Targeted MicroRNA-126 and VEGF Gene Therapy for Chronic Ischemia in Diabetes

by

Wei Jie Cao

A thesis submitted in conformity with the requirements for the degree of Master of Science

Institute of Medical Science
University of Toronto

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Master of Science
Institute of Medical Science
University of Toronto
2015

Abstract

MicroRNAs are involved in many critical biological functions, including angiogenesis. miR-126 is an important endothelial specific angiomiR which targets negative inhibitors of the VEGF-A angiogenesis pathway. We applied ultrasound mediated gene delivery (UMGD) for miR-126 and VEGF-A delivery in a clinically relevant chronic ischemia model with Fischer and Zucker diabetic fatty (ZDF) rats. In vitro, miR-126 transfected HUVECs showed greater angiogenic potential with increased tube formation and migration response. UMGD of miR-126 produced targeted vascular transfection in rat hindlimb skeletal muscle lasting for >3 days, and resulting in inhibition of SPRED-1 and PIK3R2 protein. ZDF rats showed a significant decrease of miR-126. The dual delivery of miR-126 and VEGF-A to the ischemic leg with UMGD resulted in greatest microvascular blood improvement, vessel density, enhanced arteriolar formation than either VEGF-A or miR-126 therapy alone. UMGD is therefore a promising platform for multiple gene and miRNA delivery, with applications for therapeutic angiogenesis.
Acknowledgements

First and foremost, I would like to thank my supervisor Dr. Howard Leong-Poi for the opportunity to do research at his lab. His constant encouragements has motivated me to strive for excellence and stimulated me to stay on track. He was a great supervisor who not only cared about the success of the project, but also cared about my well-being and personal growth. He has been a very supportive mentor in guiding me throughout my project. I am very grateful for the opportunities he has given me to attend and present my research at various conferences. These experiences has proven invaluable to project and my personal growth.

I would like to thank my program advising committee, Dr. Kim Connelly and Dr. Jason Fish for all the advices and criticisms that have further added depth and insights to the project. Thank you for arranging time from your busy schedules to meet and discuss project progress and research strategies with me. Additionally, through extensive questioning sessions during the meetings, you have helped me grow in critical thinking. I would like to extend my gratitude to the IMS Defense Committee members, Dr. Warren Lee, Dr. Milica Radisic and Dr. Raffi Karshafian for agreeing to participate in my thesis defense meeting.

The members of the Leong-Poi lab were critical for the completion of my project, but also their companionship and words of encouragement have motivated me to persist during rough times. Many thanks to Pratiek Matkar, Dmitriy Rudenko, Hao Chen,
Michael Kuliszewski, Yu Jin Kim, Azadeh Mofid, and Christine Liao for all your contribution and friendship.

Last but not least, I would like to thank my parents for their unconditional love and support throughout my study. Your trust on me has given me the freedom to explore my passion for science and medicine. I am grateful to have such loving and understanding parents who have stood by my side every step of this journey.
Contributions

This study was made possible from funding by Canadian Institute of Health Research (CIHR). As the primary student leading the project, I was involved in all aspects of this project including planning and execution of both \textit{in vivo} and \textit{in vitro} experiments. However, this was only possible with the help of members of the Leong-Poi lab who contributed significantly in various experiments. Pratiek Matkar was heavily involved in the \textit{in vitro} angiogenesis experiments. Dmitry Rudenko has taught me various wet lab skills that were essential for this study and have performed some of the early PCRs. Hao Chen has assisted greatly in the ligation surgeries and also broadened greatly my knowledge of diabetes through extensive intellectual discussions. Michael Kuliszewski has provided a lot of guidance with immunohistochemistry microscopic bio-imaging. Yu Jin Kim has been a great assistant during both \textit{in vivo} and \textit{in vitro} experiments. My supervisor Dr. Howard Leong-Poi has been spending countless hours to provide extensive intellectual feedback in the project and editing of the thesis. Dr. Kim Connelly and Dr. Jason Fish have been providing invaluable insights and guidance throughout the project and preparation for my defense. Figure 1 was taken with permission from Smith, AH., 2010.
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<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>ABI</td>
<td>Ankle brachial index</td>
</tr>
<tr>
<td>Ang-1</td>
<td>Angiopoeitin-1</td>
</tr>
<tr>
<td>Ang-2</td>
<td>Angiopoeitin-2</td>
</tr>
<tr>
<td>CAD</td>
<td>Coronary artery disease</td>
</tr>
<tr>
<td>CEU</td>
<td>Contrast-enhanced ultrasound</td>
</tr>
<tr>
<td>CVD</td>
<td>Cardiovascular disease</td>
</tr>
<tr>
<td>DM</td>
<td>Diabetes mellitus</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular-signal-regulated kinase</td>
</tr>
<tr>
<td>FFA</td>
<td>Free fatty acid</td>
</tr>
<tr>
<td>FGF</td>
<td>Fibroblast growth factor</td>
</tr>
<tr>
<td>FMA</td>
<td>Flourescent Microangiography</td>
</tr>
<tr>
<td>HIF</td>
<td>Hypoxia induced factor</td>
</tr>
<tr>
<td>HGF</td>
<td>Hepatocyte growth factor</td>
</tr>
<tr>
<td>HUVEC</td>
<td>Human umbilical vascular endothelial cells</td>
</tr>
<tr>
<td>IM</td>
<td>Intra-muscular</td>
</tr>
<tr>
<td>IA</td>
<td>Intra aortic</td>
</tr>
<tr>
<td>IV</td>
<td>Intravenous</td>
</tr>
<tr>
<td>NF-1</td>
<td>Neurofibromatosis type I</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MBF</td>
<td>Microvascular blood flow</td>
</tr>
<tr>
<td>MI</td>
<td>Myocardial infarction</td>
</tr>
<tr>
<td>mTOR</td>
<td>Mammalian target of rapamycin</td>
</tr>
<tr>
<td>miR</td>
<td>Micro RNA</td>
</tr>
<tr>
<td>N</td>
<td>Sample size</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric Oxide</td>
</tr>
<tr>
<td>PAD</td>
<td>Peripheral arterial disease</td>
</tr>
<tr>
<td>PAI-1</td>
<td>Plasminogen activator inhibitor 1</td>
</tr>
<tr>
<td>PDGF</td>
<td>Platelet derived growth factor</td>
</tr>
<tr>
<td>PECAM</td>
<td>Platelet endothelial cell adhesion molecule</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphoinositide-3-kinase</td>
</tr>
<tr>
<td>PIK3R2</td>
<td>Phosphoinositide-3-kinase, regulatory subunit 2</td>
</tr>
<tr>
<td>PIP2</td>
<td>Phosphatidylinositol 4,5-bisphosphate</td>
</tr>
<tr>
<td>PIP3</td>
<td>phosphatidylinositol 3,4,5 trisphosphate</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>QoL</td>
<td>Quality of life</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Real time polymerase chain reaction</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>SPRED-1</td>
<td>Sprouty-related, EVH1 domain-containing protein 1</td>
</tr>
<tr>
<td>TBI</td>
<td>Toe brachial index</td>
</tr>
<tr>
<td>TGF-B</td>
<td>Transforming growth factor B</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumor necrosis factor alpha</td>
</tr>
<tr>
<td>TRBP</td>
<td>Transactivation-responsive RNA-binding protein</td>
</tr>
<tr>
<td>UMGD</td>
<td>Ultrasound mediated gene delivery</td>
</tr>
<tr>
<td>VCAM-1</td>
<td>Vascular cell adhesion molecule 1</td>
</tr>
<tr>
<td>VEGF-A</td>
<td>Vascular endothelial growth factor-A</td>
</tr>
<tr>
<td>VEGFR1</td>
<td>Vascular endothelial growth factor receptor 1</td>
</tr>
<tr>
<td>VEGFR2</td>
<td>Vascular endothelial growth factor receptor 2</td>
</tr>
<tr>
<td>VSMC</td>
<td>Vascular smooth muscle cell</td>
</tr>
<tr>
<td>ZDF</td>
<td>Zucker diabetic fatty</td>
</tr>
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</table>
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Chapter 1 – Introduction and Literature Review

1.1 Cardiovascular complications and diabetes

The adaptation to a sedentary lifestyle in developed countries has led to increase in coronary artery disease (CAD) and peripheral arterial disease (PAD) that is fueled by an aging population (Yazdanyar & Newman, 2009) and the ongoing epidemic of obesity (Padwal & Sharma, 2010) and diabetes (Bell et al., 2009; Krempf et al., 2010). Atherosclerosis and resultant arterial narrowing, or stenoses, often lead to symptoms of angina and myocardial infarction (MI) (Grines et al., 2002) in patients with CAD, and claudication and critical limb ischemia in patients with PAD. In the event of ischemia and MI, endogenous angiogenesis is triggered in attempt to restore blood flow to the stenotic region through collateral formation (Grines et al., 2002). If the endogenous angiogenic response is insufficient to restore blood flow, chronic ischemia and angina will result. In patients with peripheral arterial disease (PAD), the hypoxia caused from formation of atherosclerotic lesions in major arteries of the limbs leads to collateral formation that is often insufficient to meet even resting metabolic requirements (Mahmud, Cavendish, & Salami, 2007; Olea et al., 2009; Rothwell et al., 2005; Testa, Pannitteri, & Condorelli, 2008). If left untreated, PAD can progress to critical limb ischemia, resulting in amputation (Olea et al., 2009) in 25% of the patients.

The complications associated with CAD and PAD decreases the quality of life in these patients and is a huge burden for the health care system. Angina pectoris, a
complication manifested from CAD, is a condition affecting over 7 million people in the United States (Grines et al., 2002). New cases of chronic limb ischemia emerge at a rate of 500-1000 cases per million people each year (Taniyama et al., 2002). Due to the overwhelming number of people affected by cardiovascular diseases (CVD), the medical expenses incurred to CVD is the highest compared to any other disease in Europe ($169 billion) and the US ($403 billion) (Leal, Luengo-Fernandez, Gray, Petersen, & Rayner, 2006). With increasing prevalence cases of diabetes and obesity in developed countries, major CVD risk factors have increased in recent years (Capewell & O'Flaherty, 2009).

1.2 Diabetes Associated Vascular Complications

Diabetes is a major risk factor of cardiovascular complications. Diabetes is associated with various micro-vascular complications such as retinopathy, nephropathy and neuropathy as well as various macro-vascular complications such as coronary heart disease, peripheral vascular disease and stroke (Golden, 2011; Jeerakathil, Johnson, Simpson, & Majumdar, 2007). Studies have shown that microalbuminuria, a marker for diabetes, is a strong predictor of total and cardiovascular mortality (Dinneen & Gerstein, 1997). Compiling results from 14 eligible studies in the total of 38,091 patients, the presence of albuminuria (either micro- or macro) increases the risk of cardiovascular events by about two-fold (Rosenson, Fioretto, & Dodson, 2011). The abnormal metabolic state of diabetes results in proatherogenic changes in the state of arterial structure and function. These proatherogenic changes include endothelial cell dysfunction, vascular smooth muscle (VSMC) dysfunction and alterations in blood component and hemostatic factors.
1.2.1 Diabetes and endothelial cell dysfunction

Vascular complications associated with diabetes often starts with endothelial
dysfunction (Avogaro, Albiero, Menegazzo, de Kreutzenberg, & Fadini, 2011). The
endothelial cell lining of the vasculature plays an integral role for mediating normal
balance of thrombosis and fibrinolysis. Abnormalities of the endothelium results in
increased susceptibility of the vascular system to atherosclerosis. Diabetic patients who
have developed PAD demonstrate endothelial dysfunction and abnormal vascular
regulation (Veves et al., 1998). In diabetes, the mediators leading to endothelial
dysfunction are numerous but they share an important common pathway leading to
decrease nitric oxide (NO) bioavailability. NO is a potent vasodilator molecule that
modulates inflammation through inhibition of platelet adhesion (Loscalzo, 2001) and
attenuation of leukocyte-vascular wall interaction (Veves et al., 1998). In diabetes, the
decrease of NO homeostasis in the endothelial cells leads to a cascade of events in the
vasculature that ultimately results in increased risks of atherosclerosis complications. The
decrease in NO is attributed to two major mediators including insulin resistance and
hyperglycemia. Insulin resistance promotes release of free fatty acids (FFA) from adipose
tissue producing protein kinase C (PKC) (Sanchez & Sharma, 2009). Activation of PKC,
in endothelial cells stimulates overproduction of reactive oxygen species (ROS).
Hyperglycemia can inhibit production of nitric oxide (NO) in endothelial and vascular
smooth muscle cells by blocking endothelial nitric oxide synthase (eNOS) and increases
oxidative stress and generation of reactive oxygen species (ROS). The increase in ROS
results in defective angiogenesis in response to ischemia (Lin et al., 2005), activation of
various proinflammatory pathways (Giacco & Brownlee, 2010) and inactivation of
antiatherosclerosis (Kaneto, Katakami, Matsuhisa, & Matsuoka, 2010) enzymes which all result in a spiral of events contributing to diabetes associated vascular complications.

1.2.2 Diabetes and VSMC dysfunction

The state of hyperglymic and hyperinsulinemia in diabetes also causes abnormalities in the VSMC functions via mechanisms similar to that of endothelial cells. VSMCs that are phenotypically differentiated, constitute to the structure of mature vasculatures and are responsible for vasoconstriction and vasodilatation. Diabetes increases VSMC proliferation, migration and accumulation in atherosclerotic lesion resulting in accelerated progression of atherosclerosis (Suzuki, Poot, Gerrity, & Bornfeldt, 2001). Hyperglycemia causes increase of oxidative stress and the upregulation of proinflammatory proteins ICAM-1, VCAM-1 and NF-κB and in VSMC (Ramana, Friedrich, Srivastava, Bhatnagar, & Srivastava, 2004). Hyperinsulinemia results in promoting VSMC migration via MAPK pathway via PI3K (C. C. Wang, Gurevich, & Draznin, 2003). These changes together result in pro-atherosclerotic activity via the proinflammatory plaque formation.

1.2.3 Impact of Diabetes on platelets and coagulation

Platelets are important for in the process of thrombosis. Similar to endothelial cells, platelets’ glucose uptake is abnormal in the settings of diabetes resulting in increased oxidative stress. As a result, platelet aggregation is enhanced in diabetic patients through the up-regulation of glycoprotein Ib and IIb/IIa receptors, which play a
critical role in adhesion and aggregation in thrombosis (American Diabetes, 2003). Diabetic endothelial and VSMC secrete blood-coagulating factors, which leads to a hypercoagulable state. Additionally, hyperglycemia is associated with increase plasminogen activator-1 (PAI-1) and decrease antithrombin which together results in impaired fibrinolysis (Ahirwar, Jain, Goswami, Bhatnagar, & Bhatacharjee, 2014). Lastly, in diabetic patients, there are abnormalities in rheology with increased blood viscosity and fibrinogen levels. Among patients with PAD, increased viscosity and fibrinogen levels are correlative with decrease in ankle-brachial index (ABI), while high levels of fibrinogen have been associated with the development of PAD (American Diabetes, 2003).

1.3 Current options for CAD and PAD patients

1.3.1 Pharmacological and surgical

Current therapeutic strategies for patients with CVD aim to reduce plaque formation/progression, to prevent clinical events such as stroke or myocardial infarction, and to restore blood flow for symptomatic benefit. The type of therapeutic approach is highly dependent on the severity of the CAD/PAD. Mild stenoses are often managed with pharmacological therapies coupled with adjustment to a healthier life style. Severe stenoses with major occlusions often require either percutaneous angioplasty and stenting or coronary arterial or peripheral arterial bypass grafting surgery. Pharmacological and surgical interventions have reduced mortality and morbidity due to CVD and resulted in overall increase of life span by 4.5 years in these patients (Ford et al., 2007). In more advanced cases of CVD, patients develop diffuse ischemia with severe stenoses that
makes pharmacological and percutaneous interventions ineffective or technically unfeasible. These patients will eventually require cardiac transplant surgery (if candidates) for CAD, and limb amputation for PAD. Despite many therapeutic advances in percutaneous revascularization and surgical arterial bypass techniques, the number of patients with CAD and PAD who are not amenable to revascularization continues to grow. As a result, new therapeutic strategies to promote vessel formation, or angiogenesis, within ischemic tissue are critical for these patients.

1.3.2 Therapeutic angiogenesis

Strategies to promote new vessel formation, or angiogenesis, within ischemic tissue have been studied, with extensive research performed in angiogenic gene and progenitor cell based therapies. With better understanding of the biology and mechanism of new vessel formation there has been promising results from pre-clinical studies. However, the translation of angiogenesis into a clinically useful therapeutic strategy for patients remains unavailable. To date all larger double-blind randomized placebo controlled clinical trials of gene therapy for angiogenesis have failed to conclusively show a significant clinical benefit (Grines et al., 2002; Henry et al., 2003; Henry et al., 2007; Kastrup et al., 2005; Losordo et al., 2002; Losordo et al., 1998; Simons et al., 2000; Stewart et al., 2009). While progenitor cell therapies have yielded positive results in early clinical trials, the magnitude of benefit remains relatively modest (Assmus et al., 2002; Schachinger et al., 2006; Strauer et al., 2002; Wollert et al., 2004). A meta-analysis of randomized controlled trials and cohort studies of bone-marrow derived mononuclear cell transplantation to treat ischemic heart disease demonstrated a significant, but small, 3.7%
improvement in left ventricular ejection fraction with cell therapy (Abdel-Latif et al., 2007). Thus, while important research into gene and cell-based therapies to treat ischemic heart and peripheral arterial disease continues, researchers continue to seek novel and effective approaches that may complement or even replace existing therapeutic strategies.

