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Epigenetic Modifications: Basic Mechanisms and Role in Cardiovascular Disease

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The term, "epigenetics," was first used to refer to the complex interactions between the genome and the environment that are involved in development and differentiation in higher organisms. Today, this term is used to refer to heritable alterations that are not due to changes in DNA sequence. Rather, epigenetic modifications, or "tags," such as DNA methylation and histone modification, alter DNA accessibility and chromatin structure, thereby regulating patterns of gene expression. These processes are crucial to normal development and differentiation of distinct cell lineages in the adult organism. They can be modified by exogenous influences, and, as such, can contribute to or be the result of environmental alterations of phenotype or pathophenotype. Importantly, epigenetic programming has a crucial role in the regulation of pluripotency genes, which become inactivated during differentiation. Here, we review the major mechanisms in epigenetic regulation; highlight the role of stable, long-term epigenetic modifications that involve DNA methylation; and discuss those modifications that are more flexible (short-term) and involve histone modifications, such as methylation and acetylation. We will also discuss the role of nutritional and environmental challenges in generational inheritance and epigenetic modifications, concentrating on examples that relate to complex cardiovascular diseases, and specifically dissect the mechanisms by which homocysteine modifies epigenetic tags. Lastly, we will discuss the possibilities of modifying therapeutically acquired epigenetic tags, summarizing currently available agents and speculating on future directions.

Epigenetic Tags: acquisition, maintenance, and inheritance

Chromatin is the complex of chromosomal DNA associated with proteins in the nucleus (for review see [1]). DNA in chromatin is packaged around histone proteins, in units referred to as nucleosomes. A nucleosome has 147 bp of DNA associated with an octomeric core of histone proteins, consisting of two H3-H4 histone dimers surrounded by two H2A-H2B dimers. N-terminal histone tails protrude from nucleosomes into the nuclear lumen. H1 histone associates with the linker DNA located between the nucleosomes. Nucleosome spacing determines chromatin structure which can be broadly divided into heterochromatin

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and euchromatin (Table 1) [1, 2]. Chromatin structure and gene accessibility to transcriptional machinery are regulated by modifications to both DNA and histone tails (Figure 1).

DNA methylation

In differentiated mammalian cells, the principal epigenetic tag found in DNA is that of covalent attachment of a methyl group to the C5 position of cytosine residues in CpG dinucleotide sequences (referred to as CpG throughout this review)[3]. Recent findings suggest that in undifferentiated stem cells, cytosines, other than those in CpG, can be methylated, as well, [4] and that methylation of non-CpG cytosines is crucial for gene regulation in embryonic stem cells in particular. CpG methylation is, however, an important mechanism to ensure the repression of transcription of repeat elements and transposons, and also plays a crucial role in imprinting and X-chromosome inactivation [5]. Transcriptional gene silencing by CpG methylation also restricts the expression of some tissue-specific genes during development and differentiation by repressing them in non-expressing cells.

During development, the pattern of CpG methylation changes in a predictable manner. In early embryogenesis, methylation is erased throughout the genome and then reestablished in all but CpG islands (regions of the genome with a concentration of CpG residues). CpG islands remain hypomethylated until later in development when some of them become methylated [6, 7]. Subsequent methylation of cytosines in CpG islands and at other CpG dinucleotides is associated with transcriptional repression [6, 8], especially when these methylated sites involve promoter or other gene regulatory regions [3]. DNA methylation may, however, be activating if it prevents binding or limits expression of transcriptional repressors. Recent studies defining the degree of methylation in mammalian promoters indicate that methylation occurs at only a small percentage of CpG dinucleotides and inhibits transcription of only a small subset of genes in differentiated cells. Many of these repressed genes are germline-specific [8], including pluripotency genes, suggesting that methylation is a crucial mechanism by which to suppress key genes during differentiation [8].

CpG methylation can supress transcription by several mechanisms. First, the presence of the methyl group at a specific CpG may directly block DNA recognition and binding by some transcription factors. For example, several studies have shown that transcriptional activation at GC-boxes is inhibited by methylation, which excludes binding of Sp1 and Sp3 transcription factors, at least in some promoter contexts [9, 10]. Methylation has also been shown to block the ability of the nuclear factor, Hif1, from inducing erythropoietin transcription under hypoxic conditions [11]. Alternatively, other factors may preferentially bind to methylated DNA, blocking transcription factor access. For example MeCP2 and other family members [12] bind to methyl CpG and contribute to transcriptional repression by the recruitment of histone-modifying proteins, such as histone deacetylases (HDAC). Subsequently, histone deacetylation promotes chromatin condensation, further repressing transcription [13, 14]. This sequence of events illustrates how DNA methylation and certain histone modifications function together to contribute to the transcriptional on or off state of genes subject to epigenetic modification (Figure 1).

