Microribonucleic Acids for Prevention of Plaque Rupture and In-Stent Restenosis

“A Finger in the Dam”

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Vascular smooth muscle cells (VSMCs), which make up the arterial medial layer, possess a phenotype switching capability. This modulation of VSMCs is important in the development of atherosclerotic vascular disease. It has been recognized that VSMCs may also have a stabilizing role in advanced atherosclerotic plaques. Moreover, reduction of the proliferative capacity of these cells may be of benefit in reducing neointimal hyperplasia following therapeutic percutaneous intervention. The biology of microribonucleic acids (miRNAs) and their ability to modify smooth muscle biology has recently emerged in a number of investigations. These studies elucidated the key role of miRNAs, miR-143 and miR-145, in particular, in the regulation of SMC homeostasis in vitro, in murine models of targeted gene deletion, and also in human vascular pathology. This review places this burgeoning knowledge within the wider context of atherosclerosis and restenosis and explores the therapeutic potential of miRNAs to change the fate of VSMCs within the plaque. (J Am Coll Cardiol 2011;57:383–9) © 2011 by the American College of Cardiology Foundation

Microribonucleic acids (miRNAs) are an emerging class of highly conserved, noncoding small RNAs (20 to 25 nucleotides) that regulate gene expression at the post-transcriptional level, inhibiting the translation of protein from messenger RNA (mRNA) by promoting the degradation of mRNA (1). miRNAs are key regulators of many cellular events, including the balance between proliferation and differentiation during tumorigenesis and organ development (1–3). More than 600 human miRNAs have been identified so far, and it is proposed that they regulate over 50% of human protein-coding genes (4). They can be located in introns of coding genes or noncoding genes or in exons and their transcription depends on the host gene (5,6). miRNAs that have their own promoters are independently expressed, and those that are organized in clusters share the same transcriptional regulation (7,8). Once they are expressed, their maturation is mediated by the 2 ribonuclease III endonucleases Dicer and Drosha (9) (Fig. 1A). The mature miRNA incorporates into the RNA-induced silencing complex (10), which directs it to the target mRNA, leading to the latter’s translational repression or degradation (9).

miR-143 and -145 in Smooth Muscle Differentiation

A breakthrough in the smooth muscle biology field occurred in 2009 with the emergence of a number of key observations, concerning the role of miRNAs (miR-143 and -145) in the regulation of smooth muscle differentiation in vitro, as well as phenotypic characterization in a murine model of targeted deletion of the miR-143/145 gene cluster (11). The conclusions from these studies are in broad agreement regarding how miR-143/145 affect the canonical balance between the synthetic/proliferative and the contractile/differentiated states in vascular smooth muscle cells (VSMCs) (11–14).

In general, contractile smooth muscle proteins such as alpha-smooth muscle actin, smooth muscle myosin heavy chain, SM22-alpha, desmin, and calponin contain CArG [CC(A/T)6GG] elements in the promoter–enhancer region of the gene (15–21). Serum response factor binds the CArG boxes and this drives transcription (20), and in VSMC differentiation, the serum response factor binds its coactivator myocardin (22) in a 2:1 stoichiometric ratio thus inducing CArG-dependent VSMC gene transcription (23). Myocardin is now considered to be the master gene regulating VSMC differentiation (24) (Fig. 1B).

In their initial report, Cordes et al. (12) identified miR-145 and -143 as having a key role in the regulation of VSMC phenotype. Myocardin up-regulates miR-143 and -145 expression and introduction of these miRNAs potenti-
sion, myofilament formation, and calcium fluxes in response to endothelin-1. The repressor of myocardin-directed smooth muscle gene expression KLF4 (which binds myocardin) was identified as a target of miR-145 in murine VSMC (12). Human KLF4 mRNA contains no binding site for miR-143 and only a low probability site for miR-145 (TargetScan 5.1, Whitehead Institute for Biomedical Research, Cambridge, Massachusetts). However, KLF5 is highly conserved across human, mouse, and rat protein sequences and does contain a binding site for miR-145. Furthermore, it has been demonstrated that vascular injury causes down-regulation of miR-145, which up-regulates KLF5 and inhibits myocardin, leading to VSMC dedifferentiation and increased neointimal lesion formation (13). Both smooth muscle marker gene and miR-145 expression were repressed by platelet derived growth factor (PDGF)-beta, a known smooth muscle proliferative stimulant, in a murine model of wire-mediated vascular injury. Accordingly, miR-145 is expressed in smooth muscle, but not endothelial cells and its expression pattern correlates with a differentiated phenotype as defined by alpha-smooth muscle actin, smooth muscle myosin heavy chain, and calponin.

