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HIGHLIGHTED TOPIC | *Epigenetics in Health and Disease*

## Epigenetics of the vascular endothelium

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**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/jappphysiol.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 monozygotic 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.

cell-specific expression; DNA methylation; histone code; endothelial nitric oxide synthase

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 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).

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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

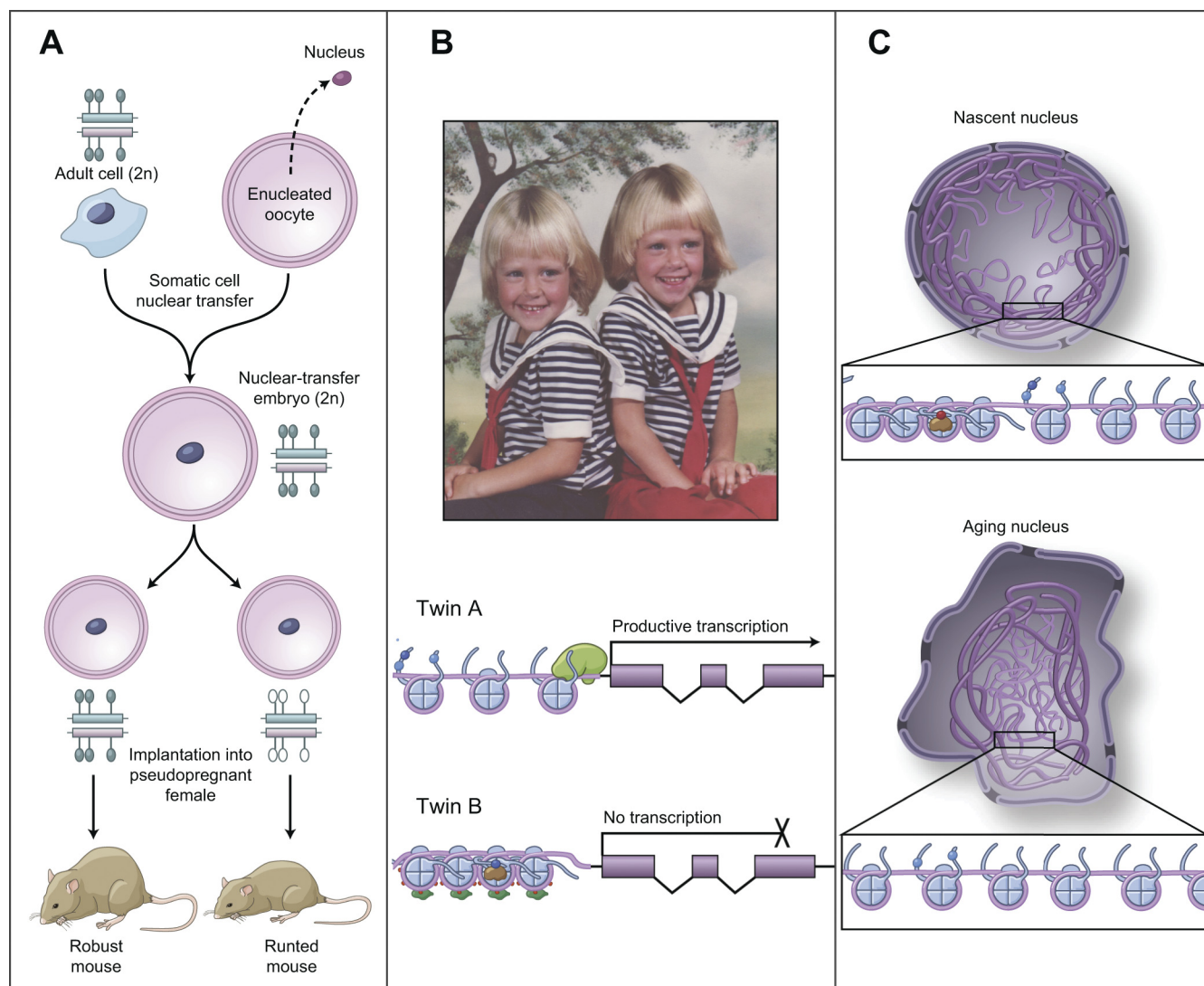


Fig. 1. Epigenetic pathways are broadly relevant to cardiovascular disease. **A**: somatic cell nuclear transfer is a cloning strategy that involves the transplantation of a donor nucleus from an adult somatic cell (blue) into an enucleated oocyte (pink). In reproductive cloning, the nuclear transfer embryo is implanted into a pseudopregnant female for the purpose of generating a genetically identical cloned animal. The prevailing model for the low efficiency of reproductive cloning and the abnormal phenotypes of cloned animals is faulty nuclear reprogramming, a fundamental process governed by the epigenetic state of the nucleus, in particular, DNA methylation (○ and ●, unmethylated and methylated CpG dinucleotides, respectively). **B**: discordance of monozygotic twins for disease phenotype in common cardiovascular disease may reflect different epigenetic states at a given genetic locus. **C**: Hutchinson-Gilford progeria syndrome and normal human aging evidences progressive epigenetic changes over time. The loss of heterochromatin is one example of these epigenetic changes.

how epigenetics is relevant in three biological settings (Fig. 1) (27, 61, 71).

