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Histone deacetylase 5 interacts with Krüppel-like factor 2 and inhibits its transcriptional activity in endothelium

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Aims Vascular endothelial dysfunction and inflammation are hallmarks of atherosclerosis. Krüppel-like factor 2 (KLF2) is a key mediator of anti-inflammatory and anti-atherosclerotic properties of the endothelium. However, little is known of the molecular mechanisms for regulating KLF2 transcriptional activation.

Methods and results Here, we found that histone deacetylase 5 (HDAC5) associates with KLF2 and represses KLF2 transcriptional activation. HDAC5 resided with KLF2 in the nuclei of human umbilical cord vein endothelial cells (HUVECs). Steady laminar flow attenuated the association of HDAC5 with KLF2 via stimulating HDAC5 phosphorylation-dependent nuclear export in HUVEC. We also mapped the KLF2–HDAC5-interacting domains and found that the N-terminal region of HDAC5 interacts with the C-terminal domain of KLF2. Chromatin immunoprecipitation and luciferase reporter assays showed that HDAC5 through a direct association with KLF2 suppressed KLF2 transcriptional activation. HDAC5 overexpression inhibited KLF2-dependent endothelial nitric oxide synthesis (eNOS) promoter activity in COS7 cell and gene expression in both HUVECs and bovine aortic endothelial cells (BAECs). Conversely, HDAC5 silencing enhanced KLF2 transcription and hence eNOS expression in HUVEC. Moreover, we observed that the level of eNOS protein in the thoracic aorta isolated from HDAC5 knockout mice was higher, whereas expression of pro-inflammatory vascular cell adhesion molecule 1 was lower, compared with those of HDAC5 wild-type mice.

Conclusions We reveal a novel role of HDAC5 in modulating the KLF2 transcriptional activation and eNOS expression. These findings suggest that HDAC5, a binding partner and modulator of KLF2, could be a new therapeutic target to prevent vascular endothelial dysfunction associated with cardiovascular diseases.

Keywords HDAC5 • KLF2 • eNOS • Transcriptional activation • Endothelial cells

1. Introduction

The vascular endothelium plays an important role in regulation of vascular homeostasis and mediates numerous vasoprotective functions, such as vasodilation, inhibition of smooth muscle cell growth, and anti-inflammatory response.[1,2] Many of these functions are largely mediated by nitric oxide (NO) from endothelial cells. The enzyme that is responsible for the synthesis of NO within the endothelium is endothelial NO synthesis (eNOS). eNOS activity is regulated by its gene transcription as well as cellular localization, protein–protein interaction, and post-translational modifications, such as acetylation and phosphorylation.[3] It has been demonstrated that fluid shear stress generated by steady laminar flow acting on endothelium increases eNOS expression and activity, which modulates vascular homeostasis, confers anti-thrombotic, anti-adhesive, and anti-inflammatory effects, and enhances endothelial survival.[4,5]

Krüppel-like factors (KLFs) are a subclass of the zinc-finger family of DNA-binding transcription factors, which have been shown to function to modulate cell differentiation and tissue development.[6] KLF2 is a shear stress-induced factor and exclusively expressed in the linear segments of

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the vessel exposed to steady laminar flow with high fluid shear stress.[7] Since the KLF2 promoter conserves a single consensus myocyte enhancer factor 2 (MEF2)-binding site, its expression is regulated by the MEF2.[8] MEF2 factors are members of the MADS box (MCM1, Agamous, Deficiens, and Serum response factor) family of transcription factors that bind to A/T-rich sequences.[9,10] The induction of KLF2 gene transcription under shear stress depends largely on the activity of MEK5/ERK5/MEF2 axis,[11] although the involvement of phosphatidylinositol 3-kinase-dependent chromatin remodelling and nucleolin has also been suggested.[12,13] Overexpression of a dominant negative MEF2 or a mutant MEK5, an upstream activator of ERK5, prevented steady laminar flow-mediated induction of KLF2 expression in endothelial cells.[11] Interestingly, MEF2C-null mice have a similar phenotype to KLF2-null mice and die in the late embryonic stage.[14] The studies using eNOS promoter deletion and mutational analysis have demonstrated that a single KLF2 site is critical for the ability of KLF2 to bind on and activates the eNOS promoter.[15] Indeed, knockdown of KLF2 prevents flow-mediated induction of eNOS.[11,16] Thus, these studies have established the role of KLF2 as a potent inducer of eNOS expression and activity. However, despite the importance of KLF2 in the vascular functions, the mechanisms of KLF2 transcriptional activation are largely unknown.

Histone deacetylases (HDACs) have emerged as crucial transcriptional co-repressors in highly diverse biological systems. Mammalian HDACs have been classified into four classes based on the homology of their catalytic domains, and the class II HDACs are further subdivided into two subclasses, classes IIa (HDAC4, 5, 7, 9, and the HDAC9 splice variant MITR) and IIb (HDAC6 and 10).[17] Class IIa HDACs contain a regulatory N-terminal domain that mediates their interaction with tissue-specific transcription factors and co-repressor, and become phosphorylated at two or three conserved serine residues in the regulatory N-terminal domain.[18] The phosphorylation status of class IIa HDACs is a critical event to determine their localization in the nucleus or cytoplasm and the ability to act as transcriptional co-repressors in the nuclear region. We have previously demonstrated that steady laminar stress stimulates phosphorylation-dependent nuclear export of HDAC5 through activating calcium/calmodulin-dependent kinase (CaMK) signalling, thereby augmenting expression of KLF2 and eNOS through stimulating transcriptional activity of MEF2 in endothelial cells.[19] In addition to the role of HDAC5 in regulating MEF2 transcriptional activity, a MEF2-binding independent function of HDAC5 has been suggested in the process of angiogenesis.[20]

In this study, we assessed the physical and functional interaction of KLF2 with HDAC5 and explored the molecular mechanism by which HDAC5 regulates laminar flow-induced eNOS expression. Our results define a novel transcriptional pathway in which HDAC5 represses KLF2 transcriptional activation and hence eNOS expression in endothelial cells.

2. Methods

2.1 Cell culture and fluid shear stress experiments

Human umbilical cord vein endothelial cells (HUVECs) were isolated from freshly and anonymously acquired as described previously.[21,22] HUVECs were cultured in Medium 200 containing 5% foetal bovine serum (FBS) and low serum growth supplement (Invitrogen, Grand Island, NY, USA). Bovine aortic endothelial cells (BAECs) were purchased from Lonza, Inc. (Allendale, NJ, USA) and cultured in Medium 199 (Invitrogen) containing 10% foetal clone III (Hyclone), MEM-vitamins (Cellgro, 25-020-CI), 1% MEM-alpha (Gibco, 11130-051), and 100 U/mL of penicillin–streptomycin (Gibco), as described previously.[23] COS7 cells were maintained in Dulbecco's modified Eagle's medium containing 10% FBS and 100 U/mL of penicillin–streptomycin. For steady laminar flow experiments, confluent HUVECs were cultured in the absence of serum for 24 h and exposed to fluid shear stress (12 dynes/cm^2).[19] HUVECs were collected in accordance with the University of Rochester human subjects review board procedures that prescribe to the Declaration of Helsinki.

