# THE ROLE OF MICRO-RNAS IN THE ANGIOPOIETIN-1/TIE-2 SYSTEM

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## I. Abstract

In adult mammals, endothelial cells (EC) retain high plasticity to sense and respond to angiogenic signals. Angiogenesis, the formation of blood vessels from pre-existing ones, can be triggered in response to tissue damage, oxygen and nutrient deprivation, and during pathological conditions like cancer, ocular diseases, and inflammation. The Angiopoietin–Tie pathway is crucial for angiogenesis and vascular homeostasis and therefore is an attractive target for therapeutic interventions. Angiopoietin-1 (Ang-1) is an oligomeric glycoprotein required for the structural integrity of blood vessels and quiescence. Ang-$1^{-/-}$ mice die at embryonic day 12.5 due to vasculature malformation and extensive apoptosis of vascular cells. Ang-1 binds primarily to Tie-2 receptors which are expressed predominantly in ECs. This binding triggers autophosphorylation of tyrosine residues on Tie-2 receptors followed by activation of downstream signaling pathways including the PI-3 kinase/AKT and members of the mitogen-activated protein kinase (MAPK) family (p38, ERK1/2 and SAPK/JNK). Ang-1 promotes migration, survival, differentiation, and inhibits apoptosis through activation of these pathways. MicroRNAs (miRNAs) have been recognized as key regulators of angiogenesis, yet the nature and functional significance of those miRNAs involved in the regulation of Ang-1-induced angiogenesis are still unknown.

Our first study focused on identification of various miRNAs whose expression is regulated by Ang-1 in ECs and at assessing the functional roles of these miRNAs in Ang-1-induced angiogenesis. We found that Ang-1 inhibited the expressions of mature miR-103b, miR-330a-5p, miR-557, miR-575, miR-1287-5p, and miR-1468-5p and that these particular miRNAs exert significant inhibitory effects on EC survival, proliferation,

migration, and differentiation. We concluded that that miR-103b, miR-330a-5p, miR-557, miR-575, miR-1287-5p, and miR-1468-5p are anti-angiogenic miRNAs.

Our second study was aimed at deciphering the functional importance of miR-1233-3p in Ang-1-induced angiogenesis in ECs since the expression of this miRNA is significantly decreased by Ang-1. We found that miR-1233-3p inhibits several Ang-1-induced angiogenic processes such as EC survival, migration, and capillary-like tube formation. We also found that miR-1233-3p interacts directly with the mRNA of p53 and DNA damage regulated protein 1 (PDRG1) and that PDRG1 expression is positively regulated by Ang-1 at the mRNA and protein levels in ECs.

Our third study was designed to assess the functional role and the mechanisms through which PDRG1 influences Ang-1-induced angiogenesis. Our experiments revealed that PDRG1 positively regulates angiogenesis and promotes the angiogenic effects of Ang-1 in cultured ECs. To assess the mechanisms through which PDRG1 mediates these effects, we immunoprecipitated PDRG1 and identified its binding partners. This analysis revealed that Tuberous sclerosis 2 protein (TSC2), a negative regulator of the mammalian target of rapamycin (mTOR) pathway, binds to PDRG1. Our experiments also revealed that PDRG1 is a positive regulator of the mTORC1 pathway since it promotes phosphorylation of P70S6K and 4E-BP1 (downstream targets of mTOR). Finally, we found that Inhibition of the mTOR pathway abolished PDRG1- induced migration, tube formation, and proliferation of ECs.

We conclude that Ang-1 promotes angiogenic processes, in part, by downregulating the expression of several anti-angiogenic miRNAs. One such miRNA is miR-1233-3p, which exerts negative influence on angiogenesis. Our studies suggest that Ang-1, by

downregulating miR-1233-3p expression, triggers significant upregulation of PDRG1 expression and that PDRG1, in turn, promotes Ang-1-induced angiogenic processes through activation of the mTORC1 pathway.

## II. Résumé

Chez les mammifères adultes, les cellules endothéliales (EC) gardent une grande plasticité pour détecter et répondre aux signaux angiogéniques. L'angiogenèse, peut être déclenchée en réponse à un dommage tissulaire, une carence en oxygène et nutriments, et lors de conditions pathologiques telles que le cancer, des maladies oculaires et des inflammations.

Le système Angiopoetin-Tie est crucial pour l'angiogenèse. Angiopoietin-1 (Ang-1) est une glycoprotéine nécessaire pour la quiescence et une intégrité structurelle correcte des vaisseaux sanguins. Les souris Ang-$1^{-/-}$ meurent au jours de développement embryonnaire 12.5 en raison de malformations vasculaires. Ang-1 se lie principalement au récepteur Tie-2 (exprimé majoritairement dans les EC). Cette interaction déclenche l'autophosphorylation récepteur Tie-2 et induit l'activation en aval de voies de signalisation dont les PI-3 kinase/AKT et des membres de la famille des Mitogen-Activated Protein Kinase (MAPK) dont p38, ERK1/2 et SAPK/JNK. Ang-1 favorise la migration, la survie, la différentiation ainsi que l'inhibition de l'apoptose in-vitro. Les micro ARN (miARNs) ont été identifiés récemment comme des régulateurs clefs de l'angiogenèse, cependant les caractère et l'importance fonctionnelles de ces miARNs impliqués dans la régulation de l'angiogenèse induite par Ang-1 sont toujours inconnus.

Notre première étude était centrée sur l'identification de miARN régulés par Ang-1 dans les EC et comment ces miARNs, en retour, régulent l'angiogenèse induite par Ang-1. Notre analyse a permis de détecter que Ang-1 inhibait l'expression des formes matures de miR-103b, miR-330a-5p, miR-557, miR-575, miR-1287-5p et miR-1468-5p et que ces miARNs exercent un effet inhibiteur significatif sur la survie, la prolifération, la

migration et la différentiation des EC.Nous en avons conclu que les miR-103b, miR-330a-5p, miR-557, miR-575, miR-1287-5p et miR-1468-5p sont des miARN anti-angiogénique.

Notre deuxième étude avait pour but de comprendre l'importance fonctionnelle de miR-1233-3p dans l'angiogenèse induite par Ang-1 dans les EC. Nos résultats ont montré que miR-1233-3p inhibe plusieurs processus d'angiogenèse induite par Ang-1 tels que la survie, la migration, la formation de tube, tout en inhibant les effet anti-apoptotiques de Ang-1. Nous avons également montré que miR-1233-3p interagit directement avec les mARNs de p53 et de la DNA damage regulated protein 1 (PDRG1), et que l'expression de PDRG1 est régulée positivement par Ang-1 au niveau des mARN et des protéines.

La troisième étude a eu pour but d'analyser le rôle fonctionnel de la protéine PDRG1. Nos expériences ont montré que PDRG1 régule positivement l'angiogenèse et favorise les effets angiogéniques de Ang-1 dans des EC en culture par rapport aux contrôles. Pour analyser le mécanisme par lesquels PDRG1 produit ces effets, nous avon immunoprécipité PDRG1 et identifié ses partenaires de liaison. Cette analyse a révélé que Tuberous sclerosis 2 protein (TSC2), un régulateur négatif de la voie mammalian target of rapamycin (mTOR), est un partenaire de liaison de PDRG1. Nos expériences ont montré que PDRG1 est un régulateur positif de la voie mTORC1 par son action favorisant la phosphorylation de P70S6K et 4E-BP1 (cibles en aval de mTOR). Pour finir, nous avons constaté que l'inhibition de la voie mTOR mène à la suppression de l'induction de la migration, de la formation de tube et de la prolifération des EC par PDRG1.

En conclusion, Ang-1 favorise le processus angiogénique, en partie en régulant négativement l'expression de plusieurs miARNs anti-angiogéniques. Une de ces régulations en question est miR-1233-3p qui, en absence de Ang-1, influence négativement l'angiogenèse. Notre étude suggère que Ang-1, en régulant négativement l'expression de miR-1233-3p, déclenche une régulation positive de l'expression de PDRG1 et que PDGR1, en retour, favorise le processus angiogénique induit par Ang-1 par l'activation de la voie mTORC1.

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## IV. List of publications

Sanchez, V., Golyardi, F., Mayaki, D., Echavarria, R., Harel, S., Xia, J. and Hussain S.N.A. (2018) Negative regulation of angiogenesis by novel miRNAs. *Pharmacological Research.*

Sanchez, V., Harel, S., Mayaki, D. and Hussain, S.N.A. Role of miR-1233-3p in Angiopoietin-1-induced angiogenesis. *Drafted*

Sanchez, V., Mayaki, D. and Hussain, S.N.A. Regulation of Aniopoieting-1-induced angiogenesis by PDRG1. *Drafted*

Desjarlais, M., Dussault, S., Rivard, F., Harel, S., Sanchez, V., Hussain, S.N.A. and Rivard, A. (2018). Reduced expression of anti-inflammatory microRNA-146b impairs ischemia-induced neovascularization in hypercholesterolemic condition. *ATVB*

Harel, S., Mayaki, D., Sanchez, V., and Hussain, S.N.A. (2017). NOX2, NOX4, and mitochondrial-derived reactive oxygen species contribute to angiopoietin-1 signaling and angiogenic responses in endothelial cells. *Vascular pharmacology.*

Harel S, Sanchez-Gonzalez V, Echavarria R, Mayaki D, Hussain S, Regulation of angiogenesis by Angiopoietin-1: Roles of miR-640 and ZFP91. To be submitted to *Blood*

Harel S, Sanchez-Gonzalez V, Sanchez-Galan J, Ismail H, Mayaki D, Huck L, Blanchette M, Hussain S, ETS1, ELK1 and ETV4 Contribute to Angiopoietin-1 Angiogenic Responses in Endothelial Cells. To be submitted to *ATVB*

Echevarria R, Mayaki D, Neel J-C, Harel S, Sanchez V, Lebrun J-J, Hussain SN. Angiopoietin-1 inhibits Toll-like receptor 4 signaling in endothelial cells: Role of miR-146b-5p. *Cardiovascular Research*, 2015 Jun 1; 106(3):465-77

Chan, W., Ismail, H., Mayaki, D., Sanchez, V., Tiedemann, K., Davis, E.C., and Hussain, S.N.A. (2016). Fibulin-5 Regulates Angiopoietin-1/Tie-2 Receptor Signaling in Endothelial Cells. *Plos One*.

## V. Contribution of authors and contribution to original knowledge

This manuscript-based thesis is composed of chapters that have been published, submitted for publication, or that will be submitted for publication in peer-reviewed journals.

Dr. Sabah N. Hussain was instrumental in the interpretation of data and generation of all the manuscripts. Veronica Sanchez Gonzalez, is responsible for carrying out most of the experiments and the analysis done on the experiments.

**Manuscript 1:** Angiopoietin-1 Signaling in Endothelial Cells: The Role of MicroRNAs

Contribution to original knowledge: Identification of various miRNAs whose expression is regulated by Ang1 exposure in ECs; identification of several novel miRNAs as negative regulators of angiogenesis processes in ECs (survival, migration, proliferation, apoptosis, and differentiation); and analysis of possible cellular networks through which these miRNAs regulate angiogenesis.

Dominique Mayaki: provided assistance for quantitative real time PCR.

Flora Golyardi, Raquel Echavarria, and Sharon Harel: performed preliminary experiments.

Janguo Xia: aided in bioinformatics analysis.

**Manuscript 2:** The role of miR-1233-3p and PDRG1 in Ang-1-dependent angiogenesis

Contribution to original knowledge: Identification of PDRG1 as a direct target of miR-1233-3p; recognition of Angiopoietin-1 as a negative regulator of miR-1233-3p expression; and identification of miR-1233-3p is a negative regulator of angiogenic processes (survival, migration, proliferation, apoptosis and differentiation).

Sharon Harel: executed short-time course Ang-1 treatment.
Dominique Mayaki: provided assistance for quantitative real time PCR.

**Manuscript 3:** PDRG1 regulates Ang-1 dependent-Angiogenesis through mTOR

Contribution to original knowledge: Identification of novel functions for PDRG1 as a pro-angiogenic protein and as a promoter of Angiopoietin-1-dependent angiogenesis; recognition of PDRG1 as a regulator of the mTORC pathway and as a direct interactor with TSC2 protein; and identification of PDRG1 as an activator of the mTORC1 pathway through negative regulation of TSC2 expression.

Dominique Mayaki: provided assistance for quantitative real time PCR.

## VI. List of abbreviations

4E-BP1: eukaryotic translation initiation factor 4E-binding protein 1

ABIN-2: A20 binding inhibitor of NF

Ago-2: argonaute 2

Ang: angiopoietin

Ang-1: angiopoietin 1

Ang-2: angiopoietin 2

ANOVA: analysis of variance

AP-1: activator protein 1

ATF: activating transcription factor

BAD: Bcl-2-associated death promoter protein

Bax: Bcl-2-associated X protein

BBB: blood brain barrier

BMP: bone morphogenetic protein

bp: base pair

BSA: bovine serum albumin

CCDC91: coiled-coil domain containing 91

cDNA: complementary deoxyribonucleic acid

CENPB: centromere protein B

CF2: chorion factor 2

CLASH: crosslinking, ligation, and sequencing of hybrids

CMV: cytomegalovirus

cPLA2: cytosolic phospholipases A2

CT: comparative threshold

CTGF: connective tissue growth factor

DBD: DNA-binding domain

DGCR8: DiGeorge syndrome critical region gene 8

DMEM: Dulbecco's Modified Eagle Medium

DN: dominant negative

DNA: deoxyribonucleic acid

dNTP: deoxyribonucleotide triphosphate

Dok-R: downstream of kinase-related

EC: endothelial cell

ECL: enhanced chemiluminescence

ECM: extracellular matrix

EDTA: ethylenediaminetetraacetic acid

EGF: epidermal growth factor

EGFR: epidermal growth factor receptor

Egr-1: early growth response 1

EMT: epithelial-mesenchymal transition

EPC: endothelial precursor cell

Eph: ephrin

ERK1/2: extracellular signal regulated kinase 1 and 2

FAK: focal adhesion kinase

FBS: fetal bovine serum

FDR: fibrinogen-like domain

FGF: fibroblast growth factor

FOXO1: forkhead box protein O1

GAP: GTPase-activating protein

GAPDH: glyceraldehyde phosphate dehydrogenase

GDP: guanosine-5'-diphosphate GEF: guanine exchange factor

GOLGA8A: Golgin A8 family member A

GOLGA8B: Golgin A8 family member B

Grb2: growth factor receptor-bound protein 2

GSK3β: glycogen synthase kinase 3β

GTP: guanosine-5'-triphosphate

HDAC1: histone deacetylase 1

HGF: hepatocyte growth factor

HHT: hemorrhagic telangiectasia

HIF1: hypoxia inducible factor 1

HITS-CLIP: High-throughput sequencing of RNA isolated by crosslinking immunoprecipitation

HMEC: human mammary epithelial cells

hMSC- human mesenchymal stem cell

HNRNPM: heterogeneous nuclear ribonucleoprotein M

HOXB3: homeobox B3

HRP: horseradish peroxidase

HSC: hematopoietic stem cell

HUVEC: human umbilical vein endothelial cell

IA: intussusceptive angiogenesis

ICAM: intercellular adhesion molecule 1

Ig: immunoglobulin

IKK: I kappa B kinase

IL-1β: interleukin 1 beta

IP-MS: immunoprecipitation mass Spectrometry

JNK1/2: c-jun nuclear kinase 1 and 2

KLF2: Kruppel-like factor 2

lncRNA: long non coding ribonucleic acid

MAPK: mitogen-activated protein kinase

MAPKK: mitogen-activated protein kinase kinase

MAPKKK: mitogen-activated protein kinase kinase kinase

MATR3: matrin 3;

MEF2: myocyte enhancer factor 2

MFF: mitochondrial fission factor

miRNA: microRNA

MMP-2: matrix metalloproteinase 2

mTOR: mechanistic target of rapamycin

mTORC1: mechanistic target of rapamycin complex 1

ncRNA: non coding ribonucleic acid

NF-κB: nuclear factor kappa b

NO: nitric oxide

NRP: neuropilins

Nt: nucleotides

Nur77: nuclear receptor 77

P70S6K: ribosomal protein S6 kinase beta-1

PAK: p21 activated kinase

PAR-CLIP: photoactivatable ribonucleoside-enhanced crosslinking and immunoprecipitation

PAZ: Piwi/Argonaute/Zwille domain

PBS: phosphate buffer saline

PCR: polymerase chain reaction

PDGF: platelet-derived growth factor

PDGFR: platelet-derived growth factor receptor

PDRG1: p53 and DNA damage regulated 1

PGI2: prostacyclin

PI: phosphoinositide

PI3-K: phosphoinositide 3-kinase

PKB: protein kinase B

PKC- protein kinase C

PKCζ: protein kinase C zeta

PMSF: phenylmethanesulfonylfluoride

Pre-miRNA: precursor microRNA

Pri-miRNA: primary microRNA

PTB: phosphotyrosine binding

PVDF: polyvinylidene difluoride

RAN-GTP: miRNA and a GTP-binding nuclear protein

RISC: ribonucleic acid induced silencing complex

RNA: ribonucleic acid

Pol II: RNA polymerase II

ROS: reactive oxygen species

RPL8: ribosomal protein L8

RPS27: ribosomal protein S27

RPS3: ribosomal protein S3

RTK: receptor tyrosine kinase

RT-PCR: real time polymerase chain reaction

Runx1: runt-related transcription factor 1

SAPK/JNK: stress-activated protein kinase/Jun-amino-terminal kinase

SDS PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis

SDS: sodium dodecyl sulfate

SE: standard error

siRNA: small interfering ribonucleic acid

snRNA: small nuclear RNA

TAK1: TGF-beta activated kinase 1

TGF-β: transforming growth factor beta

tRNA: transfer RNA

TSC2: tuberous sclerosis protein 2

TSP-1: thrombospondin-1

URI: unconventional prefoldin RPB5 Interactor

UTR: untranslated region

UV: ultraviolet

VCAM: vascular cell adhesion molecule 1

VE-cadherin: vascular endothelial cadherin

VEGF: vascular endothelial growth factor

VEGFR/KDR/Flk1: vascular endothelial growth factor receptor

WPB: Weibel-Palade bodies

WT: wild-type

XPO5: exportin 5

## VII. List of figures and tables

CHAPTER II: Angiopoietin-1 Signaling in Endothelial Cells: The Role of MicroRNAs

CHAPTER IV: PDRG1 regulates Ang-1 dependent-Angiogenesis through mTOR.

# CHAPTER I: Literature review

# 1. Structure and development of the vascular system

The cardiovasculature is the first functional system that is formed during vertebrate development. Its primary functions are to enable gas exchange, supply nutrients, and remove waste from tissues [1].

## 1.1. Formation of the vascular system

The vascular system is characterized by blood vessels composed of different cell types. The inner wall of the vessels, the endothelium, is formed by endothelial cells (ECs) that surround the lumen of the vessel in a simple monolayer rearrangement. In small vessels the endothelium is surrounded by a mixture of vascular smooth muscle cells and pericytes, known as mural cells, which stabilize the vessel [2]. Composition of large vessels, veins, and arteries is more complex, with the endothelium (tunica intima) being stabilized by a layer of smooth muscle cells and another external layer of connective tissue, collagen, and elastic fibres called the tunica media and tunica adventitia, respectively [3]. The tunica media and adventitia give stability to the vessel while allowing the regulation of blood pressure in response to environmental cues [4].

The vasculature is formed through three main processes: vasculogenesis, angiogenesis, and arteriogenesis. Vasculogenesis, the *de-novo* formation of blood vessels, is the process by which the primary plexus is established. Angiogenesis, the formation of blood vessels from pre-existing ones, allows expansion of the primary plexus. Vasculogenesis is largely restricted to early embryonic development, and angiogenesis is the major mechanism of vascular growth in later embryogenesis and in the adult [5]. This thesis is focused on angiogenesis and will not cover vasculogenesis.

## 1.2. **Angiogenesis**

In adults, vessels are quiescent and rarely form new branches [6]. However, ECs retain high plasticity to sense and respond to angiogenic signals [7]. Angiogenesis can be triggered by tissue damage, oxygen and nutrient deprivation, and during pathological conditions like cancer, ocular diseases and inflammation [8]. Two main different types of angiogenesis have been identified: sprouting angiogenesis of ECs from pre-existing vessels, and intussusceptive angiogenesis (IA), which is the formation of blood vessels by splitting [9]. The mechanism of IA remains largely unknown due to its more recent discovery in 1986, though sprouting angiogenesis has been known for more than 200 years [10].

ECs and mural cells are divided by a basement membrane formed by extracellular matrix proteins that cover the endothelial tube [11]. The function of this matrix is to prevent ECs from leaving their position in the vessels. When quiescent vessels sense angiogenic signals, pericytes that surround the ECs detach from the vessel wall and liberate themselves from the basement membrane by a process that involves proteolytic degradation. ECs then loosen their junctions and the nascent vessel dilates. Permeability of the ECs increase, causing plasma proteins to lay down a provisional extracellular matrix scaffold. The cells then migrate on this extracellular matrix surface towards the angiogenic signal. One EC, the tip cell, becomes the cell that leads the migration. The neighbor cells assume subsidiary positions as stalk cells, which divide and elongate the stalk and establish the lumen. Tip cells, characterized by filopodia, allow sensing of environmental guidance cues. Stalk cells release molecules to send spatial information about neighbor cells during tube elongation. Stalk cells have the

ability to form tubes and branches, and, compared to tip cells, produce fewer filopodia but are more proliferative. Additionally, they are equipped to form a vascular lumen (adapted from [6, 7]). The specification of the tip and stalk cells is controlled by the Notch pathway [12]. It has been found that stalk cells have high Notch activity, while tip cells have low levels. In fact, excess Notch signaling results in ECs with less filopodia extension, and inhibit these cell from having a tip position [13]. The data suggest the essential role of Notch signaling is the establishment of stalk cell specification. Instead of being permanent roles, tip and stalk cells are dynamically interchangeable and compete for the leading position of tip cell [14].

After migration, the new vessels form a lumen for blood to flow through. Lumen formation has been proposed to happen through different mechanisms. The cell-hollowing model proposes that ECs form a lumen via coalescence of intracellular vacuoles (by pinocytosis), which interconnect with other coalescing vacuoles from neighboring ECs. This model was observed in intersomitic vessels during embryo development [15, 16]. Recent studies, however, favour a different model, termed cord hollowing, where ECs adjust their shape and rearrange their junctions to open up a lumen. In cord hollowing, ECs first define apical-basal polarity, where, on the apical membrane, negatively charged glycoproteins are accumulated, producing repulsive signals that open up a lumen. Subsequent changes in ECs expand the lumen [17].

### 1.2.1. Signals regulating angiogenesis

#### 1.2.1.1. VEGFA/KDR signaling

Initiation of vessel-branching is mainly driven by VEGF, an essential factor during vasculogenesis. Upon binding to its receptor, KDR, VEGF induces the specification of ECs into tip and stalk cells [18]. At the newly forming vascular front, the cell most sensitive to VEGF will become the tip cell. This sensitivity to VEGF is determined by expression levels of KDR in these ECs [19, 20].

#### 1.2.1.2. Notch signaling

Notch signaling is involved in different developmental and disease contexts, from stem cell regulation to cancers [21]. In mammals, the Notch family is composed of four Notch receptors (1-4) and five ligands (Jagged1, Jagged2, Delta like ligand (Dll)l, Dll3, and Dll4). In the vasculature, Notch1 and Notch4 receptors are expressed in ECs. Upon ligand-mediated activation, a series of proteolytic cleavages in the receptor occurs, which leads to release of the Notch intracellular domain [22]. Once released, this domain enters the nucleus and interacts with the DNA-binding protein CBF1–Suppressor of Hairless–LAG1(CSL) and the co-activator Mastermind (MAML1) to stimulate transcription of target genes [23, 24].

Based on genetic studies, Notch signaling is involved in the regulation of angiogenesis. Notch1$^{-/-}$ embryos die during midgestation with normal establishment of the primary vascular plexus but abnormal vessel remodeling [25]. Notch1 and 4 homozygote double knockout develop relatively higher vascular disruptions compared to Notch1$^{-/-}$ alone.

These embryos show defects in large vessel formation, indicative of Notch1 and Notch 4 involvement in angiogenesis during development [25].

Notch signaling appears to be associated with VEGF signaling in angiogenesis. VEGF induces Kll4 and Notch signaling [26]. Elevated Kll4 and KDR expression is detected in tip cells compared to neighboring stalk cells [12]. Inhibition of VEGF causes decrease of Dll4 in vessels and arrest of angiogenesis [13]. Conversely, administration of VEGF leads to induction of Dll4 expression [27].

Notch signaling has also been known to regulate VEGF signaling. Indeed, Notch activation in ECs and in Dll4-deficient mice triggers significant attenuation of KDR levels [13, 28]. This finding led to the proposal that Notch may act as a negative feedback mechanism to regulate VEGF signaling. It has been proposed that KDR downregulation observed in ECs allows for local differentiation on EC tip cells prior to sprout initiation with VEGF, leading to increased Dll4 expression and activation of Notch signaling. Upregulation of notch signaling in turn downregulates the expression of KDR in the neighbouring cells [22].

#### 1.2.1.3. **Semaphorins**

The semaphorins were initially described as axon guidance factors, but they have recently been implicated in a variety of physiological and developmental functions, including regulation of immune response, angiogenesis, and migration of neural crest cells [29, 30].

More than 30 semaphorin genes have been identified, falling into seven classes. Classes 1 and 2 contain invertebrate semaphorins, classes 3–7 contain vertebrate

semaphorins, and class 8 contains viral semaphorins. All semaphorins have an amino-terminal sema domain that consists of about 500 amino acids and which is essential for their activity. The semaphorins transduce their signals by binding to one of the nine receptors from the plexin family, or, in the case of the class 3 semaphorins, by binding to one of the two neuropilin receptors [31]. Class 3 semaphorins have been shown to function as inhibitors of angiogenesis [32]. Class 3 semaphorins are produced as seven soluble secreted glycoproteins (named A-G). Upon semaphorin binding, neuropilin forms a complex with plexins that initiate intracellular signaling. Sema3F was the first class 3 semaphorin to be characterized as a direct anti-angiogenic factor and as a promoter of apoptosis of ECs [32-34]. Sema3A inhibits angiogenesis in the forelimbs and vascular branching in the brain during embryogenesis [35]. *In-vitro* studies have also shown that sema3A can induce apoptosis of ECs [34]. Adding exogenous sema3A can inhibit angiogenesis *in-vivo* [36]. Sema3E is unique among the class 3 semaphorins in that it is the only one to not use neuropilin as a receptor. Instead, it transduces signaling through (the) plexin-D receptor. During development Sema3E is highly expressed in the somites and inhibits their vascularization [37].

#### 1.2.1.4. **Slits and roundabouts**

Slit ligands are secreted chemorepellents of growing axons and migrating neurons that signal through receptors of the Roundabout (Robo) family. Slits are also involved in organ and tumor development. Three Slit ligands (Slit1–3) and four Robo receptors (Robo1–4) have been characterized in mammals on the basis of structural similarities. Slit2 and Slit3 can inhibit VEGF-induced EC migration and permeability *in-vitro* and vascular leakage in mice [38-40]. Robo4 is selectively expressed by ECs and has been

reported to control angiogenesis and blood vessel permeability [41-43]. ECs also express Robo1 and 2 receptors and Slits, in particular Slit2, which favors angiogenesis by promoting EC motility and polarity [44, 45].

#### 1.2.1.5. **Sprouty**

Members of the Sprouty (Spry) family (including four sprout proteins, Spry 1-4, and 4 Spry-related proteins) act as general antagonists of the receptor tyrosine kinase (RTK)-mediated MAPK signaling pathway, involved in many physiological and developmental processes, including angiogenesis [46, 47]. In general, Spry proteins are phosphorylated and activated by VEGF. Upon activation, spry proteins undergo nuclear translocation that leads to inhibition of the MAPK pathway [48]. Spry 1 and 2 appear to attenuate VEGF-induced MAPK signaling. Additionally, Spry4 has been shown to inhibit VEGF-induced MAPK signaling [49].

### 1.2.2. **Vessel maturation**

Following formation of a blood vessel, a series of maturation events occur that allow the vessel to become functional. Such maturation occurs at the level of the endothelium (vessel wall), but also at the level of the network. Vessels' stability and homeostasis is achieved partially through the recruitment of smooth muscle cells and pericytes to the maturing vessel wall [6]. Incomplete stabilization can result in hyperpermeable vessels that can lead to edema or increased incidence of tumor metastasis [50].

### 1.2.3. **Signals involved in vessel maturation**

#### 1.2.3.1. **Platelet-derived growth factor**

Platelet-derived growth factor (PDGF) is a family of peptides that signals through its receptor, PDGFR, on the cell surface. PDGF can stimulate many cellular functions including angiogenesis [51]. The PDGF family is composed of 4 different polypeptide chains (PDGF-A, B, C, D) encoded by 4 different genes located in 4 different chromosomes and regulated individually. The four PDGF chains assemble into disulphide-bonded dimers via homo-or heterodimerization [52]. To stabilize EC channels, ECs release PDRG-B to chemoattract pericytes expressing the receptor PDGFR-β [53]. Pericyte deficiency due to PDGF-B ablation leads to vessel instability, leakage, tortuosity, microaneurysm formation, and bleeding [54]. PDGFR-β hypomorph mice have insufficient pericytes around brain vessels that lead to defects on the blood brain barrier (BBB) and neurodegenerative damage due to leakage of toxic substances [55]. In addition, mice lacking either PDGF-B or PDGFR-β show increased levels of VEGF-A, likely contributing to further edema [56].

#### 1.2.3.2. **TGF-β**

Vessel maturation also relies on TGF-β signaling. TGF-β stimulates mural cell induction, differentiation, proliferation, and migration, and promotes production of the extracellular matrix [57]. Loss of TGF-β receptor 2, endoglin, activin or *Alk1* in mice causes vessel fragility, in part due to impaired mural cell development [58].

#### 1.2.3.3. **Angiopoietins/tie signaling**

In addition to the VEGF-VEGFR system, the angiopoietin–Tie system is another endothelial-specific receptor tyrosine kinase (RTK) pathway involved in blood and lymphatic vessel development. The VEGF-KDR signaling system was the first signaling

to be identified as an EC-specific receptor tyrosine kinase signaling system involved in blood and lymphatic vessel development [59, 60]. Together, Tie receptors and their corresponding ligands, angiopoietins, were identified as a second EC-specific receptor tyrosine kinase signaling system in the early 1990s [61]. Different from Angiopoietin/Tie signaling, VEGF-KDR appears to function as a general regulator of both vasculogenesis and angiogenesis. Angiopoietin/Tie signaling plays an essential role during angiogenesis and does not seem to be involved in embryonic vasculogenesis.

The Tie/Ang system is comprised of endothelial-specific Tie-1 and Tie-2 (Tek) receptor tyrosine kinases and the Angiopoietin (Ang) family of growth factor ligands (abbreviated as Angpt1 or Ang-1, Ang-2 and Ang-4, the latter representing a human orthologue for mouse Ang-3). Expression of Tie-1 and Tie-2 is restricted to ECs in blood and lymphatic vessels. Although less expressed, Tie-1 has been found in certain hematopoietic cell lineages, including human hematopoietic progenitor cells and a subset of megakaryocytic cells, as well as in leukemia cell lines [62, 63]. Tie-2 has also been detected in a subpopulation of monocytes (Tie-2 expressing monocytes, TEM), hematopoietic stem cells, and in muscle cell satellites located among skeletal muscle myofibres in association with the microvasculature [64]. The Ang growth factors and the endothelial Tie receptors regulate blood and lymphatic vessel development, vascular permeability, inflammation, angiogenic remodeling and tumor vascularization in adult tissues [61].

#### 1.2.3.4. **Tie receptors**

Tie-1 and Tie-2 are structurally related and their intracellular domains have a 76% identity. Both are type 1 transmembrane receptor tyrosine kinases that regulate vessel

branching and homeostasis in ECs [65]. These receptors are composed of three immunoglobulin-like (Ig) domains, three epidermal growth factor (EGF) domains, and three fibronectin type III repeats (FNIII) in the extracellular portion that are 33% identical [66, 67]. In the intracellular region, there is a structurally conserved split tyrosine kinase domain with homology to fibroblast growth factor receptor (FGFR1) [68]. A single-pass trans-membrane domain separates the ectodomain from the intracellular domain. Crystal structure and biochemical experiments showed that Ang-1 and Ang-2 bind to Tie-2 with similar affinities and on the same site (the second Ig-like domain). The binding on Ang-2 has been more studied than Ang-1. Binding of Ang-2 to Tie-2 leads to the formation of two complementary surfaces without domain rearrangement and with barely conformational changes of either molecule [66, 67].

Although Tie-1 is homologous to Tie-2, it does not associate with any of the angiopoietin ligands. Amino acid sequence comparison of the binding domain of Tie-2 reveals that many of the residues essential for ligand recognition in Tie-2 are replaced in Tie-1, resulting in unfavourable ligand binding [66, 67]. During embryonic development Tie-2 is expressed in the endocardium and the ECs [69]. Tie-1 is also expressed in ECs during embryonic development [70]. Tie-1 and Tie-2 are required for normal embryonic vascular development. Knockout studies demonstrate that *Tie-2* deletion is lethal at embryonic stage E10.5 due to reduced number of vessels and decreased vascular branching [71]. Additionally, endothelial lining of the heart appears collapsed and retracted.

Tie-1 deletion results in embryonic death starting at around E13.5, with embryos displaying lack of endothelial integrity, resulting in edema and hemorrhage. *Tie-*

*1* and *Tie-2* double knockout results in a similar but more severe phenotype than *Tie-2*$^{-/-}$ embryos [72, 73]. Unlike *Tie-2*$^{-/-}$ mice, hematopoiesis occurs normally in *Tie-1*$^{-/-}$ [74]. Embryos chimeric for the gene-targeted *Tie-1* and *Tie-2* alleles showed that Tie-1 and Tie-2 are cell-autonomously required for EC survival in the microvasculature during late embryogenesis and in essentially all blood vessels in the adult [75, 76]. Tie-1 is also critical for lymphatic development; the jugular lymph sacs of *Tie-1*$^{-/-}$ mouse embryos appear malformed, and the embryos are swollen before any signs of blood vascular defects [77, 78].

Unlike Tie-2, Tie-1 is considered to be an orphan receptor and it is unable to bind any of the angiopoietins directly. In additiona no other natural ligands have been identified [455]. This lack of a natural ligand for Tie1 has significantly complicated investigation of its function [456]. In the presence of Tie-2, Tie-1 is activated by Ang-1 [457,458]. Other studies have suggested that Tie-1 can function as a negative regulator of Tie-2 signaling; nonetheless more recent data has pointed that it can act as both, negative and positive regulator of Tie 2. For example, Tie-1 expression is stalk cells at the cell membrane stabilizes Tie1-Tie2 heteromultimers promoting ANG1/Tie2 signaling. Conversely, Tie1 expression in tip cells negatively regulates Tie2 [459]

#### 1.2.3.5. **Angiopoietin ligands**

The angiopoietins are a family of secreted glycoproteins that include Ang-1, Ang-2, Ang-3, and Ang-4. The first member to be discovered was Ang-1, due to its ability to bind to the Tie-2 extracellular domain [79]. Subsequently, Ang-2, 3 and 4 were identified through low stringency screening [80, 81]. Ang-1 and Ang-2 have been the most studied ligands. Ang-1 and Ang-2 both bind to Tie-2, but Ang-1 acts as an agonist while Ang-2

has been proposed to be an endogenous antagonist to Ang-1. It should be emphasized that the ability to bind Tie-2 receptors is cell-type and context dependent [80, 81]. In ECs, Ang-2 is unable to activate Tie-2 except at relatively high concentrations or during prolonged exposure [82]. *In-vitro* studies have revealed that Ang-2 can activate non-endothelial cells transfected with a Tie-2 receptor expression plasmid and suggested that, in this scenario, Ang-2 acts as a Tie-2 receptor agonist [83].

Mouse Ang-3 and its ortholog human Ang-4 represent the third and fourth members of the Ang family. Ang-4 can activate Tie-2 in human ECs. Ang-3 is a Tie-2 agonist in the mouse endothelium [81, 84].

Ang-1 and Ang-2 have similar overall structures. Both molecules are composed of an N-terminal super-clustering domain that is preceded by a coiled–coil motif. They also contain a short linker and a fibrinogen-like domain (FDR) at the C terminal. The FDR domain is composed of three subdomains: A, B, and P. The P subdomain contains the Tie-2 binding region and is located on the undersurface of the molecule. At the amino acid levels, sequence identity between Ang-1 and Ang-2 is approximately 60% overall and approximately 73% in the P-domains [85, 86].

The coiled–coil domain is responsible for the oligomerization of the angiopoietin monomers [86]. The super-clustering domain allows the formation of higher multimers from angiopoietin oligomers. Ang-1 and Ang-2 exist in different orders. For example, Ang-1 exists in higher order multimers while Ang-2 forms only a small portion of higher order multimers [86]. However, *in-vitro* studies have also shown that transfected Tie-2 in non-endothelial cells can be activated upon addition of dimeric Ang-1, suggesting the existence of co-receptors and activators that require the ligand to assume higher-order

multimers to be able to activate [87]. Lastly, the linker domain allows Ang-1, but not Ang-2, to bind to the extracellular matrix [88].

#### 1.2.3.6. **Angiopoietin-1**

Ang-1 is expressed by peri-endothelial mural cells (smooth muscle cells and pericytes), fibroblasts, and several types of nonvascular stromal and tumor cells [79]. This ligand has a fundamental role in regulating vessel maturation and stabilization during the mid-period of embryonic development, in vessel remodeling, and in maintenance of normal vasculature throughout life [89, 90]. The DNA sequences of both human and mouse Ang-1 cDNA clones share 97.6% identity and encode a glycosylated protein of 498 amino acids that specifically binds to Tie-2 receptors in ECs and induces tyrosine phosphorylation [79]. The importance of Ang-1 in vascular development has been shown with genetic deletion of this gene in mice. Deletion of Ang-1 in mice leads to embryonic lethality due to a phenotype that resembles that of Tie-2 null mice [75, 89]. *Ang-1*$^{-/-}$ display normal vasculogenesis but die at E12.5 due to decreased vascular complexity with dilated vessels, diminished branching, and reduced number of small vessels. Additionally, the blood vessels in these mice have less ECs, defects in the extracellular matrix, and vessel rupture. Somewhat surprisingly, conditional knockout of Ang-1 after E12.5 is dispensable, indicating that Ang-1 is required during a specific developmental window [90].

##### 1.2.3.6.1. **Cellular effects and signaling of Ang-1/Tie-2 pathway**

The primary receptor that specifically binds to and mediates angiopoietin signaling is Tie-2 [79]. Ang-1 binding to Tie-2 leads to receptor dimerization, activation of the kinase domain, and auto-phosphorylation of several tyrosine residues [91]. The phosphorylated tyrosine residues serve as binding sites for several effector proteins that activate various signaling cascades. These signaling cascades, in turn, trigger several cellular responses.

#### 1.2.3.6.2. **Anti-apoptotic and pro-survival effects of Ang-1**

Ang-1 suppresses apoptosis in various ECs, including human umbilical vein endothelial cells (HUVECs) and aortic and microvascular ECs. Additionally, these effects of Ang-1 are clearly evident during serum deprivation and hyperosmolarity [92]. In some non-ECs, including mouse cortical neurons and skeletal and cardiac myocytes, Ang-1 has also been found to inhibit apoptosis [93, 94]. However, studies show Tie-2 to be expressed only in neurons, leaving the possibility of an unknown receptor for Ang-1 in myocytes.

During serum deprivation, the anti-apoptotic effects of Ang-1 are mediated through the interaction of the p85 subunit of phosphatidylinositol 3 kinase (PI3K) with tyrosine$^{1101}$ of Tie-2 via Src homology 2 (SH2) or the phosphotyrosine-binding (PTB) domain. The PI3 kinases are a family of heterodimeric lipid kinases that are able to phosphorylate membrane-bound phosphatidylinositol to generate phosphoinositides (PIs) [95, 96]. PIs serve as docking sites for AKT [96]. Activation of the PI3K-protein kinase B (AKT) pathway in ECs leads to inhibition of Smac release from the mitochondria and upregulation of Survivin protein, leading to increased EC survival. AKT is a serine/threonine kinase that promotes survival by suppressing mediators of apoptosis,

including caspase-9 and Bcl2-associated death promoter (BAD), and inducing pro-survival proteins, including Survivin [97]. Ang-1 can also elicit anti-apoptotic effects by inhibiting forkhead transcription factor FKHR (FOXO1) through AKT activation [98, 99]. Upon activation, AKT phosphorylates FOXO1 protein at three conserved sites, resulting in the anchoring of FOXO1 protein to the cytosolic 14-3-3 protein. FOXO1 is a transcription factor that induces apoptosis and regulates expression of several genes involved in vascular destabilization and remodeling, including Ang-2 [98].

The mitogen-activated protein kinase (MAPK) pathways are also involved in the pro-survival effect of Ang-1. MAPKs are a family of enzymes conserved by evolution that connect cell-surface receptors to critical regulatory targets within cells. They regulate processes such as proliferation, migration, survival, and differentiation in response to extracellular stimuli. The mammalian MAPK family is composed of four distinctly regulated groups of MAPKs: extracellular-signal related kinases- (ERK) 1/2, Jun amino-terminal kinases- (JUNK) 1/2/3, p38 proteins (p38α/β/γ/δ), and ERK5. These MAPKs are regulated by specific MAPK kinases (MAPKK): MEK1/2 for ERK1/2, MKK3/6 for p38, MKK4/7 for JNKs, and MEK5 for ERK5. Each MAPKK can be activated by other MAPKK kinases (MAPKKK) that respond to distinct stimuli, making the pathway a complex regulatory network. Such stimuli can include small guanoside triphosphate (GTP)-binding proteins like Ras and Rho GTPases or STE20 kinases [100, 101].

P38 and JNK1/2 are stress-activated MAPK proteins. P38 activation is mediated by phosphorylation on a conserved motif that includes a glycine residue between the canonical threonine and tyrosine. Dual phosphorylation is catalyzed by mitogen-activated protein kinase kinases (MAPKKs), MKK3 and MKK6, which are activated by

several mitogen-activated protein kinase kinase kinases (MAPKKKs). P38 is activated by stress conditions and inflammatory cytokines. JNKs are activated by MKK4 and MKK7 [102, 103]. All MAPKKs (MKK3, MKK6, MKK4 and MKK7) are phosphorylated and activated by several MAPKKKs, including MEKK1, ASK1 and TAK1 [102]. Upon activation, both p38 and JNK accumulate in the nucleus, where they activate transcription factors like ATF2, p53, Elk-1, ATF1, MEF2, and Ets-1 in the case of p38 and c-Jun, NFAT, STAT-3, cMyc, and JunB in the case of JNK [102, 104, 105]. Although activation of JNK and p38 in many cell types leads to apoptosis, the biological outcome of activation of these MAPKs is diverse and is dependent on cell type. Interestingly, Ang-1/Tie-2 can simultaneously activate the pro-apoptotic effects of p38 MAPKs and the anti-apoptotic effects of PI3K; however, the activation of the anti-apoptotic effects is stronger, resulting in a net anti-apoptotic effect [106].

#### 1.2.3.6.3. **Pro-migration effects of Ang-1**

In ECs, Ang-1 stimulates migration through Tie-2, as shown by experiments in cells from Tie-2-deficient mice [107-109]. Signaling to induce migration is mediated, in part, by PI3K and the adaptor protein downstream of kinase related Dok-R. PI3K-dependent AKT activation has been shown to directly phosphorylate nitric oxide synthase (eNos), leading to nitric oxide production [110, 111]. Endothelium-derived nitric oxide (NO) is a crucial mediator in the regulation of EC migration and vessel formation [112]. Interestingly, $eNOS^{-/-}$ mice show marked impairment in postnatal angiogenesis in response to growth factor delivery [108, 113]. Dok-R, on the other hand, interacts with Tie-2, which leads to association with Dok-2 and phosphorylation of Dok-R. This phosphorylation event promotes Dok-R binding to adapter proteins, which leads to p21

activated kinase (PAK) activation. PAK activation results in reorganization of the actin cytoskeleton and Ang-1 mediated migration [114, 115].

Increased motility in response to Ang-1 also involves the GTPases RhoA and Rac1, as indicated by the activation of these proteins in response to Ang-1 exposure and by the ability of dominant-negative forms of the GTPases to suppress Ang-1-induced EC motility [116, 117]. Moreover, nicotinamide adenine dinucleotide phosphate (NADPH) oxidase can be activated by this pathway, resulting in the production of reactive oxygen species (ROS) that lead to enhanced migration of ECs [118].

Tie-2 can also interact with adapter proteins containing the Src homology domain, such as Grb2, 7, 14, and tyrosine phosphatase Shp2, which can activate the MAPK pathway. Through Grb2, the G-protein Ras is activated and in turn activates the MAPKKK protein Raf. Raf then induces the phosphorylation of serine residues in the activation loop of Mek (MAPKK), which leads to phosphorylation of ERK1/2 [119]. Active Erk phosphorylates multiple cytoplasmic and cytoskeletal proteins and translocates to the nucleus, where it phosphorylates and activates various transcription factors, including Sp1, E2F, Elk1 and AP1. This process leads to promotion of migration, survival and other processes [120].

Ang-1-induced activation of ERK1/2, JNK, and the PI3 kinase pathways also triggers the production of interleukin 8 (IL8), a chemokine associated with infiltration of inflammatory cells at sites of injury, but also promotes angiogenesis in ECs [121]. Activation of the Erk1/2, SAPK/JNK, and PI3 kinase pathways by Ang-1 causes c-Jun phosphorylation and increases DNA binding of activator protein-1 (AP1) to the promoter of IL8 [121]. Activation of IL8 mediates in part the pro-migratory effects of Ang-1 in ECs.

In addition to AP1, Ang-1 induces the expression of early growth response-1 (Egr1), a zinc finger transcription factor known to be induced by various stimuli, including growth factors and hypoxia [122, 123]. Induction of Egr1 by Ang-1 results from the activation of ERK1/2 and PI3 kinase signaling pathways and plays an important role in mediating Ang-1-induced EC migration [124].

#### 1.2.3.6.4. **Vessel reorganization and sprouting**

Ang-1 induces EC sprouting, an essential process in angiogenesis, partly through cytoskeletal changes and secretion of proteinases. This effect of Ang-1 is dependent on the PI3 kinase pathway [125]. Ang-1 treatment of porcine pulmonary artery ECs induces phosphorylation of focal adhesion kinase (FAK), secretion of plasmin and matrix metalloproteases, and suppresses the secretion of tissue inhibitor of metalloproteinase-2 (TIMP-2) [126]. Matrix metalloproteinases (MMPs) are a group of zinc-dependent endopeptidases capable of cleaving extracellular matrix proteins in the vessel wall, such as collagen, elastin, gelatins, and casein [127].

Ang-1-dependent phosphorylation of eNOS through the PI3 kinase/AKT pathway plays a role in vessel-sprouting, tip cell formation, and EC remodeling. Sinha *et al.* demonstrated that activation of the eNOS/NO/cGMP axis prompts endothelial ring formation *in-vitro* [128]. Moreover, *eNOS* knockout mouse experiments confirmed that functional eNOS is crucial for optimal tip cell sprouting. The same group concluded that eNOS-derived NO mediates tip cell sprouting via the soluble guanylate cyclase sGC–cyclic GMP (cGMP) pathway [129]. NO exerts its effect by binding to the heme in sGC that activates cGMP synthesis [129].

#### 1.2.3.6.5. **Anti-vascular leakage effects of Ang-1**

To control exchange of fluids, nutrients, and leukocytes between organs and blood, ECs form a semi-permeable cellular membrane [130, 131]. In order to regulate this barrier, ECs utilize adherens and tight junctions to establish strong cell–cell contacts [132]. An additional layer of stabilization is established by tight endothelial associations with pericytes [133]. Under normal conditions, the endothelium is stable and contains limited vascular leak [134]. Destabilization occurs when interactions between junctions are disrupted. In the endothelium, these critical vascular-stabilizing interactions are provided by the adherens junctions and the protein vascular endothelial cadherin (VE-cadherin) [135]. It has been demonstrated that a VE-cadherin blocking antibody can cause a concentration and time-dependent increase in permeability in the lung and heart *in-vivo* [136]. Moreover, breakdown of VE-cadherin is mediated by phosphorylation [136]. Multiple permeability-inducing factors can cause phosphorylation of VE-cadherin, including VEGF, which activates Src, a non-receptor kinase necessary for the permeability. Src, in turn, activates the Rac pathway by phosphorylating a serine residue of the cytoplasmic tail of VE-cadherin. Such activation leads to recruitment of β-arrestin, a mediator of VE-cadherin endocytosis [137]. Endothelial junctions are another important target of Tie-2 activation because sequestration of Src, through the RhoA downstream target mDia, prevents internalization of VE-cadherin and subsequent disruption of the endothelial barrier [137].

Contrary to VEGF, Ang-1 stabilizes the vasculature against vascular leak. Overexpression of both Ang-1 and VEGF leads to enhanced neovascularization; however, stabilization of these vessels is evident by decreased Evans Blue

extravasation in the mouse ear in response to overexpression of Ang-1 [138]. Ang-1 can inhibit the permeability-inducing effects of VEGF by inhibiting VEGF activation of Src [139]. This inhibition is mediated through a Rho-dependent mechanism. Ang-1 also stimulates dimerization mDia1 and mDia2 (downstream targets of Rho signaling) and causes mDia to bind Src. Knockdown of either mDia1 or mDia2 using siRNAs inhibits the activity of Ang-1, further demonstrating that mDia1 and mDia2 are necessary for Ang-1-mediated signaling [139]. Sequestration of Src by mDdia2 prevents internalization of VE-cadherin and subsequent disruption of the endothelial barrier[137].

#### 1.2.3.6.6. **Anti-inflammatory effects of Ang-1**

Ang-1 functions as an anti-inflammatory agent [140]. During inflammation, ECs recruit leukocytes to the sites of injury in response to pro-inflammatory mediators, and adhesion molecules are induced on the surface of ECs through activation of the nuclear factor (NF)-κβ [141]. Phosphorylated Tie-2 receptors recruit A20-binding inhibitor of NF-κβ (ABIN2), resulting in the inhibition of NF-κβ-mediated expression of inflammatory molecules such as intercellular adhesion molecule1 (ICAM1), E-selectin, and tissue factor [142, 143].

In addition to its pro-angiogenic effects, VEGF enhances leukocyte adhesion to ECs through induction of ICAM, VCAM, and E-selectin adhesion molecules [144]. It has been reported that Ang-1 mediates its anti-inflammatory effects through the nuclear receptor 77( Nur77) [145]. Nur77 is a member of the ligand-independent nuclear receptor family that is expressed in response to growth factors and cytokines [146]. *In-vitro* studies revealed that Nur77 interacts with the p65 subunit of NF-κβ, resulting in the inhibition of p65 subunit binding to the promoters of pro-inflammatory cytokines [147]. In

ECs, Nur77 expression is induced in response to TNF-α and functions as a negative feedback mechanism designed to inhibit NF-κβ signaling. This effect is mediated through transcriptional upregulation of kappa B alpha (Iκβα) expression, a protein that inhibits the NF-κβ from translocating to the nucleus [148]. In ECs, Ang-1 upregulates the expression of Nur77 via PI3 kinase and ERK1/2 pathways, and Nur77 in turn leads to inhibition of VEGF-induced leukocyte adhesion as well as VCAM and E-selectin expression [145, 149]. Another mechanism through which the Ang-1/Tie-2 pathway inhibits inflammation is through upregulation of the transcription factor KLF2, which in turn mediates a strong inhibitory effect on VEGF-induced VCAM1 expression and monocyte adhesion in ECs [150].

#### 1.2.3.6.7. **Relationship between Ang-1 and VEGF during angiogenesis**

VEGR and Ang-1 target the MAPK pathway and promote migration. However, Ang-1 is considered to promote vessel quiescence and inhibition of vascular leakage while VEGF has the opposite effect on both of these processes. An interactive model has been proposed to address the interplay between these two molecules based on Ang1 assembling distinct Tie2 signaling complexes in either presence or absence of endothelial cell-cell adhesions. Ang1 induces trans-association of Tie2 at cell-cell contacts, whereas Tie2 is anchored to the extracellular matrix (ECM) by Ang1 at the cell-substratum interface. Trans-associated Tie2 and ECM-anchored Tie2 activate distinct signaling pathways [460, 461]. Under quiescent conditions angiopoietin (Ang) 1 released from mural cells induces transassociation of Tie2 at endothelial cell-cell contacts, which activates the angiostatic signaling pathway to maintain vascular

quiescence. Since a small amount of VEGF exist in this condition, VEGF induced angiogenesis is suppressed by the Ang-1/Tie-2 signal. However once VEGF is released by stressed tissue, mural cells detach from endothelial cells and disruption of inter-endothelial cells adhesion occurs. Under this conditions Ang-1 binds to Tie2 anchored to the extracellular matrix, and activates the angiogenic signaling pathway, therefore promoting angiogenesis cooperatively with VEGF.

#### 1.2.3.7. **Ang-2**

Ang-2 has been proposed to have an antagonist role to Ang-1 in phosphorylating Tie-2 [151]. Ang-1 and Ang-2 bind with similar kinetics to Tie-2 [86, 152]. Structurally, Ang-2 shares a ~60% amino acid identity with Ang-1 and contains an amino-terminal coiled–coil domain and a carboxyl-terminal fibrinogen-like domain; however, Ang-2 lacks a cysteine between the coiled–coil and fibrinogen-like domains [79, 80, 85, 86]. The antagonistic character of Ang-2 has been demonstrated by genetic mouse models. Deletion of either Ang-1 or Tie-2 leads to a similar endothelial-specific phenotype to that observed in mice overexpressing Ang-2 [80]. In other words, too much Ang-2 resembles too little Ang-1 [153].

Ang-2 production by ECs is regulated at the transcriptional level and its expression is confined to the vascular endothelium, where it is stored in Weibel-Palade bodies [154-156]. Upon induction, Ang-2 is rapidly released and acts in an autocrine fashion to compete with Ang-1 for the binding of Tie-2, leading to disturbances in endothelial junctional integrity, pericyte drop-off, and priming of the vascular bed for angiogenic

sprouting [157, 158], the latter being highly dependent on local cytokine presence. In the presence of VEGF, Ang-2 can induce vascular sprouting. However, in the absence of VEGF, Ang-2 induces EC destabilization, blood vessel regression, and apoptosis [159]. This anti-angiogenic effect is now being tested as a means to limit solid tumor growth. In fact, several pre-clinical studies in mouse models have reported that blocking Ang-2 inhibits tumor growth and tumor blood vessel sprouting [160]. Moreover, blocking Ang-2 has been reported to inhibit tumor lymphangiogenesis, increased pericyte adhesion and upregulation of EC-EC junction molecules, thereby resulting in an improved “normal” vessel phenotype [161, 162]. In addition, *in-vivo* data have shown that a subpopulation of Tie-expressing monocytes is associated with tumor blood vessels that exhibit proangiogenic activity. This activity was inhibited when Tie-2 was silenced or Ang-2 was neutralized with an antibody [163, 164].

In addition to the regulation of angiogenesis, Ang-2 promotes endothelial inflammatory processes, as is indicated by the significant upregulation of TNFα expression in ECs and increased leukocyte adhesion to ECs in response to Ang-2 exposure. *In-vitro* Ang-2 can upregulate IL8RB and IL10B in human neutrophils [165]. Ang-2 also triggers the infiltration of neutrophils and monocytes in a murine sponge/Matrigel model [166].

