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Epigenetics of HIV Infection: Promising Research Areas and Implications for Therapy

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Abstract

We surveyed current trends in epigenetics in general and epigenetics of HIV infection and AIDS in particular to pinpoint promising areas for translational research. Epigenetic mechanisms mark and affect the structure of chromatin, thereby controlling the activity of promoters. Because epigenetic changes are reversible, epigenetic drugs can be used to modulate gene activity. At present, silenced HIV genomes, the latent HIV reservoir, is a major obstacle for a curative treatment of AIDS patients. Epigenetic therapy aims at the purging of the latent reservoir by switching on transcription of silent HIV genomes. The basic idea is that the cytopathic effect of the replicating virus and the immune system may eliminate the reactivated cells, whereas HAART may block the infection of new target cells. Although current efforts concentrate on long-lived resting memory CD4+ T-cells, dormant HIV proviruses also reside in other cell types. Thus, epigenetic characterization of the various HIV-infected host cells and host cell-dependent HIV latency mechanisms is a promising research area and may facilitate the development of cell type-specific epigenetic drugs. HAART itself affects the epigenotype of host cells. This may contribute to the development of drug resistance and unwanted side effects. A pharmaco-epigenetic approach may help to elucidate and revert such phenomena. In addition to latent reservoir purging, epigenetic research offers alternative therapeutic tools as well; although not aimed at the elimination of the virus, targeted silencing of HIV transcription by epigenetic regulators may help HAART to minimize virus replication. **(AIDS Rev. 2013;15:181-8)**

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Key words

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Epigenetic regulatory mechanisms: Essentials in a nutshell

Epigenetic regulation ensures the inheritance of cell type-specific transcription patterns from cell generation to cell generation. Epigenetic modifications alter DNA and certain histone molecules, the principal components of chromatin. Binding of distinct non-histone proteins to regulatory regions of the genome may also constitute epigenetic marks that can be inherited to daughter cells. All of these changes affect the structure of chromatin, favoring either promoter silencing or activation (Table 1). Disturbances in epigenetic regulation have pathological consequences, leading to disease development and even microbial pathogens, including HIV, may dysregulate epigenetic mechanisms in their host cells[1-4].

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Promoter silencing and promoter activation

Methylation at the C5-position of cytosine is an important epigenetic regulator of transcriptional activity. In vertebrates, the control regions of inactive promoters

Table 1. Typical chromatin marks associated with regulatory sequences and promoters in differentiated mammalian cells. Promoter activity depends on open and closed chromatin states established via modifications of DNA and certain DNA-bound proteins by epigenetic marks. Such marks are deposited by "writer" molecules or complexes and interpreted by "reader" factors. Epigenetic changes are reversible, so epigenetic marks can be removed by "eraser" mechanisms.

Silent promoters, repressive chromatin

	Epigenetic players and mechanisms involved		
	Writers	**Readers**	**Erasers**
^{m}CpG	DNMT	^{m}C-binding proteins Transcription factors	Passive demethylation; Tet proteins
H3K27me3	PRC2	PRC1	HDM (UTX/KDM6A, JMJD3)
PcG complexes (PRC1 & 2)	Binding to PRE; binding to modified or variant histones?	?	?
Nucleosomes	CRC	?	CRC

Active promoters, open chromatin

	Epigenetic players and mechanisms involved		
	Writers	**Readers**	**Erasers**
CpG	–	Transcription factors	–
H3K4me3	TrxG	BPTF, ING1; PHD fingers; RAG2; TAF3 subunit of TFIID	HDM
AcH3	HAT	Bromodomain modules	HDAC
AcH4	HAT	Bromodomain modules	HDAC
TrxG	?	?	?
Depleted nucleosomes	CRC	?	CRC