A family of DNA methyltransferase enzymes (DNMTs) is involved in *de novo* DNA methylation and its maintenance. During embryogenesis, *de novo* methylation is carried out by DNMT3A and DNMT3B [15]. Although some studies suggest an ongoing role for DNMT3A and DNMT3B in maintaining methylation status in some cell types [16, 17], the ubiquitously expressed DNMT1 is predominantly responsible for maintaining cellular levels of CpG methylation. Interestingly, transcription from alternative promoters results in expression of a truncated oocyte-specific DNMT1 isoform, DNMT1o, that is essential for early embryogenesis [18]. DNMT1 functions in a complex to recognize hemi-methylated DNA and to add methyl groups to the non-methylated daughter strand formed during

replication [19]. The base pairing of CpG allows for the reciprocal maintenance of methylation during subsequent replication cycles. In this manner, a non-genetic trait (DNA methylation) can be passed from cell to cell and, with it, the contextual effects on gene expression. Thus, methylation can be considered a long-term, relatively stable, epigenetic trait, the effects of which can contribute to maintaining the cellular phenotype.

Targeting DNA methylation: Role of histone modification, DNA-binding proteins and RNA

Owing to its heritability, DNA methylation is a powerful means by which to suppress the expression of unwanted or excess genes. Several basic questions remain unanswered, such as the mechanisms that promote targeting of specific CpG sites for methylation or prevent their modification. Many of the insights into the mechanistic aspects of targeting CpG methylation come from studies of imprinting and X-inactivation, where CpG methylation represses gene expression in chromosomal regions. X-inactivation occurs in somatic cells of females to limit the expression of most X-chromosome genes to those from one chromosome. Given the random nature of X-chromosome inactivation, female carriers may display a wide variation in phenotypic expression of X-linked disorders [20]. Similarly, imprinting regulates autosomal gene expression to genes from only one parental allele in males and females. The importance of imprinting and gene dosage regulation in normal development can be appreciated by the consequences of imprinting disturbances that cause a number of human syndromic disorders, such as Prader-Willi, Angleman, Silver-Russell, and Beckwith–Wiedermann (reviewed in [21, 22]).

In imprinting, clusters of genes in a chromosomal region are coordinately inhibited by methylation of an imprinting center; these centers are also referred to as differentially methylated regions (DMRs), and DMRs often overlap CpG islands. Recent studies suggest that transcription of DMRs in the oocyte may target them for subsequent CpG modification by maintaining an open chromatin structure accessible to *de novo* methylation [23]. In both imprinting and X-inactivation, the expression of long non-coding RNAs [24], such as the Xist transcript in X-chromosome inactivation [25], may also play a regulatory role. Chromatin conformation is apparently essential for the imprinting of some maternally determined imprinted genes. In particular, histone H3-lysine 4 (H3K4) demethylase, LSD1, has been found to be essential for these processes, and deficiency of this enzyme results in embryonic stem cell lethality during early differentiation [26, 27]. To date, the mechanisms involved in specifying methylation of DMRs during imprinting include recognition of specific DNA sequence motifs in imprinting centers, the expression of factors that are oocyte-specific to mark maternal chromosomes, chromatin remodeling, and/or combinations of these events. Similar mechanisms may also target methylation of specific gene promoters during differentiation.

CpG methylation is erased in a predictable manner during gametogeneis and following zygote fertilization. Recent findings suggest that these processes require the action of cytidine deaminases, such as AID, as well as DNA repair mechanisms [28, 29]. Thus, enzymatic deamination of 5-methylcytosine leads to formation of thymine and T:G base-pair mismatches: base excision repair mechanisms subsequently delete thymine and restore C:G base pairing during epigenetic reprogramming. Spontaneous deamination of 5-methylcytosine also requires base excision repair mechanisms to repair base pairing mismatch, a process that is highly inefficient in most differentiated cells. Thus, spontaneous deamination of 5-methylcytosine has resulted in an overall depletion of CpG dinucleotide sequences in mammalian genomes (over evolutionary time)[30]. In differentiated, adult cells, CpG methylation is considered long-lasting and refractory to elimination, except by alterations in the expression or activity of DNTM1 (Figure 2) (or following spontaneous deamination and mismatch repair). Recently, however, a novel mechanism involving specific DNA demethylation in response to hormone stimulation has been discovered [31]. In

this system, the cytochrome p45027B1 (CYP27B1) gene is repressed by vitamin D-interacting repressor (VDIR)-mediated recruitment of DNA methylases and MBD4, a methyl DNA binding protein. Transcriptional suppression can be relieved by parathyroid hormone (PTH)-induced demethylation of promoter CpGs. Apparently, in the presence of PTH-induced phosphorylation, MBD4 can mediate demethylation of promoter CpGs through a base-excision repair mechanism that removes methylcytosine, apparently without deamination. This hormone-induced mechanism contrasts with excision-repair mechanisms described above in that it is targeted to a specific promoter by hormone action and it does not require deamination [32] (Figure 2). Other recent studies suggest a role for DNMT3 in active demethylation at estrogen receptor-α-responsive promoters that also exhibit hormone-induced alterations in CpG methylation status [33, 34]. Although further studies are needed to confirm these mechanisms, the concept of hormone-induced methylation switching adds a new twist to epigenetic regulation. It remains to be seen whether other genes can be similarly regulated by methylation switching utilizing these or other, as yet, unknown mechanisms.

