

Post-transcriptional gene silencing, transcriptional gene silencing and human immunodeficiency virus

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Author contributions: Méndez C, Ahlenstiel CL and Kelleher AD solely contributed to this paper.

Conflict-of-interest statement: The authors are named inventors on a provisional patent of a short RNA molecule that suppresses HIV-1 infection. Authors declare there are no conflicts of interest among them.

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Received: December 6, 2014
Peer-review started: December 6, 2014
First decision: December 26, 2014
Revised: January 24, 2015
Accepted: April 27, 2015
Article in press: April 29, 2015
Published online: August 12, 2015

Abstract

While human immunodeficiency virus 1 (HIV-1) infection

is controlled through continuous, life-long use of a combination of drugs targeting different steps of the virus cycle, HIV-1 is never completely eradicated from the body. Despite decades of research there is still no effective vaccine to prevent HIV-1 infection. Therefore, the possibility of an RNA interference (RNAi)-based cure has become an increasingly explored approach. Endogenous gene expression is controlled at both, transcriptional and post-transcriptional levels by non-coding RNAs, which act through diverse molecular mechanisms including RNAi. RNAi has the potential to control the turning on/off of specific genes through transcriptional gene silencing (TGS), as well as fine-tuning their expression through post-transcriptional gene silencing (PTGS). In this review we will describe in detail the canonical RNAi pathways for PTGS and TGS, the relationship of TGS with other silencing mechanisms and will discuss a variety of approaches developed to suppress HIV-1 *via* manipulation of RNAi. We will briefly compare RNAi strategies against other approaches developed to target the virus, highlighting their potential to overcome the major obstacle to finding a cure, which is the specific targeting of the HIV-1 reservoir within latently infected cells.

Key words: Human immunodeficiency virus 1; RNA interference; Reservoirs; Epigenetics; Latency; Transcriptional gene silencing; Post-transcriptional gene silencing

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Core tip: The lack of progress in developing an effective human immunodeficiency virus 1 (HIV-1) vaccine has motivated the pressing need for alternate therapies to cure HIV. RNAi therapeutics represent an alternate approach to a functional cure by offering specific targeting of the HIV-1 latent reservoir with the significant advantage of allowing cessation of combination antiretroviral therapy.

Méndez C, Ahlenstiel CL, Kelleher AD. Post-transcriptional

gene silencing, transcriptional gene silencing and human immunodeficiency virus. *World J Virol* 2015; 4(3): 219-244 Available from: URL: <http://www.wjgnet.com/2220-3249/full/v4/i3/219.htm> DOI: <http://dx.doi.org/10.5501/wjv.v4.i3.219>

INTRODUCTION

Human immunodeficiency virus 1 (HIV-1) infection can be successfully controlled by combination antiretroviral therapy (cART). However, the development of an effective vaccine or an alternative therapy remains the ideal solution since cART has several disadvantages. Adverse effects^[1], high costs of therapy, emergence of resistant viruses^[2,3] and in particular, the fact that life-long continuous treatment is required^[4-6] are just a few examples. Years of research pursuing an HIV-1 vaccine have shown how challenging this task continues to be, with even the most promising trials showing only marginal efficacy^[7,8].

Two main obstacles must be overcome to obtain either a vaccine or a cure. First, the high mutation rate of the virus allows extensive accumulation of genetic changes. These genetic changes generate variation with minimal compromise of the virus identity^[9-11]; Second, the virus is never eradicated from the body, even after prolonged therapy^[6]. While cART has been largely able to deal with the variability of the virus by simultaneously targeting multiple key steps of its replication cycle, it has no direct effect upon latently infected cells^[11,12]. The latter, commonly known as latent reservoirs, includes very long-lived resting memory CD4+ T cells^[13], macrophages and other cell types^[14,15], all of which carry latent proviruses. Provirus refers to the viral form that has been integrated into the cell's genome and is inherited through each cell division. Latent means it is transcriptionally inactive, but is able to re-activate after stimulation^[16-19] and is capable of causing substantial viremia when therapy ceases^[20,21].

The viral reservoir, a term used to refer to the latently infected cells as a whole, is maintained throughout the life span of an infected individual. During episodes of low-level viremia and/or homeostatic proliferation of T cells the reservoir seems to be replenished, but contribution of each of these processes is still disputed^[21-24].

Latently infected cells are considered the major obstacle to a cure for HIV. They remain immunologically and biochemically silent, becoming invisible to the immune system with no expression of viral antigens on their surface. The only known difference between latently infected cells and un-infected cells is a newly integrated "gene": the genome of the HIV provirus.

Considerable effort has been put into understanding the molecular mechanisms of latency in order to develop strategies that specifically target either the latently infected cells or directly target the provirus within them. The establishment of latency results from a variety of molecular mechanisms, mainly transcriptional interference and epigenetic mechanisms. It is believed that there is a repressive epigenetic component in most of the inducible

proviruses. This component is facultative heterochromatin, a compact yet dynamic state of chromatin that impedes proviral transcription^[25-27]. Opposing approaches, which aim to modify the repressive epigenetic profile established at the HIV promoter, have been developed. These either activate proviral transcription by inducing chromatin relaxation or obstruct transcription through stabilization of heterochromatin.

The first strategy has already been tested in cells from HIV infected (+) patients and is currently being tested in a number of clinical trials (<http://aidsinfo.nih.gov/clinical-trials/search/b/0/reservoirs> and <http://aidsinfo.nih.gov/clinical-trials/search/b/0/vorinostat>), using pharmacological drugs or cytokines that directly and/or indirectly induce activation of HIV provirus through a variety of cellular pathways^[28-30]. However, while viral transcripts from apparently latently infected cells have been detected, no significant change or reduction in the size of the latent reservoir-proviral integrated DNA has been observed^[30,31]. There is currently a debate as to whether these cell associated viral RNA transcripts represent transcripts driven by the endogenous HIV promoter, the 5'LTR or whether these are so called "read through transcripts" which arise from altered expression from the promoter of the parent gene into which HIV has integrated^[32]. The results of further trials of these agents are awaited.

The second strategy is based on RNAi and has the advantage of being specifically directed to viral mRNAs or the provirus regardless of the cell type infected. Aiming to target persistent infection in the first place, most RNAi approaches are designed to directly cleave HIV mRNAs and were first designed in the early 2000s. Significant advances have transpired in the field, beginning from those manipulating PTGS to target viral mRNAs and cellular cofactors that support HIV replication, to those using TGS to induce heterochromatin at the HIV promoter. In this review we will discuss both PTGS and TGS RNAi based approaches for HIV, and provide a brief commentary on other gene therapy alternatives currently under development.