2.2 Transfection and reporter gene assays

For experiments testing transactivation of the human eNOS promoter (kindly provided by Dr Mukesh K. Jain, Case Western Reserve University School of Medicine, Cleveland), transfections were carried out in 24-well plates. About 0.1 μg of a luciferase reporter plasmid containing the eNOS promoter (−1.62 kb to +1 base) was co-transfected with 0.3 ng of pRL-TK vector and 0.6 μg of expression plasmid for HA-KLF2, 0.1–1 μg of green fluorescent protein (GFP)-HDAC5 or empty vector control into COS7 cells. Thirty-six hours after transfection, the cells were carried out according to the instructions of the manufacturer of the dual luciferase reporter assay (Promega, Madison, WI, USA).

2.3 Western blot and co-immunoprecipitation analysis

After being washed with ice-cold phosphate-buffered saline (PBS), cells were harvested in lysis buffer and concentrations of the samples were determined using the Bradford method (BioRad, Hercules, CA, USA) with bovine serum albumin (BSA) as a reference protein. A complete description of western blot and co-immunoprecipitation assay are provided in Supplementary material online, Methods.

2.4 Small interfering RNA and reverse transcriptase–PCR

For small interfering RNA (siRNA)-mediated silencing, HUVECs were grown to ∼90% confluence and transfected with Lipofectamine 3000 reagent (Invitrogen) according to the manufacturer's instruction. siRNAs were synthesized by Thermo Scientific Dharmacon (Thermo Scientific, Rockford, IL, USA). Total RNA was isolated from cultured HUVECs and heart from HDAC5 mice using the RNeasy Mini Kit (QIAGEN) and RNeasy Fibrous Tissue Kit (QIAGEN), respectively. About 1 μg of RNA from each sample was reverse-transcribed into cDNA (Applied Biosystems, Foster City, CA, USA) and subjected to conventional PCR using the oligonucleotide primers (Eurofins MWG Operon, Huntsville, AL, USA). The sequences for siRNA and reverse transcriptase–PCR primers used in this study were provided in Supplementary material online, Methods.

2.5 Purification of glutathione *S*-transferase fusion protein and *in vitro* binding assay

Glutathione *S*-transferase (GST)-KLF2 construct was generated by subcloning full-length KLF2 cDNA into pGEX-5T2 vector (GE Healthcare Life Sciences) and transformed into *Escherichia coli* BL21 competent cells (Stratagene, La Jolla, CA, USA). The transformants were induced by 1 mM isopropylthiogalactopyranoside for 4 h at 37°C, harvested, and lysed by sonication in PBS with 0.1% Triton X-100. Equal amounts of purified GST-KLF2 proteins, as determined by Coomassie Blue staining of aliquots run on gels, were used in each assay. The GST-KLF2 fusion proteins were incubated with the whole cell extract from HEK293 transfected with a variety of Myc-HDAC5-truncated plasmids (kindly provided by Dr Eric N. Olson at the University of Texas Southwestern Medical Center, Dallas, TX, USA) along with GST-sepharose beads (GE Healthcare) at 4°C overnight. The protein complexes bound to the beads were washed extensively and released by heating to 95°C in an equal volume of 2× SDS–polyacrylamide gel (PAGE) loading buffer, followed by being resolved by SDS–PAGE and visualized using performing western blot analysis with respective antibodies.

Electrophoresed SDS–PAGE was incubated with Coomassie Blue staining solution for 30 min at room temperature.

2.6 Chromatin immunoprecipitation assay

The detailed methods for chromatin immunoprecipitation (ChIP) assay were described in Supplementary material online, Methods.

2.7 Immunocytochemistry

Cultured cells were fixed in 4% paraformaldehyde at room temperature for 20 min. After fixation, cells were washed in PBS three times and then blocked in PBS containing 10% normal goat serum, 0.1% BSA, and 0.1% Triton X-100 for 1 h at room temperature. Anti-HA as a primary antibody was diluted in blocking solution and incubated overnight with cells at 4°C. After incubation with the primary antibody, the cells were incubated with Alexa Fluor 546-conjugated goat anti-mouse IgG (Molecular Probes, Eugene, OR, USA) in blocking solution. Nuclear staining was performed with 4′,6-diamidino-2-phenylindole (DAPI) (Roche, Indianapolis, IN, USA). Images were acquired with an upright epifluorescent microscope (Olympus BX51, Olympus, Japan).

2.8 Genotyping of HDAC5 knockout mice and tissue sample preparation

The HDAC5 knockout mice on a C57BL/6J background were generously provided by Dr Eric N. Olson (University of Texas Southwestern Medical Center). Subsequent genotyping for HDAC5 mice was performed by PCR.[24] All animal procedures were performed in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) and approved by the University of Rochester Animal Research Committee. The methods for HDAC5 mouse genotype and tissue preparation were provided in Supplementary material online, Methods in detail.

2.9 Statistical analysis

All data were performed at least five independent experiments and presented as the mean ± SD. The statistical significance between samples was evaluated by the unpaired Student's *t*-test for two group comparisons. For multiple group comparison, the parametric one-way ANOVA of GraphPad Prism was used, followed by *post hoc* Tukey analysis. A *P*-value of <0.05 was considered statistically significant.

3. Results

3.1 HDAC5 interacts with KLF2

KLF2 is a crucial transcriptional regulator of endothelial anti-inflammatory activation and a key mediator of laminar flow atheroprotective action. However, the molecular mechanisms of regulation of KLF2 transcriptional activity are poorly understood. To determine whether HDAC5 directly regulates KLF2 function in endothelial cells, we investigated the physical interaction between HDAC5 and KLF2. Co-immunoprecipitation experiments were performed to define the association between HDAC5 and KLF2. COS7 cells were infected with adenovirus that expresses HA-tagged KLF2 full-length wild type along with adenoviruses for GFP-tagged HDAC5 or LacZ as a control. As shown in *Figure 1A*, the protein complex immunoprecipitated with anti-HA antibody was associated with HDAC5 as detected by anti-GFP antibody. Reversely, the immunoprecipitate with anti-GFP antibody for GFP-tagged HDAC5 was found to interact with HA-tagged KLF2 (*Figure 1B*). Therefore, these data showed the association of HDAC5 with KLF2.

3.2 Mapping the KLF2-interacting domain in HDAC5

The direct interaction of HDAC5 with KLF2 implicates the potential mechanism by which HDAC5 regulates steady laminar flow-induced KLF2 transcriptional activity. To understand the nature of this interaction between HDAC5 and KLF2, we further mapped the HDAC5 and KLF2 interaction domains (*Figures 2A* and *3A*). COS7 cells were transfected with various truncated mutants of Myc-tagged HDAC5 along with HA-tagged KLF2 full-length wild type, or the cell lysates overexpressing Myc-tagged HDAC5 were incubated with purified bacterial GST-KLF2 protein to conduct GST pull-down assay. As shown in *Figure 2B*, the co-immunoprecipitation assays showed that HDAC5

