Flow-Dependent Epigenetic DNA Methylation in Endothelial Gene Expression and Atherosclerosis

Jessilyn Dunn, Salim Thabet, Hanjoong Jo

Abstract—Epigenetic mechanisms that regulate endothelial cell gene expression are now emerging. DNA methylation is the most stable epigenetic mark that confers persisting changes in gene expression. Not only is DNA methylation important in rendering cell identity by regulating cell type–specific gene expression throughout differentiation, but it is becoming clear that DNA methylation also plays a key role in maintaining endothelial cell homeostasis and in vascular disease development. Disturbed blood flow causes atherosclerosis, whereas stable flow protects against it by differentially regulating gene expression in endothelial cells. Recently, we and others have shown that flow-dependent gene expression and atherosclerosis development are regulated by mechanisms dependent on DNA methyltransferases (1 and 3A). Disturbed blood flow upregulates DNA methyltransferase expression both in vitro and in vivo, which leads to genome-wide DNA methylation alterations and global gene expression changes in a DNA methyltransferase–dependent manner. These studies revealed several mechanosensitive genes, such as HoxA5, Klf3, and Klf4, whose promoters were hypermethylated by disturbed blood flow, but rescued by DNA methyltransferases inhibitors such as 5Aza-2-deoxycytidine. These findings provide new insight into the mechanism by which flow controls epigenomic DNA methylation patterns, which in turn alters endothelial gene expression, regulates vascular biology, and modulates atherosclerosis development. (Arterioscler Thromb Vasc Biol. 2015;35:1562-1569. DOI: 10.1161/ATVBAHA.115.305042.)

Role of Flow in Atherosclerosis and Endothelial Cell Function

Blood flow generates shear stress on the vascular walls. Unidirectional, laminar shear stress, or stable flow (s-flow), is crucial for normal vascular function, whereas disturbed flow (d-flow), characterized by low and reversing oscillatory shear stress (OS), causes endothelial dysfunction and atherosclerosis.2-4 ECs have dramatically altered gene expression patterns when exposed to d-flow versus s-flow.7-12 Atherosclerosis preferentially develops in areas of d-flow, where the dysfunctional EC phenotype initiates and perpetuates plaque accumulation.3,13,14

Flow-Mediated Gene Expression

Shear stress regulates endothelial gene expression in vitro and in vivo.5,15-20 ECs have numerous mechanoreceptors that sense changes in shear stress direction and magnitude, which initiates a complex cascade of signaling events that alters global gene expression and regulates endothelial structure and function.21-23 The net effect of s-flow is to upregulate atheroprotective genes and downregulate proatherogenic genes, whereas the net effect of d-flow is the opposite: upregulation...
of proatherogenic genes and suppression of atheroprotective genes (Figure 1). However, the mechanism by which d-flow causes changes in EC expression is still incomplete.

**Epigenetics Introduction**

Epigenetics is defined as the modification of genetic information in a sequence-independent manner, and it may be mediated by altering DNA conformation. Genomic DNA in an open, relaxed conformation (euchromatin) is associated with acetylated histones, unmethylated DNA, and active transcription, whereas condensed heterochromatin is associated with repressive marks, such as trimethylated-histone 3 lysine 9 (H3K9), H3K27, and DNA methylation. Heterochromatin generally contains repeat elements, imprinted, and transcriptionally silent genes, and its compaction predominantly occurs during development and differentiation.

Shear stress has been found to mediate chromatin remodeling and histone modifications, and this is thought to play a role in shear-induced gene expression changes. Laminar shear stress activates histone acetyltransferase activity, deactivates histone deacetylases (HDACs), and induces histone H3/H4 acetylation and H3 phosphorylation in cultured ECs. This regulates expression of the key EC transcription factors Kruppel-like Factors 2 and 4 (Klf2, Klf4) and endothelial nitric oxide synthase, via myocyte enhancer factor-2. Conversely, OS causes HDAC overexpression, and HDAC inhibition prevents OS-induced EC proliferation and EC inflammation in vivo and in vitro (Figure 1).

