ESC European Society of Cardiology

Cardiovascular Research (2023) **119**, 2244–2255
https://doi.org/10.1093/cvr/cvad110

Epigenetic regulation of vascular smooth muscle cell phenotypic switch and neointimal formation by PRMT5

Ni Zhu[1†], Zhi-Fu Guo[1], Kyosuke Kazama[1], Bing Yi[1], Nopprarat Tongmuang[1], Huijuan Yao[1], Ruifeng Yang[1], Chen Zhang[1], Yongwen Qin[2], Lin Han[2], and Jianxin Sun [1*]

[1]Center for Translational Medicine, Department of Medicine, Thomas Jefferson University, 1020 Locust St, Philadelphia, PA 19107, USA; and [2]Department of Cardiovascular Medicine, Changhai Hospital, Naval Medical University, 168 Changhai Rd, Shanghai 200433, China

Received 31 March 2022; revised 20 March 2023; accepted 8 April 2023; online publish-ahead-of-print 24 July 2023

Time of primary review: 40 days

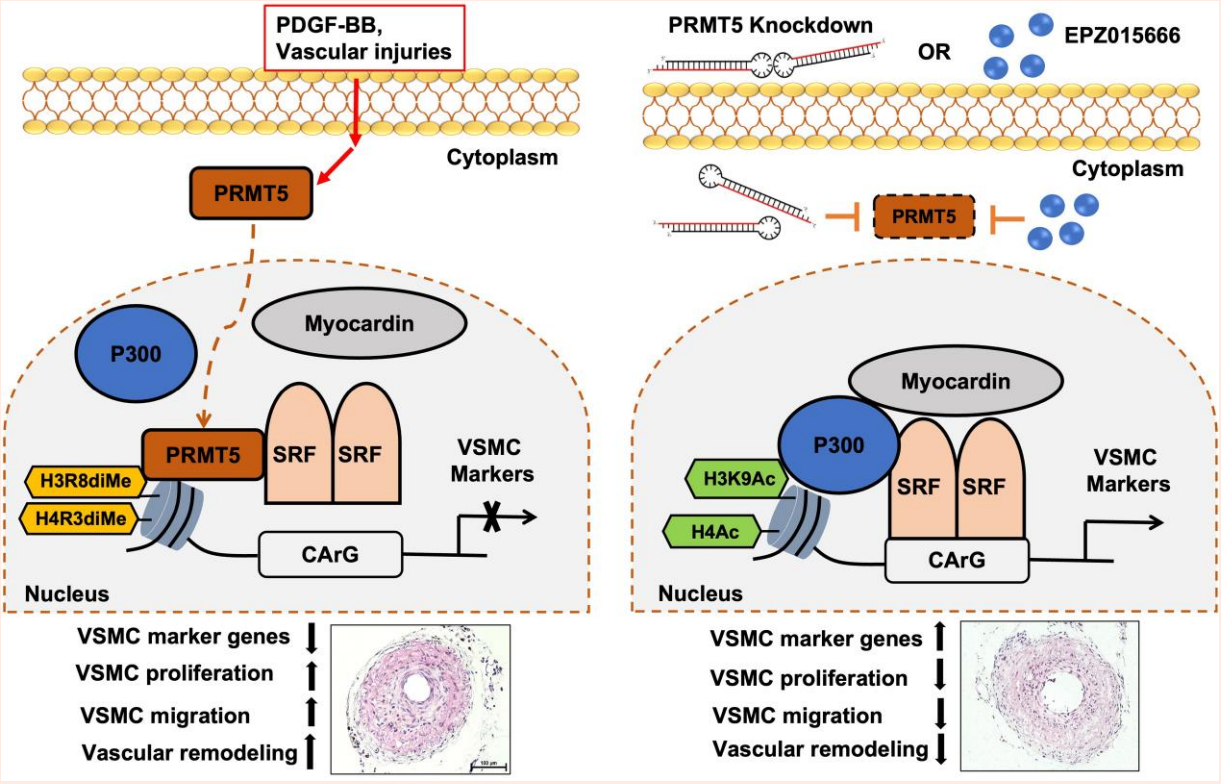
Aims Phenotypic transition of vascular smooth muscle cells (VSMCs) from a contractile to a synthetic state is involved in the development of cardiovascular diseases, including atherosclerosis, hypertension, and post-angioplasty restenosis. Arginine methylation catalyzed by protein arginine methyltransferases (PRMTs) has been implicated in multiple cellular processes, however, its role in VSMC biology remains undetermined. The objective of this study was to determine the role of PRMTs in VSMC phenotypic switch and vascular remodelling after injury.

Methods and results Our results show that PRMT5 is the most abundantly expressed PRMT in human aortic SMCs, and its expression is up-regulated in platelet-derived growth factor (PDGF)-stimulated VSMCs, human atherosclerotic lesions, and rat carotid arteries after injury, as determined by western blot and immunohistochemical staining. PRMT5 overexpression inhibits the expression of SMC marker genes and promotes VSMC proliferation and migration, while silencing PRMT5 exerts the opposite effects. Mechanistically, we found that PRMT5 overexpression led to histone di-methylation of H3R8 and H4R3, which in turn attenuates acetylation of H3K9 and H4, thus limiting recruitment of the SRF/myocardin complexes to the CArG boxes of SMC marker genes. Furthermore, both SMC-specific deletion of PRMT5 in mice and local delivery of lentivirus expressing shPRMT5 to rat carotid arteries significantly attenuated neointimal formation after injury. Likewise, pharmacological inhibition of PRMT5 by EPZ015666 markedly inhibited carotid artery ligation-induced neointimal formation in mice.

Conclusions Our results identify PRMT5 as a novel regulator in VSMC phenotypic switch and suggest that inhibition of PRMT5 may represent an effective therapeutic strategy for proliferative vascular diseases.

* Corresponding author. Tel: 215 503 9424; fax: 215 503 5731, E-mail: jianxin.sun@jefferson.edu
† Present address. Department of Cardiovascular Medicine, Changhai Hospital, Naval Medical University, 168 Changhai Rd, Shanghai, 200433, China.

Graphical Abstract



Keywords PRMT5 • Histone • Methylation • Smooth muscle cells • Phenotypic switch • Proliferation

1. Introduction

Vascular smooth muscle cells (VSMCs), which are the major component of the middle layer of the vessel wall, retain remarkable plasticity in response to changes in local environmental cues that normally regulate phenotype.[1] Unlike either skeletal or cardiac muscle that are terminally differentiated, mature VSMCs alternate between a contractile (also termed differentiated) state and a synthetic (also termed dedifferentiated) state under different circumstances. Dedifferentiated VSMCs have increased rates of proliferation, migration, and synthesis of extracellular matrix components, as well as reduced expression of differentiation markers [i.e. smooth muscle α-actin (SMA), smooth muscle 22 (SM22), and smooth muscle myosin heavy chain (SMMHC)].[1,2] Pathological phenotypic switching plays a major role in the development of cardiovascular diseases, such as atherosclerosis, post-angioplasty restenosis, and hypertension.[1–3] Particularly, platelet-derived growth factor (PDGF) has been shown to promote the synthetic VSMC phenotype and increase VSMC proliferation and migration into the neointima layer after artery injury. It has been recognized that several transcription factors and co-factors, such as serum responsible factor (SRF), myocardin, and myocardin-related transcription factors (MRTFs), are critically involved in the regulation of transcriptional expression of SMC differentiation markers.[4–6] In addition, we and others have provided evidence that microRNAs are also involved in the process of VSMC phenotypic switch.[7–9] Most importantly, VSMCs exhibit specific histone modifications during phenotypic switch.[10] For example, H3K4 di-methylation, H4 acetylation, H3K79 di-methylation, and H3K9 acetylation have been found to be enriched at the loci of CArG [the consensus nucleotide sequence CC(AT) 6GG] markers to maintain a differentiated phenotype by permitting access of the SRF–myocardin complex and the co-activator histone acetyltransferase (HAT) p300 to SMC-specific CArGs element, thus activating transcription.[2,10–13] Interestingly, the repression of SMC marker genes by PDGF is associated with the loss of activating histone modifications, such as H3/H4 acetylation and H3K79 di-methylation, hence limiting the accessibility of the SRF–myocardin complex to CArG boxes.[11,14] Together, these studies suggest that histone modification has emerged as one of the essential mechanisms in the regulation of VSMC phenotypic switch.

Protein arginine methyltransferases (PRMTs) have been characterized as critical regulators of cell homeostasis and are essentially involved in several biological processes, including RNA regulation, signal transduction, and chromatin regulation.[15] Protein arginine methyltransferase 5 (PRMT5) is the main type II PRMT that catalyzes the symmetrical di-methylation of arginine residues within target proteins.[15–17] As part of the SWI/SNF complex, PRMT5 catalyzes the histone methylation of H3R8 and H4R3 and triggers transcriptional silencing of cell cycle regulatory and tumour suppressor genes.[18,19] PRMT5 also methylates non-histone proteins, such as p53, nuclear factor kappa B (NF-κB) and E2F Transcription Factor 1 (E2F-1), which implies its versatility in the regulation of cell growth, apoptosis, and inflammation.[20,21] Recently, the roles of PRMT5 in cardiovascular biology have begun to be explored. Our recent findings indicate that PRMT5 is an important regulator of cardiomyocyte hypertrophy through direct methylation of GATA Binding Protein 4 (GATA4).[22] Furthermore, stimulus-dependent homeobox A9 (HOXA9) arginine methylation by PRMT5 has been shown to regulate pro-inflammatory responses in activated endothelial cells to promote leucocyte binding to endothelial cells.[23] Interestingly, the expression of PRMT5 is up-regulated in carotid arteries in stroke-prone dahl salt-sensitive hypertensive rats, indicating a potential

role of PRMT5 in the pathogenesis of vascular disease.[24] However, at this time, whether PRMT5 plays a role in the regulation of VSMC phenotypic switch and vascular remodelling remains elusive.

In the present study, we show that the expression of PRMT5 is significantly increased in human atherosclerotic lesions and proliferative VSMCs. PRMT5 regulates VSMC phenotypic switch by arginine methylation-mediated modulation of histone H3 and H4. Moreover, *in vivo* loss-of-function study reveals that depletion of PRMT5 markedly suppresses vascular remodelling after injury.

2. Methods

2.1 Human tissue samples

Coronary arteries specimens from patients were obtained from Specimen Bank of Cardiovascular Surgery Laboratory and Department of Pathology of Shanghai Changhai Hospital, China (five patients, three donors, age: 47–70 years). A waiver for consent was approved for surgical patients, and written informed consent was obtained from a member of the family for deceased organ donors. These specimens were processed and fixed in paraformaldehyde, and paraffin-embedded blocks were cut into 5 μm sections. All procedures performed in the studies involving human participants were conducted under ethical principles of the Declaration of Helsinki and in accordance with the standards of the Ethics Committee of Changhai Hospital.

