VAMP3 and SNAP23 mediate the disturbed flow-induced endothelial microRNA secretion and smooth muscle hyperplasia

Juan-Juan Zhu[a,b,1], Yue-Feng Liu[a,b,1], Yun-Peng Zhang[a,b], Chuan-Rong Zhao[a,b], Wei-Juan Yao[a,b], Yi-Shuan Li[c], Kuei-Chun Wang[c], Tse-Shun Huang[c], Wei Pang[a,b], Xi-Fu Wang[d], Xian Wang[a,b], Shu Chien[c,2], and Jing Zhou[a,b,2]

[a]Department of Physiology and Pathophysiology, School of Basic Medical Sciences, Peking University, Beijing 100191, People's Republic of China; [b]Key Laboratory of Molecular Cardiovascular Science, Ministry of Education, Beijing 100191, People's Republic of China; [c]Department of Bioengineering, Institute of Engineering in Medicine, University of California, San Diego, La Jolla, CA 92093; and [d]Department of Emergency and Critical Care Center, Beijing Anzhen Hospital, Capital Medical University, Beijing 100029, People's Republic of China

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Vascular endothelial cells (ECs) at arterial branches and curvatures experience disturbed blood flow and induce a quiescent-to-activated phenotypic transition of the adjacent smooth muscle cells (SMCs) and a subsequent smooth muscle hyperplasia. However, the mechanism underlying the flow pattern-specific initiation of EC-to-SMC signaling remains elusive. Our previous study demonstrated that endothelial microRNA-126-3p (miR-126-3p) acts as a key intercellular molecule to increase turnover of the recipient SMCs, and that its release is reduced by atheroprotective laminar shear (12 dynes/cm^2) to ECs. Here we provide evidence that atherogenic oscillatory shear (0.5 ± 4 dynes/cm^2), but not atheroprotective pulsatile shear (12 ± 4 dynes/cm^2), increases the endothelial secretion of nonmembrane-bound miR-126-3p and other microRNAs (miRNAs) via the activation of SNAREs, vesicle-associated membrane protein 3 (VAMP3) and synaptosomal-associated protein 23 (SNAP23). Knockdown of VAMP3 and SNAP23 reduces endothelial secretion of miR-126-3p and miR-200a-3p, as well as the proliferation, migration, and suppression of contractile markers in SMCs caused by EC-coculture. Pharmacological intervention of mammalian target of rapamycin complex 1 in ECs blocks endothelial secretion and EC-to-SMC transfer of miR-126-3p through transcriptional inhibition of VAMP3 and SNAP23. Systemic inhibition of VAMP3 and SNAP23 by rapamycin or periadventitial application of the endocytosis inhibitor dynasore ameliorates the disturbed flow-induced neointimal formation, whereas intraluminal overexpression of SNAP23 aggravates it. Our findings demonstrate the flow-pattern–specificity of SNARE activation and its contribution to the miRNA-mediated EC–SMC communication.

endothelial cells | shear stress | SNAREs | extracellular microRNA | neointimal formation

The interaction between hemodynamics and vascular endothelium is an important determinant of vascular homeostasis (1). In the straight part of the arteries, endothelial cells (ECs) are exposed to laminar blood flow with high shear stress that protects these parts of the vessels from atherogenesis, whereas in the branches and curvatures, the ECs experiencing disturbed flow with reciprocating and low shear stress stimulate the smooth muscle cells (SMCs) within the tunica media to become proliferative and migratory. The activated SMCs migrate into the intima, where they come into close contact with ECs and undergo phenotypic changes to lead to atherosclerosis (2). We recently demonstrated that vascular ECs repress expressions of forkhead box O3, B-cell lymphoma 2, and insulin receptor substrate 1 in the adjacent SMCs by producing microRNA-126-3p (miR-126-3p), and hence induce SMC turnover, and that the application of atheroprotective laminar shear (LS) at 12 dynes/cm^2 to ECs inhibits this paracrine effect (3). Although extracellular microRNAs (miRNAs) have been shown to convey messages from donor cells to recipient cells, how fluid shear stress regulates endothelial miRNA secretion remains to be elucidated.

Emerging evidence has suggested that extracellular miRNAs can serve as not only putative diagnostic or prognostic biomarkers for multiple diseases, including atherosclerosis, but also potential therapeutic tools (4–6). Extracellular miRNAs have recently been detected in membrane-bound vesicles from various body fluids and cell culture media (7–9). However, it has also been demonstrated by previous studies from us and others (3, 10–13) that donor cells can deliver miRNAs to recipient cells in forms independent of membrane-bound vesicles. Many miRNAs are released from ECs in complexes with argonaute-2 (AGO2), which protect miRNAs from ribonuclease digestion and facilitate their uptake by the recipient SMCs (3). Nevertheless, little is known about the regulatory mechanisms underlying miRNA secretion and how critical these mechanisms are for vascular homeostasis and atherogenesis. Identification of the key molecules involved in the intercellular delivery of endothelial-secreted miRNA may uncover novel targets for future diagnosis and therapeutic interventions for atherosclerosis.

