Identification of Mechanisms Regulating Endothelial Cell Capillary Morphogenesis

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ABSTRACT

In order to effectively treat disorders whose pathology is marked by neovascularization, a better understanding of the pathways that mediate the processes involved in angiogenesis is needed. To this end we have identified two important pathways that regulate endothelial cell capillary morphogenesis, a key process in angiogenesis.

We have identified the small GTPase RhoB as being induced by vascular endothelial growth factor (VEGF) in human umbilical vein endothelial cells (HUVECs). Depletion of RhoB inhibited endothelial cell VEGF-mediated migration, sprouting, and cord formation. Cells depleted of RhoB showed a marked increase in RhoA activation in response to VEGF. Defects in cord formation in RhoB-depleted cells could be partially restored through treatment with the Rho inhibitor C3 transferase or ROCK I/II inhibitors, indicating increased RhoA activity and enhanced downstream signaling from RhoA contribute to the phenotype of decreased cord formation observed in cells depleted of RhoB. Interestingly, we did not observe a significant change in RhoC activity in RhoB-depleted cells suggesting differential regulation of RhoA and RhoC by RhoB in HUVECs.

We have also identified microRNA-30b (miR-30b) as being negatively regulated by VEGF and as being a negative regulator of HUVEC capillary morphogenesis. Overexpression of miR-30b significantly reduced HUVEC cord formation *in vitro*, while inhibition of miR-30b enhanced cord formation. Neither overexpression nor inhibition of miR-30b affected migration or viability of endothelial cells. Interestingly, miR-30b regulated the expression of TGFβ2 but not TGFβ1, with overexpression of miR-30b inducing expression of TGFβ2 mRNA and protein, and inducing phosphorylaton of Smad2, suggesting TGFβ2 produced in response to miR-30b overexpression functions in an

autocrine manner to stimulate HUVECs. MiR-30b effects on TGFβ2 expression were found to be regulated to an extent by ATF2, as miR-30b overexpressing cells exhibited increased levels of phosphorylated ATF2, with depletion of ATF2 via siRNA resulting in inhibition of miR-30b-induced TGFβ2 expression. Treatment of HUVECs with TGFβ2 inhibited cord formation, while TGFβ1 had no effect, indicating a major difference in how endothelial cells respond to these two related growth factors. Inhibition of TGFβ2 with a neutralizing antibody restored cord formation in miR-30b overexpressing cells to levels similar to control cells, thus identifying TGFβ2 expression as contributing to the inhibitory effects of miR-30b overexpression on capillary morphogenesis.

Thus, we have identified two signaling pathways regulated by VEGF in HUVECs that further our understanding of the process of angiogenesis and may provide novel targets for therapeutic intervention into diseases involving angiogenesis.

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LIST OF ABBREVIATIONS

ADP	Adenosine diphosphate
αSMA	Alpha smooth muscle actin
ALK	Activin receptor-like kinase
ANG	Angiopoietin
ATF2	Activating transcription factor 2
bFGF	Basic fibroblast growth factor
BAEC	Bovine aortic endothelial cell
BMP	Bone morphogenetic protein
CCM2	Cerebral cavernous malformation 2
cDNA	Complementary deoxyribonucleic acid
DLL4	Delta-like 4
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
EBM-2	Endothelial cell basal medium 2
ECM	Extracellular matrix
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EGM-2	Endothelial cell growth medium 2
ELISA	Enzyme-linked immunosorbent assay
EMT	Epithelial-to-mesenchymal transition
EndMT	Endothelial-to-mesenchymal transition
ERK	Extracellular signal-regulated kinase
FAK	Focal adhesion kinase
FBS	Fetal bovine serum
FDA	Food and Drug Administration
FTI	Farnesyl transferase inhibitor
GAP	GTPase-activating protein
GDF	Growth differentiation factor
GDNF	Glial-derived neurotrophic factor
GDP	Guanosine diphosphate
GEF	Guanine nucleotide exchange factor
GTP	Guanosine triphosphate
GTPase	Guanosine triphosphatase
HBSS	Hank's Balanced Salt Solution
HRP	Horse radish peroxidise
Hsp90	Heat shock protein 90
HUVEC	Human umbilical vein endothelial cell
IGF-1	Insulin-like growth factor 1
JDP2	Jun dimerization protein 2
JNK	c-Jun N-terminal kinase
LAP	Latency associated peptide
LDL	Low-density lipoprotein
LTBP	Latent transforming growth factor beta binding protein

MAPK	Mitogen-activated protein kinase
miRISC	MiRNA-induced silencing complex
miRNA	Micro-ribonucleic acid
MIS	Mullerian inhibiting substance
M-MLV	Moloney murine leukemia virus
MMP	Matrix metalloproteinase
mRNA	Messenger ribonucleic acid
NF-κB	Nuclear factor-kappaB
NP	Neuropilin
NSCLC	Non-small cell lung cancer
NSPC	Neural stem/progenitor cell
PAK	p21-activated kinase
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PDGF	Platelet-derived growth factor
PDGFR	Platelet-derived growth factor receptor
PECAM1	Platelet/endothelial cell adhesion molecule 1
PKC	Protein kinase C
PLCγ	Phospholipase C gamma
PlGF	Placental growth factor
Pre-miRNA	Precursor micro-RNA
Pri-miRNA	Primary micro-RNA
PTEN	Phosphatase and tensin homolog
qRT-PCR	Quantitative reverse transcription polymerase chain reaction
R-Smad	Receptor-regulated Smad
Rho	Ras homologous
RhoGDI	Rho-specific guanine nucleotide dissociation inhibitor
RhoGDIα	Rho-specific guanine nucleotide dissociation inhibitor alpha
RNA	Ribonucleic acid
ROCK	Rho-associated protein kinase
RT	Reverse transcriptase
SEMA6A	Semaphorin 6A
siRNA	Small interfering ribonucleic acid
TAF	Tumor angiogenesis factor
TBST	Tris-buffered saline with tween
TGFα	Transforming growth factor alpha
TGFβ	Transforming growth factor beta
TGFβR	Transforming growth factor beta receptor
TKI	Tyrosine kinase inhibitor
UTR	Untranslated region
VE-Cadherin	Vascular endothelial cadherin
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor
VPF	Vascular permeability factor

1. INTRODUCTION

1.1 Angiogenesis

Blood vessels form very early in development and continue to be formed and remodelled throughout the life of an organism. The formation of blood vessels from circulating endothelial cells is often referred to as vasculogenesis, which plays a vital role in the initial stages of vascular development during embryogenesis. Angiogenesis is the process of formation of new blood vessels from pre-existing vasculature. It is a process also utilized during vascular development in embryogenesis to aid in the production of a functional vascular network that enables the continued growth and development of the embryo. Angiogenesis is also readily apparent in the adult, for example during the process of wound healing, and is a major contributor to several diseases including cancer (reviewed in Potente et al., 2011), rheumatoid arthritis (reviewed in Szekanecz et al., 2010), and ocular diseases such as age-related macular degeneration and diabetic retinopathy (reviewed in Rajappa et al., 2010).

Sprouting angiogenesis is a multi-step process involving the proliferation and migration of endothelial cells, the organization of these cells into functional lumen-containing vessels, and the recruitment of supporting cells such as pericytes, mediated by angiogenic growth factors (Fig. 1). This process differs from intussusceptive angiogenesis, whereby existing vessels are "split", thus remodelling the vasculature without an increase in the number of endothelial cells.

Quiescent vessels consist of endothelial cells interconnected through cell-cell junctions facilitated by molecules such as VE-Cadherin. These endothelial cells are covered

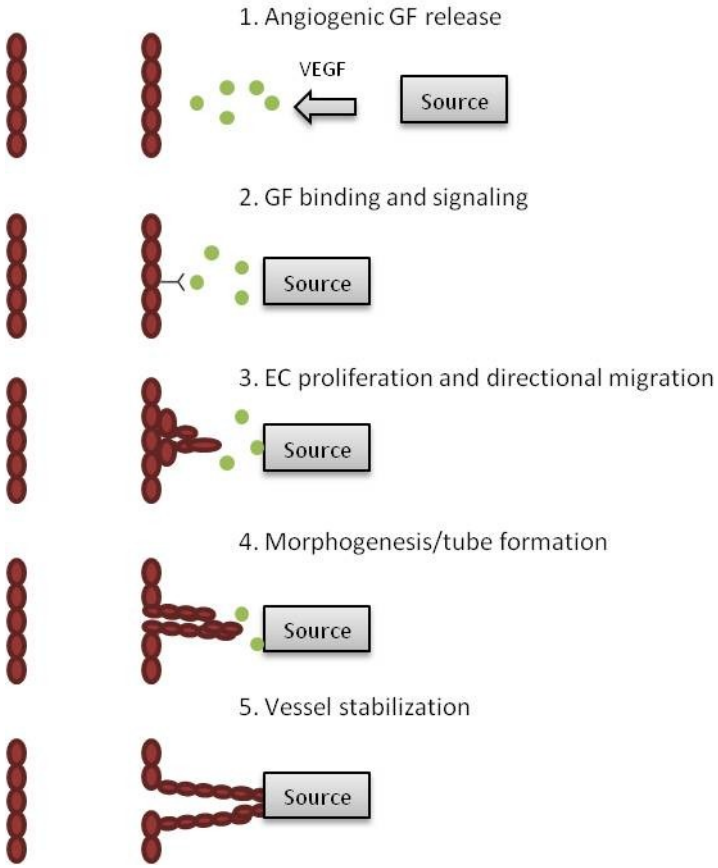

1. Angiogenic GF release
VEGF
Source
2. GF binding and signaling
Source
3. EC proliferation and directional migration
Source
4. Morphogenesis/tube formation
Source
5. Vessel stabilization
Source

Figure 1. Processes involved in angiogenesis. Angiogenesis is initiated through growth factor (GF) release from a source such as a tumor (1). These growth factors bind to cell surface receptors on endothelial cells (EC) (2) and initiate signaling pathways to promote angiogenesis. Endothelial cells proliferate and undergo directional migration towards the source of stimulation (3) with the aid of matrix degrading enzymes. Endothelial cell morphogenesis and tube formation help the branching vessel take form (4). The new vessel contacts the source of growth factor release and vessels are stabilized through pericyte deposition onto the new vessel (5).

by pericytes which help to stabilize the vessel by maintaining endothelial cell survival through the release of pro-survival molecules and by preventing endothelial cell proliferation. Growth factors, such as vascular endothelial growth factor (VEGF), released from sites requiring vascularization, such as tumor cells, signal to the quiescent vasculature to induce sprouting. Angiopoietin-2 (ANG2), released from sprouting endothelial cells, enhances pericyte detachment through antagonizing the interaction of ANG1 with the receptor TIE2 (reviewed in Augustin et al., 2009). Pericyte detachment allows for the endothelial cell designated as the tip cell to move out of the existing vessel, through the basement membrane and into the extracellular matrix (ECM). Remodelling of the ECM is necessary for sprouting angiogenesis and is facilitated by both secreted and membrane-bound matrix metalloproteinases (MMPs) (Otrock et al., 2007a). Endothelial cells migrate away from the existing vessel and towards the source of growth factor release through integrin engagement to the ECM. Migration is facilitated by a defined endothelial tip cell which guides the forming vessel directionally through a VEGF gradient and is followed by proliferating stalk cells (Gerhardt et al., 2003). Maintainance of a single tip cell guiding the formation of the new vessel is achieved through the interaction of the tip cell and its trailing stalk cells and is meant to prevent excessive branching. The tip cell expresses VEGF receptor 2 (VEGFR2), which is activated by VEGF and results in the increased expression of delta-like 4 (DLL4) by the tip cell, while DLL4 interacts with Notch receptors on stalk cells and inhibits the expression of VEGFR2 by the stalk cells, thus maintaining the stalk cell phenotype and preventing sprouting by virtue of a diminished responsiveness to VEGF (reviewed in Phng and Gerhardt, 2009). Conversely, it has been suggested that the Notch ligand Jagged 1, which is produced in stalk cells and antagonizes DLL4/Notch activation, functions to maintain the tip cell phenotype through reducing DLL4/Notch signaling and

thus maintaining VEGFR2 expression in the tip cell (Benedito et al., 2009). Interestingly, recent evidence indicates a dynamic relationship between neighboring cells within vessels, whereby the relative expression of VEGFR1 and VEGFR2 within neighboring cells determines the propensity for attaining a tip cell phenotype versus a stalk cell phenotype, again based on the resulting expression of DLL4 (Jakobsson et al., 2010). Reduced expression of VEGFR2 was shown to provide a disadvantage towards becoming a tip cell whereas reduced VEGFR1 expression was disadvantageous for possessing a stalk cell phenotype. Lumen formation in the developing mouse aorta has been shown to involve VE-Cadherin-mediated localization of CD34-sialomucins at cell-cell contacts which induce separating of the two cell surfaces; a process that also involves actin, non-muscle myosin II, and Rho-associated protein kinase (ROCK) recruitment for morphological changes necessary for complete separation of the endothelial cell surfaces and lumen formation (Strilic et al., 2009). Pericyte attachment to the new vessel is required for vessel stabilization. Platelet-derived growth factor B (PDGFB) is secreted by endothelial cells of the advancing vessel and attracts and stimulates migration and proliferation of pericytes which express PDGF receptor β (PDGFRβ) (reviewed in Gaengel et al., 2009). Attachment of pericytes to endothelial cells is also facilitated by the interaction of ANG1 released from pericytes with TIE2 on endothelial cells (reviewed in Cascone and Heymach, 2012). Adhesion between endothelial cells and pericytes is then achieved through localization of N-Cadherin in endothelial cells to areas of cell-cell contact.

The presence of extensive neovascularization surrounding tumor sites was observed more than forty years ago (Goodall et al., 1965; Warren and Shubik, 1966), and the occurrence of this proliferation in vasculature was suggested to be dependent on the tumor itself (Algire and Chalkley, 1945); however, the importance of angiogenesis to tumor growth

was first proposed by Judah Folkman in his landmark paper in The New England Journal of Medicine in 1971 (Folkman, 1971). Folkman suggested that tumor growth can be divided into two stages: (1) pre-vascularized, where tumors of a few millimeters in diameter are sustained via existing vasculature; and (2) vascularized, whereby angiogenesis is stimulated, causing penetration of new blood vessels into the tumor, allowing for tumor growth beyond a few millimeters in size. Based on this model of tumor growth, Folkman proposed that targeting the process of tumor angiogenesis, a strategy he termed "anti-angiogenesis", might arrest tumor growth at a size more susceptible to chemotherapeutic treatment and/or inhibit metastasis (Folkman, 1971). In accordance with this model of tumor growth, Brown-Pearce carcinoma cells implanted into the iris of a rabbit exhibited tumor growth coincident with neovascularization, whereas tumor cells implanted away from the iris exhibited growth consistent only with a pre-vascularized tumor (Gimbrone et al., 1972). Transplant of these pre-vascularized tumors to the iris region then allowed progressive growth of the tumor, suggesting that neovascularization was necessary for tumor growth beyond a certain size (Gimbrone et al., 1972). Although the effectiveness of anti-angiogenic therapy may not be as profound as first postulated, angiogenesis is a viable target for certain cancer treatment strategies.

1.2 Growth Factors in Angiogenesis

Early studies alluded to the presence of a diffusible factor secreted by tumor cells that could induce vascular proliferation (Greenblatt and Shubik, 1968). The presence of this diffusible factor and proof of its mitogenic activity towards endothelial cells was shown when Folkman and colleagues isolated what they termed tumor angiogenesis factor (TAF) in

1971 (Folkman et al., 1971). Subsequently, TAF was shown to be produced by a number of transformed and tumor-derived cells *in vitro* and elicit vascularization of the chorioallantoic membrane, with brain tumor-derived cells facilitating the greatest response (Klagsbrun et al., 1976). As these brain tumors were the most highly vascularized *in vivo*, a correlation between TAF production levels and the extent of neovascularization was proposed.

Since the initial discovery of a tumor angiogenic factor, a large number of growth factors have been shown to contribute to angiogenesis including: basic fibroblast growth factor (bFGF), platelet-derived growth factor (PDGF), angiopoietins (ANG), epidermal growth factor (EGF), transforming growth factor alpha (TGFα), and vascular endothelial growth factor (VEGF). FGF has been shown to be a potent inducer of angiogenesis *in vitro*, and combination of FGF with VEGF was seen to induce a synergistic response in microvascular endothelial cells (Pepper et al., 1992). The importance of FGF to tumor angiogenesis has also been shown, as inhibition of FGF signaling through the use of a soluble FGF receptor inhibited endothelial cell proliferation *in vitro* and tumor angiogenesis *in vivo* (Compagni et al., 2000). Inhibition of endothelial cell tube formation has been exhibited through the use of anti-PDGF antibody to neutralize PDGF in human serum, thus indicating the importance of this growth factor to angiogenesis *in vitro* (Battegay et al., 1994). PDGF has also been shown to be important in the recruitment of pericytes to tumor vasculature, thus indicating a role in vessel maturation and stability (Abramsson et al., 2003). Depending on the ligand, angiopoietins either promote vascular stability by enhancing the interaction of pericytes with the endothelium, or destabilize vessels through dissociation of pericytes thus allowing for sprouting angiogenesis in response to other pro-angiogenic signals (reviewed in Cascone and Heymach, 2012). EGF can stimulate endothelial cells indirectly through activation of EGF receptor (EGFR) in cancer cells resulting in subsequent

secretion of angiogenic growth factors such as VEGF from these cells which can then act on endothelial cells and promote angiogenesis (Goldman et al., 1993). Direct stimulation of endothelial cells by EGF is still disputed as conflicting reports have either confirmed or denied the existence of EGFR expression by endothelial cells of different origin (Amin et al., 2006; Hirata et al., 2002). VEGF mRNA expression has been shown to be up-regulated in a large number of human tumors (reviewed in Ferrara and Davis-Smyth, 1997) with increased expression of VEGF receptors (VEGFRs) being found specifically in tumor-associated endothelial cells (Brown et al., 1993a; Brown et al., 1993b; Plate et al., 1994; Plate et al., 1992). VEGF expression was shown to be up-regulated in glioblastoma cells (Plate et al., 1994; Plate et al., 1992) while the VEGF receptors Flt-1 (also known as VEGFR1) (Plate et al., 1992) and VEGFR2 (also known as KDR) (Plate et al., 1994) were up-regulated in tumor-associated endothelial cells, an interesting result seeing as neither receptor is normally expressed in endothelial cells of the brain. In endothelial cells of stromal blood vessels associated with adenocarcinomas of the gastrointestinal tract, Flt-1 and VEGFR2 expression was up-regulated, while the expression of these two receptors could not be identified in endothelial cells of blood vessels located away from the adenocarcinoma (Brown et al., 1993b), suggesting induced VEGFR expression is important in tumor angiogenesis. Thus a framework exists in tumors of varying origin for a paracrine signaling pathway from tumor cells to endothelial cells involving VEGF production by tumor cells and VEGFR expression on endothelial cells of tumor-associated vasculature. Furthermore, direct evidence supporting VEGF as a central mediator of tumor angiogenesis came from the treatment of human tumor cells *in vivo* with anti-VEGF monoclonal antibody leading to decreased vessel density (Kim et al., 1993), and reduced vessel diameter and vessel regression (Yuan et al., 1996).

VEGF is also of major importance to angiogenesis in contexts other than tumorigenesis. The importance of VEGF to embryonic vascular development has been shown, as heterozygous VEGF embryos exhibit impaired blood vessel formation and embryonic lethality (Carmeliet et al., 1996; Ferrara et al., 1996). VEGF has also been identified as a major contributer to angiogenesis of other pathological conditions including intraocular neovascular diseases (reviewed in Rajappa et al., 2010), and rheumatoid arthritis (reviewed in Szekanecz et al., 2010), thus identifying VEGF as a primary mediator of angiogenesis in several disease states.

Further highlighting the importance of VEGF to tumor angiogenesis has been the use of therapeutic intervention based around either inhibition of VEGF or VEGFRs. The humanized monoclonal antibody targeting VEGF, bevacizumab (trade name Avastin, Genentech/Roche), has ultimately been approved by the US Food and Drug Administration (FDA) for treatment of several cancer types including non-small cell lung cancer (NSCLC), metastatic colorectal cancer, metastatic renal cell cancer, glioblastoma multiforme, and metastatic breast cancer (Carmeliet and Jain, 2011a), although the accelerated approval for bevacizumab use in metastatic breast cancer has recently (November 2011) been removed by the FDA while approval remains accepted by the European Medicines Agency (Montero et al., 2012). Treatment with bevacizumab in combination with chemotherapy has proved necessary for increased benefit to patients with this anti-VEGF therapy. In NSCLC, combination of bevacizumab with carboplatin and paclitaxel as first-line therapy resulted in increased progression-free survival and overall survival (Johnson et al., 2004; Sandler et al., 2006). Additionally, as second-line treatment, addition of bevacizumab to docetaxel or Erlotinib resulted in increased progression-free survival and overall survival when compared to either treatment alone (Herbst et al., 2007). In treatment of Her2-negative metastatic

breast cancer, bevacizumab has shown benefit, when used as first-line treatment in combination with several chemotherapeutics including taxanes, anthracyclines, and capacitabene, with increased progression-free survival seen in combinations containing bevacizumab (Miles et al., 2010; Miller et al., 2007; Robert et al., 2011). However, these studies did not show a significant increase in overall survival. The use of current anti-angiogenic therapies targeting VEGF and its receptors, although beneficial to a degree, has failed to be the kind of significant addition to the treatment of cancer it was proposed to be. This is due to several challenges associated with this kind of therapy. Treatment with bevacizumab or VEGFR tyrosine kinase inhibitors (TKIs) is not without side effects including, but not limited to, hypertension, hemorrhage, thrombembolic events, and kidney related disfunction such as proteinuria (reviewed in Chen and Cleck, 2009). Although some of these side effects can be managed adequately in most cases, they remain pitfalls that can require the cessation of anti VEGF/VEGFR treatment. The resistance to anti-angiogenic therapies centered on VEGF/VEGFRs, that can occur due to either compensatory mechanisms such as additional pro-angiogenic factor release, and increased pericyte coverage of remaining vessels, or to intrinsic factors present in an individual's tumor microenvironment, also play a role in limiting the efficacy of these therapies (reviewed in Bergers and Hanahan, 2008). In addition, a balance between destroying tumor blood vessels to starve the tumor of vital nutrients and "normalizing" the vasculature, so as to allow better delivery of existing modes of therapy, further complicates the administration and potential of anti-VEGF therapy (reviewed in Carmeliet and Jain, 2011b). Additionally, receptor TKIs have the potential for inhibition of receptor tyrosine kinases other than the intended target and are not specific to cells of the vasculature, which can lead to unintended responses that could limit the effectiveness of these therapies as anti-angiogenics.

For these reasons it is necessary to not only further assess anti-VEGF/VEGFR therapies but to also look at the potential for development of additional therapeutics based around other molecules involved in mediation of angiogenesis by VEGF. Thus identification of signaling pathways mediated by VEGF is of significant importance.

1.3 Vascular Endothelial Growth Factor

VEGF was first identified as a tumor cell secreted factor that increased vascular permeability, and was thus termed vascular permeability factor (VPF) (Senger et al., 1983). This was followed by the independent discovery of VEGF by Ferrera and colleagues as a heparin-binding growth factor secreted by tumors that is specific for endothelial cells and capable of inducing angiogenesis (Leung et al., 1989). VEGF is related to the platelet-derived growth factor family and is intricately involved in vascular development (Keck et al., 1989). The VEGF sub-family of proteins consists of VEGF-A, VEGF-B, VEGF-C, VEGF-D, and placental growth factor (PlGF). Commonly referred to as VEGF, VEGF-A is a homodimeric glycoprotein and is the major gene product involved in the processes of vasculogenesis and angiogenesis. VEGF-A is a secreted protein that can be either freely diffusible or found bound to cell surface or extra-cellular matrix. The localization of VEGF is largely determined by the status of its heparin binding domain, which can be present or absent depending on alternative splicing.

The VEGF-A gene is composed of 8 exons, with alternative splicing producing variants of both pro- and anti-angiogenic potential (Houck et al., 1991; Keck et al., 1989; Leung et al., 1989; Tischer et al., 1991). Designation of the different isoforms is based on the number of amino acids present. Of the pro-angiogenic isoforms, VEGF165 has been

identified as the predominant isoform, with VEGF189, and VEGF121 being moderately expressed, with other, less frequently expressed isoforms including VEGF145, VEGF148, VEGF183, and VEGF206 (reviewed in Ferrara, 2004). Isoforms behave differently in terms of their bioavailability. VEGF121 is freely soluble, whereas VEGF165 and VEGF189 bind heparin to varying degrees and are functionally available after their release from the extracellular matrix (Houck et al., 1992). Interestingly, more recent evidence has indicated the presence of anti-angiogenic splice variants of VEGF-A, originating due to differences in the C-terminal sequence resulting from alternative 3' splice site selection in exon 8. The inclusion of the proximal sequence of exon 8 (exon 8a) produces isoforms with pro-angiogenic properties, whereas the use of a distal splice site that eliminates exon 8a and leaves only exon 8b produces anti-angiogenic variants. For example, VEGF165b is a splice variant created through use of the distal splice site in exon 8, producing a different isoform than VEGF165, and in fact showing anti-angiogenic properties (Bates et al., 2002; Konopatskaya et al., 2006) and the ability to reduce tumor growth *in vivo* (Varey et al., 2008). Other isoforms have been identified that also utilize the distal splice site in exon 8 (VEGF121b, VEGF145b, VEGF183b, VEGF189b). The selection of splice site in exon 8 has been shown to be mediated differently through growth factor stimulation, among other stimuli, with transforming growth factor β1 (TGFβ1) causing distal splice site selection and producing VEGF165b (Nowak et al., 2008), whereas insulin-like growth factor 1 (IGF-1) stimulation results in proximal splice site selection and the production of VEGF165 (Nowak et al., 2010; Nowak et al., 2008). A comparison of normal and malignant tissue in both the kidney (Bates et al., 2002) and colon (Varey et al., 2008) indicates a higher proportion of VEGF in normal tissue is the VEGFxxxb isoform, and upregulation of VEGF in cancerous

tissue is mainly attributable to the VEGFxxx isoform, indicating the potential importance of splice site selection in the progression of cancer.

1.4 VEGF Receptors

Signals mediated by VEGF are achieved through binding of VEGF to its cell surface receptors. The three main receptors for VEGF are the tyrosine kinase receptors VEGFR1 (Flt-1) (de Vries et al., 1992), VEGFR2 (KDR/Flk-1) (Millauer et al., 1993), and VEGFR3 (Flt-4), all of which are expressed on endothelial cells. Additionally, neuropilins 1 (NP-1) and 2 (NP-2) bind VEGF and are also present on endothelial cells (Otrock et al., 2007b). First identified as a receptor for semaphorin/collapsins, neuropilin-1 was shown to bind VEGF and enhance the interaction of VEGF with VEGFR2, thereby acting as a co-receptor, and resulting in increased mitogenic activity of VEGF towards endothelial cells (Soker et al., 1998). In addition to being expressed on endothelial cells, all four receptors exhibit non-endothelial cell expression to varying degrees (Otrock et al., 2007b; Shibuya and Claesson-Welsh, 2006). VEGF receptors 1, 2, and 3 consist of an extracellular domain with seven immunoglobulin-like domains, a transmembrane domain, and an intracellular tyrosine kinase domain with a 70-amino acid kinase insert (Otrock et al., 2007b). Although similar in structure, the receptors exhibit noticeable differences in their kinase activity, with the kinase activity of VEGFR1 being much lower than that of VEGFR2 and VEGFR3 (Shibuya and Claesson-Welsh, 2006).

The various VEGF ligands show different levels of affinity for the three receptors. VEGFR1 ligands include VEGF-A, VEGF-B and PlGF; VEGFR2 binds VEGF-A, VEGF-D, VEGF-E, and VEGF-F; VEGFR3 binds VEGF-C and VEGF-D; and NP-1 ligands are

VEGF-A, VEGF-B and PlGF (Otrock et al., 2007b). The necessity of VEGF receptors to the process of embryonic vascular development has been demonstrated. Embryos homozygous for mutation in the VEGFR1 gene die after exhibiting abnormal vascular development (Fong et al., 1995). Homozygous deletion of VEGFR2 impairs endothelial cell and hematopoietic cell development, inhibiting blood vessel formation, and resulting in the death of embryos at day E8.5 to E9.5 (Shalaby et al., 1995). Additionally, embryonic deletion of VEGFR3 results in cardiovascular failure at E9.5, but does not disrupt embryonic angiogenesis (Dumont et al., 1998). Homozygous deletion of neuropilin-1 results in vascular defects including impaired neural vascularization in mutant embryos (Kawasaki et al., 1999).

1.5 VEGF Signaling in Angiogenesis

The predominant mediator of angiogenesis in both normal and diseased states has been shown to be VEGF. Most pro-angiogenic functional signaling events in endothelial cells elicited by VEGF are mediated through VEGF binding to VEGFR2. Binding of VEGF to its receptor causes dimerization of the receptor leading to autophosphorylation and the recruitment of various adapter and interacting proteins that facilitate the VEGF signal. The cytoplasmic domain of VEGFR2 has been shown to bind a number of adapter proteins to facilitate signaling events mediating endothelial cell function. The binding of PLCγ to Tyr1175 mediates the activation of the MAPK/ERK1/2 pathway through PKC, resulting in proliferation of endothelial cells (Takahashi et al., 1999; Takahashi et al., 2001). Additionally, the PI3K/Akt pathway is induced following VEGF binding to VEGFR2, thus increasing cell survival (Gerber et al., 1998); an induction mediated by Shb interaction with phosphorylated VEGFR2 (Holmqvist et al., 2004). VEGFR2 mediated focal adhesion

turnover and migration in endothelial cells has been attributed to phosphorylation of focal adhesion kinase (FAK) and paxillin by VEGFR2 (Abedi and Zachary, 1997). The heat shock protein Hsp90 can also associate with VEGFR2 and its subsequent inhibition prevents phosphorylation of Tyr407 of FAK as well as the VEGF-induced activation of the small GTPase RhoA upstream of FAK, indicating the presence of an Hsp90-RhoA-FAK signaling cascade mediating focal adhesion turnover and migration (Le Boeuf et al., 2004). In fact, many Rho proteins have been shown to play important roles in angiogenesis.

1.6 Rho GTPases in Angiogenesis

Members of the Ras superfamily of small guanosine triphosphatases (GTPases), Ras homologous (Rho) proteins function as key regulators of cellular signaling events. Through phylogenetic analysis, Rho proteins can be divided into eight subgroups and four clusters as follows: Rho (A/B/C), Rnd (1/2/3), and RhoD/F subgroups in cluster I; Rac (1/2/3)/RhoG, Cdc42/RhoJ/RhoQ, and RhoU/V in cluster II; RhoH in cluster III; and RhoBTB (1/2) in cluster IV (Boureux et al., 2007).

Rho proteins bare similarity to other Ras superfamily GTPases as they contain intrinsic GTPase activity and express a set of conserved G box GDP/GTP binding elements (Bourne et al., 1991). The N-terminal portion of Rho proteins contains conserved residues (glycine 14, threonine 19, Phenylalanine 30, and Glutamine 63) important for GTP hydrolysis as well as two domains, switch 1 and switch 2, which undergo changes in conformation dependent on protein binding of GDP/GTP (Wheeler and Ridley, 2004). The N-terminal also contains amino acids that are modified by bacterial toxins, including asparagine 41 which is ADP-ribosylated by Clostridium botulinum exoenzyme C3

transferase (Wheeler and Ridley, 2004). Distinguishing Rho proteins from other Ras superfamily members is an insert domain located between amino acids 123 and 137 (Wennerberg and Der, 2004), which has been shown to be important in the interaction of Rho GTPases with specific effectors and regulators (Freeman et al., 1996; McCallum et al., 1996; Wu et al., 1997), and in Rho protein-mediated transformation (Wu et al., 1998; Zong et al., 2001; Zong et al., 1999). The majority of Rho family proteins contain a CAAX motif at the C-terminus which is the site of post-translational modification that creates a hydrophobic domain capable of localizing Rho GTPases to membrane compartments (Konstantinopoulos et al., 2007). The cysteine residue, 4 amino acids from the C-terminus undergoes prenylation with either a 15-carbon farnesyl group, mediated by farnesyltransferase, or a 20-carbon geranylgeranyl group, mediated by geranylgeranyltransferase I (Wheeler and Ridley, 2004).

Rho proteins are intricately involved in regulation of the actin cytoskeleton and consequently, affect cellular morphology and migration. The three most studied Rho proteins, RhoA, Rac1, and Cdc42, have been identified as regulating the formation of actin stress fibers, lamellipodia, and filopodia, respectively (Nobes and Hall, 1995). Rac1 and Cdc42 are required at the leading edge of cells in order to facilitate cytoskeletal reorganization and the formation of filopodia and lamellipodia to direct migration in response to extracellular stimuli (Raftopoulou and Hall, 2004). RhoA, on the other hand, regulates cell contractility and retraction of the rear of the cell and its activity is thus localized to the rear of migrating cells. This localization of RhoA activity may in fact be partly regulated by Rac1 at the leading edge of migrating cells, as a reciprocal relationship between Rac1 and RhoA has been identified in fibroblasts with Rac1 activation downregulating RhoA activity (Sander et al., 1999).

The control of GTPase function through cycling between a GDP-bound inactive state and a GTP-bound active state is mediated by guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs) (Fig. 2). Rho GTPase affinity for either GDP or GTP is quite high. Because of this, the dissociation rate of GDP/GTP is relatively slow (1-2 hours), considering the rapid activation of GTPase proteins upon extracellular signals (Bos et al., 2007). In order to facilitate the exchange of GDP for GTP at a rate allowing for swift signal transduction, GEFs are required. GEFs function by altering the nucleotide binding site at the switch 1 and switch 2 regions on GTPase proteins, thus lowering the affinity of the GTPase for GDP and allowing its dissociation and the subsequent binding of GTP (Bos et al., 2007). The hydrolysis of GTP through intrinsic GTPase activity is exceptionally slow and thus requires GAPs for efficient phosphate hydrolysis. Generally, GAPs stabilize amino acid residues on the target Rho protein and stabilize the transition state thus facilitating easier access to the phosphate groups (Bos et al., 2007). To highlight the importance of both GEFs and GAPs to signaling pathways mediated by GTPases, there is much information on the role of GEFs and GAPs in cancer; enough that targeting both GEFs and GAPs is being examined for therapeutic potential (reviewed in Vigil et al., 2010).

