# Neutralization of Angiostatin to Promote Therapeutic Angiogenesis

by

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# ABSTRACT

Within diseases such as coronary artery disease (CAD) and peripheral artery disease (PAD) ischemia is induced in tissues due to arterial accumulation of atherosclerotic plaque. A novel therapeutic approach to restore blood flow is to neutralize anti-angiogenic factors that could limit ischemia-induced angiogenesis. Angiostatin is a potent anti-angiogenic factor that reduces endothelial cell migration, proliferation, and survival due to inhibition of an adenosine triphosphate (ATP) synthase localized on the surface of endothelial cells. Importantly, angiostatin inhibits lung endothelial cell expression of matrix metalloproteinase (MMP)-2 and membrane-bound MMP, MT1-MMP specifically during hypoxia; these MMPs are important mediators of endothelial cell migration and angiogenesis. Angiostatin may also contribute to endothelial dysfunction and impaired angiogenic responses by decreasing endothelial nitric oxide synthase (eNOS) as observed in hypoxic lung endothelial cells. Overall, the anti-angiogenic effects of angiostatin can create a poor microenvironment for neovascularization of hypoxic tissues, thereby potentially contributing to the pathology of ischemic vascular diseases. The hypoxic-specific effects of angiostatin on cardiac-derived endothelial cells were investigated *in vitro,* while its effect on angiogenesis *in vivo* was investigated in a hind limb ischemia (HLI) model. Finally, a study to selectively neutralize angiostatin to restore the MMP-mediated angiogenic response of cardiac-derived cells was performed.

Aims: 1. To investigate if angiostatin inhibits MMP-2 and eNOS levels in cardiac-derived human microvascular endothelial cells (HMVEC-C), cells were incubated for 48 hours in normoxic or hypoxic (95% $N_2$, 5% $CO_2$) conditions.

Gelatin zymography and western blot were used to analyze MMP-2 and eNOS protein levels, respectively.

2. To investigate if administering angiostatin (30μg) intraperitoneally in transgenic eNOS-GFP mice will impair angiogenesis in a HLI model.

3. To determine if neutralizing angiostatin (600nM) using ATP synthase α and β subunits (3μM) will restore MMP-2 levels and HMVEC-C migration during hypoxia. MMP-2 protein expression was analyzed via gelatin zymography. A modified Boyden chamber assay was utilized to measure HMVEC-C migration.

4. To determine if the ATP synthase subunits interfere with fibrinolysis *in vitro*, clot formation and breakdown was induced in human platelet-poor plasma via incubation with thrombin (1U/ml) and tissue plasminogen activator (1μg/ml) in the presence of the ATP synthase α, β and δ subunits (3μM).

Results: Angiostatin decreased MMP-2 and eNOS protein expression in hypoxic HMVEC-C. In a HLI model, angiostatin-administrated mice exhibited significantly reduced blood perfusion recovery at day 14, and reduced eNOS-GFP and MMP-2 expression in ischemic tissue in comparison to control mice. Hypoxic HMVEC-C co-treated with angiostatin and the ATP synthase α subunit exhibited increased MMP-2 expression compared to angiostatin alone. Furthermore, hypoxic HMVEC-C co-treated with angiostatin and the ATP synthase α subunit exhibited an average increase in MMP-dependent migration compared to angiostatin alone. Lastly, at concentrations used to neutralize angiostatin (3μM), the ATP synthase subunits did not interfere with fibrinolysis *in vitro.*

Conclusions: During hypoxia, angiostatin reduced HMVEC-C expression of angiogenic mediators, MMP-2 and eNOS, which may be how it exerts its anti-angiogenic effects within an ischemic environment. In a murine HLI model, angiostatin impaired angiogenesis. Finally, neutralizing angiostatin using the ATP synthase α subunit increased MMP-2 expression in HMVEC-C, and resulted in an increase in endothelial cell migration all at a concentration that does not interfere with fibrinolysis. Therefore, using the ATP synthase α subunit to neutralize angiostatin could be a potential novel strategy to promote therapeutic angiogenesis.

# PREFACE

This thesis is an original work by Natasha Prashanti Govindasamy. The research project, of which this thesis is a part, received research ethics approval from the University of Alberta Research Ethics Board, Project name "EFFECTS OF ANGIOSTATIN ON THE ISCHEMIC HIND LIMB", No. AUP00000447, October 31, 2012.

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# LIST OF ABBREVIATIONS

ADP - adenosine diphosphate

AS - angiostatin

ATP - adenosine triphosphate

Bcl-2 - B cell lymphoma 2

bFGF - basic fibroblast growth factor

BSA - bovine serum albumin

CAD - coronary artery disease

cGMP - cyclic guanosine monophosphate

EBM - endothelial basal medium

ECL - enhanced chemiluminescence

ECM - extracellular matrix

eNOS - endothelial nitric oxide synthase

ERK - extracellular signal-related kinase

FBS - fetal bovine serum

FITC - fluorescein isothiocyanate

GFP - green fluorescent protein

GP - glycoprotein

H - hypoxia

HCL - hydrochloride

HIF - hypoxia inducible factor

HLI - hind limb ischemia

HMVEC-C - cardiac-derived human microvascular endothelial cells

HMVEC-L - lung-derived human microvascular endothelial cells

I - ischemic

IF1- inhibitor of F1

KO - knock-out

LLC - lewis lung carcinoma

L-NAME - L-nitro-arginine methyl ester

MMP - matrix metalloproteinase

MW - molecular weight

N - normoxia

NI - non-ischemic

NO - nitric oxide

PAD - peripheral artery disease

PBS - phosphate-buffered saline

PDGF - platelet-derived growth factor

PEX - hemopexin

PI3K - phosphoinositide 3-kinase

PVDF - polyvinylidine difluoride

SDS - sodium dodecyl sulfate

T2D - Type 2 diabetic

TGF-β - transforming growth factor-β

TIMP - tissue inhibitors of matrix metalloproteinases

tPA - tissue plasminogen activator

Trypsin-EDTA - trypsin Ethylenediaminetetraacetic acid

TSP - thrombospondin

TTBS - tween tris buffered saline

uPA - urokinase plasminogen activator

VE-cadherin - vascular endothelial cadherin

VEGF - vascular endothelial growth factor

VEGFR - vascular endothelial growth factor receptor

vWF - von Willabrand factor

# 1. INTRODUCTION

## 1.1 Angiogenesis

Coronary artery disease (CAD) and peripheral artery disease (PAD) are leading causes of death worldwide (Roth *et al.,* 2015), and are characterized by reduced blood flow to tissues due to various vascular problems, such as build-up of atherosclerotic plaque in arteries. This impairment of blood flow or ischemia creates a state of hypoxia that if left untreated, and depending on the anatomical location can lead to the loss of affected limbs, cardiac damage, and heart failure (Giordano 2005). Hypoxia is characterized as a lack of a sufficient supply of oxygen to meet the metabolic demands of a cell or tissue (Semenza 2010). This can lead to a build-up of damaging reactive oxygen species, a loss of the high-energy molecule adenosine triphosphate (ATP), and can ultimately initiate programmed cell death or apoptosis (Saikumar *et al.,* 1998). A persistent hypoxic environment can trigger the growth of new blood vessels around an obstructed artery, otherwise known as collateral blood vessels (Simons 2005, Schaper 2009). Additionally, pre-existing collateral blood vessels can be remodelled to take on the blood supply from the blocked vessel. The growth and remodelling of arteries is referred to as arteriogenesis and is mainly stimulated by an increase in vessel fluid shear stress that is exerted by blood flow along the endothelium (Heil *et al.,* 2006). These collateral vessels can provide relief from the hypoxic state and help decrease the extent of ischemic damage. This is reflected in patients with well-established collateral blood vessels demonstrating improved recovery to an ischemic event (Hansen 1989, Seiler *et al.,* 2003, Ozdemir *et al.,* 2005, Meier *et al.,* 2012, Kim *et al.,* 2016).

The growth and formation of new capillary vessels from pre-existing vasculature is referred to as angiogenesis, and this process can occur both physiologically and pathologically (Risua 1997). Angiogenesis is an essential process in developmental growth, wound healing and reproduction. However, angiogenesis can contribute to a variety of diseases such as cancer, inflammation and diabetic retinopathy (Folkman 1995, Carmeliet 2003). There are two types of angiogenesis: intussusceptive and sprouting angiogenesis. Intussusceptive angiogenesis refers to the splitting of an existing blood vessel into two vessels by which the vessel wall invades into the luminal space, and is an important process in vessel remodeling. Sprouting angiogenesis defines the process of newly forming vessels branching from pre-existing vessels, and is found to be stimulated by hypoxia (Burri *et al.,* 2004, Potente *et al.,* 2011).

Angiogenesis is carried out by endothelial cells which form the lining of all blood vessels, and are separated from the surrounding interstitial matrix by a basement membrane composed of type IV collagen, laminins, perlecan, and other macromolecules (LeBlond and Inoue 1989). This layer of endothelial cells, referred to as the endothelium, functions as a barrier between bloodstream and tissue, and as a regulator of vascular tone, coagulation, immune cell transport and the angiogenic response (Furchgott and Zawadzki 1980, Sumpio *et al.,* 2002, Pober *et al.,* 2009). The endothelium can respond to changes in shear stress, and to changes in oxygen content (Kaunas *et al.,* 2011, Krock *et al.,* 2011). A main stimulus of angiogenesis is hypoxia (Dragneva *et al.,* 2013). Initiation of angiogenesis relies on an ‘angiogenic switch’, which is regulated by the balance and contribution of pro-angiogenic factors and anti-angiogenic factors.

Under physiological conditions, anti-angiogenic factors act to maintain endothelium quiescence. However, a stimulus, such as hypoxia, can upregulate pro-angiogenic factors to an extent to where they predominate in the microvascular environment, and as a result the angiogenic switch is pushed towards initiation of angiogenesis (Hanahan and Folkman 1996). Angiogenesis occurs in two phases: the initiation phase and the stabilization phase. The initiation phase involves endothelial cell release of pro-angiogenic growth factors which stimulate endothelial cell proliferation and promote survival. In addition, proteolytic breakdown of the underlying basement membrane and remodelling of the extracellular matrix (ECM) allows for endothelial cells to migrate through interstitial tissue to form new capillary networks. The subsequent stabilization phase involves vessel lumen formation, deposition of basement membrane proteins, and the recruitment of supporting pericytes for vessel maturation. Within these stages the interaction and activities of various pro-angiogenic and anti-angiogenic factors are involved in regulating this dynamic process. (Risau 1997, Carmeliet 2000).

### 1.1.1 Initiation of Angiogenesis: Vascular Endothelial Growth Factor

Angiogenesis involves the interplay between pro- and anti- angiogenic factors and environmental conditions. In response to hypoxia, endothelial cells will accumulate oxygen sensing hypoxia inducible factor (HIF)-1α (Semenza and Wang 1992, Wang *et al.,* 1995, Lin *et al.,* 2004). HIF-1α is a subunit protein that forms a dimer with the constitutively expressed HIF-1β subunit to form the transcription factor HIF-1 (Wang *et al.,* 1995). Long term responses to hypoxia mediated by HIF-1 results in the transcriptional upregulation of pro-angiogenic factors to initiate angiogenesis. These include platelet derived growth factor (PDGF), basic fibroblast growth factor (bFGF) and a crucial and potent proangiogenic factor called vascular endothelial growth factor (VEGF) (Lin *et al.,* 2004, Byrne *et al.,* 2005, Coultas *et al.,* 2005, Shibuya and Claesson-Welsh 2006, Krock *et al.,* 2011). HIF-1 binds to the hypoxia response elements within the VEGF gene resulting in increased mRNA transcription (Ladoux and Frelin 1993, Forsythe *et al.,* 1996, Ferrara and Davis-Smyth 1997, Huang and Bao 2004). Vascular endothelial growth factor was first functionally discovered by Senger and Dvorak in 1983. It is a secreted endothelial mitogen that is critical in angiogenesis during development, as VEGF knock-out (KO) mice are embryonically lethal (Leung *et al.,* 1989, Carmeliet *et al.,* 1996, Ferrara *et al.,* 1996). Members of the VEGF family include; VEGF-A, VEGF-B, VEGF-C, VEGF-D, and placental growth factor (Hoeben *et al.,* 2004). VEGF-A, or commonly referred to as VEGF, was the first discovered and is considered the main, dominant pro-angiogenic mediator and endothelial cell mitogen in angiogenesis (Holmes and Zachary 2005). Additionally, different isoforms of VEGF-A can be generated based on where the VEGF-A gene is spliced. A few examples include $VEGF_{121}$, $VEGF_{165}$, $VEGF_{189}$, and $VEGF_{206}$ (Tischer *et al.,* 1991, Houck *et al.,* 1991).

VEGF can bind to heparan sulfate, a proteoglycan that is found expressed on cell surfaces, the basement membrane and is secreted into the ECM (Sarrazin *et al.,* 2011). The interaction with heparan sulfate can enhance stabilization of VEGF to its cell surface receptors, and prevent proteolysis and diffusion of the growth factor, thereby acting as a reserve (Olsson *et al.,* 2006, Sarrazin *et al.,* 2011). Additionally, different isoforms of the VEGF-A gene can possess distinct binding capabilities to heparan sulfate within the ECM due to presence or absence of heparin-binding domains within their structure (Houck *et al.,* 1991). VEGF mediates its angiogenic activity via binding of tyrosine kinase receptors, VEGFR-1 and VEGFR-2, which are expressed predominantly on the surface of vascular endothelial cells (Claesson-Welsh and Welsh 2013). VEGFR-2 is considered the main endothelial cell receptor responsible for VEGF mitogenic and permeability activity, while it has been suggested that VEGFR-1 acts as a negative regulator of VEGFR-2 activity as it exhibits low kinase activity in comparison (Terman *et al.,* 1994, Hoeben *et al.,* 2011). Overall, VEGF activation of its endothelial cell receptors stimulates proliferation, promotes survival, and enhances vessel permeability (Ferrara 1993, Roberts and Palade 1995).

Initiation of sprouting angiogenesis involves formation of endothelial cell sprouts, which are migrating endothelial cells forming new capillary networks. Endothelial cells comprising the sprout differentiate into tip cells at the angiogenic front of the sprout or stalk cells that form the supporting trunk and mediate lumen formation (Potente *et al.,* 2011). This tip versus stalk cell patterning has been shown to be coordinated by the transmembrane protein, Notch, and VEGF signalling (Lawson *et al.,* 2002, Gridley 2010). Furthermore, VEGF serves as a potent stimulus for sprout migration, as endothelial sprouts will follow a VEGF-gradient as they migrate through the interstitial matrix (Gerhardt *et al.,* 2003).

The role of VEGF is not limited to being a chemoattractant for endothelial sprouts, it also stimulates endothelial cell proliferation for formation of new capillary networks (Ferrara 1993). For instance, activation of VEGFR-2 on endothelial cells results in receptor dimerization and autophosphorylation which can stimulate cell proliferation via phospholipase C-γ/protein kinase C/RAS/extracellular signal-regulated kinase (ERK) pathways. Additionally, VEGFR-2 activation also stimulates cell migration via phosphoinositide 3-kinase (PI3K)/p38mitogen activated protein kinase/focal adhesion kinase signalling pathways (Rousseau *et al.,* 1997, Bernatchez *et al.,* 1999, Shibuya 2011, Hoeben *et al.,* 2011). Furthermore, VEGF contributes to hyperpermeability of the vasculature (Bates 2010). Vessel permeability is augmented by loosening of cell-cell adhesion, which can be induced by VEGF-mediated tyrosine phosphorylation of cell adherins, such as vascular endothelial (VE)-cadherin, which are localized in junctions between endothelial cells and maintain adhesive integrity to restrict endothelium permeability (Esser *et al.,* 1998, Falk 2010). As the barrier of the endothelium becomes more permeable, proteins such as fibrinogen, vitronectin and fibronectin are leaked in from the blood, and become part of a framework that serves as a supporting structure for the dynamic environment that endothelial sprouts must traverse (Davis and Sengar 2005).

Endothelial cell apoptosis can impair angiogenesis and contribute to vessel rarefaction (Dimmeler and Zeiher 2000). VEGF protects endothelial cells from apoptosis via activation of PI3K/Akt kinase survival signalling (Gerber *et al.,* 1998). Specifically, VEGF signalling increases endothelial cell expression of anti-apoptotic regulatory proteins, B-cell lymphoma 2 (bcl-2) and bcl-2 related protein, A1.

These proteins act to prevent cytochrome C release from the mitochondria, and inhibit the activity of executioner enzymes, caspases, that carry out the apoptotic cascade (Gerber *et al.,* 1998). Thus, activated endothelial cells participating in angiogenesis depend on VEGF-mediated signalling for survival in a hypoxic environment.

Finally, the angiogenic effects of VEGF during angiogenesis can be attributed to VEGF induced phosphorylation and activation of endothelial nitric oxide synthase (eNOS), an important enzyme responsible for production of another important pro-angiogenic and pro-survival mediator, nitric oxide (NO) (Kroll and Waltenberger 1998, Park *et al.,* 2002).

### 1.1.2 Nitric Oxide and Endothelial Nitric Oxide Synthase

In 1987, the endothelium-derived relaxation factor found to induce relaxation of vessels in response to acetylcholine was identified as NO independently by Moncada and Ignarro, and was named 'Molecule of the Year' in 1992 (Ignarro *et al.,* 1987, Palmer *et al.,* 1987, Moncada and Higgs 2006). It's role in cardiovascular physiology is still being investigated to this day, and it has been essential to the understanding of endothelium function and angiogenesis.

NO is a soluble gas synthesized by nitric oxide synthase (NOS) enzymes. There are three isoforms of NOS, neuronal NOS, inducible NOS, and endothelial NOS (eNOS). With relation to endothelial cells, the main source of NO under physiological conditions comes from the activity of constitutively expressed eNOS (Lamas *et al.,* 1992). This isoform of NOS can be localized within small lipid rafts in the endothelial cell plasma membrane called caveolae. Its activity is inhibited by interaction with a caveolae coat protein, caveolin-1. As intracellular calcium levels rise, eNOS dissociates from caveolin-1 to interact with calcium-activated calmodulin, thus it can exhibit calcium-dependent activation (Ju *et al.,* 1997). However, the activity of eNOS can also be regulated via calcium-independent mechanisms via phosphorylation induced by fluid shear stress pathways or mediated by VEGFR activation or heat shock protein 90 (Dimmeler *et al.,* 1999, Forstermann and Sessa 2012). Upon activation, eNOS converts its substrate, L-arginine, and co-substrates, oxygen and reduced nicotinamide-adenine-dinucleotide phosphate, into NO and L-citrulline.

NO production is essential for the proper function of the endothelium as it mediates one of the endothelial dependent vasodilation pathways, inhibits platelet aggregation, suppresses the inflammatory response, and is an important mediator of angiogenesis (Azuma *et al.,* 1986, Radomski *et al.,* 1987, Rees *et al.,* 1989, Bogdan 2001).

Its role in angiogenesis has been demonstrated in various studies, for example; eNOS-KO mice exhibit reduced wound repair and angiogenesis *in vivo*, and endothelial cells derived from eNOS-KO mice exhibit reduced migration and proliferation *in vitro* (Lee *et al.,* 1999). Furthermore, administering L-nitro-arginine methyl ester (L-NAME), a non-selective NOS inhibitor, prevents endothelial cell capillary formation in response to VEGF *in vitro* (Ziche *et al.,* 1997).

A major physiological stimulus for NO production is fluid shear stress exerted by blood flow on the endothelium surface. Therefore, under physiological conditions there is a continuous basal production of NO within the endothelium (Corson *et al.,* 1996). However, during initiation of angiogenesis several factors promote further NO production such as, VEGF and bFGF (Cooke and Losordo 2002). The close interaction between VEGF and NO is demonstrated *in vitro*, where cultured human endothelial cells release NO in response to VEGF treatment (van der Zee *et al.,* 1997, Hood *et al.,* 1998). Moreover, long-term exposure of endothelial cells to VEGF leads to increased eNOS protein levels (Papapetropoulos *et al.,* 1997). A specific interaction was delineated as VEGF mediates phosphorylation of eNOS at a serine 1177 residue via PI3/Akt kinase which leads to enzyme activation (Dimmeler *et al.,* 1999, Dimmeler *et al.,* 2000).

An important pathway through which NO exerts its physiological effects is activation of soluble guanylyl cyclase which converts guanosine triphosphate to cyclic guanosine monophosphate (cGMP) (Moncada *et al.,* 1991). cGMP is a secondary messenger that activates signalling cascades to induce smooth muscle relaxation and vasodilation (Arnold *et al.,* 1977).

This NO mediated vasodilation in a setting of angiogenesis can potentiate blood flow as an acute response to hypoxia (Krock *et al.,* 2011), while also promoting endothelial cell activation as increased blood flow has been shown to induce endothelial cell proliferation (Cho *et al.,* 1997, Hudlicka 1998). Thus, the hemodynamic effects of NO have a role in activating endothelial cells during angiogenesis.

As mentioned above, pro-survival signalling is crucial for successful neovascularization, as endothelial cell apoptosis can lead to the regression of growing vessels (Dimmeler and Zeiher 1999, Ziche *et al.,* 2000). NO promotes cell survival by interfering with key apoptotic signalling pathways. For example, NO exerts inhibitory effects on pro-apoptotic proteins, such as caspase 3 via S-nitrosylation of the enzyme's active sites (Kim *et al.,* 1997). Alternatively, it inhibits the caspase-mediated breakdown of the anti-apoptotic protein, bcl-2 (Dimmeler and Zeiher 1999). Therefore, NO's role as a pro-survival factor for endothelial cells supports its contribution to endothelium function and angiogenesis.

NO has also been associated with another critical angiogenic process, endothelial cell migration. This is demonstrated in endothelial cells derived from eNOS-deficient mice that exhibit impaired migration and tube formation, which corresponded with a reduction in expression of another mediator of angiogenesis, matrix metalloproteinases (MMPs) (Genis *et al.,* 2007).

### 1.1.3 Matrix Metalloproteinases

An early step of angiogenesis involves endothelial cell migration, whereby endothelial cells separate from the underlying basement membrane to assemble into sprouts which navigate through the ECM to form new vessels. Proteins that make up the endothelial basement membrane and the interstitial spaces include; collagen, laminin, fibrillin, fibrin, entactins, fibronectin and elastins (Mundel and Kalluri 2007). The ECM is not simply a barrier through which endothelial cells must pass; the proteins that comprise the ECM are an essential component to modulating endothelial cell signalling, survival and migration (Davis and Sengar 2005). In fact, ECM proteins have an important role in regulating angiogenesis. During initiation of angiogenesis ECM proteins such as collagen and laminin interact with endothelial cell integrins, such as integrins $\alpha1\beta1$, and $\alpha2\beta1$ that mediate cell adhesion, survival and proliferation (Lamalice *et al.,* 2007). Additionally, the ECM sequesters angiogenic factors such as VEGF, and bFGF, and as remodelling progresses these factors are liberated to modulate the angiogenic process (Kalluri 2003, Davis and Sengar 2005, Lamalice *et al.,* 2007). Remodelling is necessary for the endothelial sprout to migrate and invade into the type I collagen rich interstitial matrix to connect to other vessels (Stetler-Stevenson 1999). Major contributors of the ECM remodelling processes are a family of proteases, called matrix metalloproteinases (MMPs). MMPs are zinc-containing endopeptidases that mediate cleavage of a wide range of proteins, including collagen, that make up the basement membrane and ECM. These enzymes are synthesized in latent forms which upon proteolyzation are converted to active forms. There are 24 different human MMPs, included in Table 1, however there is a fairly conserved structure between the different subtypes.

