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MicroRNA-145, a Novel Smooth Muscle Cell Phenotypic Marker and Modulator, Controls Vascular Neointimal Lesion Formation

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***Abstract*—**Phenotypic modulation of vascular smooth muscle cells (VSMCs) plays a critical role in the pathogenesis of a variety of proliferative vascular diseases. Recently, we have found that microRNA (miRNA) miR-145 is the most abundant miRNA in normal vascular walls and in freshly isolated VSMCs; however, the role of miR-145 in VSMC phenotypic modulation and vascular diseases is currently unknown. Here we find that miR-145 is selectively expressed in VSMCs of the vascular wall and its expression is significantly downregulated in the vascular walls with neointimal lesion formation and in cultured dedifferentiated VSMCs. More importantly, both in cultured rat VSMCs in vitro and in balloon-injured rat carotid arteries in vivo, we demonstrate that the noncoding RNA miR-145 is a novel phenotypic marker and a novel phenotypic modulator of VSMCs. VSMC differentiation marker genes such as SM α-actin, calponin, and SM-MHC are upregulated by premiR-145 or adenovirus expressing miR-145 (Ad-miR-145) but are downregulated by the miR-145 inhibitor 2′OMe-miR-145. We have further identified that miR-145–mediated phenotypic modulation of VSMCs is through its target gene KLF5 and its downstream signaling molecule, myocardin. Finally, restoration of miR-145 in balloon-injured arteries via Ad-miR-145 inhibits neointimal growth. We conclude that miR-145 is a novel VSMC phenotypic marker and modulator that is able of controlling vascular neointimal lesion formation. These novel findings may have extensive implications for the diagnosis and therapy of a variety of proliferative vascular diseases. **(*Circ Res*. 2009;105:158-166.)**

Key Words: microRNAs ■ vascular smooth muscle cells ■ phenotype ■ vascular disease

Vascular smooth muscle cells (VSMCs) are not terminally differentiated and possess the ability to modulate their phenotype in response to changing local environmental cues.[1] It is well established that the transition of VSMCs from a differentiated phenotype to a dedifferentiated state plays a critical role in the pathogenesis of a variety of proliferative cardiovascular diseases such as atherosclerosis, hypertension, restenosis after angioplasty or bypass, diabetic vascular complications, and transplantation arteriopathy.[1,2] The phenotypic modulation in VSMCs is accompanied by accelerated migration, proliferation, and production of extracellular matrix components. Eventually, these cellular events result in the formation of vascular neointima, which is the common pathological lesion in these proliferative vascular diseases. It has been demonstrated that the differentiation state of VSMCs is affected by numerous environmental cues, including growth factors, cell–cell contacts, extracellular matrix components, and neuronal input. However, the final common pathway of most of these environmental influences is the change in expression of VSMC phenotypic genes. Therefore, completely understanding the regulatory mechanisms underlying VSMC phenotypic gene expression is currently the most important issue in the research area of phenotypic modulation.

Recently, the most important breakthrough regarding gene expression regulation is the discovery of microRNAs (miRNAs).[3] miRNAs comprise a novel class of endogenous, small, noncoding RNAs that negatively regulate gene expression via degradation or translational inhibition of their target mRNAs. More importantly, one miRNA is able to regulate the expression of multiple genes because it can bind to its mRNA targets as either an imperfect or perfect complement. Thus, one miRNA is functionally important as a transcription factor.[4] As a group, miRNAs may directly regulate at least 30% of the genes in a cell.[5]

In our recent study, we have found that miR-145 is the most abundant miRNA in vascular walls.[6] However, the functions of miR-145 in VSMCs and vascular disease are completely unknown. The objective of the present study is to determine whether miR-145 can be used as a novel pheno-

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typic marker for VSMCs and whether this abundant miRNA has any potential regulatory effects on the VSMC phenotype and vascular neointimal lesions using both cultured VSMCs in vitro and balloon-injured rat carotid arteries in vivo.

Materials and Methods

An expanded Materials and Methods section is available in the Online Data Supplement at http://circres.ahajournals.org.

Cell Culture

VSMCs and endothelial cells (ECs) were obtained from the rat aortas as described.[6]

Oligo Transfection, Knockdown, or Overexpression of miR-145 and KLF5 in Cultured VSMCs

Oligo transfection was performed according to an established protocol.[6–8] miR-145 was knocked down via the miR-145 inhibitor 2′OMe-miR-145 and was overexpressed via premiR-145. Krüppel-like factor (KLF)5 knockdown was performed using its small interfering (si)RNA (si-KLF5, 100 nmol/L). For KLF5 upregulation, adenovirus expressing KLF5 (Ad-KLF5) was applied (30 multiplicities of infection). Vehicle, oligo controls (Ambion Inc), siRNA control (si-control, Invitrogen), and adenovirus control (Ad-GFP) were applied.

RNA Analysis by Quantitative Real-Time PCR

Quantitative real-time (qRT)-PCR for miR-145, SM-MHC, calponin, SM α-actin, myocardin, and KLF5 was performed with a Roche Lightcycler 480 Detection System as described.[8] The sequences of the primers used are shown in Online Table I.

Northern Blot Analysis of miRNA

Northern blot analysis of miR-145 and premiR-145 was performed as described.[6,7]

Western Blot Analysis

Standard Western blot analysis was conducted using SM-MHC, calponin, SM α-actin, and KLF5 antibodies.

Construction of the Adenovirus

The adenovirus expressing miR-145 (Ad-miR-145), Ad-KLF5, and Ad-GFP were generated as described.[8]

Luciferase Assay

A construct in which a fragment of the 3′ untranslated region (UTR) of KLF5 mRNA containing the putative miR-145 binding sequence and a firefly luciferase reporter construct and transfected into HEK 293 cells with either vehicle (Vehicle control), an empty plasmid (pDNR-CMV) (0.2 μg/mL), a plasmid expressing miR-145 (pmiR-145) (0.2 μg/mL), or a control plasmid expressing an unrelated miRNA, miR-31 (pmiR-31) (0.2 μg/mL). Relative luciferase expression was measured on a scintillation counter by using a dual luciferase reporter system.[8]

Immunocytochemistry, Immunofluorescence, and In Situ Hybridization

In situ hybridization of miR-145 was performed in 10-μm vessel sections as described.[8] VSMCs were stained by immunofluorescence of SM α-actin in these sections. In addition, SM α-actin in cultured VSMCs was also determined by immunocytochemistry.

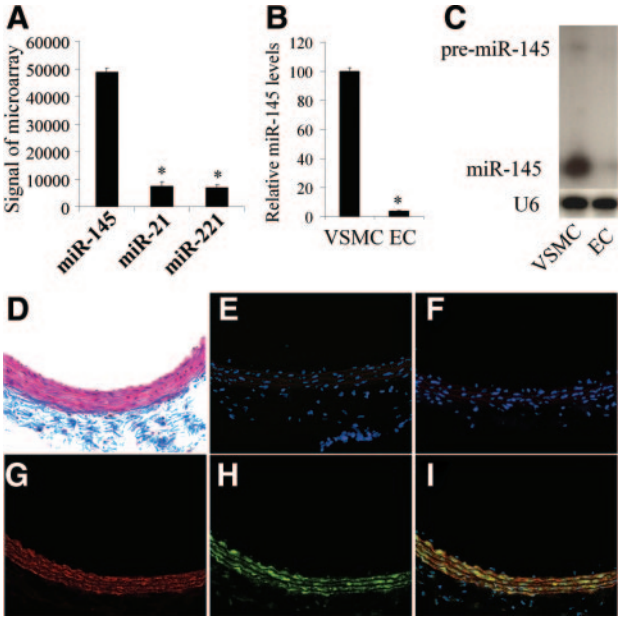

Figure 1. miR-145 is selectively expressed in VSMCs of the vascular wall. A, Expression levels of miR-145, miR-21, and miR-221 in normal rat carotid arteries (n=6). *$P<0.05$ compared with miR-145. B, Relative expression of miR-145 in rat VSMCs and ECs, as determined by qRT-PCR (n=6). *$P<0.05$ compared with that in VSMCs. C, Expression of miR-145 and premiR-145 in rat VSMCs and ECs, as determined by Northern blot analysis. D, Masson's trichrome staining of rat carotid artery. E, Negative control of In situ hybridization (no miRNA probe). F, Scrambled probe control. G, Immunofluorescence with the smooth muscle marker SM α-actin (red color). H, In situ hybridization of miR-145 (green color). I, Merged images of G and H. Blue color is the cell nuclear staining by DAPI.

Rat Carotid Artery Balloon Injury Model and Adenovirus-Mediated Gene Transfer Into the Injured Vascular Wall

Carotid artery balloon injury and adenovirus-mediated gene transfer were performed in male Sprague–Dawley rats as described.[8]

Morphometric Analysis for Neointimal Lesion Formation

Morphometric analysis via computerized image analysis system was performed in sections stained with Masson's trichrome staining as described.[6,8]

Results

miR-145 Is the Most Abundant miRNA in Normal Rat Carotid Arteries That Is Selectively Expressed in VSMCs

Our microarray analysis revealed that miR-145 is the most abundant miRNA in normal rat carotid arteries.[6] Recent studies demonstrated that miR-21 and miR-221 are also 2 abundant miRNAs in vascular walls.[6,8–10] We thus compared their expression with that of miR-145. As shown in Figure 1A, microarray analysis demonstrated that the expression of miR-145 was much higher than that of miR-21 and miR-221.