In addition to GEFs and GAPs, Rho GTPase activity is also regulated by Rho-specific guanine nucleotide dissociation inhibitors (RhoGDIs). At present, three RhoGDIs have been identified in mammals: RhoGDI1 (RhoGDIα), RhoGDI2 (RhoGDIβ), and RhoGDI3 (RhoGDIγ). RhoGDIs interact with the switch 1 and switch 2 regions of Rho proteins, thus preventing the conformational changes necessary for dissociation of guanine nucleotides and effectively sequestering GDP-bound Rho proteins in the cytosol (Garcia-Mata et al., 2011). RhoGDIs do not inhibit the binding of either GDP or GTP to nucleotide-free Rho proteins rather; they prevent the dissociation of guanine nucleotides, effectively

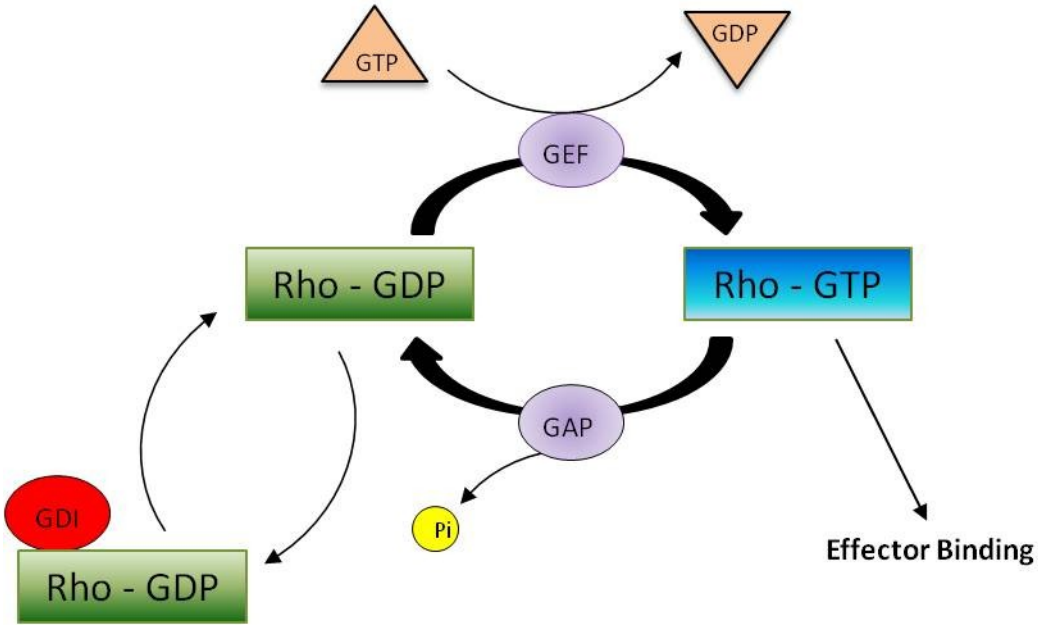

GTP
GDP
GEF
Rho - GDP
Rho - GTP
GAP
GDI
Pi
Rho - GDP
Effector Binding

Figure 2. The Rho GTPase cycle. Rho proteins affect cellular signaling by shuttling between a GDP-bound inactive state and a GTP-bound active state. Guanine nucleotide exchange factors (GEFs) facilitate the binding of a phosphate group from GTP to Rho-GDP, thus forming the active Rho-GTP with GDP as a biproduct. Rho proteins are deactivated when GTPase activating proteins (GAPs) enhance the intrinsic GTPase activity of the Rho protein to hydrolize GTP to GDP. GDP-bound Rho GTPases can bind guanine nucleotide dissociation inhibitors (GDIs) that maintain Rho proteins in an inactive form.

maintaining a pool of readily available inactive Rho GTPase. Although originally described as preventing the dissociation of GDP from Rho proteins, RhoGDIs also effectively inhibit both the intrinsic GTPase activity of Rho proteins and GAP catalyzed GTP hydrolysis, thus maintaining the Rho protein in a GTP bound state (Chuang et al., 1993). In addition to maintaining Rho proteins in a nucleotide-locked state, RhoGDIs can extract GTPases from membranes and facilitate the sequestration of the hydrophobic isoprenoid moiety at the C-terminus of Rho proteins that would otherwise cause instability of the Rho GTPase in the aqueous cytosol (Boulter et al., 2010). As a result of this, RhoGDI binding prevents the unwarranted degradation of the cytosolic pool of GTPases, thus maintaining a population that is available for timely activation in response to cellular signaling events. The release of RhoGDIs from Rho GTPases allowing for subsequent activation of the GTPase by GEFs can occur via a number of mechanisms including lipid-mediated dissociation, protein binding-mediated dissociation, and RhoGDI phosphorylation (Garcia-Mata et al., 2011). Although GDIs are often thought of as inhibitory molecules, it is clear that they are a vital part of the machinery regulating the activity of Rho GTPases and as such, their inhibition can be detrimental to cellular processes. For example, through mutation of RhoGDI binding sites in Cdc42 or depletion of RhoGDI with small interfering RNA (siRNA), transformation of fibroblasts by activated Cdc42 was inhibited; a result attributed to the necessity for functional transport of Cdc42 between membrane compartments (Lin et al., 2003). Additonally, the homozygous deletion of RhoGDIα produces viable mice that nonetheless experience age-related kidney and fertility issues (Togawa et al., 1999).

There is extensive evidence pointing to Rho proteins as essential regulators of angiogenesis, as many events during the process of angiogenesis require cytoskeletal reorganization. The generalized roles for RhoA, Rac, and Cdc42 in actin rearrangement

leading to cell migration are applicable to migration in endothelial cells (Lamalice et al., 2007). In fact, Rho proteins are involved in nearly all facets of the process of angiogenesis (reviewed in Bryan and D'Amore, 2007). RhoA is activated by VEGF in endothelial cells, with expression of dominant negative RhoA or pharmacological inhibition of the RhoA effector ROCK inhibiting VEGF-induced stress fibers (van Nieuw Amerongen et al., 2003). Additionally, the inhibition of ROCK reduced VEGF-induced endothelial cell migration and capillary tube formation in 3D fibrin gels. Cdc42 activation was elevated twofold by VEGF and expression of constitutively active Cdc42 was shown to increase actin stress fiber formation in a SAPK2/p38-dependent manner (Lamalice et al., 2004). RhoC has also been shown to be necessary for capillary morphogenesis and sprouting *in vitro* (Wang et al., 2008b). However, little is known about the relationship between VEGF and RhoB and the mechanisms guiding the actions of RhoB in endothelial cells are not fully identified, making it an interesting target for further study.

1.7 RhoB

The small GTPase RhoB is a member of the Rho family of GTPases, and along with RhoA and RhoC, makes up the Rho subclass of proteins which exhibit amino acid sequence identity of roughly 85%. RhoB is an approximately 21 kDa protein that displays a ubiquitous tissue expression in mammals. Compared to RhoA and RhoC, the RhoB gene is much smaller, containing a single exon, and is readily induced by a number of stimuli including growth factors (Wheeler and Ridley, 2004). Transcription of RhoB mRNA is rapidly and transiently induced by EGF and PDGF in rat fibroblasts (Jahner and Hunter, 1991), and by EGF in a number of breast cancer cell lines (de Cremoux et al., 1994). TGFβ1

has also been shown to induce RhoB mRNA expression within 30 minutes in Swiss3T3 and by 1 hour in HepG2 cell lines (Vardouli et al., 2008). DNA damaging agents such as ultraviolet light, increase RhoB mRNA within 30 minutes (Fritz et al., 1995); a process based on mRNA stabilization through HuR binding of AU-rich elements in the 3'-untranslated region (UTR) of RhoB mRNA (Westmark et al., 2005). RhoB expression is also regulated by growth factors at the protein level. TGFβ1-stimulated expression of RhoB protein is not dependent on up-regulation of RhoB mRNA levels rather, the stabilization of RhoB protein and prevention of its degradation (Engel et al., 1998).

Differences in the C-terminal amino acid residues of RhoB allow for modifications not present in either RhoA or RhoC. Early studies determined that the X residue of the CAAX motif at the C-terminus of a G protein was responsible for determining the nature of the prenyl group transferred by either farnesyltransferase or geranylgeranyltransferase I (Yokoyama et al., 1991). Whereas RhoA and RhoC are geranylgeranylated based on their C-terminal leucine residue, RhoB, which also has a C-terminal leucine residue, can be either geranylgeranylated or farnesylated (Adamson et al., 1992a). The ability of RhoB to be farnesylated despite its C-terminal leucine residue has been suggested to be dependent on its three carboxyl-terminal amino acids, as mutations in those residues can inhibit either geranylgeranylation or farnesylation depending on the resulting sequence (Baron et al., 2000). It has also been shown that geranylgeranyltransferase I can effectively transfer a farnesyl group to RhoB (Armstrong et al., 1995), thus the presence of a C-terminal leucine residue would not be an impediment to farnesylation, in contrast to the rules of CAAX prenylation. The C-terminus of RhoB also contains sites of palmitoylation (cysteine 189, cysteine 192) and phosphorylation (serine 185), with the status of these sites determining localization (Wang and Sebti, 2005) and activation (Tillement et al., 2008) of RhoB,

respectively. The differences in post-translational modification of RhoB compared to RhoA and RhoC largely results in differences in its intracellular localization and function. RhoB has been found to localize mainly to endosomal compartments and lysosomes (Adamson et al., 1992b), and as such has been identified as a key regulator in intracellular trafficking (Adini et al., 2003; Gampel et al., 1999; Sandilands et al., 2004). In the endosomal trafficking of the EGF receptor, alteration in prenylation of RhoB, through the use of farnesyltransferase inhibitors, has confirmed specific roles for RhoB in intracellular receptor trafficking depending on its attached prenyl group (Wherlock et al., 2004).

RhoB has an apparently divergent role in cancer progression, as compared to other Rho proteins. Unlike most other Rho family proteins that have a positive role in malignant transformation, RhoB appears to negatively regulate the process of transformation (Huang and Prendergast, 2006). Significant studies have shown that many of the growth inhibiting and tumor suppressive activities of RhoB can be linked to either its farnesylated or geranylgeranylated forms and thus depend largely on the prenylation status of the protein (Chen et al., 2000; Lebowitz et al., 1997; Mazieres et al., 2005). Based on its ability to undergo farnesylation, RhoB has been identified as a target for farnesyltransferase inhibitors (FTIs) and has been shown to mediate the inhibitory effects of FTIs on Ras transformation (Lebowitz et al., 1997; Lebowitz et al., 1995). The effects of FTIs mediated by RhoB appear to result from a gain of function, as FTIs decrease the level of farnesylated RhoB with a resultant increase in levels of geranylgeranylated RhoB (Lebowitz et al., 1997). In fact, geranylgeranylated RhoB can inhibit cell growth in Ras-transformed cells or in FTI-sensitive cells independent of Ras status (Du et al., 1999; Du and Prendergast, 1999).

A role for RhoB in angiogenesis and the mechanisms guiding its functions therein are slowly being described. The development of a RhoB knockout mouse provided evidence

supporting the importance of RhoB as a negative regulator of tumorigenesis, but did not indicate a necessity for RhoB in developmental angiogenesis as mice developed normally and were fertile (Liu et al., 2001). Although not necessary during development, it was shown that homozygous deletion of RhoB inhibits the vascularization of the retina during postnatal growth (Adini et al., 2003). This was proposed to be a result of decreased endothelial cell survival mediated by exclusion of Akt from the nucleus when RhoB was depleted. The expression of RhoB has also been suggested to mediate the inhibitory effects of microRNA-21 (miR-21) on endothelial cell migration and morphogenesis (Sabatel et al., 2011). In that study, RhoB expression was shown to be down-regulated by overexpression of miR-21, and overexpression of miR-21 resulted in similar inhibition of cell migration and morphogenesis to that of RhoB depletion. Thus, these studies lay a ground work indicating the importance of RhoB to particular angiogenic processes and warrant the further study of RhoB, in particular, in response to the major pro-angiogenic growth factor VEGF, an area which has not been described. Additionally, the study by Sabatel et al. highlights another area of increasing interest in vascular biology, that being the function of miRNAs in angiogenesis; an area of study that has only recently begun to be explored in depth.

1.8 MicroRNAs in Angiogenesis

Of increasing interest is the determination of the role of microRNAs (miRNAs) in many cellular processes including angiogenesis. In recent years, an important role for miRNAs in the regulation of angiogenesis has become evident (reviewed in Anand and Cheresh, 2011). Initial evidence that miRNAs were important to the process of vessel formation came from studies where the miRNA processing enzyme Dicer was depleted. A

study by Keubacher et al. indicated that reduction in the levels of Drosha and Dicer, the two enzymes responsible for the processing of primary miRNAs (pri-miRNAs) to mature miRNAs, inhibited human umbilical vein endothelial cell (HUVEC) sprouting, capillary morphogenesis, and migration, highlighting the importance of miRNA expression to angiogenic processes *in vitro* (Kuehbacher et al., 2007). Interestingly, this study also noted that only a subset of miRNAs were affected by Drosha and/or Dicer depletion, thus suggesting that unaffected miRNAs may possess a higher level of stability. Suarez and colleagues identified an *in vivo* role for miRNA expression when they showed that mice deficient for Dicer in endothelial cells exhibited decreased vessel formation in response to VEGF (Suarez et al., 2008).

MiRNAs are small (approximately 21 nucleotide) non-coding RNAs that can be found within both exonic and intronic locations within genes (Rodriguez et al., 2004) as well as intergenic regions, and thus can be transcribed along with other genes or as their own transcriptional unit. MiRNAs are transcribed by RNA polymerase II (Lee et al., 2004) or RNA polymerase III (Borchert et al., 2006) as pri-miRNAs and are cleaved within the nucleus by the Drosha complex to form a precursor miRNA (pre-miRNA) (Lee et al., 2003) (Fig. 3). The pre-miRNA is transported from the nucleus to the cytoplasm through the nuclear exporter Exportin-5 (Yi et al., 2003). Once in the cytoplasm, the RNase III Dicer cleaves the hairpin structured pre-miRNA to form a double stranded approximately 21 nucleotide miRNA (Bernstein et al., 2001; Hutvagner et al., 2001). The double-stranded miRNA is then loaded into the miRNA-induced silencing complex (miRISC) via its association with a complex containing Dicer, TRBP, and Ago2 (Chendrimada et al., 2005; Gregory et al., 2005; Maniataki and Mourelatos, 2005). Degradation of the passenger strand and retention of the guide strand by miRISC permits the now single-stranded miRNA to

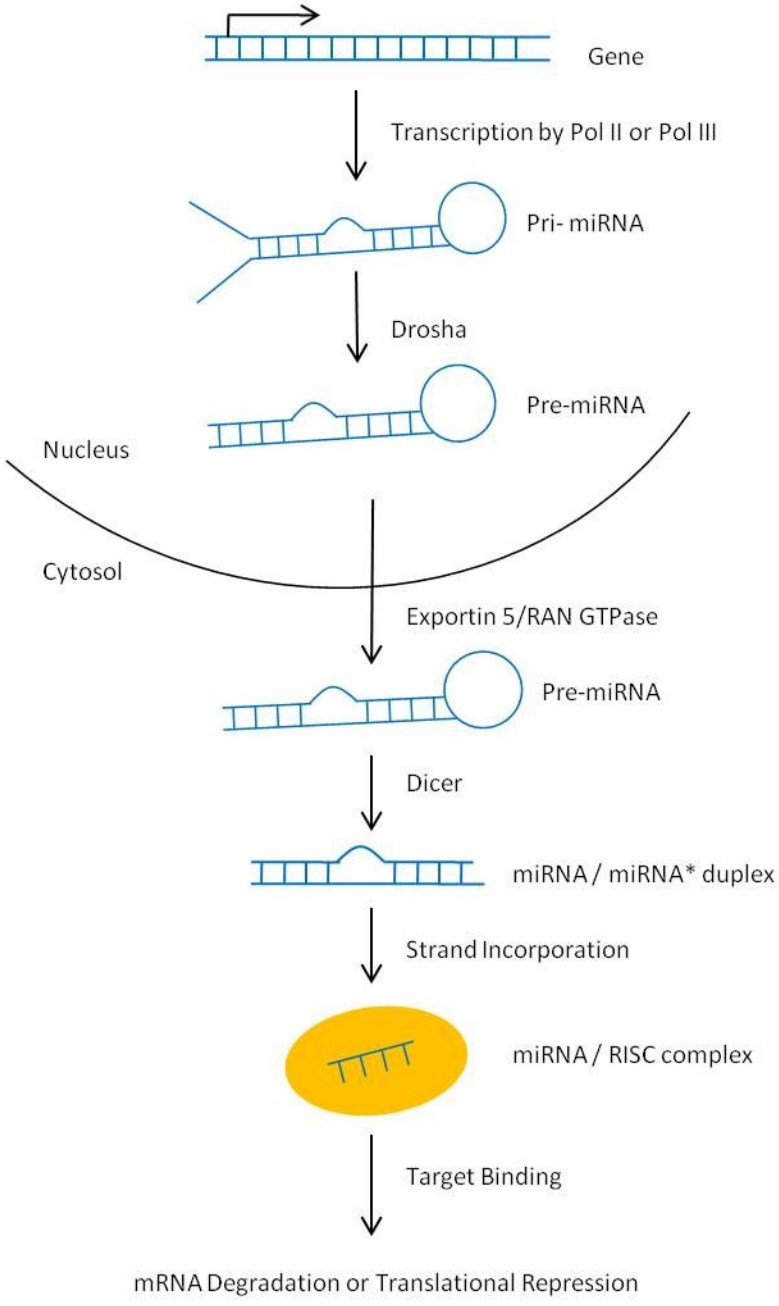

Gene
Transcription by Pol II or Pol III
Pri- miRNA
Drosha
Pre-miRNA
Nucleus
Cytosol
Exportin 5/RAN GTPase
Pre-miRNA
Dicer
miRNA / miRNA* duplex
Strand Incorporation
miRNA / RISC complex
Target Binding
mRNA Degradation or Translational Repression

Figure 3. MiRNA biogenesis. Transcription of a primary miRNA (Pri-miRNA) transcript is achieved through either RNA polymerase II (Pol II) or RNA polymerase III (Pol III). The pri-miRNA is processed by Drosha within the nucleus to form a precursor miRNA (Pre-miRNA). The pre-miRNA is transported into the cytosol by Exportin 5 and RAN GTPase. Dicer then cleaves the hairpin structure to form the miRNA/miRNA* duplex. Incorporation of one of the two strands into the RNA-induced silencing complex (RISC) allows for target mRNA binding leading to mRNA degradation or translational repression.

function by base-pairing with complementary sequences in the 3'- UTR of target mRNAs. This binding results in post-transcriptional gene silencing by sequestering and/or degrading the target mRNA through miRISC (reviewed in Fabian and Sonenberg, 2012). The seed sequence in the miRNA is located near its 5' end with nucleotides 2-7 centering the region facilitating complementary binding to target mRNA (Lewis et al., 2005). Although seemingly simple, there are additional factors that can affect the outcome of miRNA binding to target mRNA. For example, the sequence context of the 3'-UTR of the target mRNA can have a large effect on the efficacy with which a miRNA elicits repression, with factors such as AU nucleotide composition, positioning of binding away from both the center of UTRs and the stop codon, and proximity to binding sites for other miRNAs affecting the miRNA/mRNA interaction (Bartel, 2009; Grimson et al., 2007).

MiRNAs have been identified as contributing to the pathogenesis of many disease states including cancer (reviewed in Nikitina et al., 2012), cardiovascular disease (reviewed in Papageorgiou et al., 2012), liver disease (reviewed in Wang et al., 2012), and inflammatory disease such as rheumatoid arthritis (reviewed in Filkova et al., 2012). Due to their extensive number of targets, miRNA deregulation can have profound effects on the progression and treatment of these and other diseases.

The targeting of miRNAs for the treatment of cancer is a strategy that holds much promise as a multi-faceted approach due to the abundance of targets usually associated with a single miRNA. Indeed, strategies are being assessed for the delivery of such modulators of miRNA expression, with considerations for stability, cellular penetration, and tissue specific targeting among the obstacles currently holding back such therapeutic strategies [reviewed in (Pereira et al., 2013) and (Iorio and Croce, 2012)]. As angiogenesis is an important part of cancer progression, the regulation of miRNAs within the endothelium is increasingly being

explored, with the ultimate goal of determining the miRNAs that may be deregulated in tumor vasculature and/or control processes important for angiogenesis in any setting. To this end, expression profiling in endothelial cells has been performed, first within human umbilical vein endothelial cells (HUVECs) (Poliseno et al., 2006), and more recently to determine the expression profiles across endothelial cells of different origin (McCall et al., 2011). The importance of global miRNA expression to endothelial cell biology and angiogenesis has been demonstrated through the silencing of Dicer (Kuehbacher et al., 2007; Suarez et al., 2007), and the importance of individual miRNAs to the process of angiogenesis is increasingly being elucidated.

The endothelial cell-specific miR-126, which has been shown to be necessary for proper developmental angiogenesis via its role in regulating the VEGF signaling pathway (Fish et al., 2008; Nicoli et al., 2010; Wang et al., 2008a), and miR-221 whose role in endothelial tip cell function has recently been identified (Nicoli et al., 2012), exemplify the diversity of pathways regulated by miRNAs during vascular development and remodelling. Recent evidence has identified miR-27a/b as a positive mediator of embryonic vessel formation in zebrafish, as inhibition of miR-27a and miR-27b increased vascular defects and induced intracranial bleeding (Urbich et al., 2012). The same study displayed that inhibition of miR-27a/b impaired angiogenesis in adult mice, and inhibited *in vitro* sprouting through regulation of the expression of SEMA6A in endothelial cells. MiR-21, a well studied miRNA that has been shown to be highly expressed in a number of cancers and associated with increased proliferation and invasion (Asangani et al., 2008; Hiyoshi et al., 2009; Moriyama et al., 2009; Song et al., 2010; Zhang et al., 2012) has recently been shown to be negatively regulated by the pro-angiogenic growth factor bFGF in endothelial cells, suggesting an anti-angiogenic role for miR-21 in endothelium (Sabatel et al., 2011).

Conversely, miR-21 expression in human prostate cancer cells results in positive regulation of angiogenesis, as overexpression of miR-21 was shown to target PTEN, thus activating Akt and ERK1/2 and leading to increased expression of VEGF and HIF-1α, resulting in increased tumor angiogenesis (Liu et al., 2011). Recently miR-10 has been shown to be a positive regulator of angiogenesis. Depletion of miR-10 levels in endothelial cells leads to a reduction in sprouting and capillary morphogenesis, whereas over-expression of miR-10 enhances capillary morphogenesis and migration; effects that were ultimately attributed to effects on VEGFR2 mediated indirectly by miR-10 through its regulation of VEGFR1 (Hassel et al., 2012). Additionally, miR-10b levels were shown to be oppositely regulated by heparin and thrombin in microvascular endothelial cells and was suggested to be a positive mediator of angiogenesis through its regulation of homeobox D10, thus placing it as a centrally regulated component of a two-way system regulating angiogenesis (Shen et al., 2011). Interestingly, miR-10b has also been identified as being regulated by VEGF, as endothelial cells display increased levels of miR-10b upon VEGF stimulation (Plummer et al., 2013). The regulation of miRNA expression by VEGF is not surprising and increasing numbers of miRNAs are being identified as being regulated by VEGF, thus giving an indication as to their potential role in angiogenesis. Indeed, upregulation of miR-17-5p, miR-18a, and miR-20a (members of the miR-17-92 cluster) in endothelial cells by VEGF has been observed, with inhibition of these miRNAs reducing capillary tube morphogenesis, while overexpression restores morphogenesis in Dicer-deficient HUVECs (Suarez et al., 2008). Thus, VEGF upregulation of miRNA expression appears to be meaningful in this situation. Interestingly, a more recent study found that overexpression of all members of the miR-17-92 cluster inhibited both sprouting and morphogenesis in HUVECs (Doebele et al., 2010), seemingly in direct contrast to the study by Suarez et al., and suggests that the miR-

17-92 cluster may function differently depending on the status of global miRNA expression in HUVECs. The miR-17-92 cluster member miR-20a has been shown to be upregulated by VEGF, and functions in a negative feedback loop to limit VEGF-induced endothelial cell migration and angiogenesis (Pin et al., 2012). VEGF has also been shown to negatively regulate miRNA expression as in the case of miR-125b, where down-regulation in glioblastoma-associated endothelial cells through VEGF stimulation facilitates a positive feedback loop whereby VEGF expression is increased through transcriptional activation by myc-associated zinc finger protein (Smits et al., 2012). In fact, a number of miRNAs have been proposed as regulators of angiogenesis based on their regulation of VEGF and/or VEGFR expression in either endothelial cells or cancer cells (Chamorro-Jorganes et al., 2011; Choi et al., 2011; Liu et al., 2012; Liu et al., 2011; Shi et al., 2012; Xu et al., 2012). A study by Roitbak et al. identified neural stem/progenitor cells (NSPCs) as supporting endothelial cell morphogenesis *in vitro* and indicated a miRNA expression signature change in endothelial cells co-cultured with NSPCs (Roitbak et al., 2011). Of the miRNAs regulated by NSPC co-culture, miR-155, miR-100, and miR-7i exhibited effects on endothelial morphogenesis, with inhibition of all three miRNAs enhancing capillary tube formation while overexpression inhibited tube formation. Thus the cellular niche likely affects the miRNA expression profile in resident endothelial cells.

In addition to regulation of VEGF, miRNAs have been shown to regulate components of the TGFβ signaling pathway. In mouse granulosa cells, activin A was shown to suppress miR-181a expression while overexpression of miR-181a downregulated the expression of activin receptor IIA leading to reduced proliferation through reduction of cyclin D2 (Zhang et al., 2013). In addition, miR-145 and miR-224 have been shown to target activin receptor IB (Yan et al., 2012) and Smad4 (Yao et al., 2010), respectively in granulosa cells, indicating

the importance of miRNA expression to TGFβ signaling in that cell type. Recently it has been shown that FGF downregulates let-7 miRNA expression in endothelial cells and this resulted in increased expression of TGFβ ligands and receptors and overall increased TGFβ signaling in endothelial cells, leading to endothelial-to-mesenchymal transition (EndMT), a specialized process in endothelial cells similar to epithelial-to-mesenchymal transition (EMT) (Chen et al., 2012). Based on the known regulation of miRNAs during angiogenesis and described roles for miRNAs in regulating expression changes of TGFβ family members, miRNAs likely facilitate changes in TGFβ family ligands and receptors to regulate endothelial cell biological processes.

1.9 TGFβ Function in Angiogenesis

The TGFβ superfamily of proteins consists of 33 members including TGFβ proteins, Bone Morphogenetic Proteins (BMPs), Activins, Inhibins, Nodal, Lefty, Growth Differentiation Factors (GDFs), Glial-derived Neurotrophic Factors (GDNFs), and Mullerian Inhibiting Substance (MIS). TGFβ ligands form dimeric proteins that bind cell surface receptors and facilitate the assembly of a hetero-tetrameric receptor complex consisting of two type I and two type II receptor domains. The type II domains phosphorylate type I domains, enabling the recruitment and phosphorylation of intracellular substrates, of which Smad proteins are the main signaling intermediates (Fig. 4). Receptor-regulated Smads (R-Smads) for BMPs are Smad1, Smad5, and Smad8, and for TGFβ, Activin and Nodal, include Smad2 and Smad3 (Massague, 2012). After being phosphorylated, R-Smads interact with the Co-Smad Smad4, with the complex then translocating to the nucleus and interacting with other transcription factors to regulate gene expression (Miyazono, 2000). Signaling from

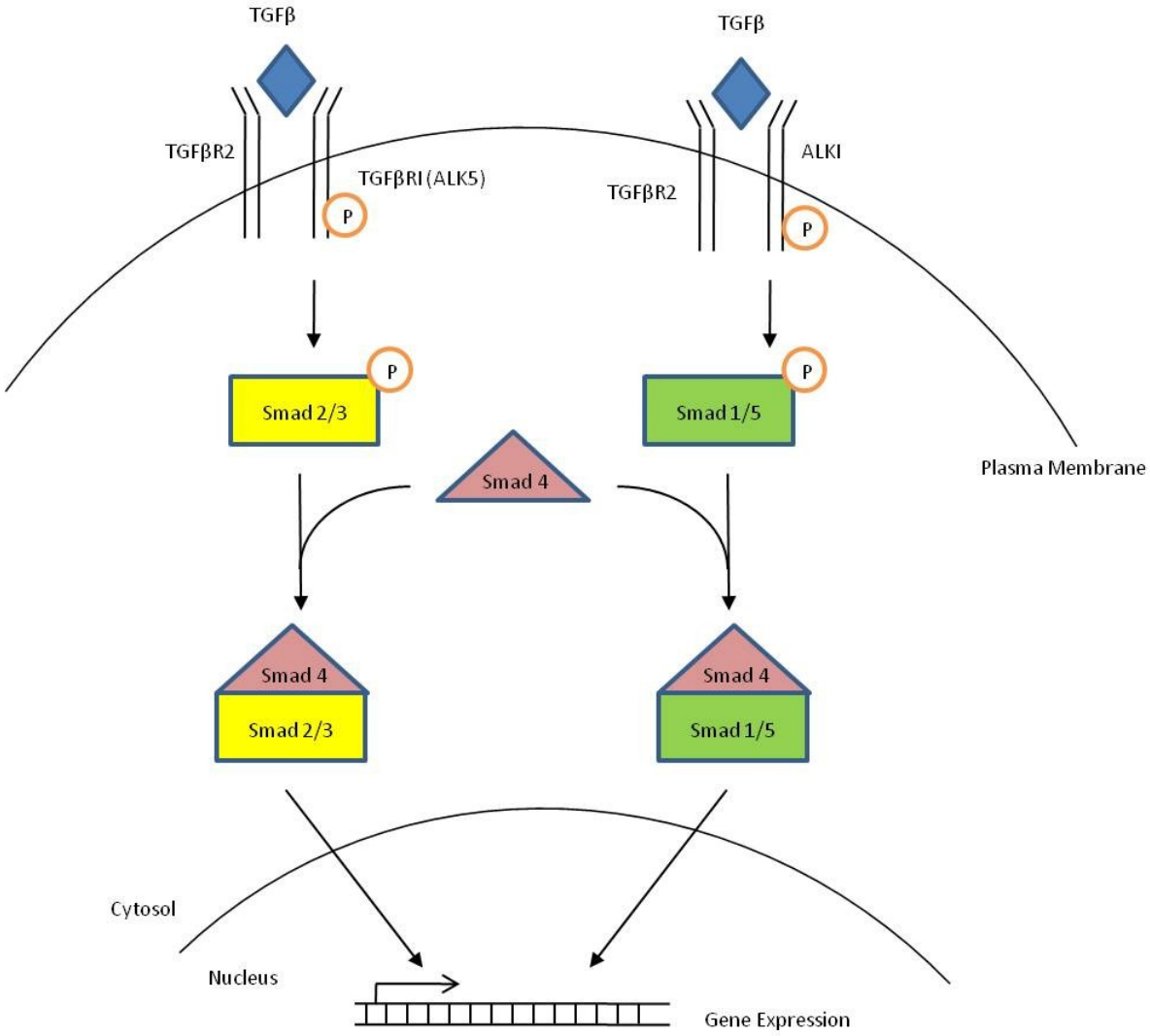

TGFβ
TGFβ
TGFβR2
TGFβRI (ALK5)
P
TGFβR2
ALKI
P
P
Smad 2/3
P
Smad 1/5
Plasma Membrane
Smad 4
Smad 4
Smad 2/3
Smad 4
Smad 1/5
Cytosol
Nucleus
Gene Expression

Figure 4. TGFβ signaling through Smad proteins in endothelial cells. TGFβ ligand binds to TGFβR2 which complexes with TGFβR1 (ALK5) or ALK1 phosphorylating them. Phosphorylation of the type I receptor causes phosphorylation of receptor Smad proteins (R-Smads). TGFβR1 signals through Smad2 and Smad3, while ALK1 signals through Smad1 and Smad5. Phosphorylated R-Smads are bound by Smad4. The formation of this complex causes nuclear translocation where Smad proteins control gene expression.

TGFβ receptors (TGFβRs) can also occur via non-canonical pathways such as the PI3K/Akt and JNK/p38 signaling pathways as well through Rho GTPases (reviewed in Zhang, 2009).

Within the TGFβ family, the TGFβ protein ligands consist of TGFβ1, β2, and β3, and exclusively utilize the type II receptor TGFβR2 and the type I receptor TGFβR1 (also known as ALK5) to signal through Smads 2 and 3 (Yoshimatsu and Watabe, 2011). The exception to this is in endothelial cells where TGFβs can also signal through activin receptor-like kinase 1 (ALK1) and Smads 1 and 5 (Oh et al., 2000). TGFβ receptors are Ser/Thr receptor kinases, making them the only known cell surface Ser/Thr kinases in humans (Massague, 2012), and the reasons TGFβ employs this type of receptor are unknown. In addition, TGFβ proteins utilize accessory receptors in order to increase their affinity for TGFβR2. Betaglycan (also known as TGFβ type III receptor) acts as an accessory cell-surface receptor and presents TGFβ to TGFβR2 (Lopez-Casillas et al., 1993) and inhibin to activin receptors (Lewis et al., 2000). Additionally, the cell surface protein Endoglin, can also function as an accessory receptor for TGFβs and has been found to be highly expressed in tumor vasculature (Burrows et al., 1995), implying an important role for Endoglin in angiogenesis. Of considerable interest is that inhibition of Endoglin may function as an effective anti-angiogenic therapy, as recent evidence has indicated that inhibition of Endoglin with either monoclonal antibodies (She et al., 2004) or siRNA (Dolinsek et al., 2013) inhibits *in vitro* endothelial cell proliferation. Interestingly, tumors treated with siRNA to Endoglin exhibited reduced tumor blood vessel growth, but the tumor cells themselves were not inhibited in their proliferative ability, suggesting that a decrease in vasculature was the cause of slowed tumor growth (Dolinsek et al., 2013).

Endothelial cell response to TGFβ can differ depending on the type I receptor used to propogate the TGFβ signal. Signaling through either TGFβR1 (ALK5) or ALK1 determines

whether the result of TGFβ stimulation will be positive or negative. Signaling through the ALK5/Smad2/3 pathway inhibits cell proliferation and migration while signaling through the ALK1/Smad1/5 pathway promotes proliferation and migration (Goumans et al., 2002). Interestingly, TGFβR1 has been shown necessary for ALK1 activation and recruitment to a TGFβ signaling complex, while ALK1 antagonizes TGFβR1/Smad2/3 signaling indicating an important cross-regulation between these two receptors in endothelial cells (Goumans et al., 2003).

The ligands TGFβ1, β2, and β3 are each produced as a proprotein consisting of an N-terminal preproprotein sequence, a prodomain known as latency associated peptide (LAP) and what is to become the mature TGFβ sequence. Prior to secretion, within the endoplasmic reticulum, the TGFβ proprotein monomer undergoes homodimerization and is cleaved by furin in the Golgi to create the latent TGFβ complex consisting of homodimers of both the LAP and mature TGFβ bound through non-covalent bonds and attached to latent TGFβ binding protein (LTBP) (reviewed in Munger and Sheppard, 2011). Latent TGFβ is secreted from cells and held inactive in the ECM through domains present in the LTBP (Horiguchi et al., 2012) until cleavage of the LAP from the TGFβ homodimer. Activation of latent TGFβ can occur through a number of mediators including integrins αvβ6 and αvβ8 (reviewed in Munger and Sheppard, 2011), Plasmin (Flaumenhaft et al., 1992), Thrombospondin-1 (Crawford et al., 1998) and several matrix metalloproteinases (MMPs) including membrane-type 1 MMP (Tatti et al., 2008), MMP-13 (Nannuru et al., 2010), and MMP-2 and MMP-9 (Yu and Stamenkovic, 2000).

