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

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ABSTRACT

**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 (SS) on the regulation.

**Methods and Results:** Co-culture 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 SS (LSS) to ECs. SMC miR-126 did not increase when treated with EC-CM from ECs subjected to inhibition of miR biogenesis, nor 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-tagged Ago2 transfected into ECs were detected in the co-cultured or EC-CM treated SMCs, indicating a direct EC-to-SMC transmission of miR-126 and Ago2. Endothelial miR-126 represses FOXO3, BCL2, and IRS1 mRNAs in the co-cultured 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 LSS.

**Keywords:** Atherosclerosis, endothelial cell, extracellular miR-126, shear stress, smooth muscle cell, atherogenesis

**Non-standard Abbreviations and Acronyms**

3’UT  3’ untranscribed region
Ago2  Argonaute2
AM126  anti-microRNA-126 inhibitor
AMC  negative control inhibitor
BAEC  bovine aortic endothelial cell
DNase  deoxyribonuclease
EC  endothelial cell
EC-CM  endothelial cell-conditioned media
HUASMC  human umbilical artery smooth muscle cell
HUVEC:  human umbilical vein endothelial cell
KO  miR-126 knockout
LSS  laminar shear stress
microRNA  miR
miRISCs  miRNA-induced silencing complex
Introduction

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. 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. During atherogenesis, activated SMCs migrate into the intima, where they come into close contact with endothelial cells (ECs) lining the vessel wall. 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. 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.

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. In addition to their primary intracellular locations, miRs have recently been found extracellularly in plasma and other body fluids of humans and mice. 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, suggesting the possibility that extracellular miRs may have diagnostic or prognostic values in cardiovascular 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. 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. MiR-126 expression in rat carotid artery is significant under normal condition and is markedly increased after angioplasty, 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 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; Argonaute2 (Ago2), the effector
component of the miRNA-induced silencing complex (miRISCs), associated with the EC-secreted miR-126 and facilitated its transmission from ECs to SMCs; knockout of miR-126 (KO) 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 Supplementary Methods.

**EC-SMC co-culture, and parallel-plate co-culture flow system.**
EC-SMC co-culture was established by plating human umbilical vein ECs (HUVECs) and human umbilical artery SMCs (HUASMCs) on the lower and upper sides, respectively, of a 10-µm-thick porous membrane with 0.4-µm pores in a transwell cell culture insert (Becton Dickinson). ECs and SMCs were maintained in their respective media supplemented with 2% FBS. 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/cm² or oscillatory SS (OSS) with a low level of mean SS at 0.5 dynes/cm² and a superimposed sinusoidal oscillation with a frequency of 1 Hz and a peak-to-peak amplitude of ±4 dynes/cm².

**Animal model.**
Animal surgical procedures were performed in accordance with National Institutes of Health guidelines and were approved by the UC San Diego IACUC. Mice were subjected to carotid artery ligation as previously described. Some mice were injected intravenously (iv) twice per week for 4 weeks with 200 µl of EC conditioned media (EC-CM) from 3-day monocultured static HUVECs or with control media. Some mice received a local administration of miR-126 mimics (PRE126) or negative control mimics (PREC) (2 µg) mixed with pluronic gel [25% (w/v), Pluronic F127, Sigma]. After 4 weeks of ligation, the ligated and unligated control carotid arteries were harvested and subjected to histology and immunostaining analyses of the vessels.

**Statistical analysis.**
Data are expressed as mean ± SEM from three independent experiments. Statistical analysis was performed by Student’s t-test for two groups of data and by one-way ANOVA for multiple comparisons. Statistical significance among multiple groups was determined by post hoc analysis (Tukey HSD test). \( P<0.05 \) was considered statistically significant.
RESULTS

The increase of miR-126 level in the SMCs co-cultured with ECs is suppressed by LSS on ECs.

