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## Regulation of Vascular Smooth Muscle Cell Turnover by Endothelial Cell–Secreted MicroRNA-126 Role of Shear Stress

Jing Zhou, Yi-Shuan Li, Phu Nguyen, Kuei-Chun Wang, Anna Weiss, Yi-Chun Kuo, Jeng-Jiann Chiu, John Y. Shyy, Shu Chien

**Rationale:** Endothelial microRNA-126 (miR-126) modulates vascular development and angiogenesis. However, its role in the regulation of smooth muscle cell (SMC) function is unknown.

**Objective:** To elucidate the role of miR-126 secreted by endothelial cells (ECs) in regulating SMC turnover in vitro and in vivo, as well as the effects of shear stress on the regulation.

**Methods and Results:** Coculture of SMCs with ECs or treatment of SMCs with conditioned media from static EC monoculture (EC-CM) increased SMC miR-126 level and SMC turnover; these effects were abolished by inhibition of endothelial miR-126 and by the application of laminar shear stress to ECs. SMC miR-126 did not increase when treated with EC-CM from ECs subjected to inhibition of miR biogenesis, or with CM from sheared ECs. Depletion of extracellular/secreted vesicles in EC-CM did not affect the increase of SMC miR-126 by EC-CM. Biotinylated miR-126 or FLAG (DYKDDDDK epitope)-tagged Argonaute2 transfected into ECs was detected in the cocultured or EC-CM–treated SMCs, indicating a direct EC-to-SMC transmission of miR-126 and Argonaute2. Endothelial miR-126 represses forkhead box O3, B-cell lymphoma 2, and insulin receptor substrate 1 mRNAs in the cocultured SMCs, suggesting the functional roles of the transmitted miR-126. Systemic depletion of miR-126 in mice inhibited neointimal lesion formation of carotid arteries induced by cessation of blood flow. Administration of EC-CM or miR-126 mitigated the inhibitory effect.

**Conclusions:** Endothelial miR-126 acts as a key intercellular mediator to increase SMC turnover, and its release is reduced by atheroprotective laminar shear stress. (*Circ Res.* 2013;113:40-51.)

**Key Words:** atherosclerosis ■ endothelial cell ■ extracellular miR-126 ■ shear stress ■ smooth muscle cell

Vascular smooth muscle cells (SMCs) are highly proliferative and migratory during the early embryonic stages of vasculogenesis but become differentiated and quiescent in adult blood vessels.<sup>1</sup> They can be activated and regain their highly proliferative characteristics under certain pathophysiological conditions such as vascular injury and atherogenesis, thus contributing to the thickening and stiffening of the arterial wall.<sup>2</sup> During atherogenesis, activated SMCs migrate into the intima, where they come into close contact with endothelial cells (ECs) lining the vessel wall.<sup>3</sup> In the atherogenic vessel, SMC apoptosis occurs and is followed by proliferation of the remaining cells, thus inducing destabilization and vulnerability of the lesion plaques.<sup>4</sup> The present study was designed to study the effects of EC-SMC interaction on SMC proliferation/apoptosis and neointimal formation and the underlying mechanisms, as well

as the actions of different patterns of shear stress (SS) on these effects.

### In This Issue, see p 2 Editorial, see p 7

MicroRNAs (miRs) are noncoding single-stranded RNA molecules of ≈22 nucleotides that regulate mRNAs by binding to target sequences of mRNAs to cause their degradation or translation repression.<sup>5</sup> In addition to their primary intracellular locations, miRs have recently been found extracellularly in plasma and other body fluids of humans and mice.<sup>6-9</sup> Changes in extracellular circulating miR level and activity have been linked to pathologies of cardiovascular disorders, including acute myocardial infarction, heart failure, and coronary artery disease,<sup>10-12</sup> suggesting the possibility that extracellular miRs may have diagnostic or prognostic values in cardiovascular

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From the Department of Bioengineering and Institute of Engineering in Medicine, University of California, San Diego, La Jolla (J.Z., Y.-S.L., P.N., K.-C.W., A.W., Y.-C.K., S.C.); Institute of Cellular and System Medicine, National Health Research Institutes, Miaoli, Taiwan (J.-J.C.); Department of Medicine, University of California, San Diego, La Jolla (L.Y.S.); and Department of Clinical Medicine, National Yang-Ming University, Taipei, Taiwan (Y.-C.K.).

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Correspondence to Shu Chien, MD, PhD, Institute of Engineering in Medicine, UCSD, 9500 Gilman Dr, La Jolla, CA 92093-0435. E-mail [shuchien@ucsd.edu](mailto:shuchien@ucsd.edu)  
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### Nonstandard Abbreviations and Acronyms

<b>3'UTR</b>	3'-untranslated region
<b>Ago2</b>	Argonaute2
<b>AMC</b>	negative control inhibitor
<b>BCL2</b>	B-cell lymphoma 2
<b>cel-miR-39</b>	<i>Caenorhabditis elegans</i> -miR-39
<b>EC</b>	endothelial cell
<b>EC-CM</b>	endothelial cell-conditioned media
<b>FOXO3</b>	forkhead box O3
<b>IRS1</b>	insulin receptor substrate 1
<b>KO</b>	knockout
<b>LSS</b>	laminar shear stress
<b>miR</b>	microRNA
<b>OSS</b>	oscillatory shear stress
<b>PCNA</b>	proliferating cell nuclear antigen
<b>PRE126</b>	microRNA-126 mimics
<b>SMC</b>	smooth muscle cell
<b>SS</b>	shear stress
<b>WT</b>	wild-type

diseases. Recent studies reveal that miRs could be delivered to recipient cells with functional targeting capabilities, leading to altered gene expression in the recipient cells.<sup>7,13,14</sup> These miRs may be involved in intercellular communication and intracellular signal transduction.

MiR-126 is highly expressed in ECs to modulate vascular development and angiogenesis.<sup>15-17</sup> MiR-126 expression in rat carotid artery is significant under normal condition<sup>18</sup> and is markedly increased after angioplasty,<sup>18</sup> suggesting a potential modulatory role of miR-126 in vascular pathogenesis. The functional relevance of miR-126 in vascular SMCs has not yet been reported. Our present study shows that (1) EC-secreted miR-126 regulated SMC gene expression and cellular functions via a paracrine effect and that laminar SS (LSS) applied to ECs attenuated this effect; (2) Argonaute2 (Ago2), the effector component of the miRNA-induced silencing complex, associated with the EC-secreted miR-126 and facilitated its transmission from ECs to SMCs; and (3) knockout (KO) of miR-126 in mouse inhibited neointimal hyperplasia in the carotid arteries induced by cessation of blood flow. These results demonstrate that miR-126 plays an important role in EC-SMC interaction to modulate SMC function and vascular homeostasis.

## Methods

The detailed methods are described in the online Data Supplement.

### EC-SMC Coculture and Parallel-Plate Coculture Flow System

EC-SMC coculture was established by plating human umbilical vein ECs and human umbilical artery SMCs on the lower and upper sides, respectively, of a 10- $\mu$ m-thick porous membrane with 0.4- $\mu$ m pores in a transwell cell culture insert (Becton Dickinson). ECs and SMCs were maintained in their respective media supplemented with 2% fetal bovine serum. The insert was incorporated into a parallel-plate flow chamber that contains a polycarbonate insert holder and is connected to a perfusion loop system. ECs were exposed to either LSS with a high level of mean SS at 12 dynes/cm<sup>2</sup> or oscillatory SS (OSS) with a low level of mean SS at 0.5 dynes/cm<sup>2</sup> and a superimposed sinusoidal oscillation with a frequency of 1 Hz and a peak-to-peak amplitude of  $\pm 4$  dynes/cm<sup>2</sup>.

### Animal Model

Animal surgical procedures were performed in accordance with National Institutes of Health guidelines and were approved by the University of California San Diego Institutional Animal Care and Use Committees. Mice were subjected to carotid artery ligation as previously described.<sup>19</sup> Some mice were injected intravenously twice per week for 4 weeks with 200  $\mu$ L of EC-conditioned media (EC-CM) from 3-day-monocultured static human umbilical vein ECs or with control media. Some mice received a local administration of miR-126 mimics (PRE126) or negative control mimics (2  $\mu$ g) mixed with pluronic gel (25% [wt/vol], Pluronic F127, Sigma). After 4 weeks of ligation, the ligated and unligated carotid arteries were harvested and subjected to histology and immunostaining analyses of the vessels.

### Statistical Analysis

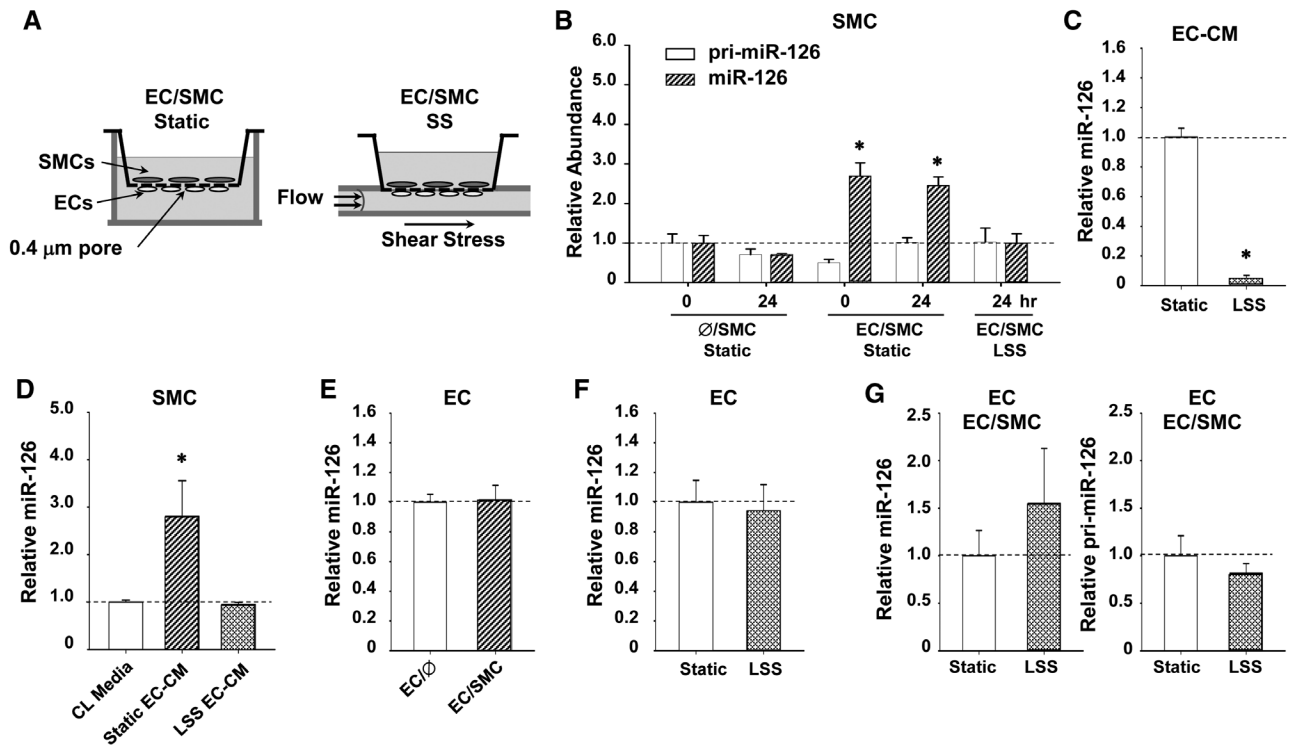
Data are expressed as mean $\pm$ SEM from 3 independent experiments. Statistical analysis was performed by Student *t* test for 2 groups of data and by 1-way ANOVA for multiple comparisons. Statistical significance among multiple groups was determined by post hoc analysis (Tukey honestly significant difference test). Values of *P*<0.05 were considered statistically significant.

## Results

### The Increase of miR-126 Level in the SMCs Cocultured With ECs Is Suppressed by LSS on ECs

Using a parallel-plate coculture flow system (Figure 1A), we determined the miR-126 levels in SMCs in response to coculture with ECs and flow shear application to ECs. Under static condition, the coculture of SMCs with ECs significantly increased the SMC mature form of miR-126 but not the primary transcript of miR-126 (Figure 1B), indicating that the effect of EC-coculture on SMC miR-126 occurs at a posttranscriptional level. Atheroprotective LSS (12 dynes/cm<sup>2</sup>) applied to ECs suppressed the level of miR-126 in the cocultured SMCs, and this suppression lasted for  $\leq 24$  hours (Figure 1B). MiR-126 level in the EC-CM was markedly attenuated by exposure of ECs to LSS for 24 hours (Figure 1C), suggesting that the effects of LSS on miR-126 in SMCs are mediated at least partially through alterations in miR-126 secretion from ECs. Treatment of SMCs with CM from static ECs caused an enrichment of SMC miR-126; this enrichment was absent with CM obtained from ECs exposed to LSS for 24 hours (Figure 1D). The miR-126 level in ECs did not show significant difference between static monocultured and cocultured ECs (Figure 1E) or between static and LSS-treated monocultured ECs (Figure 1F). These results indicate that coculture of SMCs with ECs does not induce miR-126 expression in ECs and that the inhibitory effect of LSS on miR-126 enrichment in the cocultured SMCs does not involve a downregulation of miR-126 in ECs. Together with the finding that LSS did not change the primary or mature miR-126 in the cocultured ECs (Figure 1G), our results indicate that SS and SMC cocultures have no synergistic effects on the transcriptional regulation of miR-126 in ECs. These results demonstrate that the induction of SMC miR-126 by coculture with ECs and the suppression of this induction by LSS on ECs are mediated by intercellular humoral mechanisms.

In contrast to LSS, atheroprone OSS (0.5 $\pm$ 4 dynes/cm<sup>2</sup>) augmented the accumulation of mature miR-126 and the primary miR-126 level in SMCs (Online Figure IA) without a significant change of miR-126 level in EC-CM (Online Figure IB). Although OSS caused an increase in the SMC miR-126,



**Figure 1. Atheroprotective shear stress (SS) to endothelial cells (ECs) suppresses microRNA-126 (miR-126) in the cocultured smooth muscle cells (SMCs).** **A**, Schematic diagrams of EC/SMC coculture and flow system. **B**, Human umbilical vein ECs and human umbilical artery SMCs were seeded on the lower and upper sides of a membrane, respectively (EC/SMC). Controls had SMCs but no ECs on the other side (∅/SMC). The temporal expression levels of primary (pri-miR-126) and mature (miR-126) forms of miR-126 were determined by quantitative reverse transcription–polymerase chain reaction. \* $P < 0.05$  vs ∅/SMC 0-hour static. **C**, The levels of miR-126 in the 24-hour static or sheared media. \* $P < 0.05$  vs static control. **D**, The levels of miR-126 in SMCs incubated with medium 199 supplemented with 2% fetal bovine serum (control [CL] media) or conditioned medium from static or sheared ECs (24-hour shearing) (EC-CM). \* $P < 0.05$  vs CL media. **E**, miR-126 expression in EC monoculture or SMC coculture for 24 hours. **F**, miR-126 expression in ECs exposed to laminar SS (LSS) for 24 hours. **G**, The levels of pri-miR-126 and miR-126 in EC coculture with SMCs under 24-hour static or LSS conditions.