1.3.3 Clinical trial outcomes

In the past, the diminished angiogenic response to ischemia in the setting of diabetes has been attributed to the reduced expression of angiogenic cytokines, such as VEGF, neuropilin-1, angiopoietins and their receptor, Tie-2, and the enhanced expression of anti-angiogenic proteins, such as angiotatin, thrombospondin-1, and endostatin (Boodhwani et al., 2007; Kivela et al., 2006; Schiekofer, Galasso, Sato, Kraus, & Walsh, 2005). Thus, angiogenic gene therapy could be an effective method to augment cytokine expression and enhance angiogenesis in diabetes. Multiple angiogenic factors have been studied for therapeutic angiogenesis in animal models of hindlimb ischemia and PAD (Masaki et al., 2002; Shimpo et al., 2002; Taniyama et al., 2001). Positive outcomes from these animal studies have triggered interest in human studies of therapeutic angiogenesis, leading to several small-scale clinical trials (Table 1). These clinical trials focused on gene- or protein-based therapies of pro-angiogenic factors including vascular endothelial growth factor (VEGF), fibroblast growth factors (FGF), hepatocyte growth factor (HGF) families, and the transcription factor, hypoxia inducible factor 1 (HIF-1). While some studies have shown promising results with VEGF gene therapy, other studies have failed to produce positive results, making the beneficial effect of VEGF therapy for PAD controversial. In a study by Shyu et al., the safety and effectiveness of VEGF-165 on
critical limb ischemia was demonstrated, where administration of VEGF improved ulcer healing, rest pain, and ankle–brachial index (ABI) measures, in a non-control setting (Shyu, Chang, Wang, & Kuan, 2003). In a different study by Rajagopalan et al., 54 diabetic patients with critical limb ischemia were treated with VEGF-165 therapy by adenoviral vector in double-blinded, placebo-controlled study (Rajagopalan et al., 2002). The study did not find significant improvement in the primary end point of amputation rate, but showed improvement in skin ulcer healing. Despite the promising results from animal studies and early clinical trials, subsequent more rigorous randomized placebo controlled phase II/III clinical trials have failed to produce positive results in primary endpoints (reduction in the risk of adverse cardiovascular events, prevention of major amputation, and improved survival) but did show modest improvement in secondary endpoints (relief of ischemic pain, healing of ulcers, improved quality of life) (Table 1).
<table>
<thead>
<tr>
<th>Study Author/Year</th>
<th>Year</th>
<th>Treatment</th>
<th>No. of Patients (Active/Placebo)</th>
<th>Route of administration</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Makinen et al., 2002)</td>
<td>2002</td>
<td>VEGF 165; plasmid and adenovirus</td>
<td>22/27</td>
<td>18 patients $2 \times 10^{10}$pfu VEGF-Ad; 18 patients: VEGF plasmid/liposome (2000 μg/2000 μL) IA infusion</td>
<td>- ABI improvement, but similar to control&lt;br&gt;- Increased vascularity</td>
</tr>
<tr>
<td>(Lederman et al., 2002)</td>
<td>2002</td>
<td>FGF-2; recombinant</td>
<td>59/66</td>
<td>rFGF2 30 μg/kg=single dose; rFGF2 30 x 2μg/kg=double dose (day 1 + 30); IA infusion</td>
<td>- Equal ABI, QoL&lt;br&gt;- Increased peak walking time at 90 days</td>
</tr>
<tr>
<td>(Rajagopalan et al., 2003)</td>
<td>2003</td>
<td>VEGF 121; adenovirus</td>
<td>35/19</td>
<td>Low dose $4 \times 10^9$pu; High dose $4 \times 10^{10}$pu; 20 x IM injection (unilateral PAD)</td>
<td>- No changes in peak walking time&lt;br&gt;- Equal ABI, QoL, ulcer healing&lt;br&gt;- Decrease amputation rate</td>
</tr>
<tr>
<td>(Kusumanto et al., 2006)</td>
<td>2006</td>
<td>VEGF 165; plasmid</td>
<td>72/33</td>
<td>2 sessions, 4 weeks apart, IM injection (2000 μg per session)</td>
<td>- Equal amputation rate; rest pain&lt;br&gt;- Improved skin ulcer; ABI, TBI</td>
</tr>
<tr>
<td>(Nikol et al., 2008)</td>
<td>2008</td>
<td>FGF-1; plasmid</td>
<td>259/266</td>
<td>4 sessions, 2 weeks apart, IM injection x 8 of 0.5 mg (each session)</td>
<td>- Reduce amputation rate&lt;br&gt;- Equal ulcer healing</td>
</tr>
<tr>
<td>(Powell et al., 2008)</td>
<td>2008</td>
<td>HGF; plasmid</td>
<td>78/26</td>
<td>Lower dose: 0.4mg (x3 sessions); mid-dose: 4 mg (x2 sessions); high dose: 4 mg (x3 session); IM injection</td>
<td>- Equal amputation; death; ulcer size, wound healing, TBI, ABI</td>
</tr>
<tr>
<td>(Powell et al., 2010)</td>
<td>2010</td>
<td>HGF; plasmid</td>
<td>21/16</td>
<td>3 sessions, 2 weeks apart, x 8 IM injection x 8 of 0.5 mg (each session)</td>
<td>- Equal wound healing; amputation&lt;br&gt;- Rest pain improvement&lt;br&gt;- Increased TBI in 6 months</td>
</tr>
<tr>
<td>(Shigematsu et al., 2010)</td>
<td>2010</td>
<td>HGF; plasmid</td>
<td>27/13</td>
<td>2 sessions, 4 weeks apart, x 8 IM injection x 8 of 0.5 mg (each session)</td>
<td>- Reduced ulcer size&lt;br&gt;- Increased QoL&lt;br&gt;- Equal rest pain, ABI</td>
</tr>
<tr>
<td>(Morishita et al., 2011)</td>
<td>2011</td>
<td>HGH; plasmid</td>
<td>22/0</td>
<td>2 or 4 mg x 2 IM injection</td>
<td>- Improved ABI&lt;br&gt;- Increased peak walking time in 7 patients&lt;br&gt;- Decreased ulcer size and rest pain</td>
</tr>
<tr>
<td>(Belch et al., 2011)</td>
<td>2011</td>
<td>FGF-1; plasmid</td>
<td>127/63</td>
<td>4 sessions, 2 weeks apart, IM injection x 8 of 0.5 mg (each session)</td>
<td>- Equal major amputation rate; equal death rate</td>
</tr>
</tbody>
</table>

Table 1 **Therapeutic Angiogenesis in PAD clinical trials in the last decade.**

For each trial reference information, year, treatment, number of patients, route of administration and results are included. Studies are arranged in chronological from oldest to most recent. Intramuscular (IM), intra aortic (IA), ankle brachial index (ABI), quality of life (QoL), toe brachial index (TBI).
1.4 Process of Angiogenesis

Neovascularization, the formation of functional new blood vessel, is a multistep process consisting of vasculogenesis, angiogenesis and arteriogenesis (Testa et al., 2008). Vasculogenesis generally describes the process during embryonic development where precursor angioblasts differentiate into endothelial cells and assemble the primitive vascular network (Carmeliet & Jain, 2011). Vasculogenesis can also occur in adult tissue that are in chronic state of ischemia (Carmeliet, 2000). Subsequent sprouting, branching and expansion of the primitive network into a complex and mature vasculature happen through the process of angiogenesis (Carmeliet & Jain, 2011). Angiogenesis is generally hypoxia-induced where hypoxia induced factors affect endothelial cells and starts a cascade of events that lead to the expansion of the vasculature through formation of new capillary beds. Arteriogenesis re-adjusts the vasculature to optimize blood flow (Carmeliet, 2000). Unlike angiogenesis, arteriogenesis is triggered by shear stress and is characterized by the forming collateralization of the arterial system and maturation of newly formed vasculature into arterioles or arteries through the recruitment of smooth muscle cells (Carmeliet, 2000).

I. Initiation.

In healthy vasculature, quiescent endothelial cells are maintained by ensheathed pericytes that release cell-survival signals such as VEGF and Ang-1. During hypoxia, an angiogenic signal is triggered with upregulation of nitric oxide (NO)-induced vasodilation. VEGF increases permeability of the endothelial layer to allow for the extravasation of plasma proteins that will provide a provisional extracellular scaffold for
migrating endothelial cells (Carmeliet & Jain, 2011). Permeability is increased by the formation of fenestrations and by the relocation of junction molecules such as platelet endothelial cell adhesion molecule (PECAM)-1 and vascular endothelial (VE)-cadherin (Carmeliet, 2000; Madeddu, 2005; Polverini, 2002).

II. Matrix degradation.

The conversion of plasminogen to plasmin by Urokinase plasminogen activator results in recruitment of inflammatory cells. Angiopoietin-2 (Ang-2) upregulation causes secretion of metalloproteases MMPs by inflammatory cells that will lead to digestion of the endothelial basal lamina. Matrix degradation allows for migration of endothelial cells and the release of proangiogenic factors such as insulin-like growth factor (IGF-1), basic fibroblast growth factor (bFGF) and VEGF. The antagonistic function of Ang-2 also competes with Ang-1 for binding to Tie-2 receptor and results in interrupted peri-endothelial cell support, which further contributes to vessel destabilization (Carmeliet, 2000; Madeddu, 2005; Polverini, 2002).

III. Cell migration and proliferation.

The growth factors secreted from endothelial cells and inflammatory cells recruit circulating endothelial cell to migrate and proliferate at the angiogenic site (Madeddu, 2005; Polverini, 2002).
IV. Tube formation.

The newly recruited endothelial cells begin to adhere to the preexisting endothelial layer forming solid chords and elongating the vessel. The solid chord then forms into a tubular vessel with a lumen through processes mediated by VEGF and several integrins. Then, Ang-1 contributes to promoting increase in vessel diameter. Further sprouting could occur from the newly formed venules or the venule could further divide into smaller capillaries through the process of intussusception (Carmeliet, 2000; Madeddu, 2005; Polverini, 2002).

V. Maturation.

The angiogenic growth of endothelial cells is slowed by transforming growth factor B (TGF-B). Ang-1 and NO stabilize the newly formed vascular bed through recruitment of pericytes. Platelet derived growth factor (PDGF) mediates endothelial cell into formatting organized 3 dimensional network. Integrins expressed by endothelial cells begin to mediate extracellular matrix deposition for assembly of the endothelial basal membrane (Carmeliet, 2000; Madeddu, 2005; Polverini, 2002; Yancopoulos et al., 2000). Unstable and unorganized microvessels will eventually regress to minimize resistance to flow (Schaper & Scholz, 2003). In stabilized vasculature, quiescent endothelial cells survive and are protected against insults by maintenance signals such as VEGF, angiopoietin-1 (Ang-1) and fibroblast growth factors (FGF). Vascular regression can happen if there is early endothelial senescence (Carmeliet et al., 1999).
1.5 **Vascular endothelial growth factor – A (VEGF-A)**

VEGF-A is a potent angiogenic cytokine that is highly specific for vascular endothelial cells (Neufeld, Cohen, Gengrinovitch, & Poltorak, 1999). There are various spliced isoform of VEGF-A but the most common isoforms are VEGF$_{165}$ and VEGF$_{121}$ (Polverini, 2002; Robinson & Stringer, 2001). VEGF-A is a critical molecule for promoting angiogenesis as it induces endothelial cell migration, proliferation and ultimately leads to new blood vessel formation (Ferrara, 2000). In vivo VEGF-A delivery experiments has shown that VEGF-A induces angiogenesis as well as permeabilization of blood vessels, and plays a central role in the regulation of angiogenesis (Ennett, Kaigler, & Mooney, 2006). The importance of VEGF-A as a regulator of angiogenesis has been
demonstrated in very early studies of gene disruption in mice. Animals lacking even one of the two VEGF alleles died before birth due to defects in development of the cardiovascular system (Ferrara et al., 1996). The VEGF-A cytokine binds to VEGFR-1 (flt-1) and VEGFR-2 (flk-1) tyrosine kinase receptors to mediate angiogenesis and vascular maturation (Neufeld et al., 1999). The two receptors share great homology but differ greatly in their roles. When VEGF-A binds to VEGFR-2, it mediates proangiogenic molecular pathways including cell proliferation (through Raf pathway), migration (p38/MAPK pathway) and survival (PI3K/Akt pathway). Conversely, VEGFR-1, while having a much greater affinity of VEGF-A, it has a lower tyrosine kinase activity (Sawano, Takahashi, Yamaguchi, Aonuma, & Shibuya, 1996) and acts as a “VEGF trap” to suppress VEGFR-2 pro-angiogenic signaling.

1.5.1 Role of VEGF-A in Ischemia

VEGF-A expression is potentiated during hypoxic conditions. In ischemic tissue, there is a deficiency in vascular supply of oxygen and results in increase hypoxia inducible factor (HIF). HIF then induces increased protein expression of VEGF-A protein through stabilization of VEGF-A mRNA (Ryan et al., 2000). Evidence suggests that VEGF-A expression is induced by hypoxia and is a potent mediator of hypoxia-induced angiogenesis (Shweiki, Itin, Soffer, & Keshet, 1992). In-vivo experiments, VEGF-A has also shown to function as a neuroprotector to promote neurogenesis and cerebral angiogenesis in cerebral ischemia rat models (Sun et al., 2003). However, VEGF-A alone will produce formation of immature leaky vessels and is insufficient maintaining functional vasculature. While VEGF-A induced activation of endothelial cell receptor
VEGFR-2 is important for vasculogenesis and early angiogenesis, Ang-1 and its receptor Tie-2 is critical for blood vessel remodelling and maturation (Thurston, 2002). Smith et al. have showed that while VEGF-A plasmid alone was insufficient to maintain vascular integrity, a dual therapy with temporal separation delivery of VEGF-A and Ang-1 plasmid resulted in sustained improved perfusion and vascular density in a hind limb ischemia rat model (Leong-Poi et al., 2007).

1.5.2 Role of VEGF-A in Diabetes

Due to the importance of VEGF-A in angiogenesis and vascular maintenance, disturbance of VEGF-A and its receptors can lead to complications. Studies have demonstrated that patients with diabetes and hypertension have elevated plasma VEGF-A levels that correlates with increased endothelial dysfunction and overall increased cardiovascular risk (Felmeden et al., 2003). Elevated VEGF-A contributes to the development of a proliferative diabetic retinopathy, which is common in diabetic patients. In proliferative diabetic retinopathy, high blood glucose levels damage the endothelial cell lining of the eye’s capillaries, leading to hypoxia due to poor circulation. The hypoxic state then triggers an increase in levels of VEGF-A in the vitreous, which promotes the growth of abnormal leaky capillaries (Caldwell et al., 2003). Additionally, VEGFR1 and VEGFR2 receptor expressions are abnormal in diabetic conditions. Diabetic mice have markedly lower VEGFR-2 protein that correlated with impaired perfusion recovery after ischemia insult and decreased capillary density (Dokun, Chen, Lanjewar, Lye, & Annex, 2014). Hazarika et al, utilizing type 2 diabetic mice model,
found that in the absence of ischemia, diabetes showed decreased soluble and membrane-bound VEGFR-1, decreased phospho-AKT/AKT and phospho-endothelial NO synthase/endothelial NO synthase, and no change in VEGFR-2 (Hazarika et al., 2007). After ischemia, both diabetic and non-diabetics had comparable increases in VEGF-A, VEGFR-1 and soluble VEGFR-1 expression, but the fold increase was significantly greater in diabetes (Hazarika et al., 2007). These studies suggested that diabetes associated deficient angiogenesis after ischemia is contributed by maladaptive changes in VEGF ligand/receptor expression that could account for impaired angiogenesis.

The exact mechanism and molecular pathway responsible for the changes in VEGF, VEGFR-1 and VEGFR-2 remain largely unknown. However, Warren et al. have proposed a possible mechanism to describe the changes of VEGFR-2 in the setting of hyperglycemia (Warren, Ziyad, Briot, Der, & Iruela-Arispe, 2014). In that study, Warren et al. demonstrated that in hyperglycemic conditions, increased ROS can induce ligand independent phosphorylation of VEGFR-2 in the golgi resulting in decreased VEGFR-2 at the cell surface (Warren et al., 2014). Despite the effort of various VEGF-A therapy studies for chronic ischemia in diabetes, the beneficial effects remain modest.

