MicroRNA-10a is crucial for endothelial response to different flow patterns via interaction of retinoid acid receptors and histone deacetylases

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Contributed by Shu Chien, January 9, 2017 (sent for review November 28, 2016; reviewed by Hanjoong Jo and Qingbo Xu)

Histone deacetylases (HDACs) and microRNAs (miRs) have emerged as two important epigenetic factors in the regulation of vascular physiology. This study aimed to elucidate the relationship between HDACs and miRs in the hemodynamic modulation of endothelial cell (EC) dysfunction. We found that miR-10a has the lowest expression among all examined shear-responsive miRs in ECs under oscillatory shear stress (OS), and a relatively high expression under pulsatile shear stress (PS). PS and OS alter EC miR-10a expression to regulate the expression of its direct target GATA6 and downstream vascular cell adhesion molecule (VCAM)-1. PS induces the expression, nuclear accumulation, and association of retinoid acid receptor-α (RARα) and retinoid X receptor-α (RXRα). RARα and RXRα serve as a "director" and an "enhancer," respectively, to enhance RARα binding to RA-responsive element (RARE) and hence miR-10a expression, thus down-regulating GATA6/VCAM-1 signaling in ECs. In contrast, OS induces associations of "repressors" HDAC-3/5/7 with RARα to inhibit the RARα-directed miR-10a signaling. The flow-mediated miR-10a expression is regulated by Krüppel-like factor 2 through modulation in RARα–RARE binding, with the consequent regulation in GATA6/VCAM-1 in ECs. These results are confirmed in vivo by en face staining on the aortic arch vs. the straight thoracic aorta of rats. Our findings identify a mechanism by which HDACs and RXRα modulate the hormone receptor RARα to switch miR-10a expression and hence the proinflammatory vs. anti-inflammatory responses of vascular endothelium under different hemodynamic forces.

endothelial cells | histone deacetylase | hormone receptor | microRNA | shear stress

Vascular endothelial cells (ECs) are exposed to different patterns of shear flow, including pulsatile shear stress (PS) and oscillatory shear stress (OS) (1). OS, which exists preferentially in arterial branches and curvatures, exerts proatherogenic effects to cause vascular EC dysfunction to promote atherosclerosis. In contrast, PS, which prevails in straight parts of the arterial tree, plays an atheroprotective role in regulating EC function to prevent atherosclerosis.

Epigenetics is the study of any potentially stable and ideally heritable changes in gene expression or cellular phenotype without alterations in DNA sequences (2). Epigenetic modulations, including histone modification and RNA-based mechanisms, have been identified to regulate vascular functions (2–4). Histone deacetylases (HDACs) and microRNAs (miRs) have emerged as two important epigenetic mediators in vascular pathophysiology (2–6). In previous work, we demonstrated that exposure of ECs to OS induces associations of HDAC-3/5/7 with myocyte enhancer factor-2 (MEF-2) to down-regulate the anti-inflammatory gene Krüppel-like factor-2 (KLF-2). In contrast, PS induces dissociation of HDAC-3/5/7 from MEF-2 to up-regulate KLF-2 in ECs (6). OS and PS regulate different sets of EC miRs to induce proinflammatory and anti-inflammatory responses, respectively (2–4). Although HDACs and miRs have been shown to modulate EC function in response to hemodynamic forces, little is known about the role of their interaction in regulating EC biology and pathobiology resulting from different flow patterns.

miR-10a has been identified as the miR with the lowest expression among 1,139 miRs, including miR-92a, miR-21, and miR-221, in the endothelium of atherosusceptible regions [inner curvature of the aortic arch (AA)] vs. atheroprotected regions [descending thoracic aorta (TA)] in normal adult swine in vivo, and it inhibits the proinflammatory endothelial phenotype in vitro (7). However, whether different hemodynamic forces play differential roles in regulating miR-10a expression, and the detailed mechanisms involved in these regulations, remain unclear. In tumor cells, miR-10a has been found to be regulated by retinoid acid receptors (RARs, i.e., RARα, RARβ, and RARγ) (8), which heterodimerize with any of three retinoid X receptors (RXRs, i.e., RXRα, RXRβ, or RXRγ) to bind RA-responsive elements (RAREs) in the enhancer region of target genes to regulate their expression (9). RARs also can recruit HDACs to inhibit transcriptional activity (10). In the present study, using in vitro cell culture studies on the effects of OS vs. PS on

Significance

This study demonstrates that hormone receptor RARα plays a vital role in the selective activation of proinflammatory and anti-inflammatory signaling to modulate the miR-10a/GATA6/VCAM-1 cascade in endothelial cells in response to proatherogenic oscillatory shear stress (OS) vs. atheroprotective pulsatile shear stress (PS). HDAC-3/5/7 and RXRα are induced by OS and PS to serve as mechanosensitive "repressors" and "enhancers," respectively, to associate with RARα to modulate its binding to RA-responsive element (RARE) to switch miR-10a expression. Our findings provide insight into the relationship between two different epigenetic factors (HDACs and miRs) and hormone receptors (RARα and RXRα) in the regulation of endothelial functions and elucidate new mechanisms of hemodynamic-based pathophysiology of the atherosclerotic vascular wall.