DNA methylation involves the addition of a methyl group to the 5’ carbon of a cytosine base pair (5-methylcytosine, 5mC), which occurs most frequently in the context of a CG (cytosine-guanine) dinucleotide. CpG islands are dense regions of CG sites with higher than expected CG content (generally defined as >50% with an observed versus expected ratio of >0.6, over a distance of at least 200 base pairs). Approximately 40% of human genes are associated with CpG islands, which are normally unmethylated, but transcriptionally repressed regions are generally highly methylated. CG sites are otherwise sparse throughout the genome because of a high rate of C to T mutation caused by spontaneous deamination of methylated cytosine, and only functionally important CG sites are likely to be evolutionarily protected from this mutation.

DNA methyltransferases (DNMTs) catalyze the addition of the methyl group to cytosine. DNMT1 is thought to preferentially methylate hemi-methylated DNA. Thus, the complementary strand of a CG dinucleotide, which is a mirroring GC dinucleotide, usually carries the same methylation markings as its CG partner. Although DNMT1 is classically referred to as a maintenance methylase, it also has de novo methylation capabilities. Both DNMT1 overexpression and DNMT1 deficiency are embryonic lethal, and DNMT1 deletion causes genome-wide hypomethylation, whereas DNMT1 overexpression causes genome-wide hypermethylation.

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CG methylation in a gene promoter, close to the transcription start site, generally suppresses gene expression.\textsuperscript{54–56} Promoter methylation is thought to inhibit gene transcription in two ways: first, by physically impeding transcription factor binding to the gene promoter and, second, by binding with methyl-CpG-binding domain proteins, which recruit repressive machinery, such as histone and chromatin modifiers, to the locus that cause chromatin compaction.\textsuperscript{57} The detailed crosstalk between DNA methylation and histone modifications remains a topic of extensive research.\textsuperscript{58,59} The role of DNA methylation in the gene body is less straightforward.\textsuperscript{60–62}

DNA Methylation in Endothelial Cell Development and Disease

Global DNA methylation patterns change dramatically over the course of cell differentiation, and the stable epigenetic marks of terminally differentiated cells govern their gene expression and function.\textsuperscript{63} EC biology differs across vascular beds based on the functional requirements in a given anatomic region. Differences in EC subtype gene expression are regulated, in part, by epigenetic DNA methylation and histone modifications.\textsuperscript{64} Similar differences may also be extended to endothelial dysfunction and atherosclerosis.

Aberrant epigenetic patterns of DNA methylation and histone modifications are hallmarks of diseases such as cancer. Although atherosclerosis shares many phenotypic characteristics with cancer, including hyperproliferation, migration, and inflammation, whether the same abnormal epigenetic changes also occur in atherosclerosis is unclear.

Drugs that inhibit DNMTs have proven to be promising treatment options for many cancers. 5Aza-2′-deoxycytidine (5Aza) is a well-studied DNMT1 preferential inhibitor.\textsuperscript{65} 5Aza is a nucleoside analog that works in part by trapping DNMT1 in a covalent complex with DNA, resulting in DNMT1 degradation. 5Aza, also known as Decitabine, is used as a chemotherapeutic agent for acute myeloid leukemia and myelodysplastic syndromes.\textsuperscript{66–70} However, its cytotoxicity limits its therapeutic capacity.

Flow-Dependent DNA Methylation in Endothelial Biology and Atherosclerosis

Recently, three groups working independently and using different model systems converged on a seminal finding that DNMTs are regulated by shear stress and that they regulate flow-mediated endothelial gene expression and function as well as atherosclerosis.

Jiang et al showed that flow regulates endothelial Klf4 transcription by DNMT3A-mediated DNA methylation using porcine aorta and cultured human aortic endothelial cells.\textsuperscript{71} Klf4 is a key mediator of endothelial function and has been well documented to maintain an anti-inflammatory, quiescent EC state in unidirectional flow conditions. They showed that d-flow increases expression of DNMT3A, which in turn induces Klf4 promoter hypermethylation, thereby decreasing myocyte enhancer factor-2 binding to the promoter and ultimately suppressing Klf4 transcription, in a manner reversible by the DNMT inhibitors 5Aza and RG108. Moreover, RG108 treatment reversed the d-flow–induced loss of downstream Klf4 targets endothelial nitric oxide synthase and thrombomodulin and the d-flow–induced overexpression of the monocyte chemoattractant protein-1. They validated that d-flow induces myocyte enhancer factor-2 binding site hypermethylation in the Klf4 promoter in vivo using endothelium isolated from porcine aorta. This work uncovered a novel epigenetic regulatory mechanism for the key EC gene Klf4.