1.6 Micro-RNAs (miRNAs)

In the last decade, a novel class of gene regulators called micro-RNAs (miRNAs) has gained great attention. These miRNAs represent a class of evolutionarily conserved small (~22 nucleotides long) regulatory non-coding RNAs that regulate the expression of large number of genes, by repressing gene expression post-transcriptionally by targeting
3′-untranslated regions of messenger RNAs (mRNAs) (Stark, Brennecke, Bushati, Russell, & Cohen, 2005). Genomic analysis revealed that majority of the miRNAs is located within introns of genes (Rodriguez, Griffiths-Jones, Ashurst, & Bradley, 2004). With the help of genome sequencing, computational analysis and microarrays, up to 1000 miRNAs are predicted to exist in the human genome, each of which could potentially target hundreds of mRNAs (Lamont et al., 2010; Lewis, Burge, & Bartel, 2005; Lewis, Shih, Jones-Rhoades, Bartel, & Burge, 2003). The ability of one miRNA to target multiple mRNAs, especially within the same intracellular pathway, as well as the possibility of targeting one mRNA by several miRNAs, adds further complexity to their ability to regulate gene expression (Lim et al., 2005). miRNAs have been shown to participate in a multitude of cellular process, and their dysregulation contributes to the pathophysiology development of many different human pathologies, including cancer (Esquela-Kerscher & Slack, 2006; Heneghan, Miller, & Kerin, 2010) and heart disease (M. Han, Toli, & Abdellatif, 2011; Mishra, Tyagi, Kumar, & Tyagi, 2009). Current evidence indicates that miRNAs are important regulators of cardiovascular development and disease, and coupled with their ability to specifically target particular cellular pathways, makes the possibility of exploiting miRNAs to develop diagnostic or therapeutic strategies extremely attractive.

1.6.1 miRNA biosynthesis

miRNA biosynthesis starts in the nucleus where a several kilobase pri-miRNA is transcribed (Figure 2). The nuclear RNase III Drosha then process the pri-miRNA into a hairpin-shaped premature pre-miRNA of ~70 nt. Drosha on its own exhibits little
enzymatic activity and requires the help of the regulatory subunit Pasha (a RNA binding domain) and other cofactor proteins (Denli, Tops, Plasterk, Ketting, & Hannon, 2004). The flanking region and the larger terminal loop of the pri-miRNA has been shown to be critical for recognition by Drosha and Pasha for processing (J. Han et al., 2006; Zeng & Cullen, 2005). The pre-miRNA is exported out of the nucleus into the cytoplasm by exportin5 (Exp5) and its Ran-GTP cofactor. In the cytoplasm the pre-miRNA is further processed by the cytoplasmic RNAse III Dicer and the transactivation-responsive RNA-binding protein (TRBP) into a miRNA duplex intermediate composed of two strands. The final mature miRNA is however, single stranded. Studies have suggested that the strand with less stable hydrogen bonding at its 5’ end is stabilized and eventually become the mature miRNA while the other strand is degraded. However, there is emerging evidence that in certain miRNAs, such as miR-126, both strands of the duplex can be present and having very distinct target mRNAs (Fish et al., 2008; Schober et al., 2014). The mature miRNA are then transferred to a family of conserved proteins called Argonautes and guides the whole RNA-induced silencing complex (RISC) towards the targeted mRNA to cause transcription repression (Meister et al., 2004). The targeted mRNA can be degraded or simply suppressed depending on the level of complimentary between the miRNA and the target mRNA (Bartel, 2004). One miRNA can control multiple genes and one gene can be controlled by multiple miRNAs (Bartel, 2004). Although small in size, miRNAs play critical roles in development, cardiovascular diseases, angiogenesis, oncogenesis and diabetes (Y. Wang, Stricker, Gou, & Liu, 2007).
Figure 2 microRNA biogenesis.

miRNA are transcribed by RNA polymerase II as long precursors (pri-miR). Precursors are processed twice, once in the nucleous by the Drosha enzyme that gives rise to the pre-microRNA, and finally in the cytoplasm by the Dicer enzyme in order to obtain the short duplex microRNA molecule. One of the two strands of the miR duplex is loaded in the multiprotein RISC complex and is able to recognize the target sequence (Mack, 2007).

1.6.2 Therapeutic Applications of miRNAs

The identification of specific miRNAs as key regulators of angiogenesis has opened a new avenue for cardiovascular therapeutics. There are several important advantages to miRNAs as therapeutic agents. Their small size and conserved sequence across species make them more amenable to translation to product development for human application. In addition, the ability of one miRNA to target multiple mRNAs that function in the same or closely related intracellular pathway or signaling cascade is an advantage over targeting one specific gene. Specifically, in vivo inhibitors of microRNA function and microRNA mimics (agonists) are intriguing as potential therapies for cardiovascular diseases. AntimiRs are antisense oligonucleotides with the reverse
complementary sequence of the target miRNA that effectively competes with the mRNA target, thus inactivating pathological miRNAs and avoiding down-regulation of important targets. Several chemical modifications have been performed to produce inhibition of miRNA function in vivo. For example, one class of antimiRs is conjugated to cholesterol (antagomiR) to facilitate cellular uptake and action in vivo. In this fashion, antagomiR to miR-122, whose function in the liver is required for cholesterol biosynthesis, results in upregulation of several genes targeted by miR-122 (Esau et al., 2006; Krutzfeldt et al., 2005), leading to reduced plasma cholesterol levels. Conversely, miRNA mimics (synthetic RNA duplexes in which one strand is identical to the miRNA sequence, designed to “mimic” the function of the endogenous miRNA) may lead to down-regulation of target genes (Sucharov, Bristow, & Port, 2008; Villeneuve et al., 2010; Xiao et al., 2007).

1.6.3 Role of miRNAs in Cardiovascular Diseases and Angiogenesis

Studies have demonstrated that miRNAs regulate key genetic programs in many aspects of cardiovascular biology, including cardiac development, endothelial function, lipid metabolism, ventricular hypertrophy and remodeling, and postinfarction arrhythmias. Importantly, miRNAs participate in angiogenesis and endothelial cell function, inflammatory cell recruitment, and in the response to vascular injury. The silencing or disruption of Dicer, the rate-limiting enzyme involved in the maturation of miRNAs, results in defective blood vessel formation and maintenance leading to embryonic lethality at 12.5 to 14.5 embryonic days (Giraldez et al., 2005; W. J. Yang et al., 2005). siRNA-mediated knock-down of Dicer in human endothelial cells results in
impaired development of capillary-like structures, diminished tubule formation and reduced endothelial cell migration (Kuehbacher, Urbich, Zeiher, & Dimmeler, 2007; Shilo, Roy, Khanna, & Sen, 2008). Loss of miRNA function in endothelial cells largely affects proteins that play a role in endothelial cell biology and angiogenic responses, such as Tie-2, VEGFR2 and endothelial nitric oxide synthase (eNOS). The roles of specific individual miRNAs in angiogenesis are now being elucidated. In fact, the term “angiomiR” has been adopted to describe specific miRNAs that regulate angiogenesis (S. Wang & Olson, 2009). In chronic ischemia, hypoxia inducible factor (HIF) act as a transcription factor for miRNA expression during hypoxia(Kulshreshtha, Davuluri, Calin, & Ivan, 2008). miRNA expression profiling in endothelial cells has identified miRNAs that may regulate angiogenic responses (Kuehbacher et al., 2007; Poliseno et al., 2006), including miR-126 (Fish et al., 2008), miR-210 (Fasanaro et al., 2008), miR-296 (Wurdinger et al., 2008), just to name a few. Table 2 summarizes a list of miRNAs and their functional role in angiogenesis.
<table>
<thead>
<tr>
<th>miRNA</th>
<th>Angiogenic function</th>
<th>Associated complications</th>
<th>Predicted targets</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>miR-21</td>
<td>- Induce migration of angiogenic progenitor cells.</td>
<td>- Induction of cardiomyocyte hypertrophy and restenosis.</td>
<td>PTEN, PDCD4, RhoB</td>
<td>(Fleissner et al., 2010)</td>
</tr>
<tr>
<td></td>
<td>- Induce dedifferentiation and migration of SMC.</td>
<td></td>
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<tr>
<td>miR-17/92</td>
<td>- Overexpression in tumor cells and plays an important role in tumor formation.</td>
<td>- Development of various cancers.</td>
<td>PTEN, E2F2, E2F3, TGFBRII, TSP-1, CTGF</td>
<td>(Mogilyansky &amp; Rigoutsos, 2013)</td>
</tr>
<tr>
<td></td>
<td>- Antiangiogenic in endothelial cells during ischemia</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>miR-130a</td>
<td>- Antagonizes the inhibitory effect of GAX and HoxA5 in endothelial cell migration,</td>
<td>- Increased circulating levels in atherosclerosis obliterans.</td>
<td>GAX, HoxA5</td>
<td>(Y. Chen &amp; Gorski, 2008)</td>
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<tr>
<td></td>
<td>tube formation and proliferation.</td>
<td></td>
<td></td>
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<tr>
<td>miR-320</td>
<td>- Upregulation of miR-320 in diabetic microvascular endothelial cells resulted in</td>
<td>- Decreased expression of miR-320 in ischemic reperfusion hearts.</td>
<td>IGF-1</td>
<td>(X. H. Wang et al., 2009)</td>
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<td></td>
<td>decrease IGF-1 and impaired angiogenesis in diabetes</td>
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<tr>
<td>miR-210</td>
<td>- Hypoxia induced overexpression stimulates capillarity like structure formation</td>
<td>- Upregulated in hypoxia induced expression in cancer cell lines.</td>
<td>Ephrin-A3</td>
<td>(Fasanaro et al., 2008)</td>
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<td></td>
<td>and VEGF driven migration in endothelial cells.</td>
<td></td>
<td></td>
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<tr>
<td>miR-214</td>
<td>- Involved in exosome mediated endothelial cell signaling.</td>
<td>- Upregulated in heart failure patients and hypertrophic hearts.</td>
<td>XBP1</td>
<td>(Duan et al., 2015)</td>
</tr>
<tr>
<td></td>
<td>- High miR-214 in exosomes represses endothelial senescence and blood vessel</td>
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<tr>
<td></td>
<td>formation.</td>
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<tr>
<td>miR-296</td>
<td>- Mediate angiogenesis though through decreasing expression of HGS-mediated VEGFR2</td>
<td>- Increased expression in human brain tumor endothelial cells.</td>
<td>HGS</td>
<td>(Wurdinger et al., 2008)</td>
</tr>
<tr>
<td></td>
<td>degradation in endothelial cells.</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>miR-126</td>
<td>- Mediates embryonic angiogenesis and vascular integrity</td>
<td>- Circulating levels of miR-126 increased in hyperglycemia, diabetes and coronary artery</td>
<td>SPRED-1, PIK3R2, VCAM-1</td>
<td>(Olivieri et al., 2014)</td>
</tr>
<tr>
<td></td>
<td>- Induces proangiogenesis in endothelial cell migration, tube formation and proliferation</td>
<td></td>
<td></td>
<td>(Fish et al., 2008)</td>
</tr>
</tbody>
</table>

Table 2 List of miRNAs that play a critical role in angiogenesis.

The table includes details for each miRNA’s angiogenic function, the associated complications, the predicted targets and the corresponding relevant citation.
1.6.4 Impaired Angiogenesis in Diabetes – Role of miRNAs

More recently, the role of miRNAs in the impaired angiogenic responses in diabetes has been studied. Microarray screening and miRNA network inference identified several miRNAs implicated in the epigenetic regulation of angiogenesis in human diabetes, making miRNA a good biomarker for diagnosis of pre-diabetes and early onset of diabetes (Kong et al., 2011). Endothelial cells exposed to hyperglycemic and hypoxic conditions expressed increased levels of miR-503, and expression of miR-503 in endothelial cells resulted in reduced endothelial proliferation, migration, and matrigel tubule formation (Caporali et al., 2011). Interestingly, miR-503 expression is also increased in the plasma and limb muscle of diabetic patients with critical limb ischaemia undergoing amputation compared with calf biopsies of non-diabetic/non-ischaemic controls (Caporali et al., 2011). Similarly, miR-320 is highly expressed in hyperglycemic conditions and results in decreased expression of various important endothelial growth factors including VEGF-A and insulin growth factor 1 (IGF-1) (X. H. Wang et al., 2009). The miR-221/miR-222 pair is increased in human umbilical endothelial cells (HUVECs) treated with high glucose. miR-221/miR-222 targets c-kit which is a receptor for stem cell factor, and results in decreased EPC migration and homing (Poliseno et al., 2006). More importantly, the expression of miR-126, an important endothelial specific proangiogenic miRNA, is decreased in diabetes (Zampetaki et al., 2010). In addition, high glucose concentrations reduced the miR-126 content within endothelial apoptotic bodies (Zampetaki et al., 2010). These findings would suggest that loss of miRNA-126 play a role in the pathophysiology of impaired angiogenesis in diabetes, and as such represents a potential molecular target for therapeutic angiogenesis in the setting of diabetes.
1.6.5 Role of miR-126 in angiogenesis

miRNA-126 in particular has unique properties that make it an attractive target. It is one of the only endothelial cell-restricted miRNA, and has been shown to regulate vascular integrity and developmental angiogenesis (Fish et al., 2008). Deletion of the endothelial cell–specific miR-126 impairs maintenance of vascular integrity during embryogenesis and reduces angiogenesis after myocardial infarction in mice (S. Wang et al., 2008). The pro-angiogenic effect of miR-126 has been attributed, at least in part, to the repression of Sprouty-related protein-1 (Spred-1) and phosphatidylinositol-3-kinase regulatory subunit (PIK3R2) (also known as p-85β), two negative regulators of VEGF signaling (Fish et al., 2008; Kuhnert et al., 2008), resulting in the promotion of MAP kinase and PI3K signaling in response to VEGF and fibroblast growth factor (Figure 3). In addition to its action on Spred-1 and PIK3R2, miR-126 also targets vascular cell adhesion protein-1 (VCAM-1), thereby regulating the adhesion of leukocytes to the endothelium (Harris, Yamakuchi, Ferlito, Mendell, & Lowenstein, 2008), supporting a role of miR-126 in the vascular inflammation that accompanies angiogenesis. Thus, miR-126 belongs to the family of “pro-angiomiRs” that promote angiogenesis by suppressing negative regulators in angiogenic signaling pathways. While there have been a few preliminary studies examining therapeutic strategies using miR-126 in malignant tumours (Du et al., 2014; B. Liu, Peng, Zheng, Wang, & Qin, 2009) and only several studies examining miR-126 in atherosclerosis (Du et al., 2014; Schober et al., 2014), but only one study has been performed for therapeutic angiogenesis in ischemic vascular disease (Endo-Takahashi et al., 2014). In a miRNA microarray screening experiment involving diabetic patients, it was found that endogenous miR-126 levels (among others miRNAs)
were down regulated in diabetic patients (Zampetaki et al., 2010). The abnormal vasculature in diabetes can be in part explained by the down regulation of this crucial proangiogenic miRNA, which is essential for the VEGF-A signaling to promote adequate angiogenesis. VEGF-A, however, mainly promotes the onset of angiogenesis through the sprouting and formation of immature leaky vessels (Thurston, 2002). The stabilization and maturation of the neovascular vessel is accomplished by the role of Ang-1 (Thurston, 2002). Interestingly, Sessa et al, have shown that miR126 regulates Ang-1 signaling for neovascular branching and vascular maturation (Sessa et al., 2012). This indicates that miRNA-126 not only enhances angiogenic signaling of VEGF-A but also plays a critical role in modulating vessel maturation and formation of a functional vasculature.

Figure 3 VEGF angiogenic signaling pathway involving miR-126. PIK3R2 and SPRED1 are negative inhibitors of the VEGF-A signaling pathway. miR-126 suppresses the expression of PIK3R2 and SPRED1 and therefore enhancing angiogenesis.
1.6.5.1 miR-126 target: Phosphoinositide 3-kinase (PI3K)

One of the reported targets of miR-126 is PI3KR2, the regulatory subunit of PI3K. Class IA PI3K is a key cytoplasmic signaling enzyme that has important intracellular roles such as vascular growth, proliferation, and cellular apoptosis (Hawkins, Anderson, Davidson, & Stephens, 2006; Vanhaesebroeck & Waterfield, 1999). The inhibition of PI3K in isolated mesenteric vascular beds with a synthetic inhibitor has been reported to prevent the abnormal vascular reactivity of diabetic rats (Yousif, 2008). PI3K is generally activated by tyrosine kinase or by Ras. Upon activation, PI3K phosphorylates phosphatidylinositol (4,5)-bisphosphate (PIP2) into phosphatidylinositol 3,4,5-triphosphate (PIP3). PIP3 then acts as second messenger to assist in the activation of the downstream protein kinase B (Akt). Akt is a serine/threonine kinase that is involved in downstream activation of molecular pathways such as mammalian target of rapamycin (mTOR) signaling which important for cell cycle progression, proliferation, and angiogenesis. The class IA PI3Ks is a protein complex of heterodimers consisting of a p110 catalytic subunit and a smaller p85 (PIK3R2) regulatory subunit (Hawkins et al., 2006). Upon phosphorylation of the SH2 domain by tyrosine kinase or Ras, the PIK3R2 regulatory subunit abolishes the inhibitory effect on p110 resulting in increased enzymatic activity PI3K complex (Geering, Cutillas, Nock, Gharbi, & Vanhaesebroeck, 2007; Geering, Cutillas, & Vanhaesebroeck, 2007). The targeted deletion of PIK3R2 in knockout mice exhibits enhanced PI3K signaling giving evidence for the inhibitory role of PIK3R2 in the regulation of PI3K’s catalytic activity (Ueki et al., 2003).
Endogenously, miR-126 targets and decreases the PIK3R2 expression and therefore enhancing the PI3K proangiogenic pathways upon activation by cytokines such as VEGF-A and Ang-1. Interestingly, Sessa et al. showed that an overexpression of PIK3R2 in endothelial cells inhibited Ang-1 dependent Akt activation that resulted in decreased endothelial cells angiogenic sprouting and survival. Additionally, miR-126/- knockout mice displays leaky and lack of hierarchical order vasculature that resembles the abnormalities in Ang-1/- knockout mice (Ahirwar et al., 2014; S. Wang et al., 2008). This then suggests that miR-126 is not only important for VEGF-A induced angiogenesis but is also heavily involved in the process of vessels maturation and stabilization mediated by Ang-1.

