Epigenetics of the vascular endothelium

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Epigenetics in Health and Disease

Yan MS, Matouk CC, Marsden PA. Epigenetics of the vascular endothelium. J Appl Physiol 109: 916–926, 2010. First published April 22, 2010; doi:10.1152/japplphysiol.00131.2010.—Classical models of transcription in vascular endothelial cells, specifically the cis/trans paradigm, have limitations. For instance, how does the environment have chronic effects on gene expression in endothelial cells after weeks or years? When an endothelial cell divides, how is this information transmitted to daughter cells? Epigenetics refers to chromatin-based pathways important in the regulation of gene expression and includes three distinct, but highly interrelated, mechanisms: DNA methylation, histone density and post-translational modifications, and RNA-based mechanisms. Together they offer a newer perspective on transcriptional control paradigms in vascular endothelial cells and provide a molecular basis for understanding how the environment impacts the genome to modify disease susceptibility. This alternative viewpoint for transcriptional regulation allows a reassessment of the cis/trans model and even helps explain some of its limitations. This review provides an introduction to epigenetic concepts for vascular biologists and uses topical examples in cell biology to provide insight into how cell types or even whole organisms, such as monoyzygotic human twins with the same DNA sequence, can exhibit heterogeneous patterns of gene expression, phenotype, or diseases prevalence. Using endothelial nitric oxide synthase (NOS3) as an example, we examine the growing body of evidence implicating epigenetic pathways in the control of vascular endothelial gene expression in health and disease.

Does the cure for cardiovascular disease, especially atherosclerosis, lie in our genes? The promise of the postgenome period argues that it is. In the contrary, we argue that the cure lies in defining how the genome interacts with the environment in which our cells exist. This view is significant because it encompasses epigenetics.

The International Human Epigenome Consortium (IHEC) was launched in January 2010 (1). Looking back, the proposal for sequencing the human genome seemed a daunting task for many of the scientific and public for many of the scientific and public. The International Human Epigenome Consortium (IHEC) was launched in January 2010 (1). Looking back, the proposal for sequencing the human genome seemed a daunting task when launched in 1990. The static genetic code is the same in every diploid human cell, save for germline rearrangements in the T-cell receptors and B-cell receptors in T- and B-cells, respectively, and somatic DNA mutation or copy number variations, among others. Although DNA sequence variation can have important effects on epigenetic modifications, the extent of this diversity is not fully appreciated as evidenced by ~250 distinct cell types in the human organism (1). Scientists are also unsure whether important degrees of heterogeneity exists in cells of the same lineage for epigenetic marks at identical haplotypes, sets of alleles at multiple loci on the same chromosome that are commonly transmitted together. Remembering that the term “epigenetics” was initially used to refer to the complex interactions between the genome and the environment that are involved in development and differentiation in higher organisms, reminds us that the epigenetic code is superimposed on the static genetic code. Today, the term “epigenetics” is used to refer to heritable alterations in chromatin that are not due to changes in DNA sequence per se (6). The potential exists, when taken together, therefore, for substantially higher levels of epigenetic diversity that is distributed in time and space. This is significant as we have, for some time, accepted that common diseases of the human cardiovascular system are influenced by complex interactions between the genome and environment. For example, atherosclerosis has well-defined genetic determinants as well as environmental risk factors. Although poorly understood, the epigenetic perspective is shedding new light on how the environment influences gene expression and disease susceptibility (27). Perhaps most importantly, the dynamic nature of epigenetic modification offers the possibility of therapeutic intervention. To date, the roles of these pathways in vascular endothelial biology are only beginning to be explored (61).
This review takes some topical examples from general biology to introduce how epigenetics is relevant in disparate settings (Fig. 1). We also present a conceptual framework for understanding the role of epigenetics in complex non-Mendelian diseases, including common cardiovascular diseases. We will use studies performed on the endothelial nitric oxide synthase (eNOS, NOS3) gene to illustrate key concepts that are relevant to gaining an understanding of how epigenetic pathways regulate vascular endothelial gene expression in health and disease.

