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MicroRNA Mediation of Endothelial Inflammatory Response to Smooth Muscle Cells and its Inhibition by Atheroprotective Shear Stress

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Abstract

Rationale—In atherosclerotic lesions, synthetic smooth muscle cells (sSMCs) induce aberrant microRNA (miR) profiles in endothelial cells (ECs) under flow stagnation. Increase in shear stress induces favorable miR modulation to mitigate sSMC-induced inflammation.

Objective—To address the role of miRs in sSMC-induced EC inflammation and its inhibition by shear stress.

Methods and Results—Co-culturing ECs with sSMCs under static condition causes initial increases of four anti-inflammatory miRs (146a/708/451/98) in ECs followed by decreases below basal levels at 7 days; the increases for miR-146a/708 peaked at 24 h and those for miR-451/98 lasted for only 6-12 h. Shear stress (12 dynes/cm^2) to co-cultured ECs for 24 h augments these four miR expressions. In vivo, these four miRs are highly expressed in neointimal ECs in injured arteries under physiological levels of flow, but not expressed under flow stagnation. MiR-146a, -708, -451, and -98 target interleukin (IL)-1 receptor-associated kinase, inhibitor of nuclear factor-κB (NF-κB) kinase subunit-γ, IL-6 receptor, and conserved helix-loop-helix ubiquitous kinase, respectively, to inhibit NF-κB signaling, which exerts negative feedback control on the biogenesis of these miRs. NF-E2-related factor-2 (Nrf-2) is critical for shear-induction of miR-146a in co-

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cultured ECs. Silencing either Nrf-2 or miR-146a led to increased neointima formation of injured rat carotid artery under physiological levels of flow. Overexpressing miR-146a inhibits neointima formation of rat or mouse carotid artery induced by injury or flow cessation.

Conclusions—Nrf-2-mediated miR-146a expression is augmented by atheroprotective shear stress in ECs adjacent to sSMCs to inhibit neointima formation of injured arteries.

Keywords

Atherosclerosis; endothelial cell; microRNA; shear stress; smooth muscle cell

Introduction

Atherosclerosis and its complications constitute a major cause of mortality in the Western world. Dysfunction of vascular endothelial cells (ECs) adjacent to synthetic smooth muscle cells (sSMCs) is an initial step leading to atherosclerosis, which predominantly occurs at arterial branches and curvatures, where the local flow is disturbed with low shear stress[1]. sSMCs in the neointima of atherosclerotic lesions induce pro-inflammatory signaling and gene expression in ECs[1]. We recently demonstrated that sSMCs induce adhesion molecule E-selectin expression in ECs by producing pro-inflammatory cytokines interleukin (IL)-1β and IL-6, which activate IL-1 receptor-associated kinase (IRAK) and glycoprotein-130, respectively, and hence nuclear factor-κB (NF-κB)[2]. Application of atheroprotective shear stress at 12 dynes/cm^2 to ECs co-cultured with sSMCs inhibited these EC pro-inflammatory responses[2,3]. Although sSMCs and shear stress have been shown to modulate cellular signals that affect EC gene expression at the transcription level, it is not known whether post-transcriptional regulators, e.g. small noncoding RNAs, are involved in shear-regulation of gene expression and function of ECs in response to sSMCs.

MicroRNAs (miRs) are small noncoding single-stranded RNAs that are critical for gene regulation at post-transcriptional level[4]. MiRs bind to their target sites in 3′-untranslated regions (3′-UTRs) of mRNA to cause its degradation or translational repression[4]. Several miRs have been identified to play important roles in modulating EC functions and atherosclerotic lesion development[5,6]. Fang et al.[5] demonstrated in swine the anti-inflammatory role of miR-10a in ECs. MiR-92a and -712 were shown to be highly expressed in ECs in atheroprone areas to decrease the expressions of atheroprotective transcription factor Krüppel-like factor-2 (KLF-2) and tissue inhibitor of metalloproteinase-3, respectively, thus inducing atherogenesis[6,7]. Shear stress has been shown to modulate miR expression in ECs and their functions[8-11]. Laminar shear stress at 12 dynes/cm^2 induces EC expressions of miR-19a and -23b, leading to EC cycle arrest[8,9]. Pulsatile shear stress at 12±4 dynes/cm^2 decreases EC miR-92a expression and increases KLF-2 expression to maintain EC homeostasis[10]. KLF-2 modulates shear-induction of EC miR-143/145, which can modulate SMC phenotype from synthetic toward contractile[11]. We recently found that shear stress applied to ECs inhibits the transmission of miR-126 from ECs to co-cultured sSMCs to suppress sSMC proliferation[12]. However, it is not known whether miRs are involved in sSMC-modulation of EC gene expression and function and what are the effects of shear stress on this modulation.

In this study, we found that atheroprotective shear stress augments miR-146a, -708, -451, and -98 expressions in ECs co-cultured with sSMCs. These four miRs have anti-inflammatory actions by directly targeting IRAK, inhibitor of NF-κB kinase subunit-γ (IKK-γ), IL-6 receptor (IL-6R), and conserved helix-loop-helix ubiquitous kinase (CHUK) genes, respectively, to inhibit NF-κB signaling, which exerts negative feedback control on the biogenesis of these miRs. In vivo, these four miRs are highly expressed in ECs of neointmal lesions in injured rat carotid arteries under physiological levels of flow, but not expressed under flow stagnation. We also found that NF-E2-related factor-2 (Nrf-2) is a critical transcription factor for shear-induced miR-146a transcription in co-cultured ECs. Administration of lentivirus carrying mature miR-146a (Lenti-miR-146a) inhibits neointima formation induced by injury or flow cessation in rats or mice. Our findings suggest that miR-146a, -708, -451, and -98 may serve as valuable molecular targets for intervention against vascular disorders resulting from atherosclerosis and restenosis.

Methods

Cell culture and co-culture flow system

Human aortic ECs and SMCs were obtained commercially (Clonetics, Palo Alto, CA). Details of cell culture procedures and EC/SMC co-culture flow system are provided in Supplemental Material.

Argonauts 2 (Ago2) complex immunoprecipitation

ECs were collected by scraping and lysed. An anti-Ago2 antibody (1 μg/100 μL; Cell Signaling) was added and the immune complexes were isolated with protein A/G Sepharose beads.

Chromatin immunoprecipitation (ChIP) assay

ChIP assay was performed according to the manufacturer's protocol (EZ-ChIP™; Merck Millipore). All primers are listed in Online Tables I and II.

Animals

Carotid artery balloon injury was performed in male Sprague-Dawley rats (350-500 g), and carotid artery ligation was performed in female FVB/NJ mice (12 weeks of age). Animals were euthanized with 100% carbon dioxide. All animals and experiments were maintained and performed according to the guidelines of the Animal Research Committee of National Health Research Institutes.

An expanded Methods section is provided in Supplemental Material.

Results

Atheroprotective shear stress augments miR-146a, -708, -451, and -98 expressions in co-cultured ECs

Three independent groups of experiments were performed with miR arrays, i.e., mono-cultured ECs (EC/NC), ECs co-cultured with sSMCs under static condition for 24 h (EC/

SMC), and EC/SMC co-culture with the subsequent exposure of ECs to shear stress at 12 dynes/cm^2 for 6 h (SS EC/SMC). MiR expressions with a mean co-culture/mono-culture ratio ≥2.0 (and P≤0.05) were considered positively regulated by the co-culture, whereas those with a mean ratio ≤0.5 (and P≤0.05) were considered negatively regulated. Of the 380 miRs on the array, the expressions of miR-146a and -708 in ECs were significantly induced by static 24 h co-culture with sSMCs, whereas those of miR-98, -30b, -30c, -885, -149, -654-3p and -451 were significantly decreased (Online Table III). Analysis of the time course of these nine miR expressions by qPCR showed that static co-culture with sSMCs causes initial increases of miR-146a, -708, -451 and -98 expressions in ECs followed by later declines to below basal levels (Figure 1A). MiR-146a and -708 peaked at 24 h, and then declined to below basal levels at 7 days; miR-451 and -98 declined to below basal levels at 24 h. MiR-30b, -30c, -885, -149, and -654-3p of static co-cultured ECs did not show an initial increase at 1 h and all decreased to below basal level at 24 h (Figure 1B).

Application of shear stress to co-cultured ECs for 6 h or 24 h induced miR-146a, -708, -451, and -98 expressions in these ECs (Online Table III, Figure 1C). These shear-inductions of EC miRs were declined by 24 h of flow cessation, as compared with the cells exposed to continuous flow (Figure 1D). Shear stress had no effects on these four miR expressions in mono-cultured ECs (Figure 1E).

MiR-146a, -708, -451, and -98 directly target IRAK, IKK-γ, IL-6R, and CHUK genes, respectively

Our previous study showed that sSMCs release IL-1β and IL-6 to activate IRAK and IL-6R and hence NF-κB in adjacent ECs[2]. We investigated whether miR-146a, -708, -451, and -98 can target IRAK, IL-6R, and NF-κB relevant genes. Analysis with bioinformatics algorithms PicTar, microRNA.org, and TargetScan 4.2 identified that the 3′-UTRs of IRAK, IKK-γ, IL-6R, and CHUK genes contain the putative binding sites (seed sequences) of miR-146a, -708, -451, and -98, respectively. We generated reporter constructs containing the firefly luciferase gene fused to wild-type and mutant of putative target sites of these genes (Online Figure I). Co-transfection with wild-type constructs and precursors of respective miRs (pre-miRs) into HEK293 cells reduced luciferase activities in these cells (Figure 2A); such reductions of luciferase activities were not observed by co-transfection with the mutant constructs. Transfecting ECs with pre-miR-146a, -708, -451, and -98 reduced protein (Figure 2B) and mRNA (Figure 2C) levels of IRAK, IKK-γ, IL-6R, and CHUK, respectively, in comparison to control groups. Analysis of miR-induced silencing complexes (miRISCs) immunoprecipitated with anti-Ago2 antibody showed increased mRNA levels of these genes in pre-miR-transfected ECs (Figure 2C), indicating that these EC genes are regulated by their respective miRs at the post-transcriptional level through binding to Ago2 in miRISCs. Co-culturing ECs with sSMCs induced EC expressions of IRAK, IKK-γ, IL-6R, and CHUK mRNAs in miRISCs (Figure 2D). Such sSMC-inductions of EC genes in miRISCs were inhibited by transfecting ECs with the respective antagomirs (anti-miRs).