MMPs contain a pro-domain which when attached via a cysteine switch motif coordinates with the zinc ion within the catalytic domain which prevents substrate binding, thereby maintaining the enzymes inactive zymogen form. Lastly, most MMPs contain a hemopexin-like (PEX) domain attached via a hinge region, which can mediate binding to substrates and cell surface receptors. The membrane-type MMPs have a distinct structure from other subtypes, as they contain an additional transmembrane domain. (Sternlicht and Werb 2001, Rundhaug 2005, Malemud 2006, Fragai and Luchinat 2015).

| GROUP | MMP # |
|---|---|
| | |
| **Collagenase** | |
| Collagenase 1 | 1 |
| Collagenase 2 | 8 |
| Collagenase 3 | 13 |
| Collagenase 4 | 18 |
| **Gelatinase** | |
| Gelatinase A | 2 |
| Gelatinase B | 9 |
| **Stromelysin** | |
| Stromelysin 1 | 3 |
| Stromelysin 2 | 10 |
| Stromelysin 3 | 11 |
| **Matrilysin** | |
| Matrilysin | 7 |
| Matrilysin-2 | 26 |
| **Membrane-type** | |
| MT1-MMP | 14 |
| MT2-MMP | 15 |
| MT3-MMP | 16 |
| MT4-MMP | 17 |
| MT5-MMP | 24 |
| MT6-MMP | 25 |
| Cysteine-array MMP (type II membrane type) | 23 (A and B) |
| **Others** | |
| Metalloelastase | 12 |
| Enamelysin | 20 |
| Epilysin | 28 |
| No Name | 21 |
| No Name | 27 |

**Table 1:** Family of human matrix metalloproteinases.

MMPs have been shown to have significant roles in regulating endothelial cell migration and angiogenesis. A prevalent MMP in the angiogenic process is the gelatinase, MMP-2, as inhibition of its activity in endothelial cells reduces capillary formation *in vitro* (Schnaper *et al.,* 1993). The level to which MMP-2 is expressed and activated is tightly regulated at three stages: transcription, post-translational modification via protease-mediated removal of the pro-peptide domain, and by tissue inhibitors of metalloproteinases (TIMPs) (Sternlicht and Werb 2001, Arpino *et al.,* 2015).

Transcription: Endothelial cells constitutively express MMP-2, however the expression of MMP-2 is subject to regulation by other genes, for example the tumor suppressor protein, p53, which has a binding region within the promotor of the MMP-2 gene (Bian and Sun 1997, Rundhaug 2005). Furthermore, extracellular cues such as cell-cell interactions, cell interactions with surrounding matrix proteins, and cell exposure to hypoxia can influence MMP-2 expression (Haas and Madri 1999, Kiran *et al.,* 2011). This is demonstrated in endothelial cells that express increased levels of MMP-2 mRNA when cultured in three-dimensional type I collagen matrix (Haas *et al.,* 1998), or when exposed to long term hypoxia (Ben-Yosef *et al.,* 2002).

Post-translational Modification: Pro-MMP-2 can be proteolytically activated via removal of the pro domain by a membrane-bound MMP, MT1-MMP. To be activated from its zymogen form by MT1-MMP, secreted pro-MMP-2 requires TIMP-2 as activation of pro-MMP-2 does not occur in TIMP-2 (-/-) cells expressing MT1-MMP (Morrison *et al.,* 2001). TIMP-2 forms a complex with membrane-bound MT1-MMP and pro-MMP-2. The MT1-MMP/TIMP-2 complex serves as an anchor and binds the PEX domain of pro-MMP-2, while a TIMP-2-free MT1-MMP cleaves the pro domain thereby exposing the catalytic site. (Goldberg *et al.,* 1989, Strongin *et al.,* 1995, Butler *et al.,* 1998).

Regulation by TIMPs: MMP-2 activity can be regulated by TIMPs 1-4 (Brew and Nagase 2010). TIMP-2 is an effective inhibitor of MMP-2 activity, via interactions with the MMP-2 PEX domain (Itoh *et al.,* 1998). In addition, at high concentrations TIMP-2 saturates MT1-MMPs thereby preventing a free MT1-MMP from activating latent MMP-2 (Strongin *et al.,* 1993) Furthermore, pro-MMP-2 activation can be negatively regulated via TIMP-4 inhibition of MT1-MMP (Bigg *et al.,* 2001). TIMPs themselves have been found to have both anti-angiogenic and pro-angiogenic activity, and this duality depends on the concentrations present (Qi *et al.,* 2003, Takawale *et al.,* 2017). Thus, the balance between MMP and TIMP levels can determine the overall impact on angiogenic processes (Arpino *et al.,* 2015).

A major target of MMP-2 is type IV collagen, a protein that contributes to the structure of the basement membrane. Proteolysis of type IV collagen promotes detachment of endothelial cells from the underlying basement membrane, thereby allowing for endothelial cell motility (Kalebic *et al.,* 1983). Additionally, MMP-2 co-localizes with the endothelial cell surface expressed integrin $\alpha_V\beta_3$, which mediates endothelial cell migration (Silletti *et al.,* 2001). Membrane bound MT1-MMP not only mediates MMP-2 activation; it also cleaves type I collagen, a protein that is abundant in the ECM (Sato *et al.,* 1997). Cleavage of collagen within the ECM clears a path for endothelial cells to migrate and form new vessels. Localization of MT1-MMP activity is found at the endothelial cell surface, specifically in cell processes called lamellipodia. This serves to provide a more directional and precise breakdown of the ECM, and helps regulate the extent of ECM remodelling during endothelial cell migration (Itoh 2006).

In summary, activities of both MMP-2 and MT1-MMP mediate endothelial cell migration, and ECM remodelling for new capillary formation. Mice deficient in MT1-MMP and MMP-2 are not embryonically lethal, like VEGF-deficient mice, however they do have delayed angiogenic responses (Zhou *et al.,* 2000, Ohno-Matsui *et al.,* 2003). Thus, MMPs are essential mediators within the early stages of angiogenesis.

### 1.1.4 Stabilization Phase

As angiogenesis progresses into the stabilization phase, newly formed vessels must be maintained to ensure viability and maturity. In this phase, endothelial cells begin to form vascular tubules, lumen formation occurs, and deposition of basement membrane matrix proteins is initiated to form a stable vessel (Stratman and Davis 2012). Sprouting endothelial cells are not yet a functioning capillary network without the supporting framework provided by mural cells, specifically pericytes. Pericytes are contractile cells which when closely associated with endothelial cells mediate endothelial cell survival, vessel permeability and capillary blood flow (Ribatti *et al.,* 2011). An important step in vessel maturation is the release of PDGF-B from endothelial cells which through interactions with pericyte expressed PDGF receptor-β has a role in recruiting pericytes to be incorporated into the vessels outer structure (Hellstrom *et al.,* 1999, Gaengel *et al.,* 2009). Pericyte interaction is fundamental to the stabilization of the newly formed capillaries as it promotes endothelial cell quiescence, and allows for VEGF independent survival of the endothelium (Benjamin *et al.,* 1999). Endothelial cell and pericyte contact leads to activation of transforming growth factor-β (TGF-β) signalling pathways which promote pericyte migration, proliferation, and differentiation (Pardali *et al.,* 2010). TGF-β also downregulates endothelial cell VEGFR-2 expression, and inhibits endothelial cell proliferation and migration (Orlidge and D'Amore 1987, Sato and Rifkin 1989, Mandriota *et al.,* 1996). Additionally, endothelial cells express the tyrosine kinase receptor, Tie-2, which when bound to its pericyte-derived ligand, angiopoietin-1, mediates cell survival, and decreases vessel permeability (von Tell *et al.,* 2006). In terms of the angiogenic switch, activities of pro-angiogenic factors such as VEGF and MMPs can be negatively regulated.

Pericytes induce TIMP-2 expression in endothelial cells, which at high concentrations leads to inhibition of MMP activity to reduce ECM turnover (Saunders *et al.,* 2006). Another method is entrapment with a broad spectrum MMP inhibitor, $\alpha_2$-macroglobulin, which is initially a cleavage target of MMPs, however enzyme interaction can induce a conformational change within the substrate thereby trapping the active MMP (Sottrup-Jensen and Birkedal-Hansen 1989, Baker *et al.,* 2002). VEGF pro-angiogenic activity can be negatively regulated via VE-cadherin localizing to VEGFR-2 and mediating its phosphorylation (Zanetti *et al.,* 2002, Simons *et al.,* 2016).

Alternatively, the contribution of anti-angiogenic factors becomes important to promote endothelial cell quiescence (Potente *et al.,* 2011). As the basement membrane and ECM are proteolyzed, anti-angiogenic factors are liberated and serve to negatively regulate the angiogenic process. For example, proteolysis of different types of collagen leads to the release of anti-angiogenic factors endostatin, arresten, canstatin and tumstatin, which act to promote endothelial quiescence by inhibiting endothelial cell angiogenic responses, such as migration and proliferation (O'Reilly *et al.,* 1997, Mundel and Kalluri 2007). Additional important anti-angiogenic mediators such as thrombospondin (TSP)-1 and -2 act to inhibit endothelial cell proliferation and have been shown to prevent activation and promote clearance of MMP-2 to prevent further ECM proteolysis (Bein and Simons 2000, Armstrong and Bernstein 2003).

In this phase, proliferation and migration of active endothelial cells is negatively regulated, and endothelial cells become quiescent and part of a newly formed functioning vessel network. The angiogenic process is complex and many pro- and anti-angiogenic factors can influence and regulate its progression.

Figure 1 represents the different phases of angiogenesis, and includes a few of the mediators that have a role within these distinct phases. An important anti-angiogenic factor that inhibits endothelial cell responses during angiogenesis is angiostatin.

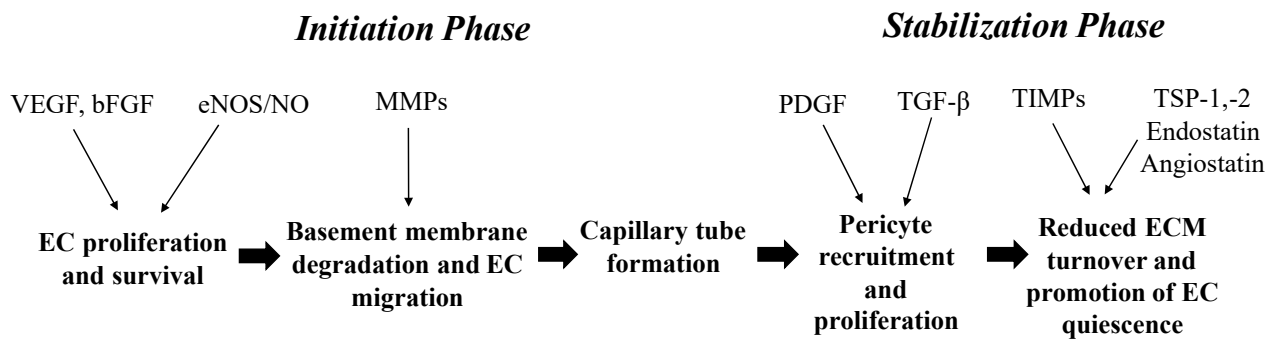


**Figure 1:** Angiogenesis: initiation phase and stabilization phase

## 1.2 Angiostatin

### 1.2.1 Discovery of An Anti-Angiogenic Factor

Angiostatin was discovered by Dr. Judah Folkman's group in 1994 in a Lewis lung carcinoma (LLC) model of concomitant resistance. In this initial study, serum and urine from LLC-bearing mice had inhibitory effects on endothelial cell proliferation. In addition, mice administered dialyzed urine from LLC-bearing mice had decreased metastatic tumor growth. Since metastases and tumor growth require angiogenesis to develop vasculature to supply nutrients and oxygen, the group proposed that a circulating anti-angiogenic factor was preventing metastatic tumor growth via inhibition of tumor-induced angiogenesis. This anti-angiogenic factor was isolated and the group termed it angiostatin (O'Reilly *et al.,* 1994). Angiostatin has been studied mostly in the context of tumor growth for its role in inhibiting tumor-induced angiogenesis (Wahl *et al.,* 2004). Many studies focus on investigating angiostatin in a tumor environment (Cao and Xue 2004), since it was first thought that angiostatin was either released by tumor cells exclusively, or that a tumor-derived factor was inducing its production in the bloodstream. However, it has now been established that angiostatin is present in the human bloodstream under normal physiological conditions (Jurasz *et al.,* 2003). Angiostatin is an internal fragment of the plasma protein, plasminogen (O'Reilly *et al.,* 1994). Plasminogen is a liver-synthesized fibrinolytic protein that can be converted to its active counterpart plasmin by tissue plasminogen activator (tPA), or urokinase plasminogen activator (uPA) (Castillino and Ploplis 2005).

Plasmin is a serine protease that functions to break down fibrin clot matrixes to fibrin degradation products, thereby mediating fibrinolysis. Plasminogen's structure is composed of five triple loop disulfide-linked domains called kringles. Cleavage at different sites by either uPA, tPA, or matrix metalloproteinases can yield various forms of angiostatin, such as angiostatin K1-3, K1-4, K1-4.5 and K1-5 (Stathakis *et al.,* 1997, Soff 2000). Additionally, it can be generated via plasmin autocatalysis following the cleavage of plasminogen to plasmin (Stathakis *et al.,* 1997). Angiostatin fragments each contain a different number of disulfide linked kringle domains. For example, angiostatin K1-3 (molecular weight: 38kDa) contains the first three kringle domains derived from plasminogen, whereas, as represented in Figure 2, angiostatin K1-4 (molecular weight: 50kDa) is composed of the first four. Integrity of the kringle structures is fundamental as disruption of the connecting disulfide bonds results in loss of angiostatin anti-angiogenic activity (Cao *et al.,* 1996). Interestingly, differences in kringle composition of angiostatin have been shown to confer differences in potencies regarding their inhibitory activities (Cao *et al.,* 1996, Ji *et al.,* 1998). For example, angiostatin K1-3 demonstrates potent endothelial cell anti-proliferative activity with a median effective dose of 70nM, compared to angiostatin K1-4 which as a median effective dose of 135nM (Cao *et al.,* 1996). However, potent anti-migratory effects are suggested to reside in kringle 4 domain of angiostatin (Ji *et al.,* 1998). Investigations into angiostatin's anti-angiogenic effects, including clinical trials investigating it as a potential anti-cancer therapy have mainly utilized angiostatin K1-3. While these trials suggest that utilizing recombinant human angiostatin K1-3 is well tolerated and feasible, they have not yet demonstrated significant differences in anti-tumor activity (Soff 2000, Beerepoot *et al.,* 2003, Cao and Xue 2004, Kurup *et al.,* 2006).

Possible reasons could be due to insufficient biological doses that can inhibit later stages of tumor growth, and/or that these studies utilize an isoform of angiostatin that is not predominantly present endogenously within the human bloodstream. In fact, the predominant isoform of angiostatin is angiostatin K1-4, and a primary source is derived from platelets (Jurasz *et al.,* 2003).

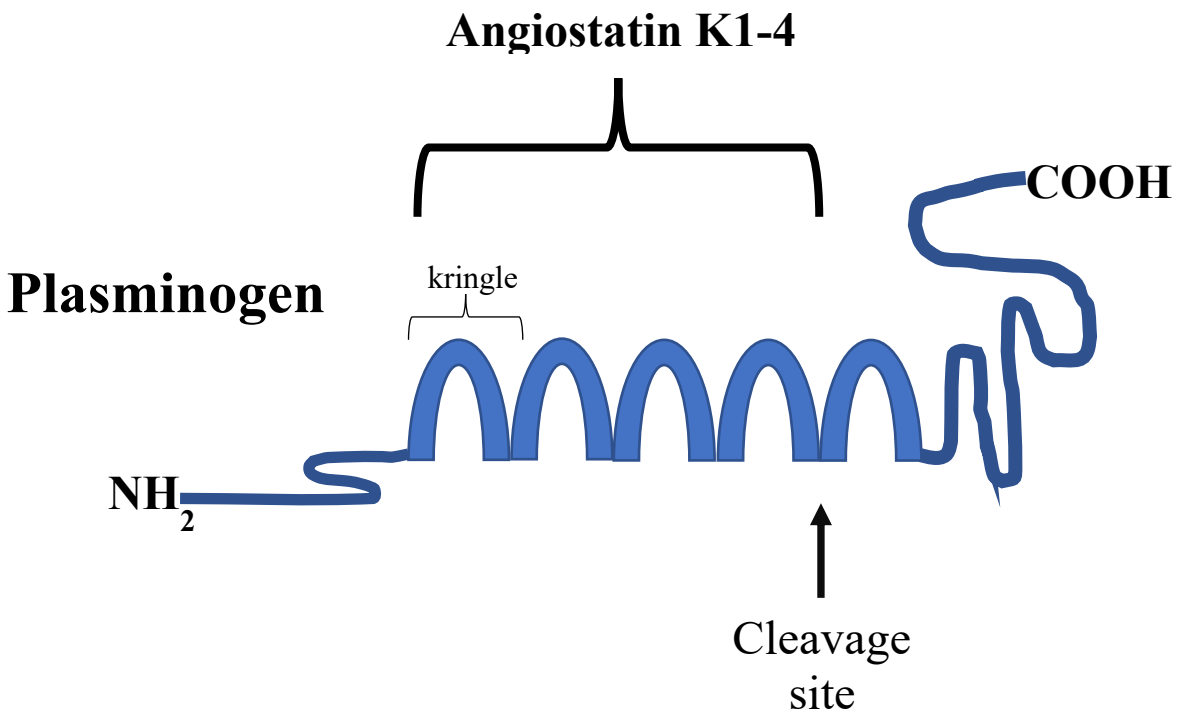


**Figure 2:** Cartoon representation of plasminogen structure indicating the cleavage site to generate angiostatin K1-4.

### 1.2.2 Platelets: An Important Source of Angiostatin

Human platelets are 2μm, disk-shaped, anuclear cells that are derived from precursor bone marrow cells called megakaryocytes. Their primary function involves maintaining hemostasis by forming clots at sites of endothelium injury. Platelets will adhere to sites of injury via interactions between platelet surface von Willabrand factor (vWF) receptors (glycoprotein (GP) Ib/IX/V) and collagen receptors (GPVI and integrin α2β1) and exposed vWF and collagen on the injured endothelium. They will also form a platelet plug by aggregating to each other through binding of GP IIb/IIIa receptors and fibrinogen. (Varga-Szabo *et al.,* 2008) Platelets contain several types of storage granules, including α-granules, dense-granules and lysosomes. Within these granules, platelets store several chemical messengers and proteins such as adenosine nucleotides, serotonin, vWF, fibrinogen, coagulation factors, growth factors and more. Upon activation, platelets release these granular contents leading to a cascade of events that mediate platelet aggregation (Timmons *et al.,*1984, Jurk and Kehrel 2005, Sangkuhl *et al.,* 2011). Additionally, platelets play an important role in angiogenesis as they have been shown to store angiogenic factors within their granules which upon activation are released into the bloodstream (Italiano *et al.,* 2008, Klement *et al.,* 2009, Radziwon-Balicka *et al.,* 2012). For example, during aggregation platelets release pro-angiogenic factors such as VEGF, bFGF, and MMPs. In addition to pro-angiogenic factors, platelets can store and produce anti-angiogenic factors, such as TSP-1, endostatin, and angiostatin (Jurasz *et al.,* 2003, Italiano *et al.,* 2008).

Angiostatin has been found to be constitutively produced by platelets. It can be generated at the platelet membrane surface and be released into the circulation, and can be sequestered and stored within α-granules of platelets which release their content during platelet activation. (Figure 3) (Jurasz *et al.,* 2003, Jurasz *et al.,* 2006). Studies have demonstrated that the physiological concentration of angiostatin within the bloodstream is approximately 600nM, however its plasma levels can almost double, to 1.2μM, for example in a platelet activated environment (Jurasz *et al.,* 2006, Jurasz *et al.,* 2010, Radziwon-Balicka *et al.* 2013). Both angiostatin K1-3 and K1-4 were identified to be produced by platelets, however the predominant form found in the human blood is angiostatin K1-4, as angiostatin K1-3 is produced at low concentrations (Jurasz *et al.,* 2006). Moreover, angiostatin is generated at platelet membrane surfaces via proteolytic cleavage of plasminogen, and subsequent plasmin autocatalysis. This has been demonstrated *in vitro* by the conversion of exogenous plasminogen to functional angiostatin by platelet membranes (Jurasz *et al.,* 2006). Furthermore, aprotinin, a serine protease inhibitor, inhibits plasmin catalytic activity, thereby reducing generation of platelet-derived angiostatin (Jurasz *et al.,* 2006). Matrix metalloproteinases (MMP-2, -3, -7, -9, -12) can generate angiostatin via MMP-mediated cleavage of plasminogen (Cornelius *et al.,* 1998). However, MMP inhibitors do not influence platelet membrane generated angiostatin, suggesting that MMPs are not the primary catalysts of platelet-derived angiostatin production (Radziwon-Balicka *et al.,* 2013). Importantly, platelet membranes incubated with a selective inhibitor of uPA exhibited reduced generation of angiostatin. This suggests that platelet *de novo* generation of angiostatin is mediated via uPA cleavage of plasminogen at platelet membrane surfaces (Jurasz *et al.,* 2006).

Furthermore, platelet release of angiostatin has been temporally characterized at different stages of platelet activation. When platelets are activated with an agonist, such as collagen, angiostatin levels are increased at later stages of platelet activation, specifically at maximal aggregation. In contrast, VEGF release from granular stores peak during platelet shape change, an early stage of platelet aggregation (Radziwon-Balicka *et al.,* 2013). This and other studies suggests that during thrombus formation temporal differences in platelet angiogenic releasates may regulate different stages of angiogenesis (Italiano *et al.,* 2008). Manipulation of platelet activity can influence sources of angiogenic regulators, such as angiostatin. For example, inhibitors of platelet aggregation, NO and prostacyclin, inhibit platelet α-granule release thereby attenuating angiostatin release (Radziwon-Balicka *et al.,* 2013). This demonstrates the contribution of platelets as important sources of angiostatin, and as regulators of the angiogenic process.

