Loss of Methyl-CpGBinding Domain Protein 2 Enhances Endothelial Angiogenesis and Protects Mice Against Hind-Limb Ischemic Injury
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Loss of Methyl-CpG–Binding Domain Protein 2 Enhances Endothelial Angiogenesis and Protects Mice Against Hind-Limb Ischemic Injury

Xiaoquan Rao, MD; Jixin Zhong, PhD; Shu Zhang, PhD; Yushan Zhang, PhD; Qilin Yu, PhD; Ping Yang, BS; Mong-Heng Wang, PhD; David J. Fulton, PhD; Huidong Shi, PhD; Zheng Dong, PhD; Daowen Wang, MD, PhD; Cong-Yi Wang, MD, PhD

Background—Despite intensive investigation, how DNA methylation influences endothelial function remains poorly understood. We used methyl-CpG–binding domain protein 2 (MBD2), an interpreter for DNA methylome–encoded information, to dissect the impact of DNA methylation on endothelial function in both physiological and pathophysiological states.

Methods and Results—Human umbilical vein endothelial cells under normal conditions express moderate levels of MBD2, but knockdown of MBD2 by siRNA significantly enhanced angiogenesis and provided protection against H2O2-induced apoptosis. Remarkably, Mbd2−/− mice were protected against hind-limb ischemia evidenced by the significant improvement in perfusion recovery, along with increased capillary and arteriole formation. Loss of MBD2 activated endothelial survival and proangiogenic signals downstream of vascular endothelial growth factor signaling characterized by an increase in endothelial nitric oxide synthase (eNOS) and vascular endothelial growth factor receptor 2 expression, along with enhanced extracellular signal-regulated kinase 1/2 activation and BCL-2 expression. Mechanistic studies confirmed the methylation of CpG elements in the eNOS and vascular endothelial growth factor receptor 2 promoter. MBD2 binds to these methylated CpG elements and suppresses eNOS promoter activity. On ischemic insult, key endothelial genes such as eNOS and vascular endothelial growth factor receptor 2 undergo a DNA methylation turnover, and MBD2 interprets the changes of DNA methylation to suppress their expressions. Moreover, MBD2 modulation of eNOS expression is likely confined to endothelial cells because nonendothelial cells such as splenocytes fail to express eNOS after loss of MBD2.

Conclusions—We provided direct evidence supporting that DNA methylation regulates endothelial function, which forms the molecular basis for understanding how environmental insults (epigenetic factor) affect the genome to modify disease susceptibility. Because MBD2 itself does not affect the methylation of DNA and is dispensable for normal physiology in mice, it could be a viable epigenetic target for modulating endothelial function in disease states.

Key Words: angiogenesis ■ DNA methylation ■ endothelium ■ MBD2 protein ■ nitric oxide synthase type III

Endothelial cells (ECs) are the initial line of defense in many vascular diseases, and frequently the first casualty. Therefore, altered endothelial function is associated with numerous human pathologies, such as atherosclerosis, allograft vasculopathy, heart failure, diabetic retinopathy, and scleroderma. Given the essential role that ECs played in angiogenesis, their functional impairment is associated with attenuated angiogenic response that features prominently in patients with poor clinical outcomes after peripheral and/or myocardial ischemia,1,2 and an epigenetic factor is implicated in the disease process.3

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As a major epigenetic mechanism, DNA methylation acts as a footprint, reflecting the consequences of environmental insults in different types of cells.4 Therefore, alterations in DNA methylome are commonly seen in animals and patients with vascular diseases,5–9 which are read by a conserved family of methyl-CpG–binding domain (MBD) proteins (ie, MBD1, MBD2, MBD3, MBD4, and MeCP2). 2–9 The MBD proteins actively implicate in DNA methylation–mediated
transcriptional repression and/or heterochromatin formation and are responsible for maintaining and interacting with DNA methylome. They are distinguished by the binding affinity to methylated DNA, and MBD2 binds with the highest affinity, but MBD3 fails to selectively recognize methylated DNA.8 Animals deficient in MeCP2, MBD1, MBD2, and MBD4 are viable; deficiency in MBD3 leads to embryonic lethality.9 Mice lacking MeCP2 or MBD1 exhibit specific neurological defects,10,11 whereas loss of MBD4 suppresses CpG mutability and tumorigenesis.12,13 In contrast, mice deficient in MBD2 are generally normal except for a minor phenotype in maternal behavior.14

In the present study, we used MBD2 as a model to dissect the effect of DNA methylation on endothelial function. We hypothesized that MBD2 deciphers DNA methylome–encoded information and modulates endothelial gene expression implicated in endothelial apoptosis and angiogenesis. We demonstrated evidence supporting the methylation of CpG elements in the endothelial nitric oxide synthase (eNOS) and vascular endothelial growth factor (VEGF) receptor 2 (VEGF-R2) promoter. We also verified that MBD2 interprets DNA methylome–encoded information by binding to the methylated CpG elements. As a result, knockdown of MBD2 significantly enhanced angiogenesis and protected ECs from H2O2-induced apoptosis. Consistently, Mbd2−/− mice were protected from hind-limb ischemic injury.

**Methods**

**Animals**
Mice deficient in MBD2 (Mbd2−/−) on a C57BL/6 background were kindly provided by Dr Adrian Bird (Edinburgh University, Edinburgh, UK).14 Eight-week-old male Mbd2−/− mice and their wild-type (WT) littersmates were used in the study. All mice were housed in a standard specific pathogen free (SPF) facility in microisolation cages. All studies were performed in compliance with the Georgia Health Sciences University and Tongji Medical College Animal Care and Use Committee guidelines.

**Cell Culture and Transfection**
Human umbilical vein ECs (HUVECs) were cultured in EBM-2 EC basal medium with the SingleQuot kit (Lonza/Cambrex, Walkersville, MD). For siRNA transfection, the cells were seeded in 12-well plates at a density of 4×10^4 cells per well. On the second day, 50 or 100 nmol/L control or MBD2 siRNA was transfected into the cells with the lipofectamine reagent (Invitrogen, Carlsbad, CA).

**Flow Cytometry Analysis of Endothelial Apoptosis**
ECs were treated with 0.2 mmol/L H2O2 for 24 hours to induce apoptosis, followed by flow cytometric analysis of apoptotic cells.

