A mechanistic role for DNA methylation in endothelial cell (EC)-enriched gene expression: relationship with DNA replication timing

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Key Point

- Promoter DNA methylation, an epigenetic process, is functionally relevant for regulating the expression of endothelial cell-enriched genes

Abstract

Proximal promoter DNA methylation has been shown to be important for regulating gene expression. However, its relative contribution to the cell-specific expression of EC-enriched genes has not been defined. We used methyl-DNA immunoprecipitation (MeDIP) and bisulfite conversion to analyze the DNA methylation profile of EC-enriched genes in ECs versus non-expressing cell types, both in vitro and in vivo. We show that prototypic EC-enriched genes exhibit functional, differential patterns of DNA methylation in proximal promoter regions of most (eg. CD31, vWF, VE-cadherin and ICAM-2), but not all (eg. VEGFR-1 and VEGFR-2) EC-enriched genes. Comparable findings were evident in cultured ECs, human blood origin ECs and murine aortic ECs. Promoter-reporter episomal transfection assays for eNOS, VE-cadherin and vWF indicated functional promoter activity in cell types where the native gene was not active. Inhibition of DNA methyltransferase activity indicated important functional relevance. Importantly, profiling DNA replication timing patterns indicated that EC-enriched gene promoters with differentially methylated regions replicate early in S-phase in both expressing and non-expressing cell types. Chromatin-based mechanisms are critical for the transcriptional regulation of EC-enriched genes both in vitro and in vivo. Collectively, these studies highlight the functional importance of promoter DNA methylation in controlling vascular endothelial cell gene expression.

Keywords: endothelium; epigenetics; DNA methylation; transcription
**Introduction**

The functional identity of an endothelial cell (EC) is dictated, in part, by its unique gene expression profile. The application of microarray profiling has reinforced the view that the concept of EC-enriched genes is valid and functionally relevant with respect to cellular phenotype\(^1\)\(^-\)\(^3\). In this regard, epigenetic processes are now appreciated to play a key role in regulating gene transcription. However, the relative contribution in ECs is not well understood\(^4\),\(^5\).

Decreased expression of constitutively active genes in ECs is a key component of endothelial cell dysfunction, such as is observed with endothelial nitric oxide synthase (eNOS) in atherosclerosis\(^6\). Defining whether perturbations of the DNA methylation status of key EC-enriched genes contributes to changes in gene expression and cellular phenotype requires a firm understanding of DNA methylation profiles of these genes under normal conditions. We previously identified a differentially methylated region (DMR) at the proximal promoter of the eNOS/NOS3 gene\(^7\). While non-EC types showed high levels of methylation, a repressive mark associated with transcriptional silencing, ECs lacked DNA methylation in this region. These and other studies have suggested that epigenetics plays an important role in the regulation of gene expression in vascular ECs (reviewed in\(^8\),\(^9\)). DNA methylation also needs to be transmitted faithfully to nascent DNA subsequent to the replication of the genetic code. Generally, early timing of DNA replication in the cell cycle correlates with global gene expression\(^10\), though less is known about whether this paradigm applies to cell-restricted genes, especially within the vascular endothelium.

The contribution of DNA methylation to EC gene regulation remains to be fully explored. We therefore wished to determine whether the epigenetic mechanisms first characterized for eNOS in ECs is a unique feature for eNOS expression, or is also applicable to a repertoire of EC-enriched genes. Discerning the epigenetic state of unique cell types is a key goal of the International Human Epigenomic Consortium (IHEC). Similarly, the Encyclopedia of DNA
Elements (ENCODE) project also aims to delineate functional elements and chromatin signatures for specific cell types\textsuperscript{11}. These genome-based approaches are, using currently existing methodologies, examining these changes in low-resolution. High-resolution data is especially needed. In this present study, we describe both \textit{in vitro} and \textit{in vivo} studies of differential DNA methylation in the proximal promoter regions of the EC-enriched genes \textit{CD31/PECAM1}, \textit{Endoglin/ENG}, \textit{ICAM-2}, P-selectin/\textit{SELP}, Tie-2/\textit{TEK}, \textit{VE-Cadherin/CDH5} and \textit{vWF} in terminally differentiated ECs versus non-ECs, in both humans and mice, and provide evidence that epigenetic modifications are functionally important for EC gene expression. Furthermore, DNA replication timing studies show that EC-enriched genes with DMRs replicate in early S-phase in both ECs and non-ECs. This was an exciting, yet unexpected finding. The aim of the study was to determine the functional relevance of promoter DNA methylation for EC gene expression. Here we describe important features regulating EC-enriched gene expression involving both chromatin-based and cell cycle pathways.

\textbf{Methods}

All animal studies were performed in accordance with the guidelines of the Canadian Council on Animal Care and were approved by the University of Toronto Animal Care Committee. This study was conducted in accordance with the Declaration of Helsinki.

\textbf{Cell Culture}

Human umbilical vein endothelial cells (HUVEC) were isolated from fresh umbilical cords as previously described\textsuperscript{12,13}. Cryopreserved human aortic vascular smooth muscle cells (HuAoVSMC) (ScienCell), human neonatal dermal microvascular endothelial cells (HMVEC), human epidermal keratinocytes, and human hepatocytes (Lonza) were maintained as recommended. Blood outgrowth endothelial cells (BOECs) were obtained from Dr. Hebbel\textsuperscript{14}. Isolation and culturing of BOECs was conducted as previously reported and assayed at passage 3-5, after 6-8 weeks in culture.
Cell Treatments

HUVEC and HuAoVSMC were treated at passages 3-5 with 5-azacytidine (Sigma) at 5 μmol/L every 48 h for 7 d, or a combined treatment of 5-azacytidine and Trichostatin A (TSA) (Sigma), where cells were treated with 5-azacytidine for 7 d, and TSA was added (1 μmol/L) for the final 24 h. For cell cycle analyses, HuAoVSMC were treated with 5-azacytidine (Sigma) at 5 μmol/L for 24 h, 48 h, 72 h or 120 h.