Our group aimed to discover whether d-flow could cause DNA methylation changes that would alter gene expression globally and promote atherosclerosis. Dunn et al performed genome-wide studies of DNA methylation and gene expression using reduced representation bisulfite sequencing (RRBS) and microarray, respectively.\textsuperscript{72} We found that d-flow regulates genome-wide DNA methylation patterns in a DNMT-dependent manner. Using partial carotid ligation surgery in a murine model to induce d-flow, we found that DNMT1 expression is upregulated by d-flow in ECs both in vivo and in vitro. Further, 5Aza and DNMT1 siRNA each markedly reduced...
OS-induced endothelial inflammation in human umbilical endothelial cells. Moreover, 5Aza treatment drastically prevented atherosclerosis development in two different models of murine atherosclerosis—the partial carotid ligation and the standard high-fat diet model. More recently, Cao et al showed a similar antiatherogenic effect of 5-Aza using LDLR−/− mice.

Zhou et al also reported that d-flow causes DNMT1 overexpression. Using human umbilical vein endothelial cells, they found that OS increases DNMT1 expression, DNMT1 nuclear translocation, and 5mC content in a 5Aza-inhibitable manner. They also used a rat partial carotid ligation model and showed that d-flow induced DNMT1 protein expression and increased 5mC content.

We performed additional studies to determine genome-wide DNA methylation and gene expression changes using the partial carotid ligation model with or without the 5Aza treatment. These two DNA methylation and transcriptome data sets were analyzed by systems biological approaches. Our initial analysis focused on finding genes that are hypermethylated in their promoter regions and have downregulated gene expression by d-flow in a 5Aza-reversible manner. This analysis revealed 11 genes: HoxA5, Klf3, Tmem184b, Adamtsl5, Cmkrl1, Pkp4, Akvr1l, Dok4, Spry2, Zfp46, and F2rl1 (Table). Interestingly, 5 of them (HoxA5, Klf3, Cmkrl1, Akvr1l, and Spry2) contain C-AMP (cyclic AMP) response elements in their promoters. HoxA5 and Klf3 encode transcription factors, and thus, the methylation status of these loci could serve as a mechanosensitive master switch in gene expression (Figure 2). The biological function of those genes is currently under investigation.

Together, these results demonstrated that d-flow controls epigenomic DNA methylation patterns in a DNMT-dependent manner, which in turn alters endothelial gene expression and induces atherosclerosis. This study generated new knowledge about the epigenetic EC flow response and uncovered novel therapeutic gene targets for further study.

Genome-wide DNA methylation studies can be used as a discovery platform to reveal novel flow-sensitive genes. Our study was the first to report that HoxA5 and Klf3 are mechanosensitive transcription factors. Homeobox genes are highly conserved, known to be important in development and dysregulated in cancer, and are controlled by DNA methylation. Homeobox family members have been implicated in vascular remodeling, angiogenesis, and disease by orchestrating changes in gene expression, the extracellular matrix, and integrins. HoxA5 is known to regulate various endothelial functions, such as migration, angiogenesis, and inflammation. Klf3, unlike the other two well-known mechanosensitive transcriptional activators Klf2 and Klf4, is known to repress transcription. Interestingly, Klf3 is suppressed in acute myeloid leukemia, and 5Aza is used to treat this disease. Acute myeloid leukemia is also treated with all-trans retinoic acid, which was shown to rescue Klf3 expression by an unknown mechanism. It would be interesting to determine whether the therapeutic effects of 5Aza and all-trans retinoic acid on acute myeloid leukemia are mediated, at least in part, by rescuing Klf3 promoter hypermethylation.