TGFβ family members are highly engaged in vascular development as well as vascular remodelling in the adult. Insight into the importance of TGFβ signaling during vascular development has been attained through knockout mouse models. Deletion of

TGFβ1 results in vascular defects in the yolk sac and haematopoiesis resulting in embryonic lethality (Dickson et al., 1995). Mice ablated of TGFβR2 expression also exhibit defects in yolk sac formation and haematopoiesis (Oshima et al., 1996). Deletion of TGFβR1 is lethal with mice exhibiting vascular defects in the yolk sac and placenta, but exhibiting normal haematopoietic potential (Larsson et al., 2001). TGFβ2-knockout mice exhibit multiple developmental defects including cardiovascular, craniofacial, eye, ear, and urogenital defects; with cardiovascular problems being postulated to be the main contributor to prenatal mortality in TGFβ2-knockout mice, while a combination of cardiovascular defects and respiratory problems result in the postnatal lethality seen in the remaining mice. (Sanford et al., 1997).

In the adult, processes such as angiogenesis in cancer can be regulated by TGFβ. Recently it has been observed that TGFβ stimulation inhibits VEGF production in colon carcinoma cells (Geng et al., 2013). Inhibition of TGFβ signaling in these cells through the use of a dominant negative TGFβR2 resulted in increased CD31 and VEGF staining in orthotopic tumors, implying increased angiogenesis, thus indicating a mechansim whereby TGFβ inhibits tumor angiogenesis by inhibiting VEGF production from tumor cells. TGFβ1 has also been shown to decrease VEGFR2 expression in endothelial cells from both microvascular and large vessel origin (Kuczynski et al., 2011; Mandriota et al., 1996), an effect that could, in theory, lead to decreased endothelial response to VEGF and inhibition of angiogenesis. Interestingly, the study by Kuczynski and colleagues (2011) showed a decrease in VEGFR2 expression in bovine aortic endothelial cells (BAECs) following their treatment with conditioned media from various colorectal carcinoma cell lines. The observed decrease in VEGFR2 could be prevented through the use of TGFβR1 inhibitors, thus implicating TGFβ1 and/or TGFβ2 signaling in control of VEGFR2 expression in

BAECs, and suggesting a potential mechanism for the heterogenous VEGFR2 expression pattern seen in colorectal tumor vessels. An inhibitory role for TGFβ signaling in endothelial cells has also been identified through the use of TGFβR kinase inhibitors. Combination of VEGF treatment with TGFβR1 kinase inhibitors to block TGFβ signaling in umbilical vein endothelial cells resulted in synergistic enhancement of endothelial cell migration and vessel sprouting *in vitro* and angiogenesis *in vivo* (Liu et al., 2009). Conversely the use of TGFβ antagonistic peptides in microvascular endothelial cells was shown to inhibit TGFβ1-induced endothelial cell invasion and morphogenesis (Serrati et al., 2009) highlighting the complex and context dependent role that TGFβ signaling plays in endothelial cells.

TGFβ is becoming increasingly described as having a role in an extreme form of endothelial cell plasticity, EndMT (Nakajima et al., 2000). In EndMT, endothelial cells gain a mesenchymal phenotype marked by loss of endothelial cell markers, gain of mesenchymal markers, and an increased migratory and invasive ability (reviewed in Potenta et al., 2008). EndMT has been suggested to play a role in angiogenic sprouting as endothelial tip cells appear to display a phenotype consistent with having undergone EndMT, such as an increased migratory and invasive ability (Potenta et al., 2008). Recently, miRNA expression has been identified as having a role in EndMT. In mouse cardiac endothelial cells, TGFβ2 induces EndMT and differentially regulates the expression of several miRNAs (Ghosh et al., 2012). TGFβ2 has also been shown to upregulate miR-21 expression in human endothelial cells resulting in miR-21-mediated decreased endothelial markers and increased fibroblast markers (Kumarswamy et al., 2012b). Additionally, TGFβ1 has been shown to up-regulate miR-29a in endothelial cells leading to decreased PTEN expression due to direct targeting of PTEN by miR-29a and resulting in increased Akt activity (Wang et al., 2013).

TGFβ family members have a documented role in processes involved in angiogenesis and have been shown to regulate, and to be regulated by, miRNAs in endothelial cells. Thus, expanding our understanding of both TGFβ signaling and miRNA biology in the context of angiogenic processess is of substantial importance.

1.10 Rationale for Study

The data presented in this thesis is the culmination of work that began with an attempt to better understand the signaling mechanisms governing angiogenic processes under the control of the major angiogenic growth factor VEGF; an endeavor that led to the identification of two signaling mechanisms responsive to VEGF stimulation and found to regulate the process of endothelial cell capillary morphogenesis. Initial results were obtained by studying the small GTPase RhoB more closely in endothelial cells while the increasingly evident importance of miRNAs to angiogenesis led us to explore VEGF regulated miRNAs in endothelial cells in more detail.

Small GTPases such as Rac, Cdc42, and RhoA all have relatively well described roles in processes important to angiogenesis. On the other hand, a well defined role for RhoB in angiogenesis was lacking. Literature indicated RhoB as being dispensible during development as mice with a homozygous deletion of the RhoB gene developed normally; however, depletion of RhoB was shown to impair the vascularization of the retina during postnatal development in mice, indicating a role for RhoB in the angiogenic process. Although an apparent function for RhoB in angiogenesis was becoming evident, there had been no studies looking at the role of RhoB in angiogenic processes specifically in response to the major angiogenic growth factor VEGF. Other growth factors with described roles in

the angiogenic process such as EGF, PDGF and TGFβ1 have all been shown to regulate the expression of RhoB. Thus, a demonstrated role for RhoB in vascular sprouting combined with the known regulation of RhoB by angiogenic growth factors provided the basis for a hypothesis that RhoB functions to regulate angiogenic processes in cultured endothelial cells in response to VEGF, and that RhoB expression itself may in fact be regulated by VEGF. Ultimately the following chapters provide evidence supporting both of these assumptions as we identify VEGF regulation of RhoB expression in endothelial cells and elucidate a novel signaling mechanism involved in the regulation of angiogenic processes mediated by RhoB.

The importance of global miRNA expression to angiogenic processes was first identified after inhibition of the miRNA processing enzyme Dicer, and since then significant roles for individual miRNAs in angiogenesis are being continuously discovered, although only a fraction of total miRNAs have been identified as having a role in angiogenesis. Thus, many unstudied miRNAs may prove to have significant roles in processes important to angiogenesis such as migration, sprouting, and cord formation. Expression of individual miRNAs has also been shown to be regulated by angiogenic growth factors such as bFGF and VEGF. Many of these miRNAs have been identified as regulating angiogenesis, indicating the potential that other growth factor regulated miRNAs may also play important roles in angiogenesis. With this in mind we aimed to identify VEGF regulated miRNAs in endothelial cells and hypothesized that these miRNAs likely possessed some degree of importance to the regulation of processes necessary for angiogenesis. The results presented in the following chapters indicate the importance of a novel signaling pathway involving VEGF regulation of miRNA expression to angiogenic processes and highlight the importance of continued exploration of the roles of individual miRNAs in angiogenesis.

Hypothesis: VEGF-induced endothelial cell capillary morphogenesis is regulated by factors such as the small GTPase RhoB and miRNA, both of which constitute putative novel therapeutic targets for treatment of diseases involving angiogenesis.

Objectives:

1) To evaluate the role of the small GTPase RhoB in endothelial cell capillary morphogenesis.

2) To investigate the effects of VEGF on miRNA expression and to further characterize the role of VEGF-regulated miRNAs in endothelial cell capillary morphogenesis.

2. MATERIALS AND METHODS

2.1 Antibodies, Growth Factors and Inhibitors

Primary antibodies used were: RhoB (C-5, SC-8048), RhoA (26 C4, SC-418), RhoC (37, SC-130339), TGFβ2 (V, SC-90), ATF-2 (C-19, SC-187), and p-ATF-2 (F-1, SC-8398) all from Santa Cruz Biotechnology (Santa Cruz, CA), Phospho-Smad2 (S465/467) from Cell Signaling Technology (3101; Danvers, MA) , Smad2 from Invitrogen (511300; Carlsbad, CA), Anti-TGFβ2 neutralizing antibody (AB-12-NA) and Normal Rabbit IgG (AB-105-C) from R&D Systems (Minneapolis, MN), monoclonal anti-β-Actin antibody (clone AC-74) from Sigma-Aldrich (A5316; St. Louis, MO). Secondary antibodies used were: goat anti-mouse IgG horse radish peroxidase (HRP) conjugate and goat anti-rabbit IgG horse radish peroxidise (HRP) conjugate, both from Calbiochem (EMD Biosciences, La Jolla, CA). Recombinant human $VEGF_{165}$ was purchased from R&D Systems (Minneapolis, MN). Cell permeable Rho Inhibitor (C3 transferase) was purchased from Cytoskeleton, Inc. (Denver, CO). ROCK I/II inhibitors H-1152 and Y-27632 were purchased from Calbiochem (EMD Biosciences, La Jolla, CA) and dissolved in dimethyl sulfoxide (DMSO). Avastin® (DIN 02270994) was from Roche (Mississauga, ON) and was used at a concentration of 1 μg/ml.

2.2 Cell Culture

Human umbilical vein endothelial cells (HUVECs) were purchased from Lonza (C2517A; Walkersville, MD) and grown in EGM-2 media [EBM-2 basal medium (CC-3156) supplemented with EGM-2 SingleQuot kit supplement and growth factors (CC-4176)] also from Lonza. Cells were routinely passaged at 80-90% confluence through cell dissociation

with 0.05% Trypsin-EDTA (Mediatech Inc., Manassas, VA) following one wash with Hank's Balanced Salt Solution (HBSS), and used for experiments at passage 6 through 10. Cells were maintained at 37 °C in 5% CO_2. For serum starvation, HUVECs were incubated in MCDB 131 Medium (Gibco by Life Technologies; Carlsbad, CA) supplemented with L-Glutamine (GlutaMAX-I; Gibco by Life Technologies, Carlsbad, CA) and 0.05% to 0.5% fetal bovine serum (FBS: Medicorp, Montreal, QC) for 16-20 hours, with additional time under starvation required under specific experimental conditions.

2.3 SiRNA Transfection

For silencing of RhoB in HUVECs, two small interfering RNAs (siRNAs) were designed as ON-TARGET reagents from Dharmacon, Inc. (Lafayette, CO). Target sequences were as follows: RhoB siRNA 1: UGCUGAUCGUGUUCAGUAA, and RhoB siRNA 2: CCGUCUUCGAGAACUAUGU. ATF2 siRNA (MQ-009871-00) was purchased as an ON-TARGET reagent from Dharmacon. Control siRNA was also purchased from Dharmacon, (siControl Non-Targeting siRNA #1; D-001210-01). For silencing experiments involving RhoB, both RhoB siRNAs and control siRNA were used at a concentration of 20 nM. For silencing of ATF2, both ATF2 siRNA and control siRNA were used at a concentration of 5 nM. HUVECs were transfected at 80% confluence in Opti-MEM® I reduced serum medium using Oligofectamine Transfection Reagent (Invitrogen, Carlsbad, CA), according to the manufacturer's protocol. Briefly, siRNA was incubated with Oligofectamine for 15 minutes and then added to cells in Opti-MEM® I. The transfection was allowed to proceed for 4 hours at 37 °C after which EGM-2 media containing 3 times the normal amount of FBS was added to the cells without removing the transfection mixture.

2.4 Transfection of MicroRNA Mimics and Hairpin Inhibitors

HUVECs were seeded onto either 6 cm or 10 cm tissue culture plates at 4 x 10^5 cells or 1 x 10^6 cells, respectively, and allowed to adhere overnight. Transfection of cells with miRIDIAN microRNA Mimics (Thermo Fisher Scientific Inc., Waltham, MA) or miRIDIAN microRNA Hairpin Inhibitors (Thermo Fisher Scientific Inc., Waltham, MA) was achieved with Oligofectamine Transfection Reagent (Invitrogen, Carlsbad, CA), according to the manufacturer's instructions. Briefly, mimics and hairpin inhibitors were incubated with Oligofectamine reagent for 15 minutes, after which the transfection mixture was added to cells incubating in Opti-MEM® I and the transfection was allowed to proceed for 4 hours at 37 °C. EGM-2 media with 3 times the normal amount of FBS was then added to the cells without removing the transfection mixture and cells were grown until harvested. Mimics used were: miRIDIAN microRNA Mimic Negative Control #1 (Thermo Fisher Scientific Inc., Waltham, MA), miRIDIAN microRNA hsa-miR-30b-5p mimic (Thermo Fisher Scientific Inc., Waltham, MA). Hairpin inhibitors used were: miRIDIAN microRNA Hairpin Inhibitor Negative Control #1 (Thermo Fisher Scientific Inc., Waltham, MA), miRIDIAN microRNA hsa-miR-30b-5p hairpin inhibitor (Thermo Fisher Scientific Inc., Waltham, MA).

2.5 Protein Isolation and Western Blotting

For protein isolation, HUVECs were washed once with phosphate-buffered saline (PBS) and lysed in appropriate amounts (65 µl for 6 cm; 90 µl for 10 cm) of lysis buffer (10 mM Tris pH 7.4, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100) containing protease inhibitor cocktail (SigmaFAST™ Protease Inhibitor Cocktail Tablet, EDTA Free; Cat# S8830; Sigma-Aldrich, St. Louis, MO), sodium orthovanadate (0.5 mM), sodium

pyrophosphate (2 mM), and sodium fluoride (2 mM). Lysate was then centrifuged at 18,000 x g for 25 minutes. Cleared lysates were stored at -80 °C until needed. Protein concentration was determined with Bio-Rad Protein Assay Dye Reagent Concentrate (Bio-Rad, Hercules, CA) and a Beckman DU® 640 spectrophotometer (Beckman Coulter Inc., Indianapolis, IN) with absorbance at 595 nm. Equal amounts of total protein per sample were diluted with NuPAGE® LDS Sample Buffer (Novex, Life Technologies, Carlsbad, CA) and reduced with dithiothreitol (DTT). Following heating at 70 °C, equal volumes were loaded onto NuPAGE® Novex® 4-12% Bis-Tris Gels (Novex, Life Technologies, Carlsbad, CA). Proteins were separated at 180 V for 1 hour and transferred to Hybond-C Extra nitrocellulose membrane (Amersham Biosciences, GE Healthcare, Piscataway, NJ) at 30 V for 1 hour and 15 minutes. Membranes were then incubated in blocking buffer [5% milk in tris-buffered saline (TBST: 10mM Tris pH 8.0, 150mM NaCl, 0.05% Tween 20)] for 1 hour at room temperature followed by incubation with primary antibody overnight at 4 °C. The following concentrations of primary antibody were used: RhoB (1:200), RhoA (1:500), RhoC (1:200), TGFβ2 (1:200), p-ATF-2 (1:200), ATF-2 (1:200), Phospho-Smad2 (1:1000), Smad2 (1:1000), β-actin (1:7000). Following primary antibody incubation, membrane was washed 3 times for 5 minutes each in TBST and incubated in appropriate secondary antibody conjugated to horse radish peroxidise (HRP) at 1:1000 dilution for 1 hour at room temperature. Membrane was then washed 6 times for 5 minutes each in TBST and incubated for 5 minutes in Immobilon Western Chemiluminescent HRP Substrate (EMD Millipore, Billerica, MA) prior to visualization using the GeneGnome detection system (Syngene, Frederick, MD).

2.6 RNA Isolation

HUVECs were washed once with PBS, followed by the addition of 700 µl QIAzol lysis reagent (Qiagen, Germantown, MD). Cell lysate was frozen at -80 °C until processing for total RNA and microRNA with the miRNeasy Mini Kit (Qiagen, Germantown, MD) according to the manufacturer's protocol. RNA was dissolved in sterile nuclease free water and stored at -80 °C. Total RNA concentration was determined with a NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA) with 260/280 absorbance ratio between 1.9 and 2.1.

2.7 Quantitative RT-PCR

Equal amounts of RNA (1 µg) were used for complementary DNA (cDNA) production. Reverse transcription was performed with Moloney murine leukemia virus (M-MLV) reverse transcriptase (RT) (Invitrogen by Life Technologies, Carlsbad, CA) according to the manufacturer's instructions. Polymerase chain reaction (PCR) was performed as individual reactions with gene specific primers and RT^2 SYBR Green ROX™ qPCR Mastermix (Qiagen, Germantown, MD). PCR was performed with a 7500 Fast Real-Time PCR System (Applied Biosystems by life Technologies, Carlsbad, CA) with the following parameters: 95 °C for 10 minutes; PCR (40 cycles), 95 °C for 15 seconds, 60 °C for 60 seconds.

The amount of RNA in each sample was normalized to β-actin levels within that sample. Relative expression was determined via delta-delta-Ct method with values displayed as $2^{-\Delta\Delta Ct}$. The primer sets used were: β-actin (forward: CCAACCGCGAGAAGATGA; reverse: CCAGAGGCGTACAGGGATAG), TGFβ1 (forward:

CACGTGGAGCTGTACCAGAA; reverse: CAGCCGGTTGCTGAGGTA), TGFβ2 (forward: CCAAAGGGTACAATCCAC; reverse: CAGATTCTGGATTTATGGTATT), ATF2 (forward: TTTGGTCCAGCACGTAATGA; reverse: CAAACCCACTTCTTCACAGTTTT), JDP2 (forward: TTTGCAGGGAGGTGCTCT; reverse: GATCTGCCCAGGCATCATA), FN1 (forward: GCGAGAGTGCCCCTACTACA; reverse: GTTGGTGAATCGCAGGTCA), Snai1 (forward: GCTGCAGGACTCTAATCCAGA; reverse: ATCTCCGGAGGTGGGATG), Slug (forward: TGGTTGCTTCAAGGACACAT; reverse: GTTGCAGTGAGGGCAAGAA), VE-Cadherin (forward: AAGCCTCTGATTGGCACAGT; reverse: CTGGCCCTTGTCACTGGT), VEGFR2 (forward: GAACATTTGGGAAATCTCTTGC; reverse: CGGAAGAACAATGTAGTCTTTGC), αSMA (forward: CTGTTCCAGCCATCCTTCAT; reverse: TCATGATGCTGTTGTAGGTGGT), PECAM1 (forward: CATTGTTCCCGGTTTCCA; reverse: CAGAGAGACCGGCTGTGG).

2.8 MicroRNA Expression Analysis

RNA samples were diluted to a concentration of 5 ng/µl in sterile nuclease free water and reverse transcription of desired mature miRNAs was performed, with up to four miRNA-specific primers in the same reaction, using TaqMan MicroRNA Assays (Applied Biosystems by Life Technologies, Carlsbad, CA) specific for individual miRNAs and the TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems by Life Technologies, Carlsbad, CA), following the manufacturer's protocol. Reverse transcription was performed with the following parameters: 16 °C for 30 minutes, 42 °C for 30 minutes, 85 °C for 5

minutes. PCR was performed with the TaqMan MicroRNA Assay PCR primer for the desired miRNA and the TaqMan 2X Universal PCR Master Mix (Applied Biosystems by Life Technologies, Carlsbad, CA). PCR was performed with a 7500 Fast Real-Time PCR System (Applied Biosystems by Life Technologies, Carlsbad, CA) with the following parameters: 95 °C for 10 minutes; PCR (40 cycles), 95 °C for 15 seconds, 60 °C for 60 seconds. Gene expression data were normalized to miR-103 unless otherwise stated. Relative expression was determined via delta-delta-Ct method with values displayed as $2^{-\Delta\Delta Ct}$.

2.9 Cell Viability Assay

HUVECs were seeded into 6-well tissue culture plates at 1×10^5 cells/well (RhoB experiments) or 9×10^4 cells/well (miR-30b experiments), in triplicate, and cells were maintained in EGM-2 growth media. To assess cell viability, cells were washed once in HBSS to remove floating debris, and then trypsinized. Viability was assessed by trypan blue exclusion using a Vi-Cell XR cell viability analyzer (Beckman Coulter, Brea, CA) at the times indicated. For transfection experiments, HUVECs were seeded for the assay at 24 hours post transfection and time points used for viable cells counts indicate 24 hour intervals post seeding.

2.10 Migration Assay

Cell migration was assessed via scratch wound assay. HUVECs were seeded into 6-well tissue culture plates at 4.5×10^5 cells/well in duplicate and allowed to grow overnight to 100% confluence. A wound of approximately 1.5 mm was made creating a gap into which

cells could migrate. For siRNA and miRNA experiments, cells were seeded at 24 hours post transfection and wounding was performed at 48 h post transfection. Images were taken at time of wounding and 24 hours post wounding with a Nikon Eclipse TE2000-U microscope. For experiments involving RhoB, cells were incubated in MCDB 131 Medium with 0.05% FBS and 50 ng/ml VEGF during the course of the experiment. For experiments involving miR-30b, cells were incubated in EGM-2 growth media for the duration of the experiment. Migration was assessed by calculating the distance migrated from 12 total measurements taken across the wound front from each of duplicate wells for each experiment.

2.11 Sprouting Assay

Fibrillar collagen I gels were generated following renaturation of PureCol® purified bovine dermal collagen (Advanced BioMatrix, San Diego, CA) as described by the manufacturer. Following overnight incubation to allow gels to solidify, gel surfaces were washed once with PBS and briefly incubated in media prior to seeding cells at 1×10^5 cells per 6 cm dish in EGM-2 growth media supplemented with 50 ng/ml VEGF. Cells were seeded at 24 hours post transfection and vessel sprouts were counted in a blinded fashion (through coding of the dishes by a third party not involved with the counting of sprouts) every two days thereafter from duplicate dishes. Counts of sprout structures were made from 10 random fields of view per dish using an Olympus CK2 microscope. Media supplemented with VEGF was replaced every two days for the duration of the assay.

2.12 Cord Formation Assay

The organization of HUVECs into capillary-like cord structures was assessed by plating cells onto Cultrex® Basement Membrane Extract (BME, Growth factor reduced, Trevigen, Gaithersburg, MD). Two hundred µl BME was polymerized at 37 °C for 30 minutes in 24-well plates followed by incubation in EGM-2 growth media for 1-2 hours. Cells were then seeded at 5×10^4 cells/well in duplicate or triplicate in EGM-2 growth media. For both siRNA and miRNA experiments, transfected cells were seeded onto BME at 48 hours post transfection. Twenty-four hours later, images were taken with a Nikon Eclipse TE2000-U microscope. Demarcation of each well into 4 quadrants allowed for a total of 4 images per well with the total number of capillary-like cord structures counted with ImageJ software (http://imagej.nih.gov/ij/), and expressed as the average number of cords per field of view. Independently performed experiments were normalized prior to means being calculated in order to control for variability in cord formation between experiments.

2.13 Rho Activation Assays

Levels of activated RhoA were determined using the RhoA G-LISA™ Activation Assay kit (Cytoskeleton Inc., Denver, CO), according to the manufacturer's instructions. Briefly, siRNA-transfected HUVECs were serum starved in MCDB 131 Medium for 5 hours at 48 hours post transfection. Cells were then treated with 10 ng/ml VEGF for the times indicated and protein lysates were collected and frozen at -80 °C for subsequent analysis. Protein lysates were then analyzed on G-LISA™ plates using RhoA specific antibody for detection of captured active RhoA according to the manufacturer's directions, and absorbance was determined with a Multiskan Ascent photometer (Thermo Scientific,

Rockford, IL). In all cases, constitutively active RhoA protein provided in the G-LISA™ kit was used as a positive control to validate that the assay was functioning appropriately.

For detection of levels of active RhoC, the G-LISA™ kit was again used according to the manufacturer's instructions with the exception that a RhoC specific antibody was used to detect the amount of captured active RhoC. For the detection of RhoC, siRNA-transfected HUVECs were starved in MCDB 131 Medium (0.5% FBS) overnight, followed by starvation in serum-free MCDB 131 for 4 hours. Cells were then treated with 50 ng/ml VEGF (hence stimulated 48 hours post-siRNA transfection) and lysates collected 5 minutes post-VEGF stimulation. For the detection of activated RhoB, the G-LISA™ kit was used according to the manufacturer's instructions with the exception that a RhoB specific antibody was used to detect the amount of active RhoB captured on the plate. Activated RhoB was detected in protein lysates from HUVECs that had been serum starved overnight in MCDB 131 Medium with 0.5% FBS followed by stimulation with 20 ng/ml VEGF for the indicated times.

2.14 ELISAs for TGFβ1 and TGFβ2

Quantikine ELISAs from R&D Systems (Minneapolis, MN) were used for the determination of TGFβ1 (cat# SB100B) and TGFβ2 (cat# SB250) in cell culture supernates. HUVECs were transfected with 20 nM of either control miRNA mimic or miR-30b mimic and at 24 hours post transfection were maintained in MCDB 131 with 0.5% FBS for 24 hours prior to collection of supernates. Cell culture supernates were collected and debris was removed through centrifugation at 1,200 x g for 5 minutes followed by storage of the supernates at -80 °C. ELISAs were run according to manufacturer's instructions in duplicate and absorbance was read at 450 nm with correction at 570 nm on a Multiskan Ascent

photometer (Thermo Scientific, Rockford, IL). A standard curve was created for the determination of TGFβ concentrations. Basal levels of TGFβ in culture media alone was subtracted as background from each cell culture sample.

2.15 Affymetrix Microarray Analysis

Gene Expression Analysis

Gene expression analysis was performed with HUVECs transfected with 20 nM control miRNA mimic or miR-30b mimic for 48 hours. Cells were lysed in Qiazol lysis reagent and total RNA was isolated with the miRNeasy mini kit (Qiagen). Total RNA concentration was determined with a NanoDrop 1000 Spectrophotometer (Thermo Scientific) with 260/280 absorbance ratio between 1.9 and 2.1. Total RNA was diluted to 100 ng/µl and was provided to StemCore Laboratories at The Ottawa Hospital Research Institute for analysis with an Affymetrix GeneChip Human Gene 1.0 ST array. Analysis of data was performed with FlexArray (http://genomequebec.mcgill.ca/FlexArray).

MiRNA Expression Analysis

MiRNA expression analysis was performed with HUVECs that were serum starved overnight in MCDB 131 with 0.5% FBS, followed by stimulation with VEGF (50 ng/ml) for 24 hours. Cells were then lysed in Qiazol lysis reagent and total RNA including miRNA was isolated with the miRNeasy mini kit (Qiagen, Germantown, MD). Total RNA concentration was determined with a NanoDrop 1000 Spectrophotometer (Thermo Scientific) with 260/280 absorbance ratio between 1.9 and 2.1. The Affymetrix FlashTag™ Biotin HSR RNA Labelling Kit was used to label 100 ng of total RNA. Confirmation of target labelling was done through an ELOSA QC assay prior to submission of labelled RNA to StemCore

Laboratories at The Ottawa Hospital Research Institute for analysis with an Affymetrix GeneChip miRNA 1.0 array. Analysis of data was performed with FlexArray (http://genomequebec.mcgill.ca/FlexArray).

2.16 Statistical Analysis

Analysis of statistical significance was performed in GraphPad Prism 3 (GraphPad Software Inc.). Comparison between two groups was performed with unpaired Student's *t*-tests. Comparisons between multiple groups were done by ANOVA with post hoc analysis. Results were considered statistically significant at $P < 0.05$.

3. RESULTS

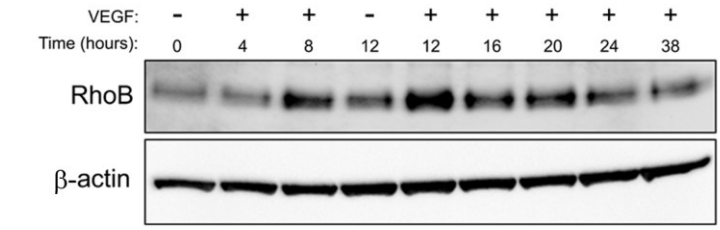
3.1 Chapter I: RhoB Regulates Endothelial Cell Capillary Morphogenesis In Part Through Negative Regulation of RhoA

Rho proteins are known to function in angiogenesis, but the extent of RhoB involvement in angiogenesis has not been thoroughly explored. RhoB is known to be regulated by growth factors and to function in growth factor signaling through receptor transport; however, the major pro-angiogenic growth factor VEGF does not have a described function in regards to RhoB. Based upon what is known about RhoB regulation we strived to describe the effects of VEGF on RhoB and determine the role RhoB plays in VEGF-mediated angiogenesis.

3.1.1 VEGF Increases RhoB Expression in Endothelial Cells

As VEGF functions as a major pro-angiogenic growth factor for endothelial cells we initially wanted to identify the expression of RhoB in HUVECs under VEGF stimulation. HUVECs were serum starved overnight and then stimulated with VEGF, with levels of RhoB protein being analyzed at the indicated time points up to 38 hours. Our results indicated increased expression of RhoB protein after 8 hours of VEGF stimulation with maximal expression being seen at 12 hours (Fig. 5A). To determine if increased protein expression of RhoB resulted in increased levels of activated RhoB, we performed analysis of RhoB activity with the G-LISA™ Activation Assay. This assay is designed for the detection of activated RhoA through the use of the Rho-binding protein Rhoteckin which binds Rho proteins in their active state. As Rhoteckin is also known to bind activated RhoB

A.


VEGF: - + + - + + + + +
Time (hours): 0 4 8 12 12 16 20 24 38
RhoB
β-actin

B.

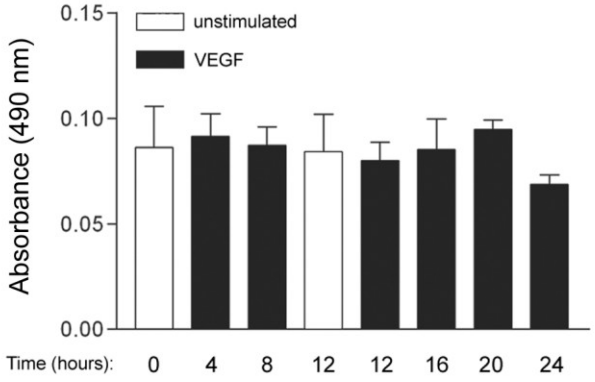

0.15
0.10
0.05
0.00
Absorbance (490 nm)
unstimulated
VEGF
Time (hours): 0 4 8 12 12 16 20 24

Figure 5. VEGF increases expression but not activity of RhoB in HUVECs. (A) Cells were serum starved overnight in MCDB 131 with 0.25% FBS. Media was then replaced with MCDB 131 with 0.25% FBS and 20 ng/ml VEGF and protein lysates were collected at the times indicated. Equal amounts of total protein were assessed by western blot for levels of RhoB with β-actin as endogenous control. (B) Cells were serum starved overnight in MCDB 131 with 0.5% FBS and then stimulated with 20 ng/ml VEGF. At the indicated time points post VEGF stimulation, protein lysates were collected and levels of activated RhoB were assessed by G-LISA as described in Materials and Methods. Data represents the mean ± SEM from duplicate wells of two independently performed experiments.

(Wheeler and Ridley, 2004), we utilized this assay for detection of activated RhoB by modifying the detection antibody for a RhoB specific monoclonal antibody. Following VEGF stimulation, HUVEC lysates were collected and equal amounts of total protein were used in duplicate wells for each condition and the level of activated RhoB was assessed. Using this method we determined that VEGF does not increase cellular levels of activated RhoB (Fig. 5B), even at time points where we have shown increased expression of RhoB protein (Fig. 5A). Thus, VEGF enhances RhoB protein expression without a concomitant increase in RhoB activity.

3.1.2 Depletion of RhoB Inhibits HUVEC Migration but not Cell Viability

To assess the function of RhoB in processes important to angiogenesis we employed a strategy of reducing cellular levels of RhoB through siRNAs designed against RhoB mRNA. To this end we designed two siRNA molecules and verified them for sequence specificity through BLAST analysis (NCBI). HUVECs were transfected with 20 nM siRNA and RhoB protein levels were assessed at 48 hours post transfection. Both siRNAs effectively reduced levels of RhoB protein in HUVECs as compared to cells transfected with control siRNA or mock transfected cells (Fig. 6), indicating that an siRNA strategy was useful for depletion of RhoB in these cells.

As both cell viability and migration are important factors in the process of angiogenesis we looked at the effects of RhoB depletion on these processes. To assess cell viability, HUVECs were transfected with RhoB siRNA and seeded into tissue culture plates 24 hours later. Cell viability was then assessed at the indicated times through quantification of viable cells by trypan blue exclusion. Results indicate no significant difference in cell

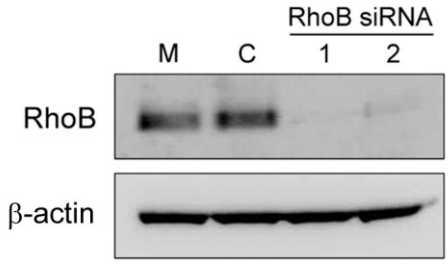

RhoB siRNA
M
C
1
2
RhoB
β-actin

Figure 6. RhoB can be effectively depleted in HUVECs through siRNA treatment. HUVECs were either mock transfected with PBS (M) or transfected with 20 nM of the indicated siRNA [Control siRNA (C), RhoB siRNA 1, RhoB siRNA 2]. Protein lysates were collected at 48 hours post transfection and levels of RhoB expression were assessed by western blot with β-actin as endogenous control. Data presented is representative of RhoB expression levels following siRNA treatment seen in three independently performed experiments.

viability between RhoB-depleted cells and control cells (Fig. 7).

We next looked at endothelial cell migration in RhoB-depleted cells by scratch wound assay. HUVECs were transfected with either of two siRNAs directed against RhoB or non-targeting control siRNA and seeded into tissue culture plates at 24 hours post transfection, then allowed to grow to confluence overnight. Cells were then "scratched" whereby a gap of approximately 1.5 mm was created in the confluent monolayer, thus creating a space into which cells could migrate. Cells were incubated in MCDB 131 Medium with 0.05% FBS and 50 ng/ml VEGF, thus making migration essentially VEGF-dependent, and measurements of the initial wound size were taken. Twenty-four hours later measurements of wound size were again taken and the distance migrated over the 24 hour period was calculated. Depletion of RhoB with either of two siRNAs resulted in similar levels of inhibition of migration of HUVECs (Fig. 8A) as indicated by a significant decrease in distance migrated over 24 hours as compared to control siRNA-treated cells ($P < 0.01$ for RhoB siRNA 1 and $P < 0.001$ for RhoB siRNA 2) (Fig. 8B). Therefore, our data indicates that VEGF-mediated migration of HUVECs requires RhoB, but in terms of HUVEC viability, RhoB is dispensable.