### *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 adequately 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 modulations 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 HGPS 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 HGPS 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

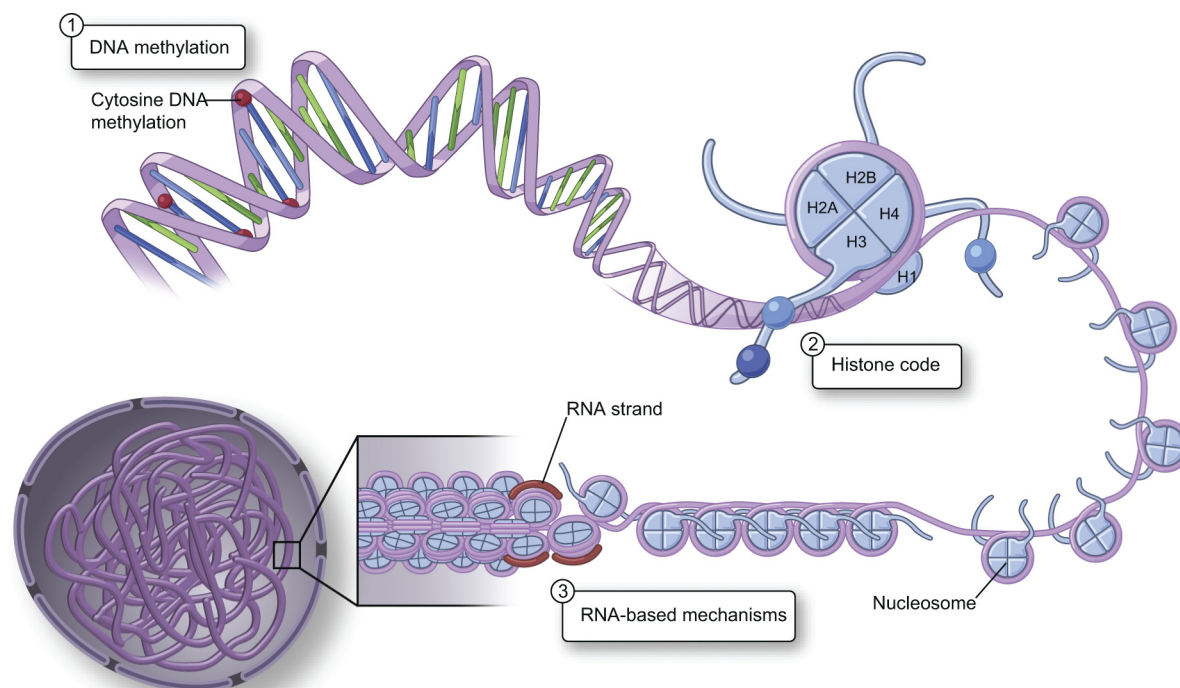


Fig. 2. Three fundamental mechanisms of epigenetic gene regulation. Epigenetic mechanisms of gene expression are subserved by three distinct, yet highly interrelated, mechanisms. 1) DNA methylation refers to the addition of a methyl group to the 5-position of cytosine in the context of CpG dinucleotides to define the “fifth base of DNA.” 2) The fundamental repeating unit of chromatin is the nucleosome comprised of an octamer of core histone proteins. Posttranslational modifications of the amino-terminal tails of histone proteins (light and dark blue balls) and the density of these proteins per unit length of DNA, can importantly affect chromatin structure and constitute a putative “histone code.” 3) RNA-based mechanisms have also recently been shown to impact on the higher-order structure of chromatin.

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-methyl-cytosine-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-1 $\alpha$  (HIF1 $\alpha$ ), 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 (acetylation, 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*

Histone Posttranslational Modification	Transcriptional Effect
Histone H3	
Acetylation (K9, K14)	↑
Methylation	
K4 (Trimethyl)	↑
K9 (Trimethyl)	↓
K27 (Trimethyl)	↓
K36 (Trimethyl)	↑
K64 (Trimethyl)	↓
R2 (Dimethyl)	↓
Phosphorylation	
S10	↑ / ↓
T6	↑
T11	↑
Histone H4	
Acetylation (K5, K8, K12, K16)	↑
Methylation	
K20 (Trimethyl)	↓
Histone H2A	
Ubiquitination (K119)	↓
Histone H2B	
Ubiquitination (K120)	↑

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

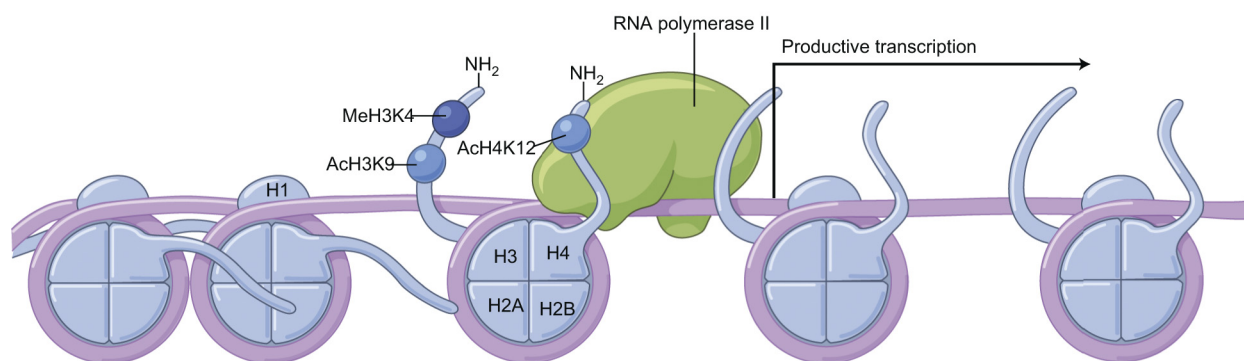
systemic and pulmonary hypertension, impaired wound healing and angiogenesis, impaired mobilization of stem and progenitor cells for neovascularization, and reduced vascular leakage during acute inflammation (2, 9, 42, 55, 72). Due to the pivotal physiological role of eNOS in the vascular endothelium, 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- $\gamma$ , 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 Sp-1, 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