Author contributions: D.-Y.L., S.C., and J.-J.C. designed research; D.-Y.L., T.-E.L., C.-I.L., Y.-H.H., and P.-L.L. performed research; D.-Y.L., J.Z., Y.-T.S., and J.-J.C. analyzed data; and D.-Y.L., S.C., and J.-J.C. wrote the paper.

Reviewers: H.J., Emory University and Georgia Institute of Technology; and Q.X., King's College London.

The authors declare no conflict of interest.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1621425114/-/DCSupplemental.

molecular signaling and in vivo investigations on atherosusceptible vs. atheroprotected regions, we have demonstrated that RXRα and HDAC-3/5/7 constitute a regulatory machinery that serves as a mechanosensitive "enhancer" and "repressor," respectively, to switch the role of hormone receptor RARα in modulating miR-10a expression in ECs in response to different shear stresses, and consequently modulate EC function/dysfunction.

Results

miR-10a Targets GATA6 to Modulate VCAM-1 Expression in ECs, with Differential Modulation by PS and OS. Several miRs have been shown to be regulated by fluid flow to modulate endothelial function/dysfunction; these "mechano-miRs" include miR-10a, -19a, -23b, -21, -663, -92a, -145, -101, -126, -155, and -148a (3). Therefore, we first examined the roles of different shear stresses in modulating EC expression of these mechano-miRs. Exposure of ECs for 24 h to PS up-regulated miR-145, -23b, -10a, -126–5p, -21, -19a, -155, and -101 and down-regulated miR-92a (Fig. 1*A*), whereas the same exposure to OS up-regulated miR-663, -92a, and -21 and down-regulated miR-10a, -126–5p, -145, and -148a (Fig. 1*B*). miR-10a is the miR with the lowest expression among all mechano-miRs examined in ECs subjected to OS, whereas its expression is relatively higher than that of other mechano-miRs in ECs exposed to PS. The opposing roles of OS vs. PS in modulating EC miR-10a expression were sustained over 24 h (Fig. 1*C*). Bioinformatics databases (Targetscan, miR.org, and Diana-Micro) predicted that GATA6 mRNA is a target of miR-10a.

We used the pMIR-REPORT system to detect whether miR-10a binds directly to the 3′-UTR of GATA6. Transfection with PreR-10a decreased the luciferase activity of reporter constructs containing wild-type GATA6 3′-UTR (WT) compared with vector control (Vet) (Fig. 1*D*). Mutation of the predicted miR-10a binding site (Mut) abolished this inhibitory effect of PreR-10a. Scramble control miR had no effect on luciferase activity. Chromatin immunoprecipitation (ChIP), gene knockdown, and luciferase assays identified GATA6 as a critical transcription factor for regulating EC VCAM-1 expression in response to different hemodynamic forces (Fig. S1). As expected, exposure of ECs to OS and PS up-regulated and down-regulated their GATA6/VCAM-1 gene expression, respectively, over the 24-h test period (Fig. 1*E*). Transfecting ECs with PreR-10a abolished the OS induction of GATA6/VCAM-1 genes, whereas AMR-10a rescued the PS inhibition of GATA6/VCAM-1 genes in these cells (Fig. 1*F*). These differential effects of OS vs. PS and PreR-10a vs. AMR-10a on the regulation of GATA6/VCAM-1 genes were also seen on their protein levels (Fig. S2). Taken together, these results indicate that PS and OS play differential roles in modulating EC expression of miR-10a to regulate its direct target GATA6 and downstream VCAM-1 expression.

