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## Epigenetics in the Vascular Endothelium: Looking from a different perspective in the epigenomics era

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### Abstract

Cardiovascular diseases (CVD) are commonly thought to be complex, non-Mendelian diseases that are influenced by genetic and environmental factors. A growing body of evidence suggests that epigenetic pathways play a key role in vascular biology and might be involved in defining and transducing CVD inheritability. In this review, we argue the importance of epigenetics in vascular biology, especially from the perspective of endothelial cell (EC) phenotype. We highlight and discuss the role of epigenetic modifications across the transcriptional unit of protein coding genes, especially the role of intragenic chromatin modifications, which are underappreciated and not well characterized in the current era of genome wide studies. Importantly, we describe the practical application of epigenetics in CVD therapeutics.

### Keywords

Epigenetics; long non-coding RNA; endothelial cells; intragenic epigenetic modifications; DNA methylation; histone post-translational modifications

### Introduction

Prevalent cardiovascular diseases (CVD), such as coronary artery disease (CAD), are thought to be complex, non-Mendelian diseases in which genetic and environmental factors figure prominently<sup>1</sup>. Traditionally, the inherited aspect of CVD is attributed to genetic variations in multiple protein-coding genes. Hence, studies have tested this hypothesis by conducting genome wide association studies (GWAS), often with subsequent meta-analysis, to identify SNPs that are present or absent in patients with CAD. Although the earliest collected studies indicate that 46 genome-wide significant loci are associated with CAD, they only account for ~10% of CAD inheritability, suggesting that other factors are

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### Disclosures

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involved<sup>1</sup>. One other major factor is epigenetics, which can be defined as alterations in chromatin that are not due to changes in the DNA sequence per se<sup>2</sup>. These chromatin-based pathways may or may not be faithfully inherited with mitotic or meiotic cell division.

## What is Epigenetics?

DNA methylation, histone density, variants, and post-translational modifications, and RNA-based mechanisms represent the 3 interrelated pathways that figure prominently in the molecular basis of epigenetics. We will briefly discuss their commonly known regulatory mechanisms and role in endothelial cell (EC) biology.

### DNA Methylation

DNA methylation is an epigenetic modification that occurs at the 5-position of cytosine to generate 5-methylcytosine and is commonly observed to occur in the context of a cytosine followed by a guanine (CpG)<sup>3</sup>. Although CpG sites are relatively sparse in the genome, due to deamination of 5-methylcytosine to thymine during the evolution of the mammalian genome, certain regions are not CpG depleted and these regions are referred to as CpG Islands (CGIs)<sup>3</sup>. The addition of a methyl group to cytosine is catalyzed by DNA methyltransferases (DNMTs), namely DNMT1, DNMT3a and DNMT3B<sup>4</sup>. In contrast, DNA demethylation can occur passively or actively<sup>4</sup>. The latter mechanism is currently under investigation and thought to be mediated, in part, by the oxidation of 5-methylcytosine to 5-hydroxymethylcytosine and subsequent oxidative products by the ten-eleven translocation enzyme family (TET1, TET2, and TET3)<sup>5</sup>. These oxidative products are thought to be substrates for base excision repair glycosylases that subsequently restores the initial cytosine<sup>6</sup>.

At gene promoters, DNA methylation mediates transcriptional repression. It is mechanistically thought to mediate repression by preventing the binding of transcription factors (TF), including the CCCTC-binding factor (CTCF), and recruiting methyl binding proteins, such as MeCP2, that can bind 5-methylcytosine to prevent the recruitment of transcriptional machinery<sup>4</sup>. Methyl binding proteins can also recruit other chromatin modifying enzymes, including histone deacetylases (HDACs) and histone methyltransferases, to further reduce chromatin accessibility<sup>4</sup>.

Although studies of DNA methylation in CVD and EC disease phenotypes are still early in their infancy, tantalizing new studies suggest that these are key next questions. For example, recent studies on the role of DNA methylation in ECs have shown that aberrant DNA methylation at the promoters of flow-inducible genes contributes to atherosclerosis. Prominently, an *in vivo* model of disturbed blood flow was performed by partial carotid ligation in mice. This model mimics the hemodynamic state of regions in the aorta that are prone to atherosclerotic plaque formation. DNMT1 was induced by disturbed flow and was associated with inflammation. Importantly, pharmacological inhibition of DNMTs in this *in vivo* model showed reduced atherosclerotic plaque growth. DNMT1 is argued to promote inflammation by hypermethylating a set of flow-sensitive genes, including HoxA5, under conditions of disturbed flow<sup>7</sup>. Consistent with these findings, a comparison of swine ECs from the athero-susceptible inner curvature of the aortic arch and the athero-protected

descending thoracic aorta found that genes with differentially DNA methylated regions (DMRs) have functions associated with atherosclerosis, including oxidative stress and inflammation<sup>8</sup>. Interestingly, the same group found that DNMT3a was induced by disturbed flow<sup>9</sup>. This was associated with increased DNA methylation of the promoter and inhibition of the transcription of KLF4, a flow-inducible factor required for maintenance of the anti-inflammatory and anti-thrombotic properties of healthy vascular ECs<sup>9</sup>. Collectively, these studies argue that DNMTs and aberrant DNA methylation promote a pro-inflammatory state for atherosclerotic plaque formation. Additional studies will need to clarify the key regulators of aberrant DNA methylation at disturbed flow-inhibited genes. Studies will also need to ask which genes are activated or repressed by flow-mediated epigenetic changes.

