Chapter 7

MicroRNAome of Vascular Smooth Muscle Cells: Potential for MicroRNA-Based Vascular Therapies

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1. Introduction

Although until recently it is presumed that the greater portion of the genome has no biological role, the current advances in genome research and RNA biology have provided evidence indicating that a large section of the human and most eukaryotic genome is transcribed as non-protein-coding RNAs or non-coding RNAs (ncRNAs)[1,2]. Only about 2% of the eukaryotic genome sequence codes for protein encoding genes and the remaining so called "junk" DNA are thought to have no functional significance [3, 4]. Based on large scale studies of human and other eukaryotic genomes it is estimated that about 98 % of the transcriptional output of their genomes is RNA that does not encode protein implying that the genomes are gorged with either inept RNA transcripts or with ncRNA transcripts that exhibit unanticipated functions in eukaryotic biology. However, recent development of new technologies in molecular biology and human genetics such as genome tiling [4,5], microarrays, and next generation RNA-sequencing (RNA-Seq) [6,7] have enabled the discovery of different types of ncRNAs that do not code for protein product [8-10]. Even though ncRNAs do not encode proteins, they play pivotal roles in the complex networks that are necessary to regulate cellular functions via transcriptional and translational regulation of protein coding genes that are crucial to normal development and physiology, and to disease [11]. Moreover, many of the ncRNAs are highly conserved and susceptible to epigenetic and genetic defects that affect normal development and disease process significantly [12-15].

There are copious non-coding transcripts that participate principally in regulating cellular protein synthesis, which are grouped into different classes based on their size, function and association with transcription start site [1, 12, 16-18]. According to their size, ncRNAs are categorized into: small ncRNAs, 20 to 200 nucleotides long, which includes microRNAs

(miRNAs), PIWI-interacting RNAs (piRNAs) and small nucleolar RNAs (snoRNAs); long ncRNAs (lncRNAs) that are longer than 200 nucleotides; and macro ncRNAs, longer than 200 nucleotides that can reach 100 kilobases (kb) longer without being processed into small ncRNAs [1,7,12,18]. Based on where they are derived from within the genome, lncRNAs can be distinguished from each other. There are intronic lncRNAs (transcribed between exons of genes), intergenic lncRNAs (transcribed from the space between two genes) and lncRNAs that are derived from the regions that overlap both exon and intron of a coding gene. Furthermore, each of these ncRNAs may also be in the sense or in the antisense direction. According to functional significance, ncRNAs can be divided into: (1) housekeeping ncRNAs and (2) regulatory ncRNAs. Housekeeping ncRNAs include constitutively expressed ncRNAs that are crucial for the normal function and cellular viability, which include transfer RNAs, ribosomal RNAs, small nuclear RNAs, and snoRNAs [18]. On the contrary, regulatory ncRNAs or riboregulators include ncRNAs such as miRNAs and lncRNAs that are expressed in response to external signals, during different cellular states such as cellular differentiation or at certain stages of development, influencing the expression of other genes at transcription and translational levels [1, 7, 12, 18]. Regarding ncRNAs that are associated with transcription start sites of genes, there are different classes of ncRNAs such as promoter-associated small RNAs (PASRs) [16], transcription start site-associated RNAs (TSSa-RNAs) [19], promoter upstream transcripts (PROMPTs) [20] and transcription initiation RNAs (tiRNAs) [21]. Even though their functional roles are poorly delineated, perhaps they have a regulatory role in transcription.

Among the ncRNAs, the most widely studied and comparatively well delineated regarding their functional relevance to normal development and physiology, and to pathogenesis of disease are, small microRNAs [1, 12, 22-25]. miRNA deficiencies or surpluses have been correlated with diverse clinically important diseases including various types of cancers, neurological diseases, metabolic diseases, cardiovascular diseases, and many others [22, 25-32]. Here, we provide an overview of the current knowledge of miRNAs that participate in the regulation of vascular smooth muscle cells (VSMC) phenotypic modulation and present the potential opportunities for miRNA-based therapeutic and diagnostic approaches for vascular proliferative diseases due to atherosclerosis and restenosis. Finally, we briefly describe our preliminary unpublished data on miRNA expression profile of VSMC in response to butyrate, a histone deacetylase (HDAC) inhibitor.

