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Epigenetic Mechanism in Regulation of Endothelial Function by Disturbed Flow: Induction of DNA Hypermethylation by DNMT1

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Abstract

There is increasing evidence that epigenetic mechanisms such as changes in DNA methylation and histone modification play an important role in regulating cellular functions in physiological and pathophysiological states. We investigated the effects of hemodynamic force disturbance, one of the risk factors for atherogenesis, on DNA methylation in HUVECs and rat carotid arteries. Our results demonstrated that athero-prone oscillatory shear flow (OS) without a clear direction induces DNA hypermethylation in comparison to the athero-protective pulsatile shear flow (PS) with a definite direction. Furthermore, OS increases the expression and nuclear translocation of DNA methyltransferase 1 (DNMT1), which is a major maintenance DNA methyltransferase that adds methyl groups to hemi-methylated DNA to repress gene expression. Pharmacological inhibition of DNMT1 by 5-Aza-2'-deoxycytidine abolished the OS-induced DNA hypermethylation. *In vivo* experiments also showed increases of DNMT1 expression and DNA methylation in the partially-ligated rat carotid arteries where the shear flow is disturbed. These *in vitro* and *in vivo* findings have provided novel evidence of the differential regulation of DNA methylation by different hemodynamic forces acting on vascular endothelium and identified DNMT1 as a key protein that governs the epigenetic changes in response to the pathophysiological stimuli due to disturbed flow.

Keywords

Oscillatory shear flow; Endothelial cells; DNA methylation; DNMT1

Introduction

Vascular endothelial cells (ECs) are constantly exposed to hemodynamic shear stress, which regulates EC function in health and disease, including atherosclerosis. The atherosclerotic

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CONFLICTS OF INTEREST

Jing Zhou, Yi-Shuan Li, Kuei-Chun Wang, and Shu Chien declare that they have no conflicts of interest.

ETHICAL STANDARDS

No human studies were carried out by the authors for this article.

All animal studies were performed in accordance with National Institutes of Health guidelines and were approved by the UC San Diego IACUC.

lesions develop preferentially in arterial branches and curvatures where local blood flow is disturbed with low and oscillatory shear (OS) and atheroprone gene expression is increased ¹. In contrast, the straight part of the arterial tree, which is exposed to sustained laminar blood flow with pulsatile shear (PS) and expresses atheroprotective genes, is generally spared from atherosclerotic lesions. Although there have been many studies on the mechanisms involved in mechanotransduction, there is a lack of information on the role of DNA methylation-mediated epigenetic regulation in modulating EC responses to the mechanical factors induced by different flow patterns.

Covalent methylation of DNA cytosine is the most widely studied epigenetic modification, which occurs almost exclusively in the context of CpG dinucleotides and often results in transcriptional repression of genes and noncoding genomic regions ². The importance of DNA methylation in maintaining normal development and biological functions is reflected by the influences of hyper- or hypo- methylation of DNA on many diseases. In cancer ², multiple sclerosis ³, Alzheimer's disease ⁴, systemic lupus erythematosus ⁵, diabetes ⁶, and cardiovascular diseases ^{7,8}, the causality roles for DNA methylation have been shown by the alterations of certain gene methylations and the disease-specific methylation landscapes. Understanding of the methylation status of EC genome, as well as EC-enriched genes can generate novel approaches for the intervention of cardiovascular diseases.

DNA methylation is mediated by DNA methyltransferases (DNMT), which constitute a family of enzymes that catalyze the transfer of a methyl group from *S-adenosyl* methionine to DNA. DNMT1 is abundant and essential for the maintenance of methylation patterns and chromatin silencing ². Aberrant expression and activation of DNMT1 have been observed in vasculature in pro-atherogenic conditions. For instance, rats fed with high-fat diet show an increase in vascular DNMT1 expression ⁹. Incubation with low-density lipoprotein cholesterol, a major risk factor for coronary heart disease, induces EC DNMT1 expression and activation ¹⁰. There is no report on the regulation of DNMT1 in vascular endothelium in response to shear stress with different flow patterns.