By utilizing a parallel-plate co-culture flow system (Figure 1A), we determined the miR-126 levels in SMCs in response to co-culture with ECs and flow shear application to ECs. Under static condition, the co-culture of SMCs with ECs significantly increased the SMC mature form of miR-126, but not the primary transcript of miR-126 (pri-miR-126) (Figure 1B), indicating that the effect of EC co-culture on SMC miR-126 occurs at post-transcriptional level. Atheroprotective LSS (12 dynes/cm²) applied to ECs suppressed the level of miR-126 in the co-cultured SMCs, and this suppression lasted for at least 24 hr (Figure 1B). MiR-126 level in the EC conditioned media (CM) was markedly attenuated by exposure of ECs to LSS for 24 hr (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 hr (Figure 1D). The miR-126 level in ECs did not show significant difference between static mono-cultured and co-cultured ECs (Figure 1E), nor between static and LSS-treated mono-cultured ECs (Figure 1F). These results indicate that co-culture 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 co-cultured SMCs does not involve a down-regulation of miR-126 in ECs. Together with the finding that LSS did not change the primary or mature miR-126 in the co-cultured ECs (Figure 1G), our results indicate that SS and SMC co-culture have no synergistic effects on the transcriptional regulation of miR-126 in ECs. These results demonstrate that the induction of SMC miR-126 by co-culture with ECs and the suppression of this induction by LSS on ECs are mediated by intercellular humoral mechanisms.

In contrast to LSS, atheroprone oscillatory SS (OSS) (0.5±4 dynes/cm²) augmented the accumulation of mature miR-126 and also the pri-miR-126 level in SMCs (Online Figure 1A), without a significant change of miR-126 level in EC-CM (Online Figure 1B). Although OSS caused an increase in the SMC miR-126, it did not change the primary or mature miR-126 in the co-cultured ECs (Online Figure 1C). 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 (TA) and the aortic arch (AA) 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 AA (Online Figure IIA), which experiences disturbed and low SS, in comparison with the TA, which is exposed to laminar flow. The expression of the endothelial marker PECAM1 in AA was lower than that in TA (Online Figure IIB), indicating that the increased miR-126 level in AA is in the media layer and not due to 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 FOXO3, BCL2, and IRS1, 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. FOXO3, BCL2, and IRS1 are miR-126 targets predicted by bioinformatics. MiRISC pull-down assay in SMCs validated the association of FOXO3, BCL2, and IRS1 mRNAs with Ago2-containing miRISCs in SMCs (Figure 2A). The association of these miR-126 targets with miRISCs was reduced by transfection of SMCs with miR-126 inhibitor (AM126) 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 up- and down-regulations of the transcripts of these genes in SMCs by AM126 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 hr post-transfection of SMCs with PRE126, there was a significant repression of the luciferase reporter activities of FOXO3, BCL2, and IRS1 wild-type 3’UTR constructs, as 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 de-repression on the wild-type (but not the mutated) 3’UTR reporter activities of those three genes when the SMCs were transfected with AM126, as compared with AMC (Figure 2B). As expected, incubation of SMCs with EC-CM collected from mono-cultured ECs significantly inhibited the reporter activities of the wild-type 3’UTRs, but not the mutated 3’UTRs (Figure 2C). Repression and de-repression of FOXO3, BCL2, and IRS1 were observed when the SMCs were co-cultured with static and sheared ECs, respectively, in comparison to the mono-cultured 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 following EC-co-culture 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 hr post-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 in comparison to 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 co-cultured SMCs (Figure 3C). In SMCs co-cultured with ECs transfected with B-miR-126 or mock control, the B-miR-126 showed a 2.19 ± 0.18 fold enrichment as compared with the mock co-cultured 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 (DNase), ribonuclease (RNase), or proteinase K (PK) digestions. The ability of EC-CM to increase miR-126 abundance in SMCs was abolished by RNase and reduced by PK, but unaffected by DNase (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 three key proteins required for the processing and maturation of miRs, in ECs through siRNA-mediated gene silencing. The impairment of miR-126 synthesis in ECs by si-Drosha/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 by using AM126 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+/+ (WT) mice or miR-126−/− (KO) mice and co-cultured those ECs with human SMCs. We found that co-culture with the WT ECs increased the miR-126 levels in SMCs, but co-culture with the KO ECs did not (Figure 4D).

We separated the extracellular vesicles from the HUVEC-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 about 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 SMC treated with the reconstructed media containing the precipitated pellet (Figure 4F). These observations are consistent with the result of Fig. 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 vs. vesicle-free components of EC-CM in the EC-induced repression of SMC genes, 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 (Figures 4G and H). In contrast, the EC-derived vesicle-containing pellet did not significantly alter the expressions of these miR-126 targets (Figures 4G and H). 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 nSMase2 with GW4869 resulted in the attenuation of cellular miR export by exosomes and a significant reduction of specific extracellular miRs 20. In this study, we found that treatment of ECs with 20 μmol/L GW4869 for 2 hr did not affect the increase of miR-126 in the co-cultured SMCs (Online Figure IV A), 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 13. We tested the involvement of apoptotic body in mediating miR-126 delivery from ECs to SMCs by
pretreatment of ECs with apoptosis inhibitor (AI) at a concentration of 50 or 100 μg/ml one hr before co-culture. AI treatment changed neither the miR-126 level in EC-CM nor that in the co-cultured 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.*

Since 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 independent of vesicles 21. We then tested the possibility that Ago2 and miR-126 can form an extracellular complex with a vesicle-excluded mechanism.

