Investigating MDM2 regulation of endothelial cell angiomir biosynthesis in response to high glucose treatment

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Abstract

MiRNAs are a class of short, non-coding RNAs that regulate gene expression at the post-transcriptional level. Dysregulation of miRNA production contributes to the pathogenesis of hyperglycemia-related complications. Although many studies have examined the effect of hyperglycemia on the expression of miRNAs, the effect of hyperglycemia specifically on the endothelial cell miRNA biogenesis machinery is poorly understood.

The aim of my project is to better characterize the effect of high glucose treatment on the miRNA biogenesis machinery and miRNA expression in endothelial cells. HDMECs were incubated with low (5mM) and high (30mM) glucose concentrations for 6 and 24 hours and subsequent miRNA expression was measured.

My results indicate that exposure to high glucose concentrations enhances MDM2:DROSHA binding and represses DROSHA expression both in vitro and in diabetic mice (db/db) muscles. Treatment with MDM2 inhibitors repealed glucose-induced DROSHA downregulation. This suggests that high glucose concentrations downregulates DROSHA expression through an MDM2-mediated mechanism.

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Table of Contents

List of Figures

List of Abbreviations

Analysis of variance	ANOVA
Argonaute	AGO
Argonaute 2	AGO-2
Basic fibroblast growth factor or bFGF	FGF-2
Bicinchoninic acid	BCA
Central domain	CED
Cluster of differentiation 36	CD36
Cluster of differentiation 47	CD47
CREB-binding protein	CBP
C-terminal tail	CTT
Cyclic guanosine monophosphate	cGMP
DiGeorge syndrome critical region 8	DGCR8
Domain of unknown function 283, a domain of DICER	DUF283
Double-stranded ribonucleic acid	dsRNA
Double-stranded ribonucleic acid-binding domain	dsRBD
Endothelial cell	EC
Endothelial nitric oxide synthase	eNOS
Fisher's least significant difference	LSD
Focal adhesion kinase	FAK
Forkhead box protein O1	FOXO1
Glycogen synthase kinase 3 beta	GSK3β
Heterogeneous nuclear ribonucleoproteins	hnRNP

Histone acetyltransferase GCN5	GCN5
Histone deacetylase 1	HDAC1
HIV-1 TAR RNA binding protein	TRBP
Horseradish peroxidase	HRP
Human dermal microvascular endothelial cell	HDMEC
Human embryonic kidney 293 cells with mutant SV40 large T antigen	HEK293T
Human umbilical vein endothelial cell	HUVEC
Hypoxia-inducible factor 1-alpha	HIF1α
Impaired fasting glucose	IFG
Impaired glucose tolerance	IGT
Mammalian or mechanistic target of rapamycin	mTOR
Matrix metallopeptidase 9	MMP-9
Matrix metallopeptidases	MMP
Messenger ribonucleic acid	mRNA
Microprocessor complex	MC
MicroRNA or miRNA	miR
Mitogen-activated protein kinase 1	ERK2
Mitogen-activated protein kinase 3	ERK1
Mitogen-activated protein kinases	MAPK
Mobile basic	MB
Nitric oxide	NO
Phosphoinositide 3-kinases	PI3K
Phosphoinositide-3-kinase regulatory subunit 2	PI3KR2

Phospholipase C gamma	PLCγ
Phosphorylation	p
Piwi argonaut and zwille	PAZ
PKR activator	PACT
Placental growth factor	PIGF
Polymerase chain reaction	PCR
Precursor microRNA	pre-miRNA
Primary microRNA	pri-miRNA
Protein Kinase B or PKB	Akt
Protein kinase C	PKC
Quantitative reverse transcription polymerase chain reaction	RT-qPCR
Reduced nicotinamide adenine dinucleotide phosphate	NADPH
Ribonuclease III	RNase III
Ribonucleic acid	RNA
RISC-loading complex	RLC
RNA-binding heme domain	Rhed
RNA-induced silencing complex	RISC
RNase III domain	RIIID
RNase III domain a	RIIIDa
RNase III domain b	RIIIDb
Single-stranded ribonucleic acid	ssRNA
Small interfering ribonucleic acid	siRNA
Small Ubiquitin Like Modifier 1	SUMO1

Soluble guanylyl cyclase	sGC
Sprouty-related, EVH1 domain-containing protein 1	SPRED1
TAR DNA-binding protein 43	TDP-43
Thrombospondin 1	THBS1
Thrombospondin-1 type I repeats	TSR
Untranslated region	UTR
Vascular endothelial growth factor	VEGF
Vascular endothelial growth factor A	VEGF-A
Vascular endothelial growth factor B	VEGF-B
Vascular endothelial growth factor C	VEGF-C
Vascular endothelial growth factor D	VEGF-D
Vascular endothelial growth factor receptor 1	VEGFR1
Vascular endothelial growth factor receptor 2	VEGFR2
Very-low-density-lipoprotein receptor	VLDLR

Section 1: Review of Literature

1.1 Introduction

Angiogenesis – the generation of new capillaries from pre-existing ones – is an important adaptation that enables the vasculature to meet changes in the oxidative and metabolic demands of the tissues (Olfert et al., 2016). This process is under tight regulation, and changes in the vasculature are dependent on the relative abundance and interplay of different pro- and anti-angiogenic signalling molecules. Angiogenic inducers promote vessel sprouting through stimulation of matrix protease production, inhibition of endothelial cell apoptosis, and promotion of endothelial cell migration and proliferation (Adair and Montani, 2010). Conversely, angiogenic inhibitors interfere with the signal transduction of the angiogenic inducers to counteract them and keep the formation of new blood vessels in check. Shifts in the balance of these angiogenic factors in the microenvironment will dictate the behaviour of the microvasculature, leading to capillary growth, maintenance or regression (Gustafsson, 2011; Olfert and Birot, 2011; Logsdon et al., 2014). This review explores the role of different angiogenic factors on angiogenesis with an emphasis on the role of angiomiR maturation.

Vascular endothelial growth factor A (VEGF-A) and thrombospondin 1 (THBS1) are two of the most well-characterized inducers and inhibitors of angiogenesis in skeletal muscle. These factors are essential in the regulation of muscle capillarity and exercise capacity and are crucial for vascular development, maintenance and adaptation. Targeted deletion of VEGF in skeletal muscle leads to decreases in capillary-to-fibre ratio and capillary density and aerobic exercise capacity (Olfert et al., 2009). A 90% reduction in gastrocnemius VEGF protein was accompanied by a 48% reduction in capillary-to-fibre ratio, a 39% reduction in capillary density, and a reduction in maximal running speed and endurance running capacity (as measured by submaximal endurance

treadmill running) by 34% and 81%, respectively (Olfert et al., 2009). Conversely, global deletion of THBS1 in mice increases basal capillary number in skeletal muscle, and the mice exhibited enhanced exercise capacity. THBS1-null mice showed an increase in maximal running speed by 11% and endurance running capacity by 67%, compared to wild-type controls. Capillary-to-fiber ratio, capillary density, and capillary contacts were all significantly increased in THBS1-null mice compared to wild-type mice (Malek and Olfert, 2009). This review focuses on VEGF-A and THBS1 because of their impact on skeletal muscle capillarization and their effect on exercise capacity in rodent models.

1.2 Key Angiogenic Factors

1.2.1 Vascular endothelial growth factor A (VEGF-A)

The vascular endothelial growth factor (VEGF) family of proteins are important regulators of blood vessel formation. The VEGF protein family includes VEGF-A, VEGF-B, VEGF-C, VEGF-D and placental growth factor (PIGF). VEGF-A is the most well-characterized protein and plays a crucial role in the migration and proliferation of endothelial cells (Amoroso et al., 1997; Connolly, 1991; Shibuya and Claesson-Welsh, 2006; Vempati et al., 2014).

Human VEGF-A is encoded by the *VEGFA* gene on locus 6p21.1 (Choi et al., 2016) and its primary mRNA transcript undergoes alternate splicing to produce several isoforms that regulate blood vessel growth. All of the VEGF-A isoforms transmit their signal through two receptors with tyrosine kinase activity: VEGFR1 and VEGFR2 (Fearnley et al., 2016). VEGF-A proteins have a higher affinity for VEGFR1 than VEGFR2; however, VEGFR1's tyrosine kinase activity is approximately 10-fold weaker than VEGFR2 (Sawano et al., 1996). Therefore, ligand-activation of VEGFR2 generates the majority of the angiogenic signal.

VEGFR2 is a 200kDa glycoprotein that regulates endothelial cell migration, proliferation, differentiation and survival as well as vessel permeability and dilation. VEGF-A binding of the VEGFR2 receptor can trigger numerous signalling cascades involving a number of effectors, including the PLCγ-PKC-MAPK and PI3K/Akt pathway (cell proliferation), Src, focal adhesion kinase (FAK) (cytoskeleton organization and migration), and endothelial nitric oxide (NO) upregulation (vascular permeability) (Cébe-Suarez et al., 2006; Claesson-Welsh and Welsh, 2013). Upon activation by VEGF-A/VEGFR2 binding, endothelial cells will release proteases to degrade the underlying basement membrane, migrate and proliferate into the interstitial space, form a lumen, recruit pericytes to generate a new basement membrane, and fuse with pre-existing vessels (Carmeliet and Jain, 2011).

Sensitivity to VEGF-A plays a crucial role in controlling sprouting angiogenesis. Release of VEGF-A by the resident tissue generates a VEGF gradient when angiogenesis is required. This gradient is recognized by specialized endothelial cells that express a higher amount of VEGFR2 and express lower VEGFR1 (Blanco and Gerhardt, 2013). These cells acquire a "tip cell phenotype." The tip cell will form numerous filopodia that extend towards the direction of the gradient and guide the cells migration. Conversely, the endothelial cells neighbouring the tip cell, the "stalk cells", will have a proliferative response to VEGF-A agonists as they maintain low VEGFR2 and high VEGFR1 (Gerhardt et al., 2003).

In addition to triggering endothelial cell migration and proliferation, VEGF-A also plays a vital role in upregulating nitric oxide (NO) bioavailability in endothelial. Endogenous NO is a vasoactive molecule produced in endothelial cells by nitric oxide synthase (and its isoforms). NO signalling contributes to angiogenic signalling by triggering cell growth and differentiation via activation of endothelial-constitutive NO synthase (eNOS), the elevation of cyclic GMP (cGMP)

transcription, activation of mitogen-activated kinase (MAPK) signalling pathway, and upregulating fibroblast growth factor-2 (FGF-2) expression. (Duda et al., 2004; Ziche and Morbidelli, 2000). Additionally, NO is also a well-described and potent vasodilator. Vasodilation is required for the initiation of angiogenesis by increasing the permeability of the vessel and degradation of the surrounding matrix; thus allowing the endothelial cells to migrate into the extracellular space (Conway et al., 2001). VEGF-A is an important driver of NO signalling via stimulation of eNOS (Fukumura et al., 2001; Milkiewicz et al., 2005; Murohara et al., 1998). By binding its receptor VEGFR2 on the endothelial cell surface, VEGF-A induces parallel pathway (via both PI3K/AKT-1 and PLCγ/AMPK pathways) phosphorylation of Ser-1117 on eNOS, which drives production of NO.

Altogether, VEGF-A is an important pro-angiogenic molecule, promoting an array of angiogenic responses, including guiding capillary sprouting, hyperpermeability, endothelial cell survival, proliferation, and migration.

1.2.2 Thrombospondin-1 (THBS1)

Conversely to VEGF, THBS1 is a potent anti-angiogenic molecule whose impact on angiogenesis has been extensively reviewed (Isenberg et al., 2009; Mirochnik et al., 2008; Zhang and Lawler, 2007). THBS1 antagonizes VEGF-A by modulating its bioavailability and signal transduction (Iruela-Arispe et al., 2004; Zhang et al., 2009). In addition to acting as a VEGF-A antagonist, THBS1 also inhibits endothelial cell migration and proliferation in a VEGF-A independent manner.

In endothelial cells, THBS1 antagonizes VEGF-A by directly interacting with it in the extracellular matrix, inhibiting its release from the extracellular matrix, and inhibition of VEGF-

A signal transduction. THBS1 inhibits the release of VEGF from the extracellular matrix through suppression of MMP activity. MMPs are a family of zinc-containing endopeptidases that degrade components of the extracellular matrix (ECM) (Rundhaug, 2003). MMP-mediated degradation of the ECM triggers the release of ECM-bound proangiogenic factors such as VEGF-A. THBS1 suppresses MMP expression, thus restricting ECM-bound VEGF release. In accordance with this, the level of MMP9 is inversely correlated with the level of THBS1 in transgenic mice overexpressing THBS1 (Rodriguez-Manzaneque et al., 2001). Moreover, Rodriguez-Manzaneque et al. (2001) detected a decrease in VEGF bound to its receptor (VEGFR2) in the presence of THBS1. Furthermore, THBS1 binds VEGF-A directly, and this interaction can mediate the uptake and clearance of VEGF-A from the extracellular space (Greenaway et al., 2007; Gupta et al., 1999). THBS1 has been shown to inhibit VEGF-A signal transduction (Zhang et al., 2009). In vitro treatment of HDMECs with TSR (thrombospondin-1 type I repeats; a THBS1 domain) decreases VEGF-A-induced phosphorylation of VEGFR2 at tyrosine-1175 in a dose-dependent fashion. This effect is also observed in vivo in mice treated with TSR (Audet et al., 2013).

THBS1 also exerts an anti-angiogenic effect in VEGF-A independent manner. Endothelial cell migration is a fundamental process involved in the formation of sprouting capillaries. THBS-1 antagonizes this process (Dawson et al., 1997). THBS1 inhibits capillary endothelial cell migration via the binding of its TSR domains to CD36 (Calzada et al., 2004). CD36 is a multi-functional membrane protein that mediates anti-angiogenic signals in endothelial cells via Src-family kinase and NADPH-mediated reactive oxygen species generation (Ramakrishnan et al., 2016). THBS1 also interacts with CD36 to antagonize the NO signalling pathway (Isenberg et al., 2007). THBS1 inhibits angiogenesis through suppression of endothelial cell cycle progression in a CD36-independent fashion (Oganesian et al., 2008; Yamauchi et al., 2007). THBS1 binds very-low-

density lipoprotein receptor (VLDLR) in the membrane of endothelial cells and triggers inhibition of cell cycle progression (Oganesian et al., 2008). Decreased Akt and MAPK phosphorylation accompanied the interaction of THBS1 with VLDLR. Additionally, THBS1 also inhibits endothelial cell cycle progression by upregulating p21 and p53 phosphorylation (Yamauchi et al., 2007). Furthermore, THBS1 also binds to and represses the activity of fibroblast growth factor-2 (FGF-2), a well-described proangiogenic factor, through its TSR (Colombo et al., 2010).

THBS1 is also an important antagonist of the NO signalling pathway via its interaction to endothelial cell surface receptors CD36 (Isenberg et al., 2007) and CD47 (Isenberg et al., 2006, 2009). Engagement of CD36 by the TSR of THBS blocks uptake of myristate and interferes with AMPK and Src signalling pathways, which are promoters of NO signalling. Chronic exposure to a THBS1 mimetic that targets the CD36 THBS1 receptor results in a reduction in skeletal muscle capillarity in mice (Audet et al., 2013). THBS1 has been shown to antagonize proangiogenic signalling by NO via its binding and activation of CD47 (Isenberg et al., 2009). Whereas both CD36 and CD47 can mediate suppression of NO signalling by THBS1, only CD47 is essential (Isenberg et al., 2006). THBS1 activates CD47, which, in turn, triggers sGC and cGMP-dependent protein kinase I activity. Altogether, THBS1 activation of CD36 and CD47 interrupts proangiogenic NO signalling.