2. Atherosclerosis and restenosis

Vascular cell activation and remodeling are the principle events in vascular pathologies such as atherosclerosis, transplant vasculopathy, post angioplasty restenosis, in-stent restenosis and bypass graft failure [33, 34]. It is realized that injury to vessel wall by various atherogenic insults sets-off inflammatory response causing endothelial cell dysfunction. Following endothelial cell dysfunction, VSMC in the media that are quiescent and contractile in nature, migrate to intima in response to local inflammation and become proliferative cells. VSMC are highly specialized cells whose principal function is to regulate the attributes of blood vessels in the body by appropriately responding to changes in the volume of blood vessels and the

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local blood pressure to facilitate distribution of oxygenated blood to different parts of the body. In adult vessels, VSMC proliferate at very low rate; display reduced synthetic activity; and express a unique compilation of proteins that is characteristic of contractile phenotype such as contractile proteins, ion channels, and signaling molecules. Yet, they still maintain remarkable plasticity and retain the ability to undergo extreme and reversible changes in phenotype in response to their local environmental signals, especially during vascular development, and in response to vascular injury as a key mechanism in wound healing. It is recognized vascular injury provoked by various atherogenic insults such as mechanical, chemical and immunological injuries triggered by different disease risk factors promote VSMC activation, migration and proliferation, which are precursors to the development of atherosclerosis and neointimal hyperplasia [34, 35]. VSMC also undergo phenotypic modification from contractile to proliferative or synthetic phenotype in conjunction with vessel remodeling by altering the cell number and composition of vessel wall as the primary pathophysiological mechanism in different clinical pathologies such as postangioplasty restenosis, in-stent restenosis, and vein bypass graft failure and transplant vasculopathy [34-37]. However, the molecular mechanisms involved in VSMC phenotypic control are still vague.

During the last few years there is an upsurge in ncRNA research specifically pertaining to a novel class of small miRNAs because of their role in various biological functions. In a variety of eukaryotic organisms miRNAs have been demonstrated to play key roles in various cellular processes including proliferation, differentiation, and apoptosis [38-40], which are central to normal development and physiology, and pathogenesis of diseases. As such, dysregulation of miRNAs has been linked to different diseases, including different cancers, neurological, cardiovascular and other diseases [22, 25-32]. Because of their effects on cellular processes as gene expression regulators, impairment of miRNAs as evidenced in many cancers, suggest involvement of miRNAs in the phenotypic modulation of VSMC both in normal and disease states. Here we briefly describe miRNAs, their biogenesis and mechanism of action and then summarize the recent progress in the functional significance of miRNAs in VSMC phenotypic modulation and response to injury.

3. miRNAs

miRNAs are endogenous, well conserved, small ncRNAs, usually 20 to 26 nucleotides, that mediate posttranscriptional gene silencing by complimentary binding to the 3′-untranslated region (3′-UTR) of their target mRNA, leading to direct target mRNA degradation or translational repression, a key phenomenon for controlling gene expression in a tissue- and development-specific manner [1, 38-40]. They were first detected in Caenorhabditis elegans as regulators of development in 1993 [41] and since then they have been found in many species of plants and animals. There are several differences between plants and eukaryotic mRNAs. In plants, transcriptional repressions require a perfect or near-perfect target match, whereas mismatched target can cause gene silencing at the translational level in eukaryotes. In eukaryotes, miRNA complementarity typically includes the 5′ bases 2-7 of the miRNAs, which is referred as miRNA “seed” region, Furthermore, one miRNA can target many different sites

on the same mRNA or many different mRNAs, and a single mRNA can be under stringent but redundant control of several miRNAs. Another difference is the location of target sites on mRNAs. In eukaryotes miRNA target sites are in the 3′-UTRs of the mRNAs. In plants, target sites are normally in the coding region but they can be present in the 3′-UTRs.

miRNAs are predicted to target about 60% of protein coding transcripts [12, 42, 43]. At present the number of miRNA sequences deposited in miRBase (Release 16) include over 15,000 miRNA loci, expressing over 17,000 distinct mature miRNA sequences from 142 species [44]. Moreover, recent appreciation in miRNA research in eukaryotes implicates that these key gene expression regulators control various biological processes as diverse as cell proliferation, cell differentiation, apoptosis, and stem cell division particularly in mammalian development [38-40, 45]. In spite of tremendous advances in miRNA research, the role of miRNAs in physiological and pathophysiological processes is just emerging. Recent miRNA expression studies demonstrate miRNAs in cardiovascular development [46], brain development [47], viral infection [48], metabolism [29], different types cancer, neurologic and cardiovascular diseases [22, 25-32] suggesting link between miRNAs and wide range of tissue development and diseases. In effect, miRNAs are considered as *trans*-acting gene regulatory molecules, similar to and as important as transcription factors in the control of gene expression [49]. Although miRNAs are considered to act as intracellular RNAs to control gene expression at posttranscriptional level, recent studies have detected miRNAs in circulating blood and in cell culture medium indicating they may be useful as biomarkers of disease [50, 51].

