HIV Latency and the Noncoding RNA Therapeutic Landscape

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Abstract The Human Immunodeficiency Virus (HIV) belongs to the subfamily of lentiviruses that are characterized by long incubation periods and chronic, persistent infection. The virus integrates into the genome of infected CD4+ cells and, in a subpopulation of cells, adopts a transcriptionally silent state, a process referred to a viral latency. This property makes it exceedingly difficult to therapeutically target the virus and eradicate infection. If left untreated, the inexorable demise of the infected individual's immune system ensues, a causal result of Acquired Immunodeficiency Syndrome (AIDS). Latently infected cells provide a reservoir that maintains viral infection indefinitely. In this chapter we explore the role of noncoding RNAs in HIV infection and in the establishment and maintenance of viral latency. Both short and long noncoding RNAs are endogenous modulators of epigenetic regulation in human cells and play an active role in gene expression. Lastly, we explore therapeutic modalities based on expressed RNAs that are capable of countering infection, transcriptionally regulating the virus, and suppressing or activating the latent state.

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Chronic HIV Infection and the Search for Novel Therapies

HIV/AIDS persists as a global health problem with little hope in the near future for an efficacious vaccine. Despite this, combinations of antiretroviral therapies (ART) have massively reduced the morbidity, mortality and transmission of HIV-related illness [1], resulting ultimately in a slow turning of the tide, and newfound optimism, in the fight against this chronic viral disease. However, current treatment regimens have significant limitations. These include drug toxicities, resistance to ART and the inability to eradicate latent viral infection. Moreover, the daily medication burden has made it difficult to ensure adequate patient compliance with treatment and the costs associated with lifelong treatment and monitoring remain a concern (particularly in developing countries). Nevertheless, the most significant limitation of ART is the inability to eliminate HIV reservoirs, which persist under lifelong treatment. At present, it is not yet feasible to discontinue ART. Latent viral reservoirs within resting memory CD4+ T cells are extremely long-lived and can persist for as long as 60 years for patients receiving ART [2]. In this chapter we explore the noncoding RNA landscape associated with HIV infection and in the establishment and maintenance of viral latency. We focus on gene therapy strategies based on RNA that are capable of transcriptionally regulating the virus, thus ultimately approaching the goal of a functional cure for HIV/AIDS.

HIV Life Cycle

HIV infects cells of the immune system, which express the relevant surface receptors necessary for interaction with the HIV transmembrane Env protein (Fig. 1). HIV enters its target cells, which include CD4+ T cells; macrophages; monocytes; dendritic cells and microglia [3–6], through interaction of the viral trimeric glycoprotein initially with the primary cellular CD4 receptor, and subsequently with the CCR5 or CXCR4 cellular chemokine co-receptors (reviewed in [7]). The gp120 surface subunit of the Env protein initially engages with the cellular CD4 receptor leading to a conformational change in gp120 which allows it to bind to a co-receptor [8]. Viral tropism is largely determined by the co-receptor to which it binds. R5 strains use the CCR5 co-receptor and are able to infect CD4+ T cells, macrophages and dendritic cells. X4 strains bind the CXCR4 co-receptor and only infect CD4+ T cells [9]. Co-receptor binding then triggers the interaction of the gp41 transmembrane subunit of the Env protein with the host cell membrane. Fusion of the cellular and viral membranes ensues [10] and the viral core is released into the cellular environment and uncoated, releasing the viral genome [11]. The viral RNA genome is reverse transcribed into cDNA in the cytoplasm by the viral Reverse Transcriptase (RT) [12] and is then transported within the pre-integration complex consisting of viral cDNA; viral RT; matrix protein; integrase and Vpr, to the nucleus. Nuclear localization signals on Vpr, integrase and matrix mediate import into the nucleus in

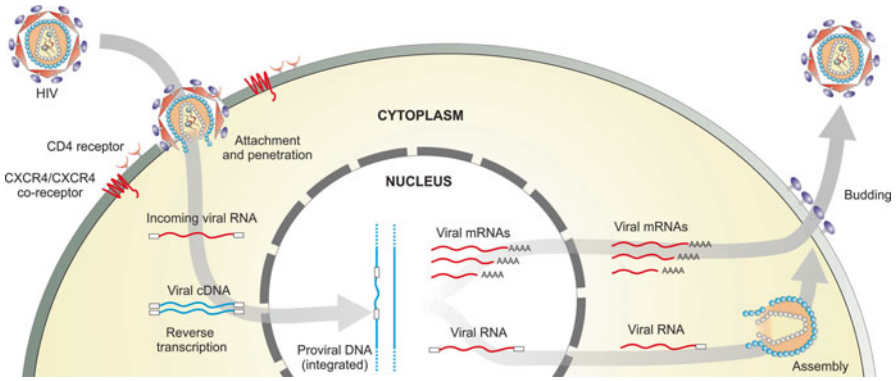

Fig. 1 HIV infection cycle. HIV infects cells and enters target CD4+ cells, through the cellular CD4 receptor, and subsequently with the CCR5 or CXCR4 cellular chemokine co-receptors. Once inside the cell, virus uncoats, releasing the viral RNA genome, which is reverse transcribed into cDNA and transported to the nucleus. Viral cDNA is subsequently integrated into the host genome to form a provirus. Tat-dependence ensures the synthesis of a full-length viral pregenomic transcript and mRNAs. Viral proteins, together with two copies of the viral genome, assemble into immature progeny virions and are released from infected cells through budding