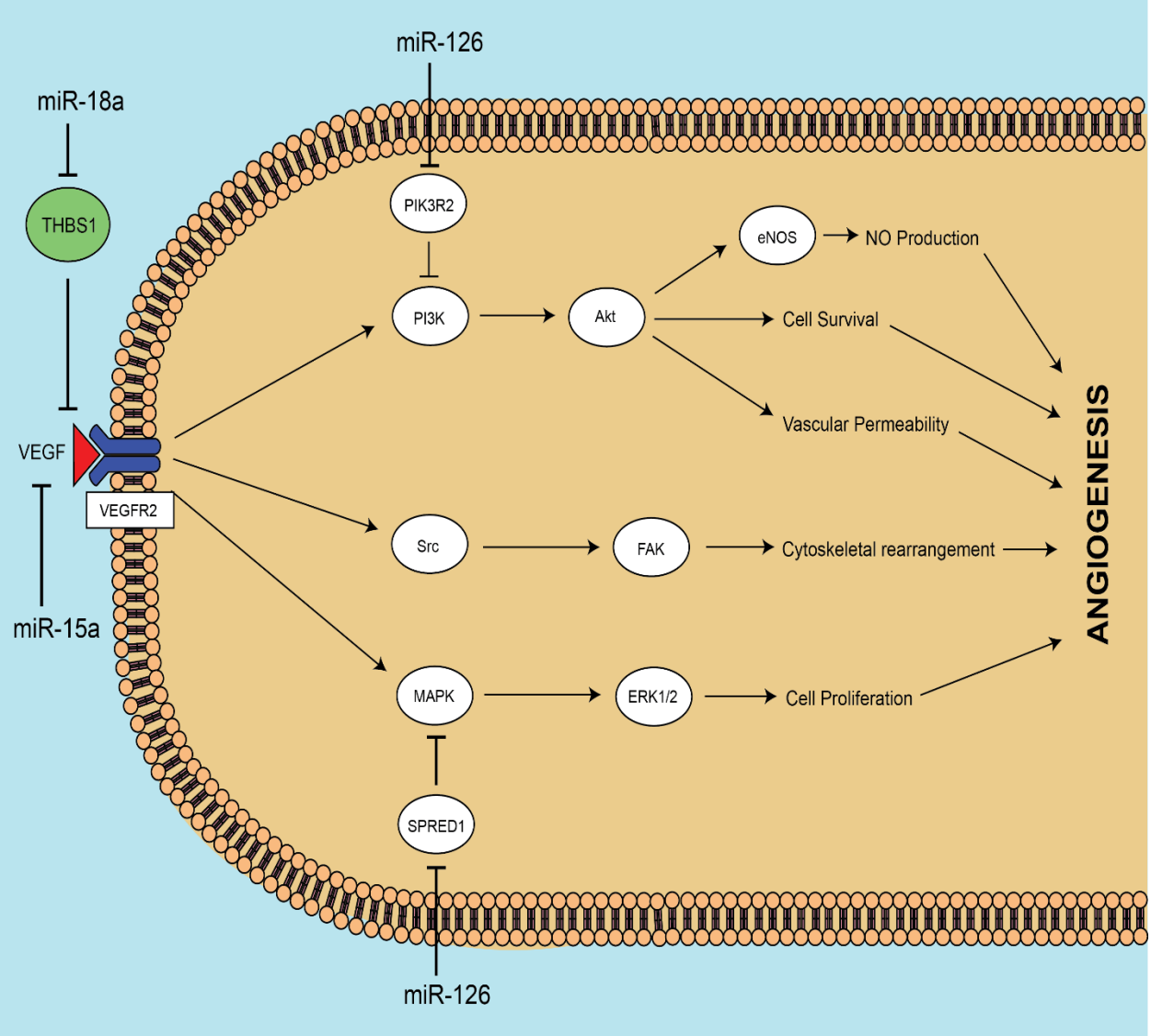


Figure 1. Angiomirs and VEGF-A signaling in endothelial cells. VEGF-A binds to VEGFR2 to initiate a signaling cascade in endothelial cells to promote vascular permeability, EC survival, and proliferation. THBS1 inhibits VEGF-A signaling by directly binding to VEGF and preventing the activation of VEGFR2. In endothelial cells, miR-126 inhibits SPRED1 and PIK3R2 to facilitate angiogenesis by activating the PI3K/Akt and MAPK/ERK pathways. miR-18a enhances VEGF signaling by inhibiting THBS1 protein expression; while miR-15a impairs angiogenesis by inhibiting VEGF-A protein expression.

1.3 MiRNAs in Angiogenesis

The angiogenic balance is tightly regulated by several complex mechanisms that control gene expression. One of the distinct mechanisms involved in the regulation of the angiogenic balance involves functional RNA molecules known as angiomiRs (Wang and Olson, 2009). AngiomiRs are a sub-category of a class of molecules known as micro-RNAs (miRNAs) and can promote or inhibit angiogenesis by regulating the protein expression of both positive and negative angio-regulatory factors (Wang and Olson, 2009). MiRNAs are small (18-24 nucleotide) RNAs that exert substantial gene regulatory effects via a posttranscriptional mechanism. MiRNAs exert potent angiogenic control by targeting critical pro- and anti-angiogenic secretory and transcriptional factors. In animal cells, miRNAs regulate their targets by binding to the 3'-untranslated regions of their target mRNAs, leading to translational inhibition by hindering ribosomal protein assembly, initiation of translation, and elongation (Filipowicz et al., 2008). Additionally, miRNA-mRNA association can lead to destabilization of the mRNA transcript (Bushati and Cohen, 2007; Wang and Olson, 2009). One of the first pieces of evidence that supports the notion of miRNA regulation of angiogenesis is the finding that homozygous deletion of the miRNA biogenesis protein, DICER, results in a phenotypic defect in angiogenesis of mouse embryo (Yang et al., 2005). DICER knockout led to global depletion of miRNA expression, resulting in severe impairment in blood vessel formation. In vitro, DICER knockdown leads to a diminished angiogenic response in endothelial cells as determined by impaired cell migration, Matrigel tube formation, and spheroid capillary sprouting (Kuehbacher et al., 2007; Shilo et al., 2008; Suárez et al., 2007).

There are now over 2000 human miRNAs identified, and it is predicted that they control the expression of one-third of the genes encoded by the entire genome (Hammond, 2015). MiRNA-

126, MiRNA-15a, and MiRNA-18a are three important angiomiRs that regulate angiogenesis by regulating VEGF-A, THBS1 and their signalling pathways.

One of the first miRNAs identified as a critical regulator of angiogenesis is miRNA-126 (Fish et al., 2008; Kuhnert et al., 2008; Wang et al., 2008). MiR-126 is a pro-angiogenic miRNA and the most abundant miRNA in endothelial cells (Fish and Srivastava, 2009). Targeted deletion of miR-126 in mice causes leaky vessels, hemorrhaging and embryonic lethality due to loss of vascular integrity and defects in angiogenesis (Wang et al., 2008). The pro-angiogenic effects of miR-126 depend on positive regulation of the VEGF pathway. MiRNA-126 targets SPRED1 (Fish et al., 2008; Wang et al., 2008) and phosphoinositide 3-kinase regulatory subunit 2 (PI3KR2) (Fish et al., 2008; Kuhnert et al., 2008). Both SPRED1 and PI3KR2 exert adverse effects on VEGF signalling via repression of the ERK1/2 and PI3K/Akt pathways, respectively.

MiRNA-15a and members of the miRNA-17-92 cluster are regulatory genes for VEGF-A and THBS1, respectively (Dogar et al., 2014; Yin et al., 2012). In mice, transgenic overexpression of MiRNA-15a led to a reduction in blood vessel formation and blood perfusion. In vitro, repression of miRNA-15a induced a significant increase in tube formation, endothelial cell (EC) migration and differentiation; while overexpression of miRNA-15a suppressed these observed effects (Yin et al., 2012). The mechanism underlying these effects involves miRNA-15a direct binding and inhibition of VEGF-A (Yin et al., 2012) and VEGFR2 (Musumeci et al., 2011) to impart an angiostatic effect. Conversely, miRNA-18a has been shown to regulate THBS1 expression (Dews et al., 2006; Doebele et al., 2010). miRNA-18a negatively regulates THBS1 post-transcriptionally in MYC-induced angiogenesis (Dews et al., 2006). Together, miRNA 15a and -18a regulates the angiogenic balance by inhibiting VEGF-A and THBS1, respectively.

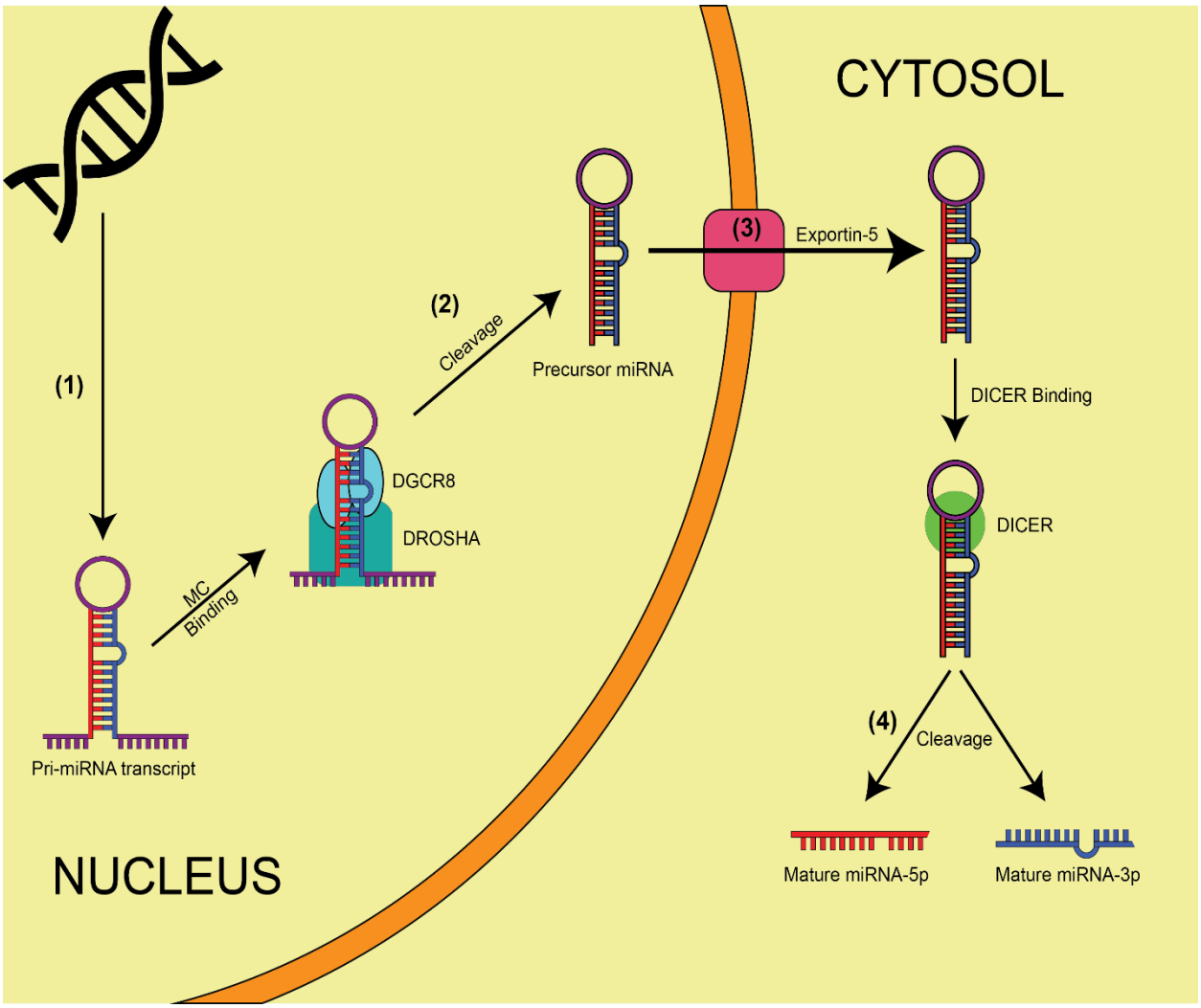


Figure 2. Canonical miRNA biogenesis pathway. (1) Primary miRNA transcript (pri-miRNA) is transcribed from the genome or spliced from introns of other genes. (2) DROSHA and DGCR8 bind and cleave pri-miRNA to produce precursor miRNA (pre-miRNA). (3) Pre-miRNA was exported into the cytoplasm by Exportin-5. (4) DICER binds and cleaves pre-miRNA into mature miRNAs.

1.4 MicroRNA Biogenesis and Regulatory Mechanisms

The expression of miRNAs is tightly regulated by a series of cleavage events that regulate their maturation into their mature functional forms. The endogenous production and maturation of miRNAs are deeply dependent on two proteins: DROSHA and DICER (Ha and Kim, 2014).

The canonical miRNA biogenesis starts with the transcription of the primary miRNA transcript (pri-miRNA) from the genome or splicing from introns of other RNAs. DROSHA, an RNase III-like enzyme will form a protein complex with its protein binding partner DiGeorge syndrome critical region 8 (DGCR8), known as the microprocessor complex (MC). The MC identifies and cleaves the pri-miRNA into precursor miRNA (pre-miRNA) (Lee et al., 2006). Following MC cleavage, the pre-miRNA is exported out of the nucleus where it will be processed by DICER into its mature functional form (Macrae et al., 2006). Following DICER cleavage, the single-stranded mature miRNA will associate with Argonaute proteins to form the RNA-induced silencing complex (RISC), a ribonucleoprotein complex that mediates post-transcriptional gene silencing through complementary base-pairing of the miRNA to target mRNAs; leading to translational repression or mRNA decay (Iwakawa and Tomari, 2015). Both DROSHA and DICER have been demonstrated to be critical to miRNA synthesis. Knockout of DROSHA and DICER in cells led to the complete ablation of canonical miRNA production and decreased miRNA production, respectively (Kim et al., 2016).

1.5 DROSHA

DROSHA is an RNase III protein that is essential for miRNA biogenesis. Within the context of angiogenesis, past studies have indicated that DROSHA plays an important role in mediating angiogenic functions of endothelial cells. Kuehbacher and colleagues demonstrated that

endothelial cells treated with DROSHA siRNA exhibited reduced sprout formation under both basal and basic fibroblast growth factor-stimulated conditions. Combined DROSHA and DICER siRNA-induced suppression did not result in further reduction of sprout formation; suggesting that the observed effect is independent of DICER processing (Kuehbacher et al., 2007). Interestingly, DROSHA-silencing does not impair endothelial cell migration (Kuehbacher et al., 2007). Additionally, DROSHA-silencing significantly increased THBS1 expression by 358%, however, surprisingly, depletion of THBS1 via siRNA did not rescue the impaired sprout formation induced by DROSHA-silencing; suggesting that DROSHA's role in mediating angiogenesis extends beyond regulation of THBS1 expression (Kuehbacher et al., 2007).

1.5.1 DROSHA Structure and Function

DROSHA is a 159kDa RNase III-like enzyme that is approximately 1374 amino acids in length. DROSHA interacts with two units of its cofactor, DGCR8, to form a heterotrimeric, microprocessor complex (MC). The MC recognizes and cleaves primary microRNA transcripts to release a short hairpin (pre-miRNA) (Gregory et al., 2004; Han et al., 2006; Lee et al., 2003). A typical pri-miRNA contains a double-stranded RNA (dsRNA) stem of approximately 35 bp that is flanked by single-stranded basal segments and an apical loop of variable size (Han et al., 2006). The cleavage site of pri-miRNAs is generally located 11 bp away from the basal junction (junction between the lower stem and basal segments) and 22 bp away from the apical junction (junction between the upper stem and the apical loop) (Auyeung et al., 2013; Han et al., 2006; Zeng et al., 2005). Within the MC, DROSHA serves as both a catalytic subunit as well as a "ruler"; determining the cleavage sites by measuring the length of dsRNA from the basal junction (Nguyen et al., 2015).

The DROSHA protein contains five major domains: the N-terminal domain, central domain, two RNase III domains (RIIIDs), and a dsRBD domain. The N-terminal of DROSHA includes a proline-rich (P-rich) region followed by domains rich in arginine and serine resides, i.e an RS-rich region. Following the RS-rich region is a highly conserved central domain (CED) that is indispensable for pri-miRNA processing (Han et al., 2004). The CED is comprised of three regions (Platform, PAZ-like domain, and Connector helix). Within the CED, the first half is known as the Platform (residues 390-713) while the C-terminal end of the CED is known as the Connector. The Platform surrounds the Connector forming a cleft that is bent towards the RNA binding site, preventing dsRNA from binding. The Platform also contains two zinc ions, forming a zinc-finger motif that stabilizes this cleft. Between the Platform and Connector helix domains is a Piwi Argonaut and Zwille (PAZ)-like domain (residues 714-849) that interacts with ssRNA basal segments. The PAZ-like domain is followed by two RIIID domains (RIIIDa and RIIIDb) that bind to the CTT domain of DGCR8 (Kwon et al., 2016).