4. Biogenesis of miRNAs

The transcription of miRNAs depends on their location within the genome. Most of the miRNA genes are located throughout the genome in introns, exons and intergenic regions with many miRNAs produced from clusters of coexpressed genes. Some miRNA transcription depends on same RNA polymerase II promoters that drive the transcription of mRNAs. miRNA genes located in intronic regions that includes half of known miRNAs genes often depend on the expression of host gene [52, 53]. Some miRNA genes with independent promoters are transcribed from their own RNA polymerase II promoters. Additionally a small number of miRNAs genes are transcribed by RNA polymerase III. Those miRNAs organized in clusters for example, miRNA-17-92-family, share the same transcriptional regulation and are grouped together in one cluster on a single unprocessed transcript and expressed together [54].

5. The pathway of miRNA biogenesis and gene silencing

The process of miRNA biogenesis starts in the nucleus as depicted in the following Figure [12, 31, 32]. miRNAs are transcribed as hundreds or thousands of base long large primary miRNA species (pri-miRNA) by RNA polymerase II or RNA polymerase III. These pri-miRNA transcripts fold into a stem loop or hairpin structures with capped 5′ end and polyadenylated

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(poly A) tail on 3′ end [55]. Following transcription by RNA polymerase II/III, pri-miRNA transcripts are trimmed to about 60 to 100 nucleotide hairpin structures with ~2 nucleotide 3′ overhang to form precursor miRNAs (pre-miRNAs) by the action of nuclear microprocessor complex. Microprocessor complexes are formed of Drosha (RNASEN), a nuclear ribonuclease RNase III enzyme and its partner DGCR8 (DiGeorge critical region 8) also called as Pasha (Partner of Drosha). The pre-miRNA transcripts are then shuttled to cytoplasm for further processing via Exportin5 and Ran-GTP6. Pre-miRNAs are processed further in the cytoplasm by the action of Dicer, another RNase III enzyme, with the assistance of double-stranded RNA binding proteins (dsRBPs) including TRBP (tar RNA binding protein), resulting in the cleavage of hairpin loop of pre-miRNAs leading to formation of ~22 nucleotide mature miRNA duplexes. Mature duplex is composed of a matured miRNA strand referred as guide strand and a complimentary strand referred as the passenger strand. The gene silencing capability depends on Dicer-mediated loading of one of the miRNA strands, usually guide strand, in the RNA-induced silencing complex (RISC) together with Argonaute (Ago) protein. The RISC guides the miRNA to bind to its complementary sequence within the 3′-UTR of its target mRNA. The degree of complementarity between the seed sequence of the miRNA and the 3′-UTR of its target mRNA determines whether to mediate mRNA degradation or to disrupt translation.

6. MicroRNAome of VSMC

miRNAs are relatively new regulatory molecules that are identified about a decade ago and demonstrated to have regulatory role in every organism and in every biological functions influencing normal biology and disease process. Once again, oncology research is in the leading position in understanding miRNA involvement in human diseases. Although most of the miRNA knowledge is coming from cancer research, during the past few years their role in other systems and diseases are emerging and rapidly being evaluated with new technologies such as deep sequencing. It is not surprising that interest in miRNA is also on the raise in cardiovascular research field. Literature on the roles and functions of miRNAs in normal cardiovascular development and in vascular pathologies is escalating [32, 46, 56-60]. Furthermore, importance of miRNAs in the regulation of VSMC development and phenotypic modification, and response to injury is swiftly being explored because VSMC proliferation and migration are important events in vascular proliferative diseases. Here we will summarize recent updates on the significance of miRNAs in VSMCs and their role in phenotypic modulation of VSMC, thus to vascular proliferative diseases [32, 57-60]. Most of the knowledge of VSMC miRNAs is coming from culture cells, animal models and blood samples of cardiovascular disease patients.