## Histone Biology

Histones are proteins that package DNA into chromatin by forming octamers composed of 2 H3-H4 and 2 H2A-H2B heterodimers. These octamers are wrapped by 146 nucleotides of DNA to form a nucleosome. Nucleosomes are connected to each other by linker DNA to form a “beads-on-the-string” chromatin structure that can be further condensed into higher order structures<sup>2, 10</sup>. Histone proteins can be modified by a myriad of post-translational modifications (PTM) to regulate transcription<sup>2, 10</sup>. The most well-characterized classes of histone PTM are lysine methylation and acetylation<sup>2</sup>.

Histone acetylation is dynamically regulated by histone acetyltransferases (HATs) and HDACs. Histone acetylation is thought to activate transcriptional initiation by neutralizing the basic charges on the lysine tails and recruiting bromodomain-containing proteins, including other HATs and chromatin remodeling complexes, to increase chromatin accessibility at the promoter and enhancer (Figure 1). Subsequently, TFs and the transcriptional machinery can be recruited to activate transcription initiation<sup>11</sup>.

Similar to histone acetylation, histone lysine methylation is regulated dynamically by histone lysine methyltransferases (KMTs) and histone lysine demethylases (KDMs)<sup>3</sup>. Unlike histone acetylation, mono-, di- and tri-methylation of histone lysine residues can occur. Importantly, at any specific lysine residue, histone acetylation and methylation are mutually exclusive. The specific lysine residue and even the number of methyl groups on that specific lysine residue can have different downstream effects on transcription initiation<sup>3</sup>. For instance, H3K4 monomethylation is localized to both promoters and enhancers (Figure 1). This modification is commonly enriched at repressed, inducible genes and it is involved in spatially restricting the recruitment of factors that recognize trimethylated H3K4, including ING1, to active promoters<sup>12</sup>. In contrast, H3K4 trimethylation is enriched at the promoter of active genes and is known to recruit factors important for transcription, including TFIID<sup>13</sup> (Figure 1).

Although most nucleosomes consist of the 4 “canonical” core histones, histone variants can be incorporated into nucleosomes, typically in a replication independent manner, to mediate transcription regulation<sup>14</sup>. One prominent histone variant is H2A.Z. H2A.Z is enriched at the promoters and enhancers of transcriptionally active genes and the promoters of inactive genes that are poised for expression<sup>14-16</sup>. The transcriptional activation of H2A.Z enriched promoters may, in part, be regulated by H2A.Z histone acetylation<sup>16</sup>.

Much effort has focused on characterizing the role of histone modifications in EC biology through studies on histone modifying enzymes, especially HDACs. Pharmacological inhibition and siRNA depletion of HDACs have shown both pro- and anti-angiogenic activity in models of postnatal angiogenesis suggesting that unique HDACs promote and inhibit angiogenesis<sup>17-19</sup>. Furthermore, HDAC activity has been shown to be important for endothelial differentiation of embryonic stem cells (ESCs) and adult endothelial progenitor cells<sup>20, 21</sup>.

Some HDACs have also been reported to contribute to atherosclerosis. HDAC3 expression was observed to increase in the aorta of ApoE<sup>-/-</sup> mice, especially at branching areas, and the inner curvature of the aortic arch in rats. These regions experience disturbed blood flow and are prone to atherosclerotic plaque formation. Consistent with this observation, ECs that are subjected to *in vitro* models of disturbed flow show increased HDAC3 expression<sup>22, 23</sup>. Importantly, HDAC3 depletion in aortic isograft models of ApoE<sup>-/-</sup> mice results in the formation of severe atherosclerotic lesions and even vessel rupture. This is interesting in that, in contrast to DNMTs, increased HDAC3 is protective against atheroprone flow. HDAC3 contributes to atherosclerosis in part, through its interaction with Nrf2 and MEF2, which prevents these TFs from binding and activating the expression of target downstream genes with anti-inflammatory and -oxidative activities<sup>22</sup>. It is currently unknown if HDAC3 can also act directly on chromatin to promote atherosclerotic progression. Similarly, HDAC2 can contribute to atherosclerotic progression as suggested by its increased expression in the inner curvature of the aortic arch in rat and ECs subjected to disturbed flow *in vitro*. Furthermore, HDAC2 can also interact and deacetylate Nrf2<sup>22</sup>. Moreover, oxidized low-density lipoprotein (OxLDL), a proatherosclerotic molecule, is found to downregulate HDAC2 expression resulting in the transcriptional activation of Arg2, which results in eNOS uncoupling and increased eNOS-dependent reactive oxidative species production<sup>24</sup>. Genetic deletion of HDAC2 in EC will need to be conducted to assess whether HDAC2 is also a protective response to atheroprone flow.