2.2 Cell culture and siRNA transfection

Human aortic SMCs (females, age: 11 and 24 years, respectively) (Lonza, Walkersville, MD) were cultured in growth media SmGM-2 (Lonza) in 5% foetal bovine serum (FBS), and 10T1/2 cells were cultured in a Dulbecco modified Eagle medium supplemented with 10% FBS. siRNA oligonucleotides for human PRMT5 (MISSION Pre-designed siRNA; Sigma) and a negative control siRNA (MISSION siRNA Universal Negative Control; Sigma) were used for the transfection of VSMCs with Lipofectamine RNAiMAX® transfecting reagent (Invitrogen) in a serum-free medium according to the manufacturer's recommendation.

2.3 Adenovirus construction

Adenoviruses harbouring FLAG-tagged PRMT5 (Ad-PRMT5) was made using AdMax (Microbix) as described previously.[22] The viruses were made and propagated in Ad293 cells and purified using $CsCl_2$ banding followed by dialysis against 20 mmol/litre Tris-buffered saline with 10% glycerol. Titering was performed on Ad293 cells using Adeno-X Rapid Titer kit (Clontech) according to the manufacturer's instruction.

2.4 Quantitative reverse-transcription polymerase chain reaction (qRT–PCR)

Total RNAs were extracted from human aortic SMCs using TRIZOL reagent kit (Invitrogen). qRT–PCR analysis was performed as we described previously. Briefly, cDNA was synthesized from total RNA using High Capacity cDNA Archive Kit (Applied Biosystem). qRT–PCR was performed using MyiQTM Single-Color Real-Time PCR Detection System (Bio-Rad) and SYBR Green Master Mix kit (Life Technology).[9] Primers used for qPCR are listed in *Supplemental Table I*. The expression levels of genes relative to 18 S RNA were determined using the $2^{-\Delta\Delta Ct}$ method.

2.5 Droplet digital PCR (ddPCR)

Total RNAs were extracted from human aortic SMCs using TRIZOL reagent kit (Invitrogen). cDNA was synthesized from total RNA using High Capacity cDNA Archive Kit (Applied Biosystem). For absolute quantification of PRMT subfamily mRNA expression in hAoSMCs, 20 μL droplet digital PCR (ddPCR) reaction mixes were prepared using 10 μL of 2× ddPCR™ Supermix for Probes (No dUTP) (Bio-Rad, Hercules, CA), 12.5 ng of cDNA, forward and reverse primers (400 nM), and probes (200 nM). Then, the ddPCR reaction mixes were transferred to DG8 cartridge and mixed with 70 μL of droplet generation oil. Droplets were then generated using the QX200 Droplet Generator (Bio-Rad). The plate was sealed and loaded on a T100 Thermal Cycler (Bio-Rad) using the following amplification protocol: 95°C for 10 min followed by 40 cycles of 30 s of denaturation at 95°C and 1 min of annealing and elongation at 55°C. When the amplification protocol was completed, the plate was read on a QX200 Droplet Reader (Bio-Rad) and data were analysed using the QuantaSoft software (Bio-Rad). Primers and probes used for ddPCR are listed in *Supplemental Table II*.

2.6 Western blot

Western blot analysis was performed as described previously.[25] Briefly, cell lyses was resolved by SDS-PAGE and transferred to nitrocellulose membrane, followed by blocking with 5% non-fat milk in phosphate-buffered saline (PBS) with 0.1% Tween20. Then, the membranes were incubated with diluted antibodies overnight at 4°C with agitation. Blots were incubated with appropriate secondary antibodies and visualized on an Odyssey Imaging System (LI-COR). Antibodies are used as follows: PRMT5 (#79998, 1:1000 dilution; Cell Signaling), SMA (sc-32251, 1:1000 dilution; Santa Cruz), SM22 (sc-53932, 1:1000 dilution; Santa Cruz), P300 (#86377, 1:1000 dilution; Cell Signaling), myocardin (#97109, 1:1000 dilution; Cell Signaling), SRF (#5147, 1:500 dilution; Abcam), Histone H3 (#39763, 1:1000 dilution; Active Motif), H3K4diMe (#39913, 1:1000 dilution; Active Motif), H3R8Me2s (ab130740, 1:1000 dilution; Abcam), H3K9Ac (#61952, 1:1000 dilution; Active Motif), H4R3Me2s (#61988, 1:1000 dilution; Active Motif), H4Ac (#39244, 1:1000 dilution; Active Motif), JMJD6 (sc-28348, 1:200 dilution; Santa Cruz), and GAPDH (sc-47724, 1:1000 dilution; Santa Cruz).

2.7 Immunofluorescent staining

VSMCs cultured on the laminin-coated glass coverslips were infected with Ad-PRMT5 or transfected with siPRMT5 (50 nM) and starved for 48 h. Cells were treated with PDGF as indicated and then followed by fixation with 4% paraformaldehyde for 10 min, washed three times in PBS, and then permeabilized with ice-cold methanol for 5 min. Following a blocking step with 10% of goat serum in PBS for 60 min, VSMCs were immunostained with a rabbit polyclonal primary antibody against PRMT5 (#79998, 1:200; Cell Signaling) and mouse monoclonal primary antibody against SMA (sc-53142, 1:200; Santa Cruz) for overnight at 4°C. After washing three times in PBS for 10 min, cells were stained with TRITC-conjugated anti-rabbit IgG (A16101, Invitrogen, USA) and FITC-conjugated goat anti-mouse IgG (#62-6511, Invitrogen, USA) second antibodies at 1:250, at 37°C for 2 h and then were rinsed three times with PBS. Cell nuclei were stained with DAPI.

2.8 Luciferase assay

10T1/2 cells seeded in 24-well plates were transfected with SRF, myocardin, p300, and PRMT5 plasmids combined with either pGL3-basic, pGL3-SMA-Luc, or pGL3-SM22-luc using FuGENE 6 transfection reagent; a reference promoter driving Renilla luciferase (pRL-TK) to normalize the data. Forty-eight hours after transfection, cells were lysed, and the reporter activity was determined with a luminescence counter (PerkinElmer Life Sciences) using the Dual-Luciferase Reporter Assay System (Promega) according to the instructions of the manufacturer. Firefly luciferase activity was normalized for transfection efficiency by corresponding Renilla luciferase activity. All transfection experiments were performed at least five times in duplicate.

2.9 VSMC wound scratch assay

VSMCs were infected with Ad-PRMT5 or transfected with siPRMT5 (50 nM) and starved in a starvation medium (0.5% FBS) for 48 h. VSMCs were incubated with mitomycin C (10 μg/mL, a potent inhibitor of cell proliferation) for 2 h.[26] After this incubation, cells were wounded with 200 μL pipette tips and then subjected to stimulation with or without recombinant

human PDGF-BB (P01127, Peprotech) at a final concentration of 20 ng/mL and monitored for an additional 24 h.[26] Still images were captured, and cells were scored based on the distance migrated from the left border of the scratch injury. Images were captured using an Olympus IX 71 microscope as we described previously.[9]

2.10 VSMC proliferation assay

VSMC proliferation was determined by MTT assay using Vybrant MTT Cell Proliferation Assay Kit (Invitrogen) according to the protocols provided by the manufacturer.[9]

2.11 Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) assays were performed as described previously.[27] Briefly, VSMCs were crosslinked for 10 min by directly adding formaldehyde (Sigma) to the culture medium at a final concentration of 1%. The fixed cells were lysed with lysis buffer (EZ ChIP; Upstate) and sonicated two times for 7 s with output 5 (Virsonic 60; Virtis) with a 2 min refractory period. For immunoprecipitation, cell lysates were incubated with an antibody against SRF (5 μg; sc-25290, Santa Cruz) or IgG (5 μg; Santa Cruz) overnight at 4°C. The immunoprecipitated and input DNAs were subjected to quantitative PCR with primers SMA (forward 5′-AGCAGAACAGAGGAATGCAGTGGAAGAGAC-3′ and reverse 5′-CCTCCCACTCGCCTCCCAAACAAGGAGC-3′) and SM22 (5′-CCCTGTCATTTCCAATACGG-3′ and 5′-CCAGCCCTTCTCATGGAGT-3′) to amplify human promoter regions containing SRF-binding sites;[28] to measure the levels of H3R8 di-methylation on SMA and SM22 promoters in human VSMCs overexpressing PRMT5 by ChIP, immunoprecipitation using anti-histone H3 symmetric dimethyl R8 antibody (H3R8Me2s, ab130740, Abcam) or IgG (#2729, Cell Signaling). The immunoprecipitated and input DNAs were then subjected to quantitative PCR with primers of SMA (5′-TCAGGCAAAGGGTAGAATCAC-3′ and 5′-GTTGTGAGGTACTGTAGATTATCCC-3′) and SM22 (5′-CAGGTACGAGCAAGATGAGAG-3′ and 5′-GAGACACTAACGCAGGAGAAG-3′).

2.12 Lentivirus construction

Lentivirus expressing rat PRMT5 short-hairpin RNA was generated by the GenePharma Company (Shanghai). Briefly, pre-designed targeting nucleotides (sense: 5′-GATCCGGTTTCCTGTTCTTTCTAAGATTCAAGAGATCTTAGAAAGAACAGGAAACCTTTTTTG; antisense: 5′-AATTCAAAAAAGGTTTCCTGTTCTTTCTAAGATCTCTTGAATCTTAGAAAGAACAGGAAACCG) were annealed and cloned into pGLVH1 vector. Plasmids were transfected into HEK-293T cells using the $CaCl_2$ method, and the viral supernatant was collected by ultracentrifugation.

2.13 Generation of the inducible SMC-specific PRMT5 KO mice

We used Cre-Lox technology to generate an inducible, smooth muscle cell-specific KO mouse line. PRMT5 floxed mice,[29] herein called $PRMT5^{fl/fl}$, were crossed with SMMHC-$CreER^{T2}$ (#019079, JAX lab), a transgenic mouse line expressing a fusion protein of the Cre recombinase with the modified oestrogen receptor binding domain ($CreER^{T2}$) under the control of the smooth muscle myosin heavy chain (SMMHC) promoter, for two generations to create SMMHC-$CreER^{T2+/-}$/$PRMT5^{fl/fl}$ (PRMT5-SMKO) mice. In this mouse strain, a tamoxifen-inducible Cre recombinase is under the control of the smooth muscle myosin heavy chain (SMMHC) promoter. The reason for employing an inducible recombination strategy was that it allowed for the determination of the consequences of PRMT5 loss in the adult mouse aortic SMC. All studies were