VSMCs and ECs are 2 of the major cell types in normal vascular walls. To determine the distribution of miR-145 expression in the vascular wall, we isolated VSMCs and ECs

from rat arteries and measured miR-145 levels in these cells. As shown in Figure 1B and 1C, both qRT-PCR and Northern blot analysis demonstrated that miR-145 was highly expressed in VSMCs; it was, however, almost undetectable in ECs. In addition, our unpublished microarray data (Yuhui Cheng and Chunxiang Zhang, 2008) revealed that miR-145 is also the most abundant miRNA in freshly isolated VSMCs and its expression was much higher than that of miR-21 and miR-221 (Online Table II).

To further confirm the cellular distribution of miR-145 in the vascular wall, we performed in situ hybridization on normal rat carotid artery. Vessel structure was demonstrated via Masson's trichrome staining in frozen sections as shown in Figure 1D. In situ hybridization of miR-145 (green color) showed that it was expressed primarily in the vessel media where VSMCs were localized (Figure 1H). In contrast, no fluorescence signal was detected in the vasculature intima where ECs were localized (Figure 1H and 1I). To confirm that miR-145 was localized in VSMCs, we performed coimmunofluorescence with the smooth muscle marker SM α-actin (red color). As expected, SM α-actin was observed in VSMCs that were located in the media (Figure 1G). Interestingly, miR-145 was clearly colocalized with VSMCs (Figure 1I, merged color with red and green). Again, no expression of miR-145 was demonstrated in intimal cells (ECs) (Figure 1I). Also, there was no miR-145 signal in 2 control sections: negative control (Figure 1E, no miRNA probe); and scrambled probe control (Figure 1F).

miR-145 Is a Novel Phenotypic Marker for VSMCs In Vitro in Cultured Cells

To explore the relationship between miR-145 and the VSMC phenotype, we applied a well-established VSMC model for phenotypic modulation in which VSMC dedifferentiation was induced by platelet-derived growth factor (PDGF).[11] As shown in Figure 2A, PDGF-BB (20 ng/mL) caused a time-dependent suppression of the mRNA levels of VSMC differentiation marker genes such as SM α-actin, calponin, and SM-MHC. Interestingly, the similar expression pattern was found in miR-145 levels in VSMCs after PDGF stimulation. PDGF-BB (20 ng/mL) caused a rapid decrease in miR-145 expression, as demonstrated by qRT-PCR (Figure 2A) and Northern blot (Figure 2B). In contrast to the high expression of mature miR-145, premiR-145 expression was very low in VSMCs both at basal and PDGF-stimulated conditions (Figure 2B). As miRNA controls, we also determined the expression levels of miR-221 and miR-221 after PDGF-stimulation. Unlike miR-145, the expression of miR-221 and miR-222 was increased by PDGF.[8] The protein levels of VSMC differentiation marker genes after PDGF-stimulation were displayed in Figure 2C and 2D. These results demonstrated that miR-145 was a novel phenotypic marker for VSMCs.

miR-145 Is Also a Phenotypic Marker for VSMCs in the Vascular Wall In Vivo

To investigate whether miR-145 is a phenotypic marker for VSMCs in the vascular wall in vivo, we applied a well-established balloon-injury model of the rat carotid artery, as

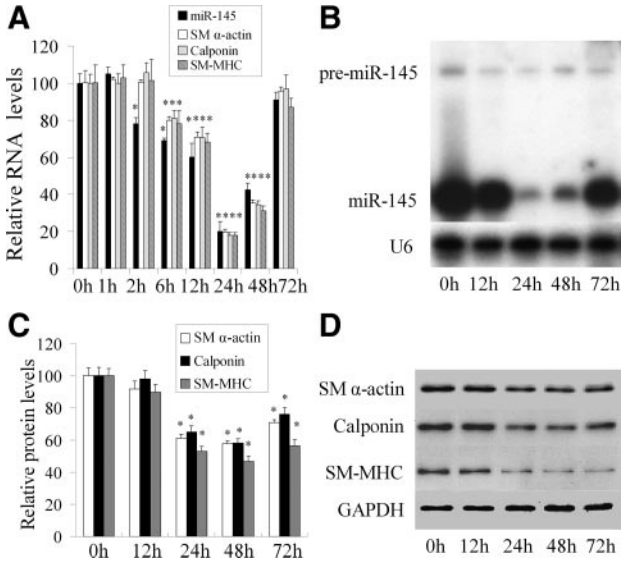

Figure 2. miR-145 is a phenotypic marker for VSMCs in vitro in cultured cells. A, PDGF-BB (20 ng/mL) caused a time-dependent suppression of miR-145 and VSMC differentiation marker genes such as SM α-actin, calponin, and SM-MHC as determined by qRT-PCR. B, miR-145 and premiR-145 in PDGF-BB–treated (20 ng/mL) VSMCs as determined by Northern blot analysis. C, Quantitative analysis of VSMC differentiation marker genes by Western blot. D, Representative Western blots in VSMCs treated with PDGF (n=6). *$P<0.05$ compared with 0 hour group.

described.[6] In this experiment, we isolated the rat right carotid arteries at 7, 14, and 28 days after angioplasty. Uninjured arteries were used as controls. As shown in Figure 3A, balloon injury resulted in time-dependent neointimal lesion formation in rat carotid arteries. Accordingly, the VSMC differentiation marker genes such as SM α-actin, calponin, and SM-MHC were downregulated both at the mRNA (Figure 3B) and protein levels (Figure 3D and 3E); however, there was a partial recovery at 28 days after injury. The results suggest that VSMCs in the vascular wall quickly changed from a differentiated phenotype to a dedifferentiated state after angioplasty. qRT-PCR (Figure 3B) and Northern blot analysis (Figure 3C) showed that miR-145 expression was significantly downregulated, with a similar time course to the VSMC differentiation marker genes. These results suggest that miR-145 is a phenotypic marker for VSMCs in the vascular wall in vivo.

miR-145 Is a Phenotypic Modulator for VSMCs In Vitro in Cultured Cells

To determine whether miR-145 affects VSMC phenotype, we addressed the following 2 questions in cultured VSMCs. First, in a PDGF-induced phenotypic modulation model, we tested whether or not miR-145 has an effect on the VSMC phenotype. If the first experiment was positive, then the next question was whether or not modulating the miR-145 level itself is sufficient to elicit phenotypic changes in quiescent, nonstimulated VSMCs.

To modulate miR-145 in cultured VSMCs, we applied both gain-of-function and loss-of-function approaches. For the miR-145 knockdown, the miR-145 inhibitor (2′OMe-miR-

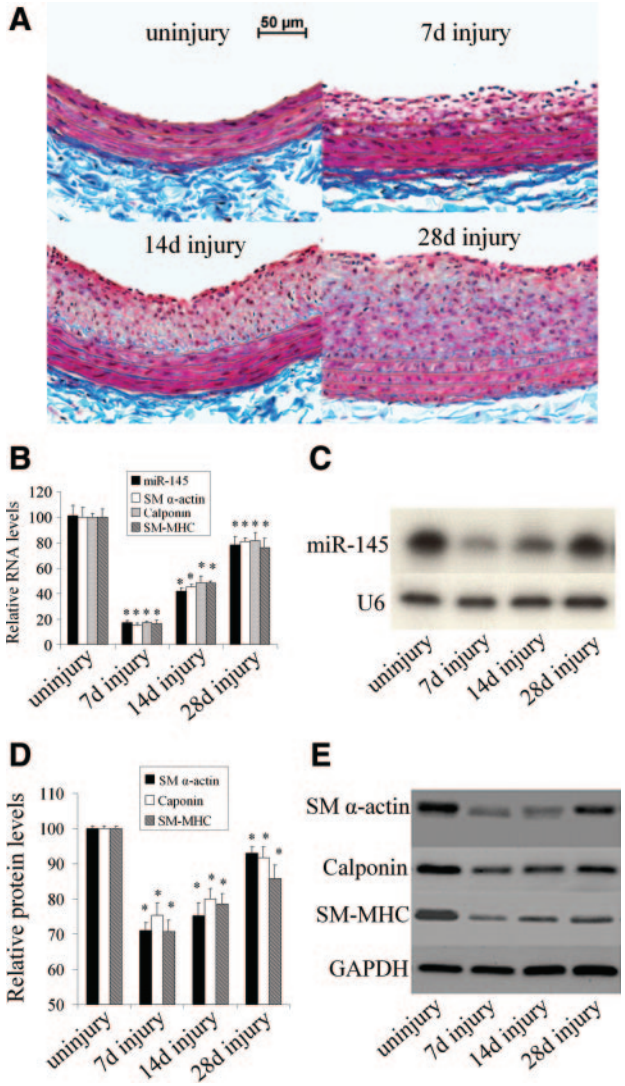

Figure 3. miR-145 is a phenotypic marker for VSMCs in the vascular wall in vivo. A, Representative Masson's trichrome staining in uninjured and injured rat carotid arteries at 7, 14, and 28 days after angioplasty. B, Expression of miR-145 and VSMC differentiation marker genes SM α-actin, calponin, and SM-MHC in uninjured and injured rat carotid arteries, as determined by qRT-PCR. C, miR-145 expression in the vascular walls determined by Northern blot analysis. D, Quantitative analysis of VSMC differentiation marker genes by Western blot. E, Representative Western blots of VSMC differentiation marker genes (n=6). *$P<0.05$ compared with uninjured control group.