The goal of the present work is to study the hitherto poorly understood connection between DNA methylation and atheroprone hemodynamic forces applied to the vascular endothelium and to identify the key protein responsible for the epigenetic changes.

Materials and Methods

Cell culture and shear experiments

Human umbilical vein endothelial cells (ECs) within passages 5–7 were maintained in Medium 199 (Gibco, Grand Island, NY) supplemented with 2% fetal bovine serum (FBS) (Gibco). An *in vitro* parallel-plate circulating flow chamber was used to impose fluid shear stress to ECs cultured on collagen I (50 µg/mL), as described previously ¹¹. The shear stress (τ) generated on the ECs seeded on the membrane was estimated as $6Q\mu/wh^2$, where Q is flow rate and μ is perfusate viscosity. ECs were exposed to either pulsatile shear (PS, 12 ± 4 dynes/cm²) or oscillatory shear (OS, 0.5 ± 4 dynes/cm²).

Fluorescent immunocytochemistry

ECs on glass slides were fixed in 4% paraformaldehyde for 15 minutes, permeabilized with cold PBS containing 0.4% Triton X-100 for 10 minutes, and incubated with blocking buffer (10% donkey serum, 3% bovine serum albumin in PBS containing 0.1% Triton X-100) for 1 hour before incubation overnight with primary antibodies against 5-methylcytosine (5-meC) (Eurogentec) or DNMT1 (Santa Cruz Biotech). For 5-meC staining, the permeabilized cells were denatured with 2 N HCl and neutralized with 100 mM Tris-HCl (pH 8.5) before blocking. The cells were washed, incubated with secondary antibodies, and mounted in fluorescent mounting medium with DAPI. The slips were then visualized by epifluorescence microscopy.

Quantitative RT-PCR

RNA was extracted from ECs by using the TRIzol reagent (Life Technologies) according to manufacturer's instructions. Isolated RNAs were reversed-transcribed into complementary DNA with M-MLV RT system (Invitrogen) followed by Real-time PCR with the specific DNMT1 primer set (forward: 5'-cgagcgagccagagatagag-3'; reverse: 5'-gtcagatgcctgctgtgt-3'). The DNMT1 expression level was normalized against GAPDH.

Immuno-slot blot assay

Genomic DNA was purified from ECs with QIAamp DNA mini kit (Qiagen) according to manufacturer's instructions. DNA was denatured with 0.4 M NaOH, 10 mM EDTA, and then neutralized with 2 M ammonium acetate (pH 7.0). 2-fold dilutions of denatured DNA samples were spotted on a nitrocellulose membrane in a Bio-Dot SF apparatus (Bio-Rad). The membrane was baked at 80°C, blocked in 5% skimmed milk in TBS containing 0.1% Tween 20, and then incubated with 1:1000 dilution of 5-meC antibody overnight at 4°C, followed by its detection using secondary antibody coupled to horseradish peroxidase (Santa Cruz Biotech) and the ECL system (Amersham). For the staining of total DNA, the blot membrane was hybridized with 0.02% methylene blue in 0.3 M sodium acetate (pH 5.2).

Western blot

ECs were lysed in the RIPA lysis buffer: 25 mM HEPES, pH 7.4, 1% Triton X-100, 1% deoxycholate, 0.1% SDS, 125 mM NaCl, 5 mM EDTA, 50 mM NaF, protease inhibitor cocktail tablets (Roche). Equal amounts of protein were separated on SDS-PAGE, transferred to nitrocellulose membranes, blocked with 5% BSA-containing PBS, and incubated with the primary antibody against DNMT1 or β -actin (Santa Cruz Biotech), followed by detection using secondary antibody coupled to horseradish peroxidase (Santa Cruz Biotech) and the ECL system (Amersham).