1.6.5.2 miR-126 target: Sprouty-related EVH1 domain (SPRED-1)

Another known target of miR-126 is SPRED-1. Spreds is a group of membrane bound proteins that is related to the Sproutys. There are three spreds identified in humans: SPRED-1, SPRED-2 and SPRED3 (Taniguchi et al., 2007; Wakioka et al., 2001). SPRED-1 has been identified as negative regulators for growth factor- and cytokine-induced ERK extracellular-regulated kinase (ERK) activation (Kim & Bar-Sagi, 2004). The Ras-Raf-MAPK-ERK activation pathway is involved in the cell proliferation, differentiation, cell movement, and apoptosis. Endogenously, Spred-1 binds to a protein called Raf, which is an upstream molecule of the MAPK pathway. The binding of the Spred-1 protein inhibits the activation of Raf, stopping the signaling through the remainder of the Ras/MAPK pathway (King et al., 2005). SPRED-1 therefore is an important negative regulator of proangiogenic signaling. The function of SPRED-1 in
MAPK signaling is supported from loss of function studies in neurofibromatosis-1 (NF1)-like phenotype in humans (Brems et al., 2007). NF1 is inherited autosomal dominant neurological disorder caused by germline mutations that affect the Ras/MAPK pathway (Denayer et al., 2011; Messiaen et al., 2009). As NF1 is caused by abnormal activation of the Ras-ERK pathway, the existence and nature of this disorder strongly support the notion that Spred is a negative regulator of the RAS-ERK pathway.

1.7 Gene therapy

Many cardiovascular complications are the result from deregulation of endogenous genes and/or miRNAs. Gene therapy is a strategy that attempts to compensate endogenous gene expression to restore normal function through exogenous administration of plasmid vectors expressing the gene of interest. Unlike the short-lived effect of pharmaceutical drug regimes, gene therapy offers a prolonged cellular protein expression for long-term therapeutic effect. The success of gene therapy largely depends on the development of vectors and delivery techniques that can selectively and efficiently deliver genes to targeted cells with minimal toxicity.

1.7.1 Vectors

Viral vectors: While viral vector delivery system is a popular method of transfection due to its ease of production, having a high functional titer and their ability to infect various cell types (Loiler et al., 2003; Work et al., 2004) the associated side effects including of cytotoxicity, high immunogenicity, an mutagenesis in the host genome (Marshall, 1999) makes this technique non desirable for clinical applications. Fatal
accidental deaths have been reported from studies after systemic administration of adenoviral vectors due to over activation of the innate immune response (Thomas, Ehrhardt, & Kay, 2003). As a result, newer non-viral vector gene delivery techniques are gaining more attention for effective gene therapy.

Non-viral vectors: The strong inflammatory response elicited by viral vector during in vivo gene delivery has boosted research into developing non-viral gene vectors that can be safe for clinical applications. The most common non-viral vectors currently available are lipid based which are economical; provide stable transfection with minimum immunogenicity. Although the transfection efficiency of non-viral vectors is lower when compared to the viral vectors, they are believed to be attractive alternatives for their lack of specific immune response, versatility, ease of large-scale production and simplicity of usage.

While promising, gene and miRNA-based therapy is faced with several challenges before developing into a viable therapeutic modality, including 1) lack of tissue specificity, 2) lack of optimal delivery methods, 3) risk of systemic or off-target effects and 4) poor tissue/organ uptake. While intravenous administration of plasmid (gene) and miRNA mimics has been a widely adapted method of systemic delivery, uptake is preferentially targeted to the liver, spleen and kidney. A non-invasive method of plasmid and miRNA delivery that results in targeted uptake with minimal systemic/off-target effects would be an important advance to the field of gene therapeutics.
1.7.2 Ultrasound Mediated Gene Delivery (UMGD)

UMGD is a novel gene delivery system developed to increase in vivo transfection efficiency of non-viral gene carriers. This technique can achieve high organ specific transfection with minimum invasiveness and immunogenicity. This in vivo delivery system employs the use of ultrasound and cationic lipid microbubbles for targeted gene delivery. The use of microbubbles in combination with ultrasound allows for targeted transfection.

Microbubbles: Microbubbles are small gas-filled lipids. The gas-filled core is made up of high molecular weight gases such as perfluorobutane or sulfur hexafluoride while the outer shell being composed of biocompatible lipids or proteins (Sirsi & Borden, 2009). The low diffusivity and solubility properties of the heavy gas core allows for prolonged stability in the circulation. The rheology of these microbubbles is comparable to that of red blood cells with a mean diameter of approximately 2-4 µm; giving them the property to freely traverse through the microvasculature. Microbubbles have been conventionally used as a diagnostic contrast agent for clinical use with ultrasound (Kaufmann, Wei, & Lindner, 2007). However, with the improvements made on newer generation of microbubbles, these bubbles are serving as a gene carrier vector for in vivo gene therapy.

UMGD: A schematic diagram of the mechanism is shown in Figure 4. First, microbubbles are generated in conjugation with the nucleic acids (plasmid, siRNAs, microRNAs or viral DNA), resulting in DNA/RNA incorporation onto the shell via
electrostatic interactions (charge-coupling). Secondly, the microbubble + nucleic acid complexes are administered intravenously. Thirdly, the complex circulates through the vascular system and a high power ultrasound targeting the desired organ is applied externally. The high frequency ultrasound causes acoustic disruption of the gas-filled microbubbles and facilitates the nucleic acid uptake by the endothelium through formation of microjets and induction of transient pores at the cellular membrane (Christiansen, French, Klibanov, Kaul, & Lindner, 2003; Kodama, Tomita, Koshiyama, & Blomley, 2006). Several other mechanisms like free radical generation, thermal changes in the surrounding liquid, endocytosis etc. have also been proposed.

**Figure 4** Mechanism of ultrasound mediated gene delivery (UMGD).
First, the nucleic acid (plasmid or miRNA) is charge-coupled to cationic lipid microbubbles. The charged coupled nucleic acid/microbubble complex is then administered intravenously, during external focused ultrasound administration to the target ischemic region. The acoustic disruption of the microbubble/miRNA complexes within the microvasculature results in carrier microbubble destruction that assists in targeted vascular transfection.
**UMGD biophysical effects:** The biophysical effects of UMGD are predominately mediated by process of microbubble cavitation, membrane sonoporation and stimulated endocytosis. Microbubble cavitation refers to the volumetric expansion and contraction of microbubbles to ultrasound waves. The behavior of microbubbles is highly dependent on the amplitude of the ultrasound used. At low mechanical index (<0.1), microbubbles experience stable linear oscillation. At high mechanical index (>0.7), the rapid expansion and compression of microbubbles during the acoustic cycle results in forcible collapse of the microbubbles and subsequent shock wave formation perturb the cell membrane integrity and increases membrane permeability (Newman & Bettinger, 2007). Additionally, sonoporation describes the process in which high power ultrasound induces increased plasma membrane permeability through the formation of reversible pores in the plasma membrane (Sundaram, Mellein, & Mitragotri, 2003). Lastly, low intensity ultrasound microbubbles oscillating near the plasma membrane can stimulate endocytosis. Meijering and colleagues have demonstrated that applying low-intensity ultrasound to primary endothelial cells in the presence of microbubbles can lead to increased endocytosis activity (Meijering et al., 2009). Together these biophysical effects of UMGD result in promoting gene uptake across the cell membrane into the targeted cells.

**UMGD In Vivo Applications:** Studies showed the efficacy of UMGD for therapeutic angiogenesis in a rat model of sever chronic hindlimb ischemia with a plasmid DNA encoding both green fluorescent protein (GFP) and VEGF in a bicistronic vector. The results comparing the transfection of UMGD with intramuscular (IM) injection of the VEGF/GFP indicated that despite higher levels of transgene messenger
RNA expression of IM injections, UMGD resulted in greater increase in improved skeletal muscle perfusion of the ischemic leg (Kobulnik, Kuliszewski, Stewart, Lindner, & Leong-Poi, 2009). Although IM injection resulted in local transfection of myocytes and perivascular regions, UMGD resulted in more diffuse transfection of the endothelium of arterioles and capillaries and surrounding myocytes. Other groups confirmed gene delivery of CMV-luciferase plasmids to the heart with UMGD, resulting in high transgene express with minimum off-target effects (Bekeredjian, Chen, Grayburn, & Shohet, 2005). A major advantage of UMGD is the noninvasive nature, which allows for delivery of multiple genes through repeated gene therapy. Using multiple UMGD delivery of plasmid DNA encoding for stem cell factor and stromal cell derived factor-1 in a rat model of myocardial infarction, Fuji et al showed increased vascular density in the peri-infarct region and greater myocardial perfusion and ventricular function compared with untreated animals (Fujii et al., 2011). The use of UMGD for in vivo gene transfer has been applied for targeted gene transfection in organs and tissues that are easily imaged by diagnostic ultrasound including skeletal, heart, kidney, pancreas and tumor (S. Chen et al., 2006; Fujii et al., 2013; Koike et al., 2005; Korpanty et al., 2005; Smith et al., 2012).

1.8  Rationale for the Study

1.8.1  UMGD of VEGF-A and miR-126 for chronic ischemia in diabetes

Given the key role of VEGF-A in promoting angiogenesis and the importance of miR-126 for embryonic neovascularization and vessel maturation, disturbances in the
expression of these two molecules are key factors that contribute to impaired angiogenesis in diabetes. In diabetes, there is a diminished increase of VEGF-A induced angiogenesis during hypoxia. Additionally, circulating levels of miR-126 are significantly decreased in diabetes and hyperglycemia. Due to the crucial role of miR-126 in enhancing VEGF-A induced angiogenesis and Ang-1 mediated vessel maturation, an exogenous source of VEGF-A and miR-126 through gene therapy could provides a novel therapeutic approach to peripheral arterial disease in the settings of diabetes.

While optimism is high for the success of gene therapy, a safe, effective and targeted delivery strategies are crucial. With the use of UMGD, we can effectively and non-invasive deliver miR-126 and VEGF-A plasmid specifically to the vascular endothelium allowing targeted transfection with good specificity and tissue uptake, coupled with minimal systemic, or off-target effects. We have shown that UMGD for gene delivery can be used to transflect chronic ischemic skeletal muscle with human VEGF-165 (hVEGF165), resulting in a significant improvement in tissue perfusion and vascular density, with minimal to no transfection in remote sites, outside the field of ultrasound application(Leong-Poi et al., 2007). UMGD gene therapy is more effective than direct intramuscular administration. As UMGD is an intravascular method of gene delivery, transfection occurs primarily within the endothelial surface of vessels, predominantly arterioles and capillaries, resulting in a more effective angiogenic response. Thus, the advantages of 1) targeted vascular delivery, 2) minimal delivery to remote regions, 3) non-invasive delivery method, 4) effective delivery (of plasmid DNA), makes the technique of UMGD uniquely suited to pro-angiogenic miR-126 and VEGF-A
delivery.

1.8.2 Animal models of PAD in diabetes

The availability of animal models to mimic the human phenotypic pathology is essential for the development of novel therapies. There are several rodent animal models currently available which closely recapitulate the features of diabetes in humans. However, due to the nephropathy resistant nature of most rodents, a model that mimics the progression to renal failure as it is seen in human patients is currently not available. The Animal Models of Diabetic Complications Consortium (AMDCC) has defined criteria for the validation of the murine models of nephropathy. These include: greater than 50% decrease in GFR, greater than 10-fold increase in albuminuria, greater than 50% thickening of the glomerular basement membrane and the presence of advanced mesengial matrix expansion, arteriolar hyalinosis and tublointerstial fibrosis (Brosius et al., 2009). Although the ideal animal model should have all these phenotypes, no current animal model meets them all. These criteria are not strict requirements but are guidelines for future development of diabetic animal models.

Zucker diabetic fatty (ZDF) rats: ZDF rats by comparison, are a well-characterized model of obese Type II diabetes. These rats have a homozygous mutation in their leptin receptor gene (fa/fa) that causes them to develop a metabolic disorder similar to Type II diabetes in humans (Takeda et al., 2005; P. Wang & Chatham, 2004). By 10-12 weeks of age, they develop hyperglycemia, hypercholesterolemia and hypertrygliceridemia, as well as hypertension (Kristiansen et al., 2004). Current evidence
supports impaired angiogenesis in Type II diabetes. Thus, ZDF rats are a more relevant animal model to test pro-angiogenic strategies in pre-clinical studies, one which will allow us to 1) gain further mechanistic insights into the altered biology of angiogenesis in the setting of diabetes, and 2) determine whether our strategies will be able to overcome these limitations to therapeutic angiogenesis in patients with chronic CAD and PAD.

Chronic hindlimb ischemia: We will test our strategies for therapeutic angiogenesis in a well-characterized animal model of chronic hindlimb ischemia (Limbourg et al., 2009), using obese Zucker diabetic fatty (ZDF) rats. A model of chronic hindlimb ischemia was created by the unilateral ligation and stripping of the femoral artery and its proximal branches that reliably results in a reduction of resting perfusion in the proximal hindlimb muscles. Contrast enhanced ultrasound (CEU) perfusion imaging has shown that resting blood flow at 4 weeks post-ligation remains chronically reduced, ~ 50-60% relative to the contralateral non-ischemic leg.
Chapter 2 – Study Objectives

2.1 Objectives and Hypothesis

The overall objective of the study was to examine the proangiogenic effects of miR-126 delivery in a model of chronic hindlimb ischemia and to study the proangiogenic effect of miR-126 and VEGF-A dual delivery therapy for chronic ischemia in a diabetic animal model. In order to achieve this, the study were divided into two sections: 1) miR-126 therapy in chronic hindlimb ischemia and 2) miR-126 and VEGF-A therapy for chronic hindlimb ischemia in diabetes.

2.1.1 miR-126 therapy in chronic hindlimb ischemia

A rat model of chronic hindlimb ischemia was used to study the proangiogenic effects of miR-126. The focus of the study was to examine functional and structural changes in the chronic ischemic model after exogenous delivery of miR-126 to the targeted ischemic muscle.

2.1.1.1 Hypothesis

The exogenous delivery of miR-126 to the ischemic muscle is expected to induce angiogenesis resulting in improved vascular architecture and microvascular blood flow. The pro-angiogenic effect will be mediated by the decrease of PIK3R2 and SPRED-1 and therefore enhancing cytokine-induced angiogenesis at the ischemic tissue.
2.1.1.2 Approach

Microvascular blood flow was measured by CEU. The perfusion measurements of the ischemic leg were compared to contralateral non-ischemic leg for % MBF. To access the vascular architecture, post-mortem FMA analysis was used to evaluate the vessel length and vessel volume of ischemic muscle across the treatment groups. Additionally, to access the mechanistic pathway of miR-126, the protein levels of PIK3R2 and SPRED1 were quantified by western blot.

2.1.2 miR-126 and VEGF-A therapy for chronic hindlimb ischemia in diabetes

The second part of the study is to develop and optimize a dual gene therapy with VEGF-A and miR-126 to treat chronic ischemia in the settings of diabetes. In order to accomplish this goal, I tested the therapy in a clinically relevant model of chronic hindlimb ischemia in the setting of diabetes, where angiogenic responses are impaired. The functional and structural changes of the ischemic tissues were examined after the treatment. The successful completion of this study offers mechanistic insights into potential miRNA therapies to promote angiogenesis in diabetic chronic ischemia.

2.1.2.1 Hypothesis

The combined miR-126 and VEGF-A angiogenic gene therapy is expected to overcome deficits in angiogenesis in diabetes through the mechanism of miR-126 to suppress angiogenic inhibitors while enhancing VEGF production by gene therapy.
2.1.1.2 Approach

The diabetic model was characterized through blood glucose monitoring and measurement of circulating miR-126 levels by qPCR. Microvascular blood flow was measured by CEU. The perfusion measurements of the ischemic leg were compared to contralateral non-ischemic leg for % MBF. To access the vascular architecture, post-mortem FMA analysis was used to evaluate the vessel length and vessel volume of ischemic muscle across the treatment groups. Additionally, to access the mechanistic pathway of miR-126, the protein levels of PIK3R2 and SPRED1 were quantified by western blot.