The RIIIDa domain contains two helices, the Bump helix and the mobile basic (MB) helix and contains the cleavage site for 3' end of the pri-miRNA. The Bump helix protrudes towards the PAZ-like domain and forms the site of bifurcation for dsRNA to ssRNA; the dsRNA can only be accommodated up to the Bump helix. The distance between the Bump helix and the catalytic site is approximately 11 base pairs, suggesting that the bump helix is responsible for the 11-bp-measuring "ruler" activity of DROSHA. The MB helix contains a large number of Arg and Lys residues and is believed to be in the vicinity of the basal junction due to its proximity to the Bump helix. Both the Bump and MB helices are crucial for pri-miRNA processing, and mutations in the MB helix abolishes DROSHA processing activity. The RIIIDb-CTT interaction is essential for stabilizing the MC-miRNA complex. The RIIIDb contains the cleavage site for the 5' end of the

pri-miRNA. The C-terminal of DROSHA contains the dsRBD domain which interacts with the RNA substrate. The dsRBD is bound to and can easily dissociate from the RIIIDb. Collectively, the CED, RIIID and dsRBD domains (390-1365) is referred to as the D3 region and is the location of DGCR8 binding and pri-miRNA processing. Due to the orientation of the D3 region, DROSHA recognizes the basal UG motif and preferentially binds to a clear ssRNA-dsRNA junction (Han et al., 2006). DROSHA alone is capable of recognizing the key structural features in the basal side of pri-miRNA. However, DROSHA alone possesses poor RNA-binding affinity (Kwon et al., 2016).

1.5.2 Non-miRNA Functions of DROSHA

In addition to its function in processing miRNAs, DROSHA also regulates aspects of RNA transcriptional activation and cleavage (Lee and Shin, 2018). The best-characterized non-canonical function of DROSHA is the post-transcriptional destabilization of mRNA by cleaving pri-miRNAs-like hairpins within them (Lee and Shin, 2018). DROSHA-mediated cleavage also contributes to the clearance of mRNAs in progenitor cells, which is essential for cell fate determination and differentiation.

DROSHA binds not only miRNAs but also the 5’ ends of many other genes. This binding is mediated by the presence of short-hairpins within the promoter-associated transcripts. Surprisingly, this binding does not result in cleavage or destabilization, rather, it promotes transcriptional activation. DROSHA’s function as a transcriptional activator is independent of its catalytic activity and is mediated through its N-terminal region.

It has also been demonstrated that DROSHA can cleave other RNAs. DROSHA can also act as a positive regulator of alternate splicing independently of its cleavage function (Havens et

al., 2014). The suppressive effect of DROSHA on splicing does not require its catalytic activity; instead, it involves sterical hinderance of the splicing machinery, impairing the recognition of the splice site (Lee et al., 2017).

1.5.3 Regulation of DROSHA Expression and Activity

DROSHA activity is modulated physiologically by posttranslational modifications, including phosphorylation and acetylation. Glycogen synthase kinase 3β (GSK3β) is a serine/threonine protein kinase, initially identified as a regulator of glycogen metabolism. GSK3β phosphorylates DROSHA at Ser300 and Ser302; leading to enhanced DROSHA activity (Fletcher et al., 2017). Phosphorylation at either of these two sites is required for DROSHA nuclear localization (Tang et al., 2010). Additionally, acetylation of DROSHA on its N-Terminus stabilizes it and inhibits its degradation by ubiquitination (Tang et al., 2013). The N-terminal, but not the C-terminal can be acetylated by multiple acetyl transferases including p300, CBP and GCN5. Moreover, TAR DNA-binding protein-43 (TDP-43) is homologous to the heterogeneous nuclear ribonucleoproteins (hnRNPs), which are involved in RNA processing. TDP-43 associates with DROSHA and directly binds a specific sub-set of pri-miRNAs. This facilitates the binding of DROSHA to the pri-miRNA and enhances the maturation of a specific subset of pre-miRNAs (Kawahara and Mieda-Sato, 2012).

1.6 DGCR8

1.6.1 DGCR8 Structure and Function

DGCR8 is an approximately 86kDa protein that serves as a cofactor for DROSHA in pri-miRNA processing. DGCR8 binds miRNA non-specifically (Roth et al., 2013). DGCR8 has five major regions: the N-terminal region that contains a nuclear localization signal (NLS), a central heme-binding domain (Rhed), two dsRBD domains, and a C-terminal tail (CTT) (Quick-Cleveland et al., 2014; Shiohama et al., 2007; Yeom et al., 2006). The central heme-binding domain ensures accurate pri-miRNA processing at the canonical site. Heme binding to Rhed promotes dimerization of DGCR8 proteins and ensures accuracy of pri-miRNA processing. When heme-bound the MC cleaves pri-miRNA exclusively at the canonical site; however, without heme, a substantial amount of unproductive cleavage products are formed as a result of MC processing (Partin et al., 2017) The Rhed region also recognizes the apical "UGU" motif on the substrate RNA; allowing for proper orientation of the substrate in the MC complex. The dsRBD interacts with the dsRNA stem non-specifically and enhances processing efficiency of the MC (Faller et al., 2007; Han et al., 2006; Nguyen et al., 2015; Yeom et al., 2006). The dsRBD interacts with successive minor, major, and minor grooves along on face of the dsRNA (Masliah et al. 2013); clamping the substrate in place to allow for accurate cleavage. The CTT domain of DGCR8 interacts with RIIID domains of Drosha and is crucial for DGCR8-DROSHA binding. The CTTs of the two DGCR8 proteins are arranged asymmetrically, creating a spatial shift between the two DGCR8 proteins by a half-helical turn. This staggers the positions of the two DGCR8 molecules along the upper stem of the dsRNA and allows the dsRBDs of the DGCR8 to interact with the upper stem without steric hindrance (Kwon et al., 2016).

1.6.2 Regulation of DGCR8 Expression and Activity

DGCR8 activity is modulated physiologically by posttranslational modifications, including sumoylation, phosphorylation, and deacetylation. Sumoylation of DGCR8 at K707 by SUMO1 increases its affinity for pri-miRNAs without altering the interaction between DGCR8-DROSHA or their miRNA biogenesis activity (Zhu et al., 2015). Phosphorylation of DGCR8 by ERK increases DGCR8 intracellular stability. All 23 phosphorylation sites are located in the N-terminal of DGCR8; this site is necessary for DGCR8 nuclear localization (Yeom et al., 2006) and for its ability to heterodimerize (Faller et al., 2007). Phosphorylation of DGCR8 also results in the generation of a pro-growth miRNA expression profile and increases cell proliferation. Because miRNA biogenesis is highly regulated, certain miRNAs appeared to be more sensitive to MC levels and/or the phosphorylation status of DGCR8: shift in miRNA profile (10a-5p and 10b-5p increased >2 fold; miR-129-5p decreased >2-fold) (Herbert et al., 2013).

Histone deacetylase 1 (HDAC1) enhances miRNA processing via deacetylation of DGCR8 (Wada et al., 2012). HDAC1 increases the affinity of DGCR8 to pri-miRNA transcripts via deacetylation of lysine residues in the RNA-binding domain of DGCR8. HDAC1 has two arms for gene silencing; transcriptional repression by promoter histone deacetylation and post-transcriptionally by increasing miRNA abundance. HDAC1 overexpression downregulated the expression of only 9.0% of mature miRNA in an array, but rather upregulated the abundance greater than 1.5-fold in 46.1%. HDAC1 enhances miRNA processing in vitro and in vivo. Acetylation of DGCR8 was greatly enhanced by p300 by abrogated by HDAC1.

1.7 DICER

1.7.1 DICER Structure and Function

DICER is a 220kDa enzyme that is highly conserved among eukaryotes. DICER plays a crucial role in the canonical miRNA biogenesis pathway; cleaving the stem-loop of precursor miRNAs to produce the mature miRNA substrates (O'Brien et al., 2018). Its structure consists of a seven distinct domains: an N-terminal putative helicase domain, a DUF283 domain, and platform domain, a piwi-argonaute-zwille (PAZ) domain, two RNase III domains, and a dsRNA binding domain (dsRBD) on the C-terminal (Kurzynska-Kokorniak et al., 2015; Lau et al., 2009, 2012; Taylor et al., 2013). In the current model of DICER ribonuclease activity, the PAZ and platform domains form two pockets that recognize and binds the substrate ends of the miRNA precursors. The PAZ domain and the platform domain recognize the 3' and 5' miRNA ends, respectively (Macrae et al., 2006; Zhang et al., 2004). The c-terminal dsRBD domain is responsible for binding and stabilizing the stem of the double-stranded precursor miRNA, and the two RIIIDs form the RNA cleavage site. It has been proposed that the length of the RNA product is determined by the distance between this cleavage site and the PAZ domain (Lau et al., 2012; Macrae et al., 2006). Lastly, helicase domain has been proposed to function as an auto-inhibitor of DICER since binding of this domain triggers substrate-dependent changes in DICER structure (Liu et al., 2015; Ma et al., 2008; Taylor et al., 2013). The function of the DUF283 domain remains unknown.

1.7.2 DICER Binding Partners

Though DICER is capable of cleaving pre-miRNA alone, its activity is modulated by two proteins: TRBP and protein activator of protein kinase R (PACT) (Chendrimada et al., 2005; Lee et al., 2013). When bound to DICER, these proteins increase the specificity of DICER as well as

adjusting the cleavage site. Under normal physiological conditions, DICER is capable of processing both dsRNA and pre-miRNAs into siRNA and miRNA, respectively. When bound to PACT, DICER increases its specificity towards miRNAs rather than siRNAs (Lee et al., 2013).

Additionally, TRBP and PACT bind to the N-terminal helicase domain of DICER, resulting in a change in DICER structure. As a result, this may change the cleavage site of DICER, triggering the production of different iso-miRNAs (isomiRs; miRNAs with sequence variations as compared to reference sequence) (Fukunaga et al., 2012; Lee et al., 2013). TRBP has also been shown to increase the rate of substrate recognition and the stability of DICER/substrate complexes (Chakravarthy et al., 2010).

Moreover, in addition to changing dicing kinetics, TRBP contributes to the assembly of large multiprotein complexes such as the RNA-induced silencing complex (RISC) and RISC-loading complex (RLC). In humans, DICER, TRBP and Argonaute-2 form the RLC (Wang et al., 2009). The DICER N-terminal helicase domain interacts with TRBP (MacRae et al., 2008), while the C-terminal catalytic domains are proximal to AGO. In a model proposed by Maniataki and Mourelatos, DICER, TRBP and Argonaute 2 form the RISC complex. DICER binds to and cleaves pre-miRNA. The resultant miRNA duplex is passed to AGO-2 where it directs the miRNA to its target mRNA (Maniataki and Mourelatos, 2005).

1.7.3 DICER and Angiogenesis

DICER is a critical regulator of endothelial sprouting and vessel growth. In vivo, DICER-deficient mice die during embryonic development after 12.5-14.5 days; showing impaired blood vessel formation (Yang et al., 2005). Kuehbacher and colleagues investigated the role of DICER in regulating angiogenic functions in endothelial cells by treating endothelial cells with siRNA

(Kuehbacher et al., 2007). Reduction in DICER expression significantly inhibited basic fibroblast growth factor (bFGF)-induced endothelial sprout and tube formation. DICER-siRNA knockdown also significantly reduced endothelial cell migration, impaired phosphorylation of Akt, and increased THBS1 protein expression 263% (Kuehbacher et al., 2007). Interestingly, in human cell clear cell renal cell carcinoma (ccRCC), DICER expression decreases cell migration, invasion and angiogenesis through suppression of MMP-2 and VEGFA expression (Chen et al., 2016). Additionally, down-regulation of DICER-1 via miR-107 increased the expression of endothelial cell-derived VEGF165, leading to endothelial cell tube formation and migration in HUVECs (Li et al., 2015). Altogether, this suggests that DICER plays a multi-faceted role in mediating angiogenesis and the effect differs among species and cell-types.

1.8 Murine double minute-2 (MDM2) and DROSHA

Recently the E3 ubiquitin ligase, murine double minute-2 (MDM2) has emerged as a potential regulator of DROSHA (Ye et al., 2015). MDM2 is a protein most well-known for its role as an important negative regulator of the tumour suppressor p53. Beyond its impact on p53 expression, our research team has identified MDM2 as a master regulator of the skeletal muscle microvasculature by controlling the expression of several angiogenesis-related genes, including VEGF-A and THBS1 (Aiken and Birot, 2016; Aiken et al., 2016a). In cancer cells, MDM2 is capable of upregulating VEGF-A expression by directly stabilizing VEGF-A mRNA (Zhou et al., 2011). Additionally, MDM2 can indirectly increase VEGF-A transcription through stabilization of hypoxia-inducible factor 1-alpha (HIF1α) in cancer cells (Bárdos et al., 2004; Carroll and Ashcroft, 2008; Ravi et al., 2000). Our research team has shown that MDM2 is an important regulator of VEGF-A production in the skeletal muscle, and reduction in MDM2 expression reduces basal muscle capillarization and impairs exercise-induced increase in VEGF-A expression

and the ability to increase capillarization (Roudier et al., 2012, 2013). Furthermore, MDM2 represses THBS1 expression (Aiken et al., 2016a) by interacting directly with Forkhead box protein O1 (FoxO1) (Aiken et al., 2016a; Milkiewicz et al., 2011; Roudier et al., 2012). Altogether, our research team has identified MDM2 as a master regulator of the angio-adaptive balance in skeletal muscle, by modulating the expression of several pro- and anti-angiogenic actors, particularly VEGF-A and THBS1.

In HEK293T cells, Myc-tagged DROSHA has been shown to bind MDM2, and MDM2-DROSHA binding facilitated DROSHA destabilization and ubiquitinylation (Ye et al., 2015). Complete starvation in glucose results in the stabilization of DROSHA. This mechanism appears to be dependent on mTOR, suggesting that an mTOR-MDM2 pathway could represent a crucial pathway to control miRNA biogenesis via DROSHA under glucose starvation (Ye et al., 2015).

1.9 Glucose and Endothelial Cells

Endothelial cells derive most of their energy through glycolysis (Krützfeldt et al., 1990; Quintero et al., 2006); therefore, it is not surprising that glucose availability and supply has a strong influence on the development and maintenance of the vascular network (Fraisl et al., 2009). High glucose conditions regulates MDM2 expression, function and activity in several different tissues and cell types including: kidney cells, β-cells, vascular smooth muscle cells, and skeletal muscles (Aiken et al., 2018; Barzalobre-Gerónimo et al., 2015; Xi et al., 2018; Ye et al., 2015), and in accordance with this, the expression of its targets, VEGF-A (Doronzo et al., 2012) and THBS1 (Maile et al., 2010) are also disturbed, resulting in a shift in the angiogenic balance. Prolonged exposure to high glucose gives rise to the development of imbalanced angiogenesis, endothelial dysfunction and poor vascular networks (Kolluru et al., 2012; Larger, 2003; Tahergorabi and Khazaei, 2012).

Recently, DROSHA was shown to be sensitive to glucose concentration, and glucose deprivation resulted in increased DROSHA protein expression in kidney cell lines (Ye et al., 2015). Endothelial cell miRNA expression is also influenced by glucose concentration and hyperglycemia-induced changes in miRNA expression can have profound diverse effects on endothelial cell function such as impairing efficient antioxidant response ((La Sala et al., 2016), suppression of endothelial cell inflammation (Tang et al., 2017; Yuan et al., 2017), and suppression of apoptosis (Bammert et al., 2017). Studies have demonstrated that dysregulation of angiomiRs may lead to abnormal angiogenesis and play an important role in the development of vascular diseases (Calin and Croce, 2006; Calin et al., 2002). Therefore, miRNAs have been proposed as markers for vascular diseases and a potential approach for endothelial injury therapy (Qu et al., 2018). As an example, blood serum miRNA-126 is downregulated in patients with impaired glucose tolerance (IGT) and impaired fasting glucose (IFG) (Liu et al., 2014). In endothelial progenitor cells, overexpression of miRNA-126 promotes proliferation, migration, and inhibited apoptosis while reduced expression resulted in impaired endothelial repair capacity (Qu et al., 2018). Following insulin, diet control and exercise, blood serum miRNA-126 increased significantly in IGT and IFG patients (Liu et al., 2014). These results support the proposal of using miRNAs as a biomarker for metabolic and vascular diseases.

1.10 Conclusion

As described above, MDM2 has recently emerged as a potential regulator of DROSHA (Ye et al., 2015). In HEK293T kidney cell lines, MDM2 interacts and ubiquitinates DROSHA. Complete starvation of glucose results in the stabilization of DROSHA. This mechanism appears to be dependent on mTOR suggesting that an mTOR-MDM2 pathway could represent a crucial pathway to control miRNA biogenesis via DROSHA.