**EPIGENETIC BASIS FOR DIFFERENCES IN GENE EXPRESSION WHEN THE DNA SEQUENCE IS IDENTICAL—APPLYING CONCEPTS TO CARDIOVASCULAR DISEASE**

Unlike familial monogenic disorders, non-Mendelian diseases share some peculiarities that are difficult to explain using current genetic paradigms. Similarly, cis/trans paradigms of gene expression, the concept of particular transcription factors (trans factors) binding to canonical promoter DNA elements to mediate a distinct transcriptional program, also cannot fully explain these peculiarities. The peculiarities that are commonly demonstrated by non-Mendelian diseases include discordance between monozygotic twins, sexual dimorphism, parent-of-origin-dependent clinical differences, progression of disease severity over time, and a relatively late age of onset (71). These characteristics are true for a number of common cardiovascular diseases, such as atherosclerosis and hypertension. The classical argument is to ascribe these disease characteristics to poorly defined environmental influences (both internal and external to the cell). We argue here that the molecular mechanisms that translate environmental influences onto the genome may well be epigenetic in basis by demonstrating...
Reprogramming Differentiated Cells

Somatic cell nuclear transfer is a cloning strategy that injects the nucleus from a donor somatic cell into a freshly fertilized enucleated oocyte. In reproductive cloning, this embryo is subsequently implanted into a pseudopregnant female to generate a genetically identical clone (46; Fig. 1A). The first mammal cloned from a somatic cell is Dolly the sheep (93). Accrued experimental evidence in a number of mammalian species has demonstrated that reproductive cloning is an extremely inefficient process, with the vast majority of cloned embryos dying in utero (102). The few nuclear transfer embryos that do survive show abnormal phenotypes, such as premature death, as exemplified by Dolly after accounting for technical failures of nuclear transfer (46). The prevailing model for the low efficiency of reproductive cloning is faulty nuclear reprogramming. This is likely because much is asked for successful reproductive cloning to occur. Namely, it requires the somatic cell to dedifferentiate to a totipotent state and redifferentiate to form a viable, adult organism (102). Mechanistically, this is mediated by pronounced changes in gene expression that reflect, in part, dynamic changes in the cellular chromatin landscape. To date, the best understood epigenetic mark in early embryonic development is DNA methylation (102). In both male and female haploid gametes, the amount of DNA methylation is high. Shortly after fertilization, the male pronucleus is specifically actively demethylated, while the female pronucleus undergoes passive demethylation (63). In mice, genome-wide de novo methylation follows at the blastocyst stage. In cloned embryos, the male and female contributions to the somatic cell nucleus might not be accurately distinguished and widespread abnormalities, such as defective genomic imprinting and X-chromosome inactivation, ensue from abnormal DNA methylation patterning (23, 46, 102). Interestingly, normal breeding of these mice yields offspring with normal phenotype. If the abnormal phenotypes were the result of genetic mutations, these would presumably be faithfully inherited across generations. Thus these data suggest an epigenetic basis for the abnormal phenotypes of these “cloned” animals (46, 102).

A further example of applied epigenetics in reprogramming is inducible pluripotent stem cell (iPS cells) generation. iPS cells are somatic cells reprogrammed to an embryonic stem (ES) cell-like state via the ectopic expression of ES cell-related transcriptional factors, such as Oct3/4, Sox2, Klf4, c-Myc, Nanog, Esrrb, and/or Lin2 (28, 41). iPS cells are ES-cell-like by virtue of their self-renewing and totipotent properties, similar cellular phenotypes, gene expression patterns, and epigenetic profiles (41, 67). Clinicians are excited because it is anticipated that patient-specific iPS cells may offer newer approaches to treat cardiovascular diseases. Although the molecular mechanism behind iPS cell generation is unclear, epigenetic pathways appear to play a fundamental role. This was demonstrated by studies in partially reprogrammed somatic cells, which are characterized by reactivation of a distinct subset of stem cell-related genes, incomplete repression of lineage-specifying transcription factors, and incomplete epigenetic remodeling (67). On treatment with 5-azacytidine, an inhibitor of DNA methylation activity, these partially reprogrammed cells undergo a rapid, stable transition to fully reprogrammed iPS cells (67). Practically, we now know that pharmacological agents that affect various chromatin modifications enhance the efficiency of iPS cell generation (41).