Regulated exocytosis of membranous secretory granules in response to stimuli is a fundamental process by which cells deliver molecules to the extracellular space (14). Enzymes, growth factors, matrix proteins, and signaling molecules can all be

Significance

Vascular homeostasis is regulated by the interactions between endothelial cells (ECs) and smooth muscle cells (SMCs). Our previous study demonstrated that endothelial microRNA-126-3p conveys the EC-to-SMC signaling that is regulated by hemodynamic shear stress to ECs. Here we show that the mechanisms by which shear stress modulates microRNA secretion include the regulations on vesicle-associated membrane protein 3 (VAMP3) and synaptosomal-associated protein 23 (SNAP23). Endothelial inhibition of VAMP3/SNAP23 reduces microRNA-126-3p secretion and rescues the SMC phenotype altered by atherogenic shear. Our findings provide a mechanistic explanation of the noncoding RNA mediation of detrimental effects of oscillatory shear on vascular function and suggest therapeutic potential of targeting the endothelial vesicular transport system in the control of smooth muscle proliferative diseases.

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[1]J.-J.Z. and Y.-F.L. contributed equally to this work.

[2]To whom correspondence may be addressed. Email: shuchien@ucsd.edu, or jzhou@bjmu.edu.cn.

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secreted by fusion of the secretory granule with the plasma membrane and the subsequent release of the vesicular contents (14). Previous studies have identified proteins that mediate the regulated exocytosis in ECs: for example, vesicle-associated membrane protein (VAMP) 3 (15), VAMP8 (15), syntaxin 4 (15), syntaxin-binding protein 5 (16), and synaptosomal-associated protein 23 (SNAP23) (17). Most of them belong to a superfamily of transmembrane proteins named SNAREs that mediate trafficking of cellular materials between intracellular compartments. During membrane docking and fusion, one SNARE molecule on a granule membrane (*v*-SNARE) binds to two counterpart SNAREs on a target membrane (*t*-SNARE), forming a stable ternary complex (*trans*-SNARE) that mediates granule exocytosis (18). Given the importance of SNAREs in the mediation of exocytosis, we investigated whether SNAREs may be involved in the machinery that mediates the flow-regulated miRNA secretion. Using microarrays and RNA sequencing to study the effects of flows on ECs, we found that both *v*-SNARE VAMP3 and *t*-SNARE SNAP23 are up-regulated by atherogenic oscillatory shear (OS) compared with atheroprotective pulsatile shear (PS). Endothelial VAMP3 resides on secretory granules such as Weibel-Palade bodies (15), whereas the only endothelial SNAP homolog, SNAP23, is mainly located at the plasma membrane (17). Based on these previous findings, we investigated in the present study the involvement of VAMP3 and SNAP23 in flow-regulated miRNA secretion, as well as their roles in EC-directed functional modulation on SMCs.

Results

OS Up-Regulates VAMP3 and SNAP23 and Induces Their Translocation in ECs. Application of OS (0.5 ± 4 dynes/cm^2, 1 Hz) to ECs for 6 h markedly increased the expressions of VAMP3 and SNAP23, compared with the application of PS (12 ± 4 dynes/cm^2, 1 Hz) (Fig. 1*A*). The most predominant phenotype observed in ECs exposed to PS was perinuclear localization of VAMP3 with a condensed appearance; in contrast, OS resulted in a much more dispersed appearance of VAMP3 (Fig. 1*B*, *Upper*). SNAP23 was located mainly in the plasma membrane and also in scattered cytoplasmic structures in ECs exposed to PS (Fig. 1*B*, *Lower*). In comparison, SNAP23 was intensively localized to the plasma membrane following OS exposure (Fig. 1*B*, *Lower*, yellow arrows). In the mouse aorta, the endothelial expressions of VAMP3 and SNAP23 were greater in the inner curvature of aortic arch (AA), where the local flow is disturbed, than in the descending thoracic aorta (TA), where the local flow is mostly laminar (Fig. 1*C*). Subcellular localization of VAMP3 in the TA was largely perinuclear and condensed (Fig. 1*D*, *Upper*); this finding is in line with the results observed in cultured ECs under PS. In contrast, VAMP3 in the AA was predominantly located in scattered punctate cytoplasmic structures, echoing the observations in cultured ECs under OS (Fig. 1*D*, *Upper*). In the TA, the vast majority of SNAP23 appeared diffuse and part of it was located at the plasma membrane (Fig. 1*D*, *Lower*). In the AA, there was a much stronger staining of SNAP23 and an increased colocalization of SNAP23 with CD31, the intercellular junction protein of ECs (Fig. 1*D*, *Lower*). Collectively, these results indicate that OS/disturbed flow up-regulates the expressions of VAMP3 and SNAP23 and induces their "intracellular-to-surface" translocation.

VAMP3 and SNAP23 Are Required for OS-Induced AGO2 Secretion. Application of OS to ECs caused an increase of SNAP23 in complex with VAMP3, compared with application of PS (*SI Appendix*, Fig. S1*A*). The increased interaction of VAMP3 with SNAP23 suggests that OS might enhance the granule exocytosis. This suggestion was confirmed by immunocolocalization study of VAMP3 and SNAP23 in ECs exposed to OS and PS (*SI Appendix*, Fig. S1*B*, *Upper*). Quantification of the degree of colocalization between fluorophores using correlation coefficients