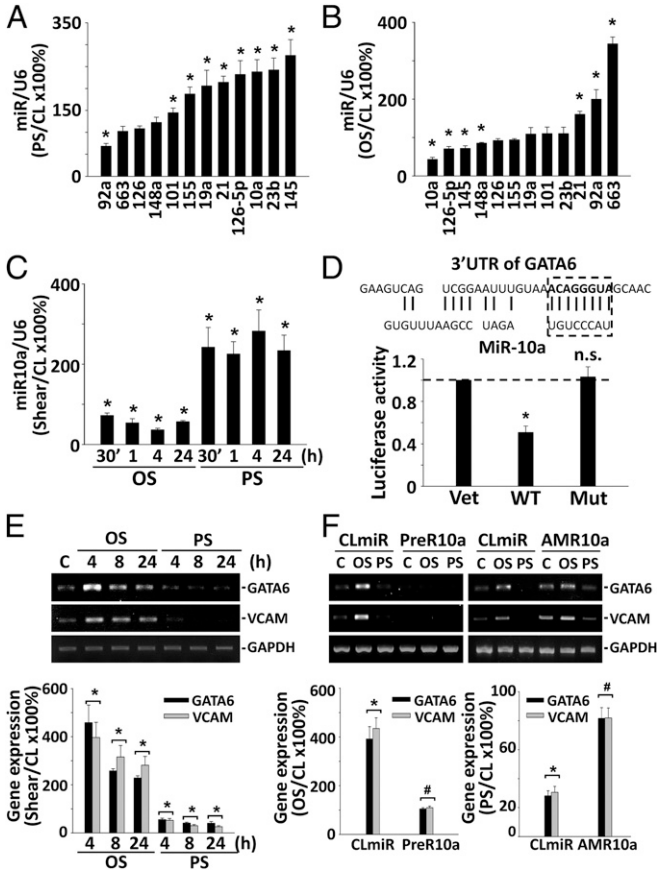

Fig. 1. PS and OS differentially regulate the expression of miR-10a, which directly targets GATA6 to modulate VCAM-1 expression in ECs. (*A–C*, *E*, and *F*) ECs were exposed to static or shear condition, and their expressions of mechano-miRs (*A* and *B*), miR-10a (*C*), and GATA6/VCAM-1 (*E* and *F*) were determined by qPCR and RT-PCR, respectively. (*D*) HeLa cells were cotransfected with PreR-10a and p-MIR-reporter plasmid with WT or Mut sequence or empty vector (Vet) to assess the miR-10a targeting of GATA6. (*F*) ECs were transfected with PreR-10a, AMR-10a, or CL-miR. Data are mean ± SEM from three independent experiments. $*P < 0.05$ vs. static control cells (*A–C*, *E*, and *F*) or Vet-transfected cells (*D*). $\#P < 0.05$ vs. sheared cells transfected with CL-miR (*F*).

PS Induction of miR-10a Is Regulated by Increased Expression and Associations of RARα and RXRα in EC Nuclei. The expression and intracellular distributions of RARs (RARα/β/γ) and RXRs (RXRα/β/γ) were determined in ECs subjected to OS or PS for 24 h, as well as static controls. The expression of RARα and RXRα, but not of RARβ, RARγ, RXRβ, and RXRγ, was increased in ECs subjected to PS compared with control and OS-exposed cells (Fig. 2*A* and Fig. S3*A*). In particular, PS induced sustained induction of RARα and RXRα in EC nuclei (Fig. 2*B* and Fig. S3*B*). A coimmunoprecipitation assay demonstrated that PS, but not OS, caused sustained increases in the association of RARα with RXRα, but not with RXRβ or RXRγ, in ECs (Fig. 2*C*). This PS-induced RARα–RXRα association was confirmed in EC nuclei by an in situ proximity ligation assay (PLA) (Fig. 2*D*). The specificity of the PLA assay was verified by negative controls using anti-RARα or anti-RXRα antibody alone. PS-mediated up-regulation of miR-10a and down-regulation of GATA6/VCAM-1 were abolished by transfections with RARα-specific siRNA in combination with RXRα-specific siRNA, but only partially inhibited by RXRα-specific siRNA alone (Fig. 2 *E* and *F* and Fig. S3*C*). RARα- and RXRα-specific siRNA (compared with control siRNA, 40 nM each) caused a 90% reduction in RARα and RXRα protein expression (Fig. S4). These results indicate that PS induces sustained increases in the expression, nuclear accumulation, and associations of RARα and RXRα in ECs. Moreover, RARα and RXRα serve as a "director" and an "enhancer," respectively, to up-regulate miR-10a and hence inhibit GATA6/VCAM-1 expression in response to PS.

OS Inhibits miR-10a Expression Through RARα-HDAC-3/5/7 Associations, with Up-Regulation of GATA6/VCAM-1 in ECs. We examined whether HDACs can modulate the RARα/miR-10a signaling cascade in ECs in response to different hemodynamic forces. Coimmunoprecipitation assays showed that OS, but not PS, induced sustained increases in association of RARα with HDAC-3/5/7, but not with HDAC-1/2, in ECs (Fig. 3*A*). This OS-induced RARα–HDAC-3/5/7 association resulted in deacetylation of RARα, whereas PS increased RARα acetylation levels (Fig. 3*A*). Transfecting ECs with any of the HDAC-3/5/7-specific siRNAs abolished OS-induced RARα–HDAC-3/5/7

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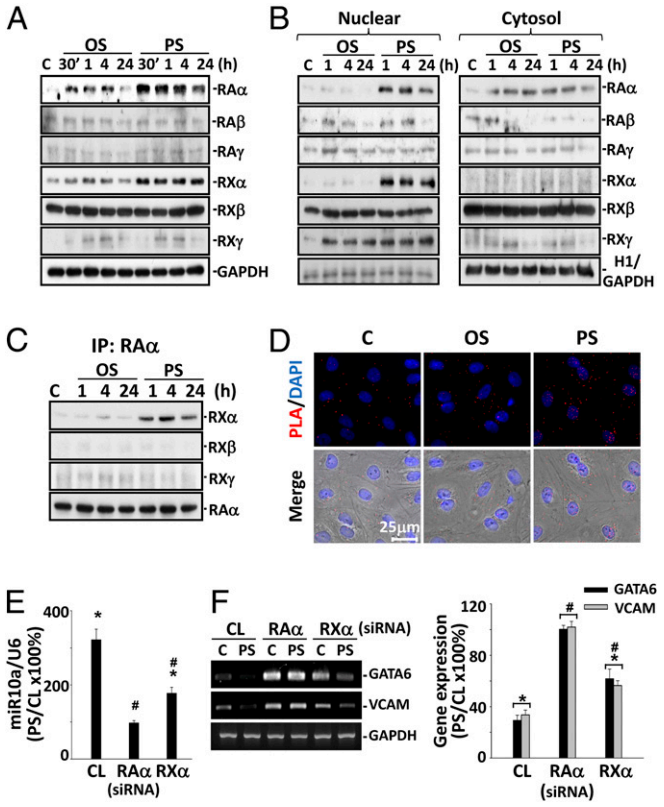