7. Evaluation of essential role of miRNAs in VSMC

Because activity of Dicer is essential for the miRNA processing, loss of Dicer activity should result in global loss of miRNAs. Importance of miRNAs for VSMC development and biolo-

gy can be validated by knocking out the miRNA processing enzyme Dicer in VSMC. To demonstrate the importance of miRNAs in VSMC development and function, a smooth muscle restricted -Dicer knockout model, SM-Dicer KO mice, is investigated recently [59,61]. Outcomes of the study indicate deletion of Dicer causes embryonic lethality due to decreased VSMC proliferation and differentiation resulting in thinner vessel walls, impaired contractility and hemorrhage as well as reduced expression of VSMC-specific genes and proteins [59, 61]. Overall, these observations suggest that Dicer-generated miRNAs are crucial for normal VSMC development, differentiation and contractile function.

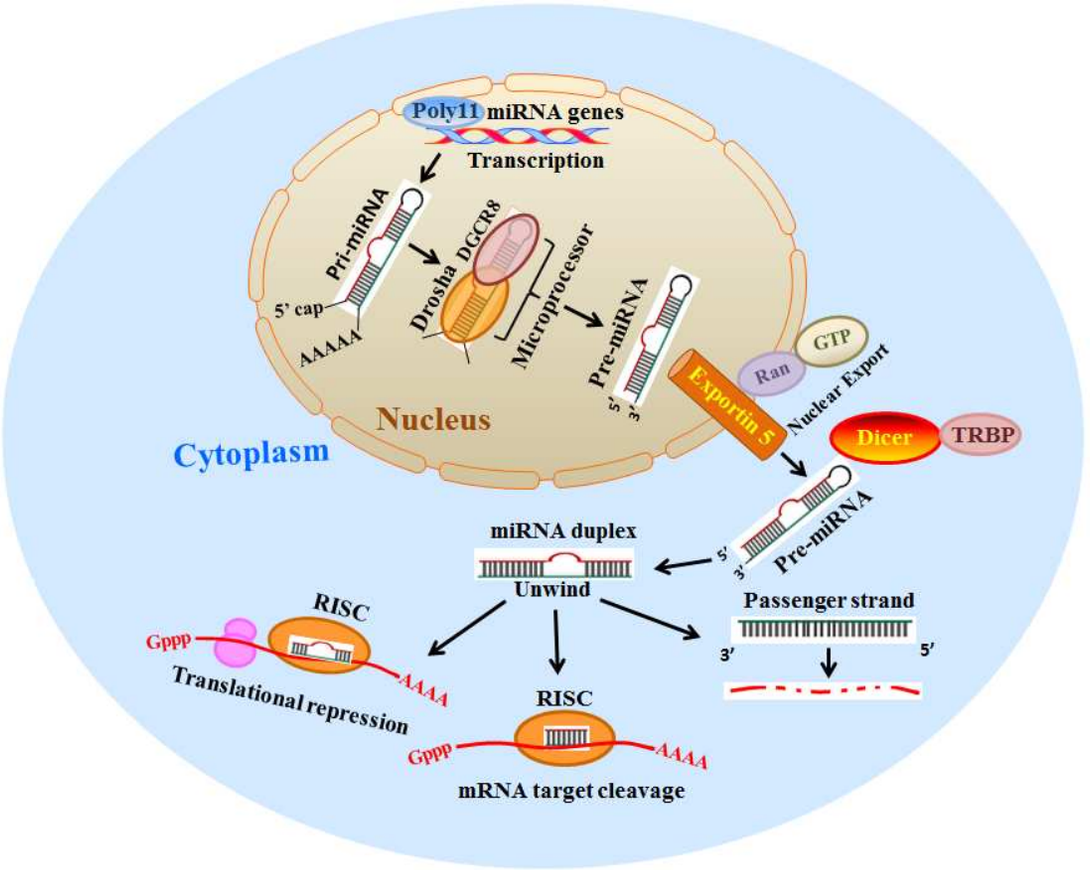

Figure 1. Biogenesis of miRNA and gene silencing pathway. The miRNA synthesis starts in the nucleus where pri-miRNA transcript is cleaved by Drosha/ DGCR8 to form ~60-100 nucleotides long hairpin loop pre-miRNA. Pre-miRNA is then transported to cytoplasm through the mediation of Exportin5 and Ran-GTP6 where it is further processed by RNase activity of Dicer to ~22 nucleotides mature miRNA duplex. The miRNA duplex then loads onto Ago in the RISC complex and undergoes strand separation. The guide strand of the miRNA mediates gene silencing by degrading the target mRNA or interfering with translational process. The passenger strand gets degraded.

