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Micro RNAs (miRNAs) are small non-coding RNAs that play a role in many biological processes. Many studies have shown that miRNAs are involved in ischemic conditions and emphasized their role as negative or positive modulators in angiogenesis. In vivo studies using various models bring light to the potential benefits of these miRNAs in perfusion recovery following surgically induced hind limb ischemia (HLI), a model of Peripheral arterial disease (PAD). PAD remains a major healthcare burden, affecting millions of Americans. With little advancement in effective therapy the morbidity and mortality of this disease continues to rise. A better understanding of how miRNAs modulate perfusion recovery may provide a potential effective therapy for PAD.

Keywords: MicroRNA, Peripheral arterial disease, Ischemia, Angiogenesis

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Introduction

Micro RNAs (miRNAs) are small (~22 nucleotide long) non-coding RNAs that regulate gene expression at the post-transcriptional level by degradation of mRNAs or inhibition of protein translation [1]. The first micro RNA, lin-4, was discovered in *Caenorhabditis elegans* (*C. elegans*) by Rosalind C. Lee et al in 1993 [2]. Currently, over 1600 miRNA precursors are thought to be included in the human genome, producing over 2,000 mature miRNAs [3]. Initially thought to have little significance it is now known that microRNAs play crucial roles in many biological processes such as cell growth, embryogenesis, apoptosis, and are also involved in numerous disease processes [4].

Peripheral arterial disease is caused by atherosclerosis leading to narrowing of the blood flow to the lower limbs. This leads to two distinct clinical manifestations, symptoms of discomfort and pain on walking known as intermittent claudication (IC) or pain at rest sometimes associated with gangrene or ulceration also known as critical limb ischemia (CLI). Risk factors for PAD include cigarette smoking, diabetes mellitus (type 1 and 2), hypertension, hy-

percholesterolemia, hyperhomocysteinemia, elevated fibrinogen levels and elevated C-reactive protein level [6]. In individuals with PAD and diabetes, the disease is more likely to develop below the knee (femoral-popliteal and tibial) while those without diabetes are more likely to develop proximal disease (aorto-ilio-femoral) [7]. Many individuals with diabetes suffer from peripheral neuropathy, resulting in impaired sensory function. As a result, the classic history of claudication may be less common in these individuals [7]. Moreover, as much as 1/3 to 1/2 of individuals with PAD remain without the 2 classic symptoms of PAD. Approximately 8 millions Americans are affected with PAD, with African Americans having higher rates [8]. PAD affects up to 10% of people younger than 70 and 15-20% of those older than 70 [9]. About 10% of individuals with IC go on to develop CLI within 5 years [10]. Within the first year after presentation an estimated 25% of patients with CLI die, 35% remain alive with amputations, 25% have resolution while 20% continue to be symptomatic [10]. The burden on the health system has been catastrophic, with an estimated 4 billion dollars spent on PAD related treatment in the year 2001 [11].

The diagnosis of PAD begins with a thorough history and examination of the patient. Several tests are available to diagnose PAD, however the most common initial test used is the ankle-brachial index (ABI). The test involves measuring the blood pressure in the ankle compared to that in the arm to give an idea of the circulation of blood. A normal ABI value ranges from 0.9-1.3, while values ≤ 0.9 are considered diagnostic and values greater than 1.4 are abnormal, indicating non-compressible arteries. In patients with rigid arteries a toe brachial index (TBI) is usually indicated. Beyond the ABI further tests may be indicated to help localize the occlusion as well as determine disease severity, these include Doppler ultrasound, magnetic resonance angiogram and arteriogram [12].

To date, the general treatment of PAD involves improving walking distance and reducing risk factors to decrease associated morbidity and mortality. Two important management strategies involve smoking cessation and exercise [13]. Medications that have been used in the treatment of PAD include cilostazol, statins, calcium channel blockers and antithrombotic therapy [14-17] How-

ever, these medications have not been very effective in treating the main defect in PAD i.e they are not very effective in improving perfusion.

