The Yin–Yang Dynamics of DNA Methylation Is the Key Regulator for Smooth Muscle Cell Phenotype Switch and Vascular Remodeling

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Objective—DNA methylation plays an important role in chronic diseases such as atherosclerosis, yet the mechanisms are poorly understood. The objective of our study is to indicate the regulatory mechanisms of DNA methylation in vascular smooth muscle cells (VSMCs) and its roles in atherosclerosis.

Approach and Results—In ApoE−/− mice fed a Western diet, DNA methyltransferase inhibitor, 5-aza-2′-deoxycytidine, significantly attenuated atherosclerotic lesions (20.1±2.2% versus 30.8±7.5%; P=0.016) and suppressed DNA methyltransferase activity and concomitantly decreased global 5-methylcytosine content in atherosclerotic lesions of ApoE−/− mice. Using a carotid ligation model, we found that 5-aza-2′-deoxycytidine also dramatically inhibited neointimal formation (intimal area: 2.25±0.14×10⁴ versus 4.07±0.22×10⁴ μm²; P<0.01). Abnormal methylation status at the promoter of ten–eleven translocation 2, one of the key demethylation enzymes in mammals, was ameliorated after 5-aza-2′-deoxycytidine treatment, which in turn caused an increase in global DNA hydroxymethylation and 5-hydroxymethylcytosine enrichment at the promoter of Myocardin. In vitro, 5-aza-2′-deoxycytidine treatment or DNA methyltransferase 1 knockdown decreased global 5-methylcytosine content and restored Myocardin expression in VSMCs induced by platelet-derived growth factor, thus preventing excessive VSMCs dedifferentiation, proliferation, and migration. Furthermore, DNA methyltransferase 1 binds to ten–eleven translocation 2 promoter and is required for ten–eleven translocation 2 methylation in VSMCs.

Conclusions—The inhibitory effects of DNA demethylation on global 5-methylcytosine content and ten–eleven translocation 2 hypermethylation in atherosclerotic aorta can recover 5-hydroxymethylcytosine enrichment at the Myocardin promoter and prevent VSMC dedifferentiation and vascular remodeling.

Key Words: aorta ■ atherosclerosis ■ DNAmethylation ■ platelet-derived growth factor ■ vascular remodeling

Atherosclerosis is a chronic and multifactorial disease mediated by complex interplay between resident endothelial cells, vascular smooth muscle cells (VSMCs), and infiltrating macrophages. Environmental exposure to risk factors such as hyperlipidemia throughout the development of atherosclerosis causes vascular remodeling and in turn reduces arterial compliance. Although many drugs inhibiting vascular remodeling and metabolic disorder offer an effective therapeutic strategy for preventing atherosclerotic progression, none of these drugs are found to totally reverse atherosclerotic plaque in animal experiments. Furthermore, clinical trials have revealed that intensive lipid-lowering treatment with rosuvastatin or atorvastatin only leads to limited plaque regression, and in some cases, plaque progression. Environmental exposure to risk factors such as hyperlipidemia throughout the development of atherosclerosis causes vascular remodeling and in turn reduces arterial compliance. Although many drugs inhibiting vascular remodeling and metabolic disorder offer an effective therapeutic strategy for preventing atherosclerotic progression, none of these drugs are found to totally reverse atherosclerotic plaque in animal experiments. Furthermore, clinical trials have revealed that intensive lipid-lowering treatment with rosuvastatin or atorvastatin only leads to limited plaque regression, and in some cases, plaque progression.
proven to counteract the depressed expression of methylation genes and is currently approved for treatment of meyloplastic syndrome.11–14 Interestingly, physiological demethylation also exists in vivo to balance the silencing effect on gene expression by DNA methylation. Even though no direct DNA demethylases were identified, current studies showed that 5-mC can be oxidized to 5-hydroxymethylcytosine (5-hmC) by ten–eleven translocation (Tet) family of enzymes.15,16 Through the base excision repair pathway, 5-hmC is then converted to unmethylated cytosine, leading to demethylation and gene reactivation.17–19

Previous studies had demonstrated that atherosclerosis occurred in familial aggregation, suggesting the role of genetic factors in atherogenesis.20 Recent evidence supported an association between DNA methylation and cardiovascular diseases, such as atherosclerosis and vascular remodeling.21–22 Dunn et al23 uncovered that inhibition of DNA methylation attenuated atherosclerotic burden through ameliorating endothelial dysfunction and restoring mechano-sensitive endothelial gene expression induced by oscillatory shear stress. On the other hand, several studies suggested that DNA methylation might participate in regulating VSMC phenotype and vascular remodeling.24 Liu et al25 reported a significant reduction in Tet2 expression in the neointima or coronary atherosclerotic plaques. Loss of Tet2 suppressed 5-hmC enrichment and affected the expression of VSMC differentiation marker genes, which in turn aggravated vascular remodeling. It is, thus, important to determine the role of DNA methylation changes in VSMCs and vascular remodeling.