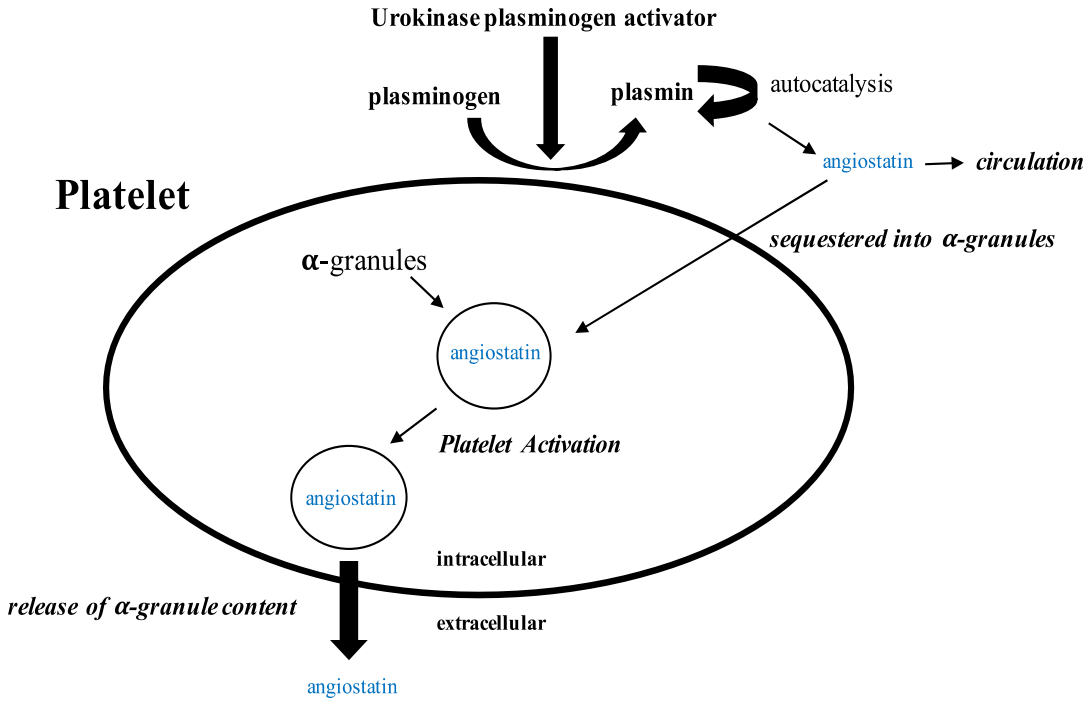


**Figure 3:** Generation of platelet-derived angiostatin.

### 1.2.3 Angiostatin's Anti-Angiogenic Effects

The physiological role of angiogenesis inhibitors is to maintain a state of quiescence within the endothelium, and during angiogenesis they serve as the brakes to prevent excessive vessel growth that can lead to unstable vasculature and contribute to pathological conditions (Folkman 1995, Carmeliet 2003). Elucidating the anti-angiogenic effects of angiostatin's can be complex as researchers often use different forms of angiostatin on different types of endothelial cells within varying environmental conditions. However, overall angiostatin has been shown to inhibit several endothelial cell processes that are important for neovascularization, including proliferation, survival and migration (Claesson-Welsh *et al.,* 1998, Ji *et al.,* 1998, Griscelli *et al.,* 1998, Troyanovsky *et al.,* 2001, Hanford *et al.,* 2003, Sharma *et al.,* 2004).

Exposure of capillary endothelial cells to angiostatin (K1-4) *in vitro* demonstrated that it inhibits endothelial cell proliferation (O'Reilly *et al.,* 1994, Claesson-Welsh *et al.,*1998). In Folkman and colleagues original study elucidating its anti-angiogenic properties, angiostatin had no inhibitory effects on proliferation of LLC cells, human fetal lung fibroblasts, mink lung epithelium cells, or bovine aortic smooth muscle cells (O'Reilly *et al.,* 1994). This led to the understanding that as an inhibitor of cell growth, angiostatin is endothelial cell specific.

Endothelial cell survival is essential in the dynamic vascular environment during angiogenesis, and depending on the concentration, exposure time and environmental conditions, angiostatin's effects can lead to endothelial cell apoptosis (Lucas *et al.,* 1998). For example; human umbilical endothelial cells undergo apoptosis after 36 hours of exposure to angiostatin (K1-4.5) (Hanford *et al.,* 2003).

Furthermore, the dose- and time-dependent anti-proliferative effects of multiple forms of angiostatin (K1-3, K1-4) were demonstrated to be due to cytotoxicity and induction of apoptosis of endothelial cells (Lucas *et al.,* 1998).

Another essential step in angiogenesis is endothelial cell migration, and formation of capillary tubule structures. Human umbilical endothelial cells incubated in a Boyden Chamber assay to assess migration capacity towards a growth factor gradient demonstrated decreased migration with angiostatin (K1-3) treatment (Claesson-Welsh *et al.,* 1998). In addition, angiostatin (K1-4) disrupts endothelial tube formation in three-dimensional angiogenesis assays (Claesson-Welsh *et al.,* 1998, Griscelli *et al.,* 1998, Troyanovsky *et al.,* 2001).

The underlying molecular mechanism of action of angiostatin has not been fully elucidated and remains unclear. Recent work has focused on the anti-angiogenic effects of angiostatin (K1-4) during hypoxia (Radziwon-Balicka *et al.,* 2013). Results from these studies revealed that angiostatin inhibits human lung-derived microvascular endothelial cell migration (HMVEC-L) specifically during hypoxia. Since MMPs mediate endothelial cell migration, upon further investigation, angiostatin was found to inhibit HMVEC-L expression of MMP-2 and MT1-MMP levels during hypoxic conditions. Furthermore, angiostatin was also found to reduce eNOS expression specifically in hypoxic HMVEC-L, suggesting a hypoxic-specific mechanism of action.

## 1.3 Angiostatin's Mechanism of Action

### 1.3.1 Angiostatin and the Microenvironment

Investigating angiostatin's anti-angiogenic mechanism requires understanding the contribution of the neo-angiogenic vascular environment and its ability to influence angiostatin activity. Hypoxia is a state characteristically induced by tumor growth due to a dysfunctional vasculature, as well as in ischemic vascular diseases due to atherosclerotic plaque buildup, or abnormal thrombus formation. Endothelial cells in a hypoxic environment will undergo anaerobic metabolism, secreting lactate thereby lowering the extracellular pH (Sorensen *et al.,* 2015, Koziel and Jarmuszkiewicz 2017). Both oxygen supply and pH of the extracellular environment have been shown to influence endothelial cell responses to angiostatin. For example; angiostatin (K1-4) inhibits endothelial cell tube formation at a low extracellular pH (6.7), an effect which is not observed at a normal pH (7.3) without using significantly higher doses of angiostatin, or longer incubation times (Wahl and Grant 2000). This demonstrated its pH-selective effects on endothelial cell angiogenic activity at certain concentrations, and may explain its potent inhibitory effects within an acidic tumor vasculature (Griscelli *et al.,* 1998). While differences exclusively in extracellular pH effect angiostatin activity, the contribution of the hypoxic environment can also influence its mechanism of action. As previously mentioned, angiostatin (K1-4) decreased MMP-2, MT1-MMP, and eNOS expression in endothelial cells during hypoxia, but had no effect during normoxia (Radziwon-Balicka *et al.,* 2013). Furthermore, angiostatin (K1-4) induces endothelial cell apoptosis at a low extracellular pH, a state that is induced by hypoxic conditions (Wahl and Grant 2000, Wahl *et al.,* 2002, Chi and Pizzo 2006, Koziel and Jarmuszkiewicz 2017).

However, angiostatin induction of HMVEC-L apoptosis during hypoxia was not observed due to hypoxia-induced VEGF upregulation and subsequent pro-survival signalling. Only when at high concentrations did angiostatin induce endothelial cell apoptosis during hypoxia that was not rescued by VEGF (Radziwon-Balicka *et al.,* 2013). This would suggest that hypoxia-induced pro-survival mechanisms may be able to overcome angiostatin's apoptotic effects. Therefore, differences in concentration of angiostatin, availability of oxygen, and extracellular pH can influence the anti-angiogenic actions of angiostatin on endothelial cells.

### 1.3.2 Targets of Angiostatin

To further elucidate angiostatin's mechanism of action researchers have focused on identifying its endothelial cell target. A few cellular targets have been discovered, however there are still unknown areas and critiques regarding these targets. Angiomotin was identified as a novel protein that interacted with angiostatin. Angiomotin has been shown to promote endothelial cell motility and tube formation (Bratt *et al.,* 2005). It was discovered to bind to angiostatin (K1-4), and mouse aortic endothelial cells transfected with angiomotin demonstrated decreased migration in response to angiostatin treatment, compared to non-transfected cells (Troyanovsky *et al.,* 2001). Although interaction between angiostatin and angiomotin may explain angiostatin-mediated inhibition of endothelial cell migration and tube formation, it does not account for its inhibitory activity on endothelial cell proliferation or induction of apoptosis.

Another target of angiostatin is an integrin found on the surface of endothelial cells, specifically integrin αvβ3. Endothelial cell adherence to the basement membrane is mediated by cell-adhesion molecules called integrins. Integrin αvβ3 is found highly expressed on the surface of activated endothelial cells and is an important regulator of angiogenesis. It is a receptor for vitronectin, fibrinogen, vWF and many other extracellular ligands. In addition, it interacts with angiogenic mediators such as MMP-2 and VEGF to activate endothelial cell proliferation and inhibit cell apoptosis. (Brooks *et al.,* 1994, Horton *et al.,* 1997, Kumar *et al.,* 2004, Liu *et al.,* 2008). Angiostatin (K1-4) was found to bind to bovine arterial endothelial cells in an integrin-dependent manner, and the predominant receptor bound was integrin αvβ3 (Tarui *et al.,* 2001).

It was proposed that the interaction between angiostatin and integrin αvβ3 prevents integrin-associated pro-survival and proliferative signaling. However, the recognition sequence arginine-glycine-aspartate motifs for integrin binding are not found within the angiostatin structure, which leaves some unanswered questions as to its molecular interactions with integrins (Wahl *et al.,* 2005).

Annexin II is a calcium-dependent phospholipid binding protein on the surface of endothelial cells that acts as a co-receptor for plasminogen and tissue plasminogen activator, and mediates conversion of plasminogen to plasmin (Hajjar and Krishnan 1999). It has been identified as an endothelial cell surface target for angiostatin (K1-4), and it was proposed that angiostatin competing for binding of annexin II with plasminogen reduces plasmin production (Tuszynski *et al.,* 2002). However, plasminogen competitively binds to annexin II, and plasminogen is far more abundant in human plasma than angiostatin (Wahl *et al.,* 2005). Furthermore, since plasminogen has no anti-angiogenic activity, annexin II binding alone does not account for the inhibitory effects of angiostatin. This would suggest that a distinct angiostatin target is more likely to be responsible for its anti-angiogenic effects on endothelial cells. The search for this target was undertaken by Pizzo and colleagues in 1999, and yielded a distinct angiostatin binding site on the surface of endothelial cells which was identified as an ectopic ATP synthase (Moser *et al.,* 1999).

### 1.3.3 Ectopic ATP Synthase

In 1999, Moser and Pizzo discovered that angiostatin bound to a distinct site from plasminogen on the surface of endothelial cells. By utilizing Sepharose affinity binding columns with angiostatin (K1-3) and human umbilical endothelial cell plasma membrane extracts, angiostatin was found to bind to the α and β subunits of an $F_1F_0$ ATP synthase. In comparison, plasminogen was found bound to its already established site, annexin II, but not to the subunits comprising the ATP synthase (Moser *et al.,* 1999). Upon further investigation, antibodies binding the ATP synthase α subunit decrease the anti-proliferative effects of angiostatin *in vitro,* while high affinity binding angiostatin-like antibodies directed against the catalytic site of the ATP synthase domain exhibit potent anti-angiogenic effects (Moser *et al.,* 1999, Chi *et al.,* 2007). These studies established that the α/β subunits of the ATP synthase on the surface of endothelial cells are a distinct binding target for angiostatin, and not plasminogen, indicating that this target may have a role in mediating angiostatin's mechanism of action.

The ATP synthase is an enzyme that exhibits a bidirectional function by synthesizing and hydrolyzing ATP. It is composed of two domains, both of which have distinct functions. The catalytic $F_1$ domain consists of α, β, δ, γ, and ε subunits and mediates ATP synthesis and hydrolysis. The extracellular catalytic domain's activity ($F_1$) is coupled to the intermembrane $F_0$ domain and functions as a proton motor (Boyer 1997, Chi *et al.,* 2007). Protons passing through the membrane drive the rotational motor which is coupled to the ATP enzymatic activity that phosphorylates adenosine diphosphate (ADP) to ATP, or dephosphorylates ATP to ADP (Jonckheere 2012).

The $F_1F_0$ ATP synthase is a protein previously thought only to be found in the mitochondrial matrix: however, Pizzo and colleagues confirmed the presence and functionality of the ATP synthase on the surface of endothelial cells (Moser *et al.,* 2001). Angiostatin inhibition of the $F_1$-ATP synthase leads to a reduction in endothelial cell proliferation, migration, and induction of apoptosis (Moser *et al.,* 1999, Moser *et al.,* 2001). ATP synthesis and hydrolysis are inhibited by angiostatin binding, and this appears to be essential for its anti-angiogenic effects (Moser *et al.,* 2001). This is demonstrated in studies where angiostatin (K1-3) competes for binding with an endogenous inhibitor of ATP synthase, called inhibitor of F1 (IF1). IF1 interferes with the ATP hydrolysis function of the ATP synthase enzyme. However, IF1 does not inhibit endothelial tube formation, as angiostatin does, and so it was proposed that both ATP synthesis and hydrolysis inhibition were required to exert this anti-angiogenic effect. (Burwick *et al.,* 2005).

An important aspect of angiostatin's activity via the ATP synthase enzyme is the contribution of the microenvironment. As mentioned previously, one important factor is the extracellular pH (Wahl and Grant 2002). Although angiostatin inhibits the ATP synthase at a wide range of pHs, only when endothelial cells are in an acidic environment do they exhibit impaired proliferation and succumb to apoptosis. This has been shown to occur due to a loss of control of intracellular pH, as *in vitro* endothelial cells suffer from acidosis in response to angiostatin treatment specifically at a low extracellular pH (Wahl and Grant 2000, Wahl *et al.,* 2002). The proton motor of the ATP synthase not only drives ATP/ADP production it also serves to regulate intracellular pH, and interference with this function may lead to pH dysregulation (Boyer 1997).

Therefore, at a low extracellular pH, angiostatin's inhibition of surface ATP synthase activity leads to intracellular acidosis of endothelial cells, which in turn contributes to apoptosis and inhibition of endothelial cell migration and proliferation (Figure 4) (Moser *et al.,* 1999, Wahl and Grant 2002, Vietonmaki *et al.,* 2004, Kenan 2005, Wahl *et al.,* 2005). It is important to keep in mind that the contribution of hypoxic-mediated survival signalling may overcome angiostatin induction of endothelial cell apoptosis (Radziwon-Balicka *et al.,* 2013). Therefore, angiostatin's inhibition of the ATP synthase in a hypoxic environment may result in disruption of other cellular pathways, such as important angiogenic mediators.

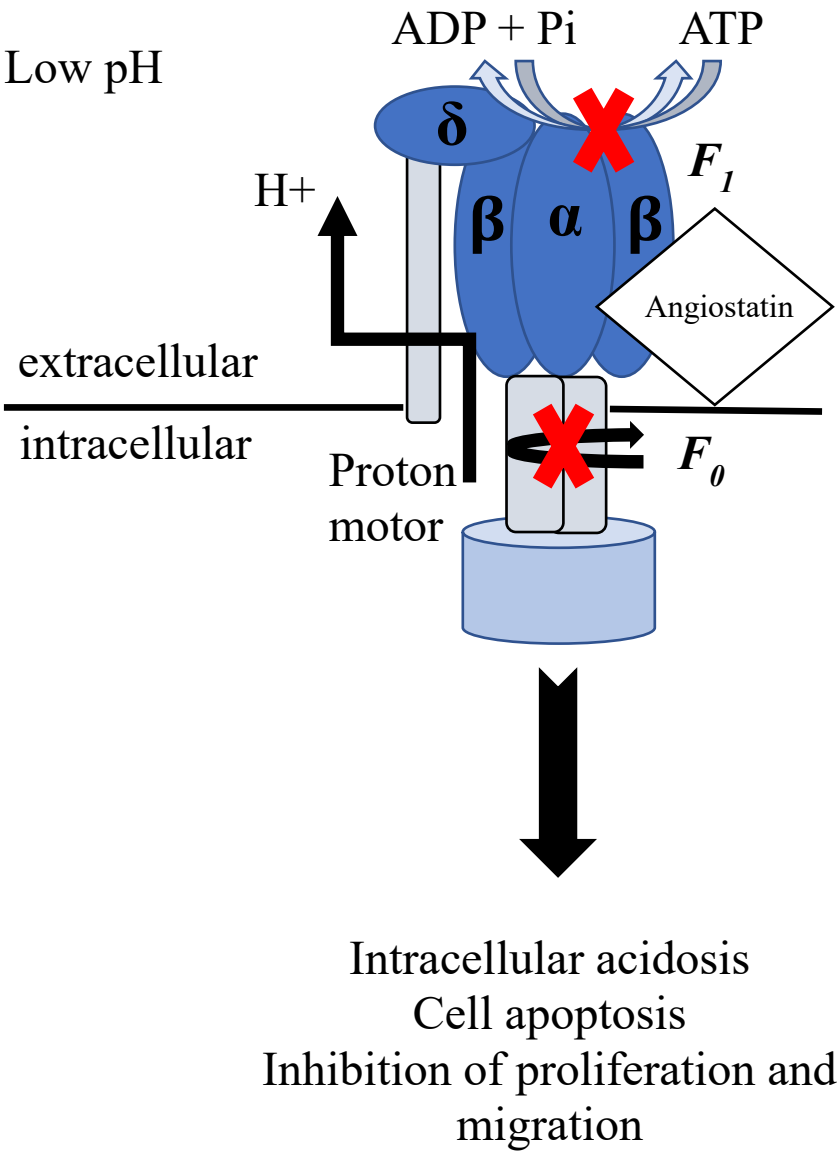


**Figure 4:** Angiostatin inhibition of ectopic endothelial cell surface ATP synthase at a low extracellular pH.

### 1.3.4 Angiostatin in a Pathological Setting

Anti-angiogenic mediators serve to maintain endothelial cell quiescence and stabilize blood vessel growth (Carmeliet *et al.,* 2003, Potente *et al.,* 2011). While physiologically anti-angiogenic mediators can serve as the brakes to regulate angiogenesis, in a pathological setting the anti-angiogenic effects of a factor, such as angiostatin, may contribute to disease progression. Quite often patients suffering from CAD or PAD have co-morbidities such as hypertension or Type 2 diabetes. A common pathological component of these co-morbidities is endothelial dysfunction, which can be caused by a systemic imbalance between vasoconstrictors and vasodilators **(**Rubanyi 1993, Deanfield *et al.,* 2005, Howangyin and Silvestre 2014). A common characteristic of endothelial dysfunction is a lack of NO bioavailability due to either impaired production or release from the endothelium, or due to excessive oxidative degradation by reactive oxygen species. A lack of NO can have negative consequences on vasodilatory responses, and can contribute to impaired angiogenesis (Bucala *et al.,* 1991, Suuronen *et al.,* 2010, Tousoulis *et al.,* 2012). Within CAD/PAD patient populations that commonly have co-morbidities that contribute to endothelial dysfunction, pro-angiogenic therapies such as VEGF gene or protein therapy may not be sufficient to promote angiogenesis to a significant extent due to insufficient NO bioavailability. Animal studies and clinical studies have demonstrated that levels of angiostatin are negatively correlated with extent of collateral blood vessel growth after an ischemic event (Matsunaga *et al.,* 2005, Dodd *et al.,* 2013). Furthermore, studies both in animals and in the clinic, have found that with a lack of NO there is an increase in angiostatin levels. For example, a study by Matsunaga and colleagues (2002) showed that treatment with L-NAME in a canine myocardial ischemia model resulted in increased angiostatin levels.

Clinically, CAD patients that have co-existing Type 2 diabetes have been found to have elevated levels of angiostatin, which was associated with a decrease in collateral blood vessel networks (Sodha *et al.,* 2009). In yet another setting of endothelial dysfunctional, blood from patients with idiopathic arterial pulmonary hypertension with increased endothelial microfragments due to endothelium injury were found to have higher levels of angiostatin within their platelets than control healthy individuals (Jurasz *et al.,* 2010).

Thus, in ischemic diseases with endothelium dysfunction, increased levels of angiostatin could be contributing to pathology as its anti-angiogenic activity would create an environment that is not conductive to recovery angiogenesis.

# 2. HYPOTHESIS AND OBJECTIVES

## 2.1 Rationale

CAD and PAD are currently leading causes of cardiovascular death worldwide (Roth *et al.,* 2015). In these ischemic disease states, angiogenesis can be an important recovery response that would be potentially therapeutic if enhanced (Meier *et al.,* 2011, Kim *et al.,* 2016). To date, clinical trials investigating pro-angiogenic therapies such as VEGF gene/protein therapy and endothelial progenitor cell/stem cell based therapies have not been significantly effective (Cao 2010, Dragneva *et al.,* 2013). This may be due to a lack of understanding of the contribution of anti-angiogenic factors that could be limiting the extent of therapeutic revascularization. Angiostatin is a potent anti-angiogenic factor which has been found to impair important angiogenic processes such as endothelial cell migration, and proliferation (Cao and Xue 2004, Wahl *et al.,* 2005). To focus on angiostatin as a target to neutralize, it would be valuable to delineate its anti-angiogenic mechanism of action within a hypoxic setting, as would occur in ischemic disease states. Previous studies have demonstrated that during hypoxia angiostatin decreases MMP-2, MT1-MMP and eNOS expression by HMVEC-L. In addition, *in vitro* studies have shown that angiostatin inhibits HMVEC-L migration specifically during hypoxia (Radziwon-Balicka *et al.,* 2013). Whether these anti-angiogenic mechanisms of angiostatin exist within endothelial cells derived from different tissues, particularly cardiac-derived tissue, is an important study to pursue for the sake of relevancy when investigating angiostatin in the context of CAD.

Investigations into angiostatin's role in reducing tumor growth via inhibition of tumor angiogenesis in mice have been performed (O'Reilly *et al.,* 1996, Griscelli *et al.,* 1998, Dell'Eva *et al.,* 2002*).* However, there is limited information on what impact excess levels of angiostatin will have on ischemia-induced angiogenesis *in vivo*. This is important as it provides a similar model to studies which correlate elevated angiostatin levels with impaired collateral vessel growth within an ischemic disease setting (Matsunaga *et al.,* 2005, Sodha *et al.,* 2009). Thus, the anti-angiogenic effects of administering angiostatin was investigated within a murine HLI model, a commonly used animal model of acute peripheral artery disease (Niiyama *et al.,* 2009).

Neutralizing angiostatin could be a novel treatment strategy to enhance ischemic-induced angiogenesis in patients who are ineligible for conventional surgical therapies (Lassaletta *et al.,* 2011). Since it has been demonstrated that angiostatin binds to the $\alpha/\beta$ subunits of the endothelial cell surface ATP synthase, recombinant forms of these subunits can be potentially used as decoy receptors to bind angiostatin. Neutralizing angiostatin, in conjunction with providing pro-angiogenic factors, could create a microvascular environment conductive to angiogenesis during ischemia. Thus, the potential neutralization of angiostatin within a hypoxic setting was investigated.

## 2.2 Hypothesis

Angiostatin will reduce expression of angiogenic mediators, MMP-2 and eNOS, in HMVEC-C during hypoxia, and impair angiogenesis in an HLI model. Neutralization of angiostatin with ATP synthase α and β subunits will restore HMVEC-C MMP-mediated angiogenic responses without interfering with fibrinolysis.

## 2.3 Objectives of the Study

1. Determine if angiostatin decreases pro-MMP-2/MMP-2 and eNOS protein expression in HMVEC-C during hypoxia.
2. Investigate the effects of administering angiostatin on angiogenesis in a mouse HLI model.
3. Investigate if angiostatin neutralization using ATP synthase α and β subunit decoy receptors will restore MMP-2 protein expression in HMVEC-C during hypoxia.
4. Evaluate if angiostatin neutralization using ATP synthase α and β subunit decoy receptors will restore HMVEC-C MMP-dependent migration during hypoxia.
5. Determine if ATP synthase α and β subunit decoy receptors interfere with fibrinolysis *in vitro*.