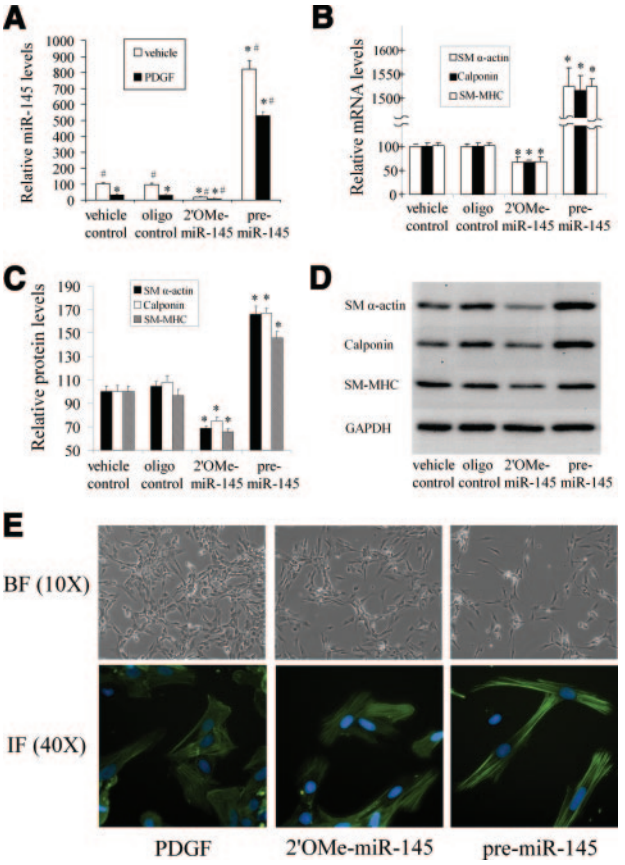

Figure 4. miR-145 modulates VSMC phenotype in vitro in cultured cells. The VSMCs were pretreated with vehicle, control oligo, 2′OMe-miR-145, or premiR-145 for 4 hours followed by PDGF or vehicle for 24 hours. A, Modulation of miR-145 expression by 2′OMe-miR-145 (100 nmol/L) and premiR-145 (100 nmol/L) in VSMCs with or without PDGF (20 ng/mL) (n=6). *$P<0.05$ compared with oligo control group treated with vehicle; #$P<0.05$ compared with oligo control group treated with PDGF. B, 2′OMe-miR-145 strengthened, whereas premiR-145 inhibited PDGF-mediated effects on VSMC maker genes as determined by qRT-PCR (n=6). *$P<0.05$ compared with oligo control. C, 2′OMe-miR-145 strengthened, whereas premiR-145 inhibited, PDGF-mediated effects on VSMC maker genes as determined by Western blot (n=6). *$P<0.05$ compared with oligo control. D, Representative Western blots of VSMC differentiation marker genes. E, Top, Representative morphological changes of primary cultured VSMCs treated with PDGF (20 ng/mL), 2′OMe-miR-145 (100 nmol/L), or premiR-145 (100 nmol/L) for 48 hours. Bottom, Representative immunofluorescence images of the VSMCs via anti–SM α-actin antibody (green color). Blue color is the cell nuclear staining by DAPI.

145) was added to the culture media at final oligonucleotide concentrations of 1, 3, 10, 30, and 100 nmol/L. 2′OMe-miR-145 was the miR-145 antisense oligonucleotide, which was modified at each nucleotide by an *O*-methyl moiety at the 2′-ribose position. 2′OMe-miR-145 decreased miR-145 expression in a dose-dependent manner (Online Figure I, A). For miR-145 upregulation, premiR-145 was added to the culture media at final oligonucleotide concentrations of 1, 3, 10, 30, and 100 nmol/L. premiR-145 increased miR-145 expression in a dose-dependent manner (Online Figure I, B). It should be noted that 30 to 100 nmol/L of premiR-145 is able to increase miR-145 expression to the level that is similar to freshly isolated, differentiated VSMCs. In addition, the effects of both 2′OMe-miR-145 and premiR-145 on miR-145 expression were miR-145 specific, because no effects were found on other miRNAs such as miR-125b and miR-352 (Online Figure II).

As shown in Figure 4A, 2′OMe-miR-145 (100 nmol/L) decreased miR-145 expression in VSMCs with or without PDGF-stimulation. In contrast, miR-145 was increased by premiR-145 (100 nmol/L) under these experimental conditions. Consistent with the expression changes in miR-145, 2′OMe-miR-145 strengthened the PDGF-mediated effects on VSMC dedifferentiation. However, premiR-145 inhibited the PDGF-mediated effects on VSMC dedifferentiation as shown by the expression changes of VSMC differentiation marker

genes detected at the mRNA (Figure 4B) and protein levels (Figure 4C and 4D). These results indicate that miR-145 has a regulatory effect on the VSMC phenotype.

To test whether modulating the miR-145 itself is sufficient to elicit phenotypic changes, we determined the effect of altering miR-145 on the VSMC phenotype in quiescent, nonstimulated VSMCs. As shown in Online Figure III, 2′OMe-miR-145 (100 nmol/L) suppressed the expression of VSMC differentiation marker genes. In contrast, these marker genes were enhanced by premiR-145 (100 nmol/L). These results demonstrate that modulation of miR-145 alone is sufficient to elicit phenotypic changes. Thus, the miR-145–mediated effect on the VSMC phenotype is not limited to PDGF-induced phenotypic modulation and miR-145 might be a causative regulator of the VSMC phenotype.

The causative role of miR-145 in VSMC modulation prompted us to test one of the most important issues in vascular biology: whether the miR-145 is able to keep differentiated morphometry in primary cultured cells. In this experiment, primary cultured cells were treated with PDGF (20 ng/mL), 2′OMe-miR-145 (100 nmol/L), or premiR-145 (100 nmol/L) for 2 days. As shown in Figure 4E (top), PDGF elicited a quickly flattened morphology that reflected a dedifferentiated state. 2′OMe-miR-145 led to a similar morphological change, although it was less pronounced. In contrast, premiR-145 kept a spindle-like shape that reflected a differentiated state. The morphological changes were further confirmed by immunofluorescence using anti-SM α-actin antibody (Figure 4E, bottom).

miR-145 Is a Phenotypic Modulator of VSMCs in the Vascular Wall In Vivo

To reverse the decreased miR-145 expression in the injured vascular wall, Ad-miR-145 was used. As shown in Figure 5A, at 7 days after balloon injury, miR-145 expression was significantly increased in balloon-injured rat carotid arteries treated with Ad-miR-145 compared with those in balloon-injured arteries treated with either vehicle or Ad-GFP as determined by qRT-PCR. However, the miR-145 could only be partially restored because of the limited transfection of the adenovirus (Figure 5A). Interestingly, adenovirus-mediated miR-145 expression significantly increased the VSMC differentiation marker genes compared with those from vehicle or control adenovirus-treated vessels as determined by qRT-PCR and Western blot analysis (Figure 5B through 5D).

KLF5 Is the Critical Target Gene of miR-145 That Is Responsible for miR-145–Mediated Effects on VSMC Phenotypic Modulation

Based on the cellular effect of miR-145, our bioinformatics analysis suggested that KLF5 could be a potential gene target for miR-145. Computational analysis suggested that KLF5 had a miR-145 binding site in its 3′-untranslated region (3′-UTR) that was conserved, as shown in Online Figure IV.

If KLF5 is a target gene for miR-145, its expression should be upregulated in cultured dedifferentiated VSMCs stimulated with PDGF, because miR-145 expression is downregulated by PDGF. As shown in Figure 6A, KLF5 expression was indeed increased in PDGF-stimulated VSMCs.

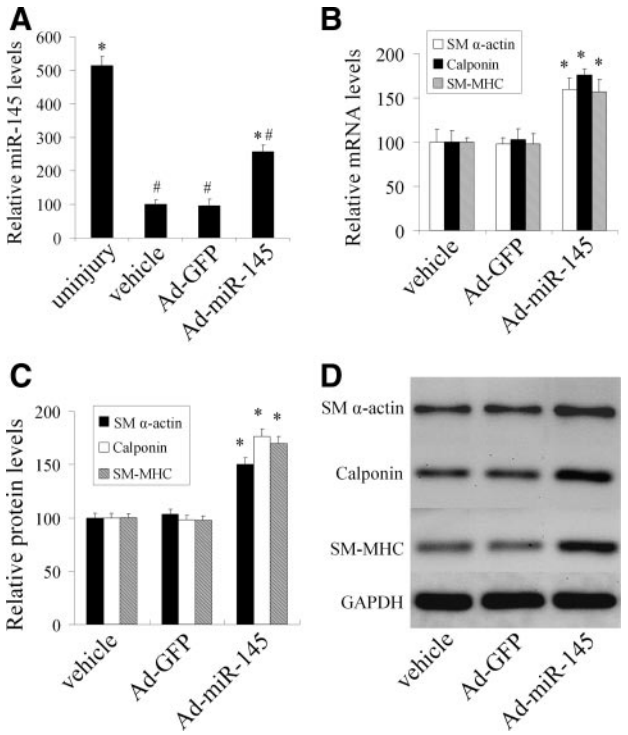

Figure 5. miR-145 modulates the VSMC phenotype in vivo in balloon injured rat carotid arteries. A, miR-145 expression in uninjured arteries and in balloon-injured arteries at 7 days after angioplasty, which were treated with vehicle, Ad-GFP, or Ad-miR-145. B, Ad-miR-145 increased the VSMC differentiation marker genes in injured arteries as determined by qRT-PCR. C, Ad-miR-145 increased the VSMC differentiation marker genes in injured arteries, as determined by Western blot. D, Representative Western blots of VSMC differentiation marker genes (n=6). *$P<0.05$ compared with Ad-GFP control; #$P<0.05$ compared with uninjured control group.

To determine whether miR-145 is able to bind to and inhibit KLF5 expression directly, we performed the luciferase assay, in which a construct in which a fragment of the 3′-UTR of KLF5 mRNA containing the putative miR-145 binding sequence was used. As we expected, pmiR-145, but not pmiR-31 or pDNR-CMV, increased miR-145 expression in HEK 293 cells (Online Figure V). Moreover, pmiR-145, but not pDNR-CMV or pmiR-31, inhibited luciferase activity (Figure 6B). In the mutated control groups, the inhibitory effect of pmiR-145 on luciferase activity disappeared (Figure 6B). The results suggest that miR-145 can bind to KLF5 directly and inhibit its expression.