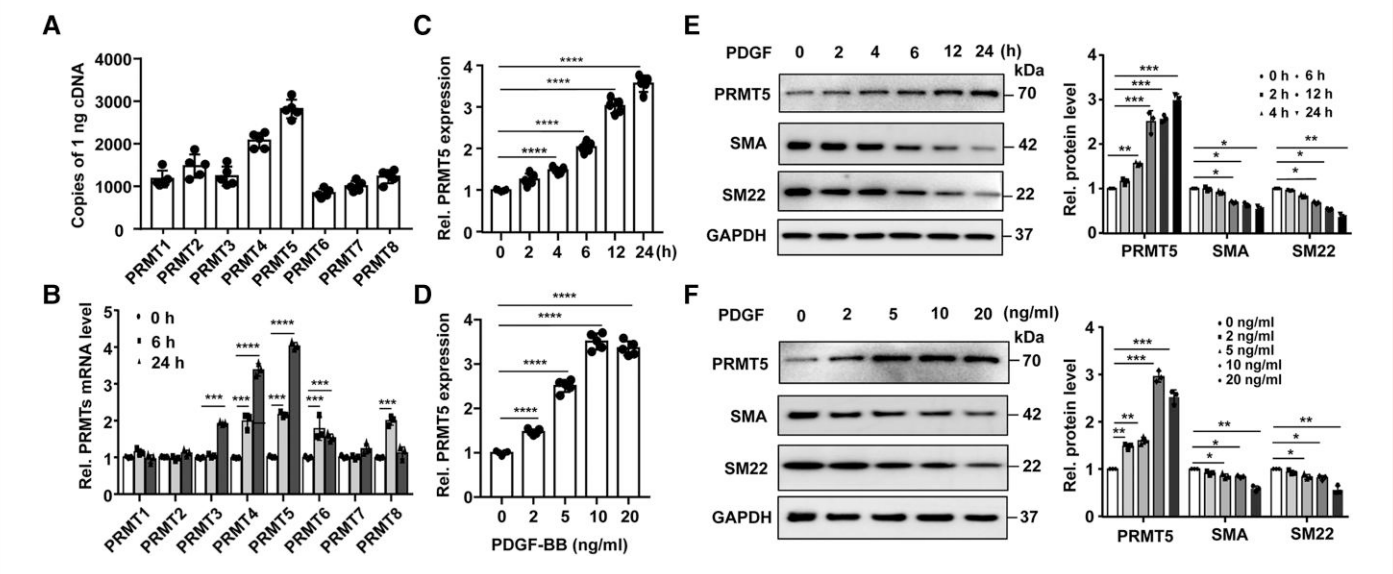


Figure 1 PRMT5 expression is increased in dedifferentiated VSMCs. (*A*) Expression of PRMTs in human VSMCs was detected by ddPCR. $n = 5$. (*B*) Human VSMCs were starved in 0.5% FBS for 48 h and then stimulated with PDGF-BB (20 ng/mL) for 0, 6, and 24 h. The expression of PRMTs was determined by qRT–PCR. ***$P < 0.001$ and ****$P < 0.0001$, two-way ANOVA. $n = 3$. (*C*) Human VSMCs were starved in 0.5% FBS for 48 h and then stimulated with PDGF-BB (20 ng/mL) for indicated time points. The expression of PRMT5 was determined by qRT–PCR. ****$P < 0.0001$, (one-way ANOVA). $n = 5$. (*D*) Human VSMCs were starved in 0.5% FBS for 48 h and then stimulated with increasing concentrations of PDGF-BB for 24 h. The expression of PRMT5 was determined by qRT–PCR. ****$P < 0.01$, one-way ANOVA. $n = 5$. *n* represents technical repeats in SMCs from different donors. (*E*) Human VSMCs were starved in 0.5% FBS for 48 h and then stimulated with PDGF-BB (20 ng/mL) for indicated time points. The expression of PRMT5, SMA, and SM22 was determined by western blot and quantitated by densitometric analysis. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$, two-way ANOVA. $n = 3$. (*F*) Human VSMCs were starved in 0.5% FBS for 48 h and then stimulated with increasing concentrations of PDGF-BB. The expression of PRMT5, SMA, and SM22 was determined by western blot and quantitated by densitometric analysis. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$, two-way ANOVA. $n = 3$.

performed in accordance with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health following protocols that were reviewed and approved by the Institutional Animal Care and Use Committee at Thomas Jefferson University.

2.14 Mouse carotid artery ligation model

Tamoxifen-treated PRMT5-SMKO mice (6–8 weeks of age) and their control littermates (*SMMHC-Cre*$^{(-)}$-PRMT5$^{fl/fl}$) were used for experiments. After anaesthesia with 1–2% isoflurane inhalation, the left and right common carotid arteries were exposed through a small midline incision in the neck as we described previously.[9] The left artery was completely ligated with a 6-0 silk suture just proximal to the carotid bifurcation to disrupt blood flow. For sham operation, a similar procedure was performed but without ligation. The animals were allowed to recover for 3–4 weeks. One day after carotid ligation, mice were treated with 50 mg/kg EPZ015666 formulated in 0.5% methylcellulose (Sigma-Aldrich) solution in water or 0.5% methylcellulose solution in water (vehicle) by oral gavage administration twice a day. Three weeks after treatment, neointimal formation was determined by immunohistochemical staining (IHC). At study end, mice were euthanized via inhalation of CO_2 (100%) followed by cervical dislocation as a secondary confirmation of death.

2.15 Rat carotid balloon injury model

Carotid artery balloon injury and lentivirus-mediated gene transfer were performed in male Sprague-Dawley rats (250 to 300 g) as described previously.[30] Briefly, rats were anaesthetized with pentobarbital (50 mg/kg, intraperitoneal). Under a dissecting microscope, the right common carotid artery was exposed through a midline cervical incision. A 2F Fogarty catheter (Edwards Lifesciences) was introduced via an arteriotomy in the external carotid artery, and the catheter was then advanced to the proximal edge of the omohyoid muscle. To produce carotid artery injury, we inflated the balloon with saline and withdrew it three times from just under the proximal edge of the omohyoid muscle to the carotid bifurcation. After balloon injury, solutions of (100 µL) Lenti-shPRMT5 (1×10^9 pfu/mL) or Lenti-shCTL (1×10^9 pfu/mL) were infused into the ligated segment of the common carotid artery for 30 min. The external carotid artery was then permanently ligated with a 6-0 silk suture, and blood flow in the common carotid artery was restored. The rats were euthanized by CO_2 inhalation at 14 days after balloon injury.

2.16 Immunohistochemistry

The segments of arteries were fixed in 4% paraformaldehyde and embedded in paraffin to generate 5 µm sections. The sections were then stained with haematoxylin and eosin (H&E) for imaging under a microscope, and the medial area and intimal area were measured using analysing software (NIH Image 1.62; http://rsb.info.nih.gov/nih-image/download.html) as previously described.[9] To examine the expression of PRMT5, Ki67, and histone H3R8Me2s, the sections were de-paraffinized, followed by antigen retrieval using a microwave. After staining with anti-PRMT5 (#79998, dilution 1:200; Cell Signaling), anti-Ki67 (#12202, dilution 1:200; Cell Signaling), and H3R8Me2s (ab130740, 1:1000 dilution; Abcam) primary antibodies followed by HRP-conjugated secondary antibody, the sections were developed using DAB substrate. The stained sections were imaged under a microscope.

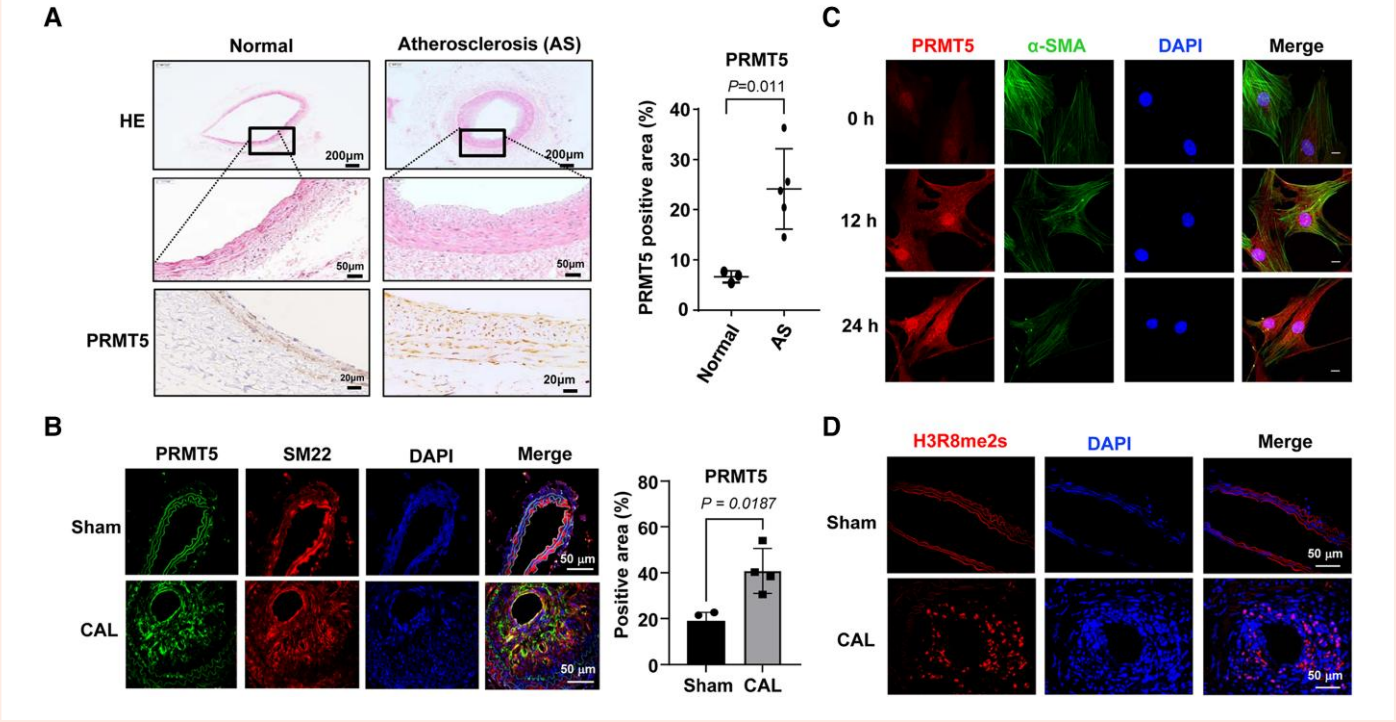


Figure 2 PRMT5 is increased in atherosclerotic and neointimal lesions. (*A*) HE staining showing neointimal formation in human atherosclerotic lesions (top). Immunohistochemistry showing increased levels of PRMT5 in five human atherosclerotic lesions compared with three control samples. PRMT5 positive area was quantitated (Student's *t*-test). (*B*) Mice were subjected to carotid artery ligation. Four weeks after ligation, the expression of PRMT5 and SM22 was determined by immunofluorescent staining. PRMT5 positive area was quantitated (Student's *t*-test). $n = 4$. (*C*) Human VSMCs were starved in 0.5% FBS for 48 h and then stimulated with PDGF-BB (20 ng/mL) for indicated time points. The expression of PRMT5 and SMA was determined by immunofluorescent staining. Scale bar: 20 µm. (*D*) Mice were subjected to carotid artery ligation. Four weeks after ligation, levels of symmetric di-methylation of H3R8 (H3R8me2s) were determined by immunofluorescent staining. Scale bar: 50 µm.

2.17 Statistical analysis

All data are expressed as the mean ± S.E.M. An unpaired two-tailed Student's *t*-test was used to compare two groups. Differences between groups were assessed by one-way or two-way analysis of variance. The Student–Newman–Keuls test was used for multiple comparisons. $P < 0.05$ was considered statistically significant.