#### 1.2.3.8. **Ang-3 and Ang-4**

Ang-3 and Ang-4 are expressed by the same gene locus in mice and humans, respectively [167]. Although these two ligands are considered interspecies orthologs, the percentage of amino acid identity between them is only 65%. In comparison, human and mouse Ang-1 have 99% amino acid identity and human and mouse Ang-2 have 87% amino acid identity [15, 80, 81]. Both Ang-3 and Ang-4 are less characterized than

Ang-1 and Ang-2. Ang-4 acts as an agonist to Tie-2, while Ang-3 was proposed to be an antagonist [81]. This is based on the observation that Ang-3 inhibits Ang-1-induced phosphorylation of Tie-2. However, Lee et al. found that treating ECs, *in-vitro* and *in-vivo,* with Ang-3 and Ang-4 recombinant proteins elicited opposing results. Although Ang-4 was a weak Tie-2 agonist, Ang-3 elicited strong activation of mouse Tie-2 [84]. That study also reported that Ang-3 and Ang-4 can elicit strong phosphorylation of AKT, promote survival of ECs, and induces corneal angiogenesis *in-vivo*. In glioblastoma multiform cells and tissues, expression of multimeric Ang-4 has been detected [233]. In this type of tumor, which also expresses Tie-2, Ang-4 promotes angiogenesis and directly activates the Erk1/2 pathway [168].

#### 1.2.3.9. **Angiopoietin signaling through integrins**

Angiopoietins binding to integrins has been described by several studies. Selective blocking of various integrins with antibodies revealed that Ang-1 binds to α2β1, α5β1, αvβ1, and αvβ1 integrins [169-172]**.** Moreover, Ang-1 binds directly to α5β1 integrin via its fibrinogen-like domain. In addition, it has been reported that Ang-1 trimers bind to soluble Tie-2-Fc chimera but do not elicit phosphorylation of Tie-2 tyrosine residues. Ang-1 dimers weakly bind to soluble Tie-2-Fc and do not elicit tyrosine phosphorylation of this protein. Ang-1 monomers barely bind to Tie-2-Fc, but they bind to α5β1 integrin [86, 87, 171]. Moreover, when a plasmid expressing an Ang-1 monomer was injected into the heart, ECs and smooth muscle cells showed significant induction of the Integrin-linked kinase, a key regulator of integrin signaling and cardiac health, while Tie-2 phosphorylation remained unchanged [173].

Like Ang-1, Ang-2 also signals via integrins to promote vessel growth. Ang-2 contains a highly conserved N-terminal fibrinogen-like receptor-binding domain that forms a functional association with certain integrin receptors [174]. Ang-1 and Ang-2 were shown to stimulate Tie-2-independent cell adhesion of ECs and fibroblasts to Ang-1- or Ang-2-coated surfaces through $\alpha_5\beta_1$ and $\alpha_v\beta_5$ integrin-mediated activation of ERK and FAK signaling [170].

#### 1.2.3.10. **Chronic inflammatory diseases and Ang/Tie-2 axis**

Physiological angiogenesis is different from pathological angiogenesis is the presence of inflammation, required to initiate angiogenesis in a pathological environment. This process contributes to the intensification of chronic inflammatory status [462]. In a K/BxN serum transfer model of arthritis using Tie-2 overexpressing mice, enhanced joint expression of IL6, IL12B, NOS2, CCL2 and CXCL10, and activation of bone marrow-derived macrophages in response to Ang-2 stimulation, was observed [463]. Both Ang-1 and Ang-2 induced the production of IL-6, IL-12p40, IL-8 and CCL-3 in synovial tissue explants of rheumatoid arthritis and psoriatic arthritis patients. In addition, Ang-2 neutralization suppressed the production of IL-6 and IL-8 in the synovial tissue of rheumatoid arthritis patients. Another study, also in rheumatoid arthritis showed that a strong expression of the receptor Tie2 and its agonistic ligand Ang-1 at sites of joint destruction [464].

## 1.3. **mTOR in angiogenesis**

### 1.3.1. **mTOR: An overview**

Target of rapamycin (TOR) is a well-conserved serine/threonine kinase important for a cell's transition between anabolic and catabolic states and is dependent on nutrient availability [175]. This kinase plays an important role in the signaling network that controls growth and metabolism in response to environmental cues. In early eukaryotes, TOR enabled these unicellular organisms to sense the availability of nutrients and promote cell growth whenever conditions were favorable [176]. With evolution and the development of multicellular organisms, the role of TOR increased, and it became a central player in controlling growth and homeostasis. Mammalian TOR (mTOR), now officially known as mechanistic TOR, is one of such molecules implicated in cellular states and diseases where regulation of homeostasis and growth is altered. As indicated by its name, TOR is targeted by a molecule name rapamycin, an anti-fungal macrolide isolated from a bacteria acquired from a soil sample from Easter Island in the 1970s [177]. In addition to its anti-fungal properties, rapamycin strongly inhibits cellular growth and proliferation. In the mid-1990s, mTOR was described as target of rapamycin [178-180].

### 1.3.2. **mTOR complexes and the downstream pathway**

mTOR belongs to the phosphoinositide 3-kinase related protein kinases (PIKK) family, which comprises large proteins that enable organisms to cope with metabolic, environmental, and genetic stress [181]. mTOR is found in mTORC1 (mTOR complex 1).

mTORC1 is known to have two substrates: S6 kinase 1 (S6K1) and eIF4E-binding protein 1 (4E-BP1). S6K1 controls protein synthesis by associating with mRNAs to regulate mRNA translation initiation and progression, thus enhancing protein synthesis

[178, 182, 183]. 4E-BP1 suppresses translation by binding to eIF4E, which, when free, brings translation initiation factors to the 5’ end of the mRNAs. However, when mTORC1 phosphorylates 4E-BP1, the latter dissociates from eIF4E [182]. S6K1 is a positive regulator of translation initiation and elongation when it is phosphorylated by mTORC1. It phosphorylates multiple substrates, including S6, eEF2K, SKAR, CBP80, and eIF4B [184-187].

Ribosomal biogenesis is an energy-intensive process that requires tight cellular control. The synthesis of both ribosomal mRNA and proteins are promoted by mTOR. Phosphorylation of S6K1 by mTOR leads to transcriptional activity of the rRNA polymerase, RNA PolI [188, 189].

Autophagy is the degradation of cellular components in a controlled manner. This degradation can be from individual proteins, termed microautophagy, to entire organelles, termed macroautophagy, and allows recycling damaged and dangerous cellular components. Autophagy can provide a source of energy when cells are undergoing a period of low nutrients [190]. An important function of mTORC1 is to suppress this process, so inhibition of mTOR results in induction of autophagy [191, 192].

### 1.3.3. **Upstream activators of mTORC1**

mTORC1 is regulated by several upstream signals that can be grouped into nutrients, growth factors, energy, stress, Rheb (Ras-homolog enriched in brain), and trimeric tuberous sclerosis complex (TSC), composed of TSC1, TSC2, and TBC1D7 (Tre2-Bub2-Cdc16-1 domain family member 7) [193, 194]. The role of Rheb is to directly bind

and activate mTORC1, but it is normally suppressed by TSC2 [195]. When GTP is bound to Rheb, it stimulates mTORC1 activity. TSC2, however, acts as a GTPase activating protein (GAP) toward active GTP-bound Rheb and converts it to GDP-bound, the inactive Rheb, which suppresses mTORC1 activity [196, 197]. Therefore, activity of mTORC1 is greatly dependent on the TSC complex, which is regulated by signals dependent on growth factor availability, cellular energy level, and oxygen presence [194].

Amino acids are known as the building blocks of proteins, but they are also necessary during DNA synthesis, glucose metabolism, and during protein synthesis [198]. It has been demonstrated that amino acids activate mTORC1; however, the actual amino acids that activate this pathway have not been identified [199-201]. Moreover, the actual level of amino acid does not directly activate mTORC1 through the TSC complex and Rheb activation [202].

Growth factors can activate downstream signaling resulting in the phosphorylation of mTORC1 via the PI3K/AKT axis [203]. Activation of mTORC1 by growth factors starts at the plasma membrane and is usually triggered by hormones like insulin and insulin-like growth factor 1 (IGF-1). These growth factors in turn activate PI3K, which culminates with the phosphorylation of AKT, which then phosphorylates and inhibits TSC [203, 204]. Availability of growth factors, AKT, and other downstream kinases, such as MAPKs and p90 RSK1, trigger the phosphorylation of TSC2, thereby causing mTORC1 activation. Exactly how TSC2 phosphorylation leads to mTORC1 activation remains unclear. Other signaling pathways activated by growth factors that regulate mTORC1 include the Ras-Raf-Erk axis, which is able to inhibit the TSC complex [205]. In addition,

the Wnt pathway has also been implicated in mTORC1 signaling, by inhibiting glycogen synthase kinase (GSK), an inducer of the TSC complex [206, 207].

### 1.3.4. **Ang-1, angiogenesis, and mTOR**

Hypoxia is an important stimulus of angiogenesis. Hypoxic stress activates hypoxia-inducible transcription factors (HIFs) that ultimately induce the expressions of VEGF, VEGFR2, FGF2, PDGF, and Ang-2 [208]. Through PI3K, mTOR induces the translation of HIF1, which enhances the expression of VEGF [209]. It has been shown that AKT-dependent angiogenesis can be inhibited by rapamycin, thereby demonstrating the importance of mTOR signaling in vascularization [210]. Moreover, hypoxia has been shown to induce mTORC1 and mTORC2 in ECs [211]. Based on these findings, inhibition of mTORC1 with rapamycin and its analogues has become a targeted therapy in inhibiting angiogenesis in solid tumors [212, 213]. These compounds have been shown to reduce VEGF production and angiogenesis in several animal models [214].

Ang-1 triggers significant activation of mTORC1 in ECs. In one study, it was described that Ang-1 attenuated iopromide-induced apopotosis in a dose-dependent manner. Inhibition of PI3K eliminated the anti-apoptotic effects of Ang-1. Moreover, it was observed that Ang-1activates mTOR/ribosomal protein p70S6K1 through PI3K, and that inhibition of mTOR suppressed Ang-1-dependent phosphorylation and reduced the anti-apoptotic effects of Ang-1 [215]. In a second study on ischemic brain injury model, addition of Ang-1 triggered angiogenesis through AKT/mTOR interaction. This interaction was independent of the MAPK pathways, and the silencing of Tie-2 receptors eliminated AKT/mTOR interaction [216]. It has also been reported that Ang-1 induces the expression of early growth response 1 (Egr1) transcription factor in ECs

through mTORC1 activation and that this transcription factor plays an important role in Ang-1-induced migration, proliferation, and capillary-like tube formation of ECs [124].

## 2. MicroRNAs and angiogenesis

### 2.1. Overview of miRNAs

Before the era of genome sequencing, many scientists believed that the human genome was composed of ~100000 protein-coding genes [217]. It was also assumed that more complex organisms would have a greater number of genes when compared to simpler ones, such as *C. elegans* [218]. With the completion of the genome project, it was found that the human genome had ~20000 protein coding genes, an equivalent to 1-2% of all the transcripts produced by the genome [219]. These data suggest that highly complex organisms like humans have about the same number of genes as much simpler organisms like *C. elegans*. The other 98% of gene-transcripts were classified as 'junk' DNA with no functional purpose [220, 221]. The RNAs were then divided in two different classes: protein-coding RNAs (composed of mainly mRNAs) and non-coding RNAs (ncRNAs) [222]. Additionally, it was noted that the number of genes was not correlated with the complexity of the organism, since humans contain roughly the same number of protein-coding genes as *C. elegans*. However, the amount of ncRNAs in an organism correlated with its complexity. These results suggested a tremendous influence of ncRNAs on the development and organization of more highly-structured animals [223].

Non-coding RNAs are divided into two classes depending on their size [224]. RNA molecules smaller than 200 bases are called small non-coding RNAS, while molecules larger than 200 bases are termed long non-coding RNAs. The two groups are

heterogeneous, with small non-coding RNA ranging from a few to 200nt and long non-coding RNA being as large as several kilobases [219, 225].

Among the non-coding RNAs, miRNAs, a class of small ncRNAs, have been the most studied because of their ability to regulate a number of biological processes, including cell proliferation, differentiation, developmental timing control, apoptosis, stress responses, and pathological states such as cancer[226-228]. miRNAs are a family of 21–25 nucleotides (small RNAs) that negatively regulate gene expression at the post-transcriptional level [229]. They have recently been implicated in the regulation of angiogenesis. miRNAs are transcribed from the genome into long RNA molecules (pri-miRNAs) [230]. These transcripts are then cleaved into a ~70nt long hair-pin loop containing molecules known as pre-miRNAs by a microprocessing complex [231]. Pre-miRNAs are then exported into the cytoplasm and are cleaved near the loop [232]. The cleavage releases small ~20bp double-stranded molecules [233]. The small duplex is then loaded into one of the Argonaut proteins to form an effector complex called RNA-induced silencing complex (RISC) [234]. The duplex is then unwounded into two single stranded miRNAs (guide and passenger strands), where the passenger strand gets degraded and the guide is bound to a complementary target. Binding to the target results in translational inhibition or degradation of mRNA [235-237].

### 2.2. **Family classification and nomenclature**

miRNA nomenclature is dependent on their discovery and is somewhat inconsistent [238]. Genes found in early genetic studies were named after their genotype. For example, Let-7, one of the first miRNAs to be identified in *C. elegans*, was named after its phenotype. Loss-of-function *let-7* animals burst through their vulvas and die, thus

indicating that the mutant is lethal (let) [239]. Other miRNAs, identified through cloning or sequencing, receive numerical names [240]. A 3-4 letter abbreviation is used at the beginning of the name to identify the species (i.e. hsa for *Homo sapiens*) [241]. The precursor miRNA is assigned ‘mir’ after the species (e.g., hsa-mir-135); mature miRNAs, on the other hand, are termed ‘miR’ [242]. Mature miRNAs whose sequences are closely related are annotated with a lower case letter at the end of the number (e.g., hsa-miR-135a and hsa-miR-135b) [242]. Distinct precursor sequences and genomic loci that express identical mature sequences are indicated with an additional number (e.g., miR-135a1 and miR-135a2) [243]. Each locus produces two mature miRNAs: one from the 3’ strand and another from the 5’ strand, which are denoted with a 3’ or a 5’ suffix respectively (i.e. miR-135-3p and miR-135-5p). Most of the time one strand will prevail over another (the guide strand), while the other is less prevalent (the passenger strand, also known as a miRNA*) [241].

According to the latest version of the miRNA database miRBase, there are 1881 precursor miRNAs and 2588 mature miRNAs (based on the genome assembly GRCh38). *C. elegans* contains 250 precursors and 434 mature miRNAs. Several miRNA loci that contain related sequences that originated from gene-duplication events lack classification. However, it is generally considered that miRNAs with identical mature seed sequence (nucleotide 2-8) belong to the same miRNA family [244]. For example, the miRNA family let-7 contains 14 paralogous loci that encode different miRNA with the same seed sequence, called “miRNAs sisters”. MicroRNA sisters are able to target the same mRNAs but are also capable of having distinct roles [245]. To date, 134 miRNA families exist that are conserved across all mammals [246, 247].

Other miRNAs share an evolutionary origin but, due to divergence, contain different seed sequences. This is the case with miR141 and miR-200c, which belong to the conserved miR-200 superfamily but differ by one nucleotide in their seed sequence. Interestingly, these two miRNAs barely overlap in their target recognition [248].

## 2.3. Biogenesis of miRNAs

### 2.3.1. Transcription from the genome

In animals, miRNAs are transcribed, mostly by polymerase II. In the nucleus, a primary RNA molecule (pri-miRNA) that contains a local hairpin structure with the miRNA sequence is transcribed. Interestingly, Polymerase III has been found to transcribe viral miRNAs [249]. miRNA sequences are found in different locations throughout the genome. In humans, most miRNAs are located in intronic regions; however, some are found in exons. In some cases, several miRNA loci are in close proximity and consequentially transcribed in a polycistronic fashion [250]. Clustered miRNAs tend to be transcribed as a unit but are later regulated at the post-transcriptional level individually. This is the case in those sequences found in the mir-100~let7~mir-7~mir-125 cluster, which play a role in vertebrate embryogenesis. During development, they are all transcribed as a unit but only Let-7 is suppressed post-transcriptionally [251, 252].

The promoter regions of most miRNA are yet to be identified. Yet, collective analysis of CpG islands and chromatin immunoprecipitation-sequencing data can give details as to where these regulatory regions can be found [253]. For example, some miRNAs sequences reside in the introns of protein-coding genes and share the

promoter region with the host gene. However, many other miRNAs have multiple transcription start sites, and their promoters are distinct from that of the gene in which they are found [254]. Epigenetic control such as DNA methylation and histone modification also contribute to miRNA gene regulation. Additionally, several transcription factors, such as p53, can positively or negatively regulate the control of miRNA expression.

### 2.3.2. **Nuclear processing**

Following transcription from the genome, the pri-miRNA, a typically long RNA molecule with more than 1Kb in length, undergoes several steps of maturation [255]. The pri-miRNA is composed of a stem loop of 35 to 55bp, a terminal loop and a single-stranded RNA segment at the 5' and 3' end [256]. Its maturation process starts with the cropping of its stem-loop, which releases a small hairpin-like RNA of ~65 nucleotides in length called precursor miRNA (pre-miRNA). The cleavage is mediated by the RNase III Drosha, which forms a complex, called Microprocessor, with cofactor DGCR8 (also called Pasha in *D. melanogaster* and PASH-1 in *C. elegans*) [231, 257-259]. Drosha is a nuclear protein that belongs to the RNase III-type endonucleases that act on double-stranded RNA specifically. The amino terminal of this protein is necessary for localization in the nucleus [260]. On the opposite end, at the carboxyl terminal, Drosha has consecutive RNase II domains and a double-stranded RNA-binding domain. Two of the RNase II domains dimerize with each other, whereby, in the centre, the RNA-molecule gets processed. One of the RNase II domain cuts at the 5' end of the pri-miRNA, and another one cuts at the 3' end, which produces a molecule with a 3'

overhang composed of only 2 nucleotides [259, 261, 262]. DGCR8 provides additional RNA-binding activity and is recruited to the middle region of Drosha.

DGCR8 is a protein found in the nucleoplasm and the nucleolus [263]. It contains two dsRNA-binding domains that recognize the pri-miRNAs, while its carboxyl terminus interacts with Drosha. Drosha recognizes the pri-miRNA hairpin 11bp away from the “basal” junction between the single stranded RNA and the double stranded RNA and 22 bases away from the “apical” junction linked to the terminal loop [264, 265]. Additional elements are also important in processing pri-miRNAs, including elements in the basal region (UG and CNNC motifs) and the terminal loop (motif UGUG). For example, SRp20, a splicing factor, binds to the CNNC motif and thus increases the processing of human pri-miRNAs [266]. At least one of these motifs is present in ~80% of human miNRAs, suggesting that other determining factors may also exist [267].

Regulation of the Microprocessor leads to efficiency of Drosha-mediated processing, resulting in regulation of miRNA abundance. Multiple mechanisms have been identified that regulate activity, level, and specificity of Drosha. For example, Drosha and DGCR8 regulate each other. Protein–protein interaction between DGCR8 and Drosha stabilizes the latter. However, Drosha destabilizes DGCR8 mRNA by cleaving it at the second exon [268-270]. This type of regulation is conserved across animals, and several researchers believe that it is necessary to enable homeostatic maintenance of Microprocessor activity. In addition, phosphorylation of Drosha by glycogen synthase kinase 3β (GSK3β) is required for nuclear localization of Drosha [271]. Moreover, acetylation of Drosha leads to inhibition of degradation and its stabilization. Regulation

of DGCR8 by histone deacetylase 1 (HDAC1) increases affinity of DGCR8 for miRNAs (68). Moreover, ERK phosphorylation of DGCR8 increases its stability [272].

### 2.3.3. **Nuclear export of miRNAs**

Following the cleavage of the primary miRNA that results in the formation of the pre-miRNA molecule, the latter is transported from the nucleus into the cytoplasm through nuclear pore complexes [273]. Transport of the pre-miRNA is mediated by Exportin 5, which forms a transport complex with the miRNA and a GTP-binding nuclear protein (RAN-GTP) [232, 274, 275]. Upon reaching the cytoplasm, the GTP is hydrolyzed, resulting in the disassembly of the complex and release of the pre-miRNA [232, 274]. The crystal structure of Exportin 5 resembles a baseball mitt structure, with miRNA placed inside, thereby allowing the interaction of miRNA stem with the positively-charged inner surfaced [276]. At the end of the structure, researchers have identified a tunnel-like conformation that strongly interacts with the 2 nucleotides overhang at the 3' end of the pre-miRNA [277, 278]. Knockdown of XPO5, the gene that encodes for exportin 5, results in a significant decrease in miRNA levels and the accumulation of pre-miRNAs in the nucleus [275]. These data suggest an additional role for exportin 5 as a miRNA protector in the nucleus against nucleolytic attack [275].

### 2.3.4. **Cytoplasmic pre-miRNA processing**

Once exported into the cytoplasm, pre-miRNAs are cleaved by Dicer at the terminal loop, which liberates a small RNA duplex [279, 280]. Like Drosha, Dicer is an RNase III type endonuclease [262]. Dicer homologues can be found in plants, animals, and fungi. Similar to Drosha, Dicer contains tandem RNase II domains at the C-terminal that

dimerize to form a catalytic center [262]. At the N terminal, a helicase domain facilitates pre-miRNA recognition by interaction with the terminal loop of the pre-miRNA [281]. Dicer is also composed of a domain of unknown function protein called DUF283 and a Piwi/Argonaute/Zwille (PAZ) domain. The PAZ domain is a double stranded RNA-terminus binding module that has a 3’ overhang binding pocket, with changing electrostatic potential and molecular surface of the pocket [282, 283]. The changing pocket may influence RNA binding by Dicer and the handing off of the miRNA molecule to other protein complexes [282].

Dicer binds to a pre-miRNA with a preference for a two nucleotide overhang at the 3’ end of the molecule [283, 284]. Cleavage occurs 21–25 nucleotides away from the 3’ overhang (known as the 3’ counting rule) [285]. This distance is dependent on the type and the species of Dicer, since some species have more than one gene with different roles [286, 287]. In mammals, Dicer binds to the 5’ phosphorylated end of the pre-miRNA and cleaves it 22nt away from the 5’ end (known as the 5’ counting rule), a process believed to be determined by an additional mechanism [288]. A mutation of the 5′ binding pocket leads to dysregulation of miRNA biogenesis *in-vivo*. Five prime end-binding occurs when the end is thermodynamically unstable, but not when it is strongly G-C paired [288].

Dicer can interact with double-stranded binding proteins that facilitate substrate recognition, cleavage fidelity, and Argonaute loading. In mammals, one such protein is trans-activation-responsive RNA-binding protein 2 (TARBP2) [289]. TARBP2 stimulates Dicer-mediated cleavage of pre-miRNAs by enhancing the stability of the Dicer-substrate complex [290]. Cells lacking TARBP2 exhibit altered cleavage sites in a

subset of miRNAs, but no effect on the general miRNA abundance or Argonaute loading [267].

### 2.3.5. **Formation of the RNA-induce silencing complex in the cytoplasm**

The small miRNA duplex generated by Dicer is subsequently loaded into an Arogonaute (AGO) protein to form an effector complex called RNA-induced silencing complex (RISC) [291]. RISC action on the miRNA duplex involves two steps—loading of the RNA duplex and unwinding it to form two single-stranded RNA molecules. AGO proteins are principal players in small non-coding RNA mediated gene silencing in various organisms. Although originally discovered in plants, AGO proteins are conserved across eukaryotes [292]. The number of AGO proteins varies between organisms [293]. For example, *D. melanogaster* has five AGO proteins, while humans have eight. Some organisms, like *C. elegans*, have as many as 27, while others, like *S. pombe*, only have one [294]. Phylogenetic analysis reveals that the eukaryotic AGO family can be divided into three major clades: the AGO clade, the PIWI clade, and the WAGO clade (only found in *C. elegans*) [295]. The AGO subfamily proteins are ubiquitously expressed and predominantly interact with microRNA (miRNA) [296]. Meanwhile, PIWI clade proteins are exclusively involved in another class of small RNA, known as PIWI-interacting RNA [297].

After being processed by Dicer, microRNA duplexes are loaded onto a particular type of AGO protein [298]. AGO proteins (AGO1-4) are associated with all miRNAs indistinguishably. All proteins interact with all miRNA duplexes and prefer mismatches in nucleotides 8-11 [299-301]. Once loaded onto RISC, a miRNA duplex is unwound to

remove the passenger strand, leaving a mature single-stranded miRNA molecule in the complex. If AGO2 is part of the risk complex, it will cleave the passenger strand at the center [302, 303]. This method of cleavage, however, is rarely used in the miRNA pathway, since most miRNA duplexes have central mismatches and human AGO1, 3, and 4 lack cleavage activity. Therefore, miRNA duplex unwinding without the presence of cleavage is the common method. Mismatches in the guide strand at nucleotide 2–8 and 13–15 promote unwinding of miRNA duplexes [301, 304].

Thermodynamic stability determines which strand of the duplex will be the passenger and which will be the guide [234, 305]. The strand with the unstable terminus at the 5' end is usually selected as the guide strand. In rare cases, 5' stability is similar in each arm of the duplex, and it is then assumed that both will be loaded at a similar frequency. In addition to thermodynamic stability, the first nucleotide sequence also determines passenger vs. guide determination because AGO proteins prefer guide strands that have uracil at position 1 [306-308].

#### 2.3.6. **Target recognition**

RISC pairs the miRNA with its target mRNA through Watson-Crick base-pairing between the guide strand and the 3'UTR of the target [309, 310]. Target recognition relies heavily on base-pairing between the seed (residues 2–8 at the 5' end) of the miRNA guide [311]. The degree and nature of complementarity determine the gene-silencing mechanism [255, 312]. In animals, complementarity in the seed regions, not over the whole miRNA, results in RNA hybrids with a characteristic bulge [255, 313, 314].

### 2.3.7. **Silencing**

It is widely accepted that miRNAs bind to their mRNA targets and inhibit their expression. One miRNA has the ability to interact with and silence different mRNAs [255]; however, the silencing mechanism remains largely unknown. Evidence points toward two possible mechanisms, namely, mRNA cleavage and translation repression (also known as slicer-dependent and slicer-independent silencing) [315, 316]. Slicer activity refers to the endonuclease cleavage of target mRNA by AGO2, which requires extensive base-pairing between the miRNA and mRNA target [299, 300, 317, 318]. Slicer-dependent and Slicer-independent silencing mechanisms have one of two downstream effects—mRNA degradation or translation inhibition. Both ultimately lead to downregulation of gene expression.

The slicer-dependent mechanism occurs when AGO2 catalyzes miRNA::mRNA cleavage. This happens when the target and the miRNA are base-paired over the seed region and bases 10–11 of the guide miRNA [299, 300, 312, 318]. Cleavage products are degraded by one of the following two processes: exosome degradation or decapping. Both begin with the deadenylation of the mRNA to remove the poly (A) tail [318, 319]. Subsequent degradation can occur via the exosome, which is a multi-protein complex with 3’-to-5’ exonuclease activity. Alternatively, the mRNA can undergo decapping by the enzymes Dcp1 and Dcp2, which facilitates 5’-to-3’ degradation by the exoribonuclease Xrn1p [318, 320].

Multiple complementary sites with imperfect base-pairing create bulges in the miRNA::mRNA duplex that inhibit the slicer activity of AGO2 [255, 309, 310, 318]. In the absence of perfect base-pairing, miRNA inhibition of translation occurs at both initiation

of translation and at the elongation steps [321]. Translation can also be regulated indirectly by the miRNA-targeted mRNA sequestered from the translational machinery into the cytoplasmic foci known as P-bodies [236, 322]. Additionally, miRNA can accelerate target mRNA deadenylation and decapping independently of slicer activity, consequently affecting translation initiation efficiency and/or transcript stability [323-325]. Transcript instability leads to mRNA decay *via* the same exosome and Xrn1p enzymatic degradation as slicer-dependent silencing [320, 323].

## 2.4. **miRNAs and angiogenesis**

### 2.4.1. **The role of Dicer**

The relevance of miRNAs in endothelial function has been clearly illustrated in mice with Dicer knockdown and in several knockout experiments performed in ECs (both *in-vitro* and *in-vivo*). Global loss of Dicer in mice, for example, leads to embryonic lethality at E7.5. These mice show depletion of pluripotent stem cells [279]. Hypomorphic mice (*Dicer* $^{ex1/2}$) also die *in utero* after day E12.5. *Dicer* $^{ex1/2}$ embryos display defective blood vessel formation, suggesting that dicer is necessary for normal mouse vascular development [326]. In zebrafish, lack of maternal and zygotic Dicer is associated with severe defects in gastrulation, brain morphogenesis, and cardiac development due to impaired blood circulation in the embryo proper and in the yolk sac [327]. Additionally, expression of VEGF, KDR, FLT1, and TIE-1 is altered. Taken together, these data suggest that Dicer is an important regulator of angiogenesis, probably through the processing of miRNAs [327].

Silencing of Dicer in human ECs using short interfering (si) RNA results in reduced cell growth and impaired tube formation [328-330]. Protein expression of Tie-2, KDR, eNOS, and other angiogenic factors is also altered [329]. Interestingly, expression of thrombosphondin-1, an angiogenesis inhibitor, was unregulated, which explains, in part, the observed inhibition of angiogenesis [331]. The postnatal effect of Dicer in angiogenesis was assessed by implanting human umbilical vascular endothelial cells (HUVECs) transfected with Dicer knockdown in a Matrigel® plugs in nude mice. A significant decrease in sprout formation was observed [328]. Moreover, in a limb ischemia model, inactivation of Dicer in ECs significantly reduced capillary density and blood flow recovery [330].

#### 2.4.2. **miR-126**

miR-126 is a miRNA found in intron 7 of the epidermal growth factor-like domain 7 (*EGFL7*) gene and is highly abundant in human ECs [332]. From the pre-miR-126 molecule, two mature differentially expressed strands are produced in ECs, miR-126-3p and miR-126-5p [333]. In mice, miR-126 is also found in intron 7 of *egfl7* [334]. Unlike other miRNAs, the passenger strand is not destroyed during its biogenesis, meaning that both miR-126 (now termed miR-126-3p) and miR-126* (now termed miR-126-5p) are highly expressed [333]. miR-126 miRNA is highly conserved in vertebrates. During embryonic development, it is found in ECs, hematopoietic progenitors, and EC lines. In adults, expression of miR-126 is confined to vascularized tissues like the heart, lungs, and liver [335]. Lack of miR-126 results in loss of vascular integrity and hemorrhage during embryogenesis [333]. Similarly, targeted deletion of miR-126 in mice leads to

formation of fragile and leaky vessels, lumen collapse, hemorrhage, and impaired EC proliferation and migration [332, 334].

During embryogenesis, angiogenesis regulation is achieved, in part, by targeting suppressors of VEGF. miR-126 targets Sprouty-related protein SPRED1 and p85β, suppressors of VEGF expression [333, 336]. However, miR-126 overexpression is not enough to induce endothelial commitment, indicating that other factors may also be needed [337]. During endothelial differentiation in humans, miR-126 is one of the pro-angiogenic miRNAs to be upregulated while stem cells markers are downregulated [338]. In embryonic vessels, miR-126 has a pro-angiogenic role; however, in mature vessels it tends to have the opposite effect. miR-126 selectively targets VEGF production. During embryogenesis, miR-126 promotes neo-vessel formation but inhibits VEGF overproduction during late embryogenesis, the time when Ang-1 has a more prominent role in promoting EC maturation and vascular integrity [339]. Overproduction of VEGF has been shown to induce severe cardiovascular abnormalities, vessel overgrowth and immaturity [340]. It has been shown that a two-fold increase of VEGF levels leads to embryonic death due to inability to induce organization of endothelial and hematopoietic lineages [341]. These data support the idea that embryonic survival requires a specific range of VEGF expression, and that miR-126 helps maintain those levels.

Other proangiogenic targets of miR-126 include EGFL7, which exerts pro-migratory effects. During embryogenesis, EGFL7 and miR-126 are regulated by EGFL7 promoter. In mature quiescent ECs, EGFL7 promoter is suppressed, leading to its downregulation.

Independent expression of miR-126 with no change in EGFL7 expression is also possible due to the fact that miR-126 has its own promoter [342, 343].

### 2.4.3. **miR-17-92 cluster**

miR-17-92 cluster is located in intron 3 of the C13orf25 gene [344]. This cluster contains six miRNAs, including miR-17, miR-18, miR-19a, miR-19b-1, miR-20a, and miR-92a-1, which are further divided into 4 families based on their seed sequence: miR-17 (miR-17 and miR-20a), miR-18, miR-19 and the miR-92 families [345]. All these miRNAs are coded from the same precursor transcript [346]. Based on their sequence, miR-17, miR-20a, and miR-20b target similar sets of genes. Mice lacking this cluster die shortly after birth, with severe hypoplastic lungs and ventricular septal defects [245]. Inhibition of the miR17-92 cluster leads to decreases in EC proliferation, without an influence on the apoptosis pathway [245]. Moreover, inhibition of the miR-17-92 cluster leads to diminished sprouting in a capillary sprouting assay, thus suggesting that it is proangiogenic.

*In-vivo* studies on mouse retinas revealed that endogenous deletion of the complete endothelial miR-17-92 cluster leads to vessel regression and reduced vascularization [347]. These findings highlighted for the first time the relationship of this cluster to adult angiogenesis. In line with the previous findings, miR-17-92 knockout mice showed significantly impaired angiogenesis in an ear angiogenic assay [348]. Ablation of miR-17-92 also decreased vascularization when a VEGF-encoding adenovirus was injected locally in the ear [348].

miR-18a and miR-19 directly repress the expression of the anti-angiogenic factors thrombospondin-1 (*TSP-1*) and connective tissue growth factor (*CTGF*) [349]. It has been reported that miR-17/92 is overexpressed in colon cancer [349]. In a tumor engraftment model, upregulation of the 17/92 cluster by c-Myc in colonocytes increases tumorigenesis and angiogenesis through direct repression of TSP-1 and CTGF expression by miR-18a and miR-19, respectively [231]. miR-92a is highly expressed in ECs but overexpression of miR-92a in those cells under ischemic conditions was shown to inhibit angiogenesis [350]. In a mouse model of leg ischemia, the administration of a miR-92a inhibitor led to an increase in new blood vessel formation and enhanced recovery from ischemia [350].

#### 2.4.4. **miR-296**

miR-296 was identified in a miRNA screen in normal human brain ECs as being upregulated in response to exposure to human brain glioma cells [351]. miR-296 contributes to angiogenesis by directly targeting hepatocyte growth factor-regulated tyrosine kinase substrate (HGS), which leads to reduced levels of HGS and HGS-mediated degradation of KDR [351]. The HGS protein is involved in the regulation of growth factors receptors, such as PDGFR-β and VEGFR2 [352, 353]. Upon ligand-stimulation, phosphorylation, ubiquitination, and internalization of growth factor receptors, HGS mediates the sorting of these ligand/receptor complexes to lysosomes, where they are triggered for degradation [352-355]. Upregulation of miR-296 expression was also demonstrated in tumor blood vessels isolated from human glioblastoma tumors [351]. Taken together, these results support a role for miR-296 in promoting angiogenesis in tumors [351]. More recently, adenovirus-mediated overexpression of

miR-296 in ECs has been shown to enhance capillary-like tube formation, which coincided with significant inductions of VEGF and VEGFR2 expression [356].

#### 2.4.5. **miR-210**

miR-210 is an intronic miRNA located within the genomic loci of transcript AK123483 [357]. Both miR-210 and AK123483 are induced by hypoxia (a pro-angiogenic stimulus). This finding suggests a role for miR-210 in the regulation of EC angiogenesis [358]. In addition, several studies have reported that miR-210 is induced by HIF-1α [359]. To date, many transcripts involved in cell survival, proliferation, and angiogenesis have been found to be direct targets of miR-210 [359].

Overexpression of miR-210 leads to increased glucose transporter expression. One such transporter is GLUT-1, whose presence compensates for the reduction of glucose during glycolysis. GLUT-1 can upregulate VEGF and PDGF expressions, and it also generates an environment primed for angiogenesis [360].

## 3. **miR-1233**

### 3.1. **Structure**

miR-1233 is a miRNA predicted to be located in the intronic region of genes *GOLGA8A* and *GOLGA8B*. Specifically, the chromosome location of both versions of miR-1233 is 15q14. Since miR-1233 is found in different regions of the genome, it has been subcategorized; miR-1233-1 is the miRNA found in the intronic region of *GOLGA8A*, while miR-1233-2 is found in the intronic region of *GOLGA8B*.

The precursor sequence of miR-1233 is composed of 82 nucleotides. The precursor sequence is cleaved and processed at the 3’ end into a 20nt miRNA mature sequence, miR-1233-3p. Cleaving at the 5’ end, from nucleotide 4 through 25, results in the mature miRNA 1233-5p. These two mature sequences are predicted to regulate mRNAs of distinct functions.

## 3.2. **Function**

### 3.2.1. **Cancer**

mir-1233 has not been identified in species outside great apes, making it more difficult to study. Knowledge about the function of miR-1233 remains largely unknown. When searching on PubMed for “miR-1233”, only 20 articles were retrieved (as of March 1$^{st}$, 2019). In general, miR-1233 has been implicated in various cancers and other anomalies.

The first published data that shed light on the function of miR-1233 was performed in 2011 by Wulfken et al. In this study, the expression level of 754 miRNAs was assessed in the serum of patients with renal cell carcinoma (RCC) [361]. Compared to controls, miR-1233 was significantly upregulated. When the same study was performed in a different cohort of the same disease, miR-1233 was once again upregulated. This study also denominated miR-1233 as an RCC associated oncomir, a miRNA associated with cancer and pointed to it as a possible biomarker for the disease.

In recent years, the function of miR-1233 in RCC has been explored more fully. Dias et al. reported that several RCC cell lines were able to release miRNAs into the cell medium [362]. One such miRNA was miR-1233. When detecting it in plasma, they

identified higher levels in RCC patients as compared to controls. In addition, high expression levels were associated with poor survival. Interestingly, when treating RCC cell with hypoxia, a known pro-angiogenic stimulus, higher levels of miR-1233 released into the media were observed, although the mechanism of exportation was not assessed. A different study, however, showed the presence of miR-1233 in exosomes derived from the plasma of RCC patients [363]. The identity of the extracellular vesicles was confirmed by the presence of CD9, a protein commonly used to identify exosomes. This group also identified upregulation of miR-1233 in the exosomes of RCC patients, compared to controls.

miR-1233 has also been linked to bladder, gastric, and prostate cancers. In every observed case, the expression of miR-1233 was elevated [363-365].

#### 3.2.2. **Cardiovascular conditions**

miR-1233 has been studied in the context of several cardiovascular diseases. In an arterial hypertension study, miR-1233-5p was upregulated in patients displaying arterial hypertension, compared to controls [366]. However, such results have not been confirmed through qPCR due to the low expression of miR-1233-5p. Recently, it has been shown that altered expression levels of circulating miR-1233-3p in Tetralogy of Fallot patients, a condition known to be a combination of four heart defects, may play a critical role in the disease's progression [367].

miR1233 has also been considered a possible circulating biomarker that could distinguish heart failure (HF) from non-heart failure (non-HF), and heart failure accompanied with reduced left ventricular ejection from heart failure accompanied with

preserved left ventricular ejection. In a study to assess this condition, miR-1233, among other miRNAs, was upregulated in HF compared to non-HF; however, that study failed to differentiate heart failure accompanied by reduced left ventricular ejection from heart failure accompanied with preserved left ventricular ejection [368].

In hypertensive disorder complicating pregnancy (HDCP), a condition that severely affects the life of pregnant women and their newborns, miR-1233-3p has been found to be upregulated. Zhong et al. identified higher levels of miR-1233-3p in placental tissue from HDCP patients, compared to controls [369]. It was noted that its upregulation coincided with decreased expression of HOXB3. The study identified HOXB3 as a direct target of miR-1233-3p, through binding of the 3'UTR of the mRNA. It also showed that *in-vitro* overexpression of miR-1233-3p significantly decreases proliferation and invasion abilities of trophoblast cells. HDCP is often accompanied by preeclampsia, a common pregnancy-related disease characterized by hypertension and proteinuria. Preeclampsia is a major cause of maternal mortality, morbidity, perinatal death, preterm birth, and intrauterine growth restriction [370]. In a microarray used to identify deregulations of miRNAs, miR-1233 was significantly upregulated in serum from women with preeclampsia [371].

### 3.2.3. **Functions of GOLGA8A and GOLGA8B**

Golgins are a major class of membrane-tethering proteins found in the cytoplasmatic surface of the Golgi apparatus. It is believed that the main function of these proteins is to capture transport vesicles, since they are found in distinct regions of the Golgi and their cytoplasmatic coil–coil domains can extend 300nm away, making them ideal for this task [372] [373]. The specific functions of Golgin A8 family members A and B

(GOLGA8A and GOLGA8B) remain to be elucidated. However, a recent study demonstrated that GOLGA8A was upregulated in the plasma of patients with obsessive compulsive disorder (OCD) [374]. Another study by Merino-Zamorano et al. identifies GOLGA8A as unregulated in patients who suffered from primary intercerebral hemorrhage [375]. GOLGA8B, on the other hand, has been predicted to be associated with cell survival, but no functional test has been performed to confirm such results [376].

## 4. PDRG1

### 4.1. PDRG1 gene and protein structure

p53 and DNA Damage-Regulated Gene 1 (PDRG1) is a small gene composed of 5 exons separated by 4 introns. It is conserved in various species, including fish, mice, and humans. In humans, exon 1 is predicted to contain the transcription start site (codon ATG) at nucleotide position 120. The stop codon is found at position 85 of exon 5 [377]. The chromosomal location of PDRG1 is 20q11.21 in humans and in mice at chromosome 2. In humans, exons 1 and 5 are the largest, at 209bp and 924bp in length, respectively. In mice, exons 1 and 5 are also the largest, at 130bp and 847bp in length, respectively. In both species, exons 2, 3, and 4 are 76bp, 75bp, and 81bp, respectively. Intron sizes are similar in humans and mice, with intron 3 being the largest (~2000bp) and intron 4 being the smallest (~500bp). Each intron contains a 5’ splice site donor and a 3’ splice site acceptor. To date, however, no splice variant has been identified during the transcription of PDRG1.

PDRG1 is a 133 amino acid-long protein with a molecular mass of 15.5 and 15.3 kDa for humans and mice, respectively. Prediction motif searches made by Luo et al., who first cloned PDRG1, identify a helix-turn-helix sequence at the C-terminal end of both human and mouse PDRG proteins. PDRG1 protein structure is also characterized by a β-prefoldin-like domain [377].

In addition to being present in many vertebrates, PDRG1 protein is highly conserved, with more than 90% shared protein identity between many mammals. For example, the human protein has a similarity of 92% to the mouse protein [377].

### 4.2. **Expression**

PDRG1 is expressed in various human tissues, including, brain, heart, liver, lung, spleen, stomach, testis and skeletal muscle. The highest relative expression is found in the testes. In rat tissue, PDRG1 mRNA expression has been found in spleen, heart, cerebellum, brain, liver, intestine, smooth muscle, lung, pancreas, kidney, testis, and vein. The highest expression was detected in the cerebellum, brain, and testes. In both, human and rat tissues, the lowest expression was found in liver tissue [377, 378].

### 4.3. **Localization of PDRG1**

The location of PDRG1 was initially identified by Luo et al. in 2003 by generating two gene constructs with GFP fused to the N-terminal or the C-terminal of PDRG1 [377]. PDRG1 was then found in the cytosol of cells using fluorescence microscopy and transfection of NIH3T3 and HCT116 cells followed by confirmation with western blotting. Aggregation of the signal in certain cellular areas suggested that PDRG1 could be localized to a subcellular organelle. Similar results were observed when GFP was

positioned on the N- or the C-terminal ends of PDRG1. Co-staining with mitochondrial or endoplasmic reticulum-specific dyes did not show co-localization with PDRG1-GFP or GFP-PDRG1. In the end, they concluded that PDRG1 was not present in the ER, mitochondria, or the nucleus. A more recent study performed by Perez et al. identified MATα1 as a binding partner of PDRG1 [379]. *MATα1* is involved in the process of transmethylation, which occurs in the nucleus. The gene encodes for the catalytic subunit of homotetrameric methionine adenosyltransferase (MAT) I and homodimeric MAT III.

MAT is an essential enzyme responsible for the synthesis of S-adenosylmethionine, the principal methyl donor to substrates like DNA in mammalian cells [380]. Two different mammalian MAT genes exist, *MAT1A* and *MAT2A,* and they *e*ncode two homologous MAT catalytic subunits, MATα1 and MATα2, respectively. MATα1 is mostly expressed in the adult liver and is present in other tissues at low levels. MATα2, on the other hand, is mostly expressed in all non-liver tissues but is induced during rapid liver growth and hepatic dedifferentiation [380]. *MAT1A* expression is reduced in more than 50% of patients with cirrhosis and in patients with human hepatocellular carcinoma [381, 382]. Murine knockout of *MAT1A* results in reduction of hepatic S-adenosylmethionine and spontaneous development of hepatocellular carcinoma [383, 384].

In human cells, MAT has been found to be mostly cytoplasmic, with some expression in the nucleus [378]. In addition, subcellular fractionation followed by western blot analysis has also demonstrated the presence of MATα1 subunits in the nuclear and cytosolic fractions of several human cell lines. Retention of MATα1 in the cytoplasm appears to be dependent on signals located in the C-terminus of the protein. Specifically, the

regions of residues 313, 368, 369, and 392 are involved in cytosolic retention. Nuclear localization has been related to region comprised by residues 340, 344, and 393. Localization of these subunits is dependent on protein structure more than their actual sequence. In addition, sequence analysis has shown no evidence of classical mono- or bipartite nuclear localization signals. Both types of MATα1 subunits, tetramers and dimers, have also been detected in the nucleus. In addition, mutations in the structural region of the MATα1 showing higher localization to the nucleus have also been linked to increased H3K27 methylation.

Based on C-, N-, and CN-terminal mutations of PDRG1, researchers have demonstrated that interaction of PDRG1 and MATα1 happens through the structural core of PDRG1 [379]. The same group also identified PDRG1 being present in the cytoplasm of CHO, COS-7, H35, and N2a cell lines, with higher expression detected in the nucleus of all cell lines except N2a. These results were confirmed by subcellular fractionation followed by western blotting. Other studies have also shown that PDRG1 interacts with nuclear complexes in the prostate cancer cell line, LNCAP [385].

To date, research data suggest that the cellular localization of PDRG1 is cell-dependent, although lack of knowledge about the function of this protein indicates that more studies need to be performed.

### 4.4. **Function**

PDRG1 is a relatively recently discovered protein and remains largely unexplored. However, some research has started to help researchers understand its function (see below)

### 4.4.1. **PDRG1 and methylation**

As previously described, PDRG1 interacts with MATα1, one of the subunits of MAT. MAT is an enzyme responsible for the cytosolic synthesis of S-adenosylmethionine, a DNA methyl donor involved in methylation processes. MATα1 subunits is incorporated into homo-tetramers (MAT I) and homodimers (MAT III) in the cytosol, whereas in the nucleus only MAT I and MATα1 monomers have been described [378]. PDRG1 interacts with MATα1 in the nucleus, and both are incorporated into a protein complex larger than the two proteins. Analytical gel filtration chromatography has shown that when PDRG1 and MATα1 are cotransfected, a combination of peaks corresponding to HA-PDRG1 or FLAG-MATα1 homo-oligomers becomes visible, with an additional new and much larger peak. The new peak demonstrates the presence of PDRG1 and MATα1 in a larger association state, with an estimated molecular mass of 360KDa (PDRG1 is 15.5KDa, MAT1A is 55KDa). Interestingly, the mRNA expression patterns of *Pdrg1* and *Mat1a* are different. In rat tissues, PDRG1 is relatively highly expressed in the cerebellum and brain, while the lowest expression is detected in the liver and pancreas. This pattern is almost the opposite to that of *Mat1a*, which has been linked to hepatocellular carcinoma and cirrhosis. A study in a Wilson disease rat model found normal PDRG1 expression but *Mat1a* expression is mildly reduced [386]. In contrast, in acute liver injury and H35 cells with strong reductions in *Mat1a* expression (~70% and >95%, respectively), *Pdrg1* mRNA levels are upregulated compared to normal livers.

Furthermore, overexpression of PDRG1 was not found to significantly alter the production of S-adenosyl methionine, the methyl donor, although a tendency to decrease was observed. When purified recombinant MATα1 and MATα2 homo-

oligomers was incubated with increasing concentrations of PDRG1, the synthesis of S-adenosyl methionine was reduced. Overall, the data have shown that in the presence of PDRG1, a reduction of MAT activity is seen.

Downregulation of PDRG1 by shRNA showed no effects on cell growth. However, microarrays performed in hepatic cells with PDRG1 knocked down, showed that few genes were upregulated in response to starvation. Upregulated genes in these cells included *Sema3c*, *Id1*, *Cxcl1* and *Ctgf*. Such genes have previously been identified as modulators of migration, invasion, and cell survival of cancer cells [387-389].

### 4.4.2. **PDRG1 and RNA transcription**

The machinery required for gene transcription includes RNA polymerase II (RNAP II), its general transcription factors, and mediator complexes [390-392]. Many other proteins that regulate the activity of RNAP II have also been identified. PDRG1 was identified in a study that surveyed key components of the RNAP II machinery. PDRG1 binding partners were identified using tandem affinity purification of several subunits of RNAP II, followed by liquid chromatography-tandem mass spectrometry. A computational model that reduces false positives was used to generate a network of PDRG1 binding partners. This network includes known transcription factors, RNA processing molecules, and protein complex assembly molecules in addition to a subset of proteins with unknown function. This analysis also revealed a group of RNA II- associated proteins (RPAPs) that interact with XAB1 to connect RNAP II to regulators of protein complex formation. RPAPs-XAB1 complex is associated with a number of additional uncharacterized (at the time) proteins, including PDRG1 [393].

In an another study, quantitative mass spectrometry-based proteomics and fluorescence microscopy were used to characterize the mechanism of RNAP II assembly in human cells [394]. Several assembly intermediates were identified using this approach. Some intermediates included a cytoplasmic complex containing subunits Rpb1, the largest RNAP II subunit, and Rpb8, which is associated with the HSP90 co-chaperone hSpagh (RPAP3) and the R2TP/prefoldin-like complex. Through independent interaction studies, some other RNAP II subunits were also studied. In particular, Rpb5 was found to interact with PDRG1.

### 4.4.3. **PDRG1 and radioresistance**

PDRG1 was first identified in a study that aimed at characterizing novel molecular markers that affect cellular responses to genotoxic stress [377]. Upon UV irradiation, PDRG1 levels increased significantly. In several human cell lines, positive and negative for p53, UV irradiation had the same effect. Interestingly, repression of p53 repression resulted in increased PDRG1 expression. In support of this study, Jiang et al. demonstrated that other genotoxic stressors like adriamycin and etoposide induce upregulation of PDRG1 levels, while non-genotoxic stressors like paclitaxel and thapsigargin had no effects on PDRG1 expression [395]. Based on these results, PDRG1 has been termed an oncogene that promotes radioresistance [396].

Recently, the effects of oleuropein (OL), the main component of olive leaf extract and a known anti-oxidant, were studied in a radiosusceptibility settings [397]. OL treatment in cells along with exposure to UV irradiation resulted in decreased cell survival. It was discovered that OL alters the expression levels of several miRNAs and genes. In particular, PDRG1 and HIF1α were downregulated in these contexts, and miR-519d

was upregulated. Luciferase reporter assays confirmed the relationship between miR-519d and PDRG1. It was found that miR-519d binds to the 3'UTR of PDRG1 and represses its translation. This interaction was identified as being HIF1α-dependent; OL would inhibit HIF1α, block its effects on miR-519d, and subsequently not allow PDRG1 to exert its radioresistant properties [397].

#### 4.4.4. **PDRG1 and nutrient sensing molecules**

TIP49a and TIP49b are proteins that belong to an evolutionarily conserved family of $AAA^+$ ATPases that are involved in multiple protein complexes. These proteins are components of at least four multiprotein complexes that play roles in SRCAP chromatin remodeling, hINO80, and TRRAP/TIP60 complexes, and the nutrient sensing URI/prefoldin complex [382, 398-400].

Sardiu et al. performed a study designed to identify the different complexes in which TIP49a/b are components [401]. Multidimensional protein identification technology (MudPIT) was used to generate a local database of interacting proteins of 27 different epitope-tagged components of the SRCAP chromatin remodeling, hINO80, and TRRAP/TIP60 complexes, and the nutrient sensing complex URI/prefoldin. PDRG1 was identified as an additional component of the URI/prefoldin complex, alongside already-known components [400]. In mammalian cells, URI is a phosphorylation target of the nutrient-sensitive mTOR pathway [400]. In mammalian cells, URI phosphorylation is influenced by signals that affect mTOR activity, such as insulin or IGF1 treatment. These signals produce hyperphosphorylation of URI, an effect blocked by rapamycin. When using PDRG1 as bait, the following proteins were identified as interactors: POL3A, PPP1CA, PPP1CC, PFDN2, HKE2, RPB5, PPP1CB, NPM1, UXT1, H2BS,

H2BR, RPB5MP, FLJ21908, BC014022, H2AZ, NOP5/NOP58, FLJ20643. TUB6, HSP70/1, HSP70/1B, BAF53, TIP49a, TIP49b had low probability scores [401]. Another study used a mass spectrometry approach to conduct an unbiased screen of URI nuclear interactors in prostate cells [385]. URI was described to selectively bind R2TP/prefoldin-like complex, composed in part by PDRG1, RPB5, and Art-27. URI-PDRG1 interaction was confirmed by immunoprecipitation. Interestingly, overexpression of URI increases PDRG1 protein but not mRNA expression, suggesting a post-transcriptional effect. This analysis further characterized a chaperone-like complex suggested earlier to have been a mediator of RNA polymerase II complex assembly in the cytoplasm of the cells [394].

#### 4.4.5. **PDRG1 function in cancer**

PDRG1 has been linked to cancer. In fact, more than 700 single nucleotide polymorphisms have been identified, most of which have yet to be studied [386]. PDRG1 mRNA has been found to be overexpressed several human malignancies, including colon, rectum, and ovarian cancer [395].

Jiang et al. found that PDRG1 mRNA and protein was unregulated in colon cancer samples. By using shRNA knockdown experiments of PDRG1 in human colon cells, they showed that cell growth was negatively affected. To gain insight into the mechanism by which cell growth was affected, binding partners of PDRG1 were assessed by a yeast two-hybrid system. Three proteins were identified, including PDCD7 (programmed cell death 7), CIZ1 (Cip1 interacting zinc finger), and MAP1S (microtubules-associated protein 1S). PDCD7 has been implicated in modulating apoptosis and also has been reported to be a component of the U12-type spliceosome

[402]. CIZ1, through interaction with p21, is believed to regulate cell cycle progression via translocation of p21 and itself between the nucleus and cytoplasm [403]. In addition, it has been linked to S-phrase progression [404]. MAP1S is a microtubule-binding protein that interacts with the tumor suppressor protein RASSF1A to modulate cell cycle progression [405]. Immunoprecipitation of PDRG1 identified PDCD7 as an interacting protein. The other two proteins have yet to be validated.

In bladder cancer, miR-214 has been shown to be attenuated and, in turn, be correlated with, cancer progression and poor prognosis [406]. Further analysis identified PDRG1 as a direct target of miR-214 through its 3'UTR. Overexpressing this mRNA was shown to reduce the mRNA and protein levels of PDRG1. PDRG1 knockdown experiments showed significant proliferation inhibition and increased cell death. The same trend was observed when miR-214 was overexpressed. Interestingly, a transwell assay with Matrigel coating demonstrated that siRNA against PDRG1 elicited an inhibitory effect on bladder cancer cell invasion compared to the control group.