**In Vitro Angiogenesis Assay**
Twenty-four hours after transfection, the ECs (2×10^4 cells) were added into each well of a 96-well plate and cultured for additional 24 hours. Endothelial tubes were then examined under a light microscope every other 4 hours by inspection of the overall tube length and branch points. [3H]-thymidine incorporation was used for analysis of endothelial proliferation and presented as counts per minute.

**Hind-Limb Ischemic Model and Assessments**
After anesthetization, a 1-cm-long incision was made in the skin at the medial thigh to expose the femoral artery. The proximal end of the femoral artery was occluded with double knots. The segment of femoral artery between the distal and proximal knots was then transected. Hind-limb blood flow in mice that had undergone hind-limb ischemia was measured with a laser Doppler imaging system (Perimed, Stockholm, Sweden).

**Immunostaining of Methyl-CpG–Binding Domain Protein 2, Capillaries, and Arterioles**
A sheep anti-MBD2 antibody and a FITC-conjugated bovine anti-sheep IgG were used to detect MBD2 expression. Staining of CD31-positive cells was used to define capillary formation, and α-actin–positive cells were used to evaluate arteriole formation.

**Western Blotting**
An enhanced chemiluminescence Western blotting kit (Millipore, Temecula, CA) was used for analyses of total and phosphorylated protein levels.

**Chromatin Immunoprecipitation Assay, Electrophoretic Mobility Shift Assay, and Endothelial Nitric Oxide Synthase Promoter Reporter Assay**
Chromatin immunoprecipitation (ChIP) assay was carried out with a ChIP assay kit (Upstate Biotechnology, Lake Placid, NY). The primers used in ChIP assay are summarized in Table I of the online-only Data Supplement. Electrophoretic mobility shift assay was carried out with a LightShift chemiluminescent electrophoretic mobility shift assay kit (Thermo Scientific, Rockford, IL), and a dual luciferase reporter system (Promega, Madison, WI) was used for eNOS promoter reporter assays.

**DNA Bisulfite Sequencing Analysis**
Genomic DNA from each preparation was first undergone bisulfite conversion with an EZ DNA Methylation kit, followed by polymerase chain reaction amplification of targeted sequences. The resulting polymerase chain reaction products were directly cloned into a TA vector. The methylation state of each targeted sequence was then analyzed by DNA sequencing.

**Statistical Analysis**
For pairwise comparisons, the data were analyzed by use of a Student t test. Comparison between multiple experimental groups was accomplished by a Bonferroni test with SPSS 17.0 for Windows. For the blood flow imaging data, repeated measures ANOVA was used. A 3-way repeated measures ANOVA was used to analyze the blood flow of Mbd2−/− and WT mice with or without N^6^-nitro-L-arginine methyl ester (L-NAME) treatment. All data are presented as mean±SEM. In all cases, values of P<0.05 were considered statistically significant.

A detailed version of Methods is available in the online-only Data Supplement.

**Results**

**Knockdown of Methyl-CpG–Binding Domain Protein 2 Promotes Angiogenesis and Protects Endothelial Cells From H2O2-Induced Apoptosis**
The HUVECs were transfected with either an MBD2 or a control siRNA to examine the potential influence of MBD2 on endothelial function. Moderate levels of MBD2 were detected in HUVECs under control conditions, whereas the MBD2 siRNA dose dependently suppressed MBD2 expression. When HUVECs were transfected with 100 nmol/L MBD2 siRNA, MBD2 was almost undetectable (Figure 1A). We next seeded siRNA-transfected HUVECs into culture plates preconditioned with growth factor–reduced Matrigel (BD Bioscience). Knockdown of MBD2 expression in HUVECs led to a significantly enhanced capacity for tube formation (Figure 1B). Tube formation was observed as early as 4 hours after seeding of HUVECs lacking MBD2, whereas almost no discernable tube formation was observed in HUVECs transfected with MBD2.
formation was observed for control siRNA-transfected cells (data not shown). The average total tube length 24 hours after transfection in each randomly selected field was significantly higher in MBD2 siRNA-transfected cells than control siRNA-transfected cells (Figure 1B).

To examine the impact of MBD2 on HUVEC proliferation, siRNA-transfected cells were cultured in the presence of 3H-labeled thymidine, followed by measurement of the incorporated radioactivity. Knockdown of MBD2 significantly enhanced HUVEC proliferation compared with control siRNA-transfected cells (Figure 1C). To investigate whether MBD2 regulates endothelial apoptosis, the transfected HUVECs were treated with H2O2 (0.2 mmol/L) for 24 hours to induce apoptosis. Interestingly, knockdown of MBD2 significantly protected HUVECs from H2O2-induced apoptosis (Figure 1D). Together, these results suggest that MBD2 negatively regulates angiogenesis and sensitizes ECs to H2O2-induced apoptosis.

Loss of Methyl-CpG–Binding Domain Protein 2 Protects Mice Against Hind-Limb Ischemia

Individuals with cardiovascular disease have an attenuated angiogenic response to ischemia that is associated with poor clinical outcomes.15,16 Given the observation that MBD2 negatively regulates endothelial angiogenesis in cultured cells, we next examined its impact on perfusion recovery after...
ischemic injury in vivo. We first confirmed the absence of MBD2 protein in blood vessels of Mbd2−/− mice (Figure 2A). We next examined MBD2 temporal expression changes in WT mice after femoral artery excision. Under control conditions, MBD2 was very low in the hind limb. However, a steady increase in MBD2 was noticed after ischemic induction; the highest level was observed 4 days after ischemic surgery. After that point, MBD2 underwent a steady decrease and then returned to a relative low level after postsurgery day 14 (Figure 2B). Immunostaining revealed that MBD2 expression (green) in the nonischemic muscle sections was limited to the location of blood vessels and was significantly improved perfusion after hind-limb ischemia.

To determine the role of MBD2 in perfusion recovery, unilateral hind-limb ischemia was induced in Mbd2−/− and WT male mice. All mice survived the surgical procedure, and blood flow in the ischemic (left side) and nonischemic (right side) limbs was monitored with a laser Doppler imaging system. Blood flow in the ischemic hind limbs was almost undetectable in both Mbd2−/− and WT mice right after femoral artery excision. Remarkably, Mbd2−/− mice showed partial blood flow restoration 2 days after induction, and blood flow was restored to ∼50% at day 7 and almost completely recovered at day 14 after the ischemic insult (Figure 3A). However, no visible blood flow restoration was observed in WT mice until day 4, and blood flow was restored to only ∼30% and 60% at days 7 and 14 after ischemic surgery, respectively.