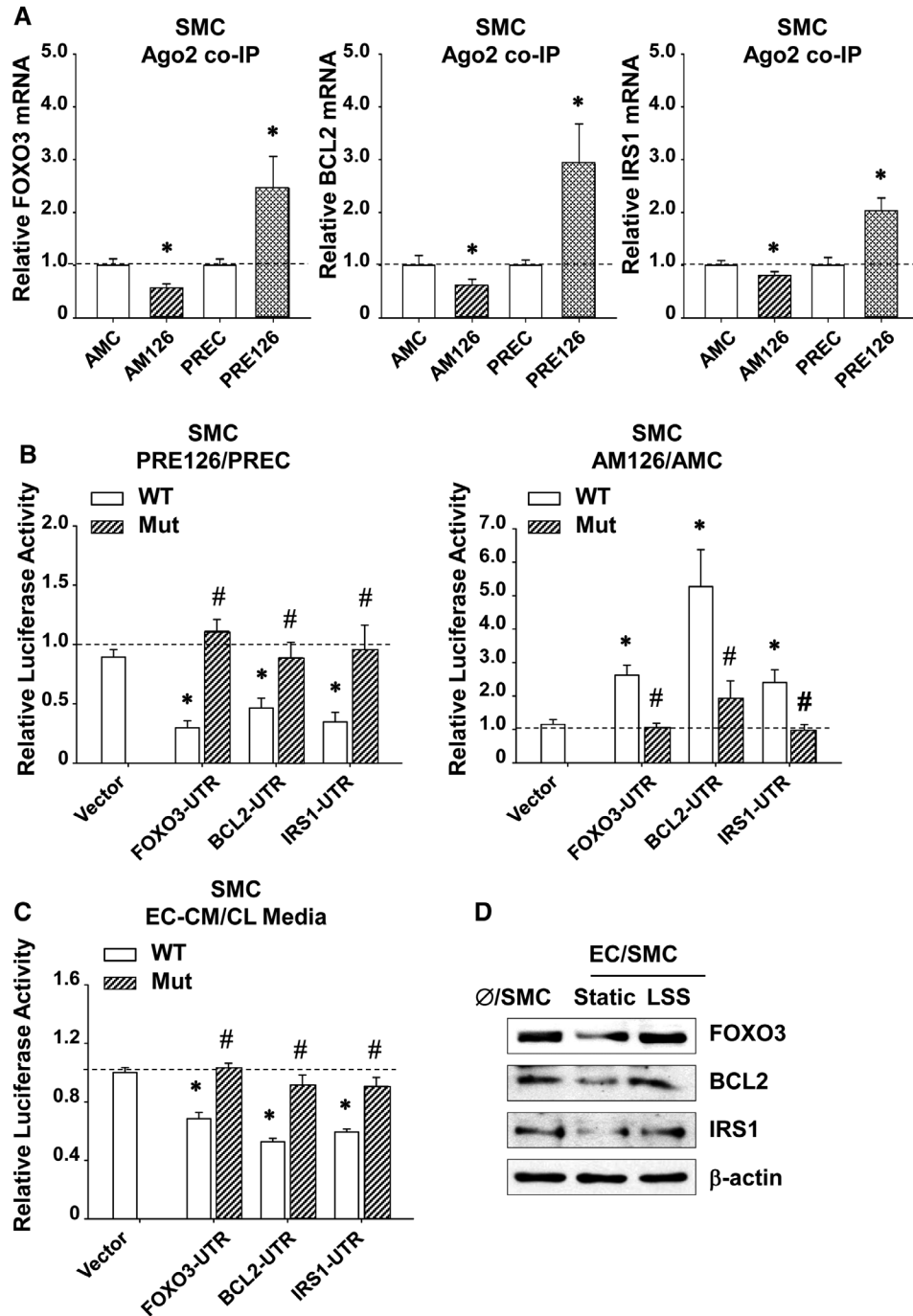
it did not change the primary or mature miR-126 in the cocultured ECs (Online Figure IC). These results suggest that OSS engenders a different regulatory machinery to modulate miR-126 secretion from ECs.

We also tested the flow regulation of miR-126 enrichment in vivo. RNAs isolated from the descending thoracic aorta and the aortic arch of mice were subjected to determination of miR-126 levels in the vessel walls. Endothelia were denuded by perfusion with PBS buffer followed by air massage before excision of the vessels. A 3-fold-higher expression of miR-126 was observed in the athero-susceptible aortic arch (Online Figure IIA), which experiences disturbed and low SS, compared with the thoracic aorta, which is exposed to laminar flow. The expression of the endothelial marker platelet EC adhesion molecule 1 in the aortic arch was lower than that in thoracic aorta (Online Figure IIB), indicating that the increased miR-126 level in the aortic arch is in the media layer and not a result of contamination from the undenuded endothelia. These results validate the differential modulations of SMC miR-126 levels by different flow patterns in vivo.

### ECs and SS Regulate Expressions of Forkhead Box O3, B-Cell Lymphoma 2, and Insulin Receptor Substrate 1, the Direct Targets of miR-126, in SMCs

We tested whether the modulations of SMC miR-126 lead to consequential changes of the miR-126–targeted gene

expressions in SMCs. Forkhead box O3 (FOXO3), B-cell lymphoma 2 (BCL2), and insulin receptor substrate 1 (IRS1) are miR-126 targets predicted by bioinformatics. miRNA-induced silencing complex pull-down assay in SMCs validated the association of FOXO3, BCL2, and IRS1 mRNAs with Ago2-containing miRNA-induced silencing complex in SMCs (Figure 2A). The association of these miR-126 targets with miRNA-induced silencing complex was reduced by transfection of SMCs with anti-miR-126 inhibitor and enhanced by miR-126 mimics (PRE126; Figure 2A). The role of miR-126 in the repression of FOXO3, BCL2, and IRS1 mRNAs in SMCs was also demonstrated by the upregulation and downregulation of the transcripts of these genes in SMCs by anti-miR-126 inhibitor and PRE126, respectively (Online Figure III). These effects of miR-126 were further validated by the responses of FOXO3, BCL2, and IRS1 3′-untranslated region (3′UTR) reporters to the manipulation of miR-126 in SMCs. At 24 hours after transfection of SMCs with PRE126, there was a significant repression of the luciferase reporter activities of FOXO3, BCL2, and IRS1 wild-type (WT) 3′UTR constructs compared with the control vector without 3′UTR, but this repression was not seen with the mutated 3′UTR (miR-126 seeding sites substituted with scrambled sequences; Figure 2B). In complementary experiments, there was a significant derepression on the WT (but not the



**Figure 2. Endothelial cells (ECs) and shear stress (SS) regulate expressions of microRNA-126 (miR-126) targets in smooth muscle cells (SMCs).** **A**, The mRNA levels of forkhead box O3 (FOXO3), B-cell lymphoma 2 (BCL2), and insulin receptor substrate 1 (IRS1) in Argonaute2 (Ago2) immunocomplexes in SMCs 48 hours after transfection with microRNA-126 mimics (PRE126) or anti-microRNA-126 inhibitor (AM126). \* $P < 0.05$  vs cells transfected with negative controls (negative control mimics [PREC] or negative control inhibitor [AMC]). **B**, The relative luciferase levels in SMCs cotransfected with PRE126 (left), AM126 (right), or the negative control and luciferase reporter plasmids: empty vector only, wild-type (WT) 3'-untranslated region (3'UTR) of FOXO3, BCL2, or IRS1 and mutants of 3'UTR of FOXO3, BCL2, or IRS1. **C**, The relative luciferase levels in SMCs transfected with the indicated luciferase reporter plasmids and then incubated with control media or EC-conditioned media (CM). \* $P < 0.05$  vs cells transfected with empty vector. # $P < 0.05$  vs cells transfected with plasmids harboring the WT 3'UTRs. **D**, The protein levels of target genes in SMCs monocultured ( $\emptyset$ /SMC) or cocultured with ECs (EC/SMC) kept under static condition or exposed to laminar shear stress (LSS). Images are representative of triplicate experiments with similar results.

mutated) 3'UTR reporter activities of those 3 genes when the SMCs were transfected with anti-miR-126 inhibitor, compared with negative control inhibitor (Figure 2B).

As expected, incubation of SMCs with EC-CM collected from monocultured ECs significantly inhibited the reporter activities of the WT 3'UTRs but not the mutated 3'UTRs

(Figure 2C). Repression and derepression of FOXO3, BCL2, and IRS1 were observed when the SMCs were cocultured with static and sheared ECs, respectively, compared with the monocultured SMCs (Figure 2D). These results indicate that ECs and SS regulate expressions of FOXO3, BCL2, and IRS1, the direct targets of miR-126, in SMCs.

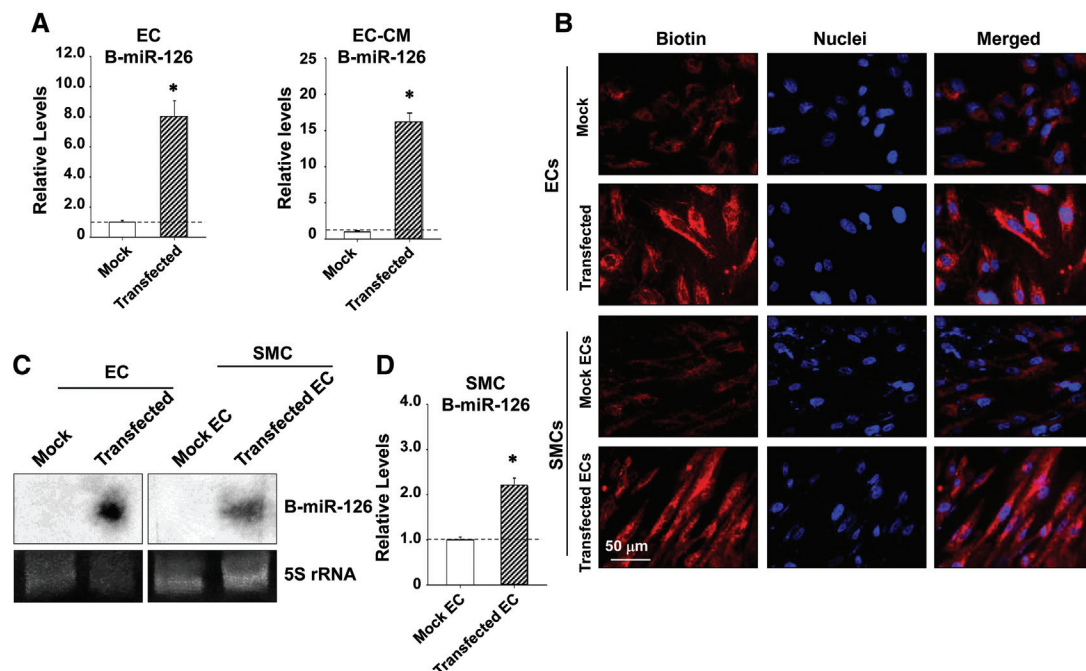
### Transmission of miR-126 from ECs to SMCs in a Paracrine Manner

We hypothesized that the miR-126 enrichment in SMCs after EC-coculture is induced by a humoral transmission of miR-126 from ECs to SMCs. To test this hypothesis, a synthetic miR-126 (a 22-oligomer labeled with psoralen-biotin conjugate) was transfected into ECs, and the culture media were refreshed 8 hours after transfection to remove the free oligonucleotides. The EC-CM was collected 24 hours after the refreshment. Transfection of ECs with this biotinylated miR-126 (B-miR-126) led to a marked enrichment of B-miR-126 in ECs and in the EC-CM compared with the mock transfection (Figure 3A). Incubation of SMCs with the EC-CM derived from ECs transfected with the B-miR-126 led to the presence of B-miR-126 in SMCs (Figure 3B). The fluorescent images identified the cytoplasmic presence of B-miR-126 in both ECs and SMCs (Figure 3B), suggesting a direct EC-to-SMC transmission of miR-126. Northern blot confirmed the presence of B-miR-126 in both ECs and cocultured SMCs (Figure 3C). In SMCs cocultured with ECs transfected with B-miR-126 or mock control, the B-miR-126

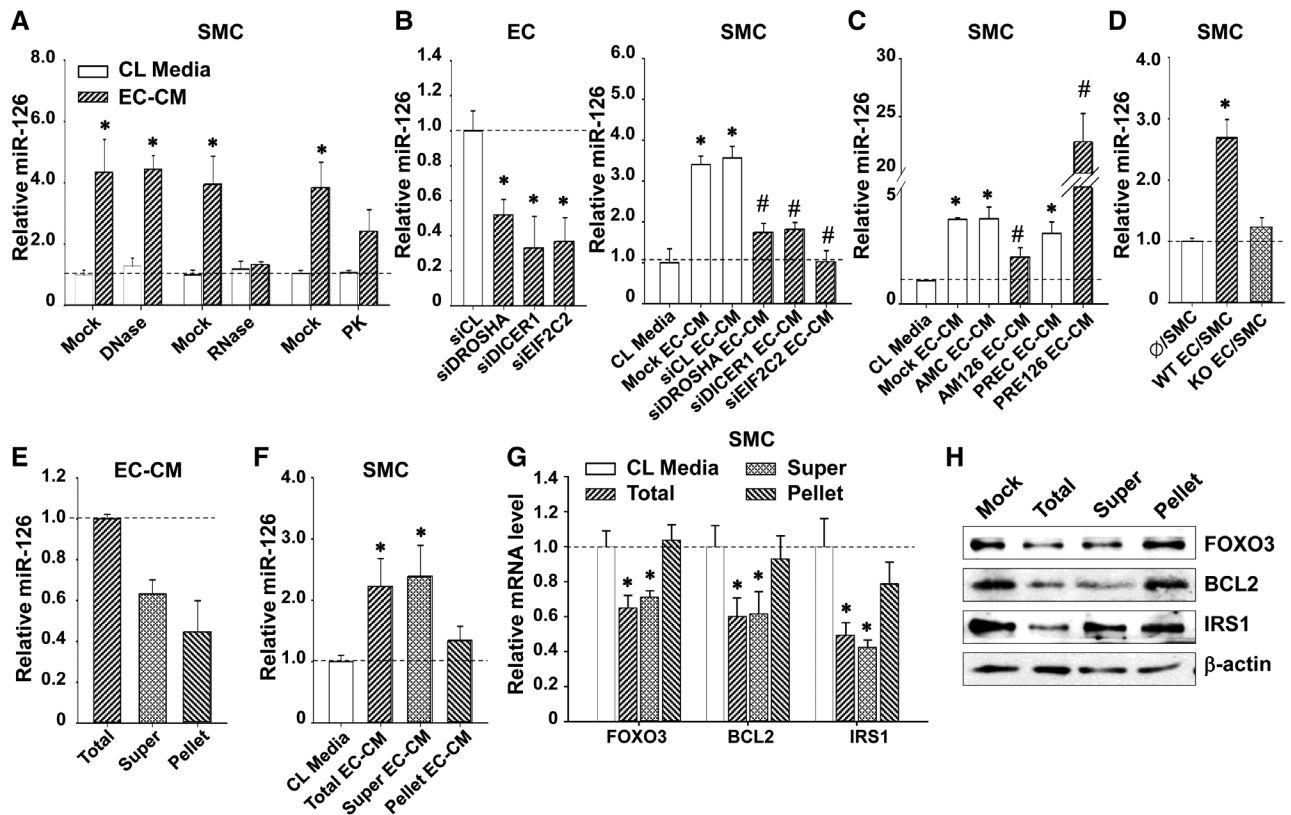
showed a  $2.19 \pm 0.18$ -fold enrichment as compared with the mock cocultured SMCs (Figure 3D).

### EC-Secreted Vesicle-Free miR-126 Regulates SMC Gene Expression

To determine the possible EC-secreted constituents responsible for the increase of miR-126 in the recipient SMCs and to characterize the form of the transmitted miR-126, we exposed the EC-CM to deoxyribonuclease, ribonuclease (RNase), or proteinase K digestions. The ability of EC-CM to increase miR-126 abundance in SMCs was abolished by RNase and reduced by proteinase K but unaffected by deoxyribonuclease (Figure 4A). These results suggest that RNAs and RNA-protein complexes are transmitted into SMCs but not DNA components or the vesicle-protected RNA. To study the involvement of miR biogenesis, we knocked down Drosha, Dicer, and Ago2, which are the 3 key proteins required for the processing and maturation of miRs, in ECs through small interfering RNA (siRNA)-mediated gene silencing. The impairment of miR-126 synthesis in ECs by siDrosha/Dicer/Ago2 (EIF2C2) blocked the miR-126 enrichment in SMCs in response to EC-CM (Figure 4B). We also found that the manipulation of miR-126 in ECs with an anti-miR-126 inhibitor or PRE126 significantly decreased or increased, respectively, the miR-126 enrichment in SMCs treated with EC-CM (Figure 4C). To further verify the transmission of miR-126 from ECs to SMCs, we isolated the lung ECs from either miR-126<sup>+/+</sup> (WT) mice or miR-126<sup>-/-</sup> (KO) mice and cocultured those ECs with human SMCs. We found that coculture with the



**Figure 3. Endothelial cells (ECs) transfer exogenous microRNA-126 (miR-126) to smooth muscle cells (SMCs).** SMCs were incubated with conditioned media (CM) derived from ECs transfected with biotinylated synthetic miR-126 (B-miR-126) or no-miR mock control (Mock) for 3 hours. **A**, The levels of B-miR-126 in ECs or the EC-CM were determined by pull-down experiments with streptavidin-agarose beads followed by quantitative reverse transcription-polymerase chain reaction, **(B)** and B-miR-126 in ECs and SMCs was detected with QDot-605-streptavidin conjugates. \* $P < 0.05$  vs mock control. **C** and **D**, SMCs were cocultured with ECs transfected with B-miR-126 or mock control for 24 hours; the levels of B-miR-126 in SMCs were determined by Northern blot **(C)** or pull-down experiments with streptavidin-agarose beads **(D)**. \* $P < 0.05$  vs mock control.