Co-immunoprecipitation 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 (BAECs) expressing eGFP-Ago2 fusion proteins were co-cultured with SMCs for 6 hr, 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, BAECs co-transfected with synthetic miR-126 and FLAG-Ago2, or the negative controls FLAG-histone deacetylase 4 (HDAC4) (FLAG-CL1) and FLAG-HDAC5 (FLAG-CL2) that do not contain miR-126 seeding sequences, were co-cultured 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 -CL2 constructs (Online Figure VA). We detected a significantly larger amount of miR-126 associated with FLAG-Ago2 in SMCs as compared to FLAG-CL1 or -CL2, which exhibited a basal miR-126 enrichment that is probably attributable to the overexpression of synthetic miR-126 in the co-cultured ECs (Online Figure VB). The immunoprecipitation specificity was confirmed using anti-FLAG antibody and IgG 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 4B), we co-transfected 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 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 in protecting miR-126 from RNase digestion. Synthetic miR-126 was incubated with the recombinant human Ago2 (rAgo2), BSA control, or left alone, and then subjected to RNase digestion. A marked increase of RNase resistance was seen when miR-126 was incubated with rAgo2, compared with
the miR-126/BSA 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 pre-incubated with rAgo2 or BSA. The resulted 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). BSA also moderately increased the entry of cel-miR-39 into SMCs (Online Figure VIC), suggesting a non-specific effect of RNA-protein interaction in mediating the transmission of miRs between cells. Complementary experiments indicated that endogenous miR-126, -21, -221, and -155 were associated with Ago2 in EC-CM, and that these associations were not decreased by the treatment of ECs with 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.**

As LSS regulates miR-126 secretion in ECs and hence the EC-to-SMC transmission of miR-126 (Figures 1B and C), 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 (Figures 5E and F). 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 due to 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 Figures. VIII A-C), 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 co-culture 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. Since FOXO3, BCL2, and IRS1 are involved in cell proliferation, cell cycle progression, and apoptosis 22-24, we sought to identify the link between miR-126 transmission and the cellular functions modulated by EC-secreted miR-126 in SMCs. SMCs co-cultured with ECs had increased cell percentage with strong nuclear expression of proliferating cell nuclear antigen (PCNA), as compared with mono-cultured 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 co-culture up-regulated cyclinA and down-regulated 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 co-culture, but actually decreased it to a level lower than that of the mono-culture control. Inhibition of miR-126 in ECs also reversed the cell cycle progression resulting from EC co-culture (Figure 6C). We also found that the percentage of apoptotic SMCs was increased by co-culture 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 co-cultured 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 three direct targets of miR-126, FOXO3, BCL2, and IRS1, were overexpressed in SMCs, which were then co-cultured with ECs. Overexpression of any of these three genes attenuated the EC-co-culture induced expression of PCNA, but not CyclinA, and reversed the effects of co-culture on expression of p21 (Online Figure XA). Overexpression of FOXO3 abolished the increased percentage of SMCs in S phase in the co-culture (Online Figure XB), whereas overexpression of BCL2 inhibited the increased percentage of SMCs undergoing apoptosis induced by co-culture (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 employed the miR-126 knockout (KO) mice with a ligation-induced neointimal growth model to further determine the effect of miR-126 on the formation of intimal lesion. The left common carotid artery of miR-126 wildtype (WT) or KO mice was ligated near the 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 post-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 (Fig. 7b), and induced neointimal thickening. The presence of endothelium on the luminal surfaces of ligated and unligated carotid arteries was verified by immunostaining for PECAM1 (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 SM-actin (SMA) and PCNA immunostaining, in comparison with those of WT mice (Figures. 7D and E).