3.1.3 Capillary Sprouting and Cord Formation are Inhibited in RhoB-Depleted HUVECs

To assess the contribution of RhoB to vessel formation we looked at two *in vitro* assays that assess characteristics necessary for endothelial cells to form vessels. We first looked at the effects of RhoB on luminal vessel-like formation. In this assay, HUVECs are seeded onto collagen I gel-coated tissue culture dishes where they are induced with VEGF

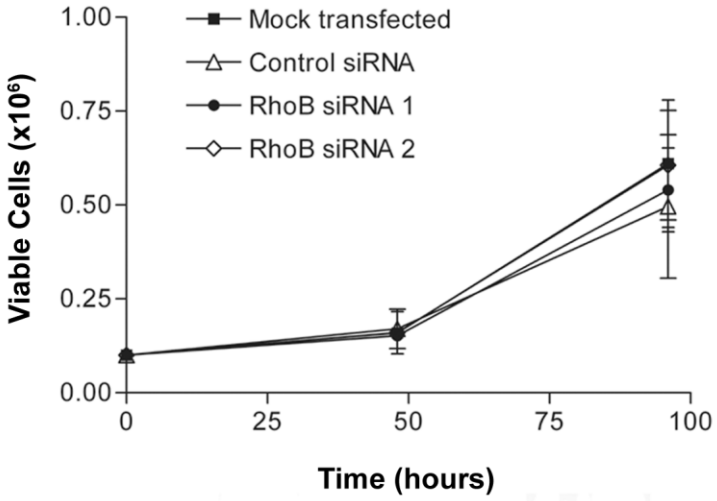
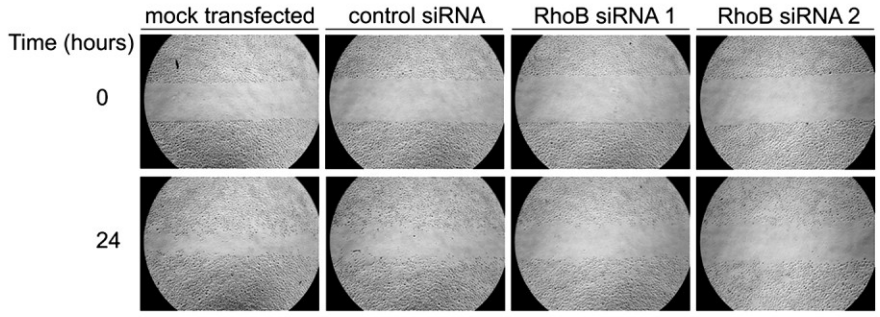

Mock transfected
Control siRNA
RhoB siRNA 1
RhoB siRNA 2
Viable Cells (x10⁶)
1.00
0.75
0.50
0.25
0.00
0
25
50
75
100
Time (hours)

Figure 7. Depletion of RhoB does not affect HUVEC viability. HUVECs were either mock transfected with PBS or transfected with 20 nM siRNAs, as indicated. Cells were seeded at 1 x 10^5 cells/well in EGM-2 growth media at 24 hours post transfection and cell counts were made every 48 hours thereafter by trypan blue exclusion using a Beckman Coulter Vi-Cell XR. Each experiment was performed in triplicate. Data presented is the mean ± SD of two independently performed experiments.

A.


mock transfected
control siRNA
RhoB siRNA 1
RhoB siRNA 2
Time (hours)
0
24

B.

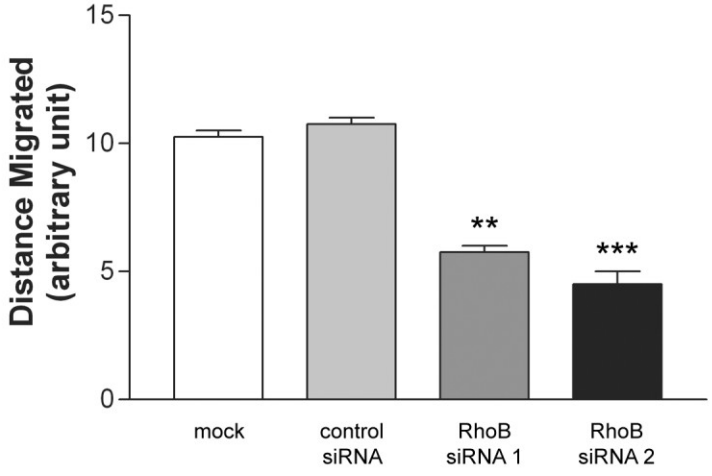
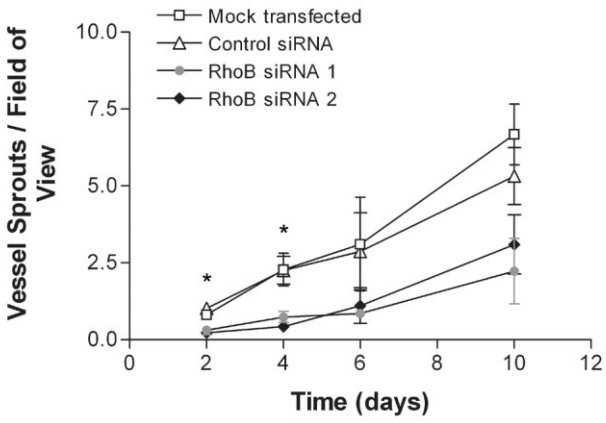

Distance Migrated
(arbitrary unit)
15
10
5
0
**
***
mock
control
siRNA
RhoB
siRNA 1
RhoB
siRNA 2

Figure 8. Depletion of RhoB inhibits HUVEC migration. (A) HUVECs were either mock transfected with PBS or transfected with 20 nM siRNA (control siRNA, RhoB siRNA 1, RhoB siRNA 2) and seeded into 6-well tissue culture dishes at 4.5 x 10^5 cells/well in MCDB 131 with 0.05% FBS and 50 ng/ml VEGF. Cells were wounded to create a gap into which cells could migrate and images were taken at the time of wounding (Time = 0 h) and 24 hours post wounding. (B) Analysis of cell migration from (A). Data presented represents the mean ± SEM for distance migrated after 24 hours. Note: ** denotes $P < 0.01$, *** denotes $P < 0.001$ as determined by ANOVA with post hoc analysis for each RhoB siRNA condition as compared to control siRNA.

to form vessel-like structures that contain lumens. HUVECs transfected with either RhoB-specific siRNA or non-targeting control siRNA were seeded onto collagen I coated tissue culture dishes at 24 hours post transfection and stimulated with VEGF (50 ng/ml) to induce sprouting. Over the course of 10 days, the number of vessel sprouts was quantified from 10 random fields of view in duplicate dishes. Figure 9 shows a significant reduction in the number of vessel sprouts in RhoB-depleted cells by day two of the assay ($P < 0.05$) as compared to control cells, with inhibition of sprouting being observed up to day ten of the assay in RhoB-depleted HUVECs (Fig. 9A). Again, both RhoB siRNA reagents indicated similar levels of inhibition of sprouting in this assay.

We next assessed the ability of HUVECs treated with RhoB siRNA to form capillary-like cord structures on basement membrane extract (BME); an assay that is dependent on endothelial cell morphogenesis and involves processes such as adhesion to the ECM, migration, elongation, and the formation of cell-cell contacts. HUVECs were transfected with one of the two siRNAs targeting RhoB or control siRNA and cells were seeded onto growth factor reduced BME at 48 hours post transfection and incubated for 24 hours. Each well was demarcated into quadrants, images were taken and total number of capillary-like cord structures was counted in each quadrant. Our results show a significant reduction in cord formation for cells treated with RhoB siRNA ($P < 0.05$), although siRNA 2 produced a greater reduction than siRNA 1 in this assay (Fig. 9B). Thus, taken together, our data identifies RhoB as mediating *in vitro* capillary sprouting and morphogenesis in HUVECs.

A.


Mock transfected
Control siRNA
RhoB siRNA 1
RhoB siRNA 2
Vessel Sprouts / Field of View
10.0
7.5
5.0
2.5
0.0
0
2
4
6
8
10
12
Time (days)
*
*

B.

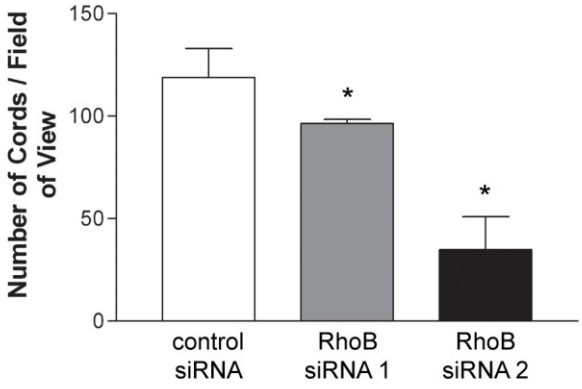

Number of Cords / Field of View
150
100
50
0
*
*
control siRNA
RhoB siRNA 1
RhoB siRNA 2

Figure 9. RhoB is required for *in vitro* vessel sprouting and cord formation.
(A) HUVECs were either mock transfected with PBS or transfected with 20 nM control or RhoB siRNAs and cells were seeded onto collagen I gels at 24 hours post transfection. At 48 hours post transfection (Time = 0 days), cells were placed in EGM-2 growth media supplemented with 50 ng/ml VEGF to induce sprouting. Media was refreshed every 2 days and the number of vessel sprouts was counted from 10 random fields of view from duplicate plates. Data presented is the normalized mean ± SEM from two independently performed experiments. Note: * denotes $P < 0.05$, as determined by ANOVA with post hoc analysis for both RhoB siRNA conditions compared to control siRNA at each time point. (B) HUVECs were transfected with 20 nM siRNA as indicated and cells were seeded onto growth factor reduced BME at 48 hours post transfection in EGM-2 media. Cord formation was assessed as the number of cords present from each of duplicate wells. Each well was divided into 4 quadrants and the total number of cord-like structures was counted from each quadrant using ImageJ (National Institutes of Health) and averaged. Data presented represents the normalized mean ± SEM from two independently performed experiments. Note: * denotes $P < 0.05$, as determined by ANOVA with post hoc analysis for comparison of RhoB siRNA conditions to control siRNA condition.

3.1.4 RhoB-Depleted HUVECs Exhibit Increased RhoA Activity upon VEGF Stimulation

Rho proteins are known regulators of endothelial cell cytoskeletal dynamics and migration (Lamalice et al., 2007), and as such, given our results indicating a role for RhoB in both endothelial cell migration and capillary-like cord formation, we set out to identify the potential mediators of RhoB's effects on these processes. Interestingly, regulation amongst Rho family members is common (Alberts et al., 2005; Nimnual et al., 2003; Rosenfeldt et al., 2006; Wildenberg et al., 2006), and in fact, regulation of RhoB protein stability by RhoA has been observed (Ho et al., 2008), although direct evidence for regulation of RhoA by RhoB had been lacking until recently (Howe and Addison, 2012). Given the propensity for Rho proteins to regulate one another and a known role for RhoA in cytoskeletal dynamics and cell migration, we looked at whether the depletion of RhoB in HUVECs affected the expression or activity of RhoA. To assess RhoA activity under conditions of decreased RhoB expression, HUVECs were transfected with either RhoB-specific siRNA or control siRNA and stimulated with VEGF (10 ng/ml). Protein lysates were collected and RhoA activity was assessed through the loading of equal amounts of total protein in the G-LISA™ Activation Assay which captures activated RhoA through binding to Rhoteckin and quantifies activity through detection with a RhoA-specific antibody. Results indicated that cells depleted of RhoB showed a significantly higher level of RhoA activity upon VEGF stimulation as compared to control transfected cells (Fig. 10A). Interestingly, even under basal conditions (Time = 0 minutes), RhoB-depleted cells exhibited slightly higher levels of RhoA activity, thus lending support to the theory that the expression of RhoB alone can regulate RhoA activity in HUVECs (Fig. 10A). Increased activation of RhoA in RhoB-depleted HUVECs

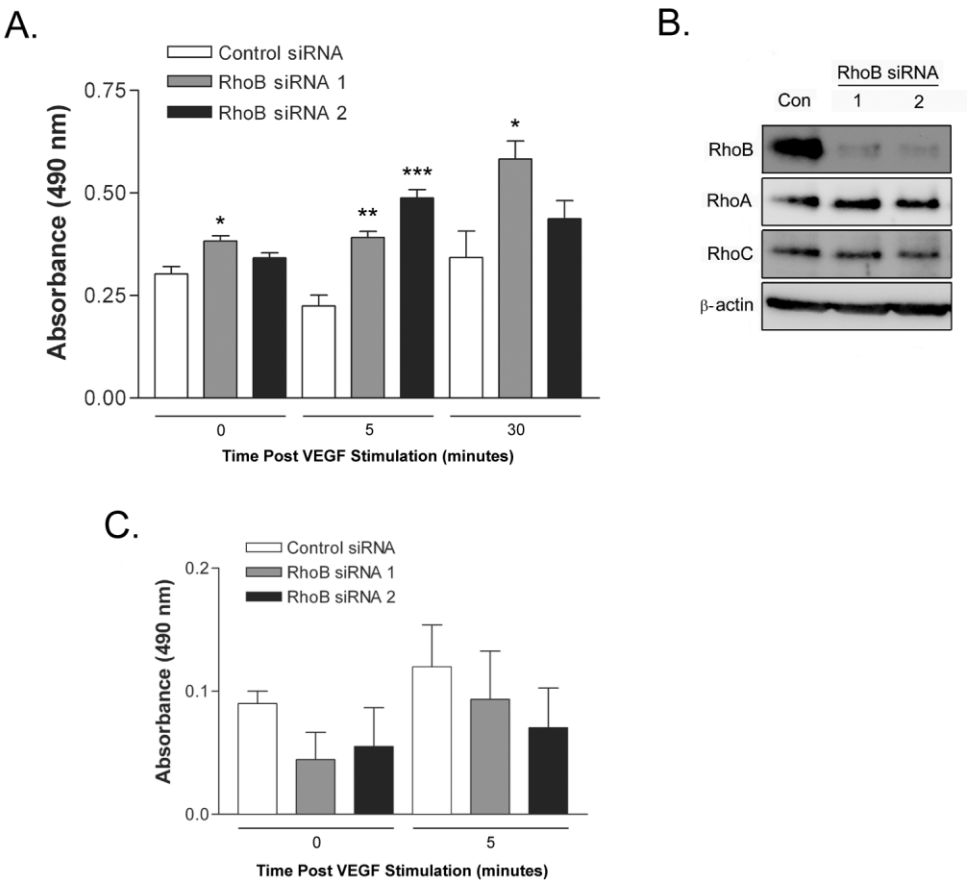

A.
Control siRNA
RhoB siRNA 1
RhoB siRNA 2
Absorbance (490 nm)
0.75
0.50
0.25
0.00
*
**
***
*
0
5
30
Time Post VEGF Stimulation (minutes)
B.
RhoB siRNA
Con
1
2
RhoB
RhoA
RhoC
β-actin
C.
Control siRNA
RhoB siRNA 1
RhoB siRNA 2
Absorbance (490 nm)
0.2
0.1
0.0
0
5
Time Post VEGF Stimulation (minutes)

Figure 10. Depletion of RhoB modulates the activity of RhoA in HUVECs. (A) Cells were transfected with 20 nM of the indicated siRNAs and at 48 hours post transfection, were serum starved in MCDB 131 with no FBS for 5 hours. Cells were then either left unstimulated (Time = 0 minutes) or were stimulated with 10 ng/ml VEGF and protein lysates collected at the times indicated. Activation of RhoA was assessed with the G-LISA assay as described in Materials and Methods. Data represents the mean ± SEM from an experiment performed in triplicate. Note: * denotes $P < 0.05$, ** denotes $P < 0.01$, *** denotes $P < 0.001$ as determined by ANOVA with post hoc analysis for RhoB siRNA treatments as compared to control siRNA treatment at each time point. (B) HUVECs were transfected with 20 nM of control (Con) or RhoB siRNAs 1 or 2, and protein lysates were collected at 48 hours post transfection. Expression levels of RhoA, RhoB, and RhoC were assessed by western blotting with β-actin serving as endogenous control. (C) HUVECs were transfected with 20 nM siRNA as indicated and cells were serum starved overnight in MCDB 131 with 0.5% FBS followed by an additional 4 hours in MCDB 131 with no serum (0% FBS). Cells were then stimulated for 5 minutes with 50 ng/ml VEGF and protein lysates collected for analysis of RhoC activity with the G-LISA assay modified for detection of RhoC as described in Materials and Methods. Data represents the mean ± SEM from duplicate samples of two independently performed experiments.

was shown to be independent of RhoA protein levels as cells transfected with RhoB siRNA did not alter the level of RhoA protein (Fig. 10B).

As RhoA and RhoB make up only two-thirds of a highly similar Rho subfamily, with RhoC being the other member, we decided to look also at the expression level and activity of RhoC in HUVECs to determine if RhoB exerts control over RhoC in addition to RhoA. To do this, we again utilized the G-LISA™ Activation Assay as RhoC has also been shown to bind Rhoteckin (Wheeler and Ridley, 2004). We modified the assay through the use of a RhoC-specific monoclonal antibody for detection of activated RhoC. We did not observe a statistically significant difference in the level of activated RhoC in RhoB-depleted HUVECs under basal conditions or after stimulation with VEGF (Fig. 10C). In addition, depletion of RhoB did not affect the level of RhoC protein in HUVECs (Fig. 10B).

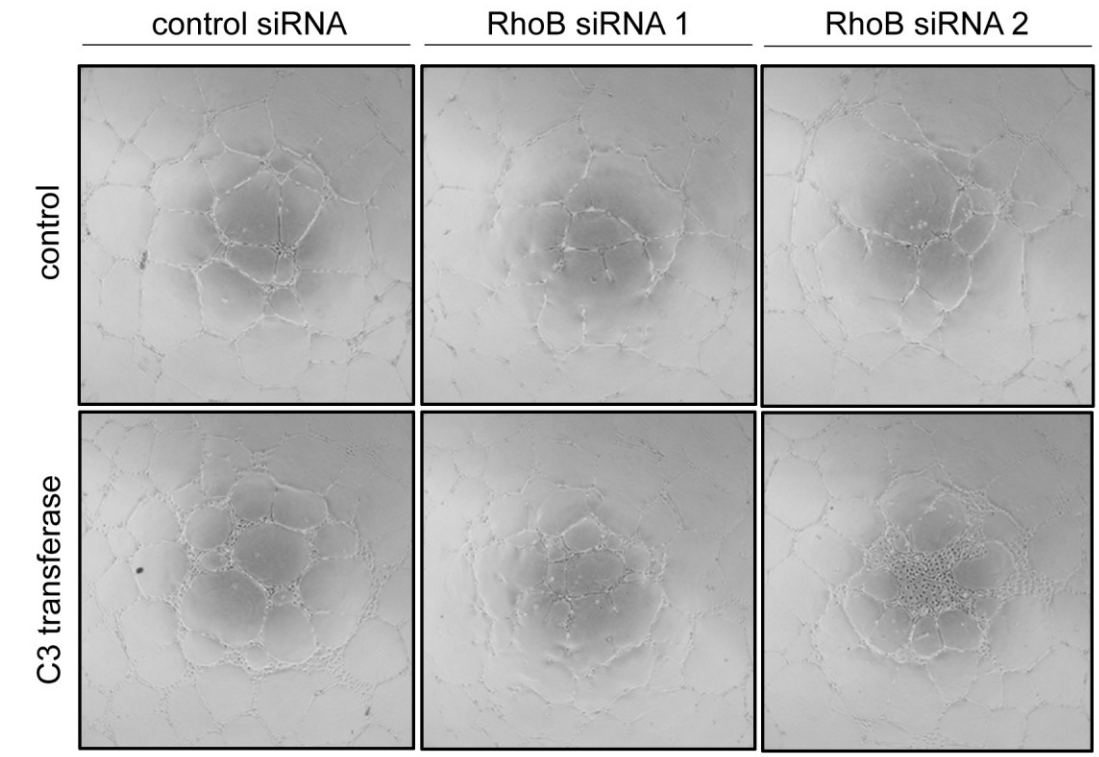
Thus, these results identify RhoB as regulating RhoA in HUVECs at the level of activation, and warrant the further study of RhoA as a potential mediator of the effects on morphogenesis seen in RhoB-depleted HUVECs.

3.1.5 Inhibition of RhoA Activity Partially Restores Cord Formation in RhoB-Depleted HUVECs

As we had identified that reduced RhoB expression resulted in increased RhoA activity, we set forth to determine whether this increased activation of RhoA was responsible for the inhibition in capillary-like cord formation seen in RhoB-depleted cells. Thus, HUVECs were transfected with RhoB-specific siRNA and seeded onto growth factor reduced BME in the presence of the cell permeable Rho inhibitor C3 transferase to inhibit RhoA activity. C3 transferase is an ADP ribosyltransferase that selectively inhibits the

activation of RhoA, RhoB, and RhoC through ribosylation. Although C3 transferase functions to ribosylate all three Rho proteins, under the conditions of RhoB depletion in HUVECs, RhoB levels are already low and RhoC activation appears unchanged, thus function of C3 transferase should mainly be observed through its effect on the inhibition of RhoA activity. As had been previously observed, the level of capillary-like cord formation in RhoB-depleted cells was significantly reduced ($P < 0.05$ for RhoB siRNA 1 and $P < 0.01$ for RhoB siRNA 2) (Fig. 11A and B). Upon treatment of control siRNA transfected HUVECs with C3 transferase, we observed a small increase in the number of capillary-like cords formed, thus indicating that the inhibition of Rho activity has a positive effect on cord formation in this assay (Fig. 11B). Importantly, RhoB-depleted cells that were treated with C3 transferase exhibited cord formation more consistent with levels found in control cells (Fig. 11B). Specifically, cells transfected with RhoB siRNA 1 saw an almost complete restoration of cord formation to levels seen in control transfected cells (Fig. 11B), while cells transfected with RhoB siRNA 2 also exhibited a restoration in cord formation, with RhoB-depleted cells treated with C3 transferase only exhibiting a 25% reduced level of cord formation as compared to the 45% reduction in cord formation seen without C3 transferase (Fig. 11B). Although we cannot rule out the possibility that inhibition of RhoC activity through our use of C3 transferase to some extent contributes to the restoration in morphogenesis, these results strongly implicate RhoA activity as a contributing factor in reduced capillary-like cord formation seen in RhoB-depleted cells.

A.


control siRNA
RhoB siRNA 1
RhoB siRNA 2
control
C3 transferase

B.

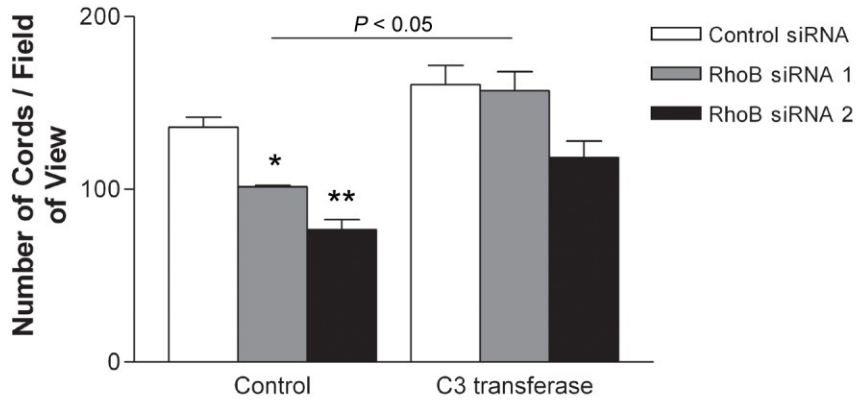

Number of Cords / Field of View
200
100
0
P < 0.05
*
**
Control
C3 transferase
Control siRNA
RhoB siRNA 1
RhoB siRNA 2

Figure 11. Inhibition of RhoA activity partially restores cord formation in RhoB-depleted HUVECs. (A) Cells were transfected with 20 nM siRNAs as indicated and seeded onto growth factor reduced BME in EGM-2 media with either 0.25 μg/ml C3 transferase or DMSO as vehicle control. Images were taken after 24 hours from triplicate wells. (B) Each well was divided into 4 quadrants and cord formation was assessed as the average number of cord-like structures from all 4 quadrants of each well, using ImageJ software. Data is presented as the normalized mean ± SEM from two independently performed experiments. Note: * denotes $P < 0.05$, ** denotes $P < 0.01$ as determined by ANOVA with post hoc analysis for RhoB siRNA treated conditions as compared to corresponding control siRNA treated condition.

3.1.6 Pharmacological Inhibition of ROCK I/II Partially Restores Cord Formation in RhoB-Depleted HUVECs

To further our understanding of how increased RhoA activity contributes to the reduced cord formation seen in cells depleted of RhoB we decided to inhibit downstream signaling from RhoA. Rho-associated kinases ROCK I and ROCK II facilitate signaling from activated RhoA and thus we looked at inhibition of this pathway and the potential for that to restore cord formation in RhoB-depleted HUVECs. HUVECs were again transfected with either RhoB-specific siRNA or control siRNA and cord formation was assessed on growth factor reduced BME in the presence of either H-1152 or Y-27632 (Fig. 12A and C), two inhibitors of ROCK I/II. Treatment with ROCK I/II inhibitors resulted in a slight increase in cord formation in control transfected cells, a result similar to what was seen upon treatment with C3 transferase ($P < 0.05$ for Y-27632) (Fig. 12B and D). In RhoB-depleted HUVECs, inhibition of ROCK I/II with either inhibitor partially restored cord formation (Fig. 12B and D). Cells transfected with RhoB siRNA 1 exhibited a more complete restoration of cord formation than cells transfected with RhoB siRNA 2 as levels were almost completely returned to those seen in control siRNA-transfected cells (Fig. 12B and D). In cells transfected with RhoB siRNA 2 and treated with ROCK I/II inhibitors, cord formation was restored to within 32% of control cells, while in the absence of ROCK I/II inhibition, RhoB-depleted cells exhibited a 43% reduced level of cord formation as compared to control cells (Fig. 12B and D), thus indicating the effectiveness of ROCK I/II inhibition to the restoration of cord formation in cells treated with RhoB siRNA 2. These results identify ROCK I/II as mediators of decreased cord formation in RhoB-depleted cells

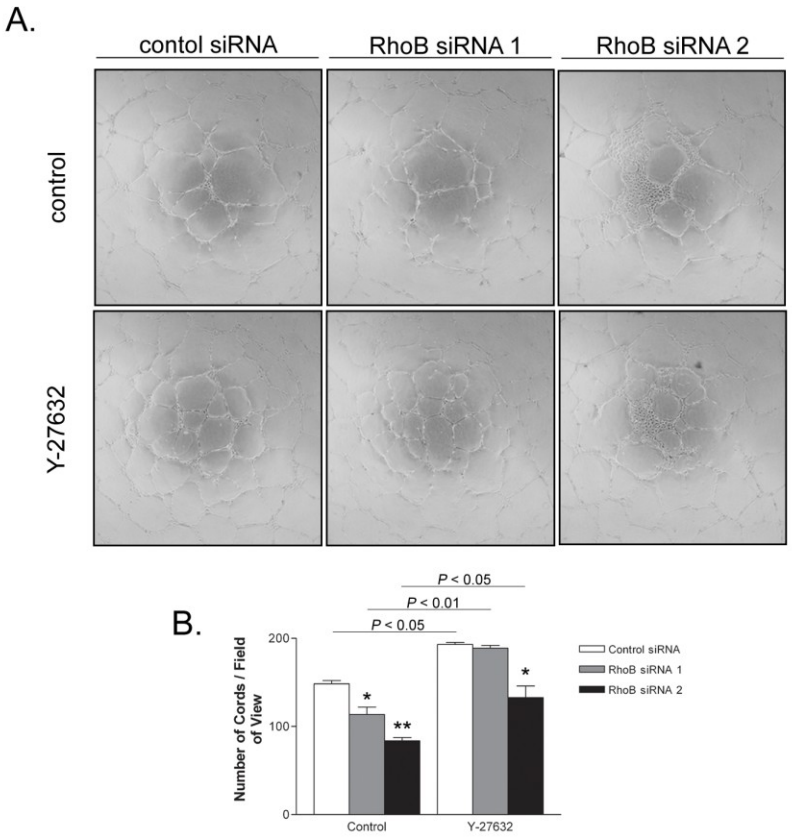

A.
contol siRNA
RhoB siRNA 1
RhoB siRNA 2
control
Y-27632
B.
P < 0.05
P < 0.01
P < 0.05
Number of Cords / Field of View
200
100
0
*
**
*
Control
Y-27632
Control siRNA
RhoB siRNA 1
RhoB siRNA 2
C.
contol siRNA
RhoB siRNA 1
RhoB siRNA 2
control
H-1152
D.
P < 0.001
Number of Cords / Field of View
200
100
0
*
**
**
Control
H-1152
Control siRNA
RhoB siRNA 1
RhoB siRNA 2

Figure 12. Inhibition of ROCK I/II activity partially restores cord formation in RhoB-depleted cells. HUVECs were transfected with 20 nM siRNAs as indicated and seeded onto growth factor reduced BME in EGM-2 growth media in the absence or presence of (A) 10 μM Y-27632 or (C) 1 μM H-1152 for 24 hours prior to images being collected from duplicate wells. Cells not treated with either Y-27632 or H-1152 were treated with an equivalent volume of DMSO as control. (B and D) Cord formation was assessed by averaging the total number of cord-like structures from each of 4 quadrants from duplicate wells. Data presented is the normalized mean ± SEM from two independently performed experiments. Note: * denotes $P < 0.05$, ** denotes $P < 0.01$ as determined by ANOVA with post hoc analysis for RhoB siRNA treated conditions as compared to the corresponding control siRNA treated condition.

and indicate the importance of RhoB control over the RhoA/ROCK signaling pathway in mediating endothelial cell morphogenesis *in vitro*.

3.2 Chapter II: Endothelial Cell Capillary Morphogenesis is Inhibited by MicroRNA-30b Through Enhanced TGFβ2 Expression

The relevance of miRNAs to many cellular processes and the increasing awareness of their contribution to disease states are prompting extensive studies on miRNA regulation. However, only a fraction of the total miRNAs present in humans has been specifically studied, and those that have been studied likely contribute to additional cellular events. MiRNA function in angiogenesis is largely unstudied and the regulation of miRNA expression by angiogenic growth factors is only recently being resolved. To this end, we decided to look at VEGF regulation of miRNA expression in HUVECs in order to ascertain candidate miRNAs that may function in response to VEGF to affect angiogenesis.

3.2.1 VEGF Regulates Expression of miR-30b in Endothelial Cells

In order to identify candidate microRNAs in HUVECs that may function in angiogenesis, we first performed a microarray for microRNA expression on VEGF stimulated cells. HUVECs were serum starved overnight and stimulated with VEGF (50 ng/ml) for 24 hours. Total RNA, including miRNA, was isolated and labelled with the FlashTag™ Biotin HSR RNA labelling kit (Affymetrix), and samples analyzed on an Affymetrix GeneChip miRNA 1.0 Array. VEGF modified the expression of a number of miRNAs to a fold change of ± 2 (Appendix: Tables 1 and 2). Of interest was miR-30b

which was identified as being downregulated by VEGF to the greatest extent in HUVECs (Appendix: Table 2, fold change = 0.12). Additionally, the RhoB 3'-UTR has a miR-30b binding site [assessed by TargetScan (http://www.Targetscan.org)] and is thus a potential target for miR-30b regulation; an interaction that could potentially explain how VEGF regulates RhoB expression. As the effect of miR-30b on angiogenesis had at that point not been studied, and its downregulation by VEGF suggested it may act as an anti-angiogenic miRNA, we decided to look further at miR-30b in HUVECs. To validate the results of the microarray analysis we performed qRT-PCR on VEGF treated samples using the Taqman MicroRNA Assay for miR-30b. HUVECs were serum starved overnight in MCDB 131 with 0.5% FBS and then stimulated with VEGF (50 ng/ml) for 24 hours. RNA was isolated and reverse transcription was performed with miR-30b specific RT primer and miR-103 RT primer as an endogenous control, followed by PCR. It was observed that VEGF consistently downregulated miR-30b by an average of approximately 12% ($P = 0.0024$, n = 6) (Fig. 13). As smaller changes in miRNA levels are more susceptible to minor changes in the level of endogenous control used to normalize the samples, we confirmed, through the use of a second endogenous control (RNU24), that VEGF decreases miR-30b expression ($P = 0.014$, n = 3) to the same extent as seen with miR-103 as endogenous control (Appendix: Fig. A1). Thus VEGF stimulation of HUVECs reduces the level of endogenous miR-30b via, an as of yet, unknown mechanism.

3.2.2 MiR-30b Regulates Endothelial Cell Cord Formation

To study the effect of miR-30b on angiogenesis, a double-stranded miRNA mimic was transfected into HUVECs in order to overexpress miR-30b. At 48 hours post

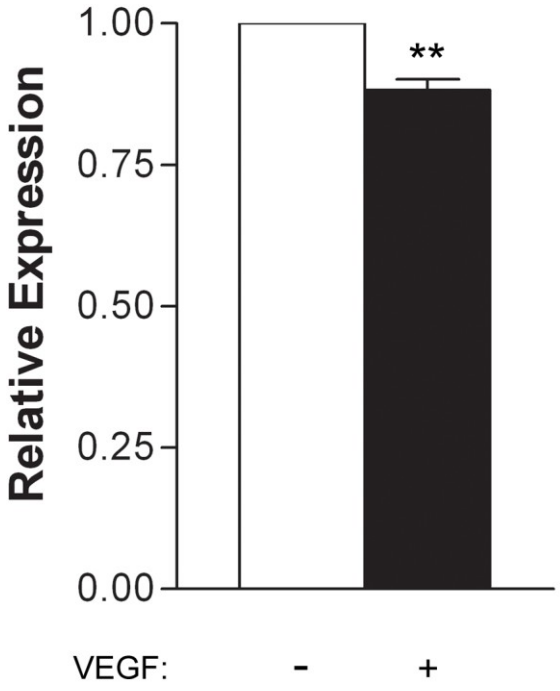

Relative Expression
1.00
0.75
0.50
0.25
0.00
**
VEGF:
-
+

Figure 13. VEGF reduces miR-30b expression in HUVECs. Cells were serum starved overnight in MCDB 131 with 0.5% FBS and were then left untreated or stimulated with VEGF (50 ng/ml) for 24 hours. Total RNA was extracted and subjected to qRT-PCR to assess the expression of miR-30b. Levels of miR-30b are expressed as mean ± SEM (n = 6) relative to the endogenous control miR-103. A statistically significant decrease in miR-30b expression is observed upon VEGF stimulation as compared to the untreated condition. Note: ** denotes $P = 0.0024$ as determined by unpaired Student's *t*-test.

transfection cells were lysed and RNA was isolated for assessment of miR-30b levels. MiR-30b-transfected HUVECs exhibited significantly higher levels of miR-30b (1 nM, P = 0.0038; 2 nM, P = 0.0025; 5 nM, P = 0.013; 20 nM, P = 0.029) as compared to cells transfected with a control miRNA mimic used at the same concentration (Fig. 14). HUVECs exhibited a dose dependent increase in miR-30b levels from approximately 250 fold at 1 nM miR-30b mimic concentration to approximately 1900 fold at 20 nM concentrations. Interestingly, cells overexpressing miR-30b (20 nM mimic) exhibited a difference in morphology compared to control transfected cells, with miR-30b overexpressing cells appearing more elongated (Fig. 15).