SIRT1, which belongs to an unique family of TSA-insensitive HDACs with a requirement for nicotinamide adenine dinucleotide (NAD<sup>+</sup>), may have an atheroprotective role<sup>25</sup>. Importantly, SIRT1 expression is reduced in atherosclerotic plaques. Furthermore, studies in ApoE<sup>-/-</sup> SIRT1<sup>+/-</sup> mice show that SIRT1 prevents endothelial activation<sup>26</sup> SIRT1 is thought to mediate its atheroprotective role by various mechanisms. This suggests that decrease in SIRT1 in atheroprone flow is mechanistically important. SIRT1 can deacetylate eNOS protein to increase nitric oxide production and promote an anti-inflammatory and -oxidative environment under atheroprotective flow conditions<sup>27</sup>. At the transcriptional level, SIRT1 can activate the expression of anti-oxidant genes in ECs by deacetylating and increasing the activity of the TFs FoxO3a and PGC-1 $\alpha$  at these genes<sup>28</sup>. Surprisingly, reduced H4K16 acetylation and increased elongating Pol II recruitment occur at these genes under oxidative stress conditions<sup>28</sup>. This observation suggests that the H4K16 deacetylase activity of SIRT1 may possibly activate these genes by reducing spurious transcript production. Additionally, the loss of SIRT1 in atherosclerotic plaque may de-repress pro-atherosclerotic genes in a H4K16 acetylation dependent manner as suggested for plasminogen activator inhibitor-1<sup>29</sup>. These latter concepts are important in that they highlight a key point. Later in this review,

we expand on the concept of intragenic epigenetic modification and contrast their functional importance with epigenetic modifications in the proximal promoter regions. Increases or decreases in HDACs may have contrasting effects on transcription if they are recruited to the proximal promoter versus the intragenic regions.

### Long Noncoding RNA (LncRNA)

LncRNAs are distinct from short noncoding RNAs, such as miRNAs, which are also known to play a role in EC function and atherosclerosis and are thought to predominantly regulate gene expression through post-transcriptional mechanisms in the cytoplasm<sup>30</sup>. LncRNAs are arbitrarily defined by their length of >200 nucleotides. This newly described heterogeneous class of transcribed regions of the non-protein coding genome may or may not be 5'-capped, spliced, or polyadenylated<sup>31</sup>. They are in the process of being characterized and are currently observed to act in *cis* or *trans*. They can be localized and functional in either the nucleus or cytoplasm. LncRNAs functions can be mediated by RNA-RNA, RNA-DNA, and RNA-protein interactions<sup>31</sup>. An important number of them regulate chromatin function in the nucleus<sup>31</sup>. Hence, they can be considered to function in a chromatin-based fashion.

Although current efforts show that epigenetic modifiers play a critical role in ECs under disease and physiological conditions, little is known about the repertoire of unique EC-enriched lncRNAs, their function and mechanisms of action, and the extent of their contribution to CVDs. However, studies have begun to identify lncRNAs that play a prominent role in ECs. A recent genome-wide analysis was conducted to identify the most highly expressed lncRNAs in ECs<sup>32</sup>. One of these lncRNAs is metastasis-associated lung adenocarcinoma transcript 1 (MALAT1). Although highly expressed, MALAT1 is widely expressed in tissues representing all germ layers and is found to be upregulated in various tumours<sup>33</sup>. Therefore, it is not EC-enriched. In ECs, MALAT1 is thought to regulate cell proliferation *in vitro* and *in vivo* and modulate cell migration<sup>32</sup>. Importantly, MALAT1 is upregulated in ECs under hyperglycemic conditions and contributes to diabetic retinopathy in rodent models of diabetes mellitus, in part, by increasing the expression of inflammatory cytokines in ECs<sup>34</sup>. The mechanistic role of MALAT1 is currently not clear. However, MALAT1 binds intragenic regions of target expressed genes and associate with chromatin modifiers involved in transcription activation, such as SET2, and pre-mRNA splicing factors<sup>35, 36</sup>. Future studies will have to identify EC-enriched lncRNAs and assess if they are functionally important epigenetic modifiers of EC gene expression.

### Epigenetic regulation of a prototypic endothelial-enriched gene: eNOS

Early studies have focused on characterizing the role of epigenetic pathways in the vascular endothelium by focusing on the promoters of EC-expressed genes. One of the most well-characterized examples of this approach is eNOS (*NOS3*)<sup>3</sup>. eNOS plays a prominent role in vascular biology as shown by eNOS null mice that are characterized by systemic and pulmonary hypertension, abnormal vascular remodeling, defective angiogenesis, and pathological healing to vascular injury<sup>37</sup>. Importantly, eNOS is thought to be atheroprotective and its expression is reduced in the ECs of atheroprone regions of mouse aorta<sup>38</sup>. EC-enriched expression of eNOS is, in part, regulated by an unique epigenetic signature. Specifically, the eNOS proximal promoter is enriched with H3K9Ac, H4K12Ac,

H3K4me2, H3K4me3, and the H2A.Z histone variant in ECs, relative to non-ECs<sup>3</sup>. These histone modifications and histone variant are functionally important for eNOS transcription activation. In contrast, the eNOS proximal promoter is DNA hypermethylated in non-EC to mediate transcription repression. Importantly, dynamic changes in the epigenetic signature of the eNOS promoter are involved in the activation and repression of eNOS expression in ECs subjected to environmental stimuli, especially hypoxia<sup>3</sup>. These early studies suggest that epigenetic pathways play a functional role in healthy and diseased vascular endothelium. As discussed below, future studies should not be limited in their bias towards *cis* regulatory elements at the proximal promoter, which are thought to be important for gene expression. They should also address intragenic epigenetic modifications.