To investigate differences in neovascularization, we examined capillary (CD31-positive cells) and arteriole (α-actin–positive cells) formation. The numbers of CD31-positive cells (ECs; Figure 3B) and α-actin positive cells (smooth muscle cells; Figure 3C) were significantly higher in the ischemic sections of Mbd2−/− mice than in WT mice. Taken together, our results demonstrate that loss of MBD2 promoted both angiogenesis and arteriogenesis and was associated with significantly improved perfusion after hind-limb ischemia.

**Suppression of Methyl-CpG–Binding Domain Protein 2 Activates Endothelial Cell Survival and Proangiogenic Signals**

To dissect the mechanisms by which MBD2 regulates endothelial survival, we examined 2 major endothelial survival signals, the extracellular signal-regulated kinase 1/2 (ERK1/2) and the serine-threonine kinase Akt, in siRNA-transfected HUVECs. Interestingly, MBD2 siRNA did not show a significant impact on Akt expression and activation (Figure I in the online-only Data Supplement). However, MBD2 siRNA promoted ERK1/2 activation, as revealed by the presence of higher phosphorylated (p-) ERK1/2, although total ERK1/2 did not change (Figure 4A). In line with the role of p-ERK1/2 in BCL-2 stabilization,17 higher BCL-2 expression was noticed in MBD2 siRNA-transfected cells on H2O2 treatment (Figure 4B). Surprisingly, no perceptible difference was detected after knockdown of MBD2 for Mn superoxide dismutase and Cu/Zn superoxide dismutase, the 2 enzymes detoxifying reactive oxygen species (Figure II in the online-only Data Supplement). Our results suggest that MBD2 regulates endothelial apoptosis probably by modulating survival signals relevant to ERK1/2 and BCL-2 signaling.

To study the mechanism underlying MBD2 regulation of angiogenesis, we analyzed the activity of eNOS and VEGF-R2, the 2 key proangiogenesis signals.18,19 Remarkably, knockdown of MBD2 increased both total and activated eNOS (p-eNOS) and VEGF-R2 compared with control siRNA-transfected HUVECs (Figure 4C). Importantly, blood vessels isolated from Mbd2−/− mice also showed enhanced eNOS and VEGF-R2 expression compared with their control counterparts (Figure 4D); a similar trend was noticed in ischemic tissues of Mbd2−/− mice (Figure III in the online-only Data Supplement). In con-
MBD2 did not show a significant impact on total p38, but higher p-p38 was detected in MBD2 siRNA-transfected HUVECs (Figure 4E), and studies in Mbd2−/−/H11002 blood vessels showed similar results (Figure IV in the online-only Data Supplement). Together, loss of MBD2 facilitates the activation of cell survival and proangiogenic signals to enhance endothelial survival and to promote angiogenesis.

**Endothelial Nitric Oxide Synthase Synergizes With Vascular Endothelial Growth Factor Receptor 2 to Promote Endothelial Survival and Angiogenesis**

To demonstrate the connection of the signals characterized above, we treated HUVECs with VEGF in the presence of blockades for either VEGF-R2 or eNOS. Stimulation with VEGF did not affect total VEGF-R2, eNOS, and ERK1/2 expression but promoted VEGF-R2, eNOS, and ERK1/2 activation, along with enhanced BCL-2 expression (Figure 5A). As expected, VEGF-R2–neutralizing antibody or inhibitor (SU1498) suppressed VEGF-induced VEGF-R2 activation and diminished VEGF-induced ERK1/2 activation and BCL-2 upregulation. Blockade of VEGF-R2 also led to a modest decrease in p-eNOS (Figure 5A). Notably, blockade of eNOS by L-NAME not only inhibited eNOS activation (p-eNOS) but also suppressed VEGF-induced p–VEGF-R2, along with a significant decrease in p-ERK1/2 and BCL-2 expression (Figure 5A). These results suggest that ERK1/2 and BCL-2 are downstream molecules of eNOS and VEGF-R2 signaling. Our data also suggest crosstalk between eNOS and VEGF-R2 signaling in which eNOS plays a predominant role by synergizing with VEGF-R2 to enhance endothelial survival and to promote angiogenesis.

To confirm the above conclusion, we next treated Mbd2−/− and control mice after hind-limb ischemic surgery with the eNOS inhibitor L-NAME. Administration of L-NAME significantly impaired blood flow restoration in both Mbd2−/− and control mice (Figure V in the online-only Data Supplement).
ment). More significantly, L-NAME treatment completely abolished the protective effect seen in Mbd2/H11002/H11002 mice as manifested by the similar extent of impaired perfusion after ischemic injury (Figure 5B).

Methyl-CpG–Binding Domain Protein 2 Binds to the Methylated CpG Elements in the Endothelial Nitric Oxide Synthase and Vascular Endothelial Growth Factor Receptor 2 Promoter

Previous studies suggested evidence supporting a role for DNA methylation regulating eNOS and VEGF-R2 transcription.2-20,21 We therefore hypothesized that MBD2 represses eNOS and VEGF-R2 expression by directly binding to the methylated CpG elements in their promoter. Although no typical CpG island exists in the eNOS promoter, a region containing CpG elements is found in the 5'-flanking region (Figure 6A). On the contrary, bioinformatic analysis characterized 2 putative CpG islands in the VEGF-R2 promoter (Figure 6B). To test our hypothesis, we first treated HUVECs with 5-aza-2'-deoxycytidine (5-azadC), a DNA methylation inhibitor. Remarkably, knockdown of MBD2 by siRNA had no perceptible effect on 5-azadC–treated HUVECs (Figure VI in the online-only Data Supplement), indicating that the effect of MBD2 involves DNA methylation. Then, ChIP was used to pull down the MBD2/DNA complexes. Primers flanking the entire eNOS promoter/5'-flanking region and the putative CpG islands of VEGF-R2 were used to amplify the MBD2-targeted DNA with the resultant precipitates. No amplification was observed for all primers in the eNOS promoter, but primers (F9/R9 and F10/R10) in the 5'-flanking region yielded positive results (Figure 6C). Surprisingly, among the 3 primer pairs used to flank the putative CpG islands for VEGF-R2, only primers (F3/R3) covering the region between 64 and 166 yielded positive results (Figure 6D).