The adaptive process of neovascularization following vascular occlusion is an important mechanism involved in perfusion recovery [18]. The area distal to the occlusion suffers from ischemia, which promotes proliferation of preexisting collateral arteries and formation of new capillaries [19]. An approach to the treatment of CLI known as therapeutic angiogenesis has gained some attention. The idea of therapeutic angiogenesis involves promoting the growth of new blood vessels or changes in existing vessels in order to improve blood flow. Although a number of angiogenic factors have been successfully used for therapeutic angiogenesis in pre-clinical models (eg. VEGF, FGF, and HGF), these agents have had very limited success in human clinical studies [6,20,21]. Therefore, other approaches are being investigated to explore the possibility of bringing the promise of therapeutic angiogenesis to fruition. One approach includes the investigation of miRNAs as a potential factor that could be manipulated to improve microvascular blood flow in PAD. This review aims to summarize different approaches that have been used to identify miRNAs involved in PAD and the potential for miRNAs as a therapy for PAD.

MicroRNA expression in proangiogenic cells of CLI patients

The vascular system develops during the process of embryogenesis. This vasculogenesis was initially thought to occur only during embryonic development, however it is now known that this process occurs to some degree in the postnatal life [22]. Situated within the bone marrow are non-hematopoietic progenitors, including endothelial progenitor cells (EPCs)/ Proangiogenic Cells (PACs) [23]. These EPCs are capable of being recruited to sites of arterial injury to participate in neovascularization [24]. Specific markers expressed by these cells include VEGFR2, VE-cadherin, CD31 and CD133 [25]. Spinetti et al. screened the PACs of CLI patients with and without diabetes and controls for 28 miRNAs that potentially modulated angiogenesis and found eighteen differentially regulated miRNAs. Two miRNAs, miR-15a and 16 were chosen for further analysis based on their potential role in angiogenesis and ischemia. PCR analysis revealed an elevation of miR-15a and 16 in the PACs as well as in the serum of CLI patients with or without diabetes. When hypoxia was used to stimulate ischemia *in vitro*, increased miR15a and 16 expression was noted in healthy PACs. Overexpression of both miRNAs in PACs increased apoptosis, while inhibition corrected it. Additionally, *in vivo* studies confirmed that ischemic mouse adductor muscles treated with anti-miR-15a/16 PACs had increased blood flow recovery and arteriole density, while those receiving pre-miR-15a/16 PACs displayed impaired perfusion recovery and a reduced number of capillaries compared to negative controls [26]. This model provides preliminary data that PACs enriched with miR15a/16 may serve as a potential therapeutic target in PAD.

MiRNAs in ischemic vs. non-ischemic muscle

A cluster of microRNAs, miR-17~92, has been shown to be upregulated in tumors and promotes tumor angiogenesis [27,28]. Bonaeur et al. showed that this microRNA cluster, particularly miR-92a, is expressed in human endothelial cells. Using a mouse hind-limb ischemic model, they showed an upregulation in the expression of miR-92a in the ischemic limb compared to the non-ischemic limb, with maximal effects seen within the first three

days following injury. Furthermore, mice injected with an inhibitor, antagomir-92a, had a significant reduction in toe necrosis and improved perfusion compared to those injected with a negative control [29].

Similar results were observed in a different ischemic condition, where acute myocardial infarction was also found to upregulate miR92a. Bonaeur et al. injected antagomir-92a or a control at various days following left coronary artery occlusion. Mice treated with antagomir-92a exhibited improved left and right ventricular function compared to controls. Additionally, infarct size and number of apoptotic cells were also reduced. Despite being proangiogenic in tumor cells, miR92a was found to be antiangiogenic in ischemic endothelial cells.

Grundman et al. performed a microarray analysis on RNA isolated from adductor muscle of C57BL/6J mice at baseline and multiple time points following femoral arterial ligation.