Generation of Methyl 450K Bead Array and ChIP-seq data

DNA methylation 450K Bead Array data were generated by R. Myers and D. Absher labs at the HAIB (HudsonAlpha Institute for Biotechnology, Huntsville, AL) and HUVEC Pol2 ChIP-seq data were generated by the M. Snyder lab at Stanford University (Stanford University, Stanford, CA). These data were collected as part of the ENCODE consortium (http://www.genome.gov/10005107) and is available on the University of California, Santa Cruz Genome Browser. RepeatMasker data was generated by Arian Smit’s RepeatMasker program (http://www.repeatmasker.org).

BrdU Labeling and Flow Activated Cell Sorting (FACS)

Asynchronous populations of HUVEC and HuAoVSMC were pulse labeled with 5-bromo-2'-deoxyuridine (BrdU) as previously described. Briefly, BrdU (Sigma) was added to cells (50 μM) and incubated for 1 h at 37°C. Equal numbers of cells (50,000) were sorted into cell cycle fractions based on DNA content (G1, S1, S2, S3, S4, G2). Sorted cells were collected directly into lysis buffer and DNA was extracted using the DNeasy Blood and Tissue Kit (Qiagen). 20 μg of salmon sperm DNA (Invitrogen) and 100,000 copies of BrdU-labeled E. coli DNA was added to each fraction prior to extraction to control for DNA recovery and immunoprecipitation efficiency. DNA was eluted in 500 μL TE. BrdU-labeled cells and extracted DNA was manipulated in the dark to prevent photolysis of BrdU-incorporated DNA.
**Sodium Bisulfite Genomic Sequencing and Pyrosequencing**

Genomic DNA from human cultured cells (5 μg) was subjected to sodium bisulfite treatment as described previously\(^7,16,17\). 500 ng of murine genomic DNA was bisulfite converted using EZ DNA Methylation-Direct™ kit (Zymo Research, Irvine, CA) and subjected to nested PCR amplification (Table S3). For pyrosequencing analysis, 10 pmol of primers per reaction and a biotinylated reverse primer was used (Tables S2-S3, EpigenDx Inc.).

**Statistics**

Unless otherwise stated, all experiments were performed a minimum of three times, and data represent the mean ± S.E.M. Statistical analyses were performed using a Student’s *t* test or analysis of variance, as appropriate. A *p* value less than 0.05 was considered to be statistically significant.

**Detailed methods are described in the Online Supplemental Material.**

**Results**

*EC-enriched Gene Promoters are Differentially Methylated in Human ECs Versus Non-ECs*  
*Types: Low-Resolution and High-Resolution Mapping*

We first analyzed promoter DNA methylation for key EC-enriched genes using data generated by the ENCODE consortium\(^11\). We utilized Methyl 450K Bead Array data to define regions of methylation at proximal promoters of key EC-enriched genes in ECs and non-ECs. The region surrounding the start of transcription displayed no methylation in HUVEC, while the same region was partially methylated or methylated in AoSMC or hepatocytes, for the genes *NOS3/eNOS* and *CDH5/VE-Cadherin* (Figure 1). Regions of differential methylation were not located within repetitive elements. Importantly, unmethylated regions corresponded to an enrichment in Pol2 binding in HUVEC and the transcriptional start site (TSS) of the gene (Figure
1). Analysis of downstream coding regions of eNOS and VE-cadherin did not reveal other DMRs in ECs vs. non-ECs (data not shown).

We used high resolution assays to examine DNA methylation of promoter regions around the TSS of EC-enriched genes using methyl-DNA immunoprecipitation (MeDIP), followed by qPCR, a robust method for assessing DNA methylation at specific loci. The genomic regions analyzed and citations used to determine the TSS are summarized in Table S4. MeDIP analysis of several EC-enriched genes demonstrated clear DNA methylation differences between HUVEC and HuAoVSMC. The EC-enriched genes eNOS, VE-Cadherin, CD31 and vWF showed higher relative levels of promoter methylation in HuAoVSMC compared to HUVEC (Figure 2A-D).

Using gold standard, high-resolution sodium bisulfite sequencing and single strand DNA analysis or quantitative pyrosequencing in EC and non-EC types, we confirmed our screen of promoter methylation of EC-enriched genes. Consistent with MeDIP assays, bisulfite analyses identified robust differences in DNA methylation between HUVEC and HuAoVSMC, at proximal promoter regions of EC-enriched genes. Pyrosequencing analyses for CD31 in the EC types HUVEC, HMVEC and BOEC, and in non-EC types HuAoVSMC, human saphenous vein vascular smooth muscle cells (HuSVVSMC), keratinocytes and hepatocytes was conducted. CD31 displayed very low levels of DNA methylation in all EC types, while high levels of methylation were observed in non-ECs (Figure 3A). The findings from quantitative pyrosequencing assay are confirmed by single strand analysis results (Figure S2).

Genomic regions corresponding to exon 1 of vWF are known to be functionally important. The 4 CpG sites downstream of the vWF TSS in exon 1 were assessed and low levels of methylation in HUVEC and HMVEC were found, in comparison to dense methylation in the non-EC HuAoVSMC, HuSVVSMC, keratinocytes and hepatocytes (Figure 3B). The eNOS promoter also showed dense methylation in both hepatocytes and keratinocytes (Figure S3A). Methylation patterns in the ICAM2 promoter was similar to other EC-enriched genes, as HUVEC
were 0% methylated and HuAoVSMC were densely methylated, apart from two CpG sites at -24 and -12 (Figure S3C). The EC-enriched genes VE-Cadherin and Tie2 showed no methylation in HUVEC, whereas high levels of DNA methylation were observed in HuAoVSMC (Figure 3C-D). Differential methylation of VE-Cadherin was restricted to a region downstream of the TSS, with methylation in HuAoVSMC observed at positions +103 to +229 (Figure 3C). Though there were low levels of DNA methylation seen in the proximal promoter of the Tie2 gene, clear differences in DNA methylation across cell types was evident (Figure 3D).