To confirm the above ChIP assay results, we specifically examined the methylation states of 13 CpG elements in the eNOS 5'-flanking region by bisulfite DNA sequencing. Indeed, we detected CpG methylation in both the physiological and ischemic condition (Figure VII in the online-only Data Supplement). We also characterized a significant DNA meth-
ylation turnover on ischemic insult (hypoxia plus serum starvation) in HUVECs as evidenced by a 1-fold increase in the methylation rate of these CpG elements (Figure 6E). Electrophoretic mobility shift assay was then carried out to further confirm that MBD2 binds to the methylated CpG elements within this region. Biotin-labeled polymerase chain reaction products flanking 31 to 197 were first methylated by SssI methylase and then used as the probe to detect MBD2 binding activity. It was found that MBD2 bound to those methylated polymerase chain reaction products with high affinity (Figure VIII in the online-only Data Supplement). To address that MBD2 represses eNOS transcription by binding to those methylated CpG elements, eNOS promoter (−1100 to +1700) was subcloned into a pGL-2 vector (pGL-eNOS). A mutated eNOS promoter reporter (pGL-eNOSm), in which cytosines in all CpG elements between −200 and 300 were mutated to adenosine, was also constructed. Luciferase assays were then performed with those in vitro methylated reporters along with a pcDNA-MBD2 plasmid in HUVECs, respectively. Consistently, only those methylated reporters showed differences for luciferase activities, in which loss of CpG elements resulted in a 1-fold increase in the reporter activities. In contrast, both pGL-eNOS and pGL-eNOSm showed similar reporter activities in the unmethylated condition (Figure IX in the online-only Data Supplement). Taken together, these results suggest that MBD2 represses eNOS and VEGF-R2 transcription by directly binding to the methylated CpG elements in their promoter region.

Methyl-CpG-Binding Domain Protein 2
Repression of Endothelial Nitric Oxide Synthase Transcription Involves Chromatin Remodeling and Is Confined to Endothelial Cells

It has been suggested that MBD2-mediated transcriptional repression is associated with chromatin remodeling that involves histone deacetylases (HDACs). To check whether MBD2 repressing eNOS transcription also involves chromatin remodeling, we exposed cells to trichostatin A (TSA), an HDAC inhibitor that inhibits eNOS transcription, into HUVECs transfected with MBD2 or control siRNA. Surprisingly, the effect of MBD2 siRNA on eNOS expression was completely abolished by TSA treatment, as revealed by the same levels of eNOS expression in both MBD2 and control siRNA-transfected HUVECs (Figure 7A).

Because TSA has been shown to induce eNOS expression in non-EC types, we wondered whether loss of MBD2 would lead to eNOS expression in non-ECs. We then treated siRNA-transfected HeLa cells with either TSA or dimethyl sulfoxide (vehicle). Surprisingly, knockdown of MBD2 failed to induce eNOS expression in HeLa cells, but eNOS was detected in the same cells after TSA treatment (Figure 7B). Studies in HEK293 cells yielded similar results (data not shown). Next, we analyzed eNOS expression in splenocytes originated from Mbd2−/− and control mice and found that eNOS was absent in both Mbd2−/− and WT splenocytes. However, eNOS was induced in all cells after TSA treatment (Figure 7C). Together, our results indicate that MBD2 repressing eNOS expression involves chromatin remodeling and that this effect is likely confined to ECs.

Discussion

Tissue- or organ-specific gene expression patterns after embryonic development are maintained and controlled by epigenetic changes instead of DNA sequence alterations. DNA methylation as a major epigenetic mechanism of the genome provides a new perspective on transcriptional control paradigms in ECs. Despite intensive investigation, how DNA methylation influences endothelial function remains poorly understood. In the present report, we used MBD2, an epige-
netic interpreter, to decipher DNA methylome–encoded information in the regulation of endothelial function in both physiological and disease states. For the first time, our studies provided direct evidence that DNA methylation regulates endothelial survival and proangiogenic signals, which constitutes the molecular basis for understanding how an epigenetic factor affects the genome to modify disease susceptibility.

An interesting finding was that MBD2 was dispensable for the normal physiology of ECs and animals. Endothelial cells under physiological condition expressed only moderate levels of MBD2, but loss of MBD2 significantly enhanced angiogenesis and protected ECs from $\text{H}_2\text{O}_2$-induced apoptosis (Figure 1). Hind-limb ischemia was then used to address the importance of MBD2 in disease pathogenesis. In the hind-limb ischemic model, ischemic injury was positively correlated with MBD2 expression (Figure 2). As a result, loss of MBD2 afforded significantly improved recovery of perfusion after ischemic surgery, along with increased capillary and arteriole formation (Figure 3). Given the fact that MBD2 itself does not affect the methylation of DNA and is dispensable for routine physiological activities, our results highlight the potential of MBD2 to be an epigenetic therapeutic target relevant to disease states.

Survival signals conducted by ERK1/2 and Akt signaling have been increasingly recognized to be critical for endothelial viability. Interestingly, MBD2 only selectively influences the activation of ERK1/2, as evidenced by higher levels of $\text{p-ERK1/2}$ (Figure 4A) but no perceptible changes in $\text{p-Akt}$. Because ERK1/2 has been found to phosphorylate BCL-2 and to prevent its ubiquitin-dependent degradation, we also detected significantly higher levels of BCL-2 after knockdown of MBD2 (Figure 4B). These results suggest that ECs with reduced MBD2 expression exhibit enhanced ERK1/2 activity, which antagonizes proapoptotic stimuli by increasing BCL-2 expression.

Studies in MBD2 siRNA-transfected HUVECs and $\text{Mbd2}^{-/-}$ mice consistently demonstrated that MBD2 negatively regulates eNOS and VEGF-R2 expression (Figure 4C and 4D). The importance of eNOS in endothelial function is underscored by the observation that $\text{eNOS}^{-/-}$ animals develop systemic and pulmonary hypertension, altered vascular remodeling, abnormal angiogenesis, impaired wound healing,
Methyl-CpG–binding domain protein 2 (MBD2) repression of eNOS expression is confined to endothelial cells (ECs). A, Trichostatin A (TSA) treatment abolished the effect of MBD2 siRNA on endothelial nitric oxide synthase (eNOS) expression in human umbilical vein ECs (HUVECs). Transfected HUVECs were treated with TSA or control vehicle for 48 hours and then subjected to Western blot analysis. Similar levels of eNOS were detected in MBD2 and control siRNA-transfected HUVECs on TSA treatment. B, Knockdown of MBD2 failed to induce eNOS expression in HeLa cells. No detectable eNOS was observed in HeLa cells transfected with either MBD2 or control siRNA; however, the addition of TSA induced eNOS expression in both transfected cells. C, Splenocytes derived from Mbd2−/− mice did not express eNOS, but TSA induced eNOS expression in both Mbd2−/− and wild-type (WT) splenocytes. Splenocytes derived from both Mbd2−/− and WT mice were treated with TSA for 48 hours before Western blot analysis.