MiR-146a, -708, -451, and -98 modulate NF-κB signaling, which exerts negative feedback control on the biogenesis of these miRs in co-cultured ECs

Static co-culturing ECs with sSMCs induced p65 and IκBα phosphorylations in ECs over the 24-h period tested (Figure 3A). Such sSMC-activations of EC p65 and IκBα were reduced after 1 h of flow exposure. Overexpressing optimal concentrations of pre-miR-146a, -451, -98 (20 nmol/L each), and -708 (1 nmol/L) in ECs also reduced sSMC-activations of EC IκBα (Figure 3B) and p65 (Figure 3C). These effects of EC pre-miR overexpression were negated by the respective anti-miRs (Figure 3C, Online Figure II). Co-culturing ECs with sSMCs induced EC E-selectin expression (Figure 3D) and monocyte adhesion (Figure 3E). These sSMC-induced responses were inhibited by overexpressing ECs with the four pre-miRs, but anti-miRs had no effect.

sSMC-inductions of EC miR-146a, -708, -451, and -98 were inhibited by transfecting ECs with p65-specific siRNA (Figure 4A) and by pre-treatment with the specific phospho-IκB inhibitor BAY117082 (Figure 4B). Co-culturing ECs with sSMCs induced time-dependent induction of EC primary-miR-146a (Figure 4C), whose promoter region contains three NF-κB binding sites[13]. Co-culture of sSMCs with ECs transfected with the constructs containing promoter region of miR-146a (547 bp) and reporter gene luciferase induced luciferase activities (Figure 4D). These responses were not seen when ECs were transfected with the constructs containing mutated NF-κB binding sites (Figure 4D). ChIP assays using anti-p65 antibody and NF-κB-specific primers for miR-146a promoter showed that sSMC-co-culture induced NF-κB binding to miR-146a promoter region (Figure 4E). Taken together, our results indicate that increased activation of NF-κB in ECs co-cultured with sSMCs induces EC biogenesis of miR-146a, -708, -451, and -98, which in turn target NF-κB signaling to cause declined expression of these miRs in co-cultured ECs.

Nrf-2 is critical for shear-induction of miR-146a in co-cultured ECs

The promoter region of miR-146a contains four putative binding sites for Nrf-2 (sequence: TGActcAGCa) (Online Figure III), which was shown to be activated by shear stress[14]. We investigated whether Nrf-2 is involved in shear-induction of miR-146a in co-cultured ECs. Nuclear fractionation assay showed that shear stress applied to co-cultured ECs induces Nrf-2 translocation into EC nuclei (Figure 5A). Electrophoretic mobility shift assay (EMSA; Figure 5B) and ChIP assay (Figure 5C) demonstrated that shear stress increases Nrf-2 binding activity in cell-free system and Nrf-2 binding to miR-146a promoter region in EC nuclei. As controls, co-culturing ECs with sSMCs under static condition did not induce these responses. We further generated a mutant construct containing the promoter region of miR-146a, with a mutation in Nrf-2 binding site (at -193 bp). Application of shear stress to co-cultured ECs transfected with a wild-type construct induced a 2.2-fold increase in luciferase activity in comparison to static control cells (Figure 5D). This shear-induced response was absent in ECs transfected with mutant constructs. Transfecting co-cultured ECs with Nrf-2-specific siRNA (compared with control siRNA, 20 nmol/L each) inhibited shear-inductions of miR-146a and -708, but not miR-451 and -98 (Figure 5E) in these ECs. Treatment of co-cultured ECs with a specific Nrf-2 activator sulforaphane (10 μmol/L) under static condition for 24 h can mimic shear stress effects to induce Nrf-2 binding to the promoter region of miR-146a (Figure 5F) and its promoter activity (Figure 5G) and hence

expression (Figure 5H) in these ECs. However, sulforaphane had no effect on EC miR-451, -98, and -708 expressions (Figure 5H). These results indicate that Nrf-2 is involved in shear-induction of miR-146a, but not miR-451, -98, and -708, in co-cultured ECs.

Integrins regulate shear-inductions of miR-146a, -708, -451, and -98 in co-cultured ECs

ECs were pre-treated with poly-L-lysine (100 μg/mL) and RGDS (Arg-Gly-Asp-Ser) (500 μg/mL) for 2 h to block the integrin-ECM interactions, and then co-cultured with sSMCs under static conditions or in response to flow for 24 h. In addition, ECs were transfected with β_1- and β_3-integrin-specific siRNAs (20 nmol/L each), which reduced the expression of the respective integrins by ≈70% (compared with control siRNA). The results showed that β_1 and β_3 integrins are involved in shear-inductions of these four miRs in co-cultured ECs. Detailed results are provided in Supplemental Material and Online Figure IV.

MiR-146a, -708, -451, and -98 are highly expressed in neointimal ECs in injured arteries under physiological levels of flow, but not expressed under flow stagnation

To investigate whether the SMC- and shear-modulations of miR-146a, -708, -451, and -98 expressions found in cultured ECs in vitro also occur in vivo, we used a rat carotid artery balloon injury model to create close interactions between ECs and sSMCs. In addition to studies under physiological levels of flow, in some experiments the affected arteries were subjected to a partial ligation (≈60% constriction of the artery diameter) at the proximal edge (Figure 6A). Ultrasonography demonstrated that this injured carotid artery model resulted in flow stagnation, with virtually no detectable level of flow velocity (Figure 6B). H&E staining showed that the intima-to-media thickness ratio of the constricted injured arteries is higher than that of unconstricted injured arteries (Figure 6C). *In situ* hybridization of the four miRs and immunohistochemical staining on Nrf-2 in serial sections of affected arteries (Figures 6D and 6E, Online Figure V) showed that these four miRs and Nrf-2 are not expressed in neointimal ECs in constricted injured arteries under flow stagnation. However, they are highly expressed in ECs in unconstricted injured arteries under physiological levels of flow. There was no detectable level of these four miRs in control vessels. These results indicate that miR-146a, -708, -451, -98 are highly expressed in ECs of neointimal lesions with sSMCs in the presence of physiological levels of flow in vivo, which may play inhibitory roles in preventing neointima formation in injured arteries. These findings validated our in vitro findings that increases in shear stress induce these four miR expressions in ECs co-cultured with sSMCs.

Nrf-2 is involved in shear-induction of EC miR-146a in vivo

To investigate whether Nrf-2 is involved in shear-induction of EC miR-146a in vivo, lentiviral-mediated RNA interference was performed two weeks after balloon injury on carotid arteries in rats (n=5), where the denuded EC layer had recovered from injury, and the animals were sacrificed after additional two weeks. The lentiviral-mediated Nrf-2 silencing abolishes miR-146a expression in ECs of unconstricted injured arteries under physiological levels of flow (Figure 7A), and this is accompanied by a concomitant increase in neointima formation, in comparison to control shRNA-infected animals (n=5, Figures 7A and 7B). Administration of anti-miR-146a in unconstricted injured arteries to knockdown EC

miR-146a expression (Figure 7C) also increased neointima formation (Figures 7C and 7D). In contrast, overexpressing miR-146a by using the shMIMIC™ strategy (see details in the next section) resulted in increased expression of miR-146a in ECs (Figure 7E) and decreased formation of neointimal lesions in constricted injured arteries under flow stagnation (Figures 7E and 7F). These results indicate that Nrf-2-induction of EC miR-146a under physiological levels of flow plays inhibitory roles in neointima formation in injured arteries.

Administration of Lenti-miR-146a reduced neointima formation in a mouse carotid artery ligation model

To confirm the therapeutic effect of miR-146a on neointima formation in vivo, we performed the shMIMIC™ lentivirus-mediated miR overexpression strategy by using a mouse carotid artery ligation model, in which neointimal lesions were created with the maintenance of endothelial integrity. Lenti-miR-146a containing mature miR-146a sequences and cDNA encoding GFP was constructed and infected into ECs under static conditions for 24 and 48 h (5×10^6 TU/mL each). ECs showed ≈70% infection efficiency, as determined from the percentage of GFP-positive cells relative to total cells (Figure 8A). MiR-146a expression in Lenti-miR-146a-infected cells was increased by 60-80 folds in comparison to Lenti-null-infected cells (Figure 8B). This miR-146a overexpression resulted in ≈60% reductions in endogenous IRAK mRNA (Figure 8C) and protein (Figure 8D) levels.

To test in vivo overexpression efficiency of Lenti-miR-146a, the Lenti-miR-146a construct (5×10^7 TU/mL in 100 μL) was administrated through the tail vein of mice (n=5) weekly after carotid artery ligation. Control animals received Lenti-null or saline injection (n=5 each). *En face* staining of luminal surface of the carotid arteries with an anti-GFP antibody two weeks after lentivirus injection showed high levels of in vivo infection efficiency of Lenti-miR-146a and Lenti-null in intact EC layers (Online Figure VIA). *In situ* hybridization of arterial cross-sections (Figure 8E) and *en face* staining (Online Figures VIB and VIC) demonstrated that miR-146a expression in intact EC layer was increased by Lenti-miR-146a injection, with concomitant decreases in IRAK expression, as compared with Lenti-null and saline groups. Ligation of left common carotid arteries in Lenti-null-infected mice (n=5) for 4 weeks increased neointimal lesion formation of the arteries (Figures 8F and 8G). This increased neointima formation in ligated arteries was inhibited by lentivirus-mediated miR-146a overexpression, confirming the inhibitory role of miR-146a in arterial neointima formation in vivo.