Monozygotic Twins

Human monozygotic (MZ) twins provide a natural experimental system to explore the contributions of epigenetic mechanisms to phenotypic variance (Fig. 1B). Classically, twin studies are used to determine the relative contributions of genetic and environmental factors to a disease phenotype. MZ twins are genetically identical, whereas dizygotic (DZ) twins share approximately half of their genetic code with equal contributions from each parent. A strong genetic component of disease is inferred if disease prevalence is increased among MZ versus DZ twins and this is quantified in the metric heritability (61). Although heritability estimates a strong genetic contribution (30–50%) for common cardiovascular diseases, MZ twin pairs frequently show low concordance rates for disease phenotype (38, 62). The classical explanation for this apparent paradox is the differential effect of the environment on genetically identical individuals, which is arguably regulated by epigenetic pathways.

In 2005, Fraga et al. (32) catalogued the global and locus-specific differences in DNA methylation and histone H3 and H4 acetylation in a large cohort of MZ twins of various ages. Comparison of the epigenetic profiles within twin pairs revealed several key observations. First, the most epigenetically similar and dissimilar twins (at least for the three epigenetic marks surveyed) were the youngest and oldest pairs, respectively. Second, twin pairs with the most discordant epigenetic profiles spent less of their lifetime together and/or reported the greatest differences in natural health/medical history. Finally, the degree of epigenetic discordance within twin pairs appeared to correlate with the degree of intra-twin pair differential mRNA expression.

Although the study of this MZ twin study did not allow correlation with disease discordance, studies by others have demonstrated that discordance for some diseases, such as Alzheimer’s disease, are associated with global or loci-specific differences in DNA methylation status (53, 60, 100). It would be of interest to directly assess epigenetic changes temporally in individual MZ twins. Nonetheless, studies support the notion that environment-dependent epigenetic modifications acquired throughout an individual’s life span might affect human gene expression and health (32, 61).

Hutchinson-Gilford Progeria Syndrome and Human Aging

Although common cardiovascular diseases demonstrate non-Mendelian patterns of inheritance, much can be learned about them by studying monogenic disorders. One example is the Hutchinson-Gilford progeria syndrome (HGPS), a childhood disease of premature aging that occurs in 1 of 4 million live births. Affected children are diagnosed at a young age with failure to thrive and prototypical skin abnormalities reminiscent of aging, such as prominent cutaneous vasculature. These children develop severe atherosclerosis and die from myocardial infarction and stroke at ~13 yr of age (66). HGPS results from a specific mutation (a C-to-T substitution, 1824C→T) in
the LMNA gene encoding lamin A, the major structural component of the nuclear lamina juxtaposed between the inner membrane of the nuclear envelope and chromatin (25). This genetic mutation activates a cryptic splice donor site resulting in a new mRNA species that is translated to a novel protein, progerin, with a 50 amino acid internal deletion. Progerin induces dysmorphic nuclei with nuclear blebbing that progress over time (25, 34). Although how progerin causes HGPS is unclear, evidence suggests that changes in epigenetic pathways are seminal. Similar to normally aged cells, the normal organization and structure of chromatin is disrupted in HGPS nuclei (25, 34, 75, 76). In particular, these cells demonstrate dramatically reduced heterochromatin, regions of limited transcription that are preceded by the progressive loss of repressive epigenetic marks including trimethylated H3K9 and H3K27 (79; Fig. 1C). This loss of epigenetic control appears to be correlated with widespread abnormalities in gene expression (76, 79). Since dramatic epigenetic changes occur in HGPS and normal aging, and HGSP patients exhibit accelerated atherosclerosis, we argue that studies of epigenetic pathways in human atherosclerosis are warranted and timely. This is further supported by the fact that progerin activates the effectors of the Notch signaling pathway, which plays a role in endothelial dysfunction (35, 74). It is noteworthy that the abnormal phenotype of HGSP in cell culture and transgenic mice models can be reversed by inhibiting progerin expression, thereby underscoring the dynamic nature of epigenetic pathways (73, 76).

Taken together, epigenetic pathways exert considerable influence on the genome’s structure and function from the earliest time in development, throughout the normal and abnormal process of aging and nuclear reprogramming (summarized in Fig. 1). Specifically, in the three examples presented here, epigenetic mechanisms appear to be involved in regulating phenotypic characteristics that cannot be fully defined by genetics or cis/trans regulation of gene expression. By translating the effects of environmental stimuli into coordinated gene expression programs for cellular adaptation, epigenetic pathways are potentially the mechanistic link between the genome and environment that is important in understanding common cardiovascular diseases.