To the best of our knowledge, it remains unknown whether the MDM2-DROSHA pathway can control how endothelial cells regulate angiomiR expression and the angiogenic balance in response to high glucose conditions. The rationale for this work comes from the potential of MDM2-mediated regulation of DROSHA and a gap in knowledge in the expression of the miRNA maturation machinery in endothelial cells. Since endothelial cells are the chief cell type involved in angiogenic response and endothelial dysfunction underlies a multitude of diabetic complications (Bitar, 2019; Kolluru et al., 2012; Kota et al., 2012; Muñoz-Chápuli et al., 2004; Tahergorabi and Khazaei, 2012), our study seeks to investigate the link between glucose concentration, the miRNA biogenesis machinery, and angiogenesis.

Section 2: Study

2.1 Introduction

The vascular system is comprised of a network of vessels that moves the blood throughout the body and plays an integral role in the maintenance of homeostasis (Pugsley and Tabrizchi, 2000). This vascular network delivers oxygen and nutrients to tissues, removes metabolic waste products, and provides a pathway for communications between distant cells in the body (Clegg and Mac Gabhann, 2015).

The network of vessels in the vascular system is divided into two broad categories: the macrovasculature and the microvasculature. The macrovasculature network is composed of the large blood vessels (arteries and veins) and regulate the bulk flow of blood throughout the body. The microvasculature is composed of a network of small vessels (the arterioles, venules, and capillaries) and is integral to the perfusion of the tissues. The capillaries, the smallest and most abundant blood vessels, serve as the interface for oxygen and nutrient delivery with the local tissues (Olfert et al., 2016).

The capillary network has been shown to exhibit extraordinary plasticity in response to physiological, metabolic and mechanical stress (Hoppeler, 2016). Physiological and pathological stressors such as changes in skeletal muscle activity, thermal stress, mechanical stress, nutrient supply, and hypoxia can lead to changes in the microenvironment of the capillary endothelium (Logsdon et al., 2014). Changes in the microenvironment can prompt vascular remodelling, leading to an expansion of the capillary network to improve its capacity for the exchange of gases, nutrients, and waste products (Haas and Nwadozi, 2015). This adaptation is known as angiogenesis

and enables the microvasculature to meet changes in the oxidative and metabolic demands of the tissues (Olfert et al., 2016).

Angiogenesis – the generation of new capillaries from pre-existing ones - is dependent on the balance and interplay of numerous pro- and anti-angiogenic factors. Shifts in this balance dictate the behaviour of the microvasculature, leading to capillary growth, maintenance or regression (Gustafsson, 2011; Olfert and Birot, 2011; Logsdon et al., 2014). An abundance of pro-angiogenic factors favours the growth of new capillaries while an excess of anti-angiogenic factors limits capillary growth and can potentially lead to the regression of the existing capillary network (Haas and Nwadozi, 2015).

Vascular endothelial growth factor-A (VEGF-A) and thrombospondin-1 (THBS1) are two well-established factors that regulate the angiogenic balance. VEGF-A is the most widely investigated pro-angiogenic molecule. VEGF-A promotes an array of angiogenic responses, including guiding capillary tip cell sprouting, capillary hyperpermeability, endothelial cell growth, endothelial cell migration, and enhanced glucose transport (Amoroso et al., 1997; Connolly, 1991; Shibuya and Claesson-Welsh, 2006; Vempati et al., 2014). Conversely, THBS1 is a potent anti-angiogenic molecule whose impact on angiogenesis has been extensively reviewed (Isenberg et al., 2009; Mirochnik et al., 2008; Zhang and Lawler, 2007). THBS1 can modulate VEGF-A activity, inhibit endothelial cell proliferation and migration and induce cell apoptosis (Iruela-Arispe et al., 2004; Zhang et al., 2009). The balance between these positive and negative angio-regulatory factors is integral in determining the overall angiogenic response (Arnold et al., 1987; Iruela-Arispe and Dvorak, 1997; Olfert, 2016; Zhang et al., 1994).

The angiogenic balance is tightly regulated by several complex mechanisms that control gene expression. One of the distinct mechanisms involved in the regulation of the angiogenic balance includes functional RNA molecules known as angiomiRs (Wang and Olson, 2009). AngiomiRs are a sub-category of molecules known as micro-RNAs (miRNAs), which promote or inhibit angiogenesis by regulating the protein expression of both positive and negative angio-regulatory factors (Wang and Olson, 2009). MiRNAs are a class of short, non-coding RNAs that regulate gene expression at the post-transcriptional level. In mammalian cells, miRNAs act on their targets by binding to the 3'-untranslated regions of their target mRNAs, leading to translational inhibition and mRNA destabilization (Bushati and Cohen, 2007; Wang and Olson, 2009). Functionally, miRNAs, along with DNA methylation and histone modification, are the main epigenetic actors known to regulate gene expression (Peschansky and Wahlestedt, 2014).

MiRNAs are involved in many physiological and pathological processes, including cell growth and proliferation, apoptosis, and carcinogenesis (Morales et al., 2017). The expression of miRNAs are tightly controlled by a series of cleavage events that regulate their maturation into their mature functional forms. The endogenous production and maturation of miRNAs are deeply dependent on two proteins: DROSHA and DICER (Ha and Kim, 2014).

Initially, miRNAs are transcribed from the genome or spliced from introns as a primary miRNA transcript (pri-miRNA). Subsequently, DROSHA, an RNase III-like enzyme will cleave the pri-miRNA into pre-cursor miRNA (pre-miRNA) (Lee et al., 2006). Following DROSHA cleavage, the pre-miRNA is exported out of the nucleus where it will be processed by DICER into its mature functional form (Macrae et al., 2006). Both DROSHA and DICER have been demonstrated to be critical to miRNA synthesis. Knockout of DROSHA in cancer cells led to complete abolishment of canonical miRNA production while DICER knockout resulted in

decreased miRNA production (Kim et al., 2016). While DICER function, activity, and selectivity has been extensively studied (Chavali et al., 2014; Hoffend et al., 2016; Ristori et al., 2015), very few studies have been conducted on DROSHA regulation of miRNAs expression. Previously, DROSHA has been shown to form a complex with HIF1α under hypoxic conditions. HIF1α: DROSHA interaction enhanced post-transcriptional miR-215 biogenesis in the glioma initiating cells (Hu et al., 2016). This finding suggests that DROSHA may display greater selectivity for their miRNA targets than previously believed.

MDM2 is a protein most well-known for its role as a negative regulator of the tumour suppressor p53. Beyond its impact on p53 expression, our research team has identified MDM2 as a master regulator of the skeletal muscle microvasculature by controlling the expression several angiogenesis-related genes, including VEGF-A and THBS1 (Aiken and Birot, 2016; Aiken et al., 2016). Recently the E3 ubiquitin ligase, MDM2 has emerged as a potential regulator of DROSHA (Ye et al., 2015). In cancer cells, MDM2 binds and ubiquitinates DROSHA when glucose is present in sufficient quantity, prompting the degradation of DROSHA.

The expression, function and activity of MDM2 has been reported to be impacted by high glucose conditions (Aiken et al., 2018; Barzalobre-Gerónimo et al., 2015; Xi et al., 2018; Ye et al., 2015). High glucose concentrations have also been reported to influence endothelial cell miRNA. High glucose-induced changes in miRNA expression can have a profound impact on endothelial cell function such as impairing efficient antioxidant response (La Sala et al., 2016), suppression of endothelial cell inflammation (Tang et al., 2017; Yuan et al., 2017), and suppression of apoptosis (Bammert et al., 2017). Studies have demonstrated that dysregulation of angiomiRs may lead to abnormal angiogenesis and play a vital role in the development of vascular diseases (Calin and Croce, 2006; Calin et al., 2002).

To date, the effect of high concentration of glucose on the MDM2-DROSHA-miRNA axis has not been examined. DROSHA and MDM2 are essential regulators of miRNA biogenesis and angiogenesis, respectively. This study aims to investigate the relationship between MDM2 and DROSHA to elucidate our understanding of the epigenetic regulation of miRNA biosynthesis in response to high glucose concentrations.

2.2 Hypotheses

1. Exposure to high glucose conditions will decrease angiomiR expression in human dermal microvascular endothelial cells, shifting the cells towards an angiostatic phenotype.
2. Exposure to high glucose conditions will increase the MDM2-DROSHA protein interaction, resulting in the ubiquitination and degradation of DROSHA. This leads to suppression in the expression of all canonical miRNAs.

2.3 Study Objectives

1. To examine the effect of high glucose treatment on angiomiR expression in primary human dermal microvascular endothelial cells.
2. To investigate whether the activity of the miRNA maturation machinery controlling angiomiR expression (DROSHA and DICER) activity is modified by high glucose treatment.
3. To investigate MDM2 as a potential mechanistic actor in high glucose-induced endothelial angiomiR expression through its regulation of DROSHA protein expression.

2.4 Materials and Methods

Cell Culture

Primary human dermal microvascular endothelial cells (HDMECs) were purchased from ScienCell Research Laboratories (cat# 2000; Carlsbad, CA, USA). The cells were maintained in endothelial cell media (ECM) (Sciencell; cat# 1001) supplemented with 5% FBS (Sciencell; cat# 0025), 1% endothelial cell growth supplement (Sciencell; cat# 1052), and antibiotic solution containing 100 units/mL penicillin and 100 µg/ml streptomycin (Sciencell; cat# 0503). HDMECs were cultured until they reached passage numbers 6-8 before treatment with D-glucose or D-mannitol.

Glucose and Mannitol Treatment

Cell cultures were plated on 6-well dishes (Starstedt; cat# 83.3920.005) or 60 mm cell culture dishes (Starstedt; cat# 83.3901) at a density of 125,000 cells per well or 300,000 cells per dish. Once the cells reach approximately 80% confluence, 10% D-glucose solution (Sigma-Aldrich; cat# 47829) or 10% D-mannitol (Sigma-Aldrich; cat# M4125-100G) solution in ddH_2O was added to the wells/dishes to bring the total glucose/mannitol concentration to 5, 10, 20, 30 or 40mM. Cells were incubated for 3h, 6h and 24h at 37°C and 5% CO_2 before harvesting.

MDM2 Inhibitor Treatment

HDMECs were treated with 10 µM Nutlin-3 (Sigma-Aldrich, cat# N6287-5MG), 10 µM MX69 (Selleckchem; cat# S8403) or 10 µM RG-7112 (Selleckchem; cat# S7030) for 1h before glucose treatment. Cells were subsequently treated with 5 or 30mM glucose for 6 or 24h before cell lysis.

Db/Db Mice Model

Gastrocnemius muscles harvested from db/db mice were obtained courtesy of Dr. Tara Haas' lab. The db/db mouse model of leptin receptor deficiency is currently the most widely used mouse model of type 2 diabetes (Kobayashi et al., 2000; Mohammed-Ali et al., 2017). Db/db mice have a mutation in the gene encoding for the leptin receptor and genetic susceptibility to diabetic complications (Alpers and Hudkins, 2011). Mice were grown until 4 or 13 weeks of age before they were sacrificed and the gastrocnemius and tibialis anterior were harvested. Prior to muscle collection, common femoral artery ligation (4 days) was performed on one of the legs. The non-ligated muscle was used in this study. All mice used in this study were male and littermates. Resting blood glucose data for the 4-weeks old mice were provided by Dr. Tara Haas' lab.

Cell Culture Protein Extraction

Cells protein homogenates are obtained by harvesting cells in lysis buffer consisting of tris base 50mM, NaCl 100mM, EDTA 5mM, Sodium Deoxycholate 1%, Triton X-100 1%, pH 8.0, and 1mM PMSF protease inhibitor, phosphatase inhibitors (NaF 1mM, Na_3VO_4 1mM), a protease inhibitor cocktail (Roche Diagnostics; cat# 04906845001) and a phosphatase inhibitor cocktail (Roche Diagnostics; cat# 11836153001). The cell lysate was incubated in the lysis buffer for 20 minutes at 4℃, centrifuged at 16000g for 15 minutes and the supernatant was collected and stored at -80℃ until further analysis.

Protein Concentration Determination

Total protein concentration of cell lysates was determined using the bicinchoninic acid (BCA) assay. BCA working reagent was prepared with a bicinchoninic acid solution (Sigma-Aldrich; cat#

B9643) and copper (II) sulfate solution (Sigma-Aldrich; cat# C2284). Samples were incubated at 37℃ for 30 minutes before absorbance measurement at 562 nm (Gen5 plate reader, Biotek).

Immunoblot Analysis

Immunoblot analyses were conducted on protein extracts from mice gastrocnemius muscles and primary HDMECs. Blots were probed with the following primary antibodies: Mdm2 clone SMP14 (Santa Cruz Biotechnology; sc-965), Mdm2 clone 2A10 (non-commercial), p-Ser166-Mdm2 (Cell Signaling Technology; cat# 3521), Drosha (Cell Signaling Technology; cat# 3364S), Dicer (Cell Signaling Technology; cat# 5362), Argonaute 1 (Cell Signaling Technology; cat# 5053), Argonaute 2 (Cell Signaling Technology; cat# 2897), THBS1 (Invitrogen; clone A6.1, cat# MA5-13398), VEGF (Santa Cruz Biotechnology; cat# sc-507), αβ-tubulin (Cell Signaling Technology; cat# 2148), and β-actin (Santa Cruz Biotechnology; cat# sc-47778). After incubation with secondary antibody [horseradish peroxidase (HRP)-linked anti-rabbit antibody, (Cell Signaling Technology; cat# 7074), HRP-linked anti-mouse antibody (Dako, cat# P0260); or light chain specific HRP-linked anti-mouse antibody (Cell Signaling Technology; cat# 55802), proteins were visualized with enhanced chemiluminescence (ThermoFisher Scientific or Millipore) on Imaging Station 4000MM Pro (Carestream Health) or X-ray film (CL-XPosure Film, #34090). Blots were analyzed with Carestream software.

Immunoprecipitation

HDMECs were treated with 20 μM MG132 (EMD Millipore, cat# 474790-5MG) and 5mM or 30mM glucose for 6h before cell lysis, to block proteasomal degradation. Drosha was immunoprecipitated by overnight incubation (Santa Cruz Biotechnology, sc-393591 AC). Normal mouse (Santa Cruz Biotechnology, sc-2342) IgG was used as a species-specific control primary

antibody. Cell lysates were precleared with normal mouse IgG for 30 minutes before overnight incubation with Agarose-Conjugated Antibody complex. Supernatants were analyzed by Western blot for Drosha (Cell Signaling Technology; D28B1, cat# 3364), Mdm2 (Santa Cruz Biotechnology; SMP14, cat# sc-965, or non-commercial 2A10), and P-MDM2-Ser-166 (Cell Signaling Technology; cat# 3521).

Cell Culture RNA Isolation

Cell culture media was taken from HDMEC after 24 hours of glucose treatment. Cells were lysed and total RNA was collected using the QIAzol Lysis Reagent (Qiagen). RNA was isolated using the miRNeasy Micro Kit (Qiagen; cat# 217004). 1000 fmol of cel-mir-39a was added to each RNA isolate to be used as an exogenous control for mature miRNA.

RT-qPCR

The RNA isolates were quantified and their purity was evaluated with a spectrophotometer using cDNA that was synthesized using High-Capacity RNA-to-cDNA Kit (ThermoFisher Scientific; cat# 4387406) or Taqman® Advanced miRNA cDNA Synthesis Kit (ThermoFisher Scientific; cat# A28007), for mRNA and pri-miRNA or mature miRNA, respectively according to manufacturer's protocol. Real-time PCR was conducted in triplicate using TaqMan® Gene Expression, TaqMan® MicroRNA or TaqMan® Advanced MicroRNA Assays and a TaqMan® Universal Master Mix II for 40 cycles of denaturation at 95°C for 3s, and annealing and extension at 60°C for 30s. DROSHA, DICER, THBS1, VEGF-A, MDM2, pri-miRNA-126, pri-miRNA-17-92, pri-miRNA-15, MIR-126-3p, MIR-126-5p, MIR-18a, and MIR-15a were measured. Data were normalized with the HPRT gene (for mRNA and pri-miRNA transcripts) and cel-mir-39a (for mature miRNA).

Statistical analysis

Statistical analyses were performed with Student's *t*-test and 1- and 2-way ANOVAs with Prism5 (GraphPad, San Diego, CA, USA). For 1- and 2-way ANOVAs, Bonferroni *post hoc* tests and Fisher's LSD's multiple comparisons were used, respectively. Correlation analyses between variables were performed with nonparametric 2-tailed Pearson-correlation with the coefficient of determination r and r^2. $P < 0.05$ was considered to be statistically significant.