## ENCODE and Intragenic Modifications

Defining the cell-specific epigenetic modifications that encompass EC-enriched genes, including their proximal promoters, enhancers, and intragenic regions, is a current goal of many groups. Although studies that focus on the promoter of protein coding genes, such as eNOS, are valuable, we need a comprehensive road map for how endothelial cells are distinct from other cell types. This cannot be inferred indirectly from studying peripheral blood genomic DNA or other solid cell types. Is the heterogeneity of EC phenotypes mediated by *cis/trans* paradigms, epigenetic marks or both? Recent whole genome profiles of various epigenetic modifications have been generated for cells of the cardiovascular system, including the vascular endothelium. These studies begin the process of addressing this question and circumvent the bias of assessing the role of epigenetic modifications at the promoter<sup>7, 8, 39, 40</sup>. One of the most prominent efforts in dissecting the epigenetic landscape of the vascular endothelium came from the Encyclopedia of DNA elements (ENCODE) project<sup>41</sup>.

It is estimated that the ~20,000 – 25,000 protein-coding genes in the human genome is represented by 1.5% of the genome. The primary goal of the ENCODE project was to determine the role of the remaining non-protein coding components. In a series of landmark papers, the ENCODE consortium argued that 80% of the genome had biochemical function, much of which was involved in controlling the expression of the protein-coding genome<sup>41</sup>. This finding is based on the chromatin-based datasets that were generated in the ENCODE project. These include, but are not limited to, RNA sequencing, TF binding, long-range interaction, DNA methylation and histone modification profiles. By integrating the ENCODE epigenetic datasets, cell-specific promoters and putative enhancers have been identified in ECs that play a role in the EC gene expression profile<sup>39</sup> (Figure 1). In particular, the EC specific promoters and putative enhancers that are identified by their epigenetic profiles are associated with genes that are enriched for EC functions, including angiogenesis and blood vessel morphogenesis<sup>39</sup>. Although a large amount of epigenomic data was generated for ECs, the role of epigenetic pathways in EC received relatively modest attention compared to other cell types. For instance, ChIP-seq datasets for ECs from ENCODE are generated at relatively low resolution and some genes are not accurately annotated, such as eNOS. Furthermore, various datasets, including DNA methylation profiling and long-range interaction data, were not generated for ECs by ENCODE<sup>41</sup>.



Aside from characterizing promoters and putative enhancers, the vast amount of epigenomic data that have been, and will be, generated can be used to characterize under-appreciated aspects of gene regulation that may have a functional role in EC biology. One such aspect is intragenic epigenetic modifications. In the following sections, we will describe their currently known regulatory roles and potential contribution to vascular biology.

### DNA methylation

Although the role of intragenic DNA methylation is not well understood, it is likely to play a critical role in the vasculature based on the observation that the majority of human atherosclerosis-specific differentially methylated CpGs are mapped to intragenic regions<sup>40</sup>. Similarly, athero-susceptible DMRs in swine are also enriched in exons<sup>8</sup>. Surprisingly, intragenic DNA methylation is positively correlated with gene expression (Figure 1)<sup>42</sup>. However, genomic integration of a transgene with patch intragenic DNA methylation into a murine cell line show reduced expression and Pol II occupancy relative to its unmethylated counterpart<sup>43</sup>. Thus, the positive correlation of intragenic DNA methylation with gene expression might be related to its function in preventing spurious expression of repetitive elements<sup>44</sup> while a gene is actively transcribed (Figure 1). Although little is known about the function of intragenic DNA methylation, recent literature has suggested that regional intragenic DNA methylation can regulate the expression of mRNA variants<sup>42, 44–47</sup> (Figure 1).

DNA methylation has been observed in mammals to globally repress transcription from intragenic CpG islands (CGIs). The absence of this intragenic methylation allows spurious transcriptional initiation at non-canonical sites. These regions now become alternative promoters of genes, which may or may not affect the open reading frame<sup>42, 44, 46</sup>. Interestingly, differential DNA methylation at these intragenic CGIs can regulate the tissue-specific expression of mRNA variants and evidence of transcription initiation has been observed at these regions<sup>46</sup>.

Alternative splicing might also be regulated by DNA methylation. Studies on CD45, whose splice variants are tightly correlated with lymphocyte development, have shown that DNA methylation can affect exon retention through its effect on the recruitment of CTCF<sup>44, 47</sup>. This observation is interesting as CTCF is more known for its role in promoter activation and repression, blocking of enhancer-promoter interaction, and regulation of three-dimensional chromatin organization<sup>48</sup>. Exon 5 retention in CD45 is associated with the lack of methylation of its respective intragenic genomic region. The lack of methylation allows the binding of CTCF to the exon, promotes Pol II pausing, and possibly allows co-transcriptional spliceosome assembly for exon 5 inclusion (Figure 1). Although recent observations suggest that DNA methylation has little effect on CTCF binding on a global scale, methylation sensitive CTCF-binding sites have been noted and are more frequently observed in exons<sup>49</sup>. This observation suggests that DNA methylation-sensitive, CTCF-dependent regulation of splicing may occur at a unique set of genes<sup>47</sup>. Contrary to these findings, intragenic DNA methylation has been observed by others to be enriched at alternative exons that are included in many mRNA variants<sup>45</sup>. In these instances, inclusion of these alternative exon is mediated in a Methyl CpG binding protein (MeCP2)- and

HDAC-dependent manner (Figure 1). Future studies will need to address the chromatin context by which these distinct DNA methylation-dependent splicing mechanisms occur.