The successful completion of this study offers mechanistic insights into the pathophysiological processes that underlie chronic ischemia and into miRNA therapies to promote angiogenesis in diabetes.
Chapter 3 – Methods and Experimental Protocol

3.1 miRNA-126 In Vitro HUVEC Transfection

To determine the effect of miR-126 on specific angiogenic transcript and protein concentrations, human umbilical vein endothelial cells (HUVECs) were transfected and assayed using quantitative real-time polymerase chain reaction (qRT-PCR). HUVECs were cultured and transfected with miR-126 according to the manufacture’s instructions using the siPORT NeoFX Transfection Agent (Applied Biosystems Inc.). Briefly, HUVECs were grown in DMEM with 10% Fetal Bovine Serum (CellGro) to 80-percent confluency at 37°C and 5% CO₂. Adherent cells were washed and trypsinized. Trypsin was inactivated by re-suspending the cells in DMEM with 10% FBS (Invitrogen). The SiPORT NeoFX transfection agent was diluted in Opti-MEM I medium (Applied Biosystems Inc.) and incubated for 10 minutes at room temperature. miR-126 was diluted into 50µL Opti-MEM I medium at a concentration of 30nM. Diluted microRNA and diluted siPORT NeoFX Transfection agent were mixed and incubated for another 10 minutes at room temperature to allow transfection complexes to form and subsequently dispensed into wells of a clean 6-well culture plate. The HUVEC suspension was overlaid onto the transfection complexes and gently mixed to equilibrate. Transfected cells were incubated at 37°C and 5% CO₂ for 24 hours.

3.2 In Vitro Endothelial Cell Functional Analysis

In vitro endothelial cell function in response to miR-126 was assessed by standard Boyden Migration and Matrigel Tubule formation assays (Kuliszewski et al., 2013). For
migration assays, VEGF (100 ng/mL) was placed in each well of the Boyden companion plate. An 8µm (pore size) insert was placed in each well containing the HUVEC suspension. After incubation, each Boyden chamber insert was gently washed, and non-adherent cells were removed. Cells were fixed and stained using DiffQuik (Sigma) and allowed to dry overnight. The membrane was removed and mounted on a slide for quantification using light microscopy with a 20X objective (Figure 5).

*In vitro* angiogenesis / matrigel capillary tube formation assay was performed to assess the effects of miR-126 on capillary-like network formation in a 96-well plate. 60 µl of Matrigel™ (Becton Dickinson, Mississauga, ON, CA) was added to each well of the 96-well tissue culture plate (Corning, Tewksbury, MA, USA) and allowed to polymerize at 37°C, 5% CO₂ for 45 minutes under sterile conditions. 1x10⁴ HUVECs transfected with either scramble miR, miR-126 or anit-miR-126 and were suspended in 100 µl Medium 199 (Gibco, Life Technologies Inc., Burlington, ON, Canada) enriched with growth factors and serum and plated onto the surface of the matrigel. All the wells were stimulated with VEGF (50 ng/ml) to facilitate *in vitro* tube formation. Control group (n=8) was also included which did not undergo any transfection, receiving the same conditions of VEGF stimulation. The capillary-like network formation was observed at regular intervals until 28 hours and photomicrographs were recorded at each time interval for quantification (Figure 5). The total number of nodes and ends of the tubes were quantified. Tube number was quantified using the formula, tubes = (number of nodes + number of ends)/2. Data was expressed as mean ± SEM.
Figure 5 *In-vitro angiogenesis assay outline*. HUVECs were transfected with either scrambled RNA, miR-126, anti-miR-126 or non-transfected controls. The cells were then divided for matrigel capillary tube formation and Boyden migration assay.

### 3.3 *In vivo* Experimental Protocol

Unilateral hindlimb ischemia was induced by ligation and striping of a segment of the femoral artery in Fischer and Zucker diabetic fatty (ZDF) rats on day 0. This model of ischemia induces an immediate loss of blood flow and skeletal muscle oxygen tension. Animals were allowed to recover for 14 days after the ligation surgery for endogenous neovascularization. At 14 days after surgery, baseline blood flow was measured with muscle flow plateauing at approximately 50% of the contralateral non-ischemic leg. Animals were then divided into various UMGD treatment groups (Figure 6). In the control group, animals were not treated. In the VEGF, scramble and miR-126 alone group, animals were treated with UMGD of VEGF-A plasmid, scramble (oligonucleotide
sequence with no known targets) and miR-126 respectively. In the dual group, animals were treated with UMGD of VEGF-A plasmid followed by UMGD of miR-126 sequentially on the same day. At day 28 post-ligation (14 days after UMGD treatment), blood flow was reassessed and tissue from the gastrocnemius muscles was collected for histological and fluorescent microangiography. A subset of animals from each treatment group was sacrificed at day 15 and 17 for protein and mRNA expression analysis.

Figure 6 Timeline of the study design.
Unilateral ligation of the femoral artery was done at day 0. Baseline blood flow was measured by contrast-enhanced imaging (CEU) 14 days after ligation. Blood flow was measured again at day 28 post-ligation. Animals were sacrificed at 28 days post-ligation for histological and fluorescent microangiography assays. A subgroup of animals in each group was also sacrificed for protein and mRNA expression analysis (not shown).
3.4 Animal Preparation

The animal protocol was approved by the Animal Care and Use Committee at Keenan Research Center for Biomedical Science at St. Michael’s Hospital, University of Toronto. To establish the chronic hindlimb ischemia model, 103 Fischer rats (180-200g), 10 Zucker lean rats (10-12 weeks old) and 77 Zucker diabetic fatty (ZDF) rats (10-12 weeks old) were anesthetized through inhalation of 2% isoflurane and 0.2 lpm medical air and using aseptic techniques, the left femoral artery was exposed and ligated at 4 sites with sutures followed by stripping of a segment of the vessel. The incision was sutured and stapled and animals received post-op treatment with Anafen (5mg/kg) twice daily for 24 hours. For contrast enhanced ultrasound (CEU) perfusion and UMGD, animals were anesthetized in the same manner and the jugular vein was cannulated for steady intravenous infusion of the microbubbles. Post-op, animals were recovered with Anafen (5mg/kg) twice daily for 24 hours.

3.5 Circulating miR-126 levels and glucose levels

To measure the circulating miR-126 and glucose levels in Zucker lean and ZDF rats, 0.5-1.0mL of tail vein blood was obtained at a non-fasting state and stored in EDTA-containing BD vacutainers (BD Biosciences). Glucose levels were measured using OneTouch Ultra®2 glucometer. To measure miR-126 levels, the blood samples were centrifuged at high speed (2500 xg) for 20 minutes at 4°C. The supernatant layer containing the plasma was carefully collected. RNA was extracted from the plasma using TRIzol® LS reagent and Aurum Total RNA mini kit (Bio-Rad). A qScript microRNA
cDNA Synthesis kit (Quanta Biosciences) was used for reverse transcription (RT) reactions. The RT reaction consisted of two separate reactions: 1) poly(A) tailing reaction and 2) first-strand cDNA synthesis. Poly(A) tailing reaction contained 2 µL of poly(A) tailing buffer (5x), 1µg of total RNA, and 1 µL of poly(A) polymerase and was completed in following steps: 37 °C for 60 min followed by 70°C for 5 min. First-strand cDNA synthesis reaction contained 10 µL from the poly(A) tailing reaction, 9 µL of microRNA cDNA reaction mix and 1 µL of qScript reverse transcriptase together incubated at 42 °C for 20 min followed by 85 °C for 5 min. PerfeCta Universal PCR primer® and miR-126-3p primers (UCGUACCGUGAGUAAUAAUGCG) were used to detect miR-126-3p levels with ViiA 7 RT-PCR (Life technologies). Each reaction was carried out in a total volume of 10µL containing: 1µL RT product, 0.2 µL of universal primer, 0.2 µL of miR-126-3p primer, 5µL of iScript SYBR Green reagent (Bio-Rad), and 3.6 µL nuclease free water. Real time PCR was completed at the following temperatures: activation at 95°C for 2 minutes followed by 40 cycles at 95°C for 5 seconds and 60°C for 30 seconds. The melt curve was developed based on 95°C for 15 seconds, 60°C for 30 seconds and 95°C for 5 seconds. The relative expression level of each individual miRNA after normalization to miR-RNU- 6B was calculated using the 2-ΔΔCT method.

3.6 Ultrasound-Mediated Gene Delivery

3.6.1 Microbubble Preparation

Cationic lipid microbubbles were created by sonating an aqueous dispersion of 1 mg•ml⁻¹ polyethyleneglycol-40 stearate (Sigma), 2 mg•ml⁻¹ distearoyl
phosphatidylcholine (Avanti) and 0.4 mg•mL⁻¹ 1,2-distearoyl-3-trimethylammoniumpropane (Avanti) with decafluorobutane gas. These microbubbles have a zeta potential of +60 mV. Microbubble concentrations were determined using a Coulter Multisizer IIe (Beckman-Coulter), prior to intravenous administration. The size distribution of the microbubbles ranged from 1 to 4 μm (<2 μm: ~55%, 2-3 μm ~31%, 3-4 μm: ~9%, >4 μm: 5%).

3.6.2 Microbubble and nucleic acid preparation

For gene delivery, cationic lipid microbubbles (1x10⁹) were charge-coupled to either 500ug of VEGF-A plasmid DNA or 2.5ug of miR-126 mimics. Microbubbles with a cationic lipid shell (zeta potential of +60mV) were incubated with VEGF-A plasmid DNA or miRNA, resulting in approximately 6,700 plasmids or 60,000 of miRNA covering each microbubble. The VEGF-A plasmid vectors were constructed by incorporating the gene for human VEGF₁₆₅ into the vector pcDNA3.1(+) containing a cytomegalovirus (CMV) promoter. Mature miR-126 mimics had the sequence of UCGUACCAGUGAUAAUUGCG and were purchased from Life Technologies.

3.6.3 MicroRNA-Microbubble Conjugation Assay

Cationic microbubbles were charge-coupled to a varying concentration range of miR-126 under different conditions to optimize for miR-microbubble binding. For miR-microbubble binding assays, cationic microbubbles were prepared as described above and washed twice with 10mL of 0.16M phosphate-buffered saline (Cellgro). Cationic microbubbles and miR-126 were conjugated by adding 0μg, 1μg, 2.5μg, 5μg or 10μg of
miR-126 to $1 \times 10^9$ microbubbles in a solution containing 1mL 0.16M PBS. The miR126-microbubble solutions were incubated for 30 minutes on a flat rocker (VWR) to facilitate miR-microbubble interaction. Following miR-microbubble binding, the supernantant containing unbound miR-126 was discarded and the remaining miR-microbubble were washed twice using 0.16M PBS to completely remove unbound miR. Microbubble concentrations were determined using a Beckman Coulter Multisizer (Beckman-Coulter) to standardize concentrations and monitor microbubble loss during the binding and washing process. The microbubble-miR126 mixtures were disrupted through vortexing to the microbubbles for 1 minute. The bound miR-126 concentration was determined using the NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific) at 260nm. The concentration of miR-126 was then normalized for the number of microbubbles in solution to determine the number of miR-126 molecules associated with each microbubble. The data was modelled to the equation $y = \frac{B_{\text{max}}x}{K_d + x}$ to determine $B_{\text{max}}$ (maximum number of miR-126 molecules bound per microbubble) and $K_d$ (the dissociation constant) by computer software (GraphPad).

3.6.4 In-vivo delivery

In-vivo UMGD of VEGF-A plasmid and miR-126 UMGD was conducted with a Sonos 5500 ultrasound machine (Philips Healthcare, Andover, Massachusetts) using ultraharmonic imaging (single pulse technique). The S3 probe was positioned to target a transverse section of the distal hindlimb gastrocnemius muscle and release single frames of high power (120 V, mechanical index $> 1.6$) ultrasound every 5 sec. At 1.3 MHz and a transmit power of 0.9 W, peak negative acoustic pressure by needle hydrophone was –
2100 kPa (assuming tissue attenuation coefficient of 0.3 dB/cm/MHz, at tissue depth of 1 cm). A 0.5 mL bolus of the microbubble/nucleic acid solution was injected, then 1 mL was continuously infused over 10min (infusion pump Model AS50, Baxter). The cannula and stopcock were then flushed with 1 mL saline (0.4 M) and ultrasound delivery continued for an additional 10 min to destroy any residual circulating microbubble/nucleic acid complexes. During delivery, the probe was adjusted, scanning slightly proximally and distally along the limb to distribute transfection throughout the distal hindlimb muscle. For dual delivery animals, miR-126 was delivered 10 min after VEGF-A plasmid delivery.

### 3.6.5 Time Course of miR-126 Circulation

The stability of miR-126 in-vivo was measured at various time points with qRT-PCR after the intravenous injuection of either miR-126 with or without microbubble conjugation in Fischer rats. Rats were anesthetized and an intravenous catheter was inserted for microbubble administration. Prior to exogenous miR-126 and microbubble administration, 0.5 mL of blood was sampled from the tail vein to determine the endogenous baseline concentration of miR-126 in peripheral venous blood. Then the animals were injected with either microbubble (MB) alone, miR-126 alone, miR-126 and microbubble (MB + miR-126) or received no injection (control). Circulating levels of miR-126 were measured at 0, 15, 30, 60, and 180 minutes after administration by sampling 0.5mL of venous peripheral blood at each time point. Plasma RNA was isolated and miR-126 levels were measured as described in section 3.5.
3.7 Perfusion Imaging – Contrast-enhanced Ultrasound

3.7.1 Microbubble Preparation

For contrast-enhanced ultrasound (CEU) perfusion imaging, octafluoropropane gas micrubbubble (Definity) were used. A Coulter Multisizer IIe (Beckman- Coulter) was used to measure the microbubble concentration by electrozone sensing prior to intravenous administration.

3.7.2 Contrast-enhanced ultrasound

Blood flow was measured through CEU in the distal hindlimb gastrocnemius muscles with gated pulse inversion imaging (HDI 5000, Philips Ultrasound), using a linear array transducer (L7-4), a mechanical index of 1.0 and a transmission frequency of 3.3 MHz. A mechanical arm was used to secure the transducer to maintain a fixed image. Frames were recorded on magnetic-optical disks for analysis using HDI Lab, DigiLink1.7.2 and MCE2.9.4 software programs.

3.7.3 Resting blood flow

Background images were acquired as baselines before microbubble infusion. With the continuous intravenous infusion of microbubbles, triggered imaging frames were captured at increasing pulsing intervals (PI), specifically 0.2, 0.5, 1, 2, 3, 5, 7, 10, 20 and 40 sec. Five averaged background frames were digitally subtracted from averaged contrast-enhanced frames at each PI. PI versus signal intensity (SI) data were fit to the
function \( y = A(1 - e^{-\beta t}) \), \( Y \) is the signal intensity at pulsing interval \( t \). \( A \) is the plateau signal intensity representing an index of maximum microvascular blood volume. \( \beta \) is the rate constant, or the time to reach plateau, used as a measure of microvascular blood velocity. Microvascular blood flow was calculated by the product of \( A \) and \( \beta \). Flow in the ischemic left leg was normalized to the contralateral non-ischemic leg to account for differences in microbubble concentration and volume of distribution between animals. The ratio of ischemic limb flow to normal limb flow was calculated and compared between the treatment groups.

3.8 Vascular Architecture – Fluorescent Microangiography

3.8.1 Adductor muscle tissue collection and preparation

Fluorescent microangiography was performed as previously described. After sacrificing the animal with T-61 euthanasia solution, the lower limbs were pressure perfused with 100 mL of heparinized saline via the abdominal aorta. Then, a mixture of FluoSpheres carboxylate-modified microspheres (10% solution, 0.2 \( \mu \)m and 0.02 \( \mu \)m diameter; Invitrogen) with low melting point agarose (1% solution; Sigma) at 45°C was slowly perfused into the hindlimb. Limbs were covered with ice to rapidly cool and solidify the solution, forming an arterial cast. The right and left gastrocnemius muscles were removed and preserved in 10% buffered formalin (VWR) at 4°C. Tissue was sectioned (200 \( \mu \)m; Leica VT1000S) and mounted onto a slide with Vectashield Mounting Medium (Vector Laboratories).
3.8.2 Confocal microscopy and 3D analysis

To image the fluorescent cast of the vasculature in the hindlimb, Confocal microscopy (Biorad, Radiance 2100) was used. A stack series of 20 images (5 μm apart, total 100 μm) was taken for each muscle section and uploaded onto NeuroLucida Software to create a 3D reconstruction. The 3D reconstruction were analyzed using Neuroexplorer for various parameter including total length of vessels per tissue volume, length of vascular trees (continuity), branch order ratios, segment length, tortuosity, diameter and volume. Maximum length of a vascular tree was used to quantify branching between vessels (i.e. collateral growth), referred to as continuity. Branch order ratios were calculated to indicate vessel network patterning (Peirce, Price, & Skalak, 2004), comparing capillaries to arterioles (order 0 to order 1) according to Neuroexplorer’s Microvascular ordering system. This is similar to Strahler’s ordering scheme, with 0 at the most distal branches and increasing order number as branches converge (Engelson, Skalak, & Schmid-Schonbein, 1985; Peirce et al., 2004); however, the Microvascular system considers branch diameter in addition to the number of converging branches. The arteriole to capillarity ratio was compared across the various treatment groups.