The regulatory effect of miR-145 on KLF5 in VSMCs was determined using both loss-of-function and gain-of-function approaches. As shown in Figure 6C and 6D, KLF5 expression was upregulated by 2′OMe-miR-145 and was downregulated by premiR-145 at the protein level, but not at the mRNA level (Online Figure VI). The results suggest that KLF5 is a target gene of miR-145 in VSMCs.

We then determined the effect of KLF5 on VSMC phenotype. As shown in Figure 6E, KLF5 was knocked down by its siRNA (si-KLF5) and was upregulated by Ad-KLF5. Interestingly, downregulation of KLF5 increased, whereas up-

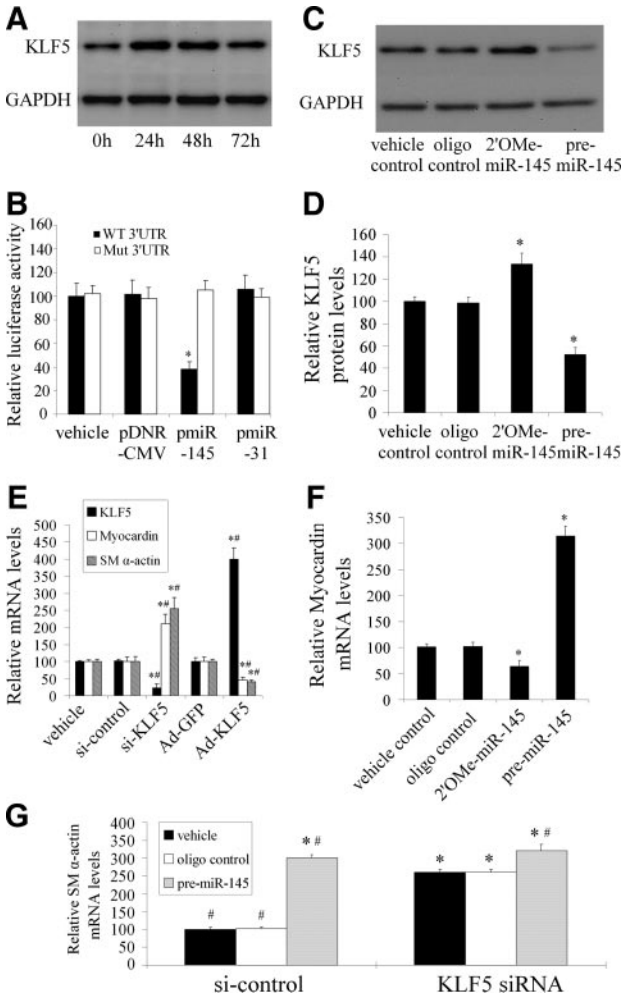

Figure 6. KLF5 is the critical target gene of miR-145 that is responsible for miR-145–mediated effect on VSMC phenotypic modulation. A, KLF5 expression was increased in VSMCs after PDGF stimulation (20 ng/mL). B, pmiR-145, but not pDNR-CMV or unrelated pmiR-31, inhibited luciferase activity. In the mutated control group, the inhibitory effect of pmiR-145 on luciferase activity was abrogated (n=5). *$P<0.05$ compared with vehicle control. C, Representative Western blots of KLF5 in miR-145 modulated VSMCs. D, 2′OMe-miR-145 increased, whereas premiR-145 decreased, KLF5 expression in cultured VSMCs (n=6). *$P<0.05$ compared with oligo control. E, The effects of KLF5 on the expression of myocardin and SM α-actin (n=5). *$P<0.05$ compared with siRNA control (si-control); #$P<0.05$ compared with Ad-GFP. F, The effect of miR-145 on the expression of myocardin (n=6). *$P<0.05$ compared with oligo control. G, The effects of premiR-145 on the expression of SM α-actin in VSMCs with or without KLF5 depletion (n=6). *$P<0.05$ compared with oligo control in siRNA control (si-control)–treated groups; #$P<0.05$ compared with oligo control in KLF5 siRNA-treated groups.

regulation of KLF5 decreased VSMC differentiation marker gene, SM α-actin.

One recent report indicated that myocardin, a key modulator for differentiation marker genes,[12] might be a downstream molecule of KLF5.[14] As shown in Figure 6E, we found that downregulation of KLF5 increased, whereas upregulation of KLF5 decreased the expression of myocardin in VSMCs. In addition, as shown in Figure 6F, 2′OMe-miR-145 (100 nmol/L) decreased, whereas premiR-145 (100 nmol/L) increased the expression of myocardin.

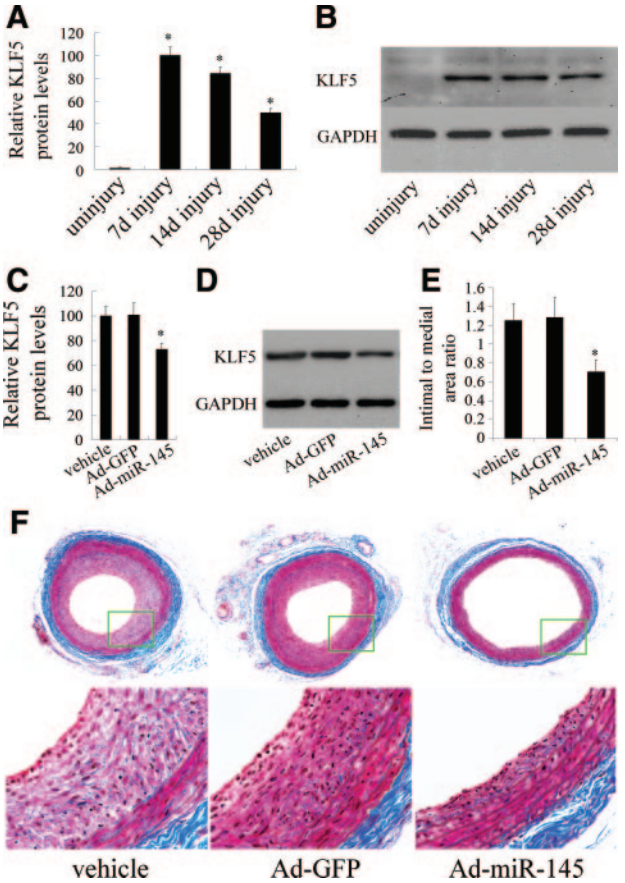

Figure 7. miR-145 modulates KLF5 expression and vascular neointimal lesion formation in vivo in balloon-injured rat carotid arteries. A, The expression of KLF5 in uninjured and balloon-injured rat carotid arteries (n=6). *$P<0.05$ compared with uninjured control. B, Representative Western blots of KLF5 in uninjured and balloon-injured rat carotid arteries. C, The effect of Ad-miR-145 on the expression of KLF5 in balloon-injured arteries (n=5). *$P<0.05$ compared with Ad-GFP. D, Representative Western blots of KLF5 in balloon-injured rat carotid arteries treated with vehicle, Ad-GFP, or Ad-miR-145. E, The effect of Ad-miR-145 on vascular neointimal lesion formation in rat carotid arteries at 14 days after angioplasty (n=9). *$P<0.05$ compared with Ad-GFP. F, Representative Masson's trichrome–stained photomicrographs of rat carotid arteries treated with vehicle, Ad-GFP or Ad-miR145.

Finally, to further verify that KLF5 was a functional target gene related to miR-145–induced effects on the VSMC phenotype, we depleted KLF5 expression in cultured VSMCs via its siRNA. As shown in Figure 6G, knock down of KLF5 and upregulation of miR-145 induced a similar effect on the VSMC differentiation marker gene SM α-actin. In the VSMCs that were depleted of the target gene, KLF5, the additional premiR-145–mediated effect on SM α-actin was significantly attenuated (Figure 6G).

miR-145 Modulates KLF5 Expression and Vascular Neointimal Lesion Formation In Vivo in Balloon-Injured Rat Carotid Arteries

In this experiment, we first determined the expression levels of KLF5 in rat carotid arteries with or without injury. As shown in Figure 7A and 7B, in uninjured vessels, the

expression of KLF5 was very low. However, after angioplasty, the expression of KLF5 was significantly upregulated. We then modulated miR-145 level via Ad-miR-145 in balloon-injured vessels. As shown in Figure 7C and 7D, KLF5 was downregulated by Ad-miR-145 in vivo. The results suggest that KLF5 is indeed a target gene of miR-145 in vivo.

Phenotypic change of VSMC plays a critical role vascular neointimal development. We thus determined the effect of miR-145 on neointimal growth. As shown in Figure 7E, restoration of the downregulated miR-145 via Ad-miR-145 in balloon-injured rat carotid arteries inhibited neointimal lesion formation significantly. Representative Masson's trichrome stained photomicrographs of rat carotid arteries from vehicle, Ad-GFP, and Ad-miR-145–treated groups were shown in Figure 7F.

Discussion

In the present study, we found that miR-145, which is the most abundant miRNA in vascular walls and in freshly isolated VSMCs, is selectively expressed in VSMCs of the vascular wall. However, its expression is almost undetectable in ECs from the normal vessels. The result is consistent with 2 recent reports in which no miR-145 expression is found in human ECs.[13,14] Thus, miR-145 may be used as a novel marker for VSMCs in the vascular walls.