Procedures for partial ligation of arteries and vessel harvesting

All animal studies were performed in accordance with National Institutes of Health guidelines and were approved by the UC San Diego IACUC. The surgical procedures are modified from the previous report^{12, 13}. Briefly, the rats were anesthetized with intraperitoneal ketamine (100 mg/kg body weight) and xylazine (10 mg/kg body weight). The left carotid bifurcation was exposed following a neck incision. Three branches (external

carotid, internal carotid, and occipital) of the left carotid artery were ligated with a 6-0 silk suture, and the superior thyroid artery was left intact. Sham-operation was performed as control. One-week post-operation, the animal was sacrificed and the vessels were perfused with a fixative (4% paraformaldehyde, PFA) under pressure (100 mmHg). The arteries were dissected out and further fixed by immersion in 4% PFA solution for 16 hours before being embedded in Tissue-Tek OCT compound and submerged into pre-chilled 2-methyl butane until frozen completely. Serial sectioning (10 μm /section) was performed in UCSD Histology Core facility. For the detection of 5-meC content and DNMT1 expression, the sections were fixed and permeabilized with cold acetone and then blocked with a blocking buffer (10% donkey serum, 3% bovine serum albumin in PBS containing 0.1% Triton X-100) for 1 hour, followed by incubation with antibodies against 5-meC and DNMT1 overnight at 4°C, and then with secondary antibodies at room temperature. For 5-meC staining, sections were denatured with 2 N HCl and neutralized with 100 mM Tris-HCl (pH 8.5) before blocking. After mounting, images were acquired with an epi-fluorescence microscope.

Statistical Analysis

Data are expressed as mean \pm SEM from three independent experiments. Statistical analysis was performed with Student's *t*-test for comparison between two groups of data.

Results

OS promotes DNA cytosine hypermethylation in ECs

To investigate the effects of hemodynamic forces on DNA methylation, the ECs cultured in the flow chamber were subjected to athero-protective PS (12 ± 4 dynes/cm²) and athero-prone OS (0.5 ± 4 dynes/cm²) for 24 hours, followed by immunostaining and slot blot using an antibody against 5-meC. OS caused a strong induction of 5-meC in EC nuclei (Fig 1A upper panel), whereas PS did not have much effect (Fig 1A lower panel). These results are confirmed by slot blot studies on DNA isolated from sheared EC (Fig 1B left panel), i.e., 5-meC levels are much stronger in ECs under OS than PS at lower DNA levels without signal saturation (Fig 1A). Methylene blue staining was used as control to demonstrate equal loading of DNA samples (Fig 1B right panel). These results reveal the differential regulations of DNA methylation under different flow patterns.

OS induces the expression of DNA methyltransferase 1

DNA methyltransferase 1 (DNMT1) is essential for the maintenance of DNA (cytosine) methylation patterns and chromatin silencing². Determination of DNMT1 expression by RT-PCR demonstrated that OS induced an increase of 1.89 ± 0.38 -fold in comparison to PS (Fig 2A). Since the subcellular functional compartment for DNMT1 is in the nuclei, we tested the localization of DNMT1 in ECs under different flow patterns. As shown in Fig 2B, OS caused a strong nuclear translocation of DNMT1 (upper panel); whereas under PS, DNMT1 is mainly located in EC cytosol (lower panel). These results show the differential regulations of DNMT1 expression and translocation to modulate DNA methylation in response to different flow patterns.

The OS-induced DNA methylation is mediated by DNMT1

Based on the results that the OS-induced DNMT1 expression and translocation are associated with DNA hypermethylation, we hypothesized that the elevated DNMT1 in EC under OS leads to the increase of the 5-meC. This hypothesis was tested by using a DNMT1-specific inhibitor 5-Aza-2'-deoxycytidine (5-Aza), which is an epigenetic modifier that inhibits DNA methyltransferase activity through proteasomal degradation of free (non-chromatin bound) DNMT1 to result in DNA demethylation (hypomethylation)¹⁴. ECs were pre-treated with 5-Aza (10 μ M) for three days prior to being subjected to shear experiments. After 24 hours of PS or OS, ECs were lysed and subjected to western blotting for the determination of DNMT1 expression, or fixed and subjected to immunostaining for the measurement of 5-meC levels. The results demonstrate that the 5-Aza treatment inhibited the OS-induced DNMT1 expression (Fig 3A) and essentially abolished the OS-induced DNA methylation (Fig 3B). These findings indicate that DNMT1 plays a significant role in regulating the OS-induced DNA methylation.