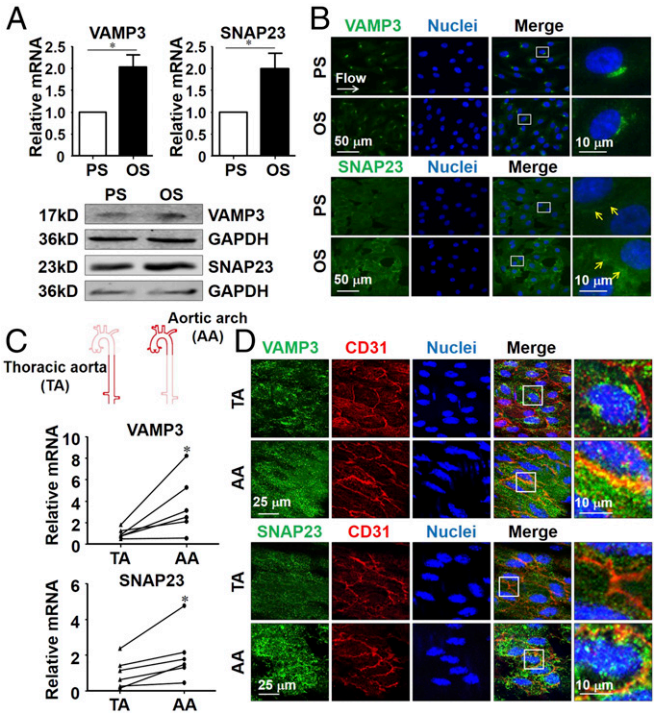

Fig. 1. PS and OS differentially modulate the expressions and subcellular localizations of VAMP3 and SNAP23. (*A* and *B*) ECs were exposed to PS (12 ± 4 dynes/cm^2) or OS (0.5 ± 4 dynes/cm^2) for 6 h, expressions of VAMP3 and SNAP23 were analyzed (*A*), and the subcellular localizations of VAMP3 and SNAP23 were assessed (*B*). In *B*, the white arrow, *Upper*, indicates the flow direction and the yellow arrowhead, *Lower*, indicates plasma membrane where SNAP23 localizes. (*C*) Expressions of VAMP3 and SNAP23 in endothelium from the mouse descending TA and AA. (*D*) Subcellular localizations of VAMP3 and SNAP23 in endothelium from the straight segment of the TA or from the inner curvature of the AA of mice were assessed by *en face* immunofluorescence. Results in *A*, *B*, and *D* are representative of triplicate experiments with similar results. Each symbol in *C* was obtained from five animals and statistical analysis was performed by paired *t* test. $^*P < 0.05$.

(CC) and intensity correlation quotient (ICQ) indicate that the colocalization of VAMP3 with SNAP23 was much higher under OS than under PS (*SI Appendix*, Fig. S1*B*, *Lower*). Next, we applied flows to ECs with VAMP3 and SNAP23 in ECs knocked down by siRNAs (*SI Appendix*, Fig. S2), and examined the cells by transmission electron microscopy. The ECs exposed to PS showed only occasionally small membrane-bound granules (70–100 nm in diameter) in the cytoplasm (*SI Appendix*, Fig. S3, open arrowheads). In contrast, OS induced a marked accumulation of granules in the cells; this accumulation became more apparent at the plasma membrane (*SI Appendix*, Fig. S3, dotted line) following VAMP3 and SNAP23 knockdown (*SI Appendix*, Fig. S3), suggesting that those granules failed to fuse with the plasma membrane and became trapped in the cells because of the deficient SNARE machinery.

Our previous research demonstrated that OS to ECs increased the secretion of the miR-126-3p/AGO2 complex. We next sought to investigate the role of VAMP3 and SNAP23 in OS-evoked AGO2 secretion. Part of AGO2 colocalized with VAMP3 or SNAP23 in the static cells (*SI Appendix*, Fig. S4*A*), suggesting a constitutive colocalization of AGO2 with VAMP3 or SNAP23. In comparison with PS, OS led to a stronger colocalization of AGO2 with VAMP3 at the perinuclear area (*SI Appendix*, Fig. S4*B*, *Left*) and with SNAP23 at the cell periphery (*SI Appendix*, Fig. S4*C*, *Left*). CC and ICQ supported the increases of colocalization (*SI Appendix*, Fig. S4 *B* and *C*, *Right*). Moreover, knockdown of