Histone regulation: readily reversible epigenetic changes

DNA methylation tags promote the persistence of certain histone states, such as deacetylation, thus providing a mechanism for perpetuating post-translational histone modifications. Histones can be post-translationally modified to restructure chromatin in many ways, including phosphorylation, ubiquitination, acetylation, and methylation[35, 36]. In fact, the ‘Histone Code Hypothesis’ suggests that different combinations of histone modifications may regulate chromatin structure and transcriptional status [37, 38]. Details, such as the location of nucleosomes relative to the transcriptional start site of a gene, together with specific combinations of sites, types, and extents of histone modifications, add to the complexity of the histone code (reviewed in [1, 39]). Nonetheless, efforts have been made to characterize patterns of histone modifications that contribute to cell-type specific regulation of genes in differentiated cells, such as those associated with smooth muscle-specific genes regulated by serum response factor binding to the CArG box [40].

Of the many described histone modifications, histone acetylation, at the ε-amino group of lysine residues in H3 and H4 tails, is most consistently associated with promoting transcription. However, this description oversimplifies a complex process, as acetylated, open-chromatin structure may also allow access of transcriptional repressors. For example, some bromodomain-containing factors, such as BRG1 and Brd4, target to acetylated histones where they can mediate the formation of repressor (or activator) complexes [41, 42]. Acetylation is targeted to regions of chromatin by the recognition and binding of DNA sequence-specific transcription factors that recruit one of a growing family of histone acetyl transferase (HAT) cofactors such as CREB binding protein (CBP), and p300, MYST, and GNAT (see Table 2 for a list of histone modifying enzymes) [43]. Disruption of the normal acetylation activity of CBP/p300 family members is associated with Rubenstein-Taybi syndrome, an autosomal dominant syndrome [44, 45], highlighting the importance of these cofactors in regulating the proper expression of gene combinations important in development and differentiation.

Deacetylation of histones correlates with CpG methylation and the inactive state of chromatin. There are 4 classes of histone deacetylase enzymes (HDACs), with members capable of deacetylation of histones and/or other protein targets [46]. These regulatory proteins are themselves subject to regulation by acetylation, phosphorylation, and sumoylation [47], which can affect their function, subcellular distribution, and protein-protein associations [48]. Several studies suggest that interactions with sequence-specific DNA binding proteins and co-repressor complexes can target certain HDAC proteins to histones in a gene-specific manner [49, 50].

Histone lysine methylation patterns and their effects on transcription are more complex than acetylation, in that some methylation sites are associated with transcriptionally permissive chromatin (euchromatin) and some are repressive, fostering heterochromatin formation. In addition, ε-amino groups of lysine residues can be mono-, di-, or tri-methylated. Overall, the H3K27me3 and H3K9me states are associated with silencing, whereas the H3K4me3 and H3K36me3 states are transcriptionally permissive modifications (see Table 3 for a list of histone methylation sites). Importantly, methylation marks recruit effector proteins that play essential roles in maintaining the transcriptional state of the chromatin, for example H3K9me recruits HP1, contributing to heterochromatin formation.

Most histone lysine methyltransferases have a SET homology domain, a vast family of proteins that can be grouped into 7 subfamilies based on their structural similarities [39]. SET1 family members specifically foster active chromatin by methylating H3K4. Other histone lysine methyltransferase families can methylate several histone targets. In addition, some of these methyl transferases have additional domains that specify binding to methylated DNA or to other proteins, such as CBP [39].

Until recently, histone methylation was considered a long-term epigenetic marker as the only mechanism for its removal was histone turnover; however, recent studies confirm the existence of multiple histone demethylases capable of demethylating histone lysine methyl groups. These enzymes include lysine-specific demethylase 1-(LSD1), which removes mono- or di-methyl groups from H3K4. The tri-methylated modification is targeted for removal by the Jumonji C-(JmjC) domain-containing demethylases [51] (see Table 2). Similar to histone methylases, LSD1 and JmjC family proteins may demethylate histones in a gene-specific manner, directed, in part, by interactions between demethylases and DNA sequence-specific nuclear factor complexes. In addition, recent studies show that specific histone demethylases may regulate androgen-mediated transcriptional responses and osteoblast differentiation [52–54].

Non-coding RNA, transcriptional gene silencing, and epigenetic modifications

As discussed above, long non-coding RNAs (lncRNAs) play an essential role in imprinting and X-chromosome inactivation. The gene silencing effects of these lncRNAs and other lncRNAs, such as HOTAIR in the human homeobox loci, are due, in part, to their recruitment of remodeling complexes such as the polycomb complex that foster histone methylation (notably the inhibitory H3K27me3) [55–57]. In addition, lncRNAs can also suppress transcription by other mechanisms, such as the recruitment of RNA-binding proteins that interfere with histone deacetylation or exclude TFIID promoter association [58, 59].