AcH3: acetylated histone H3; AcH4: acetylated histone H4; BPTF: bromodomain and PHD domain transcription factor, the largest subunit of the ATP-dependent chromatin remodeling complex; NURF: nucleosome remodeling factor; BRD4: bromodomain containing protein 4; CpG: a linear sequence of a cytosine nucleotide (C) and a guanine nucleotide (G) separated by one phosphate; CRC: chromatin remodeling complex; H3K4me3: histone H3 trimethylated at lysine 4; H3K27me3: histone H3 trimethylated at lysine 27; HAT: histone acetyltransferase; HDAC: histone deacetylase; HDM: histone demethylase; ING1: inhibitor of growth 1; ^{m}CpG: a linear sequence of 5-methylcytosine (^{m}C) and a guanine (G) nucleotide separated by one phosphate; PcG: polycomb group proteins; PHD: plant homeodomain; PRC1: polycomb repressive complex 1; PRC2: polycomb repressive complex 2; PRE: polycomb response element; RAG2: recombination activating gene 2; TAF3: TATA binding protein associated factor 3; TFIID: transcription initiation factor TFIID; TrxG: trithorax group protein.

are frequently methylated at cytosines located within CpG dinucleotides. Highly methylated genomic regions associate, as a rule, with a "closed" chromatin structure suppressing transcription[5]. In humans, cytosine methylation patterns are maintained by DNA methyltransferase 1 (DNMT1) that restores the methylation pattern of the parental strands on the initially un-methylated daughter strands during semi-conservative DNA replication. *De novo* DNA methyltransferases (DNMT3A, DNMT3B) act preferentially on un-methylated DNA strands[5]. They are involved in the establishment of DNA methylation patterns during embryonic development.

Methyl-cytosine binding proteins attach to hypermethylated DNA sequences with high affinity and attract histone deacetylases that remove the acetyl moieties of histone tails, thereby eliciting chromatin compaction[6,7]. Therefore, silent promoters are frequently associated with histone H3 and H4 that are devoid of acetylation and carry, in addition, repressive modifications, such as H3K9me3 (histone H3 trimethylated on lysine 9) (Table 1). Thus, histone proteins are not only the building blocks of nucleosomes that ensure ordered coiling and packaging of DNA, but also influence and mark local chromatin states.

Polycomb group (PcG) protein complexes with histone lysine methyltransferase (HKMT) activity (polycomb repressive complex 2, PRC2, trimethylating histone H3 lysine 27) or histone ubiquitinase activity (polycomb repressive complex 1, PRC1) also associate with inactive promoters[8,9]. The repressive mark left on the chromatin by PRC2 (histone H3 trimethylated on lysine residue 27, H3K27me3) serves as a recruitment site for PRC1. Heterochromatin-associated protein 1 (HP1) also binds to a specific mark, H3K9me3[10]. The

HP1 invites HKMT and induces "spreading" of the H3K-9me3 mark to unmodified, newly synthesized histone H3 molecules located to adjacent nucleosomes following DNA replication[8].

The DNA methylation and histone modifications are reversible. In pluripotent embryonic stem cells, "pioneer" transcription factors bind to highly methylated sequences and induce cytosine demethylation. Their association with tissue-specific enhancers precedes transcriptional activation of the flanking genes[11,12]. The DNA demethylation can be either active or passive. The active pathway is mediated by the Tet family of dioxygenases: conversion of 5-methylcytosine (5mC) to 5-hydroxymethylcytosine (5hmC) and further to 5-carboxylcytosine (5caC) is followed either by decarboxylation[13] or excision by thymine-DNA glycosylase (TDG) that triggers the base excision repair (BER) pathway, replacing thereby 5mC with C[14]. The passive pathway involves the inhibition of DNMT1 activity during successive cell cycles. Active promoters are frequently un-methylated and located to "open" chromatin areas rich in acetylated histones and other activating histone modifications, such as H3K4me3 (histone H3 trimethylated on lysine 4) produced by HKMT of the trithorax group (TrxG)[8] (Table 1).

