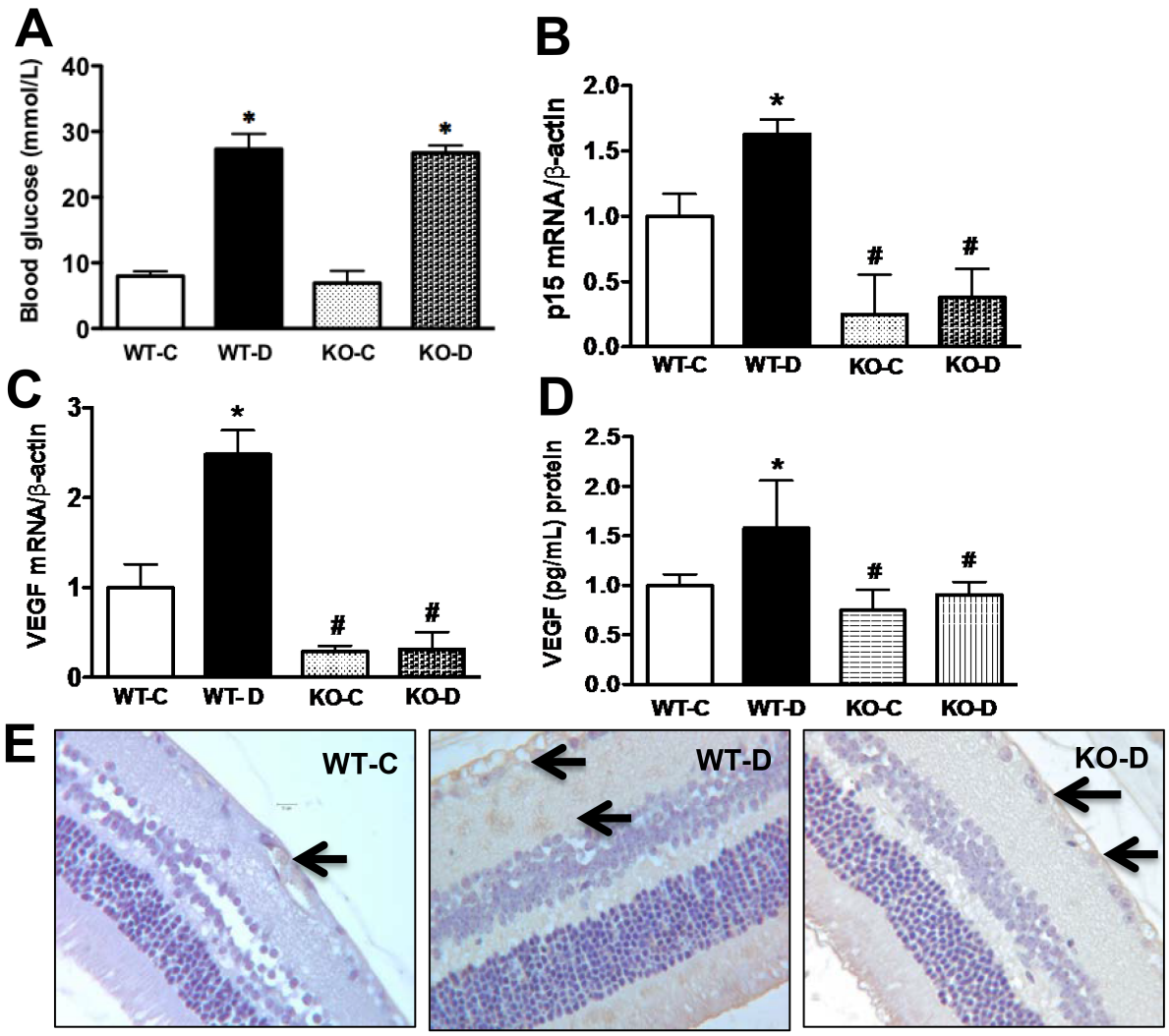

Figure 2.3: ANRIL regulates retinal VEGF in diabetic mice. (A) Diabetic wild-type (WT-D) mice and diabetic ANRILKO (KO-D) mice showed hypergylcemia and reduced body weight (data not shown). (B) Basal and diabetes-induced ANRIL RNA expression in the retina, measured by p15, its surrogate marker, was significantly reduced in the control ANRILKO mice (KO-C) mice and KO-D mice. Diabetes-induced elevation of VEGF (C) mRNA and (D) protein expression levels were also prevented in retina of KO-C and KO-D animals. (E) Immunohistochemical stain on mouse retina using anti-IgG antibody showing increased extravascular diffuse stain, indicating increased extravasation (score 3) in the WT-D compared with wildtype controls (WT-C) (score 0). Such changes were prevented in KO-D (score 1). Messenger RNA expressions are presented as a ratio of b-actin, normalized to WT-C. Protein levels are normalized to WT-C. *,#P < 0.05 versus WT-C or WT-D, n . 8.

2.3.4 ANRIL causes VEGF upregulation through PRC complex

We carried out additional in vitro experiments to gain mechanistic insight of ANRIL's actions as seen above. We have previously shown that glucose-induced miR200b-mediated VEGF upregulation works through PRC2 complex (9). PRC2 is part of transcriptional-repressive complexes PRCs (PRC1, PRC2) and consists of many subunits (eg. EZH2, EED, SUZ12 and RpAp46/48) (46). These complexes are critical in the epigenetic regulation of the CDKN2A/B locus (31,47). Hence, we examined whether ANRIL has any regulatory relationship with PRC2 complex in diabetic retinopathy. We measured expression of EZH2, which was upregulated in HRECs on exposure to HG, and in the retina of diabetic animals (Fig 2.4A and D) (9). ANRILKO resulted in a significant downregulation of EZH2 levels (Fig 2.4A and D). EED expression levels were similarly altered, whereas SUZ12 levels remained unaffected (Fig 2.4B,C,E,F).

To establish a cause effect relationship, we used DZNEP, a global methylase blocker, to inhibit PRC2 activity. All components of the PRC2 complex were suppressed by this methylase blocker (Fig 2.4G-I) (48). This blockade resulted in reduction of mRNA expression levels for both VEGF and ANRIL (Fig 2.4J and K), suggesting regulatory effect of PRC2 on ANRIL and VEGF.

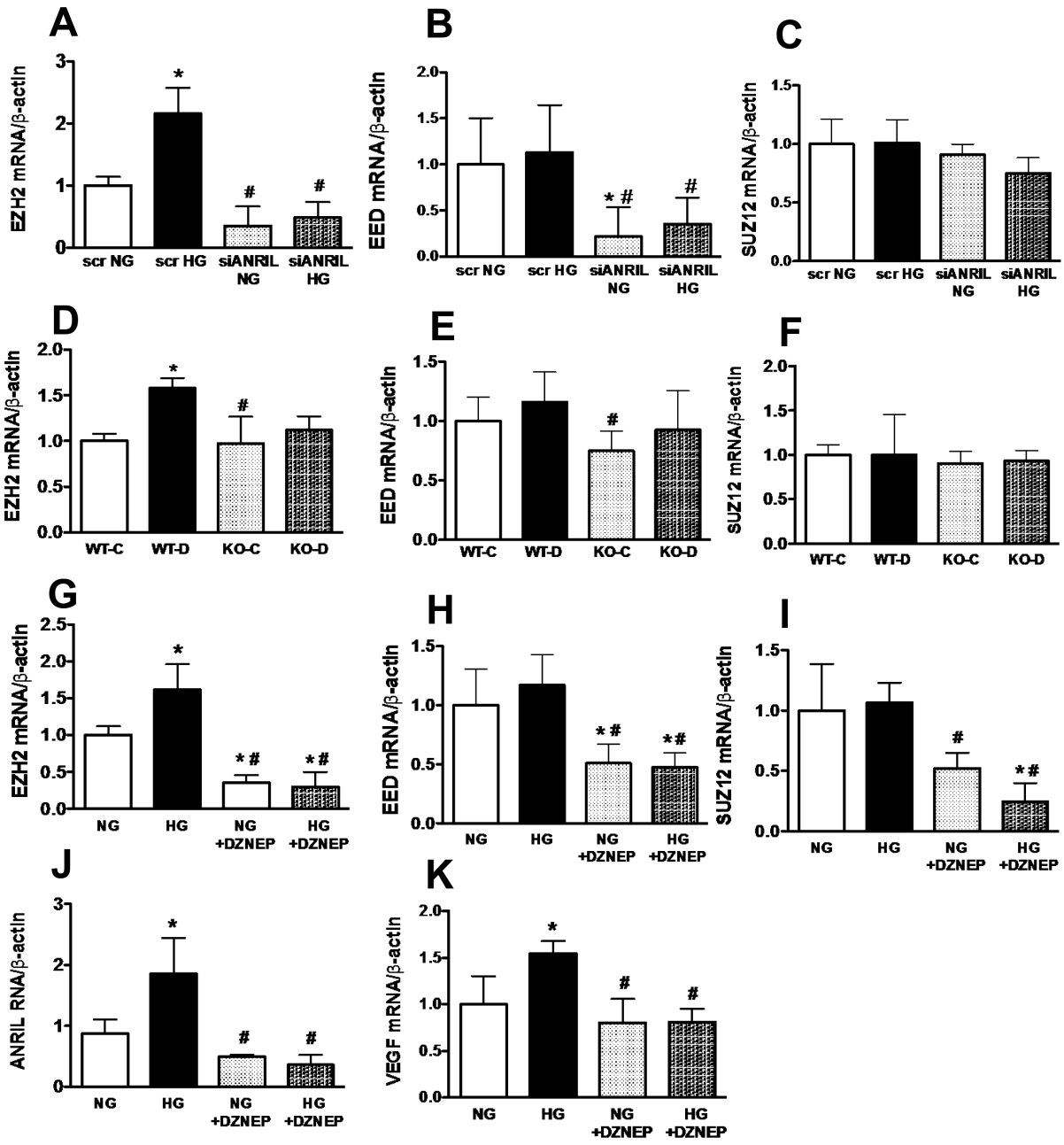

Figure 2.4: ANRIL–PRC2 complex interaction. Transfection of HRECs (48 hours) with siANRIL reduced HG (25 mM glucose)-induced (A, B) upregulation of EZH2 and EED mRNA, whereas SuZ12 mRNA (C) remained unaltered. (D–F) Diabetes-induced increased retinal mRNA expression of EZH2 and EED was similarly reduced in ANRILKO mice [controls (KO-C) and diabetic (KO-D)], whereas SUZ12 levels were unaffected. (G) EZH2 mRNA overexpression in HG were blocked by the global histone methylation inhibitor DZNEP. (H, I) 3-Deazaneplanocin A also reduced upregulation of EED and SUZ12. (J, K) 3-Deazaneplanocin A also reduced glucose-induced ANRIL and VEGF mRNA upregulation

(WT-C, WT-D, NG [5 mM glucose]). Messenger RNA expressions are presented as a ratio of b-actin, normalized to NG/WT-C. *P < 0.05 versus scrNG/WT-C, #P < 0.05 versus scrHG or WT-D, and n. 8 or more per group.

2.3.5 ANRIL acts through p300 and miR200b

We have previously shown that VEGF is also regulated by miR200b through p300 (8). We explored the relationship of ANRIL in this context. Interestingly, we found that reduction in ANRIL expression using siRNA, resulted in correction of glucose-induced upregulation of p300 (Fig 2.5A). In retinal tissues of control and diabetic animals, ANRILKO also significantly reduced expression of p300 (Fig 2.5B). These findings indicate that ANRIL regulates glucose-mediated increase in p300 in diabetic retinopathy (8,34). To further examine possible feedback regulation of ANRIL by p300, we silenced p300 in HRECs (such silencing led to ≈75% reduction in p300 mRNA expression) and observed no effect of this alteration on ANRIL expression (Fig 2.5C). On the other hand, ANRIL silencing also reduced glucose-induced miR200b downregulation (Fig 2.5D).

We further established possible interaction of ANRIL with EZH2 component of PRC2 and p300 thorough RNA-IP (RIP) assay in HRECs. ANRIL expression levels were determined by real time RT-PCR on the samples immunoprecipitated using specific antibodies to EZH2 and p300 (Fig 2.5E and F). Binding of ANRIL to p300 was significantly elevated when exposed to high glucose levels. There was also increase in EZH2 binding to ANRIL under HG. This data strongly indicates that ANRIL directly binds to both p300 and EZH2 when exposed to high levels of glucose.

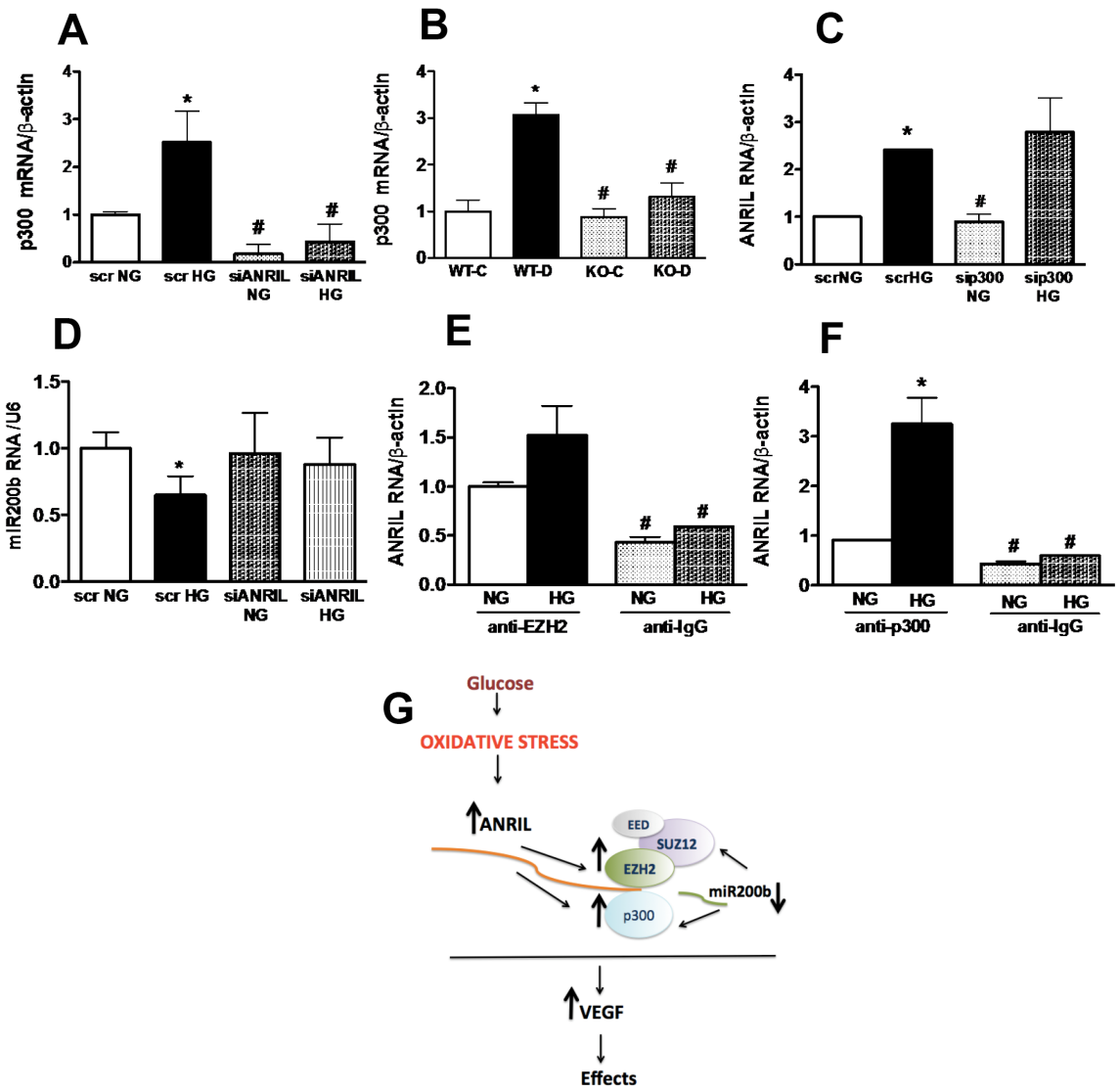

Figure 2.5: Interaction of ANRIL with the histone acetylator p300 and miR200b.Transfection of HRECs (48 hours) with siANRIL reduced HG (25 mM glucose)-induced (A) upregulation of p300 and (D) downregulation of miR200b versus control (NG; 5 mM glucose). (B) Diabetes-induced elevated p300 mRNA expressions were prevented in the retinal tissues of diabetic ANRILKO mice (KO-D; n=8/group). (C) However, silencing of p300 by siRNA did not alter ANRIL mRNA expression in HRECs. (E, F) RNA-IP with anti-EZH2 and anti-p300 performed in HRECs showed glucose-induced increased binding of ANRIL with the molecules (anti-IgG as negative control). (G) A schematic outline of the mechanism related to ANRIL upregulation and its interactions with p300, miR200b and PRC2 complex in mediating VEGF upregulation in diabetic retinopathy. In this process ANRIL binds with EZH2 and p300. It further regulates miR200b expression. A combination of all these mechanisms ultimately cause glucose-induced

VEGF upregulation. (WT-C= wildtype controls, WT-D=wildtype diabetic, KOC=ANRILKO controls, mRNA expressions are presented as a ratio of b-actin, normalized to NG/WT-C. *P < 0.05 versus WT-C or scrNG or anti-p300NG, #P < 0.05 versus WT-D or scrHG or anti-EZH2HG or anti-p300HG. N= 3 or more per group of cells.

2.4 Discussion

In this study, we have demonstrated that lncRNA ANRIL is upregulated in response to high levels of glucose. ANRIL regulates glucose-mediated upregulation of VEGF through its interaction with p300 and PRC2 components in glucose-exposed ECs and in the retinal tissue of diabetic animals.

Present-day treatments for proliferative diabetic retinopathy revolve around destructive treatments of laser photocoagulation, vitreo-retinal surgery and blocking VEGF signaling (49). Targeting the transcriptional process may constitute a novel approach. Majority of the human genome sequences studied till date are protein-coding which comprises only 1.5% of the entire genome (50). Recently, focus has been drawn onto the remaining non-coding area of the genome and its importance in normal development and relevance in diseases (51). ncRNAs generated from non-coding areas, when functional, exerts regulatory activities independent of the protein-encoding route. Hence, they constitute potential drug targets (52). Here we focus on a specific lncRNA in diabetic retinopathy.

The various roles of lncRNAs include modulation of alternate splicing, chromatin remodeling, *cis/trans*-acting regulators of gene expression and RNA metabolism (53-56). Dysregulation of target genes leads to abnormal lncRNA expression responsible for cellular defects and disease progression including carcinogenesis and neurodegeneration (51,57). Our study demonstrates an important and novel, regulatory role of lncRNA ANRIL in VEGF regulation in diabetic retinopathy.