In contrast to the other EC-enriched genes examined where genomic regions surrounding the TSS evidenced no DNA methylation, P-selectin displayed low but evident levels of methylation in HUVEC. In contrast, dense methylation was seen in HuAoVSMC (Figure S3D).

Murine EC-enriched Genes are Differentially Methylated in EC and non-EC Types

We examined murine promoters in vivo, namely the EC-enriched genes eNOS, CD31, VE-cadherin and vWF, in descending thoracic aortic EC and descending thoracic AoVSMC. We used pyrosequencing analysis of bisulfite-converted DNA to assess the methylation status and found that eNOS, VE-Cadherin, CD31 and vWF gene promoters were hypomethylated in EC and heavily methylated in AoVSMC (Figure 4), consistent with our findings in human ECs and non-ECs. Therefore, the DMRs of EC-enriched genes identified in cultured human cells are in agreement with our in vivo methylation analyses in the mouse.

EC-enriched Gene Promoters are Not Methylated in Human Blood Outgrowth ECs

We wished to determine the methylation in a cell type capable of initiating stable outgrowth ECs. Blood outgrowth endothelial cells (BOEC) are an EC type, derived from cultured peripheral blood and are capable of establishing mature outgrowth ECs14. We used well-validated methods to isolate and characterize these cells, as previously described14. Since eNOS, as well as the EC-enriched genes CD31 and vWF, are fundamental to the proper functioning of BOEC, we
were interested in determining the DNA methylation status of these EC genes along this EC differentiation cascade. Using quantitative pyrosequencing of bisulfite-converted DNA from blood outgrowth EC, the promoters of eNOS, vWF and CD31 were analyzed. All EC-enriched genes displayed hypomethylation in BOEC (Figure 3A-B and Figure S3A,C-D), displaying a methylation pattern similar to mature ECs.

Promoter Activity of EC-enriched Genes in Non-EC Types

We have previously demonstrated robust expression of episomal eNOS promoter/reporter constructs in cell types in which eNOS is not normally expressed. Surprisingly, these same genomic regions exhibited exquisite cell-specificity when stably integrated into the genome in insertional transgene murine promoter/reporter studies. This implied that eNOS episomal vectors or naked DNA did not faithfully recapitulate expression of the native gene, which highlighted functional relevance of chromatin-based mechanisms. We used episomal constructs containing the regions -487/+247 and -1912/+1, of human vWF and VE-cadherin, respectively, given that the same regions are very EC-enriched in insertional promoter transgenes. These episomal constructs were transiently transfected into ECs and non-EC types that do, or do not express eNOS, vWF or VE-cadherin, respectively (Table 1; Figure S1). These data indicate that VE-cadherin and vWF promoter reporter espisomal constructs are transcriptionally active even when the native chromatin-based genes are not, as we previously noted for eNOS.

Functional Role of Methylation in Repressing EC-enriched Genes in non-EC Types

The presence of DNA methylation at proximal promoter regions of these EC-enriched genes suggested that this chromatin mark may silence the expression of these genes in non-expressing cell types. Re-expression of epigenetically silenced genes can be achieved by inhibiting DNA methyltransferase or histone deacetylase (HDAC) activity. Treatment with 5-azacytidine, a DNA methyltransferase inhibitor, alone or in combination with TSA, an inhibitor of
HDAC activity, can be sufficient to reactivate some genes\textsuperscript{23}. For other genes, combined treatment of 5-azacytidine and TSA results in synergistic activation as compared to each treatment alone\textsuperscript{24,25}. To demonstrate a functional role for DNA methylation at the proximal promoter regions of EC-enriched genes in non-ECs, we treated both HUVEC and HuAoVSMC with 5-azacytidine, alone or in combination with TSA. The EC-enriched genes eNOS and vWF showed modest upregulation in mRNA expression in HuAoVSMC when treated with 5-azacytidine alone (Figure 5A,D). CD31 exhibited a robust response to long-term treatment with 5-azacytidine (Figure 5C). The greatest increase in expression was observed when cells were treated in combination with 5-azacytidine and TSA (Figure 5A-D). Manipulation of epigenetic processes is sufficient to induce expression of normally repressed EC-enriched genes in non-ECs. Collectively, these data indicate a functional role for promoter methylation in the repression of EC-enriched genes in a non-EC type like HuAoVSMC.

**VEGFR-1 and VEGFR-2 Promoters Are Not Differentially Methylated in ECs Versus Non-EC Types**

Surprisingly, not all EC-enriched genes examined were differentially methylated between HUVEC and HuAoVSMC. The promoter region of VEGFR2 did not display an enrichment of methylation in HuAoVSMC, as determined by MeDIP analysis (Figure 2E). The absence of proximal promoter methylation was confirmed by examining Methyl 450K Bead Array data at the promoters of VEGFR1 and VEGFR2 in ECs and non-ECs, in addition to single strand DNA analysis and pyrosequencing methods (Figure S4A-D). Bisulfite analysis demonstrated that the VEGFR2 promoter was also not methylated in keratinocytes or hepatocytes (Figure S4D). Importantly, VEGFR2 did not show any changes in expression when treated either with 5-azacytidine alone, or in combination with TSA (Figure 5E). These findings suggest that DNA methylation is not important for the transcriptional regulation of VEGFR1 and VEGFR2.
eNOS is Not Hydroxymethylated in ECs and non-ECs

The recent discovery of 5-hydroxymethylcytosine (5hmC) as a modified base in mammalian DNA, catalyzed by the TET family of proteins, has led to newer insight into the epigenetic regulation of genes\(^{26}\). The modified cytosine species 5hmC and 5-methylcytosine (5mC) are indistinguishable upon sodium bisulfite conversion and importantly, DNA containing 5hmC is not efficiently amplified by PCR following bisulfite conversion\(^{27}\). As such, using an antibody directed against 5-hydroxymethylcytosine in a technique similar to MeDIP, or hydroxymethyl-DNA immunoprecipitation (OH-MeDIP), we analyzed the promoter region of the EC-enriched genes eNOS, CD31, VE-cadherin and vWF in HUVEC (Figure S5). In contrast to robust levels of methylation at the promoter of eNOS in HuAoVSMC, we failed to detect appreciable steady-state levels of hydroxymethylation at the eNOS promoter in this same cell type (Figure S5A). The same was true for the promoters of CD31, VE-cadherin and vWF (Figure S5B-D). We can therefore be confident the observed bisulfite findings addresses 5-methylcytosine and not 5-hydroxymethylcytosine.