both actively dividing and quiescent cells through the nuclear pores [13–15]. Viral cDNA is subsequently integrated into the host genome, usually within active euchromatin [16], to form the provirus. The provirus is flanked by the 5′ LTR which serves as a promoter for transcription and the 3′ LTR which provides the termination site. The synthesis of full-length transcripts requires the interaction between the regulatory Tat protein and the Transactivating Response (TAR) loop. In the absence of Tat, only short attenuated RNA transcripts are produced [17]. Tat is a multiply spliced protein synthesized early in the viral life cycle. The interaction between Tat and the regulatory TAR loop is enhanced by the positive elongation factor (P-TEFb). Tat binds to the cyclin T1 subunit of P-TEFb and thereby recruits the cyclin dependent kinase 9 (CDK9) subunit to the LTR. CDK9 phosphorylates RNA Pol II, enabling the transition of initiation to elongation and the consequent synthesis of full-length viral transcripts (reviewed in [18, 19]).

Early phase transcripts encoding the Tat, Rev and Nef proteins are completely spliced and are exported from the nucleus. Unspliced transcripts including genomic RNA, Gag-Pol precursors, and incompletely spliced mRNAs encoding Env, Vif, Vpr, and Vpu, require the interaction between the regulatory Rev protein and the Rev responsive element present within these transcripts for nuclear export (reviewed in [20]). Following translation, viral proteins together with two copies of the viral genome assemble into immature progeny virions within lipid rafts localized at the cell membrane. Env proteins are processed into their respective subunits, which also accumulate at the cell membrane. Virions are released from infected cells through a budding process which results in virus particles coated with the host cell membrane embedded with trimeric Env glycoproteins. Maturation of progeny virions occurs following extracellular processing of the Gag and Gag-Pol polyprotein precursors [21].

Viral Latency

HIV-1 infection consists of an initial acute phase followed by a chronic phase. The acute phase is characterized by an increase in viral RNA in the blood (viral load) and the consequent decline in CD4+ T cells [22]. The activation of the immune system subsequently results in the suppression of viremia to a low steady state level termed the viral set point, and an increase in CD4+ T cells. During the chronic phase, viral loads as well as the number of CD4+ T cells may remain constant for several years with the patient remaining largely asymptomatic. During the chronic phase of viral infection, viruses may also enter into a dormant latent state.

Total eradication of HIV continues to present a serious challenge in the struggle against infection with this virus. Cocktails of chemically synthesized antiretroviral drugs, ART, have been successfully used to control viremia during the acute phase of infection. However, despite the significant decrease in morbidity and mortality offered by ART, problems associated with these treatment regimens persist. Although viral replication may be effectively suppressed, current drug regimens are incapable of completely eradicating the virus. Latent reservoirs are characterized by a complete lack of viral gene expression. The underlying molecular mechanisms of latency however are extremely complex and poorly understood, and the exact locations of these latent viral pools are still disputed (reviewed in [23]). There are currently two dominant theories that help to explain what drives latency. One view suggests that viruses are capable of infecting CD4+ T cells that are transitioning into a memory state from a previously active state (reviewed by Siliciano and Greene [24]). Memory CD4+ T cells are a well-defined reservoir and are thought to be the main source of residual viremia [2, 25, 26]. Therefore, viral latency is simply a consequence of natural heterochromatin-mediated epigenetic silencing of transcription in these cells. Another view suggests that latency is the product of stochastic transcriptional "noise" [27]. Since viral transcription is dependent on a Tat-mediated positive feedback circuit, variance in cellular levels of Tat and other transcriptional regulators can greatly impact viral replication in any specific cell [28]. This latter theory has gained much more prominence with new data suggesting that the latent reservoir, comprising replication-competent non-induced provirus, is much larger than previously thought [29]. Even during maximum T cell activation, proviral activation remains largely stochastic [29].

HIV Self-Mediated Transcription Regulation

Growing evidence suggests that an intricate relationship naturally exists between viral infection and the endogenous RNA interference (RNAi) pathway [30–34]. Intriguing data have shown that a number of viruses, including HIV-1, may either exploit the RNAi pathway to their advantage or, alternatively, activate mechanisms which repress the pathway. Furthermore, the expression levels of both viral and host derived microRNAs (miRNAs) may be modulated during viral infection for host or viral gene regulation [35]. In addition, it has been proposed that the

interaction between HIV and the RNAi pathway may also contribute to the multifaceted mechanism underlying viral latency [36, 37]. It is clear that an RNAi-based HIV-host relationship exists, yet a better understanding of this interplay at the molecular level is imperative for the development of novel therapeutics, which exploit this relationship.