Fig. 2. PS induces sustained increases in the expressions, nuclear accumulations, and association of RARα and RXRα, which modulate miR-10a and downstream GATA6/VCAM-1 expressions in ECs. ECs were kept under static (C) or shear condition. (*A* and *B*) The expression of RARs and RXRs was determined by Western blot analysis (*A*), and their subcellular localization was determined by cell fractionation assay (*B*). (*C* and *D*) The association of RARα with RXRα was detected by coimmunoprecipitation (*C*) and in situ PLA (*D*). (*E* and *F*) ECs were transfected with control siRNA or specific siRNAs of RARα and RXRα before flow experiments. The expressions of miR-10a (*E*) and GATA6/VCAM-1 (*F*) were determined. Results in *A*–*D* are representative of three independent experiments with similar results. Data in *E* and *F* are mean ± SEM from three independent experiments. $*P < 0.05$ vs. static control cells. $\#P < 0.05$ vs. sheared cells transfected with control siRNA. RA, RAR; RX, RXR.

associations without increasing RARα–RXRs associations (Fig. 3*B*). HDAC-3-, -5-, and -7–specific siRNAs (compared with control siRNA, 40 nM each) caused 90% reductions in HDAC-3, -5, and -7 protein expression, respectively (Fig. S4). This HDAC knockdown-mediated abolition of RARα–HDAC-3/5/7 association was accompanied by increased RARα acetylation levels (Fig. 3*C*), which rescued miR-10a expression (Fig. 3*D*) to inhibit OS induction of GATA6 and VCAM-1 in ECs (Fig. 3*E*). These results indicate that HDAC-3/5/7 serve as "repressors" to associate with RARα to cause its deacetylation, which down-regulates miR-10a expression and up-regulates GATA6 and VCAM-1 in ECs in response to OS.

Differential Regulation of miR-10a in ECs by PS and OS Is Attributable to Their Differential Effects on RARα–RARE Binding Through RXRα and HDAC-3/5/7, Respectively. Many of the 39 mammalian Homeobox (HoxB) genes are regulated by retinoids through RARE. miR-10a is located in the 3′ genomic region of HoxB4, and DR5 type10 RARE is a candidate target sequence in the enhancer region of miR-10a for regulation of its expression (8). Our ChIP assay using a RARα-specific antibody and the DR5 type10 RARE-specific primers showed that RARα binding to the DR5 type10 RARE in ECs was induced by PS, but decreased by OS (Fig. 4*A*). Transfecting ECs with RXRα-specific siRNAs partially inhibited the PS-induced RARα–RARE binding activity (Fig. 4*B*). Transfecting ECs with any of HDAC-3/5/7-specific siRNAs abolished the OS-inhibited RARα–RARE binding activity (Fig. 4*C*). These results indicate that RXRα serves as an enhancer to induce RARα binding to RARE in the regulatory region of miR-10a in ECs in response to PS, whereas HDAC-3/5/7 serve as repressors to inhibit this binding activity of RARα–RARE in ECs exposed to OS.

KLF-2 Is Involved in Flow-Mediated miR-10a Expression in ECs. We examined whether KLF-2 is involved in flow-mediated miR-10a expression in ECs. Transfecting ECs with either RARα- or RXRα-specific siRNA had no effect on PS-induced EC KLF-2 expression (Fig. S5*A*); however, knockdown of KLF-2 inhibited PS-induced RARα–RARE binding (Fig. S5*B*) and miR-10a expression in ECs (Fig. S5*C*), and rescued the PS-mediated down-regulation of GATA6 and VCAM-1 (Fig. S5*D*). In contrast, overexpression of KLF2 rescued the OS reduction of RARα–RARE binding and miR-10a expression to inhibit OS-induced GATA6 and VCAM-1 expression in ECs. These results indicate