# 3. MATERIALS AND METHODS

## 3.1 Reagents

Native human angiostatin K1-4 isolated from plasma was obtained from Thermofisher Scientific (Rockford, IL, USA). ATP synthase α, β, and δ subunits were obtained from MyBiosource.com (San Diego, California). Recombinant VEGF, recombinant human tPA, and recombinant human MMP-2 standard was ordered from R&D Systems (Minneapolis, MN, USA). Thrombin was purchased from Chrono-Log Corporation (Havertown, PA, USA) and aprotinin from Sigma-Aldrich (St. Louis, MO, USA). HMVEC-C were purchased from Lonza (Walkersville, MD, USA). Primary antibodies were obtained as follows: monoclonal eNOS (Clone #: eNOS/NOS Type III) antibody and mouse VE-Cadherin antibody (Clone#: 11D4.1) from BD Biosciences (Mississauga, ON, Canada). Two monoclonal eNOS antibodies (Clone#: M221 and 6H2), and polyclonal mouse MMP-2 antibody from abcam (Toronto, ON, Canada). Unless otherwise indicated, all other reagents were obtained from Sigma-Aldrich (St. Louis, MO, USA).

### 3.2 Cell culture

Primary cardiac-derived human microvascular endothelial cells (HMVEC-C) were cultured in endothelial cell basal medium (EBM-2, Lonza, Walkersville, MD, USA) supplemented with hydrocortisone and growth factors: human fibroblastic growth factor-b, VEGF, R3-insulin-like growth factor-1, ascorbic acid, human epidermal growth factor, GA-1000 and 5% fetal bovine serum (FBS) (EGM-2 MV Singlequotes, Lonza, Walkersville, MD, USA). Cells were grown in a humidified cell incubator supplied with 5% $CO_2$ at 37°C. Fresh medium was exchanged every second day, and cells were passaged once approximately 80% confluent. Cells from passages 4-7 were used in experiments. To detach cells from flasks, they were incubated with Trypsin-Ethylenediaminetetraacetic acid (Trypsin-EDTA) for five minutes with gentle shaking. Trypsin-EDTA was neutralized with twice the volume of EGM-2 medium. Collected cells were pelleted by spinning at 130 relative centrifugal force for 7 minutes at 18°C in an Eppendorf 5810R centrifuge (Eppendorf, Hamburg, Germany), and re-suspended in appropriate medium or buffer for further experiments.

### 3.3. HMVEC-C treatment with angiostatin in normoxic and hypoxic conditions

HMVEC-C were cultured and detached as described under methods, section 3.2. An equal volume of cells was seeded into 24-well plates (Falcon Corning, Tewksbury, MA, USA) and grown in EGM-2 medium. Once approximately 80% confluent, cells were serum starved for 8 hours with 0.5% FBS in EBM-2 (growth factor-free medium). Cells were treated with either angiostatin (600nM) or phosphate-buffered saline (PBS), and one group of cells was incubated for 48 hours in normoxic (74% $N_2$, 21% $O_2$, and 5% $CO_2$) conditions, while the other in hypoxic conditions. Hypoxic conditions were simulated by incubation in a Billups-Rothenberg chamber (Billups-Rothenberg Inc., San Diego, CA, USA), continuously gassed with 95% $N_2$ and 5% $CO_2$ at 37°C. After 48 hours, all floating and attached cells were collected and pooled together, and washed once in PBS. Washed cells were pelleted down by spinning at 10 000gs for 5 minutes, and pellet was frozen at -80°C until analyzed by gelatin zymography or western blot.

### 3.4 Gelatin zymography analysis of HMVEC-C pro-MMP-2/MMP-2 protein expression.

All sample preparation was performed on ice. Cell pellets were thawed and re-suspended in 20µl of sodium dodecyl sulfate (SDS) free-homogenizing buffer (Tris hydrochloride (HCL)/base 50mM + 3.1 mM sucrose + 10% protease inhibitors). Homogenized lysates were sonicated using a Misonix Ultrasonic Liquid Processor (Misonix Inc., Farmingdale, NY, USA) for five seconds, left to rest for 20 seconds on ice, then sonicated for another five seconds. Loading buffer (lacking β-mercaptoethanol) (5µl) was added to each sample to make a final sample volume of 25µl. The entire sample volume was loaded on to a 20mg/ml gelatin-incorporated 8% SDS-polyacrylamide gel. As a molecular weight reference, 15µl of Precision Plus Protein Ladder (Bio-rad, Hercules, CA, USA) was loaded adjacent to samples. Recombinant human MMP-2 was loaded as a standard. Gel electrophoresis was performed using a Bio-Rad PowerPac HC (Bio-rad, Hercules, CA, USA) at 150 volts on ice (at 4°C).

According to a previously published protocol (Govindasamy *et al.,* 2017), after run completion the gel was cut at the 50kDa mark, using the molecular weight marker as a reference. The portion >50kDa was washed three times for 20 minutes in 2% Triton-X-100 (Thermofisher Scientific, Rockford, IL, USA) followed by two 20 minute washes in zymography buffer (2M Tris HCl, 0.15M NaCl, 5mM $CaCl_2 \cdot 2H_2O$, 0.5% $NaN_3$). The gel was incubated in fresh zymography buffer at 37°C overnight to allow for enzyme activity to degrade gelatin substrate. To image degraded portions of gelatin, the gel was stained with Coomassie Blue for two hours followed by de-staining using 4% methanol + 8% acetic acid solution. Gels were imaged using a VersaDoc Imaging System (Bio-rad, Hercules, CA, USA), and QuantityOne software was used to analyze densitometry of degraded bands of gelatin.

The gel section <50kDa mark was transferred on to a 0.45μm thick polyvinylidine difluoride (PVDF) (Bio-rad, Hercules, CA, USA) membrane using a semi-dry transfer method (Trans-Blot SD, 25 volts, 30 minutes). After transfer, the PVDF membrane was blocked overnight in 5% skim milk in Tween tris buffered saline (TTBS), and then probed with a monoclonal β-actin antibody conjugated with horseradish peroxidase (1:40 000 dilution) for 30 minutes followed by four wash cycles in TTBS. Chemiluminescence was detected using an enhanced chemiluminescence (ECL) prime western blotting detection reagent (GE Healthcare, Chicago, IL, USA), and imaged using a VersaDoc Imaging System. Band densitometry was analyzed using Quantity One software. Band densitometry from the upper portion gel (zymogram >50kDa) was normalized to the respective β-actin bands from the lower portion of gel (<50kDa). Data was then normalized and expressed as mean of % of normoxic control.

### 3.5 Western blot analysis of HMVEC-C eNOS protein expression

Cell pellets were thawed and re-suspended in homogenizing buffer (Tris HCL/base 50mM + 3.1 mM sucrose + 10% protease inhibitors + 0.1% SDS). Homogenized lysates were sonicated three times for five seconds. Samples were further denatured by boiling for five minutes, and 5µl of loading buffer (containing denaturing agent: 8% SDS and reducing agent: 16% β-mercaptoethanol) was added to make a final sample volume of 25µl. The entire sample volume was loaded per lane on to an 8% SDS-polyacrylamide gel. As a molecular weight reference 15µl of Precision Plus Protein Ladder was loaded adjacent to samples. Gel electrophoresis was performed using a Bio-Rad Powerpac HC system set at 150 volts. After run, protein was transferred from the gel on to a 0.45µm PVDF membrane using a semi-dry method (Trans-Blot SD, 25 volts, 30 minutes). After protein transfer, PVDF membranes were cut at the 50kDa molecular weight marks. The top membrane (>50kDa) was blocked in 5% skim milk in TTBS for one hour at room temperature followed by overnight incubation at 4°C with anti-eNOS M221, anti-eNOS 6H2, and anti-eNOS/NOS Type III (each antibody added at 1:1000 dilution). After incubation with primary antibodies each membrane was washed four times for five minutes in TTBS, then probed for one hour at room temperature with anti-mouse IgG conjugated to horse radish peroxidase (1:2000 dilution). The lower membrane (<50kDa) was blocked in 5% skim milk in TTBS at 4°C overnight, followed by a 30-minute incubation with a monoclonal β-actin antibody conjugated to horse radish peroxidase (1:40 000 dilution). This was followed by a final four washes with TTBS for all membranes, and developed using an ECL kit. Specificity of all antibodies have been previously established in our lab (Radziwon-Balicka *et al.,* 2013).

Chemiluminescence and band densitometry was measured and analyzed as described under methods, section 3.4. Protein levels were calculated by normalizing densitometry of eNOS protein bands to their respective β-actin bands. Data was normalized and expressed as mean of % of normoxic control.

### 3.6 Murine hind limb ischemia model

Transgenic eNOS-GFP (C57BL/6 background) mice were obtained from Dr. Kram's Lab from Imperial College, London, UK, and bred by the Jurasz laboratory. These mice express mouse eNOS as well as a functional human eNOS gene fused to green fluorescent protein (GFP) which allows for visualization of eNOS-GFP in tissue (van Haperen *et al.,* 2003). All procedures, medications and protocols were approved by the University of Alberta Health Sciences Welfare Committee and were performed in strict adherence to the guidelines set by the Canadian Council of Animal Care.

Male hemizygous eNOS-GFP mice (12-14 months old) were anesthetized using 1.5%-2% isoflurane to obtain a surgical plane of anesthesia, and animals were placed on a 37°C water-heating pad to maintain body temperature. Sterile PBS or angiostatin (30μg) was administered via intraperitoneal injection. Dose represents a physiological concentration of angiostatin (30μg/ml) once administered. Mice were shaved of hair around the lower abdomen and hind leg areas. A 1cm incision was made just proximal of the right knee joint, and the femoral artery was exposed and isolated from the nerve bundle and femoral vein. A small section of the femoral artery between the superficial epigastric artery and popliteal bifurcation was tied with 10.0 sutures and the artery was excised in between suture ties, refer to Figure 5. The incision was closed using subcutaneous sutures and superficial application of tissue glue (3M Vetbond, St. Paul, U.S.A.).

Laser Doppler Scan Protocol: Mice were anesthetized using 1.5%-2% isoflurane gas. Animals were kept on a 37°C water-heating pad to maintain body temperature during scans. The lower abdomen area and hind limbs were shaved of hair. A Moor Laser Doppler Line Scanner (Moor Instruments Inc., Wilmington, DE, USA) was used to measure blood perfusion in both the ischemic and non-ischemic hind limbs. Laser Doppler scans were performed at the following time points: pre-operatively, post-operatively, day 7, 14, 21 and 28. At the 28-day post-operative mark, mice were euthanized via ketamine injection and as a secondary euthanizing method cervical dislocation was performed. Area of blood perfusion was calculated from both the ischemic and non-ischemic hind limbs. Data expressed as mean perfusion ratio between the ischemic and non-ischemic hind limb (I/NI).

### 3.7 Confocal microscopy

To further determine the effects of angiostatin on angiogenesis in the HLI model, angiogenic markers, eNOS and MMP-2, were assessed via confocal microscopy of gastrocnemius muscle tissue. To confirm eNOS-GFP fluorescence was endothelium derived, immunofluorescent microscopy of VE-cadherin, an endothelial cell marker, was performed. At the 28-day endpoint for the HLI experiment, gastrocnemius muscle tissue (Figure 5) from both hind limbs of each mouse were dissected and flash frozen in liquid nitrogen. Tissue samples were cryoembedded in optimal cutting temperature compound, and cryosectioned on to slides for microscopy (histology performed on a service by fee basis by HistoCore at University of Alberta). To image eNOS-GFP, slides were mounted using Prolong Diamond Antifade Mountant medium (Life Technologies, Carlsbad, CA, USA) and coverslipped. Immunofluorescent microscopy of VE-cadherin and MMP-2 was performed as follows. Tissue slides were blocked in 5% bovine serum albumin (BSA)+0.02% Tween20 in sterile PBS for one hour, followed by staining with primary antibodies against either mouse VE-cadherin (1:100 dilution) or mouse MMP-2 (2μg/ml) overnight at 4°C. The next day slides were washed three times for five minutes with sterile PBS, followed by staining with appropriate secondary antibodies (VE-cadherin: secondary Alexa Fluor 568 goat anti-rat IgG (1:200 dilution) (Thermofisher Scientific, Rockford, IL, USA) or MMP: secondary Alexa Fluor 647 donkey anti-rabbit IgG (4μg/ml) (abcam, Toronto, ON, Canada)) for two hours at room temperature. After a final wash cycle with sterile PBS, slides were mounted using Prolong Diamond Antifade Mountant medium and coverslipped.

Control slides for VE-cadherin and MMP-2 were obtained by performing the protocol described above, with exclusion of the primary antibodies. All tissue sections were imaged using magnifications of 10x or 20x on a Leica TCS SP5 confocal microscope (Leica Microsystems, Concord, ON, Canada).

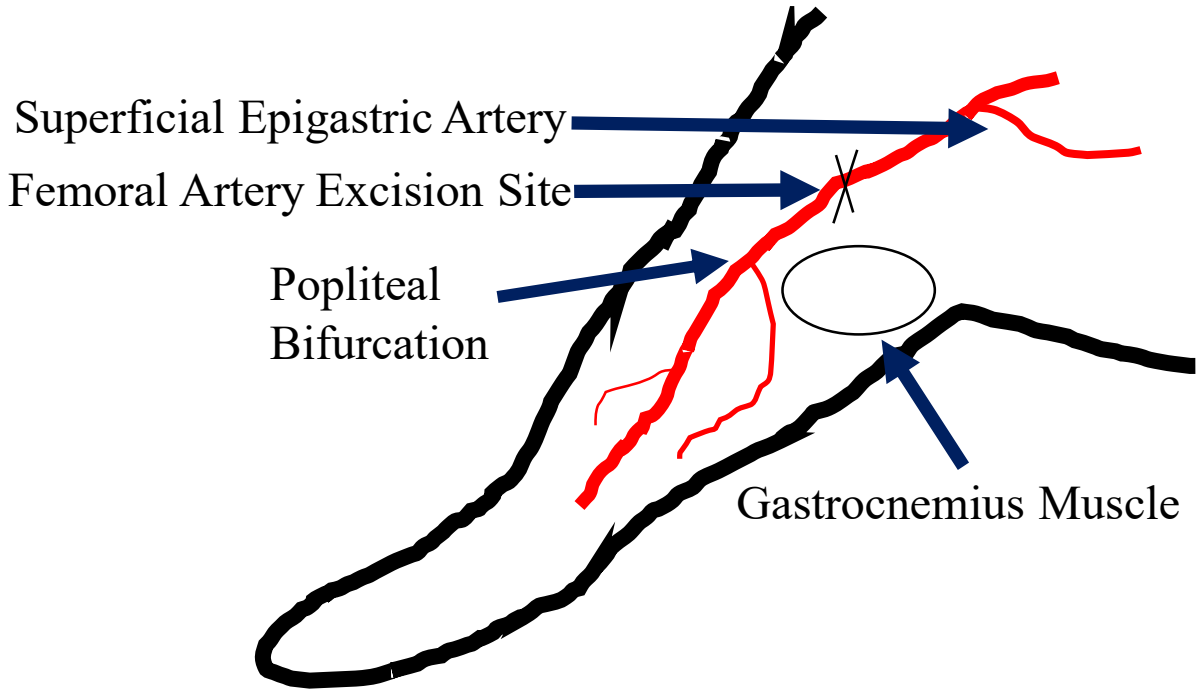


**Figure 5:** Diagram of approximate site of femoral artery ligation in the right hind limb. The femoral artery in the right hind limb was excised between the superficial epigastric artery and popliteal bifurcation to induce acute ischemia. At experiment endpoint (day 28), gastrocnemius muscle tissue from both hind limbs were harvested for confocal microscopy.

### 3.8 Processing of the ATP synthase subunits

ATP synthase subunits were processed under sterile conditions before use in experiments to remove the glycerol preservative that previously caused cell contamination. ATP synthase α, β, and δ subunits were first dialyzed using EBM-2 medium as dialysis buffer in 3.5 K MWCO Slide-a-lyzer dialysis devices (ThermoScientific, Rockford, IL, USA). Subunits were dialyzed for two hours at 4°C, then dialysis buffer was replaced with fresh buffer and dialyzed again overnight at 4°C. After dialyzing, all subunits were sterile filtered through 2μm filters. To re-concentrate the subunits, they were spun in Amicon Ultra Centrifugal Filter units (EMD Millipore, Billerica, MA, USA) at 14 000gs for one hour at 4°C. To determine the final concentration of the processed subunits, a 1μl sample of each was diluted 15x in double distilled $H_2O$ and loaded on to an SDS-polyacrylamide gel along with serial dilutions of BSA (0.125-1mg/ml) to generate a protein standard curve. Gel electrophoresis was performed using a Bio-Rad PowerPac HC, set at 150 volts. Upon run completion, the gel was stained in Coomassie Blue for two hours followed by overnight de-staining in 4% methanol + 8% acetic acid solution. Figure 6 is a representative gel after staining/de-staining procedures. Stained protein bands in the gel were imaged using a VersaDoc Imaging System, and densitometry was analyzed using QuantityOne Software. Utilizing the BSA standard curve, the protein concentration of the subunits was determined.

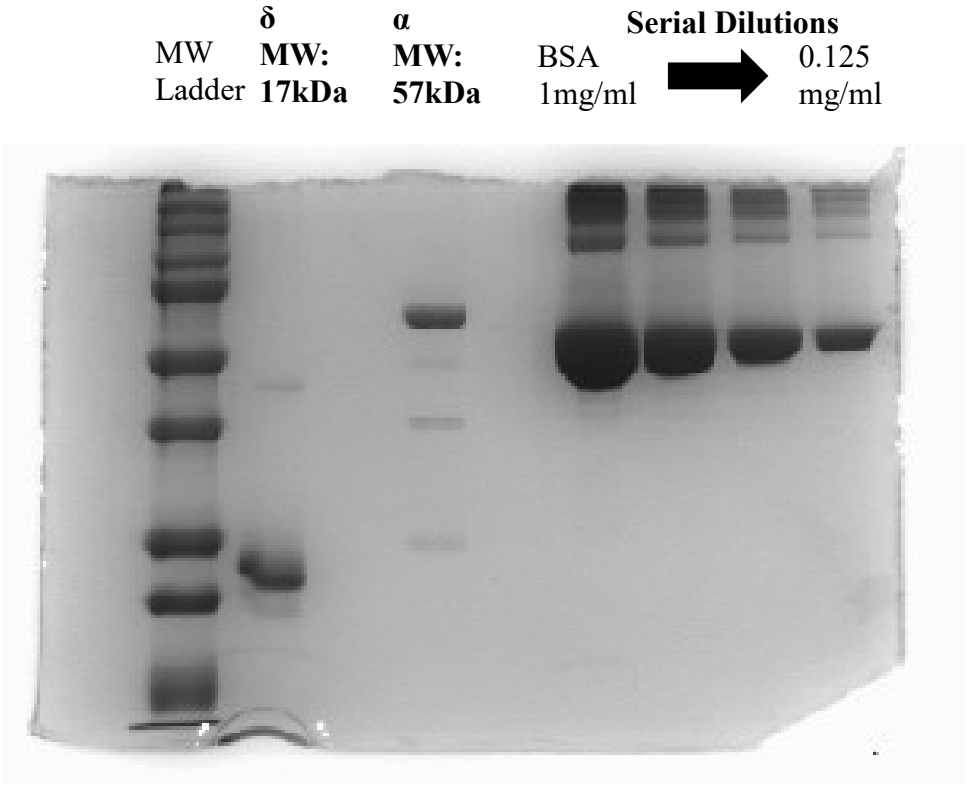


**Figure 6:** Representative gel with processed ATP synthase α and δ subunits. ATP synthase α and δ subunits loaded after dialyzing and concentrating, and BSA serial diluted from 1mg/ml to 0.125mg/ml loaded as a protein standard curve (MW: molecular weight).

### 3.9 Gelatin zymography analysis of HMVEC-C pro-MMP-2/MMP-2 protein expression in response to neutralization of angiostatin during hypoxia

Cell Treatment: HMVEC-C were grown under standard conditions as described under methods, section 3.2. Once approximately 80% confluent, cells were serum starved for 8 hours in 0.5% FBS in EBM-2. Cells were treated with PBS, angiostatin (600nM), angiostatin (600nM) + ATP synthase α subunit (3μM), angiostatin (600nM) + ATP synthase β subunit (3μM), and angiostatin (600nM) + ATP synthase δ subunit (3μM). To ensure any results are not due to the subunits interacting with the endothelial cells, the ATP synthase δ subunit, which does not bind to angiostatin, was used as a control (Moser *et al.,* 1999). HMVEC-C were incubated for 48 hours in hypoxic conditions (95% $N_2$, 5% $CO_2$). After incubation period, floating and attached cells were collected and pooled together, washed once in PBS, and pelleted. Cell pellets were frozen at -80°C until ready for analysis.

Gelatin Zymography: Cell lysate processing, gel electrophoresis protocol, and zymogram/β-actin analysis was performed as described under methods, section 3.4. Data was normalized and expressed as % of PBS control.

### 3.10 Modified Boyden chamber assay

Preparation of Cell Culture Inserts: Transparent 8.0 μM porous 24-well plate inserts (Falcon Corning, Tewksbury, MA, USA) were coated with 1mg/ml gelatin and incubated at 37°C for 2 hours to allow gelatin to solidify. Excess gelatin was aspirated and inserts were left to dry overnight under UV light, then gently washed once with 100μl of PBS.

Once approximately 80% confluent, HMVEC-C were serum starved for 8 hours in 0.5% FBS in EBM-2. Cells were detached as described under methods, section 3.2, and seeded into the top chamber of the gelatin-coated inserts at a concentration of 25 000 cells/insert. Recombinant VEGF (10ng/ml) in 0.5% FBS in EBM-2 was added to the corresponding bottom chambers to serve as a chemoattractant. HMVEC-C were treated with PBS, angiostatin (600nM), angiostatin (600nM) + ATP synthase α subunit (3μM), angiostatin (600nM) + ATP synthase β subunit (3μM), and angiostatin (600nM) + ATP synthase δ subunit (3μM). The system was incubated in hypoxia (95% $N_2$, 5% $CO_2$) for 48 hours. After incubation, the top layer of each insert was scraped gently using a Q-tip to remove the non-migrated cells. Migrated cells at the bottom of the insert were fixed in 4% formaldehyde in PBS for five minutes, followed by staining using Diffquik stain (Siemens Healthcare Diagnostics Inc., Newark, DE, USA). Stained cells from five fields of view of 24-well inserts were imaged at 10x magnification using an Olympic CKX41 microscope (Olympus America Inc., Melville, NY) equipped with an *Infinity* 1 digital camera. Figure 7 indicates the locations of the five fields of view captured from each 24-well insert. Cells were counted using ImageJ software, and expressed as total migrated cells from five-fields of view normalized to PBS control.

