MicroRNA-145 Targeted Therapy Reduces Atherosclerosis

Fina Lovren, PhD; Yi Pan, MD; Adrian Quan, MPhil; Krishna K. Singh, PhD; Praphulla C. Shukla, PhD; Nandini Gupta, BSc; Brent M. Steer, BSc (Hons); Alistair J. Ingram, MD, FRCPC; Milan Gupta, MD, FRCPC; Mohammed Al-Omran, MD, MSc, FRCSC; Hwee Teoh, PhD; Philip A. Marsden, MD, FRCPC; Subodh Verma, MD, PhD, FRCSC, FAHA

Background—MicroRNA are essential posttranscriptional modulators of gene expression implicated in various chronic diseases. Because microRNA-145 is highly expressed in vascular smooth muscle cells (VSMC) and regulates VSMC fate and plasticity, we hypothesized that it may be a novel regulator of atherosclerosis and plaque stability.

Methods and Results—Apolipoprotein E knockout mice (ApoE−/−) mice were treated with either a microRNA-145 lentivirus under the control of the smooth muscle cell (SMC)-specific promoter SM22α or a SM22α control lentivirus before commencing the Western diet for 12 weeks. The SMC-targeted microRNA-145 treatment markedly reduced plaque size in aortic sinuses, ascending aortas, and brachiocephalic arteries. It also significantly increased fibrous cap area, reduced necrotic core area, and increased plaque collagen content. Cellular plaque composition analyses revealed significantly less macrophages in ApoE−/− mice treated with the SMC-specific microRNA-145. These mice also demonstrated marked increases in calponin levels and α-smooth muscle actin–positive SMC areas in their atherosclerotic lesions. Furthermore, lentiviral delivery of microRNA-145 resulted in reduced KLF4 and elevated myocardin expression in aortas from ApoE−/− mice, consistent with an effect of microRNA-145 to promote a contractile phenotype in VSMC.

Conclusions—VSMC-specific overexpression of microRNA-145 is a novel in vivo therapeutic target to limit atherosclerotic plaque morphology and cellular composition, shifting the balance toward plaque stability vs plaque rupture. (Circulation. 2012;126[suppl 1]:S81–S90.)

Key Words: atherosclerotic plaque ■ myocardin ■ remodeling ■ vascular smooth muscle cells

Atherosclerosis remains the number 1 cause of death and disability worldwide, and it involves the progression of vascular lesions from early fatty streaks to more advanced plaques characterized by arterial intimal thickening, inflammatory cell and vascular smooth muscle cell (VSMC) accumulation, as well as extracellular lipid and fibrous tissue deposition.1–3 The pathogenesis of atherosclerosis is a result of complex interactions between circulating inflammatory factors and various cell types in the vessel wall, including endothelial cells, monocytes, lymphocytes, and VSMC.4,5 Accumulating evidence implicates alterations in VSMC structure and function in the initiation, progression, and eventual rupture of atherosclerotic plaques.6,7 VSMC possess remarkable plasticity and reversibly modulate their phenotype during postnatal development. Vascular injury, in the form of endothelial dysfunction, mechanical or hemodynamic stress, or inflammation, promotes VSMC phenotypic modulation defined as a switch from a normal, quiescent, and contractile state to a synthetic phenotype, whereby genes that define a contractile function are suppressed and genes that promote proliferation, migration, and inflammation are induced, which can lead to the synthesis of large quantities of extracellular matrix, proteases, and cytokines.6,7 In addition to higher rates of migration and proliferation, synthetic VSMC express greater proportions of very low-density lipoprotein, low-density lipoprotein, and scavenger receptors that facilitate pathological lipid uptake and foam cell formation, marking the initiation of atherosclerosis. Ongoing production of extracellular matrix and proteoglycans further enrich lipid uptake by macrophages, thus accelerating lesion progression. Finally, these phenotypically modified VSMC also appear to play an important role in defining the cellularity, necrotic core area, and fibrous cap stability of atherosclerotic plaques, key indices of plaque rupture and acute coronary syndromes. Given the importance of VSMC phenotypic modulation in lesion initiation, progression, and eventual rupture, identify-
ApoE limit neointimal formation in response to vascular injury,15–11 Evidence to date suggest that microRNA-145 (mi-
not only limits plaque burden in atherosclerosis15,16 and aneurysms.17 Transient local overexpression of microRNA-145 has been demonstrated to limit neointimal formation in response to vascular injury,15 and it has been suggested that microRNA-145 regulates VSMC differentiation via several mechanisms, including, but not limited to, KLF4, myocardin, calmodulin kinase IIβ, angiotensin-converting enzyme, and, more recently, actin polymerization.9