In our recent study, we found that miR-145 expression was significantly suppressed in the vascular walls with neointimal lesion formation.[6] This phenomenon was accompanied by phenotypic changes of VSMCs within the vascular lesions; thus, we tested the relationship between miR-145 expression and the VSMC phenotype. In cultured VSMCs stimulated with PDGF, we found that the expression changes of miR-145 are consistent with the expression changes of VSMC differentiation marker genes such as SM α-actin, SM-MHC, and calponin. This discovery was further confirmed in vivo using balloon-injured rat carotid arteries. These results demonstrated for the first time that the noncoding RNA miR-145 was a novel phenotypic marker for VSMCs.

We then sought to determine the potential role of miR-145 in VSMC phenotypic modulation. First, we found that overexpression of miR-145 was able to inhibit PDGF-induced VSMC dedifferentiation. In contrast, PDGF-induced VSMC dedifferentiation was strengthened by miR-145 inhibition. Our time course studies revealed that the decrease in miR-145 expression after PDGF-stimulation was earlier than that of VSMC differentiation marker genes (Figure 2A). Moreover, the process of protein expression recovery of VSMC differentiation marker genes after PDGF stimulation was slower than that of miR-145 and mRNAs of these marker genes (Figure 2C and 2D). The result suggests that the recovery process of VSMC differentiation is from miR-145 to mRNAs of VSMC differentiation marker genes and then to protein expression of these marker genes. Second, we investigated whether miR-145 expression plays a causative role in VSMC phenotypic modulation. We tested the effects of 2′OMe-miR-145 and premiR-145 on the VSMC phenotype in quiescent, non–PDGF-stimulated VSMCs. Both the gain-of-function and the loss-of-function experiments revealed that the miR-145 itself is sufficient to elicit phenotypic changes. Furthermore, the differentiated morphometry of primary cultured VSMCs was kept via overexpression of miR-145. Finally, the regulatory effect of miR-145 on VSMC phenotype was further verified in vivo in balloon-injured rat carotid arteries via Ad-miR-145. Thus, the present study revealed that miR-145 is a novel phenotypic modulator for VSMCs both in vitro and in vivo.

In the present study, computational analysis suggests that KLF5 is a miR-145 target gene. The negative correlation between miR-145 expression and KLF5 expression in both PDGF-stimulated VSMCs and balloon-injured arteries indicated that KLF5 could be a potential target gene of miR-145 in VSMCs. To verify this, we first confirmed that miR-145 was able to bind to KLF5 and regulate its expression directly using a construct in which a fragment of the 3′-UTR of KLF5 mRNA, containing the putative miR-145 binding sequence. We then found that miR-145 was sufficient to regulate KLF5 expression in cultured VSMCs and in balloon-injured arteries. In addition, downregulation of KLF5 and upregulation of miR-145 were able to induce a similar effect on VSMC phenotype. Furthermore, in KLF5-deleted VSMCs, premiR-145–mediated additional effect on VSMC phenotype was attenuated because of the lack of its target gene. For example, in the siRNA control group, premiR-145 increased the expression of SM α-actin by $\approx$300%. However, in KLF5-depleted cells, the expression of SM α-actin was only increased by $\approx$30% (Figure 6). The results suggest that, although KLF5 is not a sole target gene, it is indeed a critical functional target gene that is involved in miR-145–mediated effect on the VSMC phenotype.

How does KLF5 work as a negative regulator for VSMC differentiation marker genes? Two previous reports from Owens and colleagues demonstrated that KLF5 is able to increase SM α-actin transcription in cultured 10T1/2 cells and NIH3T3 cells.[15,16] Obviously, the direct effect of KLF5 on SM α-actin in these non-VSMCs does not match the effect of KLF5 on SM α-actin in VSMCs as demonstrated in the present study. However, 1 recent study suggested that the effects of KLF5 on gene expression are cell specific.[17] The direct effect of KLF5 on the transcription of SM α-actin in VSMCs should be determined in a future study. One recent study demonstrated that KLF5 has a negative effect of on myocardin.[18] Because myocardin is a key modulator of VSMC differentiation marker genes,[12] we hypothesized that KLF5-induced downregulation of VSMC differentiation marker genes might be related to the downstream molecule, myocardin. Indeed, we found that downregulation of KLF5 increased, whereas upregulation of KLF5 decreased the expression of myocardin in VSMCs. Moreover, the expression of myocardin was also upregulated by premiR-145 and downregulated by miR-145 inhibitor. These results suggest that miR-145–mediated effect on VSMC phenotype is through KLF5/myocardin pathway (Figure 8).

There appear to be dramatic differences in mRNA expression between PDGF-treated and untreated cells that have overexpression of miR-145 (Figure 4B; Online Figure III, A). We think this phenomenon may be induced by PDGF-

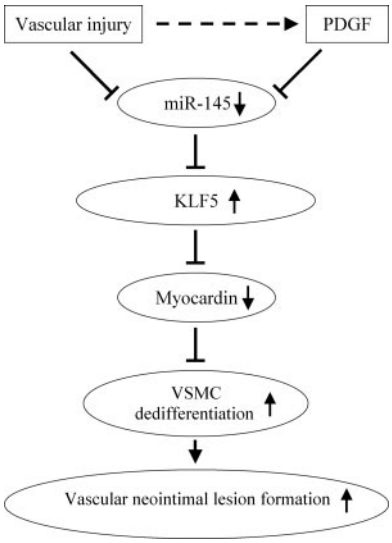

Figure 8. Molecular mechanisms of miR-145–mediated effects on VSMC phenotypic modulation and vascular neointimal lesion formation.

mediated sensitization. However, the baseline levels of the marker mRNAs are very low in PDGF-treated cells, the absolute amounts of these marker mRNAs after overexpression of miR-145 are not substantially different between PDGF-treated and untreated cells. This may explain, at least in part, why the protein levels of these marker genes are not very different in these 2 groups (Figure 4C; Online Figure III, B). The results also suggest that there may be unknown underlying mechanisms in normal cells that limit a gene from over responding to a miRNA.

Dedifferentiated VSMCs often have accelerated proliferation that plays a critical role in vascular neointimal development. Indeed, we also found that PDGF-induced VSMC proliferation was significantly inhibited by overexpression of miR-145 (Online Figure VII). Finally, we determined the role of miR-145 in neointimal growth via adenovirus-mediated miR-145 gene transfer. As expected, we found that miR-145 is an important negative controller for vascular neointimal lesion formation. It should be noted that Ad-miR-145 was transfected primarily into VSMCs in the balloon-injury model, because ECs had been removed during the angioplasty procedure.

Our microarray data have revealed that multiple miRNAs are aberrantly expressed in the vascular walls after balloon injury.[6] Until now, we have found that miR-21, miR-221/222, and miR-145 have a modest effect on neointimal growth.[6,8] Based on our limited data, we have found that their major cellular effects are antiapoptosis, proproliferation, and antidedifferentiation, respectively. Although the interactions among the aberrantly expressed miRNAs after angioplasty have not been studied, we predict that they may have additive, synergistic, or antagonistic interactions in the process of neointimal formation. In the present study, we have prioritized the effect of miR-145, miR-21 and miR-221 on VSMC phenotype. As shown in Online Figure VIII, premiR-145 induced the largest increase in the expression of the VSMC differentiation marker gene SM α-actin. Although premiR-21 also increases the expression of SM α-actin, the effect is much smaller than that induced by premiR-145. In contrast, premiR-221 decreased the expression of SM α-actin. Thus, among these 3 miRNAs, miR-145 is the most important miRNA in keeping the VSMC differentiation. In addition, we have compared the effect on VSMC differentiation induced by premiR-145 alone with that induced by premiR-145 and premiR-221. We have found that the premiR-145–induced increase in SM α-actin expression was significantly decreased via adding of premiR-221 (Online Figure IX). Clearly, there is an antagonistic interaction between miR-miR-145 and miR-221 in modulating VSMC phenotype. Other interactions between miRNAs in regulating cellular functions and neointimal formation should be investigated in future studies.

In summary, the present study reveals that vascular injury or PDGF is able to quickly decrease miR-145 expression in VSMCs. The decreased miR-145 will increase the expression of its target gene KLF5. The increased KLF-5 will then decrease VSMC differentiation marker genes via its downstream molecule, myocardin. The dedifferentiated VSMCs will have increased proliferation and result in the increased neointimal lesion formation. The findings that the noncoding RNA miR-145 is a novel phenotypic marker and modulator of VSMCs that controls vascular neointimal lesion formation may have extensive implications for the diagnosis and therapy of a variety of proliferative cardiovascular diseases.

Sources of Funding

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Disclosures

None.

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Supplement Material

Expanded Materials and Methods

Cell culture. VSMCs were obtained from the aortic media of male Sprague-Dawley rats (5 weeks old) using an enzymatic dissociation method as described (1). VSMCs were cultured with DMEM containing 10% fetal bovine serum (FBS). Endothelial cells (ECs) were obtained from the rat aortas (Cell Applications, Inc) and cultured with M199 containing 10% FBS.

Oligo transfection, knockdown or overexpression of miR-145 and KLF5 knockdown and overexpression, in cultured VSMCs. Oligo transfection was performed according to an established protocol (1-3). Briefly, cells were transfected using a transfection reagent (Qiagen, Chatsworth, CA) for 4 h. Transfection complexes were prepared according to the manufacturer's instructions. For miR-145 knockdown, the miR-145 inhibitor, 2'OMe-miR-145 (Integrated DNA Technologies), was added to the complexes at final oligonucleotide concentrations of 1, 3, 10, 30, and 100 nmol/L. For miR-145 overexpression, pre-miR-145 (Integrated DNA Technologies) was added to the complexes at final oligonucleotide concentrations of 1, 3, 10, 30, and 100 nmol/L. KLF5 knockdown was performed using its siRNA (si-KLF5, 100 nM, Invitrogen). For KLF5 upregulation, adenovirus expressing KLF5 (Ad-KLF5) was applied (30 MOI). The transfection medium was replaced 4 h posttransfection by the regular culture medium. Vehicle, oligo controls (Ambion, Inc.), siRNA control (si-control, Invitrogen), and adenovirus control (Ad-GFP, 30 MOI) were applied.