Originally identified from familial melanoma patients, ANRIL is a potential target for cardiovascular diseases (24). It has been known to be associated with diabetes, open angle

glaucoma and various cancers (58). We found that ANRIL binds to both p300 and EZH2 of the PRC2 complex to regulate VEGF expression, characteristic of retinal degeneration in diabetes. Microarray analysis followed by cellular and tissue data confirmed the glucose-mediated upregulation of ANRIL. Interestingly, ANRIL showed perinuclear localization in HRECs. In contrast, ANRIL has been previously reported to show nuclear expression in gastric cancer (33). However, lncRNAs have been observed to show cell-to-cell variability in expression patterns (59).

Histone methylation and acetylation are well characterized epigenetic marks implicated in diabetic complications (60,61). Histone methylation involves transfer of methyl groups to amino acid residues by histone methyltransferases such as EZH2 (PRC2 complex) (62). In some tumors, increased EZH2 promotes VEGF stimulation and subsequent angiogenesis (63). In our experiments, in ANRIL silenced HRECs and in ANRILKO mice, reduced levels of VEGF were accompanied by EZH2 reduction. EZH2 blockade resulted in reduction in ANRIL and VEGF RNA expression, showing a cause-effect relationship. We have previously demonstrated interaction of miR200b with SUZ12 of PRC2 complex in the regulation of VEGF (9). The upregulation of miR200b in siANRIL transfected HRECs suggests another interaction of ANRIL with mir200b in the regulation of VEGF.

Diabetic ANRILKO animals and HRECs in HG following ANRIL siRNA transfection showed reduction in p300 mRNA expression. We have previously demonstrated that p300 is upregulated in diabetes and it controls multiple gene expressions (64). We have also previously shown miR200b-dependent increased production of p300 in diabetic retinopathy (8) and glucose-induced upregulation of VEGF was prevented by p300siRNA (34). Retinal tissues from ANRILKO diabetic animals showed downregulated expression of p300 along with the lowering of VEGF. Furthermore, RNA-IP assay revealed significantly stronger binding of ANRIL with p300 after exposure to HG. Hence, another route of ANRIL regulating VEGF maybe mediated through its effect on the transcriptional regulator, p300. To the best of our knowledge this is also the first direct demonstration of ANRIL mediated p300 regulation in diabetes. A schematic outline of the regulatory process as observed in this study is outline in Fig 2.5G.

Numerous lncRNAs associate with and possibly target histone-modifying activities (59).

LncRNA scaffolds are now known to organize the concerted activities of chromatin-modifying complexes spatially and temporally (65,66). For example, HOTAIR associates with PRC2 and Lys-specific demethylase 1(LSD1) (67). PRC2 and LSD1 are responsible for the deposition of the repressive histone marks and removal of active histone marks, respectively. Recently, in other systems overexpression of ANRIL is known to change p300, mediating epigenetic modifications (32). Hence a pattern of combined interaction of ANRIL with PRC2 and p300 might be an important mechanism in its regulation of VEGF in diabetic retinopathy. Hence ANRIL-mediated VEGF upregulation is not direct and is mediated by the aforesaid molecules. However, it is possible that additional molecules, including other lncRNAs and other mediators may potentially be involved in such regulations. Such pathways need additional investigations.

Although there are no previous reports on ANRIL alterations in diabetic retinopathy, recently another lncRNA MIAT has been shown to be upregulated in diabetic retinopathy (68). However, exact pathways for ANRIL upregulation in the genome are not clear. LncRNAs are under the same regulatory process as that of coding genes (69). Hence, hyperglycemia induced oxidative stress and subsequent alterations of transcriptional machinery may also potentially regulate such process.

In summary, we have shown that in the retinal endothelial cells, glucose causes upregulation of ANRIL. This upregulation is responsible for altered VEGF expression and function. We further confirmed these novel findings in ANRILKO mice with STZ-induced diabetes. The regulatory effect of ANRIL on VEGF was mediated by interactions of ANRIL with components of PRC2 complex and histone acetylator, p300. The current data from this study sheds light on a potentially new, targeted method to prevent diabetic retinopathy using an RNA based approach.

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Chapter 3

3 ANRIL regulates production of extracellular matrix proteins and vasoactive factors in diabetic complications[b]

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3.1 Introduction

Diabetes comprises of various metabolic conditions involving cell's inability to transport and utilize glucose. According to *International Diabetes Federation Atlas*, worldwide diabetic population was 438 million in 2015 and predicts a rise to 642 million in 2040; with majority of the new diabetic population emerging from Asia (31). As the number of people with diabetes steadily rises, chronic diabetic complications remain the significant cause of morbidity. At present, the treatment involves dietary modifications, exercise and weight loss, oral medications and insulin. Recent years have seen important advances in the field of pathogenesis in diabetes, affecting cardiovascular, renal and nervous systems as well as vision and lower extremities (4).

Diabetic nephropathy (DN) continues to be leading cause of end-stage renal failure requiring renal replacement therapy (27,62). Excess mortality is observed in people with DN, with impaired glomerular filtration rate (eGFR) and/or albuminuria (1,29). For unknown reasons, 20% to 40% of patients with diabetes develop DN associated with micro or macro-albuminuria and subsequent renal failure (50). Furthermore, there is an increased risk for the development of ventricular dysfunction leading to cardiac hypertrophy and failure in diabetic patients, known as diabetic cardiomyopathy (DCM) (11,46). Alterations in myocardial structure, calcium signaling and metabolism are features that precede the above-mentioned changes (9).

Multiple mechanisms contribute to the initiation and progression of DN and DCM. These include hyperglycemia induced hemodynamic changes such as activation of vasoactive factors such as VEGF as well as genetic predisposition, setting the stage for injury and organ failure (7). Overall effects of hyperglycemia lead to structural and functional changes in the target organs. The renal tissues undergo alterations such as glomerular basement membrane thickening, increased mesangial matrix synthesis and compromised selective glomerular permeability; eventually leading to glomerulosclerosis and

interstitial fibrosis (26). The abnormal vessels formed due to enhanced VEGF signaling are associated with glomerular hypertrophy, increased capillary occlusions, tubulointerstitial injury and urinary albumin excretion in the kidneys (38). In the heart, diabetes increases fatty acid metabolism, and alters intracellular signaling leading to impairment in excitation–contraction coupling and inefficient production of energy. These changes are likely to augment development of left ventricular hypertrophy, increased sensitivity to ischemic injury and likelihood of heart failure (37).

Interstitial fibrosis is a robust indicator of disease progression during diabetes (5). It involves accumulation and deregulated remodeling of extracellular matrix (ECM) proteins such as fibronectin (FN), novel chains of laminin and type IV collagen (Col1α4) (20). The excess accumulation of ECM in the heart mirrors occurrences in the kidneys and peritoneum during diabetes. Changes to cardiac tissues include myocardial stiffening as a result of cross-linking and ECM deposition, hypertrophy and neuronal abnormalities culminating in diastolic dysfunction (3). In kidneys, there is gradual scarring of glomerulus (glomerulosclerosis) as a result of ECM accumulation in the mesangial interstitial spaces (44). The continued augmented synthesis, deposition and reduced degradation of these ECM proteins and their post-translational modifications contribute to development of fibrosis (12,33).

Ethnicity and differences in inherited genetics has been proposed as response to the damage in both kidneys and heart due to diabetes (34,60). The molecular basis underlying these complications remains unknown (34). Recent evidence has suggested important roles of epigenetics leading to DN and DCM. The epigenetic modifications include DNA methylation at cytosine, posttranslational modifications (PTMs) of histones like acetylation and methylation, and alterations of noncoding RNAs (ncRNAs) (43). ncRNAs; such as microRNAs and long noncoding RNAs (lncRNAs) are important parts of the epigenetic realm due to their ability to regulate gene expression at both transcriptional and translational levels (54). The long transcripts of lncRNA (200-2000nt) lack protein-coding capability (13). They can regulate local and distal genes by various mechanisms and play key roles in diverse biological processes (59).

lncRNA ANRIL consists of 19 exons, spanning 126.3kb, and is situated in p15/CDKN2B-p16/CDKN2A-p14/ARF (*INK4b-ARF-INK4a*) gene cluster, in the antisense direction (2). We have recently reported that ANRIL is upregulated in human retinal endothelial cells (HRECs) and retinal tissues of diabetic mice. Upregulated ANRIL regulates VEGF through interaction with EZH2 of polycomb repressive complex 2 (PRC2) and histone acetylator, p300 (53). ANRIL is known to function as a recruiter of PRC complexes to facilitate altering of chromatin structure and set the stage for gene regulation (61). Recent "allele-specific" functional genomic studies showed preferential binding of p300 led to increased interaction of chromatin with ANRIL promoters, thereby activating transcription of the lncRNA (39). Others and we have described the regulatory role of ANRIL on the expression of p300, a well-known regulator of FN in diabetic complications (15, 24, 53). ANRIL locus is a hotspot for numerous disease-associated polymorphisms and DNA alterations where they regulate genomic neighbors in both *cis* and *trans* (2). It has been steadily associated with cardiovascular diseases as well as cancer, diabetes, glaucoma and other conditions (17)**.**

Here we investigated the specific role of ANRIL in regulating the production of ECM protein and VEGF in DN. We further expanded this study to examine whether similar changes occur in DCM. The study was focused on investigating alterations in ANRIL expression in the kidneys and heart of streptozotocin (STZ)-induced diabetic mice. To examine specific role of ANRIL, we used a unique tool, i.e ANRIL$^{-/-}$ mice with STZ-induced diabetes. Subsequently we determined if the alterations resulted in pathologically significant modifications to the structure and function of these organs. We further investigated if ANRIL's regulation of ECM proteins and VEGF is modeled through p300 or PRC2- mediated pathway in DN and DCM.

3.2 Materials and Methodology

3.2.1 Animals

All animals were cared for according to the guiding principles in the care and use of animals. The experiments were approved by Western University and Animal Care and Veterinary Services. All experiments conform to the guide for care and use of laboratory animals published by the NIH (NIH publication no. 85-23, revised in 1996).

ANRIL knockout (129S6/SvEvTac-Del(4C4-C5)1Lap/Mmcd) mice, acquired from Mutant Mouse Resource & Research Centre (MMRC, Davis, CA). ANRIL had been identified to be located within the *p15/CDKN2B-p16/CDKN2A-p14/ARF* gene cluster (INK4 locus) and occurs anti-sense to these genes in the cluster. As the mouse RNA for ANRIL hasn't been characterized yet, ANRILKO mouse model was created on a 129S6/SvEv background where 70kb region on Chr 4 of the mouse gene aligning to human 58-kb non-coding CAD risk interval was removed by targeted deletion. The deletion was confirmed as the mice had significantly reduced expression levels of the neighboring genes CDKN2A and CDKN2B (located on the INK locus along with ANRIL), whereas there was no substantial expression change of other neighboring genes, Mtap and Dmrta1 (located outside of the INK locus) (57). We have also confirmed ANRILKO by measuring mRNA expression of CDKN2B (p15) in the mice tissues (Fig 3.1 A).

Diabetes was induced with STZ (five doses) intraperitoneally (50mg/kg in citrate buffer, pH5.6) on consecutive days (22). Age and sex-matched littermate controls received equal volume of citrate buffer. Blood glucose (>20mmol/L) measured from tail vein bi-weekly over the course of the study using a glucometer, confirmed diabetes. Animals were monitored for variations in body weight and blood glucose. Mice were sacrificed after 8 weeks and tissues were collected.

3.2.2 Echocardiography

Echocardiography was used to measure left ventricular hypertrophy (LVH), a marker for heart failure and myocardial infraction. Diabetic and control animals (wild types and ANRILKO) were lightly anesthetized (1.5% inhaled isoflurane) and subjected to echocardiography on a warm handling platform. A 40-MHz linear array transducer (MS-550D) and Vevo 2100 preclinical ultrasound system (VisualSonics, ON, Canada) were used for the experiment. Left ventricular fractional shortening (FS) was used as cardiac contractile function index. Pulse waved color flow-guided Doppler recordings of maximal early (E) and late (A) diastolic transmittal flow velocities and Doppler tissue imaging recordings of peak E' velocity and peak A' velocity was collected. Mitral inflow patterns (E/A ratio) and mitral annulus velocities (E'/A') were used to assess diastolic dysfunction as reported previously (22).

3.2.3 RNA analysis

TRIzol reagent (Invitrogen, Canada) was used to extract total RNA as previously described (23)**.** cDNA for PCR was synthesized with high-capacity cDNA reverse-transcription kit (Applied Biosystems, Burlington, ON, Canada). mRNA expression was detected through real time RT-PCR using LightCycler (Roche Diagnostics). Housekeeping gene β-actin was used to normalize the data.

3.2.4 ELISA

Total protein was extracted from kidney and heart tissues and concentrations measured by bicinchoninic acid (BCA) method. ELISA was performed to measure expression of FN and Col1α4 protein in mouse kidney tissues using a commercially available kit (Bosterbio, CA, USA) according to the manufacturer's instructions (15).

3.2.5 Luminex Assay

Multiplex luminex assay for angiogenesis was used to measure VEGF and hepatocyte growth factor (HGF) protein concentration in tissues extracted from heart and kidney of

diabetic and control mice. Tissues were extracted with RiPA buffer followed by precipitation with 80% ice cold acetone (8). Acetone precipitation was performed to ensure samples were devoid of possible colour from the extracted tissue that might interfere with the assay. Protein concentration of tissue lysate was measure by BCA assay. The samples (<500 mg/ml) were assessed using Luminex technology with Milliplex Mouse MAP Angiogenesis kit (Millipore, MA,USA) containing multiplex magnetic bead-based antibody detection kit according to manufacturer's protocol (55).

3.2.6 Urine albumin assay

24 hr urine was collected from the animals by placing them in metabolic cages before sacrifice with care so that feaces did not contaminate the urine samples. Measurement of urinary albumin and creatinine was performed by ELISA (Albuwell M; Exocell, Philadelphia, PA, USA). All procedures were performed using manufacture's protocol and the data was used to calculate urinary albumin:creatinine ratio (UACR) (16).

3.2.7 Histology and Immunohistochemistry

Renal cross-sections were fixed and embedded in paraffin and sectioned at 5μm thickness on positively charged slides. The sections were de-paraffinized in xylene and stained with periodic acid-Schiff (PAS) for assessment of glomerulosclerosis and mesangial expansion and Masson's trichome-stain for evaluation of interstitial expansion as described elsewhere (48). The stains were arbitrarily scored (0-3) in a masked fashion (42).

For immunohistochemical staining, the de-paraffinized kidney and heart sections were antigen-retrieved in citrate buffer and incubated with primary antibodies overnight at 4°C. The primary antibodies (Abcam, MA, USA) used were rabbit anti-mouse FN and Col1α4 (1:100). The slides were incubated in ImmPRESS^{TM} (Peroxidase) anti-rabbit IgG Reagent kit (Vector Lab, CA,USA) for 30 mins, mounted and viewed under microscope (40).

3.2.8 Statistical analysis

Statistical significance was determined by Student's *t*-test or one-way ANOVA followed by post-hoc test when appropriate. Data are expressed as mean ± SEM. A p value of 0.05 or less was considered to be significant and results are expressed as average of $n = 5$-8 animals per group. GraphPad Prism 5 (GraphPad, CA, USA) software was used for statistical analysis.

3.3 Results

3.3.1 ANRIL expression is elevated in kidneys of diabetic animals

We observed a protective effect of ANRIL nullification in HRECs and retina of diabetic mice in our previous study (53). Similar phenomena were investigated in diabetic kidneys in this research. Since ANRIL mouse RNA sequence remains uncharacterized, as per standard practice, its expression in mice was measured using p15, as a surrogate marker for ANRIL (Fig 3.1A) (57). Following STZ-induction, wildtype and ANRILKO animals were monitored for a period of 2 months. Hyperglycemia was evident in the diabetic animals (Fig 3.1B), along with polyuria, glycosuria and reduced body weight (Fig 3.1C), distinctive of poorly controlled diabetes. Interestingly, although ANRILKO had no effect on the above parameters, the urine volume in the diabetic ANRILKO animals were significantly less than in wildtype diabetics (Fig 3.2A).

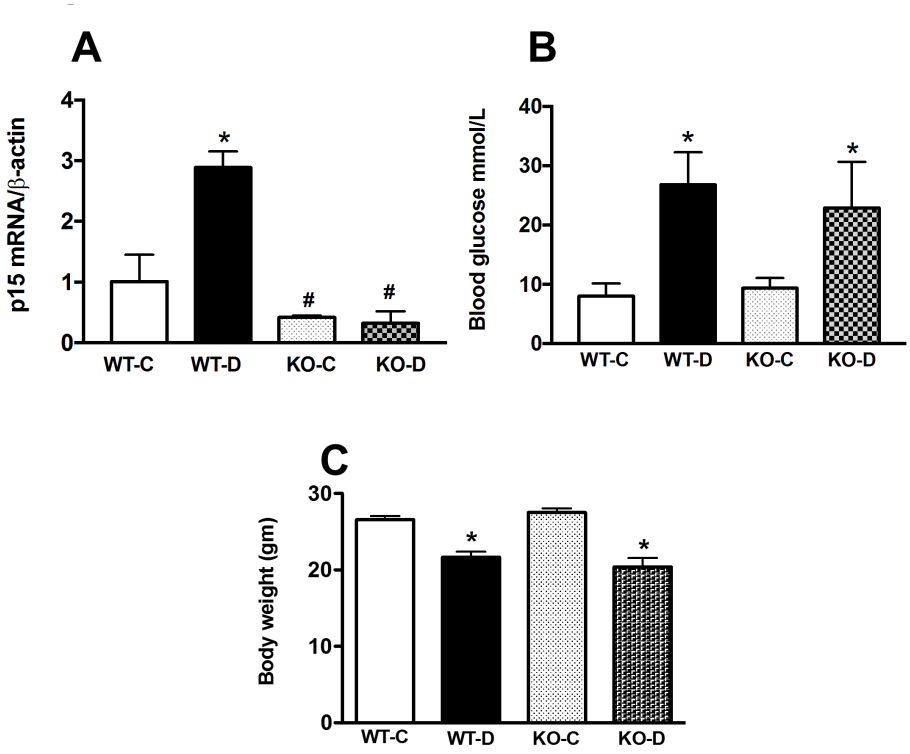

Figure 3.1: ANRIL expression in diabetes. (A)ANRIL mRNA expressions were increased in the kidneys of wild-type diabetic mice (WT-D) compared with the wild-type nondiabetic controls (WT-C). Both basal and diabetes-induced upregulated ANRIL expression were reduced in ANRIL-knockout (ANRILKO) controls (KO-C) and ANRILKO diabetics (KOD), respectively [ANRIL expression was measured by p15, its surrogate marker]. WT-D mice and KO-D animals showed hyperglycemia (B) and reduced body weight (C) gain following 2 months of diabetes compared with controls (WT-C and KO-C). (*P 0.05 vs. WT-C, #P 0.05 vs.WT-D; n 8/group).