The identification of microRNA-145 as a critical gatekeeper of VSMC differentiation and regulator of the contractile vs synthetic function phenotype makes it an attractive therapeutic target to limit aberrant vascular injury and atherosclerosis. We demonstrate herein, for the first time to our knowledge, that VSMC-specific overexpression of microRNA-145 not only limits plaque burden in atherosclerosis-prone apolipoprotein E knockout mice (ApoE⌬/−) mice but also reduces plaque inflammation and increases features of plaque stability in a fashion consistent with promotion of the VSMC contractile phenotype. These data strengthen the translational basis for novel microRNA-based gene therapy in atherosclerosis and related disorders.

Methods

Generation of Recombinant Lentivirus MicroRNA-145

Mouse microRNA-145 lentivirus under the control of the mouse smooth muscle cell (SMC)-specific promoter SM22α (mSm22α-mmumir-145) and mouse SM22α control lentivirus (mSm22α-mmumir-ctrl) were purchased from Biosettia (Biosettia Inc, San Diego, CA). To generate the former lentivirus, mouse microRNA-145 was cloned within the intron of the human EF1α promoter region and the human EF1α promoter was replaced with the VSMC-specific minimal mouse SM22α promoter18,19 in a self-inac-
tivated lentiviral vector containing the red fluorescent puromycin-
N-acetyl transferase gene. The resultant pLV-microRNA vector was cotransfected with lentiviral packaging vector mix into HEK 293T cells to synthesize the final stock of microRNA-145 lentivirus that coexpresses the minimal mouse SM22α promoter.

Experimental Animals and Lentiviral MicroRNA-145 Transduction

All animal procedures were in accordance with the guidelines of the Canadian Council on Animal Care and approved by the Institutional Animal Care Committee at St Michael’s Hospital. Studies were performed on age-matched ApoE−/− and C57Bl/6J control mice (The Jackson Laboratory, Bar Harbor, ME). Eight-week-old ApoE−/− mice were injected with either saline or lentivirus via their tail veins. The dose and dosing interval for the delivery of microRNA-145 were optimized in preliminary experiments to arrive at the regimen used in this study (data not shown). In total, 5 injections were administered, on alternate days, over a period of 9 days. For each injection, 0.2 mL of the concentrated viral suspension (plaque containing) the carotid bifurcation. MicroRNA-145 (C) and KLF4 (D) expression in carotid artery samples were evaluated by quantitative PCR and normalized against the corre-
sponding RNU6, or glyceraldehyde-3-phosphate dehydrogenase expression. n=5. *P<0.05 vs normal control group.

Human Carotid Artery Studies

Carotid artery segments were collected from below (normal control) and at study termination, after which their hearts, various arterial vessels, plasma, and serum were isolated and analyzed by standard quantitative polymerase chain reaction (PCR) and in situ hybridization methods. The remaining mice were euthanized at 1 of the study time points mentioned, after which their hearts, various arterial vessels, plasma, and serum were isolated and processed for a range of study assessments.

Cell Culture

Human aortic SMC (HASMC; 5×10^5 cells in 6-well plates), cultured in Dulbecco modified Eagle medium supplemented with 10% fetal bovine serum, were transfected with 2 mmol/L of the miRIDIAN mimic microRNA-145 (Thermo Fisher Scientific, ON,
Real-Time PCR for Detection of MicroRNA-145 and KLF4 Expression

MicroRNA were extracted from the aortas of ApoE<sup>−/−</sup> mice and human carotid artery sections with the miRNasy Mini Kit (Qiagen, ON, Canada) and reverse-transcribed with the RT<sup>2</sup> miRNA First Strand Kit (Qiagen, ON, Canada) as per the manufacturer’s instructions. Real-time PCR was performed on the StepOnePlus Real-Time PCR System (Applied Biosystems Canada, ON, Canada) using the RT<sup>2</sup> SybrGreen quantitative PCR Mastermix (Qiagen, ON, Canada). Commercially available murine primers for microRNA-145 and RNU6 were from SABiosciences. Those for human microRNA-145, RNU6, and KLF4 were from Qiagen. Custom-designed primers for gycleraldehyde-3-phosphate dehydrogenase, 5′-CACCAGGCTGCTTTTAACCTCTG-GTA-3′ and 5′-CCTTGACGGTGCCATGGAAATTGC-3′, were purchased from Operon (Huntsville, AL). Gene expression data were analyzed by the ΔΔct threshold.