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 to 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. While 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 co-cultured SMC to modulate its gene expression and function toward atherogenic phenotype. The paracrine humoral transmission from EC to SMC is proven by using the exogenous B-miR-126 (Figures. 3A and B), 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 a miR. We also showed that the increase in SMC miR-126 upon co-culture 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 (Figures. 4B - D), thus further confirming that the EC is the origin of the miR-126 enrichment in co-cultured SMCs. The inhibition of FOXO3, BCL2, and IRS1 mRNAs in SMCs by miR-126 and co-culture 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. Dimmeler and colleagues showed that ECs transfer the vesicle-packaged miR-143/145 to SMCs to enhance the repression of de-differentiation-associated genes in SMCs. Weber and colleagues reported that EC-derived apoptotic bodies convey paracrine alarm signals to the nearby recipient ECs. Our data indicate that RNAs and RNA-protein complexes are transmitted into SMCs, but not DNA components or the vesicle-protected RNA (Figure 4A). Treatments of SMCs with the fractions of the ultra-centrifuged 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, -221, and -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 -145 was undetectable (Online Figure VII), consistent with previous report indicating that EC-secreted miR-143 and -145 were packaged into vesicles. The current study provides an alternative mechanism that is distinct from those in previously reports in which the EC-specific miRs are exported and delivered through exosomal or apoptotic pathways.

Turchinovich et al. and Arroyo et al. reported that most miRs in human peripheral blood are ultrafiltratable together with Ago2 and that miR-16, -21, -24 or miR-16, 92a, let-7a are associated with Ago2 in the culture media of MCF7 cells or in the human plasma. Other proteins that bind miRs and deliver miRs to recipient cells independent of vesicles include NPM1 (nucleophosmin) and HDL. Our results showed that Ago2 exhibited a strong association with miR-126 in the CM (Figure 5A) and the recipient SMCs (Figure 5B), and also carried miR-21, -221, -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 exosomes- 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 D, 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 (Figures 5E and F). These findings may provide a mechanistic explanation of how LSS regulates the miR-126 release as well as its transmission from ECs to SMCs. Our study along with the previous findings 6-8,24 support a notion that both vesicle-dependent and -independent pathways may be involved in the humoral transmission of miRs.

In our EC/SMC co-culture flow system, ECs and SMCs were separated by a porous membrane with a 0.4-μm 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 3, 26. Co-culture of static ECs with SMCs is known to induce SMC activation 3, 27-29, 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 EC or inhibition of miR-126 in either ECs (Fig. 6) or SMCs (Online Figure IX). However, overexpression of miR-126 by PRE126 alone in mono-cultured SMCs did not activate the SMCs (data not shown). Such non-symmetrical phenotypic effects have been reported in other miR studies that although miR depletion caused phenotypical 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 30; Inhibition of miR-222 in cultured SMCs increased expressions of p21 and p57, but overexpression of miR-222 had no impact on p57 mRNA level 31. 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 co-culture vs. 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 4, 32. The lesion originates and develops preferentially at sites of curvature, branching, and bifurcation in arteries where complex hemodynamic conditions are prominent 33. In this study, a 3-fold higher expression of miR-126 was observed in the athero-susceptible AA (Online Figure II), which experiences disturbed and low SS, in comparison with TA, where the SS is laminar and non-disturbed. Cessation of blood flow in carotid arteries also up-regulates the level of miR-126 in the vessel wall (Figures 7A and B). 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 (Figures 7C - F). 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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FIGURE LEGENDS

**Figure 1.** Atheroprotective SS to ECs suppresses miR-126 in the co-cultured SMCs. (A), Schematic diagrams of EC/SMC co-culture and flow system. (B), HUVECs and HUASMCs were seeded on the lower and upper sides of a membrane, respectively (EC/SMC). Controls had SMCs but no ECs on the other side (Ø/SMC). The temporal expression levels of primary (pri-miR-126) and mature (miR-126) forms of miR-126 were determined by quantitative RT-PCR. * P < 0.05 vs. Ø/SMC 0 hr Static. (C), The levels of miR-126 in the 24-hr 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% FBS (CL Media), or conditioned medium from static or sheared ECs (24-hr shearing) (EC-CM). * P < 0.05 vs. CL Media. (E), miR-126 expression in ECs mono-culture or SMC-co-culture for 24 hr. (F), miR-126 expressions in ECs exposed to LSS for 24 hr. (G), The levels of pri-miR-126 and miR-126 in ECs co-culture with SMCs under 24 hr-static or -LSS conditions.