Discussion

This study has identified miR-146a, -708, -451, and -98 as novel anti-inflammatory miRs in ECs co-cultured with sSMCs; the expression of these miRs is sustainably induced by atheroprotective shear stress. MiR-146a, -708, -451, and -98 directly target IRAK, IKK-γ, IL-6R, and CHUK genes, respectively, to inhibit NF-κB signaling, which exerts negative feedback control on the biogenesis of these four miRs (summarized in Figure 8H). Shear-induction of these miRs in co-cultured ECs is mediated by β_1 and β_3 integrins. In addition, miR-146a is regulated by Nrf-2 at the transcriptional level in response to flow. Previous

studies using EC mono-cultures have identified EC miR-712[7], -19a[8], -23b[9], -92a[10], -143/145[11], -21[15] as shear-responsive miRs that play functional roles. Our study using EC/SMC co-culture system has shown the novel results that EC miR-146a, -708, -451, and -98 can be augmented by atheroprotective shear stress in close adjacency to sSMCs to serve as negative regulators for sSMC-induced EC inflammation. These miRs may serve as therapeutic molecules against vascular disorders resulting from atherosclerosis and restenosis.

The induction of anti-inflammatory miR-146a, -708, -451, and -98 in ECs by static co-culture with sSMCs may be considered as a compensatory response for ECs against sSMC-induced inflammation. However, this sSMC-induction of these four miRs is declined when the duration of co-culture is lengthened, suggesting a limitation of the compensatory response of static ECs in the face of sustained action from sSMCs. Application of shear stress to co-cultured ECs for 24 h induces these four miR expressions in comparison to static co-cultured ECs. This shear-induction of these four miRs was declined by flow cessation in comparison to continuous flow. In vivo, these four miRs are not expressed in ECs of neointimal lesions in injured arteries under flow stagnation, but become highly expressed under physiological levels of flow. Thus, the compensatory responses for these four miRs are limited in capacity (duration) and they may fail over time, and need the rescue by laminar shear stress.

Our previous study showed that sSMCs release IL-1β and IL-6 to induce E-selectin expression in adjacent ECs[2], and that shear stress applied to ECs inhibits these sSMC-induced pro-inflammatory responses[2,3]. The present study advanced the new notion that miR-146a, -708, -451, and -98 are involved in regulating sSMC-induced EC inflammation under flow. MiR-146a and -98 were shown to be negative regulators against pro-inflammatory responses in ECs[16] and other cell types[13,17]. However, whether miR-708 and -451 can modulate cell inflammatory function has not been reported. Our study provides the first evidence to show that miR-708 and -451 exert anti-inflammatory effects on ECs by directly silencing IKK-γ and IL-6R genes, respectively, thus inhibiting EC NF-κB activation, E-selectin expression, and monocyte adhesion. The notion that miR-451 plays anti-inflammatory roles in ECs was also substantiated by our recent findings that vascular cell adhesion molecule-1 is a direct target gene that can be silenced by miR-451 in ECs (Online Figure VII).

In this study, overexpressing miR-146a, -708, -451, and -98 in co-cultured ECs inhibited sSMC-activation of EC p65 (Figure 3C). However, transfecting with anti-miRs had no effect on p65 signaling in co-cultured ECs in comparison to scramble controls. Such nonsymmetrical effects of pre-miRs and anti-miRs on cell signaling have also been reported. For example, Huang et al.[18] showed that overexpressing miR-146a by its pre-miR decreases oscillatory pressure-activation of NF-κB in human epithelial cells; however, inhibition of miR-146a by its anti-miR did not enhance NF-κB activation. Inhibition of miR-222 in cultured SMCs increased their p57 expression, but overexpressing this miR had no effect on p57 mRNA level[19]. These nonsymmetrical effects of pre- and anti-miRs might reflect the complication of regulatory pathways triggered by different physiological and pathophysiological microenvironments, which may not lead to opposite effects.

The putative promoter regions of miR-146a, -708, -451 and -98 contain one or more NF-κB binding sites. Our results on NF-κB-regulation of sSMC-induction of these miRs in co-cultured ECs suggest that the putative NF-κB binding elements in the promoter regions of these miRs are functional. NF-κB was shown to regulate the expressions of miR-9, -155, and -146a, which in turn target the components of NF-κB pathway to inhibit its activation by a negative feedback loop[13,20]. Our study has discovered new miRs that serve as potential negative regulators of inflammation by inhibiting the NF-κB pathway through negative feedback control.

Among the four miRs we studied, only miR-146a has been identified with its promoter sequences, which contain several Nrf-2 binding domains[13]. An important new finding of this study is to identify Nrf-2 as a critical transcription factor for regulating miR-146a transcription in co-cultured ECs in response to flow. Recent studies indicated that KLF-2 and Nrf-2 govern ≈70% of shear-elicited gene sets in ECs[21]. The involvement of KLF-2 in shear-modulation of miR-143/145 transcription in ECs has been reported[11]. However, whether Nrf-2 can modulate miR expression in ECs in response to shear has not been explored. Our study represents the first demonstration that Nrf-2 plays important roles in modulating shear-induction of miR transcription in co-cultured ECs. Thus, our findings have closed a loop to indicate that both KLF-2 and Nrf-2 can modulate not only the shear-elicited genes, but also the shear-elicited miRs in ECs.

ECs and SMCs may affect each other through secreted miRs. Hergenreider et al.[11] showed that shear stress regulates EC expression of miR-143/145, which can be transferred by phospholipid vesicles to SMCs to modulate their phenotype. Our recent study showed that miR-126 can be secreted by ECs to affect sSMC proliferation, and that this EC secretion of miR-126 is inhibited by shear stress[12]. In this study, we found that pre-treating EC/SMC co-culture with GW4869, which is a neutral sphingomyelinase inhibitor that suppresses the secretion of exosomal miRs, had no effect on sSMC- and shear-modulations of EC miR-146a, -708, -451, and -98 (data not show). Thus, sSMC- and shear-modulations of these EC miRs are not attributable to the secretion of exosomal miRs from sSMCs.

The nature of blood flow plays crucial roles in the pathogenesis of vascular disorders[1]. Our rat carotid artery constriction experiments demonstrated the importance of physiological levels of blood flow in the inductions of miR-146a, -708, -451 and -98 in ECs of neointimal lesions in injured carotid arteries. These four miRs did not show significant expression in ECs of control vessels, but they were highly expressed in injured carotid arteries, in which SMCs change their phenotype toward a synthetic state and migrate into the subendothelial layers to have close interactions with ECs. These high levels of miRs in ECs situated in close adjacency to sSMCs were reduced by partial ligation of the arteries, which created flow stagnation with more severe neointimal lesion formation than in the unligated arteries exposed to higher levels of flow. Thus, physiological levels of flow and shear stress induce miR-146a, -708, -451 and -98 expressions in ECs adjacent to sSMCs to exert anti-inflammatory effects to inhibit neointima formation of injured arteries.

Among the four miRs we studied, miR-146a has several unique features. First, miR-146a showed the largest increase in its expression in co-cultured ECs in response to shear stress in

comparison to other miRs on the arrays. Second, it exhibited remarkable induction in ECs of neointimal lesions in injured arteries. Third, its expression was more stable in co-cultured ECs (Online Figure VIII). Thus, we performed miR-146a therapeutic experiments by using two animal models. In the rat carotid artery constriction model, Lenti-miR-146a was locally injected into the lumen of constricted carotid artery after balloon injury for 2 weeks, where the denudated EC layer had been recovered. In the mouse carotid artery ligation model, neointimal lesions were created in carotid artery by flow cessation, with the maintenance of an integral EC layer. Examinations on cross-sections of Lenti-miR-146a-affected arteries revealed that miR-146a induction in these arteries is more specific in the intact EC layer than the SMC regions (Figures 7E and 8E). This lentivirus-mediated overexpression of mature miR-146a in intact EC layer inhibited neointimal lesion formation of these arteries, indicating that miR-146a is a potential hemodynamic-based therapeutic target for atherosclerosis and restenosis.

Our recent data indicate that changes in EC miR-146a expression may influence phenotype of sSMCs, which form neointima. Shear stress stimulation of co-cultured ECs did not affect miR-146a expression in sSMCs in comparison to static control cells (Online Figure IXA). However, in concert with our previous findings[14], shear stress stimulation of co-cultured ECs, which induced EC miR-146a expression, increased contractile marker smooth muscle α-actin (SMα-actin) expression and decreased retinoblastoma protein (Rb) phosphorylation in sSMCs (Online Figure IXB). Transfecting co-cultured ECs with pre-miR-146a under static condition can mimic shear stress effects to increase SMα-actin expression and decrease Rb phosphorylation in sSMCs. In contrast, transfecting co-cultured ECs with anti-miR-146a abolished the shear stress effects on sSMCs. These results indicate that laminar shear stress applied to ECs may modulate sSMC phenotype toward a contractile state through EC miR-146a induction. This EC miR-146a-modulation of sSMC phenotype in response to flow may contribute to the therapeutic effect of miR-146a on arterial neointima formation under flow.

In addition to ECs, miR-146a may play protective roles against vascular inflammation through other cell types, such as white blood cells (WBCs). MiR-146a was reported to inhibit lipopolysaccharides-induction of interferon-γ in lymphocytes[22]. Genetic knockout of miR-146a has been shown to induce activation and maturation of monocytes and macrophages, resulting in several immune defects, such as myeloid malignancies[23]. These results indicate that miR-146a may play important roles in regulating innate immune and inflammatory responses through WBCs. While the present study has clearly identified the action of miR-146 on ECs in its protective effect on neointima formation, the possible involvement of other types of cells in this protective effect warrants further investigations.

Several types of surgical interventions, such as percutaneous balloon angioplasty and stent placement, have been developed with the aim of restoring blood flow in vascular occlusive diseases[1]. These interventions, however, may themselves induce stagnant flow condition, thus leading to neointimal hyperplasia and atherosclerotic lesions[1]. Increase of blood flow is a critical strategy for preventing neointimal hyperplasia after percutaneous coronary intervention (PCI)[1,24]. Our findings that shear-induction of EC miR-146a not only can exert anti-inflammatory effects on ECs adjacent to sSMCs, but also can modulate sSMC

phenotype toward a contractile state, suggest the clinical perspective of miR-146a-related strategies and drugs for neointimal hyperplasia prevention after surgical interventions, such as PCI.