### A eNOS promoter in vascular endothelial cells



### B eNOS promoter in vascular smooth muscle cells

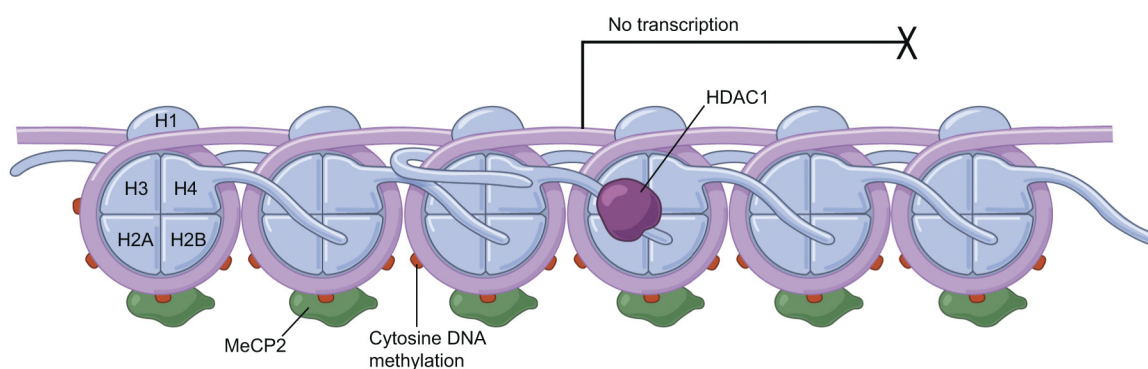


Fig. 3. eNOS—a model system for studying epigenetic pathways in the vascular endothelium. *A*: proximal eNOS promoter in expressing endothelial cells is defined by an open chromatin configuration, lack of DNA methylation, and the preferential enrichment of activating posttranslational histone modifications. This enables the efficient recruitment of the transcriptional machinery, including RNA polymerase II, to the eNOS proximal promoter and productive transcription. *B*: in contrast, the proximal eNOS promoter in nonexpressing cell types demonstrates a closed chromatin configuration. DNA methylation is a prominent feature with the recruitment of repressive proteins, for example, methyl-CpG-binding protein, MeCP2. Activating histone posttranslational modifications are absent, and the gene is not actively transcribed.

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 trans-

ected 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*<sup>-/-</sup> 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* transcriptional 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-

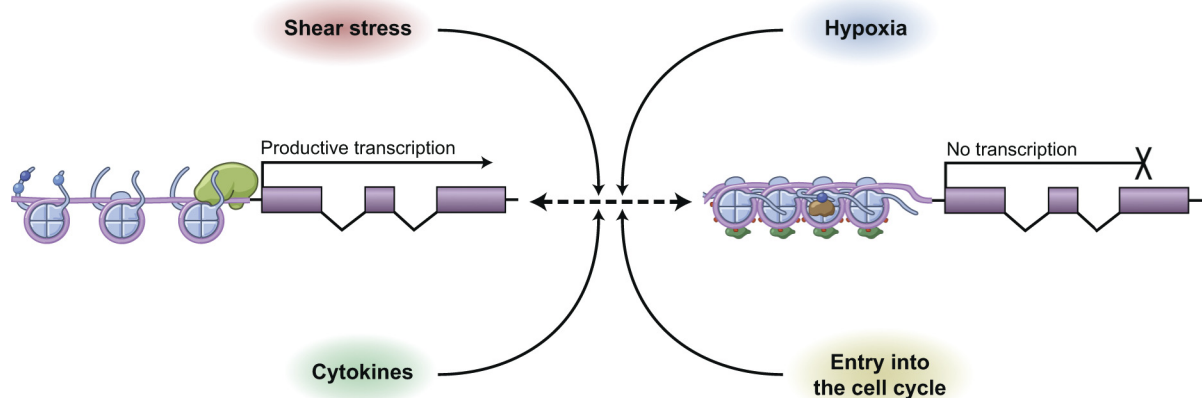


Fig. 4. The epigenetic perspective on human cardiovascular disease. Epigenetic pathways, which are important in the transcriptional control of gene expression, are responsive to various physiological and pathophysiological cues relevant to the health of the vascular endothelium. Some of these cues are 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.