**Figure 4. Functional microRNA-126 (miR-126) transmission from endothelial cells (ECs) to smooth muscle cells (SMCs) is independent of vesicles.** **A**, The levels of miR-126 in SMCs incubated with control (CL) media or the EC-conditioned media (CM) treated with DNase I, RNase, or proteinase K (PK) for 3 hours. \* $P < 0.05$  vs CL media. **B**, The levels of miR-126 in ECs transfected with control small interfering (si)RNA; siCL) or siRNA against Drosha (siDROSHA), Dicer (siDICER1) or Argonaute2 (siEIF2C2) or in SMCs incubated with CL media or EC-CM derived from those transfected ECs. **C**, The levels of miR-126 in SMCs incubated EC-CM derived from ECs transfected with anti-miR-126 inhibitor anti-miR-126 inhibitor (AM126), miR-126 mimics (PRE126), or the negative controls. \* $P < 0.05$  vs siCL or CL media. # $P < 0.05$  vs the negative control inhibitor (AMC) or negative control mimics (PREC) EC-CM. **D**, The levels of miR-126 in SMCs cocultured with lung ECs isolated from wild-type (WT) or miR-126 knockout mice (KO). \* $P < 0.05$  vs  $\emptyset$ /SMC (SMC monocultured). EC-CM was ultracentrifuged to fractionate the components in spin-down pellets (Pellet; which was reconstituted with M199), or the remaining supernatant (Super). The levels of miR-126 in **(E)** the fractionated EC-CM or in **(F)** SMCs incubated with total or fractionated EC-CM for 3 hours. **G**, The transcript levels or **(H)**, protein levels of indicated genes in SMCs incubated with the fractionated EC-CM. Images are representative of triplicates with similar results. \* $P < 0.05$  vs CL media.

WT ECs increased the miR-126 levels in SMCs, whereas coculture with the KO ECs did not (Figure 4D).

We separated the extracellular vesicles from the human umbilical vein EC-derived CM by ultracentrifugation and assayed the miR-126 levels in the vesicle-containing pellets and the vesicle-poor supernatant. Approximately 60% of the extracellular miR-126 was found to be present in the supernatant, and  $\approx 40\%$  was in the pellet (Figure 4E), demonstrating that the EC-derived extracellular miR-126 is not restricted to the vesicles. Treatment of SMCs with the supernatant led to an increase of SMC miR-126 level comparable to that resulting from the unseparated EC-CM, but such an increase of miR-126 was not observed in SMCs treated with the reconstructed media containing the precipitated pellet (Figure 4F). These observations are consistent with the results in Figure 4A in suggesting that the EC-derived vesicle-free miR-126 is the main contributor to the miR-126 enrichment in SMCs.

To further examine the roles of miR-126 in the vesicle versus vesicle-free components of EC-CM in SMC gene repression, the SMCs were treated with total EC-CM, supernatant, or pellet. The total EC-CM and the vesicle-free supernatant reduced

the transcript and protein levels of FOXO3, BCL2, and IRS1 in SMCs (Figure 4G and 4H). In contrast, the EC-derived vesicle-containing pellet did not significantly alter the expressions of these miR-126 targets (Figure 4G and 4H). These results show that ECs transmit functional vesicle-free miR-126 to the recipient SMCs to regulate the miR-126-targeted genes.

A recent study demonstrated that suppression of the ceramide signaling pathway through chemical inhibition of nS-Mase2 with GW4869 resulted in the attenuation of cellular miR export by exosomes and a significant reduction of specific extracellular miRs.<sup>20</sup> In this study, we found that treatment of ECs with 20  $\mu\text{mol/L}$  GW4869 for 2 hours did not affect the increase of miR-126 in the cocultured SMCs (Online Figure IVA), indicating that the ceramide-triggered secretion of exosomes is not required for the transmission of endogenous miR-126 from ECs to SMCs. It has also been reported that apoptotic body can mediate the transmission of miR-126 from serum-starved apoptotic ECs to the nearby ECs.<sup>13</sup> We tested the involvement of apoptotic body in mediating miR-126 delivery from ECs to SMCs by pretreatment of ECs with apoptosis inhibitor at a concentration of 50 or 100  $\mu\text{g/mL}$  1

hour before coculture. Apoptosis inhibitor treatment changed neither the miR-126 level in EC-CM nor that in the cocultured SMCs (Online Figure IVB), indicating that miR-126 transmission from ECs to SMCs is not mediated by apoptotic body.

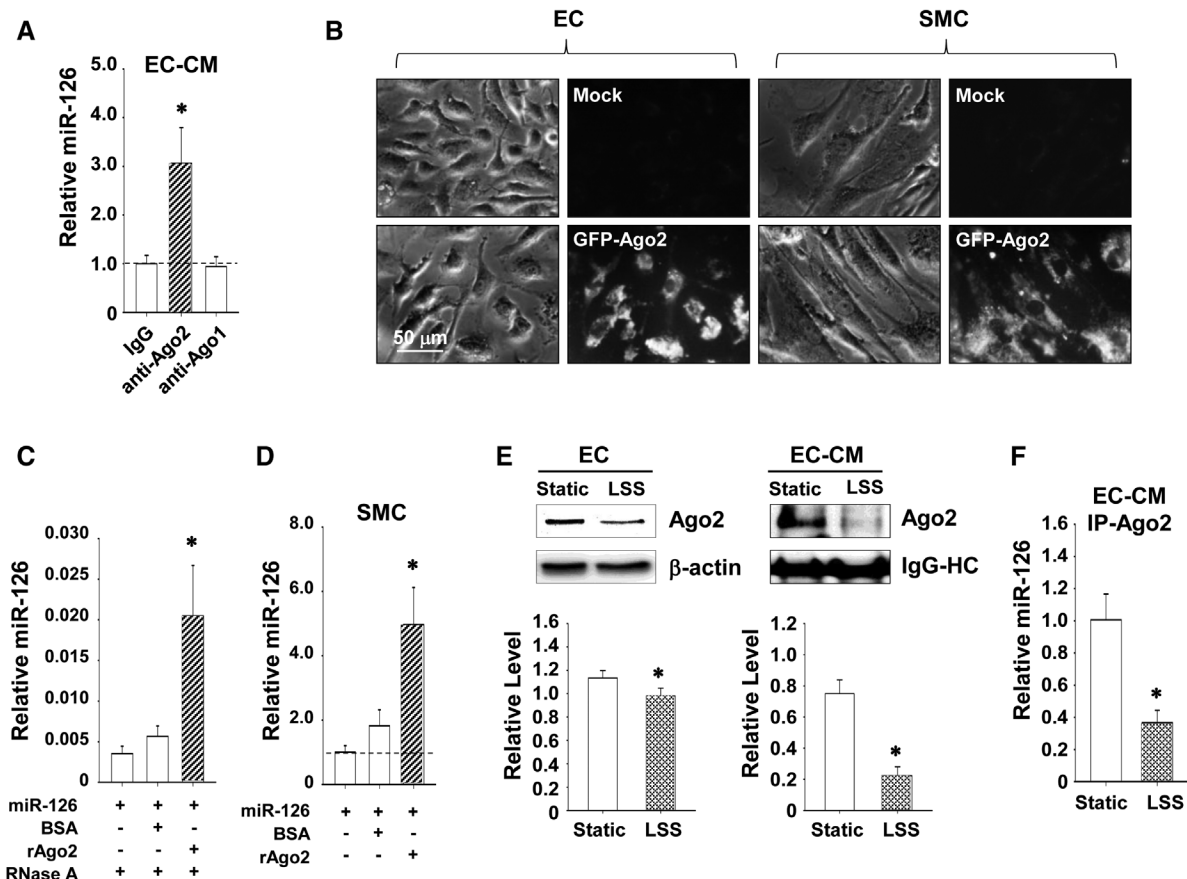
### Ago2 Carries miR-126 and Facilitates Its Intercellular Transmission

Because protease-sensitive components are involved in the transmission of miR-126 from ECs to SMCs (Figure 4A), we next identified the RNA-binding proteins that may stabilize the miRs secreted by ECs and thereby facilitate its uptake by SMCs. Ago2 has been reported to associate with miRs in the circulating blood independently of vesicles.<sup>21</sup> We then tested the possibility that Ago2 and miR-126 can form an extracellular complex with a vesicle-excluded mechanism.

Coimmunoprecipitation experiments revealed that Ago2, but not Ago1, exhibited a strong association with miR-126 in the EC-CM (Figure 5A), suggesting that extracellular miR-126 is bound to Ago2. Moreover, when the bovine aortic ECs expressing eGFP-Ago2 fusion proteins were cocultured with SMCs for 6 hours, the eGFP fluorescence can be detected in both ECs and SMCs (Figure 5B), indicating the transmission

of Ago2 from ECs to the recipient SMCs. To confirm the Ago2-coordinated delivery of miR-126 from ECs to SMCs, bovine aortic ECs cotransfected with synthetic miR-126 and FLAG-Ago2 or the negative controls FLAG-histone deacetylase 4 (FLAG-CL1) and FLAG-histone deacetylase 5 (FLAG-CL2) that do not contain miR-126 seeding sequences were cocultured with SMCs and subjected to immunoprecipitation. As expected, anti-FLAG antibody was effective in capturing cellular miR-126 in ECs transfected with the FLAG-Ago2 construct but not the FLAG-CL1 or FLAG-CL2 constructs (Online Figure VA). We detected a significantly larger amount of miR-126 associated with FLAG-Ago2 in SMCs as compared with FLAG-CL1 or FLAG-CL2, which exhibited a basal miR-126 enrichment that is probably attributable to the overexpression of synthetic miR-126 in the cocultured ECs (Online Figure VB). The immunoprecipitation specificity was confirmed using anti-FLAG antibody and immunoglobulin G control (Online Figure VC).

To determine whether Ago2 affects miR-126 enrichment in SMCs via miR-126 transmission from ECs to SMCs in addition to its inhibition of miR-126 synthesis in ECs



**Figure 5. Argonaute2 (Ago2) associates with microRNA-126 (miR-126) and facilitates its transmission.** **A**, The levels of miR-126 in endothelial cell-conditioned media (EC-CM) immunocomplexes of Ago2 or Ago1 or control immunoglobulin G (IgG). \* $P < 0.05$  vs IgG control. **B**, Smooth muscle cells (SMCs) were cocultured with bovine aortic ECs transfected with a plasmid encoding the eGFP-Ago2. The enhanced green fluorescent protein-Ago2 proteins were visualized in live ECs or SMCs by microscopy. **C**, The protection of miR-126 with preincubation of bovine serum albumin (BSA) or recombinant human Ago2 protein (rAgo2) for 30 minutes after RNase A digestion for 15 minutes. \* $P < 0.05$  vs miR-126 only. **D**, The enrichment of miR-126 in SMCs treated with miR-126 preincubated with BSA or rAgo2 for 3 hours. \* $P < 0.05$  vs miRs only. **E**, The levels of Ago2 in ECs (left) or EC-CM (right) under static or laminar shear stress (LSS) conditions for 24 hours. **F**, The levels of miR-126 in Ago2 immunocomplex in EC-CM under static or LSS conditions. Images are representative of triplicates with similar results. Semiquantification results are shown in the lower panels. \* $P < 0.05$  vs static.



(Figure 4B), we cotransfected ECs with synthetic miR-126 and Ago2-specific siRNA (siEIF2C2) to circumvent the miR-126 biosynthesis. SMCs were treated with EC-CM collected from ECs transfected with miR-126/siEIF2C2 or negative control siRNA, control without miR-126, or mock transfection. Depletion of Ago2 in ECs attenuated the increase of miR-126 in SMCs in response to EC-CM with high miR-126 content (Online Figure VIA), suggesting that Ago2 affected miR-126 transmission in addition to its synthesis. Complementary experiments revealed that knockdown of Ago2 with siEIF2C2 also impaired the delivery of *Caenorhabditis elegans*-miR-39 (cel-miR-39) from ECs to the SMCs (Online Figure VIB), indicating the requirement of Ago2 in mediating the extracellular transfer of another miR from EC to SMC.

To address whether Ago2 contributes to the stability of miR-126 in the extracellular environment, we tested its ability to protect miR-126 from RNase digestion. Synthetic miR-126 was incubated with the recombinant human Ago2 or bovine serum albumin control or was left alone and then subjected to RNase digestion. A marked increase of RNase resistance was seen when miR-126 was incubated with recombinant human Ago2 compared with the miR-126/bovine serum albumin mixture or miR-126 only (Figure 5C). To investigate whether Ago2 facilitates miR uptake by the recipient cells, SMCs were treated with synthetic miR-126 or cel-miR-39 that had been preincubated with recombinant human Ago2 or bovine serum albumin. The resulting high levels of miR-126 and cel-miR-39 in SMCs indicate that Ago2 positively contributed to miR-126 and cel-miR-39 uptakes by SMCs (Figure 5D and Online Figure VIC). Bovine serum albumin also moderately increased the entry of cel-miR-39 into SMCs (Online Figure VIC), suggesting a nonspecific effect of RNA-protein interaction in mediating the transmission of miRs between cells. Complementary experiments indicated that endogenous miR-126, miR-21, miR-221, and miR-155 were associated with Ago2 in EC-CM and that these associations were not decreased by the treatment of ECs with the nSMase2 inhibitor GW4869 (Online Figure VII), suggesting that the Ago2-facilitated miRs transmission may not be miR-126 specific.

### SS Regulates Expression and Secretion of Ago2 in ECs

Because LSS regulates miR-126 secretion in ECs and hence the EC-to-SMC transmission of miR-126 (Figure 1B and 1C), we reasoned that LSS may also regulate Ago2 secretion in ECs. LSS slightly decreased the expression level of Ago2 in ECs but markedly reduced the abundance of Ago2 and the Ago2-bound miR-126 level in the EC-CM (Figure 5E and 5F). Thus, the results suggest that the LSS-induced reduction of miR-126 release from ECs and the subsequent decrease of its uptake by SMCs are likely a result of the decreases in EC expression and secretion of Ago2. Although OSS also decreased Ago2 expression and secretion and the Ago2-bound miR-126 in the EC-CM (Online Figure VIIIA through VIIIC), it did not decrease miR-126 level in CM (Online Figure IB); it is possible that OSS also regulates other pathways to modulate miR-126 transmission.