RNAi

RNAi is an evolutionarily conserved mechanism that is present from lower eukaryotes through to mammals. Because it is beyond the scope of this review to discuss each of these, we will mainly focus on the mammalian RNAi pathways. However, we will also include some other species-specific examples to illustrate pertinent points.

The first evidence of RNAi was reported in transgenic tobacco plants expressing antisense or sense RNAs from the coat-protein gene of the tobacco etch virus (TEV)^[33,34]. The plants did not show evidence of infection after challenged with TEV, suggesting the presence of a protective nucleic acid-dependent mechanism that was later proved to spread throughout the plant in a systemic way (reviewed in^[35]). The precise mechanism was described in the worm *Caenorhabditis elegans* (*C.*

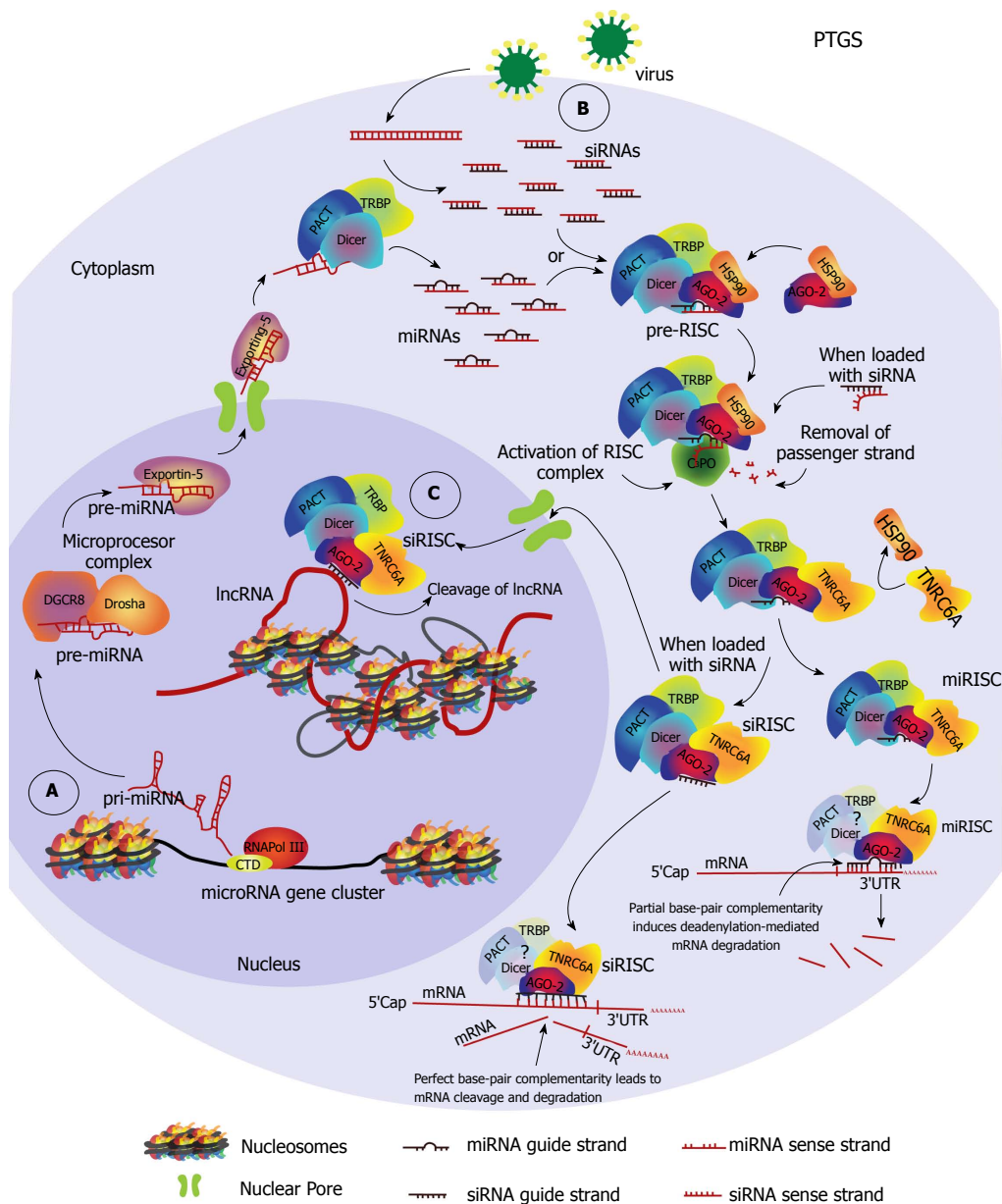


Figure 1 Cytoplasmic and nuclear post-transcriptional gene silencing pathways. A: A primary-microRNA (pri-miRNA) is transcribed by the RNA Polymerase III (RNA Pol III) from a miRNA gene cluster. The pri-miRNA is then processed by the microprocessor complex into the precursor-miRNA (pre-miRNA) which is exported to the cytoplasm by exportin-5. In the cytoplasm, dicer in complex with Tar-RNA-binding protein (TRBP) and protein kinase R activator of transcription (PACT), process the pre-miRNA into miRNA duplexes. MiRNA duplexes are loaded into argonaute (AGO) proteins 1-4 with help from heat shock protein 90 (HSP90), forming the miRNA pre-RNA-induced silencing complex (pre-miRISC). The pathway is shown for AGO-2. The pre-RISC complex is activated after removal of the passenger strand from the duplex by CPO, becoming the miRISC. TNRC6A becomes part of the complex. MiRISC finds a target region within the 3'UTR of an mRNA and induces deadenylation-dependent mRNA degradation; B: During viral infections double-strand RNA (dsRNA) intermediates of viral replication are processed by DICER/TRBP/PACT and are loaded into AGO-2 to form the siRISC complex after removal of the passenger strand. Complete complementarity between the guide strand siRNA and the target region induces cleavage of the targeted mRNA. MiRNAs can also induce mRNA cleavage if this condition is satisfied; C: A nuclear post-transcriptional gene silencing pathway can occur when an activated siRISC is imported into the nucleus and identifies a target within a nuclear RNA such as a Long-non-coding-RNA (lncRNA) resulting in cleavage of the RNA molecule.

elegans), in which interference of endogenous gene expression through inoculation of homologous dsRNA molecules was demonstrated and the involvement of a catalytic and an amplification event was suggested^[36-38]. It was further demonstrated that RNA interference, as it began to be known, resulted in genetic silencing and co-suppression of the targeted gene^[37,38]. Following this discovery, vast exploitation of RNAi for discovery of gene function in reverse genetics of mammalian cells

began and soon after was developed as a therapeutic tool, with several clinical trials currently underway for a variety of human diseases (<http://www.clinicaltrials.gov/ct2/results?term=RNAi&Search=Search>)^[39]. This RNAi pathway is known as PTGS (Figure 1). It functions in the cytoplasm and impedes translation of an mRNA into protein, by direct cleavage or by initiating degradation of the targeted mRNA sequence.