As viability, migration, and the morphogenesis required to form capillary-like cord structures are all processes important in angiogenesis, HUVECs overexpressing miR-30b were tested in assays to assess these processes. To investigate HUVEC viability in response to overexpression of miR-30b, viable transfected HUVECs were counted over the course of 72 hours. Cells were seeded into 6-well tissue culture plates, trypsinized at 24 hour intervals and cell viability was assessed by trypan blue exclusion with a Vi-Cell XR cell viability analyzer. Although a modest decrease in the viability of HUVECs was detected over the 72 hour experiment in miR-30b overexpressing cells (Fig. 16), these differences were determined to be not statistically significant, indicating elevated levels of miR-30b does not significantly affect cell viability in HUVECs.

To determine if miR-30b functions in regulation of endothelial cell migration, a scratch-wound assay was performed, whereby HUVECs overexpressing miR-30b were plated into tissue culture dishes at 24 hours post transfection and allowed to grow to confluence overnight. The following day (48 hours post transfection) a gap of approximately 1.5 mm was created in the confluent monolayer, and images were collected at time of

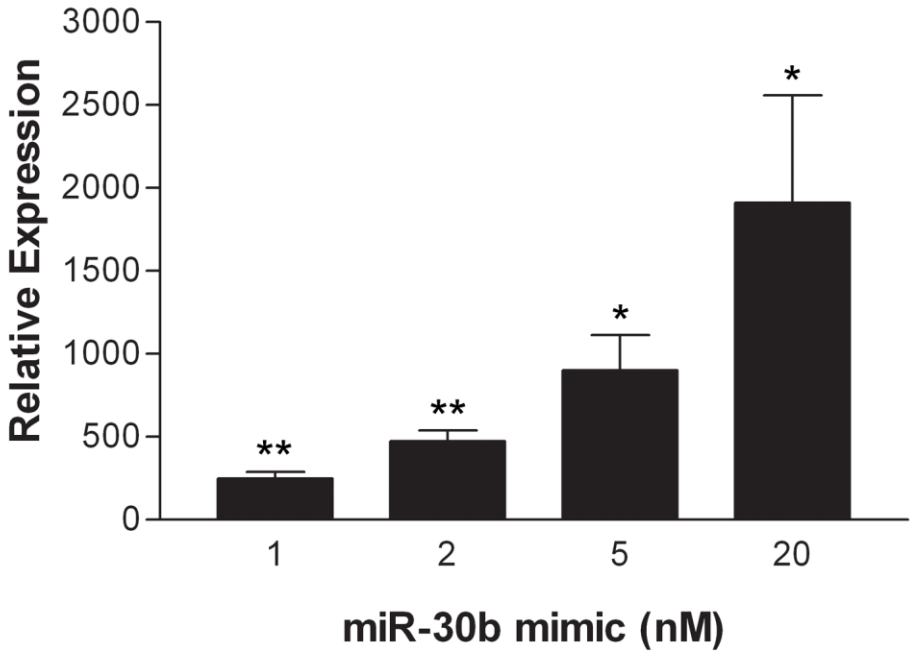

3000
2500
2000
1500
1000
500
0
Relative Expression
**
**
*
*
1
2
5
20
miR-30b mimic (nM)

Figure 14. MiR-30b mimic effectively increases the level of miR-30b in HUVECs. Cells were transfected with either control miRNA mimic or miR-30b mimic at the concentrations indicated and cell lysates were collected 48 hours post transfection. Total RNA was isolated and subjected to qRT-PCR to assess miR-30b expression with miR-103 used as endogenous control. Data presented for each concentration of miR-30b mimic is the mean ± SEM (n = 2) relative to the expression of miR-30b in control miRNA mimic transfected cells at the same concentration set to 1. A significant increase in the level of miR-30b is observed at all concentrations of miR-30b mimic tested, as compared to control miRNA mimic at the same concentration. Note: * denotes $P < 0.05$, ** denotes $P < 0.01$ as determined by unpaired Student's *t*-test.

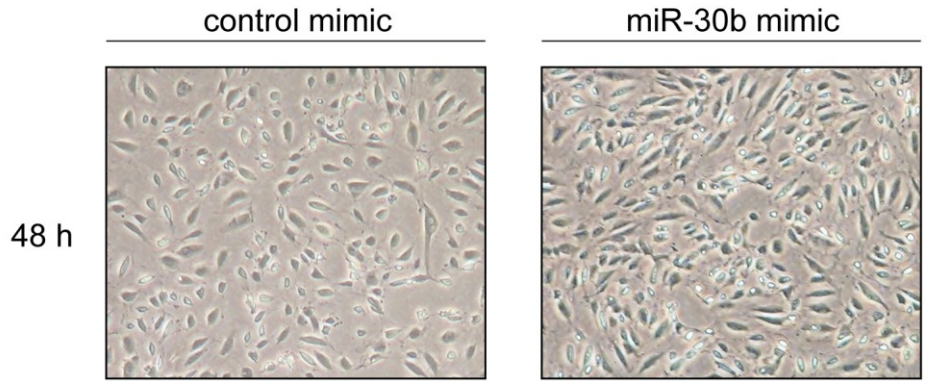

control mimic
miR-30b mimic
48 h

Figure 15. Morphology of HUVECs transfected with miR-30b mimic. Cells were transfected with either 20 nM control miRNA mimic or miR-30b mimic and phase contrast images were obtained at 48 hours post transfection with a Nikon Eclipse TE2000-U microscope.

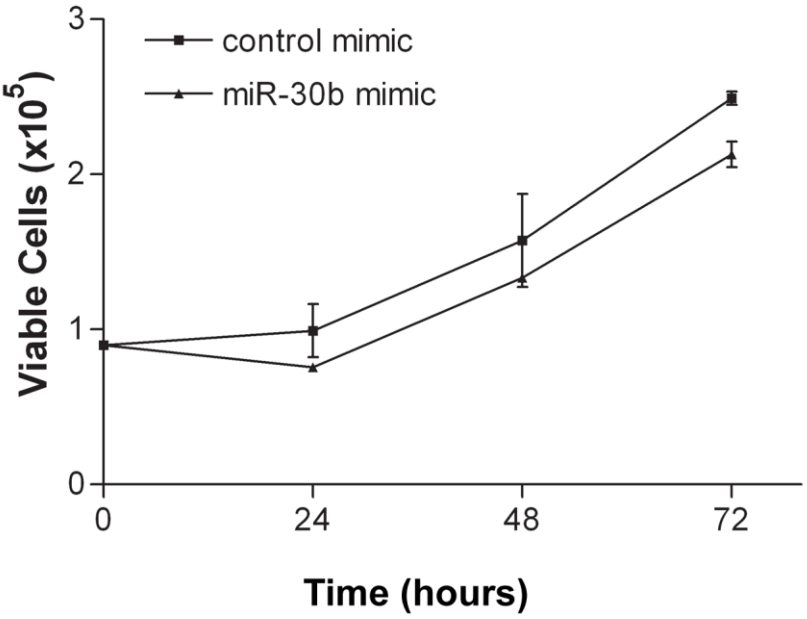

control mimic
miR-30b mimic
Viable Cells (x10^5)
3
2
1
0
0
24
48
72
Time (hours)

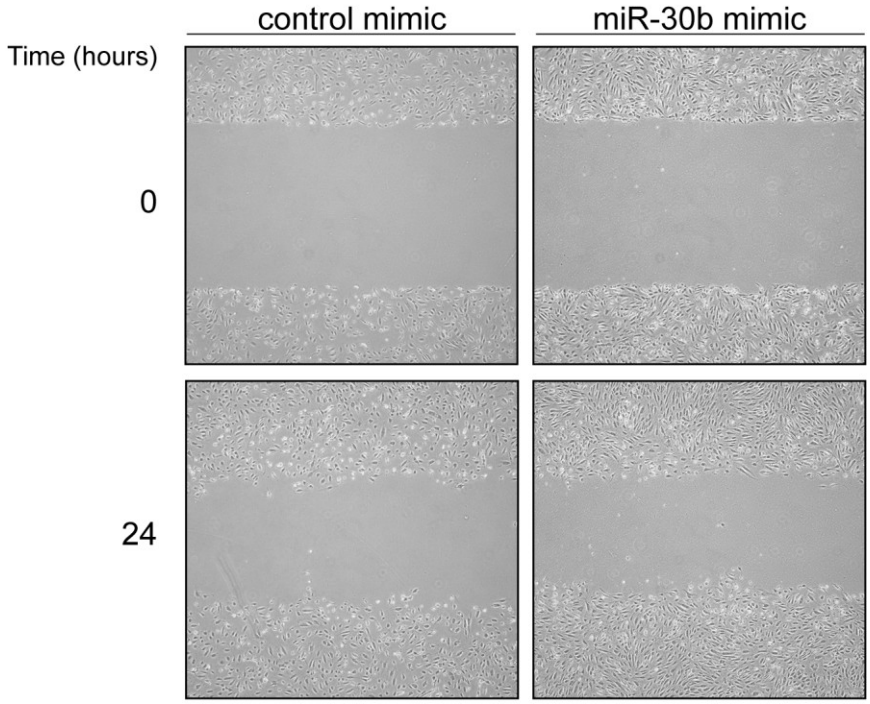
Figure 16. Overexpression of miR-30b does not affect HUVEC viability. HUVECs were transfected with 20 nM control miRNA mimic or miR-30b mimic and seeded into 6-well tissue culture plates 24 hours post transfection at 9 x 10^4 cells/well and grown in EGM-2 growth media. Cell viability was assessed at 24 hour intervals thereafter by trypan blue exclusion with a Vi-Cell XR cell viability analyzer. Number of viable cells is expressed as mean ± SEM from duplicate wells of two independently performed experiments. There are no statistically significant differences between the number of cells in control miRNA mimic transfected cells and cells overexpressing miR-30b at any of the time points tested as determined by unpaired Student's *t*-tests performed for each time point.

wounding and at 24 hours to assess the wound size (Fig. 17A). As seen in Figure 17, overexpression of miR-30b does not affect the migratory ability of HUVECs as the total distance migrated did not significantly differ between HUVECs overexpressing miR-30b and control miRNA mimic expressing cells (Fig. 17B).

We next evaluated the effect of miR-30b overexpression on endothelial cell morphogenesis. Transfected HUVECs were seeded onto growth factor reduced basement membrane extract (BME) where they underwent morphological changes to form cord-like structures. Twenty-four hours after seeding, images from duplicate wells per condition were obtained and total number of cords was quantified from two of four quadrants per well. Although the morphology of existing cord-like structures appeared unchanged between miR-30b overexpressing cells and control cells (Fig. 18A and C), overexpression of miR-30b (20 nM mimic) resulted in a 33% reduction in the number of cords ($P = 0.001$, n = 3) present after 24 hours (Fig. 18B), indicating negative regulation of cord formation by miR-30b. Importantly, we also observed a 28% reduction in the number of cord-like structures in cells transfected with a much lower amount of miR-30b mimic (1 nM) (Fig. 18D), thus indicating that a wide range of miR-30b overexpression results in similar defects on cord formation *in vitro*.

To further assess the role of miR-30b in processes important to angiogenesis we looked at the effects of inhibition of endogenous levels of miR-30b. HUVECs were either transfected with a hairpin inhibitor of miR-30b or with a control hairpin inhibitor and miR-30b expression was assessed at 48 hours post transfection. In the presence of miR-30b hairpin inhibitor the level of endogenous miR-30b was reduced for all tested concentrations of inhibitor, from approximately 20% with 0.1 nM inhibitor to approximately 70% with 50 nM inhibitor (5 nM, $P = 0.034$; 10 nM, $P = 0.004$; 20 nM, $P = 0.0007$; 50 nM, $P = 0.014$)

A.


control mimic
miR-30b mimic
Time (hours)
0
24

B.

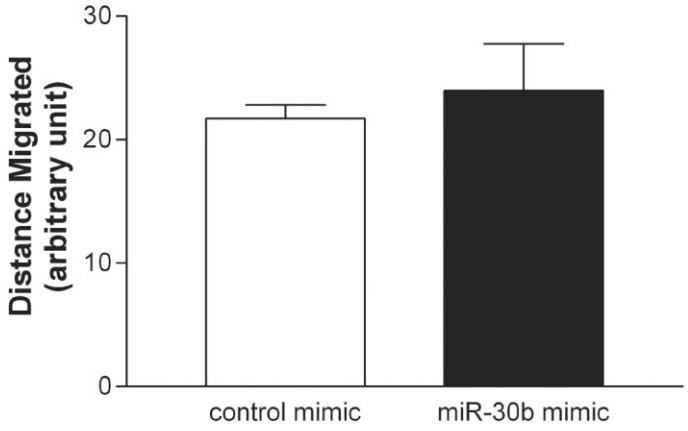

Distance Migrated
(arbitrary unit)
30
20
10
0
control mimic
miR-30b mimic

Figure 17. Migration of HUVECs is not affected by overexpressing miR-30b. HUVECs were transfected with 20 nM control miRNA mimic or miR-30b mimic and seeded into 6-well tissue culture plates 24 hours post transfection at 4.5 x 10^5 cells/well in EGM-2 growth media. At 48 hours post transfection a gap in the confluent monolayer was created and cells were allowed to migrate into the gap over the course of 24 hours. Images were collected (A) and measurements of wound size were taken at time of wounding (0 h) and at 24 hours (24 h) post wounding from duplicate wells. Data presented represents mean ± SEM of distance migrated (B) from two independently performed experiments. No significant differences in migration were observed between the two conditions tested as determined by unpaired Student's *t*-test.

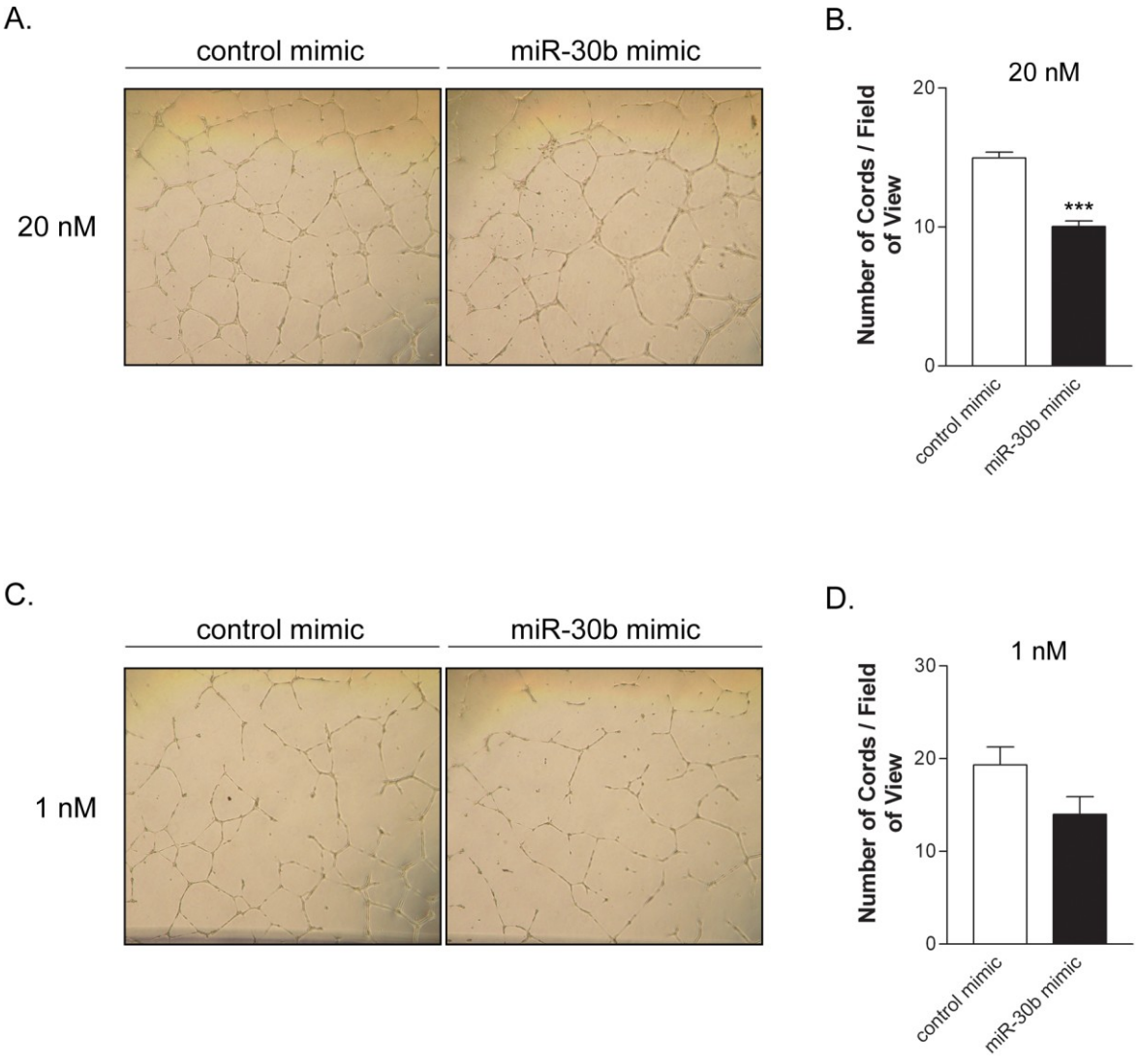
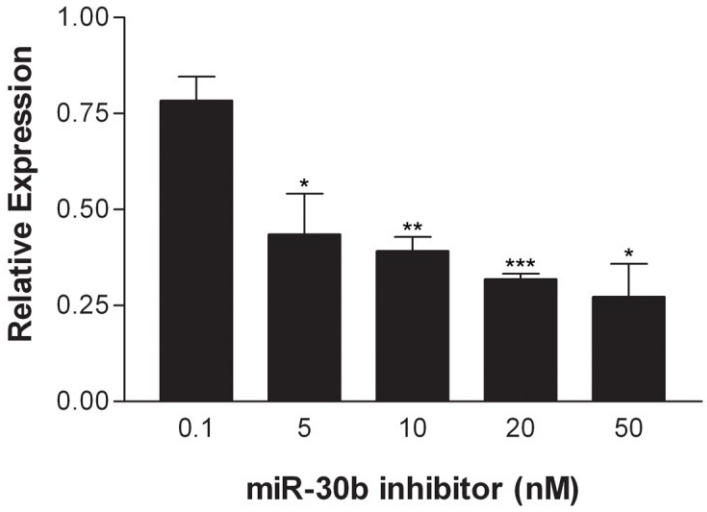

A.
control mimic
miR-30b mimic
20 nM
B.
20 nM
Number of Cords / Field of View
20
10
0
***
control mimic
miR-30b mimic
C.
control mimic
miR-30b mimic
1 nM
D.
1 nM
Number of Cords / Field of View
30
20
10
0
control mimic
miR-30b mimic

Figure 18. Overexpression of miR-30b inhibits *in vitro* cord formation. HUVECs were transfected with control miRNA mimic or miR-30b mimic at either 20 nM (A, B) or 1 nM (C, D) concentration and seeded onto growth factor reduced BME 48 hours post transfection in EGM-2 growth media. Images were collected from duplicate wells after 24 hours. Two of four quadrants from each of duplicate wells were assessed for total number of capillary-like cords. (A) and (C) are representative images of control miRNA and miR-30b mimic transfected cells. Data presented in (B) and (D) represents the mean ± SEM normalized from three independently performed experiments. Capillary-like cord formation is significantly inhibited in cells overexpressing miR-30b. Note: *** denotes $P = 0.001$ as determined by unpaired Student's *t*-test.

(Fig. 19A). In contrast to the morphological changes seen with overexpression of miR-30b, depletion of endogenous miR-30b (50 nM inhibitor) does not affect cell morphology (Fig. 19B). Additionally, reduction of miR-30b levels resulted in only a modest increase in cell viability (Fig. 20); a result that was determined to be not statistically significant. Likewise there were no statistically significant differences in the migratory ability of HUVECs depleted of endogenous miR-30b as compared to control cells (Fig. 21A and B), thus indicating that depletion of miR-30b does not significantly affect either cell viability or migration in HUVECs.

To evaluate the necessity of endogenous miR-30b for endothelial cell cord formation, transfected HUVECs were seeded onto growth factor reduced BME and total number of cord-like structures was quantified after 24 hours. Inhibition of miR-30b (50 nM inhibitor) enhanced cord formation (Fig. 22A), resulting in a 70% increase in cord number over cells with control hairpin inhibitor ($P = 0.0091$, n = 3) (Fig. 22B). Additionally, HUVECs transfected with a low concentration of inhibitor (0.1 nM) that results in only a 20% reduction in miR-30b (Fig. 19A) also exhibited a significant increase in cord number ($P = 0.0052$, n = 2) (Fig. 22C and D), indicating that a small decrease in endogenous miR-30b expression can result in significant changes in HUVEC cord formation. Taken together, the results of both overexpression and reduction of miR-30b indicate that miR-30b has a regulatory role in endothelial cell cord formation but does not significantly affect cell viability or migration.

A.


1.00
0.75
0.50
0.25
0.00
Relative Expression
*
**
***
*
0.1
5
10
20
50
miR-30b inhibitor (nM)

B.

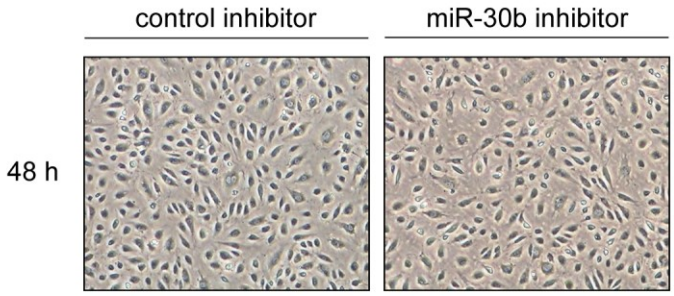

control inhibitor
miR-30b inhibitor
48 h

Figure 19. Endogenous levels of miR-30b can be effectively depleted with miR-30b inhibitor. HUVECs were transfected with either control miRNA inhibitor or miR-30b inhibitor at the concentrations indicated and cell lysates were collected 48 hours post transfection. Total RNA was isolated and subjected to qRT-PCR to assess miR-30b expression with miR-103 used as endogenous control. (A) Data presented for each concentration of miR-30b inhibitor is the mean ± SEM (n = 2) relative to the expression of miR-30b in control miRNA inhibitor transfected cells at the same concentration set to 1. Expression of miR-30b is observed to decrease at all concentrations of miR-30b inhibitor tested, as compared to control miRNA inhibitor at the same concentration. (B) The morphology of HUVECs transfected with 50 nM miR-30b inhibitor does not differ from that of control miRNA inhibitor transfected cells. Note: * denotes $P < 0.05$, ** denotes $P < 0.01$, *** denotes $P < 0.001$ as determined by unpaired Student's *t*-test.

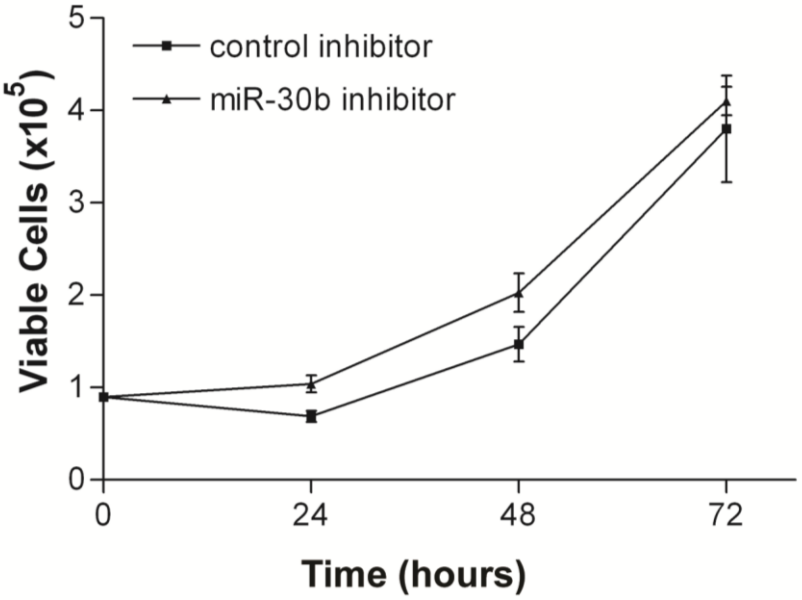
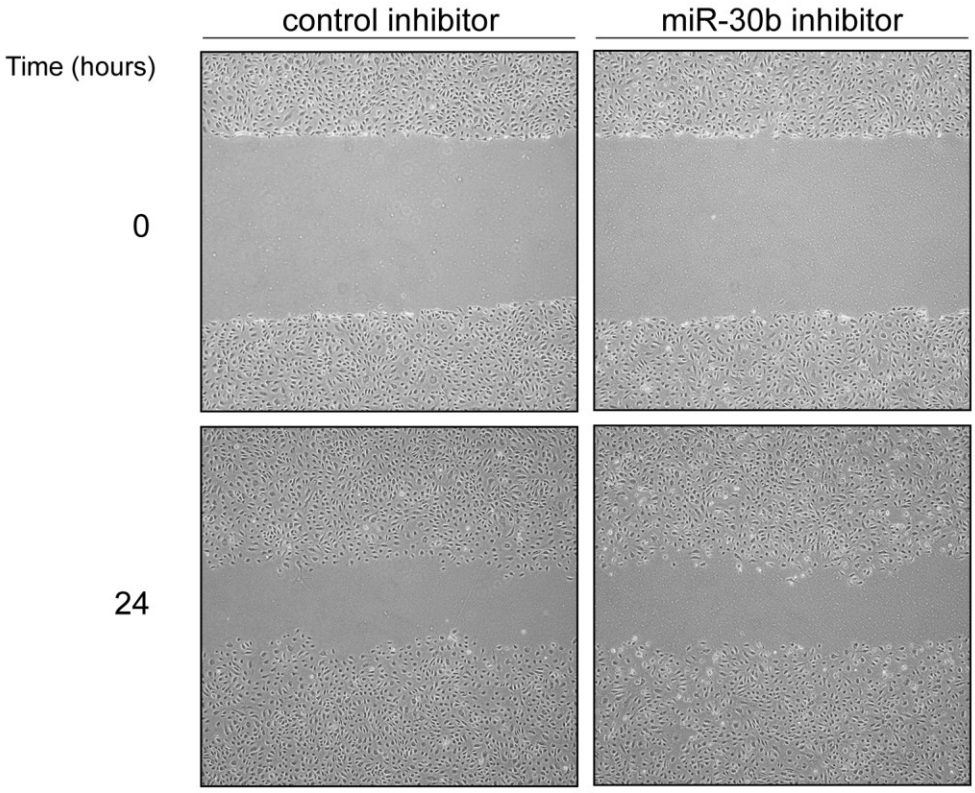

control inhibitor
miR-30b inhibitor
Viable Cells (x10^5)
5
4
3
2
1
0
0
24
48
72
Time (hours)

Figure 20. Reducing endogenous levels of miR-30b does not affect HUVEC viability. HUVECs were transfected with 50 nM control miRNA inhibitor or miR-30b inhibitor and seeded into 6-well tissue culture plates 24 hours post transfection at 9 x 10^4 cells/well and grown in EGM-2 growth media. Cell viability was assessed at 24 hour intervals thereafter by trypan blue exclusion with a Vi-Cell XR cell viability analyzer. Number of viable cells is expressed as mean ± SEM from duplicate wells of two independently performed experiments. There are no statistically significant differences between the number of cells in control miRNA inhibitor transfected cells and cells with reduced levels of miR-30b at any of the time points tested as determined by unpaired Student's *t*-tests performed for each time point.

A.


control inhibitor
miR-30b inhibitor
Time (hours)
0
24

B.

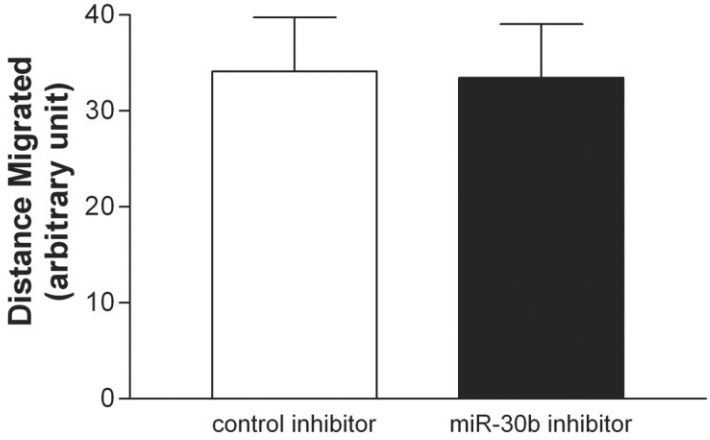

Distance Migrated
(arbitrary unit)
40
30
20
10
0
control inhibitor
miR-30b inhibitor

Figure 21. Depletion of miR-30b does not affect HUVEC migration. HUVECs were transfected with 50 nM control miRNA inhibitor or miR-30b inhibitor and seeded into 6-well tissue culture plates 24 hours post transfection at 4.5 x 10^5 cells/well in EGM-2 growth media. At 48 hours post transfection a gap in the confluent monolayer was created and cells were allowed to migrate into the gap over the course of 24 hours. Images were collected (A) and measurements of wound size were taken at time of wounding (0 h) and at 24 hours (24 h) post wounding from duplicate wells. Data presented represents mean ± SEM of distance migrated (B) from two independently performed experiments. No significant differences in migration were observed between the two conditions tested as determined by unpaired Student's *t*-test.

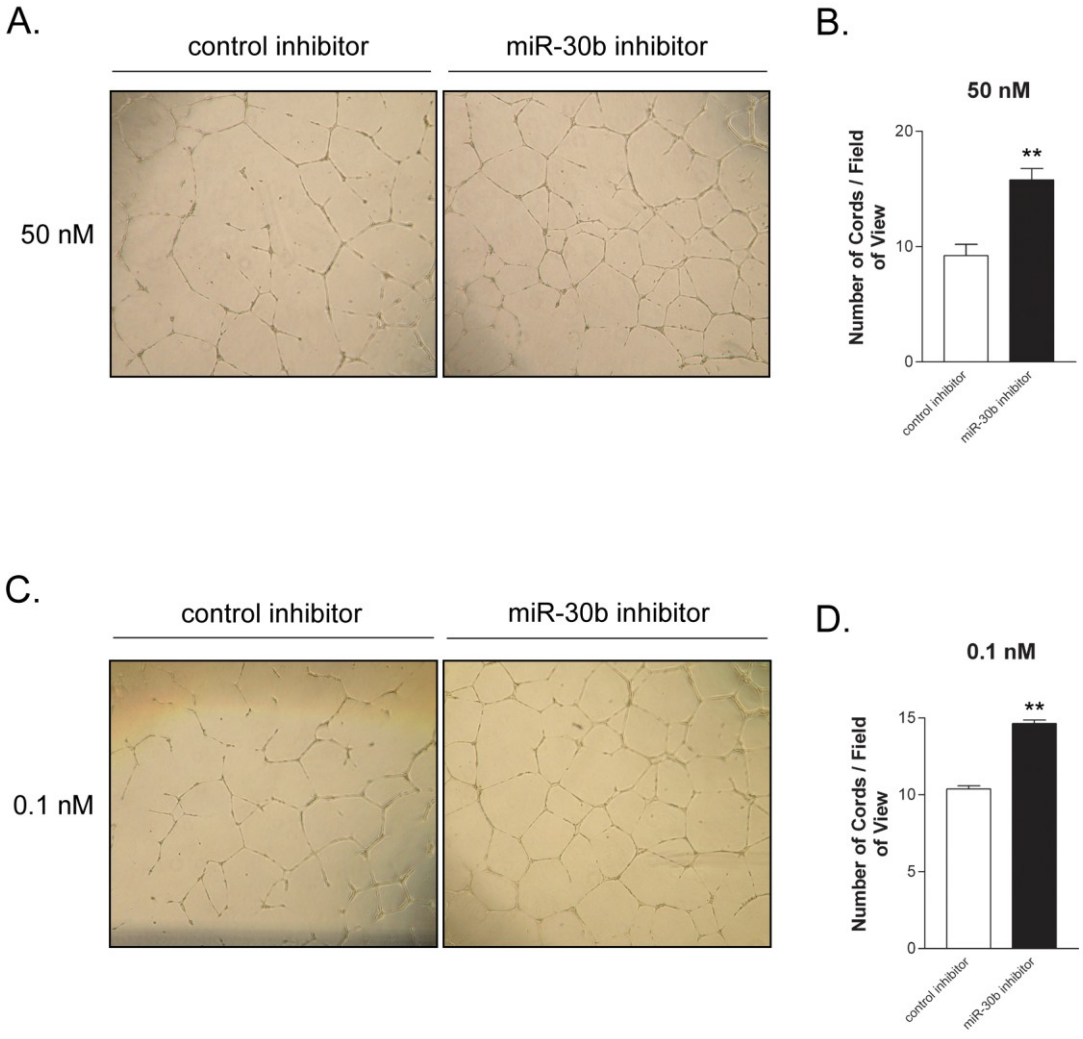

A.
control inhibitor
miR-30b inhibitor
50 nM
B.
50 nM
Number of Cords / Field of View
20
10
0
**
control inhibitor
miR-30b inhibitor
C.
control inhibitor
miR-30b inhibitor
0.1 nM
D.
0.1 nM
Number of Cords / Field of View
15
10
5
0
**
control inhibitor
miR-30b inhibitor

Figure 22. Depletion of miR-30b enhances HUVEC cord formation *in vitro*. HUVECs were transfected with either 50 nM control miRNA inhibitor or miR-30b inhibitor (A, B) or 0.1 nM control miRNA inhibitor or miR-30b inhibitor (C, D) and seeded onto growth factor reduced BME 48 hours post transfection in EGM-2 growth media. Images were collected from duplicate wells after 24 hours. Two of four quadrants from each of duplicate wells were assessed for total number of capillary-like cords. Representative images of control miRNA and miR-30b inhibitor transfected cells are presented in (A) and (C). Data presented in (B) and (D) represent the mean ± SEM normalized from three independently performed experiments performed with 50 nM inhibitor and from two independently performed experiments performed with 0.1 nM inhibitor. Capillary-like cord formation is significantly enhanced in cells with reduced levels of miR-30b. Note: ** denotes $P < 0.01$ as determined by unpaired Student's *t*-test.