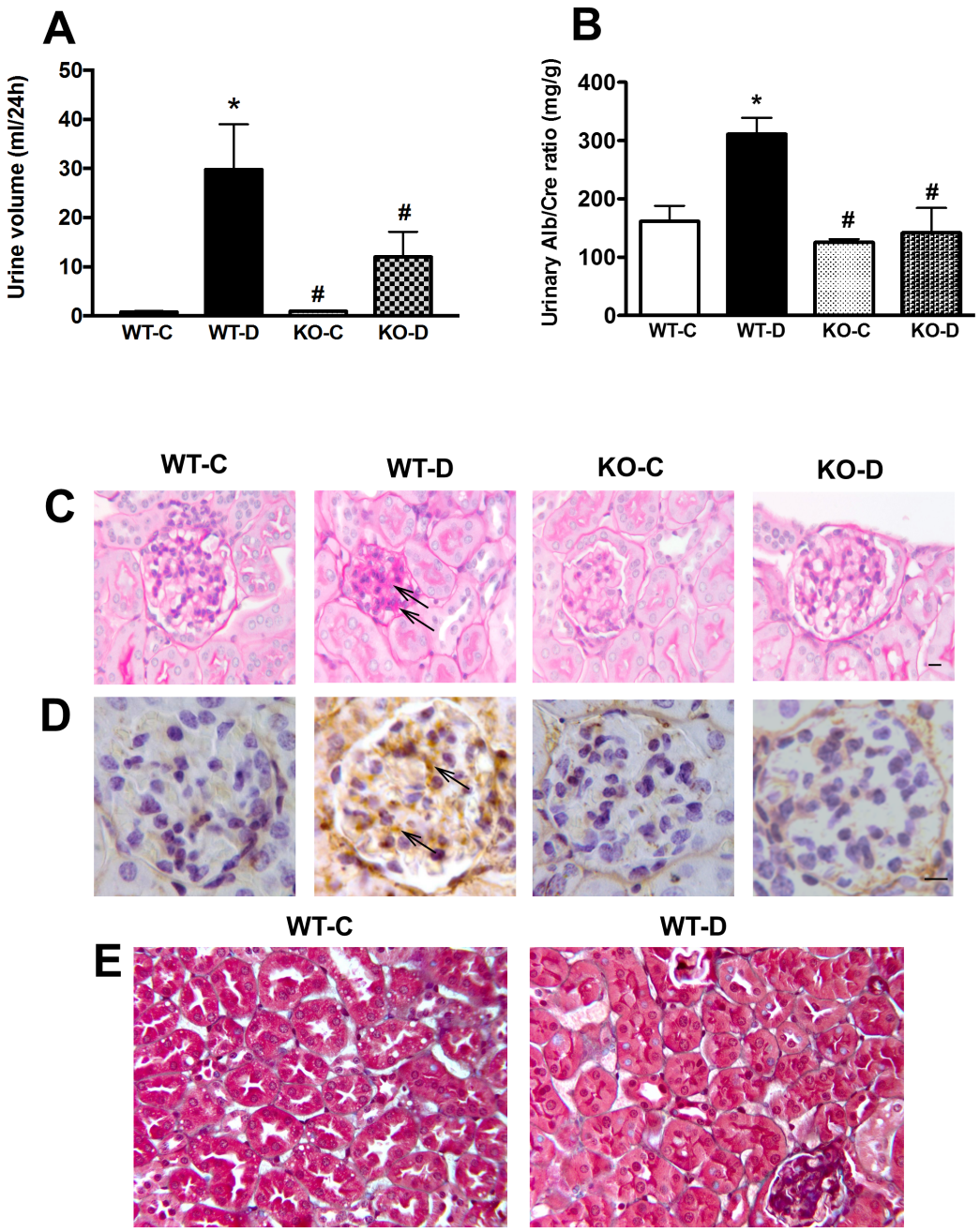

Figure 3.2: ANRIL regulates structural and functional changes in the kidneys of diabetic animals. Diabetes-induced elevated urine excretion (polyuria; A) and increased renal albumin/creatinine ratio (B) were prevented in the diabetic animals lacking ANRIL (KO-D). (C) Kidneys of wild-type diabetic (WT-D; score 3) mice showed mesangial expansion (periodic acid-Schiff stain; arrows show pink extracellular matrix protein deposition) compared with wild-type controls (WT-C; score 0) and ANRILKO controls

(KO-C; score 0). Such changes were prevented in the diabetic animals lacking ANRIL (KO-D; score 1). Immunohistochemical staining of kidney sections (D) for fibronectin (FN) showed significantly elevated positive stain (arrows) in the WT-D animals, which were prevented in KO-D mice. (E) Kidneys of wildtype diabetic (WT-D; score 0) and diabetic animals lacking ANRIL (KO-D) showed no significant increase in tubule-interstitial scarring (dark staining of Trichome stain) compared with wild-type controls (WT-C; score 0). *P 0.05 vs. WT-C, #P 0.05 vs. WT-D. Bar 10μm, same magnification for all micrographs; n 8/group.

3.3.2 ANRIL regulates renal structure and function in the diabetic mice

Kidney dysfunction generally reflects pathological changes in the kidneys in diabetes. We estimated urine volume and albumin-creatinine levels, which showed renal protection in the ANRILKO, as this ratio is characteristically elevated in wildtype diabetics (Fig 3.2B). We further stained the kidney sections with PAS stain. The stains showed increased mesangial matrix protein deposition in the wildtype diabetics. The increased mesangial matrix depositions were prevented in diabetic ANRILKO animals (Fig 3.2C). Trichome staining of the tissues did not show any significant increase in tubule-interstitial scarring in the diabetic animals as these changes could be more prevalent at later time points (Fig 3.2E).

3.3.3 ECM proteins are regulated by ANRIL in diabetic animals

The finding of reduced urine volume and Alb/Cre ratio directed us to think of a protection in the ANRILKO animals against hyperglycemic insults. Increased production, deposition and reduced breakdown of ECM proteins are key features of DN. We measured the FN and Col1α4 levels to find whether such protection was due to reduced deposition of these proteins. Immunohistochemical stains showed significantly elevated FN levels in the wildtype diabetic groups compared to non-diabetics and diabetic ANRILKO animals (Fig 3.2D). Similarly, increased levels of mRNA and protein

expression of ECM proteins, FN and Col1α4, observed in wildtype diabetic animals were prevented in the ANRILKO diabetic animals (Fig 3.3A-D).

3.3.4 ANRIL regulates VEGF production in the kidneys in diabetes

ANRIL regulates vasoactive factors such as VEGF in the retina during diabetes (45). We further explored the possibility of such regulation in the kidneys. The mRNA and protein levels of VEGF were increased in diabetic wildtypes compared to controls. mRNA alterations were prevented in ANILKO diabetic animals (Fig 3.3E and F). In contrast, mRNA expression of hepatocyte growth factor (HGF) remained elevated in both wildtype and ANRILKO diabetic animals (Fig 3.3G). This observation indicates ANRIL's role to be specifically acting through regulation of ECM proteins and VEGF.

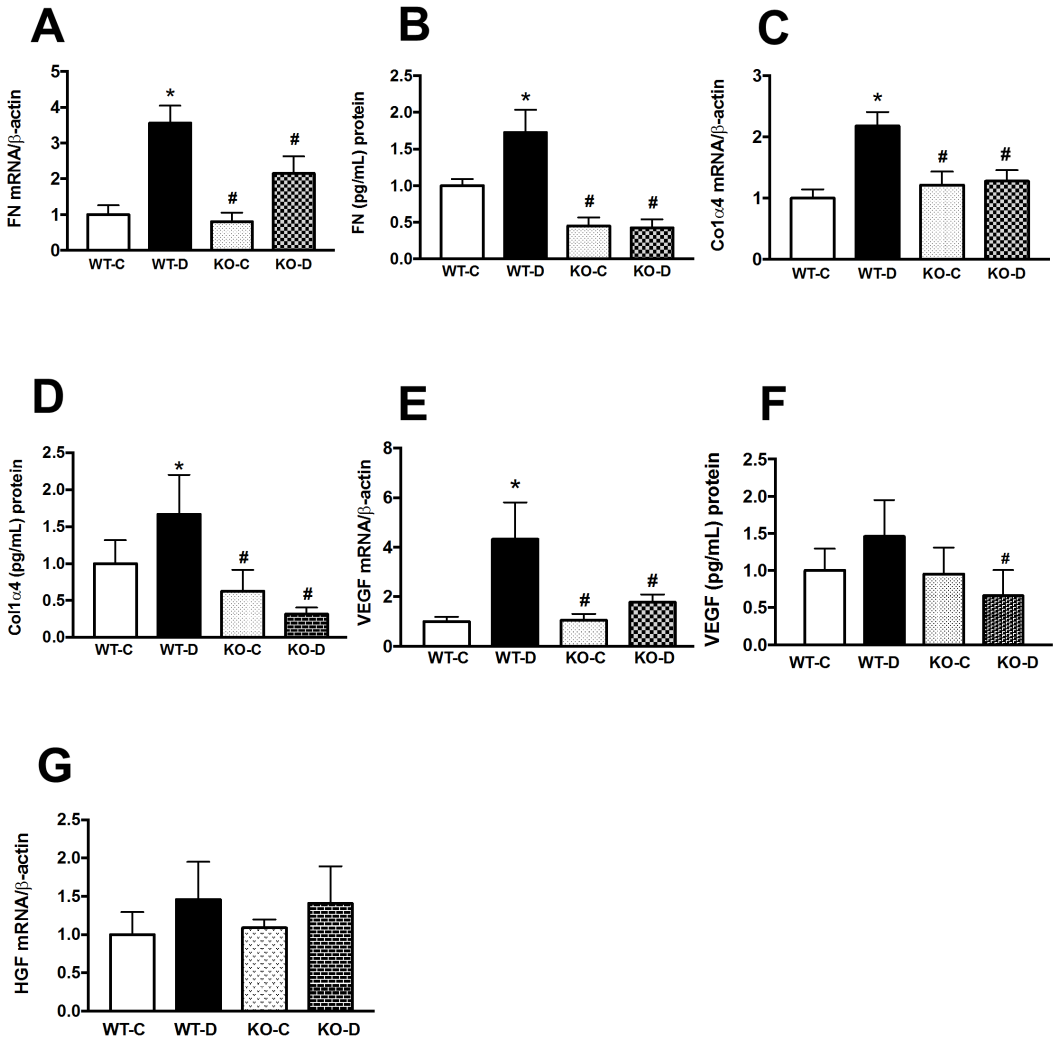

Figure 3.3: ANRIL regulates extracellular matrix (ECM) proteins and VEGF in kidneys of diabetic animals. (A–D) Diabetes induced elevation of ECM proteins; FN mRNA (A), FN protein (B), type IV collagen (Col14) mRNA (C), and Col14 protein (D) levels were prevented in the kidneys of ANRILKO diabetic animals (KO-D). Increased expression of VEGF at the mRNA (E) and protein (F) levels were significantly prevented in the KO-D animals. G: the elevated mRNA levels of hepatocyte growth factor (HGF) in diabetes remained unaffected in KO-D. WT-C, wild-type controls; WT-D, wild-type diabetics; KO-C, ANRILKO control. *P 0.05 vs. WT-C, #P 0.05 vs. WT-D; n 8/group.

3.3.5 ANRIL works in association with other epigenetic regulators

ANRIL works in concert with other epigenetic regulators, such as p300, a histone acetylator and EZH2, histone methyltransferase component of PRC2 complex (53). As expected, these components were increased in the kidneys of wildtype diabetic mice and were corrected in the diabetic ANRILKO kidney tissues (Fig 3.4A and B). The other components of PRC2, namely embryonic ectoderm development protein (EED) and suppressor of zeste (SUZ12) were not altered in the ANRILKO mice compared to wildtypes (Fig 3.4C and D).

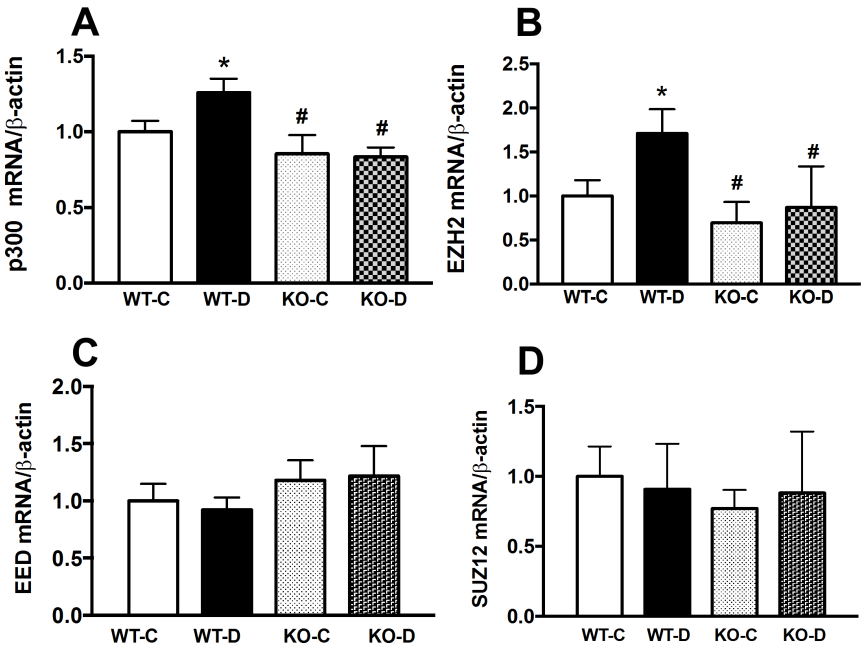

Figure 3.4: ANRIL's action is mediated by epigenetic mechanism. Diabetes-induced increased renal mRNA expressions of p300 (A) and enhancer of zeste 2 (EZH2; B) were prevented in diabetic ANRILKO animals (KO-D), whereas embryonic ectoderm development protein (EED; C) and suppressor of zeste (SUZ12; D) mRNA levels were unaffected. WT-C, wild-type controls; WT-D, wild-type diabetics; KO-C, ANRILKO controls. *P 0.05 vs. WT-C, #P 0.05 vs. WT-D; n 8/group.

3.3.6 ANRIL-mediated ECM and VEGF regulation in diabetic hearts

We extended our study to examine if other organs, namely cardiac tissue, were similarly affected. Heart tissue was analyzed to elucidate possible regulatory role of ANRIL in development of DCM and subsequent structural alterations. We observed similar alterations in expression levels of FN, Col1α4 and VEGF as seen in kidneys (Fig 3.5A-D). Immunohistochemical analysis showed elevated FN and Col1α4 protein expression in wildtype diabetic groups compared to control groups and diabetic ANRILKO animals (Fig 3.6A and B). However, trichome stain failed to show any obvious scarring (data not shown). We also performed echocardiographic examination on the live animals to examine any functional changes in the cardiac functional changes among various groups (Fig 3.6C-E). This lack of functional changes in the diabetic animals may, at least in part be explained by lack of significant scarring in this species.

Our analysis of mRNA expression levels of p300 and EZH2 of the PRC2 complex suggested that ANRIL may regulate ECM and vasoactive proteins in heart by similar epigenetic mediators (Fig 3.7A and B). The other two components of PRC2 complex; EED and SUZ12 remained unaffected by altered ANRIL levels (Fig 3. 7C and D).

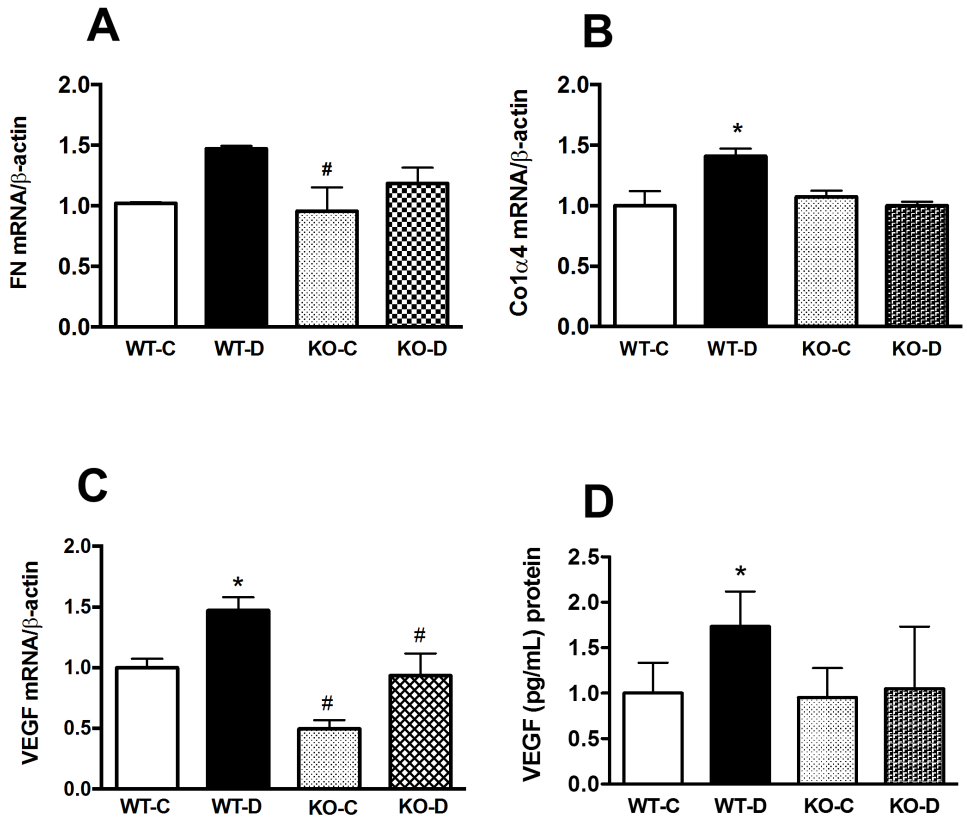

Figure 3.5: ANRIL regulates ECM proteins and VEGF levels in cardiac tissues of diabetic mice. Diabetes-induced elevation of FN (A), Col14 (B), and VEGF (C) mRNA levels as well as VEGF protein expressions (D) was prevented in hearts of diabetic ANRILKO animals (KO-D). WT-C, wild-type controls; WT-D, wild-type diabetics; KO-C, ANRILKO controls. *P 0.05 vs. WT-C, #P 0.05 vs. WT-D; n 8/group.