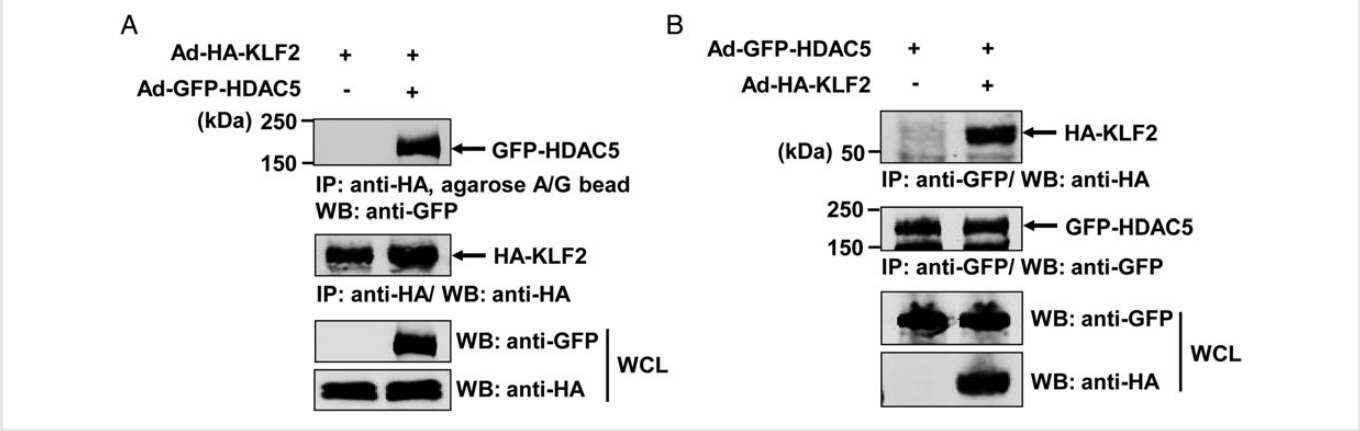

Figure 1 HDAC5 interacts with KLF2 in COS7 cells. COS7 cells were infected with adenovirus (Ad)-KLF2 tagged with HA (Ad-HA-KLF2) or Ad-HDAC5 tagged with GFP (Ad-GFP-HDAC5) as indicated conditions. Thirty hours after infection, the cells were harvested and lysed to conduct co-immunoprecipitation. (*A*) Complexes were immunoprecipitated with an anti-HA monoclonal antibody conjugated with an agarose A/G bead at 4°C overnight and then analysed by western blot analysis with anti-GFP (upper panel) or anti-HA (second panel). (*B*) Protein complex was immunoprecipitated with anti-GFP antibody (upper and second panels). Expression of each protein in whole cell lysate (WCL) was shown as an input. Five independent experiments were performed ($n = 5$).

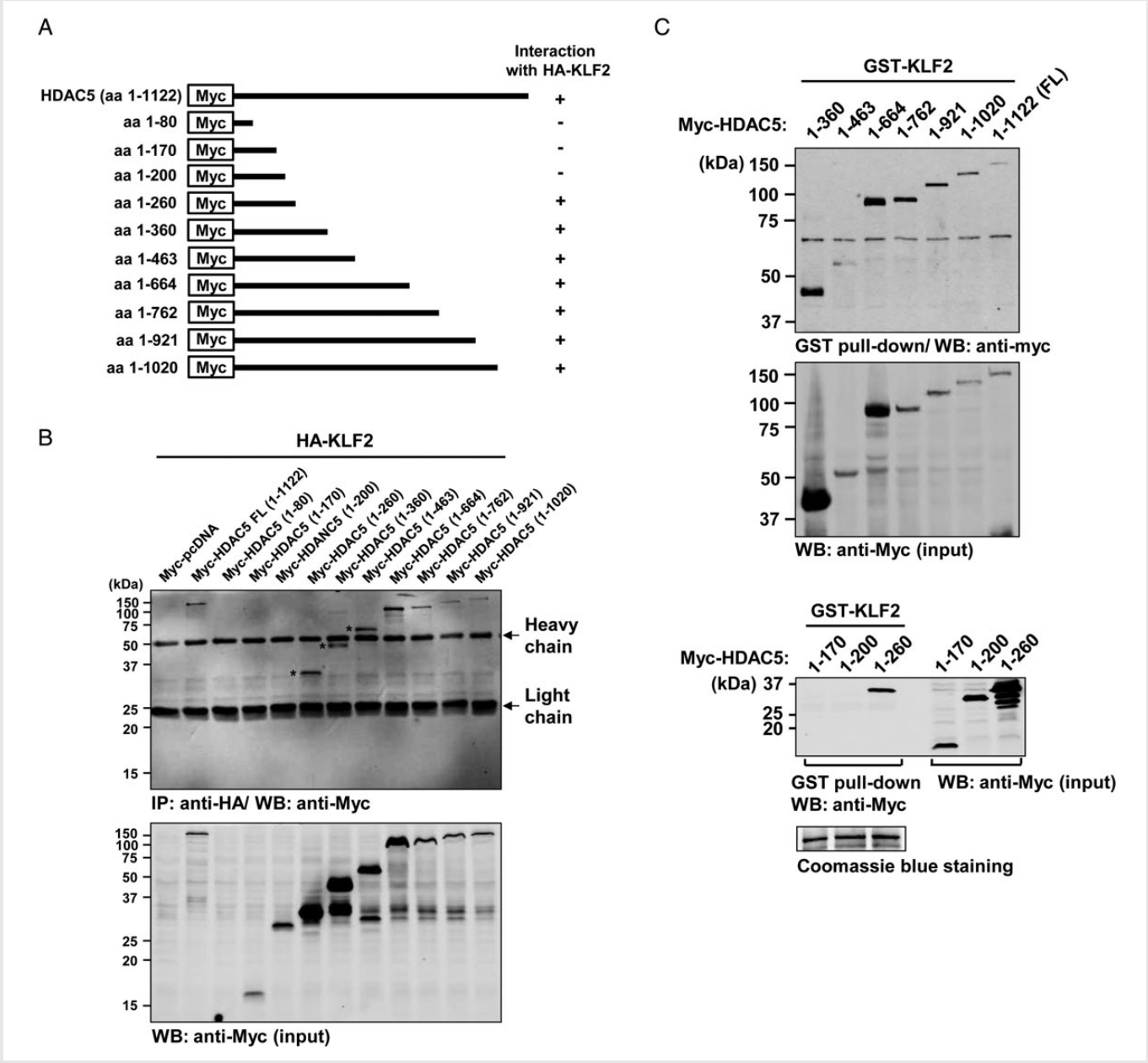

Figure 2 Mapping the KLF2-interacting domain in HDAC5. (*A*) Schematic representation of truncated constructs of human HDAC5. (*B*) Mapping the HDAC5 interactive domain that binds to full-length KLF2 by co-immunoprecipitation experiments (upper). COS7 cells were transiently transfected with various truncated forms of Myc-tagged HDAC5 along with HA-tagged KLF2. The cells were lysed after 36 h of transfection. The cell lysates were subjected to immunoprecipitation with anti-HA antibody, followed by separating on SDS–PAGE and western blot analysis with anti-Myc antibody. The N-terminus of HDAC5 (amino acids 201–260) contributes to interaction with HA-KLF2. (*C*) GST pull-down assay that a variety of Myc-HDAC5 fragments interact with purified recombinant GST-KLF2 protein. Each plasmid encoding Myc-HDAC5-truncated mutants was transiently transfected in COS7 cells. GST-fused KLF2 constructs were introduced into *E. coli* BL21, and the fusion proteins were induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) at 37°C for 4 h. About 5 μg of purified GST-tagged KLF2 protein was incubated with 500 μg of each whole cell lysate overexpressing Myc-HDAC5 fragments for 5 h at 4°C and subsequent to separate on SDS–PAGE, followed by performing western blot analysis with anti-Myc antibody. Coomassie blue staining showing the expression of fragments aa 1–170, aa 1–200, and aa 1–260 (bottom). Five independent experiments were performed ($n = 5$).

deletion mutants expressing amino acids 1–260 and beyond associated with KLF2, while those expressing amino acids 1–80, 1–170, and 1–200 failed to associate with KLF2. Similar results were obtained by using GST pull-down assay (*Figure 2C*). Taken together, these data indicate that the KLF2-interacting domain of HDAC5 resides between amino acids 201 and 260.