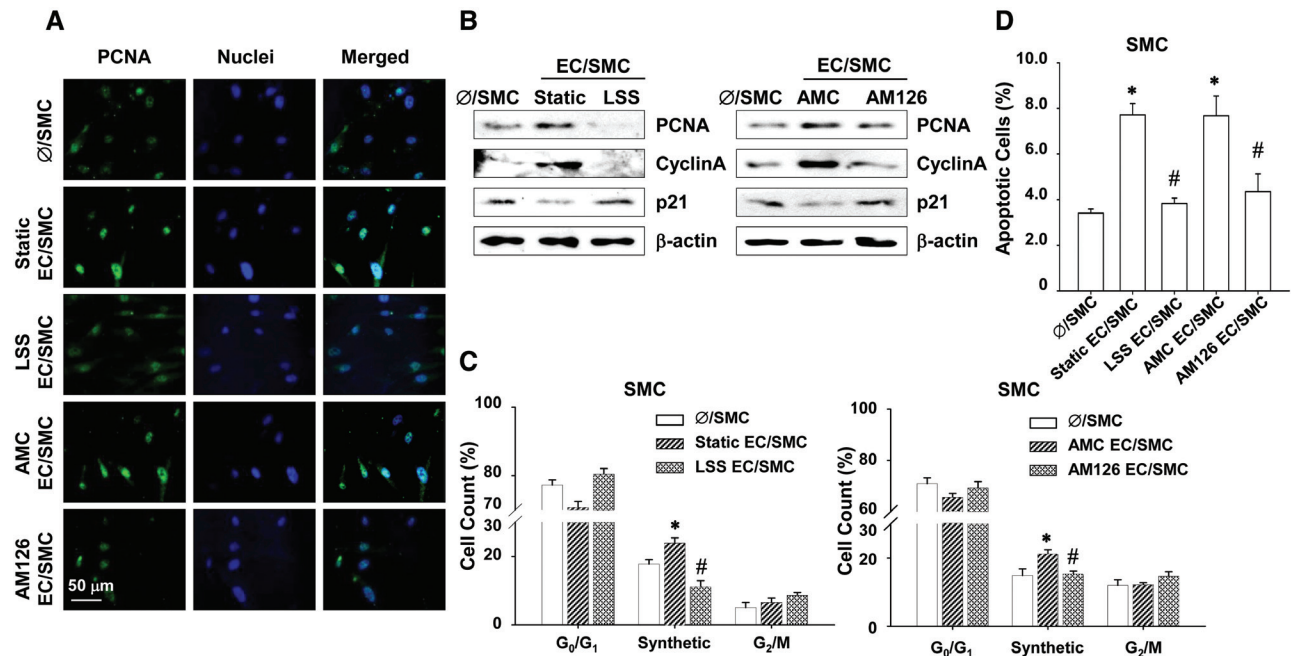
### EC-Coculture and LSS Modulate SMC Turnover via miR-126

Our data indicate that the EC-secreted miR-126 inhibits its targets FOXO3, BCL2, and IRS1 in the recipient SMCs. Because FOXO3, BCL2, and IRS1 are involved in cell proliferation, cell cycle progression, and apoptosis,<sup>22–24</sup> we sought to identify the link between miR-126 transmission and the cellular functions modulated by EC-secreted miR-126 in SMCs. SMCs cocultured with ECs had increased cell percentage with strong nuclear expression of proliferating cell nuclear antigen (PCNA) as compared with monocultured control SMCs (Figure 6A). Inhibition of miR-126 in ECs or exposure of ECs to LSS significantly reduced nuclear PCNA expression in SMCs (Figure 6A). The miR-126-mediated alteration of PCNA expression was confirmed by Western blot (Figure 6B). Furthermore, EC-coculture upregulated cyclinA and downregulated the cyclin-dependent kinase inhibitor p21 in SMCs (Figure 6B). These EC-induced changes in gene expression of SMC were reversed by LSS or inhibition of miR-126 in the ECs (Figure 6B) or in the SMCs (Online Figure IXB). Cell cycle analysis indicates that application of LSS to ECs not only abolished the increased percentage of SMCs in S phase in the coculture but also actually decreased it to a level lower than that of the monoculture control. Inhibition of miR-126 in ECs also reversed the cell cycle progression resulting from EC-coculture (Figure 6C). We also found that the percentage of apoptotic SMCs was increased by coculture with ECs and that this increase was attenuated by LSS or inhibition of miR-126 in ECs or SMCs (Figure 6D and Online Figure IXD). Taken together, these results suggest that the EC-derived miR-126 increases cell proliferation, cell cycle progression, and apoptosis of cocultured SMCs. Under LSS, these impaired SMC functions are corrected by the inhibition of humoral transmission of miR-126 from ECs to SMCs.

To characterize the mechanisms by which miR-126 promotes SMC turnover, the 3 direct targets of miR-126, FOXO3, BCL2, and IRS1,<sup>25</sup> were overexpressed in SMCs, which were then cocultured with ECs. Overexpression of any of these 3 genes attenuated the EC-coculture-induced expression of PCNA, but not cyclinA, and reversed the effects of coculture on expression of p21 (Online Figure XA). Overexpression of FOXO3 abolished the increased percentage of SMCs in S phase in the coculture (Online Figure XB), whereas overexpression of BCL2 inhibited the increased percentage of SMCs undergoing apoptosis induced by coculture (Online Figure XC). These results suggest that the miR-126-induced SMC proliferation, cell cycle progression, and apoptosis are at least partially attributable to its repression of FOXO3, BCL2, and IRS1.

### MiR-126 Promotes Neointimal Formation in Mouse Carotid Artery After Ligation

We used the miR-126 KO mice with a ligation-induced neointimal growth model<sup>19</sup> to further determine the effect of miR-126 on the formation of intimal lesion. The left common carotid artery of miR-126 WT or KO mice was ligated near the



**Figure 6. Shear stress (SS)-imposed endothelial cells (ECs) modulate smooth muscle cell (SMC) turnover through microRNA-126 (miR-126).** SMCs were monocultured or cocultured with (1) ECs kept under static condition or exposed to laminar shear stress (LSS) for 24 hours; or (2) ECs transfected with negative control inhibitor (AMC) or anti-miR-126 inhibitor (AM126). **A**, Proliferating cell nuclear antigen (PCNA) in the SMCs was detected by immunostaining. **B**, Protein levels of PCNA, cyclinA, and p21 in SMCs were assayed by Western blot. **C** and **D**, SMC proliferation and apoptosis analyzed for propidium iodide-stained DNA content and Annexin V-stained membrane flip, respectively. \* $P < 0.05$  vs  $\emptyset$ /SMC (SMC monocultured). # $P < 0.05$  vs Static EC-SMC or AMC EC-SMC.

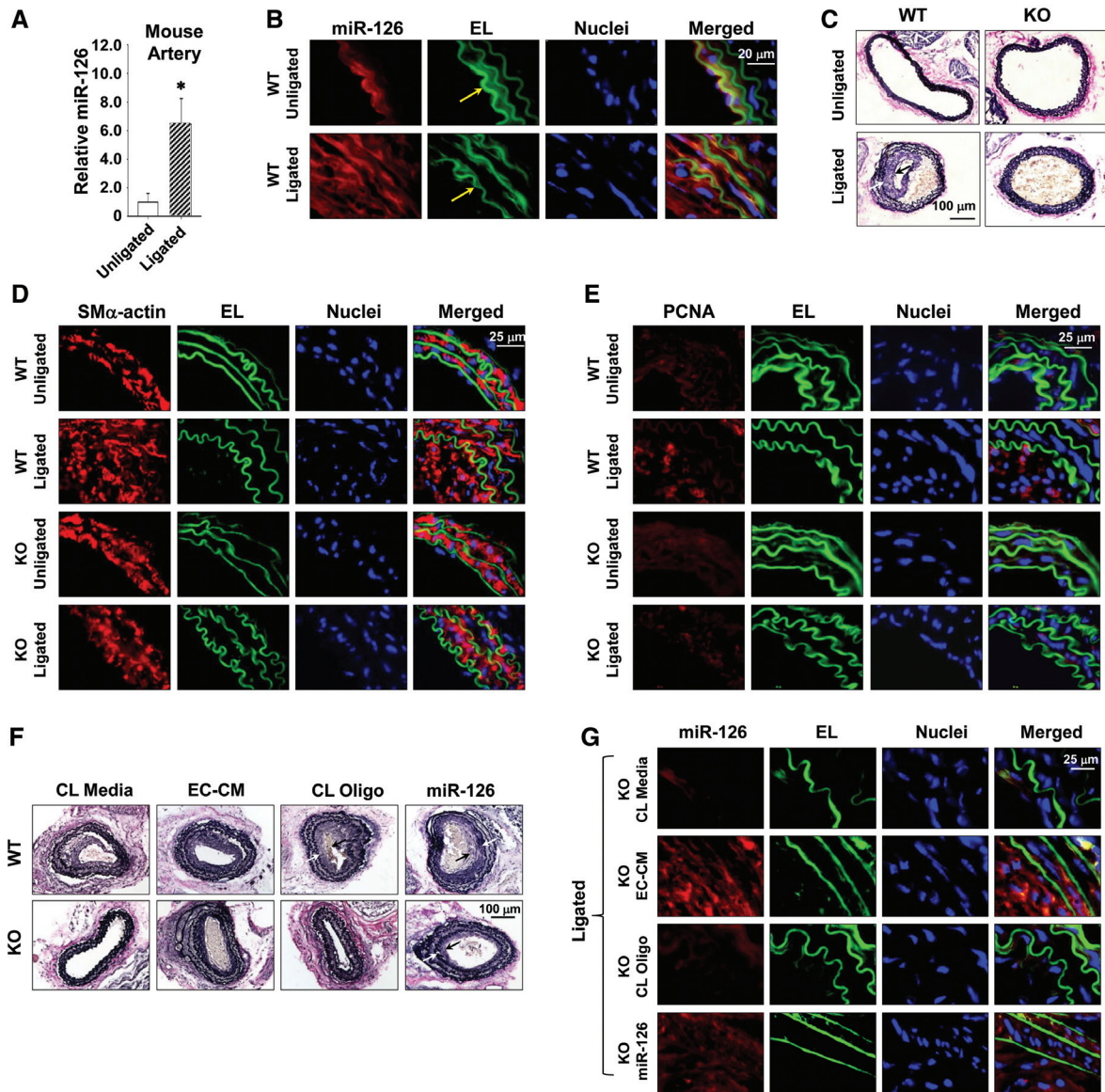
bifurcation to stop the blood flow, and the right carotid artery was sham operated as a control. Both sides of carotid arteries were isolated 4 weeks after surgery for the measurements of miR-126 and other molecules and the analysis of histology. In the WT mice, ligation of carotid arteries increased the miR-126 level in the vessels (Figure 7A), promoted the abundance of miR-126 in the intima-media layer (Figure 7B), and induced neointimal thickening. The presence of endothelium on the luminal surfaces of ligated and unligated carotid arteries was verified by immunostaining for platelet EC adhesion molecule 1 (Online Figure XI). In the KO mice, the ligated arteries displayed a marked reduction in neointimal growth, as indicated by elastic staining (Figure 7C), and the proliferation of SMCs, as indicated by smooth muscle  $\alpha$ -actin and PCNA immunostaining, compared with those of WT mice (Figure 7D and 7E).

We reintroduced miR-126 into KO mice by repetitive intravenous injection (twice a week) of CM from 3-day cultured ECs or local delivery of pluronic gel-loaded PRE126 to the ligated arteries beginning immediately after ligation. Carotid arteries of the KO mice that received long-term treatment with EC-CM exhibited an increase in neointimal growth in response to ligation compared with those treated with control media (Figure 7F). Local delivery of miR-126 in KO mice also enhanced neointimal formation (Figure 7F). Those outcomes coincide with the abundance of miR-126 in the arteries, as indicated by in situ hybridization detection in carotid arteries from KO mice with or without the administration of miR-126 (Figure 7G). The results from these experiments establish the atheroprone role of miR-126 in SMC function.

## Discussion

Endothelial miR-126 has been shown to play a role in governing vascular integrity and angiogenesis.<sup>15,16</sup> Although miR-126 serves as a beneficial regulator for EC homeostasis, the functions of miR-126 in the neighboring SMCs remain unclear. In the present study, we have demonstrated that miR-126 serves as a paracrine mediator secreted by the EC to act on the cocultured SMC to modulate its gene expression and function toward atherogenic phenotype. The paracrine humoral transmission from EC to SMC is proven using the exogenous B-miR-126 (Figure 3A and 3B), which provides direct evidence that the EC is the origin of miR-126 transmission and that cell-cell communication between ECs and SMCs can be mediated by the humoral transmission of an miR. We also showed that the increase in SMC miR-126 on coculture with EC or in response to EC-CM was changed in the expected directions by manipulating the amount or biogenesis of miR-126 in ECs (Figure 4B through 4D), thus further confirming that the EC is the origin of the miR-126 enrichment in cocultured SMCs. The inhibition of FOXO3, BCL2, and IRS1 mRNAs in SMCs by miR-126 and coculture with ECs (Figure 2) indicates that the transmission of miR-126 from ECs to SMCs mediates the EC regulation of SMC gene expression.

Several recent studies found that vesicle-mediated transfer of miRs can transduce signals between cells.<sup>9,13,14,20</sup> Hergenreider et al<sup>14</sup> showed that ECs transfer the vesicle-packaged miR-143/miR-145 to SMCs to enhance the repression of dedifferentiation-associated genes in SMCs.<sup>14</sup> Zerneck et al<sup>13</sup> reported that EC-derived apoptotic bodies convey paracrine alarm signals to the nearby recipient ECs. Our data indicate that RNAs and RNA-protein complexes



**Figure 7. Depletion of microRNA-126 (miR-126) in mice inhibits smooth muscle cell (SMC) proliferation and neointimal formation in arteries after cessation of blood flow.** **A**, The miR-126 expression levels in mouse carotid arteries with or without ligation. Vascular endothelial cells (ECs) were denuded before RNA isolation. \* $P < 0.05$  vs unligated control. **B**, In situ hybridization of miR-126 in ligated or unligated carotid arteries from miR-126 wild-type (WT) mice. **Yellow arrows**, elastic lamina (EL). **C**, Representative images of modified Verhoeff-Van Gieson–stained ligated or unligated carotid arteries from WT or miR-126 knockout (KO) mice. **Arrows** indicate the thickened neointima. **D** and **E**, Representative images of immunofluorescent-stained proliferating (proliferating cell nuclear antigen [PCNA]) SMC (smooth muscle- $\alpha$  actin) in ligated or unligated carotid arteries from WT and KO mice. **F**, Representative images of modified Verhoeff-Van Gieson–stained ligated carotid arteries from WT or KO mice treated with control (CL) media or EC-conditioned media (EC-CM); or with local delivery of CL oligo or miR-126 mimics (PRE126). **Arrows** indicate thickened neointima. **G**, In situ hybridization of miR-126 in ligated carotid arteries from KO mice with treatment of EC-CM, miR-126, or the respective controls.

are transmitted into SMCs, but not DNA components or the vesicle-protected RNA (Figure 4A). Treatments of SMCs with the fractions of the ultracentrifuged EC-CM showed that the vesicle-free supernatant caused an increase in SMC miR-126 (Figure 4F), indicating that the EC-derived vesicle-free miR-126 is the main contributor to the miR-126 enrichment in SMCs. Besides miR-126, miR-21, miR-221, and miR-155 were also detected to be associated with Ago2 in EC-CM (Online Figure VII), supporting the hypothesis that the Ago2-facilitated miRs transmission is a general mechanism regulating cell-cell communications. The extracellular Ago2-associated

miR-143 or miR-145 was undetectable (Online Figure VII), consistent with a previous report indicating that EC-secreted miR-143 and miR-145 were packaged into vesicles.<sup>14</sup> The current study provides an alternative mechanism that is distinct from those in previous reports in which the EC-specific miRs are exported and delivered through exosomal or apoptotic pathways.

Turchinovich et al<sup>6</sup> and Arroyo et al<sup>21</sup> reported that most miRs in human peripheral blood are ultrafiltratable together with Ago2<sup>6,21</sup> and that miR-16, miR-21, miR-24,<sup>6</sup> or miR-16, 92a, and let-7a<sup>21</sup> are associated with Ago2 in the culture media

of Michigan Cancer Foundation-7 cells or in the human plasma. Other proteins that bind miRs and deliver miRs to recipient cells independently of vesicles include nucleophosmin 1<sup>8</sup> and high-density lipoprotein.<sup>7</sup> Our results showed that Ago2 exhibited a strong association with miR-126 in the CM (Figure 5A) and the recipient SMCs (Figure 5B) and carried miR-21, miR-221, miR-155 (Online Figure VII), and probably other miRs in the EC-CM. The amount of miR-126 associated with Ago2 was not decreased when the exosome- or apoptotic body-related miR export pathways were chemically inhibited in ECs (Online Figure IV). Moreover, our results show that Ago2 positively contributed to the stability of miRs and their uptake by SMCs (Figure 5C and 5D; Online Figure VIC). The enhancement of cel-miR-39 uptake by Ago2 indicates that this effect of Ago2 is not limited to miR-126. Our data also demonstrate that LSS inhibits the expression and secretion of Ago2 in ECs (Figure 5E and 5F). These findings may provide a mechanistic explanation of how LSS regulates the miR-126 release and its transmission from ECs to SMCs. Our study and previous findings<sup>6–8,24</sup> support a notion that both vesicle-dependent and vesicle-independent pathways may be involved in the humoral transmission of miRs.