Several studies suggest that once integrated into the host genome, the provirus is subject to transcriptional regulation by the host epigenetic regulatory machinery. The activation of latent virus has been well characterized and requires the recruitment of histone acetyltransferases as well as other chromatin remodeling proteins to the activated viral promoter/LTR [38, 39]. Conversely, enrichment of silent state chromatin marks (such as histone 3 lysine 9 trimethylation) and HDAC-1 have been observed at the LTR of transcriptionally inactive proviruses [40–45]. These reports suggest a distinct role for chromatin remodeling, and thus the host epigenetic response, in viral latency. However, the cellular factors responsible for guiding host epigenetic complexes to specific loci are currently unknown.

Noncoding RNAs and Gene Regulation

Recent advances in transcriptomic studies have revealed that a much larger portion of the genome is transcribed than previously anticipated. Studies from the ENCODE consortium have demonstrated that a large proportion of the human genome is transcriptionally active [46]. In addition to protein coding messenger RNA (mRNA) and the well-studied noncoding RNAs (ncRNAs) such as transfer RNA (tRNA); ribosomal RNA (rRNA); small nuclear RNA (snRNA); small nucleolar RNA (snoRNA), many other ncRNAs, in particular long noncoding RNAs (lncRNAs) greater than 200 bp have recently been annotated. Although the functionality of many of these ncRNAs is still fiercely debated [47], it is becoming clear that certain ncRNAs can play a crucial role in gene regulation via multiple mechanisms [48]. Furthermore, these ncRNAs may represent diagnostic biomarkers as well as novel therapeutic targets, thus adding a layer of complexity to the role of ncRNAs in different disease states.

Noncoding RNA has been shown to guide epigenetic complexes to targeted gene promoters resulting in transcriptional gene silencing (TGS) of the targeted gene (reviewed in [49]). Noncoding RNAs antisense to low-copy promoter-associated RNA are able to direct transcriptional silencing complexes containing HDAC1, Ago1 and DNMT3a to a targeted promoter leading to histone modifications and heterochromatin formation [50–56]. This suggests that antisense noncoding RNAs are actively involved in the epigenetic regulation of gene expression (Fig. 2). A growing body of evidence indicates that HIV-1 expresses noncoding RNAs in both the sense and antisense orientation to the viral LTR and viral mRNA [57–59]. It is therefore likely that these RNA species may guide epigenetic silencing complexes to the LTR and thus play a crucial role in the establishment and maintenance of latency.

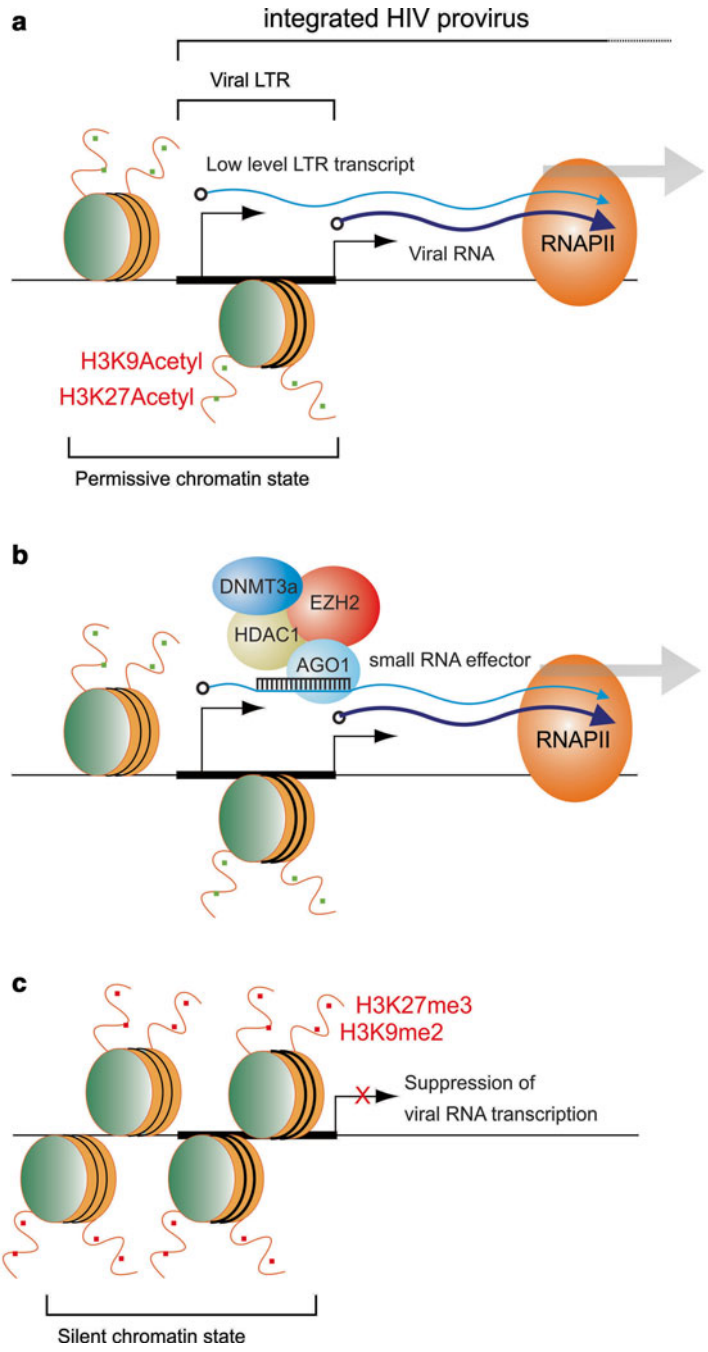

Fig. 2 Inhibition of HIV by transcriptional gene silencing. (**a**) The 5′ LTR of the integrated HIV provirus drives transcription of downstream viral RNAs and is associated with active histone modifications (*green circles*). Additionally, low levels of transcription also occur across the LTR region itself. (**b**) A small RNA effector guides AGO1 to the low copy LTR transcript and thereby recruits the histone deacetylase HDAC1, histone methyltransferase EZH2 and DNA methyltransferase DNMT3A to the LTR chromatin. (**c**) Replacement of active chromatin marks with silent modifications (*red circles*) leads to heterochromatinization and inhibition of viral transcription

