Epigenetic regulation of key vascular genes and growth factors

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Abstract

The role of small RNAs in epigenetic regulation is an emerging field. This research may also open novel treatment strategies based on manipulation of the epigenetic status of the target tissues. Our objective is to review epigenetic regulation of key vascular genes and growth factors. Vascular endothelial growth factor A (VEGF-A) is one of the key players in regulating and maintaining cardiovascular functions and pathology. Although its epigenetic regulation is still not completely understood, expression of the VEGF gene can be manipulated by epigenetic mechanisms using small RNAs that are targeted to the gene promoter which results in the alteration of histone code. VEGF exerts its effects mostly through two receptors, VEGFR1 and VEGFR2, and their expression is also regulated by promoter DNA methylation in various cancer cells. These findings suggest the importance of epigenetic mechanisms in the regulation of vascular functions.

Keywords

Epigenetics • Methylation • Histone code • VEGF • Small RNA • KDR

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1. Epigenetics

Epigenetics includes all heritable information that does not come from DNA sequence itself.1 Epigenetic inheritance takes place through mechanisms involving modifications of chromatin and proteins associated with it. It can be divided into two main mechanisms: DNA methylation and covalent or non-covalent modifications of histones (Figures 1 and 2).2 These events control gene expression independently of the DNA sequence, have profound effects on the cellular repertoire of expressed genes, and are involved in many physiological and pathological conditions.1,3

In eukaryotes, DNA is packaged into a chromatin, a higher-order structure in which the nucleosome is a fundamental unit. Two of each histone proteins H2A, H2B, H3, and H4 form an octamer around which 145–147 bp of DNA is wrapped around twice forming the nucleosome core.4,5 Histones are targets of post-translational modifications, which include methylation, acetylation, ubiquination, and SUMOylation of lysine residues, methylation of arginine residues, and phosphorylation of serines. Histone modifications are considered to form a histone code that is read by chromatin-associated proteins and translated into a transcriptionally active or repressed genetic state.6,7 This histone code interacts with proteins and has varying effects on chromatin structure and gene accessibility. The epigenetic histone modifications are predominantly located at specific positions in the amino-terminal tails of histones.2 These protease-sensitive tails protrude from the chromatin surface and comprise approximately 25% of the mass of core histones, thus providing an exposed surface for interactions with other proteins.8

DNA methylation occurs mostly on cytosine residues on the CpG islands characterized by high density of CG dinucleotides.2 These areas are typically located on promoter regions.9 The CpG islands and their methylation status are associated with tissue-specific gene expression. Monoallelic methylation is present in gene imprinting and inactivation of the X chromosome. DNA methylation also occurs in repetitive elements, such as transposons. DNA methylation can affect gene expression by two different mechanisms. DNA methylation itself can prevent binding of transcription factors to CpG-containing DNA-binding elements, as in the case of Myc10 and hypoxia-inducible factor-1α.11 Alternatively, certain methyl-CpG-binding proteins, such as MBD1, MBD2, MBD4, MeCP2, and Kaiso, specifically recognize mammalian methylation marks.12 These proteins can either directly repress transcription or recruit enzymes that catalyse posttranscriptional modifications of histones. They can also recruit chromatin-remodelling complexes that affect the chromatin structure and promote transcriptional repression.13

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The modifications of histones are dynamic and there are histone-modifying enzymes that add and remove these modifications. For acetylation and methylation, the histone acetyltransferases (HATs) and histone methyltransferases (HMTs) add the acetyl and methyl groups to histone residues, respectively. Accordingly, the histone deacetylases (HDACs) and histone demethylases remove these modifications. Many coactivators contain the HAT activity (e.g. p300/CBP), whereas some global transcriptional repressors are associated with HDACs (e.g. complex of NCoR and Sin3), which is strongly correlated with the fact that acetylation of histone residues marks active genes. HMTs seem to be the most specific of the histone-modifying enzymes, such as SUV39H1, which only methylates H3K9. PRMT family of HMTs catalyse methylation reactions on arginine residues only, whereas SET domain family proteins are lysine methyltransferases. Examples of demethylating proteins are the family of Jarid1 of JmjC proteins and the nuclear amine oxidase homologue lysine-specific demethylase 1 (LSD1). The mono-, di-, and trimethylation layer of modifications complicates the action of enzymes further. It seems like Jarid1B demethylates di- and trimethylated H3K4 and the LSD1 protein specifically targets the mono- and dimethylated forms of the same lysines.

The levels of histone modifications seem to be quantitatively related to gene expression levels. Gene expression levels can be predicted by analysing a set of histone modifications on the gene promoter. Recently, Ernst and Kellis introduced a computational method for annotating genome with different chromatin states, including diverse epigenetic information. They were able to differentiate chromatin states (e.g. promoter-associated states, enhancers) using genome-wide ChIP-Seq data for 38 histone methylation and acetylation marks, histone variant H2AZ, RNA polymerase II, and CTCF. genome-wide ChIP-Seq data for 38 histone methylation and acetylation marks, histone variant H2AZ, RNA polymerase II, and CTCF.