RNA analysis by quantitative real-time PCR (qRT-PCR). Briefly, RNAs from VSMCs, ECs, and rat carotid arteries were isolated with a RNA Isolation Kit (Ambion, Inc.). qRT-PCR for miR-145 was performed on cDNA generated from 50 ng of total RNA using the protocol of the mirVana qRT-PCR miRNA Detection Kit (Ambion, Inc). qRT-PCR for SM-MHC, calponin, SM α–actin, myocardin, and KLF5 was performed on cDNA generated from 200 ng of total RNA using the protocol of a qRT-PCR mRNA Detection Kit (Roche). Amplification and detection of specific products were performed with a Roche Lightcycler 480 Detection System. As an internal control, U6 was used for miR-145 template normalization and GADPH was used for other template normalization. The sequences of the primers used are shown in online table I. Fluorescent signals were normalized to an internal reference, and the threshold cycle (Ct) was set within the exponential phase of the PCR. The relative gene expression was calculated by comparing cycle times for each target PCR. The target PCR Ct values were normalized by subtracting the U6 or GADPH Ct value, which provided the ΔCt value. The relative expression level between treatments was then calculated using the following equation: relative gene expression = $2^{-(\Delta Ctsample-\Delta Ctcontrol)}$.

Northern blot analysis of miRNA. Ten micrograms of total RNA from snap-frozen tissues and VSMCs were loaded onto a precast 15% denaturing polyacrylamide gel (Bio-Rad) (1, 2). The RNA was then electrophoretically transferred to Bright-Star blotting membranes (Ambion, Inc.). The probe sequences of miR-145 and U6 are shown in supplementary table. Probes were end-labeled with [γ-^{32}P]ATP by T4 polynucleotide kinase. Prehybridization and

hybridization were carried out in Ultrahyb Oligo solution (Ambion, Inc.) containing 10^6 cpm/ml probes overnight at 37°C. The most stringent wash was with 2x SSC and 1% SDS at 37°C. For reuse, blots were stripped by boiling and reprobed. U6 was used as a loading control to normalize expression levels (1).

Western blot analysis. Proteins were isolated from cultured VSMCs and carotid arteries and protein levels were determined by western blot analysis. Briefly, equal amounts of protein were subjected to SDS-PAGE. Standard western blot analysis was conducted using SM-MHC (Santa Cruz Biotecnology), calponin (Sigma), SM α–actin (Sigma), and KLF5 antibodies (Abcam). GADPH antibody (1:5000 dilution; Cell Signaling) was used as a loading control.

Construction of the adenovirus. The adenovirus expressing miR-145 (Ad-miR-145), Ad-KLF5, and Ad-GFP were generated using the Adeno-X™ Expression Systems 2 kit (Clontech, CA) according to the manufacturer's protocols as described (3). The resulting adenoviruses were further amplified by infection of HEK293A cells and purified by cesium chloride gradient ultracentrifugation. The Ad-miR-145, Ad-KLF5, and Ad-GFP were titrated using a standard plaque assay.

Luciferase assay. A construct in which a fragment of the 3'-UTR of KLF5 mRNA containing the putative miR-145 binding sequence was cloned into a firefly luciferase reporter construct and transfected into HEK 293 cells with either vehicle (Vehicle control), an empty plasmid (pDNR-CMV) (0.2 μg/ml), a plasmid expressing miR-145 (pmiR-145) (0.2 μg/ml), or a control plasmid expressing an unrelated miRNA, miR-31 (pmiR-31) (0.2 μg/ml) following the transfection procedures provided by Invitrogen. The constructs with mutated fragment of the

3'-UTR of KLF5 mRNA without the putative miR-145 binding sequences were used as the mutated controls. Relative luciferase expression was measured on a scintillation counter by using a dual luciferase reporter system (3).

Immunocytochemistry, immunofluorescence and *in situ* hybridization. *In situ* hybridization of miR-145 was performed in 10-μm vessel sections (3). Tissue sections were cut using a cryostat and transferred to SuperFrost/plus slides (Fisher). Slides were stored at −80° C until ISH. Vessel sections were fixed in 4% paraformaldehyde and acetylated in acetic anhydride/triethanolamine, followed by washes in PBS. Sections were then pre-hybridized in hybridization solution (50% formamide, 5x SSC, 0.5 mg/mL yeast tRNA, 1x Denhardt's solution) at 25°C below the predicted T_m value of the LNA probe for 30 min. Probes (3 pmol) (LNA miRCURY probe; Exiqon) were DIG-labeled (DIG Oligonucleotide 3' Tailing Kit; Roche Applied Sciences) and hybridized to the sections for 1 h at the same temperature as pre-hybridization. After post-hybridization washes in 0.1x SSC at 55°C, the ISH signals were detected using the tyramide signal amplification system (PerkinElmer) according to the manufacturer's instructions. Slides were mounted in Prolong Gold containing DAPI (Invitrogen). To determine co-localization of miR-145 and VSMCs, VSMCs were stained by immunofluorescence of SM α–actin in these sections. In addition, SM α–actin in cultured VSMCs was also determined by immunocytochemistry using anti-SM α–actin antiboday (Sigma). All the fluorescence images wereanalyzed with a Nikon microscope equipped with a CCD camera and image software.

Rat carotid artery balloon injury model and adenovirus mediated gene transfer into the injured vascular wall. Carotid artery balloon injury and adenovirus mediated gene transfer were performed in male Sprague-Dawley rats (250 to 300 g) as described in our previous studies (2, 3). Briefly, rats were anesthetized with ketamine (80 mg/kg)/xylazine (5 mg/kg). Under a dissecting microscope, the right common carotid artery was exposed through a midline cervical incision. A 2F Fogarty catheter (Baxter Edwards) was introduced via an arteriotomy in the external carotid artery, and the catheter was then advanced to the proximal edge of the omohyoid muscle. To produce carotid artery injury, we inflated the balloon with saline and withdrew it three times from just under the proximal edge of the omohyoid muscle to the carotid bifurcation. After balloon injury, solutions of (100 μl) Ad-miR-145 (5×10^9 pfu/ml) or Ad-GFP (5×10^9pfu/ml) were infused into the ligated segment of the common carotid artery for 30 min (3, 4). The external carotid artery was then permanently ligated with a 6-0 silk suture, and blood flow in the common carotid artery was restored. All protocols were approved by the Institutional Animal Care and Use Committee at the UMDNJ and were consistent with the Guide for the Care and Use of Laboratory Animals (NIH publication 85-23, revised 1985).

Morphometric analysis for neointimal lesion formation. Morphometric analysis via computerized image analysis system (Scion Image CMS-800) was performed in sections stained with Masson's trichrome staining as described (1, 3). Six sections (5 μm thick) sectioned at equally spaced intervals of injured carotid arteries were used. The intimal to medial area ratio (I/M) of each section

was calculated. The average I/M of the six sections was used as the I/M of this animal.

Statistics. All data are presented as mean ± standard error. For relative gene expression, the mean value of the vehicle control group is defined as 100%. Two-tailed unpaired Student's *t* tests and ANOVA were used for statistical evaluation of the data. Sigma Stat Statistical Analysis Program was used for data analysis. A p value < 0.05 was considered significant.

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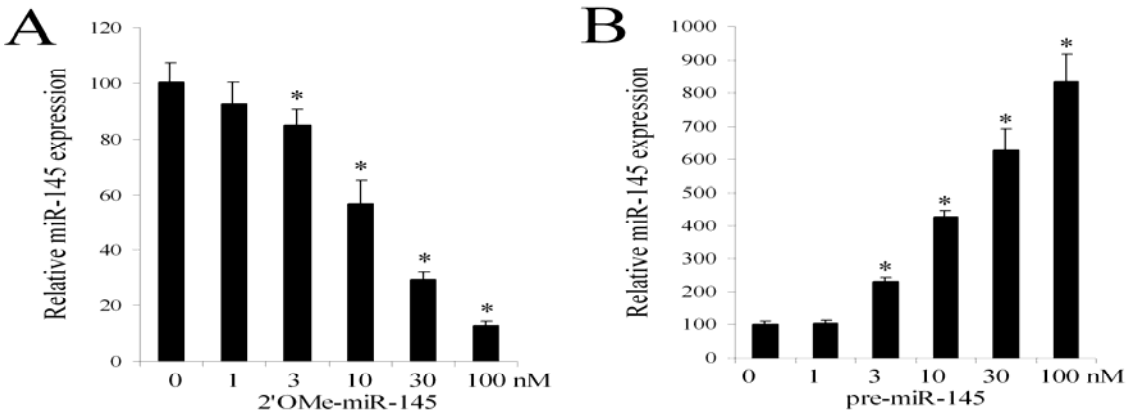
Online Table I : Northern Blot Probe and PCR Primer Sequences