**OVERVIEW OF EPIGENETIC MECHANISMS**

The molecular foundation of epigenetic theory is comprised of three highly interconnected pathways: DNA methylation, histone posttranslational modifications, and RNA-based mechanisms (Fig. 2). Together, they modulate the structure and accessibility of DNA, thereby providing an important regulatory level of transcriptional control. Specifically, the three mechanisms are involved in the formation of euchromatin and heterochromatin. These older terms are still useful in conveying concepts. In general, euchromatin represents decondensed chromatin that is actively transcribed and affiliated with activating epigenetic marks. In contrast, heterochromatin is condensed chromatin that has limited transcription and is associated with repressive epigenetic marks (85). Over the last 20 years, significant progress has been made in understanding the epigenetic marks, the processes that create and erase them, and...
the readers that interpret them. This section provides a brief review of epigenetic pathways in mammals.

DNA Methylation

DNA methylation refers to the addition of a methyl group to the 5-position of cytosine to create 5-methyl-cytosine (68). There is an inverse correlation between DNA methylation at promoter regulatory regions and gene transcription (64). As such, this review will focus on its repressive function.

DNA methylation has functional roles in X chromosome inactivation, genomic imprinting, embryonic development, and lineage specification (7, 68). Its dysregulation, in part, defines the tumor cell phenotype. DNA methylation at cytosine residues occurs almost exclusively in the context of the sequence CpG. However, non-CpG methylation has been observed in early development (58), endogenous LINE-1 retroelements (95), and integrated plasmid DNA (17). DNA methylation is catalyzed by three distinct enzymes that are collectively known as DNA methyltransferases (DNMTs). DNMT1, the “maintenance” methyltransferase, is believed to transmit DNA methylation patterns during mitotic cell division. In contrast, DNMT3a and DNMT3b function act as de novo methyltransferases that establish DNA methylation patterns during embryonic development (68). The mechanisms responsible for DNA demethylation remain poorly defined, but include both passive (replication dependent) and active (replication independent) processes (68). Interestingly, 5-methyl-cytosine can be hydroxylated into 5-hydroxymethylcytosine in murine ES cells and the purkinje and granule cells of the brain. 5-Hydroxymethylcytosine might be an intermediate of either DNA demethylation processes (52, 81).

Three general mechanisms have been proposed for 5-methylcytosine-mediated gene repression. First, 5-methyl-cytosine can sterically interfere with transcription factors to their cis-DNA binding elements. This mechanism has been described for several transcription factors, including hypoxia-inducible factor-1α (HIF1α), but not others (5, 20, 37, 92). Second, methyl-CpG binding proteins, such as MeCP2, can interfere with the recruitment of transcriptional machinery, such as DNA-binding trans factors. Third, methyl CpG binding proteins can recruit large chromatin modifying complexes that reduce DNA accessibility (68). For example, MeCP2 can recruit HDACs, histone methyltransferases, and the ATP-dependent Swi/Snf chromatin remodeling complex (68).

Histone Protein

In the nucleus, DNA is packaged into chromatin as repeating units of nucleosomes, which form a “beads-on-a-string” structure that can compact into higher order structures to affect gene expression. Nucleosomes are composed of 146-bp DNA wrapped in histone octamers (composed of two H2A, H2B, H3, and H4) and are connected by a linker DNA, which can associate with histone H1 to form heterochromatin (86). Histone proteins contain a globular domain and an amino-terminal tail, with the latter being posttranslationally modified. Currently, more than 60 modifications have been described, including the posttranslational modification of lysine (acylation, methylation, ubiquitination, sumoylation), arginine (methylation), and serine and threonine (phosphorylation) (7, 89). Many of these modifications are known to play functional roles in transcription (Table 1).