Figure 7. Methyl-CpG–binding domain protein 2 (MBD2) repression of eNOS expression is confined to endothelial cells (ECs).

A key question is how MBD2 regulates eNOS and VEGF-R2 expression. We assumed that MBD2 executes this function by binding to the methylated CpG elements in their promoter region. Although a previous study suggested evidence for DNA methylation in restricting eNOS expression to the vascular endothelium, direct evidence indicating the methylation of CpG elements in its promoter is lacking. Treatment of MBD2 siRNA-transfected HUVECs with 5-azadC yielded the first line of evidence supporting that MBD2 regulation of eNOS and VEGF-R2 expression implicates the methylation of CpG elements (Figure VI in the online-only Data Supplement). Indeed, bisulfite sequencing analysis of the eNOS 5′-flanking region demonstrated direct evidence confirming the methylation of these CpG elements (Figure VII in the online-only Data Supplement). The eNOS 5′-flanking region (−135 to 419) contains 13 CpG elements. Under the ischemic condition, the average methylation rate of these CpG elements increased by 1-fold compared with that in the physiological condition (Figure 6E). Our subsequent ChIP, electrophoretic mobility shift assay, and eNOS promoter reporter assays provided convincing evidence that MBD2 directly binds to the methylated CpG elements in the eNOS 5′-flanking region and suppresses its transcription (Figure 6B and 6D and Figures VIII and IX in the online-only Data Supplement). Collectively, our data suggest that ECs in the ischemic condition undergo a DNA methylation turnover that encodes the information in favor of disease pathologies and that MBD2 deciphers the change of DNA methylome by binding to the methylated CpG elements of targeted genes.

Previous studies have revealed that MBD2 responds to methylated DNA by recruiting HDACs and other transcription repression factors to the chromatin. Unexpectedly, administration of TSA, a specific inhibitor of HDACs, completely abolished the effect of MBD2 siRNA on eNOS expression in HUVECs (Figure 7). Strikingly, unlike TSA, which induces eNOS expression in non-ECs, the effect of MBD2 silencing on eNOS expression was observed only in ECs. For example, HeLa cells or HEK293 cells with reduced eNOS, but TSA induced eNOS expression in both Mbd2−/− and wild-type (WT) splenocytes. Splenocytes derived from both Mbd2−/− and WT mice were treated with TSA for 48 hours before Western blot analysis.

and defective mobilization of stem and progenitor cells. Similar to eNOS, VEGF-R2 transduces most of the VEGF-mediated mitogenic, survival, and vascular permeability signals critical for angiogenesis. We first demonstrated evidence suggesting that MBD2 regulates angiogenesis by affecting VEGF downstream signaling because VEGF stimulation of HUVECs activated signals similar to those of knockdown of MBD2 expression (Figure 5A). Interestingly, blocking either eNOS or VEGF-R2 signaling inhibited VEGF-induced ERK1/2 activation and BCL-2 expression, indicating that ERK1/2 and BCL-2 are downstream molecules of the eNOS and VEGF-R2 signaling. Given the fact that MBD2 did not show a perceptible impact on ERK1/2 expression, this result suggests that the enhanced ERK1/2 activation and BCL-2 expression after knockdown of MBD2 could be indirect effects resulting from increased eNOS and VEGF-R2 expression. Of important note, studies in VEGF-stimulated HUVECs also suggested evidence supporting crosstalk between eNOS and VEGF-R2 signaling in which eNOS synergizes with VEGF-R2 to enhance endothelial survival and angiogenesis; therefore, blockade of eNOS by L-NAME completely abolished the protective effect against hind-limb ischemia seen in Mbd2−/− mice (Figure 5B).

We also noticed higher p38 activation after knockdown of MBD2 expression (Figure 4E). The exact role for p38 in endothelial function, however, remains controversial. It is likely that the activation of p38 may serve a number of different functions, depending on the cellular context or rather the activation state of other signaling pathways. The role of p38 in the epigenetic pathways affected by MBD2 remains unclear in the present study; therefore, dissecting the role of p38 signaling in MBD2 deficiency–related phenotypes would be a critical focus of future studies.
that MBD2 regulation of eNOS expression is probably confined to ECs. In contrast to this conclusion, we also noticed an enhanced arteriogenesis in Mbd2−/− mice after ischemic insult as evidenced by the increase in α-actin–positive cells (Figure 3C). However, this phenotype is probably an indirect effect resulting from enhanced eNOS expression because eNOS also plays an essential role in inducing arteriogenesis.34,35

Conclusions
The present report demonstrates evidence that MBD2 deciphers DNA methylome–encoded information to modulate endothelial function. On environmental insults, ECs undergo a DNA methylation turnover that encodes the information in favor of disease pathologies. MBD2 deciphers the change in DNA methylene by binding to the methylated CpG elements of targeted genes. As a result, inhibition of MBD2 enhances angiogenesis and protects ECs from H2O2-induced apoptosis. Mice deficient in MBD2 also show significantly improved perfusion after hind-limb ischemic injury. Given the fact that MBD2 itself does not modify the patterns of DNA methylation and appears to be dispensable for normal physiology, our results suggest that MBD2 could be a viable epigenetic target to modulate endothelial function in disease states such as targeting MBD2 to prevent endothelial dysfunction during the course of atherosclerosis and diabetic nephropathy.

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Disclosures
None.