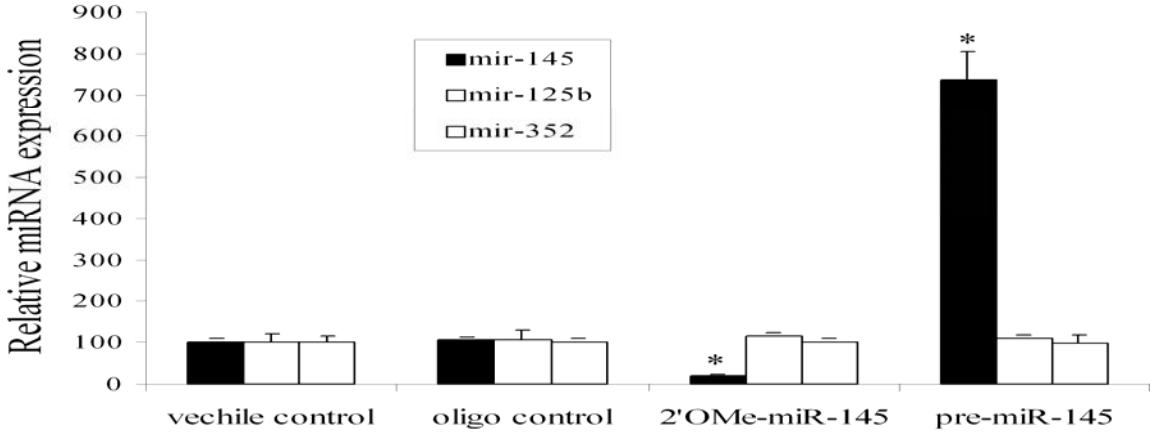
Name	Sequences
mir-145 probe	5' AAGGGATTCCTGGGAAAACTGGAC 3'
U6 probe	5' GCAGGGGCCATGCTAATCTTCTCTGTATCG 3'
Rat calponin forward primer	5' ACCAAGCGGCAGATCTTTGA 3'
Rat calponin reverse primer	5' CATCTGCAAGCTGACGTTGA 3'
Rat SM-MCH forward primer	5' GCCGCTGCCTATGACAAACT 3'
Rat SM-MCH reverse primer	5' CGCTGGTTGTCCAAGTCCA 3'
Rat GAPDH forward primer	5' AAGCTCACTGGCATGGCCTT 3'
Rat GAPDH reverse primer	5' CGGCATGTCAGATCCACAAC 3'
Rat SM α-actin forward primer	5' CTGCCTTGGTGTGTGACAATGG 3'
Rat SM α-actin reverse primer	5' CGGGTACTTCAGGGTCAGGATTC 3'
Rat KLF5 forward primer	5' GCTTGCTGTCATGCTCACTACTAG 3'
Rat KLF5 reverse primer	5' CCCATACTGAGATGCGACTGC 3'
Rat Myocardin forward primer	5' GTGCCTTGTTGGAGTAAGAGTGC 3'
Rat Myocardin reverse primer	5' GTCAGTCTATGTCCCGATAATGCC 3'
Rat KLF5 3' UTR forward primer	5' TTCTCGAGTCCGCTCGCCATCCTTTA 3'
Rat KLF5 3' UTR reverse primer	5' TTGCGGCCGCTGCCAAGCGGGTTTGTTTATCCT 3'

Online Table II: The expression of miR-145, miR-21 and miR-221 in fresh isolated VSMCs from normal rat carotid arteries determined by qRT-PCR.

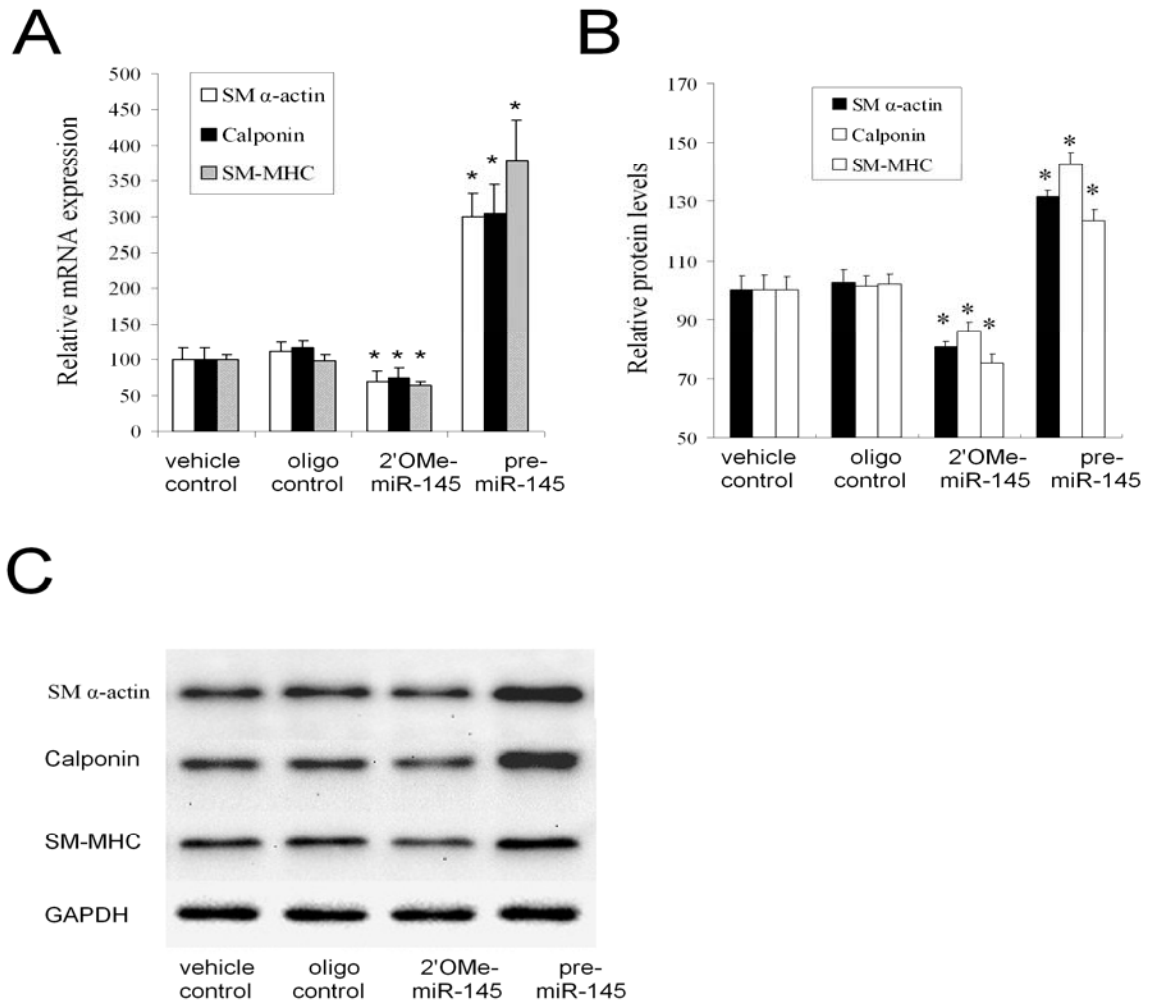
miRNA assay	C_T Mean	C_T SD
miR-145	19.20	0.02
miR-21	23.75	0.04
miR-221	26.50	0.14



Online Figure I. Modulation of miR-145 expression in cultured VSMCs. (A). miR-145 inhibitor 2'OMe-miR-145 decreased miR-145 expression in VSMCs. n=5; *P<0.05 compared with vehicle control (0). (B). Pre-miR-145 increased miR-145 expression in VSMCs. n=5; *P<0.05 compared with vehicle control (0).



Online Figure II. The effects of 2'OMe-miR-145 and pre-miR-145 on the expression of miR-145, miR-125b and miR-352 in cultured VSMCs. *P<0.05 compared with vehicle control.



Online Figure III. Modulating of miR-145 alone is sufficient to elicit phenotypic changes in quiescent, non-stimulated VSMCs. (A). 2'OMe-miR-145 (100 nM) and pre-miR-145 (100 nM) regulated the VSMC phenotype as determined by qRT-PCR. n=6; *P<0.05 compared with oligo control. (B). 2'OMe-miR-145 and pre-miR-145 regulated the VSMC phenotype as determined by western blot. n=6; *P<0.05 compared with oligo control. (C).Representative western blots of VSMC differentiation marker genes.