Flow disturbance increases DNMT1 and DNA methylation in rat carotid arteries

To validate our *in vitro* findings that DNMT1 and DNA methylation in ECs are differentially regulated by different flow patterns, we performed *in vivo* experiments on the flow-regulation of 5-meC and DNMT1 using a carotid partial-ligation model described by Korshunov et al^{12, 13}. The blood flow was reduced and disturbed in the partially ligated vessels in comparison with the control, as validated by ultrasonography (data not shown). One week post-surgery, the ligated and control common carotid arteries near the bifurcation were harvested, fixed, and sectioned, followed by immunostaining of 5-meC and DNMT1. The vascular cells were identified by DAPI staining of the nuclei. Stainings of 5-meC (yellow arrowheads in lower panel of Fig 4A) and DNMT1 (yellow arrowheads in lower panel of Fig 4B) were prominent in the nuclei of ECs (verified by vWF staining in Fig 4C) in the ligated vessels, but were relatively weak or undetectable in the control vessels (white arrowheads in upper panel of Fig 4A, and white arrowheads in upper panel of Fig 4B, respectively). These results demonstrate the differential regulation of DNA methylation in blood vessels subjected to athero-prone flow vs. athero-protective flow.

Discussion

The epigenetic mechanisms in cardiovascular pathophysiology have emerged as a major player interfacing genotype and phenotype variabilities. Three highly related epigenetic regulators, i.e., microRNAs (miRs), histone deacetylases (HDACs), and DNA methyltransferases (DNMTs), have been identified to coordinate the interactions between DNA sequences and transcription machinery in modulation of physiological functions and pathological disturbances. Others and we have previously reported that miRs^{15–19} and HDACs²⁰ play important roles in flow-regulation of EC functions. There is a lack of information of the role of DNA methylation and DNMTs in the flow regulation of vascular function. Here we report that OS *in vitro* and the disturbed flow generated by partial ligation of rat carotid artery *in vivo* induced DNA hypermethylation in the endothelium. DNA methylation and the other epigenetic regulators (miRs and HDACs) may form a network with reciprocal interconnections to modulate cellular functions.

There has been an increase in appreciation for the potential of treating diseases by modulating epigenetic pathways. Pharmacological reversal of aberrant methylation is a prospective therapeutical target through epigenetic reprogramming. DNA methyltransferase inhibitors such as 5-Aza can suppress tumor cell growth, invasion, metastasis, as well as tumor angiogenesis, *in vitro* and *in vivo* by demethylating and hence reactivating epigenetically silenced genes^{21–24}. A recent report showed that treatment of ECs with 5-Aza abrogated the LDL-induced downregulation of endothelial Kruppel-like Factor 2, a transcription factor vital to EC-dependent vascular homeostasis¹⁰. Together with our current finding that 5-Aza reverses DNA hypermethylation in ECs induced by the atheroprone OS, these results indicate that 5-Aza may be a beneficial therapeutic agent in normalizing vascular endothelial functions in pathophysiological conditions.