They identified numerous miRNAs that were upregulated and downregulated, however, miR-100 was the most consistently downregulated miR at day three after femoral artery ligation. To confirm that these changes in expression were related to vascular structures collateral arteries from hind limb at day 3 after femoral artery ligation were isolated. Both the growing collateral arteries of the ischemic limb and the non-proliferating arterial anastomosis of the non-ischemic limb expressed miR-100. However, the expression of miR-100 was significantly decreased in the growing collateral arteries of the ischemic limb compared to non-proliferating, quiescent vessels from ischemic limb. No differences were noted between the growing and quiescent vessel of the non-ischemic limb. Moreover, endothelial cells were isolated and revealed a decrease miR-100 expression in ischemic endothelial cells compared to non-ischemic endothelial cells at day 3 following ligation [30].

To investigate targets of miR-100, endothelial cells over expressing miR-100 were analyzed by micro array which revealed hundreds of potential mRNA targets. These potential targets were entered in two bioinformatics algorithms and 8 targets were common to both, including the mammalian target of rapamycin (mTOR), a proangiogenic gene. Furthermore, inhibition of miR-100 with an antagomir injection given to C57BL/6J mice before and for two days following hind-limb ischemia resulted in enhanced perfusion recovery measured by Doppler imaging [30]. Mice receiving an mTOR inhibitor, rapamycin, resulted in reduction of blood flow recovery following 7 days after femoral artery ligation.

Differentially regulated microRNAs in two phenotypically distinct mouse strains

It has been established that two mouse strains, BALB/cJ and C57BL/6J, recover differently following hind-limb ischemia (HLI). Whereas the C57BL/6J strain recovers well following HLI, BALB/cJ mice exhibit poor perfusion recovery [31,32]. With this knowledge, Hazarika et al. performed a miRNA micro-array analysis of ischemic gastrocnemius muscles of these two strains and found that C57BL/6J had a greater upregulation of miR-93 following ischemia. The expression of miR-93 was increased in ischemic muscle of C57BL/6J at a time point when perfusion recovery in both strains were similar (day 3), and remained elevated at day 7 post-surgery. Moreover, the C57BL/6 strain also expressed higher levels of miR-93 in the non-ischemic muscle compared to BALB/c [33].

In vivo studies involving gain of function and loss of function further solidified the role of miR-93 in perfusion recovery. C57Bl/6J mice treated with antagomir-93 at day 0, 7 and 14 post HLI had impaired perfusion starting at day 14 compared to the negative control. Moreover, a gain of function technique in which BALBC/cJ mice received a local intramuscular injection of pre-miR 93, resulting in overexpression of the miR93, showed enhanced perfusion recovery as well as higher capillary density compared to the negative control. The effects of this microRNA are thought to be the result of multiple genes [33]. Taken together, this unbiased model using distinct phenotypic outcomes between two inbred mouse strains following HLI confirmed miR-93 as the most consistent differentially regulated micro-RNA based on strain and ischemia and solidifies its role in perfusion recovery.

MicroRNAs in a diabetic ischemic model

As stated before, diabetes is a major risk factor for PAD. The hyperglycemic state leads to endothelial cell dysfunction and vessel wall remodeling and thus an increase in ischemic complications [34]. Diabetics have poor recovery following ischemic insults due to impaired reparative angiogenesis [34]. Caporali et al. found an upregulation of miR-503 in cultured conditions mimicking diabetes mellitus (DM) and ischemia. This prompted further in vivo studies of this miR. They used a type 1 diabetic mice model using CD1 male mice and found that expression of miR-503 was significantly higher in the ischemic adductor muscles of diabetic mice compared to non-diabetic and/or non-ischemic controls at day 3 post ischemia [35].

Moreover, efficient inhibition of miR-503 using a decoy for miR-503, Ad.decoymir-503, improved blood flow recovery in diabetic mice as well as increased capillary and arteriolar density in ischemic muscle. The target genes, *cdc25A* and *CCNE1*, were down regulated by diabetes mellitus but returned to normal levels by inhibition of miR-503 at day 3 and 21 post ischemia. To further strengthen the role of miR-503 in diabetes and ischemia, it was found that diabetic patients undergoing amputation for CLI had an increase expression of miR-503 in calf muscle compared to non-diabetics and non-ischemic control patients undergoing saphenous vein stripping (n=11 each group, p<0.05) [35].