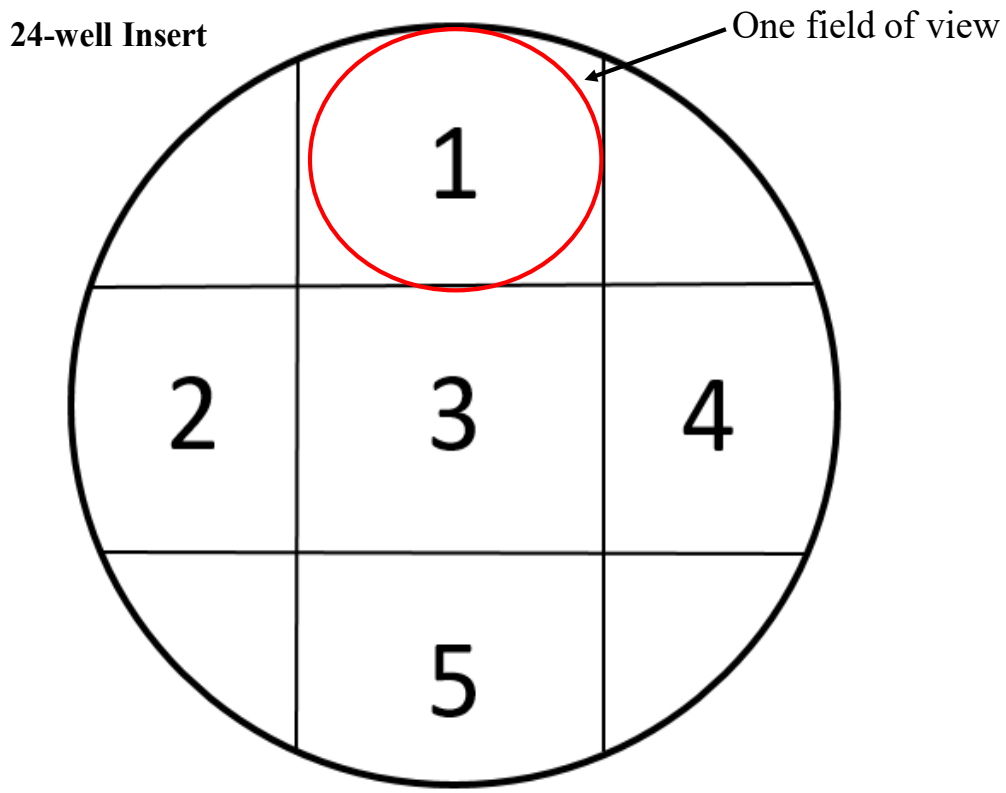


**Figure 7:** Grid representing the locations of the five-fields of view captured from 24-well inserts.

### 3.11 Fibrinolysis assay

Human platelet-poor plasma from healthy donors was collected as a by-product from experimental work approved by the University of Alberta Research Ethics Board for Dr. Jurasz's lab (Pro00029836). Briefly, platelet-poor plasma was reconstituted with 5mM $CaCl_2$. In a 96-well plate, 100µl of reconstituted platelet poor plasma was added per well and treated with thrombin (1U/ml), tissue plasminogen activator (tPA)(1µg/ml), aprotinin (10µM) as a positive control, and the ATP synthase α, β, and δ subunits (3µM). Refer to Table 2 for summary of treatment regime. The system was incubated at 37°C for 2 hours. Clot formation was measured as mean absorbance using a Bio-rad iMark microplate reader (Bio-rad, Hercules, CA, USA). Extent of fibrinolysis in tPA control group was measured as mean absorbance with thrombin alone compared to thrombin + tPA (tPA control) after two hours. Mean absorbance was then measured for all treatment groups and normalized to tPA control, and expressed as % fibrinolysis.

| Group | Thrombin 1U/ml | TPA 1μg/ml | Aprotinin 10μM | ATPα 3μM | ATPβ 3μM | ATPδ 3μM |
|---|---|---|---|---|---|---|
| 1 | √ | | | | | |
| 2 | √ | √ | | | | |
| 3 | √ | √ | √ | | | |
| 4 | √ | √ | | √ | | |
| 5 | √ | √ | | | √ | |
| 6 | √ | √ | | | | √ |

**Table 2:** Treatment array for fibrinolysis assay. Reagents added to 100μl of platelet poor plasma per group.

### 3.12 Statistics

Statistical analysis was performed using Graphpad Prism 7. Results were expressed as the mean±standard error of the mean. Comparisons of variance between multiple groups was performed using one-way analysis of variance (ANOVA), with Tukey's multiple comparisons test or Dunnett's multiple comparisons test where appropriate. Comparisons between more than one variable was performed using two-way ANOVA, with Sidak's multiple comparisons test. P values less than 0.05 were considered statistically significant. N numbers represent number of independent experiments.

# 4. RESULTS

## 4.1 Angiostatin decreases total pro-MMP-2/MMP-2, and eNOS protein expression by HMVEC-C during hypoxia

Angiostatin has been shown to inhibit the expression of MMP-2 and eNOS, by hypoxic HMVEC-L (Radziwon-Balicka *et al.,* 2013). In the present study, similar experiments were repeated in HMVEC-C, as endothelial cells derived from different tissues may have varying responses to angiostatin. Furthermore, to investigate angiostatin's mechanism of action within a context of CAD, it is more relevant to delineate its anti-angiogenic effects on endothelial cells derived from cardiac microvasculature. Therefore, HMVEC-C were treated with a physiological concentration of angiostatin (600nM) and incubated in either normoxia or hypoxia for 48 hours. Gelatin zymography analysis of HMVEC-C lysates showed that hypoxic control cells exhibited significantly increased levels of total pro-MMP-2 and MMP-2 (212.3±43%) compared to normoxic control cells (100%). However, angiostatin decreased total pro-MMP-2 and MMP-2 (101±28%) in hypoxic HMVEC-C when compared to hypoxic control (212.3±43%). This reduction was not observed in HMVEC-C treated with angiostatin in normoxic conditions (94.8±19%) as shown in Figure 8, as they had similar total pro-MMP-2 and MMP-2 protein as normoxic control (100%). Western blot analysis of eNOS protein expression by HMVEC-C indicated that cells treated with angiostatin during hypoxic conditions had significantly decreased levels of eNOS (48.8±12%) compared to angiostatin-treated cells in normoxic conditions (119.6±27%).

In normoxic HMVEC-C, eNOS protein expression in response to angiostatin treatment (119.6±27%) was not significantly different than control (100%) as depicted in Figure 9. Data expressed as mean of % of normoxia control (N-PBS) ± standard error of the mean.

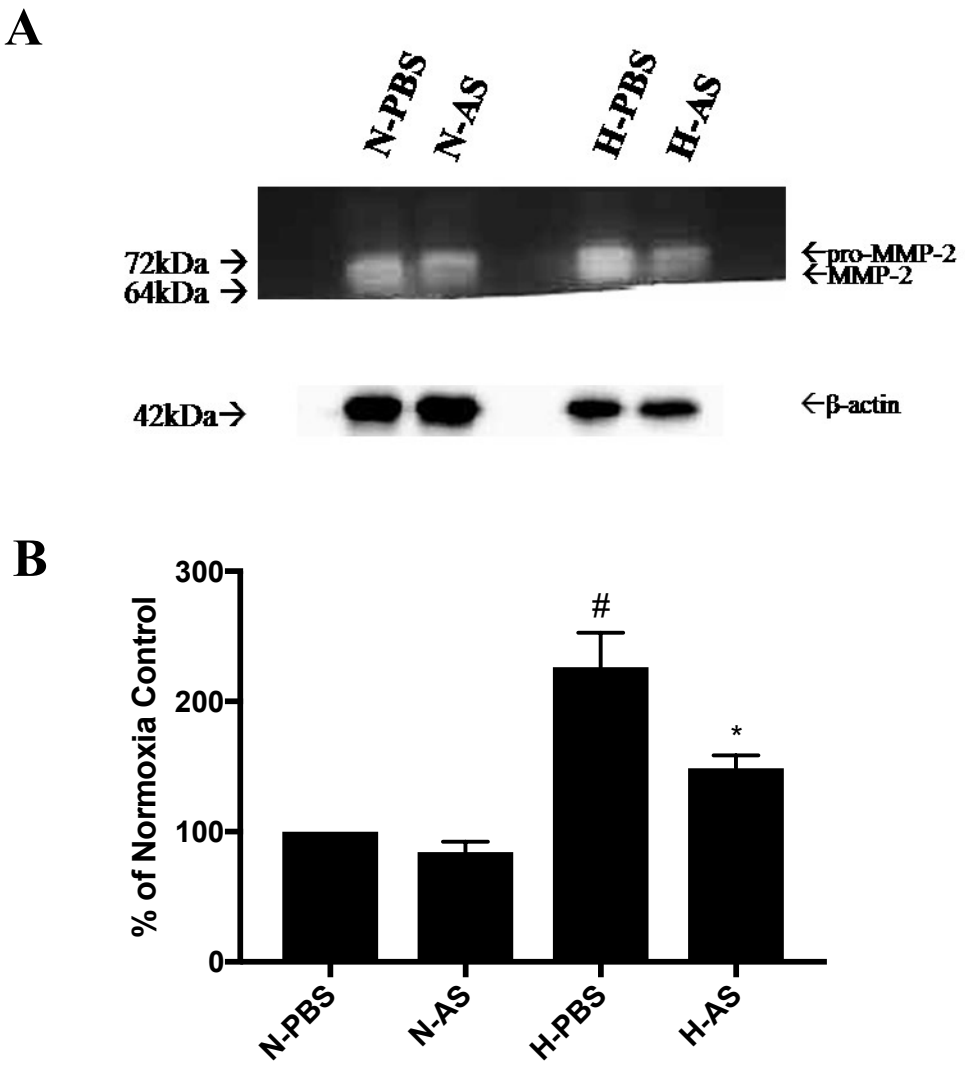


**Figure 8: (A and B)** Representative zymogram with corresponding western blot of β-actin loading control and summary bar graph of data measuring total pro-MMP-2/MMP-2 protein expression by HMVEC-C treated with angiostatin (AS) in normoxic (N) and hypoxic (H) environments. Statistics: One-way ANOVA, parametric, Tukey's multiple comparisons test, *$p<0.05$ vs hypoxia control, #$p<0.01$ vs normoxia control, n=4.

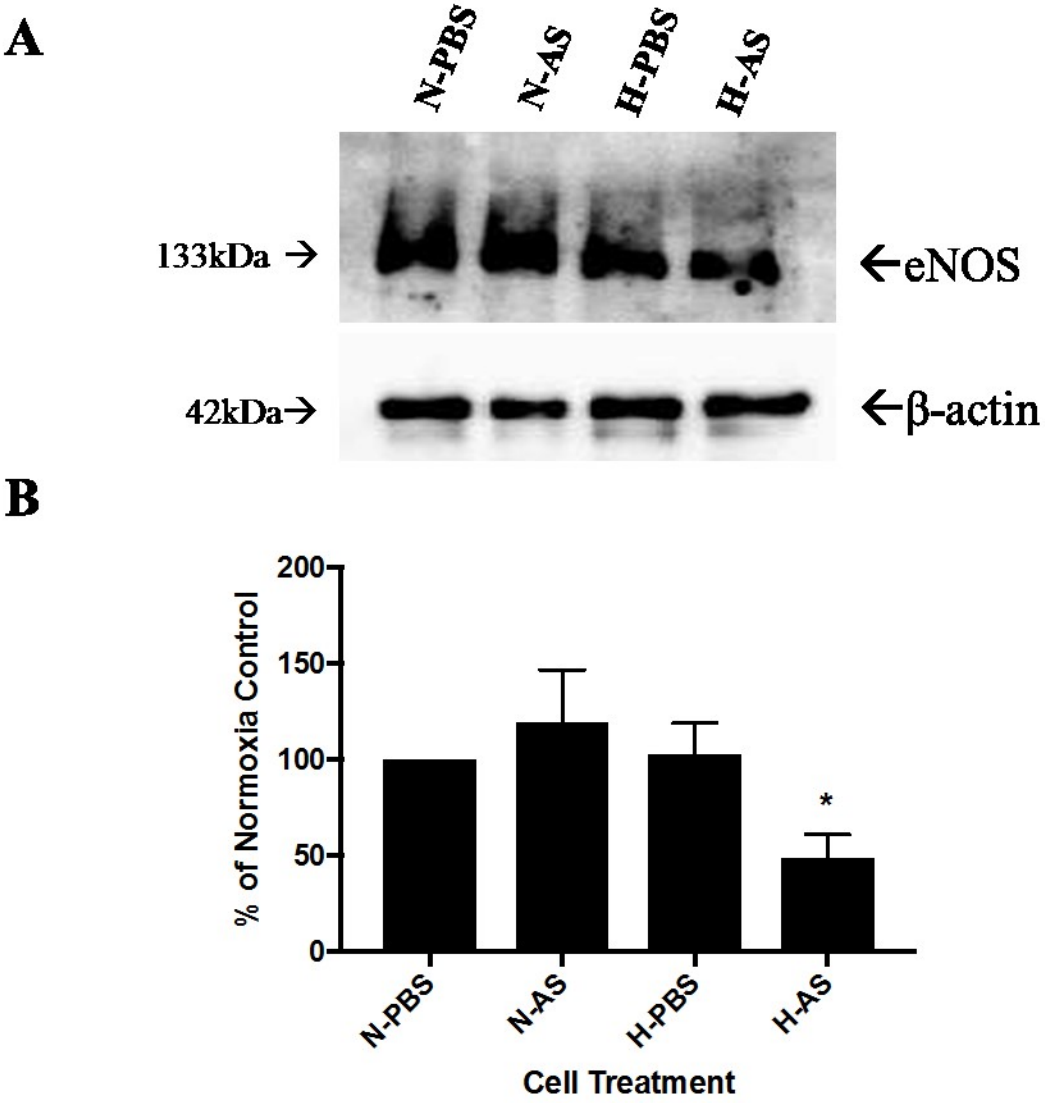


**Figure 9: (A and B)** Representative western blot and summary bar graph of data measuring eNOS protein expression by HMVEC-C treated with angiostatin (AS) in normoxic (N) and hypoxic (H) environments. Statistics: One-way ANOVA, parametric, Tukey's multiple comparisons test, *$p<0.05$ vs N-AS, n=5.

### 4.2 Angiostatin decreases blood flow recovery in a murine HLI model

In a setting of PAD, blood flow to an extremity is compromised leading to hypoxia. To mimic this disease state, a murine hind limb ischemia model was implemented. Mice produce their own endogenous angiostatin (O'Reilly *et al.,* 1994), and administering a dose of angiostatin at time of femoral artery ligation allows for investigation into whether excess angiostatin has an inhibitory effect on ischemia-induced angiogenesis.

In this model, the femoral artery in the right hind limb of male transgenic eNOS-GFP mice was ligated and excised. As a result, decreased blood perfusion to the right hind limb was observed post-operatively via laser Doppler scanner imaging as depicted in Figure 10. The ratio of blood perfusion between the ischemic (I) and non-ischemic (NI) hind limb was then measured every 7 days over a period of 28 days. Overall, treatment had an effect on recovery blood perfusion over the course of the experimental period, with angiostatin-administered mice exhibiting a significant decrease in blood perfusion ratio (0.45 ±0.07 I/NI) when compared to PBS-administered mice (0.61±0.08 I/NI) at day 14, as summarized in Figure 11. Data expressed as mean I/NI ± standard error of the mean.

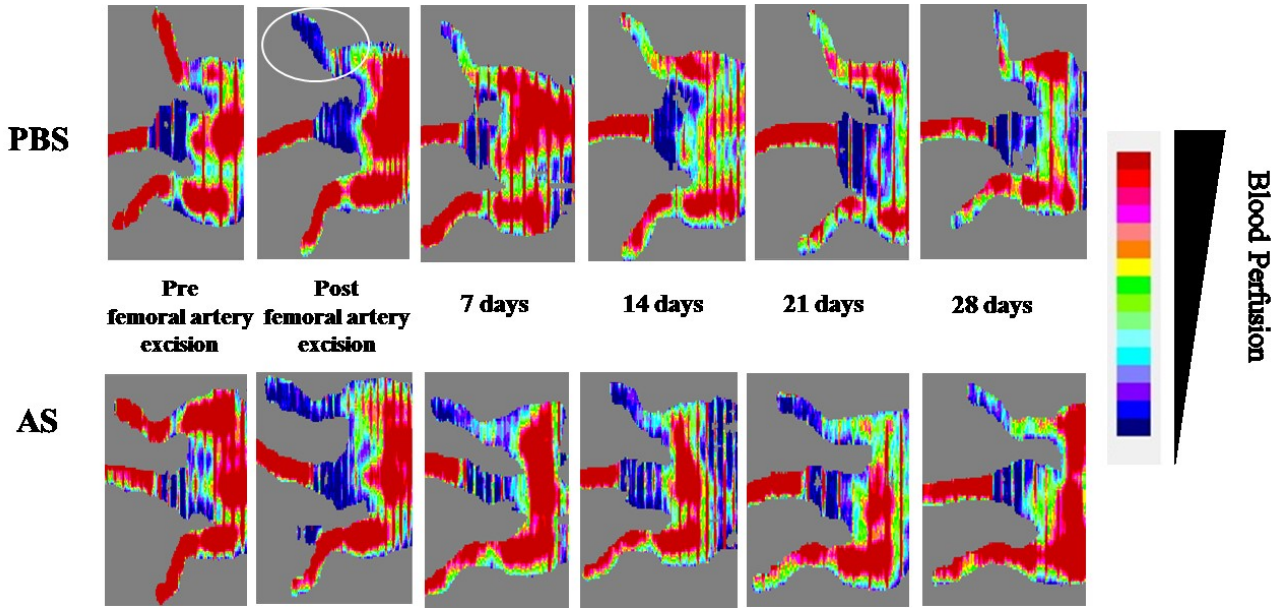


**Figure 10:** Representative laser Doppler images of hind limb blood perfusion from PBS-administered and angiostatin (AS)-administered mice. Laser Doppler scans were taken pre-operatively, post-operatively, and at day 7, 14, 21 and 28.

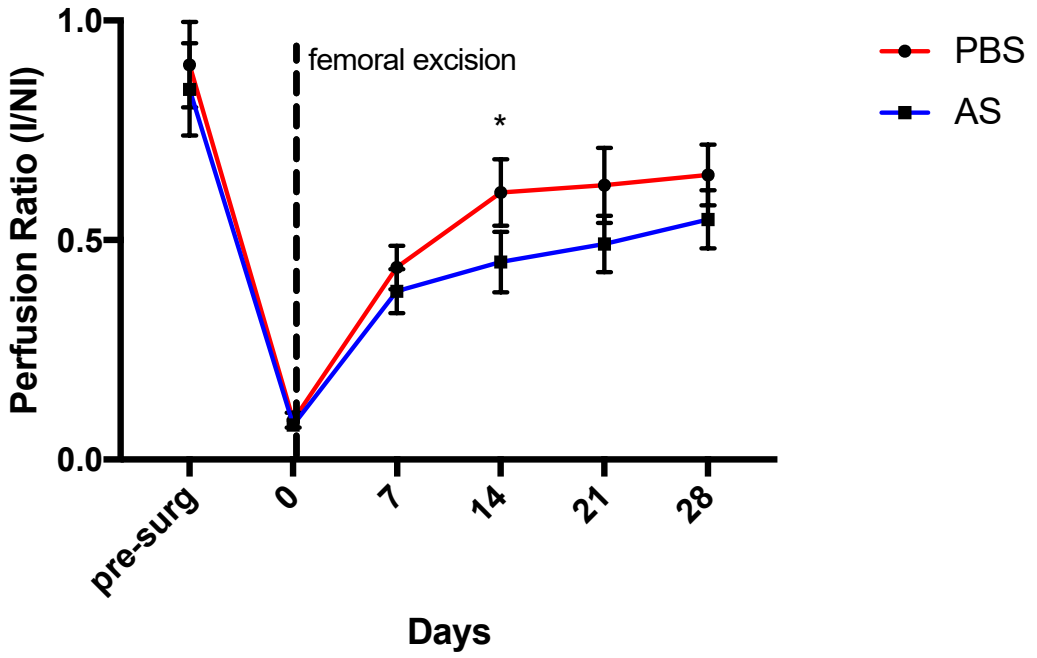


**Figure 11:** Summary graph of hind limb perfusion ratio of ischemic/non-ischemic (I/NI) limbs over 28 days from PBS-administered and angiostatin (AS)-administered mice. Statistics: Two-way ANOVA analysis, Sidak's multiple comparisons test, *$p<0.05$ PBS vs AS at day 14, n=8.

### 4.3 Ischemic gastrocnemius muscle tissue from angiostatin-administered mice exhibit reduced eNOS-GFP and MMP-2 expression

To determine if administering angiostatin in a HLI model influences revascularization, angiogenic mediators, eNOS and MMP-2, in gastrocnemius muscle tissue from the non-ischemic and ischemic hind limb were assessed via confocal microscopy. Furthermore, to determine if the eNOS-GFP signal was derived from the endothelium, tissue was immunostained for an endothelial cell marker, VE-cadherin. Gastrocnemius muscle tissue from hemizygous transgenic eNOS-GFP mice (Figure 12, Panel C) exhibited a green fluorescent signal which was not observed in wild type mice tissue (Figure 12, Panel A). In addition, eNOS-GFP fluorescence was confirmed to co-localize with staining of VE-cadherin in the gastrocnemius muscle tissue (Figure 12, Panel E). As demonstrated in Figure 13, ischemic tissue from PBS-administered mice (Figure 13, Panel B) had increased eNOS-GFP expression compared to tissue derived from the corresponding non-ischemic hind limb (Figure 13, Panel A). This pattern, however, was not observed in angiostatin-administered mice, as the eNOS-GFP fluorescent intensity was comparable between ischemic tissue and non-ischemic tissue (Figure 13, Panel D and C, respectively). Regarding MMP-2, gastrocnemius muscle tissue from the ischemic hind limb of PBS-administered mice (Figure 14, Panel C) exhibited increased staining of MMP-2 when compared to corresponding non-ischemic tissue (Figure 14, Panel B). Ischemic tissue from angiostatin-administered mice (Figure 14, Panel E), however, did not exhibit an increase in MMP-2, as the expression was comparable to non-ischemic tissue (Figure 14, Panel D). When directly comparing ischemic tissue between PBS-administered mice and angiostatin-administered mice, there was an observable decrease in eNOS-GFP and MMP-2 expression from ischemic tissue derived from angiostatin-administered mice, as depicted in Figure 15.