In a retrospective study done in rectal tumor samples, patients with elevated PDRG1 and GLUT1 gene expression had poor pathological responses [407]. The same trend was consistently observed when using other parametric analyses. However, overall survival based on PDRG1 and GLUT1 levels were not correlated.

## 5. **Objective and specific aims**

miRNAs have emerged as crucial players regulating the magnitude of gene expression in a variety of organisms. Being a relatively recent discovery, there is increasing evidence that miRNAs play important roles in the regulation of angiogenesis. The

functional roles of miRNAs in Ang-1 signaling and biological effects remain largely unexplored. Recent literature indicates that Ang-1 increases biogenesis of miRNAs in primary human dermal lymphatic ECs. In addition, it has been reported that miR-126 regulates pathways that are activated by Ang-1 and that this miRNA enhances Ang-1-induced angiogenic processes. These studies indicate that the relationship between Ang-1-dependent angiogenesis and miRNAs remains largely unexplored and that further studies in this field are warranted.

We performed pilot experiments to identify miRNAs whose expression is regulated by Ang-1 in ECs. In these experiments, we detected miRNA expression in human umbilical vein endothelial cells (HUVECs) using microarrays. These arrays detected the expression of 1,105 human mature miRNAs and 1,105 human pre-miRNAs. FlexArray® software was used to determine statistical significance which was set at changes ≥ 2.5-fold and ≤ 0.4-fold. These experiments revealed that prolonged exposure to Ang-1 triggers significant changes in the expression of several miRNAs the majority of which have not been well characterized. Among the miRNAs whose expression is upregulated by Ang-1 are miR-126 and miR-146b, two miRNAs highly expressed in ECs and have significant roles in angiogenesis and inflammation. Eight poorly characterized miRNAs whose expression was significantly downregulated by Ang-1 were selected for further analysis in terms of regulation of angiogenic processes including EC survival, migration, proliferation and differentiation. The general objective of this thesis is, therefore, to evaluate the functional roles of these miRNAs in angiogenesis and to understand the mechanisms through which these miRNAs regulate the biological functions of Ang-1/Tie-2 receptor axis in ECs. The specific objectives of this thesis include:

1) Identification of novel miRNAs that regulate Angiogenesis in general and a Ang-1-induced angiogenesis in particular.
2) Identification of the functional importance of miR-1233-3p, a miRNA whose expression is downregulated by Ang-1, and identify its molecular targets.
3) Identify the functional significance and the molecular pathway through which the protein p53 regulated and DNA damage regulated 1 (PDRG1), a target of miR-1233-3p, regulates angiogenesis.

# CHAPTER II: Angiopoietin-1 signaling in endothelial cells: The role of miRNAs

## 1. Preface

The formation of new blood vessels, angiogenesis, is an essential step in the healing of wounds and tissue regeneration. Angiogenesis is also a key factor in cancer pathogenesis and neovascular eye diseases. Growth factor receptors in ECs control the development and function of new blood vessels. One such growth factor pathway is the Angiopoietin/Tie-2 axis. In embryonic development, genetic deletion of Ang-1 or Tie-2 leads to lethality at midgestation due to poor maturation of the vascular system and widespread apoptosis of vascular cells. This pathway also plays important roles in the regulation of vascular permeability, inflammation and pathological angiogenic responses in adult tissues. For example, deregulation of the Ang-1 and Tie-2 levels has been associated with poor prognosis of solid tumors. Due to its essential role in cancer, this pathway has also become a target for drug development.

miRNAs are a large family of endogenous small non-coding RNAs. Generally, these molecules function by repressing a target mRNA through an anti-sense mechanism. In animals, these molecules bind to the 3’ UTR of the mRNA through a semi-complementary region found in bases 2 through 7, also called the seed sequence. This binding produces translation repression or direct mRNA cleaving.

miRNAs play a crucial role in various diseases by regulating translation of key growth factors and other proteins.  Increasing evidence suggests that miRNAs regulate tumor proliferation, invasion, apoptosis and therapy resistance. In addition, miRNAs have been proposed as potential disease markers and are crucially involved in the processes of vascular development. Two classes of these molecules exist in angiogenic processes: pro-angiogenic miRNAs and anti-angiogenic miRNAs.

One of the most studied miRNAs in the field of angiogenesis is miR-126 which is known to promote VEGF signaling and maintains vascular integrity, and therefore it is considered a pro-angiogenic miRNA. The polycistronic miR-17-92 gene cluster, located on human chromosome 13q31.3, encodes 6 mature miRNAs, miR-17, miR-18a, miR-19a, miR-20a, miR-19b, and miR-92a which are highly expressed in ECs. This cluster is upregulated in several types of lymphoma and solid tumors. In addition, the miR-17-92 cluster regulates vascular integrity and angiogenesis by promoting tumor neovascularization *in-vivo* by downregulating anti-angiogenic thrombospondin 1 and sprouty-2. Moreover, miR-92a also acts as anti-angiogenesis miRNA since injection of antagomirs to neutralize miR-92a results in increased angiogenesis *in-vivo.*

The involvement of miRNAs in Ang-1-mediated angiogenesis remains largely unexplored. In this chapter, we hypothesized, that Ang-1 regulates angiogenesis in part through upregulation of pro-angiogenic miRNAs and downregulation of anti-angiogenic miRNAs.

# Angiopoietin-1 Signaling in Endothelial Cells: The Role of MicroRNAs

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## 2. Abstract

Angiopoietin-1 (Ang-1) is a ligand of Tie-2 receptors that promotes survival, migration, and differentiation of endothelial cells (ECs). Recent studies have identified several microRNA (miRNA) families that either promote or inhibit angiogenesis. To date, the nature and functional importance of miRNAs in Ang-1-induced angiogenesis are unknown. Microarray screening of known miRNAs in human umbilical vein endothelial cells (HUVECs) revealed that the expressions of miR-103b, miR-330-5p, miR-557, miR-575, miR-1287-5p, and miR-1468-5p significantly decrease following exposure to Ang-1 for 24 h. Exposure to the angiogenesis factors angiopoietin-2 (Ang-2), vascular endothelial growth factor, fibroblast growth factor 2, and transforming growth factor β also inhibits miR-103b expression, but also exerts varying effects on the other miRNAs. By overexpressing miR-103b, miR-330-5p, miR-557, miR-575, miR-1287-5p, and miR-1468-5p with selective mimics, we demonstrated that the pro-survival effects of Ang-1 are eliminated, Caspase-3 activity increases, and cell migration, proliferation, and capillary-like tube formation decrease. Conversely, transfection with selective miRNA inhibitors increases cell survival, inhibits Caspase-3 activity, and stimulates migration, proliferation, and tube formation. miRNet miRNA-target gene network analyses revealed that miR-103, miR-330-5p, miR-557, miR-575, miR-1287-5p, and miR-1468-5p directly interact with 47, 95, 165, 108, 49, and 16 gene targets, respectively. Since many of these genes are positive regulators of angiogenic processes, we conclude that these miRNAs function as anti-angiogenic agents. Their down-regulation may be essential for Ang-1-induced angiogenesis to occur.

**Keywords**: angiogenesis, microRNAs, angiopoietins, endothelial cells, apoptosis, migration, differentiation, proliferation.

## 3. Introduction

Angiogenesis, or new blood vessel formation, is of paramount physiological importance to normal embryonic vascular development and adult vascular regeneration and repair. It is also essential to the deleterious vascular remodeling that is characteristic of diseases such as atherosclerosis, restenosis, idiopathic pulmonary fibrosis, and asthma. Angiopoietin-1 (Ang-1) and the endothelial-specific receptor tyrosine kinase (Tie-2) are critical regulators of angiogenesis and inflammation. Both Ang-1 and Tie-2 play essential roles in embryonic vascular development [1]. Ang-1 inhibits apoptosis, promotes migration and differentiation of endothelial cells (ECs), decreases permeability, stimulates vascular remodeling, enhances wound healing, and increases lymphangiogenesis (for review, see [2]). Tie-2 receptors initiate cell signaling by inducing the phosphorylation of key tyrosine residues [3]. They are abundantly expressed in all ECs.

The Ang-1/Tie-2 axis signals through several intracellular pathways, including the PI-3 kinase/AKT, DOK-R/PAK1, ERK1/2, p38, and JNK pathways [4–6], and activates transcription factors such as Kruppel-like factor 2 (KLF2), early growth response-1 (EGR1), and activating protein 1 (AP-1) [7,8]. In ECs, all these pathways are downstream from Tie-2 receptors and have been shown to be activated within a few minutes of exposure to Ang-1. Prolonged exposure to Ang-1 triggers significant pro-angiogenic and strong anti-inflammatory responses. For example, raising systemic Ang-1 levels for several days triggers significant decreases in bacterial lipopolysaccharide (LPS)-induced vascular leakage, leukocyte infiltration into the lungs, and mortality in

mice [9]. The signaling mechanisms through which prolonged Ang-1 exposure promotes angiogenesis and inhibits vascular inflammation remain unclear.

MicroRNAs (miRNAs) are small (22 nucleotides) molecules that regulate gene expression at the post-transcriptional level. They have recently been recognized as key regulators of several fundamental processes in the vasculature, including angiogenesis. The expressions and actions of several angiogenic factors, including vascular endothelial growth factor (VEGF), are regulated by specific sets of miRNAs in ECs, but the identities and functional significance of those involved in the regulation of Ang-1/Tie-2 receptor signaling in ECs are as yet unknown. Recently, our group reported that prolonged exposure of ECs to Ang-1 results in significant disruption of toll-like receptor 4 (TLR4) signaling and inhibition of lipopolysaccharide (LPS)-induced inflammatory responses. These responses were mediated through up-regulation of miR-146b, which selectively targets two important proteins involved in TLR4 signaling (IRAK1 and TRAF6) [10]. This suggests that miRNAs are important mediators of the anti-inflammatory action that is characteristic of Ang-1, but it remains unknown as to how they are involved in the regulation of Ang-1-induced angiogenesis.

To identify those miRNAs that are affected by prolonged Ang-1 exposure, we conducted pilot experiments using miRNA arrays in which miRNA expressions were measured in human umbilical vein endothelial cells (HUVECs) exposed to Ang-1 (300 ng/ml) for 24 hours. Ang-1 exposure triggered significant decreases in the expressions of six mature miRNAs (miR-103b, miR-330-5p, miR-557, miR-575, miR-1287-5p, and miR-1468-5p). As these miRNAs have not previously been known to be expressed in ECs, their functional importance to the regulation of angiogenesis in general and to Ang-1/Tie-2

pathway signaling in particular remains to be investigated. Accordingly, we hypothesized that miR-103b, miR-330-5p, miR-557, miR-575, miR-1287-5p, and miR-1468-5p function as anti-angiogenic miRNAs by negatively regulating several steps in the process of angiogenesis, including EC survival, proliferation, migration, and differentiation, and that down-regulation of their expressions may be a prerequisite to Ang-1/Tie-2 promotion of angiogenesis.

## 4. Materials and methods

**Materials:** Human umbilical vein endothelial cells (HUVECs) were purchased from Lonza Group (Basel, Switzerland). Recombinant human Ang-1, Angiopoietin-2 (Ang-2), fibroblast growth factor 2 (FGF2), transforming growth factor β (TGFβ), and vascular endothelial cell growth factor (VEGF) proteins were purchased from R&D Systems (Minneapolis, MN). An RNA Miniprep Kit was purchased from Sigma-Aldrich (St. Louis, MO). Lipofectamine RNAiMAX reagent and NCode miRNA First-Strand cDNA Synthesis and qRT-PCR Kits were purchased from Invitrogen (Burlington, ON). Synthetic mature miRNA mimics, inhibitors, and respective controls were purchased from Shanghai Genepharma (Shanghai, China). RNeasy and miRNeasy Mini Kits were purchased from QIAGEN (Hilden, Germany).

**Cell culture:** HUVECs were grown in complete MCDB131 medium (Life Technologies, Rockville, MD) supplemented with 20% fetal bovine serum (FBS), EC growth supplement, glutamine (2mM), heparin, and gentamicin sulfate (Invitrogen) and incubated at 37°C and 5% $CO_2$. In experiments where EC survival, Caspase-3 activity, cell cycle phases, and migration were assessed, cells were maintained in basal MCDB131 containing gentamicin sulfate and either 0% FBS or 2% FBS but no growth

supplement, glutamine, or heparin. In experiments where EC proliferation and differentiation were assessed, cells were maintained in Lonza EGM™-2 BulletKit™ medium containing 2% FBS, growth factors, and gentamicin.

**Microarray experiments:** HUVECs were maintained for 6 h in basal MCDB131 medium containing gentamicin sulfate and 2% FBS then exposed for 24 h to basal medium containing aliquots of phosphate buffered saline (PBS, control) or Ang-1 (300 ng/ml final concentration). Cells were harvested, and total RNA was extracted using a RNeasy Mini Kit, following the manufacturer's instructions. Quantification and purity of total RNA was assessed by $A_{260}/A_{280}$ absorption and by 1.2 % agarose gel electrophoresis. Three PBS- or Ang-1-exposed cell samples were pooled into a single sample, which were then subjected to miRNA detection (Genome Québec) using GeneChip® miRNA 2.0 Arrays (Affymetrix, Santa Clara, CA). These arrays provide complete coverage of miRBase V15 (15,644 probe sets, including those of mature and precursor miRNAs, snRNAs, and scaRNAs). Probe labeling and hybridization were performed according to a standardized protocol supplied by Affymetrix. Probe intensities were processed using their robust multi-array analysis (RMA) algorithm, which is a log2 scale linear additive model that summarizes perfect match probe values after correcting for background and quantiles, thus normalizing probe level data across arrays. RMA expression values were subsequently filtered using Microsoft Excel.

**Angiogenesis factor regulation of miRNA expression:** HUVECs were maintained for 6 h in basal MCDB131 medium then exposed for 24 h to basal medium containing aliquots of PBS (control), Ang-1 (300 ng/ml), Ang-2 (300 ng/ml), VEGF (40 ng/ml), FGF2 (10 ng/ml) or TGF-β (2 ng/ml). Concentration of these factors was determined

based on lab historical data and peer reviewed publications. Total RNA was extracted. miRNA expressions were measured as described below.

**Hypoxia experiment:** HUVECs were cultured in an incubator chamber (Billups-Rothenberg, Del Mar, CA) for 24 h in complete MCDB131 medium under normoxic or hypoxic (5% $CO_2$ and 95% $N_2$) conditions.

**Extracellular vesicle (EV) isolation:** HUVECs were grown to confluence in complete MCDB131 medium, maintained in basal MCDB131 medium for 6 h, then exposed for 24 h. to basal medium containing aliquots of PBS (control) or Ang-1 (300 ng/ml). EVs excreted from cells into media were isolated using an Exiqon miRCURY™ Exosome Isolation Kit (Vedbæk, Denmark). Total RNA was extracted from isolated EVs and miRNA expressions were measured as described below.

**Transfection with siRNA, miRNA mimics, and LNA-inhibitors:** Cells were transfected with 15 nM of synthetic mature miRNA mimics or 25 nM of Locked Nucleic Acid (LNA™) miRNA inhibitors using Lipofectamine™ RNAiMAX reagent (Invitrogen), according to the manufacturers' instructions. Cells were allowed to recover for 48 h in complete MCDB131 medium before undergoing experiments.

**Cell counting:** miRNA mimic- or inhibitor-transfected cells were seeded (30,000 cells per $cm^2$) and maintained in complete, basal, or basal MCDB131 medium containing Ang-1 (300 ng/ml). Cells were counted 24 h later using a hemocytometer.

**Caspase-3 activity:** miRNA mimic- or inhibitor-transfected cells were plated in 12-well plates and maintained in complete, basal, or basal MCDB131 medium containing Ang-1

(300 ng/ml). Caspase-3 activity was measured 24 h later using an EnzChek® Caspase-3 Assay Kit with Z-DEVD-AMC as a substrate (Molecular Probes, Eugene, OR).

**Cell proliferation:** miRNA mimic- or inhibitor-transfected cells were plated in 96-well plates at 7,000 cells per well and maintained in complete Lonza EGM™ medium (minus VEGF). One hour after plating, a bromodeoxyuridine (BrdU) (Millipore, Etobicoke, ON) assay was performed according to the manufacturer's instructions. BrdU absorbance was measured 24 h later.

**Cell cycle analysis:** miRNA mimic- or inhibitor-transfected cells were maintained for 24 h at 80% confluence in basal MCDB131 medium containing 2% FBS then trypsinized, washed, fixed in 70% ethanol, washed twice, re-suspended in PBS, then stained with propidium iodide. Samples were run through a FACSCalibur™ flow cytometer. At least 20,000 events were acquired. Results were subsequently analyzed using FloJo software.

**Cell migration**: A scratch (wound) healing assay was used, as previously described [7]. In brief, confluent cell monolayers were wounded using a 200µl pipette tip and maintained for 8 h in basal MCDB131 medium containing 2% FBS and aliquots of PBS (control) or Ang-1 (300 ng/ml). Wounded areas were visualized using an Olympus inverted microscope, quantified using *Image-Pro Plus*™ software (Media Cybernetics, Bethesda, MD), and reported using the following formula: % wound healing = [1 – (wound area at $t_{8\,h}$/wound area at $t_0$)] x 100, where $t_0$ is the time immediately following wounding.

**Capillary-like tube formation:** miRNA mimic- or inhibitor-transfected cells were seeded onto 96-well plates pre-coated with growth factor-reduced Matrigel® ($1x10^4$ cells per well) and maintained for 24 h in Lonza EGM™-2 medium (minus VEGF) containing aliquots of PBS (control) or Ang-1 (300 ng/ml). Whole-well images were captured using an Olympus inverted microscope (40X) and analyzed using Image-Pro Plus™ software. Angiogenic tube formation was determined by counting average and total tube length in each field, as previously described [7].

**miRNA expression:** Total RNA was extracted using QIAzol® lysis reagent and a miRNeasy Mini Kit according to the manufacturers' protocols. Mature miRNAs were detected using an NCode™ miRNA qRT-PCR Kit (Invitrogen) and real-time PCR with specific primers (Table 1), SYBR® green, and a 7500 Real-Time PCR System. All experiments were performed in triplicate. Relative miRNA expression was determined using the $C_T$ method where $C_T$ values of individual miRNA data were normalized to $C_T$ values of U6 snRNA, as previously described [10]. snRNA U6 was used as housekeeping RNA.

**miRNA-target gene network analysis:** miRNet (http://www.mirnet.ca) [11], a recently published web-based software tool, was used to investigate potential functions of miR-103b, miR-330-5p, miR-557, miR-575, miR-1287-5p, and miR-1468-5p. miRNA-target gene interaction networks are primarily detected using high-throughput cross-linking immune-precipitation (CLIP) technologies.

**Data analysis:** Data are expressed as means ± standard error (SEM). Statistical significance was determined using Two-Way Analysis of Variance with a Newman-Keuls *post hoc* test. A P value of 0.05 or less was considered statistically significant.

## 5. Results

**Regulation of miRNA expression:** Microarray experiments confirmed that miR-126 is the most abundantly expressed miRNA in HUVECs. They also showed that Ang-1 exposure downregulates miR-103b, miR-330-5p, miR-557, miR-575, miR-1287-5p, and miR-1468-5p expressions to 0.28, 0.41, 0.42, 0.40, 0.53, and 0.39-fold, respectively, of those measured under control conditions. To our knowledge, these miRNAs have never before been detected in ECs. Using qPCR in cells maintained in complete MCDB131 medium (supplemented with 20% FBS), we estimated that miR-557 and miR-575 were equivalent to 36% and 35%, respectively, of miR-126 levels, while miR-103b, miR-330-5p, miR-1287-5p, and miR-1468-5p were relatively less abundant (Supplementary figure 1).

Figure 1 shows the effects of various angiogenesis factors on the expressions of each miRNA. Relative to controls, Ang-1 decreased the expressions of all six miRNAs under investigation, thereby confirming microarray results, while Ang-2 decreased miR-103b, miR-330-5p, miR-557, and miR-575 expressions, but exerted no effects on miR-1287-5p and miR-1468-5p. VEGF decreased the expressions of miR-103b, miR-1287-5p, and miR-1468-5p. FGF2 decreased the expressions of miR-103b and miR-557. TGFβ decreased the expressions of miR-103b and miR-1287-5p but had no effect on any of the others. These results demonstrate that miR-103b expression in ECs, in addition to being directly downregulated by Ang-1, is also downregulated by Ang-2 and the angiogenic growth factors VEGF, FGF2, and TGFβ. In contrast, other than by Ang-1, miR-330-5p, miR-557, miR-575, miR-1287-5p and miR-1468-5p are more selectively regulated.

To evaluate whether downregulation of mature miR-103b, miR-330-5p, miR-557, miR-575, miR-1287-5p, and miR-1468-5p in response to Ang-1 exposure was due to their increased secretion in EVs, expressions were measured in EVsisolated from HUVECs exposed to Ang-1 for 24 h. Supplementary figure 2 shows that their exosomal levels decreased in response to Ang-1 exposure, indicating that increased exosomal secretion is not the mechanism underlying the inhibitory effects of Ang-1 on these miRNAs.

To evaluate whether miR-103b, miR-330-5p, miR-557, miR-575, miR-1287-5p, and miR-1468-5p levels in ECs are regulated by hypoxia, a strong angiogenic stimulus, HUVECs were exposed to hypoxia for 24 h, and mature miRNA levels were measured using qPCR. Hypoxia increased the expression of miR-1468-5p but had no influence on the expressions of the others (Supplementary figure 3).

**miRNA regulation of EC survival:** Cells transfected with control or miRNA mimics were maintained for 24 h in complete medium, basal medium (0% FBS), or basal medium containing an aliquot of Ang-1 (300 ng/ml final concentration). With the control mimic, cell numbers decreased under serum deprivation conditions (basal medium) (Figure 2A). The addition of Ang-1 to basal medium resulted in an increase in cell number relative to that which was observed with basal medium alone (Figure 2A). Increases in cell number were also observed in miR-575 mimic-transfected cells exposed to Ang-1 but not in those transfected with miR-103b, miR-330-5p, miR-557, miR-1287-5p, or miR-1468-5p mimics (Figure 2A).

To investigate whether proportional changes in the number of apoptotic cells relates to the ameliorative effects of mimics on total cell number, Caspase-3 activity was measured. With the control mimic, Caspase-3 activity increased under serum

deprivation. The addition of Ang-1 eliminated this increase, thereby confirming that Ang-1 exerts an anti-apoptotic effect on ECs (Figure 2B). With miR-103b, miR-330-5p, miR-557, miR-575, miR-1287-5p, and miR-1468-5p mimics, Caspase-3 activity increased under serum deprivation to levels higher than those observed with the control mimic. Caspase-3 activity did not decrease under serum deprivation when Ang-1 was added to mimic-transfected cells (Figure 2B).

Effects of endogenous miR-103b, miR-330-5p, miR-557, miR-575, miR-1287-5p, and miR-1468-5p on EC survival were assessed by transfecting HUVECs with miRNA inhibitors. With the control inhibitor, cell number decreased and Caspase-3 activity increased under serum deprivation (Figure 3A). With miR-103b, miR-330-5p, miR-557, miR-575, miR-1287-5p, and miR-1468-5p inhibitors, cell numbers decreased under serum deprivation, as compared to complete medium, but were higher than with the control inhibitor. With the control inhibitor, Caspase-3 activity increased under serum deprivation, as compared to complete medium (Figure 3B). With miR-103b, miR-557, miR-1287-5p, and miR-1468-5p inhibitors, relative increases in Caspase-3 activity under serum deprivation were smaller than they were with the control inhibitor (Figure 3B). With miR-330-5p and miR-575 inhibitors, there were no increases in Caspase-3 activity under serum deprivation (Figure 3B). These results indicate that endogenous levels of miR-103b, miR-330-5p, miR-557, miR-575, miR-1287-5p, and miR-1468-5p exert inhibitory effects on EC survival.

**miRNA regulation of EC proliferation:** To investigate whether miRNAs regulate EC proliferation, BrdU incorporation was measured in miRNA mimic- or inhibitor-transfected HUVECs maintained in complete Lonza EGM™ medium. With miR-103b, miR-330-5p,

miR-557, miR-575, miR-1287-5p, and miR-1468-5p mimics, BrdU incorporation decreased relative to the control mimic (Figure 4A), indicating that their overexpression had a negative effect on EC proliferation. With miR-103b, miR-330-5p, miR-575, miR-557, miR-1287-5p, and miR-1468-5p inhibitors, BrdU incorporation increased relative to the control inhibitor, confirming that endogenous levels of these miRNAs exert inhibitory effects on proliferation (Figure 4B).

We also investigated the influence of miRNA mimics on cell cycle phases. As compared to the control mimic, decreases in S and G2/M phase populations and increases in G0/G1 phase populations were observed with miR-103, miR330-5p, miR-575, miR-1287-5p, and miR-1468-5p mimics, but not with the miR-557 mimic (Figure 5).

**miRNA regulation of EC migration:** With miR-103b miR-330-5p, miR-557, miR-575, miR-1287-5p, and miR-1468-5p mimic-transfected HUVECs in basal medium containing PBS, basal cell migration decreased as compared to that observed with the control mimic (Figure 6). Exposure to Ang-1 increased cell migration from a mean value of 26% with the control mimic to a mean value of 48% (Figure 6). This stimulatory effect of Ang-1 on cell migration was also observed with miR-103b miR-330-5p, miR-557, and miR-575 mimic-transfected cells, but not with 1287-5p or miR-1468-5p (Figure 6).

To evaluate whether endogenous miRNA levels regulate EC migration, a wound-healing assay was used to measure cell migration in control and inhibitor-transfected cells. miR-103b, miR-330-5p, miR-557, miR-575, miR-1287-5p, and miR-1468-5p inhibitors increased migration in basal medium containing PBS or Ang-1, suggesting that endogenous levels of these miRNAs exert a negative influence on cell migration (Figure 7).

**miRNA regulation of EC differentiation:** Capillary-like tube formation assays were used to evaluate the functional regulatory roles of miRNAs on EC differentiation in cells exposed to PBS (control) or Ang-1. Relative to PBS exposure, average tube length increased by ~1.6 fold in control mimic-transfected cells exposed to Ang-1 (Figure 8A–B). In miR-103b, miR-330-5p, miR-557, and miR-575 mimic-transfected cells exposed to Ang-1, average tube length increased by 1.21, 1.40, 1.29, and 1.24-fold, respectively, of that observed with PBS; Ang-1 exposure elicited no significant effect on tube formation in miR-1287-5p or miR-1468-5p mimic-transfected cells (Figure 8C). It should be emphasized that the degree of Ang-1-induced tube formation in miRNA mimic-transfected cells was lower than that observed in control mimic-transfected cells.

To evaluate whether endogenous miRNA levels regulate EC differentiation, tube formation was measured in control and miRNA inhibitor-transfected cells exposed to PBS (control) or Ang-1. In PBS, tube formation in inhibitor-transfected cells was higher than that observed in control inhibitor-transfected cells, suggesting that endogenous levels of miR-103b, miR-330-5p, miR-557, miR-575, miR-1287-5p, and miR-1468-5p exert negative effects on cell differentiation (Figure 9A). Relative to PBS, tube formation increased in control and miRNA inhibitor-transfected cells exposed to Ang-1, although there were no significant differences between control and miRNA inhibitors as to the degree of Ang-1-induced tube formation produced (Figure 9B). This was likely due to the fact that because Ang-1 already suppresses the expressions of miR-103b, miR-330-5p, miR-557, miR-575, miR-1287-5p, and miR-1468-5p, any further suppressive effects brought about by inhibitors was unlikely to affect the degree of Ang-1-induced differentiation.

**miRNA-target gene network analysis:** Using the miRNet web tool to analyze miRNA interactions with various genes, an interaction network comprised of 479 gene targets with 496 interactions was revealed (Supplementary figure 4). Specifically, miR-103, miR-330-5p, miR-557, miR-575, miR-1287-5p, and miR-1468-5p directly interact with 47, 95, 165, 108, 49, and 16 gene targets, respectively. These target genes, their functions, and the specific processes with which they are associated are listed in supplementary tables 1–6. Seventeen genes are targeted by more than one miRNA: XIAP, SMU1, MYO10, and PTCD2 are targeted by miR-557 and miR-575; C17orf85, ZNF556, ZNF212, and WIPI2 are targeted by miR-103b and miR-575; ZNF154, ZNRF2, and RBM47 are targeted by miR-330-5p and miR-557; MMAB is targeted by miR-557 and miR-1468-5p; FRK and HSD17B12 are targeted by miR-103b and miR-557; and POTED is targeted by miR-557 and miR-1287-5p (Supplementary figure 4).

## 6. Discussion

In this study, we characterized the expression, regulatory roles, and functional importance of several miRNAs as they relate to Ang-1 signaling in cultured human ECs. Our study reveals for the first time that Ang-1 inhibits the expressions of mature miR-103b, miR-330a-5p, miR-557, miR-575, miR-1287-5p, and miR-1468-5p, and that these miRNAs exert significant inhibitory effects on EC survival, proliferation, migration, and differentiation. From these results, we can conclude that they function as negative regulators of angiogenesis.

**miR-103b:** Mature miR-103b is the antisense strand of miR-103. Except for a difference of only one nucleotide in the 5' region, miR-103 and miR-107 share an identical seed sequence. Chen et al. [12] have reported that both miR-103 and miR-107 are

upregulated in ECs exposed to hypoxia. In the present study, we report for the first time that miR-103b is a) found in primary ECs; b) is downregulated by several angiogenesis factors, including Ang-1; and c) is not affected by hypoxia. The difference between miR-103 and its antisense strand in relation to its response to hypoxia is not surprising because previous analyses of the expression patterns of miRNA-miRNA pairs that can form miRNA-miRNA duplexes, such as miR-103/miR-103b, have found that there are pronounced differences in the degree of expression between pair members [13].

Our study demonstrates that miR-103b exerts strong anti-angiogenic properties, as evidenced by the inhibitory effects of its mimic and the promotory effects of its inhibitor on EC survival, proliferation, migration, and differentiation. Taken together, these results indicate that endogenous miR-103b functions as a negative regulator of angiogenesis. The mechanisms through which it achieves this function remain unclear. Our analyses of miR-103b-gene interaction networks show that miR-103b directly interacts with 47 genes (S2 Table, supporting document). We suggest that miR-103b selectively targets genes that positively regulate EC survival, proliferation, migration, and differentiation, including two pro-angiogenic transcription factors, ELK1 and E2F3. Both have been shown to be positive regulators of angiogenesis. ELK1 promotes EC survival, proliferation, and migration [14], while E2F3 promotes proliferation [15]. Other important miR-103b targets include Fyn-related Src family tyrosine kinase (FRK), which promotes cell survival, proliferation, and differentiation [16], gelsolin (GSN), which strongly inhibits EC apoptosis [17], and laminin α4 (LAMA4), which promotes EC migration and differentiation [18].

**miR-330-5p:** Gauer et al. [19] reported that miR-330 expression is downregulated in human tumor-derived cell lines and hypothesized that it functions as a potential tumor suppressor. Until now there has been no information regarding its expression in vascular cells, but we can report here that miR-330-5p a) is expressed in ECs; b) is downregulated by Ang-1 and Ang-2 but not by VEGF, FGF2, or TGFβ; and c) exerts strong anti-angiogenic properties, as is indicated by its inhibitory effects on several angiogenic processes, including basal and Ang-1-induced EC survival, proliferation, cell cycle, migration, and differentiation. S3 Table (supporting document) indicates that miR-330-5p interacts with 95 genes. Many of these genes, such as AKT, KRAS and cyclin D1 (CCND1), are known to be positive regulators of EC survival, proliferation, migration, and differentiation.

Anti-angiogenic effects of miR-330-5p are likely mediated through direct interactions with proteins that regulate the angiogenic processes listed above. For example, Jeyapalan et al. [20] have reported that miR-330 inhibits *in-vitro* EC differentiation and *in-vivo* cancer angiogenesis and that these effects are mediated in part through direct targeting of CD44 and its downstream target, CDC42. CD44 is a trans-membrane glycoprotein that regulates a wide range of cellular functions, including cell-to-cell and cell-to-matrix interactions, migration, and differentiation. It has also been shown that miR-330 promotes apoptosis in prostate cancer cells through direct targeting of the transcription factor E2F1 and attenuation of AKT phosphorylation [21], and inhibits proliferation and promotes G1 cell cycle arrest and apoptosis in colorectal cancer cells through direct targeting of integrin α5 and CDC42 [22,23]. It is possible that these genes are also targeted by miR-330-5p in ECs.

**miR-557:** Investigations into the expression of miR-557 have mainly been conducted using cancer cells. In the breast, Chen et al. have shown that its expression is downregulated in ductal carcinoma *in situ* (DCIS) [24] and, very recently, Qiu et al. [25] have reported that it works as a tumor suppressor in human lung cancers, possibly by inhibiting the expression of lymphocyte enhancement factor 1 (LEF1). We report here that miR-557 is abundantly expressed in ECs and that its expression is downregulated by Ang-1, Ang-2, and FGF2, but not by VEGF or TGFβ. We also report that miR-557 eliminates the pro-survival effects of Ang-1, increases caspase-3 activity, inhibits EC proliferation and basal migration, and strongly attenuates EC differentiation.

Our analyses of miR-557-gene interaction networks show that it interacts with 157 genes (S4 Table, supporting document), including the transcription factor ETS-1. Several pro-angiogenic growth factors, including VEGF and FGF2, activate ETS-1 to upregulate integrins αV and β3, VE-cadherin, von Willebrand factor (vWF) and the pro-angiogenic miR-126, thus promoting angiogenesis [26]. It is possible that inhibition of ETS-1 is the mechanism by which miR-557 exerts its anti-angiogenic effects in ECs. miR-557 also interacts with several important regulators of proliferation, migration, differentiation, and the cell cycle, including β actin (ACTB), cyclin T2 (CCNT2), FRK, budding uninhibited by benzimidazoles 1 (BUB1), spindlin 4 (SPIN4), cAMP-regulated phosphoprotein 19 (ARPP19), and KLF7 (S4 Table, supporting document). In relation to apoptosis regulation, miR-557 interacts with X-linked inhibitor of apoptosis (XIAP), which is a member of the inhibitors of apoptosis (IAP) family of proteins that promotes cell motility through direct interaction with Rho GDP-dissociation inhibitor (RhoGDI) [27]. We hypothesize that downregulation of XIAP by miR-557 might explain its inhibitory

effects on EC survival, proliferation, and migration, as well as its promotion of Caspase-3 activity.

**miR-575:** Investigations into the expression of miR-557 have also mainly been conducted using cancer cells. For example, it has been reported that it is present in gastric carcinoma cells [28], decreases survival and motility of breast cancer cells, increases apoptosis and enhances the expressions of the anti-apoptotic proteins BAX and phosphorylated p53 in JEG-3 human choriocarcinoma cells, and upregulates the expressions of the anti-apoptotic regulator Bcl2 and the angiogenesis factors VEGF and Ang-2 [29]. We report here that miR-575 is abundantly expressed in HUVECs and is downregulated by Ang-1 and Ang-2 but not by FGF2, TGFβ, or VEGF. Overexpression of miR-575 decreases Ang-1-induced EC survival, increases Caspase-3 activity, and substantially attenuates EC proliferation, migration, and differentiation. This suggests that miR-575 serves as an anti-angiogenic miRNA and that its expression is selectively regulated by the Ang-1/Tie-2 pathway rather than by other angiogenesis-related pathways.

Little is known about miR-575 targets in ECs. In JEG-2 cells, Xia et al. have reported that miR-575 directly targets superoxide dismutase 2 (SOD2), a strong antioxidant enzyme [29]. S5 Table (supporting document) lists 108 genes that directly interact with miR-575. Among these are several genes that inhibit apoptosis and promote cell proliferation and differentiation, including XIAP, apoptosis inhibitor 5 (API5), BCL2L1 (a member of the anti-apoptotic BCl2 protein family), cyclin A2 (CCNA2), which promotes transition through G1/S and G2/M phases of the cell cycle, cell division cycle 45 (CDC45), which prevents G1 phase arrest and enhances cell

proliferation, and myocyte enhancer factor 2D (MEF2D), which enhances angiogenesis downstream from hypoxia inducible factor 1α [30]. It is possible that inhibition of these genes by miR-575 is the mechanism by which it inhibits EC survival, proliferation, and differentiation.

**miR-1287-5p:** It has been reported that the expression of miR-1287 is downregulated in laryngeal cancer cells but upregulated in colorectal cancer tissues [31,32]. Here we report that miR-1287-5p is expressed in HUVECs and is downregulated in response to Ang-1, Ang-2, VEGF, and TGFβ. We also report that miR-1287-5p possesses anti-angiogenic properties, as is indicated by its inhibitory effects on EC survival, proliferation, migration, and differentiation, and its stimulatory effect on Caspase-3 activity.

To our knowledge, no information is available regarding the mechanisms through which miR-1287-5p regulates EC angiogenesis. In glioma cells, Wolter et al. [33] have reported that miR-1287 interacts with and inhibits the expression of epidermal growth factor receptor (EGFR). Our analyses of miR-1287-5p-gene interaction networks show that it directly interacts with 49 genes (S6 Table, supporting document), among which are several genes that regulate cell survival, proliferation, migration, and differentiation. For instance, miR-1287-5p interacts with LAMTOR3, which is a scaffold protein that localizes to the endosomes, binds specifically to ERK1 and ERK2, and regulates the pro-proliferative and pro-migratory roles of these two genes [34]. We should emphasize that ERK1 and ERK2 play very important roles in Ang-1-induced angiogenesis in ECs. miR-1287-5p also interacts with DSN1, which is required for proper kinetochore assembly and progression through the cell cycle. In cancer cells, depletion of DSN1

leads to cell cycle arrest and decreased migration [35]. miR-1287-5p also directly interacts with GFP dissociation inhibitor 1 (GDI1), which regulates the GDP-GTP exchange reaction of the Rab and Ras family of proteins; mutation of GDI1 in cancer cells leads to depressed cell migration [36]. miR-1287-5p also interacts with MAP3K9, which is an upstream activator of JNK signaling. Knockdown of this protein in cancer cells leads to inhibition of cell proliferation, increased apoptosis, and enhanced Caspase-3 activity [37].

**miR-1468-5p:** Little information is available regarding either the expression or functional significance of miR-1468-5p. It has been noted that its expression in lung adenocarcinoma and hepatocellular carcinoma samples positively correlate with survival rate of patients [38,39]. Here we report that miR-1468-5p is expressed in HUVECs and that its expression is downregulated in response to Ang-1 and VEGF, but not by Ang-2, FGF2, TGFβ, or VEGF. We also found that that miR-1468-5p possesses relatively strong anti-angiogenic properties, as evidenced by the inhibitory effects of its mimic on EC survival, proliferation, and differentiation. Moreover, miR-1468-5p inhibits basal migration of ECs and eliminates the pro-angiogenic effects of Ang-1 on EC migration.

The mechanisms through which miR-1468-5p regulates angiogenesis are unknown. S7 Table (supporting document) lists 16 genes that directly interact with miR-1468-5p. Among these is LYR motif containing 7 (LYRM7), which is a chaperone protein critical for the assembly of complex III of the mitochondrial respiratory chain. Downregulation of LYRM7 triggers significant dysfunction of mitochondrial respiration and enhanced apoptosis [40]. Another gene that directly interacts with miR-1468-5p and

may explain its anti-angiogenic effects is plexin domain containing 1 (PLXDC1), also known as TEMP-7 (S7 Table). This gene codes for a protein highly expressed in the vasculature of tumors, and its knockdown in cultured ECs results in significant inhibition of differentiation [41]. Mir-1468-5p also interacts with semaphorin-3D (SEMA3D), which is a secreted protein-coding gene (S7 Table). The semaphorin-3D protein plays a critical role in cardiovascular development, and a recent study indicates that it promotes EC migration and morphology through regulation of ACTIN organization [42]. Downregulation of SEMA3D by miR-1468-5p might be the mechanism by which it exerts its inhibitory effect on EC migration.

## 7. Conclusions

Our study reveals that exposure of ECs to Ang-1 triggers significant downregulation of mature miR-103b, miR-330a-5p, miR-557, miR-575, miR-1287-5p, and miR-1468-5p expressions, and that these miRNAs inhibit the survival, proliferation, migration, and differentiation of ECs. We conclude that these miRNA function as negative regulators of angiogenesis.

## 8. Acknowledgements

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## 10. Figures

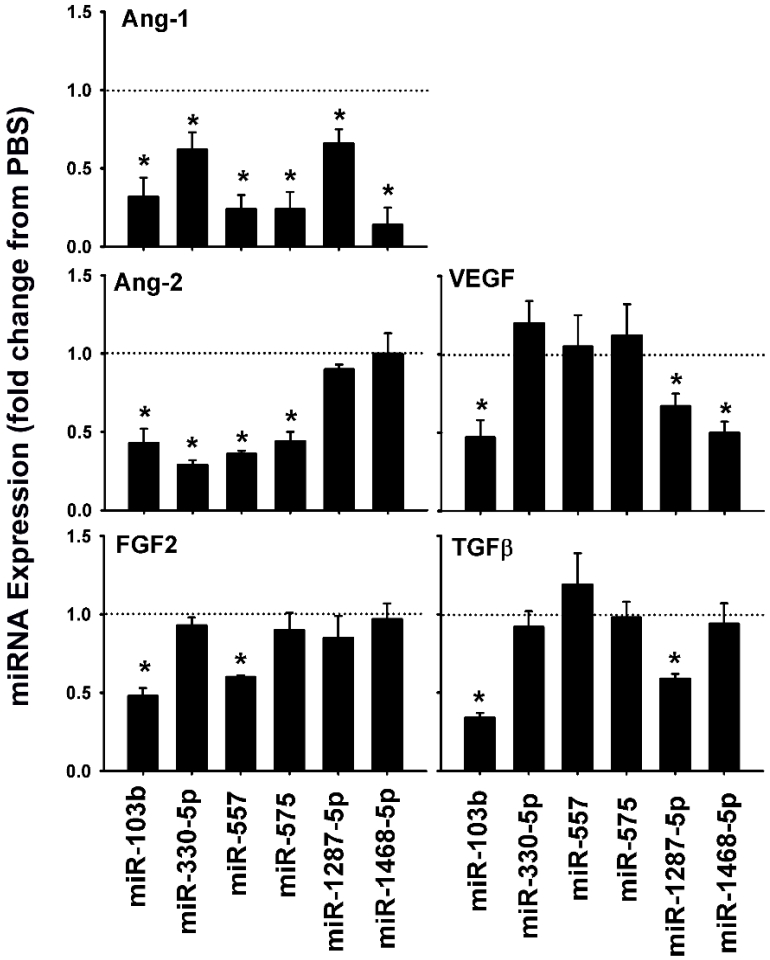



**Figure 1: Effects of angiogenesis factors on miRNA expression in ECs.**

Expression of miR-103b, miR-330-5p, miR-557, miR-575, miR-1287-5p, miR-1468-5p in HUVECs exposed to Ang-1, Ang-2, VEGF, FGF2, or TGFβ for 24 h. Values are means ± SEM, expressed as fold change from control (PBS). *P<0.05, compared to control.

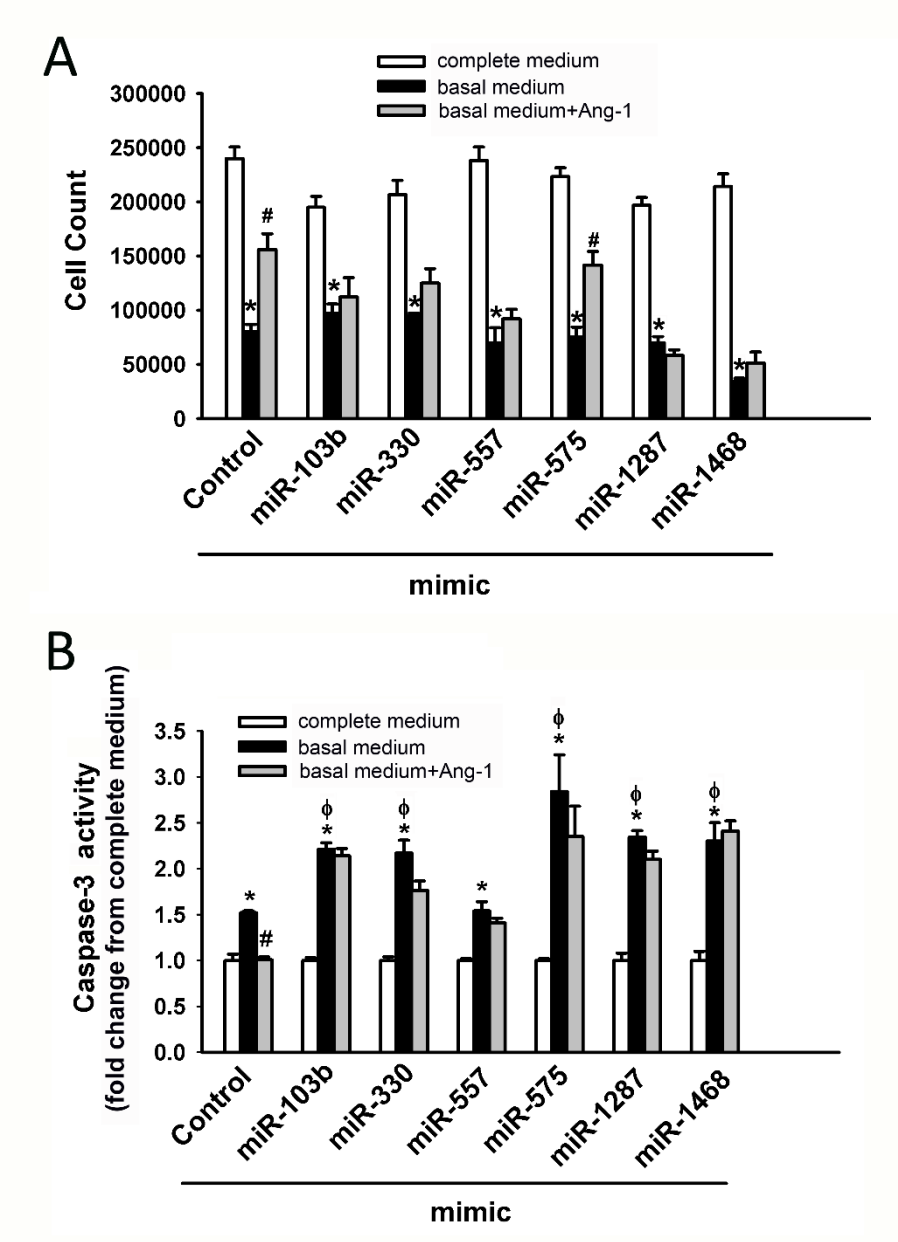



**Figure 2: Effects of miRNA mimics on EC survival and Caspase-3 activity.**

A&B: HUVECs were transfected with control or miRNA mimics. After 48 h of recovery, equal numbers of cells were maintained in complete (20% FBS), basal (0% FBS), or basal medium+Ang-1 (300 ng/ml). Cell counts and Caspase-3 activities were measured 24 h later. Values are means ± SEM. *P<0.05, compared to cells maintained in complete medium. #P<0.05, compared to cells maintained in basal medium. ϕP<0.05,

compared to cells transfected with control mimic and maintained in basal medium. N=6 per group.

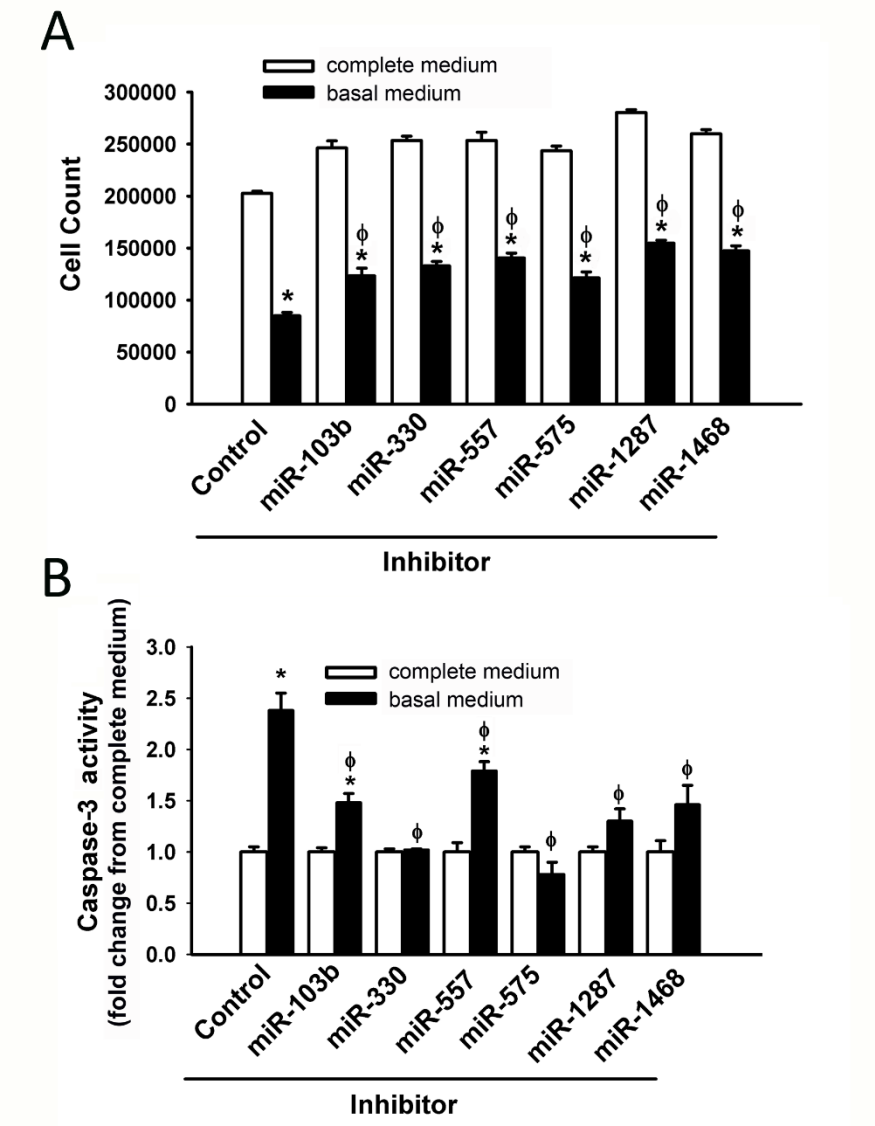



**Figure 3: Effects of miRNA inhibitors on EC survival and Caspase-3 activity.**

A&B: HUVECs were transfected with control or miRNA inhibitors. After 48 h of recovery, equal numbers of cells were maintained in complete (20% FBS) or basal medium (0% FBS). Cell counts and Caspase-3 activities were measured 24 h later. Values are means ± SEM. *P<0.05, compared to cells maintained in complete medium. $^{\phi}$P<0.05, compared to cells transfected with control inhibitor and maintained in basal medium. N=6 per group.

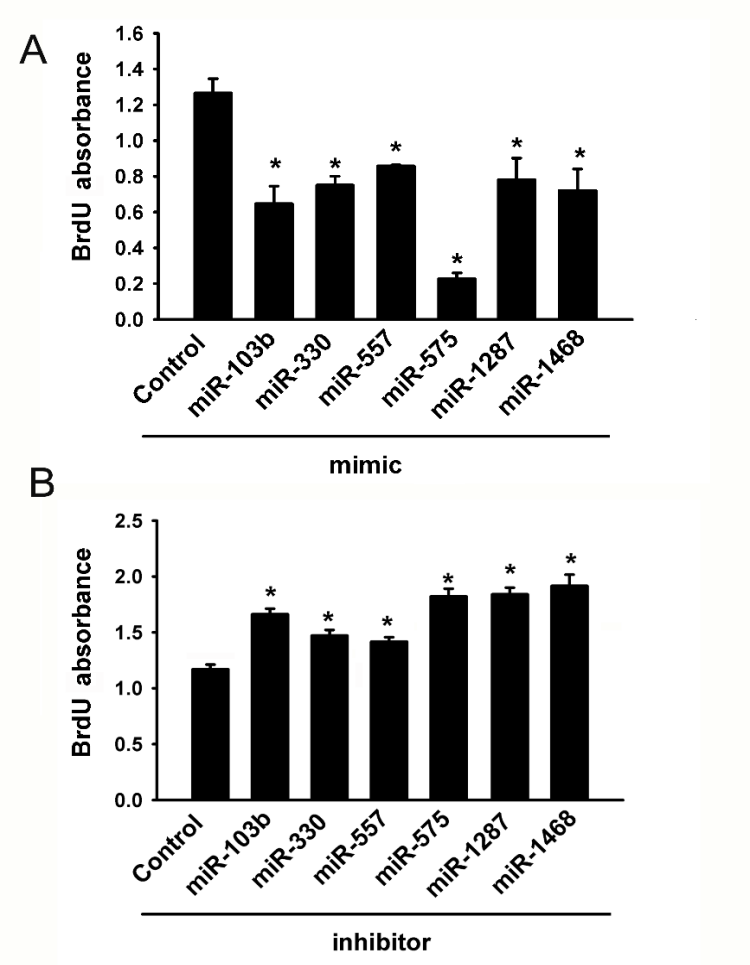



**Figure 4: Effects of miRNA mimics and inhibitors on EC proliferation.**

HUVECs were transfected with miRNA mimics (panel A) or inhibitors (panel B). After 48 h of recovery, cells were plated in 96-well plates and maintained in complete Lonza EGM™ medium (minus VEGF). One h after plating, BrdU was added. BrdU absorbance was measured 24 h later. Values are means ± SEM.*P<0.05, compared to cells transfected with control mimic or inhibitor. N=6 per group.

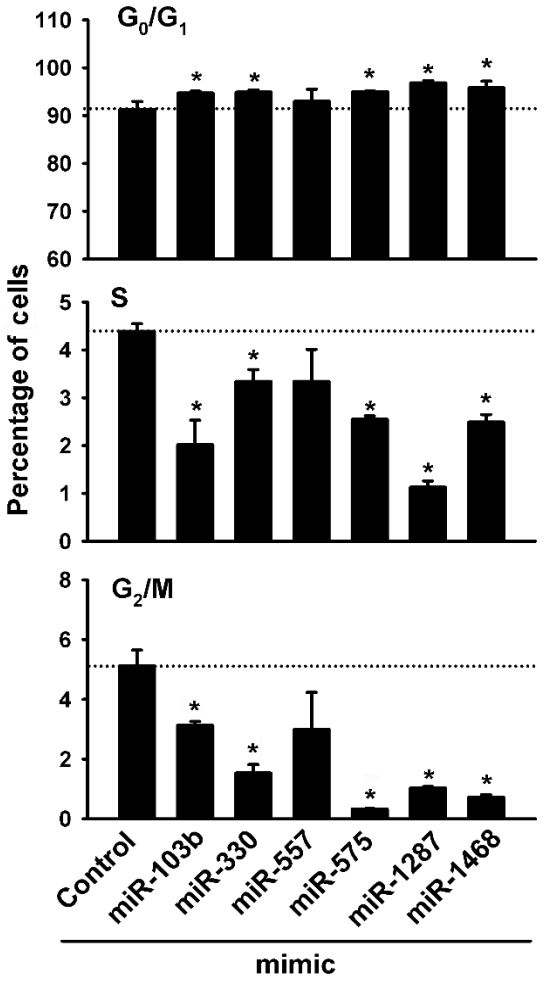



**Figure 5: Effects of miRNA mimics on cell cycle.**

Percentage of HUVECs in G0/G1, S, and G2/M phases in response to transfection with control or miRNA mimics. Values are means ± SEM.*P<0.05, compared to control mimic. N=8 per group.