8. miRNA regulation of VSMC phenotype

VSMC exhibit remarkable plasticity by adapting to local conditions via phenotypic modulation. Phenotypic modulation of VSMC is a highly complex process regulated by transcription

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factors and other gene products and multiple pathways that are still vaguely understood. Recently several reports have demonstrated the involvement of miRNA-mediated gene silencing in the regulation of VSMC proliferation, migration and differentiation in normal vascular development and in vascular pathologies. A list of a few selected miRNAs that regulate VSMC proliferation and differentiation in cell cultures and animal models with angioplasty is shown in Table 1 along with factors that regulate miRNA expression, their validated target proteins and function of the target proteins. While some of these miRNAs promote VSMC proliferation, others stimulate differentiation.

microRNA	Inducer/ Regulator	Target Proteins	Cellular Functions of Target Proteins	References
miRNA 21	Vascular injury	PTEN, Bcl2	Increase proliferation, apoptosis	62
miRNA 221/222	Injury, PDGF	p27kip1, p57kip2	Increase proliferation	63
miRNA -146a	KLF5	KLF4	Increase proliferation	64
miRNA 26a	Serum deprivation	Smad 1, Smad 4	Decrease proliferation	68
miRNA 143	p53, SRF/ Myocardin	PDGFR, Elk1	Decrease proliferation, stimulate differentiation	58
miRNA 145	SRF/ Myocardin	CamKIIδ, KLF4, KLF5	Decrease proliferation stimulate differentiation	58,65

Table 1. miRNAs regulating vascular smooth muscle cell phenotype

9. miRNAs in the mediation of VSMC proliferation

Some miRNAs, such as miR-21, miRNA-221, miRNA-222 and 146a are demonstrated to promote VSMC proliferation in balloon-injured rat carotid arteries and cultured rat VSMC by silencing their target proteins (Table 1). Among these, miRNA-21 is the first miRNA that is recognized to regulate VSMC growth and survival by silencing phosphatase and tensin homolog (PTEN), a tumor suppressor protein and increasing B-cell lymphoma 2 (Bcl-2), which increased VSMC proliferation and survival [32,59-62]. Interestingly, this same miRNA is shown to regulate features of both proliferative and contractile phenotype by separate mechanisms. Through the regulation of processing of the miRNA-21 primary transcript to the mature miRNA -21 transcript, transforming growth factor-β (TGF-β) and bone morphogenetic proteins (BMPs) increased the miRNA-21. This increased miRNA-21 is shown to promote VSMC differentiation by upregulating VSMC restricted contractile proteins by silencing programmed cell death 4, a tumor suppressor protein [63].

Other miRNAs that stimulated proliferative phenotype include miRNA-221 and -222. Their proliferative effect on VSMC is mediated through silencing of their target proteins, p27kip1 and p57kip2, respectively, both of which are negative regulators of cell cycle progression [32,

64]. miRNA-146a is shown to directly target Krupple-like factor-4 (KLF-4) and promote VSMC proliferation in cultured rat VSMC and vascular neointimal hyperplasia [32, 60, 65]. KLF-4 and miRNA-146a appear to exhibit a feedback relationship regulating each other's expression. While miRNA-146a inhibits KLF-4 expression by targeting the 3'-UTR region of KLF-4, KLF-4 inhibits miRNA-146a at the transcriptional level. KLF-5, another member of KLF family promoted the transcription of miRNA-146a. It appears these molecules form a regulatory control to appropriately modulate VSMC proliferation [32, 60].