It was not until 2004 that the nuclear RNAi pathway

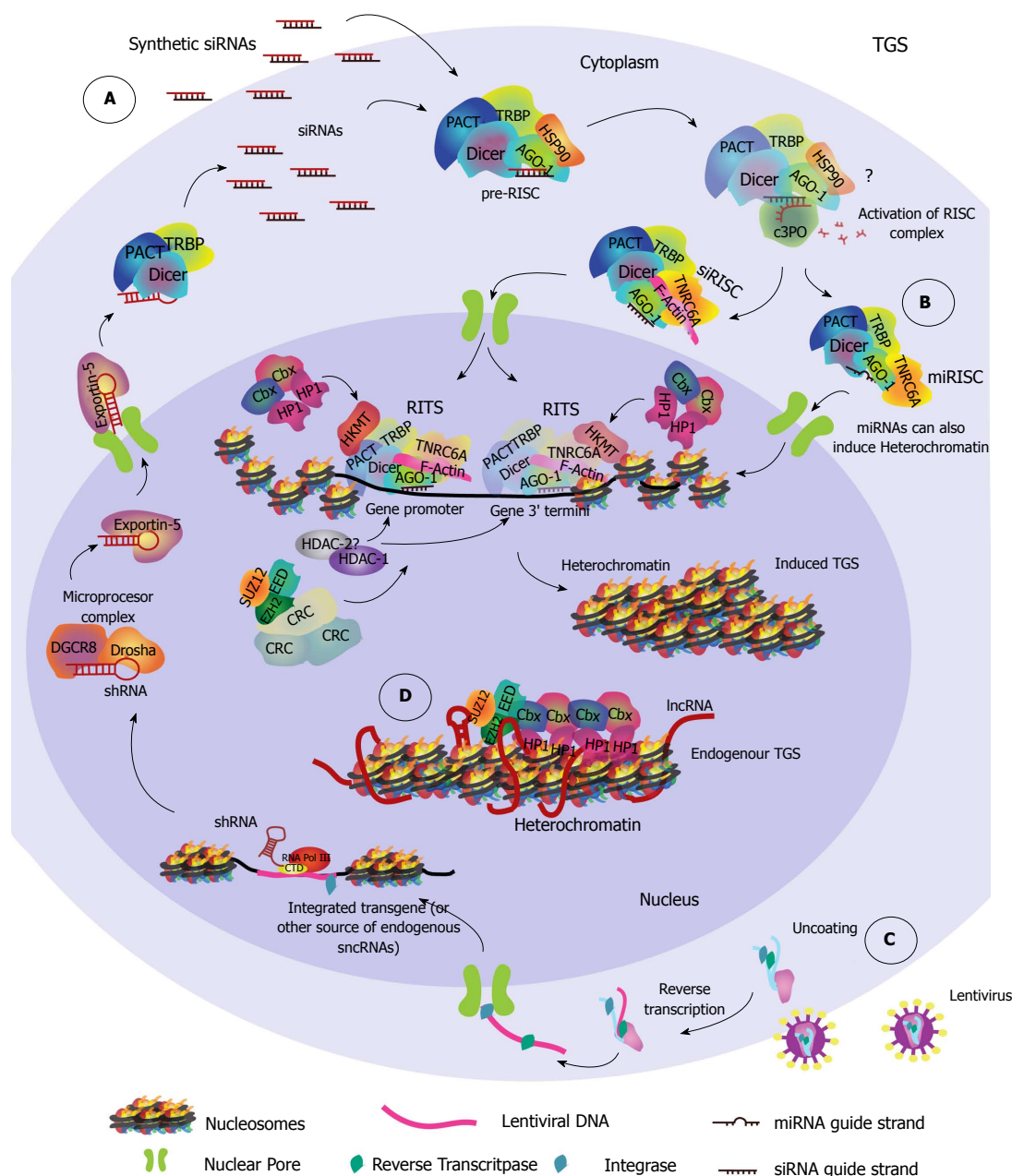


Figure 2 Endogenous and induced transcriptional gene silencing pathways. A: Synthetic siRNAs that have been designed to target the promoter region or the 3' end termini of a gene are loaded into AGO-1, forming the pre-RISC complex. It is currently unknown if removal of passenger strand is required, nonetheless if it occurs it probably takes place in the cytoplasm following the same steps as used in PTGS. F-actin participates in the nuclear import of the RISC complex which, once in the nucleus becomes the RITS complex as histone-lysine-methyltransferases (HKMTs) and other epigenetic related proteins such as Histone-deacetylases (HDAC), DNA methyltransferases (DNMTs), Histone Protein 1 (HP1) and others assemble with it. It is unknown whether RISC related proteins remain in the RITS complex. The RITS complex may vary in its composition depending on the chromatin microenvironment and the small non-coding RNA (sncRNA) target region, therefore only some proteins are shown as an example. Establishment of repressive epigenetic marks (not highlighted for simplicity) and further recruitment of chromatin remodeling complexes (CRCs) results in heterochromatin formation and induces transcriptional gene silencing (TGS). Two independent target regions are pictured together to show the different regions of a gene that can be targeted to induce TGS; B: miRISC complexes whose guide strand targets a promoter region may be exported into the nucleus, form a RITS complex and induce TGS in the same way as was described for siRISC complexes; C: Lentiviruses can be used to drive transgene integration of a DNA cassette designed to express a shRNA that induces TGS. ShRNAs are transcribed by RNA Pol III and are processed through the microRNA pathway. In the cytoplasm they are converted to siRNA duplexes that are loaded into RISC complexes and follow the same import pathway to induce TGS as explained in A; D: Endogenous transcriptional gene silencing is induced by a long non-coding RNA (lncRNA) whose secondary structure is recognized by members of the Polycomb Group repressive complex 1 (PRC1), such as enhancer of zeste 2 (EZH2), embryonic ectoderm development (EED) and suppressor of zeste 12 (SUZ12). The interaction recruits HP1 and other proteins of PRC1 complexes like the (Chromobox) Cbx family that contain a chromodomain able to induce heterochromatin formation.

TGS, involving chromatin compaction, was identified^[40] (Figure 2). Presently, both PTGS and TGS have been found to be functional in the nucleus of mammalian cells, but only TGS seems to repress gene transcription

directly through chromatin remodeling.