**Figure 2.** ECs and SS regulate expressions of miR-126 targets in SMCs. (A), The mRNA levels of FOXO3, BCL2, and IRS in Ago2-immunocomplexes in SMCs 48 hr post-transfection with PRE126 or AM126. * P < 0.05 vs. cells transfected with negative controls (PREC or AMC). (B), The relative luciferase levels in SMCs co-transfected with PRE126 (Left), AM126 (Right), or the negative control and luciferase reporter plasmids: empty vector only, wild type 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-CM. * P < 0.05 vs. cells transfected with empty vector. * P < 0.05 vs. cells transfected with plasmids harboring the wild type 3’-UTRs. (D), The protein levels of target genes in SMCs mono-cultured (Ø/SMC) or co-cultured with ECs (EC/SMC) kept under static condition or exposed to LSS. Images are representative of triplicate experiments with similar results.

**Figure 3.** ECs transfer exogenous miR-126 to SMCs. SMCs were incubated with CM derived from ECs transfected with biotinylated synthetic miR-126 (B-miR-126) or no-miRs mock control (Mock) for 3 hr. (A), The levels of B-miR-126 in ECs or the EC-CM were determined by pull down experiments with streptavidinagarose beads followed by quantitative RT-PCR, (B) and B-miR-126 in ECs and SMCs were detected with QDot-605-streptavidin conjugates. * P < 0.05 vs. mock control. (C and D), SMCs were co-cultured with ECs transfected with B-miR-126 or mock control for 24 hr; 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 miR-126 transmission from ECs to SMCs is independent of vesicles. (A), The levels of miR-126 in SMCs incubated with CL media or the EC-CM treated with DNase I, RNase A, or PK for 3 hr. * P < 0.05 vs. CL Media. (B), The levels of miR-126 in ECs transfected with control siRNA (siCL) or siRNA against Drosha (siDROSHA), Dicer (siDICER1), or Ago2 (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 AM126, PRE126 or the negative controls. *
P < 0.05 vs. siCL or CL media. *P < 0.05 vs. the AMC or PREC EC-CM. (D), The levels of miR-126 in SMCs co-cultured with lung ECs isolated from wild-type (WT) or miR-126 knockout mice (KO). * P < 0.05 vs. ∅/SMC. 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-ECM for 3 hr. (G), the transcript levels, or (H), protein levels of indicated genes in SMCs incubated with the fractioned EC-CM. Images are representative of triplicates with similar results. * P < 0.05 vs. CL media.

**Figure 5. Ago2 associates with miR-126 and facilitates its transmission.** (A), The levels of miR-126 in EC-CM immunocomplexes of Ago2 or Ago1, or control IgG. * P < 0.05 vs. IgG control. (B), SMCs were co-cultured with BAECs transfected with a plasmid encoding the eGFP-Ago2. The eGFP-Ago2 proteins were visualized in live ECs or SMCs by microscopy. (C), The protection of miR-126 with pre-incubation of BSA or recombinant human Ago2 protein (rAgo2) for 30 min followed by RNase A digestion for 15 min. * P < 0.05 vs. miR-126 only. (D), The enrichment of miR-126 in SMCs treated with miR-126 pre-incubated with BSA or rAgo2 for 3 hr. * P < 0.05 vs. miRs only. (E), The levels of Ago2 in ECs (left) or EC-CM (right) under static or LSS conditions for 24 hr. (F), The levels of miR-126 in Ago2-immunocomplex in EC-CM under static of LSS conditions. Images are representative of triplicates with similar results. Semi-quantification results are shown in the lower panels. * P < 0.05 vs. Static.

**Fig. 6. SS-imposed ECs modulates SMCs turnover through miR-126.** SMCs were mono-cultured or co-cultured with: (1) ECs kept under static condition or exposed to LSS for 24 hr or (2) ECs transfected with AMC or AM126. (A), 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. ∅/SMC. # P < 0.05 vs. Static EC/SMC or AMC EC/SMC.