In summary, this study used a combination of in vitro EC/SMC co-culture flow system and in vivo experimental animal studies to demonstrate the anti-inflammatory effects of miR-146a, -708, -451, and -98 on ECs adjacent to sSMCs. These miRs become highly expressed in these ECs in response to atheroprotective shear stress. Nrf-2 regulates shear-induction of miR-146a in these ECs at the transcriptional level. In addition, miR-146a plays a protective role against neointimal lesion formation in arteries. Our findings suggest miR-146a to be a valuable therapeutic miR for intervention against vascular occlusive diseases resulting from atherosclerosis and restenosis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Nonstandard Abbreviations and Acronyms

Anti-miR anti-microRNA

CHUK	conserved helix-loop-helix ubiquitous kinase
EC	endothelial cell
ECM	extracellular matrix
EMSA	Electrophoretic mobility shift assay
FN	fibronectin
GFP	green fluorescence protein
IKK-γ	inhibitor of NF-κB kinase subunit-γ
IL	interleukin
IL-6R	interleukin-6 receptor
IRAK	interleukin-1 receptor-associated kinase
KLF-2	Krüppel-like factor-2
miR	microRNA
miRISC	microRNA-induced silencing complex
NF-κB	nuclear factor-κB
Nrf-2	NF-E2-related factor-2
Pre-miR	precursor microRNA
SMC	smooth muscle cell
Wt	wild-type

Novelty and Significance

What Is Known?

- In atherosclerotic lesions, synthetic smooth muscle cells (sSMCs) induce pro-inflammatory signaling and gene expression in endothelial cells (ECs) under conditions of reduced blood flow.
- Increases in blood flow and shear stress result in favorable signaling and gene modulations to mitigate the sSMC-induced EC inflammation.
- MicroRNAs (miRs) are noncoding small RNAs that play important roles in regulating EC function.

What New Information Does This Article Contribute?

- The levels of EC miR-146a, -708, -451, and -98 are elevated by atheroprotective shear stress in close adjacency to sSMCs to serve as negative regulators for sSMC-induced EC inflammation.
- NF-E2-related factor-2 (Nrf-2) modulates the shear-induction of miR-146a in ECs in close adjacency to sSMCs at the transcriptional level.
- Overexpressing miR-146a inhibits neointima formation of arteries induced by injury or blood flow cessation.

A better understanding the molecular mechanisms by which sSMCs modulate EC gene expression and function and elucidation of the effects of shear stress on this modulation could provide new molecular targets for intervention against vascular disorders resulting from atherosclerosis and restenosis. We report that in ECs adjacent to sSMCs, miR-146a, -708, -451, and -98 become highly expressed in response to atheroprotective shear stress and play anti-inflammatory roles in vitro and in vivo. In addition, we found that NF-κB and Nrf-2 are involved in sSMC- and shear-modulations of these EC miR expessions at the transcriptional levels, respectively. MiR-146a serves as a valuable therapeutic miR for intervention against neointimal lesion formation that leads to atherosclerosis and restenosis.

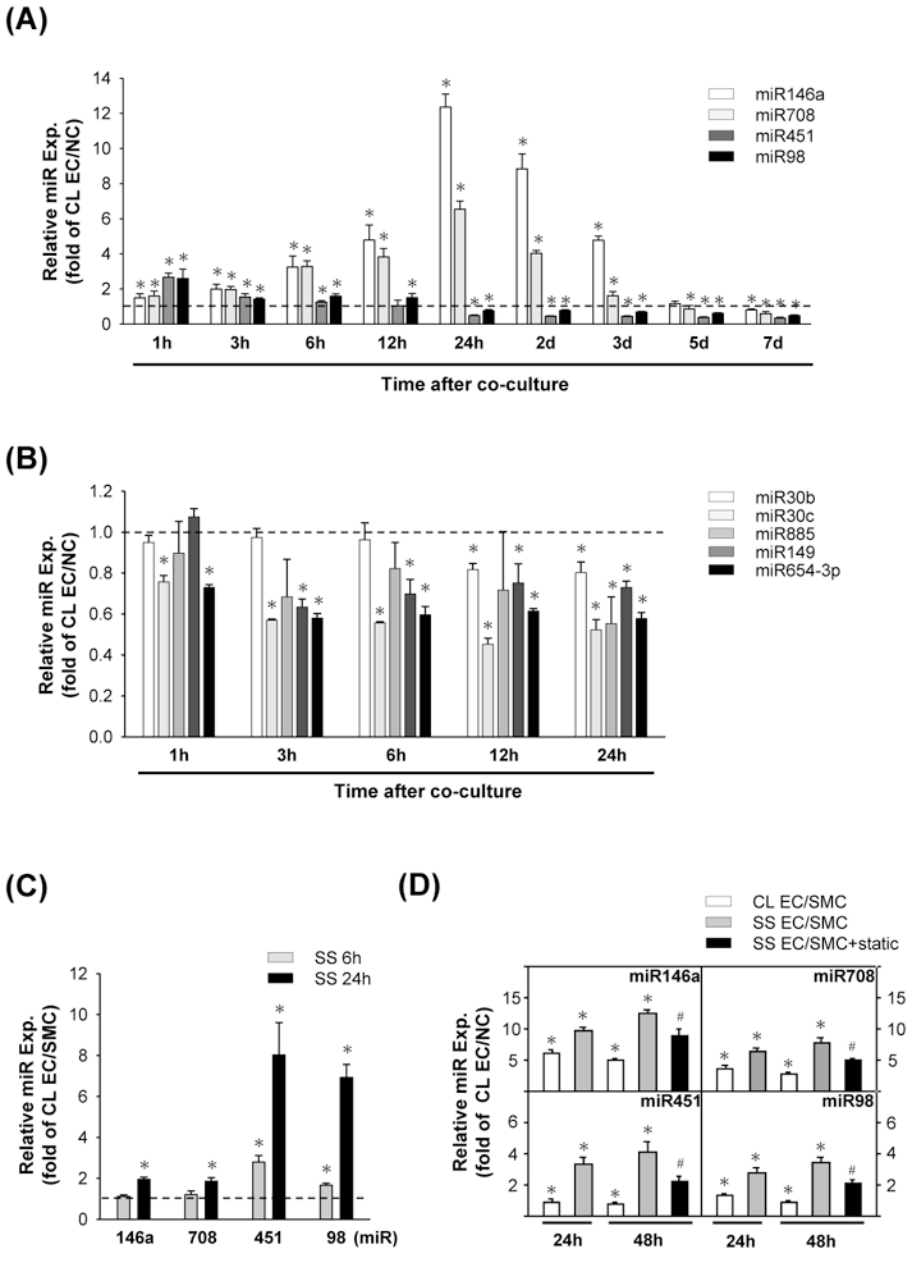


Figure 1-1

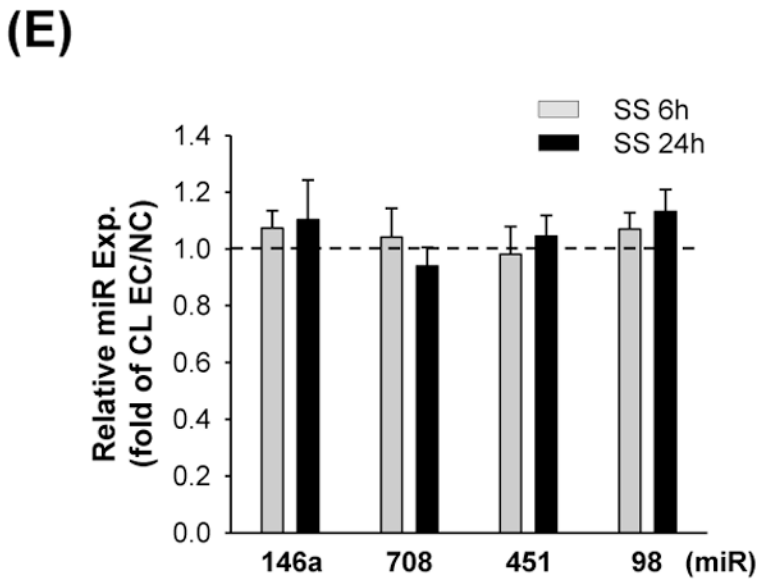


Figure 1. MiR expression profiles in co-cultured ECs under static condition and in response to shear stress

(**A** and **B**) ECs were cultured alone (EC/NC) or co-cultured with sSMCs (EC/SMC) under static conditions (CL). The EC miR expression was examined by qPCR. (**C** and **D**) Co-cultured ECs were kept under static condition or exposed to shear stress at 12 dynes/cm^2 (SS) for 6 h (**C**), 24 h (**C** and **D**), or 48 h (**D**). Co-cultured ECs were subjected to shear stress for 24 h, followed by the flow cessation for additional 24 h (**D**). (**E**) Mono-cultured ECs were kept under static condition or exposed to flow for 6 h or 24 h. Data are means±SEM from three independent experiments. $^{*}P<0.05$ *vs.* CL EC/NC. $^{\#}P<0.05$ *vs.* SS EC/SMC 48h.