During RNAi small non-coding RNAs (sncRNAs) are used as guides through sequence homology to target either mRNA transcripts or gene promoters^[41-43]. These

sncRNAs are loaded into Argonaute proteins forming the main effector complex; however, other cellular cofactors are required for the process to occur. There are three major kinds of sncRNAs involved in RNAi: small interfering RNAs (endogenous- and exogenous-siRNAs), microRNAs (miRNAs) and piwi-associated RNAs (piRNAs)^[44], which we will describe briefly in the next section. The Argonaute proteins are further subdivided into the Argonaute subfamily (AGO-1, AGO-2, AGO-3 and AGO-4, in humans), and the Piwi subfamily (HILI or PIWIL2, HIWI₁ or PIWIL1, HIWI₂ or PIWIL4 and HIWI₃, in humans)^[44,45]. There are also species-specific AGO proteins that we will not discuss (recently reviewed in^[46]), with the exception of specific examples.

Recently, many novel non-canonical sncRNAs involved in RNAi have been discovered^[47-49]. However we will only focus on the three major classes previously mentioned. SncRNAs are generally classified depending on their biogenesis (Dicer/Drosha dependent or independent), their size (about 21-30 nt) and the Argonaute protein they bind (AGO 1-4). They can be endogenous or exogenous depending on their origin. The endogenous sncRNAs are produced from transcription units (Figure 1A), protein coding genes (exons and introns), convergent promoters, long non-coding RNAs (lncRNAs), gene clusters, repetitive elements or retro-elements, such as transposons, while the exogenous sncRNAs are either synthetic or of viral origin (Figure 1B).

SncRNAs

SiRNAs: Exo-siRNAs and endo-siRNAs: SiRNAs are about 21-nt long duplexes generated in the cytoplasm by cleavage of endogenous or exogenous long dsRNA precursors (*e.g.*, lncRNA) by the endonuclease Dicer (Figure 1B). These siRNAs are then loaded onto a specific AGO protein. When exogenous, synthetic siRNAs may be delivered to the cells by transfection/nucleofection protocols or may originate from expression of artificial integrated constructs (lentivirus transduction), such as short-hairpin RNAs (shRNAs). Naturally occurring exo-siRNAs in mammalian cells were not discovered until very recently, and were found to originate from dsRNA intermediates of viral replication in mouse embryonic stem cells^[50]. On the other hand, mammalian endo-siRNAs were identified in somatic tissue and found to be processed through a non-canonical Drosha-independent mechanism, from Dicer cleavage of a long nuclear hairpin RNA expressed from short interspersed nuclear elements (SINES)^[51].

Generally, siRNAs direct the cleavage of their cognate mRNA through PTGS when they mutually base pair with perfect complementarity^[52-54]. Mutations in the siRNA or in the target region within the mRNA sequence usually reduce or abolish silencing, which is why RNAi is considered a very specific mechanism^[55,56]. In addition, siRNAs can also induce TGS, a nuclear RNAi pathway, whenever they target a complementary sequence within the promoter or the 3' end of a gene^[57,58]. Additionally, siRNA-directed transcriptional gene activation (TGA) has also been reported for several genes^[57,59].

miRNAs: In their canonical pathway miRNAs are first transcribed as primary-miRNAs (pri-miRNA) by RNA Pol II, and are then processed by the nuclear RNase III protein Drosha - an RNase type III enzyme (Figure 1A). Drosha and co-factor DiGeorge syndrome critical region gene 8 (DGCR8) form the Microprocessor complex. This complex generates precursor-miRNAs (pre-miRNA) that are further processed, exported to the cytoplasm by Exportin 5, and cleaved by Dicer. Dicer generates 22-nt miRNA duplexes that are analogous to siRNA duplexes. Non-canonical pathways exist which are Drosha, Dicer or DGCR8 independent. Importantly, unlike siRNA-duplexes, miRNA-duplexes frequently contain mismatches and about the first 2-7 nts at the 5' end of the guide strand, known as the seed region, may target the 3' untranslated (3' UTR) region of multiple mRNAs. Based on this multiple targeting ability, other biochemical characteristics and evolutionary conservation, microRNAs are clustered into families (<http://www.mirbase.org/index.shtml>). In miRNAs, complete base pair complementarity with target mRNA is found within this seed region, which allows for mismatches towards the 3' end. MiRNAs predominantly direct deadenylation-dependent mRNA-decay that results in translational repression, but they are also able to induce sequestration. While common in plants, in mammals on rare occasions when miRNAs show complete complementarity to the target region they can induce cleavage of the mRNA^[60-62]. Deadenylation and other ways of translational repression and sequestration result from partial complementarity between miRNA and the target mRNA^[44,47,63,64]. In a similar way to siRNAs, when mature miRNAs show complete homology to a promoter region, they are able to induce TGS^[65-67] (Figure 1C). However, this miRNA pathway is not well described due to the few cases that have been reported.

piRNAs: PiRNAs are longer (about 25-31 nt) than siRNAs or mature miRNAs and have fundamental roles in maintenance of stemness, transgenerational inheritance and genome instability through targeting of repetitive sequences (*e.g.*, endogenous retroviruses and transposon elements), among other functions (reviewed in^[68]). In mammals, piRNAs are expressed in germ cells and somatic germ cells (SGC), but their role in somatic stem cells, such as hematopoietic stem cells, remains controversial^[69,70]. Even though there is expression of piwi-pathway-specific AGO proteins in human CD34+ stem cells^[71], further evidence is still required to confirm a functional piRNA pathway in somatic stem cells different to SGCs.

Interestingly, piRNAs direct specific genome rearrangements in ciliates and this precise genome editing results in either somatic elimination^[72] or retention^[73], indicating the versatility of this particular RNAi pathway. PiRNAs can target mRNAs through a PTGS-like mechanism, however they may also induce TGS by directing heterochromatin formation at the target regions^[74,75]. Intriguingly, members of the piRNA pathway are highly expressed in certain human cancer cells (reviewed in^[76]), though it is still

unknown whether they are the cause or the effect. To our knowledge there are no reports regarding the use of synthetic piRNAs and since they have not been manipulated for human therapy we will not explore these further. However, the ability of piRNAs to establish a permanent, stable and inheritable silencing through directed epigenetic chromatin modifications and other mechanisms makes them of great interest for future study, especially since silencing mediated through their activities is inherited to every single cell of a multicellular organism.