Non-covalent modifications of chromatin involve mechanisms such as chromatin remodelling and the substitution of normal histone proteins with their variants. Chromatin remodelling is regulated by ATP-dependent complexes, which alter histone–DNA interactions by possibly sliding or ejecting nucleosomes and thus altering chromatin accessibility. It has been found that histone dimers are important as deposition entities for de novo nucleosome assembly, histones have variant forms with differing characteristics, and chromatin activity can be modified by the choice of a variant.

Figure 1 Histones are subject to post-transcriptional modifications, which occur in histone tails. The best-known post-transcriptional modifications (acetylation and methylation) are shown. The number under each amino acid represents its position. Greek ‘Epi’ means ‘over, on-top or above’, therefore Epigenetics refers to something above genetics itself. Epigenetic marks are not in DNA sequence itself but on top of them, as chemical additions on DNA stretch (DNA methylation, Figure 2) or on those proteins in which the DNA is wrapped around (Histones, Figure 1). These modifications act as switches turning gene expression on or off. Functional epigenome is necessary for health of a cell or organism. Epigenome is likely an easier target for therapeutic modifications when compared with genome itself.

2. Epigenetic regulation of vascular endothelial growth factor and related genes

Vascular endothelial growth factor A (VEGF-A) is critical for the differentiation of endothelial cells and morphogenesis of the vascular system during development. Mice with only one VEGF-A allele do not develop proper vascular network and die early in embryogenesis. On the other hand, only two-fold excess of VEGF-A results in embryonic lethality. These findings underline the importance of gene dosage of VEGF-A during development. Furthermore, importance of VEGF-A in various pathological states such as cancer, inflammation, retinopathies, and arthritis is well known. For these reasons, it is understandable that the expression of VEGF-A is very tightly regulated at multiple levels. The biological activities of VEGF-A are mediated by two tyrosine kinase receptors, VEGFR1 (Flt-1) and VEGFR2 (KDR). Kim et al. have recently shown that VEGFR1 and VEGFR2 are regulated by epigenetic mechanism in stomach cancer, colon cancer, and hepatocellular carcinoma. Authors showed a negative correlation between promoter methylation and expression of VEGFR1 and VEGFR2. However, they did not find any methylation of the VEGF-A gene in studied cancer cells.
We have shown that the epigenetic state of the VEGF-A promoter can be manipulated using promoter-targeted small RNAs and this results in either increased or decreased VEGF-A expression.34 These epigenetic changes happen by alteration of histone code and not through DNA methylation, at least within a month after RNA delivery. As an indirect evidence, the epigenetic regulation of VEGF-A through histone code, the blockade of p300, a transcriptional coactivator with histone acetyl transferase activity, has been shown to prevent glucose-induced upregulation of VEGF-A and other vaso-active factors in human umbilical cord endothelial cells (HUVEC).35 Therefore, it could be that possible regulation of VEGF-A through epigenetic mechanisms happens mostly through changes in the histone code rather than DNA methylation.

Recently, it was shown that VEGF-A induces epigenetic reprogramming of the promoter regions of Rex1 and Oct4 genes.36 Authors studied whether VEGF-A is capable of eliciting epigenetic modifications in these stemness genes and analysed changes in DNA methylation in their promoter regions. Upon treatment with VEGF-A, methylation patterns in promoters of both genes were diminished in endothelial progenitor cells.37 VEGF-A expression can therefore lead to epigenetic modifications in promoters of other genes. Hypermethylation of anti-angiogenic factors in cancer is a common mechanism by which tumour promotes a pro-angiogenic state.37 For example, the PlGF gene promoter is methylated in human tumours, and methylation may be one of the mechanisms that contributes to the low PlGF expression level in human lung and colorectal tumour tissues and cell lines.38 Interestingly, also the opposite effect where expression of pro-angiogenic gene is stimulated by epigenetic mechanism has been observed: the lymphangiogenic gene VEGF-C is known to be overexpressed in gastric cell line due to demethylation.39 Currently, there is little insight in how the vascularity is epigenetically regulated since many studies are descriptive of nature from patient material and, therefore, the issue of causality should be raised.