10. miRNAs in the suppression of VSMC proliferation

Certain miRNAs including miRNA-143, miRNA-145 and miRNA-26a alter VSMC phenotype by causing suppression of VSMC proliferation (Table 1). Among these miRNAs, miRNA-143 and -145 are considered master regulators of contractile phenotype by promoting contractile protein expression [32, 58, 60]. Moreover, miRNA-145 not only stimulates differentiation of adult VSMC, but also promotes differentiation of multipotent neural crest stem cells into VSMC [57]. In normal vessel walls the miRNA-143/145 cluster is lavishly expressed. However, both miRNAs are dramatically reduced not only in injured carotid arteries following angioplasty [32, 58, 60, 66] but also downregulated in different cancer cell lines [67]. Further studies proved that miRNA-145 is a critical modulator of VSMC differentiation via its target gene KLF-5. Consistent with this, while the use of miRNA-145 oligonucleotide mimics upregulated the expression of VSMC differentiation marker genes such as SM α-actin, calponin, and SM-MHC, both at gene and protein levels, overexpression of KLF-5 reduced the gene expression of SM α-actin implicating a relationship between miRNA-145 and KLF-5 gene in VSMC differentiation.

Analysis of growth arrested human aortic VSMC by miRNA array screening identified upregulation of miRNA-26a in differentiated VSMC, which is associated with reduction in SMAD activity [59, 60, 68]. This miRNA is dramatically downregulated in two murine models of aneurysm.

Embryonic stem cells are known to differentiate to VSMC and one of the factors that induces VSMC differentiation is all trans retinoic acid, which in addition to regulating a wide variety of protein coding genes it also regulates expression of miRNAs that affect smooth muscle cell differentiation. It is found that expression of miRNA-10a contributes to retinoic acid-induced VSMC differentiation by negatively regulating its target histone deacetylase 4 [69]. Involvement of miRNAs in stem cell and vessel wall progenitor cell differentiation has significant implications in the pathogenesis of atherosclerosis, the response to vascular injury and vascular remodeling.

11. Circulating miRNAs

Recently presence of miRNAs is demonstrated in circulating blood, which may be useful as biomarkers for diseases [51]. Analysis of serum or plasma for circulating levels of miRNAs in

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normal individuals and in patients with coronary artery disease revealed circulating levels of angiogenesis-related miRNA-126 and miRNA-92a, the inflammation-associated miRNA-155; and VSMC-enriched miRNA-145 and miRNA-17 are significantly reduced in patients with coronary artery disease compared to normal individuals [51]. Whereas cardiac muscle-enriched miRNAs, miRNA-133a and -208a are elevated in patients with disease. These observations suggest that circulating miRNAs can be used as biomarkers for diagnosis of cardiovascular diseases.

12. miRNAs in atherosclerosis and neointimal hyperplasia

Although roles of various miRNAs and their participation in biological processes have been recognized in various cultured cells or animal models, and expression profiles of circulating miRNAs in patients of cardiovascular diseases [50, 51], involvement of miRNAs in human atherosclerotic plaques has received little attention. However, one of the recent studies investigated miRNA/mRNA expression profiles of human atherosclerotic plaques from peripheral arteries in comparison to nonatherosclerotic left internal thoracic arteries to determine the relationship between miRNA/mRNA expression profiles and biological processes in atherosclerosis [70]. Results of this study revealed significant amounts of miRNA-21,-34a, -146a, -146b-5p, and -210 expressions in atherosclerotic lesions. Consistent with this there was downregulation of several predicted targets of these miRNAs in atherosclerotic plaques. According to the combination of miRNA/mRNA profiles and bioinformatic analysis, nine KEGG pathways including immunodeficiency, metabolism, p53 and cell proliferation signaling pathways enriched with predicted targets were significantly upregulated. On the contrary, VSMC contraction and purine metabolism were downregulated.

13. miRNAs in restenosis

Role of miRNAs in restenosis is mainly studied using the common rat carotid artery balloon injury animal model. miRNA profiles in the carotid artery is determined by using miRNA arrays [62]. One of the miRNA that was aberrantly overexpressed in injury-induced neointimal lesions is miRNA-21. miRNA-21 promotes VSMC proliferation and inhibits apoptosis of VSMC by directly targeting PTEN and programmed cell death 4, respectively [32]. Similarly miRNA-221 and -222, which are encoded by a gene cluster on X chromosome, share the same seed sequence, identical targets and similar functions were upregulated in balloon-injured carotid arteries. Consistent with their upregulation, their target genes, p27kip1 and p57kip2 were downregulated [32, 64]. Additionally, miRNA-143 and miRNA-145 that promote VSMC differentiation and expressed highly in vascular tissue, were significantly reduced in apolipoprotein E knockout mice where vascular injury was induced by hypercholesterolemic diet [71]. Cooperatively, both miRNA-143 and miRNA-145 target a network of transcription factors such as Elk1, KLF-4 and myocardin to stimulate differentiation and inhibit proliferation of

VSMC. Taken together, these studies indicate significant role of miRNA-143/miRNA-145 in VSMC differentiation and vascular disease.