In this study, we sought to study the DNA methylation state in different stages of atherosclerosis. We found that DNA methylation balance regulated by DNMT1 and Tet2 influenced VSMCs phenotype and vascular remodeling.

Materials and Methods
Materials and Methods are available in the online-only Data Supplement.

Results
DNMT Inhibitor Attenuated Atherosclerosis in ApoE−/− Mice
Because 5-aza has been proven to inhibit DNA methylation, we administrated 5-aza to ApoE−/− mice to confirm the effect of DNA demethylation on atherosclerosis. At first, we found that body weights were comparable at the indicated time points between vehicle- and 5-aza-treated group, indicating that drug treatment was well tolerated (Figure I in the online-only Data Supplement). In addition, 5-aza treatment for 12 weeks had no remarkable effect on the plasma levels of lipid profiles and fasting glucose (Figure II in the online-only Data Supplement). We then examined the effect of 5-aza on atherosclerosis. After 18 weeks exposure of 5-aza, atherosclerotic lesion area was significantly attenuated when compared with that in vehicle-treated ApoE−/− mice as demonstrated by en face Oil red O-positive area (20.1±2.2% versus 30.8±7.5%; P=0.016; Figure IA and IB) and by quantification of lesion area at the aortic root (Figure IC and ID).

We further examined whether 5-aza affected plaque composition. Masson staining demonstrated that necrotic core was smaller in 5-aza-treated ApoE−/− mice compared with vehicle-treated ApoE−/− mice (22.0±4.2% versus 50.8±4.5%; P<0.01; Figure 1E through 1G). As shown in Figure 1E and IF, 5-aza significantly reduced plaque macrophage content by >50% compared with vehicle-treated group (1.1±0.1% versus 2.8±0.2%; P<0.01). The relative contents of α-smooth muscle actin were similar between vehicle- and 5-aza-treated groups (Figure 1H and 1I). However, the distribution of α-smooth muscle actin staining was diffuse in vehicle-treated ApoE−/− mice, which was restored in 5-aza-treated ApoE−/− mice (Figure 1J and 1K).

DNMT Inhibition Dynamically Regulated DNA Methylation and Tet2 Expression in Atherosclerotic Aorta
Because epigenetic drift occurred during aging of certain tissue types, we next evaluated global methylation and hydroxymethylation levels in the aorta of ApoE−/− mice at different ages. In line with the findings derived from other tissues, we found that global DNA methylation, as opposed to global DNA hydroxymethylation, was gradually decreased with age both in aorta DNA of wild-type and ApoE−/− mice, which were fed with Western diet (Figure 2A and 2B).

Because DNMT1, DNMT3a, and DNMT3b are the principal mediators of DNA methylation in mammalian cells,26,27 we determined the expression of DNMT proteins in atherosclerotic plaques. In the serial sections of aortic sinus stained with α-smooth muscle actin or F4/80 and immunofluorescence images, we found that DNMT1 was predominantly expressed in VSMCs, rather than in macrophages, in the plaque of ApoE−/− mice (Figure 2C and 2D). DNMT3a was equivalently expressed in VSMCs and macrophages in plaque, whereas DNMT3b was undetectable in the cross sections from wild-type and ApoE−/− mice (Figure 2C).

The inhibitory function of 5-aza depends on trapping DNMT1 in a covalent complex with DNA and also preferentially targets DNMT1 via ubiquitin-dependent proteasomal degradation.13 The protein expression of DNMT1 and DNMT3a was higher in vehicle-treated ApoE−/− mice when compared with wild-type mice but was not affected by 5-aza treatment (Figure 2E and 2F). However, DNMT3b was expressed in low level in 3 groups but rich in mouse heart (Figure 2E). This intriguing phenomenon prompted us to investigate the consequence of the enzyme activity of DNMT3b.
after 5-aza treatment. As shown in Figure 2G, the aortas dissected from vehicle-treated ApoE−/− mice had higher enzyme activities than the wild-type samples. Administration of 5-aza in turn suppressed the excessive DNMT activity by 2.76-fold. In keeping with the changes of DNMT activity, 5-mC content was significantly increased in vehicle-treated ApoE−/− mice but declined close to normal level when treated with 5-aza (Figure 2H). Collectively, these results suggested that the effect of 5-aza on DNA demethylation of the aorta containing atherosclerotic plaques was mediated via suppression of DNMT enzyme activity.