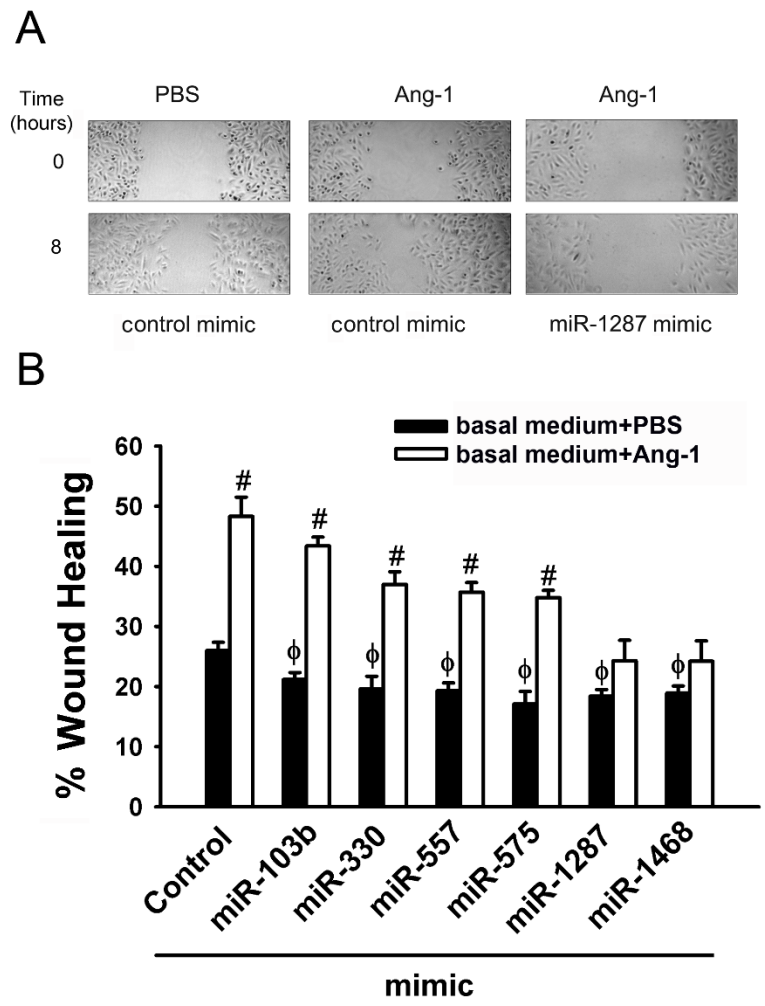



**Figure 6: Effects of miRNA mimics on EC migration.**

**A:** Representative images of wound healing in HUVECs at times 0 and 8 h. HUVECs were transfected with control or miR-1287-5p mimic and maintained in basal MCDB131 medium (2% FBS) containing PBS or Ang-1 (300 ng/ml).

**B:** Percentage of wound healing in HUVECs transfected with control or miRNA mimics. After 48 h of recovery, cells were maintained in basal MCDB131 medium (2% FBS) containing PBS or Ang-1. Wound healing was measured 8 h after wounding with a pipette tip. Values are means ± SEM. $^{\#}P<0.05$, compared to basal medium+PBS.

$^{\phi}P<0.05$, compared to cells transfected with control mimic and maintained in basal medium+PBS. N=6 per group.

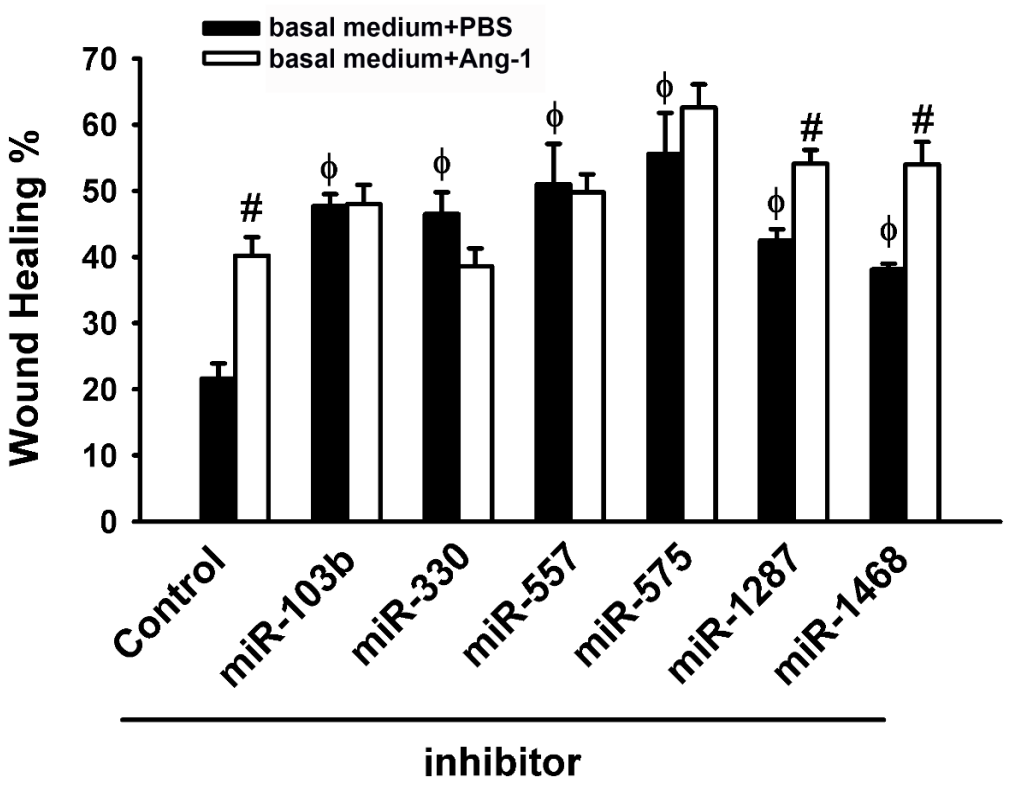



**Figure 7: Effects of miRNA inhibitors on EC migration.**

Percentage of wound healing in HUVECs transfected with control or miRNA inhibitors. After 48 h of recovery, cells were mantained in basal MCDB131 medium containing PBS or Ang-1. Wound healing was measured 8 h after wounding with a pipette tip. Values are means ± SEM. $^{\#}P<0.05$, compared to basal medium+PBS. $^{\phi}P<0.05$, compared to cells transfected with control mimic and maintained in basal medium+PBS. N=6 per group.

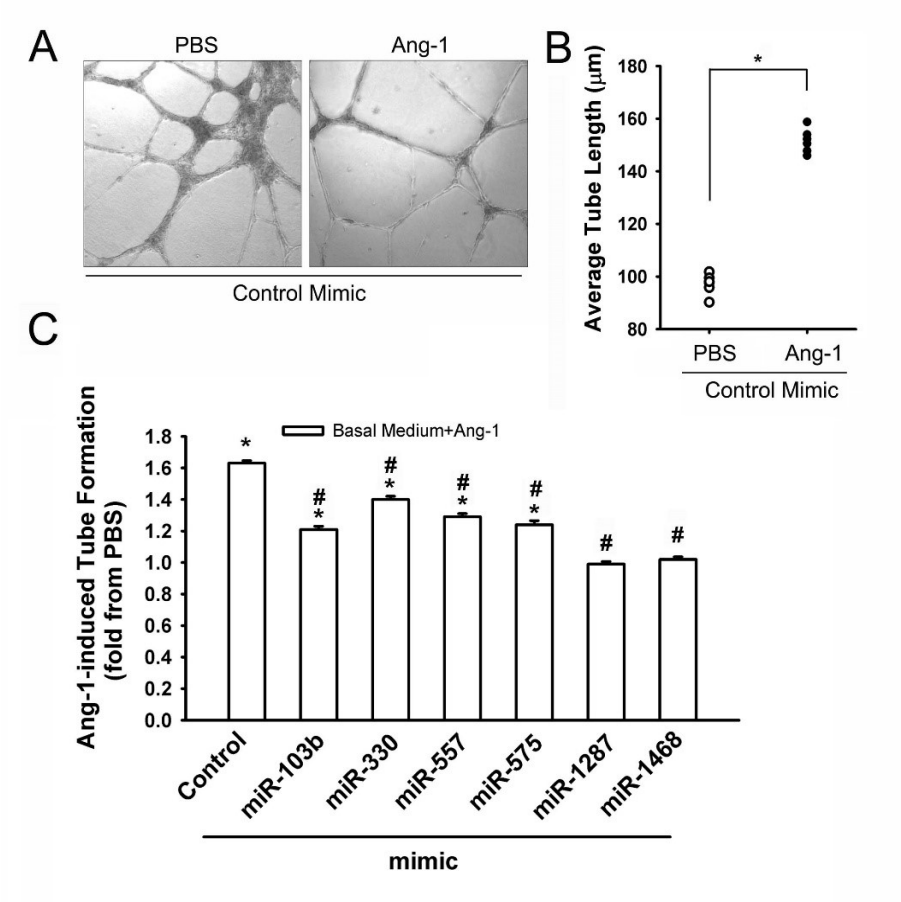



**Figure 8: Effects of miRNA mimics on EC differentiation.**

**A:** Representative images of HUVECs transfected with control mimic and maintained for 24 h in 96-well plates pre-coated with growth factor-reduced Matrigel® and basal medium containing PBS or Ang-1. Note the influence of Ang-1 on tube length, as compared to PBS.

**B:** Average tube lengths of HUVECs transfected with control mimic and maintained for 24 h in basal medium containing PBS or Ang-1. Values are means ± SEM. *P<0.05, compared to PBS. N=5 per group.

**C:** Ang-1-induced tube formation (expressed as fold change from that observed with PBS) in HUVECs transfected with control mimic or miRNA mimics and allowed to

recover for 48 h. Values are means ± SEM. *P<0.05, compared to PBS. #P<0.05, compared to control mimic. N=6 per group.

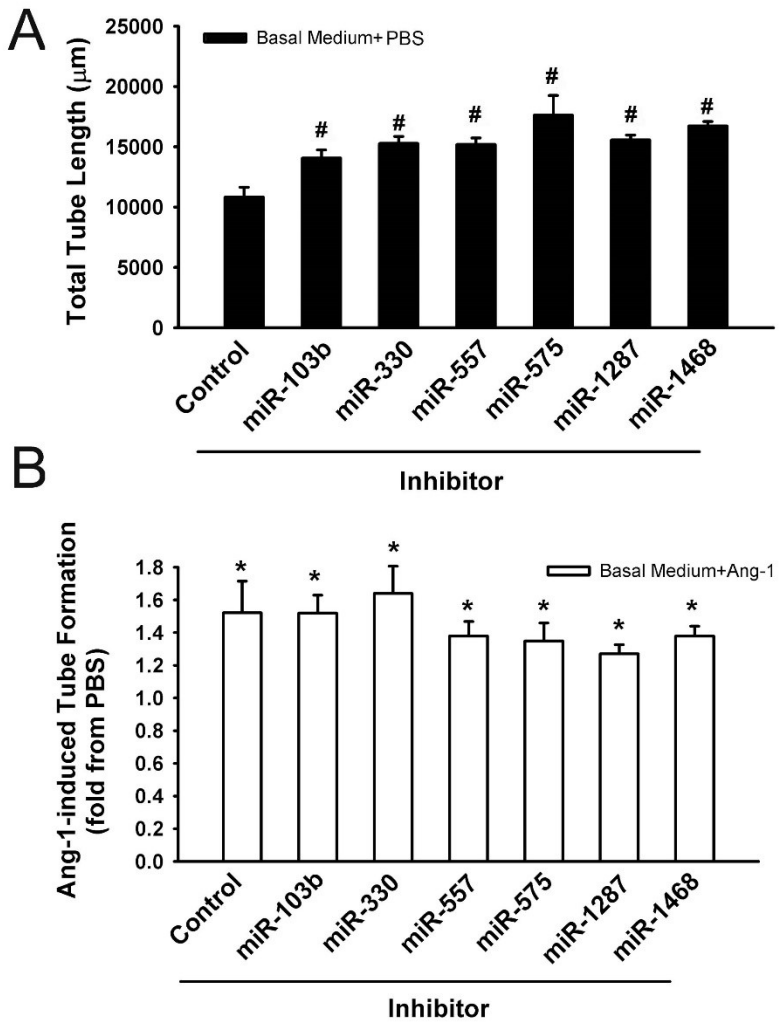



**Figure 9: Effects of miRNA inhibitors on EC differentiation.**

**A:** Average tube lengths of HUVECs transfected with control inhibitor and miRNA inhibitors. Cells were maintained for 24 h in basal medium containing PBS. Values are means ± SEM. #P<0.05, compared to control inhibitor. N=6 per group.

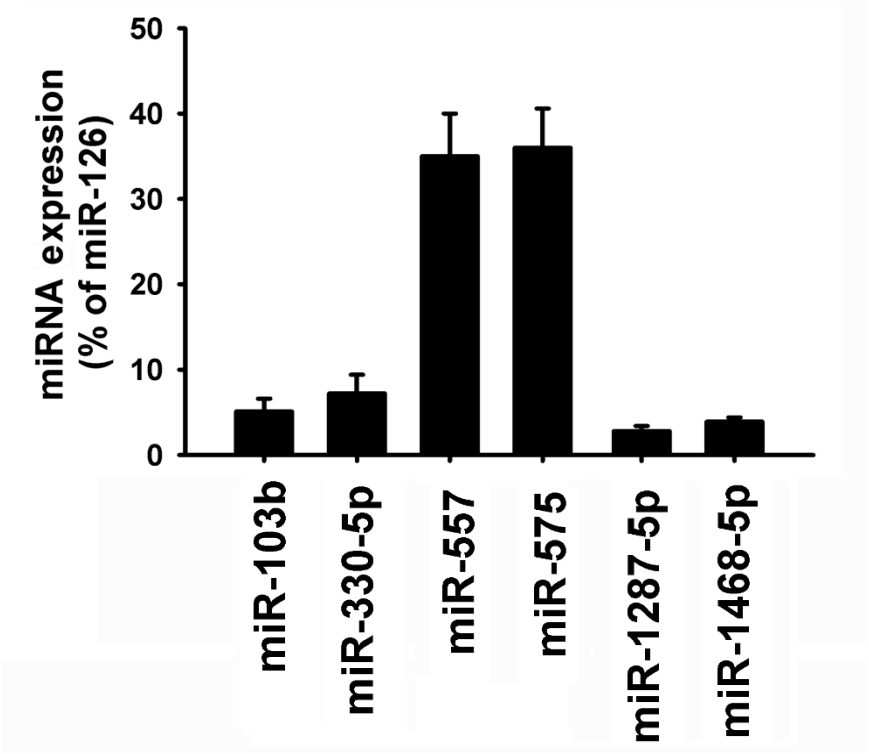
**B:** Ang-1-induced tube formation (expressed as fold from that observed with PBS) in HUVECs transfected 48 h earlier with control inhibitor or miRNA inhibitors. Values are means ± SEM. *P<0.05, compared to PBS. N=6 per group.

## 11. Tables

**Table 1: Primers used for real-time PCR experiments**

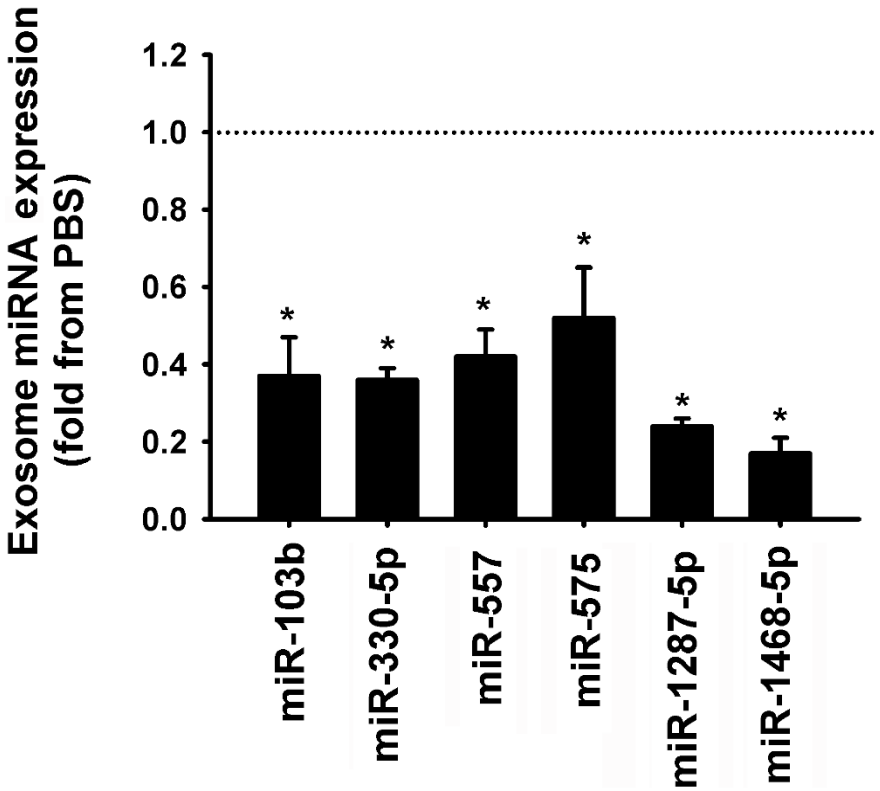
| Gene | Sequence (5'→ 3') | Accession Number |
|---|---|---|
| **Hsa-miR-03b** | TCATAGCCCTGTACAATGCTGCT | MIMAT0007402 |
| **Hsa-miR-126-3p** | TCGTACCGGAGTAATAATGCG | MIMAT0000445 |
| **Hsa-miR-330-5p** | TCTGGGCCTGTGTCTTAGGC | MIMAT0004693 |
| **Hsa-miR-557** | TTGCACGGGTGGGCCTTGTCT | MIMAT0003221 |
| **Hsa-miR-575** | GAGCCAGTTGGACAGGAGC | MIMAT0003240 |
| **Hsa-miR-1287-5p** | TGCTGGATCAGTGGTTCGAGTC | MIMAT0006789 |
| **Hsa-miR-1468-5p** | CTCCGTTTGCCTGTTTCGCTG | MIMAT0006789 |
| **U6 snRNA** | ACTAAAATTGGAACGATACAGAGA | NR_004394.1 |

12. **Supplementary figures**



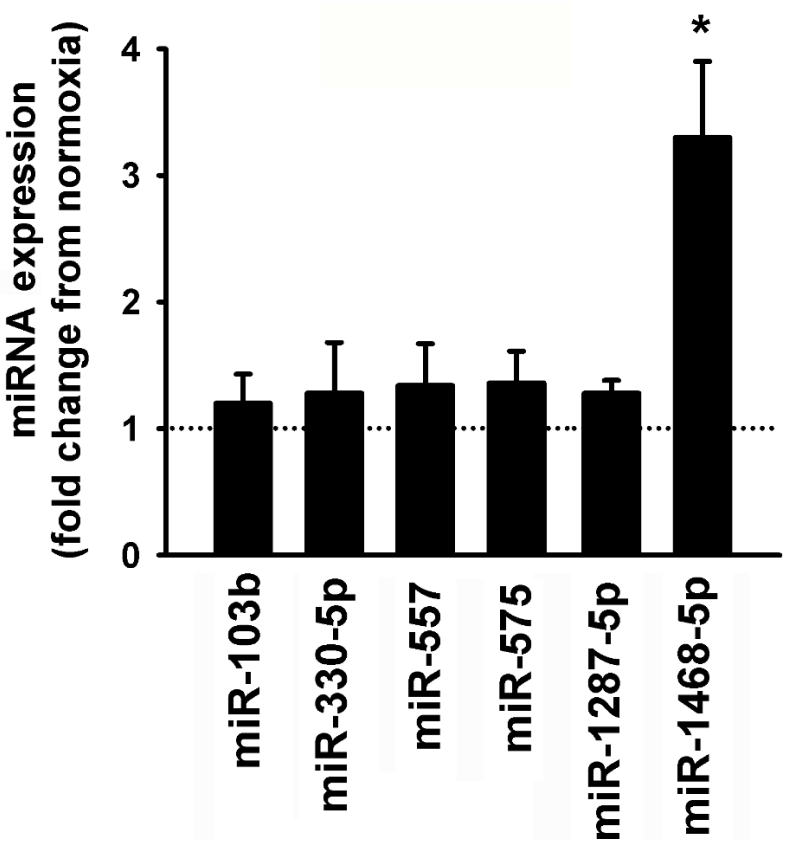

**Supplementary figure 1:** miRNA expression in complete medium.

qPCR expressions of miR-103b, miR-330-5p, miR-557, miR-575, miR-1287-5p, and miR-1468 in HUVECs maintained in complete medium. Values expressed as percentage miR-126 expression. Values are means ± SEM. N=8.



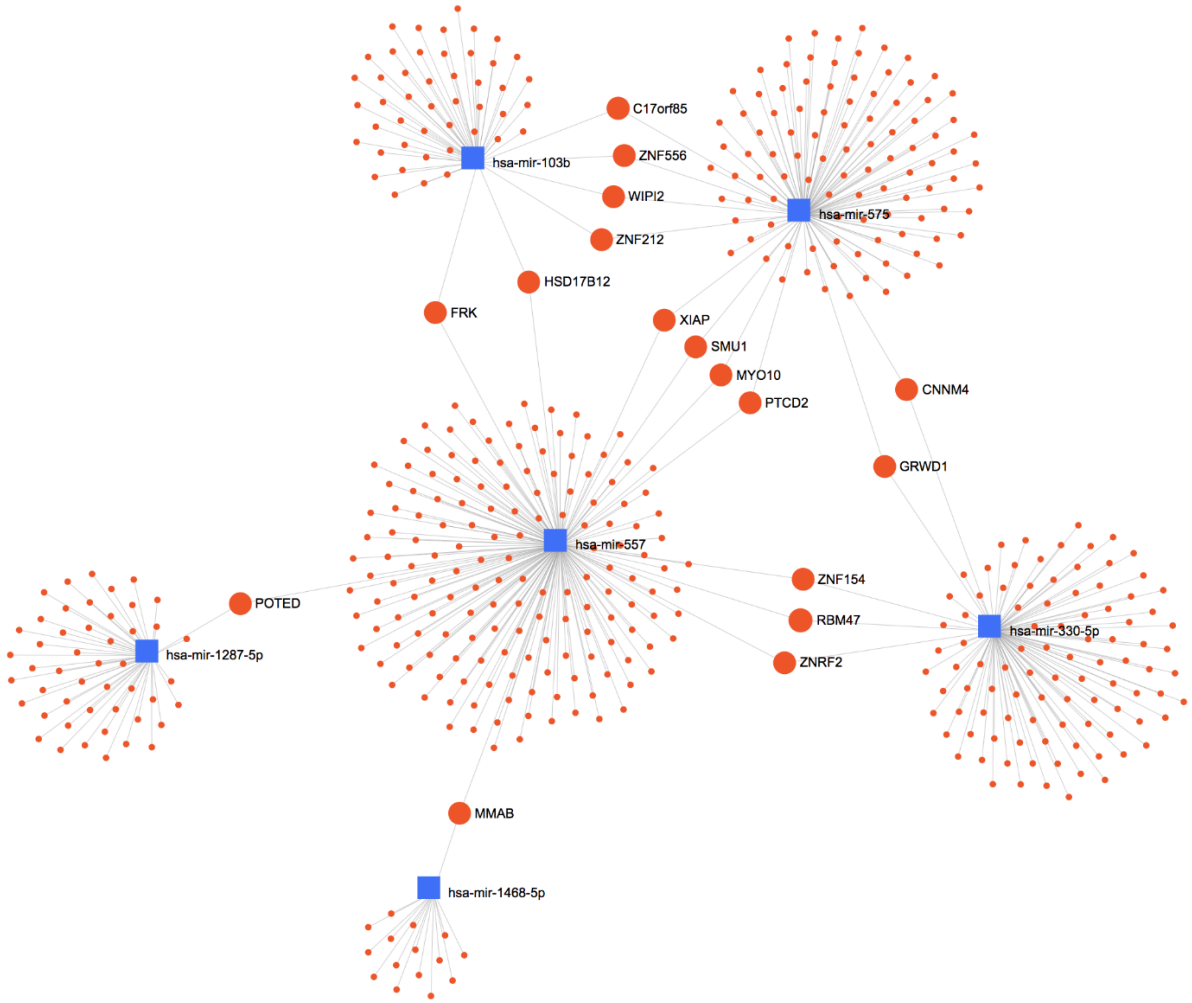

**Supplementary figure 2: Exosomal miRNA expression in response to Ang-1.**

qPCR expressions of miR-103b, miR-330-5p, miR-557, miR-575, miR-1287-5p, and miR-1468 in EVs of HUVECs exposed to Ang-1 for 24 h. Values are means ± SEM, expressed as fold change from cells exposed to PBS. *P<0.05, compared to PBS. N=8.




**Supplementary figure 3: miRNA expression during hypoxia.**

qPCR expressions of miR-103b, miR-330-5p, miR-557, miR-575, miR-1287-5p, and miR-1468 in HUVECs maintained in normoxic or hypoxic conditions for 24 h. Values are means ± SEM, expressed as fold change from normoxic cells. *P<0.05, compared to normoxia. N=6.




**Supplementary figure 4**: miRNA interaction network

miRNA-gene interaction networks detected by miRNet web tool. Small orange circles designate specific genes that interact with a given miRNA. Large orange circles designate genes that interact with two miRNAs.

## 13. Supplementary tables

**Supplementary table 1: miRNet identification of miR-103b gene targets**

| ID | Gene | Method | Function | Process |
|---|---|---|---|---|
| hsa-mir-103b | AKR7L | HITS-CLIP | Aldo-keto reductase activity | Oxidation-reduction process |
| hsa-mir-103b | ELG | HITS-CLIP | RNA binding | mRNA transport |
| hsa-mir-103b | C17orf85 | PAR-CLIP | mRNA binding | 7-methylguanosine mRNA capping |
| hsa-mir-103b | CA12 | PAR-CLIP | Carbonate dehydratase activity | Bicarbonate transport |
| hsa-mir-103b | CD2AP | PAR-CLIP | Protein binding | Cell cycle, cell division, cell adhesion |
| hsa-mir-103b | CEP89 | HITS-CLIP | Protein binding | Chemical synaptic transmission |
| hsa-mir-103b | CMBL | HITS-CLIP | Hydrolase activity | Xenobiotic metabolic process |
| hsa-mir-103b | CRY2 | HITS-CLIP | DNA binding, protein binding | Circadian regulation of gene expression |
| hsa-mir-103b | DCAF17 | HITS-CLIP | Protein binding | Post-translational protein modification |
| hsa-mir-103b | DMTF1 | PAR-CLIP | Sequence-specific DNA binding | Cell cycle, cell differentiation |
| hsa-mir-103b | E2F3 | PAR-CLIP | Sequence-specific DNA binding | Cell cycle, cell proliferation |
| hsa-mir-103b | ELK1 | HITS-CLIP | Sequence-specific DNA binding | Cell differentiation, proliferation |
| hsa-mir-103b | FOXK1 | PAR-CLIP | Sequence-specific DNA binding | Cell differentiation, protein deubiquitination |
| hsa-mir-103b | FRK | PAR-CLIP | ATP binding | Cell migration, proliferation, differentiation |
| hsa-mir-103b | GLDN | PAR-CLIP | Protein binding | Cell-cell adhesion |
| hsa-mir-103b | GNAT1 | PAR-CLIP | GTP binding, GTPase activity | G-protein coupled receptor signaling |

| | | | | |
|---|---|---|---|---|
| hsa-mir-103b | GSN | HITS-CLIP | Actin binding | Actin filament capping, polymerization |
| hsa-mir-103b | HCFC2 | PAR-CLIP | Transcription coactivator activity | Regulation of transcription |
| hsa-mir-103b | HSD17B12 | HITS-CLIP | Collagen binding | Extracellular matrix organization |
| hsa-mir-103b | IL12B | HITS-CLIP | Cytokine receptor activity | T-helper 1 type immune response |
| hsa-mir-103b | LAMA4 | PAR-CLIP | Extracellular matrix constituent | Cell adhesion, migration |
| hsa-mir-103b | LIN54 | PAR-CLIP | DNA binding, protein binding | Cell cycle |
| hsa-mir-103b | MSL1 | HITS-CLIP | Protein acetylation | Histone H4-K16 acetylation |
| hsa-mir-103b | NPY4R | PAR-CLIP | Protein binding | G-protein coupled receptor activity |
| hsa-mir-103b | NUFIP2 | PAR-CLIP | RNA and protein binding | Unknown |
| hsa-mir-103b | OSBPL10 | PAR-CLIP | Cholesterol binding | Phospholipid transport |
| hsa-mir-103b | PIGP | HITS-CLIP | Phospholipid transferase activity | Assembly of GPI anchor in ER membrane |
| hsa-mir-103b | PLXDC2 | HITS-CLIP | Protein binding | Anti-angiogenic activity |
| hsa-mir-103b | PLXNA2 | PAR-CLIP | Protein binding | Centrosome localization, cell migration |
| hsa-mir-103b | PPIL3 | PAR-CLIP | Protein binding | mRNA splicing, protein folding |
| hsa-mir-103b | PPM1A | PAR-CLIP | Phosphatase activity | Cell cycle, Wnt signaling |
| hsa-mir-103b | PPM1L | HITS-CLIP | Phosphatase activity | MAPK cascade |
| hsa-mir-103b | RPL7L1 | PAR-CLIP | RNA binding | Cytoplasmic translation |
| hsa-mir-103b | SAE1 | PAR-CLIP | ATP-dependent protein binding | Protein targeting to mitochondria |
| hsa-mir-103b | SESTD1 | PAR-CLIP | Phosphatidic acid binding | Calcium ion transmembrane transport |
| hsa-mir-103b | SPG20 | PAR-CLIP | Ubiquitin protein ligase binding | Cell division, BMP signaling |
| hsa-mir-103b | SREK1IP1 | PAR-CLIP | Nucleic acid and protein binding | RNA splicing |

| | | | | |
|---|---|---|---|---|
| hsa-mir-103b | SRP9 | PAR-CLIP | RNA and protein binding | Regulation of translation elongation |
| hsa-mir-103b | STON2 | HITS-CLIP | Protein binding | Hemapoietic progenitor cell differentiation |
| hsa-mir-103b | TDRD1 | HITS-CLIP | Metal ion binding | Meiotic cell cycle, DNA methylation |
| hsa-mir-103b | TSPAN1 | PAR-CLIP | Protein binding | Cell migration, proliferation |
| hsa-mir-103b | WIPI2 | PAR-CLIP | PI-3,5-biphosphate binding | Autophagosome assembly |
| hsa-mir-103b | ZNF212 | PAR-CLIP | DNA binding | Regulation of transcription |
| hsa-mir-103b | ZNF281 | PAR-CLIP | Sequence-specific DNA binding | Negative regulation of gene expression |
| hsa-mir-103b | ZNF556 | HITS-CLIP | DNA binding | Regulation of transcription |
| hsa-mir-103b | ZNF567 | PAR-CLIP | Transcription factor activity | Regulation of transcription |
| hsa-mir-103b | ZNRF3 | PAR-CLIP | Frizzled binding | Wnt signaling |

HITS-CLIP: High-Throughput Sequencing of RNA isolated by CrossLinking and ImmunoPrecipitation; PAR-CLIP: Photoactivatable Ribonucleoside-enhanced CrossLinking and ImmunoPrecipitation; CLASH: CrossLinking, Ligation and Sequencing of Hybrids.

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**Supplementary table 2: miRNet identification of miR-330-5p target genes**

| ID | Gene | Method | Function | Process |
|---|---|---|---|---|
| hsa-mir-330-5p | ABCC6 | PAR-CLIP | Protein binding | Membrane transport |
| hsa-mir-330-5p | ACAP2 | HITS-CLIP | GTPase activator activity | Protein localization to endosome |
| hsa-mir-330-5p | AKT1 | PAR-CLIP | Protein kinase activity | Cell survival, proliferation, differentiation |
| hsa-mir-330-5p | ANXA5 | CLASH | $Ca^{2+}$ binding | Apoptosis, blood coagulation |
| hsa-mir-330-5p | AR | PAR-CLIP | Androgen binding | Androgen receptor signaling pathway |
| hsa-mir-330-5p | ARHGDIA | PAR-CLIP | GTPase activator activity | Apoptosis, cell adhesion, actin organization |
| hsa-mir-330-5p | ARL5B | PAR-CLIP | GTP binding | GTPase mediated signaling |
| hsa-mir-330-5p | ARRB2 | CLASH | Angiotensin receptor binding | Wnt signaling, cell chemotaxis |
| hsa-mir-330-5p | BAD | CLASH | 14-3-3 protein binding | Apoptosis |
| hsa-mir-330-5p | BCL11B | PAR-CLIP | Metal ion binding | T cell differentiation |
| hsa-mir-330-5p | BMP7 | HITS-CLIP | BMP receptor binding | BMP signaling pathway |
| hsa-mir-330-5p | BVES | HITS-CLIP | cAMP binding | Heart and skeletal muscle development |
| hsa-mir-330-5p | C1R | HITS-CLIP | $Ca^{2+}$ ion binding | Complement activation |
| hsa-mir-330-5p | CARD11 | CLASH | CARD domain binding | B cell differentiation |
| hsa-mir-330-5p | CCND1 | PAR-CLIP | Cyclin-dependent kinase | Cell division, cell cycle |
| hsa-mir-330-5p | CCT3 | CLASH | ATP binding | Protein folding |
| hsa-mir-330-5p | CNNM4 | PAR-CLIP | $Mg^{2+}$ transporter activity | $Mg^{2+}$ homeostasis |
| hsa-mir-330-5p | DHX40 | PAR-CLIP | RNA helicase activity | RNA metabolism |

| | | | | |
|---|---|---|---|---|
| hsa-mir-330-5p | EPG5 | HITS-CLIP | Protein binding | Autophagosome maturation |
| hsa-mir-330-5p | EPHB3 | HITS-CLIP | ATP binding | Angiogenesis, cell migration |
| hsa-mir-330-5p | F9 | HITS-CLIP | $Ca^{2+}$ binding | Blood coagulation |
| hsa-mir-330-5p | FAM127B | PAR-CLIP | Unknown | Unknown |
| hsa-mir-330-5p | FAM167B | HITS-CLIP | Unknown | Unknown |
| hsa-mir-330-5p | FAM193A | HITS-CLIP | Unknown | Unknown |
| hsa-mir-330-5p | FAM216B | HITS-CLIP | Unknown | Unknown |
| hsa-mir-330-5p | FAM26E | PAR-CLIP | Cation channel activity | Cation transmembrane transport |
| hsa-mir-330-5p | FBXL16 | HITS-CLIP | Ubiquitin transferase | Protein polyubiquitination |
| hsa-mir-330-5p | FTSJ3 | CLASH | RNA binding | rRNA maturation |
| hsa-mir-330-5p | GDE1 | HITS-CLIP | Phosphodiesterase | Lipid metabolic process |
| hsa-mir-330-5p | GOSR2 | HITS-CLIP | SNARE receptor activity | ER to Golgi vesicle transport |
| hsa-mir-330-5p | GRWD1 | HITS-CLIP | Histone binding | DNA replication |
| hsa-mir-330-5p | GYS1 | HITS-CLIP | Glycogen synthase activity | Glycogen biosynthesis |
| hsa-mir-330-5p | HNRNPA1 | HITS-CLIP | RNA binding | RNA export from nucleus |
| hsa-mir-330-5p | HNRNPA1L2 | HITS-CLIP | RNA binding | RNA splicing |
| hsa-mir-330-5p | HOOK3 | HITS-CLIP | Microtubule binding | Cytoplasmic microtubule organization |
| hsa-mir-330-5p | HSPA1B | CLASH | AT P binding | Cellular response to heat |
| hsa-mir-330-5p | IHH | PAR-CLIP | $Ca^{2+}$ ion binding | Cell maturation, bone resorption |
| hsa-mir-330-5p | IP6K3 | CLASH | ATP binding, kinase activity | Inositol Phosphate biosynthesis |
| hsa-mir-330-5p | IRAK1 | CLASH | Kinase activity | MyD88-dependent TLR signaling |

| | | | | |
|---|---|---|---|---|
| hsa-mir-330-5p | IRS4 | CLASH | Insulin receptor binding | Insulin receptor signaling |
| hsa-mir-330-5p | KLHL9 | CLASH | Ubiquitin transferase | Cell cycle, protein ubiquitination |
| hsa-mir-330-5p | KLK2 | HITS-CLIP | Endopeptidase activity | Extracellular matrix disassembly |
| hsa-mir-330-5p | KRAS | PAR-CLIP | GTPase activity | MAPK cascade |
| hsa-mir-330-5p | LIF | CLASH | Cytokine activity | Blood vessel remodeling |
| hsa-mir-330-5p | LSM10 | HITS-CLIP | U7 snRNA binding | RNA splicing |
| hsa-mir-330-5p | MAGEB4 | HITS-CLIP | Protein binding | Unknown |
| hsa-mir-330-5p | MED18 | HITS-CLIP | Transcription co-activator | Regulation of transcription |
| hsa-mir-330-5p | MOCS3 | HITS-CLIP | ATP binding | Protein urmylation |
| has-mir-330-5p | MTHFD2 | HITS-CLIP | $Mg^{2+}$ binding | Folic acid metabolism |
| hsa-mir-330-5p | MYRF | CLASH | DNA binding, peptidase | CNS Myelin maintenance |
| hsa-mir-330-5p | NAA30 | HITS-CLIP | N-Acetyltransferase activity | Peptidyl-methionine acetylation |
| hsa-mir-330-5p | NDEL1 | HITS-CLIP | Alpha-tubulin binding | Cell migration, activation of GPTase activity |
| hsa-mir-330-5p | NHP2 | CLASH | RNA binding | rRNA processing |
| hsa-mir-330-5p | NUP98 | HITS-CLIP | RNA binding | RNA export from the nucleus |
| hsa-mir-330-5p | PATZ1 | PAR-CLIP | DNA and chromatin binding | Regulation of transcription |
| hsa-mir-330-5p | PDE1B | HITS-CLIP | cAMP-phosphodiesterase | Apoptosis, cAMP & cGMP catabolism |
| hsa-mir-330-5p | PDE3A | HITS-CLIP | cAMP-phosphodiesterase | cAMP catabolic process, signaling |
| hsa-mir-330-5p | PEX5L | PAR-CLIP | Peroxisome binding | Protein import into peroxisome |
| hsa-mir-330-5p | PFAS | CLASH | ATP binding | IMP biosynthetic process |
| hsa-mir-330-5p | PFKL | CLASH | Phosphofructokinase | Glycolysis |

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|---|---|---|---|---|
| hsa-mir-330-5p | PHF8 | PAR-CLIP | Chromatin binding | Histone demethylation |
| hsa-mir-330-5p | POFUT1 | PAR-CLIP | Fucosyltransferase activity | Notch signaling, angiogenesis |
| hsa-mir-330-5p | POMGNT1 | CLASH | Glucosaminyltransferase | Protein-O-linked glycosylation |
| hsa-mir-330-5p | PRKCSH | CLASH | $Ca^{2+}$ binding | N-glycan processing |
| hsa-mir-330-5p | PRRC2A | CLASH | RNA and protein binding | Cell differentiation |
| hsa-mir-330-5p | RBM47 | PAR-CLIP | Unknown | Unknown |
| hsa-mir-330-5p | RFT1 | CLASH | Lipid transferase activity | Carbohydrate and lipid transport |
| hsa-mir-330-5p | RPL4 | CLASH | RNA binding | Protein translation |
| hsa-mir-330-5p | RPTN | HITS-CLIP | $Ca^{2+}$ ion binding | Cornification |
| hsa-mir-330-5p | SF3B3 | PAR-CLIP | RNA and protein binding | RNA splicing |
| hsa-mir-330-5p | SLC27A4 | HITS-CLIP | Fatty acid transport activity | Fatty acid transport |
| hsa-mir-330-5p | SLC47A1 | PAR-CLIP | Proton antiporter activity | Transmembrane transport |
| hsa-mir-330-5p | SORCS2 | PAR-CLIP | Neuropeptide receptor activity | Neuropeptide signaling |
| hsa-mir-330-5p | SPTBN2 | CLASH | Actin and cadherin binding | Actin filament capping |
| hsa-mir-330-5p | SPTLC1 | PAR-CLIP | Protein binding | Regulation of lipophagy |
| hsa-mir-330-5p | SSBP2 | HITS-CLIP | DNA binding | Regulation of transcription |
| hsa-mir-330-5p | STX1B | CLASH | SNAP receptor activity | Exocytosis of neurotransmitter |
| hsa-mir-330-5p | SYNRG | HITS-CLIP | Protein binding | Endocytosis |
| hsa-mir-330-5p | TBL1XR1 | PAR-CLIP | Beta catenin binding | Wnt signaling |
| hsa-mir-330-5p | TOMM5 | PAR-CLIP | Protein binding | Mitochondrial membrane import |
| hsa-mir-330-5p | TRAPPC10 | CLASH | Protein binding | Endosome to Golgi transport |

| | | | | |
|---|---|---|---|---|
| hsa-mir-330-5p | TRPC4AP | HITS-CLIP | $Ca^{2+}$ channel activity | $Ca^{2+}$ transmembrane transport |
| hsa-mir-330-5p | TUFT1 | CLASH | Protein binding | Bone mineralization |
| hsa-mir-330-5p | UBA2 | CLASH | SUMO binding | Protein sumoylation |
| hsa-mir-330-5p | UBE4A | PAR-CLIP | Ubiquitin ligase activity | Protein polyubiquitination |
| hsa-mir-330-5p | ULBP3 | HITS-CLIP | Lectin-like receptor binding | Natural killer cell activation |
| hsa-mir-330-5p | USH1G | PAR-CLIP | Protein binding | Inner ear morphogenesis |
| hsa-mir-330-5p | VASP | CLASH | Actin and cadherin binding | Actin polymerization |
| hsa-mir-330-5p | ZDHHC9 | CLASH | Palmitoyltransferase activity | Protein palmitoylation |
| hsa-mir-330-5p | ZNF154 | HITS-CLIP | DNA binding | Regulation of transcription |
| hsa-mir-330-5p | ZNF394 | PAR-CLIP | DNA binding | Regulation of transcription |
| hsa-mir-330-5p | ZNF462 | CLASH | DNA binding | Regulation of transcription |
| hsa-mir-330-5p | ZNF772 | PAR-CLIP | DNA binding | Regulation of transcription |
| hsa-mir-330-5p | ZNRF2 | PAR-CLIP | Ubiquitin ligase activity | Protein polyubiquitination |
| hsa-mir-330-5p | ZYX | CLASH | RNA and protein binding | Cell adhesion |

HITS-CLIP: High-Throughput Sequencing of RNA isolated by CrossLinking and ImmunoPrecipitation; PAR-CLIP: Photoactivatable Ribonucleoside-enhanced CrossLinking and ImmunoPrecipitation; CLASH: CrossLinking, Ligation and Sequencing of Hybrids.

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(9) Kishore S, Jaskiewicz L, Burger L, Hausser J, Khorshid M, Zavolan M. A quantitative analysis of CLIP methods for identifying binding sites of RNA-binding proteins. *Nat Methods* 2011 May 15;8(7):559-64.

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**Supplementary table 3: miRNet identification of miR-557 target genes**

| ID | Gene | Method | Function | Process |
|---|---|---|---|---|
| hsa-mir-557 | AAGAB | HITS-CLIP | Protein binding | Protein transport |
| hsa-mir-557 | AASDHPPT | PAR-CLIP | Acyl-carrier-protein synthase | Lysine biosynthetic process |
| hsa-mir-557 | ABCC12 | HITS-CLIP | ATP binding | Transmembrane transport |
| hsa-mir-557 | ACSL4 | PAR-CLIP | ATP binding | Fatty acid transport |
| hsa-mir-557 | ACSS3 | PAR-CLIP | Acyl-coA synthetase | Unknown |
| hsa-mir-557 | ACTB | PAR-CLIP | ATP and protein binding | Cell movement, cell junction |
| hsa-mir-557 | ADAM19 | HITS-CLIP | SH3 domain binding | Extracellular matrix organization |
| hsa-mir-557 | AKAP1 | PAR-CLIP | RNA and protein binding | Regulation of protein kinase A signaling |
| hsa-mir-557 | AKAP10 | PAR-CLIP | Protein kinase A binding | Regulation of protein kinase A signaling |
| hsa-mir-557 | AKR7A2 | PAR-CLIP | Aldo-keto reductase activity | Cellular aldehyde metabolism |
| hsa-mir-557 | ANKEF1 | PAR-CLIP | Unknown | Unknown |
| hsa-mir-557 | ARHGAP1 | HITS-CLIP | GTPase activator activity | Rho protein signal transduction |
| hsa-mir-557 | ARHGEF3 | PAR-CLIP | Rho GTP exchange activity | Rho protein signal transduction |
| hsa-mir-557 | ARID1A | PAR-CLIP | DNA binding | ATP-dependent chromatin remodeling |
| hsa-mir-557 | ARPP19 | PAR-CLIP | Phosphatase inhibitor activity | Cell division, cell cycle |
| hsa-mir-557 | BACH1 | PAR-CLIP | DNA binding | Regulation of transcription |
| hsa-mir-557 | BACH2 | HITS-CLIP | DNA binding | Regulation of transcription |
| hsa-mir-557 | BCL7A | PAR-CLIP | Protein binding | Regulation of transcription |
| hsa-mir-557 | BID | HITS-CLIP | Death receptor binding | Apoptosis |

| | | | | |
|---|---|---|---|---|
| hsa-mir-557 | BOD1 | HITS-CLIP | Protein binding | Cell cycle, cell division |
| hsa-mir-557 | BRD4 | HITS-CLIP | Chromatin binding | Positive regulation of mitotic cell cycle |
| hsa-mir-557 | BUB1 | PAR-CLIP | ATP binding | Apoptosis, cell division, and proliferation |
| hsa-mir-557 | C4orf26 | HITS-CLIP | Protein binding | Positive regulation of enamel mineralization |
| hsa-mir-557 | CALM1 | PAR-CLIP | Adenylate cyclase binding | G2/M transition of mitotic cell cycle |
| hsa-mir-557 | CALM2 | PAR-CLIP | Adenylate cyclase binding | G2/M transition of mitotic cell cycle |
| hsa-mir-557 | CAND1 | PAR-CLIP | Protein binding | Cell differentiation, iron homeostasis |
| hsa-mir-557 | CBX4 | HITS-CLIP | SUMO and chromatin binding | Chromatin modification, Apoptosis |
| hsa-mir-557 | CCDC39 | HITS-CLIP | Protein binding | Dynein complex assembly |
| hsa-mir-557 | CCNT2 | PAR-CLIP | Protein kinase activity | Cell cycle, cell division |
| hsa-mir-557 | CENPK | HITS-CLIP | Protein binding | Mitosis progression |
| hsa-mir-557 | CERK | HITS-CLIP | Ceramide kinase activity | Ceramide metabolism |
| hsa-mir-557 | CJAD2 | HITS-CLIP | Protein binding | Angiogenesis, proliferation |
| hsa-mir-557 | CLOCK | HITS-CLIP | DNA and chromatin binding | Circadian regulation of gene expression |
| hsa-mir-557 | CNBP | PAR-CLIP | RNA and DNA binding | Cell proliferation |
| hsa-mir-557 | COL9A2 | HITS-CLIP | Protein binding | Extracellular matrix organization |
| hsa-mir-557 | COLEC10 | PAR-CLIP | Chemoattractant activity | Complement activation, chemotaxis |
| hsa-mir-557 | CRIPT | HITS-CLIP | Microtubule binding | Microtubule organization |
| hsa-mir-557 | CTNS | HITS-CLIP | L-cystine transporter activity | L-cystine transport |
| hsa-mir-557 | DAZAP2 | PAR-CLIP | Protein binding | WW domain binding |
| hsa-mir-557 | DNAJC15 | HITS-CLIP | Protein binding | Mitochondrial electron transport |

| | | | | |
|---|---|---|---|---|
| hsa-mir-557 | DUSP14 | PAR-CLIP | phosphatase activity | Inactivation of MAPK activity |
| hsa-mir-557 | DYM | HITS-CLIP | Protein binding | Golgi organization |
| hsa-mir-557 | EDN1 | HITS-CLIP | Endothelin receptor binding | Smooth muscle contraction |
| hsa-mir-557 | EI24 | HITS-CLIP | Protein binding | Macroautophagy, apoptosis |
| hsa-mir-557 | EMC1 | HITS-CLIP | Protein binding | ER protein folding |
| hsa-mir-557 | ETS1 | HITS-CLIP | DNA binding | Angiogenesis, cell motility |
| hsa-mir-557 | EXPH5 | HITS-CLIP | Rab GTPase binding | Intracellular protein transport |
| hsa-mir-557 | FAM102B | PAR-CLIP | Unknown | Unknown |
| hsa-mir-557 | FAM46C | PAR-CLIP | Unknown | Unknown |
| hsa-mir-557 | FAM9C | HITS-CLIP | Protein binding | Cell cycle |
| hsa-mir-557 | FER | HITS-CLIP | EGF receptor binding | Cell proliferation, adhesion, differentiation |
| hsa-mir-557 | FKBP15 | HITS-CLIP | Actin binding | Endocytosis |
| hsa-mir-557 | FNBP1L | PAR-CLIP | GTPase binding | Autophagy, endocytosis |
| hsa-mir-557 | FNIP2 | HITS-CLIP | ATPase inhibitor activity | Apoptotic signaling pathway |
| hsa-mir-557 | FRK | PAR-CLIP | ATP binding | Cell differentiation and migration |
| hsa-mir-557 | GABBR2 | HITS-CLIP | GABA receptor activity | GABA signaling pathway |
| hsa-mir-557 | GDNF | PAR-CLIP | Growth factor activity | MAPK cascade, apoptosis |
| hsa-mir-557 | GM2A | PAR-CLIP | Lipid transporter activity | Lipid transport |
| hsa-mir-557 | GOLPH3 | HITS-CLIP | Enzyme binding | Golgi organization |
| hsa-mir-557 | GPATCH8 | HITS-CLIP | RNA and protein binding | Unknown |
| hsa-mir-557 | GPC4 | PAR-CLIP | Proteoglycan binding | Wnt signaling, cell proliferation |

| | | | | |
|---|---|---|---|---|
| hsa-mir-557 | GSTM4 | PAR-CLIP | Glutathione binding | Glutathione biosynthesis |
| hsa-mir-557 | GTF2E2 | PAR-CLIP | DNA and RNA binding | Regulation of transcription |
| hsa-mir-557 | GTF2H1 | HITS-CLIP | DNA-dependent ATPase | DNA repair, mRNA capping |
| hsa-mir-557 | GUCD1 | PAR-CLIP | Unknown | Unknown |
| hsa-mir-557 | HBS1L | HITS-CLIP | GTPase activity | Protein translation |
| hsa-mir-557 | HCN4 | PAR-CLIP | Voltage-gated $K^+$ channel | $K^+$ transmembrane transport |
| hsa-mir-557 | HMGB1 | HITS-CLIP | Chemokine binding, DNA binding | DNA repair, apoptosis |
| hsa-mir-557 | HNRNPA3 | HITS-CLIP | RNA binding | RNA splicing |
| hsa-mir-557 | HNRNPF | PAR-CLIP | RNA binding | RNA splicing |
| hsa-mir-557 | HSD17B12 | HITS-CLIP | CoA reductase activity | Estrogen biosynthesis |
| hsa-mir-557 | IMP4 | HITS-CLIP | Protein binding | rRNA processing |
| hsa-mir-557 | IMPAD1 | HITS-CLIP | Phosphatase activity | Chondrocyte development |
| hsa-mir-557 | JAZF1 | HITS-CLIP | Transcription repressor | Lipid metabolic process |
| hsa-mir-557 | KANK4 | HITS-CLIP | Unknown | Unknown |
| hsa-mir-557 | KBTBD13 | HITS-CLIP | Ubiquitin transferase | Protein ubiquitination |
| hsa-mir-557 | KCNIP3 | HITS-CLIP | DNA binding | Apoptosis |
| hsa-mir-557 | KCNJ6 | HITS-CLIP | $K^+$ channel activity | $K^+$ transport |
| hsa-mir-557 | KIAA0408 | PAR-CLIP | Protein binding | Unknown |
| hsa-mir-557 | KLF7 | HITS-CLIP | Transcription co-activator | Axon guidance |
| hsa-mir-557 | LIMS1 | PAR-CLIP | Protein kinase binding | Cell junction assembly |
| hsa-mir-557 | LRRC40 | HITS-CLIP | Protein binding | Unknown |

| | | | | |
|---|---|---|---|---|
| hsa-mir-557 | LRRC55 | HITS-CLIP | Ion channel binding | Axonogenesis |
| hsa-mir-557 | MAPK8 | PAR-CLIP | JUN kinase activity | JNK cascade |
| hsa-mir-557 | MLLT6 | HITS-CLIP | Metal ion binding | Regulation of transcription |
| hsa-mir-557 | MMAB | HITS-CLIP | Cobalamin binding | Cobalamin biosynthesis |
| hsa-mir-557 | MRPS10 | HITS-CLIP | Protein binding | Mitochondrial protein translation |
| hsa-mir-557 | MYO10 | PAR-CLIP | Actin filament binding | Regulation of cell shape and adhesion |
| hsa-mir-557 | NUDT3 | PAR-CLIP | Hexaphosphatase activity | Diadenosine hexaphosphate catabolism |
| hsa-mir-557 | NWD1 | PAR-CLIP | ATP and RNA binding | RNA splicing |
| hsa-mir-557 | ONECUT3 | HITS-CLIP | DNA binding | Cell differentiation |
| hsa-mir-557 | P2RY1 | HITS-CLIP | A1 adenosine receptor binding | Purinergic receptor signaling pathway |
| hsa-mir-557 | PAQR3 | HITS-CLIP | Receptor activity | Negative regulation of MAP kinase activity |
| hsa-mir-557 | PAQR8 | HITS-CLIP | Protein binding | Steroid hormone receptor activity |
| hsa-mir-557 | PARN | HITS-CLIP | RNA binding | RNA modification |
| hsa-mir-557 | PARP15 | HITS-CLIP | Transcription repressor activity | Negative regulation of transcription |
| hsa-mir-557 | PER2 | HITS-CLIP | Transcription co-activator activity | Circardian regulation of gene expression |
| hsa-mir-557 | PEX26 | HITS-CLIP | ATPase binding | Protein import into the peroxisome |
| hsa-mir-557 | PGM3 | HITS-CLIP | Acetylglucosamine mutase activity | Carbohydrate metabolism |
| hsa-mir-557 | PITPNC1 | PAR-CLIP | Phosphatidylinositol binding | Phospholipid transport |
| hsa-mir-557 | PLIN1 | HITS-CLIP | Lipid binding | Lipid catabolic process |
| hsa-mir-557 | POGK | PAR-CLIP | DNA and protein binding | Regulation of transcription |
| hsa-mir-557 | POTED | PAR-CLIP | Protein binding | Apoptotic process |

| | | | | |
|---|---|---|---|---|
| hsa-mir-557 | PRRC2B | HITS-CLIP | RNA and protein binding | Cell differentiation |
| hsa-mir-557 | PTCD2 | HITS-CLIP | RNA binding | mRNA processing |
| hsa-mir-557 | PTPRM | HITS-CLIP | Protein tyrosine phosphatase | Endothelial cell migration and proliferation |
| hsa-mir-557 | PURB | PAR-CLIP | Single strand DNA binding | Cell differentiation and proliferation |
| hsa-mir-557 | RAB10 | PAR-CLIP | GTPase activity | Golgi to plasma membrane transport |
| hsa-mir-557 | RABGEF1 | PAR-CLIP | Rab GTPase binding | Endocytosis |
| hsa-mir-557 | RAET1E | HITS-CLIP | Lectin-like receptor binding | T cell mediated cytotoxicity |
| hsa-mir-557 | RAPH1 | PAR-CLIP | Protein binding | Axon extension |
| hsa-mir-557 | RBM47 | HITS-CLIP | RNA binding | Cytidine to uridine editing |
| hsa-mir-557 | RDH13 | PAR-CLIP | Retinol dehydrogenase activity | Eye phosphoreceptor development |
| hsa-mir-557 | REEP1 | HITS-CLIP | Microtubule binding | ER tubular network organization |
| hsa-mir-557 | REL | HITS-CLIP | DNA, protein and chromatin binding | NFκB signaling |
| hsa-mir-557 | RLIM | PAR-CLIP | Transcription corepressor activity | Negative regulation of transcription |
| hsa-mir-557 | RPL30 | PAR-CLIP | RNA and protein binding | Cytoplasmic translation |
| hsa-mir-557 | RPLP0 | PAR-CLIP | RNA and protein binding | Cytoplasmic translation |
| hsa-mir-557 | RRP7A | PAR-CLIP | RNA and protein binding | rRNA processing |
| hsa-mir-557 | RYBP | HITS-CLIP | DNA and protein binding | Positive regulation of apoptotic process |
| hsa-mir-557 | SATB1 | PAR-CLIP | DNA and protein binding | CD4+ cell differentiation |
| hsa-mir-557 | SBNO1 | HITS-CLIP | DNA binding | Notch signaling |
| hsa-mir-557 | SCML2 | HITS-CLIP | DNA binding | Anatomical structure morphogenesis |
| hsa-mir-557 | SEMA6A | PAR-CLIP | Protein binding | Regulation of neuron migration |

| | | | | |
|---|---|---|---|---|
| hsa-mir-557 | SESN3 | PAR-CLIP | Oxidoreductase activity | TORC2 signaling |
| hsa-mir-557 | SF3A1 | HITS-CLIP | RNA and protein binding | mRNA processing |
| hsa-mir-557 | SKI | PAR-CLIP | SMAD binding | BMP signaling pathway |
| hsa-mir-557 | SLBP | PAR-CLIP | Histone pre-mRNA binding | Histone mRNA processing |
| hsa-mir-557 | SLC30A10 | HITS-CLIP | $Mn^{+}$ transport activity | EGF receptor signaling, $Mn^{+}$ Transport |
| hsa-mir-557 | SLC43A2 | HITS-CLIP | Amino acid transport activity | L-Amino acid membrane transport |
| hsa-mir-557 | SLC8A1 | PAR-CLIP | Ankyrin binding, $Ca^{2+}$ binding | $Ca^{2+}$ ion homeostasis |
| hsa-mir-557 | SLFN12L | PAR-CLIP | ATP binding | Integral component of cell membrane |
| hsa-mir-557 | SMU1 | HITS-CLIP | RNA binding | mRNA processing |
| hsa-mir-557 | SNED1 | HITS-CLIP | $Ca^{2+}$ binding | Cell-matrix adhesion |
| hsa-mir-557 | SPIN4 | HITS-CLIP | Protein binding | Cell survival and proliferation |
| hsa-mir-557 | SPRY1 | PAR-CLIP | Protein binding | Negative regulation of MAPK signaling |
| hsa-mir-557 | ST8SIA3 | HITS-CLIP | Sialytransferase activity | Ganglioside biosynthetic process |
| hsa-mir-557 | STARD3NL | HITS-CLIP | Cholesterol binding | Steroid hormone biosynthetic process |
| hsa-mir-557 | STRN3 | PAR-CLIP | Calmodulin binding | Negative regulation of estrogen signaling |
| hsa-mir-557 | TADA3 | HITS-CLIP | DNA binding transcription | Positive regulator of gene expression |
| hsa-mir-557 | TBXA2R | HITS-CLIP | Thromboxane A2 receptor activity | Positive regulation of angiogenesis |
| hsa-mir-557 | TMEM218 | HITS-CLIP | Protein binding | Unknown |
| hsa-mir-557 | TMEM59 | HITS-CLIP | Endopeptidase activity | Positive regulation of autophagy |
| hsa-mir-557 | UBE2Z | HITS-CLIP | Ubiquitin ligase activity | Apoptosis |
| hsa-mir-557 | UBTF | HITS-CLIP | RNA binding | Positive regulation of RNA transcription |

| | | | | |
|---|---|---|---|---|
| hsa-mir-557 | UNC5D | HITS-CLIP | Receptor binding activity | Apoptosis, neuron differentiation |
| hsa-mir-557 | UNKL | PAR-CLIP | Ubiquitin-protein transferase | Protein polyubiquitination |
| hsa-mir-557 | USP6NL | PAR-CLIP | Rab GTPase binding | Regulation of Golgi organization |
| hsa-mir-557 | WISP1 | HITS-CLIP | IGF binding, integrin binding | Wnt signaling, regulation of cell growth |
| hsa-mir-557 | XIAP | HITS-CLIP | Endopeptidase inhibitor activity | Negative regulator of apoptosis |
| hsa-mir-557 | YIPF6 | PAR-CLIP | Protein binding | Epithelial cell development |
| hsa-mir-557 | ZBTB5 | PAR-CLIP | Transcriptional repressor | Negative regulation of transcription |
| hsa-mir-557 | ZDHHC7 | HITS-CLIP | Palmitoyltransferase activity | Protein palmitoylation |
| hsa-mir-557 | ZFHX3 | HITS-CLIP | DNA binding transcription | Myogenic and neural differentiation |
| hsa-mir-557 | ZFP62 | PAR-CLIP | DNA binding transcription | Myogenic differentiation |
| hsa-mir-557 | ZNF154 | PAR-CLIP | DNA binding transcription | Unknown |
| hsa-mir-557 | ZNF226 | PAR-CLIP | DNA binding transcription | Unknown |
| hsa-mir-557 | ZNF25 | HITS-CLIP | DNA binding transcription | Osteoclast differentiation |
| hsa-mir-557 | ZNF410 | PAR-CLIP | DNA binding transcription | Unknown |
| hsa-mir-557 | ZNF562 | HITS-CLIP | DNA binding transcription | Unknown |
| hsa-mir-557 | ZNF701 | PAR-CLIP | DNA binding transcription | Unknown |
| hsa-mir-557 | ZNF703 | PAR-CLIP | Repressing transcription activity | Cell migration and differentiation |
| hsa-mir-557 | ZNF813 | PAR-CLIP | DNA binding transcription | Unknown |
| hsa-mir-557 | ZNRF2 | PAR-CLIP | Ubiquitin-protein ligase activity | Protein polyubiquitination |
| hsa-mir-557 | ZNF525 | PAR-CLIP | DNA binding transcription | Unknown |

HITS-CLIP: High-Throughput Sequencing of RNA isolated by CrossLinking and ImmunoPrecipitation; PAR-CLIP:

Photoactivatable Ribonucleoside-enhanced CrossLinking and ImmunoPrecipitation; CLASH: CrossLinking, Ligation and Sequencing of Hybrids.