There is also a growing literature on the role of small noncoding RNAs (sncRNAs) and their effects on transcriptional gene silencing. This group of RNAs includes the dicer-dependent microRNAs (miRNAs) and small inhibitory RNAs (siRNAs) formed by RNA-interference pathways, as well as the piRNAs, that are formed in a dicer-independent manner and specifically associate with the PIWI subfamily of argonaute proteins. Although each of these classes of sncRNAs have been shown to mediate epigenetic DNA and histone modifications, the piRNAs appear to have a distinct function of repressing transposon expression in germline cells by fostering *de novo* DNA methylation [60]. A role for siRNA in RNA-mediating DNA methylation and transcriptional gene silencing was first discovered in plants [61] and has been found to exist in many species, including mammals [62]. Recent findings in mammalian cells suggest that synthetic siRNAs and endogenous miRNAs that target gene promoters may direct transcriptional gene silencing by recruiting specific argonaute proteins and forming epigenetic remodeling complexes that suppress gene expression by fostering histone deacetylation, histone methylation (H3K9 and H3K27), and

DNA methylation [63–65]. In fact, although the mechanism has not been completely elucidated, dicer-deficient ES cells exhibit defects in differentiation that correlate with a loss of *de novo* DNA methylation and loss of miRNAs[66], suggesting a role for endogenous miRNAs in regulating necessary epigenetic changes during differentiation. Other studies indicate that some siRNAs targeted to TATA-box sequences may block transcriptional inititation without causing epigenetic modifications to histones and DNA [67]. Overall, these studies illustrate the important role of ncRNAs in modulating gene transcriptional silencing.

Role of Epigenetic Changes in Cardiovascular Diseases

It has been suggested that epigenetic changes may account for the missing heritability determinants of complex diseases, such as atherosclerosis, hypertension, metabolic syndrome, and diabetes, that, to date, have not been accounted for by genetic studies of sequence variation [68, 69]. In a recent study, the influence of parental origin on disease association was examined by following the inheritance of single nucleotide polymorphisms (SNPs) near known imprinted genes. These results identified 6 SNPs in which parental origin of a gene alters risk [70]. One of these SNPs that was associated with type 2 diabetes correlated directly with methylation status, as well. Thus, these findings suggest that additional, nonsequence-dependent variations may contribute to heritable traits. Below we review the relationships between epigenetics and genetics, epigenetics and nutrition, and how these relationships may influence cardiovascular disease.

DNA methylation and single nucleotide polymorphisms

Theoretically, SNPs that create CpG sites may be targets for epigenetic modifications, just as loss of these sites will prevent DNA methylation. The consequences of a polymorphism resulting in a CpG in the promoter region of the NDUFB6 gene illustrates this intersection between genetic and epigenetic regulation. NDUFB6 is a respiratory chain protein with diminished expression in type 2 diabetes. In muscle biopsies from elderly patients, NDUFB6 expression inversely correlates with the degree of DNA methylation, suggesting that the presence of a CpG site confers more risk for decreased expression (and potentially disease risk) than not having the site [71]. Taken together, these findings support the concept that epigenetic modifications can influence risk in complex diseases.

Epigenetics, nutrition, and environment

Barker and colleagues hypothesized that environmental factors in crucial periods of early life (during fetal development, for instance) can influence risks for cardiovascular and metabolic diseases later in life. This concept is supported by a number of studies that have associated low birth weight in human populations with increased risk of cardiovascular disease (see for instance reviews [72, 73]). For example, individuals prenatally exposed to famine during the Dutch Hunger Winter (1944–45) experienced higher prevalence of obesity and coronary heart disease as adults, when compared to adults born before or conceived after that period [74]. In Barker's seminal work, it was found that low birth-weight babies who survived infancy had an increased risk of coronary heart disease later in life, and that increasing birth weight was associated with a graded decrease in risk [75]. In addition, *in utero* exposure to hypercholesterolemia has been associated with higher incidence and accelerated progression of lesions in humans, rabbits, and mice [76, 77]. Exposure to different behavioral patterns during early postnatal life has also been shown to influence epigenetic modifications in experimental animal models [78]. Thus, it has been suggested that these long-lasting changes arise, at least in part, from epigenetically mediated alterations in gene expression that occur very early in life [79]. Applying these concepts to human populations, it has recently been proposed that social and environmental stresses during development may

influence epigenetic processes that contribute to the adult race-based US health disparities in diseases like hypertension, diabetes, stroke, and coronary heart disease [80].