3. Results

3.1 Increased expression of PRMT5 in human dedifferentiated VSMCs

PRMTs have been characterized as critical regulators in various biological processes, including development, cell growth, survival, and DNA repair response.[31,32] To determine the expression pattern of all three types of PRMTs in human VSMCs, we conducted a digital quantitative PCR to analyse the absolute levels of PRMT mRNAs. Among all PRMTs, the expression of PRMT5 mRNA was the highest compared to other PRMTs, while the PRMT9 mRNA was barely detected in VSMCs (*Figure 1A*). Next, we investigate whether expression of PRMTs was up-regulated when stimulated with PDGF-BB, which is a key regulator for VSMC phenotypic modulation.[33,34] As shown in *Figure 1B*, the mRNA levels of PRMT4 and PRMT5 mRNA were increased in PDGF-BB-treated VSMCs in a time-dependent manner. Because PRMT5 is a major type II PRMT that abundantly expressed in human VSMCs, we further investigated the expression of PRMT5 in response to PDGF-BB stimulation. As shown in *Figure 1C* and *D*, PDGF-BB stimulation increased the expression of PRMT5 in a time and dose-dependent manner, as determined by qRT–PCR. Likewise, western blot showed that PRMT5 protein levels were up-regulated by PDGF-BB stimulation, in a dose- and time-dependent manner (*Figure 1E* and *F*). Accordingly, the protein levels of VSMC differentiation markers SMA and SM22 were decreased. Our time course studies show that up-regulation of PRMT5 occurs before the down-regulation of SMA and SM22 after PDGF stimulation. Based on these results, we now speculate that PRMT5 plays a role in regulating VSMC phenotypic switch.

3.2 Increased PRMT5 expression in human atherosclerotic lesions and mouse carotid arteries after ligation

To further substantiate the pathological significance of PRMT5 in vascular remodelling, we examined the expression of PRMT5 in human coronary artery atherosclerotic and mouse neointimal lesions. As shown in *Figure 2A*, HE staining revealed coronary artery lesion formation in patients with coronary artery disease. PRMT5 expression was increased primarily in the medial areas of atherosclerotic lesions. Likewise, PRMT5 is highly expressed in the neointimal area of mouse carotid arteries at 4 weeks after carotid artery ligation (*Figure 2B*). Immunofluorescent staining showed that increased PRMT5 is predominantly localized in the nucleus

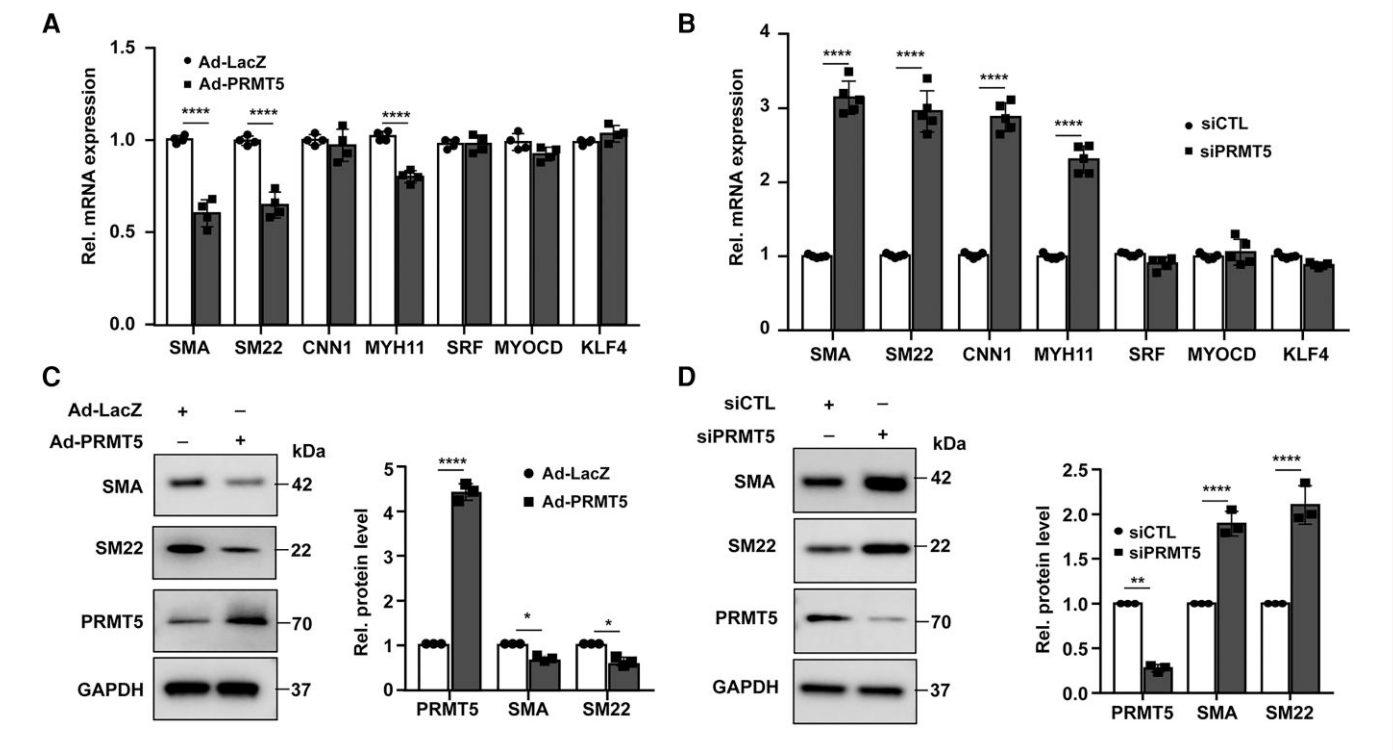


Figure 3 PRMT5 regulates the expression of SMC marker genes. (*A*) Human VSMCs were transduced with Ad-LacZ or Ad-PRMT5 (MOI = 50). Twenty-four hours after transduction, cells were starved in 0.5% FBS for 48 h and the expression of VSMC marker genes, transcriptional factors, and co-factors was determined by qRT–PCR. ****$P < 0.0001$, Student's *t*-test. $n = 4$. (*B*) Human VSMCs were transfected with either control siRNA (siCTL) or PRMT5 specific siRNA (siPRMT5) for 24 h. Cells were then starved in 0.5% FBS for 48 h and then stimulated with PDGF-BB (20 ng/mL) for 24 h. The expression of SMC marker genes, transcriptional factors, and co-factors was determined by qRT–PCR. ****$P < 0.0001$, Student's *t*-test. $n = 5$. (*C*) Human VSMCs were transduced with Ad-LacZ or Ad-PRMT5 (MOI = 50). Twenty-four hours after transduction, cells were starved in 0.5% FBS for 48 h and the expression of PRMT5, SMA, and SM22 was determined by western blot. *$P < 0.05$, ****$P < 0.0001$, Student's *t*-test. $n = 3$. (*D*) Human VSMCs were transfected with either control siRNA (siCTL) or PRMT5 specific siRNA (siPRMT5) for 24 h. Cells were then starved in 0.5% FBS for 48 h and then stimulated with PDGF-BB (20 ng/mL) for 48 h. The expression of PRMT5, SMA, and SM22 was determined by western blot. **$P < 0.01$ and ****$P < 0.0001$, Student's *t*-test. $n = 3$.

of VSMCs (*Figure 2C*). Since PRMT5 has been shown to symmetrically dimethylate histone H3R8 in the nucleus, we performed immunofluorescent staining in mouse carotid artery using anti-Histone H3 (dimethyl R8) antibody. We found that methylation of histone H3R8 is increased in neointimal lesion after injury (*Figure 2D*). Together, these results demonstrated the pathological significance of PRMT5 in vascular remodelling *in vivo*.

3.3 PRMT5 regulates the expression of VSMC marker genes

To further investigate whether PDGF-BB-mediated VSMC phenotypic modulation is PRMT5 dependent, we performed both gain and loss of functional studies and examined expression of SMC contractile genes by both qRT–PCR and western blot. As shown in *Figure 3A* and *B*, adenovirus-mediated overexpression of PRMT5 (Ad-PRMT5) significantly decreased the expression of SMA, SM22, and MYH11, while siRNA-mediated knockdown of PRMT5 increased the expression of SMA, SM22, CNN1, and MYH11 in the presence of PDGF-BB stimulation, as determined by qRT–PCR. The effects of PRMT5 on the expression of SMA and SM22 were further confirmed by western blot under both PRMT5 overexpression and knockdown conditions (*Figure 3C* and *D*). Moreover, PRMT5 overexpression and knockdown barely affected the expression of transcriptional factors, such as SRF, myocardin, and KLF4. Taken together, these results suggested that PRMT5 is implicated in modulating VSMC phenotypic switch.

3.4 PRMT5 promotes VSMC proliferation and migration

Dedifferentiated VSMCs present with higher proliferation and migration rates.[1] To investigate whether these characteristics were regulated by PRMT5, we conducted MTT and monolayer cell scratch assays. Both overexpression of PRMT5 and PDGF-BB stimulation significantly increased the quiescent VSMC proliferation rate by two to three-fold. Importantly, PDGF-BB-induced cell proliferation was further augmented in PRMT5 overexpressing cells (*Figure 4A*). PRMT5 knockdown barely affected the proliferation at baseline levels, but significantly attenuated PDGF-BB-induced proliferation by approximately 30% (*Figure 4B*). Likewise, overexpression of PRMT5 increased VSMC migration under both basal and PDGF-BB-stimulated conditions (*Figure 4C*) while PRMT5 knockdown decreased PDGF-BB-induced migration rate by approximately 35% (*Figure 4D*). Together, these results demonstrate a regulatory role of PRMT5 in VSMC proliferation and migration.