### Table 1. Histone posttranslational modifications and gene transcription

<table>
<thead>
<tr>
<th>Histone Posttranslational Modification</th>
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<td>Histone H3</td>
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<td>Acetylation (K9, K14)</td>
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<td>K4 (Trimethyl)</td>
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<td>Histone H4</td>
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<tr>
<td>Acetylation (K5, K8, K12, K16)</td>
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<td>Methylation</td>
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<td>K20 (Trimethyl)</td>
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<td>Histone H2A</td>
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<td>Ubiquitination (K119)</td>
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<td>Histone H2B</td>
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<td>Ubiquitination (K120)</td>
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K denotes lysine; R, arginine; S, serine; T, threonine.

The histone code hypothesis proposes that the combination of histone posttranslational modifications encode regulatory information interpretable by the cell (80). An increasing number of proteins that specifically recognize unique posttranslational modifications are being uncovered (89).

The functional roles of lysine acetylation and methylation on gene expression are best understood. The most important nucleosomes here are commonly at the promoter regions. Histone acetylation is associated with transcription activation and is dynamically regulated by the competing enzymatic activities of histone acetyltransferases (HATs) and histone deacetylases (HDACs), which mediate its addition and removal, respectively. Although HATs and HDACs can non-specifically regulate the acetylated states of proteins, their specificity in histone modification is achieved, in part, by their recruitment to chromatin in multiprotein complexes (89). Histone acetylation is believed to enhance transcription by neutralizing the basic charges of lysine residues and recruiting bromodomain-containing proteins, including other HATs and chromatin remodeling enzymes, that prevent chromatin compaction (78).

In contrast to histone acetylation, the impact of histone lysine methylation on gene expression is dependent on the specific lysine residue. For example, genome-wide profiles of histone methylation show that H3K4 and H3K36 methylation are associated with transcriptionally permissive chromatin, whereas H3K9 and H3K27 methylation are markers of transcriptionally silent chromatin (4). In addition, single lysine residues are variably methylated to mono-, di-, and trimethylated states. This can be contrasted with addition of a single acetyl group. Some lysine residues can be modified by either methylation or acetylation, but never both together. The different histone methylation states are functionally relevant. Active promoters are enriched in trimethylated H3K4, while enhancer elements are enriched in monomethylated H3K4 (39). Similar to histone acetylation, histone methylation status at a particular lysine is dynamically regulated by histone methyltransferases and histone demethylases (18). The molecular
mechanisms behind the functional effects of histone methylation marks are being uncovered. For instance, trimethylated H3K4 is implicated in recruiting PHD finger-containing proteins to recruit chromatin remodeling complexes and transcription machinery to promote transcription (87, 97). In contrast, trimethylated H3K9 recruits heterochromatin protein 1 to form transcriptionally silent, constitutive heterochromatin (7, 89).

Another facet of histone biology that is involved in transcriptional regulation is histone density. In general, the histone density at the transcriptional start site of expressed genes is depleted relative to non-expressed genes, suggesting that low histone density is associated with transcription (77). However, histone density can be altered to activate or repress specific genes in response to cellular activation. For instance, T-cell activation results in the loss of histones at the IL-2 promoter. This acute change in histone density is functionally relevant to enhanced IL-2 gene transcription (14). In contrast, the repression of cyclin A, a cell cycle regulator, in quiescent cells is, in part, due to the maintenance of histone density at its promoter by Brahma containing chromatin remodeling complexes (19).

RNA-Based Mechanisms

RNA-based mechanisms of epigenetic gene regulation involve the coordinated activities of noncoding RNAs (ncRNA) with other epigenetic activities, such as DNA methylation and histone posttranslational modifications. Studies suggest that long and short ncRNAs, which are distinguished by an arbitrary size cutoff of 200 nucleotides, can regulate the chromatin state of genomic loci (65). A large number of large intervening non-coding RNAs (lincRNAs) have just been described in the genomes of humans and mice. A total of ~3,300 lincRNAs have been identified with computationally predicted roles in various cellular processes, including cell-cycle regulation (36, 50). lincRNAs can recruit chromatin modifying activity and regulate gene expression at target loci (50). An excellent example is HOTAIR, which aids in HoxD silencing by recruiting polycomb repressive complex 2 and its H3K27 trimethylation activity (65). Long ncRNAs that overlap protein-coding genes can also recruit chromatin modifying complexes to regulate gene expression at target loci. Examples of this include Xist, which is involved in X chromosome inactivation. Additional examples include Air and Kcnq1ot1, which are involved in genomic imprinting, a process that mediates the expression of only one allele of a gene in a parent-of-origin-dependent manner (65). In addition, long ncRNAs can potentially mediate transcriptional activation via recruitment of the H3K4 methyltransferase MLL1 (65). Transcriptional silencing by small ncRNA in mammals may also occur, but is poorly understood. DNA methylation and repressive histone modifications can be elicited at target gene promoters following treatment of cells with exogenous administration of small interfering RNAs (siRNAs) (69). Interestingly, a similar phenomenon may be mediated by endogenous miRNAs (69). Taken together, these observations suggest a fundamental role of RNA-based mechanisms in gene regulation.