3.3 Mapping the HDAC5-interacting domain in KLF2

Reciprocally, to identify the KLF2 domain that associates with HDAC5, we generated truncated mutants of Myc-tagged KLF2 as shown in *Figure 3A*. Each of the KLF2 truncated mutants was co-transfected with FLAG-tagged HDAC5 full length into COS7 cells. Cell lysate was

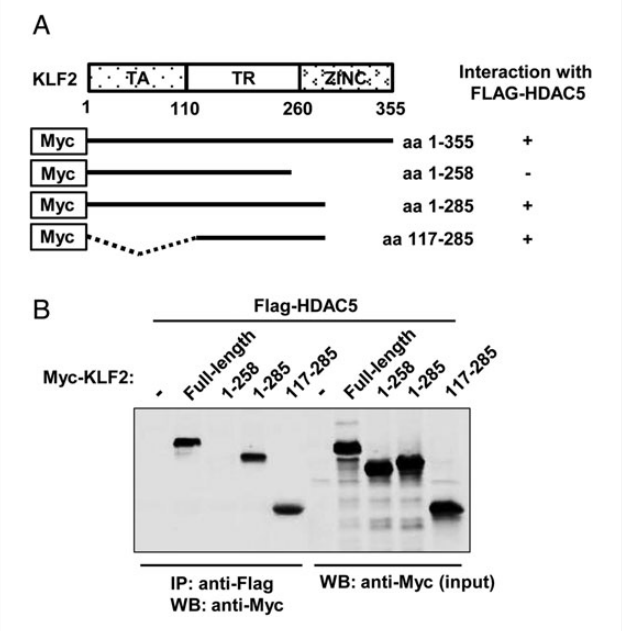

Figure 3 Mapping the HDAC5-interacting domain in KLF2. (*A*) Schematic representation of truncated constructs of human KLF2. (*B*) Mapping the Myc-KLF2 domain that interacts with full-length FLAG-HDAC5 by co-immunoprecipitation experiments. COS7 cells were transiently transfected with various truncated forms of Myc-tagged KLF2 along with FLAG-tagged HDAC5. The cells were lysed after 36 h of transfection. The cell lysates were subjected to immunoprecipitation with anti-FLAG antibody. A part of zinc-finger DNA-binding domain of KLF (amino acids 259–285) contributes to interact with HDAC5. Three independent experiments were performed ($n = 3$).

immunoprecipitated with anti-FLAG antibody, followed by the detection with anti-Myc antibody (*Figure 3B*). The results show that the zinc-finger DNA-binding domain (ZINC) of KLF2, especially, the region of amino acids 259–285, is involved in the interaction with HDAC5.

3.4 HDAC5 co-localizes with KLF2 in endothelial cells

Previously, we showed that HDAC5 is phosphorylated and exported from nuclei in endothelial cells under fluid shear stress.[19] To test whether the interaction between HDAC5 and KLF2 can be disrupted under steady laminar flow stimulation, HUVECs were infected with adenovirus encoding HA-tagged KLF2 (Ad-HA-KLF2) and FLAG-tagged HDAC5 (Ad-FLAG-HDAC5), followed by the exposure of flow (shear stress = 12 dyne/cm^2) for 1 h. HDAC5 robustly interacted with KLF2 under static condition, whereas flow disrupted the interaction between them (*Figure 4A*). Moreover, to determine whether the interaction between HDAC5 and KLF2 is phosphorylation-dependent, we infected HUVECs with both Ad-HA-KLF2 and Ad-FLAG-HDAC5 S259/498A, a phosphorylation-defective mutant on both serine 259 and 498 (*Figure 4B*). HDAC5 S259/498A remained a robust association with KLF2 after flow stimulation. To further examine the effects of steady laminar flow on the subcellular localization of HDAC5 and KLF2 in endothelial cells, we investigated the subcellular localization of KLF2 and HDAC5 wild type or HDAC5 S259/498A mutant. HUVECs were infected with Ad-HA-KLF2 along with either Ad-GFP-HDAC5 wild type or Ad-GFP-HDAC5 S259/498A, and the protein localizations were monitored as immunofluorescence analysis (*Figure 4C*). Under static condition, both GFP-HDAC5 wild type and GFP-HDAC5 S259/498A remained in the nucleus along with HA-KLF2. In contrast, after 4-h flow exposure, the majority of wild-type HDAC5 was exported to the cytoplasm, whereas HDAC5 S259/498A mutant stayed in the nucleus. KLF2 remained in the nucleus under both static and flow conditions. Therefore, these results illustrate that HDAC5 dissociates with KLF2 upon fluid shear stress-induced phosphorylation.

To further assess the physical interaction between HDAC5 and KLF2, we employed BAECs that not only endogenously express abundant KLF2 and HDAC5, but also have the applicability to arterial disease. Co-immunoprecipitation assays with respective anti-HDAC5 and anti-KLF2 antibodies showed that endogenous KLF2 and HDAC5 proteins physically interact, whereas laminar flow attenuated their association (*Figure 4D* and *E*). Taken together, our results demonstrate that HDAC5 is a novel binding partner of KLF2, indicating a potential role of HDAC5 in regulating KLF2 transcriptional activity.

3.5 HDAC5 suppresses KLF2-driven eNOS promoter activity

KLF2 is a well-known transcription factor for eNOS. The interaction between HDAC5 and KLF2 indicates that this interaction may occur at the KLF2 *cis*-element 'CACCC' region on eNOS promoter. To examine this hypothesis, we performed the ChIP assay. As shown in *Figure 5B*, HDAC5-S/A co-immunoprecipitated with a −697 to −557 region of eNOS promoter, which has been shown as a conserved KLF2-binding site and is critical for KLF2-induced eNOS promoter luciferase activity. Fluid shear stress, which has been shown to induce HDAC5 phosphorylation and disrupt the interaction between HDAC5 and KLF2, caused the dissociation of HDAC5 wild type from this eNOS promoter region. This result indicates a potential role of HDAC5 in regulating KLF2-induced eNOS expression. Furthermore, we assessed the ability of HDAC5 to modulate the chimeric Gal4-DNA-binding domain–KLF2 fusion protein on a Gal4-dependent luciferase reporter in COS7 cells. KLF2 significantly increased Gal4-luciferase activity, while HDAC5 wild type attenuated it even in the presence of KLF2 (*Figure 5C*). Furthermore, both HDAC5 wild type and serine/alanine (SA) mutant showed an inhibition on a KLF2-induced eNOS promoter (−1.62 kb to +1 base) luciferase reporter gene (*Figure 5D*), which has been shown to confer eNOS expression in endothelial cells *in vitro* and *in vivo*.[25–27] Taken together, these data show that HDAC5 inhibits KLF2 transcriptional activity on eNOS promoter through a direct interaction with KLF2.

To determine whether other HDAC family members are involved in the suppression of KLF2-driven eNOS transcription, each of the class IIa (HDAC4, 5, 7, and 9) and the class I (HDAC1 and 3) HDACs was co-transfected with HA-tagged KLF2 in COS7 cells (*Figure 5E*). All the class IIa HDACs significantly decreased KLF2-induced eNOS promoter activity. In contrast, class I HDACs showed no inhibitory effect on KLF2-driven eNOS transcription.