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mir-145      3′-UUCCCUAAGGACCCUUUUGACCUG-5′
                        ||       |||||||
Rat       UAAAAAAAAAA--AUCAAAAG--AACUGGAAAUGUAUA
Human     AAGCAAGAAAACCACAACUAA--AACUGGAAAUGUAUA
Mouse     AAAAAAAAAAA--AGCAAAGG--AACUGGAAAUGUAUA
Chicken   AAAGAAAACUA--------AA--AACUGGAAAUGUAUA
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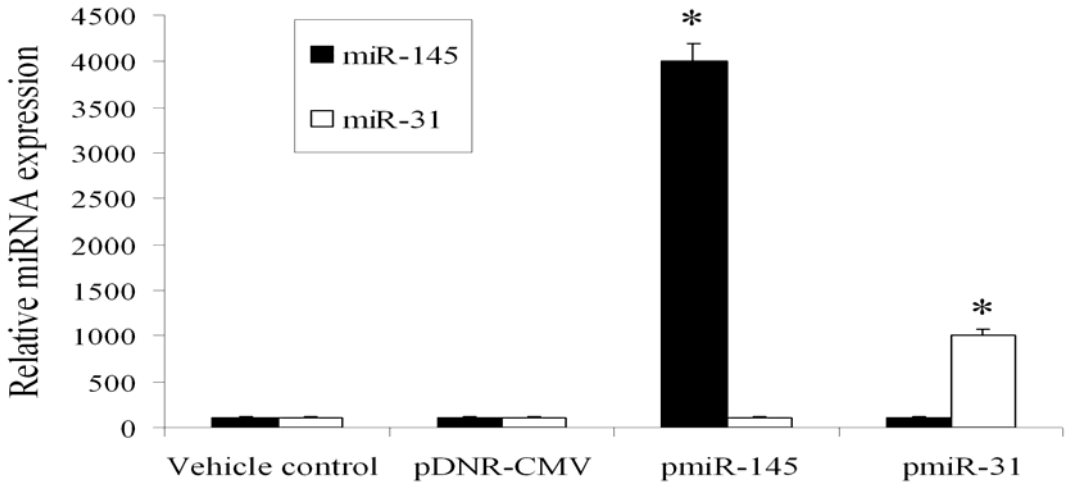
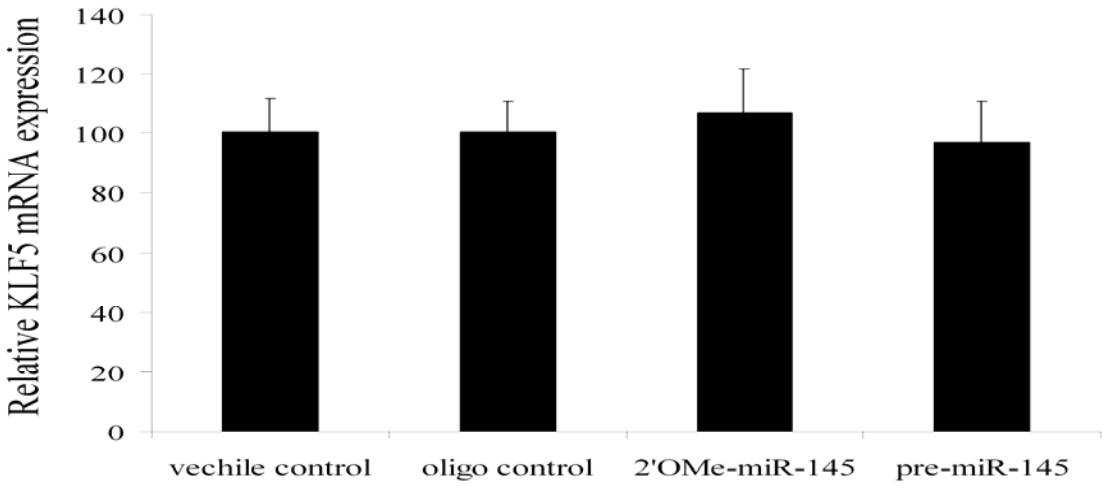
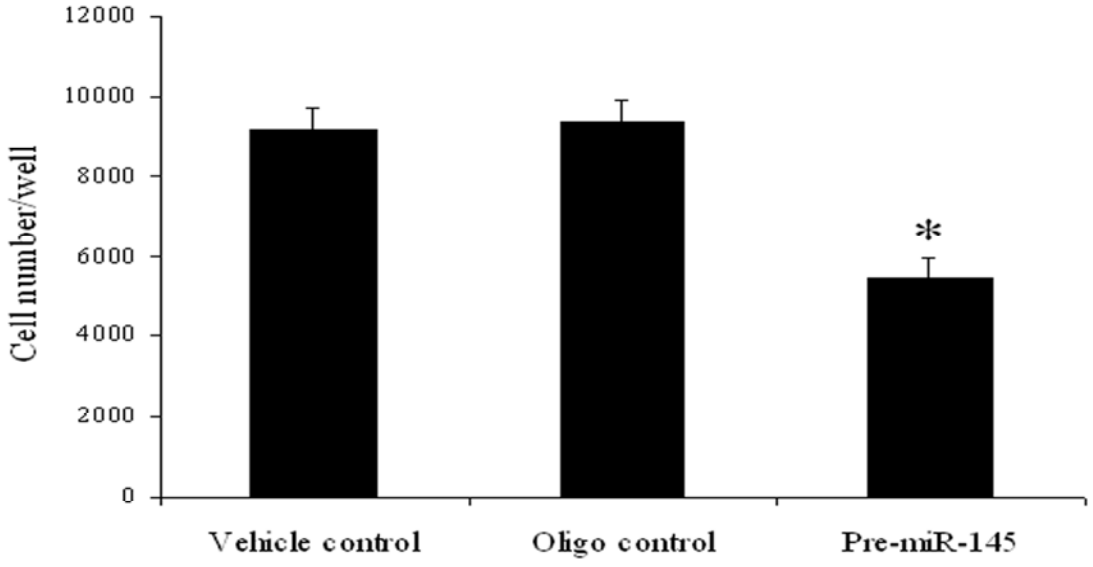
Online Figure IV. Computational analysis of miR-145 binding site in the 3'-untranslated region (3'-UTR) of KLF5.



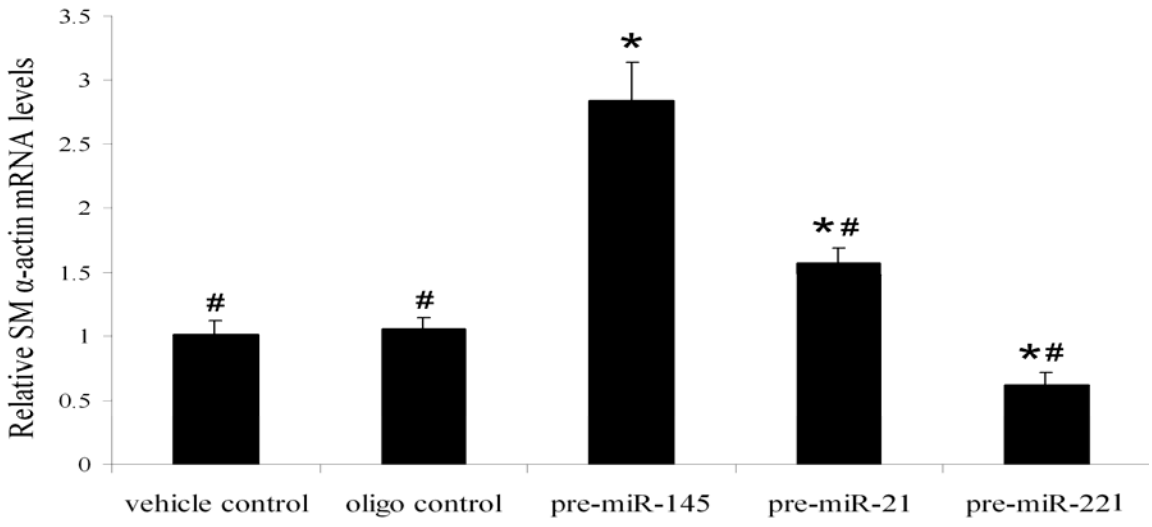
Online Figure V. The effects of transfected plasmid expressing miR-145 (pmiR-145) or miR-31 (pmiR-31) on the expression of miR-145 and miR-31 in HEK 293 cells. Vehicle (blank control) and an empty plasmid (pDNR-CMV) were used as controls. *P<0.05 compared with vehicle control.



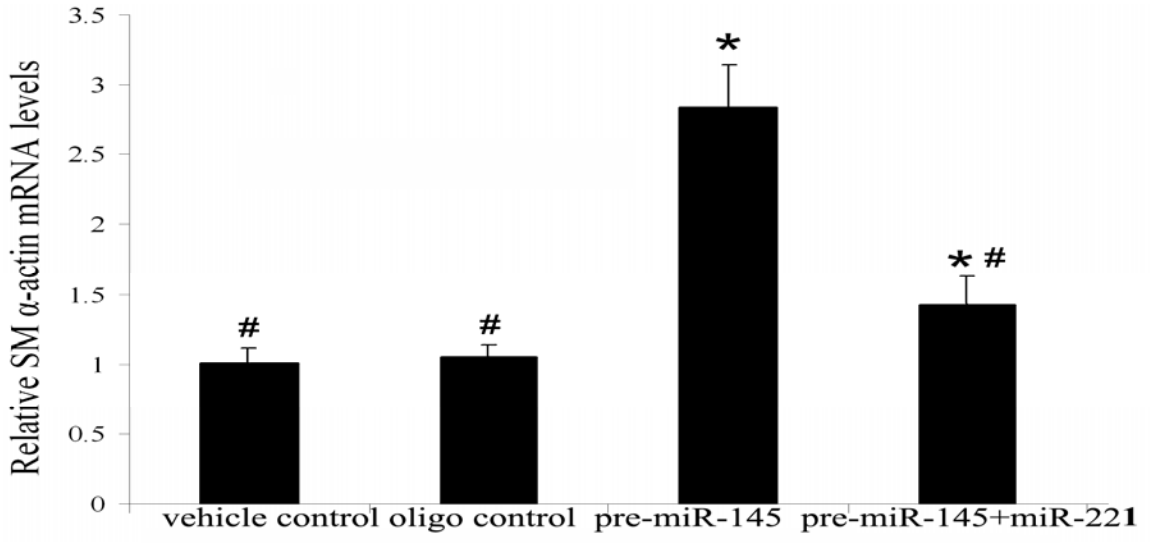
Online Figure VI. The effects of 2'OMe-miR-145 (100 nM) and pre-miR-145 (100 nM) on the mRNA expression of KLF5 in cultured VSMCs at 24 h after treatment.



Online Figure VII. The effects of pre-miR-145 (100 nM) on cell proliferation in cultured VSMCs at 24 h after treatment with PDGF (20 ng/ml). *P<0.05 compared with Oligo control



Online Figure VIII. The effects of pre-miR-145 (100 nM), pre-miR-21(100 nM), and pre-miR-221(100 nM) on the expression of VSMC differentiation marker gene, SM α-actin. *P<0.05 compared with vehicle control; # P<0.05 compared with pre-miR145.



Online Figure IX. The effect of pre-miR-221 (100 nM) on pre-miR-145 (100 nM)-mediated upregulation of SM α-actin. *P<0.05 compared with vehicle control; # P<0.05 compared with pre-miR145.