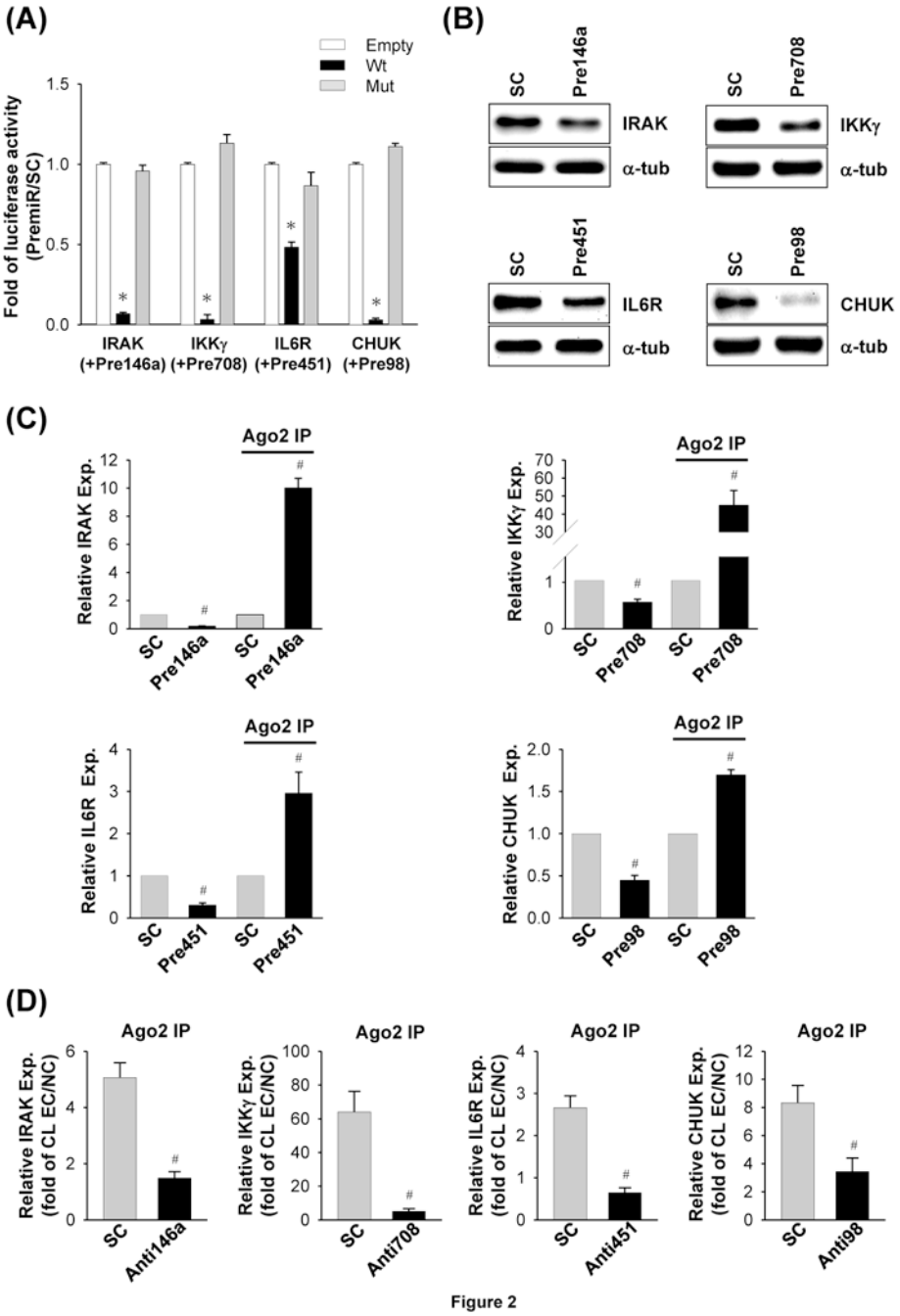


Figure 2

Figure 2. Direct target genes of miR-146a, -708, -451, and -98

(**A**) Reporter constructs containing wild-type (Wt) and mutant (Mut) of putative miR target sites of indicated genes were co-transfected with the respective pre-miRs (PremiR) or a scramble control (SC) into HEK293 cells for 24 h. (**B**) ECs were transfected with pre-miR-146a, -451, -98 (20 nmol/L each), and pre-miR-708 (0.1 nmol/L) or a scramble control for 24 h. (**C**) ECs were transfected with the indicated pre-miRs or a scramble control for 24 h, and performed with the Ago2 immunoprecipitation assay. (**D**) ECs were transfected with anti-miR-146a, -708, -451, and -98 (AntimiR; 5 nmol/L each) or a scramble control for 24 h,

and then were cultured alone (CL EC/NC) or co-cultured with sSMCs for 12 h. $^{*}P<0.05$ *vs.* empty vector. $^{\#}P<0.05$ *vs.* SC. Data in **A, C**, and **D** are means±SEM from three independent experiments. Results in **B** are representative of triplicate experiments with similar results.

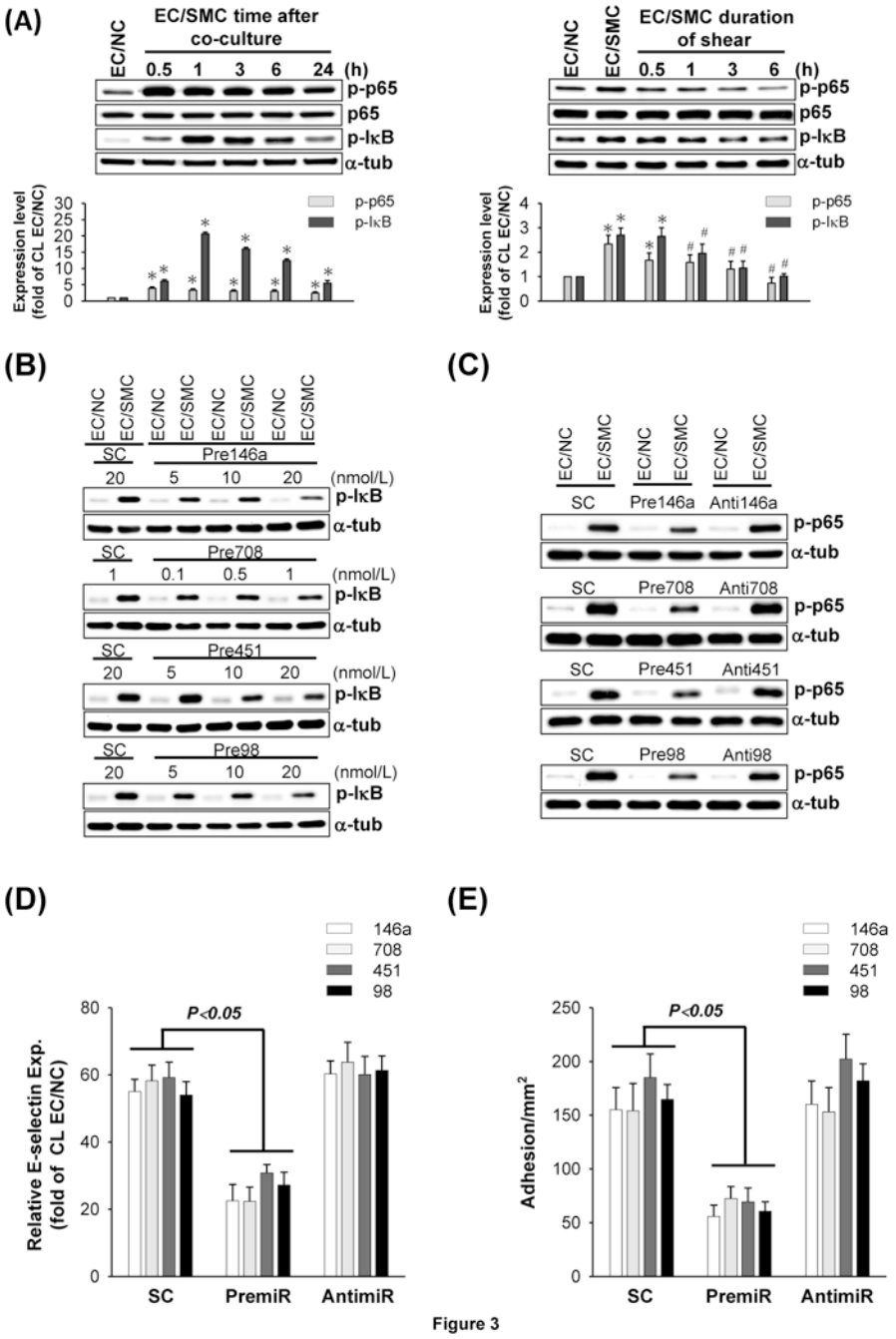


Figure 3

Figure 3. MiR inhibition in sSMC-induced pro-inflammatory responses in co-cultured ECs

(**A**) ECs were cultured alone (EC/NC) or co-cultured with sSMCs (EC/SMC) under static condition (CL). In parallel experiments, ECs co-cultured with SMCs for 24 h were subjected to shear stress. The p65 and IκBα phosphorylation levels were determined by Western blot. $^{*}P<0.05$ *vs.* CL EC/NC. $^{\#}P<0.05$ *vs.* CL EC/SMC. (**B** and **C**) ECs were transfected with the designated pre- or anti-miRs or a scramble control at the indicated concentrations (**B**) or the optimal concentrations (**C**) for 24 h. (**D**) The E-selection mRNA expression in co-cultured ECs was quantified by qPCR. (**E**) The monocytic adhesion assay was performed.

Data in **A, D**, and **E** are means±SEM from three independent experiments. Results in **B** and **C** are representative of triplicate experiments with similar results.