Given that Tet2 was the predominant DNA demethylation enzyme in arteries,24 we firstly investigated the expression of Tet2 among 3 groups. Using our panel of mouse aorta samples, we tested the abundance of RNA transcripts and protein. As shown in Figure 2I and 2J, the expression of Tet2 was significantly reduced in the aorta of vehicle-treated ApoE−/− mice compared with wild-type group and restored after administration of 5-aza, which was inversely associated with DNMT activity.

**Decreased Expression of Tet2 Attributed to Excessive Promoter Methylation**

To clarify the underlying mechanisms of Tet2 expression, we applied methylation-specific polymerase chain reaction and pyrosequence to quantify methylation percentage at target CpG sites in the promoter of Tet2. The locations of Tet2 promoter and CpG islands were shown in Figure 3A. It was reported that the fragment containing high-density CpG sites within the promoter significantly correlated with transcription repression of downstream genes. Thus, we first screened the promoter region of Tet2. Within these fragments, we performed methylation-specific polymerase chain reaction to determine the methylation status and found that the methylation level of fragment 2 was higher in the aorta of vehicle-treated ApoE−/− mice than other 2 groups (Figure 3B). Using pyrosequencing, we further confirmed that the single methylation percentages at 7 of 8 target CpG sites, as well as the average methylation percentages of all sequencing CpG sites, were markedly elevated in vehicle-treated ApoE−/− group than those in wild-type and 5-aza-treated

![Figure 1. DNA methyltransferase (DNMT) inhibitor 5-aza-2′-deoxycytidine (5-aza) suppresses atherosclerosis. A and B, En face plaque quantification of aortic arch and thoracic aorta stained with Oil red O. Values are mean±SD from 5 animals in each group. C and D, Sections and quantification of aortic sinus stained by Oil red O. Scale bar=500 μm. Values are mean±SD from 4 animals in each group. E–G, Representative images of Masson staining and quantification of the percentage of necrotic core in the plaques with and without 5-aza treatment. Values are mean±SD from 4 animals in each group. H and I, Representative images and quantification of macrophage immunostaining of the aortic roots with and without 5-aza treatment. Scale bar=500 μm. Values are mean±SD from 4 animals in each group. J and K, Representative images and quantification of α-smooth muscle actin (α-SMA) immunostaining of the aortic roots with and without 5-aza treatment. Scale bar=500 μm. Values are mean±SD from 4 animals in each group. *P<0.05 vs wild-type (WT) group; #P<0.05 vs vehicle group.](http://arch.ahajournals.org/)

![Figure 2. Changes of DNMT activity and 5-mC content after 5-aza treatment. As shown in Figure 2G, the aortas dissected from vehicle-treated ApoE−/− mice had higher enzyme activities than the wild-type samples. Administration of 5-aza in turn suppressed the excessive DNMT activity by 2.76-fold. In keeping with the changes of DNMT activity, 5-mC content was significantly increased in vehicle-treated ApoE−/− mice but declined close to normal level when treated with 5-aza (Figure 2H). Collectively, these results suggested that the effect of 5-aza on DNA demethylation of the aorta containing atherosclerotic plaques was mediated via suppression of DNMT enzyme activity.](http://arch.ahajournals.org/)
Dynamic changes in global DNA methylation and hydroxymethylation and 5-aza-2'-deoxycytidine (5-aza) inhibit DNA methyltransferase (DNMT) enzyme activity and global 5-methylcytosine (5-mC) content. A. Quantification of 5-mC content in aortas at indicated ages of wild-type (WT) and ApoE−/− mice. ApoE−/− mice were fed a high-fat diet containing 16.6% fat, 10.6% sucrose, and (Continued)
groups (Figure 3C and 3D; Figure III in the online-only Data Supplement). Moreover, compared with unmethylated vector, the methylated vector containing the fragment of Tet2 promoter suppressed luciferase activity by 56.5% in A7r5 which is a rat smooth muscle cell line (Figure 3E), reflecting that abnormal methylation status of Tet2 would repress its promoter activity and mRNA expression.