Inhibition HDAC reduce myocardial ischaemia–reperfusion injury in mice.40 By treating mice with HDAC inhibitors (TSA and scriptaid), authors could reduce the size of myocardial infarct by approximately 50% even when applying the treatment after the reperfusion. TSA is also known to significantly increase the formation of fatty streak lesions and macrophage infiltration in LDLR−/− mice.41 Dysregulation of epigenetic histone modifications may be a major underlying mechanism for metabolic memory and sustained proinflammatory phenotype in SMCs in diabetic vascular disease, which was also interestingly recently linked to miRNA expression.42 HDAC inhibitors have been shown to inhibit VEGF signalling.43 Authors showed that TSA and SAHA prevented HUVEC from invading a type-I collagen gel and forming capillary structures. These effects were cell specific and not observed in human fibroblasts nor in vascular SMCs. It was also recently shown that VEGF-mediated down-regulation of miR-101 cause pro-angiogenic effects that are partly mediated through reduced repression by miR-101 of the histone-methyltransferase EZH2, a member of the Polycomb group family, thereby increasing methylation of histone H3 at lysine 27 and transcriptome alterations.44 Furthermore, in tumour vasculature, increase in endothelial EZH2 is a direct result of VEGF stimulation by a paracrine circuit that promotes angiogenesis by methylating and silencing vasoohbin1 (vash1).45

The complex nature of atherosclerosis can be seen in the dualistic effects of epigenetic regulation of certain genes. For example, while hyperhomocysteinemia can lead to reduced DNA methylation in atherosclerotic patients,46 some promoters like FGF247 are hypermethylated. Increased methylation of the ERα promoter has been found in atheromas, as well as in the phenotypic switch from quiescent SMCs to a proliferative state.48 Furthermore, cells from the plaque area compared with non-plaque regions show a high level of methylation in the ERβ promoter.49 Although it seems that ERα and ERβ play a role in actively proliferating atheromatous SMCs, the interpretation of the exact role of ERs in vivo remains unclear. ER promoter methylation increases age-dependently and may reach 99% methylation level in the elderly.50 Global hypomethylation related to age is the dominant process, but ERα hypermethylation is not sole exception and gene-specific hypermethylation may have profound effects on the pathogenesis of atherosclerosis.

Age-related promoter hypermethylation of genes such as c-fos, c-myc, DBCCR1, E-cadherin, HIC1, IGF2, MYOD1, N33, PAX6, P15, and versican have been reported.50 Also 15-lipoxygenase, a gene implicated in the oxidative modification of low density lipoprotein, is regulated by DNA methylation.51

3. Matrix metalloproteinases and epigenetic regulation

Matrix metalloproteinases (MMPs) are enzymes responsible for extracellular matrix degradation and contribute to local and distant cell invasion during cancer progression or metastasis. Matrix metalloproteinase 2 (MMP2) and MMP9 (also known as gelatinase A and B, respectively) are known to be involved in pathological changes after balloon injury and in atherosclerosis.52 Sodium butyrate, a HDAC inhibitor, may directly influence pro-MMPs secretion.53 Authors evaluated the effect of butyrate on pro-MMP-9 and pro-MMP-2 secretion in human Jurkat and HT1080 cells, and in 36 paediatric solid tumours. They showed that Jurkat cells treated with butyrate had increases of 2-fold and 1.5-fold in pro-MMP-9 and pro-MMP-2 secretion, respectively. Also, a 50% decrease in pro-MMP-9 secretion after treatment was observed in HT1080 cells. Further, in untreated tumours, butyrate induced reduction in levels of pro-MMP-9 secretion. For cell lines and some butyrate-treated tumours, histone H4 was hyperacetylated. Thus, pro-MMPs gene expression and their secretion can be epigenetically dysregulated in tumours.53 Furthermore, also MMP-3 expression might be regulated by histone acetylation since sodium butyrate selectively enhances MMP-3 production in mesenchymal cells after TNF-α or IL-1β stimulation.54 Some findings also indicate that MMP1 and MMP13 are regulated by epigenetic mechanisms since their induction is blocked by sodium butyrate treatment in chondrocytes.55

4. Small RNAs in epigenetic regulation

Small RNA molecules have been shown to regulate gene transcription by interacting with the promoter region and modifying the histone code (Table 1).34,56–58 Transcriptional gene silencing (TGS) involves promoter-targeted small-interfering RNA and leads to silent-state epigenetic profile containing H3K9me2 and H3K27me3.39 Besides TGS, the small RNAs have been reported to induce gene activation.34,57 This is associated with a loss of H3K9me2 at the targeted promoter sequences and seems to require a member of the RISC complex, Ago2. TGS has earlier been noticed to require Argonaute proteins
Table 1 Genes that are regulated by small RNAs by an epigenetic mechanism and their possible therapeutic targets