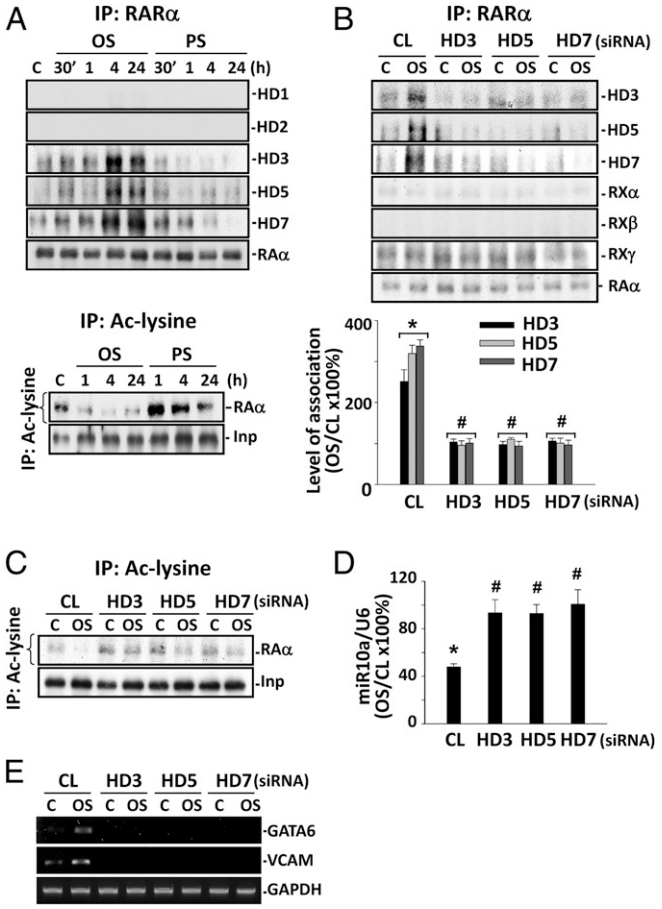

Fig. 3. OS inhibits miR-10a expression through RARα-HDAC-3/5/7 associations, with the up-regulations of GATA6 and VCAM-1 in ECs. (*A*–*C*) ECs were kept under static (C) or shear condition. ECs were transfected with control siRNA or HDAC-specific siRNAs before flow experiments. The association of RARα with HDACs (*A*), RXRs, and HDACs (*B*), and the acetylation of RARα (*A* and *C*) in ECs was detected by immunoprecipitation assay. (*D* and *E*) The expression of miR-10a and GATA6/VCAM-1 was examined. Results in *A*, *C*, and *E* are representative of three independent experiments with similar results. Data in *B* and *D* are mean ± SEM from three independent experiments. $*P < 0.05$ vs. static control cells. $\#P < 0.05$ vs. sheared cells transfected with control siRNA. HD, HDAC; RA, RAR; RX, RXR; Inp, input.

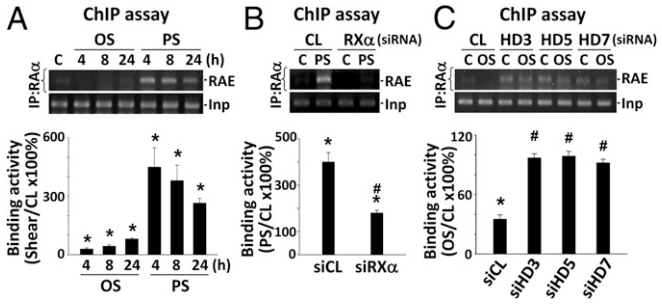

Fig. 4. RXRα and HDAC-3/5/7 serve as "enhancer" and "repressor" to modulate flow-regulated RARα-RARE binding in ECs. ECs were kept under static (*C*) or flow condition (*A*). ECs were transfected with control siRNA or specific siRNA of RXRα (*B*) or HDACs (*C*) before flow experiments. ChIP was used to detect the binding of RARα to a DR5 type 10 RARE near the miR-10a gene. Data are mean ± SEM from three independent experiments. $*P < 0.05$ vs. static control cells. $\#P < 0.05$ vs. sheared cells transfected with control siRNA. RAE, RARE; Inp, input.

that KLF-2 is involved in flow-mediated miR-10a expression through the modulation in RARα–RARE binding, with the consequent modulation of GATA6/VCAM-1 signaling in ECs.

EC Expression of RARα, RXRα, miR-10a, GATA6, and VCAM-1 in Different Flow Regions in Circulation in Vivo. We examined the differential regulations of RARα, RXRα, miR-10a, GATA6, and VCAM-1 in the AA and the straight segment of the TA of normal rats (Fig. 5*A*) by en face staining for these molecules and von Willebrand factor (vWF), with DAPI nuclear counterstaining. The expression levels of RARα (Fig. 5*B*), RXRα (Fig. 5*C*), and miR-10a (Fig. 5*D*) were high in the TA and the outer curvature of the AA, where PS exists (1), but very low in the inner curvature of the AA, where OS prevails (1). The increased RARα and RXRα expression in the PS regions were localized mostly in EC nuclei, whereas the increased miR-10a was localized in both the nuclei and cytoplasm. In contrast to these molecules, the expression of GATA6 (Fig. 5*E*) and VCAM-1 (Fig. 5*F*) was very low in the TA and the outer curvature of the AA, but high in the inner curvature of the AA. Quantitative data confirmed the differential regulations of these molecules in different areas of the vessels in vivo (Fig. 5*G*). Our previous study showed high levels of HDACs in ECs in the inner curvature of the AA, but not in the outer curvature of the AA and the TA (6). Taken together, these in vivo results are in agreement with our in vitro findings indicating that EC expression levels of RARα, RXRα, HDACs, miR-10a, GATA6, and VCAM-1 are flow pattern-specific to induce proinflammatory and anti-inflammatory responses under OS and PS, respectively.