Emerging concepts of epigenetic memory

In contrast to most transcription factors that dissociate from their binding sites during mitosis, the pioneer transcription factor FoxA1 remained attached to mitotic chromatin[15]. It turned out that binding of pioneer transcription factors to mitotic chromosomes marked distinct gene sets and ensured their expression after mitosis ("bookmarking" proteins). Variant histones, like H2A.Z, altered nucleosome occupancy and stable chromatin loops may also mark promoters for activation[16,17]. These mechanisms are regarded as novel forms of epigenetic memory, ensuring the inheritance of cell type-specific gene expression patterns.

Characterization of the epigenomes of HIV target cells using high-throughput assays

High-throughput assays, especially DNA microarrays and ChIP-Seq (the combination of chromatin immunoprecipitation and next generation sequencing) were used to characterize the epigenomes of the major HIV target cells, $CD4^+$ T-cells, monocytes, and macrophages.

There were both transcriptionally repressive and non-repressive hypermethylated regions located approximately 6 or 9 nucleosomes away, respectively, from transcription start sites in the genome of $CD4^+$ T-cells of healthy donors[18]. Methylome analysis of *in vitro* expanded conventional $CD4^+$ T-cells and regulatory T-cells revealed 100 differentially methylated regions associated mainly with promoter-distal enhancer sequences of cell type-specific genes[19]. An independent study suggested that alternative distribution of histone H3K4me3 and H3K4me1 epigenetic marks may control cell type-specific gene regulation[20]. Based on ChIP-Seq data and suitable algorithms, the chromatin boundary elements and nucleosome positions in $CD4^+$ T-cells could also be predicted[21,22]. Dynamic changes of DNA methylation were demonstrated during *in vitro* differentiation of human hemopoietic progenitor cells (HPC): differentiation-specific genes hypermethylated in HPC became demethylated both in monocytes and granulocytes[23]. In a study with antiretroviral-naive HIV patients, healthy controls, and HIV patients under antiretroviral therapy (ART), differentially expressed genes were identified in HIV-infected monocytes. Certain genes were dysregulated by ART[24]. The epigenetic background of these phenomena remains to be elucidated.

Epigenetic silencing of the HIV provirus in latently infected cells

HIV replicates with the help of reverse transcriptase (RT). The RT converts the single stranded viral RNA genome into double-stranded DNA (dsDNA) that integrates into the cellular genome (provirus). In certain target cells, the integrated HIV genomes are dormant (viral latency), due to switching off the viral promoter, located to the 5' long terminal repeat (5' LTR) sequence, by the cellular epigenetic regulatory machinery[25] (Table 2). Because the dormant state is reversible, maintenance of the latent HIV proviruses in long-lived host cells rendered curative antiretroviral therapy so far impossible[26,27]. The factors influencing the decision between promoter silencing or activation remain to be clarified[28,29]. In case of $CD4^+$ T-cells, infection of both activated and resting cells may result in the establishment of HIV latency. In addition to epigenetic mechanisms, limited availability of transcription factors or elongation factors, nuclear retention of multiply spliced viral RNA due to low expression of polypyrimidine tract binding protein (PTB), insufficient Tat activity, and cell type-dependent expression of cellular

Table 2. Mechanisms involved in HIV-1 promoter silencing

Mechanism	Consequence
Cytosine methylation	Binding of MBD2, recruitment of HDAC, build-up of repressive chromatin structure
Binding of YY1, LSF, NFκB	Recruitment of HDAC and HMT (SUV39HT) increased level of H3K9me3 and HP1 in the region, repressive chromatin structure
Binding of CBF1	Recruitment of HDAC and corepressors
Binding of c-Myc and Sp1	Recruitment of HDAC1
HIV-1 TAR microRNA	Recruitment of HDAC1
Histone H3K4me3 and H3K9me3	Recruitment of LSD1
Binding of polycomb complexes	Histone H3K27 methylation, histone H2A ubiquitination
Binding of BRD2	Association with CRC, blocking transcription
Binding of BRD4	Inhibition of Tat activity
Deacetylated nucleosomes	Blocking the transcriptional start site
Transcriptional interference by transcripts of upstream host genes	Blocking HIV-1 transcription by antisense cellular RNA