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**Supplementary table 4: miRNet identification of miR-575 target genes**

| ID | Gene | Method | Function | Process |
|---|---|---|---|---|
| hsa-mir-575 | ABHD14B | PAR-CLIP | Hydrolase activity | Phosphosulfate metabolic process |
| hsa-mir-575 | ADRBK1 | PAR-CLIP | G-protein coupled receptor activity | Heart development |
| hsa-mir-575 | AKNA | PAR-CLIP | DNA binding transcription | Positive regulation of transcription |
| hsa-mir-575 | ALG14 | PAR-CLIP | N-Acetylglucosaminyltransferase | Oligosaccharide biosynthetic process |
| hsa-mir-575 | API5 | HITS-CLIP | RNA and protein binding | Apoptotic process |
| hsa-mir-575 | ATAD5 | PAR-CLIP | ATP binding | Regulation of cell cycle |
| hsa-mir-575 | ATP2A2 | HITS-CLIP | ATPase activity | $Ca^{2+}$ uptake by the SR |
| hsa-mir-575 | BBX | HITS-CLIP | DNA binding | Regulation of bond development |
| hsa-mir-575 | BCL2L1 | PAR-CLIP | BH3 domain binding | Apoptotic process, cell proliferation |
| hsa-mir-575 | BLOC1S3 | HITS-CLIP | Protein binding | Synaptic and axonal transport |
| hsa-mir-575 | BRMS1L | HITS-CLIP | Histone deacetylase binding | Histone acetylation, cell growth |
| hsa-mir-575 | BTG2 | PAR-CLIP | Transcription repressor activity | DNA repair, cell proliferation, apoptosis |
| hsa-mir-575 | C17orf85 | PAR-CLIP | RNA binding | 7-methylguanosine mRNA capping |
| hsa-mir-575 | C19orf52 | HITS-CLIP | Protein binding | Protein transport into mitochondria |
| hsa-mir-575 | C20orf144 | HITS-CLIP | Unknown | Unknown |
| hsa-mir-575 | CCDC69 | HITS-CLIP | Protein binding | Spindle midzone assembly |
| hsa-mir-575 | CCNA2 | PAR-CLIP | Protein kinase binding | Cell cycle G1/S phase transition |
| hsa-mir-575 | CCS | HITS-CLIP | $Cu^{2+}$ binding | Positive regulation of oxidoreductase activity |
| hsa-mir-575 | CD300LG | HITS-CLIP | Protein binding | Regulation of immune response |

| | | | | |
|---|---|---|---|---|
| hsa-mir-575 | CDC45 | Microarray | 3’-5’ DNA helicase activity | DNA replication, G1/S cell cycle transition |
| hsa-mir-575 | CHCHD4 | HITS-CLIP | Protein binding | Protein import into mitochondria |
| hsa-mir-575 | CHST6 | PAR-CLIP | Sulfotransferase activity | Keratan sulfate biosynthesis |
| hsa-mir-575 | CLPP | PAR-CLIP | Peptidase activity | Protein homo-oligomerization |
| hsa-mir-575 | CMTM4 | PAR-CLIP | Cytokine activity | Chemotaxis |
| hsa-mir-575 | CNKSR3 | PAR-CLIP | Protein binding | Regulation of ERK1/2 cascade |
| hsa-mir-575 | CNNM4 | PAR-CLIP | $Mg^{2+}$ transport activity | $Mg^{2+}$ homeostasis |
| hsa-mir-575 | COX15 | PAR-CLIP | Protein binding | Cytochrome-c oxidase activity |
| hsa-mir-575 | CRCP | PAR-CLIP | Protein and nucleotide binding | Neuropeptide signaling pathway |
| hsa-mir-575 | DCAF16 | HITS-CLIP | Protein binding | Protein ubiquitination |
| hsa-mir-575 | DCTN3 | HITS-CLIP | Protein binding | Cytokinesis, mitotic cell cycle |
| hsa-mir-575 | DGKH | PAR-CLIP | ATP binding | Phosphatidic acid metabolism |
| hsa-mir-575 | DHX33 | PAR-CLIP | ATP and RNA binding | Embroygenesis, cell growth |
| hsa-mir-575 | DUSP2 | HITS-CLIP | Phosphatase activity | Negative regulation of MAPK signaling |
| hsa-mir-575 | DYNAP | PAR-CLIP | Protein binding | Activation of AKT/Protein kinase B activity |
| hsa-mir-575 | FAM118A | HITS-CLIP | Unknown | Unknown |
| hsa-mir-575 | GRWD1 | PAR-CLIP | DNA and RNA binding | Nucleosome assembly |
| hsa-mir-575 | HIAT1 | HITS-CLIP | Protein binding | Transmembrane transport |
| hsa-mir-575 | ICA1L | HITS-CLIP | Protein binding | Spermatid development |
| hsa-mir-575 | IFNLR1 | HITS-CLIP | Cytokine receptor activity | Interferon signaling |
| hsa-mir-575 | ITPRIPL2 | PAR-CLIP | Protein binding | IP3 receptor signaling |

| | | | | |
|---|---|---|---|---|
| hsa-mir-575 | KCNIP2 | PAR-CLIP | $K^+$ channel activity | Cardiac conduction |
| hsa-mir-575 | KIAA1467 | HITS-CLIP | Unknown | Unknown |
| hsa-mir-575 | KIF1C | HITS-CLIP | Microtubule motor activity | Cytoskeleton-dependent transport |
| hsa-mir-575 | KLF6 | PAR-CLIP | Transcriptional activity | B cell differentiation |
| hsa-mir-575 | KREMEN1 | HITS-CLIP | Protein binding | Wnt signaling, apoptotic process |
| hsa-mir-575 | LIX1L | HITS-CLIP | Protein binding | Autophagosome maturation |
| hsa-mir-575 | LSG1 | HITS-CLIP | GTPase activity | Nuclear export, ribosome biogenesis |
| hsa-mir-575 | LSP1 | PAR-CLIP | Actin binding | Cell movement, signal transduction |
| hsa-mir-575 | MAVS | PAR-CLIP | CARD domain binding | Activation of innate immune responses |
| hsa-mir-575 | MEAF6 | HITS-CLIP | Protein binding | Histone acetylation |
| hsa-mir-575 | MED28 | HITS-CLIP | Actin binding | Negative regulation of differentiation |
| hsa-mir-575 | MEF2D | PAR-CLIP | DNA binding transcription activity | Apoptotic process, heart development |
| hsa-mir-575 | MKLN1 | HITS-CLIP | Protein binding | Actin cytoskeleton reorganization |
| hsa-mir-575 | MRPS23 | PAR-CLIP | RNA and protein binding | Mitochondrial translational elongation |
| hsa-mir-575 | MSRB3 | HITS-CLIP | Methionine sulfoxide reductase | Oxidation-reduction process |
| hsa-mir-575 | MXD1 | HITS-CLIP | DNA binding transcription activity | Cell proliferation, organism development |
| hsa-mir-575 | MYO10 | HITS-CLIP | Motor Activity, actin binding | Intracellular transport, cell-cell adhesion |
| hsa-mir-575 | MYOZ2 | HITS-CLIP | Actin binding, telethonin binding | Myofibril assembly |
| hsa-mir-575 | NCAPG | Microarray | Protein binding | Cell division, mitotic chromosome condensation |
| hsa-mir-575 | NOLC1 | PAR-CLIP | ATP and GTP binding | Cell cycle, tRNA processing |
| hsa-mir-575 | ORAI2 | PAR-CLIP | $Ca^{2+}$ channel activity | $Ca^{2+}$ transmembrane transport |

| | | | | |
|---|---|---|---|---|
| hsa-mir-575 | OTUD5 | HITS-CLIP | Deubiquitinase activity | Protein K48- and K63-linked deubiquitination |
| hsa-mir-575 | PITX3 | PAR-CLIP | DNA binding transcription activity | Lens fiber differentiation |
| hsa-mir-575 | PLCG2 | HITS-CLIP | Phospholipase C activity | B cell differentiation |
| hsa-mir-575 | POLD3 | PAR-CLIP | DNA-directed DNA polymerase | Telomere maintenance |
| hsa-mir-575 | POLR2F | PAR-CLIP | DNA binding | mRNA capping, mRNA splicing |
| hsa-mir-575 | PPP1R3B | PAR-CLIP | Phosphatase activity | Glycogen metabolism |
| hsa-mir-575 | PRKX | HITS-CLIP | Protein kinase activity | Endothelial cell migration and proliferation |
| hsa-mir-575 | PTCD2 | HITS-CLIP | RNA binding | mRNA processing, mitochondrion organization |
| hsa-mir-575 | PTPRF | PAR-CLIP | Tyrosine phosphatase activity | Cell adhesion and migration |
| hsa-mir-575 | QPCTL | HITS-CLIP | Cyclotransferase activity | Golgi function |
| hsa-mir-575 | RAD51 | HITS-CLIP | ATP and chromatin binding | DNA recombination |
| hsa-mir-575 | RBFOX2 | PAR-CLIP | mRNA and protein binding | mRNA processing |
| hsa-mir-575 | RBM23 | PAR-CLIP | RNA and protein binding | mRNA processing |
| hsa-mir-575 | RDH10 | HITS-CLIP | NADP-retinol dehydrogenase | Embryonic organ morphogenesis |
| hsa-mir-575 | SHOC2 | PAR-CLIP | Protein phosphatase binding | RA protein signal transduction |
| hsa-mir-575 | SHROOM4 | PAR-CLIP | Actin filament binding | Actin cytoskeleton organization |
| hsa-mir-575 | SIK2 | PAR-CLIP | ATP binding | Intracellular signal transduction |
| hsa-mir-575 | SLC25A32 | HITS-CLIP | FAD transmembrane transporter | Folic acid import into mitochondrion |
| hsa-mir-575 | SLC43A1 | HITS-CLIP | Protein binding | Amino acid transport |
| hsa-mir-575 | SMG1 | HITS-CLIP | ATP and RNA binding | DNA repair, mRNA export from nucleus |
| hsa-mir-575 | SMIM12 | HITS-CLIP | Unknown | Unknown |

| | | | | |
|---|---|---|---|---|
| hsa-mir-575 | SMU1 | HITS-CLIP | Protein binding | mRNA processing |
| hsa-mir-575 | SNAPIN | PAR-CLIP | SNARE binding | Anterograde axonal transport |
| hsa-mir-575 | SNRNP48 | HITS-CLIP | Metal ion biding | RNA splicing |
| hsa-mir-575 | SNRPD3 | PAR-CLIP | RNA binding | RNA splicing |
| hsa-mir-575 | SOWAHC | PAR-CLIP | Unknown | Unknown |
| hsa-mir-575 | SYNPO2L | PAR-CLIP | Actin binding | Regulation of actin filament assembly |
| hsa-mir-575 | SZRD1 | PAR-CLIP | Unknown | Unknown |
| hsa-mir-575 | TRAF3IP2 | PAR-CLIP | Receptor binding | Positive regulation of NFκB signaling |
| hsa-mir-575 | TSG101 | PAR-CLIP | Ubiquitin binding | Autophagosome maturation, cell division |
| hsa-mir-575 | TTC22 | PAR-CLIP | Chaperon activity | Protein folding |
| hsa-mir-575 | UBN2 | HITS-CLIP | Unknown | Unknown |
| hsa-mir-575 | UBXN2A | PAR-CLIP | Protein phosphatase activity | Autophagosome assembly |
| hsa-mir-575 | UEVLD | HITS-CLIP | Protein binding | Oxidoreductase activity |
| hsa-mir-575 | USP1 | HITS-CLIP | Endopeptidase activity | Ubiquitin protein deubiquitination |
| hsa-mir-575 | VPS36 | PAR-CLIP | Ubiquitin binding | Autophagy, endosomal transport |
| hsa-mir-575 | WDR92 | HITS-CLIP | Protein binding | Apoptotic process |
| hsa-mir-575 | WIPI2 | PAR-CLIP | Protein binding | Autophagosome assembly |
| hsa-mir-575 | XIAP | HITS-CLIP | Endopeptidase inhibitor activity | Negative regulator of apoptosis |
| hsa-mir-575 | XPNPEP3 | HITS-CLIP | Peptidase activity | Cilia function |
| hsa-mir-575 | ZC3H12A | PAR-CLIP | DNA and RNA binding | Apoptosis process, cell differentiation |
| hsa-mir-575 | ZNF212 | PAR-CLIP | DNA binding transcription activity | Regulation of transcription |

| | | | | |
|---|---|---|---|---|
| hsa-mir-575 | ZNF329 | PAR-CLIP | DNA binding transcription activity | Regulation of transcription |
| hsa-mir-575 | ZNF516 | HITS-CLIP | DNA binding transcription activity | Apoptosis, cell proliferation, differentiation |
| hsa-mir-575 | ZNF556 | HITS-CLIP | DNA binding transcription activity | Regulation of transcription |
| hsa-mir-575 | ZNF677 | HITS-CLIP | DNA binding transcription activity | Regulation of transcription |
| hsa-mir-575 | ZNF680 | PAR-CLIP | DNA binding transcription activity | Regulation of transcription |
| hsa-mir-575 | ZRANB3 | PAR-CLIP | ATP binding | DNA repair |

HITS-CLIP: High-Throughput Sequencing of RNA isolated by CrossLinking and ImmunoPrecipitation; PAR-CLIP: Photoactivatable Ribonucleoside-enhanced CrossLinking and ImmunoPrecipitation; CLASH: CrossLinking, Ligation and Sequencing of Hybrids.

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(3) Xue Y, Ouyang K, Huang J et al. Direct conversion of fibroblasts to neurons by reprogramming PTB-regulated microRNA circuits. *Cell* 2013 January 17;152(1-2):82-96.

(4) Lipchina I, Elkabetz Y, Hafner M et al. Genome-wide identification of microRNA targets in human ES cells reveals a role for miR-302 in modulating BMP response. *Genes Dev* 2011 October 15;25(20):2173-86.

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**Supplementary table 5: miRNet identification of miR-1287-5p target genes**

| ID | Gene | Method | Function | Process |
|---|---|---|---|---|
| hsa-mir-1287-5p | AGBL5 | HITS-CLIP | Metallocarboypeptidase activity | Protein deglutamylation |
| hsa-mir-1287-5p | BSCL2 | PAR-CLIP | Protein binding | Fat cell differentiation |
| hsa-mir-1287-5p | C4orf32 | PAR-CLIP | Unknown | Unknown |
| hsa-mir-1287-5p | CLCN6 | PAR-CLIP | Antiporter activity | Chloride transport |
| hsa-mir-1287-5p | DNAJC10 | PAR-CLIP | Chaperon binding | Apoptotic process, cell redox homeostasis |
| hsa-mir-1287-5p | DSN1 | PAR-CLIP | Protein binding | Cell division |
| hsa-mir-1287-5p | FAM89A | PAR-CLIP | Unknown | Unknown |
| hsa-mir-1287-5p | FHDC1 | PAR-CLIP | Protein binding | Golgi ribbon formation |
| hsa-mir-1287-5p | GAS7 | PAR-CLIP | DNA binding transcription activity | Cell cycle, cell differentiation |
| hsa-mir-1287-5p | GDI1 | PAR-CLIP | GDP-dissociation inhibitor activity | Rab protein signal transduction |
| hsa-mir-1287-5p | GNG12 | PAR-CLIP | PDZ domain binding | G-protein coupled receptor signaling |
| hsa-mir-1287-5p | GPR137C | PAR-CLIP | Protein binding | G-protein coupled receptor signaling |
| hsa-mir-1287-5p | GRK7 | PAR-CLIP | ATP binding | Rhodopsin mediated signaling |
| hsa-mir-1287-5p | H2AFX | PAR-CLIP | DNA binding | DNA damage checkpoint |
| hsa-mir-1287-5p | HIST1H2BD | PAR-CLIP | DNA binding | Nucleosome assembly |
| hsa-mir-1287-5p | ITPA | HITS-CLIP | Diphosphatase activity | Chromosome organization |
| hsa-mir-1287-5p | LAMTOR3 | PAR-CLIP | Kinase activator activity | Activation of MAPKK activity |
| hsa-mir-1287-5p | LCOR | HITS-CLIP | DNA binding | Negative regulation of transcription |
| hsa-mir-1287-5p | LITAF | PAR-CLIP | Transcriptional activator activity | Positive regulation of NFκB signaling |

| | | | | |
|---|---|---|---|---|
| hsa-mir-1287-5p | LONP2 | HITS-CLIP | Peptidase activity | Peroxisome organization |
| hsa-mir-1287-5p | MAP3K9 | PAR-CLIP | JUN kinase kinase kinase activity | Activation of JUN kinase activity |
| hsa-mir-1287-5p | MED16 | PAR-CLIP | Thyroid hormone receptor binding | Androgen receptor signaling pathway |
| hsa-mir-1287-5p | MFAP2 | PAR-CLIP | Protein binding | Embryonic eye morphogenesis |
| hsa-mir-1287-5p | MYCN | PAR-CLIP | DNA binding transcription activity | Embryonic organ development, apoptosis |
| hsa-mir-1287-5p | MYO1C | PAR-CLIP | Actin binding | Microfilament motor activity |
| hsa-mir-1287-5p | NDRG1 | PAR-CLIP | Rab GTPase binding | Regulation of cell proliferation, apoptosis |
| hsa-mir-1287-5p | NME6 | HITS-CLIP | Nucleoside diphosphate kinase | Apoptotic process, mitotic nuclear division |
| hsa-mir-1287-5p | PARVB | HITS-CLIP | Actin binding | Actin cytoskeleton reorganization |
| hsa-mir-1287-5p | PLEKHA2 | HITS-CLIP | PDZ domain binding | Regulation of cell-matrix adhesion |
| hsa-mir-1287-5p | PNMA2 | PAR-CLIP | Protein binding | Apoptotic process |
| hsa-mir-1287-5p | POTED | PAR-CLIP | Protein binding | Apoptotic process |
| hsa-mir-1287-5p | PSMD7 | PAR-CLIP | Protein binding | Protein polyubiquitination |
| hsa-mir-1287-5p | PTEN | PAR-CLIP | Phosphatase activity | Apoptosis, cell proliferation, and migration |
| hsa-mir-1287-5p | RAB32 | PAR-CLIP | GTP binding | Phagosome maturation |
| hsa-mir-1287-5p | RFX7 | PAR-CLIP | DNA binding transcription activity | Regulation of transcription |
| hsa-mir-1287-5p | ROBO1 | HITS-CLIP | Axon guidance receptor activity | Axon guidance, cell adhesion |
| hsa-mir-1287-5p | SHISA9 | PAR-CLIP | PDZ domain binding | Regulation of AMPA receptor activity |
| hsa-mir-1287-5p | SOD2 | PAR-CLIP | Superoxide dismutase activity | Oxidation-reduction process, apoptosis |
| hsa-mir-1287-5p | SOX17 | HITS-CLIP | Transcription co-factor activity | Angiogenesis, Wnt signaling |
| hsa-mir-1287-5p | SULT1B1 | PAR-CLIP | Aryl sulfotransferase activity | Phosphosulfate metabolic process |

| | | | | |
|---|---|---|---|---|
| hsa-mir-1287-5p | TNFAIP8L1 | PAR-CLIP | Unknown | Unknown |
| hsa-mir-1287-5p | TVP23B | PAR-CLIP | Protein binding | Vesicle-mediated transport |
| hsa-mir-1287-5p | TVP23C | PAR-CLIP | Protein binding | Vesicle-mediated transport |
| hsa-mir-1287-5p | TXK | PAR-CLIP | ATP binding | T cell receptor signaling pathway |
| hsa-mir-1287-5p | UBB | CLASH | Protein binding | Cell proliferation, MAPK signaling |
| hsa-mir-1287-5p | UFSP2 | CLASH | UFM1 hydrolase activity | zProteolysis |
| hsa-mir-1287-5p | UTP18 | HITS-CLIP | RNA binding | rRNA processing |
| hsa-mir-1287-5p | VIM | PAR-CLIP | RNA and protein binding | Intermediate filament organization |
| hsa-mir-1287-5p | ZNF790 | HITS-CLIP | DNA binding transcription activity | Regulation of transcription |

HITS-CLIP: High-Throughput Sequencing of RNA isolated by CrossLinking and ImmunoPrecipitation; PAR-CLIP: Photoactivatable Ribonucleoside-enhanced CrossLinking and ImmunoPrecipitation; CLASH: CrossLinking, Ligation and Sequencing of Hybrids.

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**Supplementary table 6: Targets genes of miR-1468-5p identified by miRNet**

| ID | Gene | Method | Function | Process |
|---|---|---|---|---|
| hsa-mir-1468-5p | BRIX1 | PAR-CLIP | RNA binding | Ribosomal large subunit assembly |
| hsa-mir-1468-5p | C12orf73 | PAR-CLIP | Unknown | Unknown |
| hsa-mir-1468-5p | DERL2 | PAR-CLIP | Protein binding | ER unfolded protein resopnse |
| hsa-mir-1468-5p | LYRM7 | HITS-CLIP | Protein binding | Mitochondrial complex III assembly |
| hsa-mir-1468-5p | MMAB | HITS-CLIP | Cobalamin binding | Cobalamin biosynthesis |
| hsa-mir-1468-5p | MTRNR2L3 | PAR-CLIP | Unknown | Unknown |
| hsa-mir-1468-5p | MTRNR2L6 | PAR-CLIP | Unknown | Unknown |
| hsa-mir-1468-5p | MTRNR2L8 | PAR-CLIP | Unknown | Unknown |
| hsa-mir-1468-5p | PLXDC1 | HITS-CLIP | Protein binding | Angiogenesis |
| hsa-mir-1468-5p | PSORS1C2 | HITS-CLIP | Unknown | Unknown |
| hsa-mir-1468-5p | RPL27A | HITS-CLIP | RNA & protein binding | rRNA processing, translation |
| hsa-mir-1468-5p | SATB2 | PAR-CLIP | Chromatin binding | Chromatin remodeling |
| hsa-mir-1468-5p | SEMA3D | PAR-CLIP | Neuropilin binding | Cell differentiation |
| hsa-mir-1468-5p | SRSF2 | PAR-CLIP | Pre-mRNA binding | RNA splicing |
| hsa-mir-1468-5p | SYK | HITS-CLIP | ATP & protein binding | Angiogenesis |
| hsa-mir-1468-5p | MTRNR2L9 | PAR-CLIP | Unknown | Unknown |

HITS-CLIP: High-Throughput Sequencing of RNA isolated by CrossLinking and ImmunoPrecipitation; PAR-CLIP: Photoactivatable Ribonucleoside-enhanced CrossLinking and ImmunoPrecipitation; CLASH: Crosslinking, Ligation and Sequencing of Hybrids.

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(3) Gottwein E, Corcoran DL, Mukherjee N et al. Viral microRNA targetome of KSHV-infected primary effusion lymphoma cell lines. *Cell Host Microbe* 2011 November 17;10(5):515-26.

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# Chapter III: Role of miR-1233-3p in Angiopoietin-1-induced angiogenesis

## 1. Preface

Recent studies have confirmed that miRNAs play important roles in the regulation of angiogenesis. Pro-angiogenic miRNAs enhance neovascularization by inhibiting the expression of anti-angiogenic proteins while anti-angiogenic miRNAs inhibit angiogenesis by targeting pro-angiogenic factors like VEGF.

MicroRNA 1233-3p is a poorly characterized miRNA that resides in one of the introns of GOLGA8A and GOLGA8B, two highly similar genes located on chromosome 15. In addition of the previously identified miRNAs in chapter II, miR-1233-3p was also identified as being significantly downregulated in ECs in response to 24 h exposure to Ang-1. The functional roles of miR-1233-3p in angiogenesis are unknown. In this chapter, we hypothesized that miR-1233-3p is an anti-angiogenic miRNA and that Ang-1 downregulates its expression in ECs. We also hypothesized that downregulation of this miRNA may be important for the pro-angiogenic effects of the Ang-1/Tie2 axis.

# Role of miR-1233-3p in Angiopoietin-1-Induced Angiogenesis

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## 2. Abstract

Angiopoietin-1 (Ang-1) and its receptor Tie-2 promote vascular integrity and angiogenesis. microRNAs (miRNAs) are involved in the regulation of many cellular functions, including endothelial cell (EC) survival, proliferation, and differentiation. Several reports indicate that these regulatory effects mediate EC functions through modulation of angiogenesis factor signaling, including that of vascular endothelial growth factor (VEGF). To date, little is known about the roles played by miRNAs in the signaling and angiogenesis promoted by the Ang-1/Tie-2 receptor axis. Through high-throughput screening of miRNAs regulated by Ang-1 exposure in human umbilical vein endothelial cells (HUVECs), miR-1233-3p was identified as a mature miRNA whose cellular levels are significantly downregulated in response to Ang-1 exposure. Expression of miR-1233-3p in these cells was downregulated by other angiogenesis factors, including vascular endothelial growth factor (VEGF), fibroblast growth factor 2 (FGF-2), transforming growth factor β (TGFβ), and angiopoietin-2 (Ang-2). Overexpression of miR-1233-3p in HUVECs using mimics significantly attenuated cell survival, migration, and capillary-like tube formation, and promoted apoptosis. Moreover, miR-1233-3p overexpression resulted in reversal of the anti-apoptotic, pro-migration and pro-differentiation effects of Ang-1. Biotinylated miRNA pull-down assays identified p53 and DNA damage regulated 1 (PDRG1) as a direct target of miR-1233-3p in HUVECs. Exposure of HUVECs to Ang-1, Ang-2, FGF2, VEGF, or TGFβ triggers regulation of PDRG1 expression. This study highlights the role of miR-1233-3p in the regulation of Ang-1/Tie-2 signaling and angiogenesis in ECs.

**Keywords:** pre-miR-1233, pri-miR-1233, miR-1233-3p, Angiopoietin 1, migration, survival, proliferation, apoptosis, microRNA pull-down, endothelial cells, VEGF, FGF-2, TGFβ.

## 3. Introduction

Vascular development is a complex multi-step process controlled by a number of secreted vascular growth factors, such as those belonging to the family of angiopoietins [335]. The angiopoietins, of which Ang-1 and Ang-2 are best characterized, signal through the receptor tyrosine kinase, Tie-2. Ang-1 is the primary stimulatory ligand of Tie-2. Upon binding, autophosphorylation of this receptor is triggered, leading to recruitment and activation of the phosphatidylinositol-3-kinase (PI3K) and the mitogen activated protein kinase (MAPK) pathways [106, 336]. During embryonic development, Ang-1 promotes vascular development. *Ang-1*$^{-/-}$mice exhibit embryonic lethality after E9.5 and display decreased branching, increased dilation, reduced vascular complexity, and loss of heart trabeculation [89, 337, 338]. In adult vasculature, Ang-1 enhances endothelial cell (EC) integrity and survival [140] and is required for correct organization and maturation of new vessels. It also promotes quiescence and structural integrity by inhibiting apoptosis and vascular leakage [339].

EC dysfunction contributes to several pathologies, including atherosclerosis, hypertension, and diabetes. Cardiovascular diseases remain a great cause of morbidity and mortality, despite the advances in several kinds of treatment [340]. Previously believed to be “transcription noise”, non-coding RNAs (nc-RNA) compose 98% of the mammalian transcriptome, and are now considered to be crucial players in disease development [180, 341]. MicroRNAs (miRNAs), a small class of endogenous nc-RNAs, have recently been recognized as key regulators of several fundamental processes in the vasculature, including angiogenesis. miRNAs are transcribed from the genome as a primary transcript, pri-miRNA, which is then cleaved by a microprocessing complex into

a ~70nt long hair-pin loop-containing transcript (pre-miRNA) [251, 252]. The pre-miRNA is then exported to the cytoplasm, where it is cleaved near the loop [190], resulting in the production of a small ~22 bp double stranded molecule (mature miRNA) which is loaded into one of the Argonaute proteins to form an effector complex called RNA-induced silencing complex (RISC) [282]. The duplex is then unwound into two single stranded miRNAs (guide and passenger strands). The passenger strand gets degraded while the guide binds to its target mRNA, inhibiting its translation.

Several studies have shown that significant alterations in miRNA expression profile are associated with various vascular diseases [342, 343]. Gain- and loss-of-function studies have uncovered significant roles for specific miRNAs in regulating angiogenic processes, such as EC survival, extracellular matrix production, and hypoxia [257, 344, 345]. The roles of miRNAs in Ang-1-induced angiogenesis, however, remain largely unexplored. We hypothesized that the Ang-1/Tie-2 axis promotes angiogenesis through upregulation of a specific set of pro-angiogenic miRNAs and downregulation of a specific set of anti-angiogenic miRNAs. In this study, we focused on the regulation of miR-1233-3p by Ang-1 and its effects on angiogenesis. Our pilot results, using high throughput screening of miRNA expressions in HUVECs exposed to Ang-1 for 12 to 48 h, revealed that Ang-1 significantly decreases miR-1233-3p expression and that miR-1233-3p overexpression inhibits Ang-1-induced EC survival, migration, and capillary-like tube formation. In addition, we identified PDRG1 as a direct target of miR-1233-3p, whose role in angiogenesis has not been previously reported.

## 4. **Material and methods**

All experiments were conducted at least in triplicate.

**Materials**: Recombinant human Ang-1 and Ang-2 were purchased from R&D Systems (Minneapolis, MN). Vascular endothelial growth factor (VEGF), fibroblast growth factor 2 (FGF-2), and transforming growth factor beta (TGFβ) were purchased from BioShop Canada, Inc. (Burlington, ON). Antibodies for PDRG1 (cat #SAB4503242), β-ACTIN, and β-TUBULIN were purchased from Sigma-Aldrich (St. Louis, MO). HUVECs were purchased from Lonza (Basel, Switzerland). PDRG1 expression vector was purchased from OriGene (Rockville, MD, RC203661). Control and miR-1233-3p mimics (mirVana® miRNA mimics 4464066) and inhibitors (mirVana® miRNA inhibitors 4464084) were purchased from Ambion (Austin, TX).

**Cell culture**: HUVECs were grown in MCDB131 medium (Life Technologies, Rockville, MD) supplemented with 20% fetal bovine serum (FBS), EC growth supplement, 2 mM glutamine, heparin, and gentamicin sulfate (Invitrogen, Carlsbad, CA). This medium was designated as complete medium. Cells were incubated at 37°C and 5% $CO_2$. Beas2B cells (ATCC, Manassas, VA) were cultured in DMEM:F12 (Life Technologies, Rockville, MD, USA), 10%FBS, and gentamicin sulfate at 37° C and 5% $CO_2$. Ramos cells (ATCC, Manassas, VA) were incubated in RPMI-1640 (Life Technologies, Rockville, MD) supplemented with 10% FBS and gentamycin sulfate.

**Growth factor treatment:** HUVECs were seeded (30,000 cells per $cm^2$) in complete medium. The medium was then changed to MCDB131 medium without supplements, FBS, or antibiotics (basal medium). After 6 h, 2% FBS and aliquots of phosphate buffered saline (PBS, control condition), Ang-1 (300 ng/ml), Ang-2 (300 ng/ml), FGF-2 (10 ng/ml), VEGF (40 ng/ml), or TGF-β (2 ng/ml) were added to the culture medium and cells were collected at different times.

**miRNA abundance**: HUVECs were lysed and tRNA extracted using a Qiagen miRNA extraction kit. Reverse transcription (RT) was performed using an AB TaqMan® miRNA RT kit specific to miR-1233-3p and miR-126-5p. Droplet digital PCR was performed using QuantaStudio3D with 1 µl of cDNA and analyzed using QuantStudio™ 3D AnalysisSuite™ software.

**miRNA extraction and quantitative real-time PCR**: Cell were lysed with Quiazol® to extract total RNA. A miRNeasy Mini Kit was used according to manufacturer's protocols to purify miRNAs. RT-PCR was performed using a NCode™ miRNA amplification system (Invitrogen) and specific primers (**Supplementary table 1**), Platinum SYBR® (Invitrogen), and a 7500 Real-Time PCR System. TaqMan® assays (Applied Biosystems, Foster City, CA) were used to detect Pri-miR-1233. All experiments were performed at least in triplicate. miRNA expression was determined by the $C_T$ method, where $C_T$ values of individual miRNA data were normalized to $C_T$ values of U6 snRNA, as previously described [346]. miRNA-specific amplification was confirmed by a single peak in the melting-curve analysis.

**mRNA extraction and quantitative real-time PCR**: Total RNA was extracted using an Ambion PureLink RNA mini kit according to the manufacturer's protocol. Gene expression was detected using specific primers (**Supplementary table 1**), Power SYBR® (Invitrogen), and a 7500 Real-Time PCR System (Applied Biosystems). β-ACTIN was used as a housekeeping gene. All experiments were performed at least in triplicate. Relative mRNA expression was determined using the comparative threshold ($C_T$) method, as previously described [346]. For miRNA pull-down assays, GAPDH was used as a housekeeping gene.

**Tie-2 blocking**: HUVECs were seeded (30,000 cells per $cm^2$) and were pre-treated for 1 h with 50 μg/ml of neutralizing Tie-2 IgG or control IgG antibodies. Cells were then incubated in basal culture medium supplemented with 2% FBS and aliquots of PBS or Ang-1 (300ng/ml). Cells were collected 24 h later.

**Extracellular vesicle extraction:** HUVECs were seeded (30,000 cells per $cm^2$) and were maintained in basal medium without FBS for 6 h. Cells were then maintained for 24 h in basal medium containing aliquots of PBS or Ang-1 (300ng/ml). Medium was then collected, and EVs were extracted using a miRCURY™ Exosome Isolation Kit (Exiqon, Vedbaek, Denmark), according to the manufacturer's instructions. miRNAs from isolated EVs were extracted as described above.

**miRNA mimic and inhibitor transfection:** HUVECs were transfected at a confluence of 50-70% using 25 nM of control or miR-1233-3p mimic (mirVana®, 4464066), or 50 nM of control or miR-1233-3p inhibitor (mirVana®, 4464084). Mimics and inhibitors were transfected using Lipofectamine™ RNAiMAX (Invitrogen) according to the manufacturers' instructions. A PDRG1 expression plasmid was transfected into HUVECs (10 μg of plasmid per 2 million cells) using jetPRIME™ Polyplus transfection reagent (Illkirch, France) according to manufacturer's protocol. Experiments were performed 48 h post-transfection.

**Cell counting:** miRNA mimic- or inhibitor-transfected HUVECs were seeded (30,000 cells per $cm^2$) and maintained in complete, basal, or basal MCDB131 medium containing Ang-1 (300 ng/ml). Cells were counted 24 h later using a hemocytometer.

**Caspase-3 activity:** miRNA mimic- or inhibitor-transfected cells were plated in 12-well plates and maintained in complete, basal, or basal MCDB131 medium containing Ang-1 (300 ng/ml). Caspase-3 activity was measured 24 h later using an EnzChek® Caspase-3 Assay Kit with Z-DEVD-AMC as a substrate (Molecular Probes, Eugene, OR).

**Cell migration:** A scratch (wound) healing assay was used to assess cell migration. In brief, HUVEC monolayers were wounded using a 200 µl pipette tip and maintained for 8 h in basal MCDB131 medium containing 2% FBS and aliquots of PBS (control) or Ang-1 (300 ng/ml). Wounded areas were visualized using an Olympus inverted microscope, quantified using Image-Pro Plus™ software (Media Cybernetics, Bethesda, MD), and reported using the following formula: % wound healing = (1 – [wound area at t8 h/wound area at t0]) x 100, where t0 is the time immediately following wounding.

**Capillary-like tube formation:** miRNA mimic- or inhibitor-transfected cells were seeded onto 96-well plates pre-coated with growth factor-reduced Matrigel® (12,500 cells per well) and maintained for 24 h in Lonza EGM™-2 medium (minus VEGF) containing aliquots of PBS (control) or Ang-1 (300 ng/ml). Whole-well images were captured using an Olympus inverted microscope. Angiogenic tube formation was determined by measuring total tube length and average tube length using the Angiogenesis Analyzer macro with Fiji/ImageJ [347].

**Proliferation:** miRNA mimic- or inhibitor-transfected cells were plated in 96-well plates at 7,000 cells per well and maintained in complete Lonza EGM™ medium (minus VEGF). One hour after plating, a bromodeoxyuridine (BrdU) (Millipore, Etobicoke, ON) assay was performed according to the manufacturer's instructions. BrdU absorbance was measured 24 h later.

**Immunoblotting:** Cells were lysed using RIPA buffer (Santa Cruz Biotechnology, Dallas, TX). Denatured proteins were separated using SDS-polyacrylamide gel electrophoresis (PAGE) and electro-transferred onto polyvinylidene difluoride (PVDF) membranes (Bio-Rad Laboratories, Richmond, CA). Membranes were blocked with 5% (w/v) low-fat milk for 1 h at room temperature and probed with the primary antibody at 4°C overnight. After washing, membranes were incubated for 1 h at room temperature with horseradish-peroxidase-conjugated secondary antibodies (Jackson ImmunoResearch, Newmarket, UK) or Alexa Fluor® Plus conjugated secondary antibodies (Thermo Fisher Scientific, Waltham, MA). Proteins were detected using Pierce™ enhanced chemiluminescence reagents (Thermo Fisher) in the case of horseradish-peroxidase antibodies. Band intensities were quantified using ImageJ software.

**Biotin-labeled pull-down:** A biotin-labeled miR-1233-3p mimic was designed according to Wang et al. and Cloonan et al., and obtained from IDT (Skokie, IL) [348, 349]. Pull-down with target mRNAs was performed as described earlier, with some modifications [349, 350]. Briefly, HUVECs were transfected with 50 nM of biotinylated-control mimic or biotinylated-miR-1233-3p mimic. Forty-eight hours later, cells were lysed with a hypotonic lysis buffer (100 mM KCl, 5 mM $MgCl_2$, 20 mM Tris-Cl pH 7.5, 5 mM DTT, 0.3% NP-40, 60U/mL RNase OUT (Invitrogen) and 1X Complete Mini protease inhibitor (Roche, Basel, Switzerland)). Simultaneously, magnetic streptavidin beads (Dynabeads M-280 Streptavidin, #11205D, Invitrogen) were coated with 1 µg/µl bovine serum albumin and 1 µg/µl yeast tRNA (Invitrogen), and incubated with supernatants while rotating at 4°C for 2 h. Cell debris was cleared by centrifugation

(≥10,000 g at 4°C for 15 minutes). Cleared lysates were then incubated with the pre-coated beads, rotating overnight at 4°C. Beads were then washed with hypotonic lysis buffer. RNA was then released by adding 750 µl of TRIzol (Invitrogen) and 250 µl of RNase-free water. RNA was precipitated using the standard chloroform-isopropanol method and then subjected to reverse transcription and qPCR for detection of specific transcripts.

**Statistical analysis:** Statistical analyses were performed using GraphPad® Prism 5.0 software (San Diego, CA). In experiments in which more than two groups were compared, Two-way ANOVA followed by Bonferroni post-hoc analysis was utilized. For experiments in which two groups were compared, a paired student's T-test was employed. Differences were considered statistically significant at $P<0.05$.

## 5. Results

**Ang-1 regulation of miR-1233-3p:** Figure 1 illustrates the time course of miR-1233-3p expression in HUVECs in response to Ang-1 exposure. Short (Fig 1A) and long time exposures to Ang-1 (Fig. 1B) were performed in separate experiments. Ang-1 increased miR-1233-3p expression after 4 h of exposure; however, its expression decreased after 6, 24, 48, and 72 h of exposure (Figure 1A&B). In contrast to its effect on mature miR-1233-3p levels, Ang-1 exposure had no effect on pri- and pre-miR-1233 levels (supplementary figure 1A-D). Interestingly, expression of miR-1233-5p, the sister arm of miR-1233-3p, also decreased in response to 24 and 72 h of Ang-1 exposure (supplementary figure 1E-F). These data show that Ang-1 regulates the expression of both mature forms of miR-1233 but has no effect precursor or primary transcripts of miR-1233. mirR-1233 is an intronic miRNA located within GOLGA8A and GOLGA8B,

which are two highly similar genes from the same family. To test the possibility that Ang-1 regulates miR-1233-3p through transcriptional activation of GOLGA8A, GOLGA8A mRNA expression was measured after stimulation with Ang-1 (300ng/ml). Ang-1 exposure had no effect on GOLGA8A mRNA levels, as compared to PBS exposure (Figure 1C).

Exosomes contain a variety of molecules, including miRNAs, whose levels have been reported to be significantly altered under various physiological conditions [351-353]. To evaluate whether downregulation of mature miR-1233-3p is due to its exportation from the cell, its expression level was measured in EVs isolated from HUVECs exposed to Ang-1 for 24 hours. Figure 1D shows that miR-1233-3p exosomal levels were also downregulated in response to Ang-1 exposure.

The importance of Tie-2 receptors in the inhibitory effect of Ang-1 on miR-1233-3p expression was assessed using a selective Tie-2 blocking antibody. Exposure to Ang-1 for 24 h decreased miR-1233-3p expression in the presence of the control antibody but not in the presence of the Tie-2 blocking antibody, thus confirming that Ang-1 exposure decreases miR-1233-3p levels through Tie-2 receptor signaling (Figure 1E). A mild upregulation in this miRNA expression was also noticed when Tie-2 was blocked, suggesting other regulatory signals are acting upon miR-1233-3p.

To determine the effects of other angiogenic factors on the expression of miR-1233-3p, HUVECs were exposed to Ang-2, FGF-2, VEGF, or TGFβ for 24 h. TGFβ and FGF-2 increased miR-1233-3p expression while VEGF and Ang-2 decreased it (Figure 1F).

To evaluate the abundance of miR-1233-3p in ECs, we used droplet digital PCR. In HUVECs, the most abundantly expressed vascular miRNA is miR-126-5p with ~6500 copies/µl. By comparison, miR-1233-3p had only an average of ~85 copies/µl suggesting that it is not very abundantly expressed in ECs (supplementary figure 2). We also found that miR-1233-3p is expressed in several other cell types, including human microvascular endothelial cells (HMEC-1), airway epithelial cells bearing a mutation in the *CF2* gene, monocytes (THP1), B-lymphocytes (Ramos), and lung epithelial cells (Ramos) (supplementary figure 3).

**miR-1233-3p regulation of EC survival:** Ang-1 promotes survival, migration, and differentiation and inhibits apoptosis in ECs [140]. To assess the role of miR-1233-3p in Ang-1-induced angiogenic processes, HUVECs were transfected with mimics or inhibitors and exposed to Ang-1 (300ng/ml) or PBS. miR-1233-3p mimic and inhibitor effects were verified post-transfection by measuring HOXB3 levels, since HOXB3 is a direct target of miR1233-3p (supplementary figures 4 and 5). Control- or miR-1233-3p mimic-transfected cells were maintained for 24 h in complete medium, basal medium (0% FBS), or basal medium containing an aliquot of Ang-1 (300 ng/ml). With the control mimic, cell count decreased under serum deprivation conditions (basal medium) (Figure 2A). Basal medium containing Ang-1 resulted in an increase in cell number relative to that which was observed with basal medium alone (Figure 2A). With the miR-1233-3p mimic, cell count decreased in complete medium as compared to control mimic (Figure 2A). Cell number also increased in miR-1233-3p mimic-transfected cells exposed to Ang-1; however, the absolute cell number remained significantly lower than it was with control mimic exposed to Ang-1 (Figure 2A). To investigate whether proportional

changes in the number of apoptotic cells relates to the ameliorative effects of mimics on total cell number, caspase-3 activity was measured. With the control mimic, caspase-3 activity increased under serum deprivation. The addition of Ang-1 attenuated this increase, thereby confirming that Ang-1 exerts an anti-apoptotic effect on ECs (Figure 2B). With the miR-1233-3p mimic, caspase-3 activity increased under serum deprivation to levels higher than those observed with the control mimic. Caspase-3 activity did not decrease under serum deprivation when Ang-1 was added to miR-1233-3p mimic-transfected cells (Figure 2B).

Effects of endogenous miR-1233-3p on EC survival were assessed by transfecting HUVECs with a miR-1233-3p inhibitor. With the control inhibitor, cell number decreased, and caspase-3 activity increased under serum deprivation (Figure 2C&D). With the miR-1233-3p inhibitor, cell numbers in complete medium increased, relative to the control inhibitor (Figure 2C). With the miR-1233-3p inhibitor, cell numbers decreased under serum deprivation, as compared to complete medium, but were higher than with the control inhibitor (Figure 2C). With the control inhibitor, as compared to complete medium, caspase-3 activity increased under serum deprivation; the presence of Ang-1 attenuated this response (Figure 2D). With the miR-1233-3p inhibitor, caspase-3 activity under serum deprivation increased relative to complete medium; the presence of Ang-1 attenuated this increase (Figure 2D). With the miR-1233-3 inhibitor, caspase-3 activities measured in complete medium, basal medium, and basal medium+Ang-1 were significantly lower than those measured under the same conditions with cells transfected with control inhibitor (Figure 2D). These results indicate that endogenous

levels of miR-1233-3p exert inhibitory effects on EC survival and stimulatory effects on caspase-3 activity.

**miR-1233-3p regulation of EC migration:** With miR-1233-3p mimic-transfected HUVECs in basal medium containing PBS, basal cell migration decreased relative to that observed with the control mimic (Figure 2E). Exposure to Ang-1 increased cell migration in control mimic-transfected HUVECs, but not in those transfected with miR-1233-3p mimic (Figure 2E). To evaluate whether endogenous miR-1233-3p levels regulate EC migration, a wound-healing assay was used to measure cell migration in control and inhibitor-transfected cells. miR-1233-3p inhibition increased migration in basal medium containing PBS or Ang-1, suggesting that endogenous levels of miR-1233-3p exert a negative influence on cell migration (Figure 2F).

**miR-1233-3p regulation of EC differentiation:** Capillary-like tube formation assays were used to evaluate the functional regulatory roles of miR-1233-3p on EC differentiation in cells exposed to PBS (control) or Ang-1. Relative to PBS exposure, average tube length increased by ~1.5 fold in control mimic-transfected cells exposed to Ang-1 (Figure 3A). In miR-1233-3p mimic-transfected cells, average tube length in the presence of PBS increased relative to cells transfected with control mimic (Figure 3A). In miR-1233-3p mimic-transfected cells, Ang-1 failed to increase average tube length relative to PBS (Figure 3A).

To evaluate whether endogenous miR-1233-3p levels regulate EC differentiation, tube formation was measured in control and miR-1233-3p inhibitor-transfected cells exposed to PBS (control) or Ang-1. Relative to PBS exposure, average and total tube lengths increased in control mimic-transfected cells exposed to Ang-1 (Figure 3B&C). In

miR-1233-3p inhibitor-transfected cells, the total tube length measured in the presence of PBS increased as compared to cells transfected with control inhibitor, suggesting this miRNA negatively regulates EC differentiation (Figure 3B&C). In miR-1233-3p inhibitor-transfected cells, Ang-1 failed to increase average and total tube lengths relative to PBS (Figure 3B&C). This lack of response to Ang-1 could be due to the fact that that miR-1233-3p is at a basal level in controlled condition, and addition of the inhibitor does not change the effects of Ang-1. It is also possible that other signals, perhaps stronger than Ang-1 are regulating this process.

**miR-1233-3p regulation of EC proliferation:** The effects of Ang-1 on proliferation are debatable because Ang-1 has been described as having a positive effect, a negative effect, and no effect [140]. We therefore tested the effect of miR-1233-3p on EC proliferation in the absence of Ang-1. We found that mimic-induced overexpression of miR-1233-3p had no major effects on BrdU incorporation. In comparison, inhibition of miR-1233-3p in HUVECs using a selective inhibitor did trigger an increase in BrdU incorporation, suggesting that endogenous miR-1233-3p levels exert an inhibitory effect on EC proliferation (Figure 3D&E).

**PDRG1 as a direct target miR-1233-3p:** miRNAs regulate gene expression by destabilizing mRNA and by inhibiting mRNA translation. To identify mRNAs targeted by miR-1233-3p in ECs, we used several algorithms, such as miRDB, TargetScan, and DIANA, to predict that PDRG1 is a possible direct target of miR-1233-3p (Figure 4A). To confirm this prediction, HUVECs were transfected with a miR-1233-3p mimic or inhibitor and PDRG1 expression was measured. Overexpression of miR-1233-3p reduced PDRG1 mRNA expression, while transfection with a miR-1233-3p inhibitor increased

PDRG mRNA expression (Figure 4B). Several doses of mimic and inhibitor were tested, We found that 25nM produced a stronger effect on PDRG1 expression when compared to 50nM, possibly due to toxic effects of the dosage and transfection. Similarly, PDRG1 protein levels decreased in miR-1233-3p mimic-transfected cells and increased in miR-1233-3 inhibitor-transfected cells (Figure 4C-F). According to the database DIANA, the 3'UTR region of PDRGG1 mRNA contains three miR-1233-3p binding sites, two of which are somewhat conserved across species and one of which is not conserved (Figure 5A). To confirm direct binding of miR-1233-3p to PDRG1 mRNA, HUVECs were transfected with biotinylated control or miR-1233-3p mimics then biotin-labeled pull-down assays were performed as described in the methods section. Total RNA was harvested from the pull-down and input materials, and PDRG1 mRNA levels were measured using qPCR. The efficiency of miRNA-1233p3p overexpression using biotinylated mimic was confirmed (Figure 5B&C). We found that PDRG1 mRNA levels were significantly enriched in the pull-down material of cells transfected with biotinylated miR-1233-3p as compared to cells transfected with the control mimic (Figure 5D). The mRNA levels of centromere protein B (CENPB), another predicted target of miR-1233-3p, were also enriched in the pull-down material, whereas those of zinc finger protein 91 (ZFP91, not a predicted target of miR-1233-3p), were not enriched in the pull-down (Figure 5D&E). These results, which were replicated in immortalized human microvascular ECs (HMEC-1, data not shown), confirm that miR-1233-3p directly binds to PDRG1 mRNA.