2.5 Results

High glucose conditions abrogate VEGF protein expression in endothelial cells

To examine the effect of high glucose concentration on angiogenic balance, we incubated human dermal microvascular cells (HDMECs) under high (30mM) and normal (5mM) glucose concentrations for 24 hours and measured the expression of THBS1 and VEGF-A protein and mRNA. Western blotting revealed a small increase in THBS1 protein expression (+14%); however, this change was not significant ($p=0.075$) (Fig. 3A). Surprisingly, the expression of VEGF protein was completely abrogated in endothelial cells treated with 30mM glucose (Fig. 3B). Next, we assessed the expression of THBS1 and VEGF-A mRNA in HDMECs. High glucose treatment did not significantly change either THBS1 ($p=0.156$) or VEGF-A ($p=0.477$) mRNA expression (Fig. 3C, D). Together, these findings suggest that high glucose conditions shift the angiogenic balance by repressing expression of VEGF-A protein and the mechanism of repression occurs post-transcriptionally due to a lack of change in VEGF-A mRNA expression.

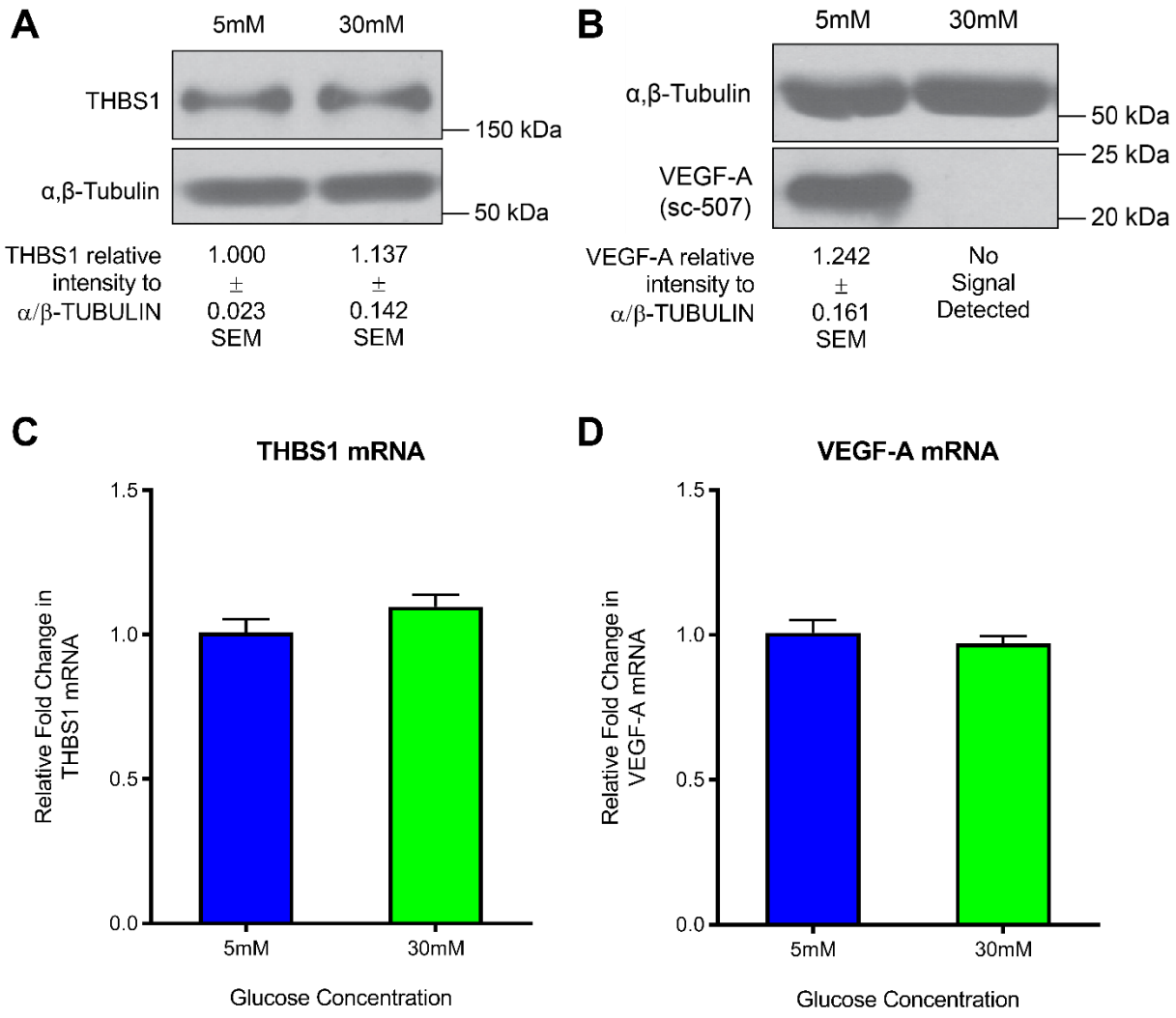


Figure 3. High glucose conditions decrease VEGF-A protein expression in human dermal microvascular endothelial cells (HDMECs). Immunoblots of (A) THBS1 and (B) VEGF-A after 24h treatment under normal (5mM) or high (30mM) glucose conditions (n=6). α,β-Tubulin was used as a loading control. mRNA levels for (C) THBS1 and (D) VEGF-A in HDMEC (n=9), relative to the *HPRT* housekeeping gene.

High glucose conditions shift endothelial cell angiomiR expression towards an anti-angiogenic profile

MicroRNAs are small non-coding RNAs that regulate gene expression post-transcriptionally. To investigate the theory that angiomiRs are mechanistic actors underlying the observed change in the THBS1 and VEGF-A balance, we measured the expression of the miRNA that regulates their expression and signalling: miRNA-126, miRNA-15a, and miR-18a.

In accordance with the observed repression of VEGF-A protein, the expression of the anti-angiogenic miR-15a is significantly upregulated (+211%) in endothelial cells under high glucose concentrations (1.05 ± 0.15 SEM vs. 3.26 ± 0.49 SEM, $p \leq 0.01$). The pro-angiogenic miRNA-18a, whose predicted target genes include THBS1, did not significantly change between normal (1.03 ± 0.18 SEM) and high glucose (1.01 ± 0.14 SEM) conditions. Conversely, expression of miRNA-126-3p and miRNA-126-5p, both of which exert pro-angiogenic activity via reinforcement of VEGF signalling, are significantly repressed under high glucose conditions. High glucose conditions decreased miRNA-126-3p by 39% (1.01 ± 0.11 SEM vs. 0.62 ± 0.08 SEM, $p \leq 0.05$) and miRNA-126-5p by 30% (0.97 ± 0.09 vs. 0.67 ± 0.09, $p \leq 0.05$) (Fig. 4A). To investigate whether these observed changes in miRNA expression are attributable to a change in transcription, we measured the expression of their corresponding pri-miRNA transcripts. There was no significant change in the expression of pri-miRNA-126, pri-miRNA-17-92 and pri-miRNA-15 (Fig. 4B). This absence of change supports the notion that the observed change in miRNA expression could be due to changes in maturation processing. These observations suggest that high glucose conditions induce a shift towards an anti-angiogenic miRNA profile.

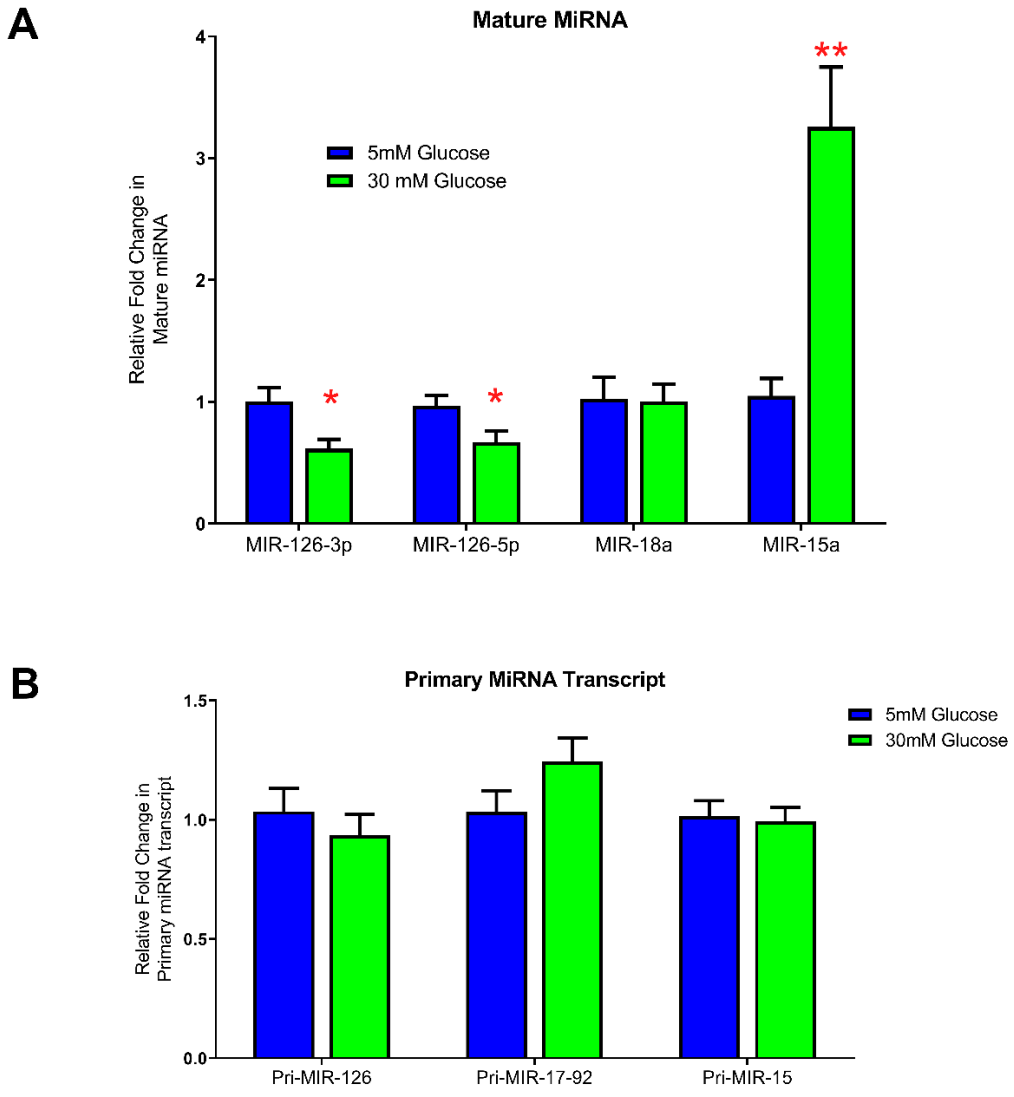


Figure 4. High glucose treatment shifts endothelial cell angiomiR expression towards an anti-angiogenic phenotype. (A) Mature miRNA and (B) primary-miRNA levels (means ± SEM) relative to *HPRT* housekeeping gene and exogenous spike-in cel-mir-39a, respectively (n=6). Student's t-test shows no effect of glucose concentration on the expression of pri-MIR-126, pri-MIR-17-92, and pri-MIR-15. High glucose concentration (30mM) decreases MIR-126-3p and MIR-126-5p, *P ≤ 0.05, and increases MIR-15a expression, **P≤ 0.01, compared to normal glucose (5mM) control.

High glucose conditions repress DROSHA protein expression

In order to investigate the mechanism underlying the altered miRNA expression, we measured the expression of the proteins involved in miRNA biogenesis and maturation. We found no significant effect of glucose concentration on DICER protein expression (Fig. 5A). Protein expression of AGO-2 protein expression also remained unchanged under high glucose conditions (Supplemental Fig. 1). Conversely, exposure to high glucose concentration for 24 hours significantly reduces DROSHA protein expression by 32% (1.00 ± 0.05 SEM under normal glucose vs. 0.69 ± 0.05 SEM under high glucose, $p \leq 0.0001$) (Fig. 5B). Assessment of mRNA expression revealed that both DICER and DROSHA mRNA levels did not significantly change in endothelial cells subjected to high glucose concentrations (Fig. 5C, D). These findings reveal that DROSHA protein expression is repressed under high glucose concentrations and suggest that the mechanism of action underlying this observed reduction occurs at the post-transcriptional level.

To further examine the effect of glucose on DROSHA and DICER protein expression, endothelial cells were treated with different concentrations of glucose for 24 hours (Fig. 6A). There is a significant effect of glucose concentration on DROSHA expression at $p \leq 0.05$ [$F(4,35) = 10.19$, $p \leq 0.0001$], suggesting that the glucose-mediated suppression of DROSHA is dose-dependent. No significant effect of glucose concentration on DICER protein expression was observed ($p=0.669$) (Fig. 6B).

Further analysis of endothelial cell cultures treated with glucose or an osmotic control reagent (D-mannitol) suggests that the effect of glucose on DROSHA expression is independent of osmotic pressure. As expected, endothelial cells treated with glucose showed a 30% decrease in DROSHA protein at 24H compared to untreated controls (0.76 ± 0.05 SEM vs 0.53 ± 0.03, $p=0.002$) (Fig. 7B). However, DROSHA protein expression remained unchanged when treated

with D-mannitol. D-glucose significantly reduced DROSHA protein expression compared to D-mannitol treatment after both 6 and 24H (6H; -39%, p=0.03 and 24H; -29%, p=0.003) (Fig. 7A, B). Therefore, DROSHA protein expression is sensitive to glucose concentration but not osmotic pressure.

Lastly, to explore the temporal effect of exposure to high concentrations of glucose on endothelial cell DROSHA protein expression, HDMECs were treated with high glucose for 3, 6 and 24H hours. There is a temporal effect on glucose-mediated downregulation of DROSHA protein [$F(2,12) = 5.12$, $p = 0.025$]. A significant decrease is observed after 6H (-21%, p=0.047) and 24H (-33%, $p = 0.002$) (Fig. 7C); however, no significant change in DROSHA protein expression is observed after 3H.

Altogether, our findings suggest that exposure to high glucose conditions decrease DROSHA protein expression in a concentration-dependent manner. This effect is independent of the effect of osmotic stress (as measured by exposure to 30mM D-mannitol) and is observable after 6H incubation. Exposure to high glucose concentrations do not appear to affect DICER protein expression.

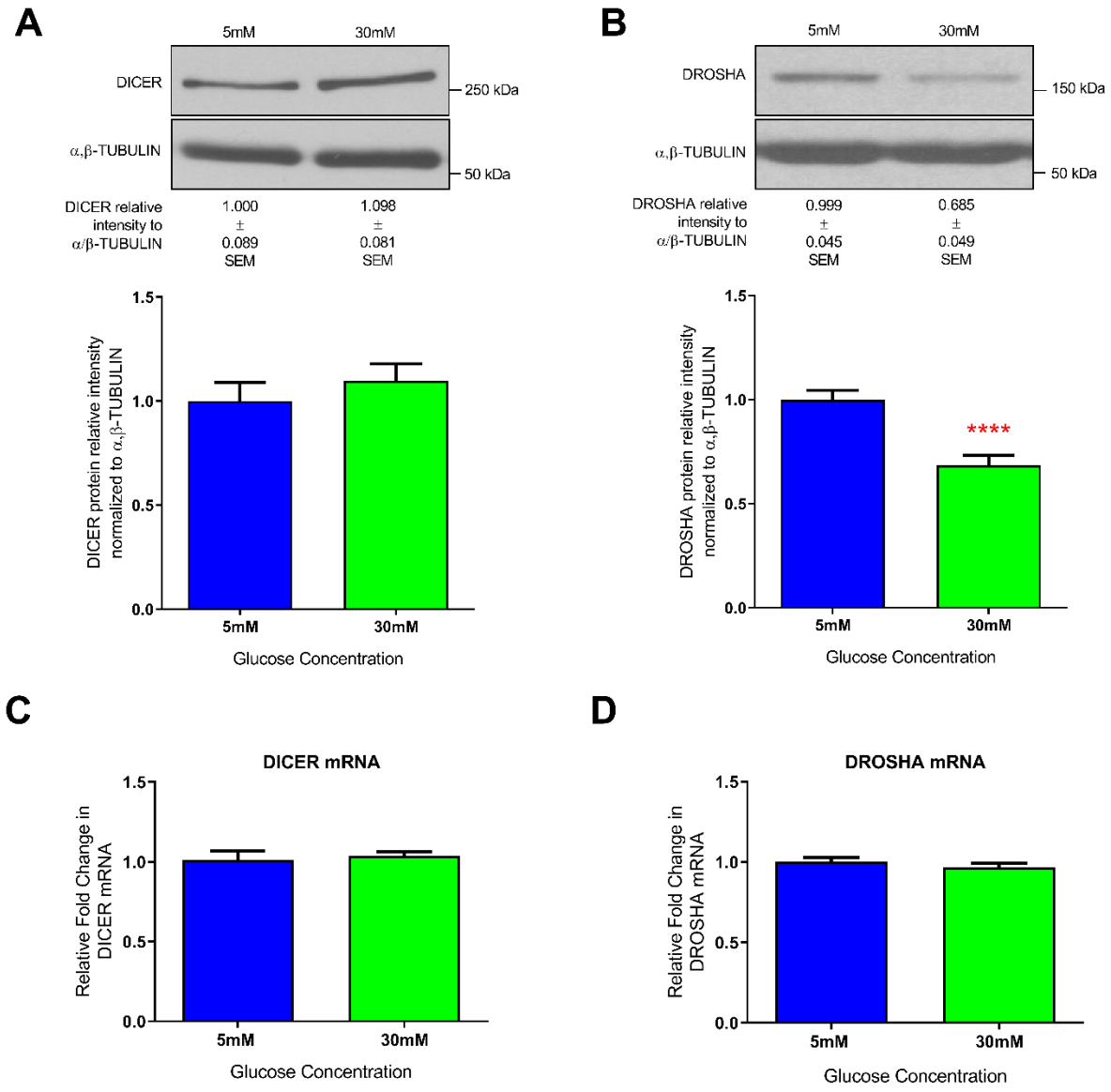


Figure 5. Effect of high glucose concentration on DROSHA and DICER protein and mRNA levels in HDMECs. Immunoblots of (A) DICER and (B) DROSHA in HDMECs following 24H treatment with high (30mM) or normal (5mM) glucose concentrations (Means± SEM) (n=12). α,β-Tubulin was used as a loading control. Expression of (C) DICER and (D) DROSHA mRNA (means ± SEM) relative to *HPRT* housekeeping gene (n=12). High glucose concentration (30mM) decreases DROSHA protein expression, ****$P \leq 0.001$.