<table>
<thead>
<tr>
<th>Gene</th>
<th>Therapeutic target</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>CDH1</td>
<td>Cancer</td>
<td>64</td>
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<tr>
<td>c-myc</td>
<td>Cancer, oncogene</td>
<td>65</td>
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<tr>
<td>CXCR4</td>
<td>Cancer</td>
<td>63</td>
</tr>
<tr>
<td>Cyclin B1</td>
<td>Cancer, cell cycle, proliferation</td>
<td>63</td>
</tr>
<tr>
<td>Ep-cadherin</td>
<td>Cancer, cell adhesion</td>
<td>57,66,67</td>
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<tr>
<td>NKX3-1</td>
<td>Cancer</td>
<td>63</td>
</tr>
<tr>
<td>p21</td>
<td>Cancer, cell cycle, proliferation</td>
<td>57,62,68</td>
</tr>
<tr>
<td>p53</td>
<td>Cancer, apoptosis</td>
<td>63</td>
</tr>
<tr>
<td>PAR4</td>
<td>Thrombosis</td>
<td>63</td>
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<tr>
<td>Progesterone receptor</td>
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<tr>
<td>Survivin</td>
<td>Cancer</td>
<td>70</td>
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<tr>
<td>Ubiquitin c</td>
<td>Skeletal muscle atrophy</td>
<td>71</td>
</tr>
<tr>
<td>VEGF-A</td>
<td>Cancer, cardiovascular disease, age-related macular degeneration, ischaemia</td>
<td>34,57</td>
</tr>
<tr>
<td>WT1</td>
<td>Cancer</td>
<td>63</td>
</tr>
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1 and 2. The more precise mechanisms for both gene activation and silencing are still largely unknown.

We recently designed some shRNAs targeted on the VEGF-A promoter and delivered them to a mouse endothelial cell lines by lentiviral transduction. We identified shRNA significantly inducing VEGF-A expression, whereas another one downregulated it. The downregulating shRNA caused demethylation of H3K4me2 and deacetylation of H3K9ac at the promoter and TSS but had no effect on the H3K9me2 level. It also enriched nucleosome positioning both at the promoter and TSS. The upregulating shRNA increased H3K4me2 at TSS but not at the targeted promoter and had no effect on nucleosome positioning. The epigenetic changes were initially observed 14 h after transduction and had further increased by 7 days. The effect is cell- and tissue-specific since the up- and downregulation and epigenetic changes were only noticed in some cell lines and not in others. We also wanted to evaluate the effects of shRNAs in vivo. Thus, we transduced mouse ischaemic hindlimbs with the lentiviral vectors encoding the up- and downregulating shRNAs. The observed effect and epigenetic profile in muscle tissue was similar to that found in the cell culture. Ultrasound analysis also showed that upregulating shRNA significantly increased vascularity and improved blood flow in the hindlimb, demonstrating that promoter-targeted shRNAs can have therapeutic effects in vivo.

It was recently shown that a long intergenic non-coding RNA (lincRNA) HOTAIR acts as molecular scaffold by binding histone methylase (polycomb repressive complex2) and demethylase (LSD1/CoREST/REST complex) with distinct domains and thus directing a specific combination of histone methylations to the target gene chromatin. The report suggests a possibility that other lincRNAs could also guide distinct histone modification patterns to specific genes and, therefore, affect the epigenetic state of the chromatin during development and disease progression.

Small RNAs have been studied in many cancer cell types. Small-activating RNA (saRNA) targeted on the p21 promoter inhibited cell proliferation and induced G1-phase arrest and apoptosis by upregulating the p21 expression in bladder cancer cells. Targeting the promoters of E-cadherin, p21, and VEGF in human cells resulted in induction of the indicated gene in the studies by Li et al. and the gene activation involved epigenetic changes in the promoter. The group also transfected African green monkey and chimpanzee cells with the same promoter-targeted small RNAs. Since the promoter sequence is highly conserved between humans and primates, the activation of the genes was also induced in these cell lines. They also tested saRNAs targeting promoters of genes p53, PAR4, WT1, RB1, p27, NKX3-1, VDR, IL2, and p52 in primate cell lines but succeeded in the induction of only p53, PAR4, WT1, and NKX3-1. Huang et al. also successfully designed saRNAs for the mouse and rat, targeting the promoters of Cyclo B1 and chemokine receptor CXCR4, respectively. Cyclo B1 is known to promote mitosis entry and the saRNAs did increase H3S10 phosphorylation, correlating with chromatin condensation in mitosis.

5. Conclusions

Our environment is in constant change and epigenetic gene regulation is the mechanism by which organisms, including humans, quickly adapt to these changes. Since epigenetic regulation of genes such as VEGF-A which play important role in regulating cardiovascular disease is now better understood, there is great demand for developing new treatment strategies based on these mechanisms. One promising approach for regulating expression of a specific gene is to use promoter-targeted small RNAs. These RNAs can be delivered either as RNA oligos or expressed as shRNA hairpins using viral vectors. It has now been shown that these RNAs can either up- or downregulate target gene expression via epigenetic mechanisms but precise mechanisms of action is under constant research. Understanding the role of small RNAs in regulating promoter activity might lead to development of novel treatment strategies for cardiovascular disease in the future.

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