Laser Capture Microdissection of Aortic SMC

Aortas isolated from ApoE<sup>−/−</sup> mice were snap-frozen in liquid nitrogen and stored at −80°C until they were processed for laser capture microdissection. Briefly, frozen aortic sections (~8 μm) were treated with the Applied Biosystems Arcturus HistoGene stain being used for SMC, as visualized with the PixCell IIe laser capture microdissection system (Arcturus, Applied Biosystems, Foster City, CA), were isolated from the aortic media. Total RNA was extracted from captured cells with the RNAqueous-micro extraction kit (Ambion, Austin, TX). DNase I digestion was included to eliminate genomic DNA contamination. Reverse-transcribed samples were prepared with the RT<sup>2</sup> miRNA First Strand Kit (Qiagen, ON, Canada) and analyzed on the StepOnePlus real-time PCR System (Applied Biosystems Canada, ON, Canada) as described.

In Situ Hybridization

Aortic samples from ApoE<sup>−/−</sup> mice were processed and stained as previously described for microRNA-145 detection. In brief, paraffin-embedded sections (6 μm) were premeabilized with proteinase K and hybridized with a 5′, 3′ double digoxigenin-labeled locked nucleic acid-modified mirCURY miR-145 detection probe or scrambled LNA oligonucleotide, which acted as the negative control (Exiqon, Vedbaek, Denmark). MicroRNA-145 signals were detected with a tyramide signal amplification system as per the manufacturer’s instructions (PerkinElmer, Waltham, MA); to confirm the localization of microRNA-145 in VSMC, sections were subsequently stained with an α-smooth muscle actin (SMA) antibody.

Blood Pressure Measurement

Systolic arterial blood pressure was monitored in anesthetized mice (1%–1.5% isoflurane) with a tail cuff (CODA System; Kent Scientific, Torrington, CT).

Lipid Analysis and Lipoprotein Profile Measurement

Plasma levels of total cholesterol and triglycerides were measured via a microplate method using the Beckman Coulter Synchron C1500 reagent. Plasma lipoprotein fractions were assessed by fast performance liquid chromatography gel filtration coupled with 2 Superox6 HR 10/30 columns (Amersham Pharmacia Biotech Inc, Piscataway, NJ).

Immunohistochemistry

Hearts, proximal ascending aortas, and brachiocephalic arteries were fixed with sucrose and embedded in OCT. Serial cross-sections (5 μm) of aortic valves, aortas, and brachiocephalic arteries were stained with hematoxylin and eosin, oil red O, trichrome, or picrosirius red. The necrotic core was identified as the part of the atherosclerotic plaque, which appeared to be acellular (hematoxylin-negative) and often contained cholesterol crystals. Areas of the SMC fibrous caps were determined in trichrome and α-SMA–stained brachiocephalic sections and the cap was identified as the immunopositive part of the plaque covering the necrotic core. Measurements were conducted with the National Institutes of Health ImageJ software and data are presented as a percentage of total plaque area as reported previously.

Actin content in the plaques was examined via routine immunofluorescent techniques by pairing an α-SMA antibody (Abcam, Cambridge, MA) with an Alexa Fluor 555-conjugated secondary antibody (Invitrogen, ON, Canada). Standard horseradish peroxidase-3,3′-diaminobenzidine immunohistochemical methods using ABC kits from Vector Laboratories were used to detect calponin content (anti-calponin from Biozzi, Woburn, MA) and the presence of macrophages (anti-Mac-3 from BD, Franklin Lakes, NJ) in the lesion area, as well as SMC proliferation (anti-Ki67 from Abcam, Cambridge, MA).