3.5 PRMT5 regulates SMA and SM22 expression through histone modification

The complex of myocardin (myocd) and SRF plays a central role in the regulation of VSMC differentiation marker expression through binding to CArG boxes.[5,35] Since increased PRMT5 is predominantly localized in the nucleus of human VSMCs, we speculate that PRMT5 may regulate the expression of SMA and SM22 through modulating the transcriptional

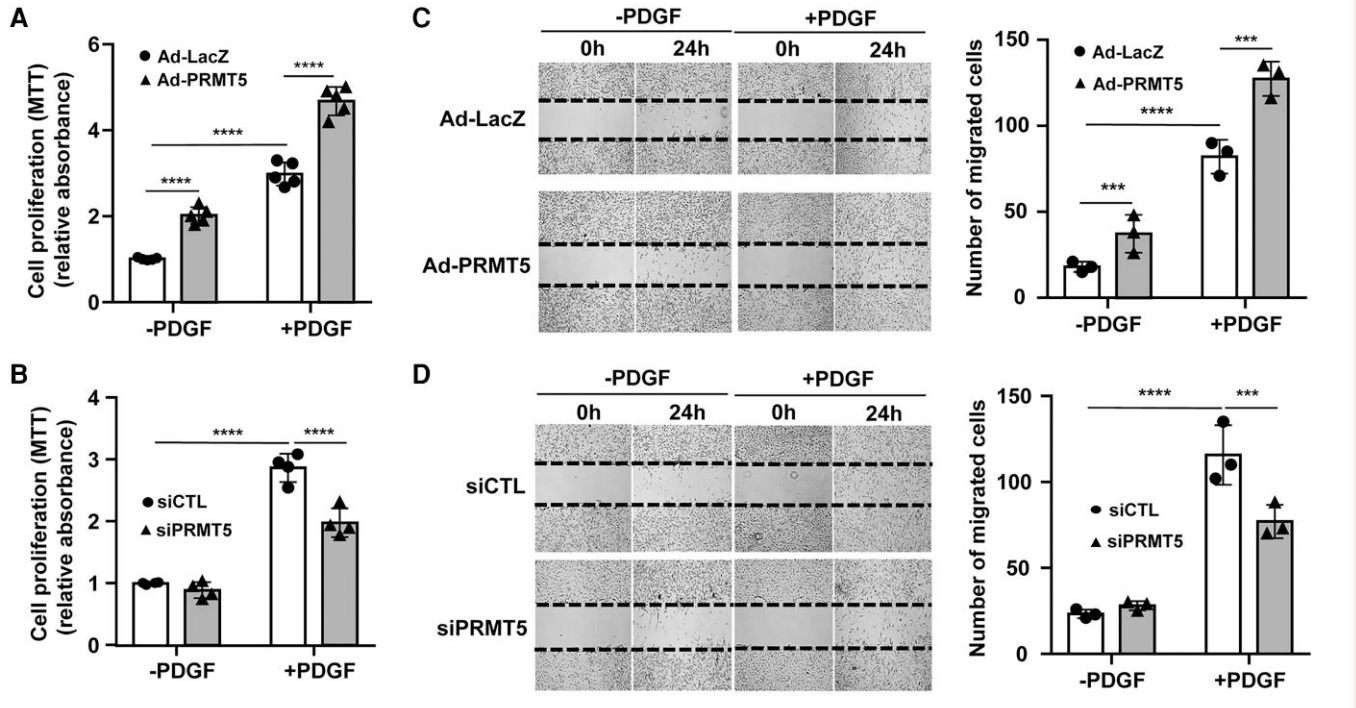


Figure 4 PRMT5 regulates SMC proliferation and migration. (*A*) Human VSMCs were transduced with Ad-LacZ or Ad-PRMT5 (MOI = 50). Twenty-four hours after virus transduction, cells were starved in 0.5% FBS for 48 h and then stimulated with or without PDGF-BB (20 ng/mL) for 24 h. The SMC proliferation was determined by MTT assays. ****$P < 0.0001$, two-way ANOVA. $n = 5$. (*B*) Human VSMCs were transfected with control siRNA (siCTL) or PRMT5 specific siRNA (siPRMT5) for 24 h. Cells were then starved in 0.5% FBS for 48 h and then stimulated with or without PDGF-BB (20 ng/mL) for 24 h. The VSMC proliferation was determined by MTT assays. ****$P < 0.01$, two-way ANOVA. $n = 4$. (*C*) Human VSMCs were transduced with Ad-LacZ or Ad-PRMT5 (MOI = 50). Twenty-four hours after virus transduction, cells were starved in 0.5% FBS for 48 h and then stimulated with or without PDGF-BB (20 ng/mL) for 24 h. The SMC migration was determined by wound scratch assay. ***$P < 0.001$, ****$P < 0.0001$, two-way ANOVA. $n = 3$. (*D*) Human VSMCs were transfected with control siRNA (siCTL) or PRMT5 specific siRNA (siPRMT5) for 24 h. Cells were then starved in 0.5% FBS for 48 h and then stimulated with or without PDGF-BB (20 ng/mL) for 24 h. The VSMC migration was determined by wound scratch assay. ***$P < 0.001$, ****$P < 0.0001$, two-way ANOVA. $n = 3$.

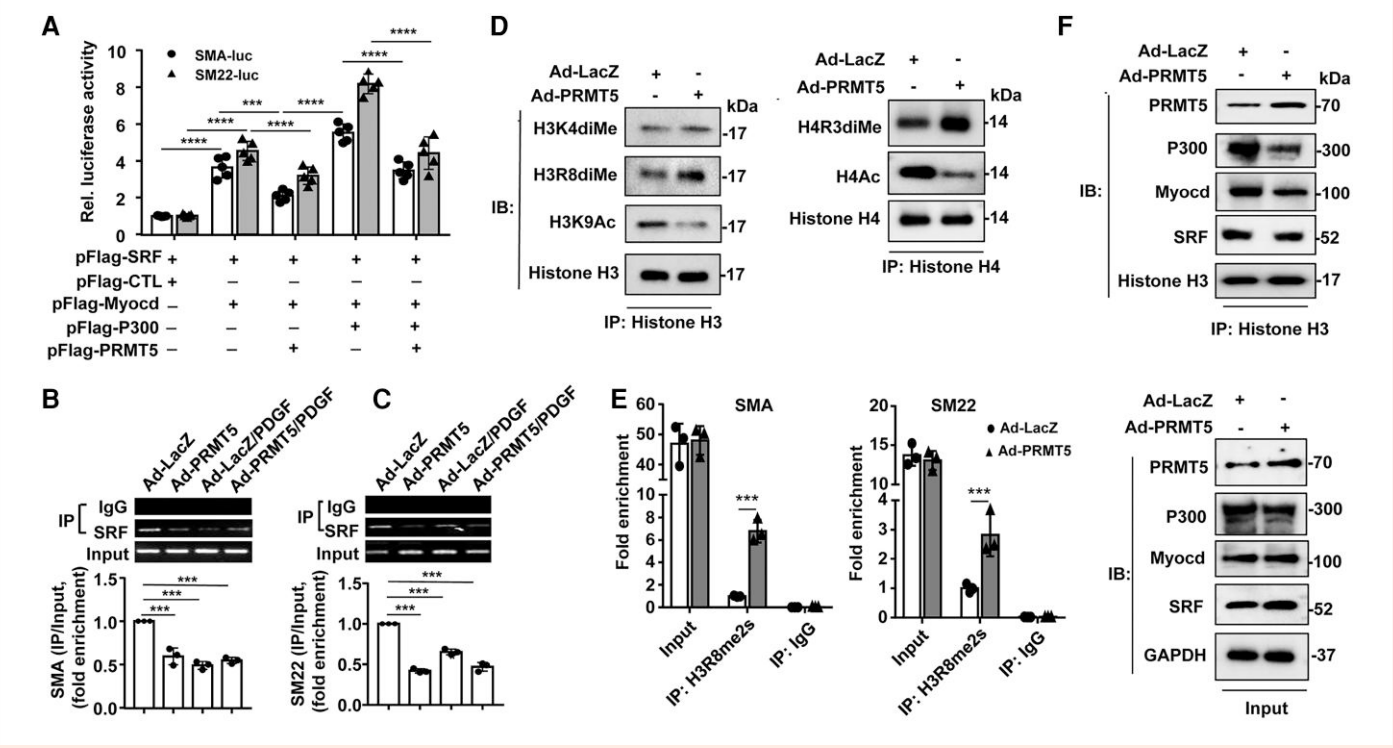


Figure 5 Epigenetic regulation of SMA and SM22 expression by PRMT5. (*A*) 10T1/2 cells were transfected with SMA-Luc or SM22-Luc reporter plasmid together with a combination of SRF, myocd (myocardin), p300, and PRMT5 expression plasmids. Twenty-four hours after transfection, luciferase activity was determined. ***$P < 0.001$, ****$P < 0.0001$, two-way ANOVA. $n = 5$. (*B* and *C*) Human VSMCs were transduced with Ad-LacZ or Ad-PRMT5 (MOI = 50). Twenty-four hours after virus transduction, cells were starved in 0.5% FBS for 48 h and then stimulated with or without PDGF-BB (20 ng/mL) for 24 h. The recruitment of SRF to the SMA promoter (*B*) and SM22 promoter (*C*) was determined by ChIP-quantitative PCR assays using anti-SRF antibody. ***$P < 0.001$, two-way ANOVA. $n = 3$. (*D*) Human VSMCs were transduced with Ad-LacZ or Ad-PRMT5 (MOI = 50). Twenty-four hours after virus transduction, cells were starved in 0.5% FBS for 48 h. Cell lysates were subjected to immunoprecipitation by anti-histone H3 or H4 antibodies and then immunoblotted with indicated antibodies. (*E*) Human VSMCs were transduced with Ad-LacZ or Ad-PRMT5 (MOI = 50). Twenty-four hours after virus transduction, cells were starved in 0.5% FBS for 48 h. The levels of H3R8me2s on the SMA and SM22 promoters were determined by ChIP assays using anti-H3R8me2s antibody. ***$P < 0.001$, Student's *t*-test. $n = 3$. (*F*) Human VSMCs were transduced with Ad-LacZ or Ad-PRMT5 (MOI = 50). Twenty-four hours after virus transduction, cells were starved in 0.5% FBS for 48 h. Cell lysates were subjected to immunoprecipitation by anti-histone H3 antibody and then immunoblotted with indicated antibodies.

factors and/or co-factors. To this end, we performed SMA and SM22 promoter-driven luciferase assays. As shown in *Figure 5A*, co-transfection of pFlag-myocd with pFlag-SRF dramatically increased both SMA and SM22 promotor driven luciferase activities and this effect was enhanced by co-expression of p300. Notably, co-expression of pFlag-PRMT5 remarkably decreased the promoter activities of SMA and SM22 stimulated by overexpression of myocd, SRF, and p300, indicating that PRMT5 may regulate the transcriptional complex formation in the promoters of SMA and SM22. To further evaluate the involvement of PRMT5 in the regulation of SMA and SM22 transcriptional activity in VSMCs, ChIP assay was performed using anti-SRF antibody. As shown in *Figure 5B* and *C*, both PDGF-BB stimulation and PRMT5 overexpression significantly reduced the binding of SRF to the promoters of SMA and SM22. Next, we examined whether PRMT5 is involved in histone arginine methylation in VSMCs. Interestingly, overexpression of PRMT5 significantly increased H3R8 di-methylation, while acetylation of H3K9 was markedly attenuated, but barely affected H3K4 di-methylation (*Figure 5D*). Further, overexpression of PRMT5 promoted H4R3 di-methylation and H4 de-acetylation (*Figure 5D*). The levels of H3R8 di-methylation on SMA and SM22 promoters were increased in VSMCs overexpressing PRMT5 as determined by ChIP assay (*Figure 5E*). Moreover, we performed immunoprecipitation using anti-histone H3 antibody to determine the binding of the transcriptional factors and co-factors to histone H3. As shown in *Figure 5F*, PRMT5 overexpression had no effect on the expression of p300, myocardin, and SRF, but significantly attenuated the binding of p300, myocardin, and SRF to histone H3. Together, these results suggest that PRMT5-mediated arginine methylation of histone H3 and H4 is involved in regulating SMC marker gene expression by the transcriptional SRF/myocardin/p300 complex.