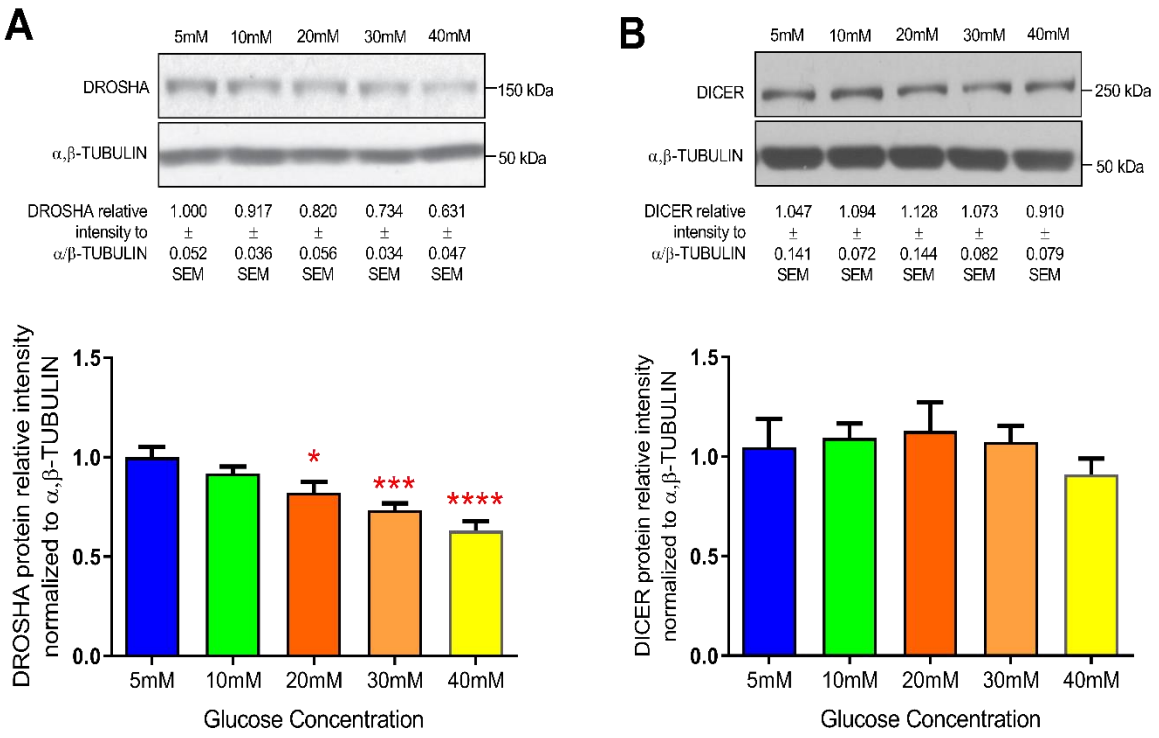


Figure 6. Differential effect of increasing glucose concentration on DROSHA and DICER protein levels. Immunoblots of (A) DROSHA and (B) DICER in HDMECs treated with different concentrations of glucose for 24H (Means± SEM) (n=8). α,β-Tubulin was used as a loading control. Downregulation of DROSHA protein expression appears to be dependent on glucose concentration. DROSHA protein is significantly reduced at 20mM ($P \leq 0.05$), 30mM (***$P \leq 0.001$), and 40mM (****$P \leq 0.0001$), compared to normal glucose (5mM) controls.

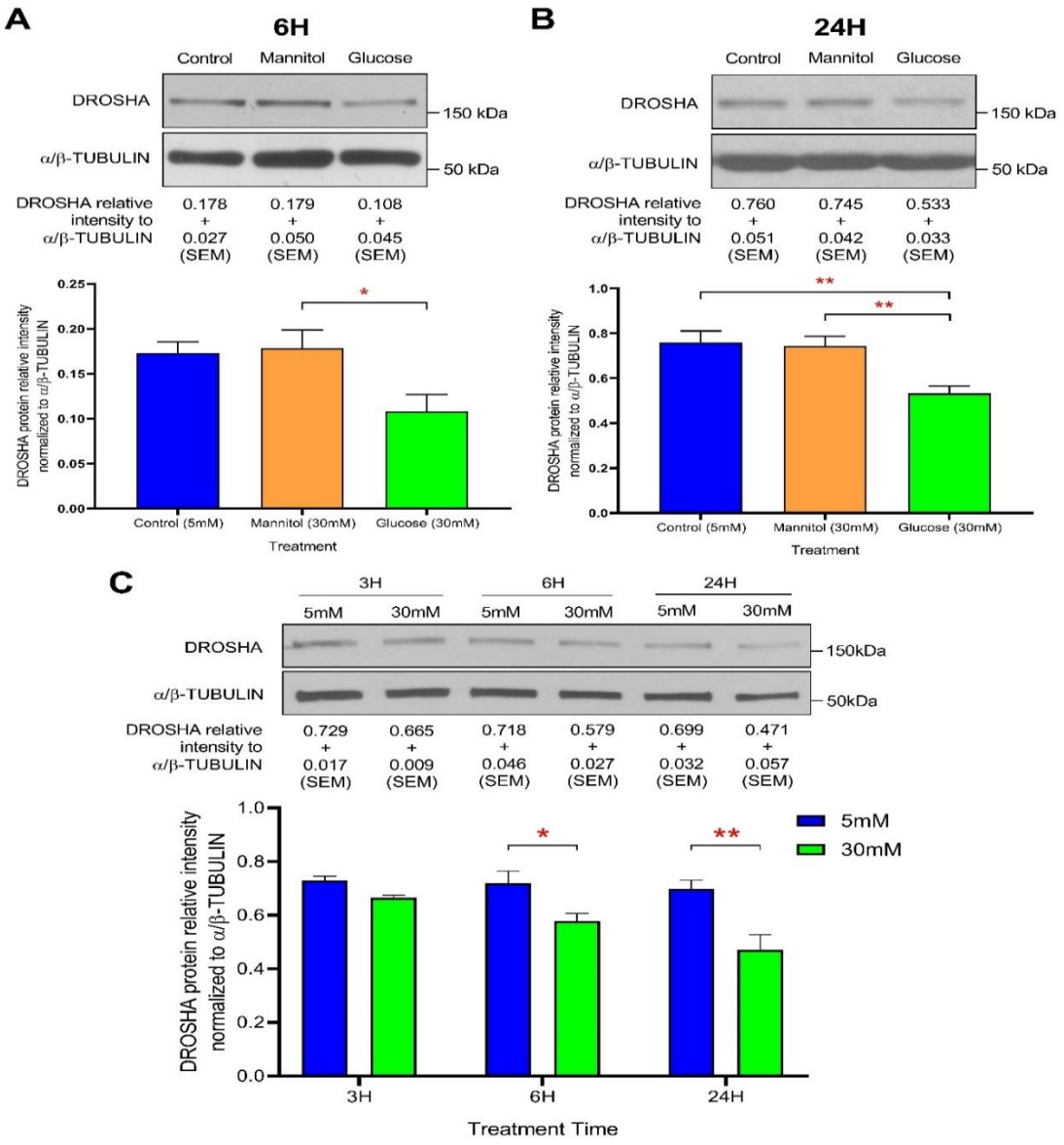


Figure 7. Time effect of glucose and mannitol on DROSHA protein levels in HDMECs. Immunoblots of DROSHA after (A) 6H or (B) 24H treatment with normal glucose (5mM), high glucose (30mM) or mannitol (30mM) (n=6). Treatment with D-mannitol was used as an osmotic control. High glucose concentration decreased DROSHA protein expression after 24H (**P ≤ 0.01), but not after 6H (p=0.056). Expression of DROSHA protein is significantly decreased in high glucose treated cells as compared to mannitol treated cells after 6H (*P ≤ 0.05) and 24H (**P ≤ 0.01). (C) DROSHA protein expression after 3, 6, and 24H exposure to 5mM and 30mM glucose (n=3). Glucose exposure decreases DROSHA expression after 6H (*P ≤ 0.05) and 24H (**P≤ 0.01).

High glucose conditions upregulate MDM2 protein expression and enhances MDM2 binding of DROSHA

To test the hypothesis that MDM2 activity could underlie high glucose-mediated DROSHA protein repression, we measured the expression of MDM2 and p-Ser166-MDM2 in glucose treated endothelial cells. After 24H incubation under high glucose concentration, MDM2 protein expression is upregulated (+62%) in endothelial cells (normal vs high glucose, 1.00 ± 0.13 SEM vs 1.62 ± 0.14, p=0.005) (Fig. 8A). In agreement with this, gene expression analysis of showed greater expression of MDM2 mRNA after high glucose treatment compared to normal glucose control (normal vs high glucose, 1.00 ± 0.04 SEM vs 1.87 ± 0.31, p=0.02); suggesting that the increase in MDM2 protein expression may be a result of increased MDM2 transcription (Fig. 8B). There was no change in p-Ser-166-MDM2 expression under high glucose conditions (p=0.928) (Fig. 8C); an in conjunction with this, the ratio of p-Ser166-MDM2 to MDM2 protein is significantly reduced under high glucose conditions (p=0.004) (Fig. 8D).

Next, we examined whether high glucose concentration changes the ability of MDM2 to bind DROSHA in endothelial cells. The ratio between the levels of MDM2 and DROSHA that were co-immunoprecipitated was greatly enhanced in cells treated with high glucose compared to normal glucose controls (0.12± 0.01 SEM vs. 0.02 ± 0.001 SEM, respectively). This finding suggests an approximately 7-fold increase in MDM2-DROSHA interaction under high glucose conditions (Fig. 8E). Interestingly, no differences were observed in p-Ser166-MDM2-DROSHA binding (Supplemental Fig. 2). This suggests that DROSHA binds preferentially to the unphosphorylated form of MDM2 on residue Serine 166.

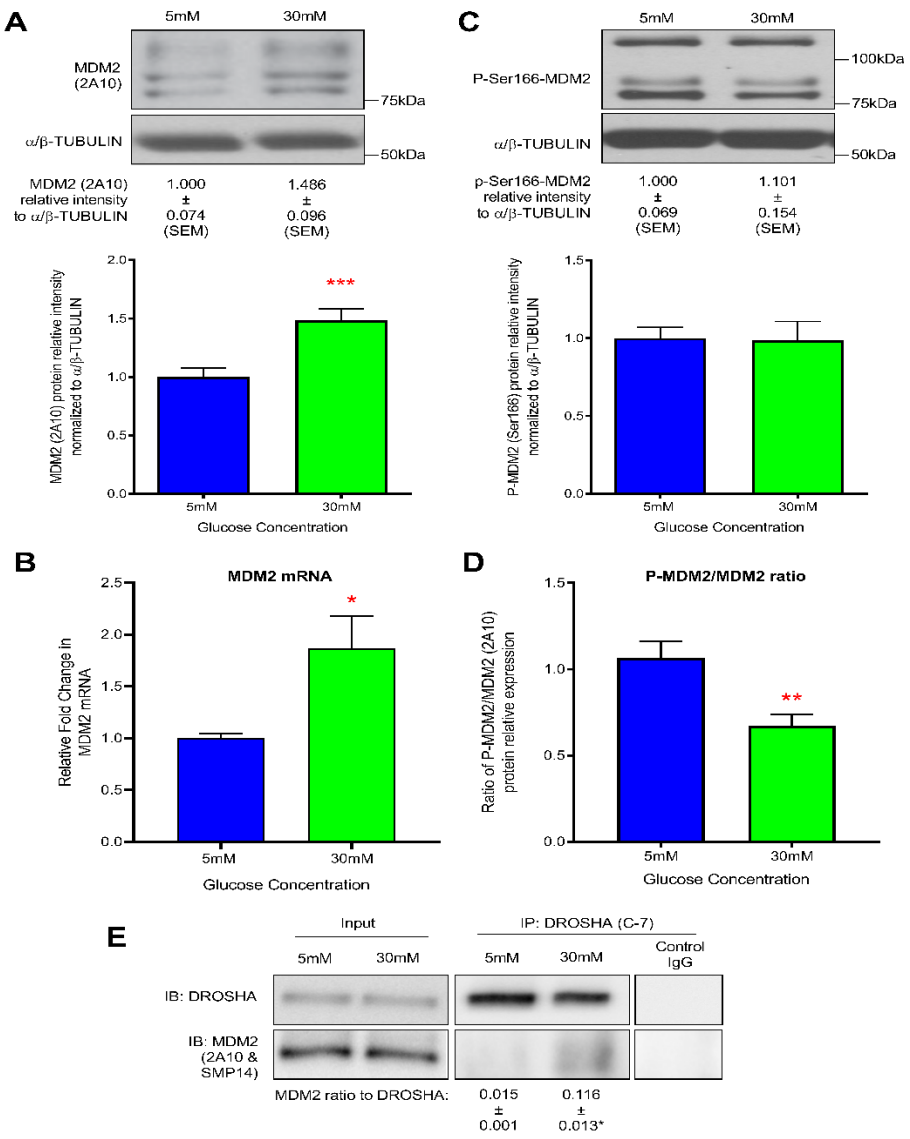


Figure 8. High glucose concentrations upregulate MDM2 expression in HDMECs and enhances MDM2-DROSHA interaction. Immunoblot of (A) MDM2 and (C) p-Ser-166-MDM2 following 24H treatment with high (30mM) or normal (5mM) glucose concentrations (Means± SEM) (n=14). Expression of (B) MDM2 mRNA (means ± SEM) relative to *HPRT* housekeeping gene (n=9). (D) Ratio of MDM2:p-Ser-166-MDM2 protein expression. (E) After immunoprecipitation (IP) of DROSHA in glucose treated HDMECs treated with MG132, levels of MDM2 and DROSHA were measured by immunoblot analysis in whole-cell lysate (input) and in the IP products (n=1 per group). Normal mouse IgG was used as a control. The MDM2:DROSHA ratio expressed in raw values between 5mM glucose and 30mM glucose conditions is indicated.

Competitive inhibition of MDM2 with Nutlin-3 and RG7112 repeals glucose-mediated downregulation of DROSHA

To further examine the role of MDM2 in repressing DROSHA protein, we measured the expression of DROSHA protein in endothelial cells treated with three different MDM2 inhibitors (MX69, Nutlin-3, and RG7112) for 1 hour prior to high and normal glucose treatment. A two-way ANOVA was conducted to compare the effect of MDM2 inhibition on DROSHA protein expression in endothelial cells under high and normal glucose conditions.