Enzyme-Linked Immunosorbent Assay Assessment of MCP-1 Levels

Frozen serum samples were thawed for quantification of MCP-1 levels with an enzyme-linked immunosorbent assay kit from Pierce (ON, Canada).

Immunoblotting

Total and nuclear protein contents were extracted from mouse aortas and HASCs with the NucBuster protein extraction kit (Merck KgaA, Darmstadt, Germany). Western blot analysis was performed according to standard procedures. Protein samples were separated on 4% to 12% Tris-glycine gels (Invitrogen) and transferred to nitrocellulose membranes (Invitrogen), which were probed with antibodies directed at α-SMA, calponin, myocardin (R & D Systems, Minneapolis, MN), KLF4 (Biosis, Woburn, MA), collagen I, and collagen III (Rockland Immunocchemicals Inc, Gilbertsville, PA), and were incubated with the appropriate horseradish peroxidase–tagged secondary antibody before visualization by enhanced chemiluminescence (GE Healthcare Biosciences, Piscataway, NJ).

Statistical Analysis

Data are presented as mean±SEM and were analyzed with GraphPad InStat (version 3.05; GraphPad Software Inc, La Jolla, CA). Data were analyzed with either the Wilcoxon–matched paired test or the 1-way ANOVA followed by the Bonferroni multiple comparisons procedure for all secondary t tests of means. Differences were considered significant at P<0.05.

Results

MicroRNA-145 Expression Is Attenuated in Experimental and Human Atherosclerosis

Figure 1A demonstrates that whereas microRNA-145 expression in the aortas of naïve ApoE<sup>−/−</sup> mice are comparable with that from age-matched (8 weeks) naïve C57Bl/6J mice, aortic microRNA-145 levels are significantly lower in 20-week-old ApoE<sup>−/−</sup> mice that have been maintained on the Western diet for 12 weeks relative to that of age-matched C57Bl/6J mice that were fed the same diet. These findings confirm that experimental atherosclerosis results in marked attenuation of microRNA-145. To determine if these observations are mirrored in human atherosclerosis, we examined carotid artery samples recovered during carotid endarterectomy and compared microRNA-145 expression in sections taken at the level of atherosclerotic plaques with that in sections from the adjacent “normal” control plaque-free area (Figure 1B). Real-time PCR revealed that relative to sections from the plaque-free areas, those from the regions containing plaques not only exhibited
significantly lower microRNA-145 levels (Figure 1C) but also exhibited greater KLF4 expression (Figure 1D).

Our treatment strategy of utilizing SMC-targeted lentiviral delivery of microRNA-145 upregulated microRNA-145 expression in whole aortas for up to 12 weeks (Figure 2A) after injection of the lentivirus and initiation of the Western diet. We report a 5-fold increase in aortic microRNA-145 expression at 4 weeks postinjection, with a persistent 2.5-fold higher expression noted at 12 weeks after injection and commencement of the Western diet. Successful lentivirus transduction was confirmed by measuring red fluorescent puromycin-N-acetyl transferase gene expression, which was upregulated in the control lentivirus as well as the microRNA-145 lentivirus-treated groups 1, 4, and 12 weeks postinjection. To confirm that the lentivirus we used delivered microRNA-145 specifically to VSMC, we isolated SMC from the aortic sections of ApoE−/− mice maintained on the Western diet for 4 weeks via laser capture microdissection and performed quantitative PCR. Our results clearly indicate overexpression of microRNA-145 in the SMC of ApoE−/− mice 12 weeks after treatment with the microRNA-145 lentivirus relative to those in SMC isolated from the aortas of ApoE−/− mice administered either saline or the control lentivirus (Figure 2B). Further corroboration of microRNA-145 delivery to the VSMC compartment is provided in Figures 2C to 2E where we found through routine in situ hybridization greater microRNA-145 signaling in the aortic walls of ApoE−/− mice that had been given the microRNA-145 lentivirus and fed the Western diet for 4 weeks (vs those treated with either saline or the control lentivirus and placed on the same diet regime). Together, these results confirm the adequacy, persistent transduction, and tissue specificity of the microRNA-145 delivery dosage and method. Notably, microRNA-145 expression in the liver was similar 1, 4, and 12 weeks after microRNA-145 lentivirus injection and initiation of the Western diet (data not shown), supporting the contention that the increased in microRNA-145 expression elicited by this treatment is likely largely confined to VSMC. Interestingly, our microRNA-145–based treatment did not appreciably affect the lipid profiles (total, low-density lipoprotein, high-density lipoprotein cholesterol levels) or the arterial blood pressure of ApoE−/− mice maintained on the Western diet for 12 weeks (Table).