**Abbreviations and Acronyms**

- CArG = CC(A/T)6GG
- ISR = in-stent restenosis
- miRNA = micro-ribonucleic acid
- mRNA = messenger ribonucleic acid
- PDGF = platelet-derived growth factor
- VSMC = vascular smooth muscle cell

**Figure 1**  Summary of miRNA Maturation and Translation, and the Effects of miR-143/145 on VSMC Function and its Therapeutic Potential

(A) Microribonucleic acid (miRNA) maturation and inhibition of messenger ribonucleic acid (mRNA) translation through formation of a miRNA-induced silencing complex, which targets specific miRNAs for degradation. (B) MiR-143/145 promotes smooth muscle cell (SMC) differentiation by inhibiting KLF5-mediated repression of myocardin/SRF-dependent vascular SMC gene expression. Dashed green arrows represent promotion; solid red lines indicate direct inhibition in a pathological vascular smooth muscle cell (VSMC) dedifferentiation context. (C) Therapeutic potential of miR-143/145 in atherosclerotic plaques: increased miR-143/145 expression may lead to increased integrin expression and focal adhesions between VSMCs in the fibrous cap, thus promoting fibrous cap integrity and, therefore, plaque stability. (D) Therapeutic potential of miR-143/145 in in-stent restenosis: a reduction in VSMC proliferative capacity induced by miR-143/145 may reduce in-stent restenosis. miRISC = micro-ribonucleic acid-induced silencing complex; pre-miRNA = previous miRNA; pri-miRNA = primary miRNA.
expression (13). Induction of miR-145 by pre-miR-145–enhanced smooth muscle marker gene expression and myofilament formation in SMCs. This role of miRNAs in VSMC differentiation is summarized in Figure 1B.

Boettger et al. (11) also demonstrated a central role for the miR-143/145 cluster in VSMC phenotypic modulation in a study that also focused on a miR-143/145 cluster knockout mouse. In their study, at baseline, SMCs from miR-143/145–/– mice lacked expression of contractile markers calponin and smoothelin compared with wild-type VSMC. Ultrastructural electron microscopic analysis showed greater presence of rough endoplasmic reticulum and less focal adhesions in miR-143/145–deficient VSMC with the converse in wild-type cells. These differences were associated with impaired contractility of femoral artery rings in response to high K+, angiotensin II, and phenylephrine. According to this report, miR-143/145–/– mice also had a tendency to develop neointimal lesions. Aortic SMCs from the double knockout mouse lacked myofilamentous cytoskeletal organization and had diffuse actin staining. Although pre-clinical studies using rodent models can yield very useful mechanistic insight, the genetic differences between these species and humans must be taken into account when translating their relevance to the clinical setting. The relevance of miR-143/145 in human vessel pathology was suggested by down-regulation of the miR-143/145 cluster in human aortic aneurysms by comparison with normal aortic tissue as assessed by quantitative reverse transcriptase polymerase chain reaction (14), suggesting a role for these microRNAs in maintaining vessel homeostasis. More thorough characterization of the miR-143/145 effects in human VSMC is now required.

**VSMCs, the Fibrous Cap, and Plaque Stability**

In humans, ruptured atherosclerotic plaques show a paucity of VSMCs compared with stable lesions (25), indicating that VSMCs contribute to plaque stability (26). Indeed VSMCs are the only cells capable of synthesizing components of the fibrous cap in plaques, which separates circulating blood from the thrombogenic plaque interior (25).