3.6 SMC-specific deletion of PRMT5 ameliorates ligation neointimal formation in mice

Our preliminary data demonstrated that constitutive SMC-PRMT5 deletion led to an embryonic lethality (data not shown). To investigate the pathological significance of PRMT5 in vascular remodelling *in vivo*, we generated inducible SMC-specific PRMT5 knockout mice by crossing PRMT5 floxed mice with SMMHC-CreER^{T2} to create SMMHC-CreER$^{T2+/-}$/PRMT5$^{fl/fl}$ (PRMT5-SMKO) mice (*Figure 6A* and *B*). Tamoxifen (3 mg/mouse/day in corn oil) was applied by intraperitoneal injections to 6–8 week old mice for 5 consecutive days to induce Cre-mediated gene deletion. After a 2 week recovery, we isolated VSMCs and examined the

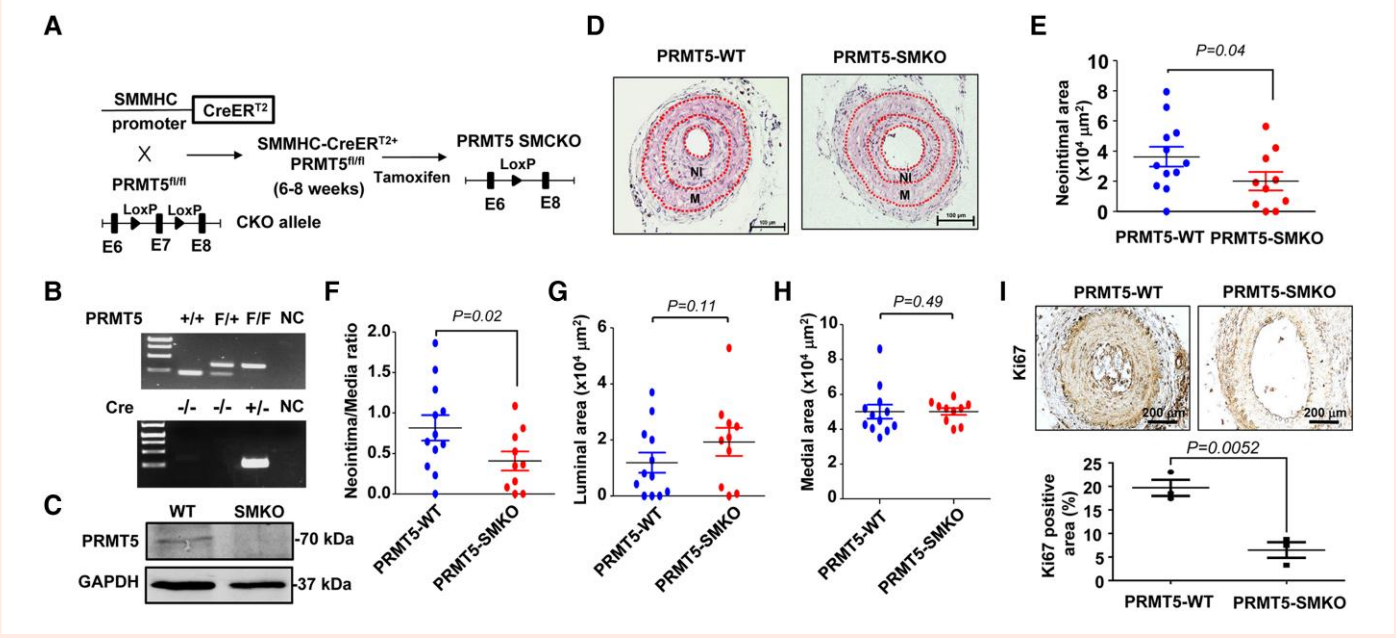


Figure 6 SMC deletion of PRMT5 prevents neointimal formation after carotid artery ligation in mice. (*A*) Breeding strategy of generating inducible SMC-specific PRMT5 knockout (PRMT5-SMCKO) mice. (*B*) Genotyping of PRMT5-SMCKO mice. (*C*) Expression of PRMT5 in aortic SMCs isolated from PRMT5-SMKO mice and their wild-type (WT) littermates. (*D*) PRMT5-SMKO mice and their WT littermates were subjected to the carotid artery ligation-induced injury. Representative haematoxylin and eosin (H&E)-stained carotid artery slices at 28 days after carotid artery ligation. (*E–H*) The effect of PRMT5 deletion on vascular neointimal lesion formation in mouse carotid arteries at 28 days after ligation injury as quantitated by neointimal area, neointima/media (N/M) ratio, luminal area, and medial area. $n = 10–12$. (*I*) Representative immunohistochemical staining of Ki67 in mouse carotid arteries at 28 days after ligation injury. $n = 3$. Statistical analyses were performed using a Student's *t*-test (*E–I*).

expression of PRMT5 by western blot. As shown in *Figure 6C*, the expression of PRMT5 was virtually absent in isolated VSMCs from PRMT5-SMKO mice after tamoxifen treatment, while the expression of other PRMTs was not affected (data not shown). PRMT5-SMKO and PRMT5$^{fl/fl}$ (PRMT5-WT) littermate mice were then subjected to carotid artery ligation.[9] As shown in *Figure 6D–H*, SMC deletion of PRMT5 dramatically reduced neointimal formation as reflected by decreased neointimal area and neointimal/media ratio, while the luminal area and medial area were not significantly impacted. In addition, Ki67 positive area was reduced from 20 to 6% in PRMT5-SMKO, suggesting that VSMC proliferation was suppressed in PRMT5-SMKO mice (*Figure 6I*). Together, these results suggest that PRMT5 promotes VSMC proliferation and neointimal formation after vascular injury in mice.

3.7 Inhibition of PRMT5 attenuates intimal hyperplasia *in vivo*

Post-angioplasty restenosis is a common complication after percutaneous coronary interventions and is mainly caused by excessive proliferation of VSMCs.[36] To establish the potential therapeutic capacity of targeting PRMT5 for the treatment of restenosis, the rat carotid balloon injury was applied. Inhibition of PRMT5 expression was achieved by a local delivery of lentivirus expressing PRMT5 shRNA (Lenti-shPRMT5) to the carotid artery wall at the time of balloon injury. As shown in *Figure 7A*, balloon injury led to a significant neointimal formation and increased expression of PRMT5 in the medial area of carotid arteries. The protein expression of PRMT5 was significant reduced after introducing Lenti-shPRMT5 as compared with Lenti-shCTL-infected arteries, as determined by IHC. Morphometric analysis of arterial cross-sections revealed that delivery of Lenti-shPRMT5 inhibited neointima area by 51% ($P < 0.01$) (*Figure 7B*) and intima/media ratio by 54% ($P < 0.01$) (*Figure 7C*) after injury as compared with Lenti-shCTL-infected arteries.

To further substantiate the significance of PRMT5 in vascular remodelling, we determined the effects of pharmacological inhibition of PRMT5 on neointimal formation in a mouse model of carotid artery ligation. EPZ015666 (GSK3235025) is an orally available specific PRMT5 inhibitor that represents a validated chemical probe for the study of PRMT5 biological function under both *in vitro* and *in vivo* conditions.[37] One day after carotid artery ligation, mice were treated with EPZ015666 (50 mg/kg, oral administration, twice a day) for 3 weeks. As shown in *Figure 7D* and *E*, treatment of mice with EPZ015666 markedly attenuated both neointimal formation and Intimal/Media ratio of intimal lesions. Together, these results show that inhibition of PRMT5 may represent a novel therapeutic approach to attenuate neointimal development.

4. Discussion

VSMCs play a vital role in maintenance of vessel homeostasis, blood pressure, and response to injury.[1,3] Despite accumulating evidence indicates that the transition of VSMCs from contractile to synthetic phenotype is essentially involved in the development of atherosclerosis, hypertension, aortic aneurysm formation, and post-angioplasty restenosis, the mechanisms underlying VSMC phenotypic switch still remain elusive.[1,3] In the present study, we identified PRMT5 as a critical regulator in VSMC phenotypic switch, proliferation, and migration. PRMT5 was highly expressed in human atherosclerotic lesions and mouse carotid arteries after balloon injury. *In vitro* gain and loss-of-function studies revealed that PRMT5 is essential for PDGF-BB-promoted VSMC migration, proliferation, and phenotypic switch. Mechanistically, we found that overexpression of PRMT5 leads to histone arginine di-methylation of H3R8 and H4R3, which in turn attenuates acetylation of H3K9 and H4. These effects further limit the access of the SRF/myocardin complexes to the VSMC marker gene CArG boxes and subsequently inhibits VSMC differentiation. Importantly, both