Noncoding RNAs Associated with HIV Infection

Noncoding RNAs derived from both the human host as well as from the HIV virus itself are emerging as important regulatory elements of host cellular functions, viral replication, pathogenesis, disease progression and host-pathogen interactions. These regulatory roles are fulfilled by various types of ncRNAs including well-characterized small ncRNAs such as miRNAs, siRNAs, and asRNAs.

Until now, three approaches existed for inhibiting HIV-replication using gene therapy modalities and included directly targeting the viral genome, targeting host dependency factors or targeting newly synthesized viral messenger RNA. Each mature virion contains two copies of the viral RNA genome, which, after fusion, are released into the cytoplasm for reverse transcription as described above. Inhibition of the incoming viral genomic RNA is an attractive target because synthesis of cDNA and establishment of the provirus is prevented, thus inhibiting the replicative cycle of HIV at a very early stage. The second possible strategy for the inhibition of HIV replication and an alternative approach to the prevention of viral entry is to suppress host cellular genes essential for the replicative cycle of HIV. In addition, inhibiting host dependency factors has the added advantage of being refractory to the emergence of viral escape mutants. The third and most obvious category of gene therapy-susceptible targets in HIV is the newly synthesized viral mRNA transcripts, which are exported to the cytoplasm from the nucleus for translation. Every possible HIV transcript has been targeted for silencing using various RNAi effectors with varying degrees of efficacy, often achieving >90 % inhibition of viral replication. The drawback of this strategy is associated with the extraordinarily high rate of error of the viral RT enzyme. HIV is a rapidly evolving virus, and in any infection a pool of viral variants exists. It is thus essential to target conserved regions within viral genes to limit the emergence of viral mutants, which are refractory to RNAi-mediated gene silencing. However, even when targeting conserved sites within the viral genome, mutations may arise under selective pressure, rendering the RNAi effector ineffective. To circumvent this problem, multiple sites should be targeted simultaneously using combinatorial RNAi. Three major platforms currently exist for the simultaneous suppression of multiple gene targets, and all three strategies have been exploited for the inhibition of HIV. These approaches include multiple shRNA expression cassettes; polycistronic miRNA shuttles and long hairpin RNAs (lhRNAs) [60–65]. Given the recent advances in transcriptome analysis, the role of noncoding RNAs in viral infection is fast becoming realized and these noncoding RNAs could thus potentially represent novel therapeutic targets.

HIV-Derived Noncoding RNAs

Small Noncoding RNAs

Small noncoding RNAs (sncRNAs) have been clearly shown to play a role in the replicative life cycle of HIV-1. Although a small number of virally derived sncRNAs have been identified and characterized which are discussed below, evidence suggests that many more low abundance HIV-1 sncRNAs may exist which can be detected with improved selection and screening protocols [66, 67]. Enrichment of low abundant sncRNAs using hybridization capture techniques showed that HIV encodes many sncRNAs of varying lengths spread throughout the viral genome [66]. Deep sequencing technologies have also allowed for a more sensitive method to detect virus-derived small RNAs [67] and together these results suggest that numerous small virus-derived RNAs are produced in HIV infected cells which may potentially play a role in viral replication.

MicroRNAs

MicroRNAs (miRNAs) are small RNA duplexes 21–24 bp in length that regulate gene expression at the posttranscriptional level. miRNAs are derived from the sequential processing of imperfect RNA stem-loop structures by the Drosha/DGCR8 microprocessor complex and by Dicer. The majority of HIV-derived small RNAs are found throughout the sense strand of the viral genome [67]. These small RNAs are thought to be processed from hairpin-like secondary structures within the sense RNA strand by Drosha and Dicer in a similar manner to endogenous miRNAs to generate virus-derived miRNAs.

The first HIV-derived miRNA was described in 2004 [31]. It was proposed that HIV encoded a miRNA precursor within its nef gene. The encoded miR-N367 was reported to target a site at the 3′ end of nef, which overlaps the U3 region of the viral LTR and was thought to play a role in transcriptional regulation of the virus [31, 33].