DNMT Inhibitor Resulted in 5-hmC Enrichment at the Myocardin Promoter

Given that Tet2 was required for conversion from 5-mc to 5-hmc, the interim cytosine to unmethylated cytosine, so we analyzed global 5-hmC content and gene-specific hydroxymethylation. As shown in Figure 4A, global hydroxymethylation level was dramatically lower in vehicle-treated mice compared with other 2 groups. Because Liu et al reported that knockdown of Tet2 caused a cascade of epigenetic changes in VSMC phenotypic genes, we wished to address the issue of whether demethylation of Tet2 promoter after 5-aza treatment mediated the methylation and hydroxymethylation status at the promoters of these genes. Gene-specific measurement of hydroxymethylation was determined by quantitative polymerase chain reaction using primers predesignated to the promoter region of Myocardin, Krüppel-like factor 4, and serum response factor (SRF). Compared with vehicle group, 5-aza treatment inhibited the methylation level at the Myocardin promoter with concomitant recruitment of 5-hmC in the corresponding region of Myocardin promoter (Figure 4B and 4C). Accordingly, the mRNA expression of Myocardin was repressed in the aorta of vehicle-treated ApoE<sup>−/−</sup> mice but significantly reversed by 5-aza (Figure 4D). On the contrary, the 5-mc and 5-hmC contents at the indicated regions of Krüppel-like factor 4 and SRF promoters were not altered among 3 groups (Figure IV in the online-only Data Supplement). In agreement with the methylation and hydroxymethylation status of Krüppel-like factor 4 and SRF promoter, the mRNA expression of Krüppel-like factor 4 and SRF remained unchanged among 3 groups (Figure IV in the online-only Data Supplement).

DNMT1 Regulated VSMCs Proliferation, Migration, and Phenotype Switching

The altered Myocardin methylation in plaque implied that DNA hypermethylation may affect VSMC marker genes expression and impair VSMC function during atherosclerotic development. Inspired by the finding and hypothesis, we then determined the role of 5-aza on VSMC function in vitro. It was known that VSMCs proliferation and migration into the intima were imperative steps for atherosclerosis and restenosis. As an initial step, we examined the effect of 5-aza on DNMT activity and global 5-mC content in rat aortic smooth muscle cells (RASMCs). RASMCs were treated with 1 and 5 μmol/L of 5-aza, which did not affect cell viability (Figure V in the online-only Data Supplement). Although administration of 5-aza did not affect the expression of DNMT1 and DNMT3a stimulated by platelet-derived growth factor (PDGF), the DNMT activity was profoundly repressed by 5-aza treatment (Figure 5A through 5D). Accordingly, incremental 5-mC content induced by PDGF was decreased after 5-aza treatment (Figure 5E). We next determined the role of 5-aza in the proliferation and migration of RASMCs. Scratch assays were performed to evaluate the role of 5-aza in RASMCs migration. As shown in Figure 5F and 5G, administration of 5-aza for 3 days significantly suppressed PDGF-induced migration of RASMCs. As shown in Figure 5H, treatment of RASMCs with 5-aza significantly inhibited PDGF-induced proliferation in a time- and dose-dependent manner.

Our in vivo observation had displayed a significant reduction of Myocardin expression in the aortas of ApoE<sup>−/−</sup> mice after 5-aza treatment. Thus, we next studied the effect of 5-aza on VSMC phenotype modulation. After treatment of RASMCs with 5-aza for ≤5 days, the mRNA expression of VSMC differentiation marker genes, such as smooth muscle α-actin, smooth muscle-myosin heavy chain, and Myocardin, was markedly reversed 1.5-fold to 4-fold when compared with the decreased expression levels induced by PDGF (Figure 5I). Accordingly, the protein expression of these genes was restored by 5-aza treatment (Figure 5J). Moreover, the morphological analysis demonstrated that PDGF induced a flattened shape and a moderately larger size that reflected a dedifferentiated state of VSMCs. In contrast, administration of 5-aza kept VSMCs in a spindle-like shape that reflected a differentiated state (Figure 5K). We then measured the cell surface area among 3 groups, finding that much larger VSMCs in PDGF-treated group compared with vehicle group and smaller VSMCs in 5-aza-treated group compared with PDGF-treated group, respectively (Figure 5L). Together, these data demonstrated that inhibition of DNA methylation retarded VSMCs proliferation and migration through suppression of VSMC plasticity.
Effects of DNMT Inhibitor on Neointimal Formation

Even though 5-aza could inhibit VSMC proliferation, migration, and dedifferentiation in vitro, it was still required to validate the effect of 5-aza on VSMC functions and vascular remodeling in vivo. For this purpose, complete ligation of common carotid artery was performed to induce extensive neointimal formation in the presence of an intact endothelial lining. First and foremost, we determined the expression levels of DNMT1 and DNMT3a in vitro. DNMT1 was highly expressed in RASMC but almost undetectable in macrophages, whereas DNMT3a was comparably expressed in RASMC and macrophages.