VSMC-Specific Lentiviral MicroRNA-145 Gene Transfer Markedly Reduces Atherosclerosis

To evaluate the potential therapeutic benefit of microRNA-145 in atherosclerosis, we evaluated atherosclerotic volumes in aortic roots, ascending aortas, and brachiocephalic arteries at the end of the 12 weeks of diet treatment. Tissues were stained with oil red O to visualize lipid-rich atherosclerotic...
plaques. The SMC-targeted microRNA-145 treatment resulted in a marked reduction of plaque size as determined in the 3 territories described (Figure 3). Of note, severe and nearly occlusive disease was observed in the brachiocephalic arteries, and microRNA-145 treatment resulted in a profound 60% reduction in plaque size (59 mm² microRNA-145 lentivirus vs 137 mm² control lentivirus; P<0.05). Morphometry analyses revealed a thicker aortic media and larger aortic media area (81.22±0.42 μm and 0.244±0.03 mm², respectively) in SMC–microRNA-145 lentivirus-treated vs control lentivirus-treated (65.50±1.55 μm and 0.194±0.04 mm², respectively) ApoE⁻/⁻ mice.

**Table. MicroRNA-145 Gene Therapy Does Not Affect Plasma Lipid Profile and Mean Arterial Blood Pressure in ApoE⁻/⁻ Mice**

<table>
<thead>
<tr>
<th></th>
<th>Saline</th>
<th>Control Lentivirus</th>
<th>microRNA-145</th>
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<tbody>
<tr>
<td>Total cholesterol (mmol)</td>
<td>19.5±1.6</td>
<td>19.4±4.4</td>
<td>18.8±3.7</td>
</tr>
<tr>
<td>VLDL (mmol)</td>
<td>8.4±2.4</td>
<td>9.4±1.2</td>
<td>8.4±0.9</td>
</tr>
<tr>
<td>LDL (mmol)</td>
<td>8.8±2.1</td>
<td>8.6±2.7</td>
<td>7.4±1.3</td>
</tr>
<tr>
<td>HDL (mmol)</td>
<td>0.5±0.3</td>
<td>0.5±0.1</td>
<td>0.4±0.1</td>
</tr>
<tr>
<td>Triglyceride (mmol)</td>
<td>1.1±0.3</td>
<td>1.5±0.5</td>
<td>1.2±0.4</td>
</tr>
<tr>
<td>MAP (mm Hg)</td>
<td>92.7±5.2</td>
<td>89.8±2.9</td>
<td>90.1±5.3</td>
</tr>
</tbody>
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HDL indicates high-density lipoprotein; LDL, low-density lipoprotein; MAP, mean arterial pressure; SEM, standard error of the mean; VLDL, very low-density lipoprotein.

Lipid profile (n=10) and MAP (n=5) were assessed in ApoE⁻/⁻ mice maintained on the Western diet for 12 weeks. Values are presented as means±SEM. P>0.05.

The SMC-targeted microRNA-145 treatment resulted in a marked reduction of plaque size as determined in the 3 territories described (Figure 3). Of note, severe and nearly occlusive disease was observed in the brachiocephalic arteries, and microRNA-145 treatment resulted in a profound 60% reduction in plaque size (59 mm² microRNA-145 lentivirus vs 137 mm² control lentivirus; P<0.05). Morphometry analyses revealed a thicker aortic media and larger aortic media area (81.22±0.42 μm and 0.244±0.03 mm², respectively) in SMC–microRNA-145 lentivirus-treated vs control lentivirus-treated (65.50±1.55 μm and 0.194±0.04 mm², respectively) ApoE⁻/⁻ mice.