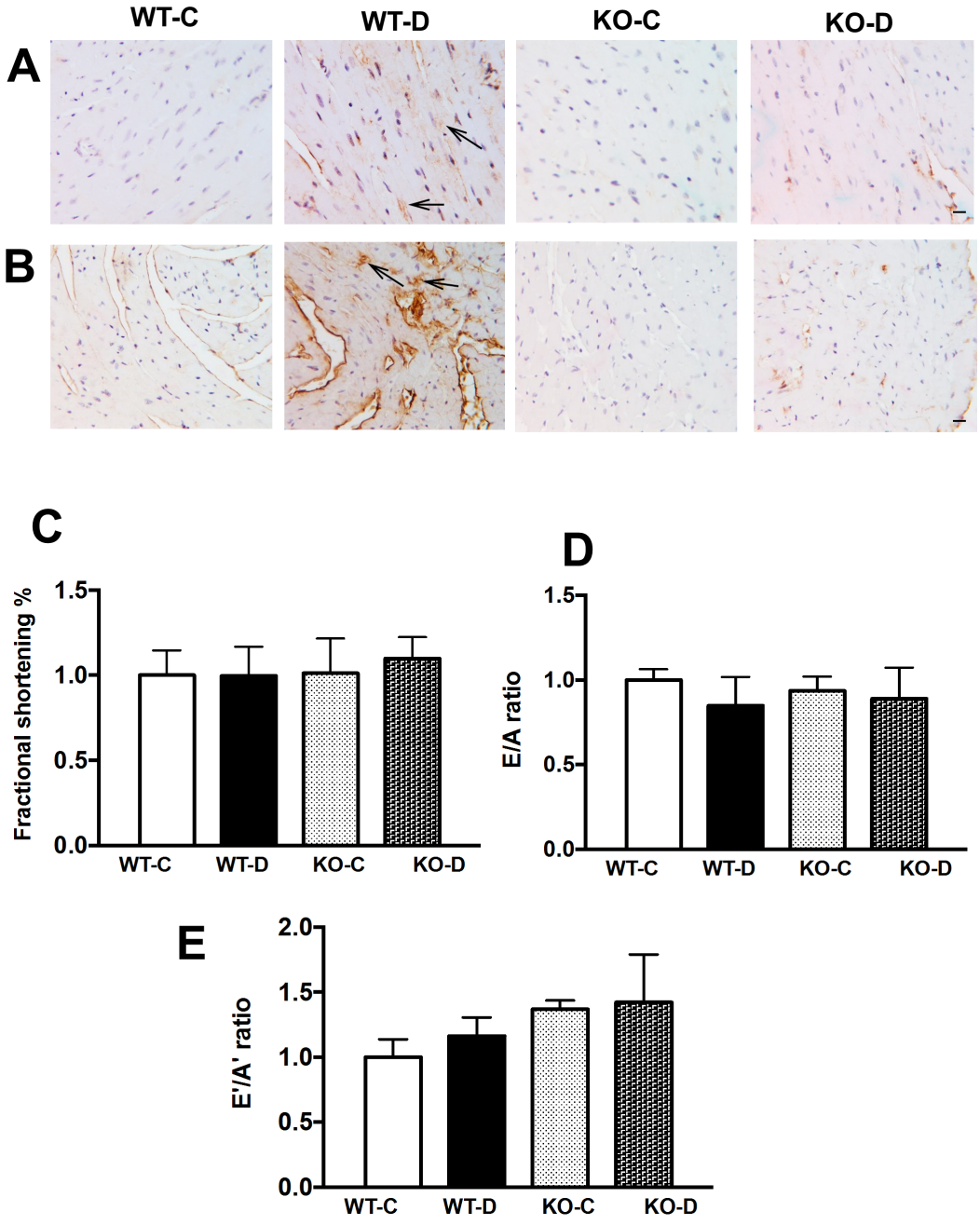

Figure 3.6: ANRIL regulates ECM protein expression in the cardiac tissues in diabetes. Immunohistochemical staining of cardiac tissue sections for FN (A) and Col14 (B) showed significantly elevated positive stain (arrows) in the wild-type diabetic (WT-D) mice. Such elevations were prevented in the ANRILKO diabetic mice (KO-D). Echocardiographic assay of mice showed no significant changes in fractional shortening (FS; C), mitral inflow pattern (E/A ratio; D), or mitral annulus velocity (E=/A= ratio; E)

levels between the groups. WT-C, wild-type controls; KO-C, ANRILKO controls. *P 0.05 vs. WT-C; #P 0.05 vs. WT-D. Bar 10μm (same magnification for all micrographs; n 8/group).

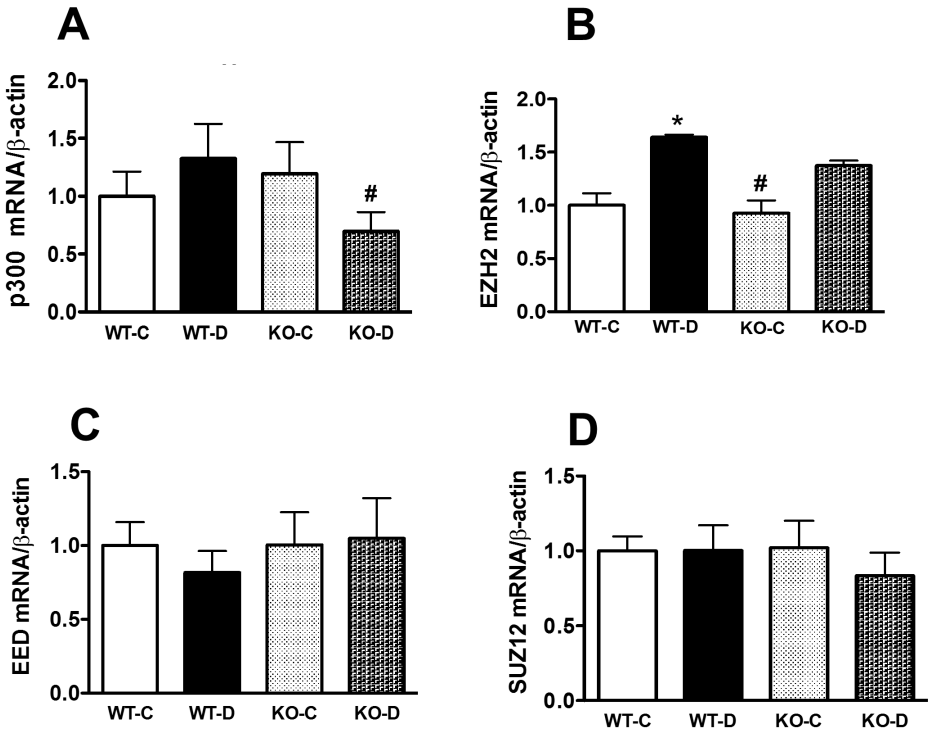

Figure 3.7: ANRIL's action is mediated by epigenetic mechanisms. The elevated levels of p300 (A) and EZH2 (B) observed in wildtype diabetic mice (WT-D) were prevented in the diabetic ANRILKO animals (KO-D). However, increased EED (C) and SUZ12 (D) levels in diabetes were unaffected in the KO-D animals. WT-C, wild-type controls; KO-C, ANRILKO controls. *P 0.05 vs. WT-C, #P 0.05 vs. WT-D; n 8/group.

3.4 Discussion

In this research, we described the role of lncRNA ANRIL in regulating ECM proteins FN and Col1α4 as well as angiogenic factor VEGF in DN and DCM. This regulation at least in part is driven by ANRIL's interaction with PRC2 complex proteins and p300 causes functional changes in murine kidneys and heart. Polyuria and albuminuria levels

characteristic of DN were prevented in the ANRILKO diabetic animals. Furthermore, increased ECM protein deposition observed in glomeruli and in the heart of wildtype diabetic mice were also absent in ANRILKO diabetics, evident through PAS stain.

Development and progression of DN, involves hyperglycemic insults on resident kidney cells including endothelial cells (ECs) and podocytes (25)**.** Similarly, ECs and cardiomyocytes are affected in DCM (58). Multifactorial causes including genetic and environmental factors can be ascribed to all chronic diabetic complications. Genetic susceptibility in the development of these complications can be attributed to familial aggregation, with a three-fold increase in risk for diabetic siblings of diabetic probands in developing chronic complications such as DN (18). The emphasis on identifying susceptible genes associated with chronic diabetes has led to widespread research including candidate gene screening and genome-wide association studies (GWAS) (6, 45). Along with various factors like transcription factor binding affinity, disruption of chromatin and post-translational modifications that are critical for modulation of gene function by genetic variants; epigenetic control and regulation of genes provides an additional layer of mechanism involved in the pathogenesis of diseases like chronic diabetic complications (32).

Previously we had reported that the expression of ANRIL was upregulated in diabetic retinopathy (53). ANRILKO led to the protection from upregulation of histone acetylator, p300 as well as EZH2 component of the PRC2 complex characteristic of diabetic complications. Similar to the retina, here we report that ANRIL controls the alteration observed in the kidneys and heart in diabetes. This study showed that certain epigenetic modulators histone acetylator, p300 and EZH2 of the PRC2 complex are also regulated by ANRIL. Histone acetylation is an important post-transcriptional modification that recruits protein complexes for the regulation of gene expression. Recent research has highlighted the contribution of p300 in the development and persistence of diabetes and its complications (36)**.** We have also shown upregulation of histone methyltransferase EZH2 of PRC2 components in diabetic complications (47,53). In our experiments, we observed the decrease in expression of FN, Col1α4 and VEGF in the ANRILKO animals

was accompanied by subsequent reduction in these epigenetic factors in the kidneys as well as heart tissues. These findings indicate that ANRIL regulates these key molecules. Our results further suggest that ANRIL's interaction with the histone modifiers EZH2 and p300 may play a role in such process.

lncRNAs including ANRIL have been shown to have a strong association with ECM protein-coding genes and are important regulators of fibrosis (30). Apart from binding of transcription factors to the promoters of the protein-coding genes, transcriptional activation or repression is linked to alteration in chromatin structure through histone modifications and subsequent nucleosome re-modeling (56). Therefore, epigenetic regulation of gene expression in the current context, involves a combined interaction between lncRNAs, histone modifiers and other regulators. We and others have shown that activation of histone acetylator, p300 is accompanied by increased expression of vasoactive factors and ECM protein in the ECs and fibroblasts (15,28). Following exposure to high glucose, there is enhanced binding of p300 to promoters of these genes in ECs (15). However, unlike acetylation, histone methylation changes are often continued down the cell lineage and thus, have received greater attention especially in glycemic memory (21,41). In their study with cultured podocytes, Siddiqi and colleagues found that deletion of histone methyltransferase, EZH2 with inhibitors or silencers rendered the cells vulnerable to deleterious effects of high-glucose, leading to elevated ROS levels and cell death (49). However, we have shown that ANRIL binds to both p300 and EZH2 and positively regulates their expression (53). Therefore, the protective effect of ANRILKO seen in the kidneys and heart could be attributed to the downregulation of p300 that might be directly regulating ECM protein and VEGF (15, 35). As for EZH2 downregulation, it might be leading to overexpression of other genes that might aid in the protection imparted by ANRILKO in diabetic complications. The pathway of ANRIL's regulation of EZH2 needs to be explored further.

Hyperglycaemia is a well-known contributor for the damage to the ECs and subsequent organ dysfunctions (14, 52). ANRIL expression has been reported in atheromatous vessels, coronary smooth muscle cells and vascular ECs (10). Recently, Song and

colleagues demonstrated that elevated ANRIL expression induced vascular cell apoptosis, exacerbated EC inflammation and reduced artheroprotective effects, leading to EC dysfunction in atherosclerosis. These features were reversed in the rat models treated with ANRIL inhibitors (51). Similarly, our previous work showed a glucose-mediated upregulation of ANRIL in HRECs and in retinal tissues, causing upregulation of key proteins such as VEGF (53). The current study also shows similar regulation of VEGF expression in DN and DCM.

We have demonstrated the regulatory interaction of lncRNA with histone acetylator and the PRC2 complex protein. Interestingly lncRNAs' effect may be mediated through microRNAs (miRNAs). Recently, lncRNA TUG1 has been reported to decrease ECM protein production by regulating miR-377 in DN (19). We have shown such interaction of ANRIL with miR200b at the cellular level (53). Further studies are needed to expose such possibilities with respect to DN and DCM. In addition, it is possible that other miRNAs, lncRNAs and additional epigenetic mechanisms are also part of this orchestra, which needs to be deciphered through additional well-designed experiments.

In summary, we have demonstrated that ANRIL may interact with epigenetic modifiers at multiple levels in regulating the expression of ECM proteins and VEGF in the pathogenesis of DN and DCM. While we acknowledge the role of other factors including other lncRNAs in these regulatory mechanisms, targeted therapies directed at ANRIL may have important implications in combatting chronic diabetic complications.

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Chapter 4

4 LncRNA H19 reverses Endothelial-Mesenchymal Transition in Diabetic Retinopathy[c]

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4.1 Introduction

The prevalence of diabetes mellitus worldwide has increased considerably and is estimated to rise from 382 million in 2013 to 592 million by 2035 (1). Over time, diabetes can lead to several life-limiting complications at both micro and macrovascular levels (2). Diabetic retinopathy (DR) is the most common microvascular diabetic complication that can cause vision-threatening retinal changes in one-third of the diabetic patient population (3). Progression of DR is strongly associated with duration of diabetes, hypertension and hyperglycemia (4). Hyperglycemia has been shown to impair endothelial function in both animal and human studies (5-7). The endothelial cells (ECs), lining the entire circulatory system, facilitate effective pumping of blood, secrete factors to regulate coagulation, fibrinolysis, platelet aggregation and vascular tone, and plays a critical role in maintaining overall homeostasis (8,9). On exposure to hyperglycemia, ECs undergo a range of intracellular occurrences that leads to endothelial dysfunction, where the endothelium loses it physiological properties (9). In diabetes, endothelial dysfunction promotes excess accumulation of fibrous connective tissue and extracellular matrix (ECM) proteins in and around the damaged tissue leading to organ dysfunction (10,11). Abnormal accumulation of ECM is accompanied by local fibroblast proliferation and its differentiation into myofibroblasts, the key cellular mediators of fibrosis (12). Damaged ECs also express markers characteristic of myofibroblast differentiation such as α-smooth muscle actin (α-SMA), smooth muscle 22 (SM22), vimentin and fibroblast specific protein 1 (FSP1); thereby, adopting a mesenchymal phenotype (8). Concurrently, EC markers such as vascular endothelial cadherin (VE-Cadherin) and cluster of differentiation 31 (CD-31) are down-regulated (8). ECs undergo this phenomenon known as endothelial-mesenchymal transition (EndMT) to gain a differentiated phenotype and attain invasive and migratory abilities in order to affect pathological processes in numerous ways (8). EndMT, similar to epithelial–mesenchymal transition (EMT) may be regulated by TGF-β superfamily of proteins including TGF-β1 and TGF-β2 through Smad-dependent and Smad-independent pathways such as MEK/ERK, PI3K and p38MAPK signaling pathways (8, 13-15).

Long non-coding RNAs (lncRNAs) are RNA transcripts >200 nucleotides in length lacking protein coding potential (16). lncRNAs are known to play important roles in physiological processes such as modulation of alternate splicing, chromatin remodeling, *cis/trans*-acting regulators of gene expression and RNA metabolism (17-19). Recent evidences show that dysregulation of target genes lead to abnormal lncRNA expression in multiple cancer types and they play critical roles in mediating TGF-β-induced EMT during tumor metastasis (20,21). One of the key lncRNAs in tumorigenesis is the oncofetal lncRNA, H19. The *H19* gene is paternally imprinted and produces a 2.3kb spliced, capped and polyadenylated lncRNA which is predominantly cytoplasmic and co-regulated by Igf2 (Insulin-like growth factor 2); a maternally imprinted gene located on the same locus (22). The specific role of H19 in tumorigenesis is disputable as it is known to harbor pro-tumorigenic properties (23) and concurrently, literature describes its tumor suppressor roles (22,24).

We have previously reported the development of EndMT in the retina and heart of diabetic animals accompanied by activation of TGF-β-mediated SMAD signaling cascade and increased expression of ECM proteins (25,26). This glucose-induced phenomenon is regulated by miRNA 200b (miR200b) through modulation of a histone acetyltransferase, p300 (25,26). H19 has been shown to alter miR-200 pathway by increasing histone acetylation upstream of this miRNA; contributing to the reversal of EMT (24). Exposure to high glucose reduced H19 expression in neonatal cardiomyocytes and in the myocardium of streptozotocin-induced diabetic rats (27). However, the role of H19 in the process of EndMT during DR remains unknown.

The purpose of the current study was to decipher the role of H19 in the development of EndMT during DR. We have used human retinal microvascular endothelial cells (HRECs) to understand if H19 regulates this phenomenon through TGF-β-mediated Smad-dependent or Smad-independent pathways and its influence on miR200b during this process. We further expanded this study to include STZ-induced diabetic mouse models and vitreous samples from diabetic patients.

4.2 Materials and Methodology

4.2.1 Cells

HRECs (Olaf Pharmaceuticals, Worcester, MA, USA) were grown in endothelial basal media-2 (EBM-2) supplemented with 10% fetal bovine serum (25). Cell were plated at 4.3 x10^5 cells/mL and following 24-hour incubation with serum free EBM-2 media were treated with various glucose levels (5 mM/L, normal glucose [NG]; 25 mM/L, high glucose [HG]) for 48 hours. All cells for transfection experiments were seeded at 7.5 x10^5 cells/mL to achieve 90% confluency. Following transfection, they were serum starved for 24 hours and treated with various glucose levels for 48 hours. Each experiment was performed with three or more replicates.

4.2.2 Animals

All animals were cared for according to the guiding principles in the care and use of animals. Western University and Animal Care and Veterinary Services approved all the experiments and they conform to the guide for care and use of laboratory animals published by the National Institutes of Health (NIH Publication 85-23, revised in 1996).

$H19^{\Delta exI}$ mice, generated on a C57/BL6-J background by cre-loxP-based deletion strategy targeted to delete exon1 from *H19*, were used for this study along with littermate controls (28). $H19^{\Delta exI}$ mice were a generous gift from Dr. Karl Pfeifer at NIH (Maryland, USA). These mice were administered with streptozotocin (STZ) intraperitoneally. They were given a total of five doses (50 mg/kg in citrate buffer, pH 5.6) on alternate days (30). Sex and age-matched littermate controls received identical volume of citrate buffer. Hyperglycemia was confirmed by measuring tail vein blood glucose (>20 mmol/L) using a glucometer. All animals were frequently monitored and placed in metabolic cages (24 hours) to collect urine following 8 weeks of diabetes. Afterwards all mice were euthanized and retinal tissues were collected.

4.2.3 Human Vitreous samples

The clinical components of this study were approved by The Western Research Ethics Board at the University of Western Ontario, London, Ontario, Canada. All patients provided informed consent prior to surgical sample procurement and all samples were handled according to the Declaration of Helsinki. Vitreous humor (VH) was collected from patients undergoing a 25-gauge pars plana vitrectomy performed by an experienced vitreoretinal surgeon. The samples were categorized into diabetic groups comprised of patients diagnosed with proliferative DR (PDR; n=8; 5 males and 3 females; mean age ± SD= 76 ± 3.77 years) and non-diabetic groups (n=5; 3 males and 2 females; mean age ± SD= 61.6 ± 8.35 years) with no previous history of diabetes mellitus and diagnosed with idiopathic macular hole or a separate non-diabetic ocular condition. All VH specimens were centrifuged at 12,000g for 10 minutes at 4°C and pellet collected for RNA extraction as described below (29). Vitreal hemorrhage indications in the VH samples were excluded from the study to avoid contaminating RNA from blood cells.