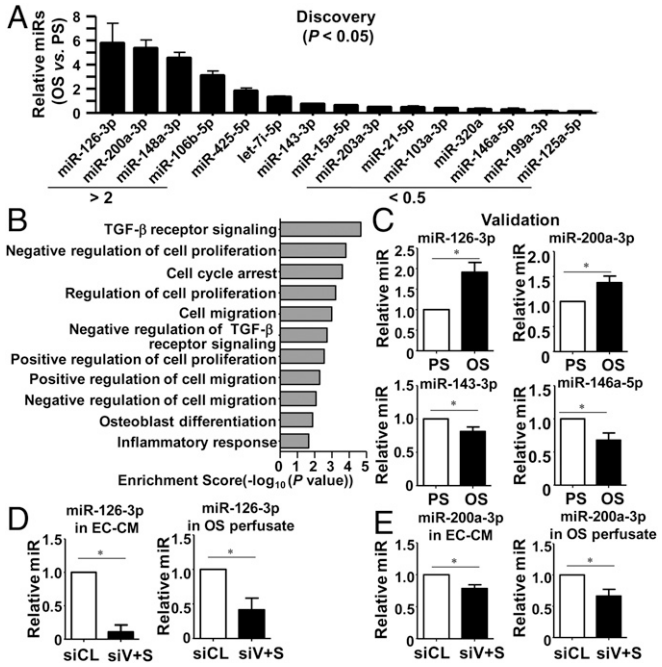

Fig. 2. VAMP3 and SNAP23 mediate the OS-induced miRNA secretion. (*A*) ECs were exposed to PS or OS for 24 h, and miRNA accumulations in the vesicle-poor flow perfusates were analyzed by qRT-PCR array ($n = 3$). (*B*) The potential targets of the 11 genes that were differentially regulated by OS vs. PS ($P < 0.05$, fold-change > 2 or < 0.5) were predicted by in silico analysis using TargetScan, and the resulting 16,738 target genes were subjected to Database for Annotation, Visualization, and Integrated Discovery analysis. (*C*) The differential regulation of endothelial secretion of miR-126-3p, miR-200a-3p, miR-143-3p, and miR-146a-5p, by OS vs. PS, was validated by qRT-PCR. (*D* and *E*) ECs were transfected with siCL (control siRNA) or siV+S (siRNAs targeting VAMP3 and SNAP23), kept under static condition or exposed to OS, and accumulations of miR-126-3p and miR-200a-3p in EC-conditioned media (EC-CM) or in the flow perfusates were analyzed. Data in *A* and *C–E* are mean ± SEM from three independent experiments. *$P < 0.05$.

VAMP3 and SNAP23 clearly reduced the AGO2 secretion under static condition as well as OS (*SI Appendix*, Fig. S5). Taken together, these results suggest potential roles of VAMP3 and SNAP23 in mediating the constitutive and regulated secretion of AGO2.

VAMP3 and SNAP23 Mediate the OS-Induced miR-126-3p Secretion. After exposure of ECs to PS or OS for 24 h, the flow perfusates were collected and subjected to sequential ultracentrifugation to precipitate the extracellular vesicles, including exosomes. qRT-PCR array with primer sets recognizing 218 endothelial-enriched miRNAs was then performed on the vesicle-poor supernatant (*SI Appendix*, Table S1). Of the 218 miRNAs on the array, 15 miRNAs were differentially regulated ($P < 0.05$) by OS vs. PS (Fig. 2*A*), with fold-changes greater than 2.0 or less than 0.5 in 11 of them. The predicted target genes of these 11 miRNAs belong to a multiplicity of biological processes (Fig. 2*B*), indicating the diversity of flow-regulated miRNA secretion and suggesting a potential importance of the secretion in regulating vascular function. Regular qRT-PCR validated the results of miR-126-3p, miR-200a-3p, miR-143-3p, and miR-146a-5p (Fig. 2*C*). We knocked down VAMP3/SNAP23 in ECs, subjected the cells to OS or static condition, and then assayed miRNA levels in the OS-perfusates or in the conditioned media. Among the four validated miRNAs, the enrichments of miR-126-3p and miR-200a-3p in both the static EC-conditioned media and the OS-perfusates were reduced by the depletion of VAMP3 and SNAP23 (Fig. 2 *D* and *E*). Interestingly, knockdown of VAMP3 and SNAP23 did not decrease the levels of miR-143-3p and miR-146a-5p in the conditioned media or OS-perfusates (*SI Appendix*, Fig. S6), nor did it cause significant changes of either the primary or the mature forms of miR-126-3p, miR-143-3p, miR-146a-5p, or miR-200a-3p (*SI Appendix*, Fig. S7), suggesting that VAMP3 and SNAP23 did not interfere with intracellular expression or maturation of miRNAs. The modulatory role of VAMP3/SNAP23 in the regulation of miRNA secretion was further studied by their overexpression. Overexpression of VAMP3/SNAP23, which per se promoted miR-126-3p secretion (*SI Appendix*, Fig. S8*A*), was able to overcome the inhibitory effect of VAMP3/SNAP23 knockdown on miR-126-3p secretion (*SI Appendix*, Fig. S8*B*).

VAMP3 and SNAP23 Are Required for the EC-to-SMC Signaling Mediated by miR-126-3p. We tested the roles of VAMP3 and SNAP23 in the EC-to-SMC transfer of miR-126-3p and their contribution to the EC-regulation of SMC phenotype and behavior. Under static conditions, coculture of SMCs with ECs increased the SMC miR-126-3p compared with monoculture; this increase in SMCs was abolished with ECs upon VAMP3 and SNAP23 knockdown and reappeared following reintroduction of miR-126-3p mimics (Fig. 3*A*). Analysis of expressions