_Vulnerable plaque_ is defined as a plaque that is at high risk of rupture and is the “short-term pre-cursor” to the culprit plaque in acute coronary syndromes (27–29). Although prediction of vulnerable plaques is still elusive, selective pharmacological modulation of VSMC phenotype may improve their stability. Promoting a quiescent VSMC phenotype may therefore lead to increased fibrous cap integrity, and in this respect, miRNAs may offer an opportunity for positive VSMC regulation (Fig. 1C).

The role of the VSMC varies depending on the stage of atherosclerotic disease, with VSMCs playing a maladaptive role in early lesion development and progression (30,31). In contrast, VSMCs may play a beneficial adaptive role within the fibrous cap by stabilizing advanced plaques in the face of end-stage disease events such as plaque rupture (32,33). The contribution of VSMCs to atherosclerosis includes production of various proteases (33), as well as extracellular matrix production (34–36), participation in chronic inflammatory responses including production of inflammatory cytokines and expression of at least some inflammatory cell markers (37–39), altered contractility, and expression of contractile proteins (40,41). Massive VSMC apoptosis occurs after injury (42) and chronic, low-level VSMC death also characterizes arterial aneurysms, where medial VSMC loss, inflammation, fragmentation of elastin, and matrix degradation lead to progressive dilation and potential rupture (43). The concept that VSMCs play an important role in plaque stability is reinforced when considering the consequences of VSMC death, which increases as atherosclerotic plaques develop (44). Together these data implicate the involvement of VSMC apoptosis in plaque rupture (45).

**Therapeutic Potential of miRs for Plaque Stabilization**

Phenotypic modulation of VSMCs is proposed as an important cellular event in the development of atherosclerotic vascular disease. If achieved using miR-143/145, 2 important clinical consequences at different stages of plaque development could result: 1) a decrease in VSMC proliferation within immature atherosclerotic plaques; and 2) fibrous cap stabilization in more advanced plaques. From a clinical viewpoint, this modulation has a potential role in prevention of coronary, and indeed peripheral, arterial disease. The consequences of plaque rupture are most clearly seen in the coronary and cerebrovascular circulations. Therefore, individuals with a heavy atherosclerotic burden, or those with significant cardiovascular risk factors, that may or may not have had a previous vascular event, would also benefit from such therapy.

Unlike many terminally differentiated cells, VSMCs can switch between differentiated and dedifferentiated phenotypes in response to changes in the local environment (46). The recent identification, by Cordes et al. (12), of miR-145 and -143 as having a key role in the regulation of VSMC phenotype, both in terms of proliferation and differentiation, provides an opportunity for targeting noncoding sequences such as miRs through gene therapy. Extrapolating from Boettger et al. (11), up-regulation of miR-145 and -143, for example, by use of pre-miR-145/143 and its flanking sequence, could potentially lead to expression of more integrins and focal adhesions, thereby strengthening the fibrous cap. Furthermore, promotion of the less synthetic, contractile SMC expressing more calponin and smoothelin markers could decrease the rate at which more immature atherosclerotic lesions develop (Fig. 1C).

Other miRNAs with the potential to modulate atherosclerotic plaques include miR-126, which confers plaque stability and decreases the rate of atherosclerosis progression (47). During plaque development, endothelial cell-derived apoptotic bodies are generated that convey para-crine alarm signals to recipient vascular cells that trigger...
the production of CXCL12. The production of CXCL12 mediated by miR-126 enabled CXCR4 to trigger an autoregulatory feedback loop that further increased the synthesis of CXCL12. Administration of apoptotic bodies, or miR-126, limited atherosclerosis, promoted the incorporation of Sca-1+ progenitor cells, and conferred features of plaque stability in a number of different atherosclerosis models (47).