Two-way analysis of variance yielded a significant main effect for MDM2 inhibition [$F(3, 38) = 4.31$, $p=0.01$] on DROSHA protein expression after exposure to high and normal glucose for 6H (Figure 9A). Consistent with our previous results, high glucose concentrations repressed endothelial cell DROSHA expression by approximately 28% in the endothelial cells that were not treated with any MDM2 inhibitor ($p=0.04$). Treatment with Nutlin-3 repeals high glucose-mediated DROSHA protein suppression at after 6H (Nutlin-3 vs. untreated; high glucose, 0.76 vs. 0.48, $p = 0.001$). RG7112 (a member of the nutlin family) treatment also repeals high glucose mediated DROSHA protein expression (RG7112 vs. untreated; high glucose, 0.70 vs. 0.48, $p = 0.02$). However, MX69 treatment did not significantly change DROSHA protein expression ($p=0.462$).

There was a significant main effect of glucose concentration [$F(1, 37) = 5.35$, $p = 0.03$] on DROSHA protein expression after 24H (Fig. 9B). Consistent with our previous results, high glucose concentrations repressed endothelial cell DROSHA expression by approximately 40% in samples that were not treated with MDM2 inhibitors ($p=0.02$). However, there was no significant main effect of MDM2 inhibition on DROSHA protein expression ($p=0.733$).

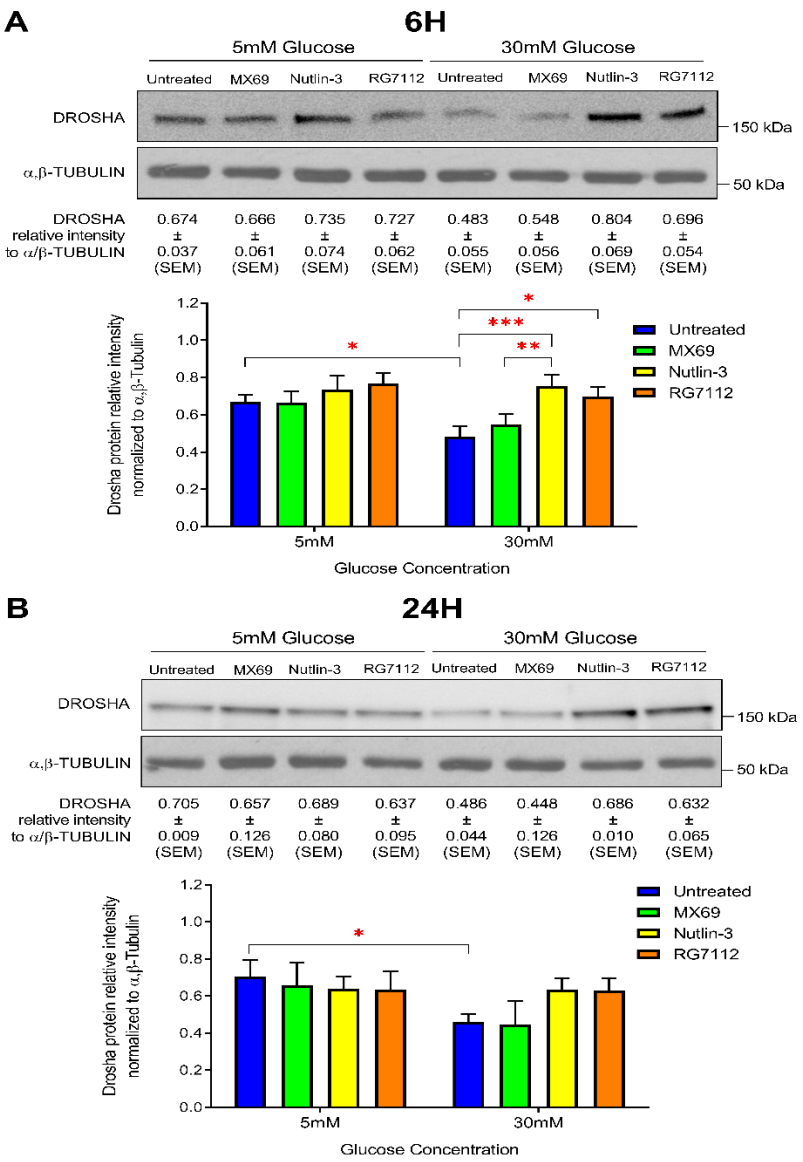


Figure 9. The MDM2 inhibitors: Nutlin-3 and RG7112, restore DROSHA protein expression under high glucose concentrations. HDMECs were treated with 30mM or 5mM glucose and an MDM2 inhibitor for 6 and 24H. Immunoblots of DROSHA after (A) 24H and (B) 6H treatment with MX69, Nutlin-3 and RG7112 under normal (5mM) and high (30mM) glucose concentrations (n=5). α,β-Tubulin was used as a loading control. Fisher's LSD multiple comparisons was used to compare the means of the different conditions. After 6H, Nutlin-3 and RG7112 treatment repeals high glucose-induced DROSHA repression (***$P \leq 0.001$ and *$P \leq 0.05$, respectively). MX69 treatment does not affect DROSHA expression in HDMECs.

DROSHA protein expression correlates with resting blood glucose

Lastly, to compare our in vitro findings to a physiological model, we measured DROSHA and MDM2 protein expression in db/db mice skeletal muscle. In the db/db mice model, the mice harboured a mutation in the gene encoding the leptin receptor, resulting in leptin-deficient mice that have a genetic background that is susceptible to hyperglycemia. Consistent with what we observed in primary endothelial cells, a similar decrease in DROSHA expression was observed in db/db mice skeletal muscle (gastrocnemius). DROSHA protein is reduced by approximately 32% in leptin-deficient (db/db) mice compared to wild-type (wt/wt) controls (leptin-deficient vs. wild-type, 0.36 ± 0.03 SEM vs 0.53 ± 0.05 SEM, p=0.02) (Fig. 10A). Surprisingly, we did not observe a significant change in MDM2 protein expression in db/db mice (Fig. 10B).

To further elucidate the relationship between resting blood glucose and DROSHA and MDM2 expression, we measured DROSHA and MDM2 protein in 4-week old heterozygous (db/wt) mice skeletal muscle. Unlike the homozygous (db/db) mice, these mice exhibit normal body weight, blood glucose and plasma insulin; however, they display increased metabolic efficiency (Ritskes-Hoitinga et al., 2012). In heterozygous (db/wt) mice gastrocnemius muscle, DROSHA protein expression is correlated negatively with increasing levels of blood glucose (Fig. 7C, $r^2 = 0.509$; $P \leq 0.01$) (Fig. 10C). There was a trend toward a negative correlation between blood glucose level and MDM2 protein expression however, this correlation was not significant (p=0.11) (Fig. 10D).

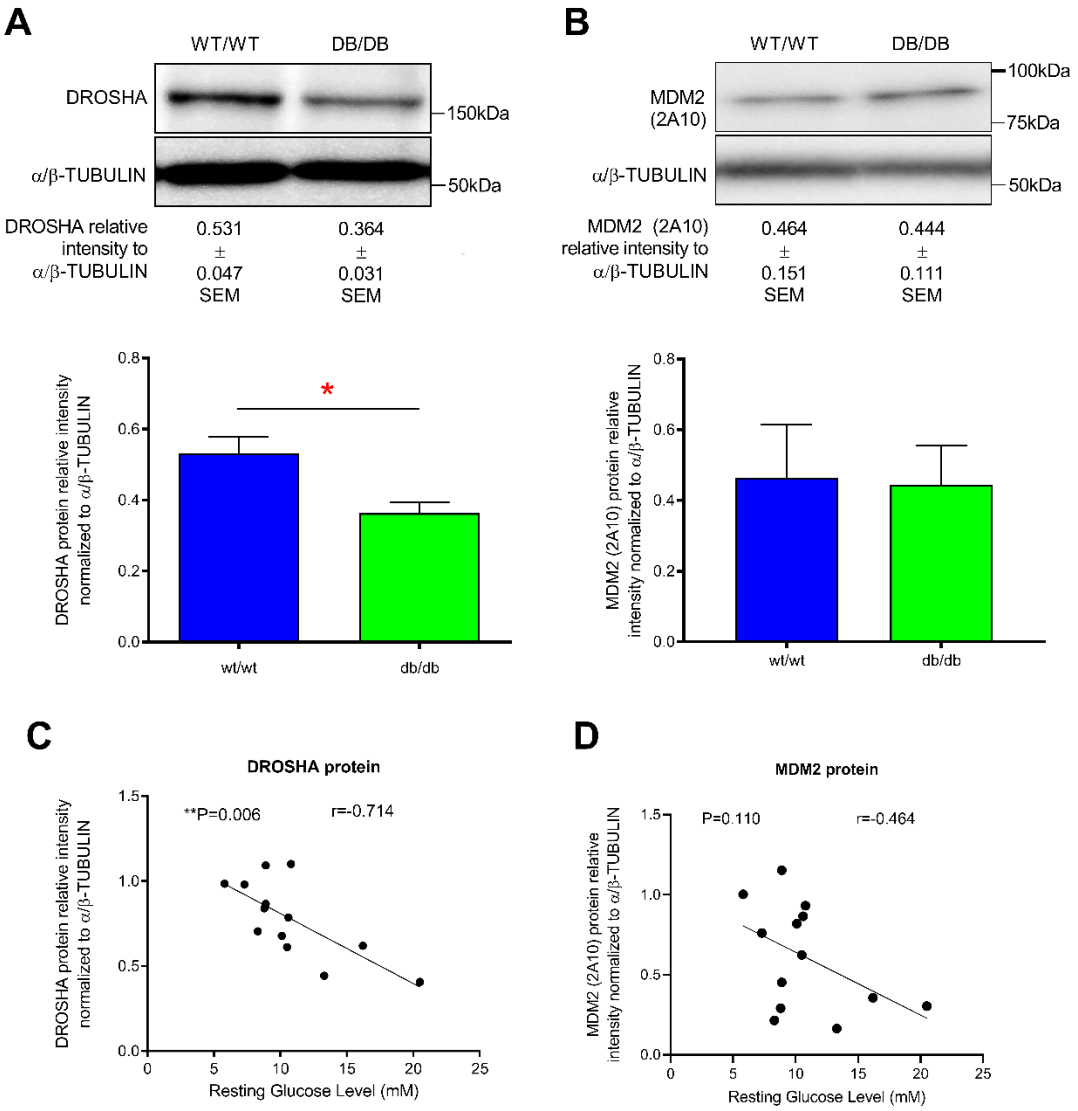


Figure 10. Blood glucose levels are strongly correlated with DROSHA protein in rodent skeletal muscle. Immunoblots of (A) DROSHA and (B) MDM2 (2A10) in wild-type (wt/wt) and leptin receptor deficient (db/db) mice (means± SEM) (n=5). α,β-Tubulin was used as a loading control. Correlation analysis between (C) blood glucose and DROSHA, and (D) blood glucose and MDM2 protein levels in heterozygous (db/wt) mice gastrocnemius muscle (n=13). The db/db mice expressed significantly less DROSHA protein compared to their wild-type littermates (*P ≤ 0.05). Resting blood glucose is negatively correlated with DROSHA protein expression in mice skeletal muscle (**P≤ 0.01).

2.6 Discussion

Hyperglycemia is a major risk factor for vascular complications in diabetes (Sena et al., 2013). Under hyperglycemic conditions, endothelial cells - the cells that line the lumen of the vasculature - become dysfunctional and undergo apoptosis (Popov, 2010). MiRNAs are small, non-coding, endogenous RNAs that can promote or inhibit angiogenesis by regulating the protein expression of both positive and negative angio-regulatory factors (Wang and Olson, 2009). Recently, circulating miRNAs have emerged as novel mediators, potential biomarkers and therapeutic targets for vascular complications in diabetes (Alexandru et al., 2016). Hyperglycemia-induced changes in miRNA expression were found to be involved in endothelial dysfunction and EC apoptosis (Silambarasan et al., 2016; Wang et al., 2019). In this manner, targeting the expression of specific miRNAs has been proposed as a therapeutic approach for improving blood flow in diabetics (Chen et al., 2017).

Though there have been many studies characterizing the functions of specific miRNA and their response to different stressors, to the best of our knowledge, the impact of high glucose concentration on the miRNA biogenesis machinery in endothelial cells has not been studied. DROSHA and DICER are proteins that are essential for the maturation of microRNAs (O'Brien et al., 2018; Treiber et al., 2018) and silencing of both DROSHA and DICER expression has been shown to negatively impact angio-adaptation in the vasculature (Kuehbacher et al., 2007). Our study was designed to investigate the influence of glucose concentration on the expression of DROSHA and DICER and the resulting impact on the endothelial cell angiogenic balance.

In this study, primary HDMECs were used to study the effect of high glucose on endothelial cells. Primary cells express the same characteristics and functions as seen in vivo (Alge et al., 2006; Pan et al., 2009); as such, observations in this model may be extrapolated to predict in vivo

endothelial cell behaviour. Previous studies conducted by our research team also revealed that HDMECs exhibit similar angiogenic responses to other endothelial cells: human adipose microvascular endothelial cells (HAMECs) and rodent skeletal muscle endothelial cells (rSMECs) when exposed to an angiogenic stimulus ($VEGF_{165}$) (Aiken et al., 2016a). These qualities distinguish HDMECs as a respectable model to examine the effect of glucose on angiomiR expression since they retain the characteristics of cells found in vivo and exhibit behaviour similar to other endothelial cell types.

Moreover, it is well known that type-2 diabetes is associated with endothelial dysfunction (Avogaro et al., 2011) and prolonged diabetes leads to impaired wound healing, as a result of defective angiogenesis (Waltenberger et al., 2000). Wound healing involves the activation of endothelial cells and robust angiogenesis in the damaged tissue (Okonkwo and DiPietro, 2017). HDMECs are the endothelial cells lining the blood vessels of the skin and actively participate in a variety of physiological processes including wound healing, temperature regulation and leukocyte trafficking. As such, studying the effect of high glucose treatment on HDMECs may provide insight on hyperglycemia-induced endothelial dysfunction.

To study the effect of high glucose concentrations on endothelial cells, we characterized high glucose conditions as a concentration of 30mM. This concentration was determined based on other hyperglycemic in vitro cell culture models used in this field of study. Previous studies investigating the effect of high glucose treatment on endothelial cell miRNA expression have employed a glucose concentration of 25-40mM (Bammert et al., 2017; Chen et al., 2018; Gao et al., 2015; He et al., 2018; Hou et al., 2018; Huang et al., 2018; Liu et al., 2018a; Lo et al., 2018; Qiu et al., 2018; Ye et al., 2017). For this study, a glucose concentration (30mM) within this range

was chosen. This glucose concentration has been previously established as an in vitro model to mimic diabetic hyperglycemia in endothelial cells (Cagliero et al., 1995).

In our study, we found that 24-hour incubation with glucose modulates the angio-adaptive balance in endothelial cells toward an angiostatic phenotype; particularly the regulation of VEGF signalling and the ratio of VEGF:THBS1. Surprisingly, we saw a complete loss of VEGF-A protein expression. This reduction is not accompanied by a change in VEGF-A mRNA expression; indicating the mechanism of action underlying VEGF-A occurs post-transcriptionally. This striking abolishment of VEGF-A protein expression brings up questions regarding the viability of cell culture. Autocrine VEGF signalling has been described to sustain vascular homeostasis and cell-autonomous VEGF signalling is important for maintaining endothelial cell viability (Lee et al., 2007). VEGF-knockout endothelial cells isolated from mice have been reported to exhibit either slower proliferation kinetics or died more frequently when compared to wild-type ECs (Lee et al., 2007). These differences in cell viability were exacerbated when the cells were subjected to stress, such as serum starvation. In this manner, the complete loss of VEGF-A protein expression observed in this study would likely be subject to slowed proliferation mechanics or apoptosis. These characteristics were not measured in this study; however, the endothelial cell culture subjected to high glucose treatment appeared nearly identical to the untreated control culture when observed under a microscope. It should be noted that the reduced endothelial cell proliferation and viability in VEGF-KO endothelial cells previously reported were only observable and statistically significant 72H post-culture. In our study design, endothelial cells were only subjected to high glucose stress for 24H. The length of glucose incubation and subsequent loss of VEGF protein expression may not be sufficient to have the same impact cell viability or induce apoptosis described by Lee et al. (2007). Furthermore, limitations in the VEGF-A detection capability of

the antibody used in this study may also explain our observation. In humans, alternative mRNA splicing of the *VEGF* gene gives rise to six distinct isoforms of VEGF-A: $VEGF_{206}$, $VEGF_{189}$, $VEGF_{183}$, $VEGF_{165}$, $VEGF_{145}$ and $VEGF_{121}$ (Robinson and Stringer, 2001). We only identified a band at 23kDa, which has been described as the $VEGF_{165}$ isoform (Cressey et al., 2005; Holmes and Zachary, 2005). As such, the observed abolishment of VEGF-A protein expression in this study might not represent a complete loss of all VEGF-A isoforms.