3.6 HDAC5 suppresses KLF2-driven eNOS expression

The inhibitory effect of HDAC5 on KLF2-driven eNOS promoter activity strongly suggests that HDAC5 performs as a repressor on KLF2-induced eNOS expression. To test this hypothesis, we assessed the level of eNOS protein expression in the presence of KLF2 together

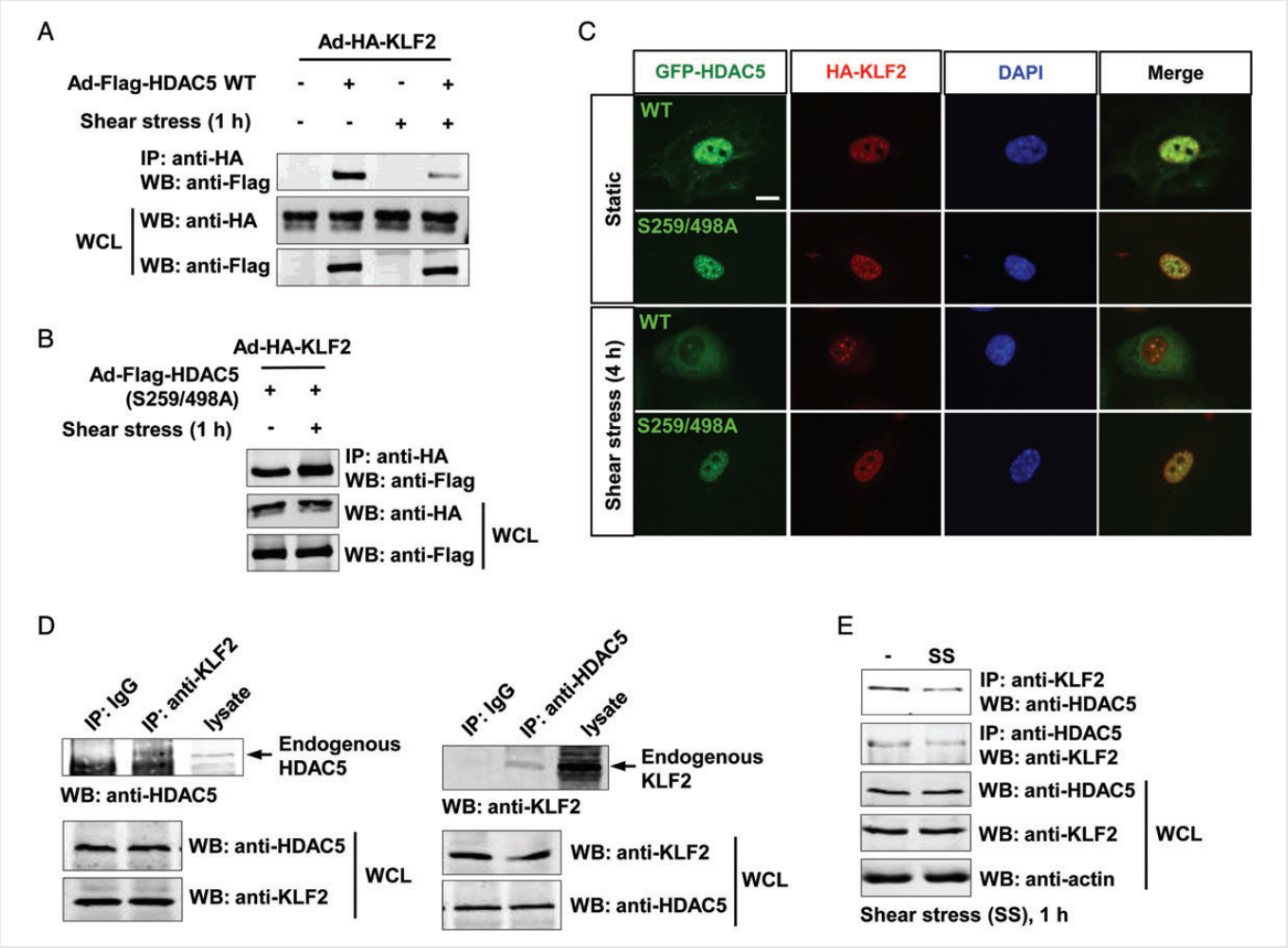

Figure 4 HDAC5 co-localizes with KLF2 in endothelial cells. (*A*) HUVECs were infected with Ad-HA-KLF2 or Ad-FLAG-HDAC5 wild type as indicated conditions. Twenty-four hours later, the cells were exposed to 12 dyne/cm^2 shear stress for 1 h. Immunoprecipitation was conducted with anti-HA antibody, and FLAG-HDAC5 was detected by probing with the anti-FLAG antibody. (*B*) HUVECs were infected with Ad-HA-KLF2 or Ad-FLAG-HDAC5 S259/498A, which were replaced serine with alanine at both residue 259 and 498, and then conditioned with static or shear stress 1 h before harvest. The complex was immunoprecipitated with the anti-HA antibody, followed by western blot analysis with the anti-FLAG antibody. (*C*) HUVECs were infected with Ad-GFP-HDAC5 wild type or S259/498A mutant along with Ad-HA-KLF2, and then incubated under static or 4-h shear stress condition. After 20 min of fixation with 4% paraformaldehyde at room temperature, the cells were reacted with the anti-HA antibody and DAPI was used to stain nucleus. Under static condition, wild-type HDAC5 was mainly localized in the nuclei and faintly in the cytoplasm, whereas HDAC5 S259/498A was exclusively distributed in the nuclei (green). In contrast, shear stress induced a majority of wild-type HDAC5 nuclear export, while the localization of HDAC5 S259/498A mutant was unchanged. Scale bar is 10 μm. (*D* and *E*) Physical interaction between KLF2 and HDAC5 was determined in BAECs. Cells were exposed to static (*D*) or flow (*E*) condition for 1 h and then lysed to conduct immunoprecipitation assay with anti-KLF2 or anti-HDAC5 antibodies, followed by western blot analysis with anti-HDAC5 or anti-KLF2 antibodies, respectively. Immunoprecipitation assay showed that both proteins directly associate and flow attenuates the interaction between these proteins. These experiments were performed at least three independent times. Three independent experiments were performed for *A*, *B*, and *C* ($n = 3$), and five independent experiments were performed for *D* and *E* ($n = 5$).

with either HDAC5 wild type or SA mutant. HUVECs were infected with adenoviruses encoding HA-KLF2 and GFP-HDAC5 wild type or SA mutant. As shown in *Figure 6A*, HA-KLF2 expression led to a 1.7- to 2-fold increase of endogenous eNOS protein, whereas both GFP-HDAC5 wild type and SA mutant attenuated KLF2-driven expression of endogenous eNOS. These similar data were observed in BAECs which were originated from the artery (*Figure 6C*). We then used siRNA targeting endogenous HDAC5 to further investigate the inhibitory effect of HDAC5 on KLF2-induced eNOS expression. Knockdown of HDAC5 by siRNA exhibited a significant reduction of endogenous HDAC5 mRNA and protein levels, as shown in *Figure 6B*. HDAC5 depletion led to a significant enhancement of endogenous eNOS expression (*Figure 6B*). Collectively, our results defined a critical role of HDAC5 in regulating KLF2 transcriptional activity and KLF2-dependent eNOS expression.