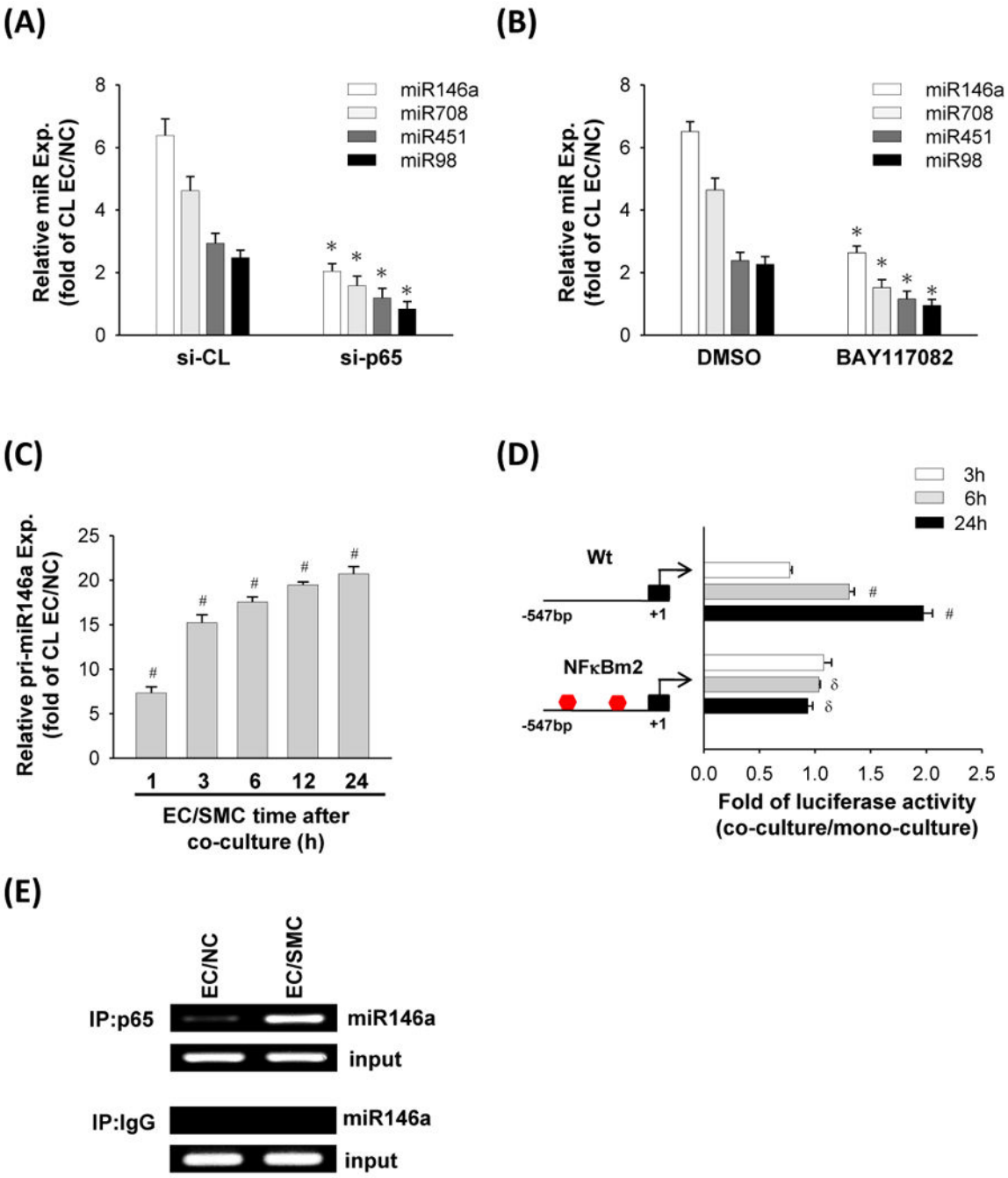
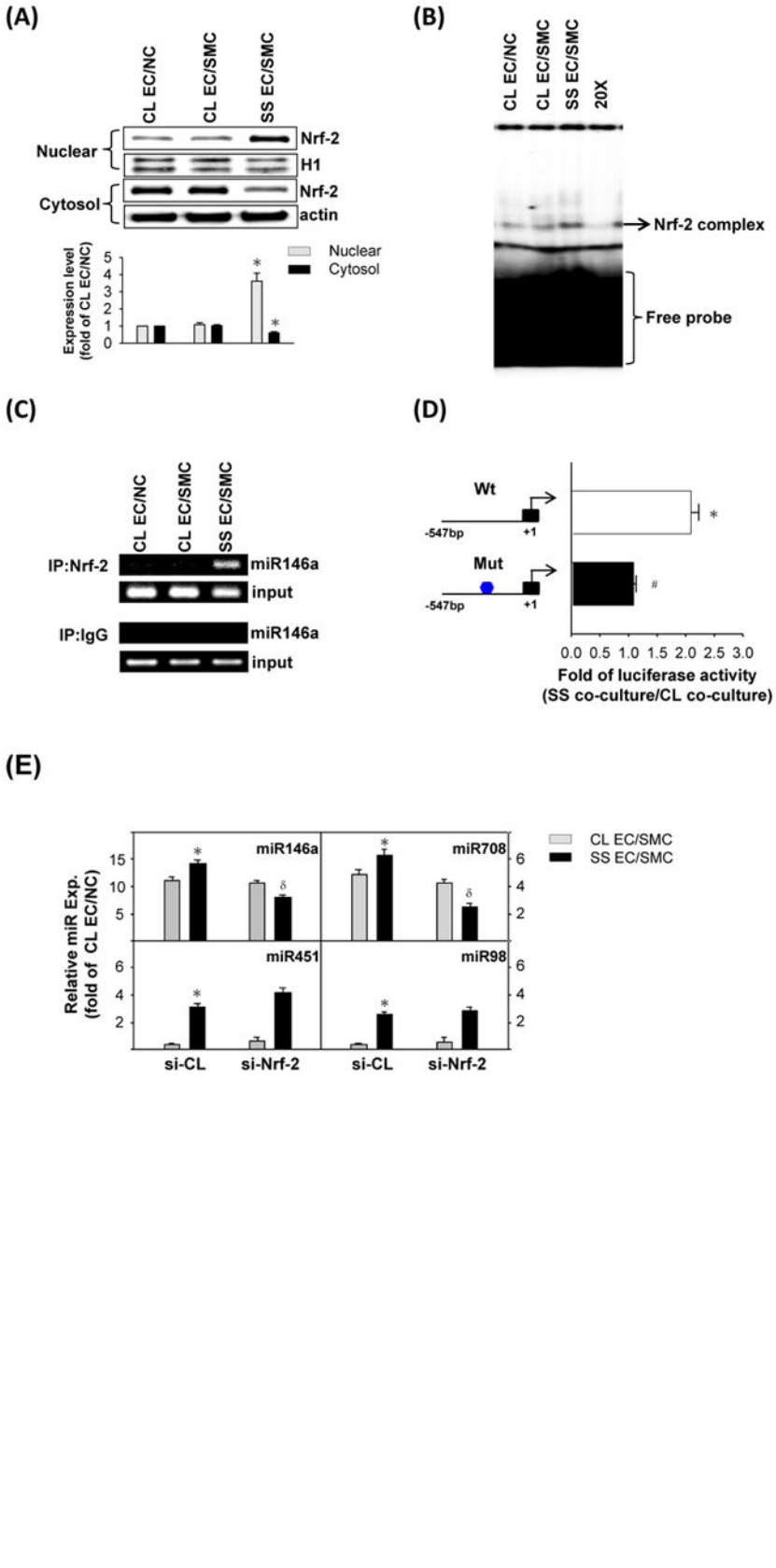


Figure 4. NF-κB is critical for sSMC-induction of miRs in ECs

ECs were transfected with control (siCL) or p65-specific siRNA (sip65) (20 nmol/L) (**A**). Cells were pre-treated with DMSO or BAY117082 for 1 h (**B**). These ECs were kept under static condition or co-cultured with sSMCs for 6 h (for miR-451/98) or 12 h (for miR-146a/708). (**C**) The expression of pri-miR-146a in co-cultured ECs was determined by qPCR. (**D**) Wild-type (Wt) promoter construct (-547 bp) of miR-146a and its mutant at two putative NF-κB binding sites (NFκBm2) were transfected into ECs. (**E**) ECs were cultured alone or co-cultured with sSMCs under static conditions for 12 h, and ChIP assay was

performed.[*]$P<0.05$ *vs.* siCL or DMSO. [#]$P<0.05$ *vs.* CL EC/NC. [δ]$P<0.05$ *vs.* Wt. Data in **A** to **D** are means±SEM from three independent experiments. Results in **E** are representative of triplicate experiments with similar results.