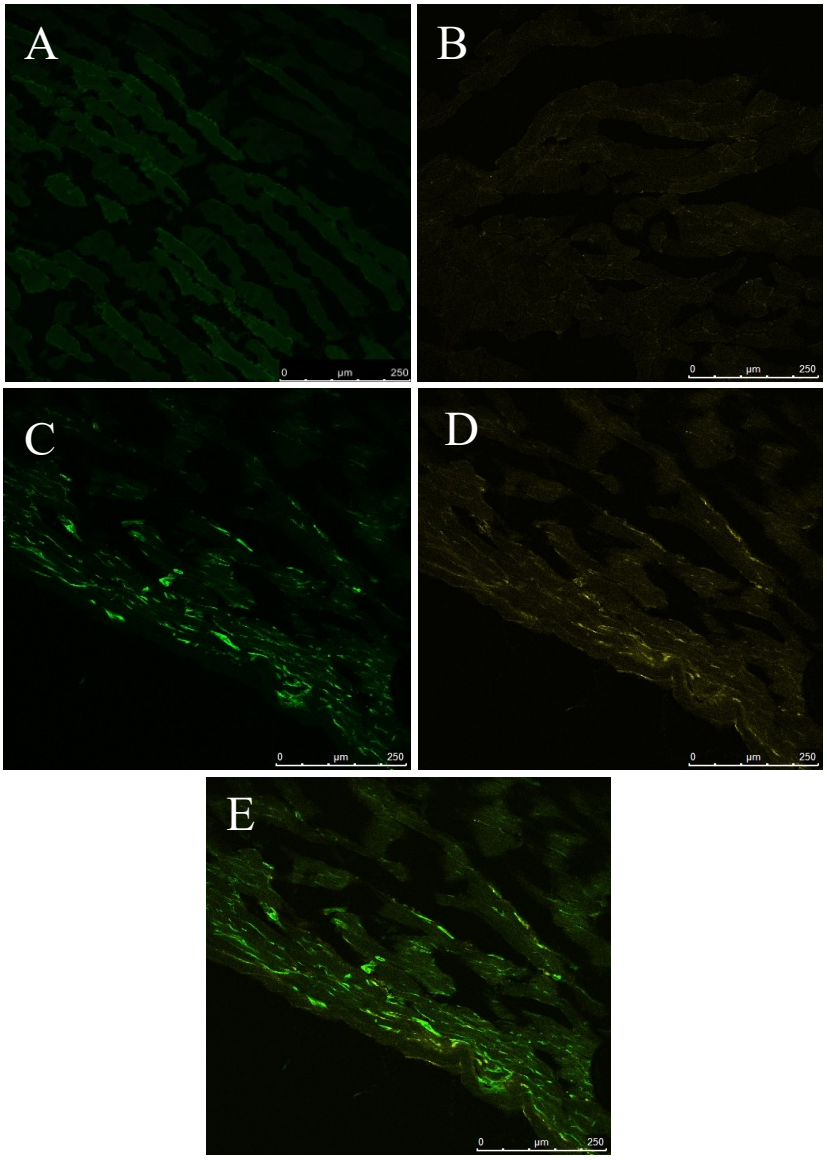


**Figure 12:** Confocal microscopy of eNOS-GFP co-localized with VE-cadherin in gastrocnemius muscle tissue. **(A)** Control: gastrocnemius muscle tissue from a wild type male mouse. **(B)** Control: primary VE-cadherin antibody excluded **(C)** Gastrocnemius muscle tissue from a transgenic hemizygous eNOS-GFP mouse. **(D)** Gastrocnemius muscle tissue stained for VE-cadherin. **(E)** Overlay of eNOS-GFP and VE-cadherin fluorescence. Images of eNOS-GFP were obtained by exciting tissue with an argon laser (488nm). Images of VE-cadherin staining were obtained by exciting tissue with a helium neon laser (543nm). All images taken with a Leica TCS SP5 confocal microscope at 20x magnification.

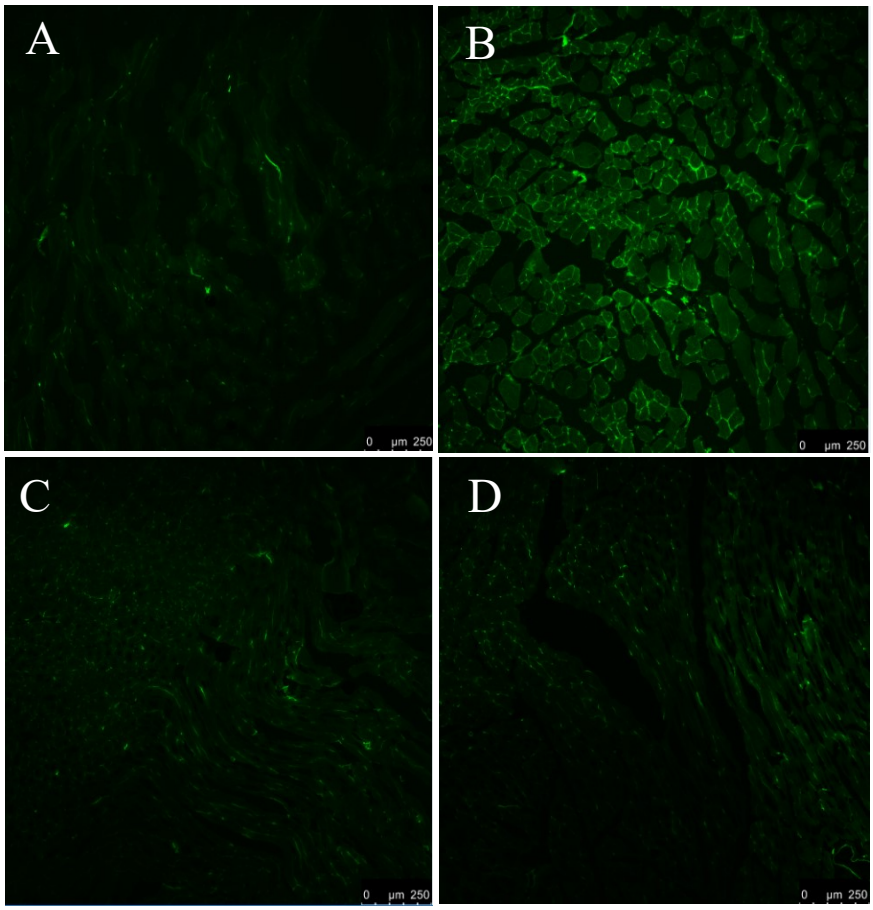


**Figure 13:** Confocal microscopy of eNOS-GFP in gastrocnemius muscle tissue derived from PBS-administered and angiostatin-administered mice. **(A)** Non-ischemic tissue from PBS-administered mouse. **(B)** Ischemic tissue from PBS-administered mouse. **(C)** Non-ischemic tissue from angiostatin-administered mouse. **(D)** Ischemic tissue from angiostatin-administered mouse. Images of eNOS-GFP were obtained by exciting tissue with an argon laser (488nm). Images taken with a Leica TCS SP5 confocal microscope at 10x magnification.

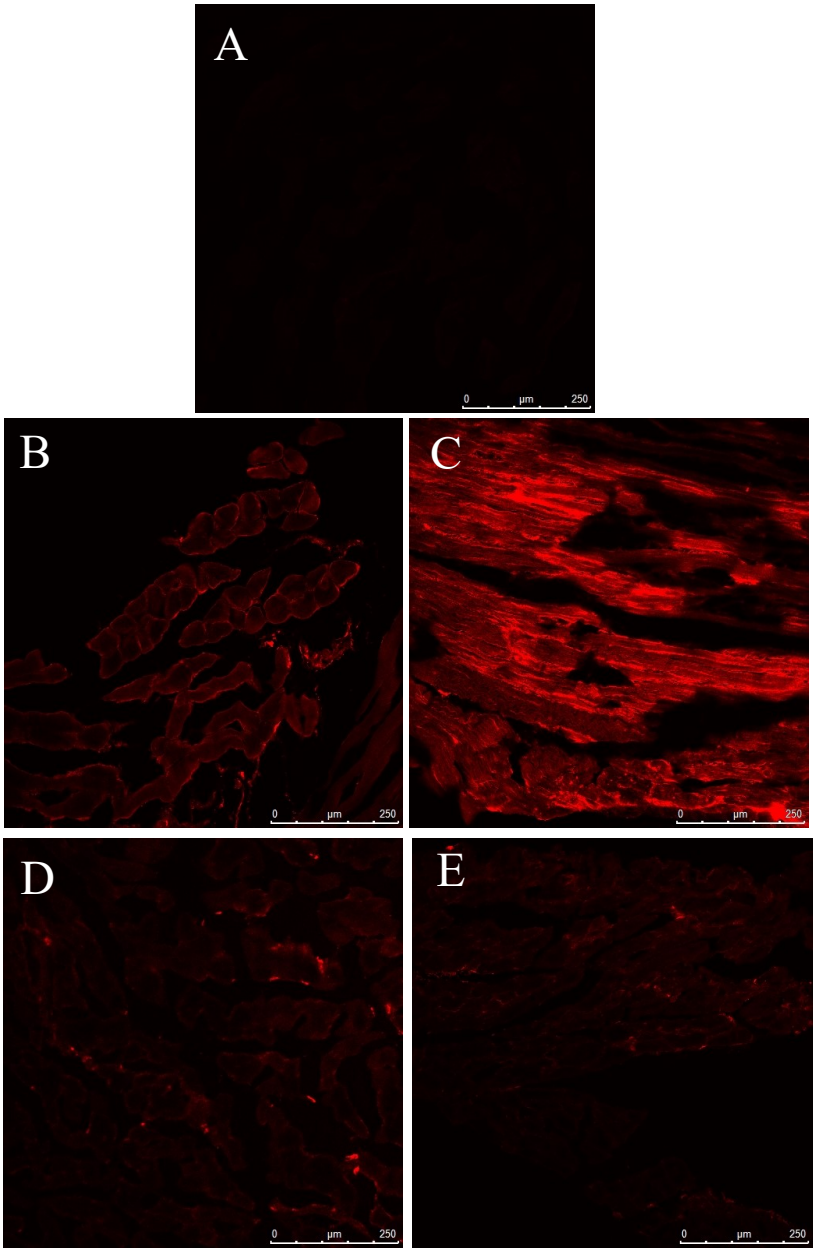


**Figure 14:** Confocal microscopy of MMP-2 in gastrocnemius muscle tissue derived from PBS-administered and angiostatin-administered mice. **(A)** Control: primary MMP-2 antibody excluded. **(B)** Non-ischemic tissue from PBS-administered mouse. **(C)** Ischemic tissue from PBS-administered mouse. **(D)** Non-ischemic tissue from angiostatin-administered mouse. **(E)** Ischemic tissue from angiostatin-administered mouse. Images of MMP-2 staining were obtained by exciting tissue with a helium neon laser (633nm). Images taken with a Leica TCS SP5 confocal microscope at 20x magnification.

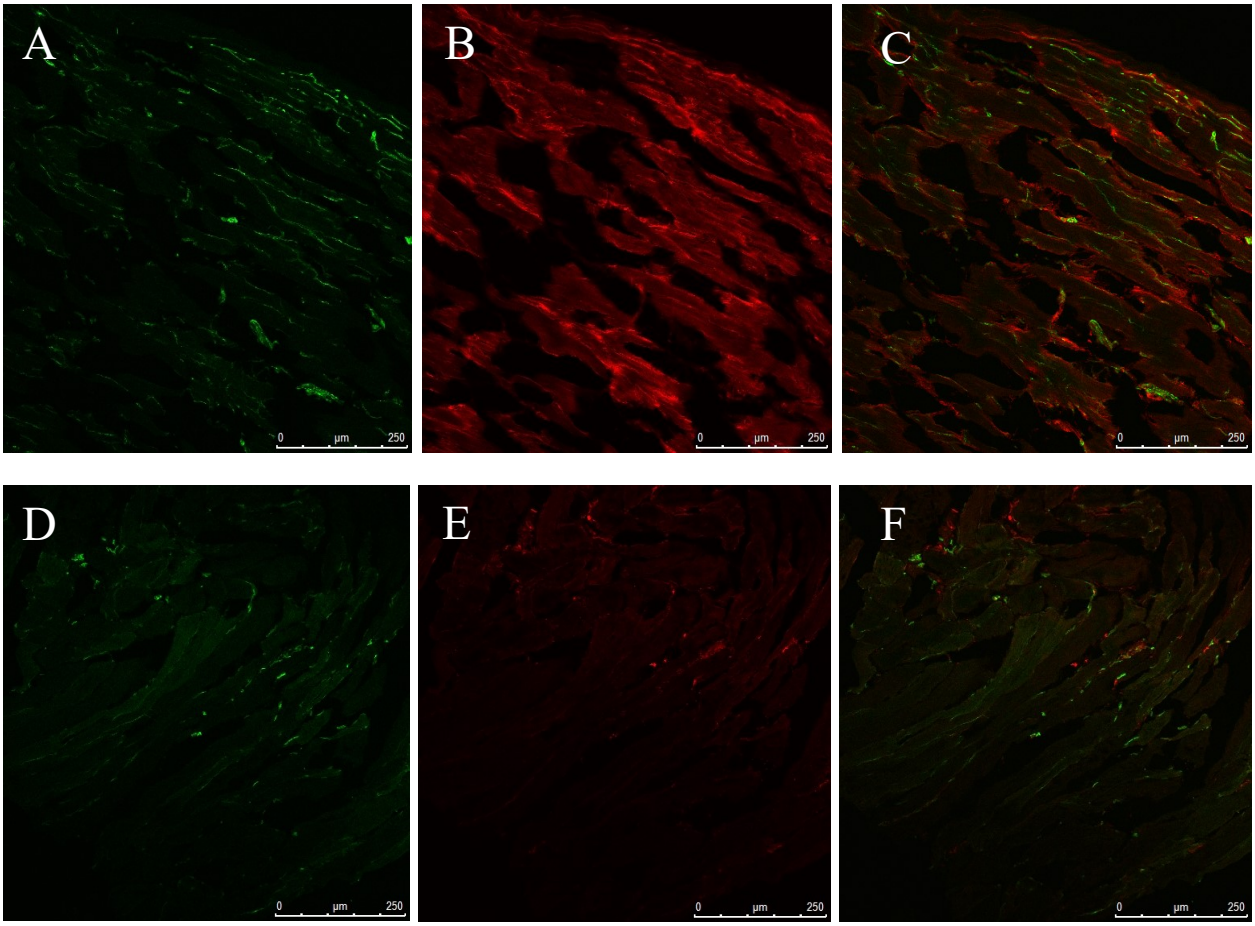


**Figure 15:** Confocal microscopy of eNOS-GFP and MMP-2 from ischemic gastrocnemius muscle tissue derived from PBS-administered and angiostatin-administered mice. **(A)** eNOS-GFP fluorescence from ischemic tissue derived from PBS-administered mouse tissue. **(B)** MMP-2 staining of ischemic tissue derived from PBS-administered mouse. **(C)** Overlay of eNOS-GFP and MMP-2 signals from ischemic tissue derived from PBS-administered mouse. **(D)** eNOS-GFP fluorescence from ischemic tissue derived from angiostatin-administered mouse. **(E)** MMP-2 staining from ischemic tissue derived from angiostatin-administered mouse. **(F)** Overlay of eNOS-GFP and MMP-2 signals from ischemic tissue derived from angiostatin-administered mouse. Images taken with a Leica TCS SP5 confocal microscope at 20x magnification.

### 4.4 The ATP synthase α subunit neutralizes angiostatin to increase total pro-MMP-2/MMP-2 in hypoxic HMVEC-C

A potential novel approach to promoting angiogenesis in a hypoxic environment is to negate the anti-angiogenic effects of angiostatin on hypoxic endothelial cells. This may be achieved by utilizing recombinant ATP synthase subunits as decoy receptors to bind angiostatin therefore preventing its inhibition of endothelial cell MMP-2 protein expression during hypoxia. HMVEC-C were treated with angiostatin (600nM) and angiostatin in the presence of either the ATP synthase α, β or δ (3μM) subunit, followed by incubation for 48 hours in hypoxic conditions. Cell lysates were analyzed via gelatin zymography for total pro-MMP-2 and MMP-2 levels. As presented in Figure 16, HMVEC-C co-treated with angiostatin and the ATP synthase α subunit had significantly higher levels of total pro-MMP-2 and MMP-2 (165.7±13%) after 48 hours of hypoxia compared to cells treated with angiostatin alone (59.3±10%), and with cells co-treated with angiostatin and the ATP synthase δ subunit (73.3±37%). Cells co-incubated with angiostatin and the ATP synthase β subunit exhibited a trend towards increased average total pro-MMP-2 and MMP-2 (127.7%±35%) compared to angiostatin alone (59.3±10%), however this was not statistically significant. Data expressed as mean of % of PBS control ± standard error of the mean.

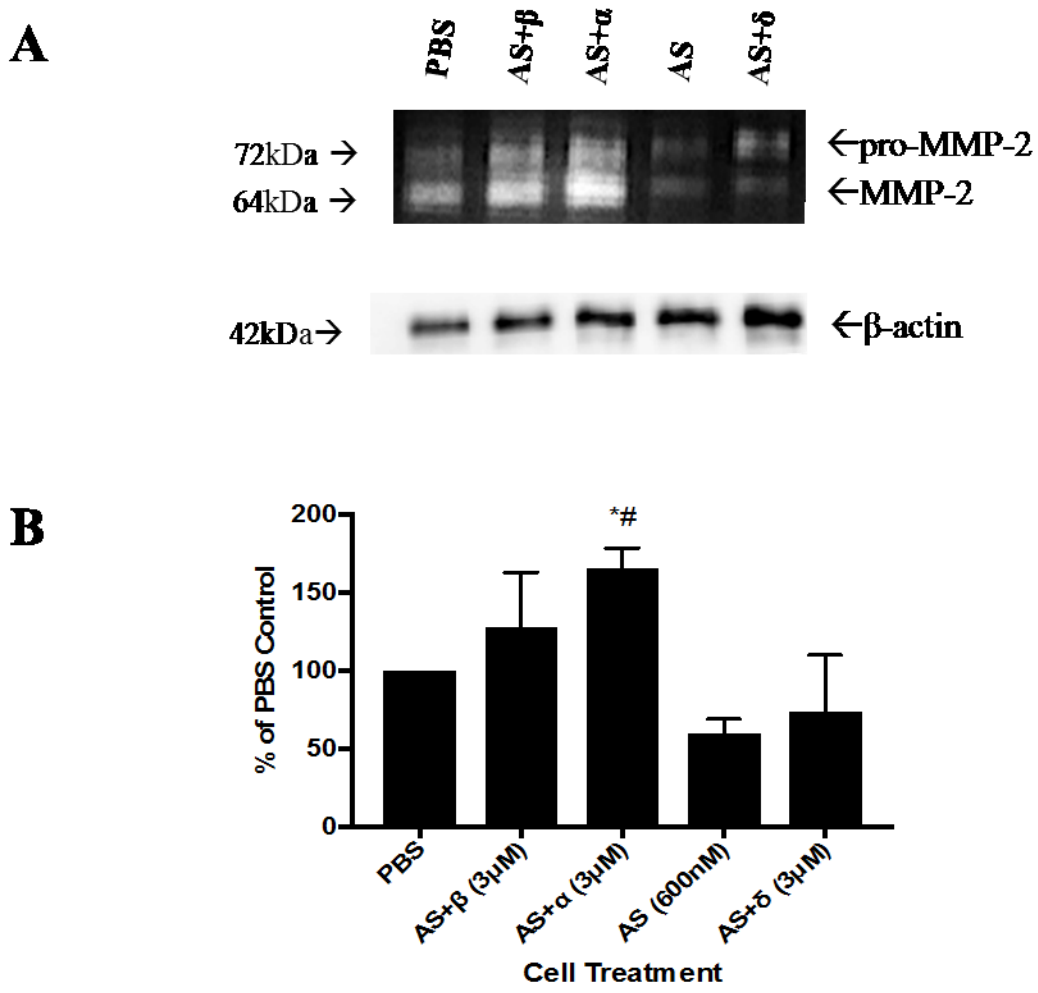


**Figure 16: (A and B)** Representative zymogram and corresponding β-actin loading control western blot and summary bar graph measuring total pro-MMP-2/MMP-2 protein expression by hypoxic HMVEC-C treated with angiostatin (AS) (600nM) in the presence of ATP synthase subunits (3μM). Statistics: One-way ANOVA, parametric, Tukey's multiple comparisons test, *p<0.05 vs AS, #p<0.05 vs AS+δ, n=3.

### 4.5 ATP synthase α subunit neutralizes angiostatin to increase HMVEC-C MMP-dependent migration during hypoxia

Angiostatin inhibits endothelial cell migration, an early step in angiogenesis (Ji *et al.,* 1998, Eriksson *et al.,* 2003, Radziwon-Balicka *et al.,* 2013). Therefore, ATP synthase subunits were used to investigate if neutralizing angiostatin would restore HMVEC-C MMP-dependent migration in hypoxic conditions. MMP-dependence within this migration assay has been established in a previous study (Radziwon-Balicka *et al.,* 2013). Utilizing a modified Boyden chamber assay, MMP-dependent endothelial cell migration towards a VEGF-gradient was analyzed in response to treatment with angiostatin (600nM) in the presence of either the ATP synthase α, β or δ (3μM) subunits during 48 hours of hypoxia. Overall, there was a significant difference in HMVEC-C migration between treatments, with HMVEC-C treated with angiostatin exhibiting an average decrease in migration (64.3±7%). While HMVEC-C co-treated with angiostatin and the ATP synthase α subunit exhibited a similar extent of migration (99±18%) to PBS control (100%). The average extent of HMVEC-C migration with angiostatin in the presence of the ATP synthase β subunit (53±17%) and the ATP synthase δ subunit (53±16%) were similar to angiostatin alone (64.3±7%) (Figure 17 and 18). Data expressed as mean % of PBS control ± standard error of the mean.

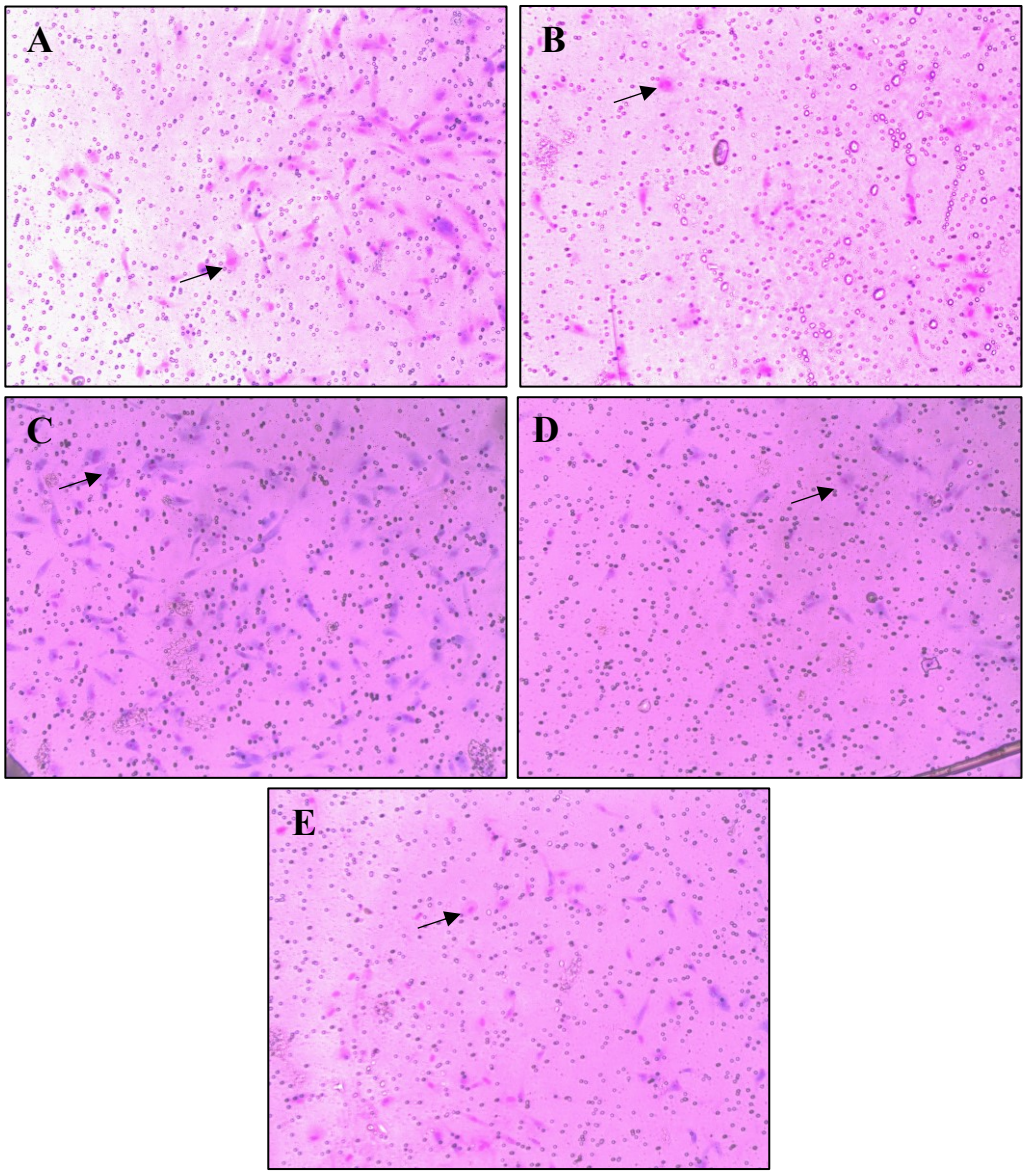


**Figure 17:** Representative images of stained migrated cells from one field of view at 10x magnification. **(A)** PBS. **(B)** Angiostatin (600nM). **(C)** Angiostatin (600nM) +ATP synthase α subunit (3μM). **(D)** Angiostatin (600nM) +ATP synthase β subunit (3μM). **(E)** Angiostatin (600nM) +ATP synthase δ subunit (3μM). Arrows denote stained migrated cells.