Discussion

The present study has elucidated the mechanisms (summarized in Fig. 6) by which HDACs and RXRα serve as key mechanosensitive molecules to associate with hormone receptor RARα to switch the control of EC miR-10a expression in shear modulation of vascular phenotypes and functions. This conclusion is based on several lines of evidence. First, endothelial miR-10a can be differentially regulated by OS and PS to play an atheroprotective role in PS by directly targeting transcriptional factor GATA6 to inhibit VCAM-1 expression in ECs. Second, PS induces sustained expression, nuclear accumulation, and associations of RARα and RXRα. PS-induced RARα and RXRα serve as a director and an enhancer, respectively, to promote RARα binding to RARE to increase miR-10a expression, thereby down-regulating GATA6/VCAM-1 signaling in ECs. Third, proatherogenic OS induces the formation of an HDAC-3/5/7–RARα repressor heterocomplex to inhibit RARα–RARE binding activity and miR-10a expression, with an up-regulation of GATA6/VCAM-1 signaling in ECs. Fourth, KLF-2 plays an important role in regulating flow-mediated miR-10a expression through regulation of RARα–RARE binding, with a consequent modulation in GATA6/VCAM-1 signaling in ECs. Finally, these in vitro results are confirmed by en face and immunohistochemical studies comparing the AA and TA of rats in vivo. Therefore, our findings provide mechanistic insight into the roles of hormone receptors (RARα and RXRα) and HDACs (HDAC-3/5/7) in switching miR-10a expression to regulate vascular functions in health and disease.

miRs have been identified as epigenetic mediators for atherosclerotic lesion development (2–4). miR-10a was recently found to be the miR with the lowest expression among 1,139 miRs in endothelia of atherosusceptible regions vs. atheroprotected regions in normal adult swine in vivo. Knockdown of miR-10a in human aortic ECs exerts a proinflammatory phenotype in vitro (7). We compared EC expression of miR-10a with that of other mechano-miRs, including miR-126–5p, -145, -148a, -126, -155, -19a, -101, -23b, -21, -92a, and -663, under different flow conditions, and confirmed that miR-10a is the miR with the lowest expression among all EC mechano-miRs examined in response to OS. In contrast, miR-10a expression is relatively

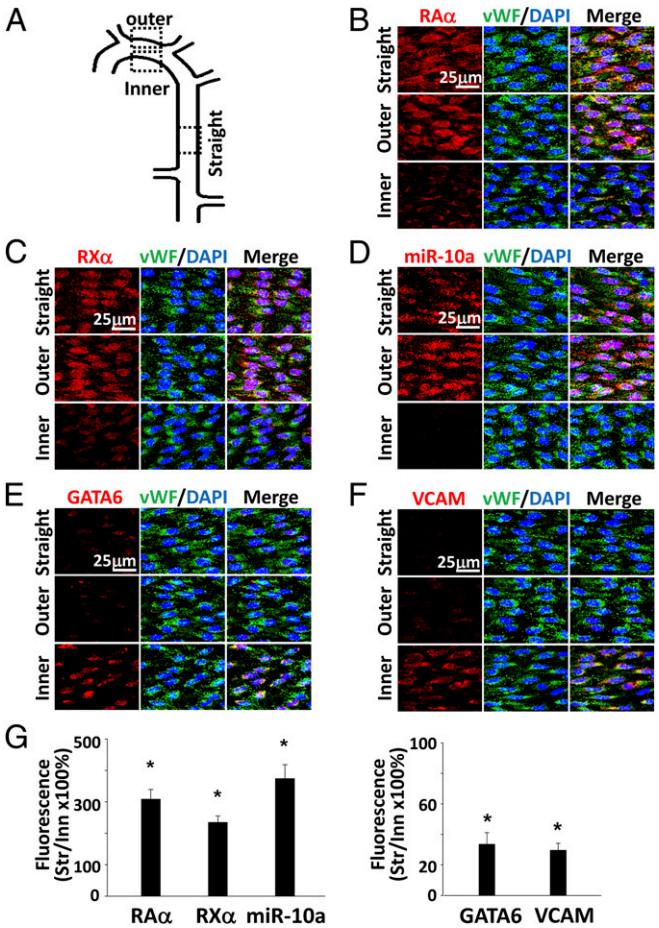

Fig. 5. Expression of RARα, RXRα, miR-10a, GATA6, and VCAM-1 is flow pattern-specific in the native circulation. (*A–F*) The inner and outer curvatures of the AA and the straight segment of the TA (*A*) of normal rats ($n = 5$) were examined by en face coimmunostaining for RARα (*B*), RXRα (*C*), miR-10a (*D*), GATA6 (*E*), or VCAM-1 (*F*), as well as vWF. Cell nuclei were counterstained with DAPI. (*G*) Samples were examined by confocal laser scanning microscopy. Data are mean ± SEM from five independent experiments. $*P <$ 0.05 vs. inner curvatures. Inn, inner; Str, straight.