MicroRNAs are highly expressed in endothelial cells and play an important role in regulating gene expression post-transcriptionally (Landskroner-Eiger et al., 2013). To explore miRNAs as a potential mechanism of action underlying the change in VEGF-A:THBS1, we measured the expression of the miRNAs that have been well described to regulate VEGF-A and THBS1 expression and function (Alhasan, 2019; Dogar et al., 2014; Shang et al., 2019; Yuan et al., 2017). MiR-126 is the most pronounced miRNA expressed in endothelial cells and acts to enhance VEGF-A/VEGFR2 signaling (Agudo et al., 2014) We observed a significant reduction in miR-126-3p and miR-126-5p. MiR-15a, the miRNA responsible for inhibition of VEGF-A translation, is greatly increased in glucose-treated endothelial cells. Together, the change in miRNA expression would support a decrease in VEGF-A expression and VEGFR2 signalling.

While we observed a change in the expression of mature miRNAs in glucose-treated endothelial cells, these changes were not reflected in the expression of the primary miRNA transcripts; suggesting that the observed changes in miRNA expression occur post-transcriptionally. DROSHA, DICER, and AGO-2 are the three central proteins involved in canonical post-transcriptional miRNA biogenesis and function. In our study, DICER and AGO-2 did not significantly change in response to increasing concentrations of glucose. However; a reduction in DROSHA protein occurred as a robust response to rising glucose concentrations. This

was observable both in endothelial cells in vitro and in diabetic mice muscle. These results expand upon the work of Ye and colleagues (Ye et al., 2015), who demonstrated that DROSHA is induced by glucose deprivation in MEF, HEK293T, A549, and HeLa cell lines. Our evidence shows that DROSHA protein expression is highly correlated with glucose concentration.

Prior work has shown that genetic silencing of DROSHA impairs the angiogenic capacity of endothelial cells through the reduction of sprout formation and an increase in THBS1 expression (Kuehbacher et al., 2007). Consistent with Kuebacher and colleagues, we observed a small (albeit non-significant) increase in THBS1 expression. Additionally, we had hypothesized that decreased DROSHA expression in response to glucose treatment would result in a universal reduction in miRNA expression. Consistent with the reduction in DROSHA expression, we observed an analogous decrease in miRNA-126-3p and -5p expression. MiRNA-126 has been well-described as a regulator of angiogenesis that enhances VEGFR2 signalling (Agudo et al., 2014; Matkar et al., 2015; Qu et al., 2018; Rogers and Herzog, 2014). The observed repression of miR-126 expression will impair VEGFR2 signal transduction through these pathways, and consequently, will lead to diminished endothelial cell survival and proliferation signaling associated with angiogenesis. Additionally, while we observed a comparable reduction in miR-126 expression when compared to DROSHA expression; surprisingly, miR-15a expression is greatly enhanced in glucose-treated endothelial cells. This suggests that miR-15a biogenesis is selectively increased under high glucose conditions. The specific mechanism that underlies this has yet to be explored; however, both DROSHA and DICER have been shown to exhibit substrate selectivity and exhibit different processing efficiencies under different physiological conditions (Feng et al., 2011; Hu et al., 2016; Lund and Dahlberg, 2006; Yao et al., 2014).

MDM2 has been described as a regulator of DROSHA expression through its E3 ligase function and has been shown to bind and ubiquitinate DROSHA in human kidney cell lines. We observed a significant increase in both endothelial MDM2 protein and mRNA expression in response to glucose exposure. Here, we show for the first time that MDM2 binds to DROSHA in human primary endothelial cells and the interaction between MDM2:DROSHA is greatly enhanced by high glucose concentration. MDM2 has been reported to be capable of binding proteins both in the nucleus as well the as cytoplasm (Yu et al., 2000). In our study, we observed little to no interaction between p-Ser166-MDM2 and DROSHA under both basal and high glucose conditions. The lack of interaction between p-Ser166-MDM2 and DROSHA indicates that nuclear localization of MDM2 might not be required for binding DROSHA, rather, it would suggest that the MDM2:DROSHA binding occurs exclusively in the cytoplasm.

Nutlin-3 and RG7112 are potent and selective MDM2 antagonists that bind and obstruct the p53-binding pocket of MDM2 (Vassilev et al., 2004; Vu et al., 2013). The glucose-driven reduction in DROSHA protein expression is diminished in endothelial cells treated with Nutlin-3 or RG7112. This more pronounced after 6H of glucose exposure as compared to 24H, rationally due to a declining temporal effect of the inhibitor. The endothelial cells were treated with inhibitors for 1 hour before 6H or 24H glucose incubation; however, the half-life of RG7112 in phase 1 studies is approximately one day (Andreeff et al., 2016). As such, MDM2:DROSHA interaction can be restored; particularly when considering the observed increase in MDM2 expression following Nutlin-3 and RG7112 treatment. Additionally, MX69 is another MDM2 inhibitor that binds to the RING protein of MDM2, leading to the destabilization, autoubiquitination, and degradation of MDM2 (Gu et al., 2016). We observed no significant effect of MX69 treatment on DROSHA protein expression in both normal and high glucose conditions. Together, this suggests

that MDM2 underlies glucose induced DROSHA protein repression since inhibition of MDM2 abolishes this effect. Additionally, it appears that MDM2's hydrophobic p53 binding pocket rather than the RING domain, is critical for glucose-induced degradation of DROSHA.

Our lab had previously reported that MDM2 phosphorylation occurs downstream of VEGF-A (Aiken et al., 2016b). In this study, we observed no significant change in p-Ser166-MDM2 expression despite a decrease in VEGF-A expression and decreased. However, there is a noticeable and significant reduction in p-Ser166-MDM2:MDM2 ratio. The change in this ratio is largely due to increased MDM2 expression. The concurrent increase in both MDM2 protein and mRNA expression under high glucose suggest that transcription of MDM2 is enhanced.

Glucose-induced DROSHA protein downregulation is also observed in vivo in leptin receptor-deficient (db/db) mice model. These mice have become identifiably obese and develop elevated plasma insulin and hyperglycemia after three to four weeks of age. In these animals, the expression of DROSHA protein is well correlated with blood glucose levels, with lower DROSHA expression observed in hyperglycemic mice. Interestingly, in db/db mouse muscle, there is a trend towards decreased MDM2 protein expression with increasing resting blood glucose levels, the opposite of what we observed in vitro. This is consistent with other studies that examine the expression of MDM2 in diabetic rodent models. Similarly, MDM2 expression is downregulated in both Zucker diabetic fatty (ZDF) mice and diabetic bio breeding rats (Aiken et al., 2019; Roudier et al., 2012). This effect is in sharp contrast to what we observed in vitro in endothelial cells.

The difference in the observed expression of MDM2 in response to glucose concentration may stem from differences in resident cells and tissues or consistency in glucose concentration. For example, in human umbilical vein endothelial cells (HUVECs) treated with constant high glucose resulted in an initial upregulation of MDM2 mRNA, peaking at 6H and diminishes over

time (Schisano et al., 2011) In this study, MDM2 mRNA remained elevated after 24H (as compared to basal level); however, MDM2 expression is dramatically lower than the levels observed at 6H. This might suggest that the increase in MDM2 expression is transient and that prolonged exposure to high glucose, as is the case with our diabetic mice models, may lead to reductions in MDM2 expression. Additionally, the difference in MDM2 expression may also be due to different cell types in the tissue. In our study, we specifically examined the effect of glucose on MDM2 expression in primary human dermal microvascular endothelial cells. It has been shown that hyperglycemia reduces MDM2 expression in adipocytes (Liu et al., 2018b). Additionally, though MDM2 was not specifically measured, hyperglycemia was reported to activate p53 (a well-known MDM2 target) in myocytes (Fiordaliso et al., 2001). This indicates that the effect of hyperglycemia on MDM2 expression is dependent on cell-type, and thus, could explain the difference in MDM2 expression between observed in this study.

In this study, we observed that high glucose concentration induces a change in the expression of the miRNA biogenesis machinery and endothelial cell miRNA. Our findings indicate that exposure to high glucose conditions significantly reduces DROSHA protein expression, and subsequently alters miRNA expression. The change in miRNA is reflected in the expression of their corresponding protein; particularly the expression of miRNA-15a and VEGF-A. However, this study has several limitations. Firstly, in this study, we examined the effect of high glucose concentration on the expression of different components of the miRNA biogenesis machinery; however, the expression of these proteins is not the only factor that may dictate the observed changes in miRNA expression. Exposure to high glucose may induce changes in the activity and functionality in the miRNA maturation machinery that alters the way DROSHA, DICER and

AGO-2 interact with miRNAs. Changes in the activity and functionality of these proteins may not necessarily be reflected in their expression.

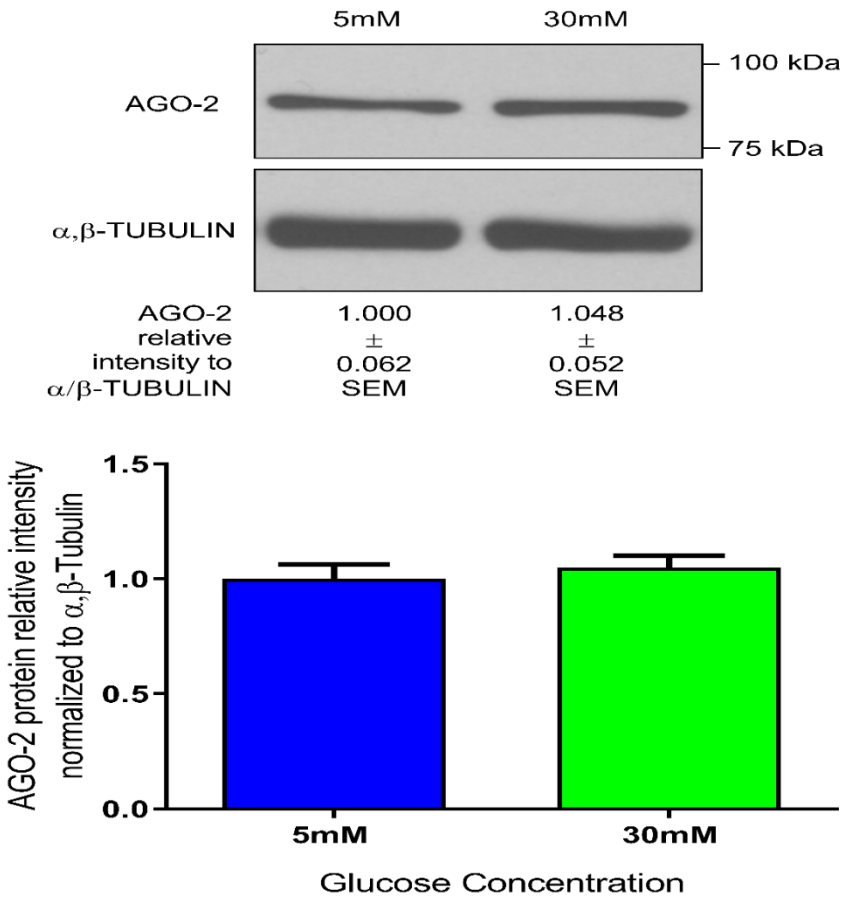
DGCR8 is a cofactor of DROSHA in the MC and has been described as an important and potentially essential component in the miRNA maturation process. Though DROSHA has been shown to be capable of cleaving pri-miRNA without DGCR8 (Partin et al., 2017), DGCR8 is critical for correcting erroneous binding events and necessary for the MC to process pri-miRNAs with high fidelity. Our study focused on the two proteins primarily responsible for the cleavage of miRNAs: DROSHA and DICER - we did not measure the expression of DGCR8. Since DGCR8 plays an important role in stabilizing the interaction between DROSHA and pri-miRNAs and in ensuring fidelity of miRNA processing, it would be beneficial for future studies to examine the influence of DGCR8 on high-glucose induced endothelial miRNA expression.

This study was limited to studying the expression of endogenous proteins and miRNAs. While the majority of miRNAs are detected within the cellular microenvironment, there are several miRNAs that have been found in the extracellular environment, including miR-126, miR-15a and miR-18a (de Gonzalo-Calvo et al., 2017; Komatsu et al., 2014; Liu et al., 2014; Sohel, 2016). Though it is beyond the scope of this study, measuring the expression of secreted endothelial miRNAs in future studies would provide a more comprehensive understanding of the effect of glucose treatment on endothelial miRNA expression.

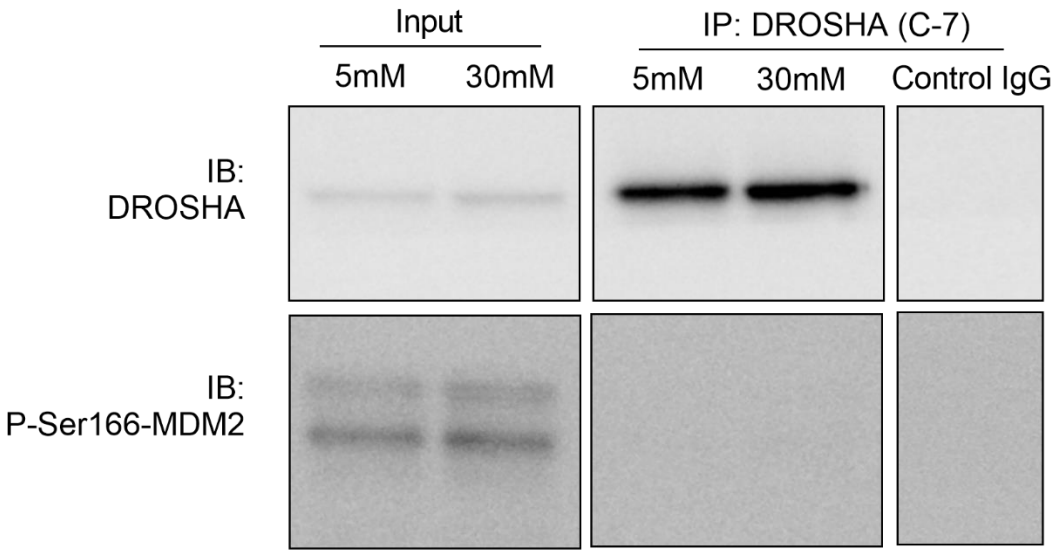
Glycemic fluctuations, characterized by oscillations in plasma glucose are an important consideration when managing type-2 diabetes and have been reported to cause vascular endothelial dysfunction (Dandona, 2017; Torimoto et al., 2013). Patients with diabetes must be mindful of fluctuations in blood glucose, and acute hyperglycemia can arise in different situations, such as acute illness (Egi et al., 2017). Under hyperglycemic conditions, endothelial cells have been

described to become dysfunctional and undergo apoptosis (Popov, 2010) and changes in miRNA expression in response to hyperglycemia were found to be involved in the development of endothelial dysfunction (Silambarasan et al., 2016; Wang et al., 2019). Here, we report that exposure to high glucose conditions influences the expression of the miRNA biogenesis machinery. Notably, acute exposure to high glucose concentrations has a robust effect on DROSHA protein expression, both in vitro and in our diabetic mouse model. In endothelial cells, exposure to high glucose increased the interaction between DROSHA and MDM2; a protein that has been suggested to regulate DROSHA. This change in DROSHA has a resultant effect on the maturation of angiomiRs, leading to an angiostatic phenotype. This study provides a foundation for future research to explore changes in the mechanisms involved in miRNA biosynthesis and helps advance current efforts in identifying potential miRNAs as biomarkers and diagnostic tools for vascular diseases.

2.7 Supplemental Figures



Supplemental Figure 1. High glucose conditions do not significantly affect AGO-2 protein expression in human microvascular endothelial cells (HDMECs). Immunoblots of AGO-2 after 24h treatment under normal (5mM) or high (30mM) glucose conditions (n=6). α,β-Tubulin was used as a loading control.



Supplemental Figure 2. High glucose concentration does not affect DROSHA:p-Ser-166-MDM2 Binding. After immunoprecipitation (IP) of DROSHA in glucose treated HDMECs treated with MG132, levels of P-Ser-166-MDM2 and DROSHA were measured by immunoblot analysis in whole-cell lysate (input) and in the IP products. Normal mouse IgG was used as a control.

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