Figure 3. 5-aza-2′-deoxycytidine (5-aza) reverses ten–eleven translocation 2 (Tet2) hypermethylation and recovers its expression in atherosclerotic aorta. A, Schematic diagram of Tet2 promoter. Upper, CG percentages within the target region of Tet2 promoter. Blue area shows cytidine phosphate guanosine (CpG) island coverage within this region. Black pillars show CpG sites detected by methylation-specific polymerase chain reaction (MSP), whereas red pillars show CpG sites detected by pyrosequencing. Green and yellow arrows indicate the primer pairs of MSP and pyrosequencing spanning fragment 2, respectively. B, The methylation status of fragment 1 and fragment 2 within Tet2 promoter in aorta among 3 groups is determined by MSP. Normal aorta DNA was treated with methylase SssI as a positive control (PC). The methylation status of PCs (2 lanes) and samples are represented. C, Illustrative CpG site methylation levels for 3 groups at the target Tet2 promoter measured by pyrosequencing. Fully methylated CpG site is colored by red, whereas unmethylated CpG site is colored by white. D, Quantification of methylation percentages from all measured CpG sites by pyrosequencing. Data are mean±SD. *P<0.05. E, Relative luciferase activity of pGL3-basic containing Ethylated (M) Tet2 fragments. Each experiment was performed in triplicate. M or unmethylated (U) Tet2 fragments is measured by dual-luciferase reporter assay and normalized with an internal pGL-TK Renilla activity. *P<0.05. TSS indicates transcription start site; and WT, wild type.
DNMT1 was widely expressed in the medial and neointimal layers, whereas DNMT3a was predominantly localized in the neointimal layer (Figure 6B). In contrast, DNMT1 and DNMT3a were not yet observed in the sham arteries. The stimulation of DNMT1 and DNMT3a in response to vascular injury promoted us to gain further insight into the effect of DNA demethylation in neointimal formation. Vehicle or 5-aza (1 mg/kg) was intraperitoneally injected daily for 3 weeks after carotid ligation. We did not find any death in 5-aza-treated group. The body weights between saline- and 5-aza-treated groups were also comparable (19.2±0.8 versus 18.6±0.9 g; \( P = 0.39 \)). After harvesting the carotid arteries, we found that 5-aza substantially inhibited neointima formation (Figure 6C), which was associated with a decrease in both neointimal area (2.25±0.14×10^4 versus 4.07±0.22×10^4 μm^2; \( P < 0.01 \); Figure 6D) and the intima-to-media ratio (0.20±0.02 versus 0.44±0.03; \( P < 0.01 \); Figure 6E). Likewise, immunohistochemistry staining and quantitative analysis showed a 4-fold increase in the proportion of Tet2-positive cells within the neointimal layer of 5-aza-treated group (Figure 6F and 6G).

Knockdown of DNMT1 Suppressed VSMCs Proliferation, Migration, and Dedifferentiation

We next sought to determine which isoforms of DNMT enzymes were involved in VSMC differentiation, proliferation, and migration. First, we confirmed the small interfering RNA (siRNA) inhibitory efficiency because the expression of DNMT1 and DNMT3a were downregulated by 63.4% and 59.9%, respectively, in protein level (Figure 7A). Indeed, when compared with DNMT3a, the knockdown of DNMT1 more efficiently abolished DNMT enzyme activities along with 5-mC content (5.6±2.7% versus 13.6±5.7%; \( P = 0.042 \); Figure VI in the online-only Data Supplement). We then examined whether DNMT1 or DNMT3a specifically mediated VSMC proliferation and migration. PDGF-induced proliferation was significantly inhibited by siDNMT1 but not siDNMT3a (Figure 7B; Figure VIIA in the online-only Data Supplement). Likewise, DNMT1 knockdown dramatically attenuated VSMCs migration stimulated by PDGF (Figure 7C and 7D). Nevertheless, no significant changes were observed in VSMC migration when treated by DNMT3a siRNA (Figure VIIB and VIIC in the online-only Data Supplement). Furthermore, the elevated expression levels of VSMCs dedifferentiation marker genes induced by PDGF were decreased after transfection with DNMT1-specific siRNA for 48 hours (Figure 7E). Nonetheless, DNMT3a siRNA had no impact on PDGF-induced VSMCs differentiation (Figure VIIID in the online-only Data Supplement).