3.9 Gene Expression – Real Time PCR

Primers specific to total (rat and human) VEGF-A and endogenous PIK3R2 and SPRED1, were designed to quantify mRNA expression by real time PCR (qRT-PCR). Exogenous VEGF could not be measured separately from endogenous because much of the sequence is conserved between species. At day 1 post-delivery (day 15 post-ligation) and day 14 post-delivery (day 28 post-ligation), animals were sacrificed and
gastronemius muscle tissue was isolated, snap frozen and stored at -80°C for RNA isolation. Gastrocnemius muscle tissue was sonicated in Trizol (1 mL) using an ultrasonic homogenizer and RNA was extracted using an Aurum Total RNA mini kit (Bio-Rad). RNA (1 μg) was reverse transcribed and the cDNA was used for qRT-PCR using PerfeCTa SYBR green (Quanta BioSciences) with the ViiA 7 RT-PCR system. Beta-actin, a housekeeping gene, was quantified as an internal control for variations in amount of total cDNA. VEGF-A, PIK3R2 and SPRED1 mRNA levels in the treatment groups were normalized to non-delivered control group.

3.10 Western Blot Analysis for PIK3R2 and SPRED1

Knockdown of miR-126 targets (PIK3R2 and SPRED1) in hindlimb tissue of normal and ischemic treatment groups were assessed by western blot. Tissue samples were cut into appropriate sections and lysed in RIPA buffer (Sigma) by sonication. Protein concentrations of each samples were determine by Bradford Assay (Bio-Rad) and 80 μg of protein was used for loading. Gel electrophoresis was performed at 120V. Transfer was done at room temperature at 80 V for 2 hours. After transfer, the membrane was stained with Ponceau Red for 30-60 seconds to detect bands and confirm the transfer. The membrane was then kept in blocking buffer for 1 hour and rinsed with washing buffer. Primary antibodies were added (anti-SPRED1 ab77079; 1:1,000, anti- PIK3R2 ab131067; 1:5,000, anti-GAPDH) and incubated overnight at 4°C. The membrane was rinsed again and anti-Rabbit HRP secondary antibody (1:5,000, Promega) was added to detect SPRED1, PIK3K2 and GAPDH. The membrane was incubated with secondary antibodies for 45 minutes at room temperature. After final washes, the blot was
developed with ECL detection kit (GE Healthcare) on a photo film (Kodak). Densitometry readings were obtained using the Quantity One software and the sample protein concentrations were normalized to the intensity of GAPDH.

3.11 Immunohistochemical Staining

Immunostaining was performed on hindlimb skeletal muscle, with antibodies against von Willebrand Factor (Santa Cruz Biotechnology, Santa Cruz, California) (endothelial cells) and Desmin (Abcam) (pericytes). Nuclei were counterstained with DAPI (Vector Laboratories) (blue). Briefly sections were embedded in OCT (Sakura Finetek) and cryosectioned at a thickness of 18um, fixed with 2% paraformaldehyde (Sigma-Aldrich) for 10min and washed with PBS. Sections were blocked with Donkey Serum (Abcam, 2%) and primary antibodies applied overnight (1:75 for VWF, 1:100 for Desmin). After overnight incubation sections were washed 3 times and secondary antibodies applied (Abcam, 1:200) FITC conjugate for CD31. Secondary antibodies were incubated for 1 hour, washed with washing buffer containing Tween 20 (Thermal Fisher Scientific, 0.1%). Finally DAPI was applied as a nuclear counter stain. Sections were covered with a cover slip and visualized under confocal microscopy the same day.

3.12 Statistical Analysis

Data are expressed as mean ± standard error of the mean (SEM), unless otherwise indicated. Comparisons between multiple treatment groups were made with one-way ANOVA analysis. When differences in the means were found, Bonferroni correction was performed. Differences were considered significant when P < 0.05.
Chapter 4 – Results

4.1 miR-126 therapy for chronic ischemia

4.1.1 miR-126 mediates angiogenesis in vitro

To determine the effect of miR-126 in angiogenesis, in vitro angiogenesis assays were used to determine the effect of miR-126 in endothelial cell migration and tube formation, two critical steps in angiogenesis. HUVECs were used for tube formation assay in Matrigel. HUVECs that were transfected with exogenous miR-126 resulted in enhanced tube formation in matrigel assay. As can be seen from the representative images, the tubule networks were notably more complex in the miR-126 transfected HUVECs as compared with control and scramble groups (Figure 6A). However, when HUVECs were transfected with anti-miR-126 (to knockdown miR-126 expression), the tube formation capacity of the endothelial cells were severely hampered when compared to scrambled and control (Figure 7A). Quantification of the matrigel assay revealed that miR-126 transfected HUVEC formed higher complexity networks with (Figure 7B) greater of tubes (*** p<0.001 vs control and scramble). Contrarily, HUVECs transfected with anti-miR-126 resulted in inhibition of network formation with decreased number of tubes (**p<0.01 vs control and scramble). In addition, HUVECs transfected with miR-126 also showed greater migration response to VEGF (Figure 7C) (*p<0.05 vs control and scramble).
Figure 7 Matrigel tubule formation and migration in vitro.
A) Representative images of matrigel tubule formation. B) Quantification of tubule formation was assessed and expressed as tubes. miR-126 transfected Human umbilical vein endothelial cells (HUVECs) showed a greater tube formation response to VEGF compared to control and scramble transfected cells. ***p<0.001 vs Control and Scramble while antimiR-126 showed a inhibited tube formation response **p<0.01 vs Control and Scramble. C) HUVECs transfected with miR-126 also showed greater migration response to VEGF compared to control and scramble (*p<0.05 vs Control and Scramble). Data expressed as mean ± SEM.
4.1.2 Charge coupling of microbubbles stabilizes miR-126 for \textit{in vivo} gene delivery

After confirmation of proangiogenic potential of miR-126 \textit{in vitro}, the next step was to study its angiogenic potential using an \textit{in vivo} animal model. In order to use UMGD for \textit{in vivo} miR-126 delivery, it was important to determine the binding affinity and stability of miR-126 to cationic microbubbles. Addition of miR-126 results in sigmoidal increase of number of miR-126 molecules per microbubble. Through miR-126 and microbubble binding assay, the optimized concentration for miR-126 to bubble ratio was 2.5μg of miR-126 to 1x10^9 cationic microbubbles that resulted is approximately 65,000 miR-126 molecules per microbubble (Figure 8A). Therefore, for the \textit{in vivo} delivery of miR-126, we have chosen a dose of 2.5μg of miR-126.

Circulating miRNAs are endogenously enclosed by lipid microvesicles to prevent their degradation. Similarity, the binding of exogenous miR-126 to cationic microbubbles prolonged the bioavailability in circulation. While intravenous administration of miR-126 alone or microbubbles alone into the bloodstream showed rapid degradation over time, when microbubble-miR-126 complexes were administered intravenously, miR-126 levels remained stable, lasting out to 3 hours after injection (Figure 8B). The binding of microbubbles to miR-126 resulted in prolonged bioavailability in circulation. The bioavailability of miR-126 in circulation was critical for UMGD to efficiently induce \textit{in vivo} transfection of miR-126 into the targeted vasculature of the hindlimb during gene delivery.
Figure 8 Stability of miR-126/microbubble complex in vivo.
miR-126 bound to microbubbles (MB) remains more stable in the systemic circulation. A) Binding of miR-126 to cationic microbubbles (1 x 10⁹) at increasing miRNA incubation doses. Maximal plateau binding occurred at 2.5 μg of miR-126, with ~60,000 miR-126 molecules per microbubble. B) Time course of miR-126 circulation in plasma after intravenous injection showing a prolonged miR half-life when bound to microbubbles. (N=6 per group). Data expressed as mean ± SEM.
4.1.3 UMGD of miR-126 results in efficient and prolong in vivo transfection

The angiogenic effect of miR-126 was tested in a hindlimb ischemia model with Fischer rats. Following ligation surgery, animals were allowed to recover for 2 weeks to develop chronic hindlimb ischemia. UMGD was performed on day 14 post-ligation surgeries with $1 \times 10^9$ cationic microbubbles charged coupled to 2.5 µg of scramble or miR-126, with controls receiving no UMGD. In the setting of chronic ischemia, miR-126 delivered animals showed higher miR-126 levels in the delivered hindlimb (Figure 9A), indicating effective transfection of miR-126 at the target site ($**p<0.01$ vs control and scramble) at day 15 (24 hours post delivery) and remained elevated until day 17 (*$p<0.05$ vs control and scramble at day 17). By day 28 (14 days post-delivery), miR-126 levels were back to basal levels, showing no difference to control and scrambled delivered groups. The transfection of miR-126 through UMGD seems to be predominantly restricted to the endothelial cells. The UMGD of a fluorescently labeled miR (Figure 9B) to chronic ischemic hindlimb showed co-localization of miR (red) and endothelial cell (green), indicating that UMGD can efficiently induce targeted transfection of miR to the endothelial cells of ischemic hindlimb muscle.
Figure 9 **miR-126 transfection to ischemic hindlimb muscle**

A) Time course of miR-126 levels in ischemic hindlimb muscle at day 15, 17 and 28 post-ligation. **p<0.01, * p<0.05 vs. Control, Scramble at day 15 and day 17 respectively. miR-126 levels peak at day 15 and remain elevated at day 17. (N=6 per time point for each group). Data expressed as mean ± SEM. B) Representative immunostaining images of ischemic muscle after UMGD of fluorescently tagged miR at day 15 post-ligation demonstrating the uptake of miR by endothelial cells. Left panels show control non-delivered ischemic muscle and right panel shows fluorescent tagged miR delivered ischemic muscle. CD31 was used as with endothelial staining (green), miR (red) and merge with nuclear counterstain DAPI. The right panels show high transfection efficiency of the fluorescent tagged miR.
4.1.4 miR-126 induces PIK3R2 and SPRED1 knockdown in vivo in ischemic hindlimbs

To verify the biological function of the exogenous miR-126, the protein levels of PIK3R2 and SPRED1 were measured in hindlimb muscles of animals across the treatment groups. With the in vivo delivery of miR-126 to the ischemic hindlimb muscle with UMGD, there was a knockdown of target proteins (Figure 10A) PIK3R2 and (Figure 10B) SPRED1, quantified by Western blot. The knockdown of protein expression persisted for at least 3 days after delivery. At day 15 (24 hours post delivery) and day 17 (3 days post delivery), miR-126 levels were elevated (Figure 9A) which correlated with a significant decrease in protein expression of both PIK3R2 (**p<0.005 vs Control and Scramble at day 15 and 17) and SPRED1 (*p<0.05 vs Control and Scramble at day 15 and 17). There is difference in the efficacy of miR-126 in knocking down PIK3R2 and SPRED1; with PIK3R2 levels being decreased more than SPRED1. The highest knockdown however, was observed at day 15 (PIK3R2: 0.61 ± 0.10, SPRED1: 0.71 ± 0.04). The protein levels of PIK3R2 and SPRED1 returned to basal levels at day 28, indicating the knockdown effect induced by the exogenous miR-126 is transient. Protein expression levels of PIK3R2 and SPRED1 were no different across scrambled and non-treated control groups.
Figure 10 *In vivo knockdown PIK3R2 and SPRED1 proteins.* Western blot quantification shows that UMGD of miR-126 resulted in decrease protein expression of PIK3R2 (A) and SPRED1 (B) in ischemic hindlimb muscle (PIK3R2: **p<0.005 vs Control and Scramble on day 15 and day 17, SPRED1: *p<0.05 vs Control and Scramble on day 15 and day 17) (N=6 per time point for each group). As can be seen from the representative blots and quantitative data presented in the graphs, target knockdown peaked at day 1 and remained significantly downregulated out to day 3. Data expressed as mean ± SEM.
4.1.5 Therapeutic Angiogenesis by UMGD of miR-126 in Chronic Ischemia

Contrast-enhanced ultrasound (CEU) was used to assess function improvements in tissue perfusion over time in all treatment groups. Representative parametric CEU perfusion images of the ischemic hindlimb muscle at day 28 in all animal groups are shown in Figure 11A. Compared to control and scramble groups, miR-126 treated group showed overall higher perfusion (red and yellow represent areas of high microvascular blood flow) throughout the muscle. Scramble treated group showed similar perfusion levels to that of control with lower perfusion that is detectable only in scattered areas. Quantitative analysis of the perfusion shows that at day 14, prior to treatment, the ischemic leg of animals in all groups had approximately 60-65% of normal flow. At day 28 (2 weeks after treatment), only animals receiving UMGD miR-126 treatment showed improvement in normalized perfusion to 0.79 ± 0.03 (**p<0.01 vs perfusion at Day 14). Flow in the ischemic leg of the control and scramble delivered groups remained at approximately 60-65% of normal flow at day 28, showing no improvement (Figure 11B).
Figure 11 **Microvascular perfusion improved after UMGD of miR-126**

A) Representative parametric CEU perfusion images of microvascular blood flow to ischemic hindlimb in all treatment groups at day 28 post-ligation (day 14 after UMGD). Red and yellow represent areas of high microvascular blood flow. B) CEU perfusion data (normalized to contralateral non-ischemic muscle) for ischemic hindlimb muscle at day 14 (before UMGD of miR-126) and day 28 (14 days after UMGD of miRNA) across treatment groups (**p<0.01 vs perfusion at Day 14). (Control N=23, Scramble N=26, miR-126 N=31). Data expressed as mean ± SEM.
4.1.6 Improvement of vascular architecture after miR-126 therapy

Fluorescent microangiography (FMA) is a post-mortem vascular casting technique that allows microvascular density and neovessel characterization. The vascular architecture of the hindlimb muscle was accessed in all the treatment groups at day 28 with FMA. Fluorescent microangiography (Figure 12A) of hindlimb muscle showed increased vessel density in miR-126 treated animals compared to control and scramble treated groups. The vasculature of the control and scramble groups is less dense with an overall disordered vascular morphology composed of disconnected vessels. The vascular architecture of hindlimb muscles from animals receiving miR-126 therapy has a more organized morphology with vessels that are well interconnected and structured. Through quantitative analysis of the vasculature in the ischemic hindlimb of animals, both the (Figure 12B) total vessel length (2225 ± 308 μm/mm$^3$) and (Figure 12C) vascular density (25442 ± 5037 μm$^2$/mm$^3$) of the miR-126 treated group was significantly greater compared to control and scramble treatment groups (**p<0.01 vs Control and Scramble). Branch order ratios (order 1 arteriole: order 0 capillary) indicated the abundance of mature vessels. By branch order analysis, miR-126 delivery by UMGD resulted in a higher (Figure 12D) arteriolar/capillary ratio (0.48 ± 0.04) compared to scramble (0.30 ± 0.04) and control (0.34 ± 0.03) groups at day 28 (*p<0.05 vs Control and Scramble).
Figure 12 Fluorescent microangiography of ischemic hindlimb.

(A) Representative fluorescent microangiographic images of the vasculature in the ischemic leg of animals from all treatment groups and their corresponding reconstructed 3D model. Quantitative fluorescent microangiography (FMA) of miR-126 treated muscle showed increased (B) total vessel length (μm/mm³), (C) vascular density (μm³/mm³), and (D) Branch order of arteriolar/capillary ratio. **p<0.01 vs Control and Scramble for vessel length and vascular density; *p<0.05 vs Control and Scramble for arteriolar/capillary ratio. (N=8 per group). Data expressed as mean ± SEM.
4.2 miR-126 and VEGF-A therapy for chronic ischemia in diabetes

4.2.1 Hyperglycaemia and decreased circulating miR-126 levels in ZDF rats

To characterize blood glucose and circulating miR-126 levels from ZDF rats, non-fasting tail vein blood were sampled from ZDF and Zucker lean (non diabetic control) rats at day 0 (prior to hindlimb ischemia) and day 14 (2 weeks after ligation surgery). ZDF animals were hyperglycemic (25.39 ± 5.09 mmol/L) while lean rats had normal blood glucose 6.93 ± 1.16 mmol/L (Figure 13A). Interestingly, the circulating miR-126 of normal ZDF rats (0.59 ± 0.05 fold of zucker lean rats) was lower compared to zucker lean rats (*p<0.05 vs normal lean rats) (Figure 13B).