RNAi pathways: PTGS and TGS

PTGS: In humans, RNAi induced mRNA cleavage is directed only by AGO-2^[54,77]. Loading of the sncRNA duplex onto the AGO proteins is well described for AGO-2 and involves the heat-shock protein 90 (HSP90) (Figure 1). HSP90 aids in the recruitment^[78] and stabilization^[79] of unloaded AGO within processing bodies (P-bodies). Inhibition of HSP90 results in unpaired siRNA- and/or miRNA-dependent silencing, respectively. These P-bodies are cytoplasmic structures that contain mRNA decay factors, untranslated mRNA, translational repressors and RNAi related factors^[80]. The active silencing complex is named RISC or miRISC, depending on the type of sncRNA (siRNAs or miRNAs, respectively) that is loaded onto the AGO protein. We will refer to both as RISC, unless specified.

The sncRNA-duplex/AGO-2 complex is called pre-RISC (pre-RNA-induced silencing complex) and requires the removal of one of the strands of the RNA, the passenger strand, in order to become an active RISC complex^[81,82] (Figure 1). The strand that remains in RISC is known as the guide strand. Passenger/guide strand selection depends on the individual thermodynamic properties of each sncRNA molecule within the duplex; these properties generally create energetic asymmetry between the duplex ends, allowing differentiation and selection of the guide strand^[83]. Asymmetry means that the duplex is energetically less stable at one 5' end and causes unwinding to begin at this site. As a result, the strand whose 5' end lies in the less stable end of the duplex will be loaded onto the AGO protein, becoming the guide strand. Whenever the energetic difference between the duplex ends is small or negligible, both strands may be randomly loaded^[83,84].

Dicer seems to play a role in sensing and positioning the guide strand, facilitating removal of the passenger strand. This ability appears to be activated through its interaction with Transactivation Response (TAR) RNA-Binding Protein (TRBP) and Protein Kinase RNA (PKR) Activator (PACT), both double-stranded RNA binding proteins (dsRBP)^[85] (Figure 1). However, there is contradictory evidence regarding this role for Dicer^[85,86], and further research may be needed to clarify these observations. Nonetheless, it is important to mention that proper selection of the guide ensures specificity towards silencing the intended target.

Pre-RISC activation requires the slicer activity

of AGO-2, specifically the nicking of the passenger strand^[87-89]. After nicking, the endonuclease component 3 promoter of RISC complex (C3PO), composed of Trax and Translin proteins in humans, is able to cleave and remove the passenger strand^[87,90,91]. This results in activation of pre-RISC into the RISC complex. Within RISC, the guide strand is used to scan mRNAs for a region with full or partial base pair complementarity. Once the region is found the mRNA is either cleaved, deadenylated or stored during translational repression^[63,92,93] (Figure 1). Storage and repression of translation may have a role in gene regulation of processes that require a quick response, as translation can be initiated from stored transcripts rather than relying on *de novo* transcription^[94].

While AGO-2 can direct either cleavage or translational repression of mRNAs, non-catalytic AGO proteins like AGO-1 seem mostly involved in translational repression, since they are unable to cleave mRNA transcripts^[46]. Furthermore, it is currently unknown how RISC activation occurs for non-catalytic AGO proteins (AGO-1, 3 and 4). However, owing to their inability to cleave mRNAs, activation of the silencing complex must either be different or rely on help from additional cofactors.

For silencing to occur, human AGO-2 requires direct binding with TNRC6A, also known as GW182, a mRNA binding protein rich in glycine/tryptophan repeats^[95,96]. Interaction between GW182 and AGO-2 proteins is crucial for miRNA-mediated silencing and appears to take place directly after the passenger strand is removed by C3PO^[96,97]. Both AGO-2 and GW182/TNRC6A have been shown to co-localize with siRNA and miRNAs within GW bodies (GWB)^[98], another term for P-bodies. Based on these observations, it has been proposed that silencing by miRNAs requires an effector complex formed of at least one AGO and one GW182/TNRC6A protein^[99] (Figure 1).

Interestingly, human GW182/TNRC6A was found to transport AGO-2 proteins to the nucleus during miRNA-induced silencing of a nuclear non-coding RNA^[100]. The latter constitutes evidence of a nuclear PTGS pathway (Figure 1C). Indeed, increasing evidence supports a functional nuclear PTGS pathway, with a recent study demonstrating not only the presence of PTGS related proteins in the nucleus of mammalian cells, but an active AGO-2-RISC complex able to efficiently cleave two nuclear lncRNAs, Malat1 and Neat1^[101]. These studies add to previous evidence indicating the existence of a nuclear RNAi pathway and suggest that PTGS and TGS may be closely related.

We have aimed to provide a detailed overview of the molecular mechanism of PTGS in order to understand the unknowns of TGS and further compare the manipulation of PTGS or TGS for HIV gene therapy. PTGS pathways have been exploited for HIV therapeutics, and several clinical trials are currently testing PTGS-based gene therapy approaches directed to cellular and viral transcripts. A major disadvantage of using PTGS to treat HIV is that PTGS requires viral transcription because it acts on mRNAs. First, this gives the virus the chance

to evolve resistance mutations and escape silencing; Second, latent proviruses will not be targeted since they are not undergoing active transcription. Improvements in siRNA/miRNA design and expression have been developed aimed at overcoming these caveats and will be discussed in the HIV-1 section.

TGS: TGS is a conserved mechanism of gene regulation across species and has been extensively studied in the plant model *Arabidopsis thaliana* (*A. thaliana*), the worm model *Caenorhabditis elegans* (*C. elegans*), and the fission yeast *Schizosaccharomyces pombe* (*S.pombe*). The first evidence for TGS was observed in plants, and it was found to require siRNA-induced DNA methylation for heterochromatin formation (recently reviewed in^[102]). However, the mechanism in *S.pombe* shed insight on the identification of TGS in mammals. In this microorganism, siRNAs generated from centromeric repeats are first processed by Dicer, then loaded onto AGO-1, and together with the proteins Chp1 and Tas3 form the silencing complex, namely the RNA-induced initiator of transcriptional gene silencing (RITS) complex. This RITS complex is analogous to the RISC complex from PTGS. RITS is then directed through siRNA base pair complementarity to a specific locus at which it induces recruitment of Clr4 (histone methyltransferase) and Swi6 (chromo domain binding protein) in order to establish and spread heterochromatin domains^[103-105].