References

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CLINICAL PERSPECTIVE

Functional impairment for endothelial cells is associated with an attenuated angiogenic response that features prominently in patients with poor clinical outcomes after peripheral and/or myocardial ischemia. Although DNA methylation has long been implicated in endothelial pathologies, the underlying mechanisms remain poorly understood. The present article reports the impact of methyl-CpG-binding domain protein 2 (MBD2), an interpreter for DNA methylome-encoded information, on endothelial function in both physiological and disease states. Moderate levels of DNA methylation were characterized for some key endothelial genes, such as endothelial nitric oxide synthase and vascular endothelial growth factor receptor 2 in the physiological condition. However, these genes undergo a significant DNA methylation turnover when endothelial cells are under ischemic condition. MBD2 deciphers this methylation turnover-encoded information by directly binding to the methylated CpG elements, leading to the suppression of their transcription in favor of endothelial pathologies. Therefore, suppression of MBD2 expression enhances endothelial nitric oxide synthase and vascular endothelial growth factor receptor 2 transcription, which then promote extracellular signal-regulated kinase 1/2 activation and BCL-2 expression. Consistent with these results, knockdown of MBD2 by siRNA enhanced endothelial angiogenesis and provided protection against H$_2$O$_2$-induced apoptosis. Mice deficient in MBD2 showed remarkably improved perfusion recovery after hind-limb ischemic surgery. Furthermore, MBD2 regulation of endothelial nitric oxide synthase expression is likely confined to endothelial cells because other types of cells fail to express endothelial nitric oxide synthase after loss of MBD2 such as splenocytes. These discoveries are important for understanding how an epigenetic factor affects the genome to modify disease susceptibility. Because MBD2 itself does not affect DNA methylation and is dispensable for normal physiology in mice, it could be a viable epigenetic target for modulating endothelial function in disease states.
Supplemental Methods

Reagents
Trichostatin A (TSA), 5-aza-2'-deoxycytidine (5-azadC) and N (G)-nitro-L- arginine methyl ester (L-NAME), were purchased from Sigma (St. Louis, MO). Antibodies for phosphor-MAPK P38, total P38, phosphor-ERK 1/2, total ERK 1/2 and phosphor-eNOS (Ser-1177) were purchased from Cell Signaling (Beverly, MA). eNOS antibody, MBD2 siRNA and control siRNA were purchased from Santa Cruz (Santa Cruz, CA). Antibody for MBD2 in ChIP assay was purchased from Bethyl (Montgomery, TX), and VEGF-R2 antibody was obtained from Upstate (Lake Placid, NY). Recombinant human VEGF165 was purchased from PeproTech (Rocky Hill, NJ). VEGF-R2 neutralizing antibody was purchased from R&D (Minneapolis, MN). SU1498 was obtained from Calbiochem (San Diego, CA).

Cell Culture and Transfection
Human Umbilical Vein Endothelial Cells (HUVECs, ATCC) were cultured at 37°C with 5% CO2 in EBM-2 endothelial cell basal medium with SingleQuot Kit (Lonza/Cambrex, Walkersville, MD) as instructed. HeLa cells were cultured in DMEM (Hyclone, Logan, Utah) with 10% fetal bovine serum (FBS) at 37°C with 5% CO2. For siRNA transfection, the cells were seeded in 12-well plate at a density of 4 x 10^4 cells per well. On the second day, 50 or 100 nmol/L of control siRNA or MBD2 siRNA (Santa Cruz, Santa Cruz, CA) were transfected into the cells using the lipofectamine reagent (Invitrogen, Carlsbad, CA) as instructed. For TSA
treatment, TSA (0.2 μg/ml) was added into the cultures and the cells were cultured for additional 48h. For 5-azadC treatment, HUVECs were cultured in the presence of 5-azadC (2 μmol/l) for 2 days, followed by siRNA transfection and subjected to Western blot analysis after 2 days. Cells cultured with same volume of DMSO were used as a control. For blocking assays, HUVECs were pre-incubated with a VEGF-R2 neutralizing Ab (1μg/ml) or SU1498 (10 μmol/l) or L-NAME (200 μmol/l) for 30 min, followed by stimulation with human recombinant VEGF165 (20 ng/ml) for 10 min.

**Flow Cytometry Analysis of Endothelial Apoptosis**

ECs transfected with control or MBD2 siRNA were treated with 0.2mmol/L H2O2 for 24h to induce apoptosis followed by annexin-V and propidium iodide (PI) (Invitrogen, Carlsbad, CA) staining. Apoptotic (annexin-V and PI positive) cells were analyzed by flow cytometry as previously reported. At least 10,000 events were collected. Data was analyzed with CellQuest v3.3 software (BD Bioscience, San Jose, CA) as instructed.

**In Vitro Angiogenesis Assay**

ECs were transfected with control or MBD2 siRNA 24h before the assay. To examine tube formation, growth factor-reduced Matrigel (BD Bioscience, San Jose, CA) was placed in 96-well tissue culture plates (100μL/well) and allowed to form a gel at 37°C for at least 30min. ECs (2 x 10^4 cells) after 24h of transfection were added into each well and incubated in 2% FBS EBM-2 endothelial cell basic medium at 37°C for 24h under a 5% CO2 atmosphere. Endothelial tubes were then examined under a light microscope every other 4h by inspecting the overall tube length and branch points. For endothelial proliferation assay, endothelial cells 24h after
transfection were trypsinized and seeded in 96-well tissue culture plates at a density of $5 \times 10^3$ cells per well. The cells were pulsed with 0.5µCi/well $^3$H-thymidine for 16h and then harvested and counted in a 1450 MicroBeta TriLux Microplate Scintillation and Luminescence Counter (PerkinElmer, Boston, MA). EC proliferation was determined by $^3$H-thymidine incorporation and present as counts per minute (cpm).

**Hindlimb Ischemia Model and Assessments**

*Mbd2*−/− or WT mice were subjected to unilateral hindlimb surgery under anesthesia with intraperitoneal administration of ketamine (85 mg/kg) and xylazine (15 mg/kg). The mice were put on a dissection microscope at 10X or 20X magnification to obtain an enlarged view of the left hindlimb region. A 1-cm long incision was made in the skin at the medial thigh to expose the femoral artery. The femoral artery in the left hindlimb was separated from the femoral vein and nerve at the proximal location near the groin. A strand of 7-0 silk suture was passed through underneath the proximal end of the femoral artery and occluded the proximal femoral artery using double knots. The segment of femoral artery between the distal and proximal knots was then transected using a pair of spring scissors. The incision was then closed using 5-0 sutures.

Hindlimb blood flow in mice undergone hindlimb ischemia was measured with a PIM 3 scanning Laser Doppler imaging system (Perimed, Stockholm, Sweden). Animals were anesthetized and maintained at 37°C on a heating plate to minimize temperature variation. Laser Doppler Blood Flow (LDBF) analyses were performed on legs and feet before/post surgery on days 0, 2, 4, 7, and 14. Blood Flow images of hindlimbs (3 per animal) were then acquired. Blood flow was presented as changes in the laser frequency, represented by different color pixels. The mean hindlimb blood flow was calculated as the ratio of ischemic side to nonischemic side. Six mice
were analyzed in each group. For treatment of mice with L-NAME, the mice after ischemic surgery were provided drinking water containing 1mg/ml L-NAME during the perfusion recovery period. Mice provided with regular drinking water were served as controls.