The Transactivating Response (TAR) element of the HIV genome is a commonly known source of HIV-derived miRNAs [30, 68–71]. TAR is a structured RNA approximately 50 nt in length found at the 5′ end of all HIV mRNA transcripts. The TAR element has been reported to be the source of two functional miRNAs; however, the regulatory role of these TAR-derived miRNAs was initially unclear [68, 69]. Klase et al. identified two host cellular genes as TAR miRNA targets: Excision repair cross complementing-group 1 (ERCC1) and Intermediate early response 3 (IER3). These genes are involved in apoptosis and cell survival and the downregulation of their expression leads to the protection of HIV-infected cells from apoptosis [70]. Another recent study has shown that these TAR-derived miRNAs utilize the same RNAi machinery as host endogenous miRNAs and in addition, further endogenous target genes were identified resulting in the elucidation of their

role in viral infection. In this study, TAR-derived miRNAs were found to target multiple additional genes related to apoptosis including Caspase 8, Aiolos, and Ikaros [71]. The TAR-derived miRNAs therefore play an important role in HIV disease progression by regulating cellular apoptosis and promoting cell survival to ensure the persistence of viral infection.

Small Interfering RNAs

Studies to identify small RNA species in HIV-infected cells have identified noncoding RNAs derived from the viral genome. An initial study looking at the potential of HIV-1 to elicit an RNAi response identified a perfectly duplexed 19 bp Dicer substrate within the HIV-1 genome. This HIV-derived short interfering RNA (siRNA) was able to specifically target its complementary transcript within the Env gene leading to a reduction in both Env mRNA and protein levels which was reversible upon inhibition of the siRNA [32]. Since then a small number of antisense RNAs emanating from the 3′ LTR region have been identified as well. These antisense RNAs may be transcribed from an internal promoter within the HIV genome or from a host promoter downstream of the integrated provirus. Either way, it is thought that these antisense transcripts may form double stranded RNA intermediates with HIV mRNA, which are able to be processed by Dicer to generate viral-derived siRNAs. These siRNAs appear to function via the endogenous RNAi pathway and are capable of modulating viral production. HIV-derived siRNAs identified in a deep sequencing study of HIV-infected cells were found to inhibit virus production by mediating cleavage of the viral transcript at the target site [67]. Furthermore, inhibition of the viral-derived siRNAs by LNA antagomirs reversed the inhibitory effect of the siRNAs on viral production resulting in increased HIV production.

Long Noncoding RNAs

The abundance of long noncoding RNAs (lncRNAs) and their wide range of functional roles in human cells are fast becoming realized. Importantly, lncRNAs have been identified as epigenetic modulators and consequently play a pivotal role in the regulation of gene expression. lncRNAs are able to guide epigenetic silencing complexes to targeted loci in the promoters of protein coding genes resulting in gene silencing in human cells and it is thus becoming apparent that antisense lncRNAs may act as endogenous effector molecules capable of utilizing RNA-based transcriptional silencing pathways in human cells [55, 72–74].

Antisense ncRNAs emanating from the HIV genome during viral infection have also been described [57–59, 75]. Promoter activity has clearly been demonstrated in the reverse orientation with transcription start sites identified in the U3 region of the 3′ LTR as well as in the nef gene sequence. Such transcripts are localized within the

nucleus and are capable of suppressing HIV gene expression over extended periods [59]. Suppression of this antisense lncRNA using small single stranded asRNAs targeted both to the lncRNA transcript as well as to the promoter driving expression of the lncRNA in cell-lines and primary CD4+ T-cells has been shown to result in the activation of viral gene expression [75]. The observed increase in viral gene expression and replication correlated to a loss of silent state epigenetic marks at the viral promoter. This HIV expressed lncRNA was found to localize to the 5′ LTR directly with DNA methyltransferase 3A (DNMT3a) and to usurp components of endogenous cellular pathways that are involved in ncRNA directed epigenetic gene silencing. Collectively, it was shown that the HIV expressed antisense lncRNA is involved in modulating HIV gene expression and that this regulatory effect is due to an alteration in the epigenetic landscape at the viral promoter [75]. This work reflects one of the mechanisms by which HIV regulates its own gene expression. HIV-derived lncRNAs may therefore play an active role in the regulation of viral transcription providing novel insight into the replicative cycle of HIV.

Host-Derived Noncoding RNAs

A subset of host cellular genes have been identified as essential in the replicative cycle of HIV and a variety of these host factors have been considered for downregulation to inhibit HIV infection. In addition to the host proteins encoded by these genes, noncoding RNAs transcribed from some of these genes may play a pivotal role in the course of infection. Furthermore, the expression levels of host derived noncoding RNAs may be modulated during viral infection for host or viral gene regulation [35].

MicroRNAs

Host cell miRNAs have in recent years been implicated in the regulation of HIV infection and the differential expression of these miRNAs has been found to play crucial roles in viral replication, pathogenesis and disease progression. One of the major clues pointing to this miRNA based regulation was uncovered when it was observed that the inhibition of Drosha and Dicer, two key enzymes involved in miRNA biogenesis, resulted in increased viral replication [76, 77]. Subsequently, a plethora of endogenous host miRNAs have been identified which are capable of regulating infection by directly targeting the virus, or by targeting host factors which play a role in the viral replicative cycle, thereby indirectly restricting viral replication.