Figure 13 Blood glucose and circulating miR-126 levels in ZDF.
(A) The ZDF rats showed hyperglycemia with mean blood glucose levels of 25.39 ± 5.10 mmol/L while lean rats showed lower mean blood glucose levels of 6.93 ± 1.16 mmol/L (***p<0.001 between ZDF and lean). (B) The circulating miR-126 levels in the plasma of ZDF animals were significantly lower than the Lean non diabetic animals. (*p<0.05 between normal lean and normal ZDF). Data expressed as mean ± SEM.
4.2.2 *In vivo transfection of miR-126 and VEGF-A plasmid*

The efficiency of UMGD for *in vivo* transfection of miR-126 and VEGF-A plasmid in ZDF rats was assessed through qRT-PCR of tissue from ischemic hindlimb muscle. UMGD of miR-126 and/or VEGF-A plasmid resulted in targeted transfection to the ischemic hindlimb muscle. At day 15 (24 hours after UMGD delivery), the miR-126 (Figure 14A) miR-126 levels in the miR-126 (1.78 ± 0.24 fold of control) and dual delivered group (1.77 ± 0.22 fold of control) were higher (*p<0.05 vs Control and VEGF-A). Similarly, the VEGF-A mRNA levels (Figure 14B) in the VEGF-A (1.59 ± 0.19 fold of control) and dual delivered group (1.57 ± 0.12 fold of control) were higher (*p<0.05 vs Control and miR-126). The increase of miR-126 to the ischemic muscle through exogenous *in vivo* transfection with UMGD corresponded to decreased mRNA expression of (Figure 14C) PIK3R2 and (Figure 14D) SPRED1 in miR-126 treatment group (PIK3R2: 0.48 ± 0.09 fold of control; SPRED1 0.54 ± 0.07 fold of control. **p<0.01 vs Control) and dual delivery group (PIK3R2: 0.61 ± 0.06 fold of control; 0.71 ± 0.03 fold of control. *p<0.05 vs Control). Additionally, miR-126 resulted in down-regulation of PIK3R2 and SPRED1 at the protein level (negative inhibitors of VEGF-A induced angiogenesis) in the miR-126 and dual delivered groups resulting in enhanced VEGF-A signaling. (Figure 14E).
Figure 14  *In vivo* knockdown of targets PIK3R2 and SPRED1 in ZDF rats
UMGD resulted in increase of (A) miR-126 levels in the miR-126 and Dual delivered group (*p<0.05 vs Control and VEGF-A) and (B) VEGF-A mRNA levels in VEGF-A and Dual delivered groups (*p<0.05 vs Control and miR-126). The increase miR-126 levels resulted in knockdown of (C) PIK3R2 and (D) SPRED1 mRNA levels (**p<0.01, *p<0.05 vs Control and VEGF-A) (N = 5-6 for each group). Western blot representative images (E) also showed decreased protein expression of PIK3R2 and SPRED1 in ischemic hindlimb muscle in miR-126 and Dual delivered groups. Data expressed as mean ± SEM.
4.2.3 Therapeutic Angiogenesis in Diabetic Chronic Ischemia

In a similar manner, CEU perfusion imaging was used to assess the tissue perfusion of ZDF rats over time in all treatment groups. Representative parametric CEU perfusion images at day 28 in all animal (blue indicating least perfusion, yellow and red indicating highest perfusion) are shown in Figure 15A. The ischemic hindlimb muscle of control animals remained fairly ischemic (mostly blue) compared to the contralateral normal leg. With treatment of miR-126 or VEGF-A alone, there are improvements in perfusion in certain areas of the muscle (white arrows) with dual delivery group showing a more dispersal overall improvement in perfusion. At day 14, prior to treatment, the ischemic leg of animals in all groups had approximately 40-50% of normal flow. UMGD of miR-126, VEGF-A to chronic ischemic hindlimb muscle resulted in significant improvement in microvascular perfusion but greatest improvement was observed in the dual delivery group (Figure 15B). At day 28 (2 weeks after treatment), animals that received UMGD of miR-126 showed improvement in normalized perfusion (0.67 ± 0.07 (**p<0.001 vs perfusion at Day 14). Similarly, animals that received UMGD of VEGF-A plasmid showed improved perfusion at day 28 (***p<0.001 vs perfusion at Day 14). However, the greatest improvement in perfusion was observed for the dual delivered animals that received miR-126 and VEGF-A sequentially ((#p<0.01 vs miR-126 and VEGF-A Day 28). The perfusion in the ischemic leg of control animals remained at approximately ~45% of normal flow at day 28, showing no improvement (Figure 15B).
Figure 15 Microvascular perfusion improvement in ZDF rats
A) Representative parametric CEU perfusion images of microvascular blood flow to ischemic hindlimb in all treatment groups at day 28 post-ligation (day 14 after UMGD). Red and yellow represent areas of high microvascular blood flow. B) CEU perfusion data (normalized to contralateral non-ischemic muscle) for ischemic hindlimb muscle at day 14 (before UMGD) and day 28 (14 days after UMGD) across treatment groups (*p<0.001 vs perfusion at Day 14). (N = 9 - 11 for the different groups). The dual delivered group showed greatest improvement of blood flow compared to single delivery of either miR-126 or VEGF-A plasmid alone (#p<0.01 vs miR-126 and VEGF-A Day 28). Data expressed as mean ± SEM.
4.2.4 Improvement of vascular architecture in diabetes after UMGD therapy

FMA analysis was used to study the therapeutic effects of miR-126 and VEGF-A in the vascular integrity of diabetic ischemic tissue. The ischemic hindlimb muscle from all treatment groups was processed with FMA at day 28, 14 days after UMGD therapy. FMA (Figure 16A) of the ischemic hindlimb muscle showed greater vessel density in miR-126, VEGF-A and dual delivery treated animals compared to controls. Through analysis of the vasculature in the ischemic hindlimb, both the vessel length (Figure 16B) and the vascular density (Figure 16C) the miR-126 and VEGF-A treated group were significantly greater compared to control and scramble treatment groups (*p<0.005 vs Control). The dual delivery of miR-126 and VEGF-A however resulted in greater vessel length (1127 ± 42 μm/mm³) and vessel volume (18924 ± 2260 μm²/mm³) than either miR-126 (length: 796 ± 92 μm/mm³; volume: 16125 ± 1051 μm²/mm³) or VEGF-A (length: 787 ± 57 μm/mm³; volume: 14337 ± 2608 μm²/mm³) delivery alone (#p<0.05 vs VEGF-A and miR-126 alone). Abundance of mature vasculature was evaluated through branch order ratios (order 1 arteriole: order 0 capillary). By branch order analysis, UMGD delivery of miR-126, VEGF-A and dual delivery all resulted in a higher (Figure 16D) arteriolar/capillary ratio compared to control (*p<0.05). Interestingly, in both miR-126 and dual delivered animals, there was a greater improvement of arteriolar/capillary ratio than VEGF-A delivered groups (#p<0.05).
Figure 16 Fluorescent microangiography in ZDF rats
(A) Representative fluorescent microangiographic images of the vasculature in the ischemic leg of animals from all treatment. Quantitative fluorescent microangiography (FMA) of miR-126, VEGF-A and dual delivery treated muscle showed increased (B) total vessel length (μm/mm$^3$) and (C) vascular density (μm$^3$/mm$^3$) in miR-126 (**p<0.01 vs Control), VEGF-A (**p<0.01 vs control) delivered but with greatest increase in the dual delivered group (**p<0.005 vs Control, #p<0.05 vs miR-126 and VEGF-A). miR-126 and dual delivered group showed increase in (D) branch order of arteriolar/capillary ratio (*p<0.05 vs Control and #p<0.05 vs VEGF-A). (N= 5-6 per group). Data expressed as mean ± SEM.
Chapter 5 – Discussion

5.1 miR-126 therapy for chronic ischemia

With very modest beneficial effect of pro-angiogenic cytokine gene therapy in clinical studies of PAD and CAD, there is a need for new therapeutic strategies. The function of miRNAs as important regulators in many cardiovascular diseases, and their ability to specifically target key cellular pathways, makes miRNAs an extremely attractive target for development of novel therapeutic strategies. miR-126 in particular is an angiomiRs that have been identified that regulate vascular development, angiogenesis and endothelial cell function. This focus of the first phase of the study was to investigate the angiogenic benefits of miR-126 UMGD therapy in a chronic hindlimb ischemia rodent model.

miR-126 plays a critical for endothelial cell proliferation and migration. In our study, the in vitro transfection of HUVECs with miR-126 resulted in significant increase in both tube formation and migration response to VEGF. This finding was consistant with literature regarding the pro-angiogenic effect of miR-126 (Jansen et al., 2013). Jansen and colleagues showed that endothelial cells exposed to miR-126 enriched endothelial microparticles (EMPs) showed greater migration and proliferation.

For in vivo therapeutic applications of miRs, it was critical to establish the ideal delivery system to prolong miRs’ bioavailability. While endogenous circulating miRs are protected in plasma by exosomes and microvesicles (Creemers, Tijsen, & Pinto, 2012; Diehl et al., 2012), short RNA sequences such as exogenously miR mimics are prone to
RNase degradation (Diehl et al., 2012). We showed that miR-126 can efficiently bind to cationic lipid microbubbles (~60,000 miRNA molecules per microbubble) and prolongs their circulatory time in vivo. While unbound miRNA was rapidly cleared, microbubble conjugation allowed the miR-126 to persist in the systemic circulation where they can undergo site-targeted transfection by focused externally applied ultrasound. Other research groups have employed similar strategies with the use of chemical modifications and lipid based carriers to prolong bioavailability and increase cellular uptake in vivo. For example, chemical modifications to anti-miR-122 oligonucleotides increased the tissue uptake and functional activity after tissue uptake, leading to a knockdown of miR-122 and decrease in cholesterol levels (Rotllan, Ramirez, Aryal, Esau, & Fernandez-Hernando, 2013) in mice. Alternatively, the use of antibody conjugated liposome carriers can further increase the therapeutic specificity of miR therapy. Liu et al., used anti-cardiac troponin I antibody modified liposomes as anti-miR-1 carriers for in vivo delivery to ischemic myocardium tissue of MI rats. This resulted in myocardial cells specific silencing of miR-1 and relieving ischemic arrhythmia (M. Liu et al., 2014).

While other studies have used high-power ultrasound and DNA-bearing carrier microbubbles to deliver plasmid vectors encoding miRNA to the kidney (Zhong, Chung, Chen, Meng, & Lan, 2011) and liver (D. Yang et al., 2013), our study examined the delivery of miRNA for cardiovascular therapeutics, specifically aimed at therapeutic angiogenesis in chronic hindlimb ischemia. In addition, this study closely described the in vivo spatial and temporal aspects of UMGD for miRNA delivery, including localization, bioavailability after transfection and target knockdown. Similar to plasmid delivery,
UMGD results in targeted transfection of miR-126 to the vascular endothelium as shown by the histological staining, and may be a key advantage of UMGD over other miRNA delivery techniques for therapeutic angiogenesis. Transfection occurs rapidly within 3 hours, and is transient, lasting 3 days, with target knockdown also occurring rapidly within 24 hours after UMGD, which may be beneficial for miRNA therapies for acute processes such as acute myocardial infarction. Use of miRNA plasmid vectors encoding miR-126 for UMGD may also prolong transfection and target knockdown, further increasing therapeutic effect, and is worthy of future studies. Importantly, remote organ uptake was minimal after UMGD of miR-126, with no significant off target effects. Thus, the advantages of targeted vascular transfection with minimal delivery to remote regions, and non-invasive delivery method makes the technique of UMGD uniquely suited to endothelial cell-specific proangiogenic miRNA delivery.

Our present study showed that miR-126 increased tubule formation and migratory capacity of endothelial cells in vitro, and that UMGD of miR-126 in the setting of chronic hindlimb ischemia in vivo resulted in increased microvascular perfusion and vascular density, with knockdown of known targets SPRED1 and PIK3R2, both negative regulators of VEGF signaling. While miR-126 transfection and target knockdown was relatively transient, the pro-angiogenic effect persisted for 2 weeks after delivery. This did not come as a surprise since VEGF-A is predominantly only important for early stages of angiogenesis. In a study that examined the time course expression of VEGF-A and other cytokines during ischemia induced angiogenesis in myocardial occlusion, it was shown that endogenous VEGF-A and Ang-2 were elevated only for first 3 days after
occlusion while Ang-1 were elevated at a later time (Matsunaga et al., 2003). Our study have analyzed the neovasculature and revealed increased arteriolar/capillary ratio and improved pericytes coverage suggesting enhanced vessel maturation. This can be explained by the study by Sessa and colleagues who showed that miR-126-induced suppression of PIK3R2 also promotes the action of Ang-1 on neovessel stabilization and maturation (Sessa et al., 2012). Thus, by simultaneously targeting both the VEGF pathway to stimulate early angiogenesis and Ang-1 signaling to promote vessel stabilization, miR-126 therapy results in a robust angiogenic response that non only promotes angiogenesis but is important for neovascular maturation.

5.2 miR-126 and VEGF therapy for chronic ischemia in diabetes

In the second phase of the study, we were interested in studying the pro-angiogenic effect of dual delivery of miR-126 and VEGF-A plasmid in the settings of diabetes. Characterization of the ZDF rats showed that they are hyperglycemic, which is a common manifestation of diabetes. Interestingly, the circulating miR-126 levels of these animals were also elevated compared to the non-diabetic zucker lean rats. This is consistent observation with literature describing a decreased circulating miR-126 levels in diabetic patients (Zampetaki et al., 2010). In a mice model of hindlimb ischemia, van Solingen and colleagues have shown that the silencing of miR-126 by injecting antimiR-126 to the ischemic hindlimb resulted in reduced angiogenic response to hypoxia (van Solingen et al., 2009). Indicating the critical role of miR-126 in ischemia induced angiogenesis. In our study with ZDF rats, the exogenous delivery of miR-126 to the ischemic muscle resulted in increased microvascular blood flow, vessel length and
vascular density. The pro-angiogenic mechanism is mediated through the knockdown of target molecules PIK3R2 and SPRED-1. Jansen and colleagues showed that the uptake of miR-126 enriched endothelial micro-particles (EMPs) by EC, resulted in promoting endothelial cell migration and proliferation mediated by downregulation of SPRED-1. Interestingly, EMPs from glucose-treated (diabetic stimulation) ECs did not show the same proangiogenic potential and showed reduced endothelial repair capacity both in vitro and in vivo (Jansen et al., 2013). Together these data suggests that miR-126 is critical for ischemia-induced angiogenesis and exogenous miR-126 to the ischemic muscle can enhance angiogenesis.

Similarly, UMGD of VEGF-A plasmid to the ischemic hindlimb of ZDF rats resulted in increased VEGF-A mRNA expression. Despite having an overall increase of circulating VEGF-A levels in diabetes (Felmeden et al., 2003), the hypoxia induce increase of muscular VEGF-A levels in ischemia is attenuated in the setting of diabetes. In a study by Yongjun and colleagues, it was showed that the increase in VEGF after ischemic injury in attenuated in mice with diet-induced type 2 diabetes at both days 13 and 20 after ligation (Li et al., 2007). Similarly in Lepr<sup>ob/ob</sup> mouse (mouse model equivalent of ZDF), there were various angiogenic growth factors under-expressed in the ischemic limbs of mice at 14 days after ischemia include the angiogenic factors VEGF-A (Schiekofer et al., 2005). These data indicate that in the settings of diabetes, endogenous ischemic response fails to correctly activate a multifaceted angiogenic. In our study, the increase of VEGF-A from exogenous VEGF-A plasmid delivery was associated with improvement of microvascular blood flow vascular length and vascular density. The
therapeutic effect could be the result of increased VEGF-A levels at the ischemic muscle, an event that is compromised in diabetes. Take together these data suggest that modulating VEGF expression remains an option as a therapeutic target even in the setting of type 2 diabetes. In a small-randomized trial, Kusumanto et al., evaluated the effect of intramuscular injections of either plasmid encoding for VEGF-165 versus placebo in 54 diabetic patients with chronic limb ischemia. There were fewer major amputations (3 [11%] versus 6 [22%]) (Kusumanto et al., 2006).

One of the major limitations of “first generation” angiogenic factors like VEGF is that resultant neovessels are immature and poorly formed (Lee et al., 2000; Schwarz et al., 2000), and might regress. Our previous studies using UMGD to deliver VEGF plasmid DNA to ischemic skeletal muscle (Kobulnik et al., 2009; Leong-Poi et al., 2007) results in an increase in blood flow and vessel density 2 weeks after VEGF delivery that does not persist at later time points. In the current study, the dual delivery of miR-126 and VEGF plasmid resulted in the greatest improvement in microvascular blood flow and vessel density. Interestingly, there was an increase in the artery/arteriole ratio, which is an indication of vascular maturity, only for the miR-126 and dual delivery group, which was absent in the VEGF-A group. miR-126 therefore not only enhances early angiogenesis through enhancing of VEGF-A signaling, but also plays a critical role in vascular maturity. A mentioned earlier, Sessa et al., has shown that miR-126 enhances Ang-1 mediated vascular maturation and stabilization through regulation of PIK3R2 (Sessa et al., 2012). In miR-126 KO zebrafish, there were vascular defects that resemble lost of Ang-1 phenotype. Our study is consistent with the literature as we have demonstrated that
a strategy of dual gene delivery of VEGF plasmid and miR-126, resulted in a more robust and functional angiogenic response.

5.3 Experimental limitations

5.3.1 Exogenous VEGF-A plasmid and miR-126

The VEGF-A plasmid used in this study encodes the human variant of VEGF-A, which has transcript homology of 89% and amino acid sequence homology of 80% of the rat variant. However, the difference in the amino acid sequence may not be as critical, as majority of the amino acid sequence being of similar properties, the function may well be preserved in rats. In previous studies that have delivered the human variant of the VEGF gene to rats, it resulted with functional improvements in angiogenesis (Leong-Poi et al., 2007). Our current study demonstrated that the VEGF gene was translatable – as demonstrated by the detection of VEGF mRNA and functional – as demonstrated by the pro-angiogenic effect during chronic ischemia in a rodent model of hindlimb ischemia.