Differentially Methylated EC-enriched Genes Replicate in Early S-phase in ECs and non-ECs

Though DNMT1 is thought to localize to the replication fork during S-phase\(^{28}\), how methylation patterns on newly replicated DNA at cell-specific genes is poorly understood. It is generally accepted that transcriptionally active genes replicate early, while inactive genes replicate late, during S-phase of the cell cycle. This principle was reaffirmed in recent whole genome analyses\(^{29}\). For example, the \(\beta\)-globin gene replicates late in non-erythroid cells and early in erythroid cells\(^{30}\), while the \(\alpha\)-globin gene replicates early in both cell types\(^{29}\). Since patterns of gene expression and alterations in the cell cycle are concomitant processes in vascular pathobiology, we argue that we need to learn more about these processes. It is surprising how little is known about DNA replication timing of tissue-specific genes, especially within the vascular endothelium.
To investigate the DNA replicating timing patterns of EC-enriched genes, we used a BrdU-pulse labeling and qPCR-based approach\textsuperscript{29}. Cyclophilin A, a housekeeping gene expressed in both ECs and non-ECs, exhibited early S-phase replication, while β-globin, which is expressed specifically in erythrocytes and not transcriptionally active in ECs and non-ECs, exhibited late S-phase replication (Figure S6C-D), as shown by others\textsuperscript{30}. We next determined the timing of DNA replication for eNOS in both expressing and non-expressing cell types. Both the proximal promoter and exon 14 region (~13 kb downstream of the TSS) of the \textit{NOS3} locus displayed early S-phase replication in HUVEC and HuAoVSMC (Figure 6B-C). This surprising finding provides evidence that the entire eNOS gene replicates early in both ECs and non-ECs. This was not expected. Replication timing analyses determined that CD31 and ICAM2 both displayed early S-phase replication in both ECs and non-ECs (Figure 6D-E), as observed with eNOS. Again, these findings were unexpected, since generally, early DNA replication timing correlates with gene expression\textsuperscript{10}. Interestingly, the EC-enriched genes VEGFR-1 and VEGFR-2 displayed early S-phase (S1) replication in HUVEC, and late S-phase (S2-S3) replication in a non-expressing cell type (Figure 6F-G). As stated, these were the only EC-enriched genes that did not display differential DNA methylation between ECs and non-ECs (Figure S4).

\textbf{The Newly Replicated eNOS Promoter has Low Levels of DNA Methylation in Early S-phase}

Since we determined that EC-enriched genes replicate in early S-phase in ECs and non-ECs, we were motivated to determine when in S-phase the promoter region of eNOS becomes methylated in HuAoVSMC. Site-specific methylation analysis at the proximal promoter of eNOS displayed an average 40% methylation in G1 and S1 phases (Figure S7A). In S2, methylation levels were observed to be double that of S1. For the remainder of S-phase, methylation levels at eNOS were comparable to the methylation level of a whole dish of cells (Figure S7A). The levels of eNOS methylation that is observed in terminally differentiated cells (Figure S3A) does not occur until late S-phase. These results indicate that while the replication timing of eNOS
occurs in S1 phase, there is a measurable lag in re-methylation of the nascent strand of the hemimethylated DNA duplex. To address a possible cause or effect relationship, we treated AoVSMC with 5-azacytidine for 24 hr, and profiled the timing of eNOS replication. It was evident that the timing of eNOS replication remained in early S-phase (Figure S7B). Thus, inhibition of DNA methylation at the eNOS promoter did not affect the replication timing of eNOS. Not all cells in S-phase are dynamically proceeding through DNA replication. We then asked whether AoVSMC arrested in S-phase have a distinct DNA methylation profile. As shown in Figure S8B, the eNOS DNA methylation profile did not differ between cells proceeding or arrested in S-phase.

**Discussion**

This report defines that the majority of endothelial cell (EC)-enriched genes show evidence of differential DNA methylation of proximal promoter regions. These genomic regions, which encompass the start sites of transcription, are unmethylated in endothelial cells that express these mRNAs, and are methylated in non-expressing cell types, such as HuAoVSMC, keratinocytes and hepatocytes. We identified differentially methylated regions (DMRs) in the EC-enriched genes PECAM1/CD31, vWF, VE-cadherin, ICAM-2, P-selectin, Endoglin and Tie2. Importantly, our *in vivo* studies of murine EC-enriched gene promoters recapitulated our findings. Use of DNA methylation inhibitors indicated that DNA methylation is functionally relevant. Inhibition of global DNA methylation, either alone or combined with inhibitors of HDAC activity, led to increases in expression of select mRNAs in cell types that do not basally express these mRNAs. We infer that DNA methylation actively represses transcription of these transcripts in non-expressing cell types. This is an important concept given that most models of cell-specific gene expression focus on what activates or enhances transcription in expressing cell types. This may help explain the promiscuous activity of episomal based promoter-reporter
constructs for EC-enriched genes in non-EC types, which do not normally express the native gene in the context of chromatin.