More recent studies in animal models have begun to characterize epigenetic modifications that are influenced by the intrauterine environment. For example, feeding a low-protein diet to pregnant rats causes low birth weight, hypertension, and endothelial dysfunction in the offspring. Studies have shown a role for the renin-angiotensin system in this phenotype as treatment of pregnant mothers with angiotensin converting enzyme inhibitors or angiotensin receptor (AT1R) antagonists [81] alleviates hypertension in the offspring. Consistent with these earlier findings, offspring of pregnant mothers fed low protein diets were found to have hypomethylated AT1bR gene promoters along with increased adrenal expression of AT1bR [82], suggesting a role for specific hypomethylation in regulating elevated blood pressure in this model. Other studies have reported that a low protein diet during pregnancy in rat results in overexpression of the hepatic glucocorticoid (GR) and peroxisomal proliferator-activated (PPARα) receptors in offspring [83, 84]. Interestingly, it had been previously reported that DNMT1 contains a GR response element [85]. Of importance, these studies established an underlying epigenetic mechanism that involves a decrease in the methylation patterns of GR and PPARα promoters, a decrease in DNMT1 expression, and an increase in the levels of transcription-permissive histone modifications. GR and PPARα receptors are key transcription factors whose altered expression may modulate the activity of a large number of metabolic pathways, and have been implicated in the pathology of numerous diseases, including obesity, diabetes, and atherosclerosis [86, 87].

As discussed further in the following section, the methyl-group responsible for DNA and histone methylation originates from *S*-adenosyl methionine (AdoMet), via Met biosynthesis through folate-dependent or -independent pathways of homocysteine (Hcy) remethylation (Figure 3). Of importance, supplementing a protein-restricted maternal diet in rats with methyl groups by addition of folate or glycine has been shown to decrease hypertension [88], improve endothelium-dependent vasodilation, and increase endothelial NO synthase mRNA levels [89], and to restore both the expression and promoter methylation status of the hepatic GR and PPARα receptors in offspring [83]. These data support the hypothesis that folate and other methyl group donors can influence fetal development and the risk of cardiovascular disease in the next generation. Interestingly, supplying folate to the offspring, rather than the pregnant mothers, increased the methylation status of some, but not all, of the genes modified by maternal protein restriction [90], suggesting that some epigenetic modifications may not be reversible by nutritional interventions in the offspring.

Other modifiers of methylation may also influence epigenetic tags. For instance, dietary status of choline (a betaine precursor that is involved in folate-independent pathways of methionine synthesis) was shown to affect DNA methylation [91]. Moreover, in a study using apo-E deficient mice to assess the efficacy of dietary intervention in retarding atherogenesis, it was shown that betaine supplementation attenuated atherosclerotic lesion formation and growth [92]. Treatments that alter epigenetic modifications may also have utility in human atherosclerosis where DNA methylation changes in target genes, such as the estrogen receptor α and β genes [93, 94], may contribute to the underlying pathogenic mechanisms (discussed further in [77]).

Regulation of vascular nitric oxide (NO) production by endothelial nitric oxide synthase (eNOS) may influence atherogenesis as well as thrombosis. Interestingly, the restricted expression of eNOS to vascular endothelium is determined, in part, by DNA methylation and histone modifications [95], as treatment of non-expressing cell types with a DNMT inhibitor induced demethylation of the *eNOS* promoter and increased the levels of *eNOS* mRNA. Furthermore, local enrichment of acetylated H3 and H4 histones across the native

eNOS locus was found in eNOS-expressing cells compared to non-endothelial cells, whose treatment with a HDAC inhibitor upregulated *eNOS* mRNA expression. Recent data suggests that NO itself may be an epigenetic factor, based on its regulatory function upon chromatin and gene expression, via modification (S-nitrosation and tyrosine-nitration) of nuclear factors, HDACs, and histones [96].

Homocysteine: a link between DNA methylation and vascular disease

Homocysteine (Hcy) is biochemically linked to the principal epigenetic tag found in DNA. Although increased circulating levels of Hcy are a risk factor for vascular disease, recent clinical trials that used folate and/or other vitamin B therapies to lower Hcy failed to reduce cardiovascular event rates, therefore casting doubt on homocysteine's direct causative role in vascular disease[97]. Most studies, however, have not explored the consequences of elevated Hcy on methylation processes, even though Hcy plays a crucial role in methyl-donor biosynthesis [98]. As depicted in Figure 3, the methyl group responsible for the establishment and maintenance of DNA methylation patterns originates from *S*-adenosyl methionine (AdoMet), an intermediate in Hcy metabolism. In addition to DNA methylation, AdoMet serves as the methyl donor for more than one hundred different cellular methyltransferase reactions, including histone methylation. Following the transfer of the methyl group, AdoMet is converted into *S*-adenosyl homocysteine (AdoHcy), which inhibits the majority of AdoMet-dependent methyltransferases. AdoHcy is further converted into Hcy and adenosine by AdoHcy hydrolase, which is widely distributed in mammalian tissues. This reaction is reversible and strongly favors AdoHcy synthesis rather than hydrolysis; however, both Hcy and adenosine are rapidly removed under physiological conditions, favoring the hydrolysis reaction. If Hcy accumulates, AdoHcy will accumulate as well, potentially inhibiting transmethylation reactions. Thus, increased Hcy may be regarded as a global DNA hypomethylation effector via AdoHcy accumulation.