MicroRNAs in Arteriosclerosis Obliterans

Li et al. designed a study which enrolled 104 patients with arteriosclerosis obliterans (ASO) and 105 age-matched controls in order to determine which miRNAs were differentially expressed between these two groups. Based on their known association with vascular biology, a selected set of miRNAs, (miR-21, miR-221, miR-222, miR-145, miR-143, miR-27b, miR-130a, let-7f, miR-378, miR-210, miR-15, miR-16 and miR-126) were examined in intima samples. Levels of miR-21, miR-130a, miR-27b, let-7f and miR-210 showed significant increase, while miR-221, miR-222 were downregulated in diseased samples compared to controls [36].

After establishing the expression levels in the intima, these miRs were then examined in the serum of the 104 ASO patients and 105 controls. Results revealed an increase in miR-21, miR-130a, miR-27b and miR-210 with no difference in the expression of miR-221, miR-222 or let-7f. Furthermore, serum levels of miR130a, miR27b and miR-210 were increased at each fontaine stage, with the expression of miR-27b significantly increasing with each advancement of fontaine stage [36]. Taken together these results suggest that these miRNAs may be used as potential risk markers for diagnosing ASO at earlier stages or serve as a severity indicator. With further studies and better understanding of the microRNAs it may be possible to manipulate their effects to establish a therapeutic agent.

Discussion

Peripheral arterial disease affects millions of individuals worldwide, yet treatment strategies remain subpar. Late diagnosis coupled with ineffective treatment has led to a large burden on the healthcare system as well as increasing mortality rate. Understanding the mechanisms involved in the pathophysiology of PAD is crucial in identifying new therapeutic agents. The ability to form new blood vessels is an important mechanism following occlusion of an artery and multiple studies have shown that many microRNAs play a role in inhibiting and stimulating this angiogenic process. Though surgical intervention has been used in patients with CLI, many patients may not be candidates for such surgical treatment and those experiencing initial success may then face complications such as graft failure or stenosis. Additionally, there is evidence that repairing occlusions of large vessels may not improve perfusion or ABI, suggesting at least in some individuals, the disease is primarily in the microvasculature.

MiRNA's have been successfully manipulated to improve out-

Table 1. Summary of microRNAs along with their targets, how they were identified and their effect on perfusion

Micro RNA	Targets	How it was identified	Effect
miR-15a/16	VEGF-A, AKT-3	PCR analysis of PACs from healthy patients and CLI patients with and without T2DM	Impairs perfusion
miR-92a	Integrin subunits $\alpha 5$ (ITGA5) and αv , S1P1, and MKK4	PCR analysis of human endothelial cells	Impairs perfusion
miR-100	Target gene mammalian target of rapamycin (mTOR)	Microarray analysis of C57BL/6J mice adductor muscles	Impairs perfusion
miR-93	P21, E2F-1, P53	PCR analysis of ischemic muscle of two phenotypically different mouse strains	Improves perfusion
miR-503	CCNE1 and <i>cdc25A</i>	PCR analysis of culture endothelial cells in conditions mimicking diabetes and ischemia	Impairs perfusion

comes in the above preclinical models of PAD. Table 1 suggests a strategy that involves overexpression of miR-93 or inhibition of miR-15a, miR-16, miR-92a, miR-100 and miR-503 may be effective. However, many factors must be taken into account in the development of these therapeutic approaches in order to achieve success. For example, the current studies have been in pre-clinical models of PAD in mice, where systemic injection or local injection of microRNA mimics or antagomirs have been used successfully. However, the sort of dosing regimen used in mice may not be translatable to humans. It would be important to determine whether systemic vs local administration would be the most effective in achieving expression of pre-miRNA or antagomirs in the lower extremities of humans with PAD. Another concern is the possibility of off target effects of these miRNAs which has the potential to increase side effects. Research involving microRNAs is evolving due to its emerging roles in disease pathophysiology. With the first therapeutic microRNA currently in phase I clinical trials for the treatment of liver cancer, manipulation of mRNAs holds promise for a potential target for improving patient outcomes in PAD.

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