3.7 HDAC5 deficiency increases eNOS expression and decreases VCAM1 expression in aortic vessels

To better understand the role of HDAC5 in regulation of endothelial function, we employed HDAC5 knockout mice to measure the

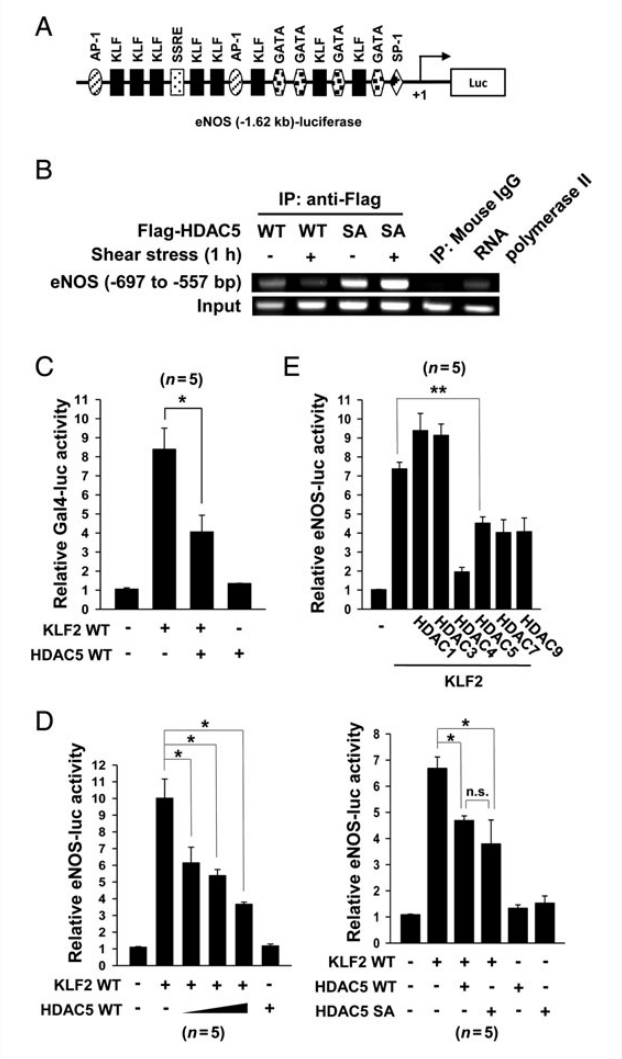

Figure 5 HDAC5 suppresses KLF2-driven eNOS promoter activity. (*A*) Schematic representation of human eNOS promoter luciferase (−1.62 kb to +1 base) construct used in this study. (*B*) ChIP assay to show the interaction between HDAC5 and KLF2 occurs at the *cis*-element on eNOS promoter. FLAG-tagged HDAC5 wild type (WT) or mutant (SA) was co-immunoprecipitated with −697 to −557 bp region of the eNOS promoter. Mouse IgG or RNA polymerase was used as a negative or a positive control, respectively. (*C*) COS7 cells were transiently transfected with expression vectors encoding the GAL4 DNA-binding domain fused to KLF2 or the GFP-HDAC5 WT along with the GAL4-luciferase construct, followed by measuring GAL4 luciferase activity after 36 h of transfection. (*D*) eNOS promoter luciferase assay showing the inhibitory effect of HDAC5 on KLF2-driven human eNOS gene expression. About 0.1 μg of a luciferase reporter plasmid containing the eNOS promoter (−1.62 kb to +1 base) was co-transfected with 0.3 ng of pRL-TK as a control for transfection efficiency, 0.6 μg of HA-KLF2 and GFP-HDAC5 WT (0.3 or 0.1–1.0 μg for increasing concentrations), or GFP-HDAC5 SA (0.3 μg) vectors. (*E*) COS7 cells were co-transfected with expression vectors for diverse HDACs isoforms, HA-KLF2, and pRL-TK. All luciferase assay experiments were performed at least five independent batches. The normalized values are calculated as mean ± SD and expressed as a relative activity of eNOS luciferase measured in appropriate empty vector control. The parametric one-way ANOVA test was used in *D* and found to be significant ($P = 0.01$), followed by post hoc Tukey analysis. *$P < 0.05$ or **$P < 0.005$ vs. appropriate control; n.s., not significant.

expression of proteins eNOS and vascular cell adhesion molecule 1 (VCAM1), two direct transcriptional targets of KLF2. In homozygous HDAC5 knockout mice, the level of eNOS expression was higher in thoracic aorta, compared with that in wild-type mice. In contrast, the level of the inflammatory responsive gene VCAM-1, whose expression has been shown to be inhibited by the KLF2 and eNOS-mediated pathways, was less than that in wild-type mice (*Figure 7B*). These data indicate that HDAC5 is an important modulator of eNOS and VCAM1 expression in intact vessels *in vivo*, suggesting the involvement of HDAC5 in regulation of endothelial function and vascular diseases.

4. Discussion

In this study, we have demonstrated that HDAC5 directly interacts with KLF2 to regulate KLF2 transcriptional activity and modulates KLF2-driven eNOS expression in endothelial cells. Our study revealed an important mechanism for maintaining vascular homeostasis, in which steady laminar flow de-represses KLF2 through stimulating HDAC5 nuclear export, resulting in an increase of eNOS expression and subsequent improvement of endothelial function.

Class IIa HDACs including HDAC4, HDAC5, HDAC7, and HDAC9 function as repressors for gene transcription and have structurally unique features. It has been shown that these HDACs have a large N-terminal extension with conserved motif to interact with the transcription factor MEF2.[17,28–31,32] Specifically, the N-terminal region of HDAC5 (HDAC5, amino acid 175–192) and the MADS/MEF2 domains of MEF2 are required for the association of HDAC5 and MEF2. The interaction between MEF2 and HDAC5 has an important role in regulation of skeletal myoblast differentiation. However, little is known about MEF2-independent mechanisms for transcriptional repression by class IIa HDACs, especially regulating in endothelial cell functions. In this study, we showed that HDAC5 associated with KLF2 and then further conducted domain mapping experiments to dissect the nature of HDAC5 and KLF2 interaction. Interaction between HDAC5 and KLF2 was observed in both overexpressed and naïve cells (*Figures 1* and *4D* and *E*). The N-terminal domain (amino acid 201–260) of HDAC5 associates with zinc-finger DNA-binding region of KLF2 (*Figures 2* and *3*). Moreover, this binding affinity was altered under steady laminar flow condition (*Figure 4A* and *B*). Fluid shear stress attenuated the interaction of KLF2 with wild-type HDAC5, but not the phosphorylation-defective HDAC5 mutant (S259/498A). This phenomenon can be interpreted as the result of fluid shear stress-induced HDAC5 phosphorylation and nuclear export.[19] Therefore, KLF2 is a new binding protein for HDAC5 with a distinct binding region compared with the interaction between HDAC5 and MEF2, suggesting that HDAC5 is a multifunctional protein that represses a number of transcriptional factors including MEF2 family members and KLF2.