### Histone Post-translational Modifications

In contrast to the studies that address genomic regions implicated in transcriptional initiation, the role of specific histone modifications in intragenic regions is not well understood. Although a myriad of intragenic histone post-translational modifications have been identified, we will focus on histone lysine acetylation and methylation.

### Intragenic Histone Acetylation

High throughput studies on genomic localization of histone acetyltransferases (HATs) and HDACs have shown that some HATs and HDACs are co-enriched at the promoter and intragenic regions of transcriptionally active genes in mammals<sup>50</sup>. Consistent with this observation, various acetylated histone residues, such as H3K14 acetylation (H3K14Ac), are enriched in the transcribed regions<sup>51, 52</sup>. These observations are relevant based on the fact that HATs that can catalyze intragenic histone acetylation also play a critical role in vessel formation. For instance, PCAF, a HAT, contributes to arteriogenesis, the remodeling of pre-existing collateral arterioles into larger arteries<sup>53</sup>. Furthermore, mice that are deficient in HBO1, a prominent H3K14 HAT, are embryonically lethal and show defects in embryonic vascular remodeling<sup>52</sup>. Future studies will have to investigate if other HATs and HDACs that catalyze intragenic histone acetylation also play a prominent role in the vasculature and are involved in EC biology.

Mechanistically, the observation that HATs and HDACs are enriched in intragenic regions suggest that dynamic histone acetylation in the intragenic region is important for transcription elongation. In agreement, hyperacetylated histone residues, such as AcH3K14, can co-enrich with Brd2 and Brd3 at intragenic regions in mammals. These bromodomain-containing proteins can bind these acetylated histone residues and facilitate transcription through chromatin *in vitro*<sup>54</sup>. This is consistent with an elongating RNA polymerase II (Pol II) transcription complex evicting histones as the DNA template is made accessible for Watson-Crick base pairing of DNA with RNA substrates (Figure 1).

Moreover, yeast studies demonstrate that dynamic intragenic histone acetylation maintains the delicate balance of efficient transcription elongation without cryptic initiation of transcription in intragenic regions. The loss of HATs Gcn5 and Elp3 in yeast result in histone H3 hypoacetylation in the intragenic region of genes and is associated with their transcriptional inhibition<sup>55</sup>. Overall inhibition of transcription by Gcn5 deficiency is due to defects in nucleosomal eviction during transcription elongation<sup>56</sup>. In contrast, mutations of HDACs, such as the Rpd3S HDAC complex, result in increased histone acetylation and intragenic cryptic transcript initiation<sup>57</sup>. Future studies will be needed to better understand the functional role and relevant protein complexes implicated in dynamic intragenic histone acetylation in mammals.



## Intragenic Histone methylation

Intragenic histone methylation also plays a prominent role in transcriptional elongation. One of the most prominent modifications is H3K36 trimethylation (H3K36me3), which is catalyzed by SET2. SET2-dependent H3K36me3 suppresses spurious intragenic transcription by recruiting the Rpd3S complex and preventing the incorporation of newly acetylated histones over transcribed intragenic regions (ORFs) in yeast<sup>57, 58</sup>. The functional role of H3K36me3 is conserved in humans and, in part, mediated by FACT-dependent histone H2B exchange<sup>59</sup>. Importantly, mice that are deficient for HYPB/Set2D, the H3K36 methyltransferase, are embryonically lethal and show defects in embryonic vascular remodeling suggesting that H3K36me3 may play a prominent role in vascular biology<sup>60</sup>.

The function of other intragenic histone methylation-based modifications is currently being investigated. Of note, H3K27me3, which commonly occurs at the promoter, is enriched at the intragenic regions of some target genes<sup>61, 62</sup>. In-depth analysis in neural stem cells have shown that intragenic region of some TGF- $\beta$ -inducible genes are enriched for H3K27me3<sup>61, 62</sup>. Importantly, TGF- $\beta$  induces the recruitment of Jmjd3 (KDM6B), a H3K27me3 demethylase, to catalyze histone demethylation and promote transcriptional elongation. TGF- $\beta$  signaling plays a critical role in developmental vessel formation and mutations in the regulators of TGF- $\beta$  signaling cause diseases with endothelial pathologies, such as Hereditary Hemorrhagic Telangiectasia<sup>63</sup>. Thus, critical TGF- $\beta$  inducible genes involved in vessel development and disease might also be regulated by intragenic H3K27me3 in ECs. Consistent with this possibility, previous Jmjd3-deficient mouse ESCs show compromised cardiovascular differentiation<sup>64</sup>. Future studies will have to address the role of intragenic H3K27me3 in ECs and define how intragenic H3K27me3 represses transcriptional elongation.

## Therapeutic potential of Targeting Epigenetic Modifiers

Alterations in the activities of critical epigenetic regulators might be integral in the therapeutic approaches of CVDs. Such therapeutic approaches include the differentiation of inducible pluripotent stem cells (iPSCs) to endothelial cell (EC)-like cells for treating CVDs and the manipulation of CVD-associated lncRNAs, such as ANRIL.