BRD2: bromodomain containing protein 2; BRD4: bromodomain containing protein 4; CBF1: C-promoter binding factor-1; CRC: chromatin remodeling complex; HDAC: histone deacetylase; histone H3K9me3: histone H3 trimethylated on lysine 9; histone H3K27: histone H3 lysine 27; LSF: late SV40 factor; MBD2: methyl-CpG-binding domain protein 2; HMT: histone methyltransferase; HP1: heterochromatin-associated protein 1; LSD1: lysine-specific demethylase 1; NFκB: nuclear factor kappa B; TAR: transactivation responsive (element); Tat: transactivator of transcription; YY1: yin yang 1.

microRNAs modulating viral protein expression also favor latency versus productive infection[29]. Epigenetic mechanisms may play an important role in HIV promoter silencing during transition of activated $CD4^+$ T-cells to resting memory $CD4^+$ T-cells, whereas during the infection of quiescent cells factor availability and post-transcriptional mechanisms may contribute significantly to the downregulation of HIV transcription[29]. The epigenetic mechanisms silencing the HIV 5' LTR were recently reviewed[30,31] (see also the references therein). Here we give only a brief summary.

CpG methylation inhibited the activity of the HIV promoter both directly, by blocking transcription factor binding, and indirectly, via attracting MBD2, a methyl-CpG binding protein that facilitated the build-up of a repressive chromatin structure through recruitment of histone deacetylases. Repressive chromatin structures were also established by yin-yang-1 (YY1), late SV40 factor (LSF), nuclear factor kappa B (NFκB), other transcription factors, and a microRNA derived from the transactivation responsive (TAR) element of the HIV-1 RNA genome. All of them recruited HDAC1, a histone deacetylase to the viral promoter. In monocytes and microglial cells, the repressor protein COUP-TF interacting protein 2 (CTIP2) also recruited HDAC2 and the histone methyltransferase SUV39HT to the proviral LTR. G9a, another histone lysine methyltransferase, as well as the PRC2 component EZH2 and the PRC1 complex played a role in HIV-1 latency, too. Bromodomain containing protein BRD4 blocked HIV transcription by interfering with Tat, the viral regulator of transcriptional elongation, whereas BRD2 acted as a Tat-independent suppressor. In addition, nucleosomal structure also affected transcriptional initiation and elongation. There were two deacetylated nucleosomes called Nuc-(0) and Nuc-(+1) flanking the enhancer region of the HIV-1 LTR. Nuc-(+1), which blocked the transcriptional start site in repressive chromatin, underwent remodeling upon binding of activating transcription factors.

HIV frequently integrates into euchromatic domains of the genome near to transcriptionally active promoters. Open chromatin favors switching on the viral promoter. In contrast, integration into heterochromatic regions around centromeres or into the repressive chromatin environment of gene deserts apparently favors the establishment of latency. Silencing of the HIV promoter may occur, however, in euchromatic domains as well, due to transcriptional interference by read-through transcripts originating at upstream host genes (Table 2).

Epigenetic silencing of HIV LTR could be reverted by various means, including DNA methyltransferase inhibitors, histone deacetylase inhibitors, inhibitors of BRD4, NFκB inducers, and Tat, the HIV transactivator[32-37]. These data formed the basis of a novel therapeutic

approach, also called "shock and kill" therapy, that aimed at the reactivation of latent HIV proviruses (shock), disrupting thereby the latent viral reservoir, combined with an intensified HAART to block the infection of new target cells by viral particles released from the reactivated cells[26]. Infected cells were expected to be destroyed (kill) by the reactivated virus or the immune system[38]. Purging of HIV-1 from the latent reservoir was attempted using HDAC inhibitors[39-41]. In *ex vivo* experiments SAHA, a selective inhibitor of Class I HDAC, could induce, indeed, HIV outgrowth in $CD4^+$ T-cells[42] and disrupted HIV-1 latency in a group of preselected patients on antiretroviral therapy[32]. The $CD4^+$ T-cells derived from patients on HAART could survive, however, the reversal of HIV latency even in the presence of autologous cytolytic T lymphocytes (CTL)[43]. Efficient killing of reactivated $CD4^+$ T-cells could only be achieved by antigen-specific stimulation of the autologous CTL prior to the disruption of HIV latency[43].