Atherosclerosis develops because of dysfunction of multiple cell types, including ECs, smooth muscle cells, immune cells, and fibroblasts, and the antiatherogenic effects of 5Aza have been shown to be mediated by not only ECs, but also by immune cells.

**Relationship Between Histone Modification, DNA Methylation, and Epigenetic Crosstalk**

Flow-mediated gene expression is regulated by both genetic (sequence-dependent transcription factor binding) and epigenetic (histone modifications and DNA methylation) mechanisms (Figure 1). Although it has not been directly demonstrated under different flow conditions in endothelial cells, it is well established that epigenetic mechanisms involving histone modifications (eg, acetylation, deacetylation, methylation, phosphorylation) and DNA methylation interact and influence one another, affecting overall gene expression activity. It is also likely that interaction of these epigenetic and genetic mechanisms leads to an orchestrated net effect of s-flow (or laminar shear stress) and d-flow (or

### Table. The Subset of Flow-Sensitive Genes Described Here Have Dramatic D-Flow–Induced Promoter Hypermethylation Corresponding to Suppressed Gene Expression, Both of Which Can Be Reversed by 5Aza Treatment

<table>
<thead>
<tr>
<th>Gene ID</th>
<th>Type</th>
<th>Known Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acvr1l</td>
<td>Serine/threonine kinase receptor</td>
<td>Type I cell-surface TGFβ receptor that may be involved in hemorrhagic telangiectasia, pulmonary arteriovenous malformations, and severe pulmonary arterial hypertension</td>
</tr>
<tr>
<td>Adamtsl5</td>
<td>Extracellular matrix-binding protein</td>
<td>Involved in fibrillin and heparin binding and metalloendopeptidase activity</td>
</tr>
<tr>
<td>Cmkrl1</td>
<td>G-protein-coupled receptor</td>
<td>Leukotriene B4 (inflammatory cytokine) receptor that can activate the transcription factor PPARα. May play a role in cardiac muscle contraction and lipid metabolism and is implicated in pancreatic cancer.</td>
</tr>
<tr>
<td>Dok4</td>
<td>Scaffolding protein</td>
<td>Docking platform for assembly of multimeric signaling complexes. May be involved in T-cell immune regulation, phospholipid binding, and insulin receptor binding.</td>
</tr>
<tr>
<td>F2rl1</td>
<td>G-protein-coupled receptor</td>
<td>Proteolytically activated receptor that modulates inflammatory responses.</td>
</tr>
<tr>
<td>HoxA5</td>
<td>Transcription factor</td>
<td>Differentiation, development, cancer, vascular biology</td>
</tr>
<tr>
<td>Klf3</td>
<td>Transcription factor</td>
<td>Predominantly acts transcriptional repressor. Involved in erythroid cell maturation and adipogenesis</td>
</tr>
<tr>
<td>Pkp4</td>
<td>Junctional protein</td>
<td>Armadillo-like protein that plays a role as a regulator of Rho activity during cytokinesis. May play a role in desmosomal junctional plaque organization and cadherin function.</td>
</tr>
<tr>
<td>Spry2</td>
<td>Receptor tyrosine kinase signaling protein</td>
<td>Bimodal regulator of epidermal growth factor receptor/MAPK signaling. May function as an antagonist of fibroblast growth factor pathways and may negatively modulate respiratory organogenesis.</td>
</tr>
<tr>
<td>Tmem184b</td>
<td>Transmembrane protein</td>
<td>Mostly unknown, but may activate mitogen-activated protein kinase (MAPK) signaling</td>
</tr>
<tr>
<td>Zfp46</td>
<td>Transcription factor</td>
<td>Mostly unknown, but may be a transcriptional repressor.</td>
</tr>
</tbody>
</table>

MAPK indicates mitogen-associated protein kinases; PPARα, peroxisome proliferator-activated receptor alpha; and TGFβ, transforming growth factor beta.
OS) on mechanosensitive gene expression in ECs. The importance and extent of these interactions remains to be determined.