**SMC-Specific MicroRNA-145 Overexpression Increases Features of Plaque Stability**

To ascertain plaque stability, we proceeded to evaluate the collagen content and fibrous cap area of the atherosclerotic plaques as well as the necrotic core area. MicroRNA-145 lentiviral treatment significantly increased fibrous cap area, profoundly reduced the necrotic core area/total plaque area ratio, and also elevated plaque collagen content as determined by staining for hematoxylin and eosin, trichrome, and picrosirius red (Figure 4A–C). Consistent with these in vivo observations, we found that microRNA-145 lentivirus treatment enhanced collagen type I and type III protein expression in HASMC (Figure 4D). Therefore, in addition to promoting overall reduction in atheroma burden, SMC-specific microRNA-145 treatment favorably alters plaque morphology and cellular composition, shifting the balance toward plaque stability vs plaque rupture.
MicroRNA-145 Administration Decreases Macrophage Infiltration and Serum MCP-1 Levels in ApoE−/− Mice

Analysis of cellular plaque composition by immunohistochemistry revealed that the relative content of Mac-3 macrophages in the lesioned brachiocephalic arterial areas was significantly decreased in ApoE−/− mice treated with SMC-specific microRNA-145 relative to the group that was administered the control lentivirus (Figure 5A, B). As shown in Figure 5C, the reduction in the Mac-3 macrophage population was associated with a significant decrease in the serum levels of proinflammatory chemokine MCP-1 (115±24 pg/mL vs 91±10 pg/mL for control lentivirus-treated vs microRNA-145 lentivirus-treated ApoE−/− mice, respectively; P<0.05). Accordingly, VSMC-targeted microRNA-145 limited plaque inflammation in our experimental model of atherosclerosis.

MicroRNA-145 Increases Expression of SMC Contractile Proteins in Atherosclerotic Plaques and HASMC

To determine whether microRNA-145 lentivirus therapy would alter lesion characteristics and, in particular, accumulation of contractile (vs synthetic) VSMC in atherosclerotic plaques, we stained cross-sections of brachiocephalic arteries from ApoE−/− mice treated with the SMC-specific microRNA-145 lentivirus demonstrated a marked increase in the α-SMA–positive SMA area in their atherosclerotic lesions (Figure 6A). Areas of plaques that stained positive for α-SMA were also positive for calponin, another marker of differentiated SMC (Figure 6B). Concordant with these in vivo observations, microRNA-145 enhanced α-SMA and calponin expression in cultured HASMC (Figure 6C). These data suggest that
the increase in smooth muscle marker expression is attributable to an increase in differentiated contractile VSMC. There was no difference in Ki67 expression, indicating that increased cell proliferation was not the primary determinant of increased plaque SMC content by microRNA-145–based treatment (data not shown).

**MicroRNA-145 Modulates SMC Differentiation Marker Genes, Myocardin, and KLF4**

SMC differentiation is accompanied by the transcriptional activation of muscle-specific genes that are controlled by the serum response factor, which also regulates genes involved in cell proliferation. Myocardin interacts with serum response

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**Figure 5.** Vascular smooth muscle cell (VSMC)-specific microRNA-145 lentiviral treatment decreases plaque inflammation in ApoE<sup>−/−</sup> mice with atherosclerosis. ApoE<sup>−/−</sup> mice were maintained on the Western diet for 12 weeks after being treated with saline, control lentivirus, or microRNA-145 lentivirus. Representative micrographs (A) and quantitative assessments (B) showing less macrophage infiltration (brown) into the lesion areas of brachiocephalic arteries from ApoE<sup>−/−</sup> mice treated with the microRNA-145 lentivirus. Magnification ×40. C, Serum MCP-1 levels were determined by enzyme-linked immunosorbent assay. n=6. *P<0.05 vs group treated with the control lentivirus.