Two cellular miRNAs, miR-29a and miR-29b, are involved in the regulation of viral replication by directly targeting the viral genome [77, 78]. These two miRNAs

target a conserved site within the viral nef gene [78] and miR-29a has been shown to suppress HIV replication through accumulation of viral mRNA in P-bodies [77]. More recent studies have also confirmed the ability of miR-29a and miR-29b to inhibit viral replication [79]. Further studies have demonstrated the presences of up to 22 target sites within the HIV genome for host cellular miRNAs and at least 5 of these synthetically generated miRNAs are capable of decreasing viral replication [80]. Taken together, the data certainly implies that host cellular miRNAs may act as a cellular defense mechanism against HIV infection.

It has also been proposed that the interaction between HIV and the RNAi pathway may contribute to the multifaceted mechanism underlying viral latency. In resting CD4+ T cells harboring latent HIV, a cluster of five host miRNAs (miR-28, miR-125b, miR-150, miR-223 and miR-382) have been shown to be upregulated [36]. These enriched cellular miRNAs inhibit HIV-1 protein translation through interactions with the 3′ end of viral mRNA transcripts and therefore appear to play a pivotal role in HIV latency. The use of a panel of miRNA inhibitors effectively facilitated viral production in resting T cells and may potentially be used to aid in the purging of latent reservoirs [36, 37].

Host cellular factors are also regulated by endogenous miRNAs as in the case of the polycistronic miRNA cluster miR-17/92, which is downregulated in HIV-infected cells. The histone acetyltransferase P300/CBP-associated factor (PCAF) is a cofactor for Tat and plays a role in procession of viral transcription. Intriguingly, PCAF is a target for miR-17-5p and miR-20a, which are two miRNA components of the miR-17/92 cluster, suggesting an intricate interplay between HIV replication and miRNA-mediated gene regulation of host factors to benefit the virus [76].

Cyclin T1 is another host factor targeted by host-derived miRNAs during HIV infection. As described above, cyclin T1 is a subunit of p-TEFb to which TAT binds for transactivation of HIV transcription. miR-198 targets the 3′ UTR of cyclin T1 mRNA and overexpression of this miRNA leads to inhibition of viral replication [81]. Furthermore, miR-198 was found to be highly expressed in monocytes and downregulated upon differentiation to macrophages suggesting a mechanism for the natural restriction of HIV replication in monocytes [81].

In addition to endogenous host miRNAs having an inhibitory effect on viral replication, host miRNAs may also enhance viral replication depending on the functional role of the miRNA target being modulated. For example, miR-132 is highly upregulated in activated CD4+ T cells and appears to augment viral replication, suggesting that a miR-132 target is a host factor with natural antiviral effects [82].

Long Noncoding RNAs

While the role of cellular miRNAs in the HIV-1 life cycle has been broadly studied in recent years, the relationship between cellular long noncoding RNAs and HIV-1 infection has not yet been fully explored. One study looked at the differential

expression of lncRNAs in HIV-infected cells and identified NEAT1 as a lncRNA that is upregulated during viral infection [83]. Further characterization of NEAT1 showed that it regulates unspliced HIV transcripts at the posttranscriptional level. The depletion of NEAT1 led to increased HIV-1 expression and also resulted in a significant reduction in the number of paraspeckle bodies suggesting that NEAT1 plays a scaffolding role in paraspeckle bodies to which unspliced and singly spliced HIV transcripts containing cis-acting instability elements (INS) bind. Additionally, it was shown that knockdown of NEAT1 leads to an increase in nuclear cytoplasmic export of unspliced Rev-dependent HIV-1 INS-containing transcripts [83].

Noncoding RNA-Based and Therapeutic Targeting of HIV Latency

miRNA Antagonism

The diverse repertoire of noncoding RNAs involved in HIV replication and viral latency present exciting opportunities for therapeutic intervention [84]. Technologies for inhibiting miRNAs are highly developed. The most advanced miRNA therapeutic (a first-in-class pharmaceutical compound) is Miravirsen, developed by Santaris Pharma A/S for treating chronic Hepatitis C Virus (HCV). Miravirsen is a phosphorothioate/locked nucleic acid mixmer antisense oligonucleotide inhibitor of miR-122, a miRNA which is required for effective HCV replication [85]. Miravirsen is currently in late stage clinical trials and has shown promise in preclinical studies in chronically infected chimpanzees [86]. It remains to be seen if miRNA inhibitors will show efficacy in inhibiting HIV replication. For example, TAR-derived miRNAs promote the survival of HIV-infected cells by inhibiting apoptosis [70] Other miRNAs therefore represent potential targets for anti-miRNA therapeutic strategies.

Small-RNA-Induced Transcriptional Gene Silencing of HIV

Exogenous small RNAs complementary to target gene promoters can induce transcriptional gene silencing (TGS) [87] by inducing silent state chromatin formation [51, 56], and promoter DNA methylation [56, 87–90]. TGS is mediated by the RNA induced transcriptional (RITS) complex which contains an argonaute protein (AGO1) [91], and is dependent on the presence of a sense-orientation promoter-associated noncoding RNA [52]. Similarly, miRNAs have also been shown to induce TGS [92–96] and RNAi components have been found to be present, and functional, in the nucleus [97, 98]. The targeted induction of epigenetic alterations at a specific promoter is particularly promising as a therapeutic approach to treating chronic viral infections. Given that HIV viral latency is believed to be mediated by

epigenetic alterations at the proviral DNA, TGS presents the opportunity to effectively 'lock' the virus in an inert, pseudo-latent state.