Our working model is that DNA methylation in the promoter regions caused by d-flow leads to chronic repression of antithromrogenic genes (epigenetic mechanisms), whereas proatherogenic gene expression is mediated by regulation of transcription factors (genetic pathways). In contrast, s-flow rescues (derepresses) expression of antithromrogenic genes by decreasing DNA methylation in the promoter regions via reduced DNMT activity and altered histone modifications. S-flow also regulates genetic pathways by regulating transcription factor binding to specific gene promoter sites, leading to increased antithromrogenic genes and decreased proatherogenic genes.

Most of the DNA CpG sites are methylated during development by DNMT3A and DNMT3B with CpG islands being subject to differential methylation affected by various conditions. There have been numerous reports describing the interaction between histone modifications, such as acetylation and methylation (eg, by G9a or SET [Su(var)3-9, Enhancer-of-Zeste, and Trithorax] domain containing histone methyltransferases), with DNA methylation and repression of transcription. DNMT3A and DNMT3B share an unmodified histone-binding site. H3K4 methylation is associated with decreased DNA methylation in multiple cell types, whereas H3K9 methylation and histone deacetylation are associated with increased DNA methylation, which leads to gene repression by forming heterochromatin.94 DNMT3A and DNMT3B also harbor domains that read H3K36me3, which is found in the bodies of certain genes, and it is thus speculated that they play a role in gene body methylation, which is abundant but not well understood.94 Moreover, DNMT1 also recognizes H3K23 monoubiquitination, which seems to play a role in maintenance of DNA methylation.94

Emerging evidence indicates that DNA methylation can also affect histone modifications via interactions of DNMTs with histone methyltransferases and deacetylases. For example, CpG methylation enhances ubiquitin-like containing PHD and RING finger domains 1, a DNMT1 recruiter, and perturbs lysine-specific demethylase 2A, a H3K36 demethylase enzyme, binding to H3K9me3-containing nucleosomes.95 Also, DNA methylation causes H3K9 demethylation, which is a marker of repressive chromatin, by interaction of G9a and DNMT1. DNA methylation also inhibits H3K4 methylation, which is a marker of active transcription.95 This is supported by the fact that unmethylated DNA is mainly assembled in nucleosomes that contain acetylated histones, whereas methylated DNA is assembled in nucleosomes containing nonacetylated H3 and H4, which leads to more compact chromatin. Moreover, there have been reports that methylcytosine-binding proteins, such as meCP2 or miD2, might help recruit HDACs and thus lead to histone acetylation.95

It is becoming clear that an interplay exists among methylation, miRNAs, and histone acetylation. Hypermethylation of multiple miRNA promoter sites (eg, miR-1, 26a, and 137) has been reported in multiple cancer types. Moreover, HDACs (HDAC1-9 and 11) are direct targets of miRNAs, whereas HDACs have been shown to regulate expression of miRNAs (eg, miR-15-a/16-1, 183, 224, 449a). In ECs, Klf4 and Klf2 are two key flow-sensitive genes that are coregulated by miR-92 and themselves regulate expression of multiple microRNAs as well.96

As we described earlier, Klf4 is also controlled by flow-dependent DNA methylation.71 This opens up the interesting possibility of cooperation between flow-mediated epigenetic events.

**Perspectives**

Emerging studies clearly demonstrate that d-flow regulates epigenomic DNA methylation by upregulated DNMTs in ECs, which in turn alters endothelial gene expression and function, ultimately contributing to atherosclerosis. The advent of new technologies to resolve genome-level methylation at the nucleotide scale combined with advanced systems biology approaches will continue to play a key role in better understanding the effects of DNA methylation in gene network regulation, endothelial biology, and vascular disease. Currently, DNA methylation studies mainly focus on studying 5mC, but other modifications, such as 5-hydroxymethylcytosine, 5-formylcytosine, and 5-carboxycytosine, are also likely to be important. In addition, demethylating enzymes, such as Ten-eleven-translocation may also play an important role in regulating gene expression in this disease.