Previous studies have revealed that intronic miRNAs may regulate the expression of their own gene. For instance, it has been reported that miR-26b

transcribed from an intron of CTDSP2 directly targets and regulates the expression of CTDSP2 and that this regulation is important for neuronal differentiation during development [354]. In this study, we report that GOLGA8A mRNA expression is not affected by miR-1233-3p overexpression, suggesting that miR-1233-3p does not regulate the expression of GOLGA8A (supplementary figure 6).

**Ang-1 regulation of PDRG1 expression:** Our results indicate that Ang-1 exposure triggers significant downregulation of miR-1233-3p in ECs and that miR-1233-3p directly targets PDRG1. To assess whether PDRG1 expression is regulated by the Ang-1/Tie-2 axis, PDRG1 mRNA and protein levels in HUVECs were measured after short (2 to 12h) and long (24-72h) exposures to Ang-1. Ang-1 exposure triggered an increase in PDRG1 mRNA after 12 and 48h and PDRG1 protein levels after 24h (Figure 6A–D). Interestingly, we observed that at 24h and at 72h time points, the levels of PDRG1 were comparable to control samples. Given the lack of information about the relationship between Ang-1, and PDRG1 in angiogenesis, it is hard to address these discrepancies in upregulation of PDRG1 under these times and conditions. Nonetheless, we speculate, that other signals are acting upon PDRG1, or regulating Ang-1 dependent downstream pathways.

The evidence shows that at 24h miR1233-3p is downregulated by Ang-1 and at the same time PDRG1 get upregulated under the same conditions (Figure 1B and Figure 6 C-D). We also observed that other growth factors, including Ang-2, FGF-2 and VEGF, upregulated the expression of PDRG1 protein levels in HUVECs (Figure 6E&F). These results suggest that the Ang-1/Tie-2 axis downregulates miR-1233-3p expression so as to enhance PDRG1 expression by removing the inhibitory effect of miR-1233-3p on

PDRG1 mRNA. These effects on miR-1233-3p and PDRG1 expression are not unique to the Ang-1/Tie-2 axis, since similar results were observed in response to Ang-2 and VEGF exposure (Figures 1&6).

## 6. Discussion

The main findings of this study are the following: 1) The Ang-1/Tie-2 axis decreases miR-1233-3p expression in ECs; 2) Overexpression of miR-1233-3p is associated with significant inhibition of Ang-1-induced EC migration, proliferation, and survival; 3) Endogenous miR-1233-3p levels exert negative effects on EC survival, migration, capillary-like tube formation, and proliferation; 4) PDRG1 mRNA is a direct target of miR-1233-3p; and 5) Ang-1 upregulates PDRG1 at the mRNA and protein levels.

It has been well established that pro- and anti-angiogenic stimuli regulate angiogenesis through modulation of miRNA levels. Perhaps the most studied pro-angiogenic stimulus is VEGF, which has been found to induce, in a time-dependent manner, the expression of several angiogenic miRNAs, including miR-191, miR-17-5p, miR-18a, and miR-20a in HUVECs [1]. Treatment with epidermal growth factor (EGF) and VEGF increases the expression of miR-296a in brain ECs [2]. Moreover, VEGF-induced angiogenesis has been shown to be mediated, in part, through downregulation of miR-101 expression, which leads to enhanced expression of histone-methyltransferase EZH2 [3].

The role of miRNAs in Ang-1-induced angiogenesis remains largely unexplored. Recently, our group reported that miR-146-5p expression is upregulated by Ang-1 in ECs and that miR-146b-5p mimic transfection into HUVECs attenuates toll-like receptor 4 signaling through selective targeting of IRAK1 and TRAF6 protein expressions [21]. In the current study, we describe for the first time the importance of miR-1233-3p in the regulation of angiogenesis. We found that the expression of this poorly characterized miRNA is downregulated by Ang-1 in ECs and that this response is independent of the expression of the genes where miR-1233-3p resides, since GOLGA8A and GOLGA8B

expressions remained unchanged in response to Ang-1 exposure. We also found that the Ang-1/Tie-2 axis-mediated decrease in cellular levels of miR-1233-3p is not the result of increased exosomal export. However, we do not exclude the possibility of miR-1233-3p being exported by other secretory mechanisms like microparticles or lipoproteins, which are known to be intercellular miRNA carriers [4].

We found that exposure to Ang-2 (competitive antagonist of Ang-1) and VEGF significantly downregulated the levels of miR-1233-3p in ECs. Body of evidence suggest that Ang-1 rapidly phosphorylates Tie-2, while Ang-2 mainly inhibits Ang-1 induced Tie-2 phosphorylation [22]. Other studies however, have shown that Ang-2 when administered at high dosage can stimulate phosphorylation of Tie-2, although not at the same level of Ang-1 [23,24]. Nonetheless, this phosphorylation was enough to activate proangiogenic processes. Ang-2 activates the PI3K/Akt pathway in ECs but with less potency than Ang-1. Downregulation of miR-1233-3p by both Ang-1 and Ang-2 can be attributed to elevated treatment of Ang-2 (300ng/mL), which might have stimulated phosphorylation of Tie-2 in endothelial cells. Similar to Ang-1, VEGF triggers significant activation of the PI3K pathway in ECs which promotes cell migration. It is possible that similar to Ang-1, VEGF downregulates miR1233-3p to promote migration through the PI3K pathway. In accordance to this idea, treatment with these three growth factors (Ang-1, Ang-2 and VEGF) upregulated PDRG1 at the protein level. Taken together the data indicates that PDRG1 is a downstream pro-angiogenic molecule that is upregulated, through downregulation of miR-1233-3p, by major angiogenic growth factors (VEGF, Ang-1 and Ang-2).

Interestingly, we found that FGF-2, a relatively strong pro-angiogenic factor, significantly upregulates miR-1233-3p expression in ECs but also PDRG1. It has been established that FGF-2 modulates EC expression of VEGF through both autocrine and paracrine mechanisms and that neutralization of VEGF with a selective antibody results in inhibition of pro-angiogenic effects of FGF-2 [23]. These observations suggest that the signaling pathways through which FGF-2 regulates miR-1233-3p expression in ECs may be more complex than those mediate Ang-1, Ang-2, and VEGF effects on this expression. Given the ability of one miRNA to regulate different and unrelated processes, it is possible that FGF-2 regulation of miR-1233-3p is strongly related to another biological process, and not targeted at PDRG1 through this miRNA but via another regulatory mechanism. Future studies aimed at elucidating signaling pathways through which various angiogenesis factors regulate miR-1233-3p expression in ECs are warranted.

Our study indicates that overexpression of miR-1233-3p using mimics leads to an increase in basal Caspase-3 activity and a significant decrease in EC survival, migration, and proliferation. These results in ECs are in agreement with those published by Zhong et al. who reported that miR-1233-3p overexpression decreased proliferation and transwell chamber invasion in BeWo cells [7]. They also observed that the expression of miR-1233-3p is elevated in placental tissue of hypertensive-disorder-complicated pregnancies. In addition, a significant correlation between elevated serum miR-1233-3p levels and the development of pre-eclampsia have been described [8]. Moreover, it has been reported that significant elevation of miR-1233-3p levels are present in tissues of patients with renal cell carcinoma (RCC) [9].

Of the numerous predicted targets of miR-1233-3p, only HOXB3 has been validated, using a luciferase construct containing the 3' UTR of this mRNA [7]. HOXB3 is a member of the mammalian HOX genes located on one of the four homologous HOX loci (HOXA, B, C, D). Several reports suggest that HOX genes play a role in regulating angiogenesis [10-14]. For example, stable expression of HOXD10 inhibits angiogenesis [13]. HOXB3, in particular, is upregulated during EC differentiation, as documented in a human bone marrow-derived mesenchymal stem cell (hMSC) model [15]. Other studies have demonstrated that HOXB3 promotes cancer cell migration and progression when upregulated [16-17]. To identify miR-1233-3p targets, we utilized pull-down assays of a biotinylated miR-1233-3p miRNA mimic and identified a direct interaction between PDRG1 mRNA and miR-1233-3 in HUVECs. Like miR-1233-3p, PDRG1 is not a well characterized protein. It consists of 133 amino acids with a molecular mass of 15511 Dalton. There is a relatively high homology between human and mouse PDRG1 protein sequences [18]. PDRG1 has a helix-turn-helix-like motif at the C-terminal end, and this motif is likely to mediate protein–protein and protein–DNA interactions. PDRG1 also has a β-prefoldin-like domain. Luo et al. showed that PDRG1 is expressed predominantly in the cytoplasm [18]. Mass spectrometry analyses have identified the R2TP/prefoldin-like complex composed of prefoldin and prefoldin-like proteins URI, art-27, PFD2, PFD6 and PDRG1 [19]. Together with HSP90, the R2TP/prefoldin-like complex is responsible for the assembly of RNA polymerase II complex (pol II) in the cytoplasm of eukaryotic cells [19]. In addition, one member of this complex, unconventional prefoldin RPB5 interactor (URI), is involved in stabilizing PDRG1. Mass spectrometry analysis and immunoprecipitation studies identified a

specific interaction between PDRG1and URI in the nucleus in prostate cells [20]. It is also hypothesized that all the prefoldin and prefoldin-like proteins in this complex, such as URI, art-27, PFD2, PFD6, and PDRG1 interact with each other through the β strands of the prefoldin-like domain [20]. In accordance with previous studies, we found PDRG1 is expressed mostly in the cytoplasm, with a relatively small fraction of the total cellular PDRG1 pool being located into the nucleus.

In summary, we report here that the Ang-1/Tie-2 axis decreases miR-1233-3p expression in ECs and that miR-1233-3p exerts a negative influence on Ang-1-induced EC migration, proliferation, survival, and capillary-like tube formation. We also report that miR-1233-3p directly target PDRG1 mRNA and that the Ang-1/Tie-2 axis significantly upregulates PDRG1 expression in ECs. These findings unveil important novel roles for miR-1233 and PDRG1 in regulating Ang-1 induced angiogenesis.

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## 8. Figures

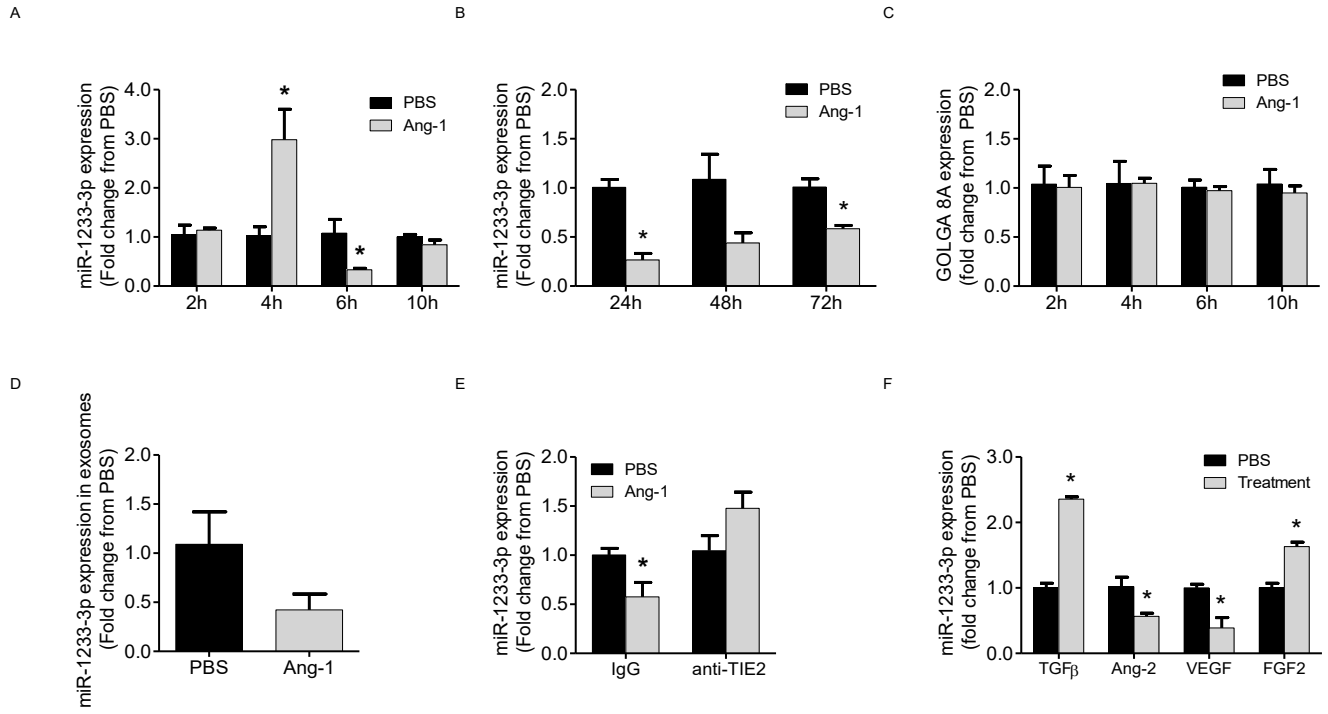



**Figure 1: Ang-1 regulates miR-1233-3p expression in HUVECs.**

**A&B:** miR-1233-3p expression in HUVECs exposed for 2, 4, 6, 10, 24, 48, or 72 h to PBS or Ang-1 (300ng/ml). **C:** GOLGA8A mRNA expression in HUVECs exposed for 2, 4, 6, and 10 h to PBS or Ang-1. **D:** miR-1233-3p expression in HUVEC exosomes exposed for 24 h to PBS or Ang-1 (300ng/ml). **E:** miR-1233-3p expression in HUVECs pre-treated with control or Tie-2 blocking antibodies and exposed for 24 h to PBS or Ang-1 (300ng/ml). **F:** miR-1233-3p expression in HUVECs exposed for 24 h to PBS or growth factors (TGFβ, Ang-2, VEGF, or FGF-2). Values are means ± SEM, presented as fold change from PBS. *P<0.05, compared to PBS.

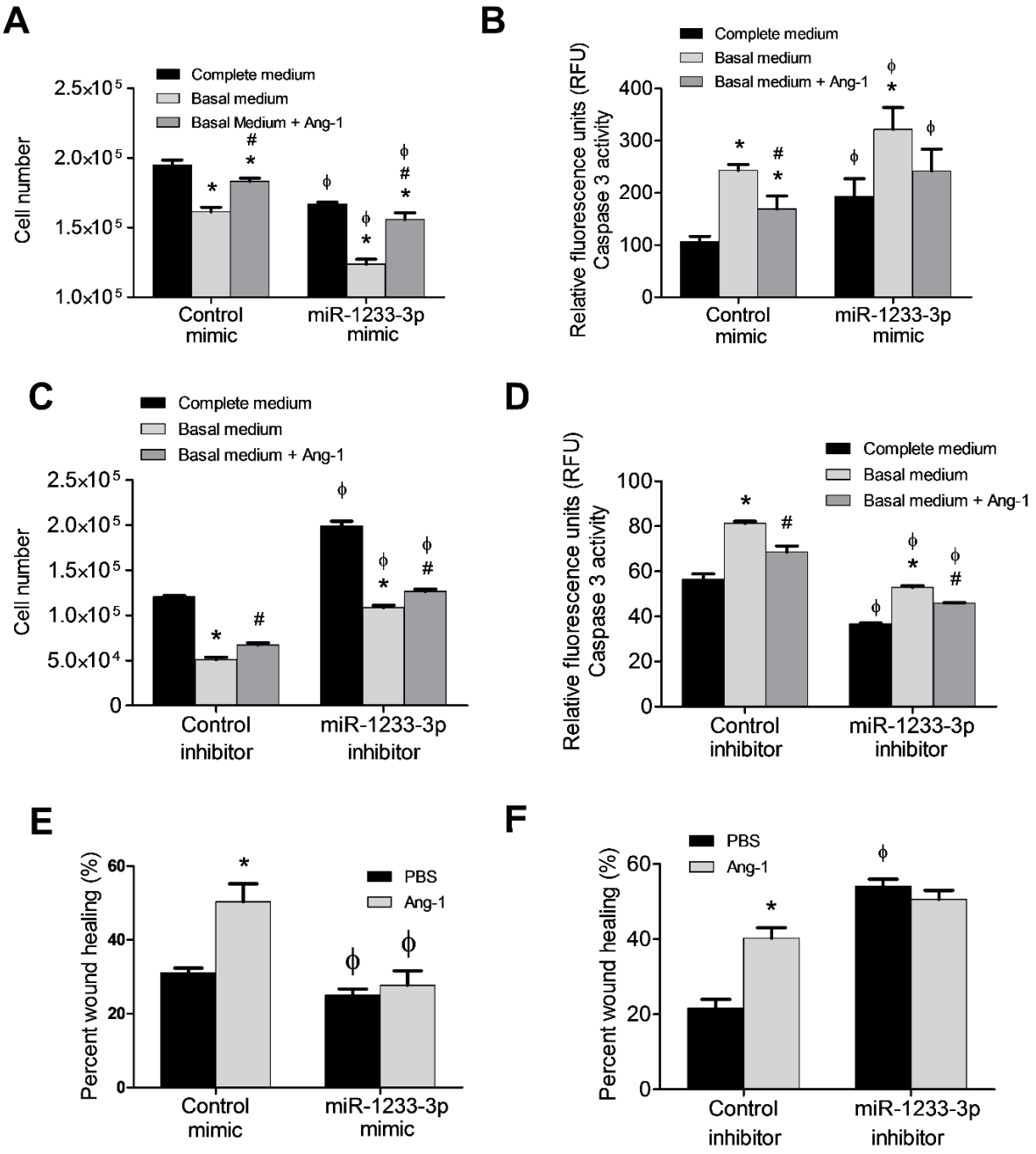



**Figure 2: miR-1233-3p regulation of EC survival and migration.**

**A&B:** HUVECs were transfected with control or miR-1233-3p mimics. Following 48 h of recovery, equal numbers of cells were maintained in complete medium, basal medium, or basal medium+Ang-1 (300ng/ml). Cell counts and Caspase-3 activities were

measured 24 h later. **C&D:** HUVECs were transfected with control or miR-1233-3p inhibitors. Following 48 h of recovery, an equal number of cells were maintained in complete medium, basal medium, or basal medium+Ang-1 (300ng/ml). Cell counts and Caspase-3 activities were measured 24 h later. **E:** Percent wound healing of HUVECs transfected with control or miR-1233-3p mimics and exposed to PBS or Ang-1 (300ng/ml) for 8 h. **F:** Percent wound healing of HUVECs transfected with control or miR-1233-3p inhibitors and exposed to PBS or Ang-1 (300ng/ml) for 8 h. Values are means ± SEM. *P<0.05, compared to PBS alone. $^{\#}$P<0.05, compared to basal medium. $^{\phi}$P<0.05, compared to cells transfected with control mimic or control inhibitor.

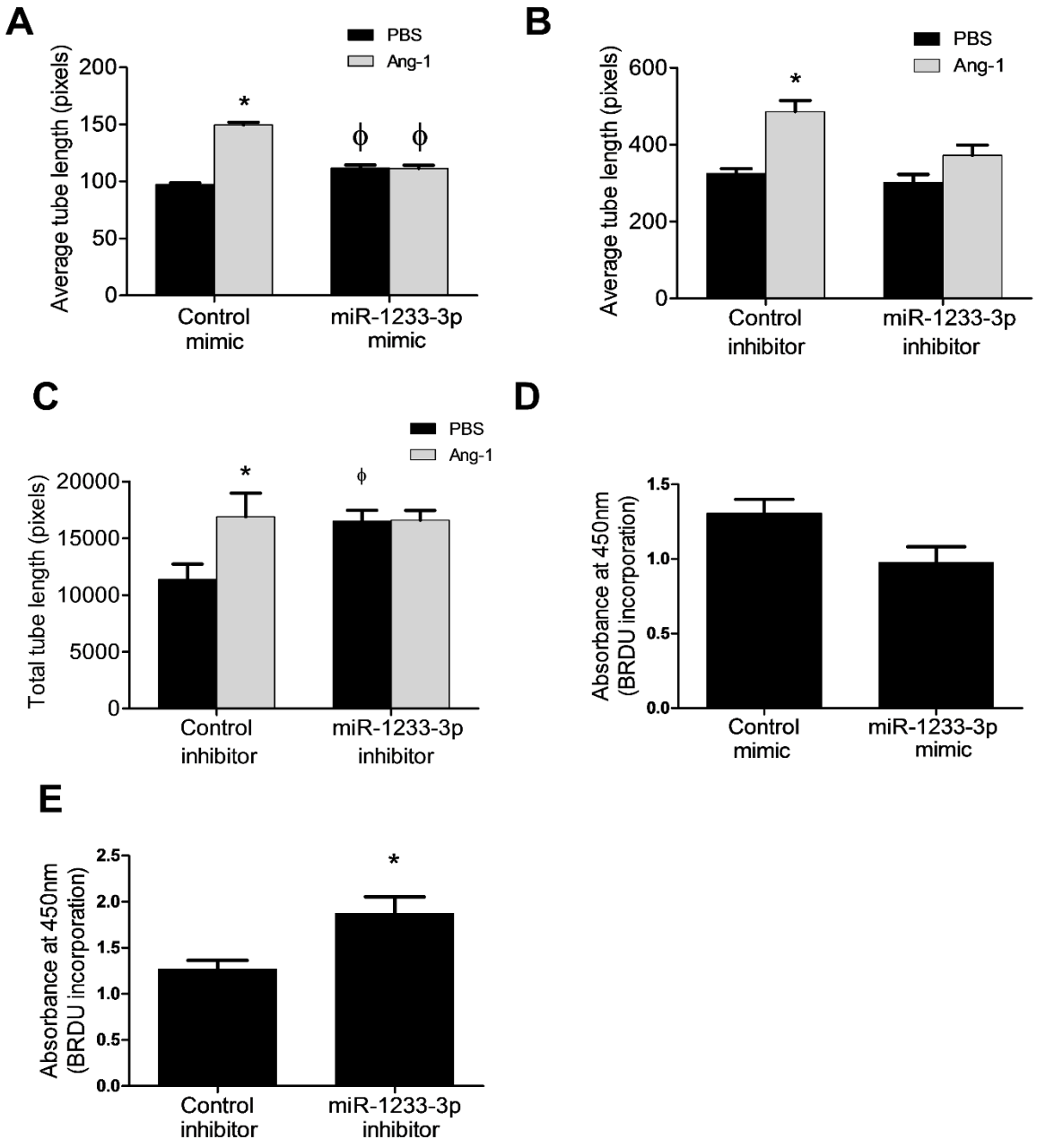



**Figure 3: miR-1233-3p regulation of EC differentiation and proliferation.**

**A–C:** Average and total tube lengths of HUVECs transfected with control and miR-1233-3p mimics or inhibitors and maintained for 24 h in Lonza EGM™-2 medium (minus VEGF) containing PBS or Ang-1. Values are means ± SEM.

**D&E:** HUVECs were transfected with control and miR-1233-3p mimics or inhibitors and maintained for 24 h in Lonza EGM™-2 medium (minus VEGF) containing PBS or Ang-1. One hour after plating, BrdU was added. BrdU absorbance was measured 24 h later. Values are means ± SEM. *P<0.05, compared to PBS alone. $^{\phi}$P<0.05, compared to cells transfected with control mimic or inhibitor.

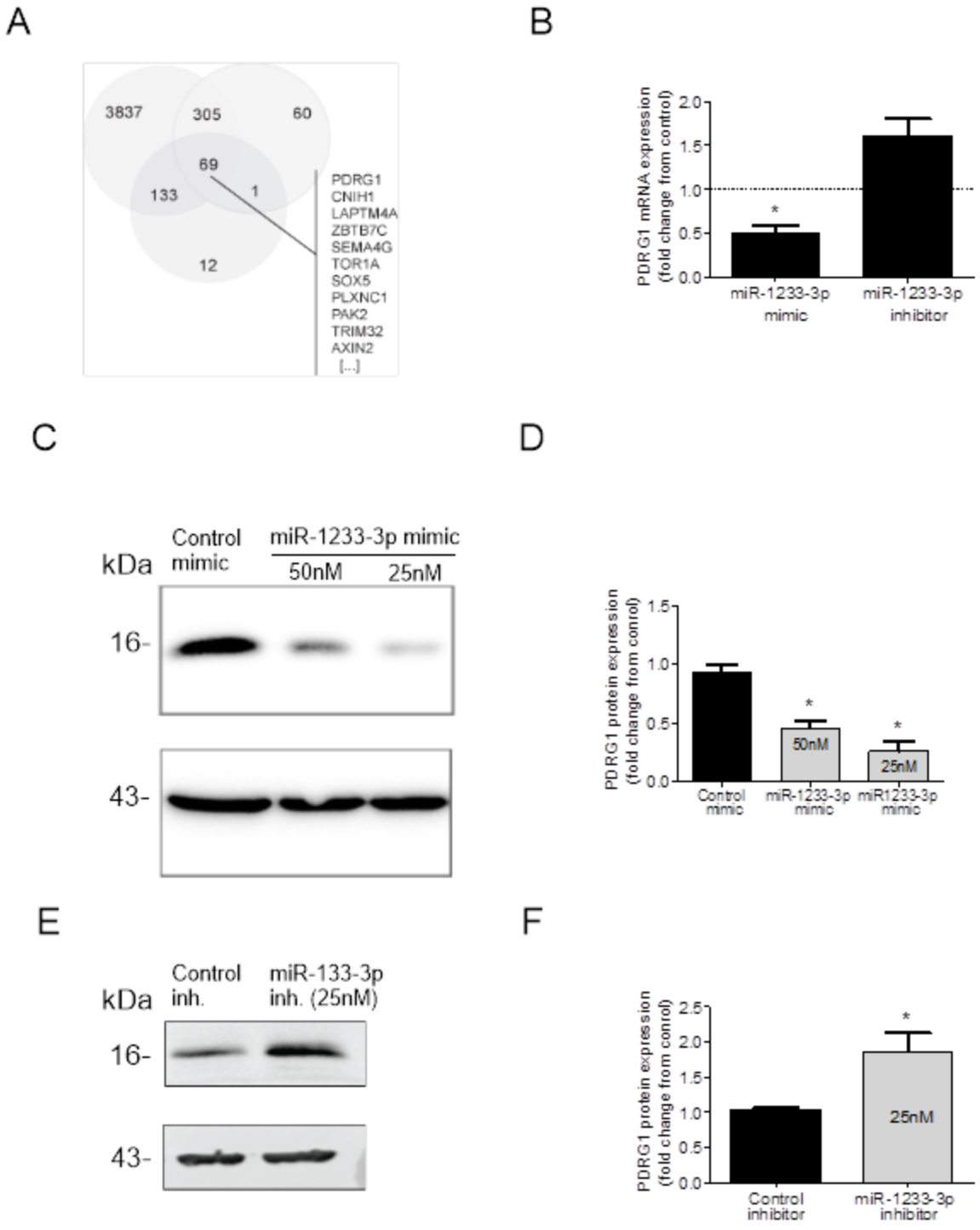



**Figure 4: Identification of PDRG1 as a target of miR-1233-3p.**

**A:** Venn diagram displaying *in-silico* predicted targets of miR1233-3p as computed by DIANA, TargetScan, and miRDB algorithms. **B:** PDRG1 mRNA expression in HUVECs transfected with miR-1233-3p mimic or inhibitor and their respective controls (denoted by dotted line). Values are presented as fold change from control mimic or inhibitor. **C&D:** Representative immunoblots of PDRG1 and β-ACTIN proteins and quantification of PDRG1 protein levels in HUVECs transfected with control or miR-1233-3p mimics. Values are means ± SEM, presented as fold change from control mimic. **E&F:** Representative immunoblots of PDRG1 and β-ACTIN proteins and quantification of PDRG1 protein levels in HUVECs transfected with control or miR-1233-3p inhibitors. Values are means ± SEM, presented as fold change from control inhibitor. *P<0.05, compared to control mimic or inhibitor.

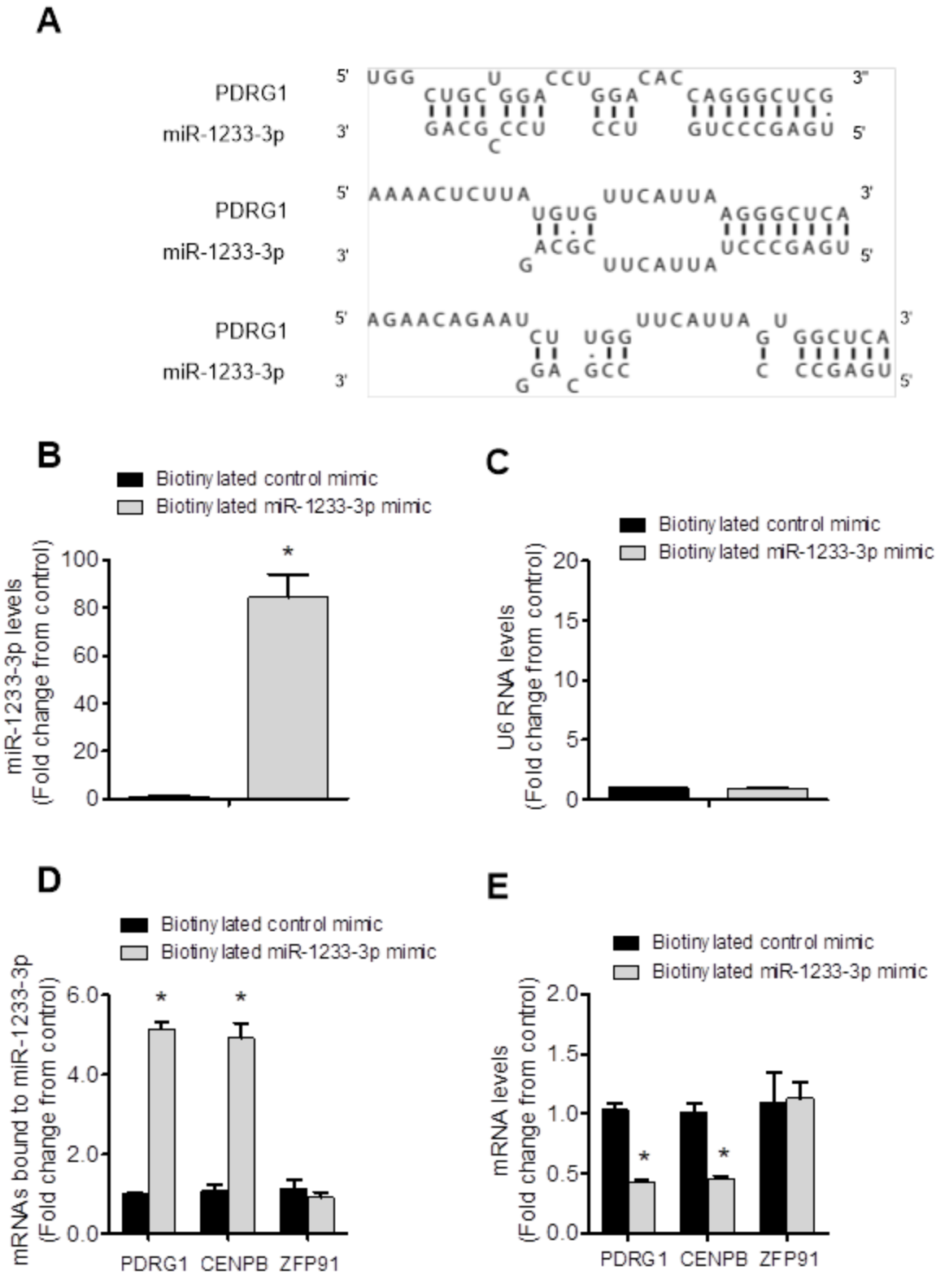

A
PDRG1
miR-1233-3p
PDRG1
miR-1233-3p
PDRG1
miR-1233-3p
B
Biotinylated control mimic
Biotinylated miR-1233-3p mimic
miR-1233-3p levels
(Fold change from control)
C
Biotinylated control mimic
Biotinylated miR-1233-3p mimic
U6 RNA levels
(Fold change from control)
D
Biotinylated control mimic
Biotinylated miR-1233-3p mimic
mRNAs bound to miR-1233-3p
(Fold change from control)
PDRG1 CENPB ZFP91
E
Biotinylated control mimic
Biotinylated miR-1233-3p mimic
mRNA levels
(Fold change from control)
PDRG1 CENPB ZFP91

**Figure 5: PDRG1 is a direct target of miR-1233-3p.**

**A:** Schematic representation of the sequence alignment of miR-1233-3p and the predicted binding sites on the 3' UTR of PDRG1 mRNA. **B–D:** Biotinylated miR-1233-3p pull-down assays. Panels B and C illustrate miR-1233-3p and U6 RNA levels in HUVECs after transfection of biotinylated control or miR-mimic (50 nM). Values presented as fold change from control mimic. Panel D shows PDRG1, HOXB3, and ZFP91 mRNA levels in pull-down materials isolated from HUVECs transfected with biotinylated control or miR-1233 mimics. Values presented as fold change from control mimic. **E:** PDRG1, HOXB3 and ZFP91 mRNA levels in total mRNA input. Enrichment calculated as follows: miR pull-down/control pull-down (X), miR input/control input (Y), fold binding = X/Y. Values are means ± SEM. *$P<0.05$, compared to biotinylated control mimic.

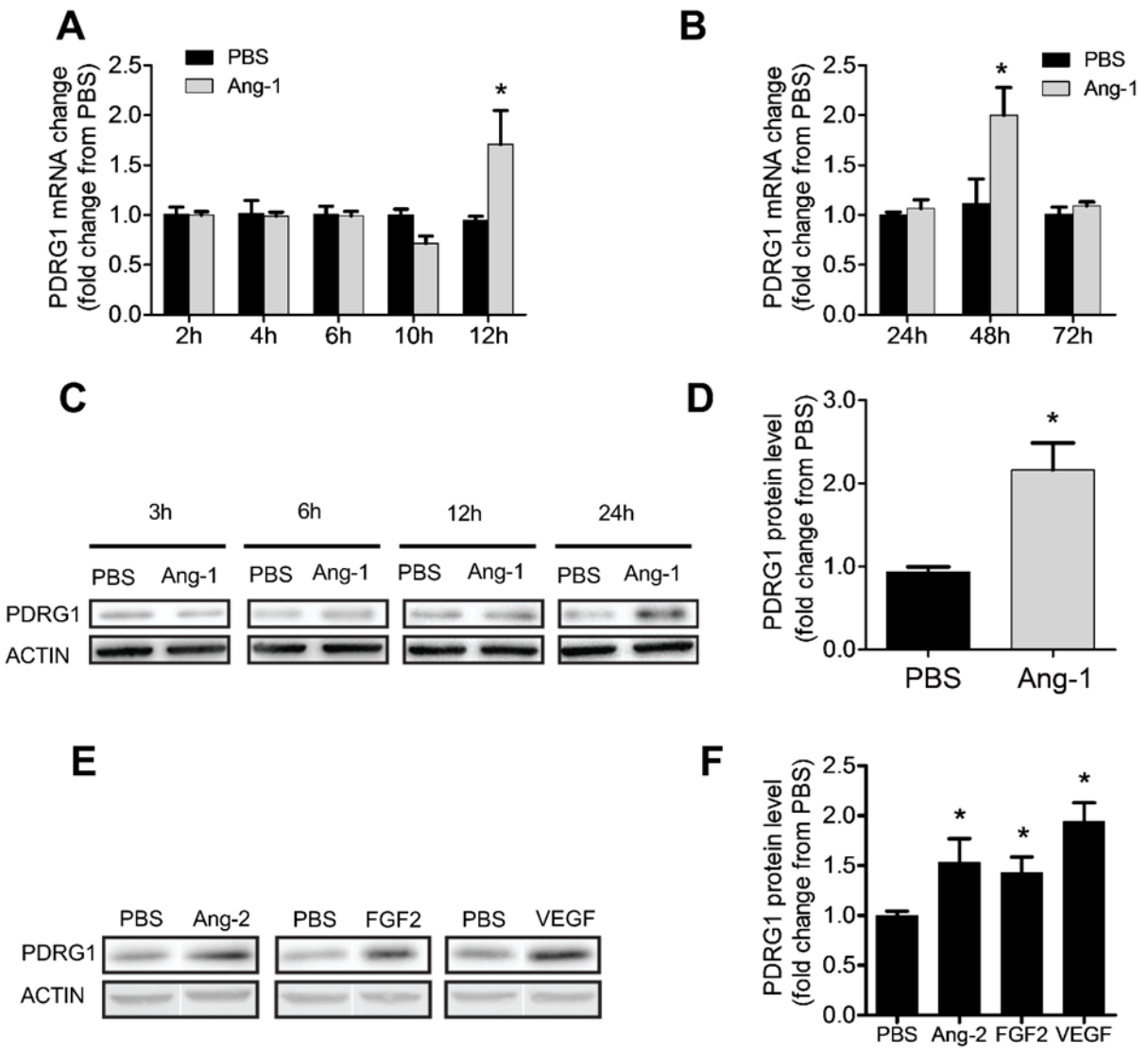


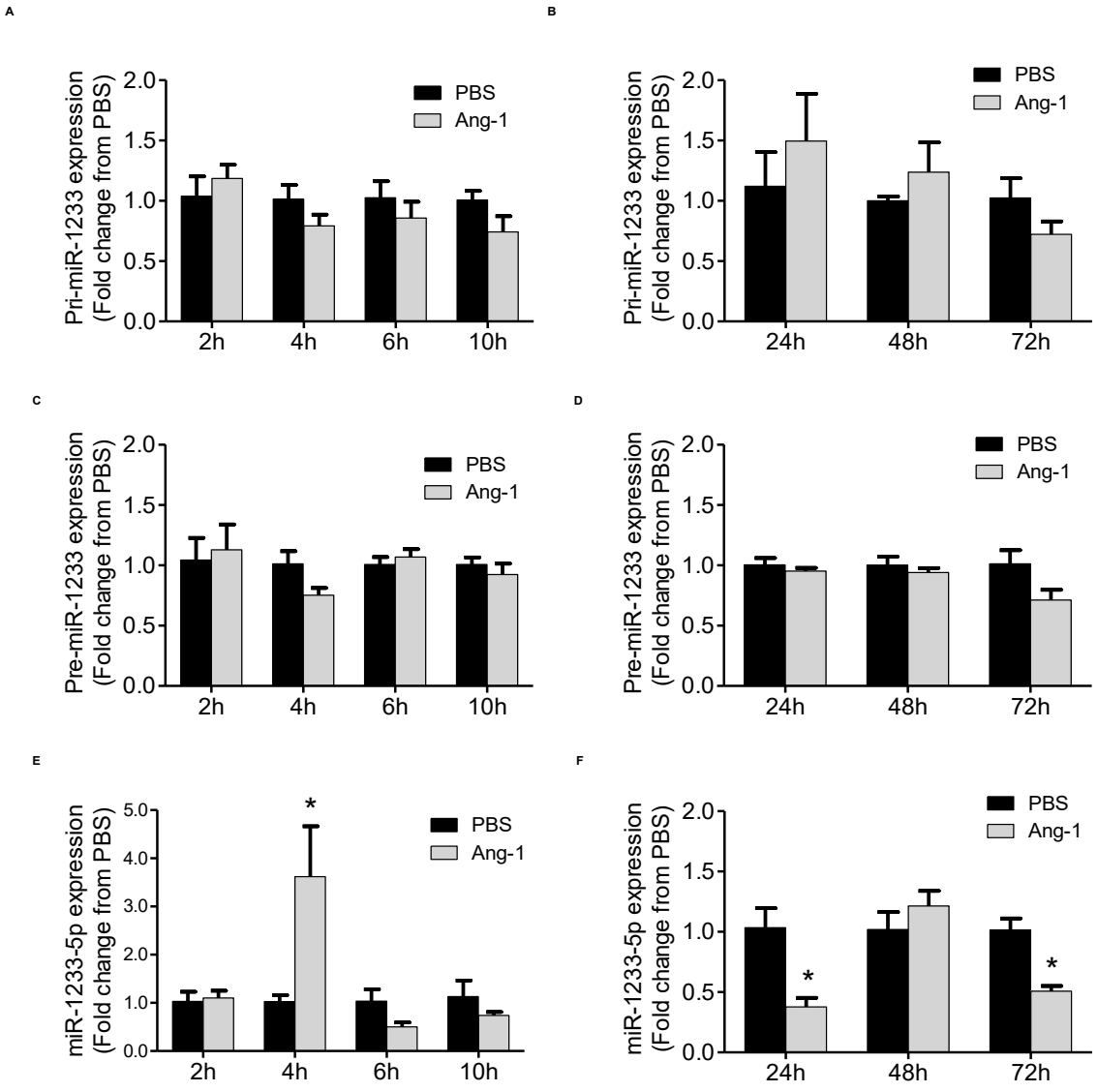

**Figure 6: Ang-1 regulates PDRG1 expression in ECs.**

**A&B:** PDRG1 mRNA levels in HUVECs exposed to PBS or Ang-1 (300ng/ml) for 2, 4, 6, 10, 12, 24, 48 or 72 h. **C:** Representative immunoblots of PDRG1 protein in HUVECs exposed to PBS or Ang-1 for 3, 6, 12 or 24 h. **D:** PDRG1 protein levels in HUVECs exposed to PBS or Ang-1 for 24 h. **E&F**: Representative immunoblots of PDRG1 and β-ACTIN proteins and PDRG1 protein levels in HUVECs exposed for 24 h to PBS, Ang-2 (300ng/ml), FGF-2 (10ng/ml), or VEGF

(40ng/ml). Values are means ± SEM, presented as fold change from PBS.

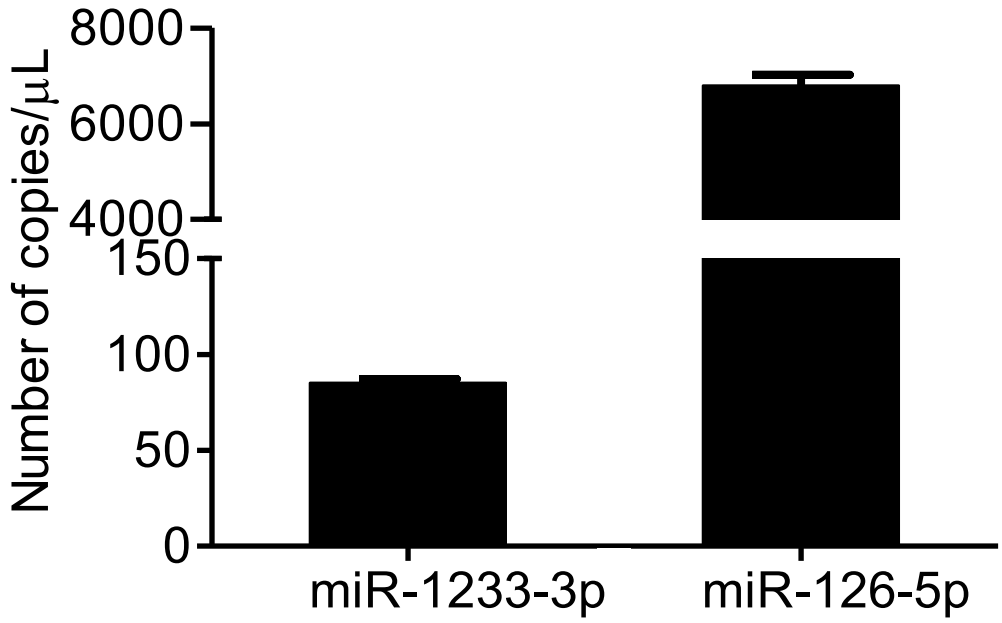
*P<0.05, compared to PBS.

## 9. **Supplementary figures**



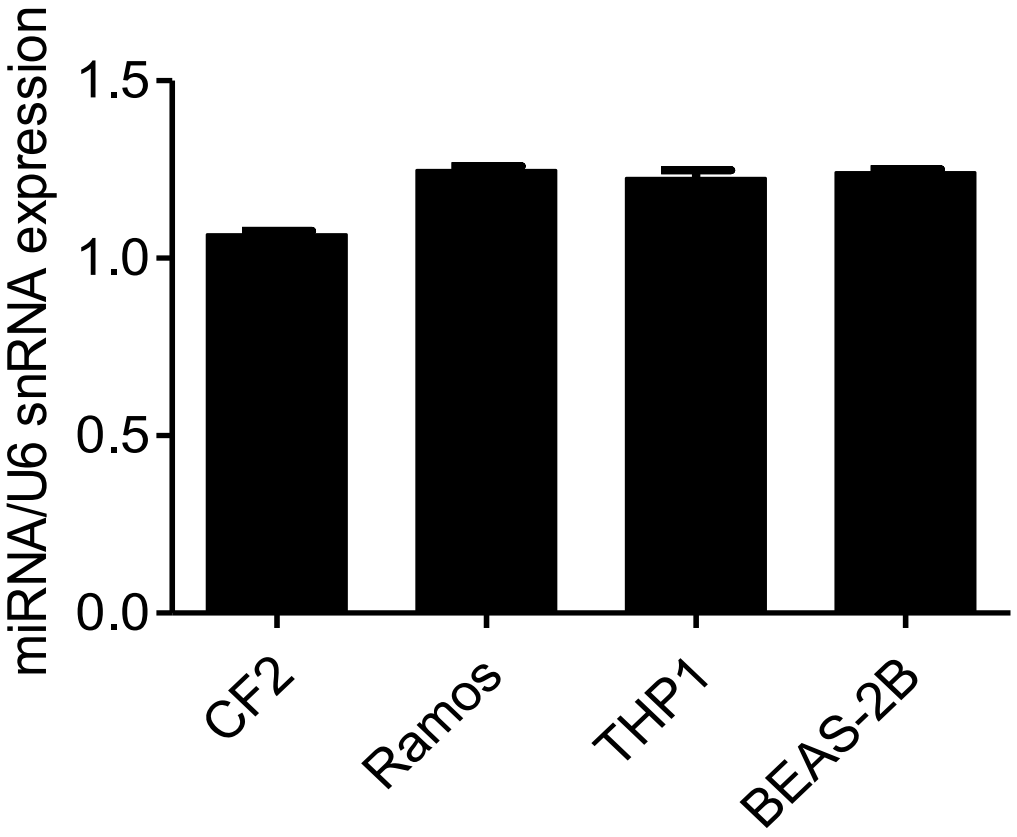

**Supplementary figure 1: Ang-1 regulation of pri-miR-1233, pre-miR-1233, and mature miR-1233-5p expression in HUVECs.**

**A-F:** pri-miR-1233, pre-miR-1233 and miR-1233-5p levels in HUVECs exposed for 2, 4, 6, 10, 24, 48, or 72 h to Ang-1 (300ng/ml) or PBS. Values are means ± SEM, presented as fold change from PBS. *P<0.05, compared to pre-incubation with PBS.



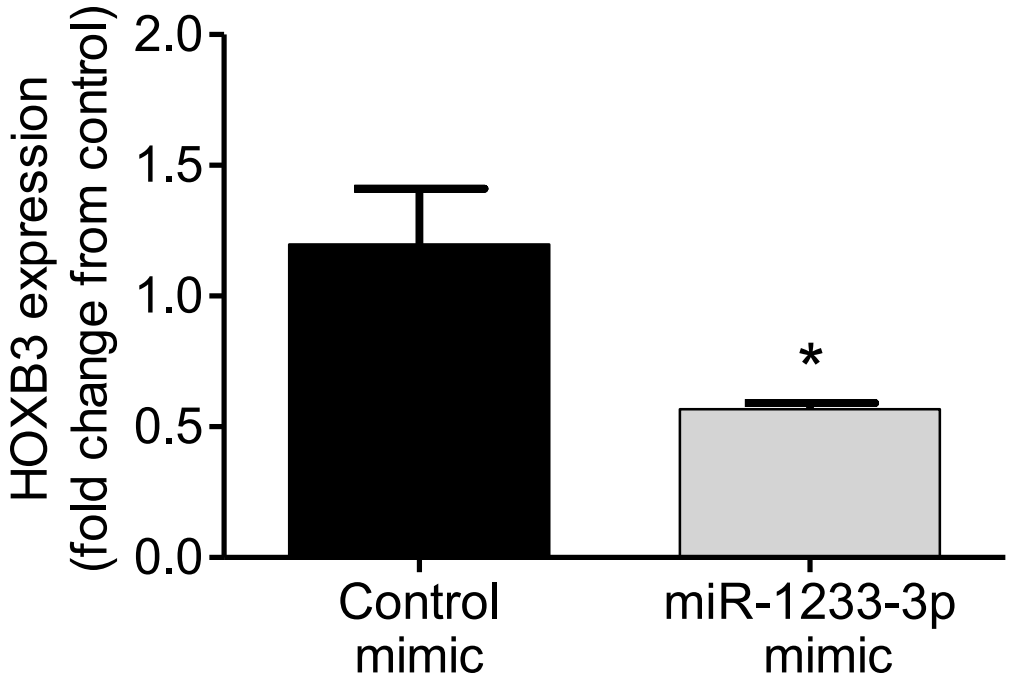

**Supplementary figure 2: Abundance of miR-1233-3p in HUVECs.**

miR-1233-3p and miR-126-5p copies per µL in HUVECs, as measured with Droplet digital PCR. Values are means ± SEM.



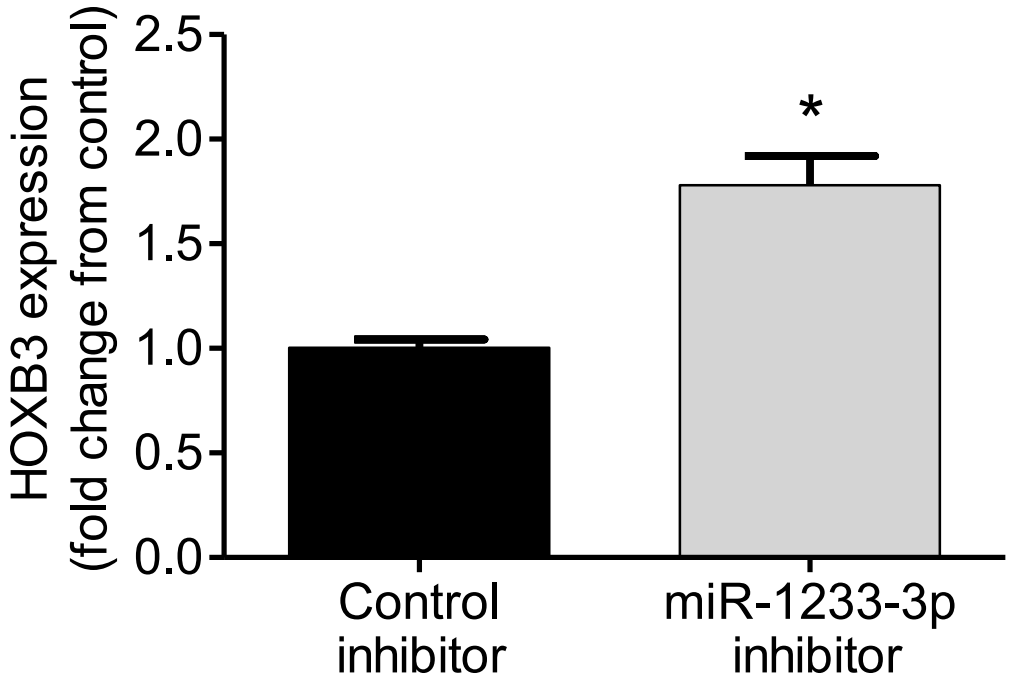

**Supplementary figure 3: Detection of miR-1233-3p in different cell types.**

miR-1233-3p levels in CF2, Ramos, THP1, and BEAS-2B cells, as measured with qPCR. Calculated as miRNA expression normalized to snoU6 expression. Values are means ± SEM



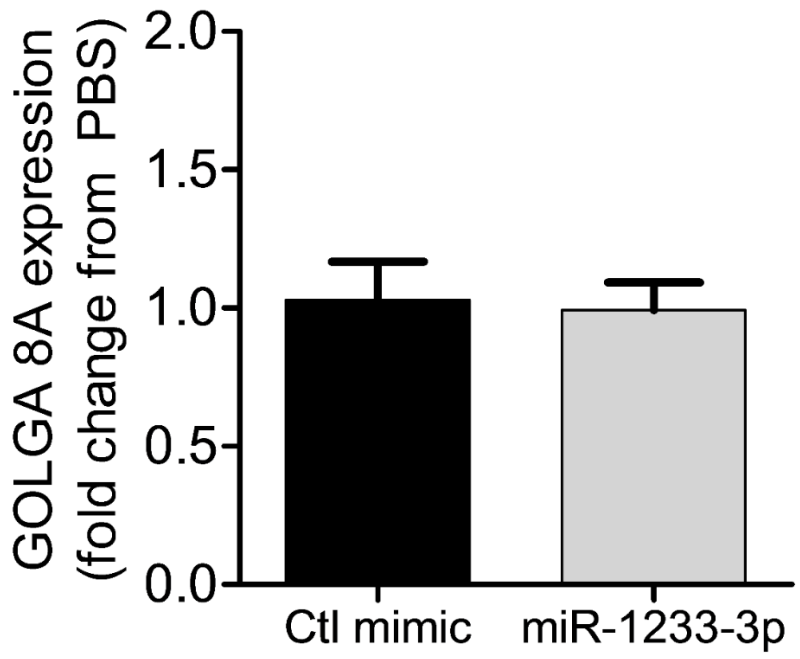

**Supplementary figure 4: Transfection efficiency of miR-1233-3p mimics in HUVECs.**

HOXB3 mRNA levels 48 h after transfection of control or miR-1233-3p mimics in HUVECs. Values are means ± SEM. *P<0.05, compared to control mimic.




**Supplementary figure 5: Transfection efficiency of miR-1233-3p inhibitor in HUVECs.**

HOXB3 mRNA levels 48 h after transfection of control or miR-1233-3p inhibitors in HUVECs. Values are means ± SEM. *P<0.05, compared to control inhibitor.




**Supplementary figure 6: Effects of miR-1233-3p on GOLGA8A and GOLGA8A expression.**

GOLGA8A mRNA levels in HUVECs transfected with control or miR-1233-3p mimics. Values are means ± SEM, presented as fold change from control mimic.