**Immunostaining of MBD2, Capillaries and Arterioles**

Tissue sections (10-μm) prepared from OCT-embedded ischemic and control hindlimbs were used for immunostaining. To detect MBD2 expression, the sections were incubated with a sheep anti-MBD2 antibody (Millipore, Bedford, MA), followed by staining with a FITC-conjugated bovine anti-sheep IgG (Santa Cruz, Santa Cruz, CA). For defining capillaries, a rat anti-mouse CD31 antibody (BD pharimagen, San Diego, CA) was used to detect endothelial cells, followed by staining with a Texas red-conjugated goat anti-rat IgG (Santa Cruz, Santa Cruz, CA). A rabbit polyclonal anti-smooth muscle α-actin (Abcam, Cambridge, MA) was used to stain smooth muscle cells in the arterioles, and the sections were next stained with a Texas red-conjugated goat anti-rabbit IgG (Santa Cruz, Santa Cruz, CA). The relative amount of MBD2-, CD31- and α-actin-positive cells were counted in 5 randomly selected high-power fields (magnification x200) with 4 mice for each group, respectively.

**Western Blotting**

Proteins were separated by 8% SDS-PAGE and then transferred onto PVDF membranes. After blocking with 5% nonfat milk in TBS-T, the membranes were incubated with indicated primary antibodies for 16h at 4 °C, followed by incubation with secondary horseradish peroxidase-conjugated antibody for 1h at RT. After extensive washes with TBS-T, the membrane was
visualized with ECL plus reagents (Pierce, Rockford, IL). All primary antibodies were used at the recommended dilutions provided by the manufacturers.

**Chromatin Immunoprecipitation (ChIP) Assay**

ChIP assay was carried out using a Chromatin Immunoprecipitation Assay Kit (Upstate Biotechnology, Lake Placid, NY) as instructed. A β-actin antibody was used to demonstrate non-specific precipitation (negative control). Briefly, 5 x 10^6 HUVECs were incubated with 1% formaldehyde to cross-link DNA-interacting protein to chromatin DNA. Cells were then lysed with 200μl SDS lysis buffer followed by sonication. DNA/protein complexes were then diluted with 1.8ml dilution buffer and pulled down with agarose beads conjugated with an anti-MBD2 or a control antibody. The complexes were then eluted with 500μl elution buffer, and the DNA was released from crosslinks by incubating with 200mM NaCl. One microliter of resulting solution was then used as template to amplify MBD2 targeted DNA sequences. One microliter of solutions before incubation with antibody was used as input. Primers that generate overlapped PCR fragments (around 100 – 240bp for each) to flank the entire eNOS promoter and the putative CpG islands of the VEGF-R2 promoter were used to detect the MBD2 targeted sequences. The CG percentage and CpG islands were analyzed using EMBOSS CpGPlot software (http://www.ebi.ac.uk/Tools/emboss/cpgplot/index.html). All primer sequences and locations are summarized in the Online Data Supplement (Table 1).

**DNA Bisulfite Sequencing Analysis**

HUVECs were cultured in the hypoxic condition with serum starvation for 16h to mimic ischemic insult in animals. Genomic DNA was then extracted using a QIAamp DNA mini kit.
(QIAGEN, Valencia, CA) from HUVECs cultured in the normal and ischemic condition, respectively. Around 0.5 to 1μg DNA from each preparation were then employed for bisulfite conversion using an EZ DNA Methylation™ Kit (Zymo Research Corporation, Orange, CA) as instructed. After bisulfite conversion, the eNOS 5'-flanking region was amplified using following primers (product size, 554bp): forward, 5'-GGA GTT GAG GTT TTA GAG TTT TTT AG-3'; reverse, 5'-CCC TTA CTC CCA ACT TTC ACC T-3'. PCR amplifications were carried out at 95°C for 10 minutes, followed by 38 cycles of 95°C for 30 seconds, 57°C for 30 seconds and 72°C for 30 seconds, with final extension at 72°C for 10 minutes. The resulting PCR products were first checked by agarose gel electrophoresis and then subcloned into a TA vector using a TOPO® Cloning Kit (Invitrogen, Carlsbad, CA). Ten positive clones originated from each PCR products were randomly selected for DNA sequencing analysis with T3 and T7 primers in an ABI 310 automated sequencing system (Perkin Elmer, Foster City, California). DNA sequencing results were analyzed by software Sequencher 4.7 (Ann Arbor, MI).

Electrophoretic Mobility Shift Assay (EMSA)

Nuclear proteins were extracted from HEK293 cells transfected with a pcDNA-MBD2 construct (provided by Dr. Wen-Cheng Xiong, Medical College of Georgia) using the NE-PER® Nuclear and Cytoplasmic Extraction Reagents (Thermo Scientific, Rockford, IL), which were then used for EMSA with a LightShift® Chemiluminescent EMSA Kit (Thermo Scientific, Rockford, IL) as instructed. A biotin-labeled double-stranded probe (position +31 to +197) was prepared by PCR amplification from HUVECs using the following primers: Biotin-F9, Biotin-5'-CAG AGT GGA CGC ACA GTA ACA T-3'; R9, 5'-CGC TGG TGG GAG TAG GGA T-3'. The resulting products were first examined on a 2% agarose gel and then methylated by SssI as detailed below.
A mixture of probe (1:200, Biotin-labeled vs. unlabeled) was used as a competitive control. Probes without biotin were prepared using primers F9 and R9 followed by SssI treatment.

**eNOS Promoter reporter Assay**

eNOS promoter (-1700 to +350) was directly amplified from HUVECs and subcloned into a pGL-2 reporter vector (Promaga, Madison, WI; pGL-eNOS) as instructed. The mutated eNOS promoter was the same as the wild-type promoter except for that cytosines in all CpG-elements between -200 to +300 were mutated to A, which was directly synthesized by GeneScript (Piscataway, NJ) and then subcloned into the pGL-2 vector (pGL-eNOSm). Both constructs were next methylated by incubating with SssI methylase (New England BioLabs, Beverly, MA) at 10mM Tris, pH7.9, 50mM NaCl, 10mM MgCl₂, 1mM DTT, and 160μM S-adenosylmethionine at 37°C for 1h. After purification, each reporter vector was co-transfected with a pcDNA-MBD2 plasmid along with an internal control reporter (pGL-Tk) into HUVECs using the lipofectamine 2000 transfection reagent (Invitrogen, Carlsbad, CA). Luciferase reporter activity was determined 48h after transfection using the Dual-Luciferase reporter assay system (Promega, Madison, WI) as previously reported. The relative luciferase activity was normalized by the internal pGL-Tk reporter.
Table 1. The sequence of primers used in ChIP.