Depletion of DNMT1 Restored Tet2 Expression and Suppressed Tet2 Methylation

We further investigate the effect of 5-aza and DNMT1 on Tet2 observed in mice model. Although PDGF inhibited Tet2 expression in a time-dependent manner, the administration of 5-aza for 3 and 5 days significantly restored Tet2 expression in RASMCs (\( P = 0.013 \) and \( P = 0.004 \), respectively; Figure 8A). The methylation levels of Tet2 promoter in RASMCs were then evaluated by quantitative methylation-specific polymerase chain reaction. Consistent with increased Tet2 expression after incubation with 5-aza, the methylation levels of Tet2 promoter were suppressed by 5-day treatment of 5-aza (Figure 8B).

Inspired by the role of 5-aza on the inhibition of 5-mC content and Tet2 expression, we further discussed the reciprocal effect between DNMT1 and Tet2. Although depletion of DNMT1 increased Tet2 expression (Figure 8C), knockdown of Tet2 did not conversely affected DNMT1 expression (Figure 8D). Likewise, when compared with control siRNA,
Figure 5. 5-aza-2'-deoxycytidine (5-aza) decreases vascular smooth muscle cell (VSMC) proliferation, migration, and dedifferentiation. A. Rat aortic smooth muscle cells (RASMCs) were cultured in serum-free medium for 24 h, then pretreated with 5-aza (5 μmol/L) for 24 h, and followed with platelet-derived growth factor (PDGF; 20 ng/mL) incubation for 0.5, 1, 3, and 5 d. Western blot analyses (Continued)
Discussion

In this study, we showed that DNA methylation was increased, whereas DNA hydroxymethylation, 1 intermediate form for demethylation, was decreased in atherosclerotic aorta. The increased global methylation could be explained by the enhanced activity of DNMT1 and reduced expression of Tet2. However, administration with 5-aza would restore aberrant hypermethylation both in global level and within the promoter of Tet2. As a consequence, 5-hmC was recaptured within the promoter of Myocardin, which promoted differentiation of VSMCs and inhibited the vascular remodeling (Figure IX in the online-only Data Supplement). Accordingly, knockdown of Tet2 also activated the suppression of RASMC dedifferentiation obtained from 5-aza treatment (Figure VIIIId in the online-only Data Supplement).

DNA methylation significantly repressed the methylation levels of Tet2 promoter induced by PDGF for 5 days in RASMCs (Figure 8E). Considering the effect of DNMT1 on Tet2 expression, we applied chromatin immunoprecipitation assays to test whether DNMT1 binds to Tet2 promoter and found that DNMT1 recognized and bound to the second fragment at Tet2 promoter (Figure 8F and 8G). More importantly, PDGF incubation for 3 days promoted DNMT1 binding to Tet2 promoter when compared with phosphate buffer saline group (P=0.017; Figure 8H and 8I). Compared with pretreatment with control siRNA, the 5-aza-induced suppression of RASMC proliferation and migration was partly counteracted by pretreatment with Tet2 siRNA (Figure VIIIa through VIIIc in the online-only Data Supplement). Accordingly, knockdown of Tet2 also activated the suppression of RASMC dedifferentiation obtained from 5-aza treatment (Figure VIIIId in the online-only Data Supplement).

The clinical relevance of DNA methylation firstly became apparent in relation to cancer. Then the abnormal DNA methylation was also found associated with other diseases such as Rett syndrome, immune deficiency, and so on. Some changes of DNA methylation can be a physiological response to environmental changes, whereas others might be associated with a pathological process such as cell phenotype change or even malignant transformation. VSMCs can revert to a synthetic phenotype featuring highly proliferative and migratory activity and increased synthesis of inflammatory factors. We find that DNMT activities differ along with atherosclerotic progression, although DNMT1 and Tet2 influence the DNA methylation equilibrium and SMC phenotype. Furthermore, to preclude other confounding factors, we apply a carotid artery ligation model in which abnormalities in VSMCs are primarily responsible for vascular remodeling. The observation that 5-aza treatment inhibits neointimal formation confirms our assumption that 5-aza inhibits vascular remodeling via DNA demethylation in VSMCs.