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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#### DISCLOSURES

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#### REFERENCES

- Abbott A. Project set to map marks on genome. *Nature* 463: 596–597.
- Aicher A, Heeschen C, Mildner-Rihm C, Urbich C, Ihling C, Technau-Ihling K, Zeiher AM, Dimmeler S. Essential role of endothelial nitric oxide synthase for mobilization of stem and progenitor cells. *Nat Med* 9: 1370–1376, 2003.
- Ballestar E, Esteller M, Richardson BC. The epigenetic face of systemic lupus erythematosus. *J Immunol* 176: 7143–7147, 2006.
- Barski A, Cuddapah S, Cui K, Roh TY, Schones DE, Wang Z, Wei G, Chepelev I, Zhao K. High-resolution profiling of histone methylations in the human genome. *Cell* 129: 823–837, 2007.
- Bell AC, Felsenfeld G. Methylation of a CTCF-dependent boundary controls imprinted expression of the Igf2 gene. *Nature* 405: 482–485, 2000.
- Berger SL, Kouzarides T, Shiekhattar R, Shilatifard A. An operational definition of epigenetics. *Genes Dev* 23: 781–783, 2009.
- Bernstein BE, Meissner A, Lander ES. The mammalian epigenome. *Cell* 128: 669–681, 2007.
- Beyer S, Kristensen MM, Jensen KS, Johansen JV, Staller P. The histone demethylases JMJD1A and JMJD2B are transcriptional targets of hypoxia-inducible factor HIF. *J Biol Chem* 283: 36542–36552, 2008.
- Bucci M, Roviezzo F, Posadas I, Yu J, Parente L, Sessa WC, Ignarro LJ, Cirino G. Endothelial nitric oxide synthase activation is critical for vascular leakage during acute inflammation in vivo. *Proc Natl Acad Sci USA* 102: 904–908, 2005.
- Casas JP, Bautista LE, Humphries SE, Hingorani AD. Endothelial nitric oxide synthase genotype and ischemic heart disease: meta-analysis of 26 studies involving 23028 subjects. *Circulation* 109: 1359–1365, 2004.
- Chan GC, Fish JE, Mawji IA, Leung DD, Rachlis AC, Marsden PA. Epigenetic basis for the transcriptional hyporesponsiveness of the human inducible nitric oxide synthase gene in vascular endothelial cells. *J Immunol* 175: 3846–3861, 2005.
- Chan Y, Fish JE, D'Abreo C, Lin S, Robb GB, Teichert AM, Karantzoulis-Fegaras F, Keightley A, Steer BM, Marsden PA. The cell-specific expression of endothelial nitric-oxide synthase: a role for DNA methylation. *J Biol Chem* 279: 35087–35100, 2004.
- Chen W, Bacanamwo M, Harrison DG. Activation of p300 histone acetyltransferase activity is an early endothelial response to laminar shear stress and is essential for stimulation of endothelial nitric-oxide synthase mRNA transcription. *J Biol Chem* 283: 16293–16298, 2008.
- Chen X, Wang J, Woltring D, Gerondakis S, Shannon MF. Histone dynamics on the interleukin-2 gene in response to T-cell activation. *Mol Cell Biol* 25: 3209–3219, 2005.
- Chen Z, Zang J, Whetstone J, Hong X, Davrazou F, Kutateladze TG, Simpson M, Mao Q, Pan CH, Dai S, Hagman J, Hansen K, Shi Y, Zhang G. Structural insights into histone demethylation by JMJD2 family members. *Cell* 125: 691–702, 2006.
- Choi JH, Nam KH, Kim J, Baek MW, Park JE, Park HY, Kwon HJ, Kwon OS, Kim DY, Oh GT. Trichostatin A exacerbates atherosclerosis in low density lipoprotein receptor-deficient mice. *Arterioscler Thromb Vasc Biol* 25: 2404–2409, 2005.
- Clark SJ, Harrison J, Frommer M. CpNpG methylation in mammalian cells. *Nat Genet* 10: 20–27, 1995.
- Cloos PA, Christensen J, Agger K, Helin K. Erasing the methyl mark: histone demethylases at the center of cellular differentiation and disease. *Genes Dev* 22: 1115–1140, 2008.
- Coisy M, Roue V, Ribot M, Philips A, Muchardt C, Blanchard JM, Dantonel JC. Cyclin A repression in quiescent cells is associated with chromatin remodeling of its promoter and requires Brahma/SNF2alpha. *Mol Cell* 15: 43–56, 2004.
- Comb M, Goodman HM. CpG methylation inhibits proenkephalin gene expression and binding of the transcription factor AP-2. *Nucleic Acids Res* 18: 3975–3982, 1990.
- De Val S, Black BL. Transcriptional control of endothelial cell development. *Dev Cell* 16: 180–195, 2009.
- De Val S, Chi NC, Meadows SM, Minovitsky S, Anderson JP, Harris IS, Ehlers ML, Agarwal P, Visel A, Xu SM, Pennacchio LA, Dubchak I, Krieg PA, Stainier DY, Black BL. Combinatorial regulation of endothelial gene expression by ets and forkhead transcription factors. *Cell* 135: 1053–1064, 2008.
- Dean W, Santos F, Stojkovic M, Zakhartchenko V, Walter J, Wolf E, Reik W. Conservation of methylation reprogramming in mammalian development: aberrant reprogramming in cloned embryos. *Proc Natl Acad Sci USA* 98: 13734–13738, 2001.
- Edelstein LC, Pan A, Collins T. Chromatin modification and the endothelial-specific activation of the E-selectin gene. *J Biol Chem* 280: 11192–11202, 2005.
- Eriksson M, Brown WT, Gordon LB, Glynn MW, Singer J, Scott L, Erdos MR, Robbins CM, Moses TY, Berglund P, Dutra A, Pak E, Durkin S, Csoka AB, Boehnke M, Glover TW, Collins FS. Recurrent de novo point mutations in lamin A cause Hutchinson-Gilford progeria syndrome. *Nature* 423: 293–298, 2003.
- Evans AJ, Russell RC, Roche O, Burry TN, Fish JE, Chow VW, Kim WY, Saravanan A, Maynard MA, Gervais ML, Sufan RI, Roberts AM, Wilson LA, Betten M, Vandewalle C, Bex G, Marsden PA, Irwin MS, Teh BT, Jewett MA, Ohh M. VHL promotes E2 box-dependent E-cadherin transcription by HIF-mediated regulation of SIP1 and snail. *Mol Cell Biol* 27: 157–169, 2007.