4.2.4 RNA analysis

Total RNA was extracted using TRIzol reagent (Invitrogen, Canada) as described previously (26)**.** cDNA for PCR was produced with high-capacity cDNA reverse-transcription kit (Applied Biosystems, Burlington, ON, Canada). Detection of mRNA expression was performed through real time RT-PCR using LightCycler (Roche Diagnostics). Housekeeping gene β-actin was used to normalize the data.

4.2.5 miRNA Analysis

miRNA extraction from HRECs were performed using mirVana miRNA isolation kit (Ambion, Austin, TX, USA). cDNA was synthesized using TaqMan microRNA Assay Reverse Transcription Primer and MultiScribe reverse transcriptase (Life Technologies, Canada) following manufacture's protocol (26). TaqMan microRNA Assay was also used to perform real time RT-PCR in a LightCycler (Roche Diagnostics). All data were normalized to U6 small nuclear RNA.

4.2.6 Fluorescence In Situ Hybridization

In order to perform fluorescence *in situ* hybridization (FISH), the cells were seeded at 75% confluency on glass coverslips in 12-well plates and treated with various glucose levels (5 mM/L, normal glucose [NG]; 25 mM/L, high glucose [HG]) for 48 hours as described previously (30). FISH was performed according to the manufacturer's protocol (biosearchtech.com/ stellaris protocols). The human H19 probes were oligonucleotides tagged with CAL Fluor® Red 610 Dye, mixed and pooled to a final concentration of 5nmol (Biosearch Technologies, Petaluma, CA, USA). The HRECs hybridized with probes were counterstained with Hoechst and mounted with Vectashield mounting medium (Vector Labs, Burligame, CA, USA). Images from the stained cells were captured with a fluorescent microscope (Olympus BX51; Olympus, Richmond Hill, ON, Canada) and analyzed with ImageJ software (National Institute of Health, Bethesda, MD, USA).

4.2.7 Immunofluorescence

The HRECs were seeded on a cover slip in 12-well plates at approximately 75% confluency. Following various treatments the cells were rinsed with PBS and fixed with methanol:acetone (4:1, vol:vol) and permeated with 0.5% Triton X-100 in PBS (25). After incubation with 5% normal serum, the cells were incubated with one of the primary antibodies: mouse anti-CD31 (Abcam, Toronto, Canada), rabbit anti-VE Cad (Santa Cruz, CA, USA), rabbit anti-FSP1 (Abcam, Toronto, Canada), rabbit anti-SM22 (Santa Cruz, CA, USA), goat anti-vimentin (Santa Cruz, CA, USA) and rabbit anti-TGF-β (Abcam, Toronto, Canada) at 1:100 dilutions. The corresponding secondary antibodies conjugated with Alexa Fluor 488 (goat anti-mouse IgG, goat anti-rabbit IgG or donkey anti-goat IgG [Invitrogen, Canada]) were used at 1:200 dilutions. A fluorescent microscope (Olympus BX51; Olympus, Richmond Hill, ON, Canada) was used to detect and capture the stains and images were analyzed with ImageJ software. Hoechst 33342 (1 mg/mL; Invitrogen) was used as the nuclear stain.

4.2.8 Luminex Assay

The components of TGF-β signaling were measured using Milliplex MAP TGF-β signaling pathway magnetic bead 6-plex-Cell Signaling Multiplex assay (Millipore, MA, USA). The kit comprises of pAkt (Ser473), pERK 1/2 (Thr185/Tyr187), pSmad 2 (Ser465/Ser467), pSmad 3 (Ser423/Ser425), Smad 4 (Total), TGFβRII (Total) analytes. The cells following treatment, were collected in ice cold 1X MILLIPLEX® MAP Lysis Buffer with freshly added protease. Particulate matter from samples were removed by filtration and total protein from the extracted lysate was measured by bicinchoninic acid (BCA) method. The samples (<500 mg/ml) were assessed using Luminex technology with Milliplex MAP TGF-β signaling kit containing multiplex magnetic bead-based antibody detection kit according to manufacturer's protocol (30).

4.2.9 Statistical analysis

The statistical significance was determined by Student's *t*-test or one-way ANOVA followed by post-hoc test when appropriate. All data were expressed as mean ± SEM and a p value of 0.05 or less was considered to be significant. All results are expressed as average of n = 5-8/per group. GraphPad Prism 5 (GraphPad, CA, USA) software was used for statistical analysis.

4.3 Results

4.3.1 H19 expression is regulated in ECs by exposure to glucose

Our first aim was to study the expression changes of lncRNA H19 in HRECs when subjected to glucose insults. We had previously performed a microarray analysis on HRECs exposed to 25 mM D-glucose (HG) for 48 hours to study expression levels of lncRNAs (30). The results of the array analysis showed one of the highest levels of downregulation of H19 in HG compared to 5 mM D-glucose (NG) controls (*data not shown*). Furthermore, duration dependent analyses of H19 expression showed one of its lowest levels after 48 hours of HG exposure. We validated glucose-mediated downregulation of H19 expression in these retinal endothelial cells through measurement

of H19 RNA expression post-HG treatment (Fig 4.1A). H19 expression was also visualized in these cells by performing RNA FISH, which helped us to delineate its sub-cellular distribution. The experiment was performed with oligonucleotide probes for human H19 that were highly specific, and the technique has been previously performed by us (30). FISH analyses showed a significant reduction in H19 expression in the HG treated cells (Fig 4.1B and C). In the control groups (NG), H19 was distributed both in the nucleus and predominantly cytoplasm. When treated with HG, although both nuclear and cytoplasmic positivity were reduced, the changes were pronounced in the nucleus. To examine whether this finding is clinically relevant, we performed similar analyses from the RNA extracted from vitrectomy samples. We found that H19 RNA expression levels were significantly lower in the diabetic vitreous samples compared to its controls (Fig 4.1D).

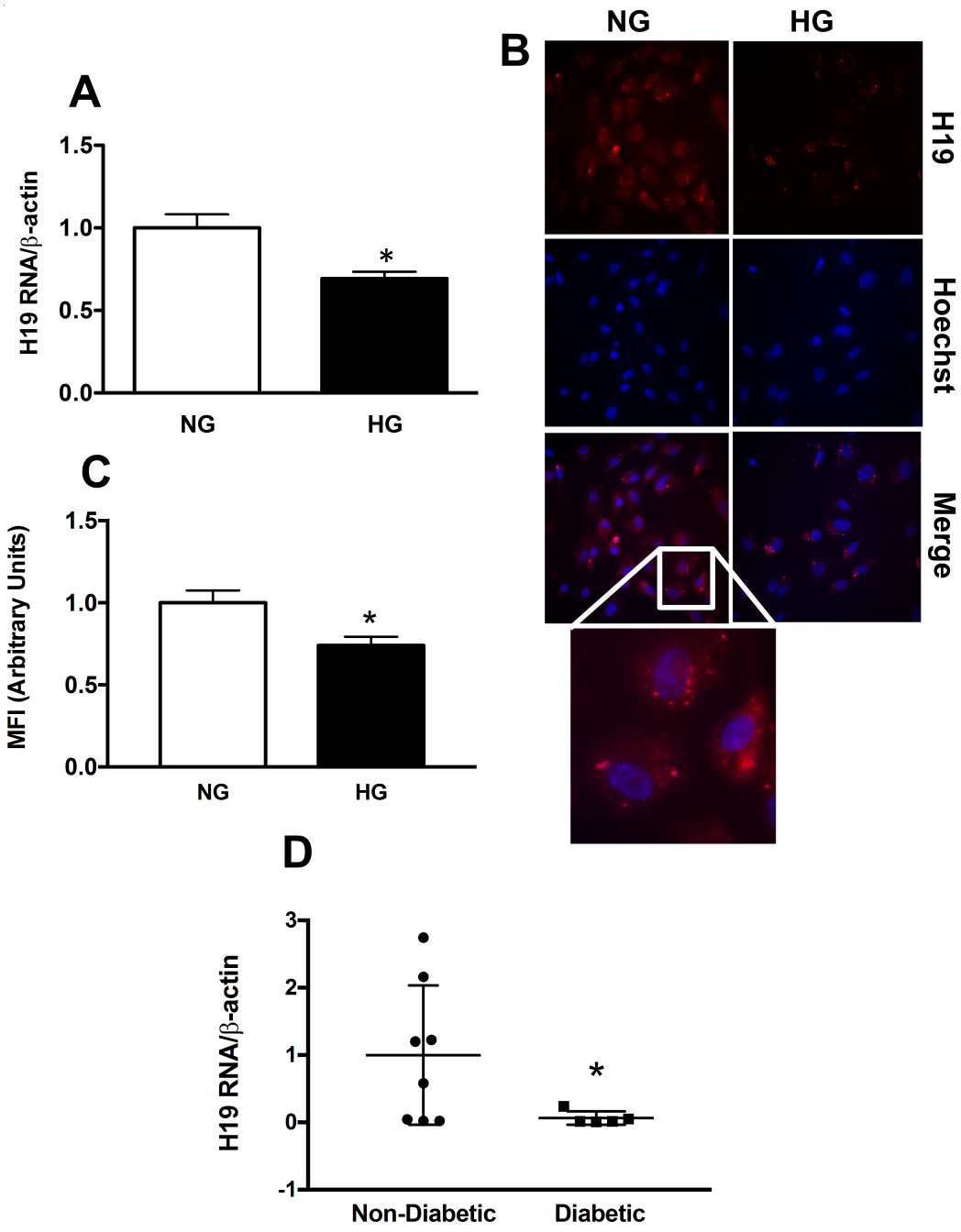


Figure 4.1: H19 expression in glucose-treated human retinal endothelial cells (HRECs). (*A*) H19 RNA expression (Mean±SE) measured using real-time RT-PCR was reduced on exposure to 25mM glucose (HG, 48 hours). Fluorescence *in situ* hybridization (FISH) using human H19 probes showed a decrease in H19 expression (*B*) in HRECs exposed to HG with largely cytoplasmic localization in comparison to 5mM glucose (NG) controls. FISH data quantification (*C*) performed using ImageJ confirmed glucose-induced H19 down-regulation (MFI- mean fluorescence intensity). RNA analysis of vitreous humor in

diabetic patients (Diabetic) indicated decrease in H19 levels (*D*) compared to non-diabetics. (RNA data expressed as ratio to β-actin, normalized to control, * $p< 0.05$ versus NG or non-diabetics, n=5 or more/group, individual values are represented as dots or squares).

4.3.2 H19 reverses glucose-mediated EndMT in HRECs and in the retinas of diabetic animals

The transition from endothelial to mesenchymal phenotype is characterized by decrease in the expression of endothelial markers and gain of a more mesenchymal phenotype. We have employed multiple markers to validate this transition. For the endothelial markers, we used cluster of differentiation 31 (CD 31) and VE cadherin (VE-cad); while the mesenchymal markers used were fibroblast-specific protein 1 (FSP1), smooth muscle-specific 22 (SM22), α-smooth muscle actin (α-SMA), and vimentin. All of these molecules represent well-characterized markers of EndMT and have been used in multiple studies including in our previous work (25,26,31,32). HG treatment showed significant downregulation of endothelial markers; CD 31 and VE-cad (Fig 4.2A and B) accompanied by upregulation of mesenchymal markers; FSP1, SM22 and α-SMA (Fig 4.2C-E). As the expression of H19 is reduced in HRECs on exposure to HG, we overexpressed H19 in HRECs to establish a cause-effect relationship. We used a pcDNA 3.1(+) harboring a full-length cDNA sequence of human H19. We transfected the HRECs with an empty vector (control) or h19-pcDNA 3.1, followed by treatment of cells with varying glucose concentrations and quantification of mRNA levels by real-time RT-PCR. Overexpressing H19 reversed the EndMT phenomenon in spite of the presence of HG environments (Fig 4.2A-E), indicating that glucose-induced EndMT changes are mediated through H19 in HRECs. As miR200b is a well-known negative regulator of EndMT (25,26), we conducted a similar experiment with miR200b mimic transfection followed by HG incubation, confirming its prevention of HG-induced EndMT (Fig 4.2A-E).

To identify a relationship of H19 with miR200b in this context, we performed additional experiments (Fig 4.2A-E). H19 overexpression increased basal and glucose-induced

miR200b downregulation (Fig 4.2F). In order to delineate a direct relationship, we performed a rescue experiment. We suppressed miR200b expression using miR200b antagomirs in NG environment and then overexpressed H19. Such intervention prevented glucose-induced EndMT suggesting that H19 may also work independent of miR200b (Fig 4.2A-E).

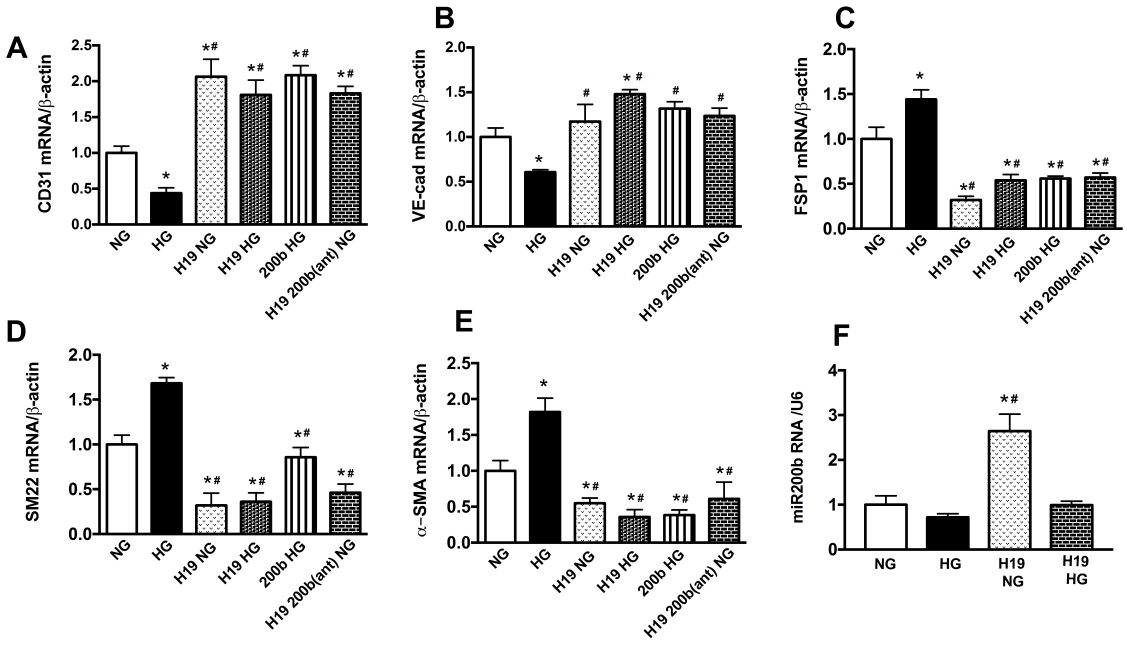


Figure 4.2: Effect of H19 overexpression on EndMT markers at mRNA level (Mean±SE). Twentyfive mM glucose (HG) treatment reduced mRNA expression of endothelial markers [CD 31 (*A*) and VE cadherin (VE-cad) (*B*)] and increased expression of mesenchymal markers [FSP1 (*C*) and SM22 (*D*) and α-SMA (*E*)] compared to 5mM glucose (NG). Overexpression of H19 (H19 NG and H19 HG) reversed such alterations of EndMT markers (*A-E)*. Similarly, miR200b overexpression [using miR200b mimics (200b HG)] corrected HG-induced alteration of EndMT markers. H19 overexpression increased basal and glucose-induced miR200b downregulation (*F*) and rescued cells from EndMT in spite of blockade of miR200b using antagomirs (H19 200b(ant) NG) (*A-E)*. (mRNA data expressed as ratio to β-actin, normalized to control. $*,^{\#}$ $p< 0.05$ versus empty vector treated with NG (NG) and empty vector treated with HG (HG) respectively, n=6 or more/group).

We extended our study to confirm the changes at protein level. HRECs transfected with empty vectors and H19 were treated with varying glucose concentrations (NG and HG). Following the treatments, fixed cells were incubated with fluorescence-tagged antibodies for endothelial markers, CD 31 and VE-cad, and mesenchymal markers, FSP1, SM22 and vimentin. In all instances, HG-induced reductions of endothelial markers were corrected with H19 overexpression (Fig 4.3A-D). Furthermore, similar correction of HG-induced increase of mesenchymal markers were seen following H19 overexpression (Fig 4.4A-D and Supplementary Figure) in keeping with the mRNA levels. siRNA mediated silencing in NG produced a glucose-like effect on EndMT markers. Interestingly, lowering of H19 expression in HG environment did not have any further effects on these markers, which may suggest that such alterations have already reached lowest biological levels following HG incubation (Fig 4.5A-E). In the animal experiments, STZ-induced wild-type and $H19^{\Delta exI}$ were monitored for 2 months. Elevated blood glucose, polyuria and reduced body weight were evident in the diabetic animals, indicative of poorly controlled diabetes (Supplementary Table). Analyses of retinal tissues from these animals showed diabetes-induced, reduced H19 in association with characteristic retinal changes of EndMT, namely, reduced endothelial and increased mesenchymal markers. All such changes were further exaggerated in the H19/KO mice (Fig 4.5F-J).