As reviewed elsewhere, 2 pathways involve the DNA demethylation. One is passive demethylation, which is largely acquired by the inhibition of DNMTs activity. On the other side, the process of active conversion and removal of 5-mC is referred to as active demethylation. About the latter, mounting evidence has accumulated that Tet enzyme family serves as keys for the conversion of 5-mC to 5-hmC. Together with the established pharmacological action of 5-aza and our current finding, the effect of 5-aza on Tet2 expression could be explained by the mechanism that 5-aza is recruited into the hypermethylated fragments, forming covalent bonds with DNMT1 and causing DNMT1 enzyme inactivation. As a consequence of 5-aza incorporation, DNMT1 fails to combine with Tet2 promoter and maintain Tet2 methylation, eventually resulting in the recovery of Tet2 expression and the elevation of global 5-hmC levels. These results suggest that DNMT inhibitor, 5-aza, reverses DNA hypermethylation in atherosclerotic aorta of ApoE−/− mice not only through inhibition of DNMT activity and traditional passive demethylation but also indirectly through recovery of Tet2 expression and active demethylation. However, based on analyses of recent evidence from our and other laboratories, we could not postulate which pathway predominates after 5-aza treatment.

DNA demethylation regulated by the Tet family is important for physiology and pathology. The decreased expression of Tet2 has been shown in human atherosclerosis. The Tet2 hypermethylation observed in our study emerges as another acceptable mechanism to explain persistently reduced Tet2 expression in atherosclerosis. In addition, Liu et al documents that induction of Tet2 in VSMCs is capable of converting 5-mC to 5-hmC at promoters of smooth muscle-myosin heavy chain, SRF, and Myocardin and lifts the repression of gene expression. Conversely, our study finds that the Myocardin is the major transcription factor regulated by DNA methylation in vivo because we only observe that a decrease in 5-mC is concomitant with an increase in 5-hmC at promoter of Myocardin after 5-aza treatment, although smooth muscle-myosin heavy chain and SRF are not found similar changes in atherosclerotic aorta.

Another goal of our study is to identify specific DNMT enzyme involved in atherosclerosis for subsequent functional analysis. In mammalian cells, DNMTs have 3
isoforms: DNMT1, DNMT3a, and DNMT3b. DNMT3a and DNMT3b mediate de novo DNA methylation, whereas DNMT1 acts on newly synthesized DNA to maintain methylation marks. Although both DNMT1 and DNMT3a localize within plaques and are upregulated after vascular injury or PDGF treatment, 5-mC content is decreased exclusively through knockdown of DNMT1 but not affected by knockdown of DNMT3a. In accordance with in vivo observations, 5-aza treatment or DNMT1 silencing by siRNA recovers Myocardin expression, thereby inhibiting VSMC

Figure 6. Inhibitory effect of 5-aza-2′-deoxycytidine (5-aza) on vascular remodeling after carotid ligation. A, Comparison of DNA methyltransferase 1 (DNMT1) and DNMT3a expression in rat aortic smooth muscle cells (RASMCs) and mouse macrophage RAW264.7 cells. B, Representative immunostaining of DNMT1 and DNMT3a in sham and ligated carotid artery. Scale bar=20 μm. Arrowheads show strong expression of DNMT1 and DNMT3a in ligated carotids. Immunohistochemical staining of normal IgG is presented as negative control experiments. C, Photomicrographs showing representative cross-sectional areas of ligated carotid artery from C57BL/6 mice with and without administration of 5-aza. Scale bar=20 μm. D and E, Comparison of intima area and intima/media (I/M) ratio in C57BL/6 mice with and without 5-aza treatment. Values are mean±SD from 4 animals in each group. *P<0.05. F, Representative immunohistochemistry images of cross-sectional areas of ligated carotids via anti-ten–eleven translocation (Tet2) antibody. Arrowheads show strong expression of Tet2 in carotid arteries. Immunohistochemical staining of normal IgG is presented as negative control experiments. G, Comparison of the percentage of Tet2-positive cells in the carotid arteries between C57BL/6 mice with and without 5-aza treatment. * P<0.05.
dedifferentiation, proliferation, and migration. Nevertheless, it is worth noting that DNMT3a, but not DNMT1, is shown to be associated with oxidized-low-density lipoprotein–induced inflammation and hypoxia-induced matrix damage in VSMCs in a previous study. Nevertheless, our study focuses only on VSMC phenotype modulation induced by PDGF. The difference with our study suggests that each DNMT is regulated by specific stimuli and plays different roles in the process of atherosclerosis. Nonetheless, because knockdown of DNMT3a mediated by siDNMT3a only partially inhibits DNMT3a expression, we could not conclusively preclude the role of DNMT3a on VSMC functions.