There are many *in vivo* examples that suggest Hcy levels may modulate global DNA methylation. For example, in healthy humans, increased levels of plasma Hcy were associated with both increased AdoHcy concentrations and DNA hypomethylation in lymphocytes [99]. This inverse relationship between Hcy plasma concentrations and DNA methylation patterns was further confirmed in other reports [100–102] (with one exception [103]) and extended to several animal models [102]. Several studies support the concept that DNA hypomethylation may be responsible, in part, for vascular complications associated with increased circulating levels of Hcy [104]. For example, vascular disease patients manifested increased levels of both plasma Hcy and intracellular AdoHcy, together with decreased DNA methylation, supporting a role for hyperhomocysteinemia (HHcy) in modulating epigenetic mechanisms [105]. This association has also been confirmed in several animal studies in which increased circulating levels of Hcy and AdoHcy were associated with endothelial dysfunction and aberrant DNA methylation patterns [106–108]. [One human study, however, documented that coronary heart disease patients had increased global DNA methylation in the presence elevated serum Hcy levels [109].] In patients with renal functional impairment (and altered homocysteine clearance), which augments the risk for vascular disease, Ingrosso and colleagues reported increased levels of Hcy together with reduced global lymphocyte DNA methylation pattern [110]. Of importance, Ingrosso and colleagues reported that global and specific DNA hypomethylation affected the expression of two genes (*SYLB*, a pseudoautosomal gene, and *H19*, an imprinted gene), and that both global DNA methylation patterns and allelic gene expression were normalized after lowering plasma Hcy levels with folate administration. Although two later reports failed to confirm these observations regarding global hypomethylation in patients with renal failure [111, 112], Ingrosso and colleagues work represents the first human study that causally linked Hcy with altered gene expression via DNA hypomethylation.

Aberrant global DNA methylation is only an index of the potential for epigenetic dysregulation. In addition to AdoHcy, a growing list of factors has been identified that can modify DNA methylation patterns. These include the rate of cell growth and DNA replication, chromatin accessibility, local availability of AdoMet, nutritional factors including folate supplementation, duration and degree of the hyperhomocysteinemic state, inflammation, dyslipidemias, oxidative stress, and aging [113]. Thus, the relation between increased Hcy and DNA global hypomethylation may be masked in the clinical setting owing to the presence of these confounders, thereby possibly explaining some contradictory and counterintuitive findings reported to date. Another important aspect to consider is that DNA methylation is unequally distributed throughout chromosomes of differentiated cells [4]. Thus, hyper- and hypomethylated regions can coexist in the genome, and global DNA methylation status need not correspond to the methylation status of specific genomic regions. For example, it has been recently shown that human cardiomyopathies of different etiologies display a unifying pattern of altered DNA methylation of three angiogenesis-related *loci* in which the differential (increased or decreased) methylation was correlated with the expression of the corresponding gene [114]. It is likely that research on single gene methylation and expression may lead to a better understanding of the vascular effects of elevated Hcy [113, 115].

Several investigators have focused on target gene methylation patterns to explain some of the deleterious effects of Hcy. For example, in cultured endothelial cells, it was shown that Hcy, at physiological relevant concentrations, inhibits cell growth with a concomitant increase in intracellular AdoHcy concentration and a significant decrease in the AdoMet/AdoHcy ratio, suggesting that cellular hypomethylation could play a role in the observed phenotype [116]. Subsequent studies suggested a role for transcriptional suppression of cyclin A in mediating Hcy-induced endothelial cell growth inhibition [117, 118], and found that Hcy triggers transcriptional inhibition of cyclin A through demethylation of a specific CpG site located in the core promoter, *viz.*, on the cell-cycle dependent element (CDE). In addition, cyclin A promoter demethylation eliminates MeCP2 binding, which, in turn, impairs HDAC association, leading to an accumulation of acetylated H3 and H4. The authors concluded that the loss of DNA methylation in the CDE repressor site and the resulting chromatin remodeling increases chromatin accessibility to repressors, resulting in inhibition of cyclin A gene transcription. Of additional interest was the observation that a physiological concentration of plasma Hcy inhibits DNMT1 activity in this cell system, providing evidence that Hcy can directly modulate specific DNA methylation reactions. In other studies, Hcy was also shown to disrupt the growth of endothelial cells by downregulating fibroblast growth factor-2 (FGF2) via an epigenetic mechanism involving transcriptional repression [119]. Apparently, the *FGF2* gene promoter encompasses a CpG island and, in contrast with the cyclin A example, the *FGF2* gene was heavily methylatedat cytosine residues despite significant AdoHcy accumulation. Normal levels of *FGF2* transcription were restored when the cells were simultaneouslyexposed to a DNA demethylating agent and Hcy. Taken together with other examples in the literature [108, 120–122], these findings suggest that Hcy and AdoHcy accumulation can have complex effects on DNA methylation targets and their transcriptional potential.