**Fig. 7. Depletion of miR-126 in mice inhibits 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 ECs were denuded prior to RNA isolation. * P < 0.05 vs Unligated control. (B), In situ hybridization of miR-126 in ligated or unligated carotid arteries from miR-126 wildtype (WT) mice. EL, elastic lamina, indicated by Yellow arrows. (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 (PCNA) SMC (SM- 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-CM; or with local delivery of CL oligo or miR-126 mimics (PRE126). Arrows indicate the 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.
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 anti-atherogenic.
- Application of laminar shear stress to endothelial cells co-cultured 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 co-cultured SMCs via a paracrine mechanism that involves the intercellular communicating molecule Argonaute 2, to promote SMC proliferation.
- Laminar shear stress applied to endothelial cells inhibits transmission of miR-126 from endothelial cells to co-cultured SMCs, thus suppressing SMC proliferation.
- Curtailment of blood flow (decrease in lamina shear stress) induces proliferative pro-atherosclerotic 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 co-cultured 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 co-cultured SMCs. This anti-atherogenic 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 wildtype mice was abrogated in miR-126-null mice. These results indicate that the athero-protective hemodynamic force acting on endothelial cells exerts its anti-atherogenic effect on SMCs by inhibiting the paracrine secretion of miR-126 by endothelial cells.
Figure 1
Figure 3
Figure 4
Figure 5

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Figure 5

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F
Figure 6
Figure 7 (continued)
Regulation of Vascular Smooth Muscle Cell Turnover by Endothelial Cell-secreted MicroRNA-126: Role of Shear Stress

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

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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 β-actin were obtained from Santa Cruz Biotechnology. Rabbit pAb against FOXO3 was from Millipore. Mouse mAb against SMα-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. 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-µm-thick porous polyethylene terephthalate (PET) membrane (Falcon cell culture inserts; Becton Dickinson) with 0.4-µm pores configured at a density of 1.6 x 10⁶ pores/cm². 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 ¹. 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% CO₂ in air. The flow of the perfusate in the channel is laminar. The fluid shear stress (τ) generated on the ECs seeded on the membrane can be estimated as $\tau = 6Q\mu/wh²$, 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/cm² for indicated hours. The oscillatory shear stress (OSS) is composed of a low level of mean flow (shear stress = 0.5 dynes/cm²) 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 ±4 dynes/cm². 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 protease 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 protease 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′-ctctcgtctgattttgggtgaaca-3′, 5′-tccgcttcaagacctcatttt-3′; BCL2, 5′-ttggatcagggagttggaag-3′, 5′-ccatgctgatgtctctggaa-3′; IRS1, 5′-gttcccagaacgaccaag-3′, 5′-tgaatgtgcatgcctgcc-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 × 10⁵ 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)², FLAG/HA-hAgo2 (10821)³, FLAG-FOXO3a (8360)⁴, FLAG-Bcl2 (18003)⁵ were obtained from Addgene. Plasmid expressing HA-tagged human IRS1⁶ 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 µg/µl, and 8 µg of Ago2- or FLAG-specific antibody or control IgG was added into 100 µl of cell lysate. In some experiments using liquid samples, 200 µl of Lysis Buffer and 10 µg of Ago2-specific antibody or control IgG was added into 800 µl 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 µl of 2×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 mmol/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 µl 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 µg/ml) for another 30 min at 37 °C. The miRs from each condition were then purified using mirVana 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 µl 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 µg/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 µm cross cryosections were prepared from 4% paraformaldehyde-fixed tissue. MiR fluorescence in situ hybridization (FISH) was performed on the sections as described previously 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 +/+ (WT) and miR-126 −/− (KO) mice (8-12 weeks old, 18-25 g) were anesthetized by intraperioneal 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 µl 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 µg of PRE126 or scrambled control were preloaded into the 50 µl, 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 µm 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-µm 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 µl 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 µg/ml Heparin (Sigma), 10% endothelial cell growth medium (ECGM), and 25 mol/L HEPES, and then plated in one T75 flask.

Supplementary References

5. Wang NS, Unkila MT, Reineks EZ, Distelhorst CW. Transient expression of wild-type or mitochondrially targeted bcl-2 induces apoptosis, whereas transient expression of endoplasmic reticulum-targeted bcl-2 is protective against bax-induced cell death. J Biol Chem. 2001;276:44117-44128
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 ± 4 dynes/cm²) 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 ± 4 dynes/cm²) 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. monocyte culture control.
Online Figure V. Exogenous Ago2 associates with miR-126 in ECs and SMCs. SMCs were mono-cultured or co-cultured with BAECs that had been co-transfected with miR-126 and pIRESngeo-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 μmol/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, cyclin A, 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 G0/G1, S, and G2/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. ∅/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 lighted or unligated carotid arteries from WT or KO mice.