We find that for the majority of EC-enriched genes that contain DMRs in their proximal promoter, DNA replication timing occurs in early S-phase in both ECs and non-ECs. This finding is not consistent with the general paradigm of replication timing patterns and gene expression. Importantly, the majority of studies are based on global approaches of replication timing analyses, and focus on developmentally controlled and tissue-specific genes that show differential DNA replication timing profiles between cell types\(^{31,32}\). We examined publicly available data on replication timing in embryonic stem (ES) cells, neural progenitor cells and lymphoblasts, and noted early replication timing for eNOS\(^{33}\) (www.replicationdomain.org). We found that eNOS is replicated early in the cell cycle and there is a small, but discernible, delay in DNA methylation. Early replication of a locus with a DMR may allow sufficient time for accurate methylation inheritance after each cell division, ensuring the faithful transmission of epigenetic signatures at tissue-specific genes. Our novel findings indicate that future studies in this area are now needed.

Our work highlights that for some genes (eNOS, vWF, CD31, ICAM2 and P-selectin), very high levels of DNA methylation were observed in non-expressing cell types, while for other genes (VE-cadherin, tie2 and endoglin), lower levels of DNA methylation are associated with gene silencing. In general, the relationship between the absolute levels of DNA methylation and its functional effects are not well understood. Another important point relates to the basal presence of DNA methylation in expressing cell types. The promoters for eNOS, vWF, Endoglin, Tie2, ICAM2 and CD31 are nearly completely unmethylated in EC. In contrast, P-selectin is partially methylated in ECs. The functional relevance of this basal methylation of P-selectin and whether it represents population heterogeneity remains to be defined.

We demonstrated that VEGFR1 and VEGFR2 did not contain DMRs in their proximal promoters. In disease settings, these genomic regions can be methylated. For instance, the
VEGFR2 promoter and exon 1 was reported to be methylated in prostate cancer and upon ischemic injury\textsuperscript{34,35}. Interestingly, VEGFR1 and VEGFR2 are among the earliest EC-enriched genes to be expressed in vascular development. Expression of VEGFR1 and VEGFR2 is first detected at ED6.5\textsuperscript{36}, and ED7.0\textsuperscript{37}, respectively. This contrasts with the later onset of other endothelial enriched genes - Tie2 (ED7.5)\textsuperscript{37}, VE-Cadherin (ED8.5)\textsuperscript{38} and vWF (ED8.5)\textsuperscript{39}. The expression of eNOS occurs even later, at ED9.5, after the establishment of robust unidirectional flow\textsuperscript{20}. Therefore, an intriguing hypothesis is that the timing of onset expression during vascular development may be an important determinant of the presence of differential methylation in EC-enriched genes.

We found that VEGFR1 and VEGFR2 replicated early in expressing cell types and late in non-expressing cell types (Figure 6). These two EC-enriched genes also did not contain differentially methylated regions in their proximal promoter regions. Studies have shown that genes with high absolute CpG-density at the promoter replicate early in mammalian cells\textsuperscript{40}. High CpG-density regions can also represent CpG islands if the observed/expected ration is high. In this regard, the promoter regions of VEGFR1 and VEGFR2 represent CpG islands. Interestingly, when the areas around the TSS of all EC-enriched genes were analyzed for the presence of CpG islands, only VEGFR1 and VEGFR2 showed an observed/expected ratio of a strong CpG island (>0.75), whereas the other EC-enriched genes were classified as poor CpG islands (Table S4). The relationship between the presence or absence of DNA methylation and timing of onset of gene expression in development, DNA replication timing in the mitotic cell cycle and the presence of CpG islands requires further study.

We assayed the methylation status of EC-enriched gene promoters in blood outgrowth endothelial cells and found comparable findings to results in HUVEC and HMVEC. This is an important finding, as BOEC have proved to be valuable for several therapeutic applications, including the ability to home to sites within the tumour neovasculature for gene delivery\textsuperscript{41,42}. Interestingly, recent studies by others have shown that in early proangiogenic cells, the eNOS
promoter is epigenetically silenced. These early proangiogenic cells are distinguishable from BOEC, especially as the DNA methylation profile of EC-enriched genes in BOEC are similar to our findings in mature vessel wall ECs.

Taken together, our findings demonstrate the first studies of promoter DNA methylation and the regulation of EC-enriched gene expression in both endothelial outgrowth cells and mature endothelial cells. We have provided evidence for the role of 5-methylcytosine in regulating the expression of a number of EC-enriched genes within the vascular endothelium. Importantly, the novel concept introduced here is that EC-enriched genes are transcriptionally repressed in non-expressing cell types by epigenetic mechanisms. These studies provide newer insight into the importance of epigenetic processes within the vascular endothelium. Given that chromatin-based pathways provide a newly appreciated mechanism by which environmental or exogenous stimuli can interact with the static DNA code, there may be translational implications for these findings. For example, hemodynamic stimuli influence EC gene expression in blood vessels. Whether hemodynamic forces modify gene expression in EC, in part, via chromatin-based pathways needs further study. However, a role for changes in 5-methylcytosine content in diseases of the vascular endothelium remains to be defined.
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Authorship

Contribution: A.V.S., R.S.B., A.G., A.K., B.J.K., M.Y., H-S.J.M. and M.S. performed experiments and collected data; R.P.H., P.O. and W.C.A. provided reagents, performed select experiments and gave critical intellectual input; A.V.S. and P.A.M. designed the research and wrote the paper.