The effects of conventional RNAi are transient, typically reaching maximal effect within 4–7 days and therefore require frequent repeat dosing. In contrast, TGS has been shown to induce long-term inhibition of the target promoter [56, 99, 100]. As a result, multiple studies have focused on the 5′ LTR of HIV-1 (and also the E6/E7 promoter of Human Papilloma Virus [100]). Two groups of researchers have used small RNAs to target the NF-κB binding motif doublet in the U3 region of the 5′ LTR of HIV-1. Suzuki et al. demonstrated silencing of HIV transcription using small interfering RNAs (siRNAs) in chronically infected MAGIC-5 cells [101]. Silencing was accompanied by methylation of proviral DNA CpG dinucleotides and lasted for 30 days post transfection. The silencing effect was accompanied by the formation of a closed chromatin structure at the viral promoter as evidenced by nucleosome repositioning and enrichment of the silent state mark H3K9me2 [54]. Additionally, AGO1 and the histone deacetylase 1 (HDAC1) were also enriched at the HIV promoter following treatment [54]. Similarly, siRNAs targeted against the simian immunodeficiency virus (SIV) were also shown to induce TGS in MAGIC-5 and CEMx174 cells [102].

When the HIV-1 promoter-targeting sequence was delivered as a short hairpin RNA (shRNA) using a retroviral vector, viral suppression was observed for at least 1 year post-transduction in an HIV infected T-cell line [99]. Silencing was accompanied by enrichment of H3K9me2 and H3K27me3 silent state chromatin modification. TGS of HIV was subsequently demonstrated to be highly specific as (a) mismatched shRNAs were unable to induce TGS, (b) HIV-1 targeting shRNAs were unable to silence HIV-2, (c) NF-κB regulated genes were unaffected by treatment with the on-target shRNA, (d) viral entry-related proteins were not affected by the treatment, and (e) no evidence of interferon induction was observed following shRNA transfections [103]. Recently, the same group demonstrated the *in vivo* potential of TGS approaches to HIV-1 inhibition [104]. Human peripheral blood mononuclear cells (PBMCs) were transduced with a retrovirus carrying the HIV-1 promoter-targeting shRNA. The transduced PBMCs were then transplanted into nonobese diabetic (NOD)/SCID/Janus kinase 3 knockout (NOJ) humanized mice and antiviral activity demonstrated [104].

In parallel, we have also targeted the NF-κB doublet of the HIV-1 5′ LTR (the target site being only four nucleotides off-set relative to that targeted by Suzuki and co-workers). This study showed that the antisense strand of a siRNA alone was sufficient to induce transcriptional silencing of a luciferase cassette driven by the HIV-1 5′ LTR [51]. Subsequently, LTR-targeting antisense RNAs (asRNAs) delivered by mobilization-competent lentiviral vectors were shown to repress viral replication for up to 1 month post-transduction with no evidence to suggest the occurrence of viral escape mutations [90]. Silencing was concurrent with enrichment of silent state chromatin modifications (H3K27me3), recruitment of AGO1, and loss of NF-κB occupancy at the 5′ LTR. Pretreatment of cultures with siRNAs demonstrated that the TGS effect was dependent on expression of HDAC1, AGO1, and the

de novo DNA methyltransferase DNMT3A. A follow-up study also demonstrated the efficacy of the LTR-targeting asRNA in primary human CD4⁺ T lymphocytes [105]. Alternatively, TGS approaches may also be an effective means of silencing HIV host factors. For example, the HIV co-receptor *CCR5* has been shown to be amenable to TGS [52, 91]. CCR5 is a particularly promising target as it is both required for viral entry and is dispensable as evidenced by the prevalence of the inactivating CCR5-Δ32 mutation in certain northern European populations [106].

Small-RNA-Induced Transcriptional Activation of HIV

Latent viral reservoirs within resting memory CD4+ T cells are extremely long-lived and can persist for as long as 60 years for patients receiving ART [2]. One promising strategy is to purge the pool of latently infected cells in the presence of ART by reactivating dormant virus. Reactivation of latent HIV purges infected cells directly (via active viral replication), or indirectly via the host immune system; ARTs can then act to prevent new infection from the released virus to extinguish the reservoir [107]. A seminal clinical study using the histone acetylase (HDAC) inhibitor vorinostat resulted in viral reactivation, but it remains uncertain whether only partial transcriptional reactivation was induced in memory CD4+ T cells [108]. Since activation from latency is largely driven by stochastic events in both active and resting memory T cells [29], HDAC inhibitors and cell-reactivation strategies alone are unlikely to reverse the mechanisms of latency for the entire reservoir [29, 109]. Novel approaches are therefore needed to target the latently infected provirus specifically for activation or suppression/elimination.