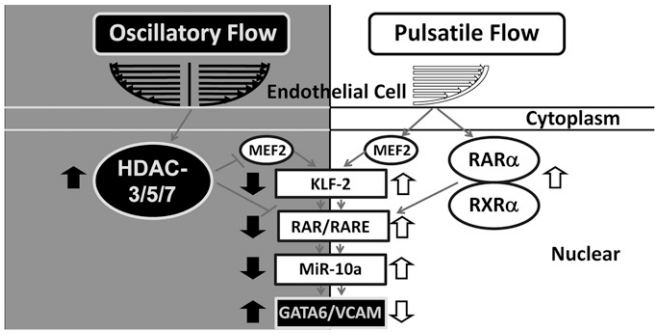

Fig. 6. Schematic diagram of the roles of hormone receptors and HDACs in modulating miR-10a expression and hence proatherogenic and anti-atherogenic signaling in EC in response to different flow conditions. Boxes with black and white shading represent proatherogenic and atheroprotective molecules, respectively.

higher than that of other shear-responsive miRs in ECs exposed to PS. miR-10a is differently regulated by OS and PS to modulate its downstream GATA6/VCAM-1 signaling by directly targeting 3′-UTRs of the transcriptional factor GATA6.

Our data on apolipoprotein E-deficient ($ApoE^{-/-}$) mice receiving control miR (CL-miR) or PreR-10a demonstrate that tail vein injection with a PreR-10a–invivofectamine mixture abrogated atherosclerotic lesion formation in the AA in comparison with CL-miR control mice by en face Oil-Red O staining of the whole aorta (Fig. S6*A*) and H&E staining of cross-sections of the AA (Fig. S6*B*). The results in cross-sections of the AA (Fig. S6*C*) show high miR-10a expression levels in the outer curvature, where PS exists, but very low miR-10a expression levels, with high GATA6/VCAM-1 expression levels, in the inner curvature, where OS prevails. Compared with CL-miR–treated mice, the expression of miR-10a in the EC layer in the AA inner curvature is increased in PreR-10a–treated mice (Fig. S7), accompanied by decreased expression of downstream molecules GATA6 and VCAM-1 (Fig. S7). These findings are in concert with our rat en face staining data showing that decreased expression of miR-10a is associated with increased expression of GATA6/VCAM-1 in ECs in the inner curvature of the AA. These results indicate that systemic delivery of PreR-10a can rescue EC miR-10a expression, thereby abolishing the OS induction of GATA6/VCAM-1 in proatherogenic regions to inhibit atherosclerotic lesion development. This information provides a causal link between flow, miR-10a, and downstream GATA6/VCAM-1 signaling in an animal model. These results indicate that miR-10a is a shear-responsive miR and functions as a major regulator of switching the expression of GATA6 and VCAM-1 to regulate the proinflammatory vs. anti-inflammatory response of vascular endothelium in response to OS vs. PS.

Recent studies indicate that RARs and their partners RXRs may play significant roles in cardiovascular biology. Whereas compound null mutations of RARs lead to significant heart malformations, RXRα gene disruption results in hypoplasia of the ventricular compact zone and muscular ventricular septal defect. Interestingly, compound null mutations of RARs with RXRα demonstrate marked synergistic effects on cardiac defects (11). Our study found that atheroprotective PS induces sustained increases in the expression, nuclear accumulation, and associations of RARα and RXRα in ECs. These PS inductions cooperate to enhance RARα–RARE binding and miR-10a expression, thereby down-regulating proinflammatory GATA6/VCAM-1 signaling in ECs. Our in vivo en face studies in rats further show that the expression levels of RARα, RXRα, and miR-10a in the atheroprotective areas with PS are much higher than those in the atherosusceptible areas with OS, whereas the relative expression levels of GATA6 and VCAM-1 are reversed. The increased RARα and RXRα in these PS regions were localized mostly in EC nuclei. Our findings indicate that RARα and RXRα serve as a director and an enhancer, respectively, to form a heterodimer in the nucleus to drive atheroprotective signaling by enhancing miR-10a to down-regulate proinflammatory GATA6/VCAM-1 signaling in ECs in response to atheroprotective PS.

HDAC is another important epigenetic factor that can modulate gene expression and cellular function by removing acetyl groups from critical signaling molecules to suppress their functions (2). Our previous study demonstrated that OS induces the expression of both class I (HDAC-1/2/3) and class II (HDAC-5/7) HDACs and their nuclear accumulation in ECs in vivo and in vitro (6); however, whether HDACs can modulate the EC expression of miRs to modulate vascular biology and pathobiology has not been reported. Our present study provides evidence that proatherogenic OS can induce associations of HDAC-3/5/7 with hormone receptor RARα to deacetylate RARα, thereby suppressing its binding to RARE and hence miR-10a expression, with the consequent induction of proinflammatory GATA6/VCAM-1 signaling in ECs. Knockout of any of HDAC-3/5/7 can totally abolish this OS-induced HDAC-3/5/7–RARα heterocomplex formation, leading to rescue from the repression of RARα–RARE binding and miR-10a expression, with the consequent abolition of GATA6/VCAM-1 induction in ECs. These results are in agreement with previous reports (12, 13), suggesting that class II HDACs may serve as a bridge to recruit HDAC-3 to form complexes that bind to selected transcription factors to regulate cellular function. Fischle et al. (12) also showed that the activity of class II HDAC is dependent on its interaction with the HDAC-3 in cell nuclei; however, knockout of any of HDAC-3/5/7 cannot induce RARα-RXRα associations. These results indicate that HDAC knockdown can only inhibit formation of the HDAC–RARα repression heterocomplex to rescue miR-10a expression to the basal level, but cannot induce RARα–RXRs association to enhance miR-10a expression. Our findings indicate a mechanism by which HDACs can form a repressor heterocomplex with a hormone receptor to regulate miR expression to promote proatherogenic signaling in ECs in response to OS.