Previously, experiments using gene-deficient mice and gene knockdown assays have shown that class IIa HDAC5 and HDAC7 play a role in regulation of endothelial proliferation and migration.[28–30] HDAC5 silencing increases the expression of fibroblast growth factor 2 (FGF2) and angiogenic guidance factors including Slit2 through HDAC5 binding to the promoter of FGF2 and Slit2, thereby repressing sprout induction.[20] Disruption of HDAC7 gene in mice leads to the failure of endothelial cell–cell adhesion and artery enlargement, whereas overexpression of HDAC7 suppresses HUVEC proliferation by preventing nuclear translocation of β-catenin and down-regulating T-cell factor1/inhibition of differentiation 2 (Id2) and cyclin D1. Knockdown of HDAC7 by siRNA up-regulates platelet-derived growth factor-B (PDGF-B) and its receptor

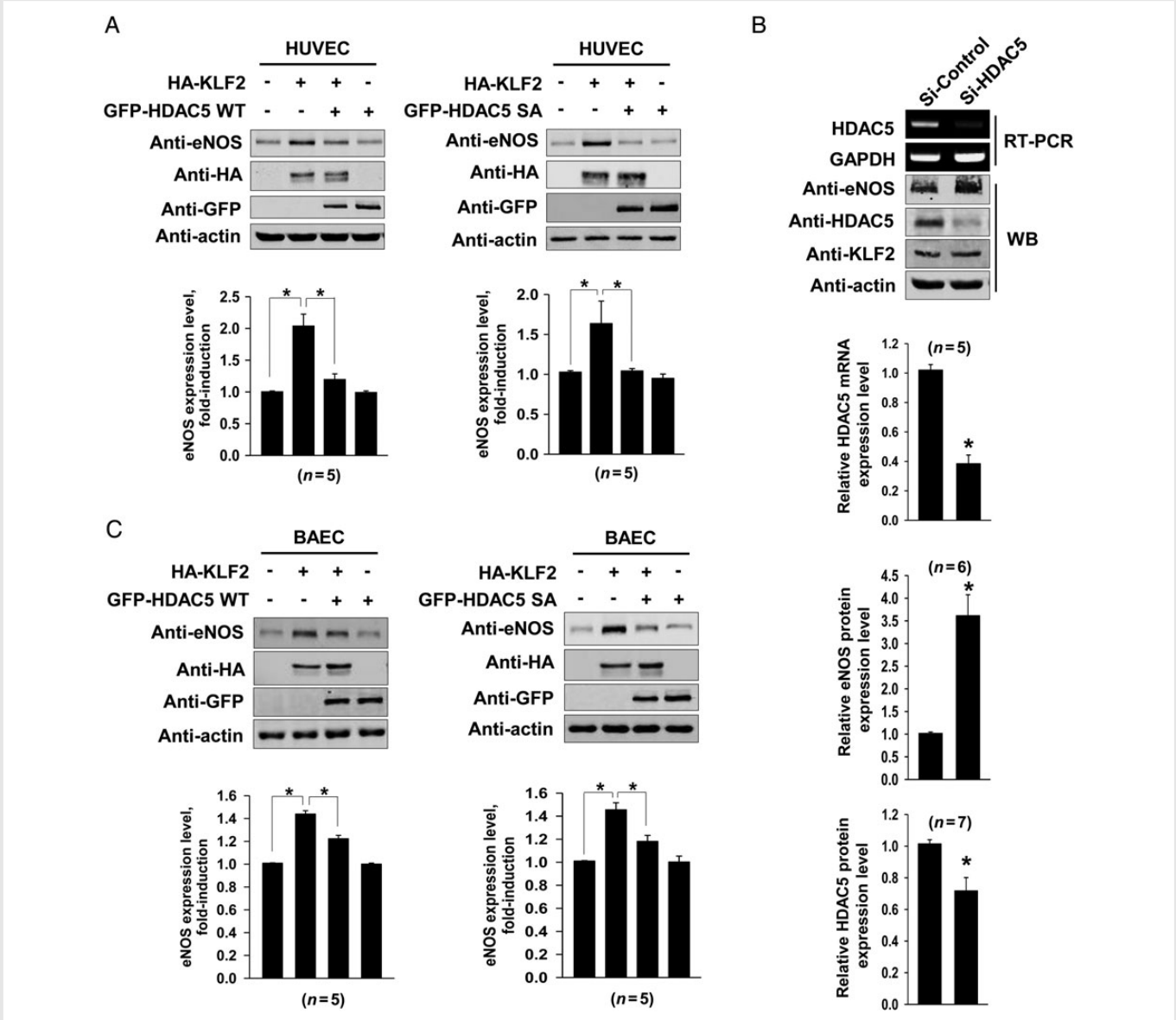

Figure 6 HDAC5 suppresses eNOS expression in endothelial cells. (*A*) HUVECs were infected with Ad-HA-KLF2 and Ad-GFP-HDAC5 wild type (WT) or mutant (SA) as indicated conditions. KLF2-driven induction of eNOS protein was suppressed by HDAC5 expression as determined by western blot analysis (upper). (*B*) HUVECs were transfected with 50 nM siRNA of HDAC5 (si-HDAC5) or scramble siRNA as a control (si-control), and then harvested to conduct RT-PCR or western blot analysis 36–40 h later. The knockdown effect of the HDAC5 siRNA (si-HDAC5) in HUVECs was observed at the levels of both mRNA and protein. GAPDH was included as a loading control. eNOS protein level in HUVECs treated with siRNA was monitored by western blot analysis. The expression level of HDAC5 or eNOS was normalized by GAPDH or actin expression level from at least five independent experiments ($n = 5–7$), respectively, and plotted (bottom). (*C*) BAECs were infected with Ad-HA-KLF2 and Ad-GFP-HDAC5 WT or SA, followed by harvest to determine KLF2-driven expression of eNOS 30 h after infection. The bar graph in *A*–*C* represents quantification of eNOS or HDAC5 relative to actin (Image J) from five independent experiments ($n = 5$). The normalized values are calculated as mean ± SD. The parametric one-way ANOVA test was used in *A* and *C* and found to be significant ($P = 0.05$), followed by post hoc Tukey analysis. *$P < 0.05$ or **$P < 0.005$ vs. appropriate control.

PDGFR-β, which are partially responsible for the inhibition of endothelial cell migration.[31] However, the role of class IIa HDACs in regulation of KLF2-dependent gene transcription and the underlying mechanisms are unknown. In this study, we showed that all members of class IIa are able to inhibit KLF2-driven eNOS transcription (*Figure 5E*), indicating the involvement of class IIa HDACs in regulation of endothelial gene expression and function. In particular, we characterized the interaction between HDAC5 and KLF2, and explored the mechanism by which HDAC5 represses KLF2 transcriptional activation in endothelial cells. The possible involvement of other members of class IIa HDACs such as HDAC7 in regulating KLF2-dependent gene expression will be explored in the future studies.

KLF2 is a steady laminar flow-responsive endothelial transcription factor whose expression is induced by fluid shear stress. Emerging evidences support a key role of KLF2 in maintaining endothelial haemostasis by promoting eNOS expression and by inhibiting the inflammatory