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References

1. Chi JT, Chang HY, Haraldsen G, et al. Endothelial cell diversity revealed by global expression profiling. *Proc Natl Acad Sci U S A*. 2003;100(19):10623-10628.
2. Aird WC. Phenotypic heterogeneity of the endothelium: I. Structure, function, and mechanisms. *Circ Res*. 2007;100(2):158-173.
3. Bhasin M, Yuan L, Keskin DB, Otu HH, Libermann TA, Oettgen P. Bioinformatic identification and characterization of human endothelial cell-restricted genes. *BMC Genomics*. 2010;11:342.
4. Lister R, Pelizzola M, Dowen RH, et al. Human DNA methylomes at base resolution show widespread epigenomic differences. *Nature*. 2009;462:315-322.
5. Irizarry RA, Ladd-Acosta C, Wen B, et al. The human colon cancer methylome shows similar hypo- and hypermethylation at conserved tissue-specific CpG island shores. *Nat Genet*. 2009;41(2):178-186.
6. Wilcox JN, Subramanian RR, Sundell CL, et al. Expression of multiple isoforms of nitric oxide synthase in normal and atherosclerotic vessels. *Arterioscler Thromb Vasc Biol*. 1997;17(11):2479-2488.
7. Chan Y, Fish JE, D'Abreo C, et al. The cell-specific expression of endothelial nitric-oxide synthase: a role for DNA methylation. *J Biol Chem*. 2004;279(33):35087-35100.
8. Matouk CC, Marsden PA. Epigenetic regulation of vascular endothelial gene expression. *Circ Res*. 2008;102(8):873-887.
9. Shirodkar AV, Marsden PA. Epigenetics in cardiovascular disease. *Curr Opin Cardiol*. 2011;26(3):209-215.
10. White EJ, Emanuelsson O, Scalfio D, et al. DNA replication-timing analysis of human chromosome 22 at high resolution and different developmental states. *Proc Natl Acad Sci U S A*. 2004;101(51):17771-17776.
11. A user’s guide to the encyclopedia of DNA elements (ENCODE). *PLoS Biol*. 9(4):e1001046.
12. Marsden PA, Schappert KT, Chen HS, et al. Molecular cloning and characterization of human endothelial nitric oxide synthase. *FEBS Lett*. 1992;307(3):287-293.
13. Flowers MA, Wang Y, Stewart RJ, Patel B, Marsden PA. Reciprocal regulation of endothelin-1 and endothelial constitutive NOS in proliferating endothelial cells. *Am J Physiol*. 1995;269(6):H1988-1997.
14. Lin Y, Weisendorf DJ, Solovey A, Hebbel RP. Origins of circulating endothelial cells and endothelial outgrowth from blood. *J Clin Invest*. 2000;105(1):71-77.
15. Azuara V. Profiling of DNA replication timing in unsynchronized cell populations. *Nat Protoc*. 2006;1(4):2171-2177.
16. Chan GC, Fish JE, Mawji IA, Leung DD, Rachlis AC, Marsden PA. Epigenetic basis for the transcriptional hyporesponsiveness of the human inducible nitric oxide synthase gene in vascular endothelial cells. *J Immunol*. 2005;175(6):3846-3861.
17. Fish JE, Matouk CC, Rachlis A, et al. The expression of endothelial nitric-oxide synthase is controlled by a cell-specific histone code. *J Biol Chem*. 2005;280(26):24824-24838.
18. Weber M, Davies JJ, Wittig D, et al. Chromosome-wide and promoter-specific analyses identify sites of differential DNA methylation in normal and transformed human cells. *Nat Genet*. 2005;37(8):853-862.
19. Guillot PV, Liu L, Kuivenhoven JA, Guan J, Rosenberg RD, Aird WC. Targeting of human eNOS promoter to the Hprt locus of mice leads to tissue-restricted transgene expression. *Physiol Genomics*. 2000;2(2):77-83.
20. Teichert AM, Scott JA, Robb GB, et al. Endothelial nitric oxide synthase gene expression during murine embryogenesis: commencement of expression in the embryo occurs with the establishment of a unidirectional circulatory system. *Circ Res*. 2008;103(1):24-33.
21. Jahroudi N, Lynch DC. Endothelial-cell-specific regulation of von Willebrand factor gene expression. *Mol Cell Biol*. 1994;14(2):999-1008.
22. Prandini MH, Dreher I, Bouillot S, Benkerri S, Moll T, Huber P. The human VE-cadherin promoter is subjected to organ-specific regulation and is activated in tumour angiogenesis. *Oncogene*. 2005;24(18):2992-3001.
23. Coombes MM, Briggs KL, Bone JR, Clayman GL, El-Naggar AK, Dent SY. Resetting the histone code at CDKN2A in HNSCC by inhibition of DNA methylation. *Oncogene*. 2003;22(55):8902-8911.
24. Zhang Y, Fatima N, Dufau ML. Coordinated changes in DNA methylation and histone modifications regulate silencing/derepression of luteinizing hormone receptor gene transcription. *Mol Cell Biol*. 2005;25(18):7929-7939.
25. Cameron EE, Bachman KE, Myohanen S, Herman JG, Baylin SB. Synergy of demethylation and histone deacetylase inhibition in the re-expression of genes silenced in cancer. *Nat Genet*. 1999;21(1):103-107.
26. Tahiliani M, Koh KP, Shen Y, et al. Conversion of 5-methylcytosine to 5-hydroxymethylcytosine in mammalian DNA by MLL partner TET1. *Science*. 2009;324(5929):930-935.
27. Huang Y, Pastor WA, Shen Y, Tahiliani M, Liu DR, Rao A. The behaviour of 5-hydroxymethylcytosine in bisulfite sequencing. *PLoS One*. 2010;5(1):e8888.
28. Goll MG, Bestor TH. Eukaryotic cytosine methyltransferases. *Annu Rev Biochem*. 2005;74:481-514.
29. Azuara V, Perry P, Sauer S, et al. Chromatin signatures of pluripotent cell lines. *Nat Cell Biol*. 2006;8(5):532-538.
30. Ginder GD, Gnanapragasam MN, Mian OY. The role of the epigenetic signal, DNA methylation, in gene regulation during erythroid development. *Curr Top Dev Biol*. 2008;82:85-116.
31. Perry P, Sauer S, Billon N, et al. A dynamic switch in the replication timing of key regulator genes in embryonic stem cells upon neural induction. *Cell Cycle*. 2004;3(12):1645-1650.
32. Kitsberg D, Selig S, Keshet I, Cedar H. Replication structure of the human beta-globin gene domain. *Nature*. 1993;366(6455):588-590.
33. Weddington N, Stuy A, Hiratani I, Ryba T, Yokochi T, Gilbert DM. ReplicationDomain: a visualization tool and comparative database for genome-wide replication timing data. *BMC Bioinformatics*. 2008;9:530.
34. Yamada Y, Watanabe M, Yamanaka M, et al. Aberrant methylation of the vascular endothelial growth factor receptor-1 gene in prostate cancer. *Cancer Sci*. 2003;94(6):536-539.
35. Rao X, Zhong J, Zhang S, et al. Loss of methyl-CpG-binding domain protein 2 enhances endothelial angiogenesis and protects mice against hind-limb ischemic injury. *Circulation*. 2011;123(25):2964-2974.
36. Fong GH, Klingensmith J, Wood CR, Rossant J, Breitman ML. Regulation of flt-1 expression during mouse embryogenesis suggests a role in the establishment of vascular endothelium. *Dev Dyn*. 1996;207(1):1-10.
37. Dumont DJ, Fong GH, Puri MC, Gradwohl G, Alitalo K, Breitman ML. Vascularization of the mouse embryo: a study of flk-1, tek, tie, and vascular endothelial growth factor expression during development. *Dev Dyn*. 1995;203(1):80-92.
38. Breier G, Breviario F, Caveda L, et al. Molecular cloning and expression of murine vascular endothelial-cadherin in early stage development of cardiovascular system. *Blood*. 1996;87(2):630-641.
39. Dumont DJ, Yamaguchi TP, Conlon RA, Rossant J, Breitman ML. tek, a novel tyrosine kinase gene located on mouse chromosome 4, is expressed in endothelial cells and their presumptive precursors. *Oncogene*. 1992;7(8):1471-1480.
40. Hiratani I, Ryba T, Itoh M, et al. Global reorganization of replication domains during embryonic stem cell differentiation. *PLoS Biol*. 2008;6(10):e245.