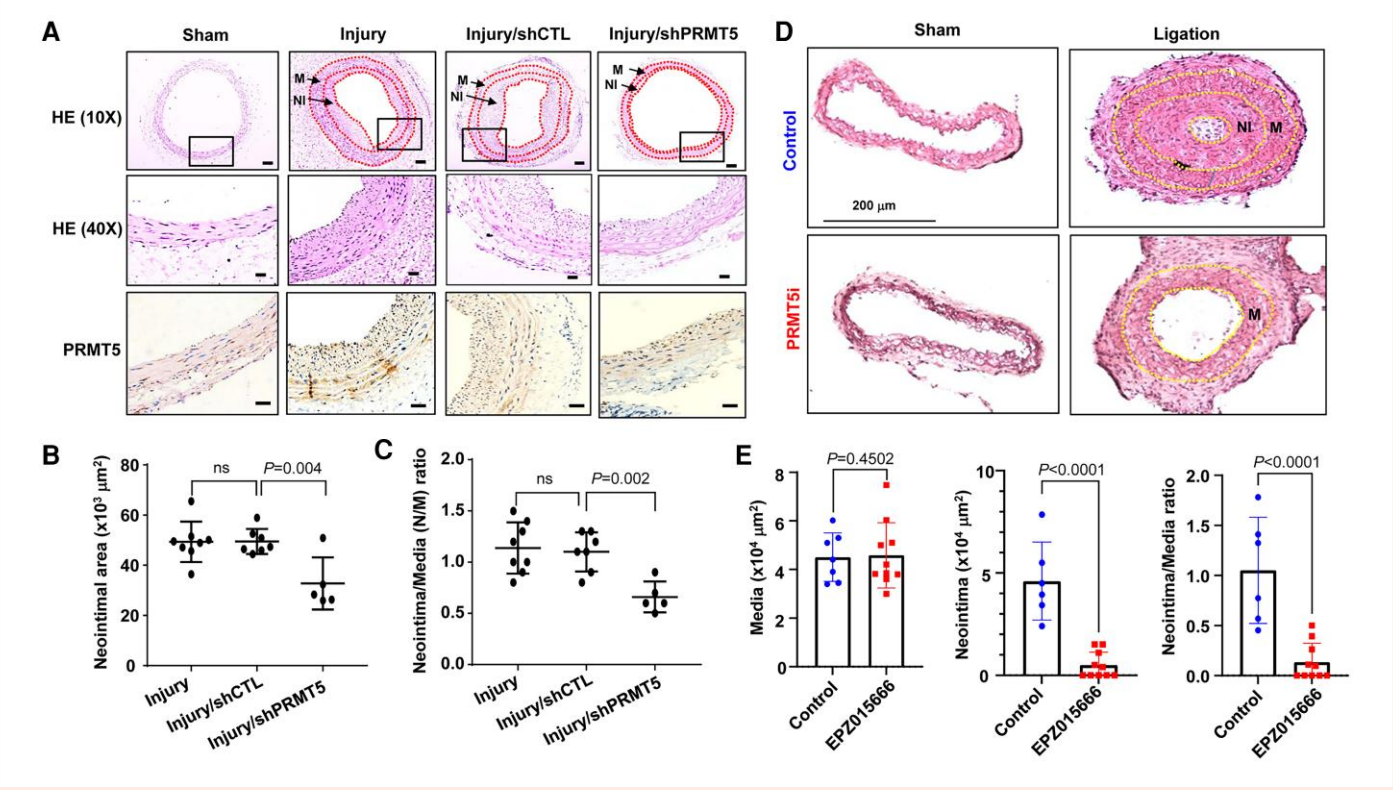


Figure 7 Inhibition of PRMT5 attenuates intimal hyperplasia *in vivo*. (*A*) Representative haematoxylin and eosin (H&E)-stained carotid artery slices at 14 days after balloon injury. Expression of PRMT5 in rat carotid arteries at 14 days after transduction of lentivirus expressing control shRNA (Lenti-shCTL) or PRMT5 shRNA (Lenti-shPRMT5) was determined by immunohistochemical staining (IHC). Scale: 200 μm (HE: 10×), 50 μm (HE: 40×), and 20 μm (PRMT5 IHC). M, media; NI, neointima. (*B* and *C*) The effect of Lenti-shPRMT5 on vascular neointimal lesion formation in rat carotid arteries at 14 days after balloon injury was quantitated by neointimal area and neointimal/media (N/M). $n = 5–8$. (D) PRMT5 specific inhibitor EPZ015666 attenuates neointimal formation in mice. C57BL/6J (8–10 weeks of old) were subjected to carotid artery ligation. One day after carotid ligation, mice were treated with 50 mg/kg EPZ015666 formulated in 0.5% methylcellulose (Sigma-Aldrich) solution in water or 0.5% methylcellulose solution in water (vehicle control) by oral gavage administration twice a day. Three weeks after treatment, neointimal formation was determined by immunohistochemical staining. Representative haematoxylin and eosin (H&E)-stained carotid artery slices at 21 days after injury. Scale: 200 μm. (*E*) The effect of EPZ015666 on vascular neointimal lesion formation at 21 days after carotid artery ligation was quantitated by media (M), neointimal area (NI), and neointimal/media (NI/M). $n = 7–10$. Statistical analyses were performed using a two-way ANOVA (*B* and *C*) or a Student's *t*-test (*E*).

SMC-specific deletion of PRMT5 in mice and local delivery of Lenti-shPRMT5 to rat carotid arteries significantly attenuated neointimal formation after injury. To our best knowledge, this is the first study to elucidate the role of PRMT5-mediated histone arginine methylation in regulating VSMC differentiation and vascular remodelling *in vivo*.

Accumulating evidence has revealed that chromatin remodelling is a vital element in the determination of VSMC differentiation state, particularly in the areas of histone acetylation and methylation.[2,10] Indeed, several epigenetic regulators, including histone acetyltransferases (HATs), histone deacetylases (HDACs), and histone and DNA methyltransferases (HMTs and DNMTs) have been shown to regulate SMC marker gene transcription through chromatin modification.[10] Indeed, differentiated vascular SMCs display several specific histone tail modifications including acetylation (H3K9, H3K14, and H4) as well as methylation (H3K4diMe and H3K79diMe) at VSMC marker gene CArG boxes.[11,38] These modifications have been shown to enable recruitment of the myocardin/SRF complexes to SMC-specific CArGs, thus leading to increased transcriptional activation of SMC marker genes.[38] Recently, histone arginine methylation, which is enzymatically catalyzed by the family of PRMTs, has been shown to play an important regulatory role in gene expression, mRNA splicing, and the DNA damage response.[31] Thus far, nine PRMTs have been identified and they are classified as type I (PRMT1, 2, 3, 4, 6, and 8), II (PRMT5 and PRMT9), and III (PRMT7) enzymes that catalyze asymmetric di-methylation, symmetric di-methylation, and mono-methylation, respectively, on arginine residues in histone and non-histone proteins.[31] PRMT5 is the major type II enzyme in mammalian cells, and it has been shown to localize to both the cytoplasm and the nucleus and methylate multiple histone and non-histone proteins, thus exerting diverse biological effects.[15,32,39] For example, methylation of histone protein by PRMT5 can regulate DNA damage repairing, gene expression, and RNA splicing.[39] Methylation of non-histone proteins, such as p53, HOXA9, NF-κB, and E2F-1, by PRMT5 has been implicated in the regulation of cell growth, apoptosis, and inflammation[20,21,23]. PRMT5 is widely expressed in different human tissues, with high expression levels observed in heart, skeletal muscle, and testis.[40] Recently, increasing evidence has shown that PRMT5 functions as an oncogene and its overexpression are associated with the progression of several types of cancers, including oropharyngeal squamous cell carcinoma, lung cancer, and breast cancer, thus, PRMT5 has become a promising target for the cancer treatment.[15,32,41] At present, the role of PRMT5 in VSMC biology has not been determined. In the present study, we found that PRMT5 is the most abundantly expressed PRMT in human aortic VSMCs, and its expression is significantly increased in proliferating VSMCs and human atherosclerotic lesions, hence highlighting a pathological significance of PRMT5 in proliferative vascular disease. It should be noted

that in addition to PRMT5, the expression of PRMT4 is also increased in response to PDGF stimulation and its roles in regulating VSMC function needs under investigation.

Epigenetic regulation including specific histone modifications plays an important role in regulating SMC plasticity and the binding of the transcriptional regulators to SMC marker gene promoters.[2,38] Histone modifications, such as acetyl-H3K9, -H3K14, and -H4, have been identified as distinguishing marks in differentiating SMCs as compared with non-SMCs.[11,38] Both H3K9 and H4 acetylation have been shown to activate SMC differentiation marker gene expression,[11,38] while methylation on histone arginine residues has been shown to either activate or repress gene transcription depending on the actual residue involved. For instance, PRMT5 induced di-methylation on histone H4R3 and H3R8 is considered as repressive marks for gene expression.[15,18] Consistent with this notion, we found that PRMT5 catalyzed the di-methylation of H3R8 and H4R3, which in turn limits access of the SRF/myocardin complex to SMC marker CArG boxes, thus repressing the expression of VSMC marker genes in human VSMCs. Moreover, we found that acetylation of H3K9 was attenuated in PRMT5 overexpressing VSMCs, suggesting a potential cross-talk between H3R8 methylation and H3K9 acetylation, as previously reported.[18] Although the underlying mechanism remains to be determined, it is attempted to speculate that PRMT5-mediated H3R8 methylation may sterically hinder the accessibility of activated acetyltransferases to H3K9, leading to decreased H3K9 acetylation. Similarly, we found that H4 acetylation was also inhibited in VSMCs overexpressing PRMT5. Nevertheless, it remains to be determined whether repression of VSMC marker gene expression by PRMT5 requires co-ordinated methylation of H3R8 and H4R3 simultaneously in VSMCs. Furthermore, SMA deficient SMCs have been recently shown to increase SMC proliferation and migration through focal adhesion (FA) rearrangement and increased ligand-independent activation of platelet-derived growth factor receptor beta (Pdgfr-β).[42] In our study, we show that PRMT5 overexpression reduced SMA expression, which may contribute to increased SMC proliferation and migration in PRMT5 overexpressing SMCs even in the absence of PDGF stimulation. Whether this process involves a ligand-independent activation of Pdgfr-β needs to be investigated in future studies.

Another important finding of this study is that the loss of function of PRMT5 reduces neointimal formation in SMC-specific PRMT5 knockout mice. Our initial studies demonstrated that constitutive deletion of PRMT5 in mouse SMCs led to an embryonic lethality (data not shown), suggesting that PRMT5 in VSMCs is indispensable for vascular development. To overcome this lethality, we generated inducible SMC-specific knockout mice to study the role of PRMT5 in vascular modelling in adult mice. After tamoxifen treatment, the expression of PRMT5 in aortic SMCs isolated from PRMT5-SMKO mice was virtually absent. Furthermore, we found that both smooth muscle specific deletion and pharmacological inhibition of PRMT5 markedly attenuated the carotid artery ligation-induced neointimal formation in mice. Using the rat carotid artery balloon injury model, we also confirmed that a local delivery of lentivirus-mediated infection of PRMT5 shRNA dramatically reduced neointima formation. Vascular inflammation is also implicated in neointimal formation. Interestingly, PRMT5 has been recently shown to mediate trimethylamine *N*-oxide (TMAO)-induced inflammatory response in VSMCs.[43] These data together with the role of PRMT5 in promoting VSMC synthetic phenotype suggest that targeted inhibition of PRMT5 may be an attractive therapeutic strategy for the treatment of vascular diseases, such as atherosclerosis and restenosis. Moreover, our current study focused on the role of PRMT5 in human aortic SMCs, whether PRMT5 plays a role in other types of SMCs, such as coronary VSMCs, needs further investigation.

In summary, PRMT5 is a novel protein arginine methyltransferase that is up-regulated in human coronary atherosclerotic plaque and rat carotid arteries after balloon injury. Overexpression of PRMT5 promotes VSMC dedifferentiation and represses the expression of VSMC marker genes mainly through arginine methylation of histone H3R8 and H4R3, thus limiting the binding of the SRF/myocardin complex to CArG boxes of SMC-specific genes. In this regard, our results identify a novel mechanism underlying the regulation of SMC phenotypic switch via PRMT5-mediated histone arginine methylation and provide a potential therapeutic target for the treatment of proliferative vascular disease.

Supplementary material

Supplementary material is available at *Cardiovascular Research* online.

Authors' contributions

N.Z., Z.G., Y.Q., and J.S. designed the research; N.Z., Z.G., K.K., B.Y., N.T., H.Y., C.Z., R.Y., L.H., and J.S. performed the research; N.Z., Z.G., K.K., B.Y., H.Y., R.Y., L.H., Y.Q., and J.S. analysed the data; and N.Z., Z.G., K.K., and J.S. wrote the paper.

Acknowledgements

We thank Dr. Stephen D. Nimer at the University of Miami for kindly providing us with PRMT5 floxed mice.

Conflict of interest: None declared.

Funding

This work was funded by National Heart, Lung, and Blood Institute (NHLBI) grants R01HL159168 and R01HL152703 (J.S.).

Data availability

All data associated with this study are present in the paper.