27. **Feinberg AP.** Phenotypic plasticity and the epigenetics of human disease. *Nature* 447: 433–440, 2007.
28. **Feng B, Jiang J, Kraus P, Ng JH, Heng JC, Chan YS, Yaw LP, Zhang W, Loh YH, Han J, Vega VB, Cacheux-Rataboul V, Lim B, Lufkin T, Ng HH.** Reprogramming of fibroblasts into induced pluripotent stem cells with orphan nuclear receptor Esrrb. *Nat Cell Biol* 11: 197–203, 2009.
29. **Fish JE, Marsden PA.** Endothelial nitric oxide synthase: insight into cell-specific gene regulation in the vascular endothelium. *Cell Mol Life Sci* 63: 144–162, 2006.
30. **Fish JE, Matouk CC, Rachlis A, Lin S, Tai SC, D'Abreo C, Marsden PA.** The expression of endothelial nitric-oxide synthase is controlled by a cell-specific histone code. *J Biol Chem* 280: 24824–24838, 2005.
31. **Fish JE, Yan MS, Matouk CC, St Bernard R, Ho JD Jr, Gavryushova A, Srivastava D, Marsden PA.** Hypoxic repression of endothelial nitric-oxide synthase transcription is coupled with eviction of promoter histones. *J Biol Chem* 285: 810–826, 2010.
32. **Fraga MF, Ballestar E, Paz MF, Ropero S, Setien F, Ballestar ML, Heine-Suner D, Cigudosa JC, Urioste M, Benitez J, Boix-Chornet M, Sanchez-Aguilera A, Ling C, Carlsson E, Poulsen P, Vaag A, Stephan Z, Spector TD, Wu YZ, Plass C, Esteller M.** Epigenetic differences arise during the lifetime of monozygotic twins. *Proc Natl Acad Sci USA* 102: 10604–10609, 2005.
33. **Gan Y, Shen YH, Wang J, Wang X, Utama B, Wang J, Wang XL.** Role of histone deacetylation in cell-specific expression of endothelial nitric-oxide synthase. *J Biol Chem* 280: 16467–16475, 2005.
34. **Goldman RD, Shumaker DK, Erdos MR, Eriksson M, Goldman AE, Gordon LB, Gruenbaum Y, Khuon S, Mendez M, Varga R, Collins FS.** Accumulation of mutant lamin A causes progressive changes in nuclear architecture in Hutchinson-Gilford progeria syndrome. *Proc Natl Acad Sci USA* 101: 8963–8968, 2004.
35. **Gridley T.** Notch signaling in vascular development and physiology. *Development* 134: 2709–2718, 2007.
36. **Guttman M, Amit I, Garber M, French C, Lin MF, Feldser D, Huarte M, Zuk O, Carey BW, Cassady JP, Cabili MN, Jaenisch R, Mikkelsen TS, Jacks T, Hacohen N, Bernstein BE, Kellis M, Regev A, Rinn JL, Lander ES.** Chromatin signature reveals over a thousand highly conserved large non-coding RNAs in mammals. *Nature* 458: 223–227, 2009.
37. **Harrington MA, Jones PA, Imagawa M, Karin M.** Cytosine methylation does not affect binding of transcription factor Sp1. *Proc Natl Acad Sci USA* 85: 2066–2070, 1988.
38. **Hayward CS, Benetos A.** Hereditary and environmental influences on arterial function. *Clin Exp Pharmacol Physiol* 34: 658–664, 2007.
39. **Heintzman ND, Stuart RK, Hon G, Fu Y, Ching CW, Hawkins RD, Barrera LO, Van Calcar S, Qu C, Ching KA, Wang W, Weng Z, Green RD, Crawford GE, Ren B.** Distinct and predictive chromatin signatures of transcriptional promoters and enhancers in the human genome. *Nat Genet* 39: 311–318, 2007.
40. **Hellebrekers DM, Griffioen AW, van Engeland M.** Dual targeting of epigenetic therapy in cancer. *Biochim Biophys Acta* 1775: 76–91, 2007.
41. **Hochedlinger K, Plath K.** Epigenetic reprogramming and induced pluripotency. *Development* 136: 509–523, 2009.
42. **Huang PL, Huang Z, Mashimo H, Bloch KD, Moskowitz MA, Bevan JA, Fishman MC.** Hypertension in mice lacking the gene for endothelial nitric oxide synthase. *Nature* 377: 239–242, 1995.
43. **Illi B, Nanni S, Scopece A, Farsetti A, Biglioli P, Capogrossi MC, Gaetano C.** Shear stress-mediated chromatin remodeling provides molecular basis for flow-dependent regulation of gene expression. *Circ Res* 93: 155–161, 2003.
44. **Illi B, Scopece A, Nanni S, Farsetti A, Morgante L, Biglioli P, Capogrossi MC, Gaetano C.** Epigenetic histone modification and cardiovascular lineage programming in mouse embryonic stem cells exposed to laminar shear stress. *Circ Res* 96: 501–508, 2005.
45. **Inoue K, Kobayashi M, Yano K, Miura M, Izumi A, Matakai C, Doi T, Hamakubo T, Reid PC, Hume DA, Yoshida M, Aird WC, Kodama T, Minami T.** Histone deacetylase inhibitor reduces monocyte adhesion to endothelium through the suppression of vascular cell adhesion molecule-1 expression. *Arterioscler Thromb Vasc Biol* 26: 2652–2659, 2006.
46. **Jaenisch R.** Human cloning—the science and ethics of nuclear transplantation. *N Engl J Med* 351: 2787–2791, 2004.