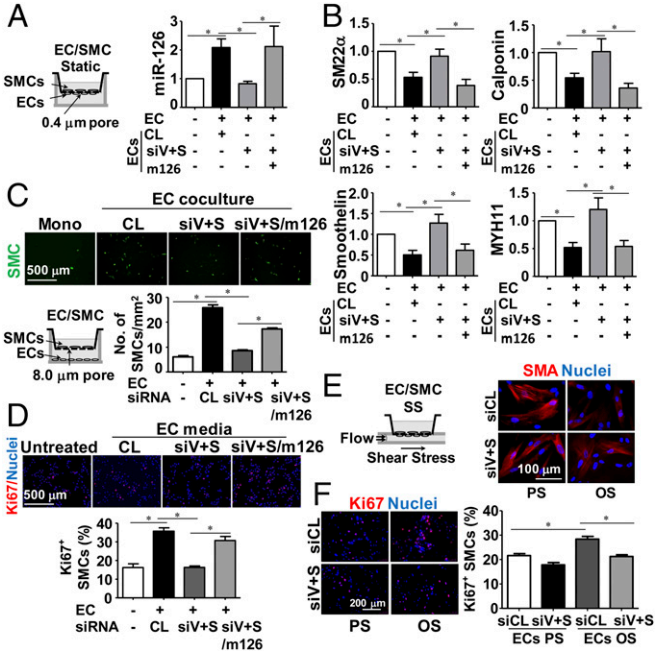

Fig. 3. Knockdown of VAMP3 and SNAP23 in ECs abolishes the regulation of ECs on SMC phenotype and behavior. In *A–C*, *E*, and *F*, SMCs were monocultured (EC–) or cocultured with ECs (EC+) and transfected with siRNAs (siV+S) and/or miRNA-126-3p mimics (m126); in *D*, SMCs were treated with conditioned media from ECs that had been transfected with siRNAs and/or miRNA mimics. (*A*) Schematic diagram of EC/SMC coculture (*Left*). Enrichment of miR-126-3p in the SMCs was analyzed (*Right*). (*B*) Expressions of smooth muscle contractile markers in SMCs from *A* were analyzed. (*C*) Schematic diagram of transwell migration assay (*Left*, *Lower*). The GFP-labeled migrated SMCs were observed microscopically (*Upper*) and quantified (*Right*, *Lower*). Mono, monoculture. (*D*) SMCs were untreated or treated with conditioned media from ECs that had been transfected with siRNAs and/or miRNA mimics, and cell proliferation was indicated by immunostaining of K_i67. (*E*) Schematic diagram of EC-SMC coculture and flow system (*Left*). Expression and organization of SMA were assayed by immunofluorescence (*Right*). (*F*) Expression of K_i67 in SMCs from *E* was assayed by immunofluorescence. Data in *A* and *B* are mean ± SEM from six independent experiments. Images in *C–F* are representative of triplicate experiments with similar results. Results in *C*, *D*, and *F* are mean ± SEM from triplicate experiments with 10 randomly selected microscopic fields of each experiment. *$P < 0.05$.

of contractile genes in the SMCs that have been cocultured with static ECs showed that the repressions of calponin, smoothelin, SM22α, and MYH11 because of EC coculture were abolished by the knockdown of VAMP3 and SNAP23 in the ECs; the repressions reappeared with the subsequent treatment of ECs with miR-126-3p mimics in the ECs (Fig. 3*B*). Transwell migration assay revealed that SMC migration was induced by ECs transfected with control siRNA (Fig. 3*C*). This effect was eliminated by the cotransfection of ECs with VAMP3- and SNAP23-specific siRNAs, but was maintained by the cotransfection of ECs with these siRNAs and miR-126-3p mimics (Fig. 3*C*). Immunostaining of the proliferative marker K_i67 indicated that treatment of SMCs with conditioned media from control ECs or from ECs with cotransfection of VAMP3- and SNAP23-specific siRNAs and miR-126-3p mimics induced SMC proliferation, but not those from ECs transfected with only the siRNAs in the absence of miR-126-3p mimics (Fig. 3*D*).

We studied the roles of EC VAMP3/SNAP23 in modulating the shear-regulated SMC phenotypic switch in our coculture and flow system. SMCs cocultured with ECs subjected to PS had clearly visible SMA stress fibers, but those cocultured with ECs subjected to OS did not display such SMA expression (Fig. 3*E*). This decrease in SMA expression by OS was ameliorated by knockdown of VAMP3 and SNAP23 in the ECs (Fig. 3*E*). Application of OS to ECs increased the percentage of K_i67^+ cells in the cocultured SMCs, and this increase was suppressed by inhibition of EC VAMP3 and SNAP23 (Fig. 3*F*).

Taken together, these results demonstrate the importance of VAMP3 and SNAP23 in modulating the miR-126-3p mediated EC-to-SMC signaling. Supporting evidence was provided by experiments showing that the EC-to-SMC miR-126-3p transfer could be blocked by treating SMCs with dynasore, a cell-permeable inhibitor of dynamin that is essential for clathrin-mediated endocytosis (*SI Appendix*, Fig. S9*A*). Dynasore-dependent inhibition of SMC endocytosis blocked the decrease of SMC contractile marker SM22α caused by the action of OS on the cocultured ECs (*SI Appendix*, Fig. S9*B*). Moreover, transfection of SMCs with miR-126-3p mimics induced SMC proliferation, migration, and down-regulation of contractile markers (*SI Appendix*, Fig. S10 and S11), reinforcing the functional relevance of miR-126-3p in phenotypic modulation of SMCs.

Rapamycin Decreases the Endothelial Secretion of miR-126-3p via Inhibiting VAMP3 and SNAP23. We and others have reported that OS activates the mammalian target of rapamycin (mTOR) and that this activation resulted in endothelial dysfunction (19, 20). mTOR controls the synthesis of proteins, including Syntaxin13 (21), which belongs to the SNARE family and forms complexes with VAMP3 and SNAP23. Pharmacological inhibition of mTOR complex 1 (mTORC1) in ECs by rapamycin decreased the expressions of VAMP3 and SNAP23 (Fig. 4*A*). In addition, treatment with rapamycin abolished the OS-induction of VAMP3 and SNAP23 (Fig. 4*B*). In luciferase reporter experiments, induction of the promoter activity was observed in cells that were transfected with the luciferase-promoter constructs of VAMP3 or SNAP23, and then treated with the mTOR activator MHY1485 (Fig. 4*C*). This induction was abolished when the transfected cells were cotreated with rapamycin (Fig. 4*C*). Rapamycin per se did not decrease the promoter activity, probably because of the low basal level of mTOR activity in the cells. These results suggest a transcriptional regulation of VAMP3 and SNAP23 expressions by mTOR.