14. miRNAs in histone deacetylase (HDAC) inhibitor arrested VSMC proliferation

Butyrate, a dietary-derived epigenetic histone modifier and a histone deacetylase (HDAC) inhibitor, is a strong inhibitor of VSMC proliferation [72-75]. Butyrate elicits many cytoprotective, chemopreventive and chemotherapeutic activities mainly through inhibition of cell proliferation, induction of cell death or stimulation of cell differentiation by selectively modulating gene expression via epigenetic changes [72-75]. Incidentally, the cellular effects that are stimulated by butyrate are also regulated by miRNAs and expression of some of these miRNAs is regulated by epigenetic mechanisms including DNA methylation and histone modification [76, 77]. Because butyrate is an established epigenetic histone modifier it is possible that butyrate may alter expression of some of the miRNAs in butyrate arrested VSMC proliferation. To explore this possibility, we recently examined expression profile of 650 miRNAs in butyrate inhibited rat VSMC proliferation by qRT-PCR array platform. Our preliminary unpublished data indicates differential expression of about 60 miRNAs. Among these, members of the miRNA-17-92 cluster are some of the miRNAs that are downregulated by butyrate in VSMC suggesting that antiproliferation action of butyrate is linked to downregulation of miRNAs of miRNA-17-92 cluster (Table 2). Studies have shown that the miRNAs of this cluster are not only involved in normal development of heart, lung and immune system but they also exhibit essential role in tumor formation by promoting cell proliferation and suppressing apoptosis [78].

miRNA-17-92 cluster mature miRNAs	**Fold change ***
rno-miR-17-1-3p	-2.77
rno-miR-17-2-3p	-2.65
rno-miR-17-5p	-2.15
rno-miR-18a*	-1.80
rno-miR-19a	-2.26
rno-miR-19a*	-2.31
rno-miR-19b	-2.32
rno-miR-19b-1*	-2.84
rno-miR-20a*	-2.40
rno-miR-92a	-2.45
rno-miR-92a-1*	-8.62

*Values represent fold changes relative to untreated rat VSMC.

Table 2. Changes in miRNA-17-92 cluster mature miRNAs levels in butyrate treated rat VSMC

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The miRNA-17-92 cluster is a polycistronic miRNA gene, which is titled as oncomir-1 in humans because of their oncogenic properties and overexpression in different cancers [79]. The miRNA-17-92 primary transcript encodes six mature miRNAs: miRNA-17,-18a, 19a, 20a, 19b-1, and 92a-1 that are tightly grouped within an 800 base-pair region of human chromosome 13 [80]. For some of these members corresponding target genes have been identified, which include cell cycle inhibitor CDKN1A (p21Cip1) and pro- apoptotic PTEN and BCL2L11 (Bim). Furthermore, transcription of miRNA-17-92 has been shown to be activated by c-myc transcription factor [78]. In our earlier studies butyrate has been shown to downregulate c-myc [81] and upregulate CDKN1A (p21Cip1) [72-75] in proliferation inhibited VSMC. Based on these observations, it appears by downregulating c-myc expression potentially via epigenetic modification, butyrate inhibits expression of miRNA-17-92 cluster with a corresponding increase in miRNA-17-92 target genes such as CDKN1A (p21Cip1). Taken together, our preliminary miRNA expression data emphasizes role of miRNAs in antiproliferative and chemoprotective effects of butyrate in VSMC. Further studies are under investigation to confirm the role of miRNA-17-92 cluster in the regulation of VSMC proliferation by investigating the effects of miRNA mimics of miRNA-17-92 cluster in reversing the effect of butyrate on VSMC proliferation and on decreasing the levels of their target proteins. Utilization of this information is beneficial in targeting miRNAs aimed to decrease the level of pathogenic/aberrantly expressed miRNAs or to increase miRNAs with valuable functions in the intervention of occlusive vascular proliferative diseases.