Similarly, when delivering exogenous miR-126 in vivo, it is vital that the miR-126 is successfully transfected to the targeted endothelial cells and is functional. While endogenous miRs are protected by exosomes or microvesicles (Creemers et al., 2012; Diehl et al., 2012), due to the small size nature of exogenous miRs, they are prone to endonuclease degradation in circulation and thus hinder its bioavailability for UMGD. However, we have shown that charge coupling of miR-126 to microbubbles protects miR-126 against degradation and prolongs bioavailability in circulation. miR-126 was also successfully transfected into the targeted endothelial cells as shown from
immunohistochemistry and qPCR data indicating sustained bioavailability up to 3 days and resulting in enhanced angiogenesis.

5.3.2 Length of Study

Our study used a chronic hindlimb ischemia model that was established 2 weeks after unilateral femoral artery ligation. This is a relatively short period of time, in comparison to the chronic effects of peripheral artery disease, which may take years to develop in humans. The femoral artery ligation is one of the many variations of surgical ligations for establishing the hindlimb ischemia model. The surgical procedures can vary from a single ligation of the femoral or iliac artery to a complete excision of the artery and sometimes even the vein and nerve are dissected too. The relative modest level of ischemia by our hindlimb ischemia model (~50% decrease of microvascular blood flow) is ideal for study of gene therapy as it permits partial perfusion of plasmid and miR bearing microbubbles to the ischemic region through the collaterals.

Another concern with the relatively short study period is that we are not able to monitor the long-term effects of the angiogenic therapy. As mentioned earlier, VEGF-A therapies have shown promising improvement of vascular function that fail to persist through long term. Even with prolonged expression of VEGF-A plasmid, the therapeutic effect will be transient. Similarly the half-life of miRs is relatively short lived as they became undetectable at 14 days after delivery. Even with repeated therapeutic administrations there are safety concerns regarding prolonged elevated VEGF-A and miR-126 levels. Elevated VEGF-A levels have been reported to be crucial for
development and metastasis of various cancers, making anti-VEGF-A strategies the focus of various anti-cancer therapies (Ellis & Hicklin, 2008). Similarly, the deregulation of miRs levels has been associated with various disease and complications (Table 2). In future studies, monitoring and determining the ideal length of gene therapy will allow for better design of therapeutic interventions for clinical practice. Additionally, it is worth exploring the benefits of temporal separated delivery of genes/miRs to optimize therapeutic potential. In a study by Smith et al., temporal separation delivery of VEGF-A early and Ang-1 at 2 weeks after, resulted in the greatest angiogenic effect with sustained vascular integrity (Smith et al., 2012).

5.3.3 Efficiency of Gene Delivery Method

Although UMGD has been proven to be an effective method of gene delivery with high tissue specificity with a diffuse delivery area (S. Chen, Shohet, Bekeredjian, Frenkel, & Grayburn, 2003; Kobulnik et al., 2009), the gene delivery efficiency is much less in comparison to direct intramuscular injection or viral delivery technique. Despite its lower transfection efficiency, UMGD gene delivery method produces gene expression pattern that induced angiogenesis, leading to enhanced functional (increased muscle perfusion) and structural (microvascular density) endpoints (Kobulnik et al., 2009). UMGD delivery method has been successfully described in a rat cardiac delivery setting by Chen and colleagues (S. Chen et al., 2003). However a major limitation of UMGD method is that tissue or organs available for this delivery method are limited to those that can be readily imaged by the ultrasound probe. Small animal studies utilizing UMGD such as our study allows for relatively efficient transfer of gene and/or miRs to the
ischemic leg as we move the probe along hindlimb muscle in a timely manner to cover the majority of the ischemic tissue. However, in large animal settings, or even clinical studies where the tissue are much larger in size, there needs to be a systematic protocol for the gene delivery method to maximize the coverage of the delivery in the tissue. Also, the limitation of the ultrasound mediated gene delivery is that it is limited by perfusion. Patients suffering from late stage PAD often suffer severe atherosclerosis (Hirsch et al., 2001), which may significantly hinder the effectiveness of the therapy. Therefore UMGD approach remains as a beneficial therapeutic strategy limited only to tissue and organ that are not highly fibrotic with moderate level of perfusion.

5.3.4 Animal model

The availability of an animal model that can mimic the pathophysiology of a human disease is critical for development of gene therapy. Despite immense effort, an animal model that completely recapitulates the human diabetic condition is not available. Accordingly, when choosing the correct animal model for our study, we selected a type II diabetic animal model to mimic the clinical situation of diabetes as accurately as possible. The ZDF rat spontaneously develops complications such as hyperlipidemia, hyperglycemia and hyperinsulinemia that are commonly manifested in human type II diabetes. Additionally, the ZDF rat has been shown to manifest various vascular complications that are common with PAD patients including reduced endothelial mediated vasodilation, resting blood flow and wound healing. However, ZDF rats do not develop atherosclerosis and macrovascular complications but have been useful for studies have microvascular complications such as diabetic retinopathy, nephropathy and
hindlimb ischemia (Castoldi et al., 2014; Kajiwara et al., 2009; Y. S. Yang, Danis, Peterson, Dolan, & Wu, 2000). A drawback from our experimental design is that glucose levels were not controlled in the ZDF rats. In clinical situations, type II diabetic patients generally have dietary restrictions that often allow them to maintain a fairly moderate level of blood glucose. The lack of controlled blood glucose in ZDF rats makes the study ambiguous regarding its therapeutic effect in the settings of diabetes and hyperglycemia.
Chapter 6 – Conclusion and Future Directions

6.1 Conclusion

In phase I of our study, we have demonstrated that UMGD therapy of the miR-126 in the ischemic muscle can improve microvascular blood flow and vascular density in a hindlimb ischemia rodent model through down regulation of PIK3R2 and SPRED1. *In-vitro* data suggested that HUVECs transfected with miR-126 had significant increase in tube formation and migration towards VEGF. Contrarily, inhibition of miR-126 resulted in decreased tube formation abilities. From our *in-vivo* hindlimb ischemia animal model, the UMGD of miR-126 resulted in targeted transfection of miR-126 to the endothelium of ischemic hindlimb muscle with knockdown of target proteins PIK3R2 and SPRED1 (two critical negative inhibitors of VEGF-A signaling). The knockdown of the target molecules was associated with enhanced angiogenesis resulting in improved microvascular blood flow and vascular density.

In phase II of the study with a clinical relevant model using the ZDF rats as the diabetic animal model, both UMGD of miR-126 and VEGF-A therapy alone resulted in improved angiogenesis. However, the greatest improvement in angiogenesis and vascular maturation was for treatment groups receiving dual therapy of miR-126 and VEGF-A sequentially. The improvement of vascular maturity was greatly improved in animals that received miR-126 and dual therapy group compared to VEGF-A therapy alone, indicating the importance of miR-126 for promoting vascular integrity. Therefore, the therapeutic
effect of miR-126 not only amplifies VEGF-A induced angiogenesis but also plays an important role in promoting vascular integrity in the settings of diabetes.

The study demonstrated that miR-126 offers great therapeutic potential for ischemic vascular disease such as CAD and PAD specially in the settings of diabetes by promoting angiogenesis through enhancement of the VEGF-A signaling pathway and promoting vascular integrity. The non-invasive nature of UMGD for targeted vascular transfection is an efficient method for gene and miR therapeutics that has great potentials for clinical translation. To conclude, the UMGD of miR-126 and VEGF-A for targeted vascular transfection therefore is a promising non-invasive technique for combination gene delivery for therapeutic angiogenesis in diabetes.

6.2 Future directions

The field of miRNA therapeutics is still relatively young and further research into its mechanism of action and epigenetic regulation could provide valuable insights for development of new miRNA therapeutic strategies. Although compelling therapeutic efficacy evidence has been surfacing from recent studies, there are still questions that remain unanswered regarding some of their biological principles. Specifically, we are only beginning to understand the mechanism of miR-126 in maintenance of vascular integrity and its importance in modulation of inflammation in the settings of diabetes. Future studies can explore some of these mechanistic and functional aspects of miR-126 in the settings of diabetes.
Mechanism of miR-126 in maintenance of vascular integrity: Our study indicated that miR-126 not only induces angiogenesis through amplification of VEGF-A signaling, but also maintained vascular integrity. However, the exact mechanism of how miR-126 promotes vascular integrity is still not fully understood. In the study by Sessa and colleagues, they have shown that miR-126 enhances Ang-1/Tie-2 mediated vascular maturation (Sessa et al., 2012). The overexpression of PIK3R2 hampered Ang-1 mediated endothelial sprouting and survival. Contrarily, miR-126 overexpression was able to down-regulate PIK3R2 and reverse the effect. This study however did not look at the vascular changes in the context of an in vivo model. It will be valuable to study effect of miR-126 in modulating the cellular changes that resulted in the improvement of vascular integrity. Some parameters that can be use to access vascular maturity is to look for VSMCs and pericytes, which are cell types that maintain vascular integrity and are found in mature vessels (Bergers & Song, 2005).

Regulation of miR-126 biogenesis in ischemia and diabetes: Despite the understanding of the general mechanism for biosynthesis of miRs, the cellular mechanism that regulates the expression levels of specific miRs remains largely unknown. The change of miRNA profiles is associated with pathophysiology development of various diseases. Despite the mounting evidence of benefits from miR therapy, there is very little understanding regarding the mechanisms that govern the expression levels of specific miRs. Only recently, we begin to understand some of the regulations of miRs expression. From recent studies, there are interesting findings demonstrating the relationship of hypoxia in miR biosynthesis. In the settings of hypoxia,
critical enzymes for miRNA biosynthesis such as Dicer and Drosha are down-regulated (Ho et al., 2012; van den Beucken et al., 2014). Ho and colleagues have shown that hypoxia resulted in decreased mRNA and protein levels of Dicer, which in turn resulted in mRNA stabilization of hypoxia-responsive genes such as HIF-1 and VEGF (Ho et al., 2012). This study was one of the first to examine the mechanism of miR expression.

Up to date, there are no studies that have investigated whether diabetic complications such as hyperglycemia affect expression of important miRNA processing enzymes such as Drosha and Dicer. Furthermore, insights from stress-induced changes in miR processing enzymes such as Drosha and Dicer only provide a general understanding of global miR profile. The ultimate breakthrough in miR research will be to understand the regulation of specific miR in disease conditions. From our study, in the settings of diabetes, we have shown that systemic miR-126 level is decreased but currently, there the mechanism that can account for this observation is largely unknown. In future studies, it will be extremely important to develop an understanding of the cellular changes that results in altered miRNA profiles in the disease state. That way, we wont be dependent on constant exogenous miRNAs therapies to compensate endogenous state but instead, we will have a better understanding of the root cause that induce the altered levels in the first place.

Other relevant targets of miR-126: Most miRNAs have multiple targets and miR-126 is no different. Although, PIK3R2 and SPRED1 are the most reported targets of miR-126, there are other predicted targets of miR-126 in the literature including VCAM-1 and
RGS16, two molecules that play critical role in inflammation and development of atherosclerosis (Harris et al., 2008; Zernecke et al., 2009). Atherosclerosis being a common vascular complication in diabetes, the study of these two molecules could broaden the understanding of diabetes associated vascular complications. VCAM-1 is an adhesion molecule that mediates leukocyte trafficking. Resting endothelial cells normally do not express adhesion molecules, but upon activation by cytokines, activated endothelial cells express adhesion molecules such as VCAM-1 to mediate leukocyte adherence to endothelial cells at sites of inflammation. Harris and colleague have shown that overexpression of miR-126 resulted in decrease VCAM-1 expression (Harris et al., 2008). Contrarily, a decrease of miR-126 levels in endothelial cells resulted in increased TNF-α-stimulated VCAM-1 expression and leukocyte adherence to endothelial cells. The anti-inflammatory function of miR-126 makes it a potential therapeutic target for prevention of atherosclerosis associated vascular complications. From another study, Zernecke and colleagues how shown that endothelial cell-derived apoptotic bodies generated during atherosclerosis is rich in miR-126 (Zernecke et al., 2009). These miR-126 enriched apoptotic bodies mediate paracrine alarm signals to neighboring vascular cells through down-regulation of regulator of G-protein 16 (RGS16). The administration of apoptotic bodies or miR-126 resulted in increased recruitment of progenitor cells that limited atherosclerosis progression. These studies together highlighted the importance of miR-126 in modulating inflammation and atherosclerosis. In the settings of diabetes, systemic miR-126 level is decreased and may be a critical contributing factor that results in increased risk of developing atherosclerotic complications in diabetic patients. Future
studies could be extended to include the study of the therapeutic benefits of miR-126 in prevention of atherosclerosis in the settings of diabetes.

**miR-126-5p:** For majority of the miRNAs, they start off as double stranded and through various processing steps by cellular RNAses, only one strands becomes the mature functional strand while the complimentary strand is degraded. However, miR-126 in particular has been shown to have two functional strands, both the sense and the antisense strands. miR-126-3p (the more widely studied strand and the strand used in our study) has been shown to be important for angiogenesis. In our study, we have demonstrated that it improves angiogenesis through down-regulation of negative inhibitors of VEGF-A. Additionally, miR-126-5p (the less common strand) has been shown to be critical suppressor of atherosclerotic lesion formation (Schober et al., 2014). Schober and colleagues showed that the proliferative ability of endothelial cells was augmented after miR-126-5p overexpression and countered the antiproliferative effects of hyperlipidemia and decreased atherosclerosis in miR-126/- mice. Taken together with findings from our study, these findings suggest that miR-126 has dual function of angiogenesis and atheroprotective endothelial regeneration. In our current study, we only studied the angiogenic effect of miR-126-3p but did not investigate the atheroprotective effects of miR-126-3p and miR-126-5p. In future studies it will be very interesting to investigate the relationship between miR-126-3p and miR-126-5p in the settings of diabetes and how they interact to modulate inflammation and atherosclerosis. Additionally, the proangiogenic effects of miR-126-3p may require basal miR-126-5p expression. Currently, there are no studies that have examine weather miR-126-5p could
augment the proangiogenic effects of miR-126-3p. Especially in settings of diabetes, where angiogenesis is compromised and atherosclerosis is a common associated complication, it will be fascinating to study whether overexpression of both miR-126-3p and miR-126-5p could enhance the overall health of the vasculature.

There are still many intriguing unanswered questions that can be explored to fully take advantage of miR-126 for therapeutic strategies to improve vasculature in diabetes. The insights from these future studies will broaden our current understanding of the complex interplay of miR-126 and its critical role in promoting angiogenesis, vascular integrity and atheroprotection in the settings of diabetes.

*Optimizing of UMGD for gene delivery*: UMGD due to its minimum invasiveness offers several advantages over the viral delivery strategy. Despite this, various limitations have been associated with it that needs to be addressed before advancing to phase I human clinical trials. For instance, UMGD can only be applied to organs or tissues that are readily accessible by ultrasound. Low transfection efficiency of exogenous genes has been considered to be a significant limitation of this technique. To overcome this, future therapies should develop novel DNA vectors to enhance transfection efficiency. Minicircle offers great potential owing to their small size and lack of bacterial elements (Jia et al., 2010). Chen and colleagues have shown that *in vivo* delivery of minicircle to liver of mice resulted in more than 40 folds increase in expression compared to standard plasmid DNA vectors (Z. Y. Chen, He, Ehrhardt, & Kay, 2003). The use of minicircle should improve efficiency of UMGD for *in vivo* gene delivery. The smaller size of
minicircle compared to standard plasmid allows for a greater amount of minicircle to be bound per microbubble. Additionally, the lack of bacterial backbone elements makes minicircle an ideal vector for gene therapy for clinical translation.

The cationic microbubbles that are used as vehicles for in vivo gene delivery with UMGD can be optimize to enhance its stability and modified to improve transfection efficiency. Several recent studies have developed and made use of ligand (Rychak, Lindner, Ley, & Klibanov, 2006) or antibody conjugated microbubbles to enhanced gene transfection specificity and efficiency (Yan et al., 2014; Zhou et al., 2015). These modifications can further improve the specificity of microbubbles to target specific cell types or targeted tissue. Yan and colleagues showed that compared to non-conjugated Timp3 bearing microbubbles, UMGD of MMP2 antibody conjugated microbubbles bearing Timp3 plasmid resulted in greater efficiency of gene delivery and expression in the myocardium in a rodent ischemia/reperfusion injury model (Yan et al., 2014). This subsequently resulted in smaller infarcts and improved cardiac function. The ability of the microbubbles to specifically target tissues will greatly improve efficiency of UMGD especially in settings with severely compromised vasculature (ischemia, necrotic tissue, infarction). With these critical facets in mind, UMGD certainly exhibits the potential to offer innovative-targeted therapy for vascular diseases such as CAD and PAD.

Insights from future molecular studies exploring miRs biosynthesis and regulation along with optimization of UMGD will certainly offer great potential to development of a noninvasive clinically applicable gene/miR therapeutic strategy that could alleviate vascular complications.
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