Among the few clinical investigations on the role of DNA methylation in cardiovascular diseases, including atherosclerosis, there are some seemingly contradictory observations. Global DNA hypomethylation was found in advanced atherosclerotic vessels and peripheral blood cells in humans, rabbit, and mice^{25–27}, and it has also been reported that CpG islands are hypomethylated in the arteries of patients with atherosclerosis, in comparison to control arteries²⁸. In contrast, the estrogen receptor β gene, which has essential roles in vascular function, was reported to be hypermethylated in coronary atherosclerotic tissues than their normal appearing arterial and venous tissues in the patients²⁹. It is possible that global hypomethylation may be predominant in the atherosclerotic arteries, whereas specific hypermethylation may occur only for certain atheroprotective genes. Since the atherosclerotic lesion includes multiple cell types, there is a need to dissect out the contributions of methylation in each cell type to the overall methylation status of the lesion. Moreover, it is important to understand the temporal modulations of global or gene-specific methylation during atherogenesis.

In order to elucidate the mechanisms of methylation changes observed in clinical setting, besides *in vitro* flow studies, the *in vivo* experiments with well-controlled diseased model were conducted. We have applied the partial carotid ligation model^{12, 13} that creates a low and oscillatory wall shear stress to study the effects of flow disturbance on DNA methylation in rat carotid arteries. The disturbed flow with low and oscillating shear stress led to a robust DNA hypermethylation and a prominent induction of DNMT1 expression in the EC nuclei of the partially ligated rat carotid arteries one week after ligation. Such changes in methylation were not seen in the control vessels with physiological pulsatile flow.

Our *in vivo* and *in vitro* results have demonstrated the differential regulation of DNMT1 and DNA hypermethylation by different hemodynamic forces and provided a novel molecular mechanism by which atheroprone hemodynamic forces act as a risk factor for atherosclerosis.

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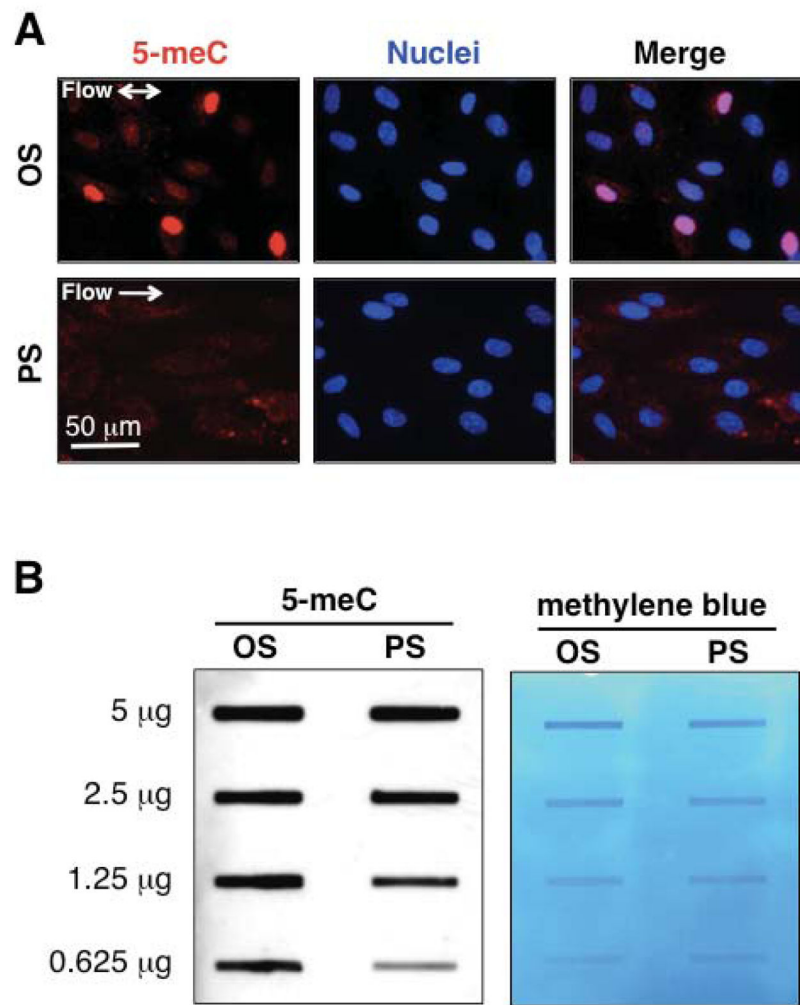