**VSMCs and In-Stent Restenosis**

VSMCs play a central role in in-stent restenosis (ISR), which is reviewed in Mitra and Agrawal (48). Briefly, the vascular injury induced by stent implantation gives rise to local apoptosis of medial VSMC (49). The ensuing regenerative repair response involves the coordination of many elements including thrombus deposition and leukocyte trafficking to the stent site, growth factors such as PDGF-beta (50), eicosanoids such as thromboxane A2 (51), local thrombin generation (52), and vasoactive agents such as angiotensin II (53), all of which are known VSMC mitogenic stimuli with the latter 2 both signaling through G-alpha-q-coupled receptors. The dysregulation of this repair response may lead subsequently to excessive VSMC proliferation and, therefore, the formation of a stenotic lesion that encroaches upon the vessel lumen (54). PDGF-beta, one of the most potent VSMC mitogens, induces a proliferation stimulus via biphasic extracellular signal-regulated kinase activation in addition to phosphoinositide-3 kinase and Akt activation, which downstream results in cell cycle progression through G1/S (55). Within the first 18 months following stenting, the VSMC content (determined by alpha-smooth muscle actin staining) of the neointimal lesion is around 66%, which reduces to ~12% thereafter, which is indicative of a greater extracellular matrix component (determined by collagen I and III staining) within the lesion (56). VSMC under transforming growth factor-beta stimulation are the major producers of proteoglycan-rich extracellular matrix in neointima (57), which contributes to lesion size in the later phases. Therefore, the initiation of the proliferative state in VSMC occurring with phenotypic switching may represent an ideal opportunity for therapeutic intervention (Fig. 1D).

**Modification of in-stent restenosis by miRs.** There are 3 miRs implicated in modification of vessel restenosis after interventional endothelial injury: miR-21, -145, and -221. Ji et al. (58) demonstrated that antisense knockdown of miR-21, which they also revealed is moderately increased after vessel injury, blunted the formation of a neointimal lesions in response to balloon injury of the carotid artery. Furthermore, miR-21 promoted VSMC proliferation after vessel injury via activation of Akt and Bcl-2 with concomitant inhibition of phosphatase-and-tensin-homology-deleted-from-chromosome-10, the latter being a putative target for silencing by miR-21 (58). A general miRNA-21 inhibition was also shown to be beneficial in terms of heart failure, especially attenuation of fibrosis (59). It should be noted that miR-21 inhibition also improved function of impaired endothelial progenitor cells especially in patients with coronary artery disease and high risk for neointima formation (60). Adenovirus-mediated overexpression of miR-145 promoted VSMC marker expression and reduced neointima formation in response to rat carotid balloon injury (13). miR-221 was induced in VSMCs on PDGF-beta stimulation, which caused: 1) down-regulation of cKit, resulting in a decrease in SMC differentiation; and 2) p27Kip1 inhibition, which increased SMC proliferation (61), both of which contribute to the formation of a neointimal lesion after arterial injury.

Therefore, knockdown of miR-21, miR-221, and over-expression of miR-145 may block neointima formation after vessel injury.

**Therapeutic Approaches for miRs: Strategies, Delivery, and Complications**

To date, very few gene therapy studies delivering an intracoronary agent have been carried out in humans (62). However, those performed suggested that this is a safe and feasible therapeutic strategy, with potential benefits in terms of anti-ischemic end points reported. The AGENT (Anti-Genic GENE Therapy)-1, -2, -3, and -4 trials (63–65) involved intracoronary delivery of a replication defective adenovirus containing a human fibroblast growth factor gene that showed some promising anti-ischemic potential in patients with angina pectoris. Intracoronary gene transfer leading to improved myocardial perfusion without increased restenosis rate at 6 months was performed in the KAT (Kuopio Angiogenesis Trial) (66), which involved local vascular endothelial growth factor gene transfer during angioplasty and stenting. Although routine use of intracoronary gene therapy is years away, the use of miRs to enhance atherosclerotic plaque stability or prevent ISR offer some promise as therapeutic agents based on the laboratory investigations already carried out.