Experimental methods to interrogate the epigenome of flow-regulated ECs involve the use of either in vivo–derived or in vitro–cultured cells. In vivo methods have a clear benefit because of biologically artificial conditions of cell culture, which are known to have epigenetic consequences. For example, ECs in vivo are constantly exposed to flow, whereas cultured ECs must be exposed to static, no-flow conditions during the culture process and nonadherent conditions during passage. These intermediate mechanical conditions can have dramatic effects on long-term epigenetic state. Gene expression studies by microarray using cultured ECs demonstrate that in vitro biological responses are often different from events that occur in vivo.97 This raises an important consideration, especially surrounding persisting epigenetic changes, for the limitations of in vitro studies and points to the major benefit of models that use in vivo–derived genomic DNA with no intermediate cell culture steps.

There are several methods available to interrogate genomewide DNA methylation patterns. The most comprehensive is whole genome bisulfite sequencing, but the large amount of data generated, including poorly understood intergenic desert regions, currently makes whole genome sequencing a less desirable method. Alternative methods with higher efficiency and practicality to resolve the endothelial DNA methylome include methylation microarrays and RRBS methods. Methylation microarrays are limited by the probe set and only examine regions defined by previous knowledge (such as promoter- and CpG island–confined CpG sites). RRBS is currently a gold standard for genome-scale methylation studies at the nucleotide resolution, although incomplete CG coverage is an inherent limitation of RRBS because of the MspI restriction enzyme digest, PCR, and sequencing steps.98 Both methylation microarrays and RRBS produce partial genomic coverage, which may result in overlooking potentially important methylation sites. Compared with currently existing technologies, RRBS is a much more comprehensive but cost-effective method to interrogate genomewide DNA methylation.

Although most work in this realm has been mainly focused on CpG methylation in the promoter region of genes, DNA
methylation in other locales, including coding or noncoding gene regulatory regions, and at other motifs, such as CpH (where H is A/T/C), are known to be important for controlling gene expression, alternative transcripts, chromatin conformation, and genome stabilization. Additionally, although DNA methylation at the 5′ carbon of a cytosine base pair is the most well-studied epigenetic DNA modification, several others are known to exist and were recently implicated to be important in gene expression regulation, although their mechanisms are unclear. These modifications include 5-hydroxymethylcytosine, 5-formylcytosine, and 5-carboxycytosine. Emerging methods of detection will enable future study of these cytosine modifications in endothelial biology and pathogenesis.

Much remains to be determined about the nature of DNA methylation changes in response to flow; how does d-flow regulate DNMT expression and what controls flow-dependent DNA demethylation? What are the functional consequences of DNA methylation at the genome-wide and individual gene levels? How does DNA methylation at a specific promoter site affect transcription of the flow-sensitive genes? It is also important to compare and translate mouse data to clinical data, with important limitations to experimental models, especially because human atherosclerosis develops over decades and is multifactorial in nature. For example, the study of epigenetic factors involved in atherosclerosis would expect to be more complicated in humans because of differences in life habits, such as smoking, sedentary life styles, and other comorbidities, such as hypertension, diabetes mellitus, and obesity. There have been conflicting reports showing global hypomethylation in human atherosclerotic plaques, whereas other studies in contrast showed hypermethylation across many genomic loci in diseased aortas, indicating that more studies are needed. Given the known side effects of using DNMT inhibitors, such as 5AzA, as therapeutics, it is important to compare and translate mouse data to clinical data.

Significance

These emerging findings surrounding the endothelial cell epigenetic response to flow provide new insight into the mechanism by which flow controls epigenomic DNA methylation, which in turn regulates endothelial gene expression, vascular biology, and ultimately atherogenesis. Based on our recent mouse DNA methylome study, it is tempting to speculate that physical inactivity increases atherosclerosis risk partly because it is associated with d-flow, which leads to chronic repression of antiatherogenic genes by epigenetic DNA methylation–dependent mechanisms. In contrast, the well-known antiatherogenic effect of aerobic exercise may be due in part to derepression of those antiatherogenic genes, mediated by DNA demethylation. The discovery of endothelial cell disease master regulators that control a large network of genes, such as DNMTs, and their specific target genes is important to enable further development of therapeutics for vascular disease treatment and prevention.

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Disclosures

None.

References


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