Figure 1. Oscillatory shear (OS) induces DNA hypermethylation in endothelial cells (ECs)
 ECs were exposed to OS (0.5 ± 4 dynes/cm²) or PS (12 ± 4 dynes/cm²) for 24 hr, and 5-meC level was determined by (A) immunofluorescent staining (images shown are representative from three independent experiments) and (B) Left: Immuno-slot blot using anti-5-meC. Right: The blot membrane was incubated with methylene blue to indicate equal loading of DNA.

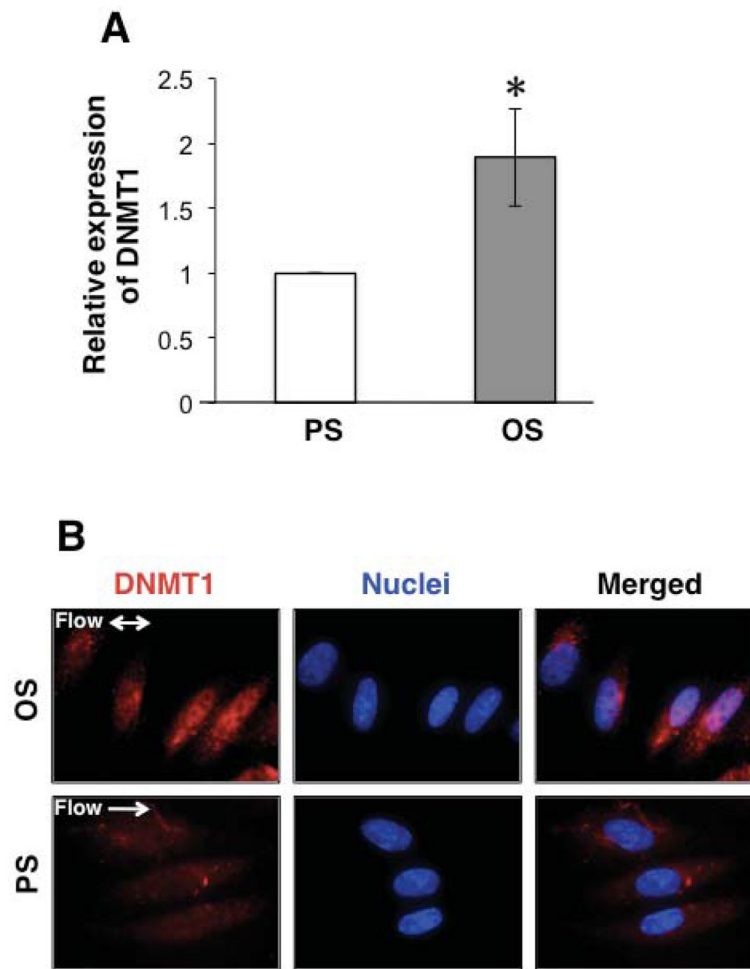


Figure 2. OS induces the expression and nuclear translocation of DNA methyltransferase 1 (DNMT1)

ECs were exposed to OS or PS for 24 hr. (A): Expression of DNMT1 was determined by quantitative RT-PCR. * $P < 0.05$ for OS vs PS. (B): Visualization of subcellular localization of DNMT1 by immunofluorescent staining. Images are the representatives from three independent experiments.