The inhibitory effects of rapamycin on EC secretion of miR-126-3p were evidenced by the decreases of the miR-126-3p levels by rapamycin both in the conditioned media from static ECs and in the OS-perfusate (Fig. 4*D*). The EC-to-SMC transfer of miR-126-3p was also attenuated by rapamycin treatment of ECs (Fig. 4*E*).

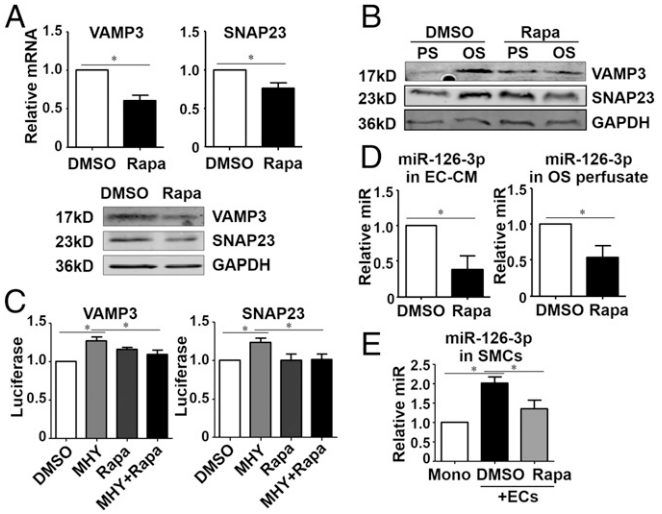

Fig. 4. Rapamycin decreases the endothelial secretion and EC-to-SMC transfer of miR-126-3p via its inhibition on VAMP3 and SNAP23 expressions. (*A*) ECs were treated with rapamycin (Rapa, 50 nmol/L) or control vehicle (DMSO) for 12 h, and expressions of VAMP3 and SNAP23 were analyzed. (*B*) ECs were pretreated with rapamycin or DMSO, exposed to PS or OS for 6 h, and expressions of VAMP3 and SNPA23 were analyzed. (*C*) Luciferase activity assay in Hela cells with the transfection of reporter constructs harboring the promoter regions of VAMP3 or SNAP23. Cells were treated with DMSO, MHY1485 (MHY, 10 μmol/L), rapamycin, or MHY1485 plus rapamycin (MHY+Rapa). (*D*) ECs were pretreated with rapamycin or DMSO, kept under static condition or exposed to OS for 12 h, and miR-126-3p accumulations in EC-CM or in the flow perfusates were assayed. (*E*) SMCs were monocultured (Mono) or cocultured with ECs, those were treated with rapamycin or DMSO, and miR-126-3p enrichments in the SMCs were detected by qRT-PCR. Data in *A*, and *C–E* are mean ± SEM from three to five independent experiments. Results in *B* are representative of triplicate experiments with similar results. $^*P < 0.05$.

Inhibition of EC-to-SMC Transfer of miR-126-3p Suppresses the Disturbed Flow-Induced Smooth Muscle Hyperplasia. Local induction of disturbed flow in vivo was achieved by partial carotid ligation surgery in which three of the four caudal branches of the left common carotid artery were ligated. Such partial carotid ligation surgery in Apolipoprotein E-deficient ($ApoE^{-/-}$) mice fed a Western diet would result in severe smooth muscle hyperplasia in 1 mo. In the present study, this model was used to investigate the functional outcome of inhibition of EC-to-SMC transfer of miR-126-3p. In some experiments, the mice received intraperitoneal injection of rapamycin or its control solvent. In others, the left common carotid arteries of mice were subjected to a local intraluminal incubation of adenovirus expressing SNAP23 (Ad-SNAP23) or its control virus before ligation. In complementary experiments, dynasore- or its control solvent-loaded pluronic gel was applied periadventitially to the operated arteries immediately after ligation. The operated mice were fed a Western diet. The carotid arteries on both sides were isolated 1 or 4 wk after surgery. Partial carotid ligation resulted in increased expressions of VAMP3 and SNAP23 in the endothelia of the common carotid arteries, in comparison with the expressions from unligated right sides, at 1 wk postsurgery (Fig. 5*A*). Repeated injections of rapamycin decreased the expressions of VAMP3 and SNAP23 in the arteries (Fig. 5*A*), in agreement with our in vitro findings (Fig. 4 *A* and *B*). H&E staining showed that in mice treated with rapamycin or dynasore the neointimal thickening were reduced by 74.8% and 46.7% of their respective controls, as indicated by the intima-to-media ratio (Fig. 5*B* and *SI Appendix*, Fig. S12*A*). In situ hybridization of miR-126-3p in the serial sections showed that there was a concomitant decrease of miR-126-3p in the neointima