In our EC-SMC coculture flow system, ECs and SMCs were separated by a porous membrane with a 0.4- $\mu$ m pore size that does not allow physical contact between ECs and SMCs but allows communications via humoral transmission through the short distance between these cells. This system has been used previously to investigate the interaction of endothelium and SMCs under physiological and pathophysiological conditions.<sup>3,26</sup> Coculture of static ECs with SMCs is known to induce SMC activation,<sup>3,27–29</sup> but the underlying molecular mechanisms and signaling pathways were unclear. Our current findings indicate that miR-126 controls SMC gene expression in a paracrine manner to mediate the EC promotion of SMC turnover in terms of cell proliferation, enhancement of synthetic phase of the cell cycle, and apoptosis (Figure 6). This EC-promotion of SMC turnover was abolished or attenuated by the application of LSS to the ECs or inhibition of miR-126 in either ECs (Figure 6) or SMCs (Online Figure IX). However, overexpression of miR-126 by PRE126 alone in monocultured SMCs did not activate the cells (data not shown). Such nonsymmetrical phenotypic effects have been reported in other miR studies that showed that although miR depletion caused phenotypic changes, miR overexpression showed little effects. For example, miR-133a KO mice had increased proliferation and apoptosis of cardiomyocytes compared with WT, but cardiac overexpression of miR-133a did not affect apoptosis<sup>30</sup>; inhibition of miR-222 in cultured SMCs increased the expression of p21 and p57, but overexpression of miR-222 had no impact on p57 mRNA level.<sup>31</sup> The mechanisms underlying the function of certain miRs seem to be context dependent. In our case, the functional outcomes caused by the alteration in miR-126 levels in SMCs resulting from EC-coculture versus miR-126 overexpression might reflect the diversity of regulatory pathways triggered by different physiological/pathophysiological microenvironments. The actions of LSS in suppressing EC miR-126 secretion and hence the consequential changes of SMC functions provide

further explanation of the miR-mediated atheroprotective effects of LSS.

Vascular SMC proliferation and apoptosis are the critical cellular events in vascular neointimal lesion formation in relation to restenosis and atherosclerosis.<sup>4,32</sup> The lesion originates and develops preferentially at sites of curvature, branching, and bifurcation in arteries, where complex hemodynamic conditions are prominent.<sup>33</sup> In this study, a 3-fold-higher expression of miR-126 was observed in the athero-susceptible aortic arch (Online Figure II), which experiences disturbed and low SS, compared with thoracic aorta, where the SS is laminar and nondisturbed. Cessation of blood flow in carotid arteries also upregulates the level of miR-126 in the vessel wall (Figure 7A and 7B). Furthermore, this study provides evidence that neointimal lesion formation after disruption of LSS in mice carotid arteries can be suppressed by systemic depletion of miR-126 (Figure 7C through 7F). These findings help to identify the contribution of miR-126 in neointimal hyperplasia and to establish the atheroprone role of miR-126 in SMCs despite its beneficial effects on endothelial functions.

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### Disclosures

None.

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## Novelty and Significance

### What Is Known?

- In endothelial cells, microRNA (miR)-126 participates in maintaining vascular integrity, enhancing angiogenesis, and inhibiting inflammation, but its action on smooth muscle cells (SMCs) is unknown.
- The laminar shear stress seen in the atheroprotected regions of the vasculature is antiatherogenic.
- Application of laminar shear stress to endothelial cells cocultured with SMCs decreases SMC proliferation and promotes a change in SMC phenotype from synthetic to quiescent state.

### What New Information Does This Article Contribute?

- MiR-126 can be transmitted from endothelial cells to cocultured SMCs via a paracrine mechanism that involves the intercellular communicating molecule Argonaute2 to promote SMC proliferation.
- Laminar shear stress applied to endothelial cells inhibits transmission of miR-126 from endothelial cells to cocultured SMCs, thus suppressing SMC proliferation.

- Curtailment of blood flow (decrease in lamina shear stress) induces proliferative proatherosclerotic changes in SMCs in wild-type mice, and this action is abrogated in miR-126 null mice.

Endothelial miR-126 has been shown to play a role in maintaining vascular integrity and facilitating angiogenesis, thus serving as an important regulator of EC homeostasis. Nevertheless, the functions of miR-126 in the neighboring SMCs are not known. We demonstrate that miR-126 can be secreted by endothelial cells and then taken up by the cocultured SMCs to promote SMC proliferation, cell cycle progression, and apoptosis. Exposure of endothelial cells to laminar shear stress inhibits its miR-126 secretion to abrogate the atherogenic actions of miR-126 on the cocultured SMCs. This antiatherogenic effect of shear stress mediated by inhibition of miR-126 secretion in vitro was confirmed in vivo in a carotid artery ligation model in which neointimal lesion formation seen in wild-type mice was abrogated in miR-126 null mice. These results indicate that the atheroprotective hemodynamic force acting on endothelial cells exerts its antiatherogenic effect on SMCs by inhibiting the paracrine secretion of miR-126 by endothelial cells.

## Supplemental Methods

**Antibodies, Oligonucleotides, Probes, and Recombinant Proteins.** Rabbit polyclonal antibody (pAb) against Ago2 was purchased from Cell Signaling Technology. Goat pAb against PCNA; rabbit pAbs against BCL2, and cyclin A; and mouse monoclonal antibodies (mAbs) against p21 and  $\beta$ -actin were obtained from Santa Cruz Biotechnology. Rabbit pAb against FOXO3 was from Millipore. Mouse mAb against SM $\alpha$ -actin was from Sigma. 5'-DIG and 3'-DIG labeled miR-126 locked nucleic acid (LNA) detection probe was from Exiqon. Dylight 594 anti-DIG antibody was from Jackson ImmunoResearch. Ago2 (eIF2C2), Dicer (DICER1), and Drosha (DROSHA)-specific and control siRNA were purchased from Santa Cruz Biotechnology. Anti-miR-126 inhibitor (AM126), miR-126 precursors or mimics (PRE126) and the respective negative control inhibitors (AMC) and mimics (PREC), with random sequences that produce no identifiable effects on known miR function, were purchased from Ambion. MiR-126 oligonucleotides were synthesized by Valuegene. Human Argonaute2 recombinant protein (rAgo2, full length) was obtained from Sino Biological.

**Cell Culture.** Human umbilical vein ECs (HUVECs) were cultured in medium 199 (Gibco) supplemented with 10% fetal bovine serum (FBS) (Omega Scientific) and 10% Endothelial Growth Medium (Cell Applications). Human umbilical artery SMCs (HUASMCs) were cultured in Nutrient Mixture F12 Ham Kaighn's Modification (Sigma-Aldrich) supplemented with 10% FBS and 50% SMC Growth Medium (Cell Applications). Bovine aortic ECs (BAECs) were maintained in Dulbecco's Modified Eagle's Medium (Gibco) supplemented with 10% FBS.

**Co-culture Modules and Parallel-plate co-culture Flow System.** EC/SMC co-culture was established by plating ECs and SMCs on the lower and upper sides, respectively, of a 10- $\mu$ m-thick porous polyethylene terephthalate (PET) membrane (Falcon cell culture inserts; Becton Dickinson) with 0.4- $\mu$ m pores configured at a density of  $1.6 \times 10^6$  pores/cm<sup>2</sup>. ECs and SMCs were maintained in medium 199 or F12 Ham Kaighn's Modification (F12K), respectively, supplemented with 2% fetal bovine serum (FBS) until fully attached to the membrane. After the completion of EC/SMC co-culture, the inserts were incorporated into a parallel-plate flow chamber containing a polycarbonate insert holder, as previously described<sup>1</sup>. The chamber is connected to a perfusion loop system, which was kept in a constant-temperature controlled enclosure, with pH maintained at 7.4 by continuous gassing with a humidified mixture of 5% CO<sub>2</sub> in air. The flow of the perfusate in the channel is laminar. The fluid shear stress ( $\tau$ ) generated on the ECs seeded on the membrane can be estimated as  $\tau = 6Q\mu/wh^2$ , where Q is the flow rate and  $\mu$  is the dynamic viscosity of the perfusate. In Laminar shear stress (LSS) experiments, ECs were exposed to LSS with a high level of mean shear stress at 12 dynes/cm<sup>2</sup> for indicated hours. The oscillatory shear stress (OSS) is composed of a low level of mean flow (shear stress = 0.5 dynes/cm<sup>2</sup>) supplied by a hydrostatic flow system to provide the basal nutrient and oxygen delivery, and a superimposed sinusoidal oscillation using a piston pump with a frequency of 1 Hz and a peak-to-peak amplitude of  $\pm 4$  dynes/cm<sup>2</sup>. In some experiments, the conditioned media collected from static or 24 hr-sheared ECs seeded on glass slides pre-coated with collagen I (BD Biosciences) were used to study the paracrine effect of factor(s) released from ECs.

**Preparation and Fractionation of Conditioned Media (CM), Deoxyribonuclease (DNase), Ribonuclease (RNase) and Proteinase Treatment.** HUVECs with or without treatment were

cultured in medium 199 supplemented with 2% FBS for 24 hr and the media were collected and centrifuged at 1000g for 10 min to remove cell debris. The supernatant (designated as EC-CM or Total EC-CM) was transferred to a new tube and spun at 120,000 g for 120 min. The final supernatant was collected. The pellets were resuspended in medium 199 supplemented with 2% FBS of an identical volume with the Total EC-CM. For the DNase, RNase, or proteinase treatment, EC-CM were incubated with DNase I (1 unit/ml, Invitrogen) or RNase A (10 µg/ml, Invitrogen) at 37 °C for 15 min, or with proteinase K (PK, 20 µg/ml, Invitrogen) at 55 °C for 15 min and then at 95 °C for 5 min to inactivate the PK.

**RNA Isolation and Quantitative RT-PCR.** RNA was extracted from cultured cells by using the *mirVana* miRNA Isolation Kit (Ambion) or TRIzol reagent (Life Technologies) according to the manufacturer's instructions. For the isolation of miRNAs from liquid samples, 5 pg of synthetic *C. elegans* miRNA *cel-miR-39* was added to each sample as a spike-in control for purification efficiency and RNA was extracted using the *mirVana* PARIS kit (Ambion) following the manufacturer's protocol for liquid samples. Isolated RNAs were reversed-transcribed into complementary DNA with M-MLV RT system (Invitrogen) by using the Taqman primer sets for miRs (Applied Biosystems) or Oligo(dT) primers for the others. Real-time PCR was performed with the Taqman Fast Universal PCR Master Mix (Applied Biosystems) or iQ SYBR Green Supermix (Bio-Rad) by using the Taqman probes (Applied Biosystems) or specific primer pairs (FOXO3, 5'-cttgcgtattgggtgaaca-3', 5'-tccgctcaagacctcattt-3'; BCL2, 5'-ttggatcagggagttggaag-3', 5'-ccatgctgatgtctctgga-3; IRS1, 5'-gtttccagaagcagccagag-3', 5'-tgaaatggatgcatcgtacc-3'). Primary and mature miR expression levels were normalized against the control RNU48 or Cel-miR-39 probes. Expressions of FOXO3, BCL2, and IRS1 were normalized against GAPDH.

**Transient Transfection and Luciferase Reporter Assay.** For gain- and loss-of-function studies of miRs, HUVECs or HUASMCs at 80% confluence were transfected with anti-miR-126 inhibitor (AM126), miR-126 precursors or mimics (PRE126), or the respective negative control molecules (30 nmol/L) using siPORT *NeoFX* transfection agent (Ambion) according to the manufacturer's instructions. For inhibition of gene expression, 40 nM of siRNA was used. For luciferase assay, the cells were co-transfected by using Lipofectamine 2000 reagent (Invitrogen) with 0.2 µg of respective DNA with or without the presence of AM126 or PRE126 or the negative control molecules per  $2 \times 10^5$  cells, following a standard protocol. For forced expression of eGFP- or FLAH/HA-Ago2 fusion proteins, bovine aortic ECs (BAECs) were co-transfected by using Lipofectamine 2000 reagent with or without the presence of miR-126 oligonucleotides. For luciferase assay, the pSV-β-galactosidase plasmid was co-transfected with the luciferase reporter vectors to normalize the transfection efficiency. Twenty-four hours post-transfection, luciferase activity was measured using the Luciferase assay system (Promega) and normalized to the β-galactosidase activity assessed using *o*-nitrophenyl-β-D-galactopyranoside.

**Plasmids.** Plasmids expressing eGFP-hAgo2 (21981)<sup>2</sup>, FLAG/HA-hAgo2 (10821)<sup>3</sup>, FLAG-FOXO3a (8360)<sup>4</sup>, FLAG-Bcl2 (18003)<sup>5</sup> were obtained from Addgene. Plasmid expressing HA-tagged human IRS1<sup>6</sup> was kindly provided by Dr. Michael J. Quon from University of Maryland. To generate the luciferase reporter constructs, the 3'-UTRs of FOXO3 (6106-6672 of its mRNA, 566 bp), BCL2 (5165-5569 of its mRNA, 403 bp), or IRS1 (3735-4215 of its mRNA, 480 bp) harboring the predicted

miR-126 binding sequences were PCR-amplified from human genomic DNA and cloned into HindIII and SpeI of the pMir-Report luciferase vector (Ambion). Mutagenesis of predicted miR-126 binding sequences was performed following Quikchange site-direct mutagenesis protocol (Stratagene).

**Immunoprecipitation and Co-immunoprecipitation Assay.** Cells were trypsinized and lysed with Lysis Buffer containing 20 mmol/L Tris-HCl, pH 7.4, 150 mmol/L NaCl, 0.5% NP-40, 0.5% sodium deoxycholate, 100 U/ml RNase inhibitor, and a protease inhibitor cocktail (Roche). Protein concentration of the lysate was adjusted to be 2-3  $\mu\text{g}/\mu\text{l}$ , and 8  $\mu\text{g}$  of Ago2- or FLAG-specific antibody or control IgG was added into 100  $\mu\text{l}$  of cell lysate. In some experiments using liquid samples, 200  $\mu\text{l}$  of Lysis Buffer and 10  $\mu\text{g}$  of Ago2-specific antibody or control IgG was added into 800  $\mu\text{l}$  of conditioned media. After incubation and rotation at 4°C overnight, the immune complexes were pulled down with protein A/G Sepharose beads and washed with the lysis buffer. For immunoprecipitation assay, 50  $\mu\text{l}$  of 2 $\times$ SDS buffer was added into each sample, which was then subjected to Western blot analysis. For co- immunoprecipitation assay, 1 ml of TRIzol reagent was added into each sample, and RNA was extracted following a standard protocol. The purified RNA was analyzed by quantitative real-time RT-PCR.