The other limitation of our study is that we cannot totally exclude the possibility that 5-aza may alter the DNA methylation of other relevant cell types for vascular remodeling such as endothelial cells. However, the in vitro experiments in SMCs confirm that the methylation condition will affect SMC phenotype definitely.

Strong evidence and our findings consistently indicate that drifts in global DNA methylation concur with aging. Although 5-hmC comes from 5-mC and acts as an intermediate during active demethylation, 5-hmC levels fluctuate during mouse preimplantation development and vary from tissue to tissue. In this regard, our work uncovered that 5-hmC accumulated, coinciding with a reduction in 5-mC, in normal aorta but remained unchanged in atherosclerotic aorta. This discrepancy could be attributed to Tet2 hypermethylation and inactivation in atherosclerotic aorta, which ultimately limited 5-hmC conversion. About gene-specific methylation, our results demonstrate that Tet2 hypermethylation is secondary to atherosclerosis, although most of previous studies looking across the genome suggest that disease-associated DNA methylation could arise before cancer onset.
Figure 8. DNA methyltransferase (DNMT1) binds to ten–eleven translocation 2 (Tet2) promoter and is required for the maintenance of Tet2 methylation. A, Rat aortic smooth muscle cells (RASMCs) were cultured in serum-free medium for 24 h, then pretreated with 5-aza-2′-deoxycytidine (5-aza; 5 μmol/L) for 24 h, and followed with platelet-derived growth factor (PDGF; 20 ng/mL) incubation for 0.5, 1, 3, and 5 d. Western blot analyses and quantitative results showed the expression of Tet2 in RASMCs at indicated time points. B, Quantitative methylation-specific polymerase chain reaction (PCR) showed the methylation levels of Tet2 promoter in response to 5-aza (5 μmol/L) treatment at indicated time points. *P<0.05 vs PDGF treatment group at the same time point. C and D, RASMCs were transfected with DNMT1 or Tet2 small interfering RNA (siRNA) followed with PDGF treatment (20 ng/mL), and the protein expression of DNMT1 and Tet2 was examined by Western blot. *P<0.05. E, RASMCs were transfected with DNMT1 siRNA followed with PDGF treatment (20 ng/mL), and quantitative methylation-specific PCR was performed to determine the methylation levels of Tet2 promoter. *P<0.05 vs control siRNA plus PDGF-treated group at the same time point. F and G, Chromatin immunoprecipitation (ChIP) assays were performed (Continued)
conceivable that prolonged environmental exposure initiates and exacerbates atherosclerosis, as well as Tet2 methylation.

**Conclusions**

DNMT inhibition prevents VSMC dedifferentiation, proliferation, and migration in vitro and eventually retards vascular remodeling and atherosclerosis. The underlying mechanisms depend on a passive demethylation pathway via suppression of global 5-mC content and Tet2 hypomethylation, which in turn initiates an active demethylation pathway, characterized as enrichment of 5-hmC in the Myocardin promoter. Therefore, stringent control of the yin–yang equilibrium of DNA methylation is critical to VSMC phenotypic modulation and artery protection.

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**Disclosures**

None.

**References**


**Highlights**

- DNA methylation dynamic changes would affect cells phenotypes and lead to physiological and pathological changes.
- In this study, we identify that DNA methylation influences vascular smooth muscle cell phenotype switch and vascular remodeling.
- In addition, we also indicate how the DNA methylation equilibrium is regulated from methylation enzyme, DNA methyltransferase, and demethylation, ten–eleven translocation 2.
- Mechanistic studies show that DNA methyltransferase 1 binds to ten–eleven translocation 2 promoter and is required for ten–eleven translocation 2 methylation, which in turn affects global 5- methylcytosine content, 5- hydroxymethylcytosine content, and the methylation status at Myocardin promoter as well.
- The yin–yang dynamics of DNA methylation suggests a new pathogenic mechanism of atherosclerosis, which will contribute to the development of effective therapy.
- In addition, it also provides insights into the understanding of complex interplay between environment and DNA modification.
The Yin–Yang Dynamics of DNA Methylation Is the Key Regulator for Smooth Muscle Cell Phenotype Switch and Vascular Remodeling
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