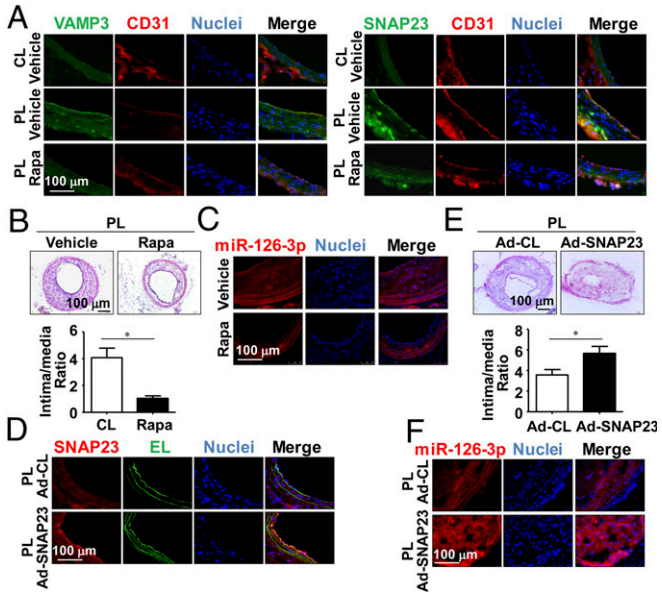

Fig. 5. Systemic application of rapamycin ameliorates the disturbed flow-induced neointimal formation, whereas local delivery of Ad-SNAP23 aggravates it. (*A*) Representative images of immunofluorescent-stained VAMP3, SNAP23, and endothelial marker CD31 in unligated or ligated carotid arteries of mice. The mice were subjected to partial ligation (PL) and were injected with rapamycin (Rapa) or control vehicle. VAMP3, SNAP23, and endothelial marker CD31 were visualized. CL, the unligated carotid arteries. (*B*) Representative images and quantification of H&E-stained ligated carotid arteries. The mice were subjected to repeated intraperitoneal injection of rapamycin or control vehicle immediately after surgery. (*C*) In situ hybridization of miR-126-3p in ligated carotid arteries from *B*. (*D*) Representative images of immunofluorescent-stained SNAP23 in ligated carotid arteries. The mouse carotid arteries were subjected to a locally intraluminal incubation with adenovirus expressing SNAP23 (Ad-SNAP23) or control virus (Ad-CL). (*E*) Representative images and quantification of H&E-stained ligated carotid arteries. The mice were subjected to a local incubation of Ad-SNAP23 or Ad-CL. (*F*) In situ hybridization of miR-126-3p in ligated carotid arteries from *E*. Results in *B* and *E* are mean ± SEM from 10 animals in each group. EL, elastic lamina. $^*P < 0.05$.

of rapamycin- and dynasore-treated arteries (Fig. 5*C* and *SI Appendix*, Fig. S12*B*), implicating a causal role of endothelial miR-126-3p in neointimal formation. Intraluminal application of Ad-SNAP23 increased the expression of SNAP23 in the endothelia (Fig. 5*D*). Neointimal thickening (Fig. 5*E*) and miR-126-3p accumulation (Fig. 5*F*) were significantly exaggerated in the Ad-SNAP23–treated arteries compared with those in controls. Collectively, our results suggest that inhibition of endothelial VAMP3 and SNAP23 and the consequent EC-to-SMC miR-126-3p transfer could suppress the disturbed flow-induced smooth muscle hyperplasia.

Discussion

Mutation, abnormal expression, and mislocalization of vesicular transport proteins have been observed in various cardiovascular diseases, including atherosclerosis (22, 23). However, there is only a limited understanding of the pathophysiology of vesicular-transport–associated cardiovascular diseases. Among those vesicular-transport proteins, *v*-SNARE VAMP3 was originally found to be required for exocytosis of the GluT4 isoform of the glucose transporter in adipocytes (24). It is enriched in the membrane of GluT4 secretory granule and undergoes translocation from the cytoplasmic subcompartment to the plasma membrane in response to insulin (24). VAMP3 forms complexes with *t*-SNARE SNAP23 that are localized predominantly to the plasma membrane to participate in exocytotic processes in multiple types of cells (15, 25, 26). In ECs, VAMP3 cooperates with SNAP23 to direct the fusion of Weibel-Palade bodies with the plasma membrane, and thus mediates the exocytosis of von Willebrand Factor (15), the release of which initiates platelet capture and thrombus formation. In this study, we demonstrated that VAMP3 cooperates with SNAP23 (*SI Appendix*, Figs. S1 and S3) to mediate the exocytosis of Ago2 (*SI Appendix*, Figs. S4 and S5) and miRNAs, such as miR-126-3p and miR-200a-3p (Fig. 2 *D* and *E* and *SI Appendix*, Figs. S7 and S8). ECs with decreased expressions of VAMP3 and SNAP23 have a reduced miR-126-3p release into the media (Fig. 2*D* and *SI Appendix*, Fig. S8) and a limited miR-126-3p uptake in the adjacent SMCs (Fig. 3*A*). These observations reinforce the potential importance of the roles of VAMP3 and SNAP23 in both constitutive and regulated EC secretion and provide an important piece of evidence that establishes the connection between SNAREs and miRNA secretion. The SNARE-mediated secretion of miRNAs, as well as other granule contents, enables ECs to respond in a tightly regulated manner to the alteration of environmental stimuli. Moreover, inhibition of VAMP3 and SNAP23 in endothelia suppresses the smooth muscle hyperplasia caused by endothelial activation (Fig. 5 *B* and *C*). These findings provide a mechanistic explanation for the relevance of the SNARE family to vascular function and for the mechanisms underlying the pathophysiology of SNAREs-associated diseases.