47. **Johnson AB, Barton MC.** Hypoxia-induced and stress-specific changes in chromatin structure and function. *Mutat Res* 618: 149–162, 2007.
48. **Johnson AB, Denko N, Barton MC.** Hypoxia induces a novel signature of chromatin modifications and global repression of transcription. *Mutat Res* 640: 174–179, 2008.
49. **Karantzoulis-Fegaras F, Antoniou H, Lai SL, Kulkarni G, D'Abreo C, Wong GK, Miller TL, Chan Y, Atkins J, Wang Y, Marsden PA.** Characterization of the human endothelial nitric-oxide synthase promoter. *J Biol Chem* 274: 3076–3093, 1999.
50. **Khalil AM, Guttman M, Huarte M, Garber M, Raj A, Rivea Morales D, Thomas K, Presser A, Bernstein BE, van Oudenaarden A, Regev A, Lander ES, Rinn JL.** Many human large intergenic noncoding RNAs associate with chromatin-modifying complexes and affect gene expression. *Proc Natl Acad Sci USA* 106: 11667–11672, 2009.
51. **Kim MS, Kwon HJ, Lee YM, Baek JH, Jang JE, Lee SW, Moon EJ, Kim HS, Lee SK, Chung HY, Kim CW, Kim KW.** Histone deacetylases induce angiogenesis by negative regulation of tumor suppressor genes. *Nat Med* 7: 437–443, 2001.
52. **Kriaucionis S, Heintz N.** The nuclear DNA base 5-hydroxymethylcytosine is present in Purkinje neurons and the brain. *Science* 324: 929–930, 2009.
53. **Kuratomi G, Iwamoto K, Bundo M, Kusumi I, Kato N, Iwata N, Ozaki N, Kato T.** Aberrant DNA methylation associated with bipolar disorder identified from discordant monozygotic twins. *Mol Psych* 13: 429–441, 2008.
54. **Laumonier Y, Nadaud S, Agrapart M, Soubrier F.** Characterization of an upstream enhancer region in the promoter of the human endothelial nitric-oxide synthase gene. *J Biol Chem* 275: 40732–40741, 2000.
55. **Lee PC, Salyapongse AN, Bragdon GA, Shears LL II, Watkins SC, Edington HD, Billiar TR.** Impaired wound healing and angiogenesis in eNOS-deficient mice. *Am J Physiol Heart Circ Physiol* 277: H1600–H1608, 1999.
56. **Lehrke M, Lazar MA.** The many faces of PPARgamma. *Cell* 123: 993–999, 2005.
57. **Lin HS, Hu CY, Chan HY, Liew YY, Huang HP, Lepescheux L, Bastianelli E, Baron R, Rawadi G, Clement-Lacroix P.** Anti-rheumatic activities of histone deacetylase (HDAC) inhibitors in vivo in collagen-induced arthritis in rodents. *Br J Pharmacol* 150: 862–872, 2007.
58. **Lister R, Pelizzola M, Dowen RH, Hawkins RD, Hon G, Tonti-Filippini J, Nery JR, Lee L, Ye Z, Ngo QM, Edsall L, Antosiewicz-Bourget J, Stewart R, Ruotti V, Millar AH, Thomson JA, Ren B, Ecker JR.** Human DNA methylomes at base resolution show widespread epigenomic differences. *Nature* 462: 315–322, 2009.
59. **Marsden PA, Heng HH, Scherer SW, Stewart RJ, Hall AV, Shi XM, Tsui LC, Schappert KT.** Structure and chromosomal localization of the human constitutive endothelial nitric oxide synthase gene. *J Biol Chem* 268: 17478–17488, 1993.
60. **Mastroeni D, McKee A, Grover A, Rogers J, Coleman PD.** Epigenetic differences in cortical neurons from a pair of monozygotic twins discordant for Alzheimer's disease. *PLoS One* 4: e6617, 2009.
61. **Matouk CC, Marsden PA.** Epigenetic regulation of vascular endothelial gene expression. *Circ Res* 102: 873–887, 2008.
62. **Mayer B, Erdmann J, Schunkert H.** Genetics and heritability of coronary artery disease and myocardial infarction. *Clin Res Cardiol* 96: 1–7, 2007.
63. **Mayer W, Niveleau A, Walter J, Fundele R, Haaf T.** Demethylation of the zygotic paternal genome. *Nature* 403: 501–502, 2000.
64. **Meissner A, Mikkelsen TS, Gu H, Wernig M, Hanna J, Sivachenko A, Zhang X, Bernstein BE, Nusbaum C, Jaffe DB, Gnirke A, Jaenisch R, Lander ES.** Genome-scale DNA methylation maps of pluripotent and differentiated cells. *Nature* 454: 766–770, 2008.
65. **Mercer TR, Dinger ME, Mattick JS.** Long non-coding RNAs: insights into functions. *Nat Rev Genet* 10: 155–159, 2009.
66. **Merideth MA, Gordon LB, Clauss S, Sachdev V, Smith AC, Perry MB, Brewer CC, Zalewski C, Kim HJ, Solomon B, Brooks BP, Gerber LH, Turner ML, Domingo DL, Hart TC, Graf J, Reynolds JC, Gropman A, Yanovski JA, Gerhard-Herman M, Collins FS, Nabel EG, Cannon RO, 3rd Gahl WA, Introne WJ.** Phenotype and course of Hutchinson-Gilford progeria syndrome. *N Engl J Med* 358: 592–604, 2008.
67. **Mikkelsen TS, Hanna J, Zhang X, Ku M, Wernig M, Schorderet P, Bernstein BE, Jaenisch R, Lander ES, Meissner A.** Dissecting direct reprogramming through integrative genomic analysis. *Nature* 454: 49–55, 2008.