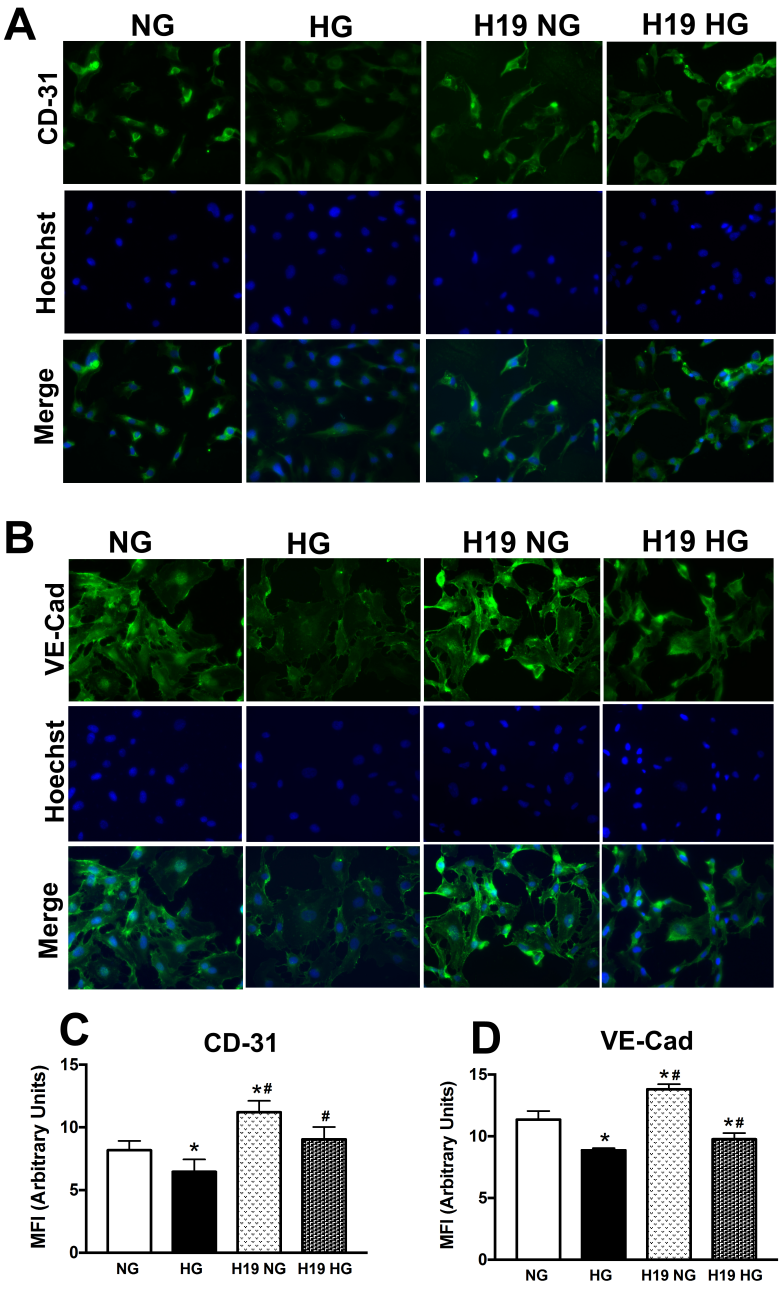


Figure 4.3: Effect of H19 upregulation on protein expression of endothelial markers. Immunofluorescence stains of human retinal endothelial cells (HRECs), showed reduced expression of endothelial markers CD 31 (*A*) and VE cadherin (VE-cad) (*B*) in 25mM glucose (HG) versus 5mM glucose (NG). These alterations were prevented in H19 overexpressed HRECs (H19 NG and H19 HG). Quantification date using ImageJ confirmed these findings (*C* and *D*) (MFI- mean fluorescence intensity. $*,^{\#}$ $p< 0.05$ versus empty vector treated with NG (NG) and empty vector treated with HG (HG) respectively, n=6 or more/group).

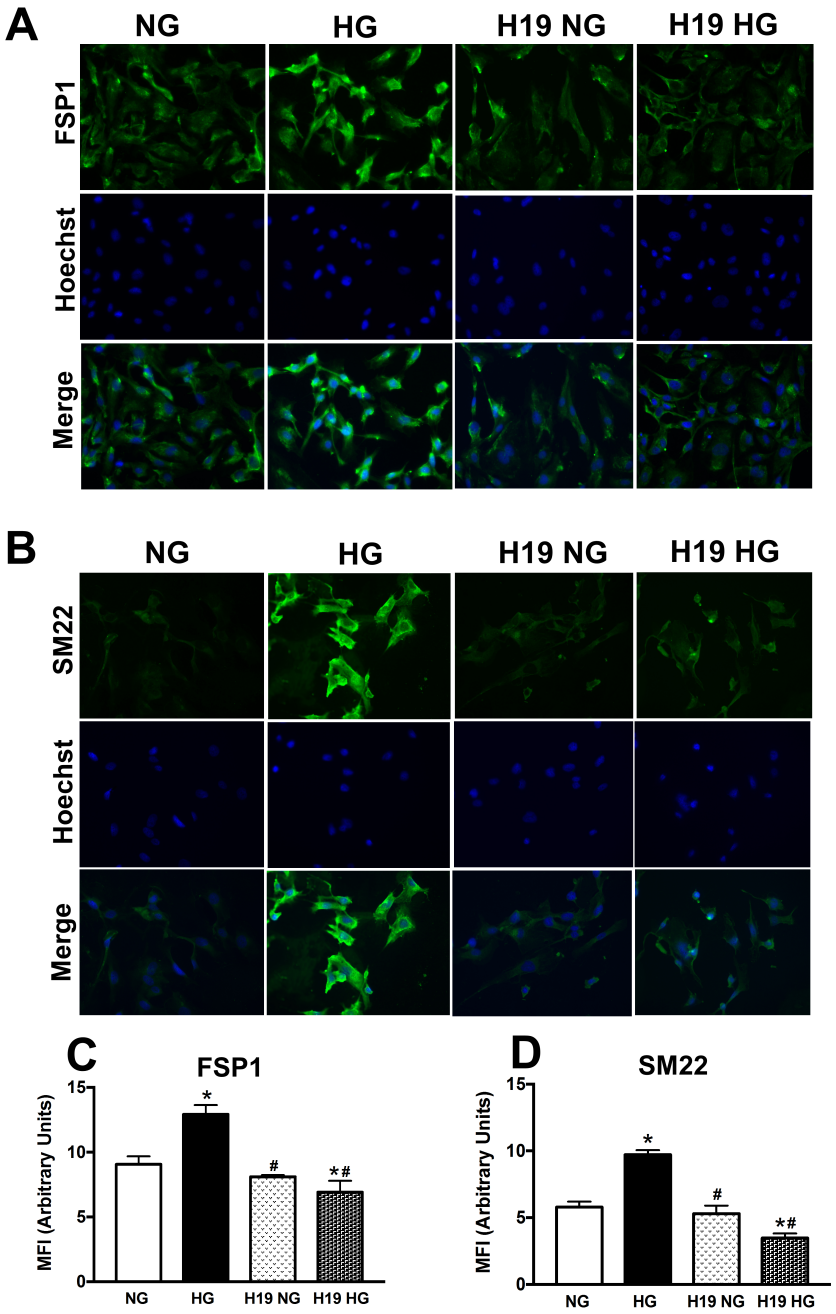
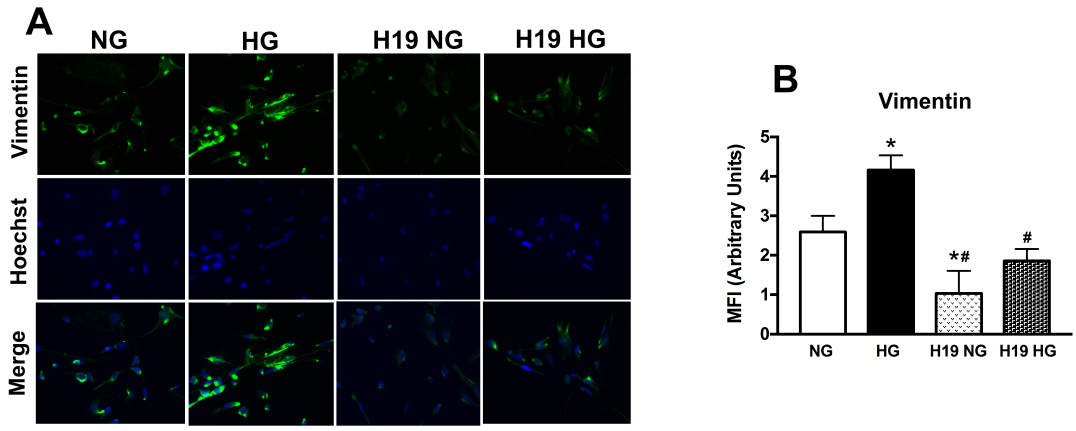


Figure 4.4: Effect of H19 on mesenchymal markers at protein level. Immunofluorescence stains of human retinal endothelial cells (HRECs) showed increased expression of mesenchymal markers FSP1 (*A*) and SM22 (*B*) in 25mM glucose (HG) treated cells versus 5mM glucose (NG). H19 overexpression downregulated these markers in HRECs (H19 NG and H19 HG). Quantification data using ImageJ confirmed these findings (*C* and *D*). (MFI- mean fluorescence intensity. $*,^{\#}$ $p< 0.05$ versus empty vector treated with NG (NG) and empty vector treated with HG (HG) respectively, n=6 or more/group).



Supplementary Figure: Effect of H19 on mesenchymal marker vimentin protein. Immunofluorescence stains showed increased expression of vimentin (*A*) with 25mM glucose (HG) treatment versus 5mM glucose (NG) in human retinal endothelial cells (HRECs). H19 overexpression downregulated vimentin in HRECs (H19 NG and H19 HG). Quantification data using ImageJ confirmed these findings (*B*). (MFI- mean fluorescence intensity. $*,^{\#}$ $p< 0.05$ versus empty vector treated with NG (NG) and empty vector treated with HG (HG) respectively, n=6 or more/group).

Table 4.1: Body weight, blood glucose levels and urine volume of all treatment groups.

Mouse genotype	n	Body weight (g)	Body glucose (mmol/L)	Urine volume (ml/24hour)
WT-C	6	26 ±0.816	10 ±0.292	<1
WT-D	6	22 ±1.92*	34 ±0.447*	38 ±2.887*
H19 KO-C	8	30 ±1.73*,#	10 ±0.873#	<1#
H19 KO-D	7	21 ±1.7*	31 ±1.966*,#	44±2.74*

$*,^{\#}$ $p< 0.05$ versus WT-C or WT-D respectively

Supplementary Table: Body weight, blood glucose levels and urine volume in various groups of mice following 2-month diabetes. (WT-C: wildtype controls, WT-D: wildtype diabetics, H19KO-C: $H19^{\Delta exI}$ controls, and H19KO-D: $H19^{\Delta exI}$ diabetic animals. $*,^{\#}$ $p< 0.05$ versus WT-C or WT-D respectively, n=6 or more/group).

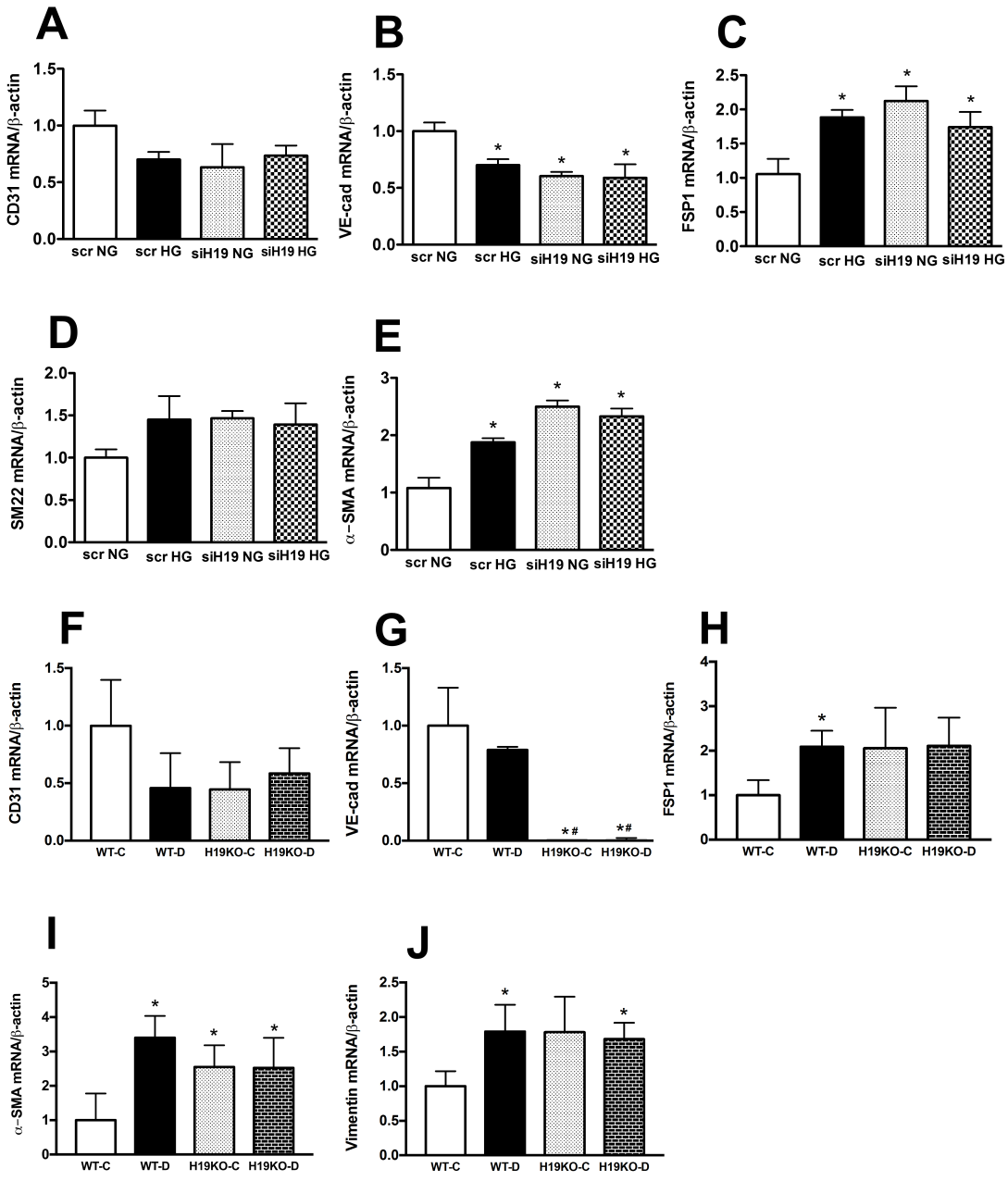


Figure 4.5: H19 siRNA transfection lead to a glucose-like EndMT phenotype. H19 siRNA transfection of human retinal endothelial cells reduced endothelial markers CD 31 (*A*) and VE cadherin (VE-cad) (*B*) and upregulated mesenchymal markers FSP1 (*C*) and SM22 (*D*) and α-SMA (*E*) in 5mM glucose (NG). No further enhancement of EndMT was seen when similar transfection was carried out in 25mM glucose (HG). Similarly, $H19^{\Delta exI}$ animals (H19KO-C) demonstrated a diabetes-like EndMT phenotype in the retina

(F-J). No further exaggeration of EndMT was seen when diabetes was induced in the KO animals. [mRNA data (Mean±SE) expressed as ratio to β-actin, normalized to control. *p< 0.05 versus scrambled NG (scrNG) or wild-type control (WT-C), WT-D=wildtype diabetic, H19KO-D= $H19^{\Delta exI}$ diabetic, n=6 or more/group].

4.3.3 H19 regulates transforming growth factor β to suppress EndMT

As TGF-β is an important regulator of EndMT, we examined the role of H19 in regulating TGF-β1 levels following glucose treatment (25). H19 overexpression had an inhibitory effect on TGF-β1 and prevented glucose-mediated upregulation of TGF-β1 at both mRNA (Fig 4.6A) and protein levels (Fig 4.6B and C). The effect of H19 was not influenced by the varying levels of glucose. miR200b regulates EndMT through TGF-β1 (25). We confirmed this with miR200b mimic transfection followed by HG incubation (Fig 4.6A). We also wanted to understand if the regulation of TGF-β1 by H19 is dependent on miR200b levels. We did so through a rescue experiment, by suppressing miR200b expression using miR200b antagomirs in NG environment and then overexpressed H19. The TGF-β1 levels continued to be downregulated in these samples, further indicating possible H19 action independent of miR200b (Fig 4.6A).

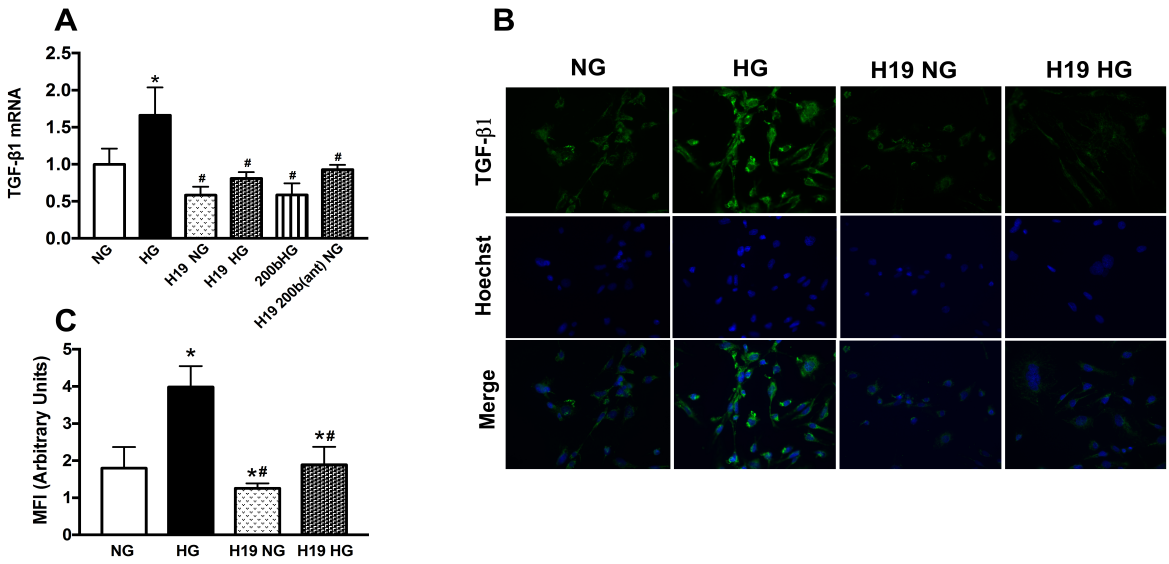


Figure 4.6: H19 prevents glucose-mediated upregulation of TGF-β1. Twenty five mM glucose (HG) treatment increased mRNA expression (Mean±SE) of TGF-β1 compared to 5mM glucose (NG) (*A*). H19 overexpression reduced TGF-β1 both in NG and HG environments (H19 NG and H19 HG). Similar reduction was seen following miR200b mimic transfection (miR200b HG). miR200b antagomirs [H19 200b(ant) NG] failed to rescue H19 overexpression-induced TGF-β1 downregulation. Exposure to HG elevated TGF-β1 protein expression in HRECs (*B*). H19 overexpression downregulated the increase in TGF-β1 protein, on exposure to varying glucose levels (H19 NG and H19 HG). Quantification data using ImageJ confirmed these findings (*C*). (MFI- mean fluorescence intensity. $*,^{\#}$ $p< 0.05$ versus empty vector treated with NG (NG) and empty vector treated with HG (HG) respectively, n=6 or more/group).

4.3.4 H19 regulates TGF-β1mediated EndMT through Smad-independent MAP-ERK 1/2 pathway

We further attempted to identify the signaling pathway affected as a result of H19's regulation of EndMT. As we had several potential candidates for TGF-β signaling encompassing both Smad-dependent and Smad-independent pathways we decided to utilize Luminex technology. This is a cutting-edge technology that utilizes multiplex assays that will facilitate simultaneous relative quantification of several phosphorylated and total TGF-β signaling proteins from cell lysates. HG caused increased production or

phosphorylation of TGFβRII (total), pSmad 2, pSmad 3 and Smad 4 (total), pAkt and pERK1/2 proteins (Fig 4.7A-F). The HRECs were also transfected with h19-pcDNA 3.1 vector and treated with NG (H19 NG) and HG (H19 HG). TGFβRII (total) was significantly reduced following H19 overexpression (Fig 4.7A). Basal levels of SMAD proteins (pSmad 2, pSmad 3, Smad 4) and pAkt were increased following H19 overexpression (Fig 4.7B-E). However, levels of these molecules in the HG environment appeared to be unaltered following H19 overexpression. Interestingly, pERK1/2 levels were significantly downregulated in samples overexpressing H19 irrespective of the influence of glucose in these groups (Fig 4.7F). This led us to conclude that H19 suppresses glucose-mediated EndMT through regulation of MAP-ERK 1/2 pathway of TGF-β signaling using a Smad-independent route.