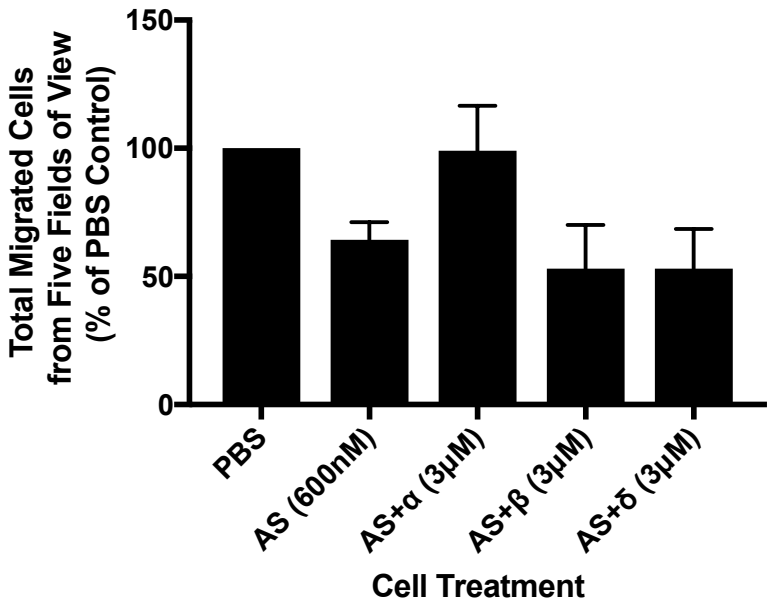


**Figure 18:** Summary bar graph measuring total HMVEC-C migration in the presence of angiostatin (AS) and the ATP synthase subunits during hypoxia. Statistics: One-way ANOVA, parametric, Tukey's multiple comparisons test, $p<0.05$ between treatments, n=3.

### 4.6 ATP synthase α, β, and δ subunits do not interfere with fibrinolysis *in vitro*

An important question to investigate in this research is whether the ATP synthase subunits interfere with fibrinolysis. Angiostatin is derived from a fibrinolytic protein, plasminogen, and as a result there are structural similarities. Therefore, it is important to determine if the ATP synthase subunits bind to plasminogen, as this would interfere with plasminogen conversion to plasmin, thus disrupting fibrin clot break down, as summarized in Figure 19. A disruption of fibrinolytic processes would be a barrier towards translating this research into a clinical setting. Fibrinolysis was assessed as % fibrinolysis compared to tPA alone (control). Aprotinin was used as a positive control, as it is a serine protease inhibitor which inhibits both plasminogen and plasmin. As presented in Figure 20, % fibrinolysis of thrombin-induced clots in the presence of aprotinin (10μM) was significantly decreased (29.7±15% of tPA control). Percent fibrinolysis of thrombin-induced clots in the presence of the ATP synthase α (96.2±4%), β (89.8±13%), and δ (104±3%) (3μM) subunits were not significantly different than tPA control (100%) (Figure 20). Data expressed as mean of % of tPA control ± standard error of the mean.

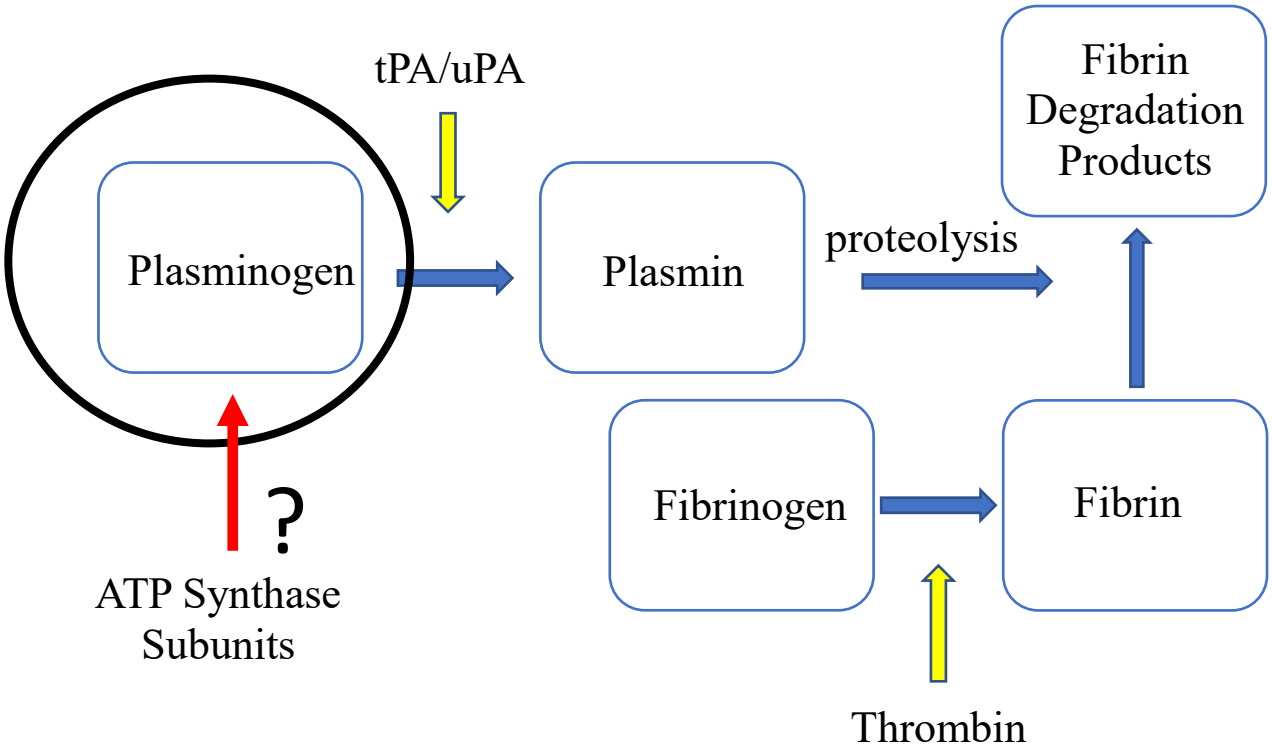


**Figure 19:** Schematic diagram of fibrin clot formation and fibrinolysis. Tissue plasminogen activator (tPA), urokinase plasminogen activator (uPA).

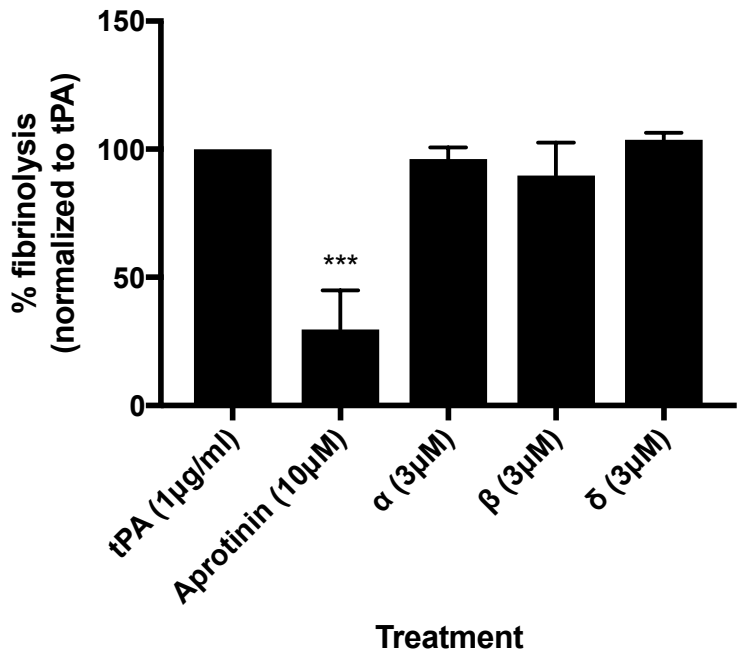


**Figure 20:** Summary bar graph of data representing % fibrinolysis. Percent fibrinolysis in tissue plasminogen activator (tPA) control group was measured as mean absorbance with thrombin alone compared to thrombin + tPA. Mean absorbance for treatment groups (aprotinin, ATP synthase α, β, and δ subunits) were normalized to tPA control. Statistics: One-way ANOVA, parametric, Dunnett's multiple comparisons test, ***$p<0.001$ vs tPA, n=3.

# 5. DISCUSSION

In ischemic diseases, such as CAD and PAD, therapeutic angiogenesis is an alternate treatment strategy for patients who are ineligible for conventional surgeries such as some patients with co-morbidities such as Type 2 diabetes (Aronson and Edelman 2010). In some patients, when arteries are obstructed due to the buildup of atherosclerotic plaque, either existing collateral vessels are remodeled or angiogenesis is stimulated to vascularize ischemic tissues (Helisch and Schaper 2003, Meier *et al.,* 2013). Clinical trials have mainly pursued the benefits of enhancing pro-angiogenic factors such as VEGF gene/protein therapy or endothelial progenitor cell/stem cell therapies. Unfortunately, results from these trials were not significantly effective in promoting therapeutic angiogenesis (Cao 2010, Dragneva *et al.,* 2013). A possible limitation of these trials is that the contribution of both pro-angiogenic and anti-angiogenic mediators need to be addressed to create an optimal environment for revascularization. Angiostatin has been identified as a platelet-derived anti-angiogenic factor which has potent inhibitory effects on endothelial cell migration, proliferation, survival and vessel tube formation (O'Reilly *et al.,* 1994, Ji *et al.,*1998, Lucas *et al.,* 1998, Jurasz *et al.,* 2003). As previously mentioned, CAD patients commonly have co-morbidities, such as Type 2 diabetes, and studies have found that these patients can have elevated levels of angiostatin (Sodha *et al.,* 2009). Even if this patient population receives pro-angiogenic factor therapy, the extent of stimulated angiogenesis may be limited due to high levels of this potent anti-angiogenic factor. In fact, investigations undertaken in CAD patients observed that elevated angiostatin levels were negatively associated with coronary collateral blood vessel growth (Matsunaga *et al.,* 2005).

Thus, angiostatin is a potential target to neutralize for the promotion of therapeutic angiogenesis.

At a low extracellular pH, angiostatin induces intracellular acidosis via inhibition of an ectopic ATP synthase on endothelial cells. By this mechanism, it has been shown to induce endothelial cell apoptosis, and inhibit endothelial cell migration (Moser *et al.,* 1999., Moser *et al.,* 2001, Burwick *et al.,* 2005). However, within a hypoxic environment, which can also induce an acidic extracellular pH, endothelial cells will upregulate HIF-1 leading to increased VEGF transcription thereby activating pro-survival mechanisms that can rescue endothelial cells from angiostatin-induced apoptosis (Wang *et al.,* 1995, Lin *et al.,* 2004, Jurasz *et al.,* 2010., Jurasz *et al.,* 2011). This would suggest that angiostatin may have alternative anti-angiogenic effects to cell apoptosis in a hypoxic setting. To understand the potential contribution of angiostatin to ischemic disease, of which hypoxia is a major component, its molecular mechanism of action on cardiac derived endothelial cells within a hypoxic setting was investigated.

Matrix metalloproteinases are crucial to the angiogenic response of endothelial cells, and they play a fundamental role in cell migration, liberation of ECM-sequestered angiogenic factors, and ECM remodelling as they cleave major ECM proteins such as collagen, laminin, elastin and fibronectin (Sternlicht and Werb 2001, Rundhaug 2005).

Previously, human microvascular endothelial cells derived from the lung (HMVEC-L) were shown to express decreased levels of MMP-2 and MT1-MMP in response to angiostatin under hypoxic conditions (Radziwon-Balicka *et al.,* 2013). To gain a more complete understanding of the mechanism of action of angiostatin in a context of CAD, I performed a similar study using HMVEC derived from cardiac microvasculature.

I hypothesized that a physiological concentration of angiostatin (600nM) would decrease HMVEC-C MMP-2 protein expression during hypoxia. Gelatin zymography analysis was utilized to analyze total pro-MMP-2 and MMP-2 protein levels from HMVEC-C. Hypoxia itself increased total pro-MMP-2 and MMP-2 levels compared to normoxic cells. This is consistent to other *in vitro* studies where prolonged hypoxia resulted in increased MMP-2 protein levels in human macrovascular cells and human umbilical vein endothelial cells (Ben-Yosef *et al.,* 2002, Ben-Yosef *et al.,* 2005). However, this result was inconsistent with previous experiments involving angiostatin hypoxic-specific inhibition of HMVEC-L MMP-2 expression (Radziwon-Balicka *et al.,* 2013). Hypoxia increased HMVEC-L MMP-2 mRNA expression, however this did not translate to enhanced MMP-2 protein expression, which the authors suggested could reflect hypoxia-mediated suppression of protein translation (van den Beucken *et al.,* 2006, Radziwon-Balicka *et al.,* 2013). The differences in MMP-2 protein expression by HMVEC-C and HMVEC-L in response to hypoxia, highlights the variations between endothelial cells derived from different tissues.

Under normoxic conditions, physiological concentrations of angiostatin did not have any significant effect on HMVEC-C total pro-MMP-2 and MMP-2 protein content. However, during hypoxia angiostatin decreased HMVEC-C total pro-MMP-2 and active MMP-2 protein levels compared to hypoxic control (Fig. 8). This is consistent to previous studies performed with HMVEC-L (Radziwon-Balicka *et al.,* 2013). A loss in active MMP-2 could be due to both a reduction in MMP-2 mRNA expression, and a reduction in MT1-MMP expression or activity, as it functions to convert the pro-MMP-2 zymogen to its active state (Deryugina *et al.,* 2001). While a decrease in pro-MMP-2 could be due to angiostatin mediated reduction in MMP-2 mRNA levels.

This has been demonstrated in HMVEC-L where angiostatin inhibited both MMP-2 and MT1-MMP mRNA and protein levels during hypoxia (Radziwon-Balicka *et al.,* 2013). Within a hypoxic environment, angiostatin-mediated inhibition of endothelial cell production of MMP-2 would contribute to impaired endothelial cell migration effectively inhibiting an early stage of angiogenesis. This has been observed in studies involving MMP-2 null mice which exhibit impaired revascularization during ischemia (Lee *et al.,* 2005).

Endothelial function and the angiogenic response are mediated by NO, a bioactive molecule produced by eNOS, an enzyme constitutively expressed by endothelial cells (Lamas *et al.,* 1992). During normoxia, eNOS protein expression was similar between angiostatin-treated and PBS-treated cells. However, a physiological concentration of angiostatin reduced eNOS protein expression by hypoxic HMVEC-C compared to angiostatin treated HMVEC-C exposed to normoxic conditions (Fig. 9). These results were consistent with studies performed in HMVEC-L whereby angiostatin decreased eNOS expression in a hypoxic environment (Radziwon-Balicka *et al.,* 2013). Angiostatin has been previously associated with inhibition of eNOS activity as it reduces endothelial-dependent vasodilation in response to acetylcholine in rat arterioles, an effect that was comparable to treatment with L-NAME (Koshida *et al.,* 2003). A loss or depletion of eNOS can contribute to a stunted angiogenic response, for example aortic segments from eNOS-KO mice demonstrate reduced endothelial cell migration, and proliferation leading to inhibition of sprout formation *in vitro* (Lee *et al.,* 1999).

Furthermore, studies *in vivo* demonstrate that eNOS-KO mice exhibit reduced wound repair, and impaired revascularization after an ischemic challenge leading to critical limb ischemia (Lee et al., 1999, Yu *et al.,* 2005). This highlights the importance of eNOS expression and activity within a setting of ischemic-induced angiogenesis.

Angiostatin's inhibition of eNOS expression within a hypoxic environment would contribute to impaired NO production of which is essential for proper endothelial function and endothelial cell responses in ischemia-induced angiogenesis (Murohara *et al.,* 1998).

However, the downstream events that occur between angiostatin-mediated inhibition of endothelial surface ATP synthase and reduction in eNOS and MMP-2 protein expression remain unclear. One proposed pathway involves the tumor suppressor protein, p53. In HMVEC-L, angiostatin was also found to inhibit p53 expression during hypoxia (Radziwon-Balicka *et al.,* 2013). The authors of this study proposed that under hypoxic conditions angiostatin's inhibition of the endothelial surface ATP synthase results in decreased ATP synthesis leading to disrupted intracellular downstream calcium signalling via a P2Y-purinoceptor. The P2Y-purinoceptor is a known extracellular receptor of ATP, and intracellular calcium levels within endothelial cells can be influenced by ATP (Mo *et al.,* 1991, Ralevic and Burnstock 1998). In summary, ATP depletion could contribute to disrupted downstream calcium signalling thereby attenuating calcium-dependent eNOS activity leading to a reduction in NO production. This is supported by a link between ATP and NO, as ATP has been shown to induce the release of NO from endothelial cells (Gordon 1990). Furthermore, NO levels are known to regulate p53 expression (Forrester *et al.,* 1996). Therefore, with a depletion of NO there may be reduced p53 expression, as observed in hypoxic HMVEC-L treated with angiostatin (Radziwon-Balicka *et al.,* 2013). As p53 is a modulator of MMP-2 transcription (Bian and Sun, 1997), a reduction in p53 could lead to attenuated transcriptional regulation of MMP-2. Moreover, involvement of p53 in the mechanism of action of angiostatin is also demonstrated by a lack of inhibition of angiogenesis by angiostatin in p53-null mice (Jimenez *et al.,* 2000).

Results from these experiments demonstrate that angiostatin acts in a hypoxic-specific manner. This environment specific mechanism of action was also observed in a study where angiostatin's inhibitory effects on angiogenesis were restricted to tumor-affected organs in mice (Lee *et al.,* 2009). The vascular environment in ischemic diseases and tumor growth share a common characteristic; poor oxygen perfusion leading to hypoxia and a resulting acidic extracellular environment due to endothelial cells switching to anaerobic metabolism (Koziel and Jarmuszkiewicz 2017).

Angiostatin's target, the ATP synthase, may exhibit changes in activity or expression within different extracellular environments. Expression of ATP synthase has been found to be increased on cell surfaces at a low extracellular pH and in hypoxic environments (Ma *et al.,* 2010). Furthermore, ATP synthesized by the enzyme activates surface P2Y receptors on endothelial cells, which have been found to transactivate VEGFR-2 to stimulate endothelial cell tubulogenesis (Rumjahn *et al.,* 2009). In settings of hypoxia and resulting acidic conditions, the surface ATP synthase function may be important for endothelial cell maintenance of intracellular pH (Wahl *et al.,* 2005), as well as an important source of ATP that mediates endothelial cell angiogenic responses, such as VEGFR-2 activation (Rumjahn *et al.,* 2009). Thus, inhibition of ATP synthase activity by angiostatin specifically in a low oxygen environment, when its activity could be crucial, could be what leads to a disruption of endothelial cell angiogenic responses.

Angiostatin administration *in vivo* has been shown to reduce tumor growth via impaired angiogenesis (O'Reilly *et al.,* 1994., O'Reilly *et al.,* 1996., Bergers *et al.,* 1999). However, few studies have been performed that delineate angiostatin's effects on ischemic-induced angiogenesis *in vivo*, which could be applied to model an ischemic disease, such as PAD.

As mentioned previously, elevated levels of angiostatin have been associated with decreased coronary collateral angiogenesis in CAD patients (Matsunaga *et al.,* 2005, Sodha *et al.,* 2009). Thus, it is reasonable to hypothesize that excess angiostatin in mice will have inhibitory effects on angiogenesis in ischemic tissue. Folkman and colleagues have established that mice produce endogenous angiostatin (O'Reilly *et al.,*1994). Therefore, angiostatin (30μg) was administered to create an environment of excess angiostatin within male transgenic eNOS-GFP mice.

Hind limb ischemia was induced by excising the right femoral artery, thereby modeling acute peripheral artery disease whereby blood flow to an extremity is obstructed. C57BL mice do exhibit collateral vessel growth in response to femoral artery ligation (Couffinhal *et al.,* 1998, Wagner *et al.,* 2004). Animals were 12-14 months old, relatively older mice which is a rare occurrence in this field of study. Utilizing aged mice in a hind limb ischemia model can provide greater clinical significance towards the middle-aged to aged patient population that conventionally suffer from ischemic diseases. Furthermore, the rate of revascularization is slower in aged mice thereby allowing for any intervention of angiogenesis to be effectively observed within the experimental allotted time span (28 days) (Rivard *et al.,* 1999, Bosch-Marce *et al.,* 2007). In control and angiostatin-administered mice, blood flow recovery was evident over time after hind limb ischemia was induced (Fig. 10). However, at day 14 post-femoral artery excision, mice administered angiostatin exhibited significantly decreased blood reperfusion in the ischemic limb. (Fig. 11). As a marker of angiogenesis, eNOS-GFP, was visualized within gastrocnemius muscle tissue via confocal microscopy. Imaged eNOS-GFP fluorescence did indeed co-localize with VE-cadherin immunostaining, indicating that expressed eNOS-GFP was derived from the endothelium.

Ischemic tissue from control mice exhibited greater eNOS-GFP fluorescent intensity and distribution when compared to non-ischemic tissues (Fig. 13). Previous studies have observed this in other tissue localizations, such as increased eNOS levels in ischemic rat and human myocardium (Bloch *et al.,* 2001). Moreover, overexpression of eNOS has been reported to be beneficial to promoting angiogenesis in porcine myocardium (Kupatt 2007). Therefore, the increase in eNOS-GFP intensity within the ischemic tissue of control mice could reflect the important role of eNOS in revascularization of ischemic tissues (Murohara *et al.,* 1998, Yu 2005). In contrast, eNOS-GFP fluorescent intensity within ischemic gastrocnemius muscle tissue from angiostatin-administered mice was reduced when compared to control mice (Fig. 13). This pattern parallels with the present HMVEC-C and previous HMVEC-L *in vitro* studies, where angiostatin treatment reduced eNOS protein expression during hypoxia (Radziwon-Balicka *et al.,* 2013). Additionally, this could explain the reduced blood flow recovery exhibited by the angiostatin-administered mice. A loss of eNOS expression and NO bioavailability would impair angiogenesis and therefore slow blood perfusion recovery. This is illustrated in eNOS-deficient mice which exhibit impaired myocardial angiogenesis during development (Zhao *et al.,* 2002), and reduced neovascularization in response to HLI (Aicher *et al.,* 2003).