Three primer pairs were used to cover the two putative CpG islands characterized in the VEGF-R2 promoter region. Ten primer pairs were used to flank the entire eNOS promoter and 5' untranslated region (5'-UTR). The promoter sequences for VEGF-R2 and eNOS are retrieved from website [http://rulai.cshl.edu/cgi-bin/TRED/tred.cgi?process=home](http://rulai.cshl.edu/cgi-bin/TRED/tred.cgi?process=home), and the accession numbers are 32514 and 38471, respectively.

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<th>Gene</th>
<th>Primer Position and Name (transcriptional starting site as +1)</th>
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<td>-709 to -532 (178bp) F1 GAATGTTGGCGAACTGGG</td>
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<td>R1 CACAAGGGAGAAGCGGATA</td>
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<td>-547 to -352 (196bp) F2 CGGCTTCTCTCTCCGTGCT</td>
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<td>R2 ACTCGGAACGGGCCGCTGA</td>
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<td>R3 CGGGAGCCGGGTCTTTTC</td>
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Supplemental Figure I
Supplemental Figure II

siRNA: Ctl MBD2

β-actin

MnSOD Cu/ZnSOD
Supplemental Figure III
Supplemental Figure IV
Supplemental Figure V

BS: the day before ischemic surgery;
AS: the day after ischemic surgery
Supplemental Figure VI
FP: forward primer; RP: reverse primer; TS: transcription site

○ unmethylated    ● methylated

Supplemental Figure VII
Lysates - + +
Biotin-Probe + - +
Unlabeled-probe - + +

Supplemental Figure VIII
Supplemental Figure IX
Supplemental Figure Legends

Figure I. Knockdown of MBD2 by siRNA shows no perceptible effect on Akt signaling.
MBD2 or control siRNA transfected HUVECs were treated with 0.2mmol/L H₂O₂ for 24h. Cell lysates were then used for Western blot analysis of total Akt and activated or phosphorylated Akt (p-Akt). No significant difference was detected for both total Akt and p-Akt between MBD2 siRNA and control siRNA transfected cells.

Figure II. Altered MBD2 expression does not affect Cu/ZnSOD and MnSOD transcription.
HUVECs 24h after MBD2 or control siRNA transfection were treated with 0.2mmol/L H₂O₂ for 24h. The cells were then harvested for Western blot analysis of Cu/ZnSOD and MnSOD expression. No significant change for both Cu/ZnSOD and MnSOD was observed after knockdown of MBD2 by siRNA.

Figure III. Mbd2−/− mice after hindlimb ischemic surgery show enhanced eNOS and VEGF-R2 expression along with higher ERK1/2 activation.
Ischemic tissues were collected from Mbd2−/− and WT mice after day 7 of ischemic surgery. Tissue lysates were then analyzed by Western blotting for the expression levels of eNOS and VEGF-R2 as well as the levels of phosphorylated ERK1/2 (p-ERK1/2). GAPGH was used for normalization. Much higher eNOS and VEGF-R2 expressions along with higher ERK1/2 activation were observed in Mbd2−/− mice than that of WT mice. The molecular changes for eNOS and VEGF-R2 expression and ERK1/2 activation were correlated with improved perfusion recovery in Mbd2−/− mice as compared with that of WT mice.
Figure IV. Enhanced p38 activation in the blood vessels of Mbd2−/− mice.

Blood vessels were isolated from both WT and Mbd2−/− mice and subjected to Western blot analysis of total and phosphorylated or activated p38 (p-p38). No significance was observed for total p38, but Mbd2−/− mice showed higher levels of p-p38 as compared with that of control mice.

Figure V. Blockade of eNOS by L-NAME abrogates the improved blood flow restoration in Mbd2−/− mice.

Mbd2−/− and WT mice after hindlimb ischemic surgery were provided with drinking water containing 1mg/ml L-NAME to block eNOS signaling during the perfusion recovery period. Mice provided with regular drinking water were served as controls. Blood flow was measured using a Laser Doppler imaging system as described earlier. Images shown here are representatives of three mice analyzed for each study group. The improved blood flow in Mbd2−/− mice was completely abolished after blockade of eNOS signaling by L-NAME.

Figure VI. Treatment of HUVECs with 5-azadC abolishes the effect of MBD2 siRNA on eNOS and VEGF-R2 expression.

HUVECs 6h after MBD2 siRNA or control siRNA transfection were cultured in the presence of 5-azadC (2 μmol/l) for 2 days, followed by Western blot analysis of the expression levels for eNOS and VEGF-R2. It was interestingly noted that 5-azadC treatment completely abolished the enhancing effect of MBD2 siRNA on eNOS and VEGF-R2 expression.

Figure VII. DNA bisulfite analysis results for the eNOS 5′-flanking region.
Ten clones originated from PCR products of HUVECs in the normal and ischemic condition were analyzed by direct DNA sequencing, respectively. The location for each CpG-element in the eNOS 5'-flanking region is indicated. The methylation state for each CpG-element is present either with an empty circle (unmethylated) or filled circle (methylated). The overall methylation rate for all of these CpG-elements was much higher in ischemic condition as compared with that of normal condition.

**Figure VIII. MBD2 binds to the methylated CpG-elements in the eNOS 5'-flanking region.**

Biotin-labeled probe (+31 to +197) was prepared using a Biotin-labeled F9 primer and a reverse R9 primer as described. The resulting PCR products were first examined on a 2% agarose gel followed by treatment with SssI methylase. Native MBD2 was harvest from pcDNA-MBD2 transfected HEK293 cells. EMSA assays were carried out in the presence of SssI treated probes. Lane 1, Negative control (biotin-labeled probe only); lane 2, biotin-labeled probe; and lane 3, 1:200 biotin-labeled probe vs. unlabeled probe (competitive control).

**Figure IX. MBD2 suppresses eNOS promoter reporter activity.**

pGL-eNOS and pGL-eNOSm were first treated with SssI methylase, and then co-transfected with a pcDNA-MBD2 palindrome into HUVECs, respectively. pGL-eNOS and pGL-eNOSm without SssI treatment were used as controls. Luciferase reporter activities were assayed 48h after transfection and were normalized with an internal pGL-Tk reporter.
Reference List