Epigenetic dysregulation in HIV-infected cells

HIV was the first human pathogen implicated in the induction of an epigenetic change in host cells[4]. Mikovits, et al. observed that HIV infection of $CD4^+$ T-cells *in vitro* downregulated interferon gamma (IFN-γ) expression[4]. In parallel, there was an increased DNA methyltransferase expression and the methylation level of the IFN-γ promoter was also elevated[4]. Switching off transcription of IFN-γ may block the host immune response. HIV infection also increased *de novo* methylation at the promoter of the *P16/INK4A* tumor suppressor gene and *GNE*, coding for UDP-N-acetylglucosamine 2-epimerase/N-acetylmannosamine kinase that generates the sialyl-donor substrate for cellular sialyl-transferases (reviewed[1]). The "early" HIV proteins, Nef and Tat were responsible for DNMT1 upregulation[44]. The HIV protein Vpr (viral protein R) bound p300/CBP (CREB binding protein), a coactivator with HAT activity[45], and recruited it to the centromeres of mitotic chromosomes, resulting in increased histone acetylation and displacement of heterochromatin binding protein 1-α (HP1-α) and HP1-γ, causing premature chromatid separation, a hallmark of aneuploidy.

Epigenetic mechanisms of drug resistance in HIV-infected cells

Mutant viral genomes are regularly generated during HIV replication, causing alterations of the viral enzymes reverse transcriptase, protease, integrase, and certain structural proteins. These changes result in the development of clinical resistance to antiretroviral drugs[46]. However, a cell-mediated mechanism of drug resistance was also described in case of nucleoside analogue reverse transcriptase inhibitors that require metabolic activation to the nucleotide form[47]. The basis of the latter phenomenon was the drug-induced silencing of the cellular thymidine kinase gene *(TK). TK* codes for the activating enzyme that phosphorylates 3'-Azido-3'dideoxythymidine (AZT), generating AZT monophosphate. Further phosphorylation by thymidylate kinase and pyrimidine nucleoside diphosphate kinase generate AZT diphosphate and the active AZT triphosphate analogue, respectively. The latter acts both as a chain terminator, blocking RT-mediated DNA synthesis, and as an inhibitor of cellular DNA replication[48]. Similarly to other drugs inhibiting cellular DNA synthesis**,** AZT induced DNA hypermethylation and inactivated *TK* in tissue culture[47]. The epigenetically altered cells (epimutants) were highly resistant to the cytotoxic effects of AZT and their capacity to activate AZT to AZT 5'-monophosphate became severely reduced. A suboptimal AZT level may favor the generation of AZT-resistant HIV variants[47,49]. Even low, nontoxic levels of AZT induced site-specific hypermethylation and silencing of *TK*[50].

AZT administered to HIV-1-infected pregnant women to prevent mother-to-child transmission of the virus altered the organization of constitutive heterochromatin in leukocytes of children born to such mothers[51]. Relocation of chromatin domains away from the nuclear periphery favors the activation of lymphoid-specific gene sets (reviewed[52]). Thus, by perturbing the structure of constitutive heterochromatin, AZT treatment may elicit epigenetic changes long after perinatal exposure[51].

Perspectives and implications for therapy

Although the epigenetics of HIV promoter silencing has been intensely studied, high-throughput characterization of the epigenomes of HIV**-**infected cells is still a promising research area. Characterization of the epigenomes of $CD4^+$ T-cells, monocytes, and macrophages and other HIV target cells before and after HIV infection may have pathogenetic and therapeutic implications. In untreated patients, the vast majority of HIV particles are produced by activated $CD4^+$ T-cells. In patients on HAART, however, monocytes are an important site of HIV-1 replication[53]. In addition,

HIV-infected peripheral blood monocytes may differentiate to tissue macrophages**.** Activation, alternative activation, or deactivation of macrophages by cytokines resulted in distinct cellular phenotypes (M1, M2, dM)[54] and possibly epigenotypes that either favor or curtail the formation of HIV reservoirs.