MMP-2 expression in an *in vivo* setting has been correlated with increased angiogenesis, and endothelial progenitor cell migration (Rojiani *et al.,* 2010., Kanayasu-Toyoda *et al.,* 2015). Therefore, as an important mediator of endothelial cell migration and angiogenesis, MMP-2 was visualized via immunohistochemistry of mice gastrocnemius muscle tissue.

In control mice, MMP-2 staining was increased in ischemic tissue versus non-ischemic tissue (Fig. 14). This result is in line with a previous study demonstrating increased MMP-2 protein expression from ischemic gastrocnemius muscle tissue within a mouse HLI model (Muhs *et al*., 2003). As observed with the eNOS-GFP, this again could reflect the role of MMP-2 in ischemia-induced angiogenesis. In comparison, MMP-2 staining was reduced in ischemic tissue derived from angiostatin-administered mice (Fig. 15). This pattern parallels to *in vitro* results, whereby angiostatin decreased MMP-2 protein expression from HMVEC-C and HMVEC-L during hypoxia (Radziwon-Balicka *et al.,* 2013). This consistency in downregulation of MMP-2 and eNOS mediated by angiostatin provides valuable insight into how it inhibits angiogenesis within ischemic environments.

In summary, as markers of angiogenesis, eNOS and MMP-2, were reduced in ischemic tissue harvested from angiostatin-administered mice that underwent HLI. These results suggest that the decrease in blood flow perfusion observed in response to angiostatin administration can be attributed to a reduction in ischemia-induced angiogenesis. This is in line with previous *in vivo* investigations demonstrating the association between elevated angiostatin levels and impaired angiogenesis. For instance, increased levels of angiostatin were correlated with impaired coronary angiogenesis in a canine myocardial infarction model (Matsunuga *et al.,* 2002). Also, rats with metabolic syndrome exhibited increased angiostatin levels which contributed to reduced coronary collateral growth in response to myocardial infarction (Dodd *et al.,* 2013). Additionally, ischemic injury in diabetic rats has been shown to elevate angiostatin levels, and decrease capillary growth responses (Liang *et al.,* 2016). Finally, neovascularization in a rabbit aortic stent model was attenuated by angiostatin delivered intravascularly (Ganaha *et al.,* 2004).

Thus, my results further contribute to delineating the inhibitory role of angiostatin on angiogenesis within a model of PAD, and provides a basis for investigating angiostatin as a target to neutralize to promote therapeutic angiogenesis.

Different approaches to neutralizing angiostatin can involve inhibition of angiostatin production, or prevention of its platelet-derived release into the bloodstream. Each approach has potential issues that if further developed may prevent clinical success. Previous studies have determined that anti-platelet drugs, such as aspirin, can prevent angiostatin release from platelet granules by inactivating platelets, however this prevented granular release of pro-angiogenic factors such as VEGF, and has no effect on *de novo* production of angiostatin from plasminogen cleavage (Radziwon-Balicka *et al.,* 2013). Aprotinin has been investigated to prevent angiostatin generation via inhibition of the plasminogen/plasmin pathway, however this would disrupt fibrinolytic processes and therefore would be invalid for use clinically as a pro-angiogenic therapy (Jurasz *et al.,* 2006).

In 1999, Moser and colleagues identified an endothelial surface target for angiostatin, an ectopic ATP synthase. ATP synthase was conventionally thought to be exclusively expressed within the mitochondria, however it was identified to be localized and functional on the surface of endothelial cells (Moser *et al.,* 1999, Moser *et al.,* 2001). Angiostatin was found to bind to the α/β subunits that make up the structure of the F1 domain of the ATP synthase enzyme (Moser *et al.,* 1999). Therefore, a novel approach was explored to utilize the ATP synthase α and β subunits as decoy receptors to bind angiostatin, thereby preventing it from exerting its anti-angiogenic effects on endothelial cells.

Results demonstrated that the ATP synthase α subunit neutralized angiostatin as hypoxic HMVEC-C had increased total pro-MMP-2 and MMP-2 protein expression when compared to hypoxic HMVEC-C treated with angiostatin alone, and with HMVEC-C co-treated with angiostatin and the ATP synthase δ subunit (Fig. 16). The ATP synthase δ subunit was not identified as a binding target for angiostatin (Moser *et al.,* 1999), and as expected HMVEC-C treated with angiostatin and the ATP synthase δ subunit did not demonstrate a difference in total pro-MMP-2 and MMP-2 expression compared to angiostatin alone. Therefore, the effects observed with the ATP synthase α subunit are not due to "non-angiostatin" specific subunit activity upon HMVEC-C. Co-treatment of HMVEC-C with angiostatin and the ATP synthase β subunit demonstrated a trend towards increased levels of total pro-MMP-2 and MMP-2 during hypoxia, however it was not increased to the same extent as observed with the α subunit, and was not statistically different than angiostatin treatment alone (Fig. 16). A possible explanation for this is differences in binding kinetics between the subunits, as angiostatin may bind more efficiently to the α subunit than the β subunit at the concentrations used.

In addition, competitive binding to the subunits by other factors present in the cell culture cannot be ruled out. For example, β-thymosin is an extracellular modulator of angiogenesis that has been shown to bind and interact with the β subunit of endothelial cell surface ATP synthase (Freeman *et al.,* 2010). A possible source of β-thymosin in the *in vitro* experiments could be the 0.5% FBS used in the cell culture medium. If angiostatin competes with β-thymosin for binding of the decoy β subunit, perhaps increasing the concentration of the β subunit would be more successful in neutralizing angiostatin.

Angiostatin has been shown to inhibit endothelial cell migration *in vitro* (Eriksson *et al.,* 2003, Radziwon-Balicka *et al.,* 2013). Endothelial cell migration through the ECM environment during angiogenesis is partly mediated by MMP-2 (Rundhaug 2005), therefore a MMP-dependent migration assay was utilized to investigate if neutralizing angiostatin would restore HMVEC-C migration during hypoxia. HMVEC-C were seeded into a modified Boyden chamber that was separated from a lower chamber via a gelatin-coated membrane. This would ensure that cell migration towards a VEGF-gradient would be partially mediated by MMP-2 activity as gelatin is a preferred substrate (Tryggvason *et al.,* 1992). Statistical analysis of data from these experiments indicated significant difference between cell treatments, with HMVEC-C co-treated with angiostatin and the ATP synthase α subunit exhibiting an average increase in migration compared to angiostatin treatment alone and angiostatin with the ATP synthase δ subunit (Fig. 18). This pattern was not observed in cells co-incubated with angiostatin and the ATP synthase β subunit. As mentioned previously, the ATP synthase β subunit at the concentration used may bind with proteins other than angiostatin, such as β-thymosin, and the binding of angiostatin to the decoy receptor may not be as efficient as the ATP synthase α subunit. In addition, there is a possibility that increasing the n number of these experiments may give more power to the statistical analysis.

Thus, utilizing the ATP synthase α subunit as an angiostatin decoy receptor to increase endothelial cell pro-MMP-2 and MMP-2 protein expression and migration within a hypoxic setting could have a significant impact on an essential early step in angiogenesis.

Previous attempts to neutralize angiostatin involved utilizing aprotinin to prevent plasminogen/plasmin derived angiostatin formation (Jurasz *et al.,* 2006). However, interfering with the plasminogen/plasmin system results in interference with fibrinolysis. Therefore, the ATP synthase subunits were tested *in vitro* to determine if they disrupted the fibrinolytic process mediated by the plasminogen/plasmin system. These results indicated that the ATP synthase subunits at the given concentrations (3μM) showed no interference with fibrin clot breakdown *in vitro* (Fig. 20).

In summary, these results are promising for future studies involving the ATP synthase subunits as decoy receptors to neutralize angiostatin within an *in vivo* setting, and for potential translational research into a clinical setting.

## 5.1 Limitations of the Study

As gelatin zymography analysis involves dissociation of TIMP/MMP complexes, only MMP-2 protein expression can be measured, while the activity of MMP-2 within a biological setting cannot be truly represented. Analyzing levels of TIMPs, such as TIMP-2, could be performed to further elucidate angiostatin's hypoxic-specific effects on balances between TIMP and MMPs thereby providing insight into the extent of MMP-2 activity. Furthermore, MMP-2 mRNA expression was not analyzed, and so the results obtained can only speak to protein expressed following protein translation. Additionally, a possible limitation of treating cultured cells *in vitro* with angiostatin is that the surface ATP synthase activity can be influenced by flow conditions (Yamamoto *et al.,* 2007). Thus, factors such as shear stress should be considered when delineating angiostatin's mechanism of action *in vitro*. Furthermore, different cell culture compositions, for example two-dimensional (monolayer growth) versus three dimensional (collagen matrix), can influence the response of endothelial cells (Haas *et al.,* 1998, Stegemann *et al.,* 2003). When analyzing MMP-dependent cell migration in a Boyden chamber assay, cell migration through the gelatin-coated membrane could be mediated partially by MT1-MMP, as extent of MMP-2 activation can be regulated via MT1-MMP (Strongin *et al.,* 1995). Therefore, it is important for future research to delineate angiostatin's effects on HMVEC-C expression of MT1-MMP during hypoxia.

During this project, a major issue occurred while ordering and receiving the ATP synthase subunits. Unfortunately, this caused a major delay as I was unable to utilize the ATP synthase subunits for almost a year due to delays with manufacturing by the supplier, followed by issues with contamination of the subunits by the supplier when received.

As a result, this limited the extent to which this study could investigate the ATP synthase subunits in neutralizing angiostatin, particularly investigating them *in vivo* utilizing the HLI model.

# 6. CONCLUDING REMARKS

Angiostatin is an anti-angiogenic factor that demonstrates inhibitory effects on endothelial cell migration, proliferation and survival (Claesson-Welsh *et al.,* 1998, Ji *et al.,* 1998, Griscelli *et al.,* 1998, Troyanovsky *et al.,* 2001, Hanford *et al.,* 2003, Sharma *et al.,* 2004). Due to complexities introduced into studies by utilizing different isoforms of angiostatin and the contribution of different microenvironments, its exact mechanism of action has yet to be fully elucidated. In this study, it was hypothesized that angiostatin acts to reduce angiogenic mediators, MMP-2 and eNOS, within cardiac-derived endothelial cells specifically in a hypoxic environment**.** Furthermore, as elevated levels of angiostatin have been associated with impaired angiogenesis (Matsunuga *et al.,* 2005, Sodha *et al.,* 2009, Dodd *et al.,* 2013), I hypothesized that administering angiostatin in transgenic eNOS-GFP mice, thereby creating an environment of excess angiostatin, would reduce recovery angiogenesis in a HLI model. Neutralization of angiostatin is a novel potential strategy to promote therapeutic angiogenesis, and would be beneficial particularly in an ischemic disease setting where elevated levels of angiostatin correlate with reduced blood vessel growth. Since an ATP synthase identified on the surface of endothelial cells is well-characterized target for angiostatin (Moser *et al.,* 1999, Moser *et al.,* 2001), ATP synthase $\alpha$ and $\beta$ subunits were investigated for their potential to selectively neutralize angiostatin to restore MMP-2 levels, and MMP-dependent endothelial cell migration at concentrations that did not interfere with fibrinolysis *in vitro*. In summary, results from this study are as follows:

1. Angiostatin decreases HMVEC-C total pro-MMP-2 and MMP-2 protein expression in hypoxic conditions.

2. Angiostatin decreases eNOS protein expression in hypoxic HMVEC-C in comparison to angiostatin treated normoxic HMVEC-C.

3. Administering angiostatin in transgenic eNOS-GFP mice significantly reduced blood flow recovery in response to HLI. This is due to impaired angiogenesis as indicated by reduced levels of angiogenic markers, eNOS and MMP-2, within ischemic tissue.

4. Neutralizing angiostatin with the ATP synthase α subunit increases hypoxic HMVEC-C total pro-MMP-2 and MMP-2 protein expression compared to angiostatin alone.

5. Through neutralization of angiostatin within a migration assay, hypoxic HMVEC-C co-incubated with the ATP synthase α subunit exhibited an average increase in MMP-dependent migration compared to angiostatin treatment alone.

6. The ATP synthase α and β subunits (3μM) did not interfere with fibrinolysis *in vitro.*

Therefore, utilizing the ATP synthase α subunit, as a decoy receptor to bind and prevent angiostatin from exerting its anti-angiogenic effects is a potential novel therapeutic strategy for promoting angiogenesis. To completely inhibit angiostatin action may lead to excessive angiogenesis, potentially in the case of an unrecognized indolent tumour. However, in patient populations where angiostatin levels are abnormally high, using decoy receptors to selectively bind excess angiostatin, in conjunction with providing pro-angiogenic therapy may have the potential to tip the 'angiogenic switch' to promote angiogenesis in ischemic vascular disease.

# 7. FUTURE DIRECTIONS

Platelets have been shown to store and release angiostatin derived from their α-granules (Jurasz *et al.,* 2003). Plasma concentrations of platelet-derived angiostatin can reach up to double its physiological concentration (600nM to 1.2μM) upon platelet activation (Radziwon-Balicka *et al.,* 2013). This may translate to pathological states where increased platelet activity may potentially lead to localized elevated levels of angiostatin. Therefore, it would be important to investigate the effects of a high concentration of angiostatin (1.2μM) on HMVEC-C during hypoxia, specifically if these high concentrations would override VEGF-mediated pro-survival signalling to induce apoptosis as observed in HMVEC-L (Radziwon-Balicka *et al.,* 2013). Additionally, further investigations with the ATP synthase subunits could determine if neutralizing angiostatin at these high concentrations would protect HMVEC-C from angiostatin-induced apoptosis.

In some CAD patients with Type 2 diabetes angiostatin levels have been found to be elevated in the blood, and correlated with impaired collateral vessel growth (Sodha *et al.,* 2009). Endothelial dysfunction, often due to loss of endothelial NO bioavailability, is a common pathological characteristic of Type 2 diabetes, and can contribute to a decrease in endothelial cell survival and impaired angiogenesis in response to ischemia. Thus, Type 2 diabetes is a high risk factor for development of cardiovascular diseases. (Tabit *et al.,* 2010, Kolluru *et al.,* 2012, Sena *et al.,* 2013, Howangyin and Silvestre 2014). Within these pathological conditions, downregulation of eNOS expression by angiostatin would further reduce NO availability, thereby contributing to the progression of endothelial dysfunction and impaired angiogenic responses.

Therefore, it was hypothesized that Type 2 diabetic (T2D) HMVEC-C would have impaired eNOS expression and as a result would be susceptible to cell death in response to angiostatin treatment during hypoxia.

A preliminary western blot analysis of basal eNOS expression from T2D HMVEC-C demonstrated reduced levels of eNOS compared to HMVEC-C derived from non-diabetic patients. I have performed preliminary studies where T2D HMVEC-C were treated with a physiological concentration of angiostatin (600nM) followed by incubation in hypoxia for 48 hours. Cell apoptosis and necrosis were measured by utilizing flow cytometry to analyze annexin V-fluorescein (FITC) and propidium iodide staining (Figure 22). Data from these experiments suggest that when compared to non-diabetic HMVEC-C, angiostatin induced apoptosis and more noticeably increased cell necrosis in hypoxic Type 2 diabetic HMVEC-C, as summarized in Figure 23. Further experiments are required to statistically analyze these results. In addition, investigating the effects of angiostatin on eNOS expression by Type 2 diabetic HMVEC-C may confirm whether Type 2 diabetic HMVEC-C are more susceptible to hypoxic challenge due to angiostatin's suppressive effects on already low eNOS levels. This route of investigation can determine if angiostatin-mediated reduction in eNOS expression within a model of endothelial dysfunction would further contribute to existing impairments in ischemia-induced angiogenesis.

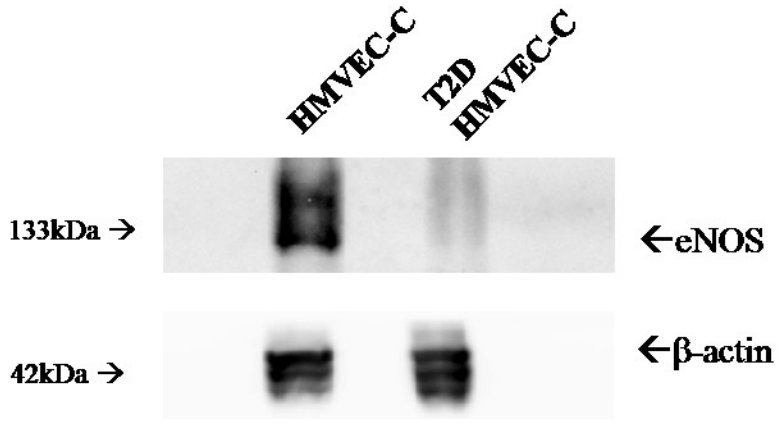


**Figure 21:** Western blot measuring basal levels of eNOS protein expression by HMVEC-C and T2D HMVEC-C.

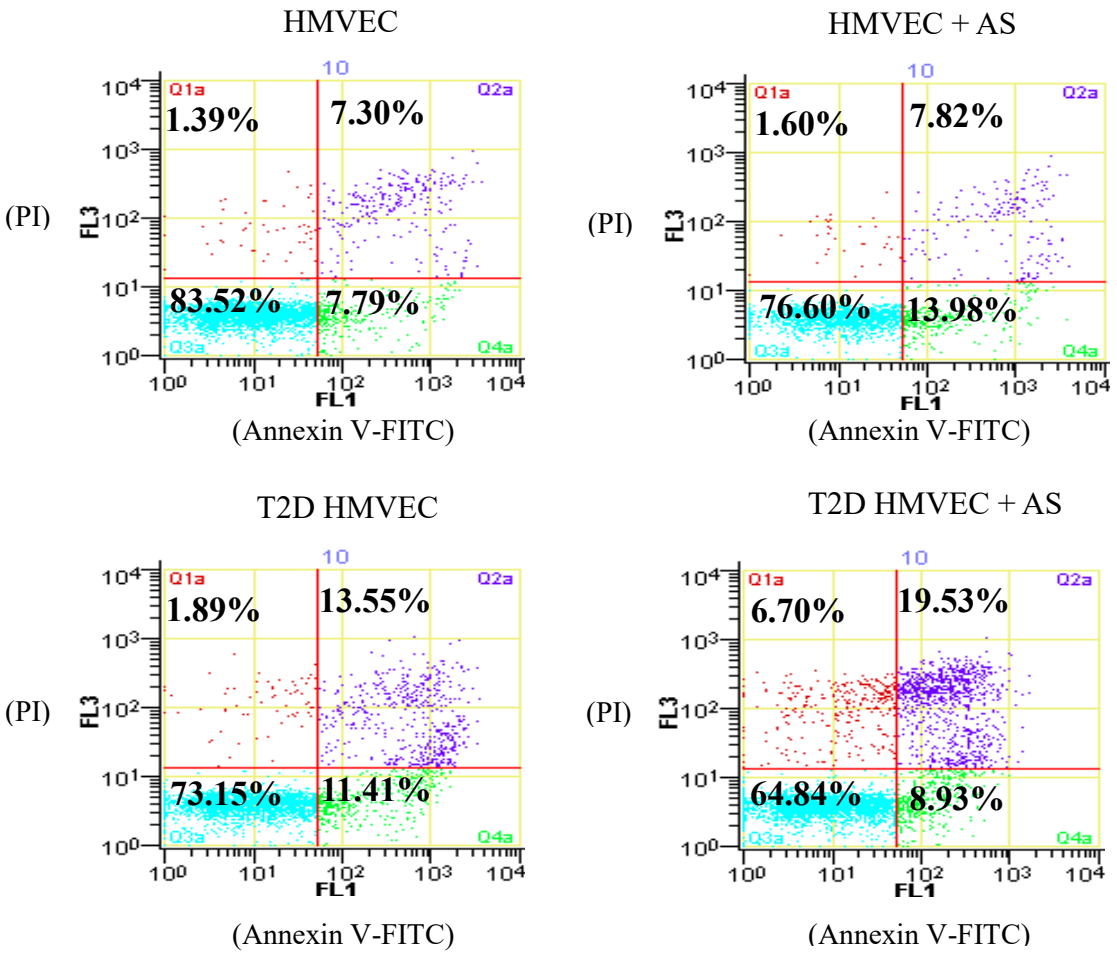


**Figure 22:** Representative dot plots of flow cytometry analysis of annexin V-FITC and propidium iodide staining. Hypoxic HMVEC-C treated with PBS or angiostatin (AS) (600nM). Hypoxic T2D HMVEC-C treated with PBS or angiostatin (600nM). Q3a: % live cells. Q4a: % early apoptotic cells. Q2a: % late apoptotic cells. Q1a: % necrotic cells.

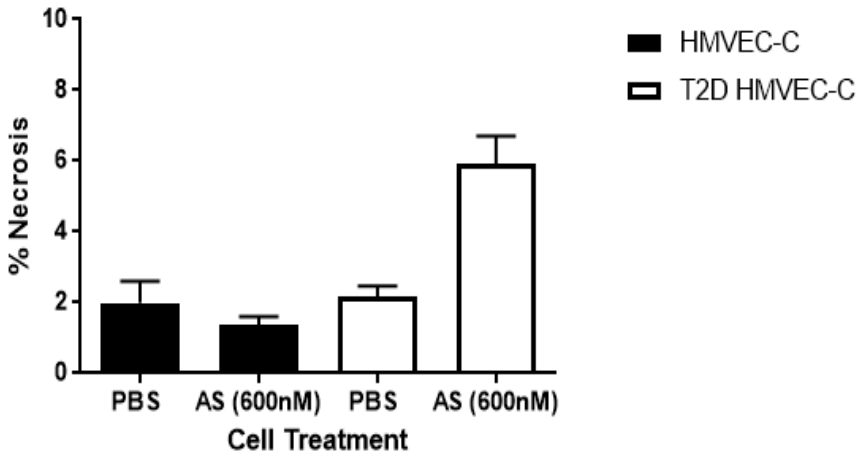


**Figure 23:** Summary bar graph of flow cytometry dot plot data measuring % necrosis from all groups. T2D HMVEC-C treated with angiostatin (AS) (600nM) exhibited a trend towards increased % necrosis compared to PBS treated T2D HMVEC-C after 48 hours of hypoxia, n=2.

Neutralization of angiostatin with the ATP synthase α subunit (3μM) yielded promising results whereby total pro-MMP-2 and MMP-2 were increased in hypoxic HMVEC-C. Future studies could involve developing a concentration-response curve, using a range such as 1μM-10μM of the ATP synthase α subunit, as well as the ATP synthase β subunit as perhaps at higher concentrations this subunit binds angiostatin more effectively.

Future experiments could include utilizing the ATP synthase subunits to neutralize angiostatin's inhibitory effects on eNOS expression by hypoxic HMVEC-C. Furthermore, since the ATP synthase subunits (3μM) did not interfere with the fibrinolytic process, there is a potential for further investigation into an *in vivo* hind limb ischemia model within transgenic eNOS-GFP mice. This could determine if neutralizing endogenous angiostatin in mice would promote angiogenesis in the ischemic limb. Additionally, studies investigating the ATP synthase subunits within a murine myocardial infarction model would be valuable to delineate if neutralizing angiostatin would promote angiogenesis in a cardiovascular disease model. To take this further, HLI experiments could be performed in T2D mice to explore the potential of neutralizing angiostatin to promote angiogenesis within a model of endothelial dysfunction.

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