3.2.3 Overexpression of miR-30b Increases TGFβ2 Expression

To assess potential targets of miR-30b overexpression, a microarray was performed on HUVECs transfected with 20 nM of either miR-30b mimic or control miRNA mimic. Cell lysates were collected at 48 hours post transfection and RNA was isolated. Microarray analysis (Appendix: Tables 3 and 4) identified TGFβ2, among other targets, as being positively regulated by miR-30b overexpression (Appendix: Table 3, fold change = 3.986). TGFβ2 was chosen for further study as a prospective mediator of the effects seen with miR-30b overexpression for several reasons. TGFβ has been shown to be inhibitory to angiogenesis in some contexts (Goumans et al., 2002; Maroni and Davis, 2011; Pepper et al., 1991), and thus increased expression of TGFβ may result in our observed defect in cord formation. In addition, the morphology of cells overexpressing miR-30b (Fig. 15) bore similarity to cells having been treated with TGFβ2 (Ghosh et al., 2012; Kumarswamy et al., 2012b) and having been identified as undergoing or having undergone the process of TGFβ-mediated endothelial-to-mesenchymal transition (EndMT), a process whereby cells obtain an elongated phenotype and exhibit changes in the expression of several markers indicative of either endothelial cells or mesenchymal cells (Potenta et al., 2008). Based on this information, we sought to confirm TGFβ2 as a putative mediator of miR-30b regulation of angiogenesis.

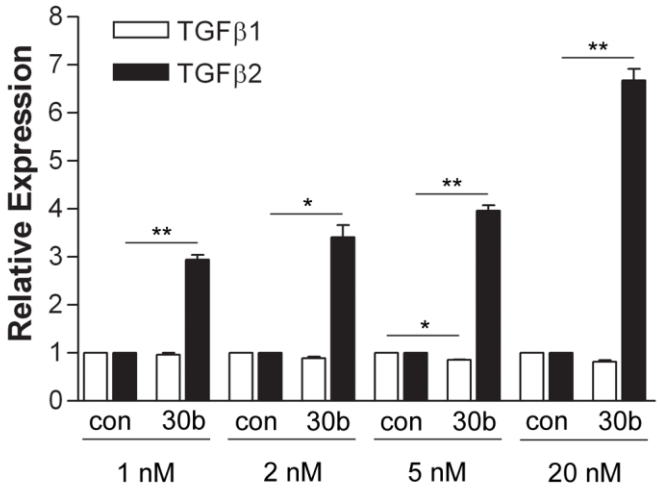
To validate the upregulation of TGFβ2 expression in endothelial cells overexpressing miR-30b, HUVECs were transfected with increasing concentrations of miR-30b mimic and levels of TGFβ2 and its closely related family member TGFβ1 were assessed by qRT-PCR. Cells overexpressing miR-30b showed a significant dose-dependent increase in expression of TGFβ2 (1 nM, $P = 0.0042$; 2 nM, $P = 0.011$; 5 nM, $P = 0.0018$; 20 nM, $P = 0.0019$) but not

TGFβ1, which was only significantly decreased at 5 nM miR-30b mimic concentration (P = 0.044) (Fig. 23A). Over the range of concentrations of miR-30b mimic tested (1-20 nM), expression of TGFβ2 mRNA was increased 3-7 fold (Fig. 23A). To confirm that increased TGFβ2 mRNA expression results in increased protein expression, TGFβ2 protein levels were assessed in HUVECs transfected with 20 nM miR-30b mimic or control miRNA mimic at 48 hours post transfection by western blot analysis. Figure 23B indicates that overexpression of miR-30b resulted in increased TGFβ2 protein expression. As TGFβs are secreted proteins we assessed the concentrations of both TGFβ1 and TGFβ2 in 24 hour cell culture supernates from either control miRNA mimic transfected cells or miR-30b mimic transfected cells. We observed a significant increase (P = 0.044) in the concentration of TGFβ2 in cell culture supernates from miR-30b overexpressing cells as compared to control cells but did not observe any differences in TGFβ1 concentration, indicating increased secretion of TGFβ2 from miR-30b overexpressing HUVECs.

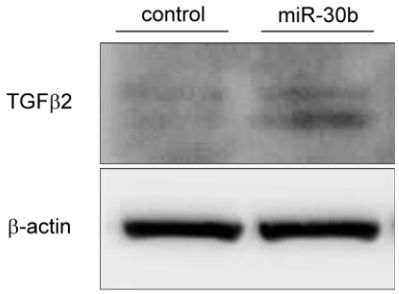
In order to determine if increased TGFβ2 production could function in an autocrine manner to stimulate HUVECs we looked at phosphorylation of Smad2, which occurs downstream of TGFβ2 activation of TGFβR1 (Yoshimatsu and Watabe, 2011), and would thus be an indicator of active TGFβ signaling. HUVECs were tranfected with 20 nM miR-30b mimic or control miRNA mimic and serum starved overnight in MCDB 131 with 0.5% FBS at 48 hours post transfection, followed by collection of protein lysates which were analyzed by western blotting. Cells overexpressing miR-30b displayed increased phosphorylation of Smad2 without affecting the total level of Smad2 (Fig. 24), implying increased signaling through TGFβ2/TGFβR1 in HUVECs overexpressing miR-30b.

To determine whether endogenous levels of miR-30b are important in the regulation of TGFβ2 expression, HUVECs were transfected with miR-30b hairpin inhibitor and levels

A.


Relative Expression
TGFβ1
TGFβ2
0
1
2
3
4
5
6
7
8
**
*
*
**
con
30b
con
30b
con
30b
con
30b
1 nM
2 nM
5 nM
20 nM

B.


control
miR-30b
TGFβ2
β-actin

C.

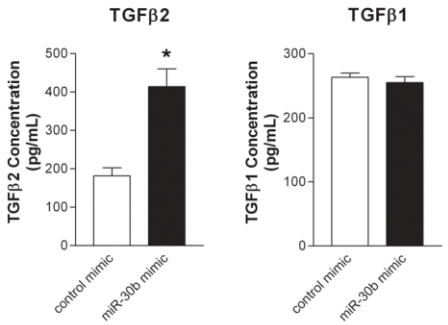

TGFβ2
TGFβ2 Concentration (pg/mL)
0
100
200
300
400
500
*
control mimic
miR-30b mimic
TGFβ1
TGFβ1 Concentration (pg/mL)
0
100
200
300
control mimic
miR-30b mimic

Figure 23. MiR-30b induces TGFβ2 expression. (A) HUVECs were transfected with the indicated concentrations of control miRNA mimic (con) or miR-30b mimic (30b) for 48 hours and levels of TGFβ1 and TGFβ2 mRNA were assessed by qRT-PCR. Expression levels of TGFβ1 and TGFβ2 in miR-30b overexpressing cells were determined relative to those of control mimic expressing cells at the same concentration. Expression levels relative to β-actin are presented as mean ± SEM (n = 2). Overexpression of miR-30b significantly induces TGFβ2 mRNA levels. Note: * denotes $P < 0.05$, ** denotes $P < 0.01$ as determined by unpaired Student's *t*-test. (B) HUVECs were transfected with 20 nM control miRNA mimic (control) or miR-30b mimic (miR-30b) and cell lysates were collected at 48 hours post transfection for the assessment of TGFβ2 protein levels by western blot. (C) ELISAs for TGFβ1 and TGFβ2 were performed on 24 hour cell culture supernates from control miRNA mimic or miR-30b mimic transfected HUVECs (20 nM mimic concentration). Data presented is the mean ± SEM from two independently performed experiments. Overexpression of miR-30b results in a significant increase in TGFβ2 in the cell culture supernate. Note: * denotes $P = 0.044$ as determined by unpaired Student's *t*-test.

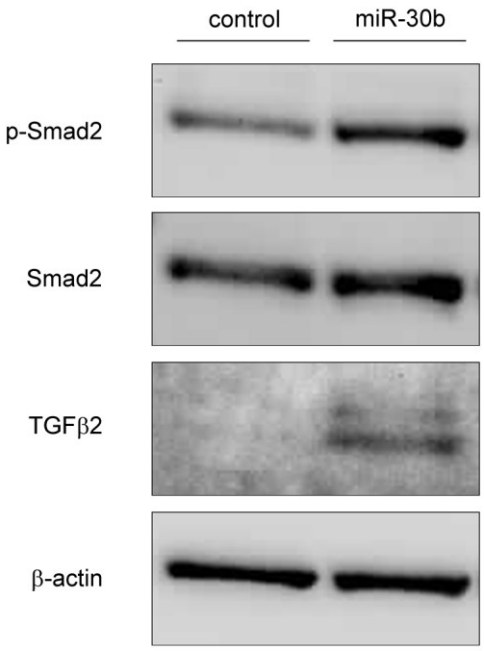

control
miR-30b
p-Smad2
Smad2
TGFβ2
β-actin

Figure 24. Overexpression of miR-30b induces phosphorylation of Smad2. HUVECs were transfected with 20 nM of either control miRNA mimic (control) or miR-30b mimic (miR-30b) and cell lysates were collected at 48 hours post transfection for analysis of Smad2 phosphorylation by western blot.

of TGFβ2 mRNA expression were assessed at 48 hours post transfection. Inhibition of miR-30b did not significantly alter the expression of TGFβ2 mRNA in HUVECs (Fig. 25). Since miR-30b is not completely removed in cells transfected with the concentration of miR-30b inhibitor used, it remains a possibility that small amounts of miR-30b can maintain TGFβ2 levels and further reduction of miR-30b may be necessary to observe a statistically significant change in TGFβ2 expression levels.

As we identified that overexpression of miR-30b increases TGFβ2 expression and microarray analysis by Ghosh et al. (2012) indicated miR-30b as being positively regulated by TGFβ2 in mouse cardiac endothelial cells (Ghosh et al., 2012), we sought to determine whether TGFβ2 induced miR-30b levels in HUVECs and thus would potentially create a positive feedback loop involving miR-30b and TGFβ2. Following overnight serum starvation, HUVECs were treated with either TGFβ1 or TGFβ2 for the times indicated and expression levels of miR-30b were assessed. We did not observe any changes in miR-30b expression after treatment with either TGFβ1 or TGFβ2 at any of the time points tested up to 48 hours (Fig. 26).

3.2.4 MiR-30b Induction of TGFβ2 is Dependent on ATF2

As overexpression of miR-30b resulted in an induction of TGFβ2, we hypothesized that miR-30b targets a repressor of TGFβ2 gene expression. Based on this model we looked at transcription factors known to regulate TGFβ2 expression and that were either predicted to be direct targets of miR-30b (as in the case of a transcriptional inhibitor of TGFβ2), or that were repressed by a predicted target of miR-30b (as in the case of a transcriptional activator of TGFβ2). Analysis of transcriptional regulators of TGFβ2 led us to activating transcription

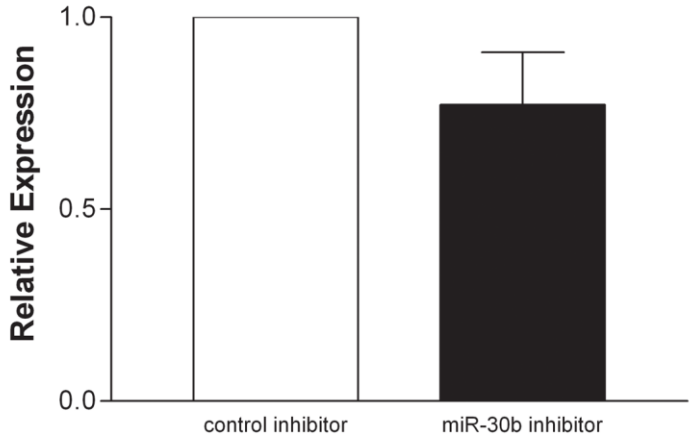

Relative Expression
1.0
0.5
0.0
control inhibitor
miR-30b inhibitor

Figure 25. Depletion of miR-30b does not significantly alter TGFβ2 expression in HUVECs. Cells were transfected with 50 nM of either control miRNA inhibitor or miR-30b hairpin inhibitor and TGFβ2 expression was assessed at 48 hours post transfection by qRT-PCR. Expression levels relative to β-actin are presented as mean ± SEM (n = 2). Depletion of miR-30b in HUVECs does not decrease TGFβ2 expression to a statistically significant level.

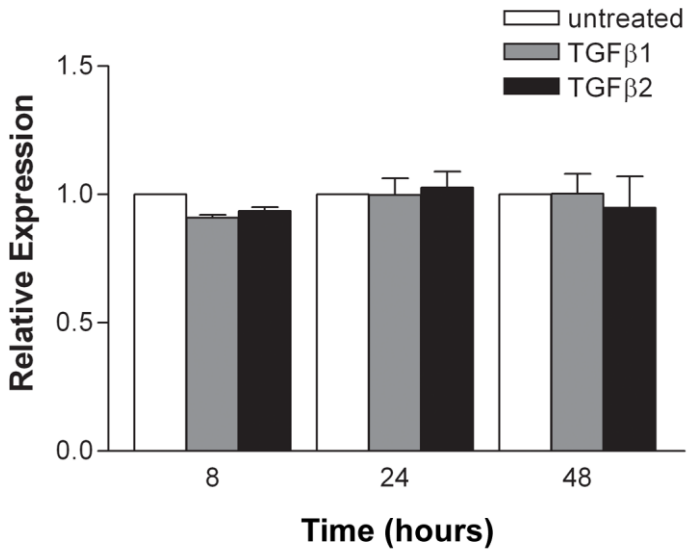

untreated
TGFβ1
TGFβ2
Relative Expression
1.5
1.0
0.5
0.0
8
24
48
Time (hours)

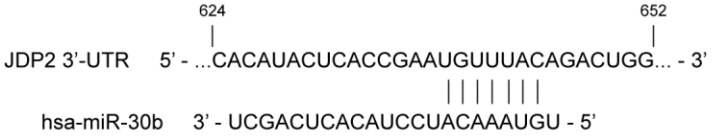
Figure 26. MiR-30b expression is unaffected by TGFβ2 stimulation in HUVECs. Cells were serum starved overnight in MCDB 131 with 0.5% FBS followed by stimulation with 5 ng/ml of either TGFβ1 or TGFβ2 for the times indicated. Data represents the mean ± SEM (n = 3) for expression of miR-30b relative to miR-30b levels in the untreated conditions at each time point. Endogenous control used was miR-103.

factor 2 (ATF2), which has been shown to positively regulate TGFβ2 expression (Kim et al., 1992), and is functionally repressed by Jun dimerization protein 2 (JDP2) (Jin et al., 2001), which is a predicted target of miR-30b (Fig. 27A). Thus, in theory, miR-30b may target JDP2 which could alleviate repression of ATF2 transcriptional activity and result in increased TGFβ2 transcription.

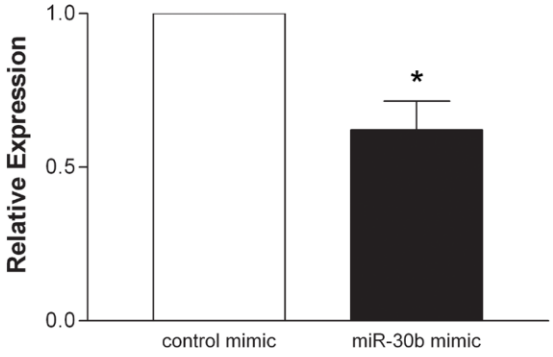
We first looked at the effect of miR-30b overexpression on JDP2 expression and found that JDP2 mRNA levels are significantly reduced in HUVECs overexpressing miR-30b ($P = 0.016$) (Fig. 27B). Additionally, we looked at the effects of reducing JDP2 levels on the expression of TGFβ2 as we would expect TGFβ2 levels may be increased in cells depleted of JDP2. Thus, HUVECs were transfected with 5 nM of either control siRNA or JDP2 siRNA and mRNA was isolated at 48 hours post transfection. Treatment of HUVECs with JDP2 siRNA reduced the levels of JDP2 by 50% (Fig. 27C), indicating that our siRNA approach was effective. In JDP2-depleted cells we did not observe a statistically significant increase in the expression of TGFβ2 mRNA suggesting that additional mechanisms aside from JDP2 expression changes may be required for maximal changes in TGFβ2 levels upon overexpressing miR-30b.

As we had observed an effect of miR-30b on JDP2, we assessed the possibility that ATF2, downstream of JDP2, was mediating the effects of increased miR-30b on TGFβ2 expression, and thus we looked at the expression of TGFβ2 in HUVECs depleted of ATF2. HUVECs were transfected with ATF2 siRNA and RNA was isolated at 48 hours post transfection. Transfection of HUVECs with ATF2 siRNA significantly reduced the mRNA expression of ATF2 by approximately 60% ($P = 0.0051$, n = 2) (Fig. 28A), indicating the effectiveness of the siRNA strategy. Cells with depleted levels of ATF2 exhibited a significant decrease of approximately 40% ($P = 0.0027$, n = 3) in the expression of TGFβ2

A.


624
652
JDP2 3'-UTR 5' - ...CACAUACUCACCGAAUGUUUACAGACUGG... - 3'
hsa-miR-30b 3' - UCGACUCACAUCCUACAAAUGU - 5'

B.


Relative Expression
1.0
0.5
0.0
*
control mimic
miR-30b mimic

C.

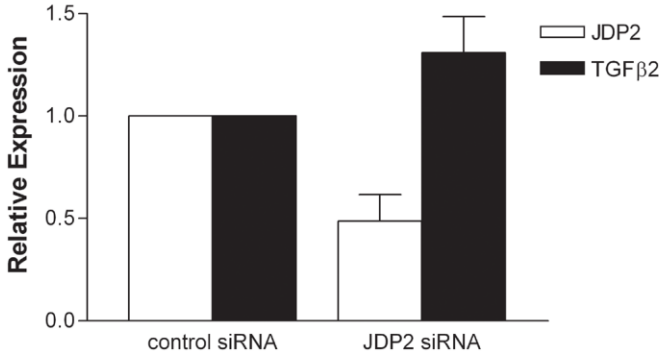
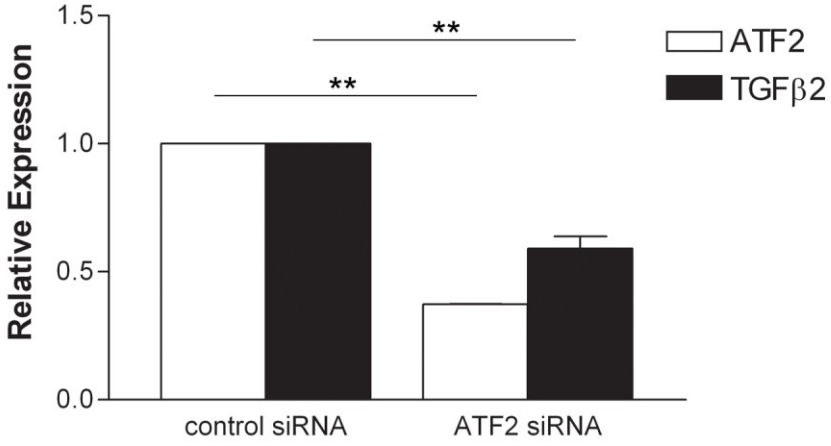
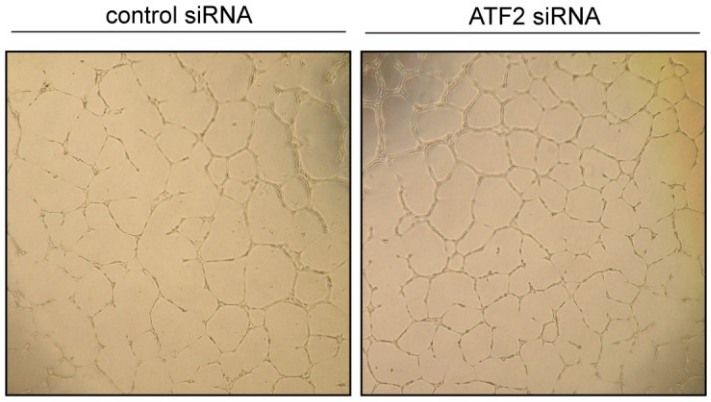

Relative Expression
1.5
1.0
0.5
0.0
JDP2
TGFβ2
control siRNA
JDP2 siRNA

Figure 27. JDP2 mRNA expression is decreased in miR-30b overexpressing HUVECs. (A) JDP2 contains a miR-30b binding site in its 3'-UTR. (B) HUVECs were transfected with 20 nM control miRNA mimic or miR-30b mimic and JDP2 mRNA expression relative to β-actin was assessed after 48 hours by qRT-PCR. Data presented are mean ± SEM (n = 3). (C) HUVECs were transfected with 5 nM JDP2 siRNA or control siRNA and TGFβ2 mRNA levels were assessed at 48 hours post transfection. Expression levels of JDP2 and TGFβ2 relative to β-actin are expressed as mean ± SEM (n = 2 for JDP2, n = 3 for TGFβ2). Note: * denotes $P = 0.016$ as determined by unpaired Student's *t*-test.

A.


1.5
1.0
0.5
0.0
Relative Expression
**
**
ATF2
TGFβ2
control siRNA
ATF2 siRNA

B.


control siRNA
ATF2 siRNA

C.

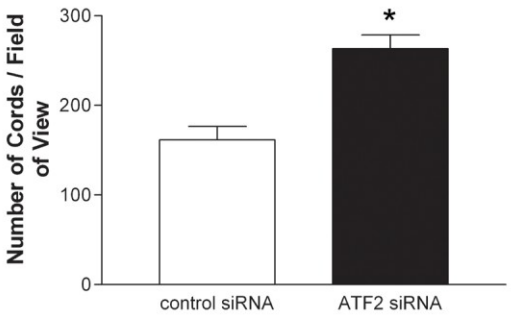
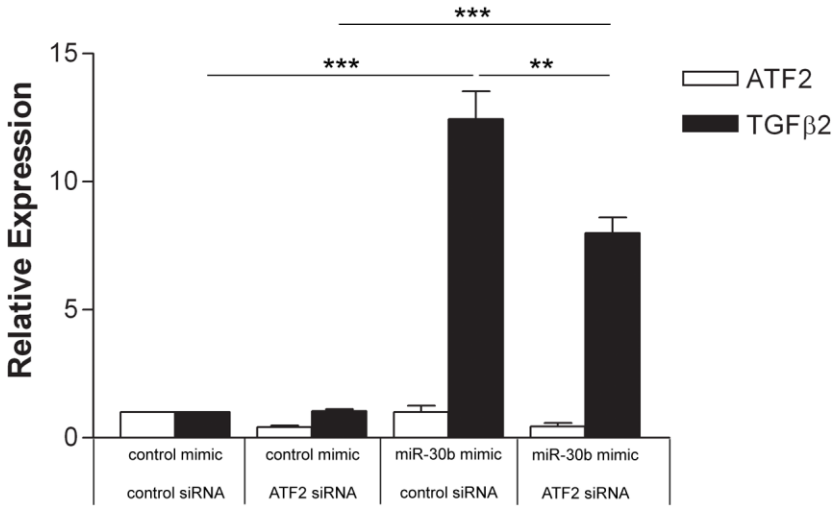

300
200
100
0
Number of Cords / Field of View
*
control siRNA
ATF2 siRNA

Figure 28. Depletion of ATF2 down-regulates TGFβ2 expression and enhances cord formation in HUVECs. (A) HUVECs were transfected with 5 nM of either control siRNA or ATF2 siRNA and RNA was isolated at 48 hours post transfection. Levels of ATF2 and TGFβ2 mRNA were assessed by qRT-PCR with β-actin as endogenous control. Data presented is mean ± SEM (n = 2 for ATF2, n = 3 for TGFβ2). Statistically significant decreases in ATF2 and TGFβ2 expression were seen in ATF2 siRNA treated cells as compared to control siRNA treated cells. Note: ** denotes $P < 0.01$ as determined by unpaired Student's *t*-tests. (B and C) HUVECs transfected with 5 nM of either control siRNA or ATF2 siRNA were seeded onto growth factor reduced BME at 48 hours post transfection and images of capillary-like structures were taken 24 hours later. Data presented in (C) is the normalized mean ± SEM for two independently performed experiments. A statistically significant increase in the number of capillary-like cords was observed for cells transfected with ATF2 siRNA. Note: * denotes $P = 0.041$ as determined by unpaired Student's *t*-test.

mRNA (Fig. 28A), indicating a requirement of ATF2 in TGFβ2 gene expression. To determine if ATF2 expression levels affect cord formation in HUVECs and thus whether ATF2 may be a contributing factor to regulation of this process by miR-30b, we plated ATF2-depleted HUVECs on growth factor reduced BME. Interestingly, HUVECs with reduced levels of ATF2 exhibited a significant increase in capillary-like cord formation when plated on growth factor reduced BME ($P = 0.041$) (Fig. 28B and C), suggesting modulation of ATF2 may contribute to the phenotypes mediated by miR-30b.

To determine if ATF2 is necessary for miR-30b upregulation of TGFβ2 mRNA in HUVECs, cells were co-transfected with miR-30b mimic and ATF2 siRNA and expression of TGFβ2 was assessed after 48 hours by qRT-PCR. Figure 29 indicates a significant induction ($P < 0.001$) of TGFβ2 mRNA in cells overexpressing miR-30b (Fig. 29A), as we have previously shown (Fig. 23A). MiR-30b overexpressing cells that were also treated with ATF2 siRNA, exhibited a 56% reduction in ATF2 levels, and showed a significantly reduced ($P < 0.01$) level of induction of TGFβ2 mRNA as compared to miR-30b overexpressing cells transfected with a control siRNA, indicating that miR-30b induction of TGFβ2 gene expression requires, at least in part, ATF2 (Fig. 29A). In addition to mRNA levels, we also looked at TGFβ2 protein expression in the presence of ATF2 siRNA. Interestingly, overexpression of miR-30b resulted in increased phosphorylation of ATF2 (Fig. 29B). ATF2 phosphorylation is required for its function as a transcription factor (Gozdecka and Breitwieser, 2012; Lau and Ronai, 2012), thus this data indicates a larger proportion of ATF2 in miR-30b overexpressing cells with capability to regulate gene expression. MiR-30b overexpressing HUVECs exhibited increased TGFβ2 protein expression and this induction was inhibited in ATF2-depleted cells (Fig. 29B), indicating a requirement for ATF2 for miR-30b induced TGFβ2 protein expression. As siRNA treatment did not completely inhibit

A.


***
***
**
15
10
5
0
Relative Expression
ATF2
TGFβ2
control mimic
control siRNA
control mimic
ATF2 siRNA
miR-30b mimic
control siRNA
miR-30b mimic
ATF2 siRNA

B.

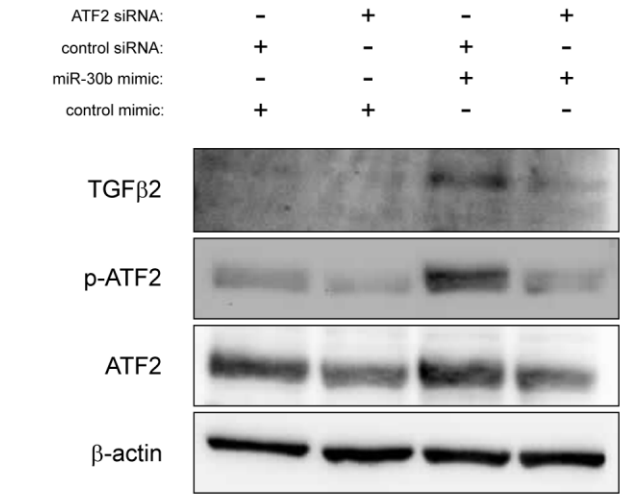

ATF2 siRNA: - + - +
control siRNA: + - + -
miR-30b mimic: - - + +
control mimic: + + - -
TGFβ2
p-ATF2
ATF2
β-actin

Figure 29. Induction of TGFβ2 expression by miR-30b requires ATF2. HUVECs were co-transfected with miRNA mimic (20 nM) and siRNA (5 nM) in the combinations displayed and cell lysates were collected at 48 hours post transfection and assessed for TGFβ2 mRNA expression (A). Data presented is mean ± SEM for two independently performed experiments. (B) HUVECs were co-transfected as in (A) and serum starved overnight in MCDB 131 with 0.5% FBS at 48 hours post transfection and cell lysates were collected and assessed for protein expression. Data presented is representative of protein expression levels seen in two independently performed experiments. Note: ** denotes $P < 0.01$, *** denotes $P < 0.001$ as determined by ANOVA with post hoc analysis.

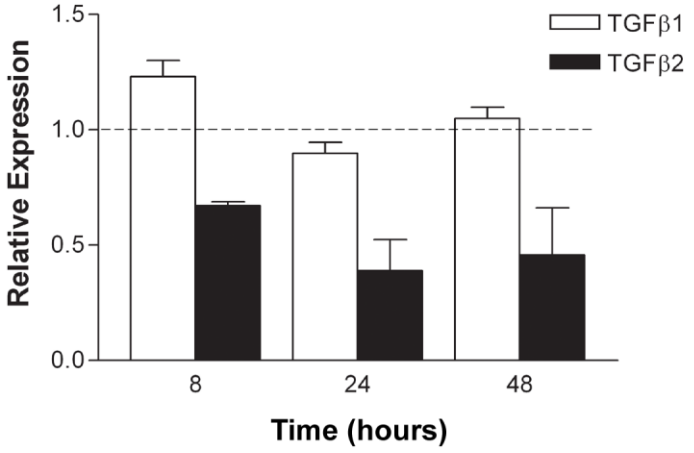
either ATF2 mRNA or protein expression (Fig. 29A and B), this could account for the incomplete inhibition of TGFβ2 induction seen in miR-30b overexpressing cells treated with ATF2 siRNA. Taken together, these results indicate that HUVECs overexpressing miR-30b exhibit increased levels of phosphorylated ATF2 and that ATF2 is required for maximal TGFβ2 expression mediated by miR-30b.

3.2.5 TGFβ2 Inhibits Endothelial Cell Cord Formation

As we have shown that miR-30b regulates the expression of TGFβ2 in endothelial cells, we aimed to determine the role that TGFβ2 plays in *in vitro* angiogenesis in HUVECs. Upon stimulation of HUVECs with VEGF, we observed a decrease in the expression of TGFβ2 but not TGFβ1, indicating a possible role for TGFβ2 suppression in response to pro-angiogenic signals (Fig. 30A). Treatment with the anti-VEGF monoclonal antibody Avastin inhibited the VEGF-mediated decrease in TGFβ2 expression ($P < 0.01$) (Fig. 30B), indicating that the VEGF ligand is responsible for inducing suppression of TGFβ2 in HUVECs.

To further understand the role that TGFβs play in angiogenesis we examined the effect of TGFβ stimulation on HUVEC cord formation *in vitro*. We first looked at the effect of TGFβs on morphogenesis by treating with TGFβ1 or TGFβ2 at the initiation of cord formation. To this end, HUVECs were seeded onto growth factor reduced BME in the presence of either TGFβ1 or TGFβ2 and total cord number was determined 24 hours later. As seen in Figure 31, neither TGFβ1 nor TGFβ2 significantly affected cord formation when administered at time of seeding (Fig. 31A), although treatment with TGFβ2 did reduce cord formation by approximately 10% (Fig. 31B), suggesting a minor effect of TGFβ2 in this

A.


1.5
1.0
0.5
0.0
Relative Expression
TGFβ1
TGFβ2
8
24
48
Time (hours)

B.

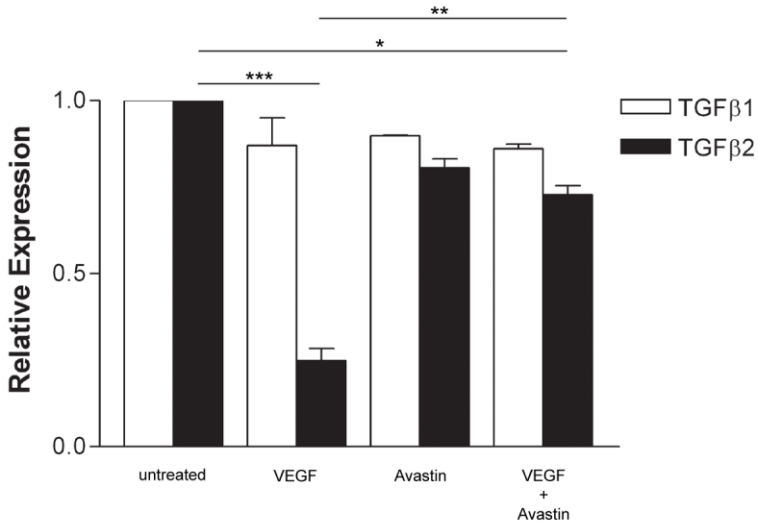
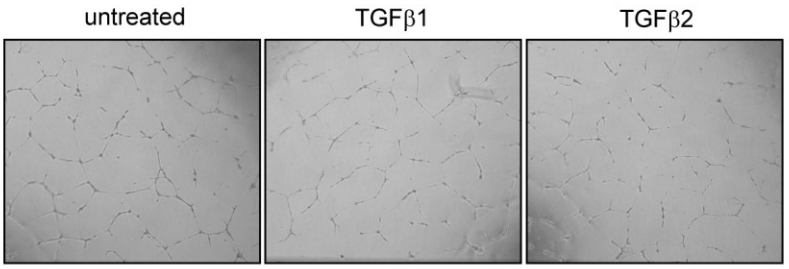
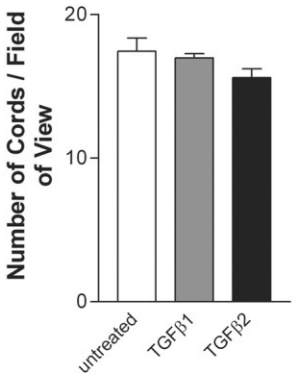

**
*
***
1.0
0.5
0.0
Relative Expression
TGFβ1
TGFβ2
untreated
VEGF
Avastin
VEGF
+
Avastin

Figure 30. VEGF reduces TGFβ2 expression in HUVECs. (A) HUVECs were serum starved overnight in MCDB 131 with 0.5% FBS and then stimulated with VEGF (50 ng/ml) for the times indicated. RNA was isolated and levels of TGFβ1 and TGFβ2 expression were assessed by qRT-PCR relative to β-actin endogenous control. Data presented represent mean ± SEM (n = 2) with each target mRNA expressed relative to the respective unstimulated condition at each time point set to an expression of 1. (B) HUVECs were serum starved overnight in MCDB 131 with 0.5% FBS and then treated with VEGF (50 ng/ml), Avastin (1 μg/ml), or a combination of both VEGF and Avastin for 24 hours. Expression levels of TGFβ1 and TGFβ2 were assessed by qRT-PCR relative to β-actin endogenous control. Data presented represents mean ± SEM for two independently performed experiments. Note: * denotes $P < 0.05$, ** denotes $P < 0.01$, *** denotes $P < 0.001$ as determined by ANOVA with post hoc analysis.

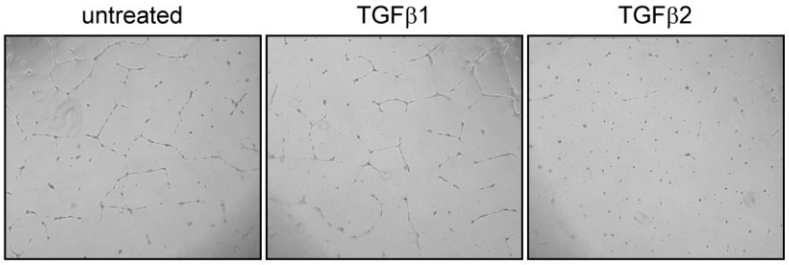
A.


untreated
TGFβ1
TGFβ2

B.