**Figure 6.** Vascular smooth muscle cell (VSMC)-specific microRNA-145 lentiviral treatment increases plaque stability in the lesion areas of brachiocephalic arteries from ApoE<sup>−/−</sup> mice maintained on the Western diet for 12 weeks and also elevates α-SMA and calponin levels in human aortic smooth muscle cells (HASMC). Representative immunofluorescent and immunohistochemical staining of brachiocephalic arterial cross-sections for the VSMC marker proteins (A) α-SMA and (B) calponin. Magnification ×40 and ×20, respectively. n=6. *P<0.05 vs group treated with the control lentivirus. C, Representative Western blots for α-SMA, calponin, and glyceraldehyde-3-phosphate dehydrogenase protein from untreated HASMC and HASMC transfected with either the transfection reagent or the double-stranded oligonucleotide microRNA-145. Blots shown are representative of 3 separate experiments. Collectively, the in vivo and in vitro evidence suggest that microRNA-145 lentivirus treatment imparts plaque stability via facilitation of VSMC differentiation toward a contractile phenotype.
factor and potently stimulates serum response factor–dependent transcription. KLF4/5 is one of the targets of microRNA-145 and is a known repressor of myocardin expression and a negative regulator of VSMC differentiation. We evaluated the effects of microRNA-145 lentiviral delivery on KLF4 and myocardin expression and report that this form of microRNA-145 treatment resulted in a reduction in KLF4 levels with a concomitant increase in myocardin expression (Figure 7A). Likewise, microRNA-145 overexpression in HASMC elicited a decrease in KLF4 expression and a parallel increase in myocardin expression (Figure 7B). The observation of a decline in KLF4 levels and an elevation in myocardin expression is consistent with an effect of microRNA-145 to promote a contractile phenotype of VSMC.13

Discussion
The main observation made in this study is that SMC-targeted microRNA-145–based therapy is associated with a marked reduction in atherosclerotic plaque burden in ApoE−/− mice, imparting features of plaque stability in a fashion consistent with phenotypic modulation of VSMC toward a quiescent vs proliferative phenotype. Given the critical importance of microRNA-145 in normal and abnormal VSM physiology, these observations underscore a critical therapeutic potential for microRNA-based therapies in attenuating atherosclerosis and potentially limiting plaque rupture.

Alterations in VSMC structure and function play an important role in the initiation, progression, and eventual rupture of atherosclerotic plaques.6,7 VSMC are capable of remarkable phenotypic modulation and it is now well-established that the phenotypic switch to a synthetic state is associated with a marked increase in VSMC elaboration of inflammatory cytokines such as IL-6, IL-8, and MCP-1, which in turn modulate monocyte and macrophage adhesion and promote plaque formation.7 In addition to higher rates of migration and proliferation, synthetic VSMC express greater proportions of very low-density lipoprotein, low-density lipoprotein, and scavenger receptors, which contribute to the beginnings of atherosclerosis. Furthermore, synthetic VSMC play an active role in altering plaque cellular composition and plaque stability.6,7 Hence, strategies aimed at limiting or restricting abnormal VSMC phenotypic modulation have great therapeutic promise to attenuate both atherosclerosis and promote fibrous cap stabilization.

MicroRNA are highly conserved, noncoding RNA that regulate gene expression ultimately inhibiting protein translation. They represent key regulators of several cellular processes via balancing proliferation and differentiation during tumorigenesis and organ development.26–29 The mature microRNA incorporates into the RNA-induced silencing complex,28,30 which directs it to the target mRNA, with resultant inhibition of translation.28,31 More than 600 human microRNA have been identified and it is suggested that these regulate ~50% of human protein-coding genes.28,32 It is estimated that 1% to 4% genes in the human genome are
microRNA, and a single microRNA can modulate as many as 200 mRNA. Furthermore, 1 microRNA is capable of regulating the expression of multiple genes because it can bind to its mRNA targets as either an imperfect or a perfect complement. There is increasing evidence suggesting that microRNA play critical roles in many key biological processes such as cell growth, tissue differentiation, cell proliferation, embryonic development, apoptosis, cellular signaling network, cross-species gene expression variation, and coregulation with transcription factors. Alterations in the expression and function of microRNA also have been linked to several chronic diseases, including cancer and cardiovascular disease.