## Epigenetics in cellular reprogramming and its application to CVD

The advent of inducible pluripotent stem cells (iPSCs) offers a promising potential for cell-based therapies of CVD. Briefly, iPSCs are somatic cells that are reprogrammed to an embryonic stem (ES) cell-like state. Although they were initially established by the combined ectopic expression of Oct3/4, Sox2, Klf4, c-Myc, it was later discovered that different cocktails of *trans* factors could facilitate this process<sup>65</sup>. The use of iPSC-differentiated EC-like cells have been shown to contribute to vascular repair in various vascular injury models, such as hind limb ischemia<sup>66, 67</sup>. These studies are promising, given that patient derived iPSC-differentiated EC-like cells may offer treatment options for critical limb ischemia through the process of modifying blood vessel formation (Figure 2).

A major challenge in applying patient derived iPSC-differentiated ECs for treating CVDs is the low efficiency (<3%) in iPSC reprogramming<sup>65</sup>. The low efficiency reflects the required,

dramatic changes in gene expression that are dependent in a major way on profound changes in their epigenetic landscape. In a comprehensive analysis of epigenetic changes during cellular reprogramming, chromatin was found to be more “open” during the early reprogramming phase and it subsequently returns to its original stem-cell like state based on overall changes in levels of the repressive H3K27me3 modification at genomic loci<sup>68</sup>. Genes that are expressed during the reprogramming process are associated with pluripotency and their expression is correlated with a decrease in H3K27me3 and the presence of H3K4me3 at their proximal promoters. In contrast, increased H3K27me3 enrichment occurs at genes that are associated with development and cell fate determination. During the later phases of iPSC reprogramming, DNA methylation patterns are also altered and associated with repression of gene expression.

Consistent with the importance of altering the epigenetic landscape, inhibition of epigenetic modifiers, such as DNA methyltransferases (DNMT) and histone deacetylases (HDACs), improve the efficiency of iPSC reprogramming<sup>69, 70</sup>. A possible reason for this improvement is that such manipulation helps transition partially reprogrammed cells to more fully totipotent iPSCs. Indeed, pharmacological inhibition of epigenetic modifiers have been used to direct the transition of partially reprogrammed cells to more totipotent iPSCs<sup>69, 70</sup>.

Little is known about the effects of altering epigenetic modifier activity on the differentiation of iPSCs to EC-like cells even though significant progress has been made in generating EC-like cells efficiently via iPSC differentiation to cord blood endothelial colony forming-like cells<sup>66</sup>. However, studies have shown that TGF $\beta$ , Wnt, and VEGF signaling play a prominent role in differentiating iPSCs to EC-like cells<sup>71</sup>. Future studies may identify critical epigenetic modifiers that improve the efficiency of EC differentiation. Furthermore, it is essential that we understand the epigenetic modifications that are prevalent at EC-enriched genes and how exogenous stimuli and developmental processes, especially TGF $\beta$ , Wnt, and VEGF signaling, set up these unique epigenetic signatures.

### The lncRNA ANRIL and its role in CVD

Intriguingly, lncRNA might reflect the interaction between epigenetic pathways and CVD genetic determinants. In CAD, for instance, many of the associated genetic variants occur in non-protein coding regions<sup>1</sup>. Although this can reflect the contribution of distal regulatory elements for protein-coding genes, it can alternatively suggest the actions of long noncoding RNAs (lncRNAs). The latter possibility is exemplified by the 9p21 chromosome locus, which contains the strongest genetic risk factor for CAD<sup>1</sup>.

The 9p21 risk variants are single nucleotide polymorphisms (SNP) commonly observed in GWAS studies on CAD. These variants are associated with CAD-related outcomes, including myocardial infarction, and are independent of covariates, such as lipids and hypertension<sup>1</sup>. Strikingly, the top SNP risk allele in the locus increases CAD risk by 36% per copy<sup>1</sup>. Aside from CAD, the 9p21 variants are associated with other vascular phenotypes, including stroke<sup>1</sup>.

These 9p21 risk variants map to a long noncoding RNA gene known as antisense non-coding RNA at the INK4 locus (ANRIL). The closest flanking protein coding genes are

*CDKN2A* and *CDKN2B*, which are about 100 kb away and encode p16INK4A/p14ARF and p15INK4B respectively. Importantly, some of these genetic variants are associated with increased ANRIL expression in cancer and vascular tissue samples<sup>72, 73</sup>. Increased ANRIL expression is observed to inhibit apoptosis, increase cell proliferation and enhance cell adhesion, which might support its pro-atherogenic function<sup>73</sup>. Importantly, ANRIL regulates the expression of genes associated with these processes<sup>73</sup>. Mechanistically, ANRIL can associate with polycomb repressive complex 2 (PRC2), which catalyzes histone H3 lysine 27 trimethylation (H3K27me3), to repress target protein-coding genes, including p15INK4B and p21<sup>72-74</sup>. ANRIL can also recruit PRC2 to repress KLF2, a laminar flow-inducible transcription factor that is important for maintaining a healthy endothelial cell (EC) phenotype, suggesting that ANRIL can potentially act similarly in CVD<sup>72</sup>.