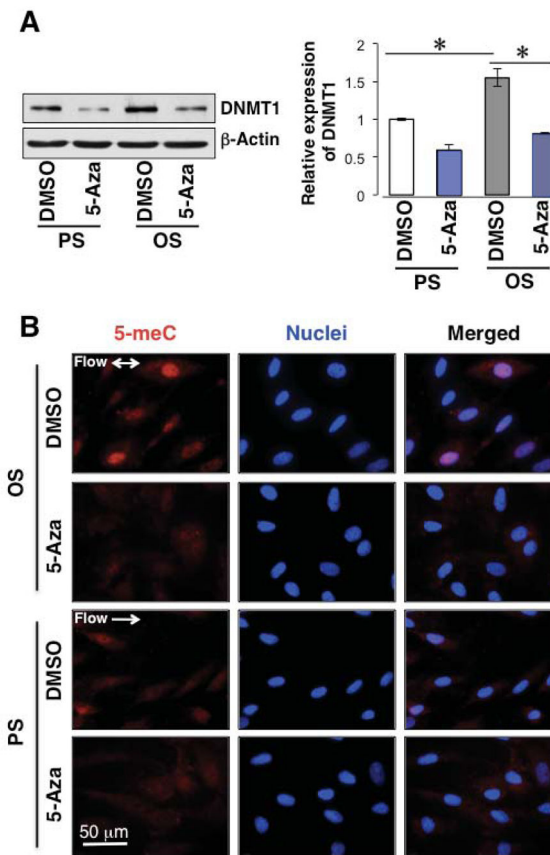


Figure 3. Inhibition of DNMT1 suppresses the OS-induced DNA hypermethylation

ECs were treated with 5-Aza-2'-deoxycytidine (5-Aza, 10 μ M) or DMSO, and then exposed to OS or PS for 24 hr. (A): DNMT1 expression in those cells was measured by Western blot and presented by representative images (left) and the bar graph (right). * P < 0.05 vs DMSO.

(B): 5-meC level was determined by immunofluorescent staining. Images are representatives from three independent experiments.

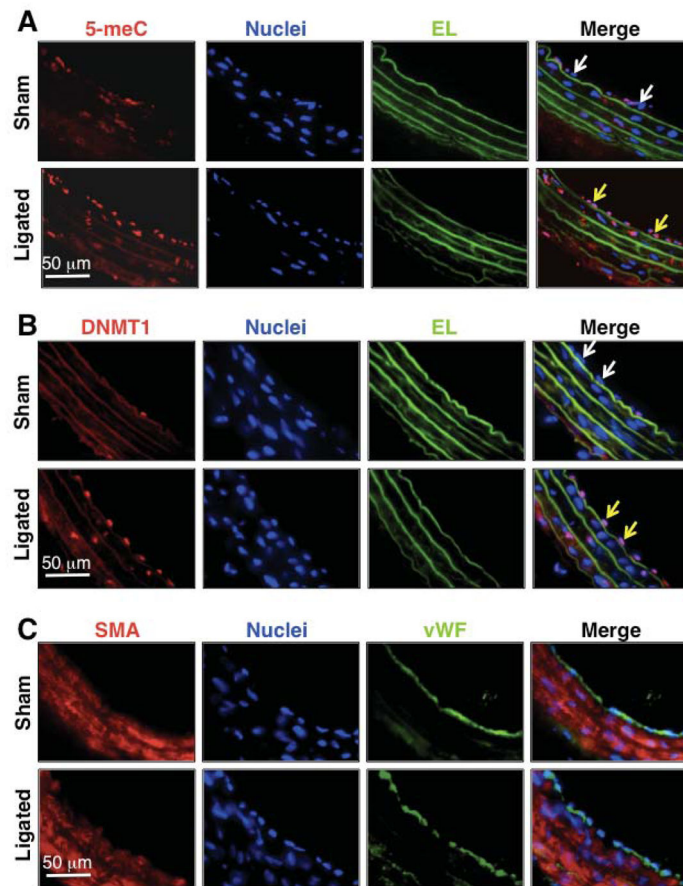


Figure 4. Flow disturbance increases DNA methylation and DNMT1 expression in rat carotid arteries

Rat carotid arteries were subjected to partial ligation or sham operation and then harvested 1 week post-operation. 5-meC (A) and DNMT1 (B) were determined in the arteries by immunofluorescent staining. ECs and smooth muscle cells were indicated by vWF and SMA (smooth muscle α -actin), respectively. EL: elastic lamina.