Small RNAs targeting noncoding regions can also induce epigenetic activation [110] by targeting antisense lncRNAs [72, 111] (Fig. 3). While the functions of many lncRNAs are currently unknown, some common themes are starting to emerge (reviewed in [112]). Given that the HIV-1 genome encodes both sense and antisense noncoding RNAs [57–59] it is possible that these transcripts act to epigenetically regulate the establishment and maintenance of viral latency. Indeed recent observations suggest that those antisense lncRNAs emanating from the HIV 3′ LTR [57–59] are indeed active epigenetic modulators of HIV expression [75]. The HIV expressed antisense lncRNA appeared to interact with and be involved in the recruitment of DNMT3a, EZH2, and HDAC1 to the 5′ LTR, resulting in epigenetic modulation of HIV transcription, similar to observations with endogenous lncRNAs in human cells [74]. Collectively, these recent observations suggest that HIV, once integrated functions synonymously to endogenous genes and is under lncRNA directed epigenetic regulation. Such insights suggest that it is possible to control viral latency, possibly inhibiting the viruses ability to enter latency by suppressing the expression of the HIV expressed antisense lncRNA (Fig. 3).

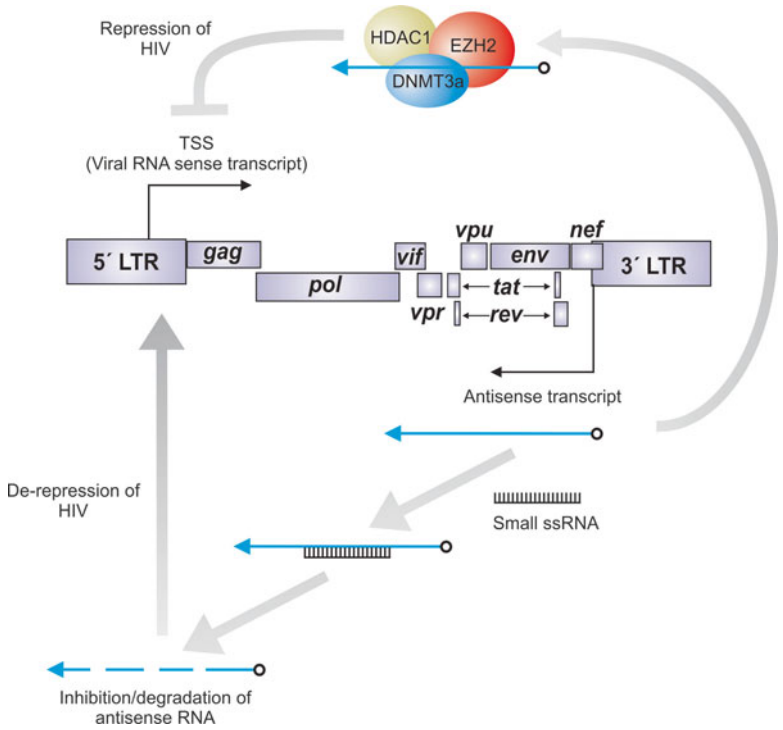

Fig. 3 Model for HIV-encoded antisense lncRNA mediated regulation of viral transcription. A long noncoding RNA (lncRNA) antisense to the viral genome is transcribed from a putative promoter within the nef gene. The lncRNA recruits chromatin remodeling proteins DNMT3a, EZH2, and HDAC-1 and guides these proteins to the viral promoter (5′ LTR). The localization of the chromatin remodeling complex at the 5′ LTR results in the formation of heterochromatin. This alteration of the chromatin state at the viral promoter leads to transcriptional shutdown and an epigenetically silenced virus. The HIV-encoded lncRNA may be inhibited by small single stranded RNAs targeted to the promoter driving expression of the ncRNA via transcriptional gene silencing, or by small RNAs targeted to the ncRNA transcript via posttranscriptional gene silencing (shown). The inhibition of the antisense lncRNA by small RNAs prevents the recruitment of chromatin remodeling proteins to the viral promoter which remains in a euchromatin state, free of silent state epigenetic marks and ultimately resulting in elevated viral gene expression

Conclusions

The success of ART cocktails has meant that the tide is now turning against a once intractable disease. However, the latent HIV reservoir, which harbors transcriptionally dormant virus, represents a unique challenge to efforts aimed at eradicating infection. The fact that current ART cocktails cannot eliminate infection makes it imperative that new therapies are sought, especially those that focus on targeting latent virus and the mechanisms that maintain viral latency. Since viral latency reactivation is stochastic, affecting the positive feedback circuit of viral activation, it is

likely that general factors associated with transcriptional and posttranscriptional control can modulate this process. Already, significant evidence points to both long and short ncRNAs as positive and negative effectors of HIV infection. Moreover, using gene therapy approaches that target these ncRNAs represents a viable approach to eradicating infection. Already a multitude of studies have demonstrated the feasibility of TGS approaches for suppressing HIV-1 replication. Also, the effectiveness of small RNA/oligonucleotide-mediated epigenetic modulation has been demonstrated in vivo [100, 104, 113–116]. This suggests that these approaches may be viable antiviral therapies in the future. Moreover, a new understanding of host and viral-derived ncRNA control of transcription has yielded novel approaches for transcriptionally activating viral gene expression. The latter represents an intriguing therapeutic possibility whereby modalities that interfere or "de-repress" the regulatory ncRNAs result in target-specific viral transcriptional activation. Both these two pathways could be utilized in combination in order to induce enhanced viral suppression or activation of latent infection, thereby working together to act as a "functional cure" of infection.

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