References

1. Owens GK, Kumar MS, Wamhoff BR. Molecular regulation of vascular smooth muscle cell differentiation in development and disease. *Physiol Rev* 2004;**84**:767–801.
2. Alexander MR, Owens GK. Epigenetic control of smooth muscle cell differentiation and phenotypic switching in vascular development and disease. *Annu Rev Physiol* 2012;**74**:13–40.
3. Gomez D, Owens GK. Smooth muscle cell phenotypic switching in atherosclerosis. *Cardiovasc Res* 2012;**95**:156–164.
4. Wang D, Chang PS, Wang Z, Sutherland L, Richardson JA, Small E, Krieg PA, Olson EN. Activation of cardiac gene expression by myocardin, a transcriptional cofactor for serum response factor. *Cell* 2001;**105**:851–862.
5. Chen J, Kitchen CM, Streb JW, Miano JM. Myocardin: a component of a molecular switch for smooth muscle differentiation. *J Mol Cell Cardiol* 2002;**34**:1345–1356.
6. Du KL, Ip HS, Li J, Chen M, Dandre F, Yu W, Lu MM, Owens GK, Parmacek MS. Myocardin is a critical serum response factor cofactor in the transcriptional program regulating smooth muscle cell differentiation. *Mol Cell Biol* 2003;**23**:2425–2437.
7. Boettger T, Beetz N, Kostin S, Schneider J, Kruger M, Hein L, Braun T. Acquisition of the contractile phenotype by murine arterial smooth muscle cells depends on the Mir143/145 gene cluster. *J Clin Invest* 2009;**119**:2634–2647.
8. Cordes KR, Sheehy NT, White MP, Berry EC, Morton SU, Muth AN, Lee TH, Miano JM, Ivey KN, Srivastava D. miR-145 and miR-143 regulate smooth muscle cell fate and plasticity. *Nature* 2009;**460**:705–710.
9. Li P, Zhu N, Yi B, Wang N, Chen M, You X, Zhao X, Solomides CC, Qin Y, Sun J. MicroRNA-663 regulates human vascular smooth muscle cell phenotypic switch and vascular neointimal formation. *Circ Res* 2013;**113**:1117–1127.
10. Spin JM, Maegdefessel L, Tsao PS. Vascular smooth muscle cell phenotypic plasticity: focus on chromatin remodelling. *Cardiovasc Res* 2012;**95**:147–155.
11. McDonald OG, Wamhoff BR, Hoofnagle MH, Owens GK. Control of SRF binding to CArG box chromatin regulates smooth muscle gene expression in vivo. *J Clin Invest* 2006;**116**:36–48.
12. Qiu P, Li L. Histone acetylation and recruitment of serum responsive factor and CREB-binding protein onto SM22 promoter during SM22 gene expression. *Circ Res* 2002;**90**:858–865.
13. Cao D, Wang Z, Zhang CL, Oh J, Xing W, Li S, Richardson JA, Wang DZ, Olson EN. Modulation of smooth muscle gene expression by association of histone acetyltransferases and deacetylases with myocardin. *Mol Cell Biol* 2005;**25**:364–376.
14. Thomas JA, Deaton RA, Hastings NE, Shang Y, Moehle CW, Eriksson U, Topouzis S, Wamhoff BR, Blackman BR, Owens GK. PDGF-DD, a novel mediator of smooth muscle cell phenotypic modulation, is upregulated in endothelial cells exposed to atherosclerosis-prone flow patterns. *Am J Physiol Heart Circulatory Physiol* 2009;**296**:H442–H452.
15. Blanc RS, Richard S. Arginine methylation: the coming of age. *Mol Cell* 2017;**65**:8–24.

16. Sun L, Wang M, Lv Z, Yang N, Liu Y, Bao S, Gong W, Xu RM. Structural insights into protein arginine symmetric dimethylation by PRMT5. *Proc Natl Acad Sci USA* 2011;**108**: 20538–20543.
17. Guccione E, Richard S. The regulation, functions and clinical relevance of arginine methylation. *Nat Rev Mol Cell Biol* 2019;**20**:642–657.
18. Pal S, Vishwanath SN, Erdjument-Bromage H, Tempst P, Sif S. Human SWI/SNF-associated PRMT5 methylates histone H3 arginine 8 and negatively regulates expression of ST7 and NM23 tumor suppressor genes. *Mol Cell Biol* 2004;**24**:9630–9645.
19. Wang L, Pal S, Sif S. Protein arginine methyltransferase 5 suppresses the transcription of the RB family of tumor suppressors in leukemia and lymphoma cells. *Mol Cell Biol* 2008;**28**: 6262–6277.
20. Jansson M, Durant ST, Cho EC, Sheahan S, Edelmann M, Kessler B, La Thangue NB. Arginine methylation regulates the p53 response. *Nat Cell Biol* 2008;**10**:1431–1439.
21. Cho EC, Zheng S, Munro S, Liu G, Carr SM, Moehlenbrink J, Lu YC, Stimson L, Khan O, Konietzny R, McGouran J, Coutts AS, Kessler B, Kerr DJ, Thangue NB. Arginine methylation controls growth regulation by E2F-1. *EMBO J* 2012;**31**:1785–1797.
22. Chen M, Yi B, Sun J. Inhibition of cardiomyocyte hypertrophy by protein arginine methyltransferase 5. *J Biol Chem* 2014;**289**:24325–24335.
23. Bandyopadhyay S, Harris DP, Adams GN, Lause GE, McHugh A, Tillmaand EG, Money A, Willard B, Fox PL, Dicorleto PE. HOXA9 methylation by PRMT5 is essential for endothelial cell expression of leukocyte adhesion molecules. *Mol Cell Biol* 2012;**32**:1202–1213.
24. Herrera VL, Decano JL, Giordano N, Moran AM, Ruiz-Opazo N. Aortic and carotid arterial stiffness and epigenetic regulator gene expression changes precede blood pressure rise in stroke-prone Dahl salt-sensitive hypertensive rats. *PLoS One* 2014;**9**:e107888.
25. Zhu N, Yi B, Guo Z, Zhang G, Huang S, Qin Y, Zhao X, Sun J. Pim-1 kinase phosphorylates cardiac troponin I and regulates cardiac myofilament function. *Cell Physiol Biochem* 2018;**45**: 2174–2186.
26. Lim HJ, Kang DH, Lim JM, Kang DM, Seong JK, Kang SW, Bae YS. Function of Ahnak protein in aortic smooth muscle cell migration through Rac activation. *Cardiovasc Res* 2013;**97**: 302–310.
27. Yan G, Zhu N, Huang S, Yi B, Shang X, Chen M, Wang N, Zhang GX, Talarico JA, Tilley DG, Gao E, Sun J. Orphan nuclear receptor Nur77 inhibits cardiac hypertrophic response to beta-adrenergic stimulation. *Mol Cell Biol* 2015;**35**:3312–3323.
28. Liu M, Espinosa-Diez C, Mahan S, Du M, Nguyen AT, Hahn S, Chakraborty R, Straub AC, Martin KA, Owens GK, Gomez D. H3k4 di-methylation governs smooth muscle lineage identity and promotes vascular homeostasis by restraining plasticity. *Dev Cell* 2021;**56**: 2765–2782 e2710.
29. Liu F, Cheng G, Hamard PJ, Greenblatt S, Wang L, Man N, Perna F, Xu H, Tadi M, Luciani L, Nimer SD. Arginine methyltransferase PRMT5 is essential for sustaining normal adult hematopoiesis. *J Clin Invest* 2015;**125**:3532–3544.
30. Cheng Y, Liu X, Yang J, Lin Y, Xu DZ, Lu Q, Deitch EA, Huo Y, Delphin ES, Zhang C. MicroRNA-145, a novel smooth muscle cell phenotypic marker and modulator, controls vascular neointimal lesion formation. *Circ Res* 2009;**105**:158–166.
31. Di Lorenzo A, Bedford MT. Histone arginine methylation. *FEBS Lett* 2011;**585**:2024–2031.
32. Kim H, Ronai ZA. PRMT5 function and targeting in cancer. *Cell Stress* 2020;**4**:199–215.
33. Raines EW. PDGF and cardiovascular disease. *Cytokine Growth Factor Rev* 2004;**15**:237–254.
34. Millette E, Rauch BH, Kenagy RD, Daum G, Clowes AW. Platelet-derived growth factor-BB transactivates the fibroblast growth factor receptor to induce proliferation in human smooth muscle cells. *Trends Cardiovasc Med* 2006;**16**:25–28.
35. Yoshida T, Sinha S, Dandre F, Wamhoff BR, Hoofnagle MH, Kremer BE, Wang DZ, Olson EN, Owens GK. Myocardin is a key regulator of CArG-dependent transcription of multiple smooth muscle marker genes. *Circ Res* 2003;**92**:856–864.
36. Komatsu R, Ueda M, Naruko T, Kojima A, Becker AE. Neointimal tissue response at sites of coronary stenting in humans: macroscopic, histological, and immunohistochemical analyses. *Circulation* 1998;**98**:224–233.
37. Chan-Penebre E, Kuplast KG, Majer CR, Boriack-Sjodin PA, Wigle TJ, Johnston LD, Rioux N, Munchhof MJ, Jin L, Jacques SL, West KA, Lingaraj T, Stickland K, Ribich SA, Raimondi A, Scott MP, Waters NJ, Pollock RM, Smith JJ, Barbash O, Pappalardi M, Ho TF, Nurse K, Oza KP, Gallagher KT, Kruger R, Moyer MP, Copeland RA, Chesworth R, Duncan KW. A selective inhibitor of PRMT5 with in vivo and in vitro potency in MCL models. *Nat Chem Biol* 2015;**11**:432–437.
38. McDonald OG, Owens GK. Programming smooth muscle plasticity with chromatin dynamics. *Circ Res* 2007;**100**:1428–1441.
39. Kanamaluru D, Xiao Z, Fang S, Choi SE, Kim DH, Veenstra TD, Kemper JK. Arginine methylation by PRMT5 at a naturally occurring mutation site is critical for liver metabolic regulation by small heterodimer partner. *Mol Cell Biol* 2011;**31**:1540–1550.
40. Hong E, Lim Y, Lee E, Oh M, Kwon D. Tissue-specific and age-dependent expression of protein arginine methyltransferases (PRMTs) in male rat tissues. *Biogerontology* 2012;**13**: 329–336.
41. Yuan Y, Nie H. Protein arginine methyltransferase 5: a potential cancer therapeutic target. *Cell Oncol (Dordr)* 2021;**44**:33–44.
42. Papke CL, Cao J, Kwartler CS, Villamizar C, Byanova KL, Lim SM, Sreenivasappa H, Fischer G, Pham J, Rees M, Wang M, Chaponnier C, Gabbiani G, Khakoo AY, Chandra J, Trache A, Zimmer W, Milewicz DM. Smooth muscle hyperplasia due to loss of smooth muscle alpha-actin is driven by activation of focal adhesion kinase, altered p53 localization and increased levels of platelet-derived growth factor receptor-beta. *Hum Mol Genet* 2013;**22**: 3123–3137.
43. Liu H, Jia K, Ren Z, Sun J, Pan LL. PRMT5 critically mediates TMAO-induced inflammatory response in vascular smooth muscle cells. *Cell Death Dis* 2022;**13**:299.