41. Lin Y, Chang L, Solovey A, Healey JF, Lollar P, Hebbel RP. Use of blood outgrowth endothelial cells for gene therapy for hemophilia A. *Blood*. 2002;99(2):457-462.

42. Jevremovic D, Gulati R, Hennig I, et al. Use of blood outgrowth endothelial cells as virus-producing vectors for gene delivery to tumors. *Am J Physiol Heart Circ Physiol*. 2004;287(2):H494-500.

43. Ohtani K, Vlachojannis GJ, Koyanagi M, et al. Epigenetic Regulation of Endothelial Lineage Committed Genes in Pro-Angiogenic Hematopoietic and Endothelial Progenitor Cells. *Circ Res*. 2011;109(11):1219-29.

44. Webster AL, Yan MS, Marsden PA. Epigenetics and cardiovascular disease. *Can J Cardiol*. 2013;29(1):46-57.

45. Won D, Zhu SN, Chen M, et al. Relative reduction of endothelial nitric-oxide synthase expression and transcription in atherosclerosis-prone regions of the mouse aorta and in an in vitro model of disturbed flow. *Am J Pathol*. 2007;171(5):1691-1704.

46. Meyer LR, Zweig AS, Hinrichs AS, et al. The UCSC Genome Browser database: extensions and updates 2013. *Nucleic Acids Res*. 2012;41(D1):D64-69.

47. Gulati R, Jevremovic D, Peterson TE, et al. Diverse origin and function of cells with endothelial phenotype obtained from adult human blood. *Circ Res*. 2003;93(11):1023-1025.

48. Hirschi KK, Ingram DA, Yoder MC. Assessing identity, phenotype, and fate of endothelial progenitor cells. *Arterioscler Thromb Vasc Biol*. 2008;28(9):1584-1595.
Table Legend

**TABLE 1. Promoter activity of EC-enriched genes in ECs and non-ECs.**

The episomal constructs eNOS pGL3-1193/+109, vWF pGL3-487/+247 and VE-Cadherin pGL3-1912/+1 were transiently transfected into the ECs HCAEC (human coronary artery endothelial cells), HPAEC (human pulmonary artery endothelial cells), HUVEC (human umbilical vein endothelial cells) and non-EC types HeLa, HEK293 and HuAoVSMC (human aortic vascular smooth muscle cells). Shown are the fold increases in luciferase expression of eNOS pGL3-1193/+109, vWF pGL3-487/+247 and VE-Cadherin pGL3-1912/+1 constructs. Studies are controlled for transfection efficiency across cell types, as previously demonstrated. Steady-state levels of eNOS, VE-Cadherin and vWF mRNAs were not detectable in HuAoVSMC, as determined by RT-qPCR (Figure S1), in contrast to the observed in vitro activity of episomal EC-enriched promoter/reporter constructs. The eNOS pGL3-1193/+109 episomal construct was active in the non-EC types HeLa, HEK293 and HuAoVSMC, as previously described. Similar to eNOS, the VE-Cadherin pGL3-1912/+1 construct demonstrated a 5.17 ± 0.45 and 6.95 ± 0.56 fold increase in expression when transiently transfected into HEK293 and HeLa cell types, respectively. Robust expression was also demonstrated in all EC types. Interestingly, the vWF pGL3-487/+247 construct also demonstrated expression when transfected into the non-ECs HeLa, HEK293 and HuAoVSMC (1.49 ± 0.15, 2.72 ± 0.29, 1.02 ± 0.09, respectively), as well as in the three EC types assayed.
## Table 1. Promoter activity of EC-enriched genes in EC and non-EC

| Cell Type   | Empty vector | pGL3-VE Cadherin-1912/+1* | pGL3-vWF-487/+247* | pGL3-eNOS-1193/+109* |
|-------------|--------------|---------------------------|--------------------|----------------------|
| HeLa        | 1.00 ± 0.09  | 6.95 ± 0.56               | 1.49 ± 0.15        | 3.72 ± 0.06          |
| HEK293      | 1.00 ± 0.08  | 5.17 ± 0.45               | 2.72 ± 0.29        | 1.95 ± 0.11          |
| HuAoVSMC    | 1.00 ± 0.07  | 1.69 ± 0.10               | 1.02 ± 0.09        | 3.91 ± 0.45          |
| HPAEC       | 1.00 ± 0.20  | 13.12 ± 1.07              | 3.66 ± 0.40        | 6.50 ± 0.10          |
| HCAEC       | 1.00 ± 0.05  | 9.59 ± 0.63               | 4.00 ± 0.51        | 9.80 ± 0.92          |
| HUVEC       | 1.00 ± 0.06  | 22.89 ± 1.05              | 11.55 ± 0.86       | 10.90 ± 0.77         |