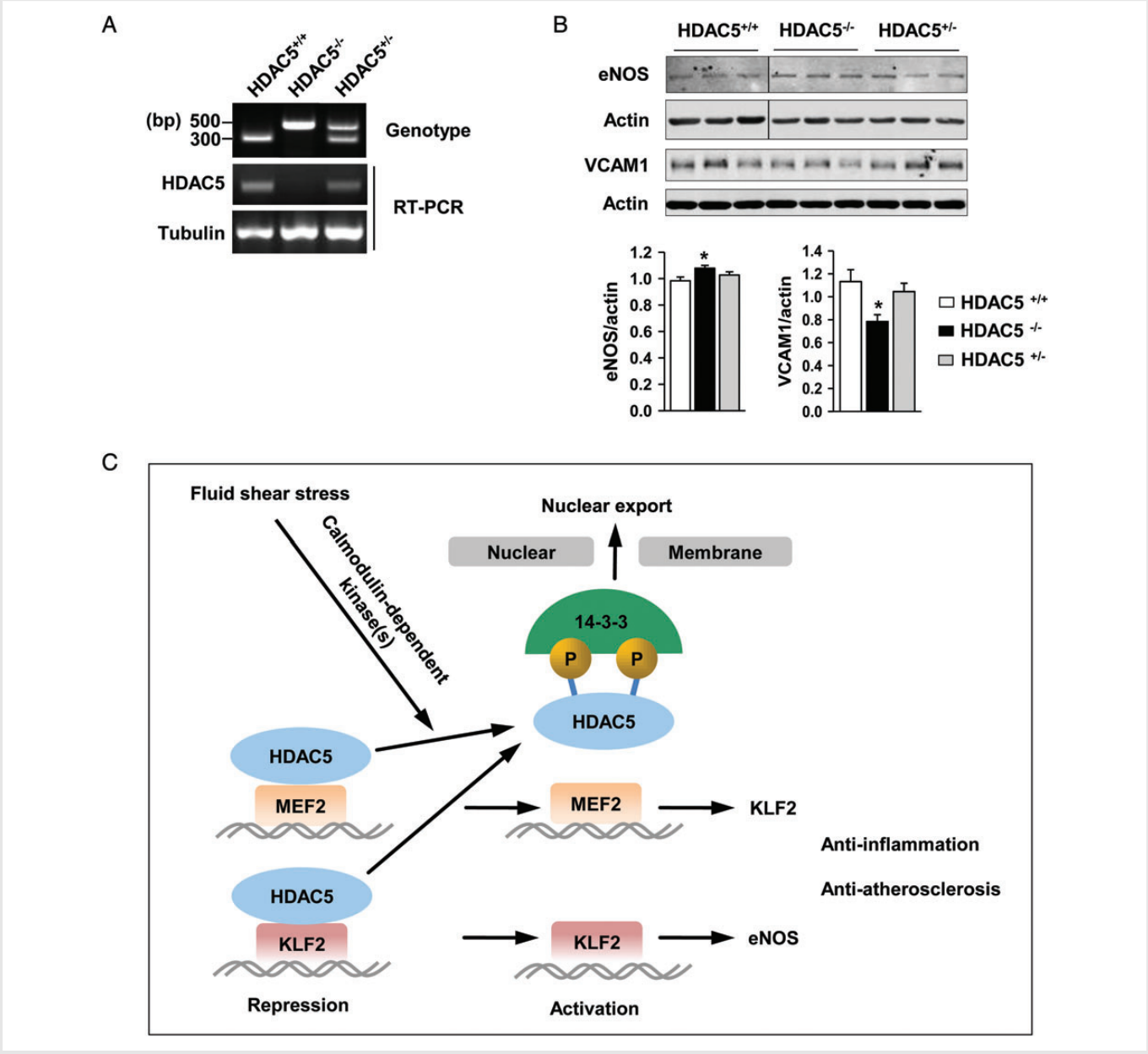

Figure 7 Analysis of the protein expression in thoracic aorta of HDAC5 knockout mice. (*A*) Data of mouse genotype and RT-PCR for HDAC5 expression. (*B*) Mouse thoracic aorta tissues were harvested and homogenized using RIPA buffer. Each homogenate was immunoblotted with the antibodies against eNOS and VCAM1, two direct transcriptional targets of KLF2. The bar graph represents quantification of eNOS and VCAM1 relative to actin (Image J) from each group of mice (eight mice for wild type, eight mice for heterozygous, and six mice for homozygous). The normalized values were calculated as mean $\pm$ SD. *$P < 0.05$ vs. wild type. (*C*) A proposed model for HDAC5-mediated expression of KLF2 and eNOS in endothelial cells. In endothelial cells, fluid shear stress induces HDAC5 phosphorylation on both serine 259 and 498 and nuclear export through a CaMK-dependent pathway. Phosphorylated HDAC5 releases its repression on both MEF2C and KLF2, resulting in an enhancement of MEF2C and KLF2 transcriptional activity, thereby triggering KLF2 and eNOS gene expressions, which mediates laminar flow atheroprotective action.

genes such as VCAM1. However, how KLF2 transcriptional activity is regulated is largely unknown. In this study, we revealed for the first time that HDAC5 regulates KLF2 transcriptional activity on the eNOS promoter (*Figure 5D*). HDAC5 dose-dependently inhibited KLF2-induced eNOS promoter luciferase activity and eNOS expression. When endothelial cells exposed to steady laminar flow, HDAC5 was phosphorylated and changed its subcellular localization from the nucleus to the cytoplasm, which de-represses KLF2 and increases eNOS expression. Steady laminar flow induced wild-type HDAC5 nuclear export (*Figure 4C*). In contrast, HDAC5 S259/498A mutant was localized predominantly in the nucleus of HUVECs, regardless of fluid shear stress stimulation. Our results suggest that while HDAC5 represses KLF2 transcriptional activation through the direct interaction with KLF2, laminar flow is able to overcome the repressive effects of HDAC5 on KLF2 and hence drives KLF2-dependent gene expression and atheroprotective action. Moreover, our *in vivo* study showed that HDAC5 deficiency

in mice induced an increase of eNOS expression and a decrease of VCAM1 expression in aortic vessels (*Figure 7B*). As noted, the changes in aortic eNOS expression were modest, but the reduction of VCAM1 was more obvious. This could be due to the contribution of eNOS-independent mechanisms, such as KLF2-mediated direct inhibition of VCAM1 expression, resulting in further decrease of VCAM1 in HDAC5 knockout mice. Taken together, these results further support that HDAC5 plays an important role in regulation of laminar flow atheroprotective genes and endothelial function.

We previously demonstrated that laminar flow-induced HDAC5 phosphorylation and nuclear export is mediated through a CaMK-dependent pathway.[19] Phosphorylated HDAC5 releases its repression on MEF2C and enhances MEF2C transcriptional activity, thereby increasing KLF2 gene expressions. In this study, we further found that HDAC5 represses KLF2 transcriptional activity and KLF2-dependent eNOS expression. This HDAC5 inhibitory activity occurs through the direct interaction of HDAC5 with KLF2 on the eNOS promoter. Taken together, we propose that HDAC5-mediated inhibition of KLF2 and eNOS in endothelial cells is regulated through two distinct KLF2-dependent mechanisms: (i) HDAC5 regulates KLF2 gene expression via the interaction between HDAC5 and MEF2 and (ii) HDAC5 modulates KLF2 transcriptional activation via the direct interaction of HDAC5 with KLF2. Laminar flow induces HDAC5 phosphorylation and nuclear export, which de-represses both MEF2 and KLF2 and promotes expression and activation of KLF2 and eNOS to mediate laminar flow atheroprotective action (*Figure 7C*).

In summary, we have demonstrated a novel role of HDAC5 in regulation of laminar flow-responsive genes KLF2 and eNOS in endothelium, in which HDAC5, as a new binding partner of KLF2, represses KLF2 transcriptional activity and KLF2-dependent eNOS expression. Endothelial dysfunction, characterized by an impairment of endothelium-dependent vasorelaxation caused by a loss of NO bioavailability in the vessel wall, has been implicated in the pathogenesis of many cardiovascular diseases, including hypercholesterolaemia, atherosclerosis, hypertension, diabetes, and heart failure. Therefore, the inhibition of HDAC5 could be a new therapeutic strategy to prevent vascular endothelial dysfunction and to treat cardiovascular diseases, such as atherosclerosis, hypertension, and ischaemic stroke.

Supplementary material

Supplementary material is available at *Cardiovascular Research* online.

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Conflict of interest: none declared.

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