We have previously demonstrated that endothelial secretion of miR-126-3p is reduced by atheroprotective LS. To date, only three studies—including ours—have analyzed the flow-regulated miRNA secretion in ECs (3, 8, 27). The other two studies indicated that the secretions of miR-143-3p and miR-145-5p in ECs were regulated by shear stress and that the mechanisms involved the induction of transcription factor KLF2 and were probably dependent on Rab GTPases Rab7a and Rab27b (8, 27). To search for other miRNAs whose export is regulated by shear stress, we expanded the scope in the current study and introduced high-throughput profiling to identify and to characterize the vesicle-independent secretory miRNAomes in response to OS vs. PS. We hypothesized that the secretory miRNAomes can be differentially regulated by atheroprotective and atherogenic shear stress to result in beneficial or detrimental outcomes, respectively, for the vasculature. After 24 h of shearing, 11 endothelial-enriched miRNAs in the vesicle-poor supernatant of flow perfusate were significantly regulated in OS compared with PS. We validated part of the array results and found that miR-126-3p and miR-200a-3p were up-regulated by OS, whereas miR-143-3p and miR-146a-5p were down-regulated (Fig. 2*C*). In vascular SMCs, miR-126-3p is proproliferative (3) and miR-200a-3p plays a proinflammatory role (28), whereas miR-143-3p (29) and miR-146a-5p (30) help the cells to maintain the highly differentiated phenotype. The flow pattern-specific regulation of the secretion of these miRNAs provides a mechanistic basis of the miRNA-mediated atherogenic effects of OS. Bioinformatics analyses have suggested that the major differences of the miRNA-targeted genes between PS and OS are in the categories of cell proliferation, migration, inflammation, and TGF-β receptor signaling (Fig. 2*B*). This dataset allows us to construct the functional targeting networks of mechano-sensitive miRNAs and to identify novel miRNA-mediated pathways.

Previously, we and others showed that the signaling molecules involved in the regulation of endothelial homeostasis in response to shear stress include an atypical serine/threonine kinase mTOR (20, 31, 32). mTOR is a critical regulator of many fundamental features responding to upstream cellular signals and participating in the control of gene expression and cell behavior (33). Pharmacological intervention of mTORC1 with rapamycin has shown clear evidence of the value of mTOR inhibition in preventing the development of atherosclerosis and restenosis (34, 35). Although mTORC1 has been linked to endothelial function and dysfunction, its downstream targets functioning in vascular dysregulation have not been examined in detail. Using

rapamycin to inhibit mTORC1, we identified a potential mechanism of the preeminent effect of rapamycin on the expressions of VAMP3 and SNAP23 in ECs and the subsequent EC-to-SMC miRNA transfer (Fig. 4). Luciferase reporter experiments revealed that VAMP3 and SNAP23 were transcriptionally regulated by mTOR (Fig. 4*C*). Our data demonstrat that VAMP3 and SNAP23 expressions are up-regulated (Fig. 1 *A* and *C*) by OS-activation of mTOR in ECs to promote miRNA export, thus providing further explanation of the miRNA-mediated detrimental effects of OS on vascular function.

We have previously reported that EC miR-126-3p serves as a functional molecule to increase SMC proliferation and apoptosis, and that its release is reduced by atheroprotective LS to ECs (3). We also reported that systemic depletion of miR-126-3p in mice inhibits smooth muscle hyperplasia induced by cessation of blood flow (3). However, we could not exclude the possibility that other endothelial secretory miRNAs whose export can be regulated by shear stress [e.g., miR-143-3p and miR-145-5p (8)] also contribute to the endothelial modulation of SMC function. In fact, the transferable miRNAs that mediate cell–cell communications have been increasingly found and their functional targeting capabilities in the recipient cells have been verified (5–8, 10, 11, 36). Therefore, the identification of key molecules or mechanisms that mediate the miRNA-dependent endothelial regulation of smooth muscle hyperplasia is of particular importance. Herein, we demonstrate that VAMP3 and SNAP23 play critical roles in regulating miRNA secretion and the subsequent neointimal formation, as evidenced by the results that pharmacological inhibition of VAMP3 and SNAP23 attenuates the disturbed flow-induced smooth muscle hyperplasia (Fig. 5*B*), whereas adenovirus-mediated overexpression of SNAP23 aggravates it (Fig. 5*E*). This study shows the functional importance of SNAREs in the regulation of vascular SMC function in response to atherogenic fluid shear stress to endothelia. Our findings provide a mechanistic explanation for the noncoding RNA-mediation of detrimental effects of oscillatory shear on vascular function and suggest a therapeutic potential of targeting the endothelial vesicular transport system in the control of smooth muscle proliferative diseases.

Materials and Methods

All animal studies were performed in accordance with the approved protocol of the Animal Care and Use Committee of Peking University. The sources of antibodies, adenovirus, plasmids, and reagents and detailed methods for EC-SMC coculture and parallel-plate coculture flow system, flow apparatus for EC monoculture, preparation of vesicle-poor EC conditioned media, transmission electron microscopy, miRNA qRT-PCR array, RNA isolation and qRT-PCR, miRNA in situ hybridization, transient transfection and luciferase reporter assay, immunoprecipitation and coimmunoprecipitation assay, Western blot assay, immunofluorescence, transwell migration assay, and statistical analyses are described in *SI Appendix, SI Materials and Methods*.

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