*Data expressed as luciferase units, relative to empty vector for each cell type. Shown is the mean ± S.E.M (n=3).
FIGURE LEGENDS

FIGURE 1. DNA methylation profiles at EC-enriched gene promoters. Promoter DNA methylation profiles of A) NOS3/eNOS and B) CDH5/VE-Cadherin genes in HUVEC, AoSMC and Hepatoctye. Levels of methylation are colour-coded, where blue represents unmethylation, purple represents partial methylation and orange represents full methylation, as identified by the Illumina Infinium Human Methylation 450K Bead Array platform. Regions of repetitive elements are indicated in black, identified by the RepeatMasker program. Signal enrichment of Pol2 ChIP-seq data in HUVEC is shown in orange, with the max signal indicated on the y-axis. Black arrow denotes the transcriptional start site. The UCSC Genome Browser was used to generate the track displays46.

FIGURE 2. MeDIP analysis of EC and non-EC enriched genes. Relative methylation levels as determined by methyl-DNA IP (MeDIP) in HUVEC and AoVSMC. EC-enriched genes A) eNOS, B) VE Cadherin, C) CD31 and D) vWF display greater levels of methylation in AoVSMC relative to HUVEC. E) VEGFR2 is hypomethylated in both HUVEC and AoVSMC. F) iNOS is known to be methylated in both cell types, which is observed here. G) E-Cadherin serves as a positive control for promoter methylation, as it is not expressed in either cell type. H) Cyclophilin A is expressed in both cell types, and hypomethylation is evident in both HUVEC and AoVSMC. IP’d DNA was normalized to total input DNA for each cell type and gene and is expressed in arbitrary units (au). Data represents mean ± SEM (n=3). * denotes statistical significance at p <0.05 of HUVEC compared to AoVSMC.
FIGURE 3. EC-enriched genes are methylated in non-ECs. Promoter methylation of the EC-enriched genes A) CD31, B) vWF, C) VE-Cadherin and D) Tie-2. EC types HUVEC (orange), HMVEC (yellow) and BOEC (red) are compared to non-EC types AoVSMC (light green), SVVSMC (dark green), keratinocytes (blue) and hepatocytes (purple). BOEC exhibit a cobblestone-shape, have high proliferative capacity, take up acetylated low density lipoprotein and are uniformly positive for several endothelial markers. BOEC are expanded from small numbers of cells after long-term culture, and are distinct from the early outgrowth colonies obtained after 4-7 days in culture, as reviewed by others. Quantitative pyrosequencing (A and B) and single strand analysis (n ≥ 15) (C and D) of bisulfite-converted DNA was used to assess methylation. Extensive mixing studies of in vitro methylated or mock-methylated templates revealed that pyrosequencing a PCR product cannot distinguish 0% from 5% methylation, or 100% from 95% methylation. Single strand plasmid clone analysis indicates that CpG sites are not methylated in ECs for these genes.

FIGURE 4. Differential promoter DNA methylation of EC-enriched genes in mouse EC and VSMC. Promoter methylation was determined using pyrosequencing of bisulfite-converted DNA. The promoters of A) eNOS, B) CD31, C) VE-cadherin and D) vWF are unmethylated in EC (grey) and densely methylated in VSMC (black). Data is presented as the mean ± SEM (n=3-7 mice studied per gene). * denotes statistical significance between the two cell types (p<0.001). Previous studies by us show that the nine CpG sites in the murine eNOS promoter (-199/-4) are completely unmethylated in ECs, while complete methylation was observed in AoVSMC.
FIGURE 5. Functional role of promoter DNA methylation of EC-enriched genes in AoVSMC. RT-qPCR analysis of mRNA levels in HUVEC and AoVSMC treated with either control (Ctl), 5-azacytidine (Aza; 5uM, 7d) or a combination of Aza and trichostatin A (TSA; 1uM, 24h). Results are displayed relative to Ctl within each cell type. A) eNOS, B) VE Cadherin, C) CD31 and D) vWF display greater expression in AoVSMC with combined Aza and TSA treatment. E) VEGFR2 expression did not change with treatment. Addition of synthetic capped polyadenylated luciferase RNA was used for RNA recovery and 1st-strand cDNA synthesis efficiency. HUVEC/AoVSMC ratios of absolute RNA copy numbers for each gene are as follows: eNOS (750:1); CD31 (2500:1), VECadherin (5000:1); VEGFR2 (10:1); vWF (10000:1). A representative experiment (mean ± SE, n=4) is shown. * denotes a statistically significant difference between treated samples and control for each cell type (p<0.05).

FIGURE 6. Replication timing profile of eNOS and EC-enriched genes in ECs and non-ECs. A) A schematic of the NOS3 locus, with primer locations used to detect eNOS. The relative abundance (percent IP) of B) the proximal promoter of eNOS and C) exon 14 of eNOS in each cell cycle fraction quantified by qPCR, in HUVEC (grey) and HuAoVSMC (black). Data represents mean ± SEM, n=4. The relative abundance (percent IP) of EC-enriched genes in each cell cycle fraction is shown for D) CD31, E) ICAM-2, F) VEGFR-1 and G) VEGFR-2, quantified by qPCR. Data represents mean ± SEM, n=3.
Figure 1.
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Figure 6.
A mechanistic role for DNA methylation in endothelial cell (EC)-enriched gene expression: relationship with DNA replication timing

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