Additional studies on ANRIL will need to be conducted in relevant cell types of CVD, especially ECs. Furthermore, the mechanism by which ANRIL enhances CVD risk also remains elusive and requires further investigation. Since the ANRIL lncRNA can recruit epigenetic modifiers to regulate a distinct set of genes, this may suggest that manipulation of lncRNA expression can be used to modulate the expression of specific genes such as those relevant to CVD.

## Conclusions

With the use of genome-wide approaches for probing the epigenome, the catalogue of pathological epigenetic changes that occur in CVDs grow ever larger. The current challenge is to understand how these epigenetic changes mechanistically alter gene expression and to identify the regulators responsible for these changes. Thus, it is important to have a greater understanding of various mechanisms of epigenetic regulation, including those mediated by intragenic epigenetic modifications and lncRNAs. Consistent with the importance of intragenic epigenetic modifications, most atherosclerosis-specific differentially methylated CpGs are mapped to intragenic regions<sup>40</sup>. Also, enzymes that catalyze these modifications play important roles in vascular biology<sup>52, 53, 60</sup>. It is important to note that the studies on intragenic epigenetic modifications and their epigenetic modifiers focus on protein-coding genes. It is unknown if similar mechanisms are involved in regulating non-protein coding genes and if lncRNAs play a role in regulating these modifications. Greater understanding of intragenic epigenetic modifications and lncRNAs may result in the identification of novel targets for pharmacological inhibition in CVD and improve the efficacy of cell-based therapies of CVDs.

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## Nonstandard Abbreviations and Acronyms

<b>lncRNA</b>	long noncoding RNA
<b>iPSC</b>	inducible pluripotent stem cells
<b>EC</b>	endothelial cells
<b>DNMT</b>	DNA methyltransferase
<b>HAT</b>	histone acetyltransferase
<b>HDAC</b>	histone deacetylase
<b>Pol II</b>	RNA polymerase II
<b>SNP</b>	Single Nucleotide Polymorphism

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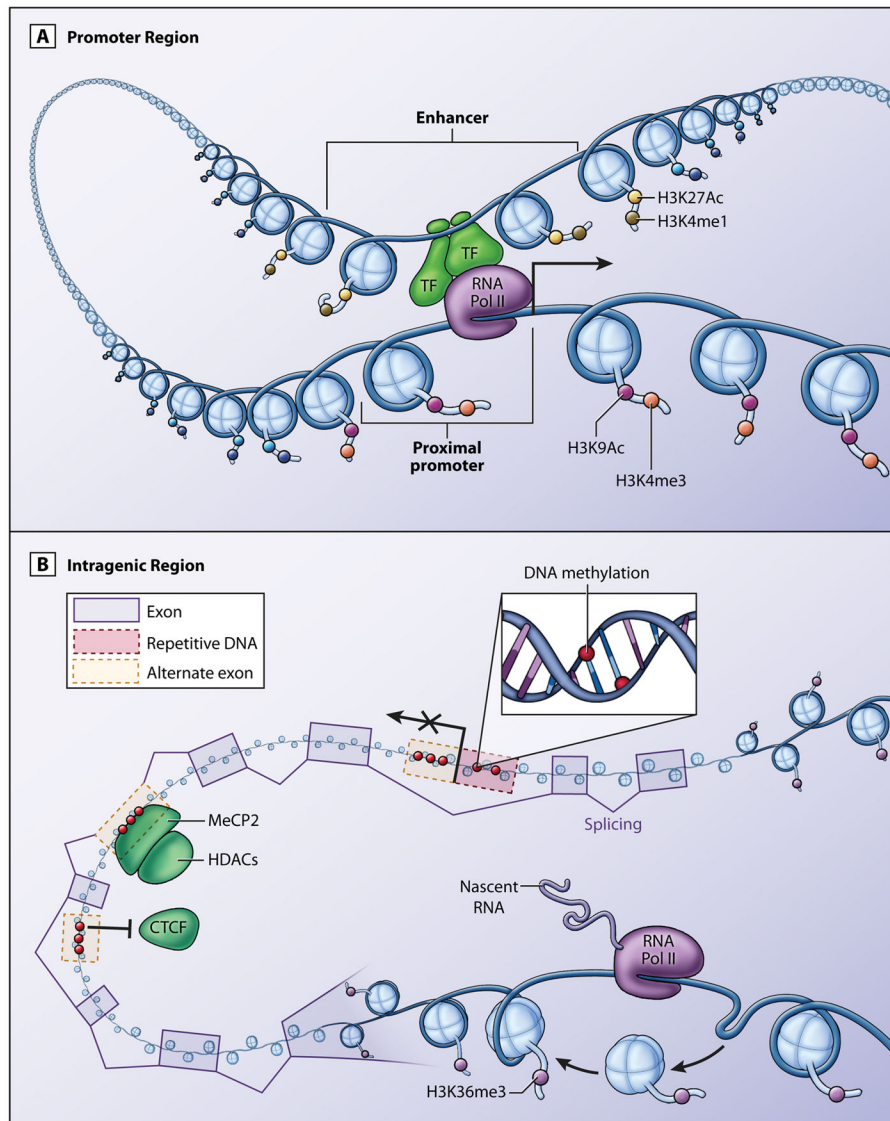


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### Significance

The emergence of whole genome approaches to investigating the role of epigenetic pathways has provided a platform for gaining further insight into under-appreciated aspects of gene regulation, including intragenic epigenetic modifications and long non-coding RNAs. These facets of gene regulation contribute to the function of the vascular endothelium and the pathological changes that occur in CVDs. Greater understanding of epigenetic pathways in the vascular endothelium provides the exciting potential to improve diagnostic and therapeutic approaches for treating CVDs, especially cell-based therapies.