At present, epigenetic therapies based on reactivation of silent HIV genomes focus on long-lived resting memory CD4+ T-cells that are regarded as a major obstacle to curative treatment[25-27,39,55-59]. However, HIV latency mechanisms may differ, depending on the host cell phenotype[31,60], which may necessitate the development and use of cell type-specific epigenetic drugs to activate silent HIV proviruses. In addition, the phenotype of the host cell may also affect its sensitivity to the cytopathic effect of the replicating virus. Tat activated apoptotic pathways in CD4+ T-cells[61], but upregulated the expression of the antiapoptotic *Bcl-2* gene in macrophages, and other cell types[62,63]. HIV-1 Nef, a pleiotropic regulatory protein[64], also protected macrophages from apoptosis[65]. Nerve growth factor (NGF) produced by HIV-infected macrophages rescued human monocytes/macrophages from the cytopathic effect of HIV infection, too[66]. Differentiation of monocytes to macrophages was associated with the development of resistance to apoptosis induction by Vpr[67]. Thus, various mechanisms may prevent killing and ensure long-term survival of HIV-infected, reactivated macrophages[68,69]. Because HIV-infected cells may escape immune-mediated killing as well, getting rid of the monocyte/macrophage reservoir seems to be a formidable task. In addition, cells carrying silent HIV proviruses may reside in compartments that are not easily accessible for epigenetic therapy and HAART[70], necessitating the development of novel drugs or carriers of drugs, efficiently penetrating such compartments[71].

Although pioneering studies on AZT, the very first drug used in ART, described how it silenced the *TK* gene in HIV-infected cells, there are no data regarding the epigenetic effects of other RT inhibitors, or drugs targeting other molecules involved in HIV replication. A pharmacoepigenetic approach[72], i.e. exploring the epigenetic consequences of ART, may help to understand the molecular basis of certain side effects of HAART. A basic step in this direction was a recent transcriptome analysis of ritonavir-exposed adipocytes[73].

In addition to latent reservoir purging, epigenetic research offers alternative therapeutic tools and strategy as well; targeted epigenetic silencing of HIV transcription may curb HIV replication in concert with HAART. Epigenetic regulators, including antisense oligonucleotides, oligonucleotide-intercalator conjugates, hairpin-loop structured oligodeoxynucleotides, polyamide nucleic acids, antisense RNA and small interfering RNA in association with or encapsulated within novel, nano- or micro-sized delivery systems ensure nontoxic and selective inhibition of the HIV promoter[74,75]. Targeting other regions of the dsDNA genome or viral RNA by antisense technologies may also block HIV replication[76-80]. Silencing of the HIV-1 LTR/promoter by antisense strand-specific small interfering RNA (siRNA) targeting the U3 region was associated with an increased level of H3K27me3[75]. Thus, one of the antisense technologies apparently induced both silencing and epigenetic modification of the HIV-1 LTR promoter. Targeted epigenetic silencing of the HIV promoter does not aim at reservoir purging. However, it may help HAART to curb HIV replication.

Conclusion

A detailed analysis of HIV proviral epigenotypes in major HIV-infected host cells may facilitate the optimization of epigenetic therapy and the development of cell type-specific epigenetic drugs or drug combinations. Because cells carrying silent HIV proviruses may reside in compartments inaccessible for current epigenetic and antiretroviral therapy, development of novel drugs or drug carriers efficiently penetrating such compartments appears to be a fruitful new research area, too. In addition, a pharmacoepigenetic approach may clarify the molecular basis for certain side effects of antiretroviral drugs and provide important clues as to their combination with epigenetic drugs. In addition to current forms of epigenetic therapy that are based on the activation of latent HIV proviruses, there are also alternative research efforts to establish selective epigenetic silencing of HIV transcription.

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