Increasing evidence indicates that alterations in lipid metabolism may play a role in vascular pathology associated with hyperhomocysteinemia (HHcy) [123–126], and many studies suggest that epigenetics may play a role in these processes. For example, changes in DNA methylation have been suggested as a potential mechanism for altered *apoA-I* and *apoA-IV* gene expression in mice with HHcy [127, 128]. In mice, both *apoA-I* and *apoA-IV* genes are contained within the apolipoprotein gene cluster on chromosome 9, and, notably, the cluster contains a CpG-rich regioncorresponding to the 3′ flanking region of the *apoA-I* gene andthe 5′ flanking region of the *apoA-IV* gene. Similarly, it has been shown that Hcy is significantly

and inversely correlated with HDL-bound cholesterol and apoA-I in both human and murine models of HHcy [129]. In murine primary hepatocyte cultures, cellular hypomethylation, induced by AdoHcy accumulation, was suggested as an explanation for the Hcy-induced inhibition of apoA-I protein synthesis [130]; however, additional analysis indicated that Hcy regulation of apoA-I synthesis may also involve other, nonepigenetic mechanisms. Additional evidence for regulation of lipid biosynthesis by the epigenetic effects of Hcy was reported by Devlin and colleagues, who showed that a reduced methylation capacity (assessed by AdoMet/AdoHcy ratio) in liver from mice with mild or moderate HHcy was associated with hepatic changes in phospholipid species and impaired long-chain polyunsaturated fatty acid metabolism that could be attributed, in part, to differential CpG methylation of genes involved in these pathways [131, 132]. Taken together, these findings indicate that Hcy may influence gene expression by modulating epigenetic pathways. As discussed above, the list of potential modifiers of methylation is long, suggesting that there are still many questions yet unresolved about Hcy's effects on methylation, gene expression, and cardiovascular disease risk.

Future Directions and Therapeutic interventions

The plasticity of certain epigenetic modifications can be followed throughout development and differentiation and in response to environmental stimuli. The fact that modifications can accumulate in aging is supported by studies in genetically identical monozygotic twins: younger twins were far more concordant in terms of the patterns of DNA methylation and histone acetylation than older twins, suggesting that these tags are acquired or modified over time [133]. Thus, it seems possible that epigenetic modifications may be amenable to pharmacological interventions. For example, 5-azacytidine, a DNMT inhibitor, has been used to increase fetal hemoglobin production by causing hypomethylation of γ-globin genes in sickle cell disease patients [134], and, as discussed above, methyl donor treatments (folate, glycine and betaine) can increase DNA methylation, altering gene expression. Recent studies have also reported a microRNA (microRNA-29b) that can cause global hypomethylation, by reducing the expression of DNMT enzymes [135]. These approaches, however, are not specific and may have undesirable consequences on the expression of genes distinct from those of primary interest.

Additional studies are necessary to unravel the mechanisms that select specific genes for epigenetic regulation prior to developing targeted therapeutic approaches to reprogram these modifications. To date, most epigenetic therapies have focused on modulating chromatin structure. For example, there has been a surge in the development of many class and isoform-selective HDAC-inhibitors [136], some of which may have utility in cancer, Huntingtons disease, sickle cell disease, or cardiovascular diseases [137, 138]. The usefulness of these approaches, again, may depend on the ability of a target HDAC to modulate subsets of genes, rather than cause global changes. This goal may not be that unrealistic: sirt6, a sirtuin deacetylase, apparently coordinately regulates the expression of multiple glycolytic genes [139], suggesting a possible target through which to regulate cellular metabolism. In addition, the specific methylation/demethylation of CYP27B1 [31] discussed above suggests novel mechanisms of regulating epigenetic tags at the DNA level by modulating proteins that mediate methylation switching. In addition siRNA-based methods may provide a targeted means to transcriptionally silence genes. Recent findings also suggest that this method may be adapted to provide long term (with epigenetic changes) or short term (without epigenetic changes) regulation of gene transcription, depending on the targeting site in the promoter; this type of flexibility may have many therapeutic advantages [62].

Since Waddington made his initial observations about the environmental influences in development [140], much progress has been made to uncover the molecular mechanisms

involved in epigenetic regulation. At the present time, additional studies are needed to define the human epigenome, its role in development and disease, and the processes that regulate its formation and dynamic modulation throughout the life of an individual.

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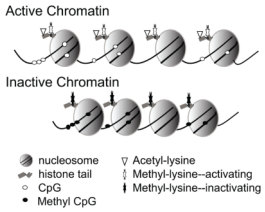

Figure 1. Epigenetic tags and chromatin structure

Chromosomal DNA is packaged around histone cores to form nucleosomes. Nucleosome spacing in open structure that is accessible to nuclear factors is maintained, in part, by post-translational modification of histone tails, including lysine acetylation and specific lysine methylation. CpG dinucleotides are unequally distributed throughout chromosomal DNA, and may be concentrated in regions called CpG islands that can overlap gene promoters. Methylation of cytosines in CpG dinucleotides is overall associated with inactive, condensed states of the chromosome. Inactive chromatin is also maintained by specific histone lysine modifications.