**Figure 1.** Epigenetic-based mechanisms of gene regulation. (A) The epigenetic signatures at the promoter and enhancer of a gene contribute to the recruitment of *trans* factors (TF) and RNA Polymerase II (Pol II) for transcription initiation regulation. Epigenetic modifications that are observed frequently at proximal promoter regions include H3K9Ac (pink) and H3K4me3 (orange). Common epigenetic modifications that are associated with an active enhancer are H3K4me1 (olive) and H3K27Ac (yellow). (B) Intragenic epigenetic modifications are critical for transcription and mRNA variant expression. H3K36me3 (purple) is commonly associated with transcription elongation by RNA polymerase II through intragenic regions. H3K36me3 is believed to be necessary for the inhibition of spurious transcription initiation. Intragenic DNA methylation (red) is positively correlated with gene expression and may prevent spurious transcript initiation from DNA repetitive elements or alternative promoters. Intragenic DNA methylation also affects alternative exon inclusion. DNA methylation may interfere with CTCF recruitment to inhibit exon inclusion.

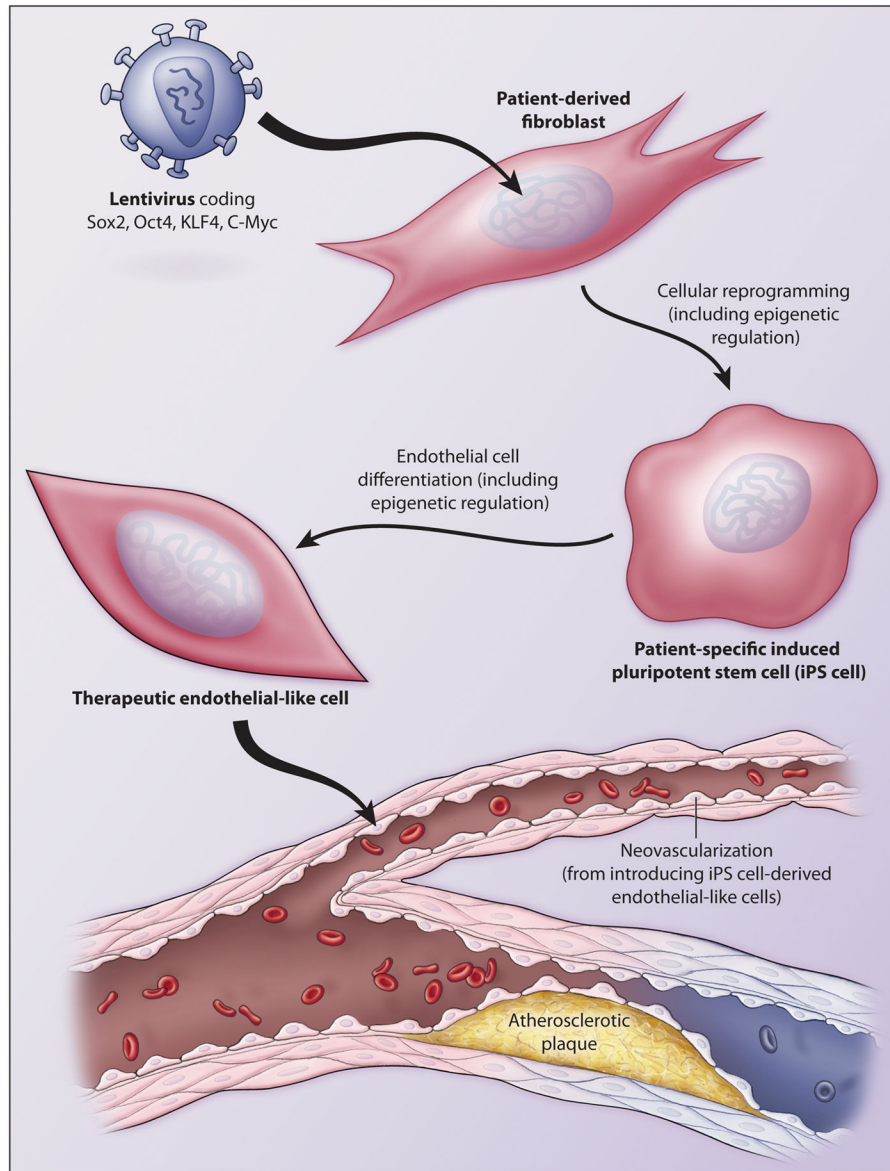
Alternatively, DNA methylation may recruit MeCP2 and HDACs to promote exon inclusion.

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**Figure 2.** Inducible pluripotent stem cell (iPSC)-based therapies for cardiovascular disease (CVD). Patient-derived fibroblasts can be reprogrammed into iPSCs and differentiated into endothelial (EC)-like cells for cell-based therapies of CVD. The processes of cellular reprogramming and endothelial differentiation involve dynamic changes in the epigenetic landscape.