eNOS: FIRST CLUE TO THE IMPORTANCE OF EPIGENETIC REGULATION OF VASCULAR ENDOTHELIAL GENE EXPRESSION

The constitutively expressed endothelial NOS (NOS3) is the main source of NO in the vascular endothelium and is pivotal for its function (61). eNOS-null mice are characterized by systemic and pulmonary hypertension, impaired wound healing and angiogenesis, impaired mobilization of stem and progenitor cells for neovascularization, and reduced vascular leakage during acute inflammation (2, 9, 42, 55, 72). Due to the pivotal physiological role of eNOS in the vascular endothelium, its regulation has been extensively studied.

eNOS is a member of a unique set of endothelial-restricted genes that define endothelial cell identity. In contrast to other cell types such as skeletal muscle or adipocytes, there are no known “master regulators” of gene expression, such as MyoD or PPAR-γ, respectively, that are specifically expressed only in ECs (56, 82). A number of transcription factors have been shown to be preferentially expressed in differentiating endothelial progenitor cells and mature ECs and have been argued to orchestrate the expression of a wide number of endothelial genes. Indeed, the promoters and enhancers of endothelial-restricted genes are commonly enriched with cis-binding elements recognized by such factors, including Sp1, forkhead, and Ets proteins, among others (21, 29). Additionally, a 44-bp enhancer containing the composite cis-binding element of Forkhead and Ets proteins has been found to be present at many endothelial-restricted gene enhancers and is sufficient for directing endothelial-specific expression (22). However, the paradox is that these transcription factors are not restricted in expression to the vascular endothelium. Thus the concept of a master transcription factor (trans factor) binding to a canonical promoter DNA element (cis element), the cis/trans paradigm, that is uniquely evident in EC-enriched genes has, to date, not been substantiated by published work. Nonetheless, is there additional regulatory information that allows ubiquitous transcription factors to distinguish and induce the appropriate expression of endothelial-restricted genes? One possible source of information is their chromatin accessibility.

eNOS is the most well-characterized example of an endothelial-restricted gene that is regulated by its chromatin accessibility. eNOS evidences a TATA-less promoter with two 5′ cis regulatory element, known as positive regulatory domain I and II, that are situated –104/–95 and –144/–115, respectively, from its single major transcriptional start site (TSS) (49, 59). In addition, eNOS has a 269-bp enhancer that is −4.9 kb from the TSS (54). Similar to other endothelial-restricted genes, the regulatory DNA elements of eNOS can bind ubiquitous transcription factors, including Sp1 and the Ets (54, 61). Although transient transfection of eNOS promoter-reporter constructs into various expressing and non-expressing cells show robust promoter activity (12), eNOS promoter-reporter transgenic mice show endothelial-restricted expression (83). These observations suggest that the chromatin context of eNOS is involved in regulating its endothelial-restricted expression.

Indeed, the chromatin structure at the eNOS promoter is transcriptionally permissive in endothelial cells and repressive in nonendothelial cells. Specifically, the eNOS promoter in endothelial cells was found to be DNA hypomethylated and enriched with activating histone posttranslational modifications, including acetylated H3K9, acetylated H4K12, di- and trimethylated H3K4, by sodium bisulfite genomic DNA sequencing analysis and ChIP analysis, respectively (12, 30) (Fig. 3). In contrast, similar analysis of the eNOS promoter in non-expressing cell types, such as vascular smooth muscle cells (VSMCs), showed DNA hypermethylation and a lack of activating histone posttranslational modifications. Consistent with the differences in the chromatin
structure of the eNOS promoter, ChIP analysis showed selected recruitment of Sp1, Sp3, Ets transcription factors and RNA polymerase II to the eNOS proximal promoter in endothelial cells, while MeCP2 and HDAC1 were specifically localized to the promoter in VSMCs (12, 30, 33) (Fig. 3). The functional importance of DNA methylation and histone posttranslational modifications at the eNOS promoter was demonstrated by pharmacological inhibition studies. Namely, treatments of VSMC with 5-azacytidine, a DNMT inhibitor, and trichostatin A, a HDAC inhibitor, upregulated eNOS mRNA levels. In contrast, eNOS expression was downregulated in endothelial cells when treated with methylthioadenosine, a H3K4 methylation inhibitor.