An interesting finding of this study is that the hormone receptor RARα is a critical regulator that can switch the expression of miR-10a in ECs in response to different types of flows by directly interacting with different mediators, i.e., RXRα and HDACs. These results indicate that RARα and its downstream miR-10a serve as a key signaling cascade that can converge anti-atherogenic and proatherogenic signals in ECs through RXRα and HDACs, respectively.

Our findings demonstrate that VCAM-1 can be negatively regulated by miR-10a through GATA6. Recently reported studies have suggested that VCAM-1 also may be negatively regulated by miR-126–3p (14) and KLF-2 (15). Nicoli et al. (16) reported that miR-126–3p is up-regulated by fluid flow in zebrafish embryos. However, several other reports have indicated that miR-126–3p is not regulated by fluid flow and hence is identified as a shear-insensitive miR in human ECs (17, 18). In concert with these previous reports in human ECs, our present study indicates that miR-126–3p is a shear-insensitive miR and is not regulated by RARα, RXRα, and HDAC-3/5/7 in human aortic endothelial cells (HAECs) in response to shear stress (Fig. S8), indicating that miR-126–3p might not be involved in the regulation of shear-eliciting RARα/miR-10a/GATA6/VCAM-1 signaling in human ECs. On the other hand, KLF-2 has been predicted to be a transcriptional regulator for several miRs, including miR-10a (4). Our previous study demonstrated that the EC expression of KLF-2 is differentially regulated by OS vs. PS in vitro and in vivo (19). OS induces the expression of HDAC-3/5/7 in EC nuclei and their association with transcription factor MEF2, thereby inhibiting KLF-2 expression. Conversely, PS stimulates the phosphorylation and nuclear export

of HDAC-3/5/7 and their dissociation from MEF2 to induce KLF-2 expression (6). In the present study, we have demonstrated that KLF-2 is involved in the context of shear-eliciting RARα/miR-10a/GATA6/VCAM-1 signaling in ECs. In combination with previous results (6, 19), our findings advance the notion that PS can induce not only the dissociation of HDAC-5/7 from MEF2 to increase KLF-2 expression, but also the accumulation of RARα and RXRα and their association in EC nuclei to enhance RARα–RARE binding and miR-10a expression, thereby down-regulating GATA6 and VCAM-1 in ECs (Fig. 6). In contrast, OS can induce the association of HDAC-3/5/7 not only with MEF2 to repress KLF-2 expression, but also with RARα to induce RARα deacetylation, with the subsequent inhibitions in RARα–RARE binding and miR-10a expression, thereby up-regulating GATA6 and VCAM-1.

In summary, this study has elucidated the molecular and cellular mechanisms by which hemodynamic forces modulate the interactions of hormone receptors, interplay of epigenetic factors, and expression of proinflammatory genes, leading to the regulation of EC functions and dysfunctions. The hormone receptor RARα serves as a hub molecule to control miR-10a expression in ECs in response to different patterns of hemodynamic forces. Atheroprotective PS induces the formation of an RARα/RXRα heterodimer complex to enhance miR-10a transcription, which down-regulates proinflammatory GATA6/VCAM-1 signaling by targeting GATA6. In contrast, proatherogenic OS induces the formation of an HDAC-3/5/7–RARα repressor heterocomplex to inhibit miR-10a expression, thereby up-regulating GATA6/VCAM-1 signaling in ECs. Our findings provide insight into the mechanisms that regulate lesion development in vascular niches with disturbed flow and may help generate new approaches for therapeutic interventions.

Materials and Methods

Animal experiments were approved by the Animal Research Committee of National Health Research Institutes. The sources of materials and antibodies and the methods for cell culture; flow apparatus experiments; miR real-time quantitative PCR (qPCR); luciferase reporter assay; VCAM-1 promoter luciferase assay; miR, siRNA, and DNA plasmid transfection; RNA isolation and RT-PCR; immunoprecipitation; Western blot analysis; in situ PLA study; ChIP assay; en face preparations and staining; and statistical analysis are described in *SI Materials and Methods*.

ACKNOWLEDGMENTS. We thank Dr. Hye Jung Kim (Gyeongsang National University) for providing the VCAM-1 promoter-luciferase plasmid and its GATA mutant. This work was supported by the Ministry of Science and Technology, Taiwan (Grants MOST-106-2633-B-009-001/105-2321-B-400-007, to J.-J.C., and MOST-103-2321-B-400-011, to D.-Y.L.), the National Natural Science Foundation of China (Grants 91539116, 31522022, and 81470590, to J.Z.), and the National Institutes of Health (Grants HL-106579/HL-108735, to S.C.).

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