68. **Miranda TB, Jones PA.** DNA methylation: the nuts and bolts of repression. *J Cell Physiol* 213: 384–390, 2007.
69. **Moazed D.** Small RNAs in transcriptional gene silencing and genome defence. *Nature* 457: 413–420, 2009.
70. **Peng Y, Jahroudi N.** The NFY transcription factor inhibits von Willebrand factor promoter activation in non-endothelial cells through recruitment of histone deacetylases. *J Biol Chem* 278: 8385–8394, 2003.
71. **Ptak C, Petronis A.** Epigenetics and complex disease: from etiology to new therapeutics. *Annu Rev Pharmacol Toxicol* 48: 257–276, 2008.
72. **Rudic RD, Shesely EG, Maeda N, Smithies O, Segal SS, Sessa WC.** Direct evidence for the importance of endothelium-derived nitric oxide in vascular remodeling. *J Clin Invest* 101: 731–736, 1998.
73. **Sagelius H, Rosengardten Y, Schmidt E, Sonnabend C, Rozell B, Eriksson M.** Reversible phenotype in a mouse model of Hutchinson-Gilford progeria syndrome. *J Med Genet* 45: 794–801, 2008.
74. **Scaffidi P, Misteli T.** Lamin A-dependent misregulation of adult stem cells associated with accelerated ageing. *Nat Cell Biol* 10: 452–459, 2008.
75. **Scaffidi P, Misteli T.** Lamin A-dependent nuclear defects in human aging. *Science* 312: 1059–1063, 2006.
76. **Scaffidi P, Misteli T.** Reversal of the cellular phenotype in the premature aging disease Hutchinson-Gilford progeria syndrome. *Nature Med* 11: 440–445, 2005.
77. **Schones DE, Cui K, Cuddapah S, Roh TY, Barski A, Wang Z, Wei G, Zhao K.** Dynamic regulation of nucleosome positioning in the human genome. *Cell* 132: 887–898, 2008.
78. **Shahbazian MD, Grunstein M.** Functions of site-specific histone acetylation and deacetylation. *Annu Rev Biochem* 76: 75–100, 2007.
79. **Shumaker DK, Dechat T, Kohlmaier A, Adam SA, Bozovsky MR, Erdos MR, Eriksson M, Goldman AE, Khuon S, Collins FS, Jenwein T, Goldman RD.** Mutant nuclear lamin A leads to progressive alterations of epigenetic control in premature aging. *Proc Natl Acad Sci USA* 103: 8703–8708, 2006.
80. **Strahl BD, Allis CD.** The language of covalent histone modifications. *Nature* 403: 41–45, 2000.
81. **Tahiliani M, Koh KP, Shen Y, Pastor WA, Bandukwala H, Brudno Y, Agarwal S, Iyer LM, Liu DR, Aravind L, Rao A.** Conversion of 5-methylcytosine to 5-hydroxymethylcytosine in mammalian DNA by MLL partner TET1. *Science* 324: 930–935, 2009.
82. **Tapscoff SJ.** The circuitry of a master switch: MyoD and the regulation of skeletal muscle gene transcription. *Development* 132: 2685–2695, 2005.
83. **Teichert AM, Miller TL, Tai SC, Wang Y, Bei X, Robb GB, Phillips MJ, Marsden PA.** In vivo expression profile of an endothelial nitric oxide synthase promoter-reporter transgene. *Am J Physiol Heart Circ Physiol* 278: H1352–H1361, 2000.
84. **Teichert AM, Scott JA, Robb GB, Zhou YQ, Zhu SN, Lem M, Keightley A, Steer BM, Schuh AC, Adamson SL, Cybulsky MI, Marsden PA.** Endothelial nitric oxide synthase gene expression during murine embryogenesis: commencement of expression in the embryo occurs with the establishment of a unidirectional circulatory system. *Circ Res* 103: 24–33, 2008.
85. **Trojer P, Reinberg D.** Facultative heterochromatin: is there a distinctive molecular signature? *Mol Cell* 28: 1–13, 2007.
86. **Van Holde K, Zlatanova J, Arents G, Moudrianakis E.** Elements of chromatin structure: histones, nucleosomes, and fibres. In: *Chromatin Structure and Gene Expression*, edited by Elgin SCR. New York: IRL Press, 1995, p. 1–26.
87. **Vermeulen M, Mulder KW, Denisov S, Pijnappel WW, van Schaik FM, Varier RA, Baltissen MP, Stunnenberg HG, Mann M, Timmers HT.** Selective anchoring of TFIID to nucleosomes by trimethylation of histone H3 lysine 4. *Cell* 131: 58–69, 2007.
88. **Vernia P, Annese V, Bresci G, d'Albasio G, D'Inca R, Giaccari S, Ingrosso M, Mansi C, Riegler G, Valpiani D, Caprilli R.** Topical butyrate improves efficacy of 5-ASA in refractory distal ulcerative colitis: results of a multicentre trial. *Eur J Clin Invest* 33: 244–248, 2003.