eNOS is also regulated by RNA-based mechanisms. A 27-nt RNA duplex produced at the variable number tandem repeat region (VNTR) of intron 4 in eNOS was found to be expressed and localized to the nucleus of endothelial cells exclusively (103, 105). Interestingly, exogenous administration of the small RNA to endothelial cells induced H3K9 and H4K12 hypoacetylation at the eNOS promoter, DNA methylation at exon 3 of eNOS, and reduced eNOS transcription (103, 104). The repressive function of the small RNA was supported by the ability to salvage eNOS expression in the small RNA transfected endothelial cells by HDACIII depletion and treatments with trichostatin A and 5-azacytidine (104). Although the biological relevance of micromanaging eNOS transcription by the 27-nt RNA duplex is unknown, it is clinically relevant that copy number polymorphism of the eNOS VNTR is associated with risk for ischemic heart disease (10).

Taken together, chromatin-based mechanisms of gene regulation ensure that eNOS expression is restricted to endothelial cells at, perhaps, an appropriate level. It is important to note that chromatin-based gene regulation is observed in other endothelial-restricted genes, including vWF, Notch4, and E-selectin (24, 70, 96).

THE EPIGENETIC PERSPECTIVE ON HUMAN CARDIOVASCULAR DISEASE

Recent years have witnessed an increased appreciation for the potential of modulating epigenetic pathways to treat disease. For example, pharmacological HDAC inhibitors are under investigation in treating cancers (40) and have shown promise in treating chronic inflammatory diseases, including rheumatoid arthritis among others (3, 57, 88). However, the
demonstration that TSA treatment of atherosclerosis-prone Ldlr−/− mice exacerbates neointimal lesions underscores the need for improving our understanding of epigenetic pathways in cardiovascular disease (16). From this perspective, the contribution of epigenetic pathways in the endothelial response to external stimuli, including the physical forces of circulation (e.g., shear stress), hypoxia, cytokines (11, 45, 90), and entry into the cell cycle (61), are being explored (Fig. 4).

Interestingly, laminar shear stress can elicit both global and gene-specific histone modification changes in cultured human endothelial cells (43). Shear stress can affect changes in global histone modification in mouse ES cells and promote their differentiation to an endothelial cell lineage (44, 99). Laminar shear stress can also induce histone modifications at specific sites in the genome as demonstrated by the dependency on p300/HAT-mediated H3 and H4 acetylation in laminar flow-induced eNOS expression (13). Since laminar flow can affect gene regulation via epigenetic pathways, disturbed flow may impinge on them to regulate gene expression. Whether epigenetic pathways contribute to the susceptibility of different regions in the vasculature to atherosclerosis is worth considering, especially since the expression of eNOS, an atheroprotective gene, is lower at regions of the mouse aorta with high probability (HP) of developing atherosclerosis compared with regions with low probability (LP) of developing the disease (84, 94).

Hypoxia has major effects on endothelial phenotype. In general, hypoxia decreases global transcriptional activity (48). The hypoxia-inducible factor (HIF) transcription paradigm is an ancient eukaryotic response that allows cells to adapt to changes in oxygen supply or availability. Evidence suggests that epigenetic pathways are also relevant. In contrast to the HIF cis/trans transcription paradigm, which is well studied, the effects of hypoxia on chromatin-based pathways is a ripe area for detailed study. Concomitant with this, hypoxia induces a global decrease in H3K9 acetylation in various cells as a possible consequence of HDAC upregulation (47, 48). However, acetylated H3K9 is enriched at the promoters of hypoxia-activated genes, such as VEGF (31, 47, 48).