Human sncRNA-directed TGS is mainly, but not exclusively directed by AGO-1 rather than AGO-2, and is generally triggered by promoter-targeted sncRNAs (Figure 2). Recent evidence suggests that it may be also triggered by sncRNAs that target the 3' termini of genes^[58,106]. While increasing evidence suggests a role for AGO-2 in nuclear gene silencing^[100], it seems to be predominantly through a nuclear PTGS that involves RNA cleavage^[101], with only few described exceptions^[107]. There is also evidence of RNA-induced nuclear silencing without heterochromatin formation, involving both AGO1 and AGO2^[108], and there seems to be various RNA-directed nuclear-pathways that control transcription at different stages^[109]. However, it is generally accepted that heterochromatin and its associated markers (*i.e.*, histone methylation and deacetylation) is a characteristic feature of TGS. Therefore, for this review we will focus on the different endogenous TGS mechanisms that involve heterochromatin formation induced by sncRNAs loaded into an AGO protein.

Heterochromatin is considered a hallmark of repressive silent chromatin, ubiquitous in eukaryotic organisms. In mammals, its establishment at a particular locus is a result of protein interactions and cross talk with multiple silencing mechanisms such as DNA methylation, genomic imprinting and Polycomb group of proteins (PcG)^[65,103,110]. The epigenetic profiles across mammalian genomes are very heterogeneous and show a wide range of silencing dynamics. Silencing extends from permanent and inheritable to inducible, dynamic silencing. The former is mainly but not restricted to, constitutive heterochromatin and is found in centromeres and telomeres^[111]; while the

latter, predominantly within facultative heterochromatin, controls specific gene expression during differentiation and development^[112].

SncRNA-directed TGS in mammalian cells has been a controversial topic since its discovery, nearly a decade ago, with some still doubting its existence. These doubts have relied on the inability to explain in detail the molecular mechanisms driving TGS. In particular, the much awaited identification and characterization of a functional nuclear mammalian RITS complex, because there are apparently no RNAi proteins with homology to Tas3 and Chp1 present in the nucleus and AGO-1 is non-catalytic. At present, most of the evidence of mammalian sncRNA-AGO-1 directed TGS relies on synthetic siRNAs or shRNAs driving TGS to control infectious agents, such as HIV-1^[113], or cellular genes that support viral replication^[114]. Nonetheless, the relatively slow accumulation of evidence has supported the existence of this functional pathway, with evidence for miRNA-induced TGS in senescence^[107] and in differentiation^[65]. We will explain the basis for the doubts and show the recent evidence supporting mammalian sncRNA-directed TGS.

The breakthrough proving the existence of a TGS mechanism in mammalian cells came with the identification of the human ortholog for Clr4, known as Suppressor of variegation (Su(var)3-9) in *D. melanogaster* and Su(var)39H in humans; and then with the ortholog for Swi6, known as Histone Protein 1 - alpha (HP1- α) (in both *D. melanogaster* and humans)^[115-117]. However, no human orthologs for Chp1 and Tas3 proteins from fission yeast RITS complex have been yet identified. At present, there are more questions than answers about the series of events in humans that result in siRNA-AGO-1 mediated heterochromatin formation and activation of the RITS complex. It is possible that both PTGS and TGS share a core multi-protein complex, which may differ in accessory subcellular or pathway-specific co-factors, because the initial steps of TGS may potentially resemble those of PTGS.

There is also controversy regarding the activation of the RITS complex during TGS. It is assumed that removal of the passenger strand occurs during TGS to allow the RITS complex to scan for the target sequence that is complementary to the guide strand. However, since AGO-1 lacks the catalytic amino acid tetrad DEDH responsible for the slicing function, it is not clear how this process occurs^[118]. AGO-1 needs to nick the passenger strand from the siRNA duplex, so C3PO or a similar complex would be able to remove the passenger strand.

On one side, it was shown *in vitro* from bacterially expressed human AGO proteins, that AGO-1 is able to cleave the passenger strand, but requires assistance for removal of the cleaved fragments^[119]. This has been interpreted as non-catalytic AGO proteins being very inefficient catalysts and having an extremely low nickase activity.

This is in agreement with findings in mouse embryonic stem cells, in which the absence of the four mammalian AGO proteins resulted in apoptosis, but the expression of any one of the other AGO proteins in isolation, was

enough to rescue the cells and restore a functional RNAi pathway, showing evidence for functional redundancy^[120]. In addition, another study showed that non-catalytic AGO proteins are loaded within the duplex but removal of passenger strand takes place approximately 2 to 3 d^[121]. The process of passenger strand removal is currently unknown.

In contrast, the crystallographic structures of human AGO-1 in association with endogenous RNA (1.75 Å) and in association with Let-7 miRNA (2.5 Å) were used to show that while highly similar to hAGO-2-RNA structures, there was an absolute requirement for the introduction of the catalytic tetrad by introduction of a single point mutation as well as the reconstitution of a loop called PL3, in order to restore the slicer functionality of AGO-1^[122]. These observations argue against a catalytic role for AGO-1.

It seems more likely that other proteins aid non-catalytic AGOs during this step. These cofactors would be present in the AGO knockout mice study and in the cells used to show removal of passenger strand after a few days, but not in the bacterial system, in which cleaved fragments remained loaded to the AGO proteins. Comprehensive studies are required to address this question definitively.

An increasing number of studies have found PTGS-related proteins in the nucleus of mammalian cells, such as GW182/TRNC6A and the endonucleases hC3PO and Dicer^[100,123-125]. These proteins appear to have functions related to both to the mechanisms underpinning PTGS in the nucleus and to the regulation of chromatin and transcription.

For example, human Dicer has been shown to interact with NU153, a non-canonical nuclear transport nucleoporin, as demonstrated by co-localization within the nucleus^[125]. In addition, human Dicer has been shown to associate with the chromatin structures of ribosomal DNA^[124]. It also has a role in termination of transcription^[126], in regulation of intergenic transcription in the human β -globin gene cluster^[127] and in regulation of nuclear receptor (NR) signaling, as evidenced by direct binding of Dicer to NR promoter regions^[128]. Further, Dicer has been reported to be required in heterochromatin formation in fission yeast^[129] and in vertebrates^[130], suggesting its presence in the nucleus of human cells could be due to an as yet unidentified role in mammalian TGS (Figure 2B).

We previously mentioned that GW182/TNRC6A shuffles AGO-2 proteins between the nucleus and cytoplasm through a non-canonical nuclear localization signal^[100]. Additionally, GW182/TNRC6 associates with all four RNA loaded-AGO proteins during PTGS. Therefore, it is a possibility that Dicer is contained within a loaded AGO-1-TNRC6A complex during the nuclear shuffling that occurs during TGS^[131] (Figure 2). Furthermore, the interaction between GW182/TNRC6 and AGO-1 occurs through binding of the GW repeats of GW182/TNRC6 to the Piwi domain of AGO-1^[77]. This is intriguing because the fission yeast RITS member protein, Tas3, has a GW-repeat-containing motif and interacts with AGO-1 to promote TGS^[132]. It is therefore possible that, the Tas3/

AGO-1 interaction in fission yeast could be analogous, not homologous, to the AGO-1 and GW182/TNRC6 interaction in humans. Consistent with this hypothesis, the plant specific PTGS-related GW protein NERD was found to be involved in TGS in *A. thaliana*^[133]. Thus, there is evolutionary evidence supporting the likelihood of a link between the two mammalian pathways in the nucleus.