**miR Tracking.** Biotinylation of miRs was carried out using BrightStar Psoralen-Biotin Kit (Invitrogen) according to manufacturer's instructions. The biotinylated miRs were transfected into HUVECs using siPORT *NeoFX* transfection agent at a concentration of 30 nmol/L. In some experiments, the ECs were washed with culture medium 8 hr post-transfection and then cocultured with SMCs for 24 hr. In other experiments, the ECs were washed with culture medium 8 hr post-transfection and incubated with fresh culture medium for another 24 hr. EC-CM were then collected from those ECs and used to treat SMCs. Direct staining of biotinylated miRs in ECs and SMCs was performed using Qdot-605 streptavidin conjugate (Invitrogen). The cells were seeded on sterilized cover slips and fixed with 4% paraformaldehyde and permeabilized with 0.5% Triton X-100 in PBS. The cells were then incubated with 10 nmol/L of Qdot-605 streptavidin conjugate in PBS and rinsed with PBS. Nuclei were stained with Hoechst 33258 (Invitrogen). The cells were dehydrated by submerging the cover slip sequentially in 30%, 50%, 70%, and 90% ethanol/water, twice in 100% ethanol, once in 100% toluene, and the final dip in 100% toluene. After mounting, the slips were visualized by fluorescence microscopy (Olympus). To capture the biotinylated miRs in cells, the transfected ECs or the cocultured SMCs were trypsinized and lysed with Lysis Buffer (as described above). The biotinylated miRs were pulled down with Streptavidin Agarose Resin (Thermo Fisher Scientific) and purified with TRIzol reagent and analyzed by quantitative real-time RT-PCR. For Northern blot of biotinylated miRs in cells, total RNA was extracted from cells with TRIzol reagent and separated by 15% denaturing urea polyacrylamide gel and then transferred to nylon membranes. The biotinylated miRs on membranes were probed with BrightStar BioDetect Kit (Ambion) according to the manufacturer's instructions.

**MiR Protection and Uptake Assay.** To analyze the ability of Ago2 protein in protecting miRs from RNase digestion, synthetic miR-126 or Cel-miR-39 (3 pmol) in 200  $\mu\text{l}$  of medium 199 was kept alone or incubated with rAgo2 (1.5 pmol) or bovine serum albumin (BSA, 1.5 pmol) for 30 min at room temperature followed by incubated with RNase A (5  $\mu\text{g}/\text{ml}$ ) for another 30 min at 37 °C. The miRs from each condition were then purified using *mir*Vana PARIS kit following the manufacturer's



protocol for liquid samples and the miR levels were determined by quantitative RT-PCR. To determine the ability of Ago2 protein in facilitating uptake of miRs by the recipient cells, synthetic miR-126 or Cel-miR-39 (5 pmol) in 100  $\mu$ l of medium 199 was kept alone or incubated with rAgo2 (1.5 pmol) or BSA (1.5 pmol) for 30 min at room temperature and then treated HUASMCs for 3 hr at 37 °C. The cells were then harvested and RNA was extracted from the cells and was analyzed by quantitative real-time RT-PCR.

**Cell Cycle Analysis.** Cells were harvested in PBS containing 2 mmol/L ethylenediaminetetraacetic acid, washed once with PBS, and fixed for 2 hr in cold ethanol (70%). They were then stained with 50  $\mu$ g/mL of propidium iodide (Roche) and 1 mg/mL RNase A for 30 min. Stained cells were analyzed by flow cytometry and the data analysis were processed by FlowJo.

**Annexin V-PI Staining and Flow Cytometry.** Cells were trypsinized from coculture membranes and subjected to Annexin V-PI staining using FITC Annexin V Apoptosis Detection Kit (BD Pharmingen) according to manufacturer's instructions to identify apoptosis. Data were analyzed by CellQuest.

**Immunofluorescence.** Cells were trypsinized from coculture membranes and seeded on sterilized cover slips. After attached well, the cells were fixed with 4% paraformaldehyde and permeabilized with 0.5% Triton X-100 in PBS and nonspecific binding was blocked by 5% BSA in PBS. The cells were probed with primary antibody, washed and then probed with secondary antibody (Santa Cruz). Nuclei were stained with Hoechst 33258. After mounting, the slips were visualized by fluorescence microscopy.

**MiR *in situ* Hybridization.** 6  $\mu$ m cross cryosections were prepared from 4% paraformaldehyde-fixed tissue. MiR fluorescence *in situ* hybridization (FISH) was performed on the sections as described previously<sup>7</sup> with minor modifications. A 5'-digoxigenin and 3'-digoxigenin labeled locked nucleic acid oligonucleotide designed to hybridize to miR-126 was incubated with the tissue sections for over 4 hr at 60 °C. MiR-126 was visualized using the Dylight 594 anti-digoxigenin antibody.

**Western Blot Analysis.** Cells were collected by scraping and lysed with a buffer containing 20 mmol/L Tris-HCl, pH 7.4, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, and a protease inhibitor cocktail (Roche). The total cell lysate was separated by SDS-PAGE (10-12% running, 4% stacking) and transferred onto a polyvinylidene fluoride membrane (Immobilon P, 0.45-mm pore size). The membrane was then incubated with the designated antibodies. Immunodetection was performed using the Gel-imaging System for Life Science (Alpha-Innotech).

**Animal Model.** All animal studies were performed in accordance with National Institutes of Health guidelines and were approved by the UC San Diego IACUC. Male miR-126<sup>+/+</sup> (WT) and miR-126<sup>-/-</sup> (KO) mice (8-12 weeks old, 18-25 g) were anesthetized by intraperitoneal injection (ip) of xylazine and ketamine HCl (5 mg/kg body weight and 80 mg/kg body weight, respectively). The left common carotid artery was dissected and ligated near the carotid bifurcation with the use of 5-0 silk. The wound was sutured and the animals were allowed to recover and showed no symptoms of a stroke. 43 WT and 43 KO mice were subjected to carotid artery ligation. For the systematical delivery of

EC-CM, immediately post-ligation, 15 WT and 15 KO mice were injected intravenously (iv) with 200  $\mu$ l of EC-CM from 3-days cultured HUVECs twice per week for 4 weeks or with control media. For the local application of miR-126, 2  $\mu$ g of PRE126 or scrambled control were preloaded into the 50  $\mu$ l, 25% F-127 pluronic gel (Sigma) at 4°C. Lipofectamine 2000 reagent (Invitrogen) was added at a final concentration of 1%. Immediately after the ligation of carotid artery, the F-127 pluronic gel loaded with those oligonucleotides was applied locally to the adventitia around the ligated artery segments of 12 WT and 12 KO mice. 4 weeks after ligation, the mice were sacrificed and fixed for 5 minutes by perfusion through left cardiac ventricle with 4% *p*-formaldehyde in PBS buffer under physiological pressure. The ligated and unligated carotid arteries were isolated and immersed in fixative solution for 16 hr at 4 °C and then embedded in OCT media. 6  $\mu$ m cross cryosections were prepared from those vessels using cryostat microtome and then subjected to histology or immunostaining assay.

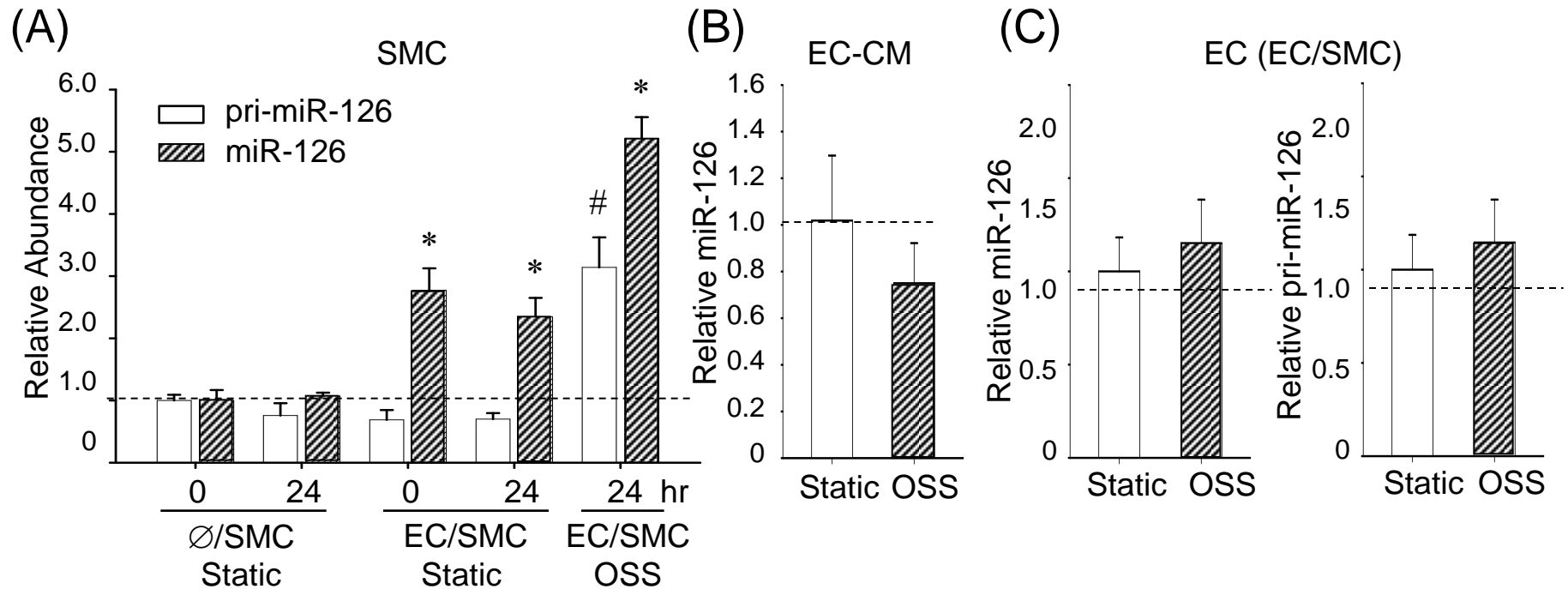
**Isolation of ECs from mouse lung.** Lungs from two young adult mouse were removed aseptically, rinsed in DMEM supplemented with 20% FBS, minced finely with scissors for 1min, and digested in 25 mL of type I collagenase (2 mg/mL) at 37°C for 45 minutes with occasional agitation. The cellular digest was filtered through sterile 70- $\mu$ m disposable cell strainer (Falcon), centrifuged at 400g for 10 minutes, and washed twice with cold PBS containing 0.1% BSA; the cell pellet was resuspended in 2 mL of cold PBS containing 0.1% BSA. Dynabeads M-450 sheep anti-rat IgG (Invitrogen) pre-incubated overnight with rat anti-mouse PECAM-1 antibody were added into the cell suspension at 15  $\mu$ l of beads per 1 ml of cell suspension. After 10 minutes at room temperature with occasional agitation, the bead-bound cells were separated with a magnetic separator, washed five times with DMEM supplemented with 20% FBS, and resuspended in DMEM supplemented with 20% FBS, 100  $\mu$ g/ml Heparin (Sigma), 10% endothelial cell growth medium (ECGM), and 25 mol/L HEPES, and then plated in one T75 flask.

### Supplementary References

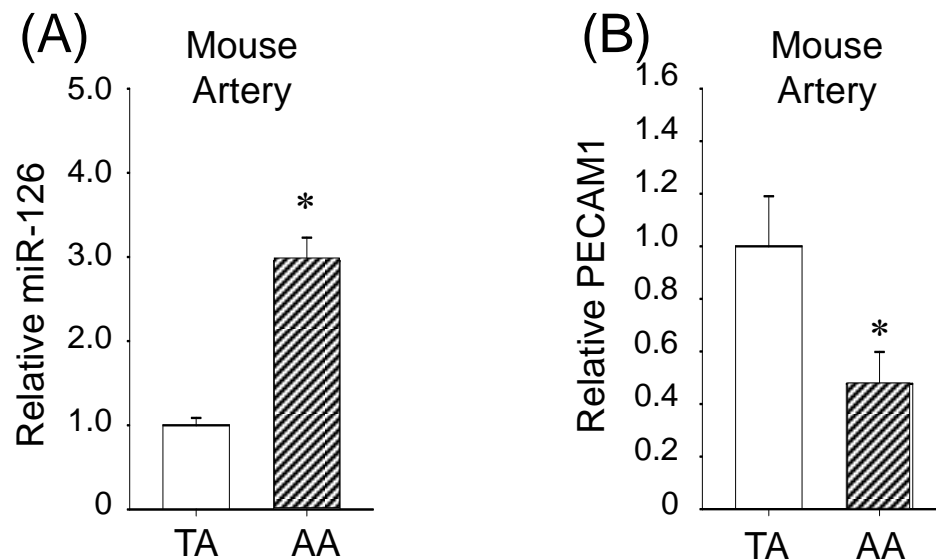
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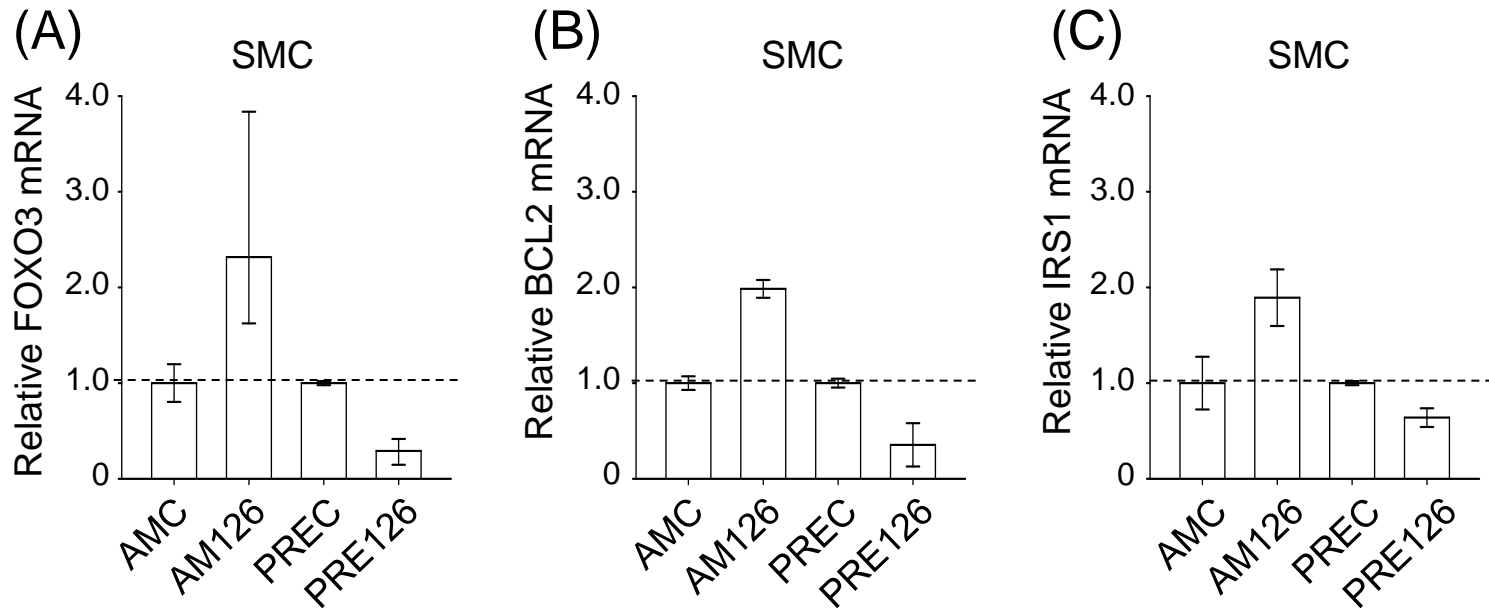
## Supplemental Figures and Figure Legends