## 10. Tables

**Table 1: Primers used for quantitative real-time PCR experiments.**

| Name | Sequence (5'→3') | Accession number | Expected size (bp) |
|---|---|---|---|
| β-ACTIN | F: AGAAAATCTGGCACCACACC<br>R: GGGGTGTTGAAGGTCTCAAA | NM_001101 | 126 |
| GAPDH | F: AAGAAGGTGGTGAAGCAGGCG<br>R: ACCAGGAAATGAGCTTGACAA | NM_02396.1 | 166 |
| GOLGA8A<br>GOLGA8B | F: TAGGCTCCCGTGCTTTTCT<br>R: AAAGCTCCCCCAAAAGGTTA | NM_181077.3<br>NM_001023567.4 | 285<br>285 |
| PDRG1 | F: TGGGTGGTTGCTGAATGAAG<br>R: GGTAAGAGGCGCATCCACTC | NM_030815.2 | 222 |
| CENPB | F: GACGTTCCGGGAGAAGTCAC<br>R: AGCCCTCGAGCTTGTCGTAG | NM_001810.5 | 215 |
| HOXB3 | F: GGTGGAGCTGGAGAAGGAGT<br>R: GGCCTTCTGGTCCTTCTTGT | NM_002146.4 | 148 |
| ZFP91 | F: CTCGCTATTTGCAGCACCAC<br>R: GCCCGAGCACAATATTCACA | NM_053023.4 | 165 |
| miR-1233-3p | F: TGAGCCCTGTCCTCCCGCAG | MIMAT0005588 | |
| miR1233-5p | F: AGTGGGAGGCCAGGGCACGGCA | MIMAT0022943 | |
| pre-miR-1233 | F: AGTGGGAGGCCAGGGCACGGCA<br>R: GCGGGAGGACAGGGCTCA | NR_036050 | |
| U6 | F: ACTAAAATTGGAACGATACAGAGA | NR_004394.1 | |
| 5S | F: ATACCGGGTGCTGTAGGCTT | D14867 | |

# Chapter IV: P53 and DNA Damage Regulated 1 (PDRG1) regulation of Angiopoietin-1-induced angiogenesis

## 1. Preface

miR-1233 is a poorly studied miRNA and little is known about its functional role in angiogenesis. Several studies have described the expression of miR-1233 in various cancers. For example, miR-1233 levels are significantly elevated in the plasma of patients with renal cell carcinoma, and this increase miR-1233 levels has been linked to patients' poor survival. It has also been suggested that increased miR-1233 expression in extracellular vesicles is a possible biomarker of cancer development. In the vascular system, plasma level of miR-1233 has been suggested as a useful biomarker of heart failure development.

The mode of action of miR-1233 and its direct targets remain largely unexplored. Our previous study (Chapter III) revealed that the Ang-1/Tie-2 axis decreases miR-1233-3p expression in ECs and that overexpression of miR-1233-3p significantly attenuates Ang-1-dependent EC migration, proliferation, and survival. We also found that miR-1233-3p directly interacts with PDRG1 mRNA.

p53 and DNA damage regulated 1 (PDRG1) is a poorly studied protein that belongs to the prefoldin subunit beta family, whose members are chaperone complex subunits. The precise intracellular localization of PDRG1 remains unclear. Several studies have concluded that the location of PDRG1 is cell dependent, and can be found in either the nucleus or the cytosol. PDRG1 expression is elevated in several human malignancies, suggesting that it may play an important role in cancer development and/or progression. The functional importance of PDRG1 in the regulation of angiogenesis is unknown.

Our previous study (Chapter III) revealed that miR-1233-3p directly interacts with PDRG1 mRNA in ECs. We also described that PDRG1 mRNA and protein levels are significantly elevated in ECs exposed to Ang-1. Our next study is focused on elucidating the functional importance of PDRG1 in regulating angiogenesis in general and Ang-1-induced angiogenesis in particular. We hypothesize that PDRG1 serves as an important positive regulator of angiogenesis and that it promotes Ang-1-induced angiogenic processes such as EC survival, proliferation, migration and differentiation.

# Regulation of Angiopoietin-1-induced angiogenesis by PDRG1 (P53 and DNA Damage Regulated 1)

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## 2. Abstract

The Angiopoietin1 (Ang-1)/Tie-2 axis is essential for embryonic vascular development and in the maintenance of adult quiescent vasculature. We reported recently that exposure of endothelial cells (ECs) to Ang-1 for 24 h elicits significant downregulation of miR-1233-3p expression and that PDRG1 (P53 and DNA damage-regulated gene 1) is a direct target of miR-1233-3p in these cells; however, the functional role of PDRG1 in Ang-1-dependent angiogenesis and the mechanisms through which PDRG1 regulates EC function are unknown. In this study, we report that PDRG1 promotes angiogenic processes, including EC survival, migration, capillary-like tube formation, and proliferation, and that it inhibits Caspase-3 activity. Immunoprecipitation and mass spectrometry analyses revealed that PDRG1 interacts with several proteins among which is TSC2 (a regulator of the mammalian target of rapamycin, mTOR). This interaction was present not only in ECs but in other cell types as well. Overexpression of PDRG1 activated mTOR complex 1 (mTORC1), as is indicated by increased phosphorylation of P70S6K and 4E-BP1 proteins. Inhibition of mTORC1 activity repressed the PDRG1 effect on mTORC1 activation, EC migration, capillary-like tube formation, and proliferation. On the basis of these results and our previously reported findings on the regulation of miR-1233-3p by the Ang-1/Tie-2 axis, we propose a model in ECs in which the Ang-1/Tie-2 axis promotes angiogenesis, in part through downregulation of miR-1233-3p expression, which results in upregulation of PDRG1 expression and enhanced interaction between PDRG1 and TSC2, in turn resulting in removal of the inhibitory effects of TSC1 and TSC2 on mTORC1 activity.

## 3. Introduction

The angiopoietin-1/Tie-2 receptor axis is essential for embryonic vascular development [1]. In adult quiescent vasculature, activation of Tie-2 receptors by Ang-1 leads to enhanced vascular integrity and endothelial cell (EC) survival [2-3]. In addition, the Angiopoietin-1/Tie-2 axis stimulates lymphangiogenesis, EC migration and differentiation, and inhibits apoptosis [2].

The functional roles of microRNAs (miRNAs) in the vascular system have been the focus of numerous studies that have revealed their association with diseases in which pathological angiogenesis is the main feature [4]. Recently, we identified miR-1233-3p as an important anti-angiogenic miRNA that inhibits the pro-migratory and pro-differentiation effects of Ang-1 in human umbilical vein endothelial cells (HUVECs). We also reported that miR1233-3p directly targets PDRG1 (P53 and DNA damage-regulated gene 1), a poorly characterized protein. The PDRG1 gene resides on the long arm of chromosome 20 and encodes for a protein consisting of 133 amino acids. Early studies of this protein found that its expression is downregulated by the tumor suppressor protein p53 but upregulated by UV-light. Clinical studies have found PDRG1 expression is elevated in several malignancies, including colon, lung, and uterine cancer [6]. In addition, PDRG1 has been recently found to promote migration and proliferation of cells and to inhibit apoptosis in bladder cancer cells [7]. The role of PDRG1 in angiogenesis in general and in Ang-1/Tie-2-induced angiogenesis remains to be elucidated. Therefore, the main objective of the current study is to characterize the functional roles of PDRG1 in angiogenesis and to explore the mechanisms through which this protein regulates angiogenesis. Our study reveals that PDRG1 promotes

basal and Ang-1-induced EC survival, migration, differentiation, and proliferation, and that it inhibits apoptosis. We also found that PDRG1 interacts with several proteins, among which are TSC2 and TSC1 (inhibitors of mammalian target of rapamycin, mTOR), and that this interaction leads to activation of the mTOR complex 1, a pro-angiogenic protein complex. Inhibition of mTORC1 by rapamycin or torin1 resulted in elimination of the pro-angiogenic effects of PDRG1. On the basis of these results, we propose a new model to explain the pro-angiogenic effects of Ang-1 in ECs. In this model, the Ang-1/Tie-2 axis regulates angiogenesis in part through downregulation of miR-1233-3p expression and removal of its inhibitory effects on PDRG1 expression, which then interacts with TSC1 and TSC2 and removes the inhibitory effects of the TSC1 and TSC2 complexes on mTORC1 activity.  Activation of mTORC1 leads to promotion of several pro-angiogenic processes, including EC survival, migration, differentiation, and proliferation.

## 4. Materials and methods

**Materials:** Recombinant human Ang-1 was purchased from R&D Systems (Minneapolis, MN). Antibodies for PDRG1 (SAB4503242), β-ACTIN, and β-TUBULIN were acquired from Sigma-Aldrich (St. Louis, MO). Antibodies against TSC2 (#3612), 4E-BP1 (#9644), P70S6K (#9202) and phospho P70S6K $Thr^{389}$ (#9205) were purchased from Cell Signaling Technology (Danvers, MA). Rapamycin and Torin1 were a kind gift from Dr. Arnold Kristof (McGill University). The PDRG1 overexpression plasmid, PDRG1-myc (RC203661), was purchased from OriGene (Rockville, MD).

**Cell culture:** Human umbilical vein endothelial cells (HUVECs) were purchased from Lonza (Basel, Switzerland). Human microvascular endothelial cells (HMEC-1) were a gift from Dr. Anie Philip (McGill University). The human fibrosarcoma cell line (2FTGH) and human embryonic kidney cells (HEK293) were a gift from Dr. Arnold Kristof (McGill University). Primary normal human long fibroblasts (NHLF) were a gift from Dr. Q. Hamid (McGill University). HUVECs were grown in MCDB131 medium (Life Technologies, Rockville, MD) supplemented with 20% fetal bovine serum (FBS), EC growth supplement, 2 mM glutamine, heparin, and gentamicin sulfate (Invitrogen, Carlsbad, CA). This medium was designated as complete medium. Cells were incubated at 37°C and 5% $CO_2$. HMEC-1 were grown in MCDB131 containing 10% FBS, 10 ng/ml epidermal growth factor (EGF), 1µg/ml hydrocortisone, 10 nM glutamine, and gentamicin sulfate. HEK293, 2FTGH, and NHLF were grown in DMEM containing 10% FBS, 2 mM glutamine, and antibiotics

**Transfection of miRNA inhibitor, siRNA and vector**: HUVECs were transfected at a confluence of 50–70% with 25 nM of siRNAs against PDRG1 (Silencer® Select

4392420, Ambion, Austin, TX) or control siRNA (Silencer® Select 4390843) using Lipofectamine™ RNAiMAX (Invitrogen) according to the manufacturer's instructions. PDRG1 expression vector was transfected at 10 μg of plasmid per $2x10^6$ HUVECs using jetPRIME™ Polyplus transfection reagent (Illkirch, France) according to the manufacturer's protocol. Transfection of 50 nM miR-1233-3p inhibitor (mirVana® miRNA inhibitor 4464084, Ambion) was performed using RNAiMAX according to the manufacturer's protocol. In all cases, experimental treatments were performed 48 h after transfection.

**Cell count:** HUVECs were seeded (30,000 cells/$cm^2$) in a 6-well plate. The cells were maintained in complete medium, basal medium (MCDB131 medium without supplements, FBS or antibiotics) or basal medium in the presence of Ang-1 (300 ng/ml) for 24 h. Cell were then trypsinized and counted with a hemocytometer. For each condition, at least 5 replicates were performed.

**Caspase-3 activity:** HUVECs were seeded (30,000 cells/$cm^2$) in a 6-well plate. The cells were maintained in complete medium, basal medium, or basal medium in the presence of Ang-1 (300 ng/ml) for 24 h. Caspase-3 activity was measured 24 h later using an EnzChek® Caspase-3 Assay Kit with Z-DEVD-AMC as a substrate (Molecular Probes, Eugene, OR) in the case of PDRG1 overexpression studies. Otherwise, Caspase-3 assays were performed as follows. Forty-eight hours after transfection, HUVECs were plated in 96 well plates. Caspase-3 Green Apoptosis Assay Reagent (IncuCyte®, Ann Arbor, MI, 4440) and NucBlue™ Live ReadyProbes™ Reagent (Invitrogen, R37605) were added to the cells according to the manufacturer's protocol for a total of 24 h. Three images per well were taken at 24 h on a Cytation 5 Reader

(BioTek, Winooski, VT) using a 10X lens. To quantify the results, the number of positive cells for cleaved Caspase-3 was normalized by the total number of nuclei in the image.

**Scratch wound healing assay:** HUVECs were plated at confluence in 96-well plates. The monolayer was wounded using a 10uL pipette tip and exposed to Ang-1 (300 ng/ml) or PBS for 6 h in 2% FBS MCDB131. Wound images were visualized at time 0 and 8 h later using an Olympus inverted microscope and quantified using Image-Pro Plus™ software (Media Cybernetics, Bethesda, MD). Percentage wound healing was calculated as: [1 – (wound area at t8/wound area at t0)] x 100, where t8 is the time (8 h).

**Capillary-like tube formation:** HUVECs were seeded (12,500 cells per well) onto 96-well plates pre-coated with growth factor-reduced Matrigel® Matrix at a concentration of 10 mg/ml. Cells were maintained for 24 h in Lonza EGM™-2 medium (minus VEGF) containing aliquots of PBS (control) or Ang-1 (300 ng/ml). Whole-well images were captured using an Olympus inverted microscope. Angiogenic tube formation was determined by measuring total tube length and average tube length using the Angiogenesis Analyzer macro with Fiji/ImageJ [347].

**Proliferation:** HUVECs were seeded (7000 cell/well) onto 96-well plates and maintained in complete Lonza EGM™ medium (minus VEGF) containing either PBS or Ang-1 (300 ng/ml). One hour after plating, a bromodeoxyuridine (BrdU) (Millipore, Etobicoke, ON) assay was performed according to the manufacturer's instructions. In summary, 1 h after plating, BRDU was added and left for a total of 24 h. The cells were then fixed and BrdU absorbance was measured 24 h later.

**Immunoblotting:** Cells were lysed using RIPA buffer (Santa Cruz Biotechnology, Dallas, TX). Denatured proteins were separated on SDS-polyacrylamide gel electrophoresis (PAGE) and electro-transferred onto polyvinylidene difluoride (PVDF) membranes (Bio-Rad Laboratories, Richmond, CA). Membranes were blocked with 5% (w/v) low-fat milk for 1 h at room temperature and probed with the primary antibody at 4°C overnight. After washing, membranes were incubated for 1 h at room temperature with horseradish-peroxidase-conjugated secondary antibodies (Jackson ImmunoResearch, Newmarket, UK) or Alexa Fluor® Plus conjugated secondary antibodies (Thermo Fisher Scientific, Waltham, MA). Proteins were detected by Pierce enhanced chemiluminescence reagents (Thermo Fisher) in the case of horseradish-peroxidase antibodies. Bands intensities were quantified using ImageJ image software.

**Immunoprecipitation (IP):** HUVEC, HMEC-1, 2fTGH, HEK293, and NHLF cells were transfected with 10 μg of PDRG1 expression plasmid, control plasmid, or left untransfected. Forty-eight hours after transfection, cells were lysed for 10 minutes on ice in 1 ml lysis buffer (150 mM NaCl, 50mM Tris-HCL pH7.4, 1% NP-40, 0.25% Na-deoxycholate, 1 mM EGTA, and 1X Complete Mini protease inhibitor). Lysates were cleared by centrifugation at 12,000 rpm for 15 minutes at 4°C, and supernatants were collected. Protein A/G agarose (Thermo Fisher) was washed 3 times with 500 µl of lysis buffer. Immunoprecipitation was performed by bringing 1 mg of clear lysates to a final concentration of 1 mg/ml in lysis buffer and incubating these lysates with gentle rotation at 4°C for 2 h with 20 μl 50% suspension of Protein A/G agarose and 1 μg/ml of mouse monoclonal PDRG1 antibody (sc-398815, Santa Cruz Biotechnology). For immunoblotting, beads were washed 5 times with 500 µl lysis buffer, 20 µl 2x laemmli

buffer was added, then beads were boiled at 95°C for 5 minutes. For mass spectrometry, beads were washed with 500µL lysis buffer 2 times and 3 times with PBS to remove salts.

**Mass spectrometry:** Mass spectrometry analysis was performed by the RI-MUHC proteomics platform. In brief, proteins bound to beads were reduced with dithiothreitol, alkylated with iodoacetic acid, and digested with trypsin. Lyophilized peptides were re-solubilized in 0.1% aqueous formic acid/2% acetonitrile. Peptides were loaded onto a Thermo Acclaim Pepmap (Thermo Fisher, 75µM ID by 2cm C18 3µM beads) pre-column and then onto an Acclaim Pepmap Easyspray analytical column (Thermo Fisher, 75µM by 15cm with 2 µM C18 beads) for separation using a Dionex Ultimate 3000 UHPLC at nl/min with a gradient of 2-35% organic (0.1% formic acid in acetonitrile) over 2 h. Peptides were analyzed using a Thermo Orbitrap Fusion mass spectrometer operating at 120,000 resolution (FWHM in MS1, 15,000 for MS/MS) with HCD sequencing all peptides with a charge of 2+ or greater. Raw data were converted into mascot generic format (*.mgf) and searched using Mascot 2.3 against human sequences (Swissprot). Database search results were loaded into Scaffold Q+ Scaffold_4.4.8 (Proteome Software, Portland, OR) for spectral counting, statistical treatment, and data visualization.

**mTOR inhibition experiments**: HUVECs transfected with vectors were maintained for 48 h in MCDB131 medium supplemented with 10 nM of torin1, 50nM rapamycin, or PBS (control). After 6 h, cells were lysed, and immunoblotting was performed as described above. For angiogenesis assays, HUVECs were transfected with vectors as described above. Following 48 h of recovery, measurements of survival, migration, differentiation,

and Caspase-3 activity assays were performed as described above. Addition of 10 nM of torin1, 50nM rapamycin, or PBS to the medium was performed at the beginning of each assay and kept for the duration of the assay. During the proliferation assay, torin (10 nM), rapamycin (50 nM), or PBS was added to endothelial cell growth medium (Lonza). For measurements of cell proliferation, BrdU was added to the media 1 h later and incubated for 24 h.

**Statistical analysis:** Statistical analyses were performed using Graph Pad Prism 5.0 software (GraphPad® Software Inc., San Diego, CA). In experiments where more than two groups were compared, Two-way ANOVA followed by Bonferroni post-hoc analysis was utilized. For experiments where only two groups were compared, a paired student's T-test was employed. Differences were considered statistically significant at $P<0.05$.

## 5. Results

**Regulation of EC survival by PDRG1:** We have recently reported that exposure of ECs to Ang-1 triggers upregulation of PDRG1 mRNA and protein levels. We determined PDRG1 localization in HUVECs by fractionating cell lysates into nuclear and cytosolic fractions and by using immunofluorescence. Immunoblotting of cytosolic and nuclear fractions of HUVECs maintained in complete medium revealed that PDRG1 protein is localized mainly in the cytosol, but some protein appeared in the nucleus (supplementary figure 1). Exposure to Ang-1 resulted in increased PDRG1 protein levels but had no effect on PDRG1 localization (supplementary figure 1). Immuofluoresence confirmed cytosolic localization of PDRG1 protein in HUVECs (supplementary Figure 1). To explore the functional roles of endogenous PDRG1 in Ang-1-induced angiogenesis, HUVECs were transfected with control or PDRG1 siRNA oligos. Downregulation of PDRG1 expression with siRNA oligos was verified (supplementary figure 2). Forty-eight hours after transfection, HUVECs were exposed to PBS or Ang-1 (300 ng/ml) and *in-vitro* angiogenic assays, including cell survival, Caspase-3 activity, proliferation, migration, and differentiation into capillary-like tubes, were performed. With the control siRNA, cell numbers decreased under serum deprivation conditions (basal medium) (Figure 1A). The addition of Ang-1 to the basal medium increased cell numbers (Figure 1A). With the PDRG1 siRNA, the cell number decreased when maintained in complete medium, compared to the control siRNA (Figure 1A). No significant increase in cell number was observed in response to Ang-1 exposure in cells transfected with the PDRG1 siRNA (Figure 1A). With the PDRG1 siRNA, the absolute cell number in basal medium and basal medium+Ang-1 was

significantly lower than with the control siRNA (Figure 1A). To assess whether proportional changes in the number of apoptotic cells relates to the effects of PDRG1 knockdown on total cell number, Caspase-3 activity was measured. With the control siRNA, Caspase-3 activity increased under serum deprivation. The presence of Ang-1 attenuated this increase, thereby confirming that Ang-1 exerts an anti-apoptotic effect on ECs (Figure 1B). With the PDRG1 siRNA, Caspase-3 activity increased under serum deprivation to levels higher than those observed with the control siRNA. Caspase-3 activity did not decrease under serum deprivation when Ang-1 was added to PDRG1 siRNA-transfected cells (Figure 1B). With the PDRG1 siRNA, Caspase-3 activity in complete medium, basal medium, and basal medium+Ang-1 was significantly higher than with the control siRNA (Figure 1B).

In addition to assessing the roles of endogenous PDRG1 in the regulation of Ang-1-induced EC survival, we evaluated the effects of PDRG1 overexpression on EC survival and Caspase-3 activity. We have verified that PDRG1 protein levels increase in response to PDRG1-myc vector transfection (supplementary figure 3). With the control vector, cell numbers decreased and Caspase-3 activity increased in response to serum deprivation (Figure 1C&D). Introduction of the PDRG1-myc vector showed that cell numbers in complete and basal medium, and upon Ang-1 treatment increased, as compared to the control vector (Figure 1C denoted by $\phi$). With PDRG1-myc vector, cell numbers decreased under serum deprivation, as compared to complete medium, but were higher than with the control vector (Figure 1C). With the control vector, Caspase-3 activity increased under serum deprivation, as compared to complete medium, and Ang-1 exposure attenuated this response (Figure 1D). With the PDRG1-myc vector,

Caspase-3 activity under serum deprivation increased, as compared to complete medium, and Ang-1 exposure attenuated this increase (Figure 1D). With the PDRG1-myc vector, Caspase-3 activities in complete and basal mediums were lower than those with the same conditions in cells transfected with the control vector (Figure 1D). These results indicate that PDRG1 promotes EC survival and inhibits Caspase-3 activity.

**Regulation of EC migration by PDRG1:** In the presence of PBS, basal migration of PDRG1 siRNA-transfected HUVECs decreased, as compared to the control siRNA (Figure 2A). Exposure to Ang-1 increased cell migration in control siRNA-transfected HUVECs but not in those transfected with the PDRG1 siRNA (Figure 2A). Overexpression of PDRG1 increased EC migration in basal medium containing PBS or Ang-1, suggesting that increasing PDRG1 levels exerts a positive effect on EC migration (Figure 2B).

**Regulation of EC differentiation by PDRG1:** Capillary-like tube formation assays were used to evaluate the functional regulatory roles of PDRG1 in EC differentiation. Relative to PBS exposure, total tube length increased in control siRNA-transfected cells exposed to Ang-1 (Figure 3A). With PDRG1 siRNA-transfected cells exposed to PBS, total tube length was lower than with the control siRNA (Figure 3A). Ang-1 increased total tube length in cells transfected with the PDRG1 siRNA, but to a lesser degree than in cells transfected with the control siRNA (Figure 3A). To evaluate whether overexpression of PDRG1 affects EC differentiation, capillary-like tube formation assays were performed in control vector- and PDR vector-transfected HUVECs. Relative to PBS exposure, total tube lengths increased in control vector-transfected cells exposed to Ang-1 (Figure 3B). With PDRG1 vector-transfected cells, total tube length in the

presence of PBS increased as compared to cells transfected with the control vector (Figure 3B). With PDRG1 vector-transfected cells, Ang-1 total tube lengths increased, as compared to PBS (Figure 3B).

**Regulation of EC proliferation by PDRG1:** The effect of Ang-1 on EC proliferation is open to debate because it has been described as having a positive effect, a negative effect, and no effect. We therefore tested the effect of PDRG1 on EC proliferation in the absence of Ang-1. BrdU incorporation in cells transfected with the PDRG1 siRNA was lower than with the control siRNA (Figure 4A). In comparison, BrdU incorporation in cells transfected with the PDRG1-myc vector was higher than with the control vector, suggesting that PDRG1 promotes EC proliferation (Figure 4B).

**PDRG1 interacts with TSC2:** To gain insight into the mechanisms through which PDRG1 regulates angiogenic processes, we identified its interacting partners using immunoprecipitation followed by mass spectrometry (IP-MS). Human microvascular endothelial cells (HMEC-1) were transfected with a PDRG1-myc vector to overexpress PDRG1, which was then immunoprecipitated from cell lysates with a polyclonal antibody. An isotype-matched non-specific IgG antibody served as control. To control for false positive results that might have been caused by PDRG1 overexpression, IP of endogenous PDRG1 protein was performed in un-transfected HMEC-1s. Prior to mass spectrometry, we validated that PDRG1 protein is immunoprecipitated with a selective antibody (Figure 5). Immunoprecipitated PDRG1 protein bands separated on polyacrylamide gels were excised and analyzed by mass spectrometry (MS). In total, 339 proteins were identified by MS. After the elimination of unspecific proteins, 46 unique proteins were identified in the two IP experiments performed on PDRG1-myc

vector-transfected HMEC-1 cells (Supplementary table 1). Fewer peptides were detected when the IP was performed on un-transfected HMEC-1 cells (supplementary table 1). Only ten proteins, including PDRG1, TSC2, MATR3, CCDC91, HNRNPM, AFB, RPS27, RPS3, MFF, and REPL8, were identified in all three IP experiments (Table 1). We then focused on TSC2 protein because of its relative abundance in PDRG1 immunoprecipitates and its known regulatory function in determining the activity of mTORC1 (Figure 6A). In addition to TSC2, TSC1 protein was detected in two IP experiments (supplementary table 2). TSC1 forms a complex with TSC2 to inhibit mTORC1 activity. Using IP and immunoblotting, we confirmed that TSC2 protein can be detected in PDRG1 immunoprecipitates in several cell types, including HEK293, HUVECs, HMEC-1, 2fTGH, and NHLF cells (Figure 6B and supplementary figure 4). These results suggest that PDRG-1 interacts with TSC2, and possibly TSC1, and that this interaction is not specific to ECs.

**PDRG1 promotes phosphorylation of p70S6K and 4E-BP1:** TSC2 inhibits mTORC1 from binding and activating its downstream effectors, including 4E-BP1 and P70S6K1. Phosphorylation of these two proteins is considered to be indicative of mTORC1 activation levels [445]. To test the role of PDRG1 in mTORC1 pathway activation, we transfected HUVECs and HEK293 cells with control or PDRG1-myc vectors and, after allowing them to recover for 48 h, the cells were lysed and P70S6K1 and 4E-BP1 phosphorylation levels were assessed with immunoblotting. In addition, we evaluated the effect of PDRG1 knockdown using siRNA oligos on P70S6K1 and 4E-BP1 phosphorylation levels. PDRG-1 overexpression triggered increases in P70S6K1 and 4E-BP1 phosphorylation as compared to cells transfected with the empty vector (Figure

7A&B). In comparison, knocking down PDRG1 expression with siRNAs decreased P70S6K1 and 4E-BP1 phosphorylation levels (Figure 7A&B). These results suggest that PDRG1 functions as a positive regulator of the mTORC1 pathway. However, it is possible that PDRG1 may regulate P70S6K1 and 4E-BP1 phosphorylation levels independently of mTORC1. To test this possibility, HUVECs were transfected with a PDRG1-myc vector or control vector. Forty-eight hours after transfection, cells were pre-treated for 6 h with rapamycin (50 nM) or torin1 (10nM), mTORC1 inhibitors. Cells were then lysed and phosphorylation of P70S6K1 and 4E-BP1 was detected with immunoblotting. Exposure to rapamycin or torin1 decreased phosphorylation of P70S6K1 and 4E-BP1 and eliminated observed increases in their phosphorylation in cells overexpressing PDRG1 (Figure 7C). These results indicate that PDRG1 promotes phosphorylation of P70S6K and 4E-BP1 through the mTORC1 pathway.

We recently found that miR-1233-3p exerts strong anti-angiogenic properties in ECs and that it directly targets PDRG1 mRNA. To test the possibility that miR-1233-3p exerts its anti-angiogenic effects through mTORC1 inhibition, we transfected HUVECs with control and miR-1233-3 inhibitors and evaluated P70S6K1 and 4E-BP1 phosphorylation levels. Figure 7D indicates that inhibition of miR-1233-3p is associated with increases in P70S6K1 and 4E-BP1 phosphorylation, thereby confirming the importance of mTORC1 as a downstream indirect target of miR-1233-3p.

**Dependence of PDRG1 induced-angiogenesis on mTORC1:** Our results suggest that the pro-angiogenic effects of PDRG1 may be mediated in part through activation of the mTORC1 pathway. To test this hypothesis, HUVECs were transfected with a control vector or PDRG1-myc, and the effects of this transfection on EC number, Caspase-3

activity, differentiation into capillary-like tubes, and tube proliferation were assessed as described above. Forty-eight hours after, cells were pre-treated with PBS, rapamycin, or torin1 for 6 h and then underwent angiogenesis assays. In the presence of PBS, overexpression of PDRG1 increased cell number when cells were maintained in complete medium or basal medium (Figure 8A). Incubation with rapamycin or torin1 decreased cell number when cells were maintained in complete medium. Overexpression of PDRG1 increased cell numbers when cells were maintained in complete medium, but not in basal medium, in the presence of rapamycin or torin1 (Figure 8A). In the presence of PBS, transfection with the PDRG1-myc vector decreased Caspase-3, as compared to the control vector, when cells were maintained in basal medium (Figure 8B). In the presence of rapamycin, PDRG1 overexpression decreased Caspase-3 activity when cells were maintained in complete or basal medium (Figure 8B). In the presence of torin1, overexpression of PDRG1 decreased Caspase-3 activity when cells were maintained in complete medium (Figure 8B). These results suggest that the pro-survival and anti-apoptotic effects of PDRG1 do not require active mTORC1.

EC migration decreased in the presence of mTORC inhibitors (Figure 8C). In the presence of PBS, overexpression of PDRG1 increased migration as compared to control-transfected cells (Figure 8C). However, in the presence of rapamycin or torin1, overexpression of PDRG1 did not elicit any significant increases in cell migration (Figure 8C).

Overexpression of PDRG1 increased capillary-like tube formation and BrdU incorporation in the presence of PBS but not in the presence of rapamycin or torin1

(Figure 8D&E). These results suggest that the stimulatory effects of PDRG1 on EC migration, differentiation, and proliferation—but not survival—are dependent on the mTOR pathway.

6. **Discussion:**

The main findings of this study are that: 1) PDRG1 promotes EC survival, migration, proliferation, and differentiation and inhibits apoptosis; 2) the presence of PDRG1 is required for Ang-1-mediated increases in EC survival, migration, and differentiation; 3) PDRG1 protein is localized mainly in the cytoplasm of ECs, although relatively weak protein expression is detectable in the nucleus; 4) TSC2 interacts with PDRG1, and that this interaction mediates the stimulatory effect of PDRG1 on mTORC1 activity; and 5) PDRG1 promotes EC migration, proliferation, and differentiation through activation of mTORC1.

The expression and effects of PDRG1 on cell proliferation have been mainly investigated in cancer cells. PDRG1 is upregulated in several tumors, including colon (32/39), rectum (22/28), ovary (17/24), lung (19/27), stomach (25/37), breast (35/56), and uterus (29/47), relative to their own control tumors [6]. In a recent study on bladder cancer cells, it was reported that siRNA knockdown of PDRG1 expression resulted in inhibition of proliferation and enhanced apoptosis [8]. In addition, PDRG1 has been reported to inhibit migration and invasion in bladder cancer cells [7]. shRNA knockdown of PDRG1 expression in RKO human colon cancer cells resulted in significant inhibition of cell growth [8]. In the present study, we report for the first time, using loss-of-function and gain-of-function approaches, that PDRG1 negatively regulates Caspase-3 activity and enhances EC survival, migration, proliferation, and differentiation into capillary-like

structures. We also found that PDRG1 is required for *in-vitro* angiogenic effects of Ang-1. These results indicate that PDRG1 is a pro-angiogenic protein and that it plays a significant role in promoting pro-angiogenic effects of Ang-1.

Localization of PDRG1 has been studied in different cell types. In NIH3T3 and HCT116 cells, Luo et al. generated two different PDRG1–GFP fusion constructs carrying GFP either at the N- or C-terminal ends of PDRG1 and transfected these constructs into NIH3T3. They reported that PDRG1-GFP expression was concentrated in discrete areas within cytosol, indicating that PDRG1 protein may be located in subcellular organelle(s). The same results were observed in HCT116 cells [10]. Another group of investigators used a subcellular fraction technique on LNCaP cells to pull down URI. Their MS analysis revealed that PDRG1 was an interacting protein, suggesting its presence in the nucleus [11]. Immunostaining of PDRG1 in CHO, COS7, and H35 transfected with PDRG1-HA and PDRG1-eGFP constructs revealed relatively stronger nuclear localization as compared to the cytosol. We used subcellular fractionation of HUVECs and immunoblotting to localize PDRG1 protein in ECs. These experiments revealed stronger cytosolic PDRG1 protein localization as compared to nuclear localization. In support, mass spectrometry after PDRG1 pull down showed this protein interacts with many cytosolic molecules but also nuclear ones such as HIST3H3. Taken all together, published data and the one produced in this study suggest that subcellular localization of PDRG1 differs in various cell types and in different conditions, thus indicated its role in different pathways and tissues. In our case, we found that PDRG1 was mostly located in the cytosolic compartment. In addition, one of its most abundant interacting partners, TSC2, is a cytosolic protein. Nonetheless, mass spectrometry also

identified interacting partners, like histones, known to be present in the nucleus of the cell. Our data and previous studies suggest the possibility that PDRG1 resides in the nucleus and the cytosol. However, its expression levels are cell- and context-dependent.

Little is known about the interactions between PDRG1 and other proteins. Much of the information we have today comes from yeast two-hybrid screening and MS analysis. For example, Jiang et al. identified PDCD7 (programmed cell death 7), CIZ1 (Cip1 interacting zinc finger), and MAP1S (microtubules-associated protein 1S) as PDRG1-interacting proteins [6]. These proteins are involved in apoptosis and cell cycle regulation. PDCD7 regulates apoptosis and is a component of the U12-type spliceosome [12, 13]. CIZ1 modulates the cell cycle by interacting with p21 and promotes DNA replication [12]. MAP1S knockdown in HeLa cells compromises spindle assembly, delays metaphase alignment and anaphase entry, and causes genome instability [15]. It is also known to interact with RASSF1A to regulate cell-cycle progression [16]. These observations suggest that PDRG1 promotes cancer cell proliferation and survival through direct interactions with PDCD7, CIZ1, and MAP1S. In addition, PDRG1 was found by SILAC MS to interact with RPB3, the protein that makes the third largest subunit of RNA polymerase II. Indeed, it has been reported that PDRG1 is part of the R2TP/prefoldin-like complex, which contains other proteins like UXT, RPB5, WDR92/Monad, and URI [17, 18]. R2TP, together with the prefoldin-like complex, is involved in the assembly of RNA polymerase II. Using a combination of quantitative MS-based proteomic analyses, it was determined that when cells were treated with the inhibitors α-amanitin and leptomycin B, an accumulation of

unassembled Rpb1, one of the largest subunits of RNA Pol II, was observed [18]. In addition, deletion of any of the subunits of RNA poly II produced cytoplasmatic accumulation of RPB1, suggesting its assembly occurred in the cytoplasm, which is essential for a proper nuclear transportation [17]. Furthermore, immunoprecipitation and MS analysis of nuclear URI, part of the R2TP/prefoldin-like complex, revealed its interaction with PDRG1. Moreover, URI was found to stabilize PDRG1 [11].

Our MS analysis of PDRG1 IP experiments did not reveal any of the previously described PDRG1-interacting proteins. Interestingly, some anti-angiogenic proteins were identified as possible interactors in the overexpression pull down, suggesting PDRG1 might promote angiogenesis by inhibiting anti-angiogenic molecules. In addition, it is possible that PDRG1-interacting proteins differ depending on the cell type (cancer, ECs, etc.). Nevertheless, our study reveals that TSC2 and TSC1 interact with PDRG1. In addition, we found several proteins that function as anti-angiogenic inhibitors to be PDRG1-interacting proteins in ECs (Table 1 and supplementary Table 2). These results suggest that PDRG1 may function in ECs to sequester and thus prevent anti-angiogenic proteins from promoting their effects. Further studies are warranted to examine the functional importance of PDRG1 interactions with the proteins listed in Table 1 and supplementary Table 2.

TSC1 and TSC2 are two distinct but conserved genes that form a complex to inhibit mTORC1 activity. mTORC1 regulates ribosomal, protein, lipid, and nucleotide synthesis and exerts positive effects on cell survival, proliferation, cell cycle progression, angiogenesis, and energy metabolism [20]. mTORC1 has two well-characterized downstream targets, P70S6K1 and 4E-BP1, which possess residues that are directly

phosphorylated by mTOR kinase [19]. In ECs, activation of P70S6K has been linked to regulation of cell proliferation, apoptosis, and protein synthesis in response to growth factors, including Ang-1 [20-22].

TSC2 interaction with PDRG1 was confirmed by IP and detected with MS. Interestingly, we were able to identify TSC2 protein in PDRG1 IP in several other cell lines, including microvascular and macrovascular ECs, lung fibroblasts, and epithelial cells from a human fibrosarcoma. These results indicate that this interaction occurs in several cells types and may be regulated by factors other than angiogenesis factors. In fact, we previously showed that PDRG1 mRNA and protein are regulated by Ang-2, VEGF, and FGF2 (data not shown). In addition, loss-of-function and gain-of-function experiments revealed that PDRG1 regulates the activity of mTORC1, as indicated by changes in the phosphorylation levels of P70S6K and 4E-BP1 proteins. They also revealed that PDRG1 exerts strong effects on EC survival, migration, proliferation, and differentiation through this stimulatory effect on mTORC1.

We have recently reported that exposure of ECs to Ang-1 triggers significant downregulation of miR-1233-3p and that this effect leads to the removal of its inhibitory effect on the expression of one of its selective targets, namely, PDRG1. We also found that inhibition of miR-1233-3p expression results in increases in EC survival, migration, proliferation, and differentiation. These results raise the intriguing possibility that the pro-angiogenic effects observed in response to miR-1233-3p inhibition may be mediated through PDRG1-depedent activation of mTORC1. To test this possibility, we transfected control or selective miR-1233-3p inhibitors in HUVECs, then measured the phosphorylation status of P70S6K1 and 4E-BP1. Figure 7D illustrates that selective

inhibition of miR-1233-3p triggers increases in phosphorylation of P70S6K1 and 4E-BP1. Based on these results, we propose a pathway by which Ang-1 promotes angiogenesis by downregulating miR-1233-3p and allowing PDRG1 to bind to TSC2. PDRG1 and TSC2, and possibly TSC1, interaction probably plays a role in preventing the TSC2/TSC1 complex from binding and inhibiting mTORC1, resulting in increased mTORC1 activity, which then mediates the pro-angiogenic effects of Ang-1 (Figure 9).

The importance of PDRG1 at the clinical level can be deduced from the fact that it resides at the long arm of chromosome 20 in the cytogenetic region 20q11.2. Several reports have described high frequency chromosome gains in 20q in several cancers, including breast, lung, liver, colon, and rectum [6, 23-25]. These gains have been linked to short survival in colorectal, ovarian, and other kinds of cancer [26]. Moreover, the chromosomal region 20q11.2–20q13.2 is often amplified in colorectal cancers [27, 28]. PDRG1 resides in the cytogenetic region often amplified in some malignancies, and has been found overexpressed in colon, lung, and uterine cancers [6]. In relation to cancer, PDRG1 is downregulated by p53 and upregulated by some genotoxic agents [6, 10]. Moreover, p53 is also known to inhibit mTOR and promote tumor suppression [29]. Future studies are warranted to investigate the involvement of PDRG1 in the regulation of cancer cell survival, proliferation and migration.

## 7. References

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8. **Figures**

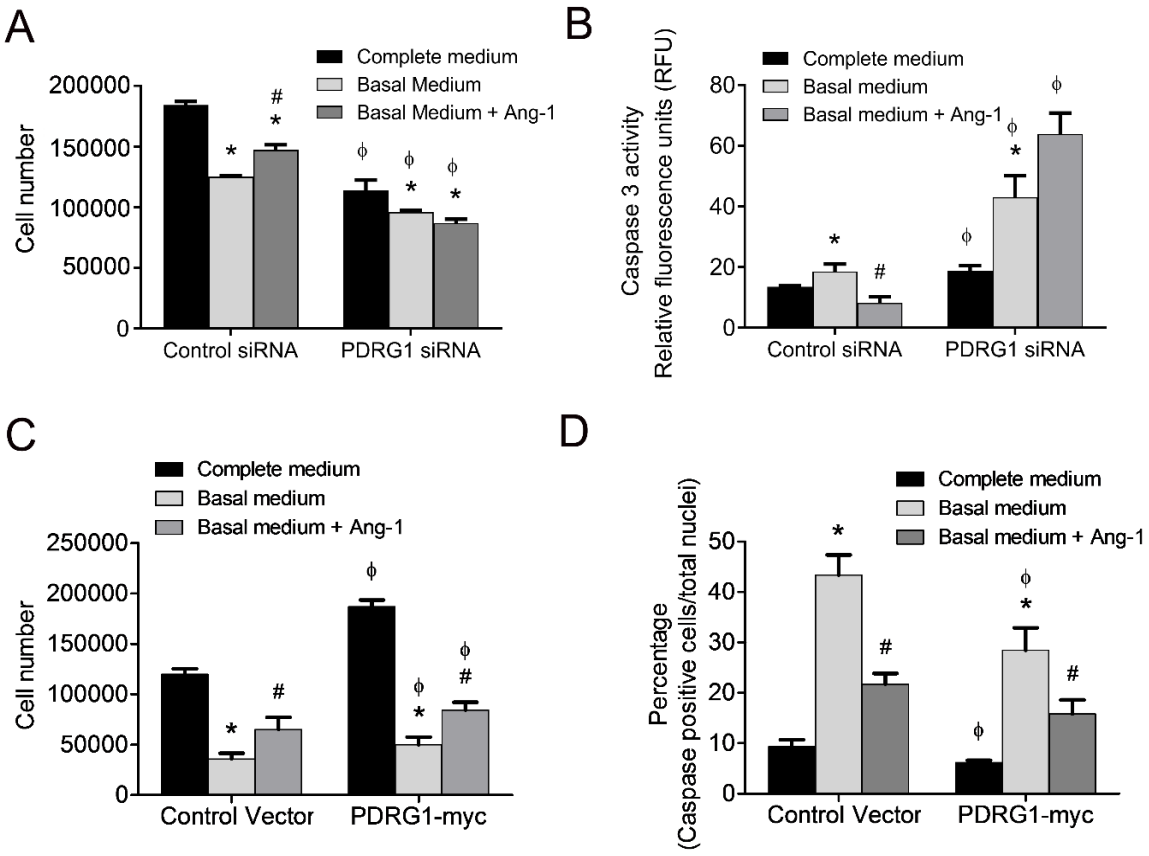



**Figure 1: PDRG1 regulation of EC survival and Caspase-3 activity.**

**A&B:** HUVECs were transfected with control siRNA or PDRG1 siRNA. Following 24 h of recovery, equal numbers of cells were maintained in complete medium, basal medium, or basal medium+Ang-1 (300ng/ml). Cell counts and Caspase-3 activities were measured 24 h later. **C&D:** HUVECs were transfected with control vector or PDRG1-myc vector. Following 24 h of recovery, equal numbers of cells were maintained in complete medium, basal medium, or basal medium+Ang-1 (300ng/ml). Cell counts and Caspase-3 activities were measured 24 h later. Values are mean ± SEM. *P<0.05, compared to complete medium; #P<0.05, compared to basal medium; ϕP<0.05, compared to control siRNA or control vector.

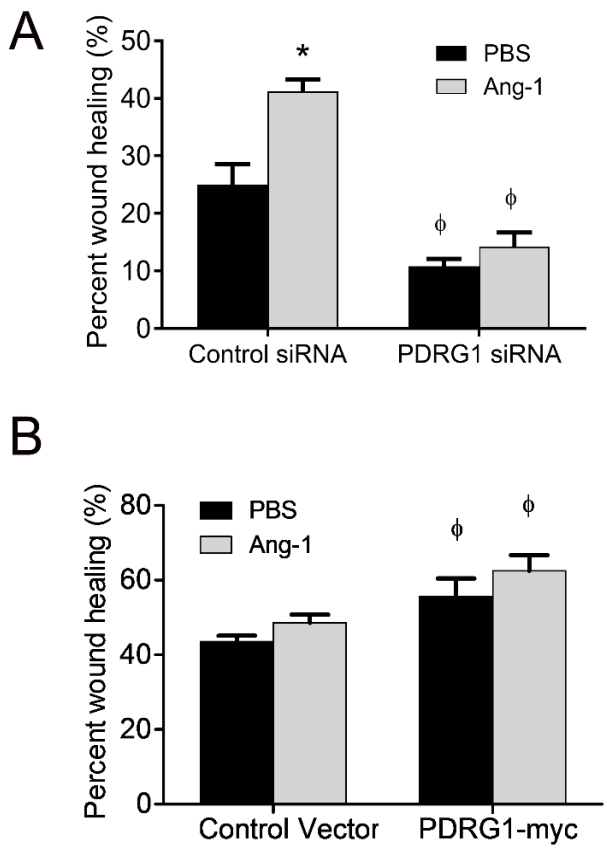



**Figure 2: PDRG1 regulation of EC migration.**

**A&B:** Percent wound-healing of HUVECs transfected with control siRNA, PDRG1 siRNA, control vector, or PDRG1-myc vector and exposed to PBS or Ang-1 (300ng/ml) for 8 h. Values are means ± SEM. *P<0.05, compared to PBS; ϕP<0.05, compared to control siRNA or control vector.

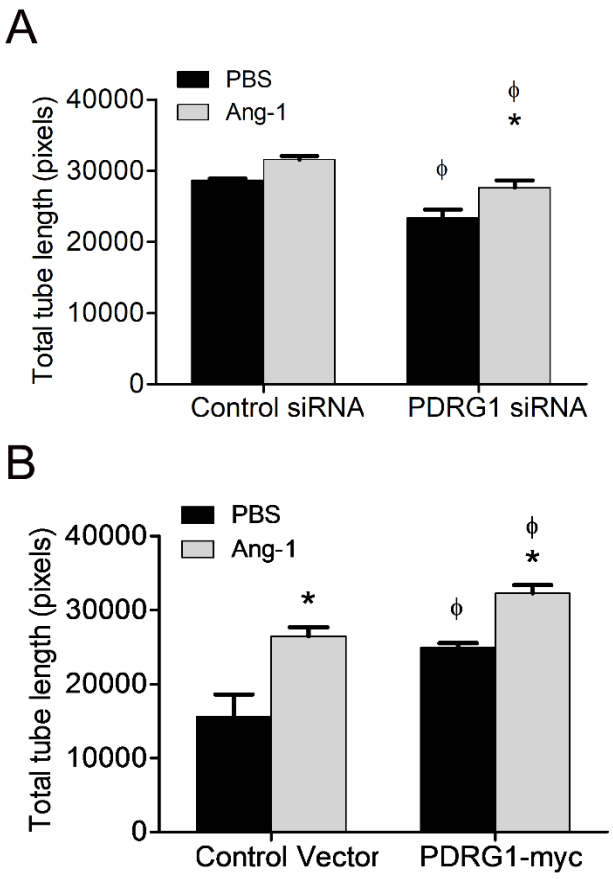



**Figure 3: PDRG1 regulation of EC differentiation into capillary-like tubes.**

**A&B:** HUVECs were transfected with control siRNA, PDRG1 siRNA, control vector, or PDRG1-myc vector. Following 24 h of recovery, cells were plated onto growth factor-reduced Matrigel®-coated plates, maintained for 24 h in Lonza EGM™-2 medium (minus VEGF), and exposed to PBS or Ang-1. Total tube length presented as pixels. Values are means ± SEM. *P<0.05, compared to PBS; ϕP<0.05, compared to control siRNA or control vector.

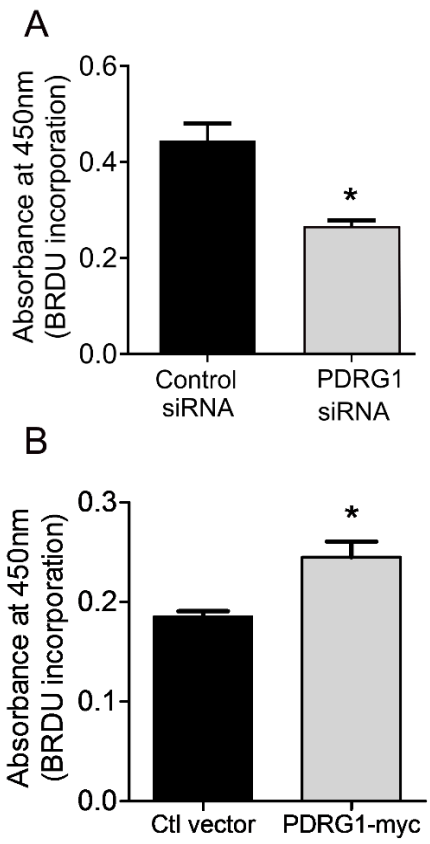



**Figure 4: PDRG1 regulation of EC proliferation.**

**A&B**: HUVECs were transfected with control siRNA, PDRG1 siRNA, control vector, or PDRG1-myc vector. Following 24 h of recovery, cells were maintained in Lonza EGM™-2 medium (minus VEGF). One h after plating, BrdU was added. BrdU absorbance was measured 24 h later. Values are means ± SEM. *P<0.05, compared to control siRNA or control vector.

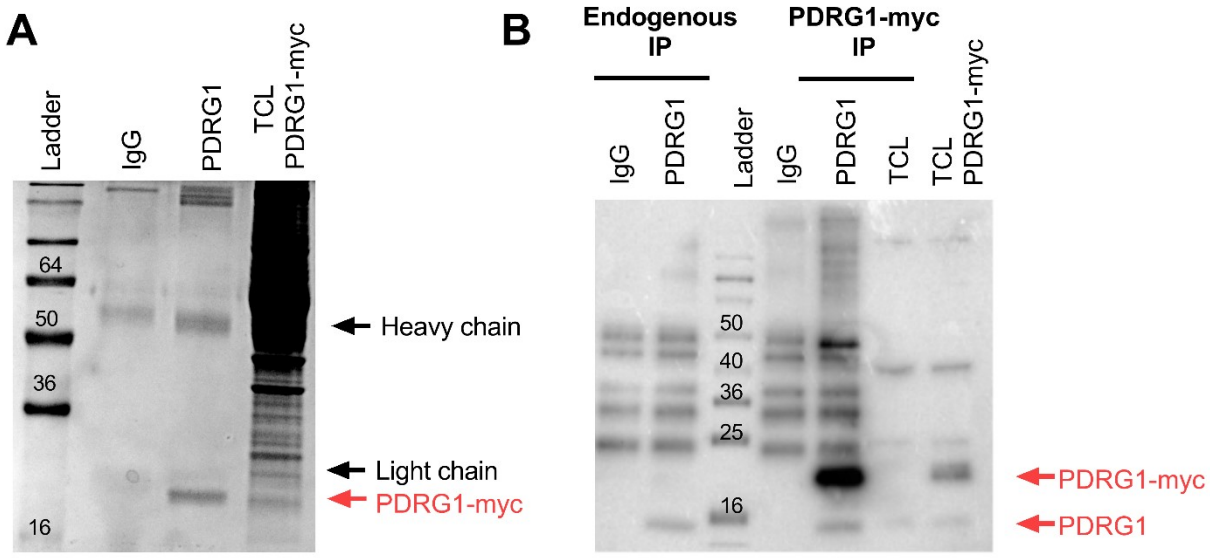



**Figure 5: Immunoprecipitation of PDRG1 in ECs.**

**A:** Coomassie stained gel showing streptavidin bead pull-down of PDRG1 in PDRG1-myc-plasmid-transfected HMEC-1. IP was performed 48 h post-transfection. **B:** Representative immunoblot of IP performed on untransfected HMEC-1 (endogenous IP, left), and PDRG1-myc-plasmid-transfected HMEC-1. Arrows indicate endogenous PDRG1 protein and PDRG1-myc fusion protein. TCL = total cell lysates. IgG = control nonspecific IgG. Ladder is shown in KDa.

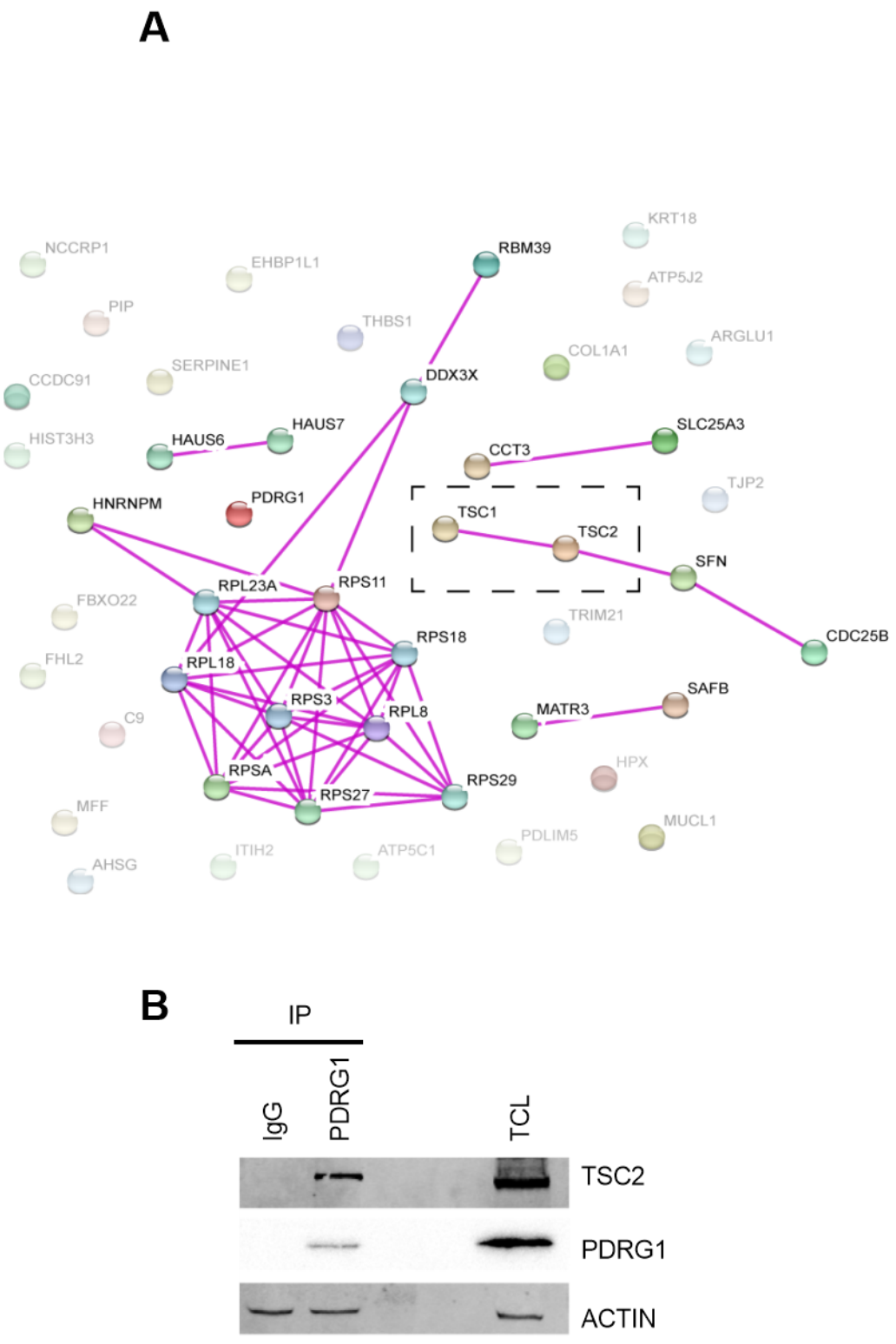



**Figure 6: Interaction of PDRG1 with TSC2.**

**A:** Interaction networks of experimentally validated protein–protein interactions detected by IP of PDRG1 in HMEC-1 cells. PDRG1 is represented in red, TSC2-TSC1 interaction is shown with a dashed box. Direct interaction between proteins are represented with a purple line.