Protein complexes containing AGO-2, TNRC6A, Dicer and TRBP have been immunoprecipitated from human isolated cell nuclei. These protein complexes were able to induce PTGS, with the specific cleavage of four different nuclear lncRNAs mediated by corresponding siRNAs^[101]. Similar complexes were immunoprecipitated with nuclear AGO-1 and found to harbor the same PTGS proteins, supporting the notion of a core complex for both pathways. However, this study did not identify proteins that have been implicated in the loading of sncRNA onto AGO proteins, such as C3PO and HSP90 within mammalian cell nuclei. In previous studies the identification of these proteins could have been the result of contamination from cytoplasmic remnants. This study highlighted the importance of ensuring that isolated nuclei are free from endoplasmic reticulum (ER) to avoid contamination with cytoplasmic AGO-containing complexes. Recently, a comprehensive protocol was developed to ensure that purified nuclei are free from ER contamination^[134].

It is important to note that the majority of studies aimed at understanding the mechanisms of loading and activation of silencing complexes incorporating non-catalytic AGO proteins have done it in the context of PTGS, either in the cytoplasm or in the nucleus. These studies have not specifically targeted genes embedded in chromatin. Therefore, a possibility remains that siRNAs or miRNAs that are only homologous to specific regions such as promoter regions, can be identified and differentially processed. In this way, complexes could share a common core, but would vary in accessory proteins that modify their function to induce either TGS or PTGS.

Consistent with this model, a recent study unveiled a sorting mechanism in humans, which directs differential loading of AGO-1 proteins for unique sncRNAs in the setting of a viral infection^[135]. However the determinants of this selection remain unknown. Nonetheless, most sncRNAs were loaded in equivalent ratios to AGO-1 and AGO-2 proteins and thus these unsorted sncRNAs may be used to scan targets in both cellular compartments. Therefore we hypothesize that when there are targets in both compartments, both pathways are likely to occur, depending how efficient each of these sncRNAs is for the pathway.

While the understanding of the molecular mechanisms of PTGS is reasonably complete, and there is some evidence of commonalities with TGS, there are far many more uncertainties in the TGS mechanism. Several important early steps in the TGS mechanism remain to be fully deciphered, including the precise mechanism that determines RITS recognition of target, the characteristics or type of target and the determinants of induction of different epigenetic heterochromatin profiles. In addition,

while human TGS can be thought of at a single cell level, its implication needs to be considered within the context of a multicellular organism. Many changes or epigenetic check points occur early during embryogenesis and development or during cell differentiation. While some changes are dynamic allowing differentiation of cells down different pathways, once certain check points are reached epigenetic profiles are more stable and are inherited to daughter cells through multiple cell divisions.

At present, there is evidence supporting two main models describing target recognition. The first is a siRNA/DNA-binding model^[65,136], during which the RITS complex binds directly to chromatin. This binding seems to be dependent on the interaction between the siRNA and its DNA-target sequence. Once the interaction has taken place it triggers the *in situ* recruitment of chromatin remodeling factors that induce heterochromatin and establish silencing (Figure 3A). We previously introduced the unresolved question of how the passenger strand is removed. In the TGS model however, each strand of the duplex will find a target on DNA, in the same location but on different DNA strands. Therefore, for the sake of identifying the target region, both strands are potentially useful. In HIV-1, a siRNA guide-strand targeting a promoter region will find two target sites. One on the 5' LTR of the sense strand, and the other in the antisense strand in the region that is complementary to the 3'LTR of the sense strand (Figure 3B).

In the second model the RITS complex binds to an RNA intermediate, finding its target in either an antisense transcript or in a sense nascent transcript (recently reviewed in^[59] and in^[137]). In this model, only one strand of the duplex acts as the guide strand (Figure 3C). Presently, there is more experimental evidence supporting the RNA model given that, owing to its similarity with lncRNAs silencing mechanisms, more studies have tested this hypothesis. Though, there are still critical gaps in the data and more evidence is required to further evaluate the DNA model. It is possible that each of the models occur under particular conditions and potentially a variety of mechanisms control the diverse and precise regulation of gene expression in humans.

Establishment of heterochromatin is a progressive process. Once the RITS complex has found its target region a series of events follow, which generally initiate with removal and or replacement of specific histone-tail post-translational modifications to alter the biochemistry and structure of the associated chromatin (Table 1). Numerous histone modifications important for histone structure and gene regulation have been described^[138], however we will only be discussing canonical acetylation and methylation marks that have been related to TGS and HIV-1. The different histone tail modifications are generated and recognized by histone deacetylases (HDACs), histone and DNA methyltransferases (HMTs and DNMTs, respectively), and chromatin modifying complexes. Ultimately, the combination of histone tail modifications and the recruitment of protein complexes make up a pattern that relates to the specific transcription

state of a gene (a recent review can be found in^[139]).

HDACs are required early in heterochromatin formation and remove acetylation (Ac) marks that are frequently found in actively transcribing chromatin. HDACs appear to be continuously recruited to epigenetically repressed loci^[140], however, in very robust silencing, HDACs may not be continuously recruited. HDACs are recruited to chromatin by different mechanisms that are in some cases dependent on DNA methylation in CpG islands (discussed below). This differential recruitment is attributed to HDACs being able to form higher order complexes that may or may not include methyl-CpG-binding domain (MBD)-containing proteins^[141].

The removal of Ac marks is necessary for the establishment of methylation repressive marks and chromatin compaction^[142]. Several lysine residues from histone tails can be methylated by specific histone lysine methyltransferases (HKMTs) in order to repress chromatin (Table 1). Methylated residues are recognized by HP1 and HKMTs, both of which bind to chromatin and dimerize to induce chromatin compaction^[143]. Nucleosome compaction exposes hidden lysine residues that become accessible to further methylation by HKMTs. Progressive methylation recruits more HP1- α and chromatin remodeling complexes. Chromatin remodeling complexes promote the establishment and spread of heterochromatin through a positive feedback loop with HP1^[144] (Figure 3A).

Heterochromatin is also the final outcome of DNA methylation, genomic imprinting^[145] and Polycomb (PcG) mediated silencing^[65,146]. Therefore, RNAi-induced TGS has the potential to induce a variety of epigenetic profiles.