15. miRNAs as new therapeutic targets for vascular proliferative diseases

Despite the substantial progress in understanding the etiology and clinical management of vascular proliferative diseases, they are still life threatening diseases responsible for the global burden of cardiovascular diseases. Clinically, medications and surgical procedures are the only methods of treatment for patients with atherosclerotic disease. Atherosclerotic patients are generally treated by angioplasty with stent replacement but it commonly leads to restenosis in significant number of angioplasty patients. Phenotypic modification of VSMC from contractile differentiated state to proliferative dedifferentiation state is the primary pathophysiological mechanism in the development of atherosclerosis and in different clinical pathologies such as postangioplasty restenosis, in-stent restenosis, vein bypass graft failure and transplant vasculopathy [33,34]. Therefore, understanding the molecular mechanisms of VSMC proliferation may offer novel insights into disease pathogenesis leading to targeted therapies. Vascular phenotypic modulation is a multifactorial process involving multiple pathways and multiple genes. Based on the current understanding of the roles of miRNAs in the normal development and in disease pathogenesis, it appears miRNA-based therapy has a potential in vascular proliferative diseases, particularly because one endogenous miRNA can target its multiple target genes. Moreover, demonstration of changes in expression of certain miRNAs that is specifically associated with particular VSMC phenotype in different models of studies, as depicted in this article, clearly suggests that expression analysis of miRNA will provide insights into vascular proliferative disease mechanisms and possibly identifies novel

targets for future vascular therapy. This information is important in targeting miRNAs aimed to decrease the level of abnormally expressed miRNAs and/or to increase miRNAs with valuable functions in the intervention of occlusive vascular proliferative diseases.

The recent demonstration that changes in expression of certain miRNAs in neointimal lesions, particularly upregulation of miRNA-21 and miRNA-221/-222 and downregulation of miRNA-145 support proliferative phenotype of VSMC suggests targeting miRNAs may represent a new form of therapy for vascular proliferative diseases [62, 64]. Furthermore, silencing of miRNA-21 and miRNA-221/222 by the local delivery of chemically engineered oligonucleotide-based miRNA inhibitors referred as "antigomirs" are efficient and specific silencers targeted for miRNA-21 and miRNA-221/222 was shown to reduce neointima formation [62, 64]. Similarly, use of an antagomir against miRNA -122, specifically silenced miRNA-122 expression in the liver, lung, intestine, heart, skin and bone marrow for more than a week after one intravenous injection [82]. In another method, silencing of mir-145 or miRNA- 143 was achieved by adenovirus-mediated delivery of these miRNAs to vascular lesion, which appears to restore miRNA profile of vascular lesion that resembles normal tissue [66, 71]. Although these studies suggest that targeting miRNAs may represent a new therapy for vascular proliferative diseases, the miRNA-based technology is still long way from being translated to clinical therapy.

16. Conclusion

Exploring the microRNAome that controls VSMC phenotype and analysis of their targets have greater possibilities for unraveling unforeseen regulatory pathways and disease mechanisms, development of novel therapeutic approaches. miRNAs in cardiovascular research are a newly emerging powerful biomolecules, which demonstrate several unique opportunities for microRNAs-based therapeutics. Although some of the studies appear to indicate targeting certain miRNAs presents a potential therapy for atherosclerosis, knowledge of full scope of miRNAs in vascular pathogenesis is limited. With 1,000 or more microRNAs encoded by the human genome, only a few of which have been analyzed appear to be linked to vascular proliferative diseases. Considering the complexity of the multifactorial vascular proliferative diseases including atherosclerosis and restenosis, there may be several miRNAs and even several clusters of miRNAs, similar to miRNA-17-92 cluster, which impact the development of vascular pathogenesis. Therefore, several issues have to be addressed prior to use of miRNA-based technology can be translated to clinical therapy such as: profile of miRNAs responsible for vascular proliferative diseases needs to be determined; detailed effects of these miRNAs in the prevention and treatment of vascular proliferative diseases requires investigation; procedures for in vivo miRNA silencing needs to be improved to minimize off-target effects; technology for miRNA upregulation in arterial vessel wall requires development; and potential toxicity of miRNA-based therapy should be determined. Developing miRNAs into therapeutics reveals other significant challenges, such as methods of delivery and duration of action. Methods for local delivery to the arteries via catheters or coated stents may avert these challenges and should minimize off-target effects on other tissues. Besides their therapeutic

potential, identification of circulating miRNAs released from injured tissues or highly expressed in patients with cardiovascular diseases suggest miRNAs can also be useful as potential biomarkers for clinical diagnosis of cardiovascular disease patients.

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