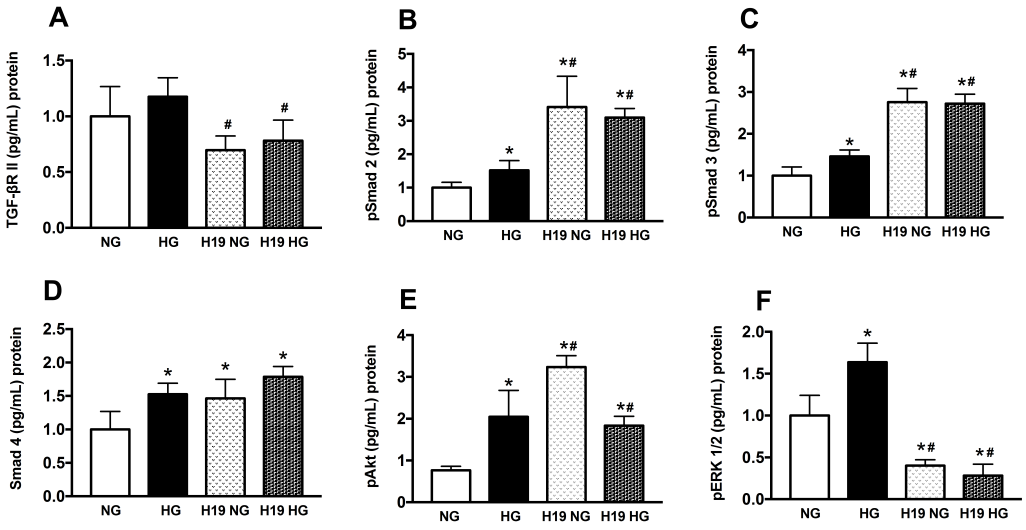


Figure 4.7: H19 regulates EndMT through Smad-independent MAP-ERK 1/2 pathway. Luminex assay panel for TGF-β signaling was used to quantify several proteins; TGFβRII (total), pSmad 2, pSmad 3, Smad 4 (total), pAkt and pERK 1/2. These proteins were upregulated in human retinal endothelial cells when exposed to 25mM glucose (HG) treatment compared to 5mM glucose (NG) (*A-F)*. H19 overexpression downregulated the increase in TGFβRII (total), both in NG and HG (H19 NG and H19 HG) (*A*). Increased H19 levels did not reduce pSmad 2 (*B*), pSmad 3 (*C*), Smad 4 (total)

(*D*) and pAkt (*E*) protein levels. pERK 1/2 protein levels were significantly downregulated (*F*) as a result of H19 overexpression in HRECs treated with NG and HG. (*,# $p < 0.05$ versus empty vector treated with NG (NG) and empty vector treated with HG (HG) respectively, n=6 or more/group).

4.4 Discussion

Our current study deciphers the role of lncRNA H19 in regulating diabetes-induced EndMT changes in HRECs. We report that H19 overexpression can prevent glucose-induced transition of endothelial cells from endothelial to mesenchymal phenotype through its regulation of TGF-β1 and subsequent TGF-β signaling though blockade of MAP-ERK 1/2 pathway. To demonstrate these changes, we conducted experiments using H19 over-expression vector and H19 silencing siRNA in HRECs in NG and HG environments. We also found that such regulatory action of H19 occurs independent of miR200b, a miRNA that we have previously shown to play a regulatory role in diabetes-induced EndMT (25,26). Our animal experiments further confirmed the role of H19 in retinal EndMT. Our findings from the human vitreous specimens showed a hyperglycemia-induced downregulation of H19 in PDR compared to non-diabetics.

lncRNAs are known to play important roles in controlling cancer metastasis through regulation of epithelial-mesenchymal transition (EMT) by targeting multiple signaling pathways including TGF-β signaling (33). However, their roles in EndMT, are relatively less studied. The lncRNA MALAT1, a well-known regulator of DR can modulate TGF-β1-induced EndMT in isolated endothelial progenitor cells (EPCs) by negatively regulating miR145 (34). A recent study revealed that hypoxia-induced EndMT in human umbilical vein endothelial cells was promoted by the lncRNA GATA6-AS, through changes in histone methylation (35). Nevertheless, the role of lncRNAs in regulating hyperglycemia-induced EndMT remain unclear. We have reported for the first time, a regulatory role of lncRNA H19 in glucose-mediated EndMT through its inhibition of TGF-β1 and its subsequent signaling pathway. H19 has been well-studied in tumor biology, but its role in diabetic complications is not certain. Our data is similar to a

previous study reporting downregulation of H19 in cardiac tissues of diabetic rats (27). Our FISH analyses revealed that although in normal cells H19 distributed across the cell cytoplasm and nucleus, HG treatment reduced its expression to few scattered spots predominantly in the cytoplasm (24). The exact reasons for such sub-cellular changes observed need further exploration. Moreover, during EMT in certain cancers, TGF-β is an important inducer of H19 through activation of PI3K/Akt signaling pathway (36). However, in our study, during EndMT in HRECs, we found a suppressive effect of H19 on TGF-β1 and TGF-βIIR. Overexpression of H19 significantly reduced TGF-β1 levels resulting in prevention of EndMT in spite of the presence of glucose. Such findings suggest alternative epigenetic regulatory mechanisms of H19 that require further studies.

Hyperglycemia is the central event leading to micro- and macrovascular pathologies in diabetes (37,38). ECs lining the blood vessels are exposed to circulating blood glucose and is the primary cell type to be affected by hyperglycemia insults (38,39). Endothelial cell dysfunction ensues as ECs lose their quiescence, elude their normal function and acquire new phenotypes (39). Increasing evidence suggests that ECs can then acquire a mesenchymal phenotype and start expressing markers characteristic of myofibroblasts, contributing to the advancement of sclerotic diseases (40,41). In patients with PDR, fibrotic and inflammatory alterations in the epiretinal membranes are characterized by α-SMA-expressing myofibroblasts and inflammatory cells in the stromal compartment (42). We and others have reported the development of hyperglycemia-induced EndMT during various diabetic complications (25,26,43). In the present study, this phenomenon was reproduced in HRECs as we observed decreases in expressions of endothelial markers, CD31 and VE-cadherin, accompanied by elevation in the expression of several mesenchymal markers, FSP1, SM22, α-SMA and vimentin, depicting a phenotype characteristic of EndMT. Furthermore, our animal data demonstrated similar changes in the retina of chronically diabetic animals, which are further pronounced following H19 knockdown. In addition, vitreous from PDR subjects showed H19 downregulation.

EndMT was originally identified as an embryonic mechanism for cardiac valve development from ECs of atrioventricular canal (44). It is also involved in angiogenic

sprouting of postnatal retina, causing mesenchymal cells to be formed at the tips of vascular sprouts mediated by VEGF-A (45). Recently, EndMT has emerged as an important contributor in various pathologies, such as organ fibrosis involving heart, kidney and retina (14,25,26). EndMT is also suggested to influence development of fibroblasts and myofibroblasts that are responsible for the progression of fibrosis and advancement of PDR (41). As the involvement of EndMT in several of these pathologies increases, a number of EndMT-inducing stimuli have been identified, including growth factors, cell-cell interactions, shear stress, environmental factors like hypoxia and hyperglycemia and recently epigenetics (46,47). There have been several reports on epigenetic modifications contributing to EndMT, which is a stable cell-phenotype alteration (47). These include aberrant promoter methylation (47), histone deacetylase (HDAC3) mediated repression (49) and several miRNAs as key regulators of EndMT by targeting the signaling pathways (49). Our previous work has also reported miR200b as a negative regulator of diabetes-induced EndMT in retinal and cardiac cells and tissues (25,26). Data from this study however suggests that H19 may influence EndMT independent of miR200b. Such pathway needs further characterization.

TGF-β is described as the major regulator of EndMT and harbors pro-fibrotic properties by inducing and propagating resident fibroblasts (50). As TGF-β1 can mediate both Smad-dependent and Smad-independent pathways, we investigated whether these pathways are regulated by H19. Our previous work on epigenetic regulation of EndMT in DR describes a Smad-dependent regulation of TGF-β1 mediated by miR200b and histone acetyltransferase, p300 (25). We employed Luminex assay that quantified phosphorylated proteins of Smad-dependent (pSmad 2 and pSmad 3) and Smad-independent (pAkt and pERK 1/2) pathways. The analysis showed no effects on Smad proteins and pAkt levels following overexpression of H19. Conversely, H19 regulated the expression of phosphorylated ERK 1/2 proteins, suggesting that H19 mediated suppression of EndMT occurs through Smad-independent pathway. However, we recognize the need for extended study into the specific pathway affecting the mechanisms involved in such epigenetic regulation.

In conclusion, our current data describes a novel role of lncRNA H19 in regulating EndMT induced by elevated glucose levels. H19 facilitates this regulation by suppressing TGF-β1 and its signaling pathways by repressing MAP-ERK 1/2 signaling pathway. We recognize that additional mechanisms may be in place regulating such pathways, which warrants further exploration. Nevertheless, our findings open up possibilities of RNA-based therapies targeting a fundamental glucose-mediated cellular phenotypic alteration.

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Chapter 5

5 Discussion and future directions

5.1 Discussion

In the research, we examined two specific lncRNAs, ANRIL and H19 and investigated their influential role in regulating key factors altered during DC. Following initial identification of ANRIL and H19 alterations by an array, we demonstrated through a series of experiments that ANRIL is upregulated on exposure to high glucose levels. ANRIL regulates glucose-mediated VEGF expression in HRECs and STZ-induced mouse models. This study of ANRIL in DR was also extended to other diabetic complications, namely DN and DCM. Here we observed that along with VEGF, ANRIL also influenced the expression of ECM proteins FN and Col1α4. This regulation is at least in part, driven by ANRIL's interaction with PRC2 complex proteins and histone acetylator, p300. These findings were revealed using ANRIL silencing with siRNA in HRECs exposed to NG and HG environments. Our results were validated *in vivo*, using ANRIL knockout animals induced with STZ.

Similarly, we deciphered the role of H19 in regulating important cellular phenotypic changes in DR. Exposure to high glucose environment downregulated the expression of H19 in HRECs and retinal samples from diabetic animals. Furthermore, evaluation of human vitreous established that similar changes occur in human DR. H19 overexpression was able to prevent glucose-induced transition of ECs from endothelial to mesenchymal phenotype (EndMT). This phenomenon, well known to be under the regulation of TGF-β1 was regulated by H19's action on TGF-β1 and subsequent TGF-β signaling though blockade of MAP-ERK 1/2 pathway. We demonstrated these alterations through experiments conducted using H19 over-expression vector and H19 silencing siRNA in HRECs during NG and HG treatments. Additionally, we observed this action of H19 can occur independent of miR200b, an important regulator of glucose-induced EndMT (1).

Our animal experiments further established the role of H19 in retinal EndMT during diabetes.

It has been well established that ECs are primary targets of glucose-induced tissue damage (2). In ECs, multiple glucose-induced biochemical alterations coverage onto the cell nucleus and changes gene transcription ultimately producing increased amounts of cellular macromolecules. It is important to have a better understanding of such cellular synthetic processes.

Multiple studies have previously been carried out examining specific genetic abnormalities as causative factors for the development of chronic diabetic complications. However, such studies have never been able to provide definitive answers (3). On the other hand, metabolic memory phenomena and legacy effects of diabetes are well established (4,5). Hence investigations in epigenetics are essential. Epigenetic mechanisms have several components including DNA and histone methylation, histone acetylation ubiquitination as well as non-coding RNA (ncRNA) alterations. ncRNAs include short ncRNAs such as miRNAs and other types such as long ncRNAs (lncRNAs) that can regulate chromosomal function and gene expression (6). Among these, the role of lncRNAs are understudied. They have an extensive role in gene regulation and work in various ways to regulate expression of targets. Their mechanism of action includes post-transcriptional gene regulation by controlling functions like protein synthesis, RNA maturation and transport. They have also been implicated in transcriptional interference, induce chromatin remodeling, generate endo-siRNAs, modulate protein activity, alter protein localization, function as structural components or act as sponges for miRNAs (7). Based on our research of the two lncRNAs, it appears that ANRIL acts through PRC2 complex and p300 in regulating VEGF and ECM proteins, while H19 regulates TGF-β1 signaling to prevent EndMT transition. Both these regulators function as modulators of protein activity.

High-throughput deep sequencing approaches have recently enabled transcriptomic sequencing with greater accuracy (8). This has helped us to identify various different

types of ncRNAs and quantify their expression in different tissues. Moreover, ncRNAs have been demonstrated to be key players in gene regulation. An increasing body of evidence points to the notion that ncRNAs interact with each other and further are co-regulated during various pathological conditions (8-10).

Several pathways can regulate the targets under investigation. We have previously shown that VEGF is regulated by miR200b, histone acetylator p300 and PRC2 complex (11,12). Our current research has demonstrated another layer of regulation of VEGF in this pathway. We have shown that ANRIL through interaction with miR200b, p300 and PRC2 complex mediate VEGF production. These data for the first time demonstrated such role of ANRIL in the context of diabetic complications. However, in other systems ANRIL has been shown to specifically bind to PRC proteins and regulate histone modification (13). Similar regulations of ECM proteins FN and Col1α4 through ANRIL were demonstrated in this research in kidney and heart tissues in diabetes. It is of further interest to note that some of these actions of ANRIL were also mediated by p300. We have previously demonstrated that p300 regulates a large number of transcripts which are induced by glucose (14). Hence p300's regulation by ANRIL may at least in part provide explanation for regulation of multiple seemingly diverse transcripts that are altered by glucose.

Similarly, for the first time we demonstrated the role of H19 in DR. H19 has been previously shown to be downregulated in neonatal cardiomyocytes exposed to high glucose and myocardium of STZ-induced diabetic (15). In tumor models like hepatocellular carcinoma, H19 has been shown to alter miR-200 pathway by increasing histone acetylation upstream of this miRNA; contributing to the reversal of epithelial-mesenchymal transition (16). Similar to ANRIL our finding with respect to H19 further identified another piece of this puzzle.

As mentioned earlier, in our work we have observed ANRIL's interaction with miR200b. However, ANRIL can also be a regulator of H19. This was evident in our *in vivo* and *in vitro* experiments where nullifying ANRIL expression led to recovery from glucose and

diabetes-induced reduction of H19 levels. Furthermore, H19 knockdown in low glucose environment lead to a glucose-like effect as evidenced by a tendency towards increased ANRIL expression. Retinal tissues of H19KO animals further showed slight reduction of ANRIL levels. Interestingly, in additional cell culture experiments, H19 overexpression led to an inclination towards reduced ANRIL expression (Figure in Appendix I). Hence a regulatory relationship exists between these two lncRNAs. Additional investigations are needed to further decipher the exact mode of interaction and subsequent effects.

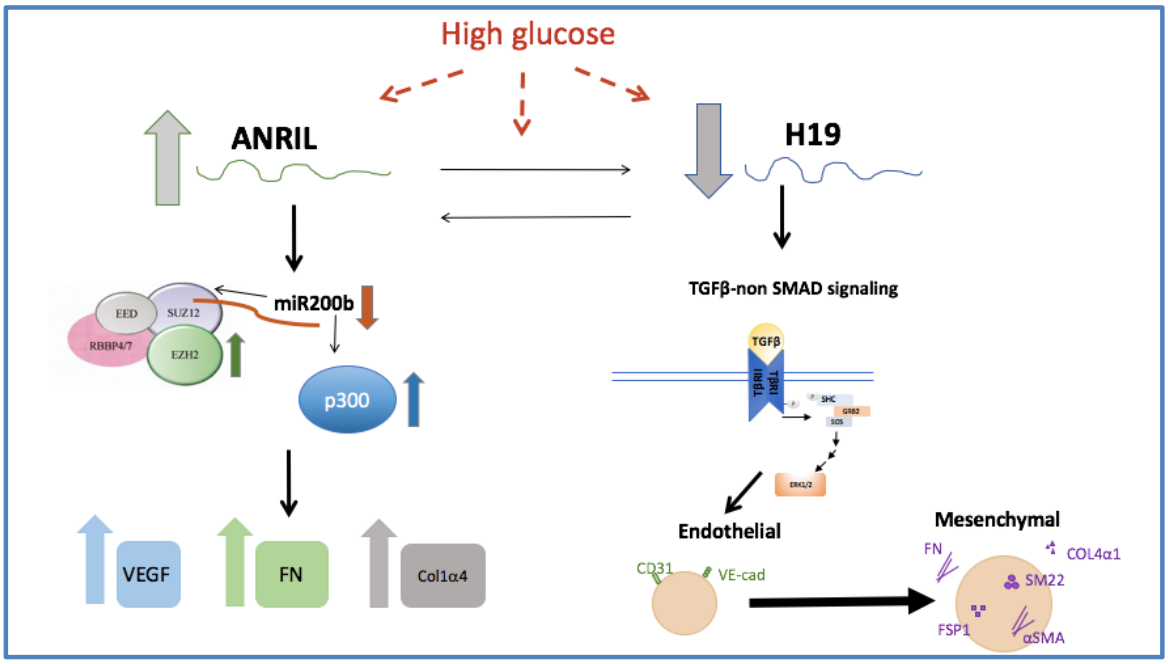

Figure 5.1: Diagrammatical representation of the key findings of this study

A recent evaluation of literature by Schwartz and colleagues suggests that a key element of all vascular diseases could be unresolved vascular remodeling (17). It raises the prospect of restoration of normal modeling as an approach to treat multiple pathological states. In diabetes, vascular remodeling is hypertrophic and inward as manifested by increase in the outer lumen diameter with accompanying decreased inner lumen diameter. Such remodeling in diabetes is driven by endothelial dysfunction and complex signaling pathways, leading to structural alterations of the arteries and tissue hypoperfusion and hypoxia (18-21). These events are attributed to the adverse effects of exposure to high glucose and oxidative stress on the vascular system. The remodeling vasculature in

contrast to stable blood vessels are associated with VEGF secretion by cells of ischemic tissues to instigate angiogenesis accompanied by metalloprotease secretion, ECM degradation accompanied by assemblage of temporary matrices and changes to key EC signaling and gene expression pathways (17,22). These events are attributed to the adverse effects of exposure to high glucose and oxidative stress on the vascular system. Oxidative stress due to increased production of ROS disrupts numerous mitochondrial and other intracellular pathways, which are associated with increase oxidants produced and reduction in antioxidant defenses (23). The disrupted normal function of the mitochondria results in blockade of insulin signaling and activation of NF-κB, resulting in increased vascular inflammation (24,25). During these inflammatory conditions, mediators like IL-1β, TNF-α and NF-κB contribute to endothelial dysfunction by activating ECs and converting them to mesenchymal-like cells through EndMT (26). EndMT has emerged as a participant in process of tissue fibrosis in various diseases including DC and mostly induced by TGFβ signaling pathway. The transformed ECs increase levels of altered ECM and have higher vascular permeability thereby compelling further inflammation (17). These incidents create a positive feedback loop- EndMT triggers inflammation and vice versa (17). These developments mediated by TGF-β further promote fibrosis, which is generally irreversible. Such feedback loops hinder restoration of a normal vasculature and forces endothelium to maintain its remodeled state, leading to disease progression (17). Thus, it is important to break the feedback loop to restore a normal model state as an effective approach to treat multiple vascular diseases. Hence an understanding of regulation of the production of cell damaging molecules at every level is essential. The data generated in this research further validates this hypothesis and contributes to better understanding of this process. We have described ANRIL's regulation of VEGF and ECM proteins as well as H19 playing a role in EndMT as examples of multiple targets regulated by lncRNAs under pathological high glucose conditions. However, additional large-scale and long-term studies are needed to further expand current findings.