Number of Cords / Field of View
20
10
0
untreated
TGFβ1
TGFβ2

C.


untreated
TGFβ1
TGFβ2

D.

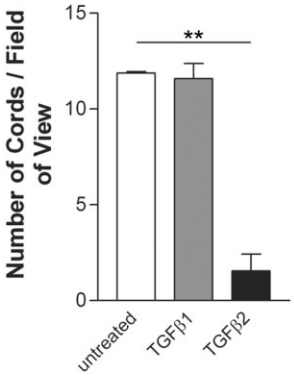

Number of Cords / Field of View
15
10
5
0
**
untreated
TGFβ1
TGFβ2

Figure 31. Prolonged TGFβ2 stimulation inhibits HUVEC cord formation.
(A, B) HUVECs were seeded onto growth factor reduced BME in EGM-2 growth media supplemented with 5 ng/ml of either TGFβ1 or TGFβ2 and cord formation was assessed after 24 hours. Representative images are displayed in (A) while data presented in (B) represents the mean ± SEM from two of four quadrants from each of duplicate wells and two independently performed experiments. (C, D) HUVECs were treated with 5 ng/ml of either TGFβ1 or TGFβ2 for three days in EGM-2 growth media prior to seeding onto growth factor reduced BME for 24 hours. Representative images are displayed in (C) and data presented in (D) is the mean ± SEM for two of four quadrants from each of duplicate wells and from two independently performed experiments. A significant decrease in cord number is observed in the TGFβ2 treated condition. Note: ** denotes $P = 0.0072$ as determined by unpaired Student's *t*-test.

context. As we hypothesized that any potential effects of TGFβ on this process may be related to cellular changes that accumulate over prolonged exposure to TGFβ and thus perhaps significant effects would not be realized after such a short exposure, we treated HUVECs for 3 days with either TGFβ1 or TGFβ2 prior to seeding cells onto growth factor reduced BME (Fig. 31C). Cells treated with TGFβ1 for 3 days showed no change in number of cord-like structures as compared to untreated cells, while cells pretreated with TGFβ2 showed a significant reduction in cord number of approximately 87% ($P = 0.0072$) (Fig. 31D), indicating that TGFβ2 has inhibitory effects on cord formation in HUVECs while TGFβ1 does not.

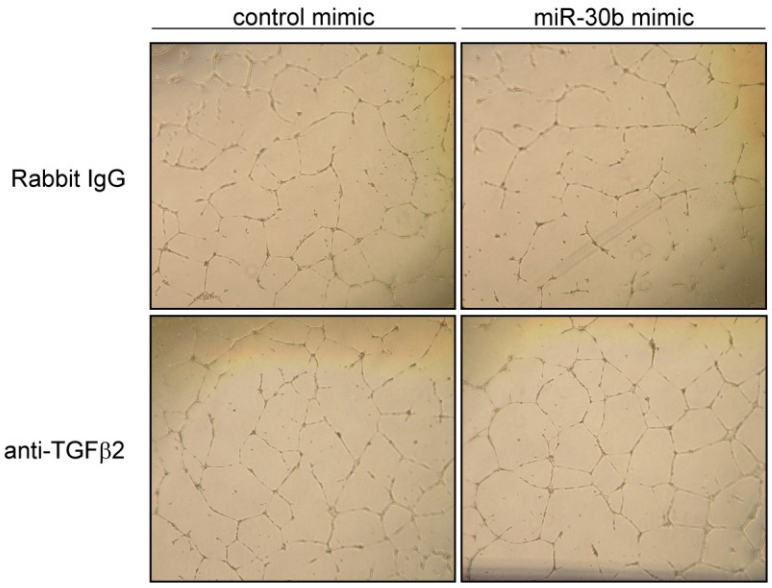
Given the observed regulation of TGFβ2, but not TGFβ1, by VEGF and the inhibition of cord formation in TGFβ2 treated cells, but not in TGFβ1 treated cells, these results identify key differences in the function and regulation of these two closely related TGFβ family members in endothelial cell angiogenesis and suggest that miR-30b regulation of TGFβ2 and not TGFβ1 is potentially important to the regulation of HUVEC capillary morphogenesis.

3.2.6 Neutralization of TGFβ2 Restores Cord Formation in miR-30b Overexpressing HUVECs

To determine if increased autocrine TGFβ2 signaling facilitated the phenotype of reduced capillary-like cord formation in miR-30b overexpressing cells, we treated cells with a neutralilzing antibody to TGFβ2. HUVECs were transfected with either control miRNA mimic or miR-30b mimic and four hours post transfection cells were incubated in media containing either normal rabbit IgG or anti-TGFβ2 neutralizing antibody. Cells were treated immediately after transfection so as to inhibit any cumulative or time-dependent effects that

TGFβ2 may have on HUVECs. Media was changed 24 hours later with fresh antibody being added once again. At 48 hours post transfection cells were seeded onto growth factor reduced BME in media containing either rabbit IgG or anti-TGFβ2 antibody and cells were incubated for 24 hours. Images were collected and the number of cord-like structures was assessed from two of four quadrants from each of duplicate wells. As we have previously seen, cells overexpressing miR-30b exhibited decreased capillary-like cord formation (Fig. 32A) as the number of cords was approximately 33% lower than that of control cells ($P < 0.05$) (Fig. 32B). Treatment of control cells with anti-TGFβ2 antibody had a very slight positive effect on cord formation, while the effect of neutralizing TGFβ2 antibody had a much more pronounced effect on cells overexpressing miR-30b when compared to treatment with rabbit IgG. In miR-30b overexpressing cells treated with anti-TGFβ2 antibody, capillary-like cord formation was almost completely restored to levels seen in control cells with cord number significantly increased compared to miR-30b overexpressing cells treated with rabbit IgG ($P < 0.05$) (Fig. 32B). Thus, decreased cord formation in miR-30b overexpressing HUVECs is a result of increased TGFβ2 expression.

A.


control mimic
miR-30b mimic
Rabbit IgG
anti-TGFβ2

B.

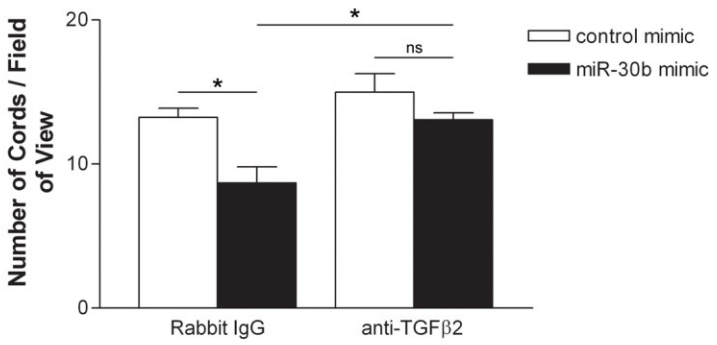

Number of Cords / Field of View
20
10
0
*
*
ns
control mimic
miR-30b mimic
Rabbit IgG
anti-TGFβ2

Figure 32. Inhibition of TGFβ2 ligand restores cord formation in miR-30b overexpressing cells. HUVECs were transfected with 1 nM of either control miRNA mimic or miR-30b mimic and 4 hours post transfection were treated with 0.8 μg/ml of either Rabbit IgG or anti-TGFβ2 neutralizing antibody. Media was refreshed after 24 hours, again with 0.8 μg/ml rabbit IgG or anti-TGFβ2 antibody and cells were seeded onto growth factor reduced BME 24 hours later (ie. 48 hours post transfection) in EGM-2 growth media with Rabbit IgG or anti-TGFβ2 antibody. Cord number was assessed after 24 hours on BME from two of four quadrants from each of duplicate wells per condition. Representative images are displayed in (A). Data presented in (B) is the mean ± SEM normalized from three independently performed experiments. Note: * denotes $P < 0.05$, ns denotes not significant as determined by ANOVA with post hoc analysis.

4. DISCUSSION

Angiogenesis is an important process in vascular development and in everyday events such as wound healing, but also contributes to pathological conditions such as cancer and ocular diseases such as age-related macular degeneration and diabetic retinopathy. Many endothelial cell functions have been identified as being important contributors to the process of angiogenesis, including matrix degradation, proliferation, migration, sprouting, and morphogenesis. Endothelial cell capillary morphogenesis is a vital component to angiogenesis involving adhesion, migration, elongation, and interactions among cells necessary for the formation of lumen-containing vessels. The assessment of endothelial cell morphogenesis can be achieved through plating cells on BME where they form networks of branching cord-like structures (Kubota et al., 1988). These structures form rapidly (within 6-20 hours), with an initial stage consisting of adhesion to the matrix, cell migration and elongation followed by the alignment of cells and formation of intercellular spaces similar to lumens (Montanez et al., 2002). The capillary-like structures contain cell-cell contacts, are polarized with nuclei localizing towards the BME surface, and have been shown to uptake acetylated low-density lipoprotein (LDL), an indicator of endothelial cell differentiation, with uptake of LDL failing to occur in cells plated on plastic or collagen I-coated tissue culture plates (Arnaoutova et al., 2009). The effects of either angiogenic or anti-angiogenic molecules or chemotherapeutic drugs on capillary-like cord formation assessed through *in vitro* assays such as the morphogenesis assay on BME has, in many cases, correlated nicely with *in vivo* assays of angiogenesis, indicating the validity of utilizing these assays for initial determination of angiogenic effects (Presta et al., 1999; Ribatti et al., 2007; Vacca et al., 1999). The *in vitro* assessment of capillary-like cord formation is thus very useful for

determination of potential for angiogenic or anti-angiogenic effects *in vivo*. Thus, we have used this *in vitro* system to investigate the process of capillary morphogenesis in HUVECs and have identified two signaling pathways involved in the formation of cord-like structures *in vitro*. In one case, we have shown that regulation of RhoA activity by RhoB is necessary for proper HUVEC morphogenesis, while we have also discovered that miR-30b functions to inhibit morphogenesis through regulation of TGFβ2 expression. In addition, we have identified the mediators of these two pathways, namely RhoB and miR-30b, as each being regulated to an extent by the major pro-angiogenic growth factor VEGF, adding support to our observations indicating an important role for these two molecules in angiogenesis.

The importance to angiogenesis for a number of Rho proteins has been identified (reviewed in Bryan and D'Amore, 2007), although data on the specific functions of RhoB in angiogenesis has only begun to come to light within the last twelve or so years. Evidence from RhoB knockout mice indicated that RhoB was not required for developmental angiogenesis, although vascular sprouting defects were observed in the retinas of adult mice, indicating a role for RhoB in sprouting angiogenesis in adult mice (Adini et al., 2003; Liu et al., 2001). RhoB also has a described function in growth factor signaling and trafficking (Gampel et al., 1999; Huang et al., 2007; Huang et al., 2011; Lajoie-Mazenc et al., 2008) which along with its function in sprouting suggested the possibility that RhoB may function in VEGF/VEGF receptor-mediated angiogenesis; a role that had not yet been studied for RhoB. VEGF is the primary growth factor mediating signaling events in endothelial cells leading to angiogenesis and as RhoB had been shown to be regulated by growth factors such as TGFβ and EGF in several human cell lines (de Cremoux et al., 1994; Vardouli et al.,

2008; Vasilaki et al., 2010), we aimed to identify the relationship between RhoB expression and VEGF stimulation in HUVECs.

Our results indicated that VEGF increases expression of RhoB protein by 8 hours post VEGF treatment with maximal expression seen after 12 hours, without increasing the level of activated RhoB in HUVECs. The lack of enhanced cellular RhoB activity in VEGF-treated HUVECs is not entirely surprising as activation of Rho proteins is a coordinated event that occurs independently from protein expression. Although the exact mechanism of upregulation of RhoB by VEGF is at this point unknown, it suggests an important role for RhoB in the cellular response to VEGF stimulation. Indeed, the stimulation of human keratinocytes with TGFβ results in increased RhoB expression, while depletion of RhoB levels inhibits TGFβ-induced migration (Vasilaki et al., 2010), suggesting an important role for RhoB expression in processes mediated by TGFβ in that cell type.

Cell viability, migration, sprouting, and morphogenesis are all processes vitally important to angiogenesis. Our results indicate an important role for RhoB in angiogenesis based on findings that identify RhoB as having a regulatory role in HUVEC migration, sprouting and cord formation. HUVECs depleted of RhoB exhibited reduced migration in response to VEGF, thus indicating the importance of RhoB to VEGF signaling during this process. Additionally, RhoB was shown to be necessary for HUVEC sprouting in response to VEGF and to cord formation *in vitro*, as reduced levels of RhoB resulted in the inhibition of both of these processes. Confirming the importance of RhoB to angiogenesis, these findings are supported by the work of others in mouse and in *in vitro* models of angiogenesis (Adini et al., 2003; Sabatel et al., 2011), although we have additionally specified RhoB as a mediator of VEGF-induced migration specifically, whereas a study by Sabatel et al. utilized

a combination of bFGF and VEGF to induce migration and thus RhoB was not identified as mediating VEGF-induced migration in that system (Sabatel et al., 2011).

Furthering the understanding of how RhoB affects VEGF-induced processes of angiogenesis, we identified increased RhoA activity as mediating effects of RhoB depletion in HUVECs. Increased RhoA activation has been shown in human endothelial cells depleted of CCM2, a gene involved in cerebral cavernous malformations, a common vascular dysplasia (Whitehead et al., 2009). Vascular defects in CCM2-depleted human microvascular endothelial cells could be rescued by inhibition of Rho activity with C3 transferase or through inhibition of ROCK I/II with Y-27632 (Whitehead et al., 2009). In mouse embryonic endothelial cells, depletion of CCM1, -2, or -3 resulted in increased RhoA activity and reduced cord formation, which could be restored through inhibition of ROCK through either shRNA or the pharmacological inhibitor Y-27632 (Borikova et al., 2010), indicating the importance for regulated RhoA activity in vascular processes, and supporting our findings. Our results indicate increased activation of RhoA in response to VEGF in HUVECs with depleted levels of RhoB, indicating potential significance for increased RhoB expression upon VEGF stimulation, which we have observed. Increased RhoA activity was found to be a contributing factor in decreased capillary-like cord formation seen in RhoB-depleted cells, as cord formation could be partially restored upon treatment with the Rho inhibitor C3 transferase. Additionally, we have linked the effects of increased RhoA activity to signaling through ROCK, as pharmacological inhibition of ROCK partially restored cord formation in RhoB-depleted HUVECs.

Given our observation that RhoB regulates RhoA activity, we were interested in looking at the potential for RhoB regulation of other Rho proteins and thus we looked at RhoC, the third member, along with RhoA and RhoB, of a Rho subclass displaying extensive

homology (Wheeler and Ridley, 2004). The potential importance of RhoC to angiogenesis has been shown through its role in the migration and cord forming abilities of human dermal microvascular endothelial cells, as depletion of RhoC with siRNA inhibited these processes (Wang et al., 2008b). In addition, reciprocal regulation of RhoA and RhoC activity has been previously observed during EMT in colon carcinoma cells and deemed functionally relevant as the effect of inhibition of each Rho protein on post-EMT cell migration correlated with the level of activation seen for each during EMT (Bellovin et al., 2006). Despite this knowledge, we did not observe a statistically significant difference in the level of activated RhoC in RhoB-depleted HUVECs, and thus modulation of RhoC activity does not likely contribute to our observed phenotype of reduced cord formation in cells depleted of RhoB.

An interesting aspect of Rho proteins is their propensity for regulation via crosstalk between Rho family members that affects the activation, expression, stability, or downstream signaling from the interacting GTPases (Guilluy et al., 2011). Indeed, reciprocal regulation of Rho proteins has been seen to regulate cell morphology and migration and has identified the shift in expression and activation of certain Rho proteins as being necessary for certain cellular events (Bellovin et al., 2006; Sander et al., 1999). Concerning Rho proteins, the interaction between RhoA and Rac1 has been extensively documented. Comprehensive evidence suggests regulation of RhoA activity by Rac1 is routinely mediated through p21-activated kinase (PAK) phophorylation of various GAPs and GEFs (Alberts et al., 2005; Barac et al., 2004; Rosenfeldt et al., 2006). Activated Rac1-mediated reactive oxygen species production results in activation of p190RhoGAP and decreased RhoA activity; a process that is necessary for Rac1-induced membrane ruffles (Nimnual et al., 2003). The control of RhoA activity by Rac1 has also been shown to require the phosphorylation of p190RhoGAP and the subsequent interaction of p190RhoGAP with p120-catenin, as the

absence of p120-catenin prevented the suppression of RhoA activity by Rac1 (Wildenberg et al., 2006). Conversely, RhoA can regulate Rac1 activity and function. The RhoA effector ROCK negatively regulates Rac1 activity, whereas the effector mDia1 appears to positively regulate Rac1, indicating the importance of effector binding to RhoA as a determinant of Rac1 activity (Tsuji et al., 2002). The RhoA/ROCK mediated inhibition of Rac1 activity has also been shown to play a role in determining cell movement phenotypes by switching cells to a RhoA/ROCK-dependent movement type (Sanz-Moreno et al., 2008). Crosstalk between Rho proteins is not limited to RhoA and Rac1, as evidence of extensive regulation between other Rho family members is increasingly being identified as important to various cellular processes (Cau and Hall, 2005; Ho et al., 2008; Howe and Addison, 2012; Huang et al., 2011). Specifically, regulation of RhoB by RhoA has been observed, as stabilization of RhoB protein in the absence of RhoA was attributed to increased binding of RhoB to the Rho guanine nucleotide dissociation inhibitor, RhoGDIalpha (Ho et al., 2008). Additionally, RhoB has been shown to affect the intracellular activation and localization of Cdc42 in response to PDGF in vascular smooth muscle cells (Huang et al., 2011). Thus, RhoB has been documented as being part of Rho protein regulatory networks; however, direct evidence for the regulation of RhoA by RhoB had not been demonstrated prior to our work. RhoB had however, been suggested to regulate RhoA activity, possibly as a result of differential prenylation of RhoB, based on a potential outcome being a change in regulator or effector binding amongst RhoA, RhoB, and RhoC (Zeng et al., 2003). Although currently unknown, the mechanism for the observed regulation of RhoA activity by RhoB could be a result of a similar mechanism to that observed by Ho and colleagues, namely, competition for RhoGDI binding, as a number of studies have highlighted RhoGDI binding to Rho proteins as being sensitive to the modulation of the Rho proteins themselves (Boulter et al., 2010; Giang Ho et

al., 2011). Our result indicating RhoB regulation of RhoA activity as important in the control of cord formation has further explained the interplay between RhoA and RhoB as being one of mutual co-regulation.

Thus, these results identify VEGF regulation of the small GTPase RhoB and highlight a role for RhoB in endothelial cell migration, sprouting, and cord formation *in vitro*. Although the mechanisms whereby RhoB funcions to control endothelial capillary morphogenesis are likely multi-factorial, relying on expression changes as well as interactions with other proteins that may be spatially regulated; the importance of RhoB expression to *in vitro* cord formation is partially a result of its negative regulation of RhoA activity, and subsequently its control over RhoA signaling, in response to VEGF (Fig. 33). Additionally, our results identify a novel form of Rho protein cross-regulation in endothelial cells with potential importance to angiogenesis.

During the course of studying RhoB in angiogenesis our lab became interested in the increasing prevalence of data indicating the importance of miRNA expression to various biological processes. Our preliminary analysis of VEGF-regulated miRNAs had suggested that one of our top regulated miRNAs could be targeting RhoB, and could in part explain the VEGF-induced RhoB expression we had previously observed. This, together with the fact that little was known and there was still much to be learned about how individual miRNAs function in the angiogenic process, led us to examine the effects of VEGF-regulated miRNAs on angiogenic processes.

The importance of miRNAs to angiogenesis was first described in cells depleted of the miRNA processing enzymes Drosha and Dicer, which indicated the importance of a functional cytosolic pool of mature miRNAs to the processes of migration, sprouting, and

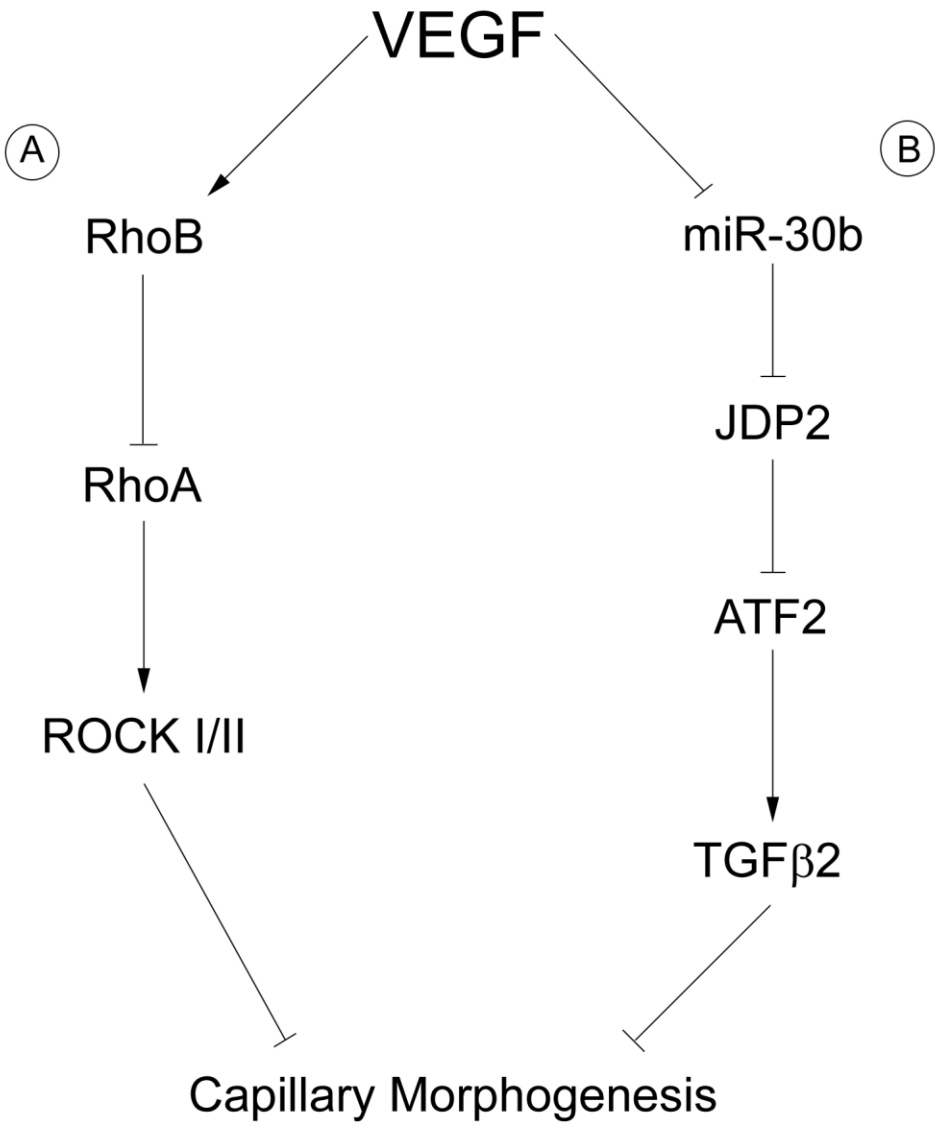

VEGF
A
B
RhoB
miR-30b
RhoA
JDP2
ATF2
ROCK I/II
TGFβ2
Capillary Morphogenesis

Figure 33. Proposed model of VEGF mediated endothelial cell capillary morphogenesis regulated by RhoB or miR-30b. (A) Capillary morphogenesis regulated by RhoB. Stimulation of HUVECs with VEGF increases RhoB protein expression without altering RhoB activity. Depletion of RhoB with siRNA reduces *in vitro* cord formation while cells depleted of RhoB also exhibit increased RhoA activation in response to VEGF. Treatment of RhoB-depleted HUVECs with C3 transferase to inhibit RhoA activity partially restores cord formation in those cells, implicating RhoA activity as a contributing factor in RhoB regulation of capillary morphogenesis. Cord formation is also partially restored in RhoB-depleted HUVECs treated with inhibitors of ROCK I/II, thus identifying the RhoA/ROCK signaling pathway as contributing to the phenotype of reduced capillary morphogenesis in HUVECs depleted of RhoB. (B) Capillary morphogenesis regulated by miR-30b. VEGF stimulation of HUVECs results in decreased miR-30b expression. Overexpression of miR-30b inhibits cord formation *in vitro* while reduction of miR-30b levels enhances cord formation. Overexpression of miR-30b reduces expression of JDP2, with cells exhibiting increased levels of phosphorylated ATF2. Overexpression of miR-30b also increases expression of TGFβ2, with maximal increase in TGFβ2 in response to miR-30b requiring ATF2. Treatment of HUVECs overexpressing miR-30b with an anti-TGFβ2 antibody partially restores cord formation, highlighting the contribution of TGFβ2 expression to miR-30b regulated capillary morphogenesis.

capillary morphogenesis in endothelial cells (Kuehbacher et al., 2007; Suarez et al., 2008). Since those studies, roles for individual miRNAs in angiogenesis are slowly being discovered. However, comparatively, the field of study into miRNA function in angiogenesis is in its infancy. While studies utilizing microarray analysis of miRNA expression have shed light on potential candidate miRNAs for future study (Suarez et al., 2008), many of those candidate miRNAs remain to be examined for a role in angiogenesis. Given this, along with the potential for use of miRNA modulation as a therapeutic approach towards combating disease (reviewed in van Rooij, 2011), we aimed to identify miRNAs whose expression was regulated by VEGF in HUVECs and would thus warrant further study as potential regulators of angiogenesis.

To identify candidate miRNAs for further study, microarray analysis of HUVECs was performed, and expression levels of miRNAs were assessed in response to VEGF treatment. Although other studies have presented array data for miRNA expression in endothelial cells (Suarez et al., 2008), we thought it prudent to perform our own microarray analysis for the system that we employ to study angiogenesis in endothelial cells for a number of reasons. Primary endothelial cells such as HUVECs are sensitive to growth conditions and serum, and miRNA biogenesis specifically is sensitive to culture conditions such as cell confluence (Hwang et al., 2009), conditions which cannot always be replicated exactly from lab to lab. The results of our microarray highlighted the induction of, as well as the reduction in, expression of several miRNAs by VEGF. The array data indicated the increased expression of miR-210 and miR-10b, confirming reports of VEGF induction of these miRNAs in various cell types (Alaiti et al., 2012; Plummer et al., 2013). Additionally, miR-19a, which has been shown to inhibit sprouting angiogenesis *in vitro* (Doebele et al., 2010), was shown to be downregulated by VEGF in our array, indicating the potential

relevance of VEGF regulation to miRNA function in angiogenesis. With this in mind, our array data indicated that miR-30b was downregulated by VEGF in HUVECs. At that time a role for miR-30b in angiogenesis had not been identified, although during the course of our study, information indicating the importance of miR-30b expression to endothelial tip cell biology was demonstrated (Bridge et al., 2012). Of additional interest to us in pursuing the study of miR-30b was the fact that RhoB was a potential target of miR-30b based on the sequence of the RhoB 3'-UTR [assessed by TargetScan (http://www.Targetscan.org)]. Although this information led us to hypothesize that VEGF regulation of RhoB protein expression could be through modulation of miR-30b levels, ultimately we did not observe regulation of RhoB by miR-30b in HUVECs (Appendix: Fig. A2). However, based on our array data, we hypothesized that miR-30b may function as an anti-angiogenic miRNA regulated by VEGF and thus we set out to describe the function of miR-30b in endothelial cells.

MiR-30b is located in an intergenic region on chromosome 8, and is processed from a primary miRNA transcript into a 21 nucleotide mature miRNA. Information on the transcriptional regulation of miR-30b is scarce although transactivation of the *miR-30b* gene and subsequently, its increased expression, has been documented to occur via the p65 subunit of NF-κB (Zhou et al., 2010; Zhou et al., 2009). However, seeing as our data indicates decreased expression of miR-30b in response to VEGF, further study of the promoter region of the *miR-30b* gene is required to determine if potential exists for VEGF downregulation of miR-30b at the transcriptional level.

Similar to a number of miRs, miR-30b has been shown to display varying degrees of expression in cancer. MiR-30b is differentially expressed in inflammatory breast cancer as compared to non-inflammatory breast cancer (Van der Auwera et al., 2010), and is

downregulated in parathyroid carcinoma compared to adenoma (Rahbari et al., 2011). Increased expression of miR-30b has been correlated with decreased overall survival in melanoma, with overexpression of miR-30b enhancing invasion *in vitro*, indicating a potential role for miR-30b in metastasis (Gaziel-Sovran et al., 2011). Interestingly, the genomic region containing the *miR-30b* gene is frequently amplified in cancer progression. In medulloblastoma cell lines a region of genomic amplification was shown to harbour the gene encoding miR-30b with expression levels correlating with copy number, suggesting a role for miR-30b in medulloblastoma development (Lu et al., 2009). The same study found miR-30b to be over-expressed in greater than 50% of primary medulloblastoma tumours tested, again suggesting miR-30b has oncogenic properties in this tumour type. Amplification of the genomic region containing the miR-30b gene is also prevalent in oral squamous cell cancers where increased expression of miR-30b is also observed (Shao et al., 2012). These studies indicate the diverse nature of processes regulated by miR-30b, as would be expected due to the large number and diversity of its predicted and confirmed targets.

MiR-30b has also been identified as having a relatively high expression level in endothelial cells, as it was in the top 25% of 168 miRNAs tested in terms of expression level (Kuehbacher et al., 2007). Upon further characterization we confirmed that VEGF consistently and significantly reduced miR-30b expression in HUVECs by more than 10%. We have increased the confidence that this result is accurate by obtaining similar levels of miR-30b reduction with the use of two endogenous controls for normalization. Although this change in expression could be considered modest, it was consistent, hence the statistical significance attached to it. It has been suggested that relatively small changes in the expression of a single miRNA may still hold biological relevance as each miRNA has a large

number of targets and thus the effects of changes in miRNA expression would not be felt by only one signaling pathway (Calin and Croce, 2006). Expanding on this idea, we must also consider the potential for additional effects due to overlapping sequence recognition sites in the 3'-UTRs of target mRNAs which could become available for miRNA binding upon downregulation of a competing miRNA. We, in fact, have shown that a decrease in miR-30b expression of less than 20% is biologically relevant as we observed a significant decrease in cord formation in cells transfected with 0.1 nM miR-30b inhibitor. Thus, the main angiogenic growth factor for endothelial cells, VEGF, decreases expression of miR-30b which suggested to us that miR-30b may function as an anti-angiogenic miRNA.

To better understand the potential role miR-30b plays in angiogenic processes and thus the potential relevance of its regulation by VEGF, we modulated the expression of miR-30b through the use of miRNA mimics and inhibitors. Neither overexpression nor reduction of miR-30b significantly affected HUVEC viability or migration. However, cells overexpressing miR-30b exhibited a reduction in the ability to form cord-like structures on BME, while cells with depleted miR-30b levels showed enhanced cord formation, indicating the importance of miR-30b regulation to endothelial cell capillary morphogenesis. Reduction in cord formation of roughly 30% was observed for cells over a wide range of increased expression levels of miR-30b, with miR-30b levels approximately 250-fold higher at the lowest mimic concentration tested (1 nM) and 1900-fold higher with 20 nM mimic concentration. The apparently high levels of exogenous miR-30b expression observed following transfection are not necessarily representative of miR-30b functionality within the cells. A recently published article by Thomson et al. (2013) indicated that the majority of exogenously provided miRNA (miR-200a in their study) following transfection is not associated with the RISC complex but is kept within vesicles localized within or at

lysosomes and is thus non-functional. In fact, the authors observed an 1176-fold increase in miR-200a after transfection with 60 nM mimic by qRT-PCR; however, RISC-associated miR-200a was found to be only 92-fold higher following mimic transfection which was an expression level similar to some highly expressed miRNAs, indicating a substantially lower amount of functional miRNA than the expression level predicted by qRT-PCR (Thomson et al., 2013). Thus, the level of functional miR-30b in our cells following transfection with miR-30b mimic may be much lower than the expression levels indicated by our qRT-PCR data and only further signifies the relevance of our observed defects in cord formation for cells overexpressing miR-30b.

As mentioned, we also observed a positive effect on cord formation when miR-30b levels were reduced with a hairpin inhibitor specific for miR-30b. Reduction of miR-30b levels by less than 20% resulted in an approximately 50% increase in cord formation. Importantly, this minor reduction in miR-30b expression with hairpin inhibitor was similar to that observed when HUVECs were stimulated with VEGF and thus signifies that VEGF downregulation of miR-30b may be a biologically important event that enhances cord formation and thus contributes to the process of VEGF-mediated angiogenesis.

Recently it was shown that the miR-30 family targets Delta-like 4 (DLL4), a Notch ligand involved in the regulation of the ratio of tip and stalk cells during sprouting angiogenesis, and overexpression of miR-30b resulted in increased *in vitro* sprouting in human endothelial cells as well as enhanced intersegmental vessel branching during zebrafish development (Bridge et al., 2012). These seemingly positive effects of miR-30b overexpression on angiogenesis in fact results in disorganized and uncontrolled sprouting as the balance between tip cells and stalk cells is shifted, resulting in loss of tip cell restriction. Thus, the authors' data complements what we have seen with modulation of miR-30b levels

whereby increased miR-30b expression is detrimental to the regulation of processes involved in angiogenesis. Interestingly, one could hypothesize that miR-30b expression may differ between tip cells and stalk cells of advancing vessel sprouts and that VEGF signaling in the advancing tip cell may mediate this difference by reducing miR-30b levels thus maintaining DLL4 production by the tip cells which then signals through binding to Notch on stalk cells resulting in the maintainance of the stalk cell phenotype.

As we had identified miR-30b regulation as playing an important role in endothelial cell cord formation, we sought to identify potential mediators of this effect. Microarray and qRT-PCR analysis of miR-30b overexpressing HUVECs indicated TGFβ2 mRNA as being upregulated, identifying miR-30b as regulating the expression of TGFβ2 in HUVECs. Importantly, we observed a significant increase in TGFβ2 concentration in cell culture supernates from miR-30b overexpressing cells, indicating that these cells secrete higher amounts of TGFβ2 than their control cell counterparts. Interestingly, increased miR-30b expression did not have an effect on TGFβ1 expression indicating specificity for the effect of miR-30b on TGFβ2 expression. Additionally, we did not observe any regulatory effect of TGFβ stimulation on miR-30b expression levels in contrast to what has been observed in mouse endothelial cells (Ghosh et al., 2012). To determine whether TGFβ2 produced by miR-30b overexpressing HUVECs was capable of eliciting a signaling response in an autocrine manner, we looked at activation of one of the main signaling intermediates for TGFβ, Smad2. We observed an increase in the phosphorylation of Smad2 in cells overexpressing miR-30b. TGFβ signaling through TGFβR1 and Smad2/3 has been implicated in mediating inhibitory signals in endothelium as opposed to signaling through ALK1 and Smad1/5 which results in promotion of proliferation and migration (Goumans et al., 2002). Thus, the observed increase in Smad2 phosphorylation fits a model whereby

increased expression of TGFβ2 in HUVECs could be functioning in an autocrine signaling pathway, as has been previously observed for TGFβ in HUVECs (Baker et al., 2008), to mediate the inhibitory effects of increased miR-30b expression on capillary-like cord formation.