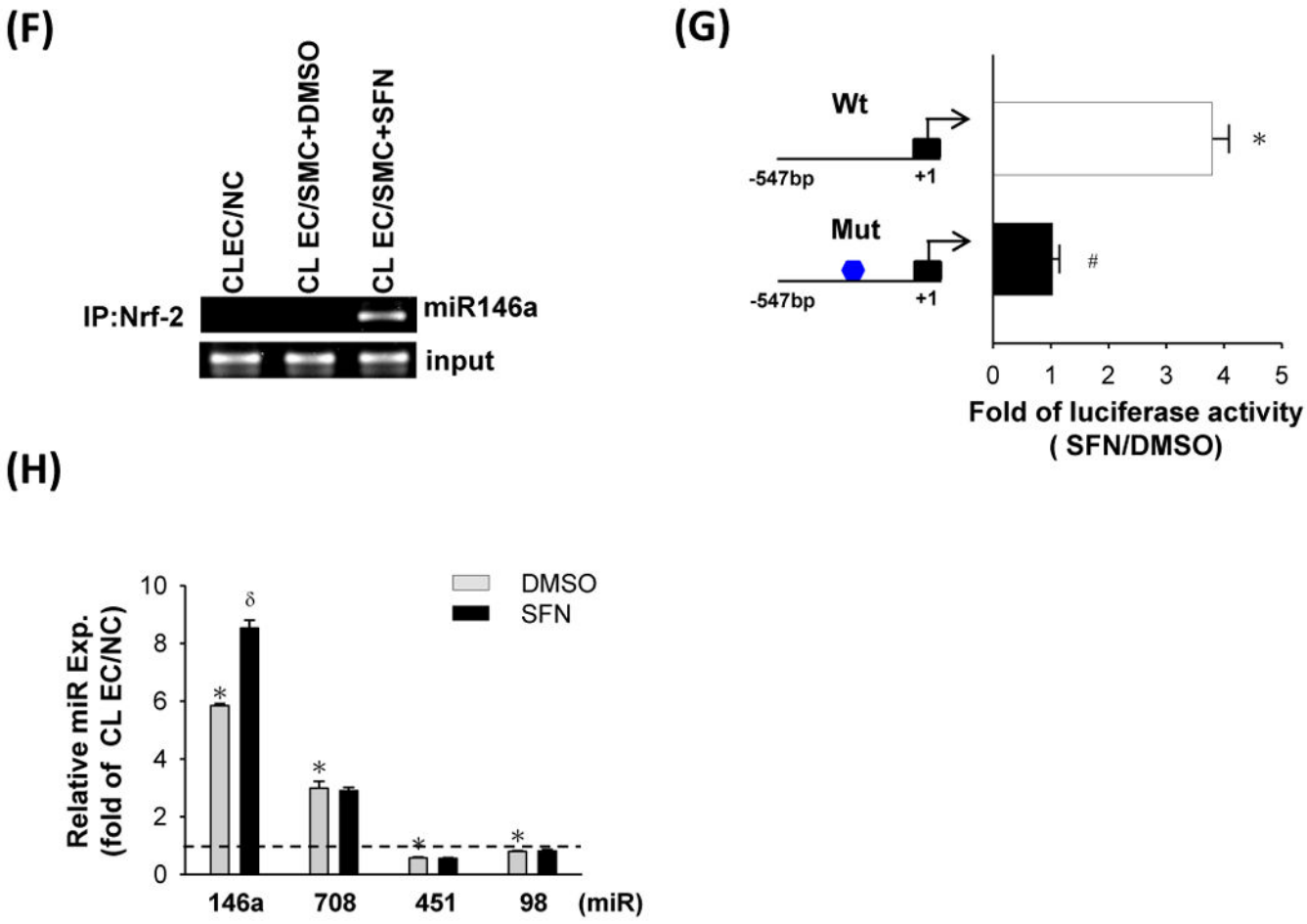


Figure 5. Nrf-2 is critical for shear-induction of miR-146a in co-cultured ECs

(**A**) ECs were cultured alone or co-cultured with sSMCs under static condition for 24 h. Co-cultured ECs were subjected to shear stress for 24 h. Nrf-2 expression in the EC nuclei and cytosol was determined by Western blot. (**B**) EMSA analysis of nuclear protein extracts from these ECs. The nuclear extracts were pre-incubated with excess unlabeled oligonucleotides containing Nrf-2 binding sequences (20X). (**C**) ChIP assay was performed. Input DNA was analyzed by PCR to normalize the extracts used for immunoprecipitation. (**D**) Wild-type (Wt) promoter construct (-547 bp) of miR-146a and its mutant at the Nrf-2 binding site (Mut) were transfected into ECs. (**E**) ECs were transfected with Nrf-2-specific siRNA (siNrf-2) or control siRNA (siCL), and then cultured alone or co-cultured with sSMCs under static condition. The co-cultured ECs were subjected to shear stress for 24 h. (**F** to **H**) Co-cultured ECs were treated with DMSO or sulforaphane (SFN) (10 μmol/L) for 24 h. The binding of Nrf-2 to the promoter region of miR-146a (**F**) and its promoter activity (**G**) and different miR expressions (**H**) were examined. $^{*}P<0.05$ *vs.* CL EC/SMC. $^{\#}P<0.05$ *vs.* Wt. $^{\delta}P<0.05$ *vs.* siCL or DMSO. Data in **A, D, E, G**, and **H** are means±SEM from three independent experiments. Results in **B, C,** and **F** are representative of triplicate experiments with similar results.

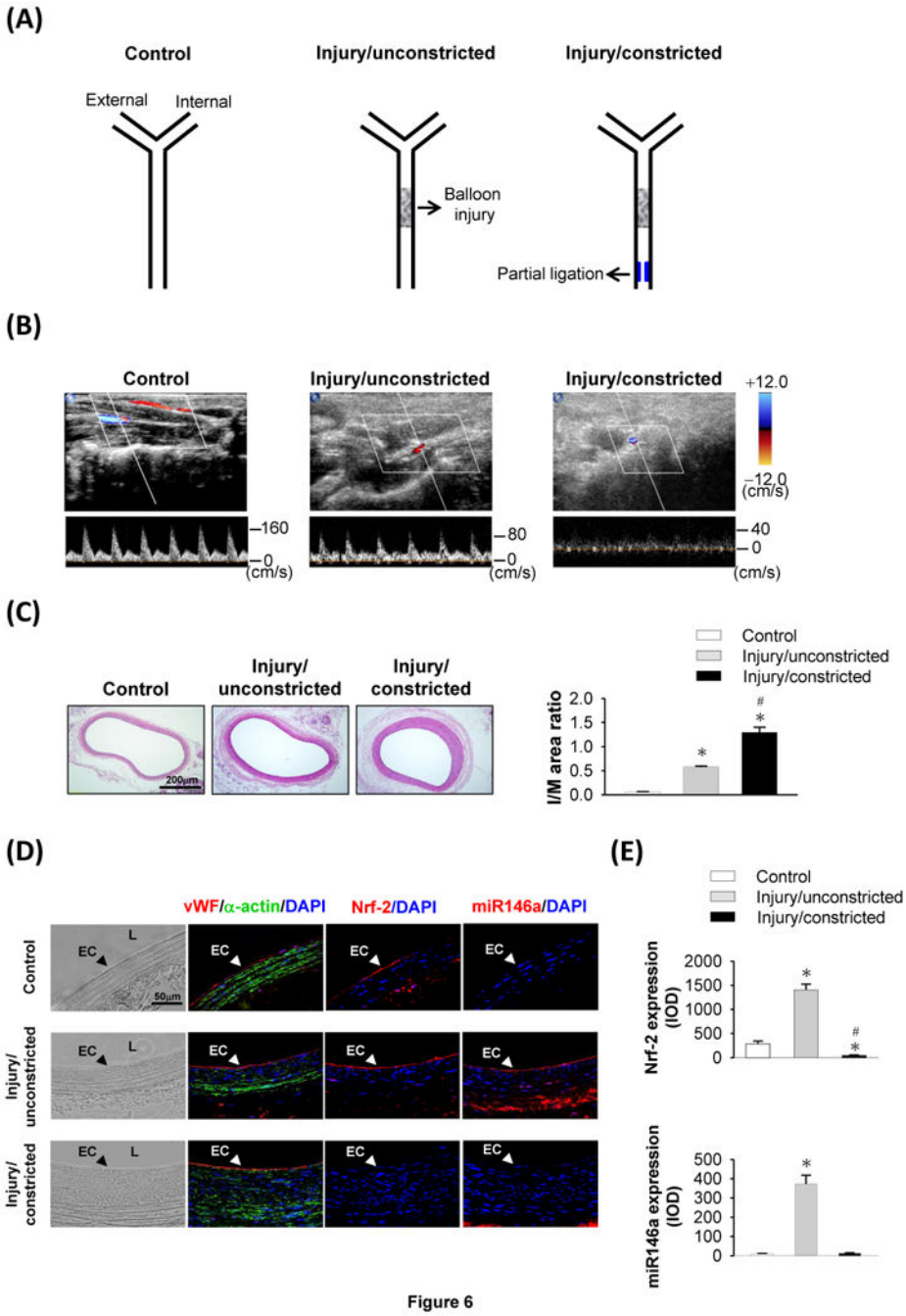


Figure 6

Figure 6. MiR-146a is highly expressed in the EC layer of neointimal lesions in injured arteries under flow

(**A**) Schematic diagrams of rat experimental models. (**B**) Ultrasound measurements on the affected carotid arteries. Upper images: vessels with the 2D and color Doppler modes. Lower images: Doppler spectral display of velocity profiles with a plus wave Doppler mode. (**C**) H&E staining of the cross-sections of affected carotid arteries 4 weeks after surgery. I/M: intima-to-media ratio (n=5 each). (**D**) Serial cross-sections of the affected carotid arteries (n=5 each) were stained for Nrf-2 and miR-146a expressions. L, lumen. (**E**)

Quantitative analysis of the results in **D**. * $P<0.05$ *vs.* control vessels. # $P<0.05$ *vs.* injury/unconstricted model. Images shown in each examination are representative of five rats with similar results.