In contrast to histone acetylation, hypoxia-mediated changes in histone methylation are more complicated and also a newer area for study. Consistent with decreased global transcriptional activity under hypoxic conditions, increased global H3K9 dimethylation, a repressive histone mark, has been observed across different cells and is attributed, in part, to increased G9a histone methyltransferase expression (47, 48). Although other repressive histone methylation marks increase globally, global di- and tri-methylated H3K4 levels, which are activating histone marks, are paradoxically elevated (48). It is tempting to attribute this to the decreased catalytic activities of oxygen-dependent JmjC-demethylase domain-containing histone demethylases. This is because structural studies on the JmjC domain of JmjD1a show a similarity to Fe(II)- and 2-oxoglutarate-dependent dioxygenases, whose catalytic activities are responsive to cellular oxygen levels (15). However, the mRNA levels of 17 of 22 JmjC-domain family members are upregulated by hypoxia (98). In fact, JmjD1A, JmjD2B, JmjD2C, JARID1B are directly regulated by the HIF transcription factor, the heterodimeric master regulator of the hypoxia-induced gene transcription program (8, 98, 101). Histone demethylase upregulation might be a compensatory mechanism for minimizing increases in global histone methylation levels as demonstrated by an increase in global trimethylated H3K4 levels in hypoxic cells with a disrupted HIF pathway (98). This is significant, as it suggests that HIF is involved in maintaining global transcriptional silencing, as well as directing gene repression at specific genes (26, 91). However, in contrary to the compensatory role of histone demethylase in global histone methylation levels, depletion of methyl H3K9 demethylases, JmjD2B and JmjD1A, does not affect global di- and trimethylated H3K9 levels (8).

How is a distinct epigenetic signature established in hypoxia-regulated gene promoters? One possibility is that the original epigenetic signature of a hypoxia-regulated gene is reset and established anew. In support of this, Fish et al. (31) demonstrated that rapid eNOS transcriptional repression in hypoxic endothelial cells is associated with a decrease in histone H3 and H4 acetylation levels at the eNOS proximal promoter. This is mediated by histone eviction and subsequent reincorporation of histones that lack substantial modifications. Although not observed at eNOS, it is possible that the reincor-

![Fig. 4. The epigenetic perspective on human cardiovascular disease. Epigenetic pathways, which are important in the transcriptional control of gene expression, are responsive to various physiological and pathophysiological cues relevant to the health of the vascular endothelium. Some of these cues are shear stress (blood flow), hypoxia, cytokines, and entry into the cell cycle. Their ability to act as molecular integrators of environmental signals internal and external to the cell forms the basis for this fundamentally new, epigenetic perspective on human cardiovascular disease.](http://jap.physiology.org/)
porated histones are modified to establish a distinct hypoxic epigenetic signature at other hypoxia-regulated genes.

The effects of hypoxia on global levels of DNA methylation are just beginning to be studied. Fish et al. (31) recently reported that acute (4 h) or chronic (24 h) hypoxia does not have a major effect on global levels of endothelial cell DNA methylation. Little is known about whether DNA methylation levels are altered at specific genes under hypoxic conditions to regulate transcription.

Our current understanding of hypoxia-regulated epigenetic pathways, as discussed above, is relatively sparse. Future genome-wide mapping of specific acetyl and methyl histone modifications, histone demethylases, histone density, and DNA methylation in hypoxic cells will be necessary to fully understand their importance in transcriptional regulation and formation of distinct hypoxia-mediated epigenetic signatures at hypoxia-regulated genes. This may be therapeutically useful as shown by the finding that TSA can blunt hypoxia-inducible angiogenesis of mature endothelial cells (51). This finding suggests that manipulation of the epigenetic pathways may be clinically relevant in inhibiting tumor angiogenesis.

SUMMARY
So pervasive is the role of epigenetic pathways in the response of endothelial cells to physiological and pathophysiological stimuli that it represents a fundamentally new perspective on human cardiovascular disease. This perspective is exciting given the possibility of therapeutic intervention by environmental and pharmacological modulation of epigenetic pathways. Additional studies that expand our understanding of chromatin-based regulation of endothelial restricted gene expression are important because of their translational implications for regenerative medicine and blood vessel diseases.

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REFERENCES