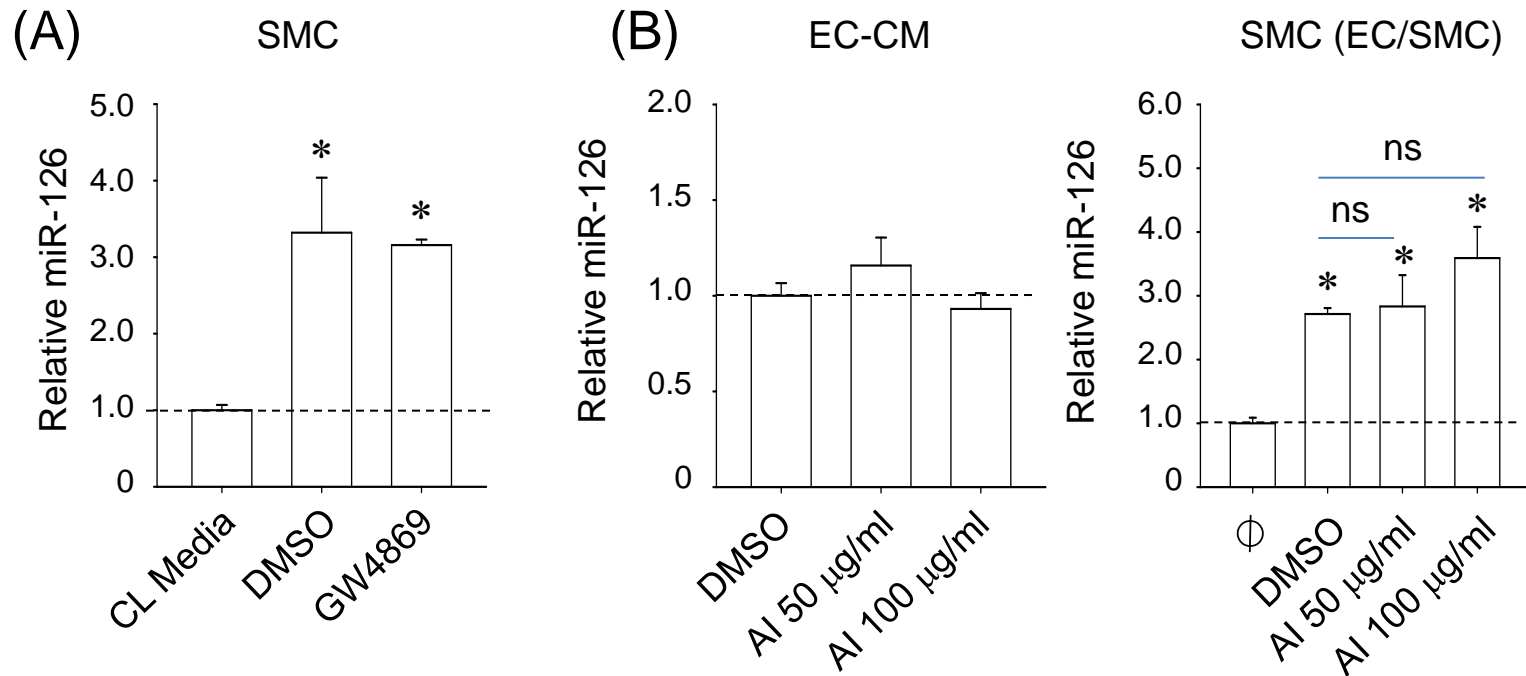
**Online Figure I. OSS to ECs does not suppress miR-126 in the co-cultured SMCs.** (A), SMCs were monocultured or co-cultured with ECs. The ECs were kept as static controls or subjected to OSS ( $0.5 \pm 4$  dynes/cm<sup>2</sup>) for 24 hrs, and the levels of primary (pri-miR-126) and mature (miR-126) forms of miR-126 in SMCs were determined by quantitative RT-PCR. \*  $P < 0.05$  vs. ∅/SMC 0 hr Static, miR-126. #  $P < 0.05$  vs. ∅/SMC 0 hr Static, pri-miR-126. (B) ECs were kept as static control or subjected to OSS for 24 hr and the levels of miR-126 in the static or sheared media were determined by quantitative RT-PCR. (C) ECs and SMCs were co-cultured and the ECs were kept as static controls or subjected to OSS ( $0.5 \pm 4$  dynes/cm<sup>2</sup>) for 24 hrs, and the levels of primary (pri-miR-126) and mature (miR-126) forms of miR-126 were determined by quantitative RT-PCR.



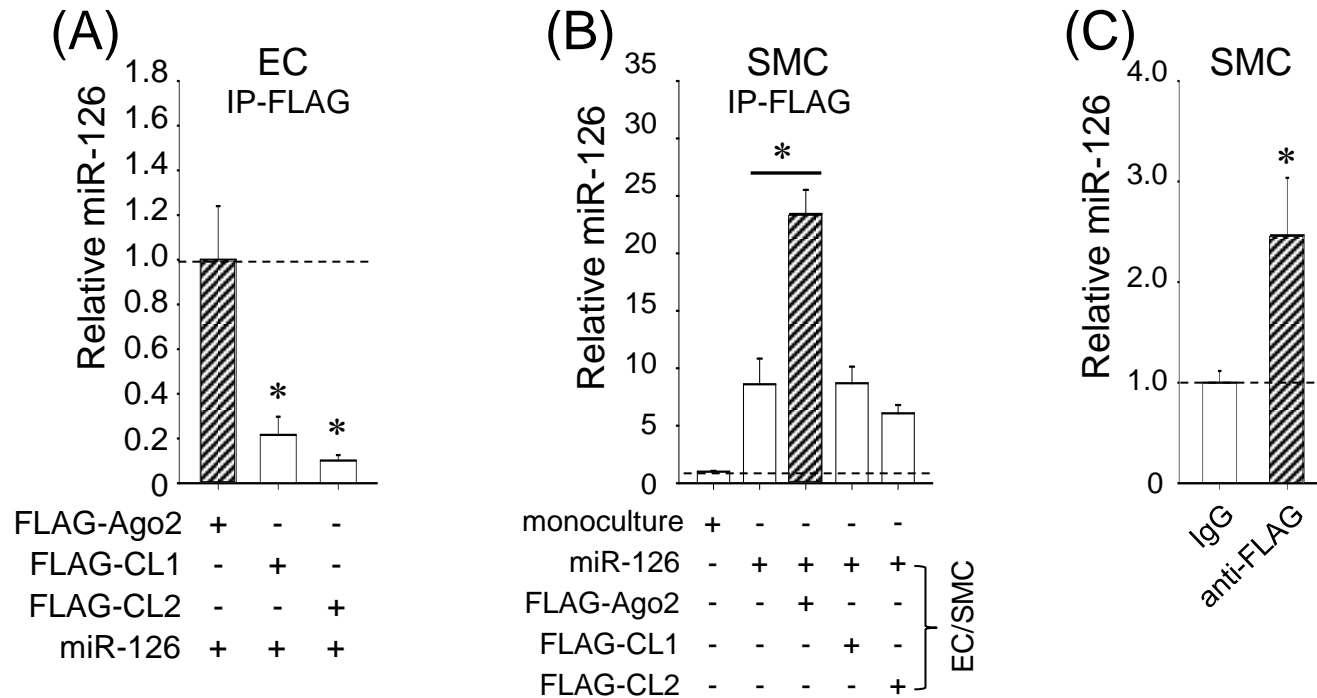
**Online Figure II. The blood vessels wall of mouse aortas from the inner curvature of the aortic arch (AA) has higher level of miR-126 than that from the nearby descending thoracic aorta (TA).** RNA was isolated from TA or AA regions of mouse aortas and the levels of miR-126 (A) and PECAM1 (B) were determined by quantitative RT-PCR. \*  $P < 0.05$  vs. TA.



**Online Figure III. MiR-126 represses FOXO3, BCL2, and IRS1 in SMCs.** SMCs were transfected with AM126, PRE126 or the negative control molecules and the expressions of FOXO3, BCL2, or IRS1 were determined by quantitative RT-PCR. Error bars represent the minimum and maximum value of data sets.

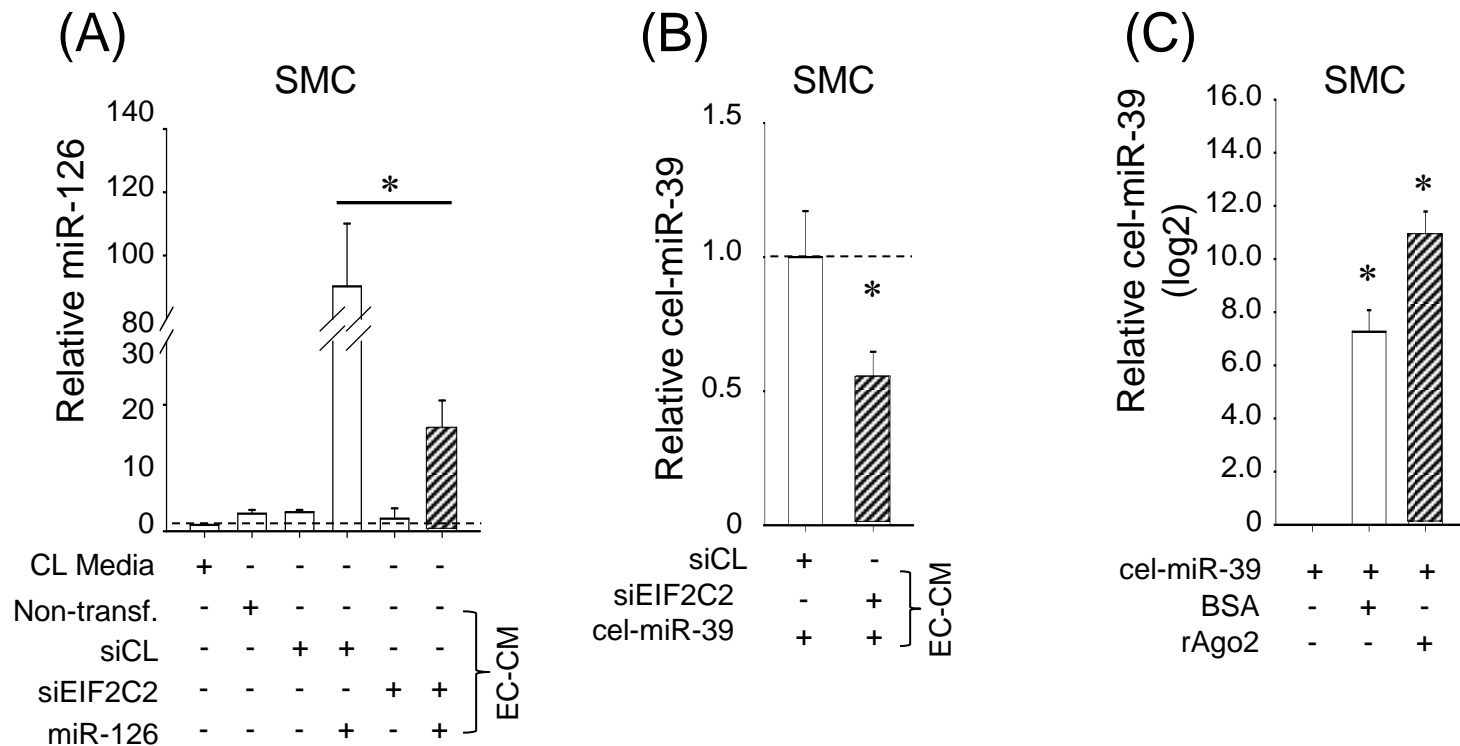


**Online Figure IV. Inhibition of the ceramide signalling pathway or apoptotic body formation in ECs doesn't impair the transmission of miR-126 from ECs to SMCs.** (A), ECs were pre-treated with nSMase2 inhibitor GW4869 (20 µmol/L) or DMSO for 2 hr and then the culture media were refreshed. SMCs were incubated with CL Media or the 24 hr-conditioned media from the pre-treated ECs for 3 hr and the levels of miR-126 in SMCs were determined by quantitative RT-PCR. \*  $P < 0.05$  vs. CL Media or DMSO control. (B), ECs were pre-treated with apoptotic inhibitor sc-205596 (50 µg/ml or 100 µg/ml) or DMSO for 1 hr and then kept for the collection of EC-CM (Left) or co-cultured with SMCs (Right), the miR-126 levels in EC-CM (Left) or in the co-cultured SMCs (Right) were determined by quantitative RT-PCR 24 hr post treatment. \*  $P < 0.05$  vs. mono-culture control.

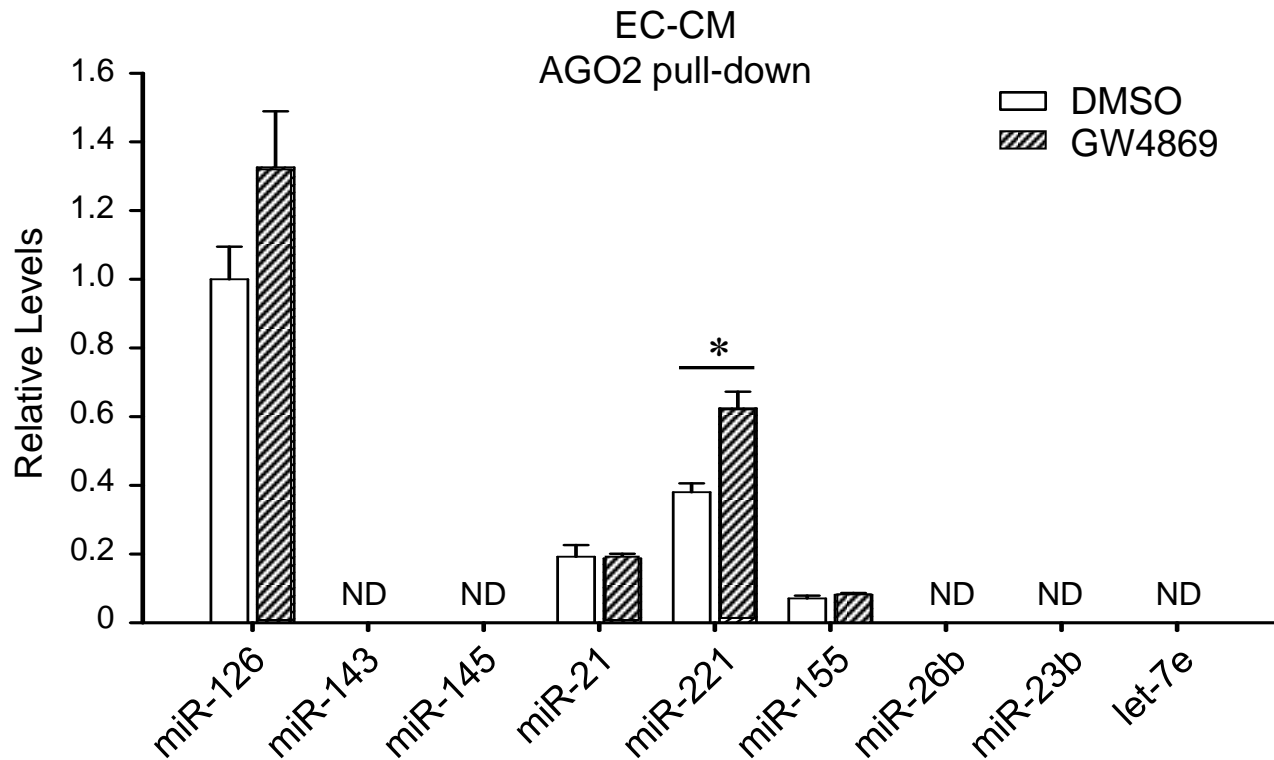


**Online Figure V. Exogenous Ago2 associates with miR-126 in ECs and SMCs.** SMCs were monocultured or co-cultured with BAECs that had been co-transfected with miR-126 and pIRESneo-FLAG/HA Ago2, a plasmid encoding the FLAG/HA-tagged human Ago2 protein (FLAG-Ago2), or two control plasmid expressing FLAG-tagged proteins (FLAG-CL1 and FLAG-CL2). Co-immunoprecipitation assay was performed on ECs (A) and SMCs (B and C) with anti-FLAG antibody or control IgG. The enrichment of miR-126 associated with FLAG-tagged proteins was determined by quantitative RT-PCR. \*  $P < 0.05$  vs. FLAG-Ago2 (A) or mono-culture control (B) or IgG control (C).