89. **Wang GG, Allis CD, Chi P.** Chromatin remodeling and cancer, part I: covalent histone modifications. *Trends Mol Med* 13: 363–372, 2007.
90. **Wang J, Mahmud SA, Bitterman PB, Huo Y, Slungaard A.** Histone deacetylase inhibitors suppress TF-kappaB-dependent agonist-driven tissue factor expression in endothelial cells and monocytes. *J Biol Chem* 282: 28408–28418, 2007.
91. **Wang Y, Roche O, Yan MS, Finak G, Evans AJ, Metcalf JL, Hast BE, Hanna SC, Wondergem B, Furge KA, Irwin MS, Kim WY, Teh BT, Grinstein S, Park M, Marsden PA, Ohh M.** Regulation of endocytosis via the oxygen-sensing pathway. *Nature Med* 15: 319–324, 2009.
92. **Wenger RH, Kvietikova I, Rolfs A, Camenisch G, Gassmann M.** Oxygen-regulated erythropoietin gene expression is dependent on a CpG methylation-free hypoxia-inducible factor-1 DNA-binding site. *Eur J Biochem* 253: 771–777, 1998.
93. **Wilmut I, Schnieke AE, McWhir J, Kind AJ, Campbell KH.** Viable offspring derived from fetal and adult mammalian cells. *Nature* 385: 810–813, 1997.
94. **Won D, Zhu SN, Chen M, Teichert AM, Fish JE, Matouk CC, Bonert M, Ojha M, Marsden PA, Cybulsky MI.** Relative reduction of endothelial nitric-oxide synthase expression and transcription in atherosclerosis-prone regions of the mouse aorta and in an in vitro model of disturbed flow. *Am J Pathol* 171: 1691–1704, 2007.
95. **Woodcock DM, Lawler CB, Linsenmeyer ME, Doherty JP, Warren WD.** Asymmetric methylation in the hypermethylated CpG promoter region of the human L1 retrotransposon. *J Biol Chem* 272: 7810–7816, 1997.
96. **Wu J, Iwata F, Grass JA, Osborne CS, Elnitski L, Fraser P, Ohneda O, Yamamoto M, Bresnick EH.** Molecular determinants of NOTCH4 transcription in vascular endothelium. *Mol Cell Biol* 25: 1458–1474, 2005.
97. **Wysocka J, Swigut T, Xiao H, Milne TA, Kwon SY, Landry J, Kauer M, Tackett AJ, Chait BT, Badenhorst P, Wu C, Allis CD.** A PHD finger of NURF couples histone H3 lysine 4 trimethylation with chromatin remodelling. *Nature* 442: 86–90, 2006.
98. **Xia X, Lemieux ME, Li W, Carroll JS, Brown M, Liu XS, Kung AL.** Integrative analysis of HIF binding and transactivation reveals its role in maintaining histone methylation homeostasis. *Proc Natl Acad Sci USA* 106: 4260–4265, 2009.
99. **Yamamoto K, Sokabe T, Watabe T, Miyazono K, Yamashita JK, Obi S, Ohura N, Matsushita A, Kamiya A, Ando J.** Fluid shear stress induces differentiation of Flk-1-positive embryonic stem cells into vascular endothelial cells in vitro. *Am J Physiol Heart Circ Physiol* 288: H1915–H1924, 2005.
100. **Yamazawa K, Kagami M, Fukami M, Matsubara K, Ogata T.** Monozygotic female twins discordant for Silver-Russell syndrome and hypomethylation of the H19-DMR. *J Hum Genet* 53: 950–955, 2008.
101. **Yang J, Ledaki I, Turley H, Gatter KC, Montero JC, Li JL, Harris AL.** Role of hypoxia-inducible factors in epigenetic regulation via histone demethylases. *Ann NY Acad Sci* 1177: 185–197, 2009.
102. **Yang X, Smith SL, Tian XC, Lewin HA, Renard JP, Wakayama T.** Nuclear reprogramming of cloned embryos and its implications for therapeutic cloning. *Nat Genet* 39: 295–302, 2007.
103. **Zhang MX, Ou H, Shen YH, Wang J, Wang J, Coselli J, Wang XL.** Regulation of endothelial nitric oxide synthase by small RNA. *Proc Natl Acad Sci USA* 102: 16967–16972, 2005.
104. **Zhang MX, Zhang C, Shen YH, Wang J, Li XN, Chen L, Zhang Y, Coselli JS, Wang XL.** Effect of 27nt small RNA on endothelial nitric-oxide synthase expression. *Mol Biol Cell* 19: 3997–4005, 2008.
105. **Zhang MX, Zhang C, Shen YH, Wang J, Li XN, Zhang Y, Coselli J, Wang XL.** Biogenesis of short intronic repeat 27-nucleotide small RNA from endothelial nitric-oxide synthase gene. *J Biol Chem* 283: 14685–14693, 2008.