MicroRNA-145 has been identified as the most abundant vascular microRNA. It is exclusively expressed in VSMCs and is markedly downregulated in response to vascular injury in human atherosclerosis and in aneurysms. Importantly, recent data implicate the microRNA-143/145 gene cluster as a critical regulator of VSMC phenotypic modulation. MicroRNA-145 has been suggested to regulate VSMC differentiation via several mechanisms, including, but not limited to, myocardin, KLF4, calmodulin kinase II, angiotensin-converting enzyme, and, more recently, actin polymerization. Myocardin is a well-established dominant driver of SMC contractile phenotype. It is a SMC-restricted transcriptional coactivator that physically associates with the MADS box transcription factor serum response factor to synergistically activate transcription of genes encoding SMC-restricted cytoskeletal and contractile proteins. Emerging data suggest that microRNA-145 serves to potentiate myocardin-induced SMC marker gene expression, myofilament formation, and calcium fluxes. However, KLF4 represses SMC genes by both downregulating myocardin expression and preventing serum response factor/myocardin from associating with SMC gene promoters. MicroRNA-145 has been suggested to promote SMC differentiation by inhibiting KLF4/5-mediated repression of myocardin/serum response factor–dependent vascular SMC gene expression. Recent data suggest that vascular injury leads to a downregulation of microRNA-145, which in turn upregulates KLF5 and reduces myocardin levels, leading to VSMC dedifferentiation with increased neointimal formation. In addition, SMC from microRNA143/145−/− mice lacked expression of the contractile markers calponin and smoothelin and demonstrated impaired vascular responsiveness, further supporting a causal role of microRNA-145 in the maintenance of normal vascular structure and function. Furthermore, it has been suggested that endothelial–VSMC cross-talk may be important in the delivery of microRNA-145, particularly under conditions of altered shear stress. Despite the accumulating evidence linking microRNA-145 as a critical regulator of VSMC phenotypic modulation in normal and abnormal vascular pathologies, our study is the first to our knowledge to evaluate the therapeutic potential of microRNA-145 in atherosclerosis. Because microRNA-145 is exclusively expressed in SMC, we applied a SMC-specific lentiviral delivery approach to atherosclerosis-prone ApoE−/− mice fed the Western diet. This approach resulted in a marked 60% reduction in plaque size with decreased plaque inflammation as evidenced by a reduction in macrophage infiltration and MCP-1 levels. Furthermore, microRNA-145 treatment significantly increased fibrous cap area, reduced the necrotic core area/total plaque area ratio, and elevated plaque collagen content, all consistent with features of greater plaque stability. The favorable phenotype observed in response to microRNA-145 treatment was associated with greater expression of α-SMA and calponin, consistent with a VSMC contractile phenotype. We also report reduced levels of the microRNA-145 target KLF4 in aortas from ApoE−/− mice treated with the microRNA-145 lentivirus with coincident increases in the expression of the downstream KLF4/5 target myocardin. Collectively, these observations suggest that microRNA-145 treatment may serve to attenuate atherosclerosis and confer plaque stability, at least in part, through restriction of VSMC phenotypic alterations.

A few limitations of the study merit mention. Despite our data demonstrating unequivocal reductions in plaque volume and plaque composition, namely an increase in differentiated VSMCs with an increase in collagen content and a reduction in necrotic core area, these are indirect yet widely accepted surrogates of plaque stability. However, plaque rupture per se was not evaluated. Second, we did not evaluate the potential role and levels of microRNA-145 in other cell types that are found in plaques. However, because previous work has demonstrated that the minimal Sm22α promoter directs transgene expression exclusively to VSMCs of large and medium arteries in adult mice with no expression in venous or visceral SMCs, it is unlikely that SMC-targeted lentivirus delivery of microRNA-145 would result in microRNA-145 upregulation in cells other than SMCs. Third, although application of microRNA-145 antagonism would have further substantiated the VSMC-specific delivery nature of the microRNA-145 lentivirus we used, this was beyond the scope of the current work. Fourth, we only evaluated 2 potential downstream targets of microRNA-145, namely myocardin and KLF4/5. Several other targets, such as angiotensin-converting enzyme, have been identified that may also contribute to the antiatherosclerotic effects of microRNA-145. Fifth, the treatment was initiated before the development of atherosclerosis and future studies will be required to evaluate whether treatment is associated with reversal of atherosclerosis.

In summary, the present study provides the first report of a microRNA-145–based therapeutic approach in experimental atherosclerosis. Long-term SMC-specific upregulation of microRNA-145 favorably alters plaque morphology and cellular composition, shifting the balance toward plaque stability vs plaque rupture. These benefits appear to be mediated through an effect of microRNA-145 to promote VSMC differentiation toward the contractile (vs synthetic) phenotype via a mechanism that involves reciprocal regulation of KLF4 and myocardin. Translational studies using microRNA-145–based therapies in atherosclerosis are warranted.

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Disclosures

None.

References

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