**B:** Representative immunoblots from IP experiments performed on un-transfected HEK293 cells designed to detect TSC2 protein enrichment in PDRG1 immunoprecipitate. ACTIN was used to verify equal loading across lanes. TCL = total cell lysates.

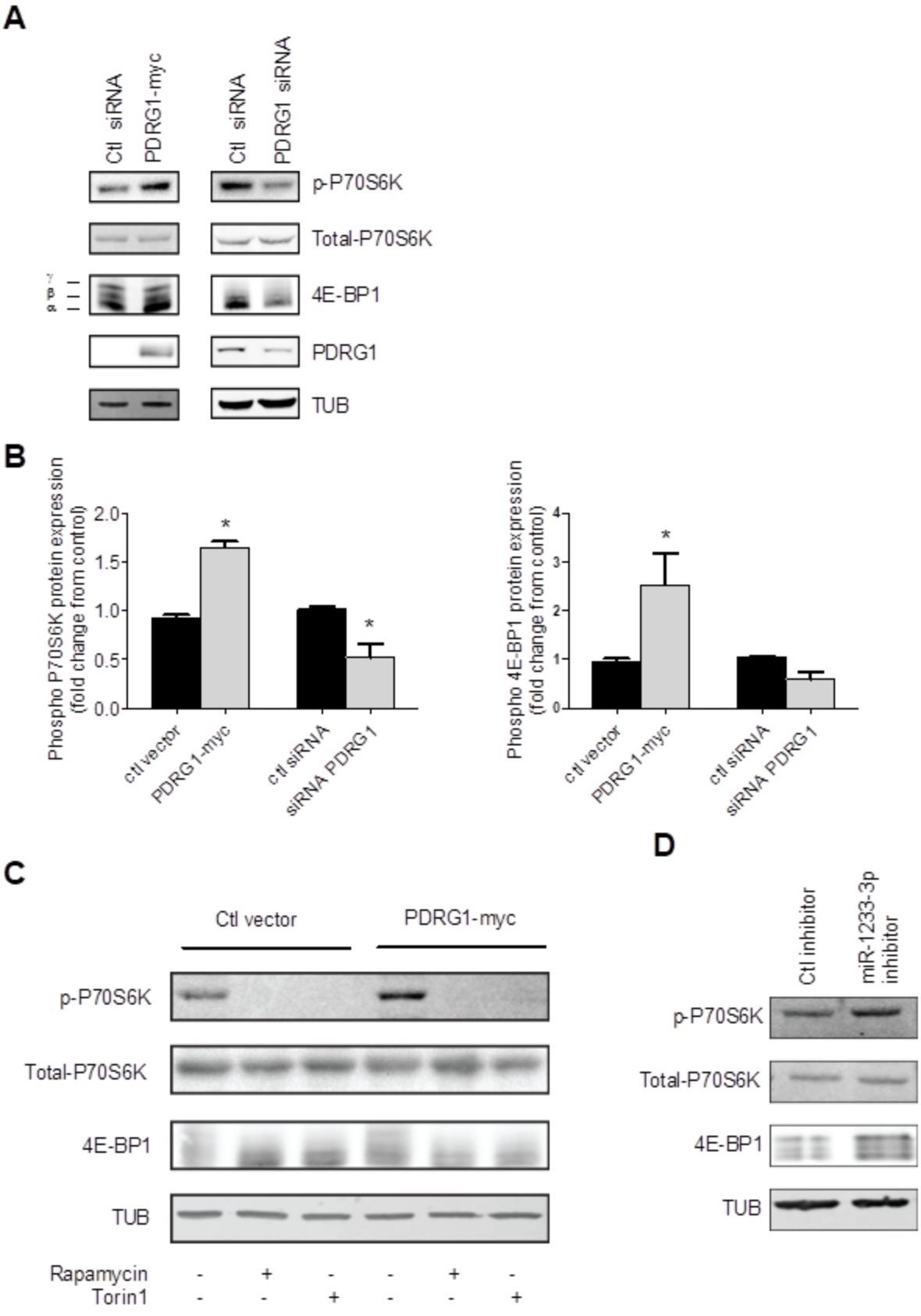

A
Ctl siRNA
PDRG1-myc
Ctl siRNA
PDRG1 siRNA
p-P70S6K
Total-P70S6K
4E-BP1
PDRG1
TUB
B
Phospho P70S6K protein expression (fold change from control)
2.0
1.5
1.0
0.5
0.0
*
*
ctl vector
PDRG1-myc
ctl siRNA
siRNA PDRG1
Phospho 4E-BP1 protein expression (fold change from control)
4
3
2
1
0
*
ctl vector
PDRG1-myc
ctl siRNA
siRNA PDRG1
C
Ctl vector
PDRG1-myc
p-P70S6K
Total-P70S6K
4E-BP1
TUB
Rapamycin - + - - + -
Torin1 - - + - - +
D
Ctl inhibitor
miR-1233-3p inhibitor
p-P70S6K
Total-P70S6K
4E-BP1
TUB

**Figure 7: PDRG1 regulates the mTORC1 activity.**

**A&B:** Representative immunoblots and optical densities of phosphorylated and total P70S6K and 4E-BP1 proteins in HUVECs transfected 24 h earlier with control vector, PDRG1-myc vector, control siRNA, or PDRG1 siRNA. Values are means ± SEM. *P<0.05, compared to control siRNA or control vector. TUB = β-TUBULIN.

**C:** Representative immunoblots of phosphorylated and total P70S6K and 4E-BP1 proteins in HUVECs transfected 48 h earlier with control or PDRG1-myc vector and treated for 6 h with PBS, rapamycin (50 nM), or torin1 (10 nM).

**D:** Representative immunoblots of phosphorylated and total P70S6K and 4E-BP1 proteins in HUVECs transfected 48 h earlier with control or miR-1233-3p inhibitor.

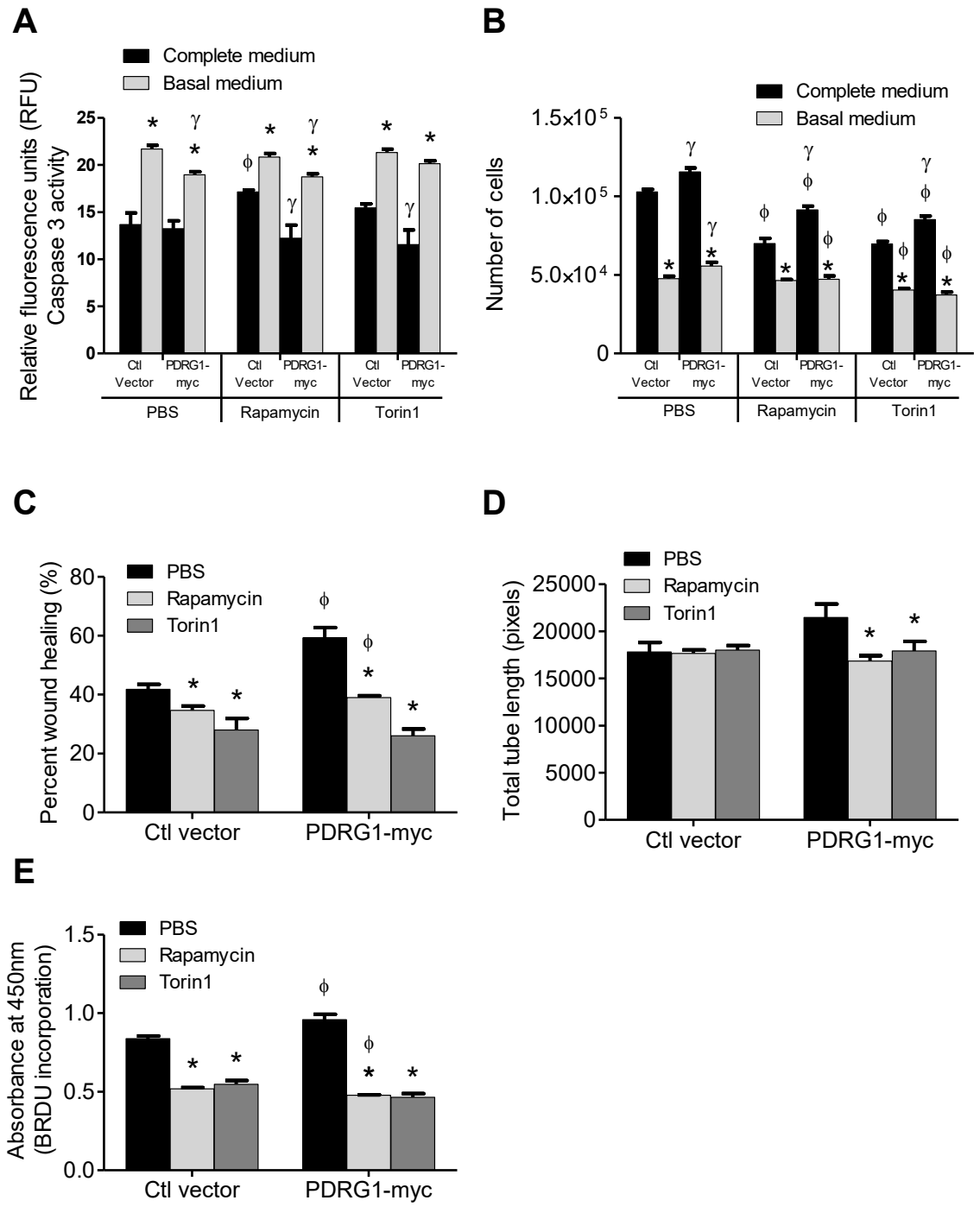



**Figure 8: Dependence of PDRG1-induced angiogenesis on mTORC1.**

**A&B:** HUVECs were transfected with control or PDRG-1 vector. Following 24 h of recovery, equal numbers of cells were maintained in complete or basal medium containing PBS, rapamycin (50 nM), or torin-1 (10 nM). Cell count and Caspase-3 activity were measured 24 h later. Values are means ± SEM. *P<0.05, compared to complete medium; $^{\phi}$P<0.05, compared to PBS; $^{\gamma}$P<0.05, compared to control vector within treatment.

**C:** Percent wound-healing of HUVECs transfected with control or PDRG1-myc vector and wounded at time 0. Cells were maintained for 8 h in medium containing PBS, rapamycin (50 nM), or torin-1 (10 nM). *P<0.05, compared to PBS; $^{\phi}$P<0.05, compared to control vector within treatment.

**D:** Total tube length of HUVECs transfected with control or PDRG1-myc vectors (presented as pixels). Following 24 h of recovery, cells were plated on a layer of growth factor-reduced Matrigel®-coated plates in the presence of PBS, rapamycin (50nM), or torin-1 (10nM) for 24 h. *P<0.05, compared to PBS.

**E:** BrdU incorporation in HUVEC cells transfected with control or PDRG1-myc vectors. Following 24 h of recovery, cells were seeded and 1 h later BrdU was added for 24 h in the presence of PBS, rapamycin (50 nM), or torin-1 (10 nM). *P<0.05, compared to PBS; $^{\phi}$P<0.05, compared to control vector within treatment.

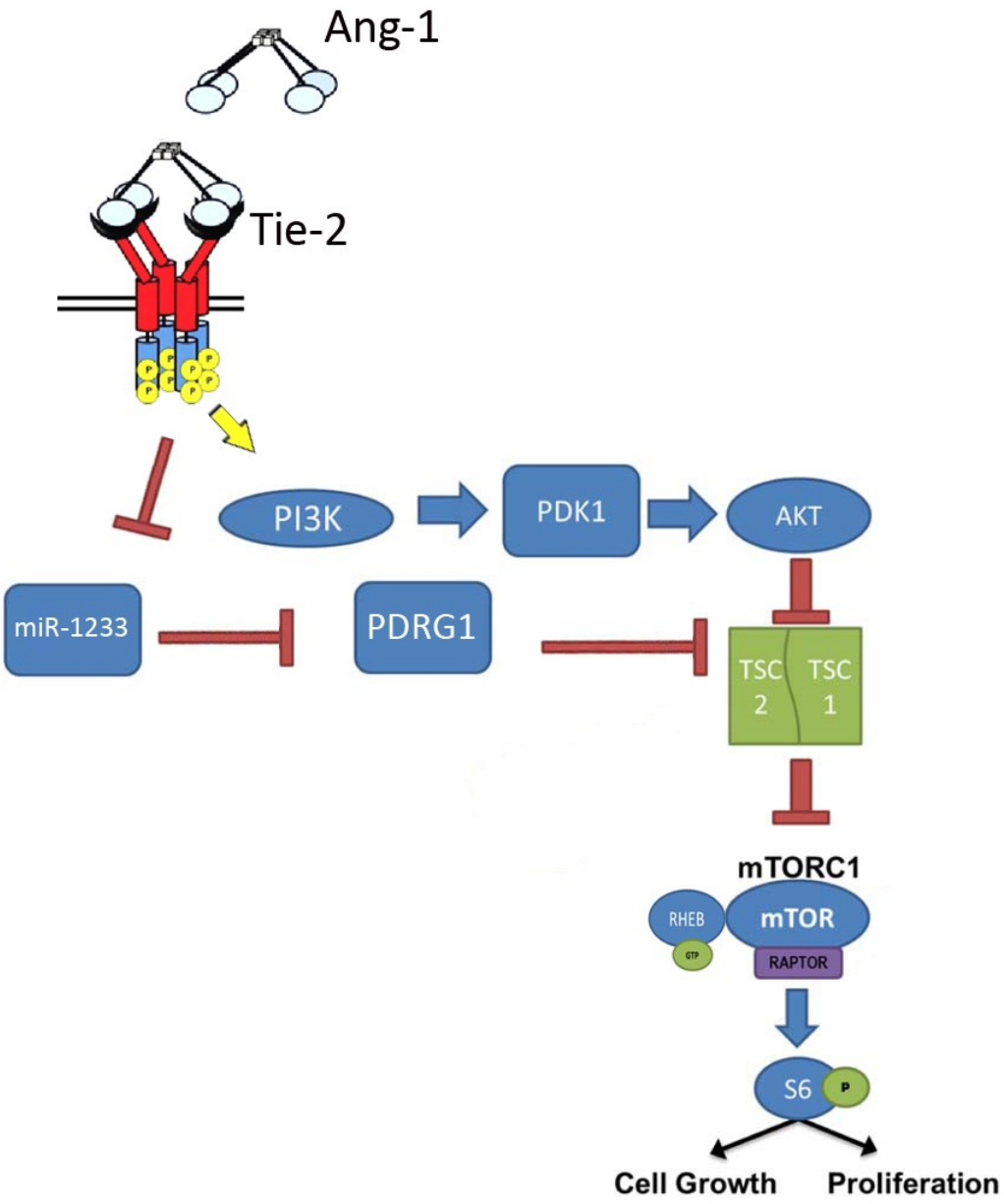
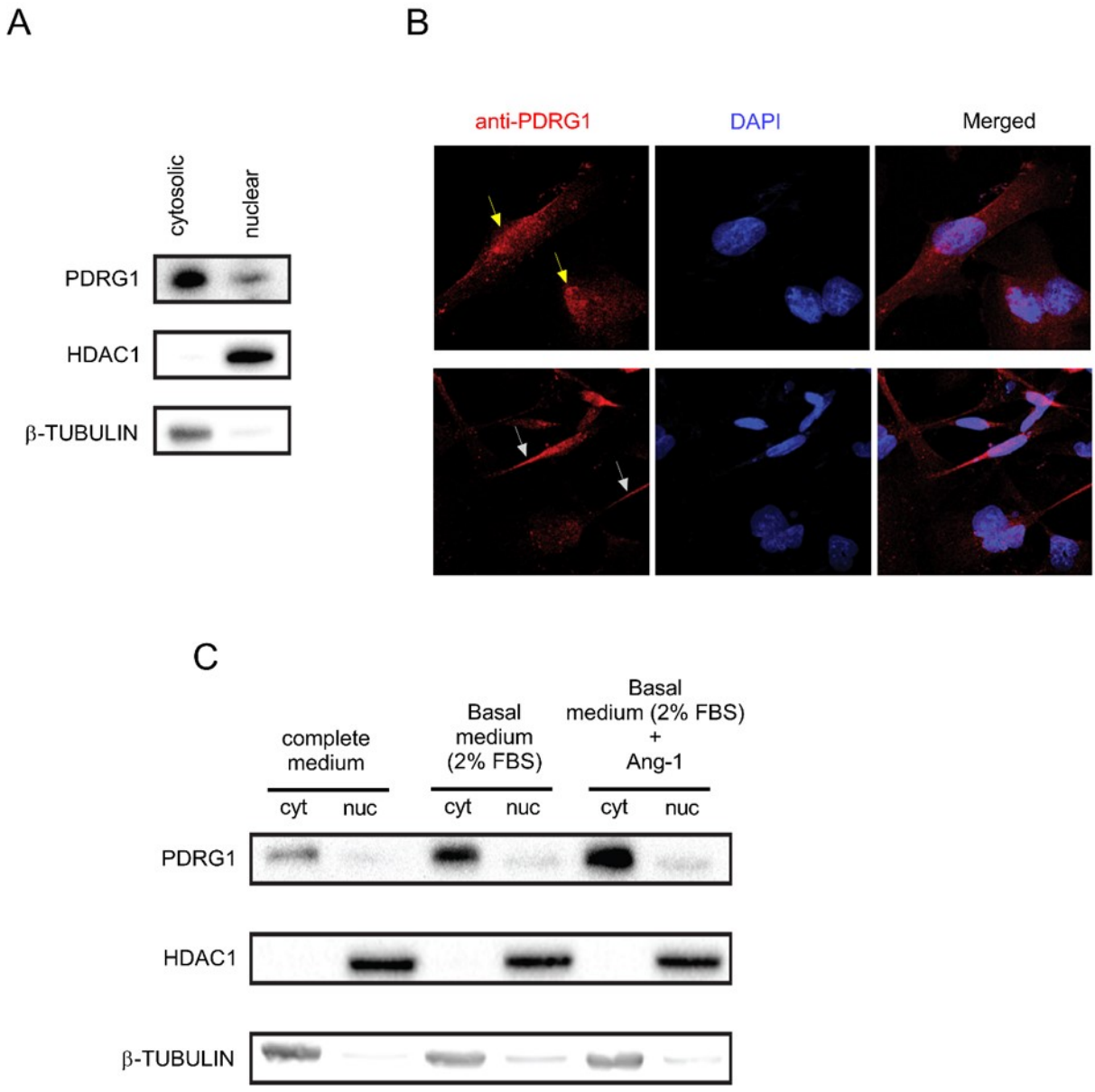



**Figure 9: Proposed model of the roles of miR-1233-3 and PDRG1 in Ang-1-induced angiogenesis.**

9. **Supplementary figures**

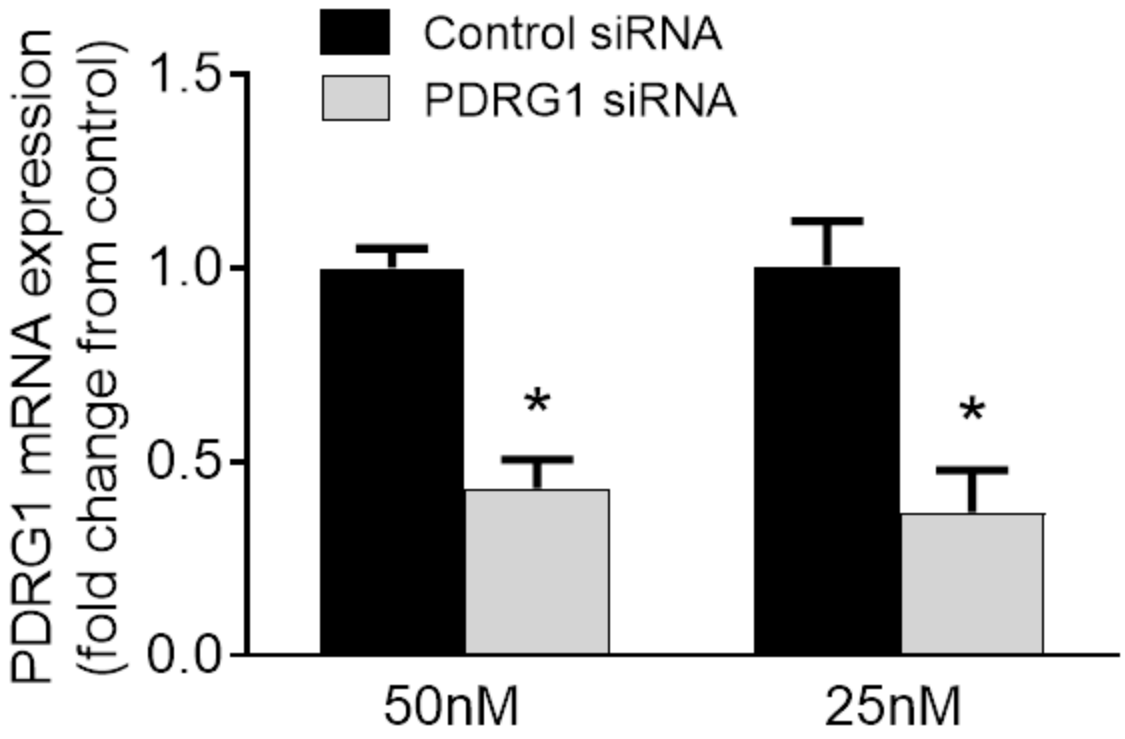



**Supplementary Figure 1: PDRG1 protein localization in ECs.**

**A:** Detection of PDRG1 protein with immunoblotting in the cytosolic and nuclear fractions of HUVECs.

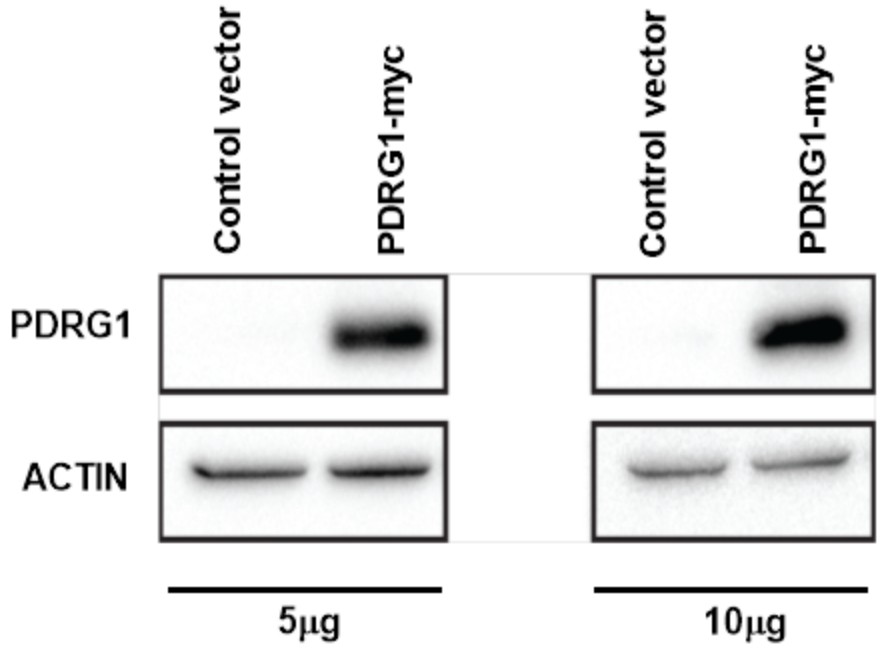
**B:** Detection of PDRG1 protein (red) and nuclei (DAPI, blue) with immunostaining in HUVECs. Images are representative of the cross-section of several HUVECs. Yellow arrows indicate nuclear localization of PDRG1; white arrows indicate cytoplasmic localization of PDRG1.

**C:** Detection of PDRG1 protein with immunoblotting in cytosolic and nuclear fractions of HUVECs maintained for 24 h in complete medium, basal medium (2%FBS), or basal medium containing Ang-1 (300ng/mL) for 24 h. cyt = cytosolic fraction; nuc = nuclear fraction.



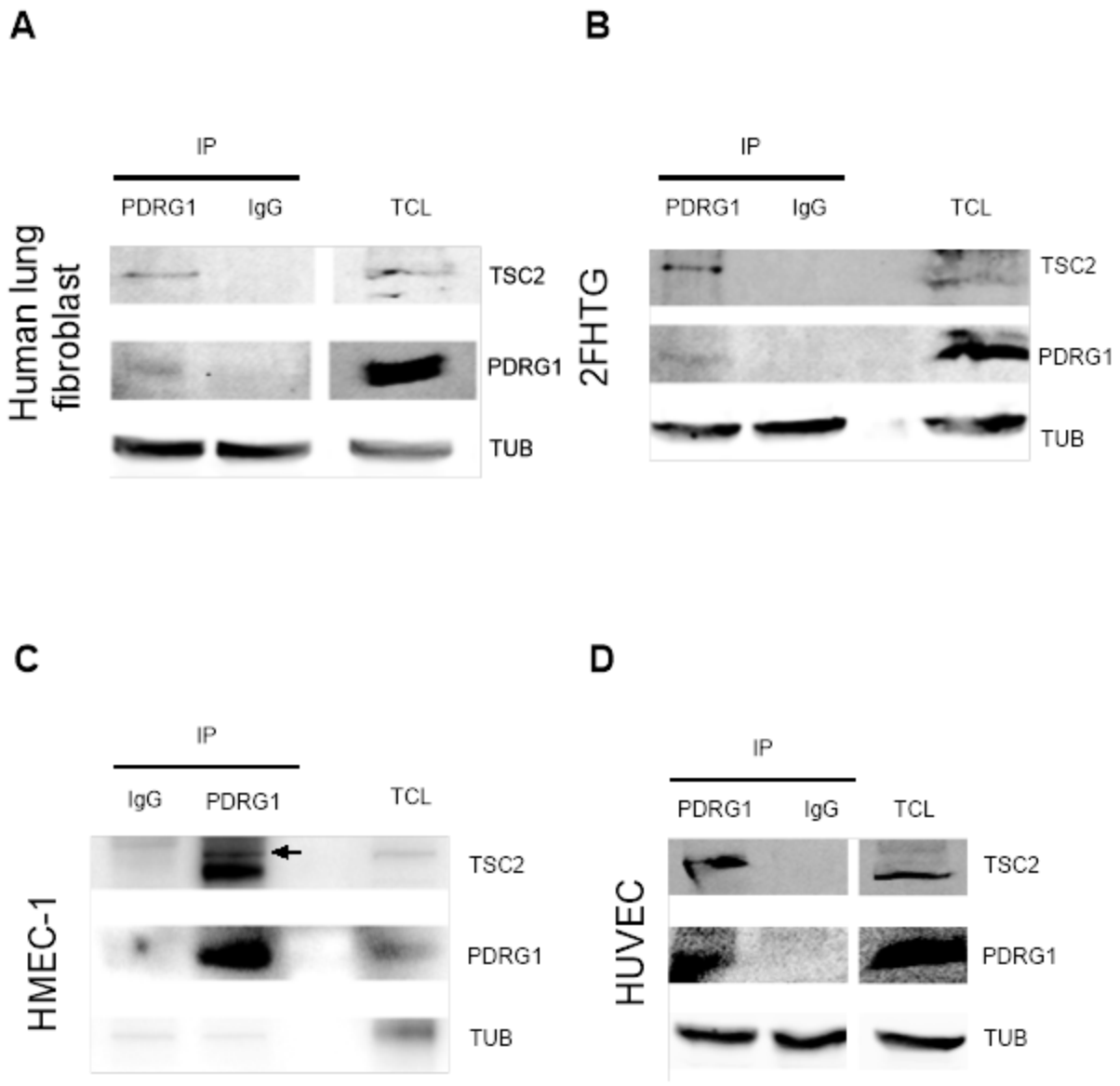

**Supplementary Figure 2: Knockdown of PDRG1 in HUVECs.**

PDRG1 mRNA levels in HUVECs transfected 24 h earlier with either 50nM or 25nM of control or PDRG1 siRNA oligos.



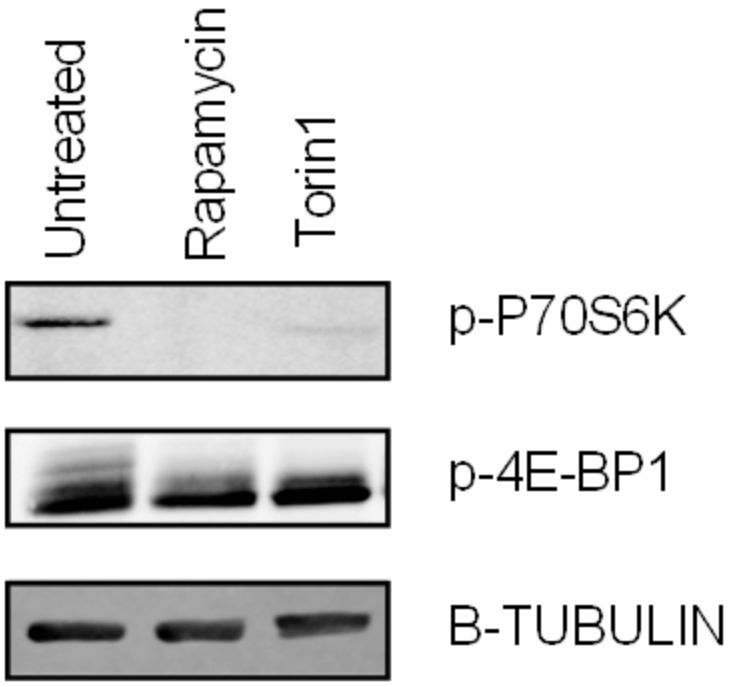

**Supplementary Figure 3: Overexpression of PDRG1 in HUVECs.**

Representative immunoblots of PDRG1 and ACTIN proteins in HUVECs transfected 24 h earlier with 5 μg or 10 μg of control or PDRG1-my vector.




**Supplementary Figure 4: PDRG1 interaction with TSC2 in various cell types.**

Immunoprecipitation (IP) of endogenous PDRG1 and control (IgG) in human lung fibroblasts, 2FHTGs, HMEC-1s, and HUVECs, performed to verify PDRG1 interaction with TSC2.




**Supplementary Figure 5: Inhibition of the mTOR pathway by rapamycin and torin1.**

Representative immunoblots of total and phosphorylated P70S6K and 4E-BP1 proteins in HUVECs treated for 6 h with rapamycin or torin1.

## 10. Tables

**Table 1: Peptides identified by MS of three independent PDRG1 IP experiments.**

| Protein | Accession number | Molecular weight (KDa) | Unique Peptides | | | Percent Coverage (%) | | |
|---|---|---|---|---|---|---|---|---|
| | | | IP1 | IP2 | IP3* | IP1 | IP2 | IP3* |
| PDRG1 | B2R511 | 16 | 4 | 10 | 2 | 35 | 77 | 25 |
| TSC2 | B4DIL8 | 201 | 26 | 18 | 8 | 22 | 16 | 7 |
| MATR3 | B7ZAV5 | 95 | 15 | 25 | 2 | 25 | 31 | 5.1 |
| CCDC91 | A0A024RAW6 | 50 | 10 | 13 | 2 | 27 | 26 | 6.3 |
| HNRNPM | Q8WZ44 | 78 | 8 | 12 | 1 | 13 | 16 | 1.4 |
| AFB | B7ZLP5 | 103 | 2 | 7 | 3 | 4.5 | 9.5 | 5.1 |
| RPS27 | Q5T4L6 | 9 | 1 | 3 | 1 | 15 | 40 | 9.5 |
| RPS3 | B2R7N5 | 27 | 1 | 1 | 1 | 4.9 | 9.8 | 2.5 |
| MFF | A0A0A0MS29 | 38 | 5 | 1 | 2 | 19 | 32 | 15 |
| RPL8 | A8K094 | 28 | 1 | 1 | 1 | 6.2 | 6.2 | 6.2 |

*Endogenous PDRG1 immunoprecipitation

## 11. Supplementary Tables

**Supplementary table 1: Primers used for quantitative real-time PCR experiments.**

| Name | Sequence (5'→3') | Accession number | Expected size (bp) |
|---|---|---|---|
| β-ACTIN | F: AGAAAATCTGGCACCACACC<br>R: GGGGTGTTGAAGGTCTCAAA | NM_001101 | 126 |
| PDRG1 | F: TGGGTGGTTGCTGAATGAAG<br>R: GGTAAGAGGCGCATCCACTC | NM_030815.2 | 222 |

**Supplementary table 2: Peptides identified in at least two of the three independent PDRG1 IP-MS.**

| Protein | Accession number | Molecular weight (KDa) | Unique Peptides | | | |
|---|---|---|---|---|---|---|
| | | | IP1 | IP2 | IP3* | IgG |
| PDRG1 | B2R511 | 16 | 4 | 10 | 2 | 0 |
| TSC2 | B4DIL8 | 201 | 26 | 18 | 8 | 0 |
| MATR3 | B7ZAV5 | 95 | 15 | 25 | 2 | 0 |
| CCDC91 | A0A024RAW6 | 50 | 10 | 13 | 2 | 0 |
| HNRNPM | Q8WZ44 | 78 | 8 | 12 | 1 | 0 |
| AFB | B7ZLP5 | 103 | 2 | 7 | 3 | 0 |
| RPS27 | Q5T4L6 | 9 | 1 | 3 | 1 | 0 |
| RPS3 | B2R7N5 | 27 | 1 | 1 | 1 | 0 |
| MFF | A0A0A0MS29 | 38 | 5 | 1 | 2 | 0 |
| RPL8 | A8K094 | 28 | 1 | 1 | 1 | 0 |
| HPX | B2R957 | 52 | 0 | 8 | 0 | 0 |
| THBS1 | B9EGH6 | 129 | 1 | 9 | 0 | 0 |
| AHSG | B2R7G1 | 39 | 2 | 6 | 0 | 0 |
| CDC25B | B3KS38 | 65 | 7 | 7 | 0 | 0 |
| EHBP1L1 | Q8TB89 | 162 | 9 | 6 | 0 | 0 |
| HAUS6 | B3KPK4 | 109 | 4 | 6 | 0 | 0 |
| TRIM21 | P19474 | 54 | 5 | 6 | 0 | 0 |
| SERPINE1 | A0A024QYT5 | 45 | 3 | 5 | 0 | 0 |
| ITIH2 | Q14659 | 106 | 1 | 4 | 0 | 0 |
| PIP | A4D2I1 | 17 | 0 | 3 | 1 | 0 |
| ARGLU1 | B4E0Y3 | 33 | 2 | 3 | 0 | 0 |
| C9 | P02748 | 63 | 1 | 2 | 0 | 0 |

| | | | | | | |
|---|---|---|---|---|---|---|
| HIST3H3 | B2R5K3 | 16 | 2 | 2 | 0 | 0 |
| IGHG4 | P01861 | 36 | 2 | 3 | 0 | 0 |
| KRT18 | A0A024RAY2 | 48 | 6 | 3 | 0 | 0 |
| RBM39 | A5D8W2 | 59 | 3 | 2 | 0 | 0 |
| RPSA | A0A024R2P0 | 33 | 1 | 3 | 0 | 0 |
| TJP2 | B7Z954 | 134 | 26 | 2 | 0 | 0 |
| TSC1 | B7Z897 | 130 | 1 | 2 | 0 | 0 |
| CCT3 | B3KX11 | 61 | 1 | 2 | 0 | 0 |
| FHL2 | Q14192 | 32 | 1 | 2 | 0 | 0 |
| MUCL1 | Q96DR8 | 9 | 0 | 2 | 1 | 0 |
| PDLIM5 | B7Z481 | 64 | 1 | 2 | 0 | 0 |
| RPL18 | A0A024QZD1 | 22 | 1 | 2 | 0 | 0 |
| RPS11 | B2R4F5 | 18 | 1 | 2 | 0 | 0 |
| RPS18 | P25232 | 18 | 6 | 2 | 0 | 0 |
| SFN | Q96DH0 | 28 | 1 | 2 | 0 | 0 |
| SLC25A3 | A0A024RBH9 | 40 | 1 | 2 | 0 | 0 |
| ATP5C1 | A8KA31 | 33 | 2 | 1 | 0 | 0 |
| ATP5J2 | C9J8H9 | 11 | 1 | 1 | 0 | 0 |
| COL1A1 | Q7KZ34 | 139 | 1 | 1 | 0 | 0 |
| DDX3X | A8K538 | 73 | 1 | 1 | 0 | 0 |
| FBXO22 | Q8IXW3 | 45 | 1 | 1 | 0 | 0 |
| HAUS7 | B4DUH6 | 41 | 3 | 1 | 0 | 0 |
| RPL23A | B2R5B2 | 18 | 1 | 1 | 0 | 0 |
| RPLP0P6 | Q8NHW5 | 34 | 1 | 1 | 0 | 0 |
| RPS29 | A8MZ73 | 7 | 1 | 1 | 0 | 0 |
| NCCRP1 | Q6ZVX7 | 31 | 1 | 0 | 1 | 0 |

*Endogenous PDRG1 immunoprecipitation

# CHAPTER V: DISCUSSION AND OPPORTUNITIES FOR FUTURE RESEARCH

The general purpose of this dissertation is to understand how Ang-1 regulates angiogenesis. A large amount of work has been done on signaling pathways and subcellular mechanisms through which angiopoietins regulate angiogenesis. Substantial literature exists regarding the expression of angiopoietins in various types of cancer [408]. In addition to their roles in physiological angiogenesis required for wound healing and tissue regeneration, there is evidence that angiopoietins also contribute to pathological angiogenesis such as that in solid tumors, vascular eye diseases, and cardiac diseases [409-411]. Despite the major progress made in exploring the mechanisms of angiopoietin-induced angiogenesis, little information is available regarding non-coding RNAs in regulating Ang-1-mediated angiogenesis. The work presented in this thesis is aimed at understanding the roles of various miRNAs in the regulation of angiogenesis in general and Ang-1-induced angiogenesis in particular.

Our studies, in which we profiled miRNA expression in ECs exposed to Ang-1 revealed that Ang-1 elicits significant alterations in the expression of several poorly characterized miRNAs. A specific set of downregulated miRNAs were selected for further analyses in terms of functional relevance to angiogenesis. The results indicate that several miRNAs whose expression is downregulated by Ang-1 appear to serve as anti-angiogenic miRNAs that suppress EC survival, proliferation, migration, and differentiation. Additional analyses of one of these miRNAs (miR-1233-3p) revealed that the anti-angiogenic effects of this particular miRNA are related to specific targeting of poorly characterized protein, namely, PDRG1.

1. **miRNAs**

    1.1. **miRNAs as therapeutic targets**

As a result of the discovery of miRNAs, several novel therapies to treat deregulation of angiogenesis have mainly targeted the ligands they bind to. By far the most commonly used anti-angiogenesis agents are those targeting the VEGF pathway, as in the case of Bevacizumab. This drug is used in combination with chemotherapy to treat colorectal, non-small cell lung, breast, and renal cell cancers, and glioblastoma [412]. Unfortunately, anti-VEGF agents failed to improve overall survival, either as a monotherapy or in combination treatments in many cases. In conclusion, the efficacy of these anti-VEGF therapies is suboptimal and the duration of response is often short-lived, as it has been reported that many tumors do not respond to anti-VEGF therapies or regain growth by developing evasive resistance [413].

Over the past several years, several strategies of miRNA-based tumor treatment have been developed. These can be classified into two categories [414]. The first inhibits proliferation or induces apoptosis in tumor cells. The second inhibits the function of miRNAs that are overexpressed in tumors by applying an antisense oligonucleotide strategy, including anti-miRNA oligonucleotides (AMOs), miRNA antagomirs, locked-nucleic-acids antisense oligonucleotides (LNAs), miRNA sponges, multiple-target anti-miRNA antisense oligodeoxyribonucleotides (MTg-AMOs), and nanoparticles [415-418].

To date, more than twenty clinical trials have been initiated using miRNA- and siRNA-based therapeutics [419]. The possibility of miRNAs as a therapeutic agent was somewhat confirmed in our studies. We report that overexpressing specific mature miRNAs using mimics triggered significant decrease in angiogenic processes such as EC proliferation, migration and differentiation. Ideally, miRNAS that have been identified would be further studied in an *in-vivo* angiogenesis models to confirm our results that

we obtained in cultured ECs. Perhaps the best-known miRNA therapy is compound SPC3649, an inhibitor of miR-122, a product developed by Santaris Pharma in Denmark [420]. This product is the only one in this category that has entered a clinical trial. Another ongoing trial using the liposomal miR-34 mimic, MRX34, has shown that this molecule has anti-tumor effects [421]. Initial *in-vivo* and *in-vitro* studies showed that miR-34 was able to regulate more than thirty oncogenes. The targeted diseases have been primary liver cancer, lymphoma, melanoma, multiple myeloma, and renal cell carcinoma [422].

One limitation of our studies is that we only tested the angiogenic properties of a single miRNA at a given time but we never combined two or more miRNAs to evaluate their combinatorial effects on angiogenic process. Recent studies have revealed that diseases like cancer are not a single-molecule problem but are linked to a combination of growth factors, miRNA, long non-coning RNAs, and other epigenetic modulators. In our case, it would be interesting to study the effects of mir-103b, miR-330, miR-557, miR-575, miR-668, miR-1287, miR1468, and miR-1233 when used together as a treatment in models of pathological angiogenesis. Moreover, it would be interesting to evaluate the effects of these miRNAs on vessel regression when all are introduced at the same time

### 1.2. **miRNA as biomarkers**

Circulating miRNAs have been studied as biomarkers under several conditions. In fact, several studies have reported the deregulation of miRNAS in different human diseases such as cancers and cardiovascular diseases [423]. Many non-coding RNAs are located in areas of the human body known for their instability and often associated with

malignancies. In addition, the modifying profile of several miRNAs has been implicated in several diseases. The miRNAs studied in this thesis, however, have not been identified as biomarkers of diseases. Future studies designed to evaluate miR-103, miR-330, miR-557, miR-575, miR-1287, miR-1468, and miR-1233 as possible biomarkers of vascular pathologies or cancers are warranted.

Circulating miRNAs have been identified in extracellular vesicles, including exosomes. Exosomes are vesicles derived from the endocytic membrane of cells and sometimes from the actual cell membrane. Numerous studies have shown the potential of exosomes in disease diagnostics and monitoring. Multivesicular bodies can release exosomes into body fluids [424]. Exosomal miRNA has been detected in the peripheral blood as well as in other bodily fluids, making them prime candidates for diseases, since they express the same proteins as their parental cells [425]. It would be interesting to evaluate the expression of miR-103, miR-330, miR-557, miR-575, miR-1287, miR-1468, and miR-1233 in plasma exosomes of patients with vascular diseases and cancer to evaluate their potentials as biomarkers.

### 1.3. **Ang-2 and other MAPK regulating factors**

Clinical research regarding the therapeutic potentials of targeting Ang-1 is far less widespread than those targeting VEGF and Ang-2. Several reports have indicated that targeting Ang-1 is not sufficient to limit solid tumor angiogenesis as compared to targeting VEGF, [426]. Trebananib (AMG386) is a first-in-class selective antagonist peptide-Fc fusion protein inhibiting the interactions between Ang-1, Ang-2, and Tie-2 [427]. In xenograft models, this agent demonstrated potent anti-tumor activity, accompanied by suppressed EC proliferation [428]. Phase 1 clinical trials of trebananib

in patients with advanced solid tumors showed favorable tolerability and preliminary anti-tumor activity [429]. However, using AMG386 was no more effective than blocking Ang-2 alone with nesvacumab. Moreover, the effects of using aflibercept to inhibit VEGF decreased vascular permeability and the presence of tumor-associated macrophages is enhanced when Ang-1 supplements nesvacumab, rather than when using nesvacumab alone [430].

We demonstrated in this thesis that the expressions of miR-103, miR-330, miR-557, miR-575, miR-1287, miR-1468, and miR-1233 miRNAs are downregulated by Ang-1 in ECs. However, the decrease in some these miRNAs was also observed in response to other angiogenesis factors including Ang-2, VEGF, and FGF-2. All these factors have in common the ability to activate several members of the MAPK family. For instance, Ang-2 is able to bind t Tie-2 receptors as well as integrins and its role in tumor angiogenesis has been previously described [431]. There is strong evidence that Ang-2, like Ang-1, activates the ERK1/2 and p38 members of the MAPK family [118]. Ang-2 has also been shown to induce the activation of the ERK/MSK1/CREB pathway to impart cell survival [432]. Similarly, VEGF is a mitogen for ECs that stimulates proliferation through VEGFR2-induced activation of the RAS/RAF/ERK/MAPK pathway [433]. VEGF can also stimulate p38, which results in activation of MAP kinase activated protein kinase-2/3 and phosphorylation of the F-actin polymerization modulator and heat shock protein 27 (HSP27) [434]. Fibroblast growth factor 2 (FGF2) is one of the most well-studied members of the fibroblast growth factor superfamily, and it has been implicated in cellular processes and paradigms as diverse as mitogenesis, differentiation, proliferative lifespan, survival, oncogenesis, and stem cell self-renewal,

among others [435, 436]. FGF2 activates FGFRs and several downstream signaling pathways including several members of MAPK family including ERK1/2 and p38 [437-439]. On the basis of these studies, we speculate that the downregulation of anti-angiogenic miRNAs observed in response to Ang-1, Ang-2, VEGF, and FGF-2 may be mediated in part through the MAPK pathways. The involvement of other signaling pathways downstream from Tie-2, VEGFR1, VEGFR2 and FGFRs such as the PI3 Kinase/AKT pathway in the regulation of miRNA expression in ECs cannot be excluded.

## 2. **miR-1233**

miR-1233 was identified during our screening for miRNAs that were downregulated by Ang-1. We decided to explore the functional role of miR-1233 in the regulation of Ang-1-induced angiogenesis on the basis of literature that linked it to angiogenesis [361, 362, 369, 440].

### 2.1. **Understanding miR-1233**

Few studies have described the expression of miR-1233-3p in various cells but its biological function remains largely unidentified. Zhong et al. [369] reported that miR-1233 expression is significantly elevated in placental tissue derived from hypertensive disorder complicating pregnancy. These authors also reported that miR-1233-3p selectively targets HOXB3 [369]. HOXB3 is a transcription factor that has been linked to enhanced cancer cell migration and progression [441, 442]. Together these results suggest that miR-1233-3p may negatively regulate cancer cell migration and invasiveness by selectively targeting HOXB3.

To understand more about the cellular pathways regulated by miR-1233-3p, one needs to study how the whole transcriptome and proteome are altered by this miRNA. The transcriptome is the complete set of mRNAs in a cell, and their quantity. This information allows the construction of a gene expression profile in a specific developmental stage or physiological conditions [443]. Understanding the transcriptome is essential for interpreting the genome and for understanding various biological processes. Several technologies have been developed to quantify changes in the transcriptome. Such tools are mostly hybridization-based or sequencing- based. Hybridization approaches usually involve incubation with fluorescently labeled cDNA with an array of probes [444]. Although relatively inexpensive, these methods rely on pre-existing knowledge of the genome sequence and are prone to high background levels, which can be difficult when exploring low-expression genes. The other major technology directly determines the cDNAs through sequencing. RNA-sequencing, a sequencing-based technology does not required pre-existing knowledge of the genomic sequence to be performed. In fact, this technology can be used to identify *de-novo* gene isoforms and, in some cases, understand the location of newly identified genes. In addition, RNA-seq can reveal genome variations like single nucleotide polymorphisms in transcribed regions [445, 446]. In addition, compared to hybridization methodologies, RNA-seq has very low background and does not have an upper limit of quantification corresponding to the number of sequences obtained. RNA-Seq has also been shown to be highly accurate for quantifying gene expression levels [447]. The transcriptome of ECs measured in response to miR-1233-3p overexpression or knockdown can reveal potential pathways of angiogenesis or cell survival and differentiation that are regulated

by miR-1233-3p. In addition, RNA-seq can point to regulation of other non-coding RNAs that could be regulated by miR-1233-3p.

The proteome, the expression of proteins expressed by a cell, can help support the results produced by the RNA-seq approach. This other piece of information can help determine how translational repression produced by miR-1233-3p changes the translation of other proteins, considering that changes at the mRNA level do not always correlate with changes at the protein level.

### 2.2. **miR-1233-3p clinical relevance as biomarker in vascular diseases**

Much of the studies of miR-1233-3p have linked its elevated expression to malignancies, suggesting that it can be used as a biomarker. Nonetheless, no investigation has been done to relate miR-1233-3p to vascular diseases. The closest piece of information in the vascular field has been through studies in hypertension that found miR-1233-5p to be upregulated [366]. However, the fact that both transcripts originate from the same genomic region, miR-1233-3p and miR-1233-5p target different mRNA molecules and therefore participate in different pathways. Studies in pregnancy-related illness have shown miR-1233-3p to be deregulated [369]. Some of these conditions are actually vascular related, indicating that miR-1233-3p may be linked to vascular tumors or malformations.

Multiple miRNAs have been found to be linked to several vascular anomalies. For example, studies in stenotic and atherosclerotic arteries have found several miRNAs to be deregulated. In particular, miR-21, miR-145, and miR-146 have been highly implicated in animal models but also in clinical settings [448, 449]. In addition, many of

these miRNAs target molecules are directly linked to angiogenesis. The fact that miR-1233-3p is not conserved in rodents renders its evaluation in animal models of vascular diseases quite difficult. Such a scenario leaves the evaluation of the functional roles of miR-1233-3p to be performed only in *in-vitro* settings. Another approach to explore the functional roles of miR-1233-3p is evaluate the expression of this miRNA in tissues obtained from patients with vascular diseases or those with highly vascularized tumors. These studies are likely to shed an important light on the potentials of miR-1233-3p as an anti-angiogenic therapy.

3. **PDRG1**

In this thesis, we identified PDRG1 as a direct target of miR-1233, and reported that PDRG1 functions as promoter of angiogenic processes through its effect on mTORC1 activation. The interactions between PDRG1 and the mTORC1 pathway were identified using miRNA pull-down technique in which biotin-labeled miRNA was transfected and was then pulled down using streptavidin-coated beads. All miRNA::mRNA complexes are extracted and further identified using Mass Spectrometry. We also used another technique in which a luciferase expression vector that contains the 3' UTR of PDRG1 mRNA was transfected into ECs and then miR-1233-3p mimic was introduced in these cells. This technique identifies the regions of PDRG1 mRNA which interact directly with miR-1233-3p.

PDRG1 attracted research interests when its link to the well-studied p53 was first identified. Its protein structure and motifs have been identified. Despite the progress in identification of its structure, there is still a debate about its intracellular localization. One group of researchers provided evidence that PDRG1 is a cytosolic protein, while

others suggested it could also be expressed in the nucleus [379, 385]. In fact, most of the identified functions of PDRG1 are related to nuclear processes. In our case, we identified PDRG1 in two intracellular domains of ECs. While most of PDRG1 protein can be detected in the cytoplasm, relatively weak expression can also be detected in the nucleus. This finding is in agreement with previous reports [379, 395]. We should emphasize that our study is the first to link PDRG1 to the mTORC1 pathway and is the first to identify a potential interaction between PDRG1 and TSC proteins. The major limitation of our study is that it was performed entirely on cultured ECs, and, therefore there is a need to verify our findings including the possible interactions between PDRG1 and the mTORC1 pathway in in-vivo vascular tissues.

Unlike miR-1233, PDRG1 is conserved in many species, which enables investigators to study its function in animal models of angiogenesis [395]. Interestingly, its gene has yet to be knocked out to assess its function in embryonic development. With the advent of newly identified CRISPR/Cas9 technique, it should be very feasible to perform such as assessment. The CRISPR/Cas9 technology, developed in mammals in the laboratory of Dr. Feng Zhang of MIT, is simpler and less costly than previous techniques for in vivo gene knockout. Unlike conventional cloning and homologous recombination, CRISPR technology is able to modify genomic sequences much faster fashion and can also target more than one gene simultaneously. CRISPR was first discovered in archea and later in bacteria, in a laboratory in Spain, where it was proposed to be part of the bacterial immune system defense against viral attacks [450]. This technology relies on small sequences used as guidance to find matching sequences in the genome, the target [451]. When the target is found, another component of the system, an

endonuclease, binds to the DNA and cuts it. The genome undergoes a process of repair, which is known to be error prone, thus introducing missense mutations that can lead to gene knockout or protein products that are truncated or lack functionality. However, when introducing CRISPR, a complementary sequence to the targeted site can also be introduced [451]. This sequence can serve as a template during gene repair, thus allowing one to change the genome to a desired code. This technique allows for correction of gene mutations into wild type sequences.

The use of CRISPR could be extended to create knockout rodent models, in which angiogenesis could be studied *in-vivo*. In addition, the gene mutation could be rescued to assess the specificity of PDRG1 in regulating angiogenesis. This knockout could also help in studying the relationship of other molecules known to regulate angiogenesis, such as VEGF and FGF, which were found to regulate PDRG1 at the protein level. Moreover, it could help further study the mTOR pathway and its downstream regulation of other pathways.

### 3.1. **Therapeutic opportunities for PDRG1**

Expression and functional roles of PDRG1 in ECs have never been investigated. Previous studies have focused on assessing the expression and the regulation of cell growth by PDRG1 in various types of cancer including colon, rectum, ovary, lung, stomach, breast, and uterus cancers. The positive regulation of cancer cell growth by PDRG1 has been documented in one study in which Inhibition of PDRG1 expression with shRNAs in human colon cancer cells significantly affected cell growth [395]. Moreover, in bladder cancer cells, knocking down PDRG1 results in increased cleaved Caspase-3 levels suggesting that apoptosis is activated [452]. Our studies show that

overexpression of PDRG1 promotes angiogenesis in ECs as indicated by increased proliferation, cell survival, migration, and differentiation, and inhibition of cleaved Caspase-3 activity. To identify the mechanisms through which PDRG1 influences angiogenic processes in ECs, we used immunoprecipitation to pull down PDRG1 proteins and we then performed mass spectrometry of the immunoprecipitates. Our analysis revealed that TSC2 protein was one of the most abundant in the PDRG1 pull down suggesting that there is an interaction between these two proteins. However, whether this interaction is direct or whether the two proteins are parts of a yet to be determined protein complex need further clarification. Another protein which was also detected in the PDRG pull down is TSC1 although its abundance in immunoprecipitates is much lower than that of TSC2. The fact that both TSC1 and TSC2 are likely to interact with PDRG1 and that this interaction may lead to enhanced mTORC1 activity suggest that PDRG1 may not be a feasible target for cancer therapy. This scenrario is because mTORC1 pathway is downstream from various growth factors that targeting growth factors responsible for regulating major cellular processes has been associated with serious adverse events [453]. Such is the case of VEGF [454]. VEGF is expressed in almost all tissues and is a positive regulator of EC survival, migration, and proliferation, among other processes [149]. Inhibition of the VEGF pathway impairs angiogenesis, disrupt vascular integrity, and disturb normal EC interaction with platelets and surrounding tissues. A similar case is encountered when other major growth factors such as FGF2 and angiopoietins are targeted. Targeting a less global molecule more specifically linked to a particular pathway may help solve this problem. In the case of PDRG1, to be a useful therapeutic target more studies would need to be performed to

fully understand its functions and the risks associated with its deregulation. However, its ability to regulate the mTOR pathway suggests that it could be a molecule whose function is critical for normal cellular function.

## 4. Conclusions

In this thesis, we uncovered important roles for miRNAs in the signaling and pro-angiogenic effects of Ang-1. We identified several miRNAs whose expression is significantly downregulated by Ang-1 in human ECs. We also report that these miRNAs which are downregulated by Ang-1 function as anti-angiogenic agents through inhibition of basic angiogenic processes such as EC survival, proliferation, migration, and differentiation. One such miRNA is miR-1233-3p, which we studied in more details and identified PDRG1 as its direct target. We also investigated the role of PDRG1 in angiogenesis and found that Ang-1 upregulated its expression and that PDRG1 interacts with mTOR inhibitors including TSC2, and TSC1, and that it promotes the activation of the mTORC1 complex.

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