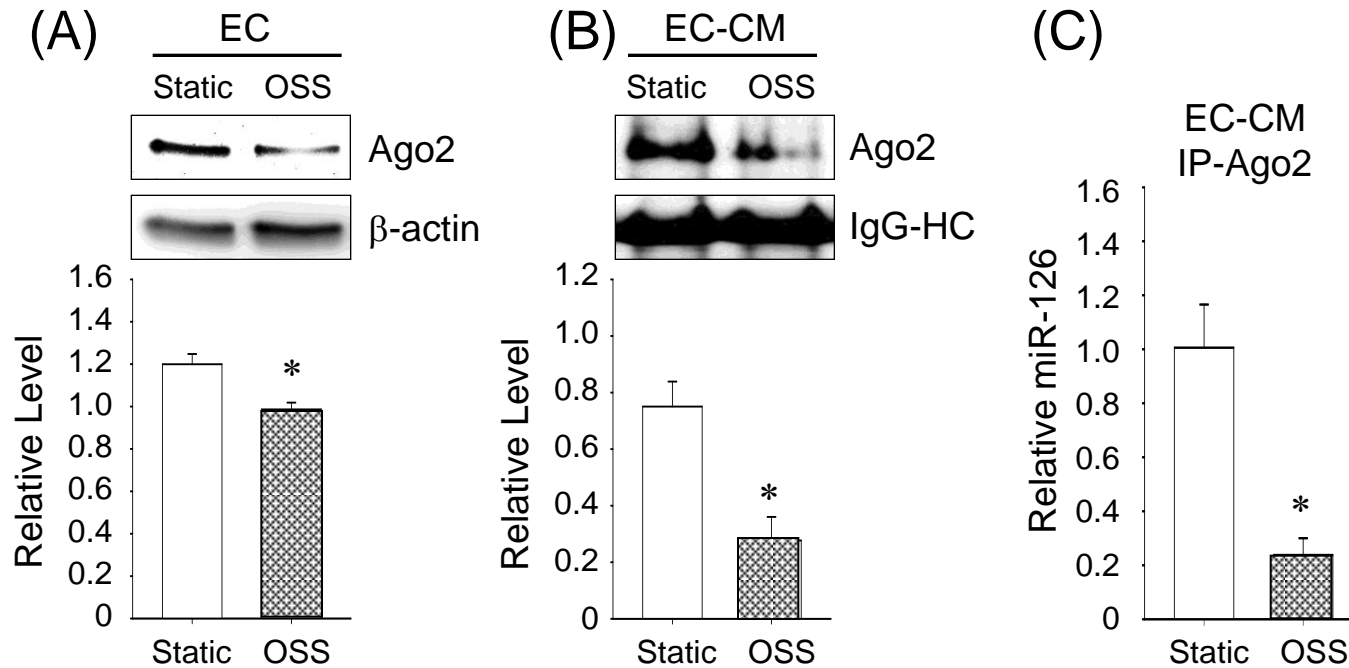




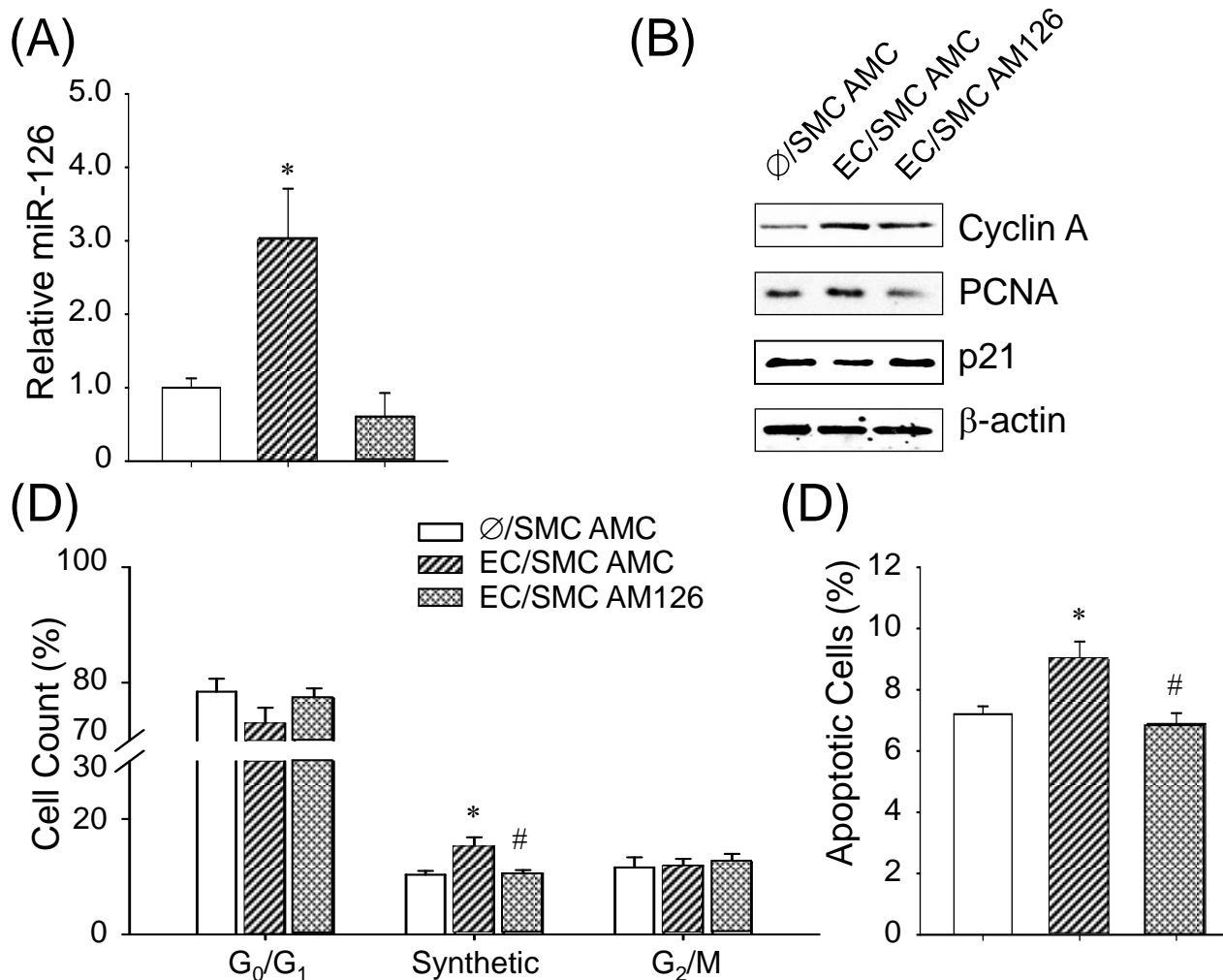
**Online Figure VI. Ago2 facilitates the transmission of miR-126 and cel-miR-39 from ECs to SMCs and the uptake of cel-miR-39 by SMCs.** (A) and (B), SMCs were incubated with CL media or EC-CM from ECs co-transfected with miR-126 or cel-miR-39 and control siRNA (siCL) or specific siRNA of Ago2 (siEIF2C2), and the levels of miR-126 (A) or cel-miR-39 (B) in SMCs were determined by quantitative RT-PCR. \*  $P < 0.05$  vs. siCL. (C), Synthetic cel-miR-39 was kept alone or pre-incubated with BSA or rAgo2 for 30 min, and then used to treat SMCs for 3 hr. The enrichment of cel-miR-39 in SMCs was determined by quantitative RT-PCR. \*  $P < 0.05$  vs. miRs only.



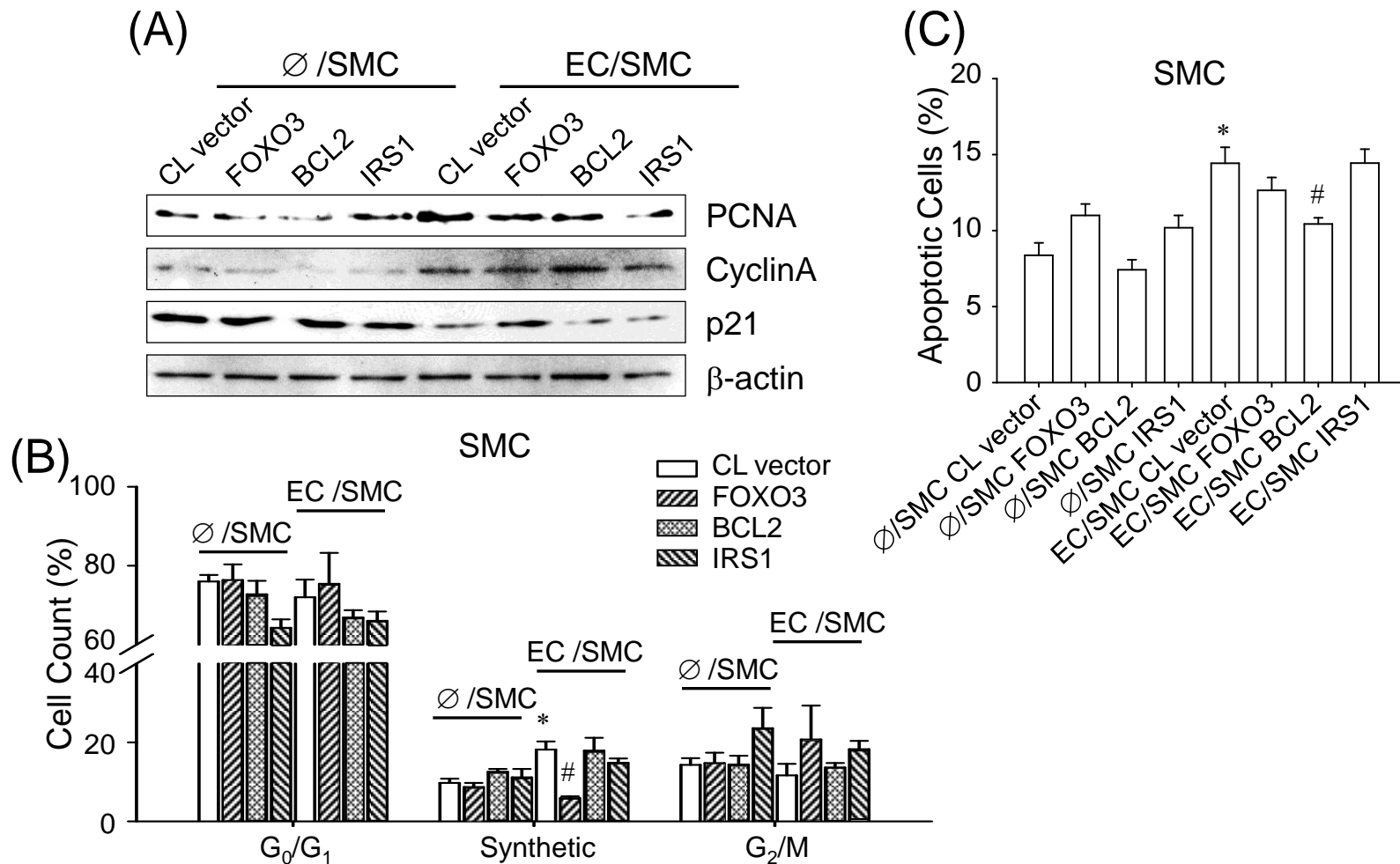
**Online Figure VII. Inhibition of the ceramide signalling pathway in ECs doesn't impair the association of miRs with Ago2.** ECs were pre-treated with nSMase2 inhibitor GW4869 (20  $\mu$ mol/L) or DMSO for 2 hr and then the culture media were refreshed. 24 hr later, EC-CM were collected and the association of indicated miRs with Ago2 were determined by Ago2 pull-down and followed by quantitative RT-PCR. ND: undetectable. \*  $P < 0.05$  vs. DMSO control.



**Online Figure VIII. OSS regulates expression and secretion of Ago2 in ECs.** ECs were kept as static controls or subjected to OSS for 24 hr, and the levels of Ago2 in the cells (A) or the EC-CM (B) were determined by Western blot or immunoprecipitation followed by Western blot, respectively. Images are representative of triplicates with similar results. Semi-quantification results are shown in the lower panels. (C), The levels of miR-126 associated with Ago2 in the EC-CM were determined by co-immunoprecipitation followed by quantitative RT-PCR. \*  $P < 0.05$  vs. Static.

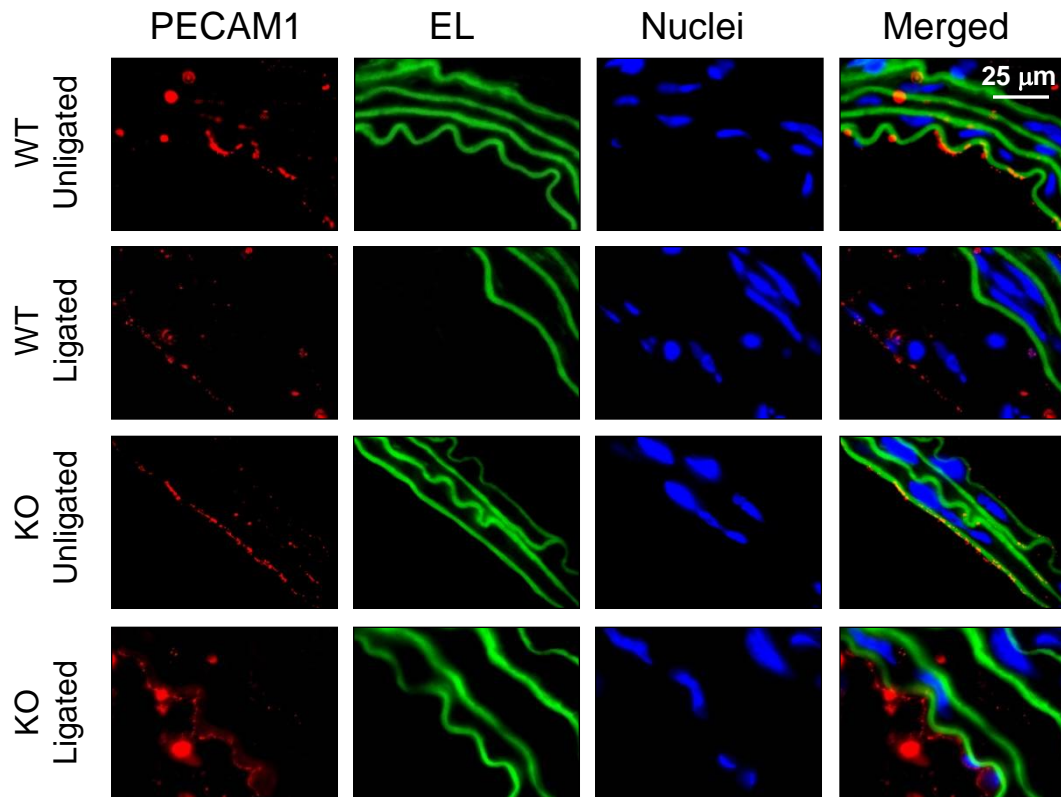


**Online Figure IX. Inhibition of miR-126 in SMCs abolished EC co-culture-induced SMC turnover.** SMCs were transfected with AMC or AM126 and then mono-cultured or co-cultured with ECs. MiR-126 levels in SMCs were determined by qRT-PCR (A). Protein levels of PCNA, cyclinA, and p21 in SMCs were assayed by Western blot (B). The SMCs were stained with propidium iodide and analyzed for DNA content by flow cytometry to show cell cycle distribution (C), or double stained with Annexin V and propidium iodide and analyzed to show cell apoptosis (D). \*  $P < 0.05$  vs.  $\emptyset$ /SMC transfected with AMC. #  $P < 0.05$  vs. SMCs transfected with AMC and co-cultured with ECs.



**Online Figure X. Overexpression of miR-126 targets differentially affects turnover of SMCs co-cultured with ECs.**

SMCs were transfected with control (CL) vector or plasmids overexpressing FOXO3, BCL2, or IRS1, and then were mono-cultured or co-cultured with ECs. The levels of PCNA, cyclinA, and p21 in SMCs were assayed by Western blot (A). The SMCs were stained with propidium iodide and analyzed for DNA content by flow cytometry to show percentages of cells in  $G_0/G_1$ , S, and  $G_2/M$  phases of the cell cycle (B). The SMCs were double stained with Annexin V and propidium iodide and analyzed by flow cytometry to show percentages of cells underwent apoptosis (C). \*  $P < 0.05$  vs.  $\emptyset$ /SMC transfected with CL vector. #  $P < 0.05$  vs. SMCs co-cultured with ECs.



**Online Figure XI. Endothelium is present in ligated or unligated mouse carotid arteries.** Representative images of immunofluorescent stained PECAM1 of ligated or unligated carotid arteries from WT or KO mice.