There are several possibilities to overcome the obstacles that exist in the delivery of miR-143/145-based therapeutics. Various chemical modifications of the nucleotides can enhance stability in vivo (67), such as in the first mammalian anti-miR knockdown reported by Krützfeldt et al. (68) using cholesterol-conjugated, 2′-O-methyl–modified “antagomiRs” that resulted in derepression of putative target mRNAs. So far in animal studies, intravenously injected antagomiRs and miR mimetics have been the primary method of systemic delivery (59,69–73) and have demonstrated effects on myocardial remodeling; however, they are preferentially targeted to the liver, kidney, and spleen. Therefore, enrichment of miR-based agents in the coronary vasculature may be a major challenge. Strategies might include conjugation of the nucleic acid to targeting molecules such as peptides, antibodies, or other bioactive molecules, which may promote homing of the miRNA–based agents to the site of vascular injury. The specific application of the agent to the coronary vasculature during
cardiac catheterization may also be an effective therapeutic approach.

Another significant challenge is the platform for delivery. Whereas previously, the delivery of miRNAs would have relied on direct naked/nonviral deoxyribonucleic acid plasmid injection or viral-mediated gene transfer (74,75), synthetic vehicles consisting of a double-stranded polynucleotide, a lipid moiety to facilitate entry into the cell, and pH labile bonds allowing release of the polynucleotides may allow efficient in vivo delivery of miRNA-based therapeutics (76). The most relevant miR human study up to now involved delivery of oligodeoxynucleotides (77) targeting proliferation in autologous vein grafts, by interfering with the E2F transcription factor that regulates cell cycle progression effectively “ex vivo” (78). This resulted in inhibition of graft occlusion associated with selective inhibition of proliferating cell nuclear antigen and c-myc expression. In the PREVENT II (Program in Ex Vivo Vein Graft Engineering Via Transfection II Study: E2F Decoy in CABG) randomized, double-blind, placebo-controlled trial (79), the safety and feasibility of E2F decoy oligonucleotides in preventing autologous vein graft neointimal hyperplasia and atherosclerosis after coronary artery bypass surgery was demonstrated. In the acute infarct setting, however, therapeutic administration, either before or after reperfusion remains a challenge. However, this is a clinical scenario that demands careful attention and holds potentially the greatest reward. Use of a polymer-coated stent coupled with a synthetic miRNA-vehicle-complex would allow delivery of the miRNA at the time of infarct, with a potential 3-fold benefit—stabilizing the ruptured plaque, slowing progression of “target vessel” disease, and prevention of ISR—simultaneously addressing the most significant issues of the mechanical coronary intervention era.

Given the far-reaching and ever-expanding roles of miRNAs, it will be necessary to explore the potential adverse consequences of therapeutic miRNA manipulation. Specific miRNAs have been linked to cancer, and interestingly, mature miRNA levels of miR-143 and -145 are reduced in colorectal tumors as well as many cancer cell lines (80,81), suggesting that therapies that increase levels of these miRNAs, theoretically at least, will not have neoplastic potential. Furthermore, the tumor-suppression activity of miR-145 has been shown to be expressed through the phosphoinositide-3 kinase/Akt and p53 pathways (82). miR-126, which inhibits progression of atherosclerosis and phosphoinositide-3 kinase/Akt and p53 pathways (82).

The conclusions on miRNA therapy will depend on the specific miRNAs implicated in VSMC phenotypic switching and have not been targeted therapeutically. Given the recent discovery that miRNAs regulate the genome to a much greater degree than originally thought, there must be a degree of caution when manipulating such influential non-coding sequences. Although tumorigenesis is one of the greatest concerns associated with miRNA manipulation, up-regulation of miR-143/145 appears to be a theoretically safe option and may have multiple potential benefits in terms of plaque stabilization and reduction in atherosclerosis.

Manipulation of VSMC-associated miRNAs, therefore, presents an excellent opportunity for cardiologists and vascular biologist alike and is an exciting potentially new therapeutic option for atherosclerotic plaque and its complications including ISR.

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