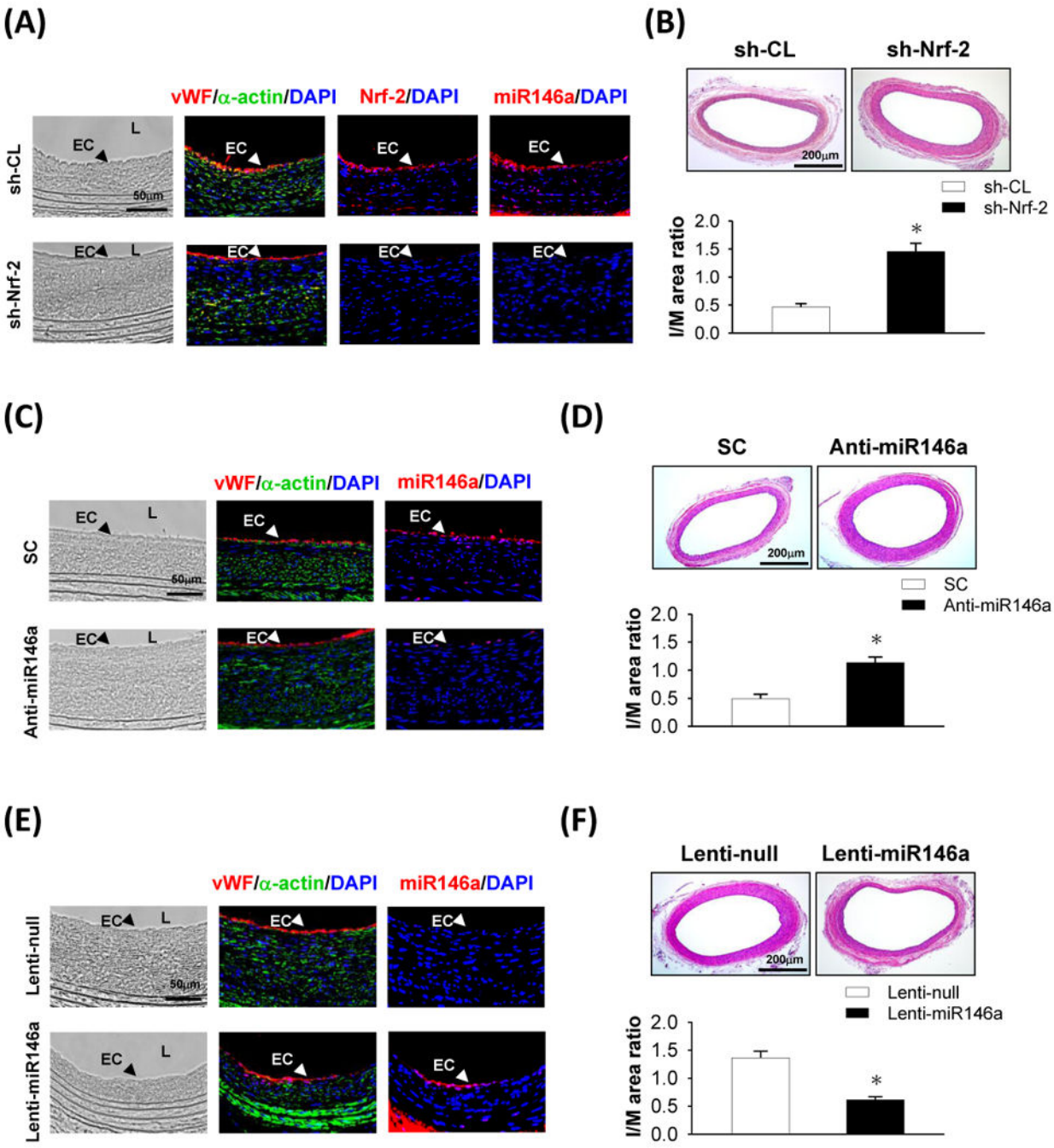
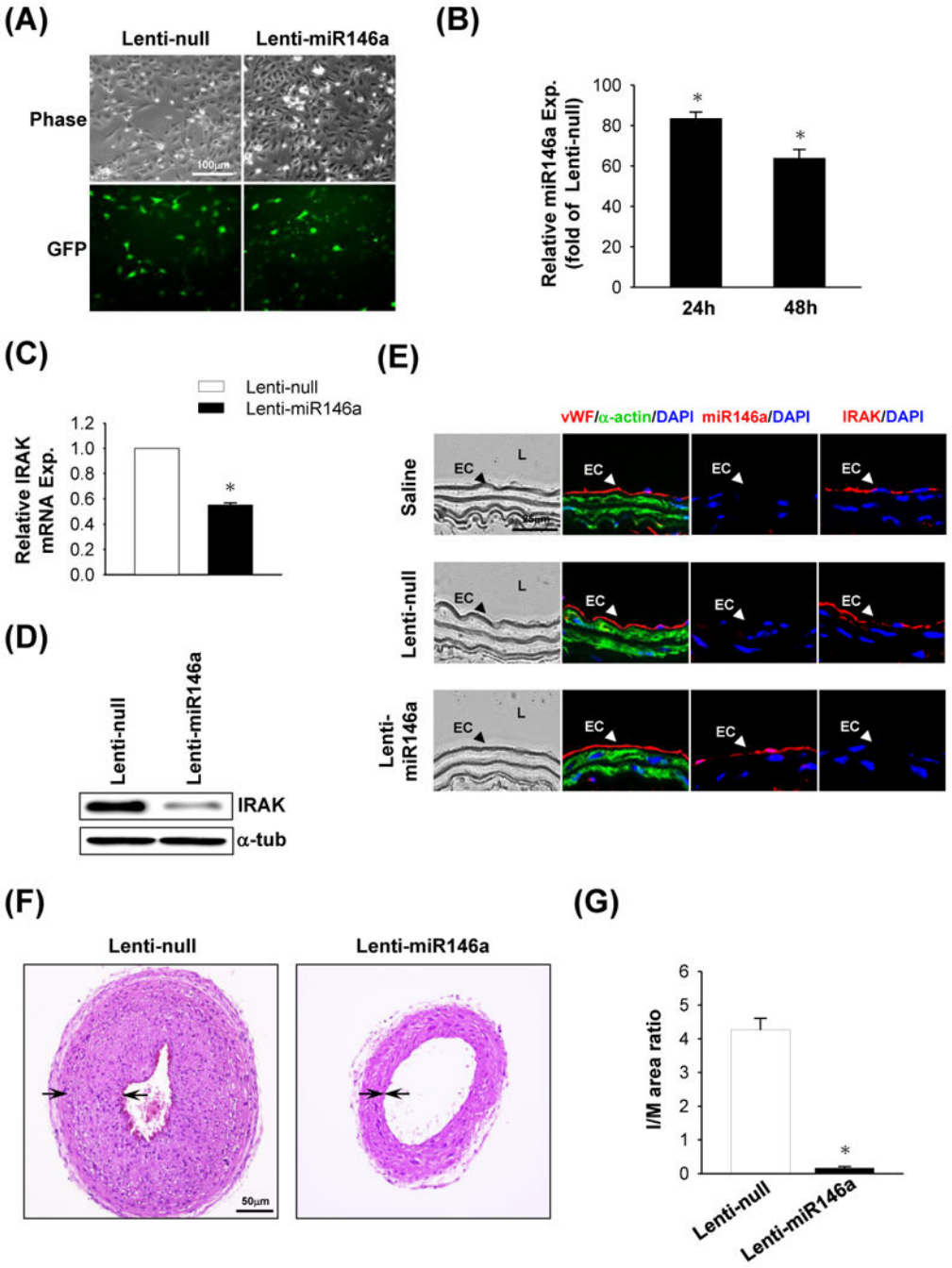


Figure 7. Nrf-2 and miR-146a play crucial roles in modulating neointimal lesion formation in vivo

Two weeks after balloon injury in unconstricted carotid arteries in rats (n=5), 50 μL of lentiviral control (shCL) or Nrf-2-specific shRNA (shNrf-2) (1×10^6 TU/mL each) (**A** and **B**), 10 μg of chemically-modified anti-miR-146a or scramble controls (**C** and **D**), or Lenti-null or Lenti-miR-146a (5×10^6 TU/mL each) (**E** and **F**) was respectively infused into the local injured sites. The rats were sacrificed 2 weeks after local injections of agents. (**A, C**, and **E**) Serial cross-sections of the affected carotid arteries were stained for Nrf-2 and

miR-146a. L, lumen. (**B, D**, and **F**) H&E staining of the cross-sections of affected carotid arteries. I/M: intima-to-media ratio (n=5). $^{*}P<0.05$ *vs.* control group. Images shown in each examination are representative of five rats with similar results.



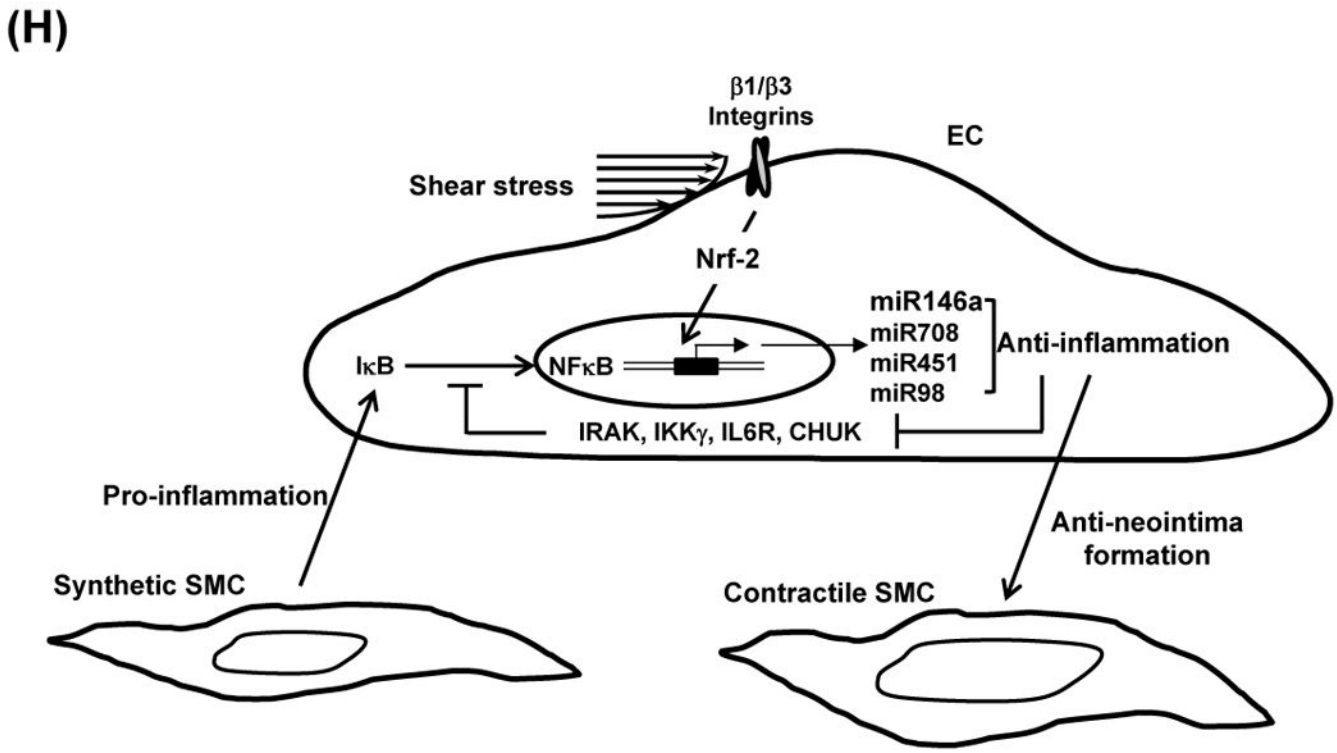


Figure 8. Lenti-miR-146a inhibits neointimal lesion formation in a mouse carotid artery ligation model

(**A**) Cultured ECs were infected with Lenti-null or Lenti-miR-146a (5×10^6 TU/mL each) for 48 h. (**B**) MiR-146a expression was analyzed at 24 h and 48 h after infection. IRAK mRNA (**C**) protein (**D**) expressions in infected ECs were determined. (**E**) Serial cross-sections of the affected carotid arteries were stained for miR-146a and its direct target IRAK. L, lumen. H&E staining (**F**) and quantitative analysis (**G**) of neointimal lesions in the affected arteries. * $P<0.05$ *vs.* Lenti-null. Images shown in each examination are representative of five mice with similar results. (**H**) A schematic diagram of the mechanisms underlying sSMC- and shear-modulations in EC miR expression.