5.2 Conclusion

Today more diabetics face morbidity and mortality due to chronic complications. Hence there is a need for specific therapeutic approaches for diabetic complications. This can only be achieved by better understanding of the pathogenic mechanisms. In this study, we have shown novel roles of lncRNAs, ANRIL and H19 in regulating key molecules (VEGF, ECM proteins FN and Col1α4) and cellular phenotypes (endothelial to mesenchymal transition) altered during diabetic complications. This understanding can contribute to further development of RNA-based or other epigenetic mechanisms-based therapies for chronic diabetic complications.

5.3 Limitations of the study

Although the study has achieved its aims, we understand that there are no studies without limitations. This study has predominately focused on short-term T1D diabetes and we have formulated our work with ECs and animal models around short-term diabetes (48 hours and 2 months, respectively). We are yet to decipher the roles of these lncRNAs under conditions of long-term T1D. Furthermore, we haven't looked at the role of these lncRNAs in T2D, although genomic-wide study of ANRIL has identified it to be a hotspot for T2D (15). We were unable to extent our study past T1D due to time limitations of this thesis and feel that it is one of the first topics to be addressed in future studies.

Furthermore, some specific mechanisms need additional investigations. In our study, we have shown that ANRIL binds to both EZH2 (PRC2 complex) as well as p300 and positively regulates these proteins. However, p300 is histone acetylator and is known to activate gene expression, whereas EZH2 is a histone methyltransferase predominantly know to act as a negative regulator of gene expression. Therefore, ANRIL's regulation on EZH2 may additionally involve other factors and pathways, eventual leading to activation of gene expression. Hence the pathway of ANRIL's regulation of EZH2 needs to be further exploration.

We have employed an H19 knockout (KO) mouse model for elucidation of H19's role in DR. We acknowledge that use of a knockout model for an lncRNA that is downregulated under high glucose conditions is not the best approach. However, STZ- induced H19KO mouse models were helpful in understanding the effect of further reduction of H19 under high glucose. H19 overexpressing mouse models are rare and usually involve ectopic expression of H19 in nude mice, mostly suitable to study tumorigenic potentials of cells like in heptocellular carcinoma (27, 28). However, the transgenic mice that overexpress H19 shows several phenotypic changes eg. smaller birth weight and embryonic growth retardation at E16.5. This was due to H19's regulation of a network of imprinted genes (IGN) including Igf2 (Insulin growth factor 2). Igf2 is known to share with H19, important regulatory elements important for imprinting (29). Hence development of H19 overexpressing mouse models continues to be a challenge and at the current stage such animals were deemed unsuitable for our study.

Another limitation would be possible off-targets effects of lncRNA knockouts in the mouse models. Although, we were unable to observe any such effects in the various tissue sections of the animals, but the possibility of small off target effects that were not obvious can't be completely excluded.

5.4 Future direction

Based on the limitations mentioned above we plan to extent our study to additional longer time points and in other types of diabetes. The mechanism of action of the lncRNAs described here are part of the big picture, many segments of which needs to be deciphered through careful and well-designed experimental studies. We also acknowledge that there could be other factors, such as other lncRNAs playing roles in these regulatory mechanisms that need further elucidation. They will eventually play specific roles in together combatting chronic diabetic complications.

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Appendix 1

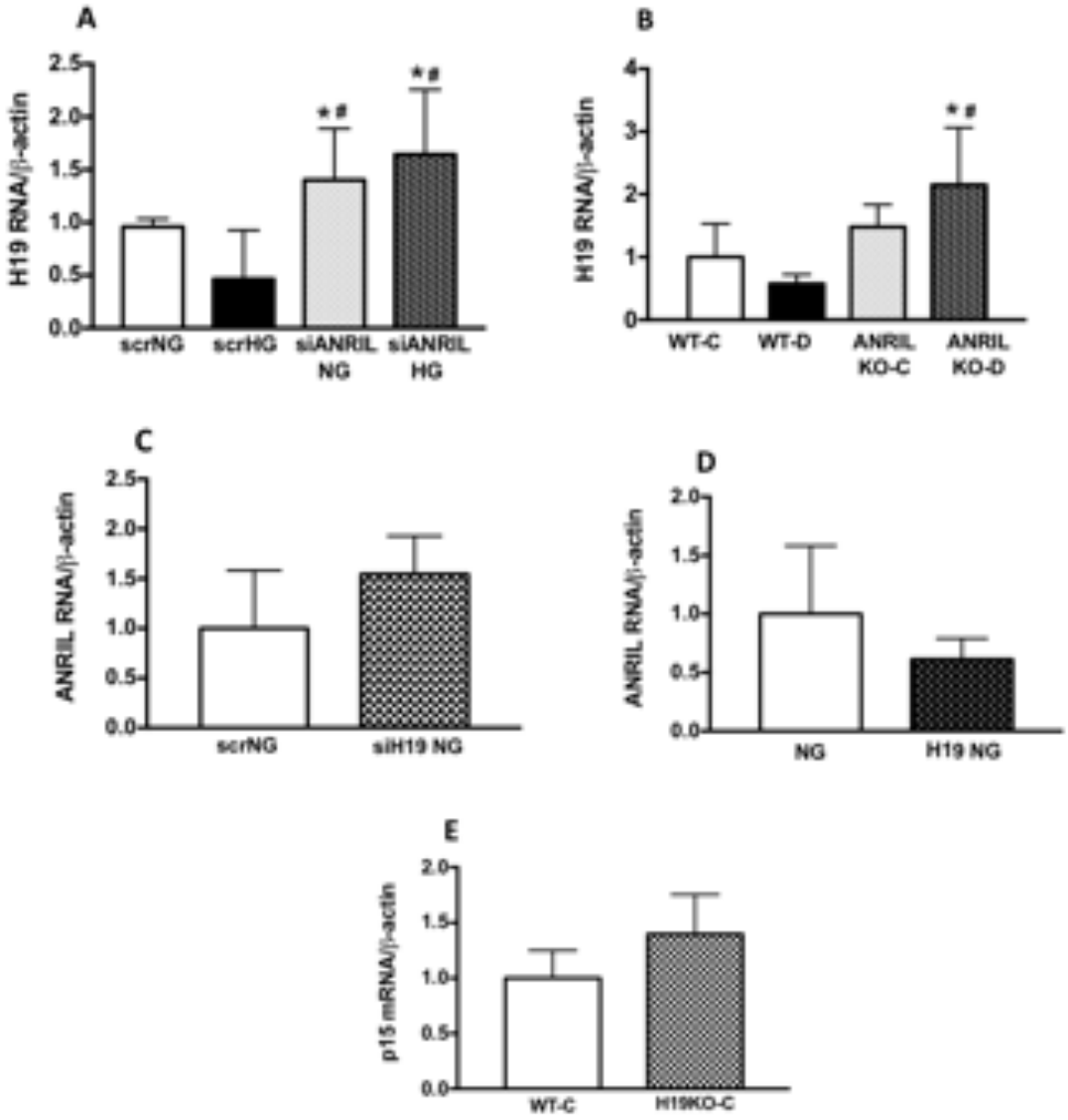

Figure: Interactions of lncRNAs, ANRIL and H19. RNA expressions analysis in (A) ECs transfected with ANRIL siRNA (siANRIL) and the (B) kidney tissues of ANRILKO mice (ANRILKO) showed upregulation of H19 compared to scrambled siRNA transfection and wildtype (WT) mice. (C) ANRIL RNA levels showed a tendency towards increase siH19 transfected ECs (siH19) in a normal glucose environment. (D) H19 overexpression in ECs (H19) showed a tendency towards reduction of basal ANRIL expression. (E) p15 mRNA (surrogate marker of ANRIL) was similarly showed a tendency towards increase in H19KO animals (H19KO) versus WT-C. (Scr=scrambled RNA, NG=5mMglucose, HG=25mM glucose, C= nondiabetic control, D= diabetic). RNA expressions are presented as a ratio of β-actin, normalized to NG/WT-C. (*$p < 0.05$ versus ScrNG/WT-C, #$p < 0.05$ versus ScrHG/WT-D, n=5 or more per group).

Appendix 2

Subrata Chakrabarti - FW: eSirius Notification - New Animal Use Protocol is APPROVED2010-001::5

From: AUSPC
To: "'Subrata Chakrabarti'"
Date: 2016/02/08 3:35 PM
Subject: FW: eSirius Notification - New Animal Use Protocol is APPROVED2010-001::5
Attachments: image001.gif; image002.png; image004.png; image005.png

From: eSiriusWebServer
Sent: May 21, 2014 3:40 PM
To:
Cc:
Subject: eSirius Notification - New Animal Use Protocol is APPROVED2010-001::5

Western

AUP Number: 2010-001
PI Name: Chakrabarti, Subrata
AUP Title: Pathogenesis of Diabetic Retinopathy
Approval Date: 05/21/2014

Official Notice of Animal Use Subcommittee (AUS) Approval: Your new Animal Use Protocol (AUP) entitled "Pathogenesis of Diabetic Retinopathy" has been APPROVED by the Animal Use Subcommittee of the University Council on Animal Care. This approval, although valid for four years, and is subject to annual Protocol Renewal.2010-001::5

1. This AUP number must be indicated when ordering animals for this project.
2. Animals for other projects may not be ordered under this AUP number.
3. Purchases of animals other than through this system must be cleared through the ACVS office. Health certificates will be required.

The holder of this Animal Use Protocol is responsible to ensure that all associated safety components (biosafety, radiation safety, general laboratory safety) comply with institutional safety standards and have received all necessary approvals. Please consult directly with your institutional safety officers.

Submitted by: Copeman, Laura
on behalf of the Animal Use Subcommittee
University Council on Animal Care

Subrata Chakrabarti - FW: eSirius Notification - New Animal Use Protocol is APPROVED2015-091::1

From: AUSPC
To: "'Subrata Chakrabarti'"
Date: 2016/07/15 9:54 AM
Subject: FW: eSirius Notification - New Animal Use Protocol is APPROVED2015-091::1

From: eSiriusWebServer
Sent: February 16, 2016 4:11 PM
To:
Cc:
Subject: eSirius Notification - New Animal Use Protocol is APPROVED2015-091::1

Western

AUP Number: 2015-091
PI Name: Chakrabarti, Subrata
AUP Title: Vasoactive And Cardioactive Factors In Diabetic Cardiomyopathy

Approval Date: 02/16/2016

Official Notice of Animal Use Subcommittee (AUS) Approval: Your new Animal Use Protocol (AUP) entitled "Vasoactive And Cardioactive Factors In Diabetic Cardiomyopathy

" has been APPROVED by the Animal Use Subcommittee of the University Council on Animal Care. This approval, although valid for four years, and is subject to annual Protocol Renewal.2015-091::1

1. This AUP number must be indicated when ordering animals for this project.
2. Animals for other projects may not be ordered under this AUP number.
3. Purchases of animals other than through this system must be cleared through the ACVS office. Health certificates will be required.

The holder of this Animal Use Protocol is responsible to ensure that all associated safety components (biosafety, radiation safety, general laboratory safety) comply with institutional safety standards and have received all necessary approvals. Please consult directly with your institutional safety officers.

Submitted by: Copeman, Laura
on behalf of the Animal Use Subcommittee
University Council on Animal Care

Appendix 3

Western Research

Research Ethics

Western University Health Science Research Ethics Board
HSREB Annual Continuing Ethics Approval Notice

Date: May 12, 2017
Principal Investigator: Dr. Subatra Chakrabarti
Department & Institution: Schulich School of Medicine and Dentistry\Microbiology & Immunology,Western University

Review Type: Delegated
HSREB File Number: 108005
Study Title: Pathogenesis of diabetic retinopathy
Sponsor: Canadian Diabetes Association

HSREB Renewal Due Date & HSREB Expiry Date:
Renewal Due -2018/05/31
Expiry Date -2018/06/07

The Western University Health Science Research Ethics Board (HSREB) has reviewed the Continuing Ethics Review (CER) Form and is re-issuing approval for the above noted study.

The Western University HSREB operates in compliance with the Tri-Council Policy Statement Ethical Conduct for Research Involving Humans (TCPS2), the International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use Guideline for Good Clinical Practice (ICH E6 R1), the Ontario Freedom of Information and Protection of Privacy Act (FIPPA, 1990), the Ontario Personal Health Information Protection Act (PHIPA, 2004), Part 4 of the Natural Health Product Regulations, Health Canada Medical Device Regulations and Part C, Division 5, of the Food and Drug Regulations of Health Canada.

Members of the HSREB who are named as Investigators in research studies do not participate in discussions related to, nor vote on such studies when they are presented to the REB.

The HSREB is registered with the U.S. Department of Health & Human Services under the IRB registration number IRB 00000940.

Ethics [illegible] on behalf of Dr. Joseph Gilbert, HSREB Chair
EO: Erik [illegible] ile ___ Grace Kelly ___ Katelyn Harris___ Nicola Morphet___ Karen Gopaul ___

Curriculum Vitae

Name: Anu Alice Thomas

Post-secondary Education and Degrees:

(Dr. GRD College) Bharathiyar University
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2005-2007 M.Sc (Biochemistry)

Western University
London, Ontario, Canada
2009-2011 MESc (Biochemical Engineering)

Western University
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2013-2018 Ph.D. Pathology & Laboratory Medicine

Honours and Awards:

- Travel award from Diabetes Canada to attend 2017 Diabetes Canada/CSEM Professional Conference and Annual Meetings, Edmonton AB.

- Dr. P.C. Raju & Jyoti Shah Graduate Education Prize- Best Publication 2017, Dept. Pathology & Laboratory Medicine, Western University.

- Best Basic/Clinical Science Collaborative Poster Presentation Award – Pathology and Laboratory medicine research Day April 2016. Dept. Pathology & Laboratory Medicine, Western University.

- Dutkevich Memorial Foundation travel award, Dept. Pathology & Laboratory Science, Western University to attend the following conferences:
  - Diabetes Canada/CSEM Professional Conference-Edmonton, November 2017
  - CDA/CSEM Professional Conference-Ottawa, October 2016
  - CDA/CSEM Professional Conference Winnipeg October 2014

- Alzheimer's Society Research Program scholarship 2013-2016 (declined)

Research Support

- Western Graduate Research Scholarship (WGRS)
  September 2013-August 2017
  September 2011-April 2013
  May 2009-April 2011

Related Work Experience

Teaching Assistant, September 2009- April 2011
(Biochem Eng. & Organic Chemistry)
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2009-2011

Publications

- **Thomas AA**, Biswas S, Gonder J, Chakrabarti S. LncRNA reverses Endothelial-Mesenchymal transition in Diabetic Retinopathy (Submitted).
- Biswas S*, **Thomas AA***, Chakrabarti S. LncRNAs in diabetic cardiomyopathy: proverbial genomic "junk" or key epigenetic regulators in fibrosis? Front Cardiovasc Med, 2018. 5: 1-13. (* equal contribution).
- Biswas S, Feng B, **Thomas AA**, Chen S, Aref-Eshghi E, Sadikovic B, Chakrbarti S. Endothelin-1 regulation is entangled in a complex web of epigenetic mechanism in diabetes. Physiol Res, 2018
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Conferences

- Thomas AA, Fei L, Chakrabarti S. H19 regulates glucose-induced EndMT in chronic diabetic complications. Poster at 2017 Diabetes Canada/CSEM Professional Conference, Edmonton, AB.
- Thomas AA, Feng B, Chakrabarti S. Alteration of Long noncoding RNA ANRIL in Diabetic Retinopathy. Poster at 2017 London Health Research Day, London, ON.
- Thomas AA, Feng B, Chakrabarti S. Alteration of Long noncoding RNA ANRIL in Diabetic Retinopathy. Poster at 2017 Pathology and Laboratory Science Research day, Western University, London, ON.
- Thomas A, Gordan A, Biswas S, Feng B, Chakrabarti S. Alterations in long noncoding RNA cause pathogenic changes in Diabetic Retinopathy. Poster at 2016 CDA/CSEM Professional Conference, Ottawa, ON.
- Thomas AA, Feng B, Chakrabarti S. ANRIL: polycomb complexes regulate VEGF expression in diabetic complications. Poster at 2016 TRENDs RNA conference, Toronto, ON.
- Thomas AA, Feng B, Chakrabarti S. ANRIL mediates diabetes-induced endothelial dysfunction through upregulation of vascular endothelial growth factor. Poster at 53rd EASD Annual Meeting 2016, Munich, Germany.
- Thomas AA, Chakrabarti. Thomas AA, Feng B, Chakrabarti S. Long noncoding RNA ANRIL regulates VEGF-mediated angiogenesis in Diabetic Complications. Poster at 2016 London Health Research Day, London, ON
- Thomas AA, Feng B, Chakrabarti S. Long noncoding RNA ANRIL regulates VEGF-mediated angiogenesis in Diabetic Complications. Poster at 2016 Pathology and Laboratory Science Research day, Western University, London, ON.
- Thomas AA, Feng B, Chakrabarti S. ANRIL: regulator of VEGF mediated angiogenesis in Diabetic Retinopathy. Poster at 2015 International Diabetes Federation-World Diabetes Conference, Vancouver, BC.
- Thomas AA, Feng B, Chakrabarti S. Role of lncRNAs in Diabetic Complications. Poster at 2015 London Health Research Day, London, ON.
- Thomas AA, Feng B, Chakrabarti S. Role of lncRNAs in Diabetic Complications. Poster at 2015 Pathology and Laboratory Science Research day, Western University, London, ON.
- Thomas AA, Feng B, Chakrabarti S. lncRNA mediated regulation of angiogenesis in Diabetic Complications. Poster at 2015 Diabetes Research Day, St. Joseph's Health Care and Western University, London, ON.
- Thomas AA, Puthenveetil PP, Chen S, Feng B, Chakrabarti S. H19 regulates glucose-induced alteration in Diabetic Complications. Poster at 2014 CDA/CSEM Professional Conference, Winnipeg, MB.
- Thomas AA, Puthenveetil PP, Chen S, Feng B, Chakrabarti S. H19 regulates glucose-induced alteration in Diabetic Complications. Poster at 2014 Diabetes Research Day, St. Joseph's Health Care and Western University, London, ON.
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