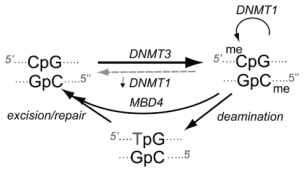

Figure 2. DNA methylaton and demethylation

DNA methylation at the cytosine in CpG dinucleotides is initiated de novo by the DNA methyl transferases (DNMT) 3A and 3B. Following replication, DNMT1 plays a primary role in maintaining the methylation state in the daughter strands. Demethylation is thought to occur by reduction of DNMT1 activity or by excision repair mechanisms following deamidation of methyl cytosine (^{me}C) to create a T:G mismatch. Recent findings (discussed further in the text) suggest that the methyl-DNA binding protein 4 (MBD4) may mediate demethylation by an hormonally regulated mechanism that does not involve deamination of ^{me}C, rather it involves the DNA glycosylase activity of MBD4 followed by a base-excision repair mechanism.

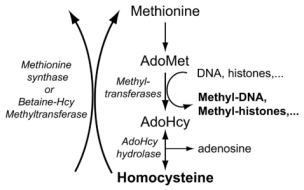

Figure 3. Homocysteine and methylation reactions

S-adenosyl-methionine (AdoMet) is the primary source of methyl groups for hundreds of transmethylases that methylate DNA, RNA, histones, other proteins, and small biological molecules. Following transfer of methyl groups, S-adenosyl-homocysteine (AdoHcy) is formed. Accumulation of AdoHcy can inhibit methyltranferases. The hydrolysis of AdoHcy yields homocysteine (Hcy) and adenosine. Intracellular homocysteine can be removed from the cell, reform AdoHcy, become further metabolized in the transsulfuration pathways (not illustrated), or become methylated to form methionine by the folate-dependent methionine synthase or the folate-independent betaine-Hcy methyltransferase.

Table 1

Chromatin Domains

Heterochromatin: transcriptional inactive, densely packed nucleosomes.
constitutive: highly repetitive DNA sequences, such as centromeric and telomeric domains, hypoacetylated nucleosomes, H3K9me*
facultative: includes silenced genes, such as inactive X chromosome or imprinted regions, or transcriptionally repressed genes, hypoacetylated nucleosomes, H3K27me
Euchromatin: transcriptional permissive chromatin, less densely packed. Accessible to nuclear factors and nuclear repressors, acetylated nucleosomes, H3K4me, H3L36me

* histone methylation sites are listed in abbreviated forms, for example H3K9me, histone lysine 9 methylation.

Table 2

Histone Modifying Enzymes

Category*	Properties
Histone acetyltranferase families	
GNAT MYST CBP/p300	Promotes open conformation of chromatin and gene activation, transcriptional-coactivators recruited by nuclear factors to specific genes
Histone deacetylase families	Promotes condensation of chromatin and gene repression, recruited by transcriptional repressors to specific genes
Class I HDAC	Zn-dependent metallohydrolases
Class II HDAC	
Class IV HDAC	
Class III HDAC (sirtuins)	NAD+-dependent deacetylases
Histone lysine methyl-transferases	
Set domain proteins (partial list):	***Histone specificity, transcriptional effect***
Set1, MLL, Set7/9, SMYD3	H3K4 specific, gene activation
G9a; Suv91, SetB1, PRD14, CLL8, GLP, Suv39h1, Suv39h2	H3K9 specific, mostly repressive
EZH2	H3K27, gene silencing
NSD1	H3K36, gene activation H4K20, gene silencing
Suv4-20h1, Suv4-20h2, Set8/PR-SET7	H4K20, gene silencing
Histone lysine methyl-transferases	
Dot1 domain protein	
Dot1L	H3K79, gene activation
Histone lysine demethylases	
LS	amine oxidase superfamily; FAD-dependent
LSD1	H3K4 me1/2, important in development
Histone lysine demethylases	
Jumonji (JmjC) domain proteins (partial list)	Hydroxylase activity requires iron and α-ketoglutarate cofactors
JMJD2A	H3K9me2/3; H3K36 me2/3
JHDM1A	H3K36me1/2
JAR1D1A	H3K4me2/3

* subtypes of enzymes involved in histone lysine acetylation and methylation.

Table 3

Histone methylation sites

Histone	position	modification	Transcriptional effect/gene or chromatin location *
H3	K4	di-methyl	gene activation
		tri-methyl	gene activation/5′ end transcriptionally active genes
	K9	mono-methyl	gene silencing/euchromatin
		di-methyl	gene silencing/euchromatin
		tri-methyl	gene silencing/promoters & heterochromatin
		tri-methyl	gene activation/gene coding region
	K27	mono-methyl	gene silencing/heterochromatin
		tri-methyl	gene silencing/inactive X-chromosome, imprinted regions & homeotic genes
	K79	di-methyl	gene activation
		tri-methyl	gene activation
H4	K20	di-methyl	gene silencing/heterochromatin
		tri-methyl	gene silencing/heterochromatin

* most common effects and gene or chromatin region are listed, if known