As we observed an increase in TGFβ2 expression upon overexpression of miR-30b it stood to reason that miR-30b must facilitate this change in expression by targeting either a transcriptional repressor of *TGFβ2* or by targeting a repressor of a transcriptional activator of *TGFβ2*. Based on this hypothesis, we identified the transcription factor ATF2 as a prospective mediator of the effects of miR-30b on TGFβ2 expression. This was based on the fact that *TGFβ2* has an ATF2 binding site in its promoter region and ATF2 has been shown to contribute to the positive regulation of TGFβ2 expression (Kim et al., 1992). Importantly, ATF2 has an identified repressor, Jun dimerization protein 2 (JDP2) (Jin et al., 2001), which has a predicted miR-30b binding sequence in its 3'-UTR [assessed by TargetScan (http://www.targetscan.org)], thus indicating a potential mechanism whereby miR-30b could lead to TGFβ2 expression through the release of ATF2 from functional inhibition by JDP2. It was determined that reduction of ATF2 levels with siRNA resulted in decreased TGFβ2 expression and that miR-30b overexpressing HUVECs that were co-transfected with ATF2 siRNA exhibited a significant reduction in the expression level of TGFβ2 compared to miR-30b overexpressing cells with normal levels of ATF2, thus indicating that ATF2 is required for maximal TGFβ2 expression in response to miR-30b. Additionally, we observed that depletion of ATF2 levels in HUVECs resulted in an increase in capillary-like cord formation on growth factor reduced BME, suggesting that ATF2 may have a negative regulatory role in this process in endothelial cells. Interestingly, it was observed that overexpression of miR-30b resulted in a substantial increase in ATF2 phosphorylation. As phosphorylation of

ATF2 on residues Thr69 and Thr71 is required for its dimerization and transcriptional activity (Lau and Ronai, 2012), this result indicates the possibility that miR-30b targets a protein whose absence facilitates increased activation of ATF2 in HUVECs. Although we did observe a decrease in JDP2 mRNA expression in miR-30b overexpressing HUVECs, we did not see a significant increase in TGFβ2 expression in cells depleted of JDP2 by siRNA as would be expected if a reduction in JDP2 facilitated increased binding of ATF2 to the *TGFβ2* promoter. However, it is likely that additional signaling events are required to phosphorylate ATF2, and that reduced JDP2 expression may simply create a pool of ATF2 that is available for activation but not directly result in increased ATF2 phosphorylation. Thus, we would not necessarily expect that TGFβ2 expression would increase simply due to depletion of JDP2, if phosphorylation of ATF2 is required for our observed increase in TGFβ2 expression in miR-30b overexpressing cells. Thus, while we have identified ATF2 as being involved in the regulation of TGFβ2 expression by miR-30b, we have not yet confirmed JDP2 as either a direct target of miR-30b or as a contributing factor to increased ATF2 phosphorylation in miR-30b overexpressing HUVECs.

TGFβs are known in certain contexts to inhibit processes important to angiogenesis (Goumans et al., 2002; Pepper et al., 1991) including *in vitro* capillary-like cord formation (Maroni and Davis, 2011), and given our observations that miR-30b enhances expression of TGFβ2 and increases TGFβ signaling through Smad2 phosphorylation we hypothesized that TGFβ2 might be mediating the effects of miR-30b overexpression on cord formation. To this end we looked at the effects of both TGFβ1, which we have observed as not being regulated by miR-30b, and TGFβ2 on cord formation in HUVECs. Our results indicated that neither TGFβ1 nor TGFβ2 had significant effects on cord formation when HUVECs were stimulated for short periods of time at the initiation of cord formation. However, following 3

days of treatment with TGFβ2 prior to initiation of cord formation, HUVECs did exhibit a significant reduction in the number of cord-like structures produced, while TGFβ1 again had no effect. Interestingly, TGFβ1 has been shown to inhibit capillary-like cord formation in bovine microvascular endothelial cells of the corpus luteum following stimulation at the initiation of cord formation (Maroni and Davis, 2011). Although performed with a different concentration of TGFβ, our contrasting results in this regard highlight that differences exist in how specific types of endothelial cells respond to TGFβ1, as well as indicating a key difference in the effects of TGFβ1 and TGFβ2 specifically in HUVECs. Differential effects of TGFβ1 and TGFβ2 on angiogenic processes in vascular cells has been previously observed (Merwin et al., 1991), and our results indicated that the effects of miR-30b on cord formation might also be specifically a result of TGFβ2 expression. Interestingly, we observed a significant decrease in the expression of TGFβ2 but not TGFβ1 in HUVECs in response to VEGF, suggesting that reduction of TGFβ2 may be an important pro-angiogenic response. To test whether miR-30b induction of TGFβ2 is involved in our observed reduction in cord formation we treated miR-30b overexpressing HUVECs with anti-TGFβ2 neutralizing antibody and observed a restoration in cord formation in miR-30b overexpressing cells. Thus, we have shown that miR-30b elicits its inhibitory effects on cord formation by increasing TGFβ2 expression.

Based on our results indicating decreased capillary-like cord formation with TGFβ2 stimulation only after cells had been treated with TGFβ2 for extended periods of time prior to the initiation of cord formation, it is possible that inhibition of cord formation in this case is due to cumulative cellular changes that occur after prolonged exposure to TGFβ2. HUVECs overexpressing miR-30b exhibited a change in morphology seemingly consistent with what had been seen by others after prolonged stimulation of endothelial cells with

TGFβ2 (Ghosh et al., 2012; Kumarswamy et al., 2012b). The elongated phenotype associated with these cells was suggested as an outcome of the process of EndMT whereby cells obtain a mesenchymal phenotype and become more motile and invasive (Potenta et al., 2008), and TGFβ signaling is a known mediator of EndMT (Nakajima et al., 2000). Although we did not see an increase in the migratory ability of cells overexpressing miR-30b as would be expected with cells having undergone EndMT, we did look at the expression of a number of markers associated with the transition from endothelial-to-mesenchymal cell phenotypes (Appendix: Fig. A3). The results indicated increased expression of the mesenchymal marker fibronectin ($P = 0.0066$, n = 2), although there was no significant change in the level of Snai1 expression, and Slug ($P = 0.01$, n = 2) and αSMA ($P = 0.013$) expression were seen to be decreased in miR-30b overexpressing cells. Additionally, there were no significant changes in the level of expression of the endothelial markers VEGFR2, VE-Cadherin, or PECAM1. The observed increase in fibronectin expression could be directly due to increased TGFβ2 signaling in these cells as the human fibronectin promoter contains three CRE sites (Bowlus et al., 1991) and TGFβ2 has been shown to induce fibronectin expression via activation of the c-Jun N-terminal kinase (JNK) pathway (Hocevar et al., 1999). The decrease in Slug expression observed could be due to direct targeting by miR-30b as the 3'-UTR of Slug has a miR-30b binding site [assessed by TargetScan (http://www.targetscan.org)] and although Snai1 expression is known to be induced by TGFβ (Cho et al., 2007) and function to promote EMT (Cano et al., 2000), the 3'-UTR of Snai1 also has a miR-30b binding site which has been shown to facilitate miR-30b induced reduction of a luciferase reporter (Kumarswamy et al., 2012a), and thus these two effects could effectively cancel each other out resulting in the maintainance of Snai1 levels. Thus, although it has been shown that the process of EndMT can produce cells that co-express

endothelial and mesenchymal markers, our results cannot conclusively indicate whether the observed morphology of miR-30b expressing cells is indicative of cells undergoing the process of EndMT; however, observing similar changes in gene expression upon stimulation of HUVECs with TGFβ2 would conceivably identify potential downstream mediators of the effect of TGFβ2 on capillary-like cord formation.

It is of additional interest that VEGF negatively regulates both miR-30b and TGFβ2, but not TGFβ1. TGFβ regulation of VEGF expression in a number of cell lines has been observed (Fang et al., 2012; Jeon et al., 2007), with TGFβ induced expression of VEGF in peripheral blood mononuclear cells being shown to enhance *in vivo* angiogenesis (Fang et al., 2012). Although VEGF has been shown to regulate the expression of TGFβ1 (Lee et al., 2008), evidence for the importance of VEGF-mediated regulation of TGFβ to angiogenic processes is lacking. As TGFβ2, but not TGFβ1, exhibits anti-angiogenic effects in our system of study, it stands to reason that VEGF-mediated down-regulation of TGFβ2 is an important pro-angiogenic event for capillary-like cord formation and further highlights the importance of regulated miR-30b expression to angiogenesis.

These results identify miR-30b as being negatively regulated by the pro-angiogenic growth factor VEGF and suggest, based on miR-30b depletion studies, that reduction in miR-30b expression may be a mechanism of VEGF-enhanced capillary morphogenesis (Fig. 33). Additionally, miR-30b was shown to regulate TGFβ2 expression and increased TGFβ2 was shown to mediate the negative effects of miR-30b overexpression on cord formation. Although the model presented in figure 33 is simplistic in its description of VEGF-regulated capillary morphogenesis and does not specifically acknowledge the presence of a multitude of other interactions that most certainly play a role in how miR-30b can regulate complex processes such as those described in this work, it nevertheless describes a novel signaling

pathway whereby miR-30b functions as an anti-angiogenic miRNA in the context of capillary morphogenesis, a process vitally important to angiogenesis.

5. CONCLUSIONS

This study has identified both the small GTPase RhoB and the miRNA miR-30b as being regulated by VEGF in HUVECs. VEGF positively regulated RhoB protein expression and negatively regulated miR-30b expression. Depletion of RhoB with siRNA resulted in inhibition of endothelial cell migration, sprouting, and cord formation *in vitro*. RhoB-depleted cells exhibited increased levels of activated RhoA and this increased activity was shown to contribute to the reduction in capillary-like cord formation seen in cells depleted of RhoB. Treatment of RhoB-depleted HUVECs with the Rho inhibitor C3 transferase or with inhibitors of ROCK I/II kinases downstream of Rho proteins confirmed the contribution that RhoA signaling through ROCK I/II plays in defective cord formation when RhoB expression is reduced, as cord formation was partially restored. In addition, modulation of miR-30b levels in HUVECs was also shown to regulate capillary morphogenesis. Reduced miR-30b expression resulted in enhanced capillary-like cord formation which suggests a potential biological relevance to VEGF downregulation of miR-30b as part of the pro-angiogenic effect of VEGF. In addition, overexpression of miR-30b was shown to inhibit cord formation and induce TGFβ2 expression and secretion from HUVECs. Increased TGFβ2 expression was confirmed as mediating the inhibitory effects of miR-30b on cord formation as neutralization of TGFβ2 activity with an anti-TGFβ2 antibody restored cord formation in miR-30b overexpressing cells. Thus, we have identified novel targets of regulation by VEGF and shown these targets to be important mediators in pathways controlling the angiogenic process of capillary morphogenesis.

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7. STATEMENT OF CONTRIBUTION OF COLLABORATORS

Experiments contributing to data presented for miR-30b regulation of JDP2 mRNA expression were performed by Kayla Kazda under my guidance. FlashTag™ labelling of RNA for miRNA expression analysis with Affymetrix GeneChip miRNA Array was performed by Huijun Zhao. Antibodies to RhoA and phosphorylated ATF2 were provided by Dr. Jim Dimitroulakos. Antibodies to Smad2 and phosphorylated Smad2 were provided by Dr. Barbara Vanderhyden.

8. APPENDIX

Table 1. MiRNAs positively regulated by VEGF in HUVECs. HUVECs were serum starved overnight in MCDB 131 with 0.5% FBS and subsequently stimulated with VEGF (50 ng/ml) for 24 hours. RNA was isolated and miRNA profile was assessed through Affymetrix GeneChip miRNA 1.0 Array following the labelling of RNA with the Affymetrix FlashTag™ Biotin HSR RNA Labelling Kit. MiRNAs listed had fold changes of greater than 2 in VEGF-stimulated cells as compared to unstimulated cells.

miRNA	Fold Change
hsa-miR-21_st	13.75412
hsa-miR-497_st	12.88785
hsa-miR-337-5p_st	9.875666
hsa-miR-887_st	8.688956
hsa-miR-330-3p_st	8.587995
hsa-miR-421_st	8.332844
hsa-miR-1280_st	7.425486
hsa-miR-30c-2-star_st	7.252907
hsa-miR-210_st	7.037436
hsa-miR-150-star_st	6.096927
hsa-miR-505-star_st	5.824558
hsa-miR-199a-3p_st	5.458229
hsa-miR-10b_st	5.231699
hsa-miR-1292_st	5.119662
hsa-miR-339-3p_st	5.114975
hsa-let-7g_st	5.010549
hsa-miR-18b_st	4.891865
hsa-miR-132_st	4.870783
hsa-miR-1281_st	4.536199

hsa-miR-15b_st	4.313564
hsa-miR-625_st	4.299848
hsa-miR-24-2-star_st	4.246825
hsa-miR-431_st	4.238237
hsa-miR-193a-3p_st	4.003842
hsa-miR-501-3p_st	3.824213
hsa-miR-31-star_st	3.781058
hsa-miR-30d_st	3.631782
hsa-miR-30c_st	3.617433
hsa-miR-769-5p_st	3.600259
hsa-miR-99b-star_st	3.559581
hsa-miR-498_st	3.442684
hsa-miR-629_st	3.424896
hsa-miR-181c_st	3.402351
U53_st	3.361188
hsa-miR-606_st	3.297939
hsa-miR-199b-3p_st	3.238943
hsa-miR-181a-2-star_st	3.200879
hsa-miR-125b-1-star_st	3.079914
hsa-miR-181a-star_st	3.051721
hsa-miR-152_st	2.807005
hsa-miR-220c_st	2.728575
hsa-miR-631_st	2.712162
hsa-miR-302c_st	2.672344

hsa-miR-197_st	2.667439
hsa-miR-195-star_st	2.6134
hsa-miR-128_st	2.576006
ENSG00000207217_st	2.526592
hsa-miR-19b_st	2.515011
hsa-miR-595_st	2.511065
U46_st	2.502163
hsa-miR-671-3p_st	2.472202
hsa-miR-93-star_st	2.459738
hsa-miR-548n_st	2.436886
hsa-miR-505_st	2.407744
hsa-miR-455-5p_st	2.365078
hsa-miR-494_st	2.354543
hsa-miR-323-5p_st	2.347347
hsa-miR-181c-star_st	2.321312
ACA23_st	2.295385
hsa-miR-663b_st	2.292487
hsa-miR-16-1-star_st	2.28653
HBII-85-29_st	2.27143
hsa-miR-324-3p_st	2.259921
hsa-miR-329_st	2.212592
hsa-miR-520h_st	2.197685
hsa-miR-362-5p_st	2.196784
hsa-miR-378-star_st	2.194537
hsa-miR-519d_st	2.178623

hsa-miR-191-star_st	2.176403
hsa-miR-129-3p_st	2.163192
hsa-miR-596_st	2.163192
hsa-miR-222-star_st	2.150516
hsa-miR-18b-star_st	2.150516
hsa-miR-331-5p_st	2.128767
HBII-438A_s_st	2.100382
ENSG00000212558_st	2.057868
14qII-21_x_st	2.057868
hsa-let-7i-star_st	2.033159
hsa-miR-411_st	2.027974
hsa-miR-758_st	2.011666
hsa-miR-550-star_st	2.010927
hsa-miR-1244_st	2.003244

Table 2. MiRNAs negatively regulated by VEGF in HUVECs. HUVECs were serum starved overnight in MCDB 131 with 0.5% FBS and subsequently stimulated with VEGF (50 ng/ml) for 24 hours. RNA was isolated and miRNA profile was assessed through Affymetrix GeneChip miRNA 1.0 Array following the labelling of RNA with the Affymetrix FlashTag™ Biotin HSR RNA Labelling Kit. MiRNAs listed had fold changes of less than 0.5 in VEGF-stimulated cells as compared to unstimulated cells.

miRNA	Fold Change
hsa-miR-30b_st	0.1239
hsa-miR-132-star_st	0.133806
hsa-miR-885-3p_st	0.138507
hsa-miR-425-star_st	0.139603
hsa-miR-938_st	0.168774
hsa-miR-19a_st	0.236134
hsa-miR-92a-2-star_st	0.246788
hsa-miR-708-star_st	0.251374
hsa-miR-1247_st	0.285673
hsa-miR-484_st	0.30521
hsa-miR-92b-star_st	0.30558
hsa-miR-1257_st	0.306998
hsa-miR-937_st	0.335584
hsa-miR-92a-1-star_st	0.336753
hsa-miR-140-5p_st	0.338115
hsa-miR-1288_st	0.359301
hsa-miR-744-star_st	0.362812
hsa-miR-1202_st	0.390677
hsa-miR-544_st	0.399654

hsa-miR-380-star_st	0.400464
hsa-miR-941_st	0.409091
hsa-miR-940_st	0.414406
hsa-miR-548d-5p_st	0.417267
hsa-miR-615-3p_st	0.418113
hsa-miR-483-3p_st	0.418355
hsa-miR-642_st	0.431468
hsa-miR-326_st	0.435657
ENSG00000201502_st	0.435657
ENSG00000201827_x_st	0.435657
hsa-miR-374a_st	0.441584
hsa-miR-21-star_st	0.443349
hsa-miR-346_st	0.447746
hsa-miR-553_st	0.449229
hsa-miR-500-star_st	0.45019
hsa-miR-593-star_st	0.450839
hsa-miR-1234_st	0.450907
hsa-miR-580_st	0.451011
hsa-miR-890_st	0.451099
hsa-miR-767-3p_st	0.45621
hsa-miR-376a-star_st	0.471084
hsa-miR-139-3p_st	0.472039
hsa-miR-525-5p_st	0.476054
hsa-miR-150_st	0.481162
ENSG00000212423_x_st	0.485175

ENSG00000212421_st	0.48594
hsa-miR-9-star_st	0.486352
14ql-7_st	0.487216
hsa-miR-1267_st	0.487409
hsa-miR-205_st	0.491846
ENSG00000206637_x_st	0.499022

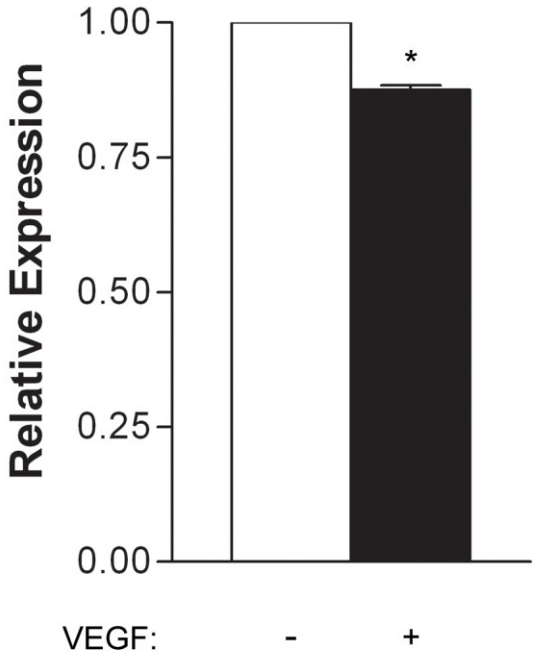

1.00
0.75
0.50
0.25
0.00
Relative Expression
*
VEGF:
-
+

Figure A1. MiR-30b expression following VEGF stimulation with RNU24 used as endogenous control. HUVECs were serum starved overnight in MCDB 131 with 0.5% FBS and then stimulated with 50 ng/ml VEGF for 24 hours. MiR-30b levels are expressed as mean ± SEM (n = 3) relative to RNU24 endogenous control. MiR-30b expression is significantly decreased following VEGF stimulation as compared to the untreated condition. Note: * denotes $P = 0.014$ as determined by unpaired Student's *t*-test.

Table 3. Genes positively regulated in miR-30b overexpressing HUVECs. HUVECs were transfected with 20 nM of either control miRNA mimic or miR-30b mimic and RNA was isolated at 48 hours post transfection. Analysis of gene expression was performed with an Affymetrix GeneChip Human Gene 1.0 ST array. Data presented is for gene expression with fold change greater than 2.

Gene Symbol	Fold Change	Gene Title
TAGLN	10.2962	transgelin
PCSK7		proprotein convertase subtilisin/kexin type 7
CD69	7.030757	CD69 molecule
CXCL10	5.783008	chemokine (C-X-C motif) ligand 10
TGFβ2	3.985968	transforming growth factor, beta 2
TGFβI	3.973664	transforming growth factor, beta-induced, 68kDa
IFITM1	3.43619	interferon induced transmembrane protein 1 (9-27)
CXCL12	3.426605	chemokine (C-X-C motif) ligand 12 (stromal cell-derived factor 1)
ITGA4	3.199907	integrin, alpha 4 (antigen CD49D, alpha 4 subunit of VLA-4 receptor)
CERKL		ceramide kinase-like
PKP2	3.171928	plakophilin 2
CD34	2.935711	CD34 molecule
IL7R	2.818153	interleukin 7 receptor
CD274	2.772466	CD274 molecule
UGCG	2.592565	UDP-glucose ceramide glucosyltransferase
IFI44L	2.574389	interferon-induced protein 44-like
SNORD13P1	2.566852	small nucleolar RNA, C/D box 13 pseudogene 1

COL8A1	2.566265	collagen, type VIII, alpha 1
C8orf4	2.55806	chromosome 8 open reading frame 4
MX1	2.547055	myxovirus (influenza virus) resistance 1, interferon-inducible protein p78 (mouse)
IFIT1	2.420659	interferon-induced protein with tetratricopeptide repeats 1
LOC151760	2.385977	hypothetical LOC151760
OAS2	2.382226	2'-5'-oligoadenylate synthetase 2, 69/71kDa
VCAN	2.354989	versican
HTR1D	2.300265	5-hydroxytryptamine (serotonin) receptor 1D
OAS1	2.298193	2',5'-oligoadenylate synthetase 1, 40/46kDa
IGF1	2.291814	insulin-like growth factor 1 (somatomedin C)
IRF6	2.270075	interferon regulatory factor 6
MYL9	2.232097	myosin, light chain 9, regulatory
C1QTNF9B	2.168218	C1q and tumor necrosis factor related protein 9B
PCOTH		prostate collagen triple helix
GRPEL2	2.152167	GrpE-like 2, mitochondrial (E. coli)
ABCA4	2.133793	ATP-binding cassette, sub-family A (ABC1), member 4
NOG	2.114637	noggin
CLDN1	2.073747	claudin 1
SCD	2.034113	stearoyl-CoA desaturase (delta-9-desaturase)
CCND2	2.023875	cyclin D2
GADD45B	2.021477	growth arrest and DNA-damage-inducible, beta
TNFSF18	2.008419	tumor necrosis factor (ligand) superfamily, member 18
IL1A	2.007945	interleukin 1, alpha

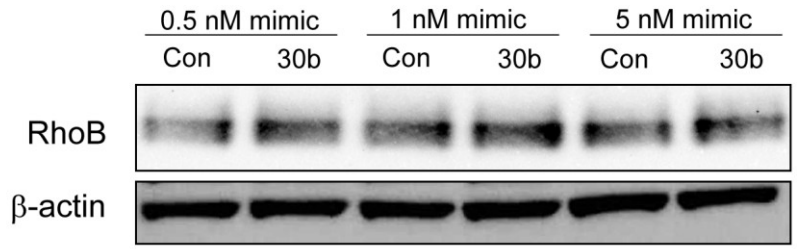
Table 4. Genes negatively regulated in miR-30b overexpressing HUVECs. HUVECs were transfected with 20 nM of either control miRNA mimic or miR-30b mimic and RNA was isolated at 48 hours post transfection. Analysis of gene expression was performed with an Affymetrix GeneChip Human Gene 1.0 ST array. Data presented is for gene expression with fold change less than 0.5.

Gene Symbol	Fold Change	Gene Title
SERPINB10	0.221198	serpin peptidase inhibitor, clade B (ovalbumin), member 10
SERPINB2		serpin peptidase inhibitor, clade B (ovalbumin), member 2
POSTN	0.2269631	periostin, osteoblast specific factor
MMP16	0.2303788	matrix metallopeptidase 16 (membrane-inserted)
MMP1	0.2591807	matrix metallopeptidase 1 (interstitial collagenase)
RGS7BP	0.2668887	regulator of G-protein signaling 7 binding protein
LYVE1	0.2826659	lymphatic vessel endothelial hyaluronan receptor 1
MMP16	0.292008	matrix metallopeptidase 16 (membrane-inserted)
LRRC17	0.2972482	leucine rich repeat containing 17
FAM38B2	0.2982037	family with sequence similarity 38, member B2
FAM38B		family with sequence similarity 38, member B
ENTPD1	0.3313138	ectonucleoside triphosphate diphosphohydrolase 1
CCL14	0.3475253	chemokine (C-C motif) ligand 14
CCL15		chemokine (C-C motif) ligand 15
CCL14-CCL15		CCL14-CCL15 read-through transcript
GLT8D2	0.351474	glycosyltransferase 8 domain containing 2
ELMOD1	0.3765935	ELMO/CED-12 domain containing 1

ADAMTS9	0.3788005	ADAM metallopeptidase with thrombospondin type 1 motif, 9
PPAP2B	0.4066924	phosphatidic acid phosphatase type 2B
PEG10	0.4198512	paternally expressed 10
DOK5	0.433622	docking protein 5
C18orf58	0.436623	chromosome 18 open reading frame 58
FAM38B		family with sequence similarity 38, member B
SELL	0.4374864	selectin L
ARHGAP28	0.4405629	Rho GTPase activating protein 28
STC1	0.4413668	stanniocalcin 1
TMEM156	0.4418229	transmembrane protein 156
MYRIP	0.4435412	myosin VIIA and Rab interacting protein
RUNX1T1	0.4460879	runt-related transcription factor 1; translocated to, 1 (cyclin D-related)
ADAMTS1	0.446747	ADAM metallopeptidase with thrombospondin type 1 motif, 1
SLC6A6	0.447975	solute carrier family 6 (neurotransmitter transporter, taurine), member 6
DUSP4	0.4482079	dual specificity phosphatase 4
LOX	0.4614352	lysyl oxidase
MRAP2	0.4667656	melanocortin 2 receptor accessory protein 2
KCNAB1	0.4692342	potassium voltage-gated channel, shaker-related subfamily, beta member 1
IDH1	0.4695791	isocitrate dehydrogenase 1 (NADP+), soluble
FST	0.4737868	follistatin
ANKRA2	0.4826837	ankyrin repeat, family A (RFXANK-like), 2

HEY2	0.4846147	hairy/enhancer-of-split related with YRPW motif 2
EXTL2	0.4875831	exostoses (multiple)-like 2
C6orf138	0.4888657	chromosome 6 open reading frame 138
GABBR2	0.4913081	gamma-aminobutyric acid (GABA) B receptor, 2
MST131	0.4929728	MSTP131
B3GNT5	0.4931574	UDP-GlcNAc:betaGal beta-1,3-N-acetylglucosaminyltransferase 5
NR5A2	0.4996778	nuclear receptor subfamily 5, group A, member 2

A.


0.5 nM mimic
1 nM mimic
5 nM mimic
Con
30b
Con
30b
Con
30b
RhoB
β-actin

B.

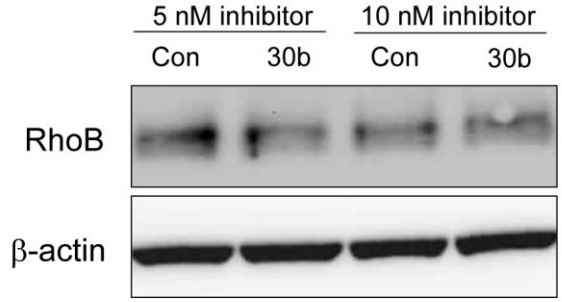

5 nM inhibitor
10 nM inhibitor
Con
30b
Con
30b
RhoB
β-actin

Figure A2. MiR-30b does not regulate RhoB expression in HUVECs. Cells were transfected with various concentrations of either (A) control miRNA mimic (Con) or miR-30b mimic (30b) or (B) control hairpin inhibitor (Con) or miR-30b inhibitor (30b) and RhoB protein expression was determined at 48 hours post transfection through western blotting with β-actin as endogenous control. Neither overexpression nor depletion of miR-30b resulted in changes in RhoB protein expression in HUVECs.

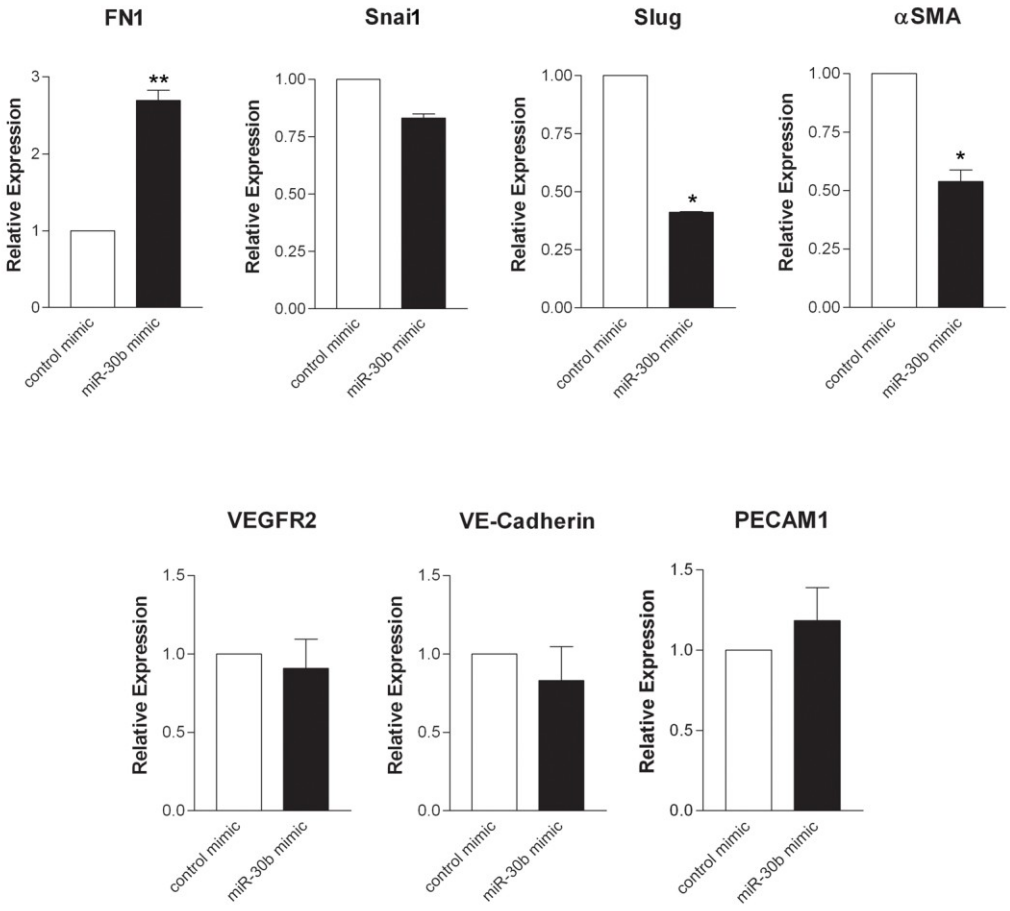

FN1
Snai1
Slug
αSMA
Relative Expression
control mimic
miR-30b mimic
**
*
VEGFR2
VE-Cadherin
PECAM1

Figure A3. Expression of EndMT markers in miR-30b overexpressing HUVECs. HUVECs were transfected with 20 nM control miRNA mimic or miR-30b mimic and RNA isolated at 48 hours post transfection. Expression of EndMT markers by qRT-PCR is presented relative to β-actin endogenous control. Note: * denotes $P < 0.05$, ** denotes $P < 0.01$ as determined by unpaired Student's *t*-test.

9. CURRICULUM VITAE

Grant Alexander Howe

Education

University of Ottawa
Candidate for Doctorate in Philosophy in Biochemistry, June 2013

University of Ottawa
Bachelor of Science in Biochemistry (Honours), May 2005

Experience

May 2006 – Present **Centre for Cancer Therapeutics, The Ottawa Hospital, Ottawa, ON**

- Undertook novel research leading to peer-reviewed publication.
- Designed, performed and analyzed experiments for multiple research projects.
- Responsible for the guidance of undergraduate research projects including experimental design and analysis.
- Experience writing both research and review articles
- Well-established ability for critical thinking and analysis

Sept. 2009 – Jan. 2013 **Teaching Assistant, University of Ottawa, Ottawa, ON**

- Teaching assistant in the Department of Chemistry for courses in both general and organic chemistry.
- Responsible for directing students in the completion of laboratory experiments and for providing a theoretical background for the experiments being performed.
- Evaluated students' work and provided constructive feedback necessary academic improvement.
- Proven leadership abilities and interpersonal skills through interactions with students of varying abilities and levels of understanding.
- Ability to engage students and foster learning through guided analysis of experimental results.

Awards

- Recipient of a Queen Elizabeth II Graduate Scholarship in Science and Technology (formerly Ontario Graduate Scholarship in Science and Technology) from September 2009 to September 2011.

- Recipient of University of Ottawa Undergraduate Admission Scholarship, September 2001.

Publications

- Howe, G.A. and Addison, C.L. (2012) RhoB controls endothelial cell morphogenesis in part via negative regulation of RhoA. Vasc Cell 4, 1.

- Howe, G.A. and Addison, C.L. (2012) β1 integrin: an emerging player in the modulation of tumorigenesis and response to therapy. Cell Adh Migr. 6(2), 71-77.

Presentations

Poster Presentations

- Keystone Symposia, Angiogenesis and Lymphangiogenesis in Cancer, January 2009, Big Sky, Montana.
- Departmental Research Day, May 2007, 2009, and 2011, Department of Biochemistry, Microbiology and Immunology, University of Ottawa, Ottawa, ON.
- OHRI Research Day (yearly), 2007 – 2011, The Ottawa Hospital Research Institute, Ottawa, ON.

Oral Presentations

- Cell Signaling Seminar Series, 2008, University of Ottawa, Ottawa, ON.
- Work in Progress meetings (yearly), 2006 – 2012, Centre for Cancer Therapeutics, The Ottawa Hospital, Ottawa, ON.
- Departmental Research Seminar Day, February 2008, 2010, Department of Biochemistry, Microbiology and Immunology, University of Ottawa, Ottawa, ON.

Charitable Work

Organizer for an annual charity hockey game at The Ottawa Hospital Centre for Cancer Therapeutics. Over the past two years this event has raised more than $9,000 for the Patient Emergency Fund, which, among other endeavors, provides financial assistance to families with members undergoing treatment at The Ottawa Hospital.

Interests

Adventure Travel, Woodworking, Golf, Hockey.