CpG islands (CGIs) are genomic regions that are unusually high in their CG or CpG content when compared to the genomic average of these nucleotides. CGIs are predominantly found in promoter regions and are demethylated during active gene transcription^[40]. Conversely, methylation of promoter CGIs is associated with epigenetic gene repression. Thus, DNA methylation accounts for another layer of control of gene expression. It is well known that DNA-nucleotide-methyl-transferases (DNMT) methylate CpG residues^[147] and seem to catalyse the reverse reaction^[148]. However, the Ten-Eleven-Translocation enzymes (TET) are considered the main CpG DNA demethylases^[149] while proteins containing DNA-methyl-CpG-binding domain (MBD) recognize the methylated status^[150] in order to induce heterochromatin. However, it is not known how methylation is selectively established at precise promoters.

Genomic DNA methylation of CpG islands is fundamental for the programmed repression of genes during embryogenesis in mammalian cells. The methylation pattern is erased in the early embryo in order to establish the totipotent state, but is re-established during implantation with pluripotency genes being methylated and thus repressed^[151,152]. Methylation of CGIs is then recognized by HKMTs that contain a methyl-binding domain (MBD) domain, in this case G9a. G9a establishes H3K9me3 and recruits HDACs, inducing HP1- α binding and local heterochromatinization. Heterochromatinization of HP1 promotes *de novo* DNA methylation by DNMT3 and

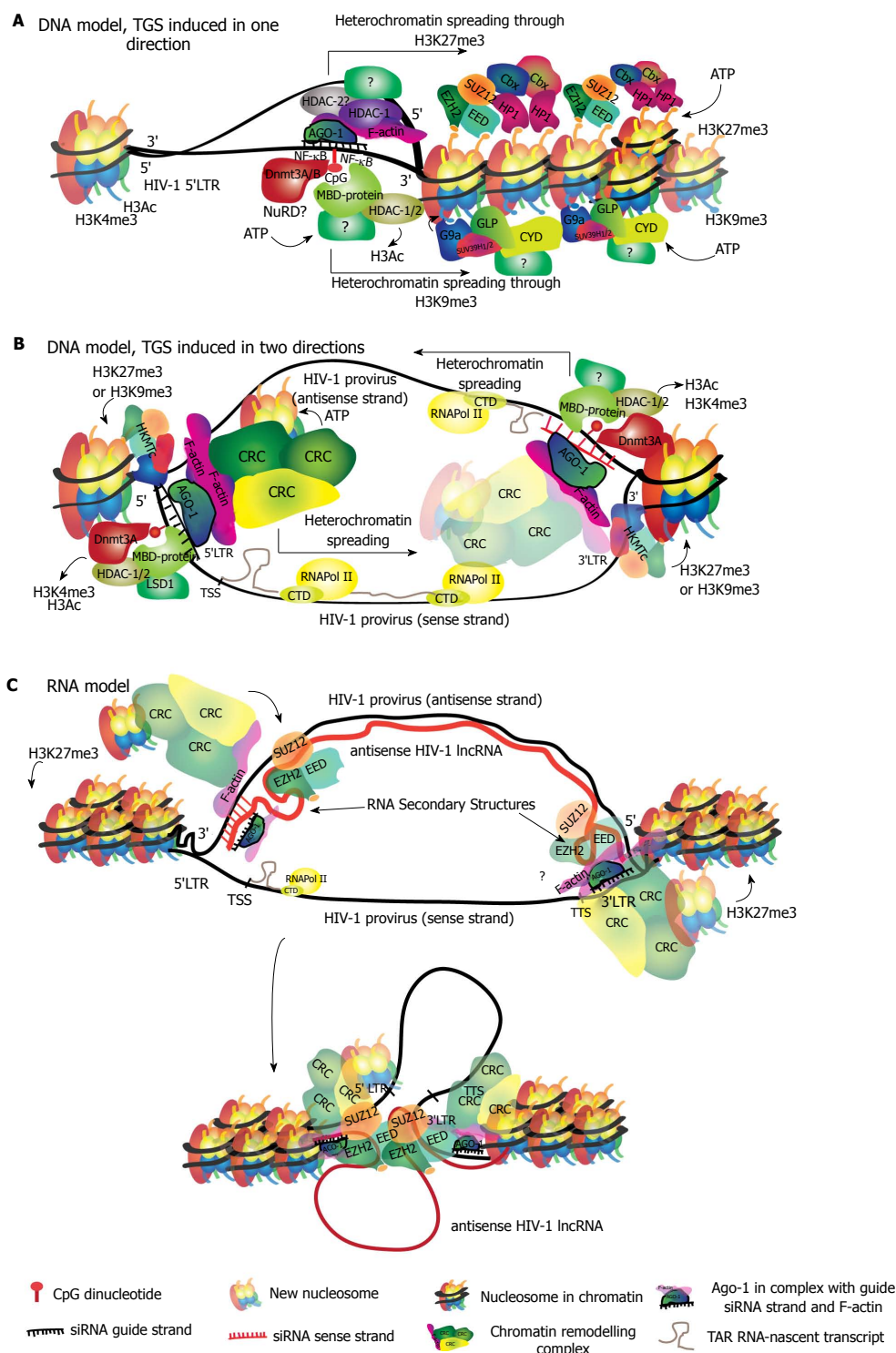


Figure 3 Models describing possible molecular mechanisms of siRNA-induced transcriptional gene silencing in human immunodeficiency virus 1. A: DNA model in which the siRNA guide-strand finds its target in the 5'LTR promoter of the HIV-1 genome binding directly to the DNA. This binding triggers the recruitment of HDACs and HKMTs, which further recruit CRCs to induce chromatin compaction. Two mutually exclusive pathways are shown simultaneously, for simplicity. While both pathways may be initiated with the DNA methylation of CpG dinucleotides, they differ in the proteins that are recruited to the locus. The pathway characterized by H3K27me3 is shown above and involves initial recruitment of PCR2 (EZH2-SUZ12-EED) followed by the specific CRCs readers of H3K27me3. The H3K9me3 recruits G9a or SUV39H1/2 followed by specific CRCs as well. In this model heterochromatin is likely to spread in only one direction; B: In this DNA model, both strands of the siRNA duplex find a target on opposite DNA strands given that both, the 5'LTR and 3'LTR from the HIV-1 genome, have the same sequence. Regardless of the epigenetic pathway that is induced, heterochromatin will spread in the 5' to 3' direction from each end of the HIV-1 genome; C: In the RNA binding model, antisense transcription generates a HIV-1 specific lncRNA that covers all the HIV-1 genome. The siRNA guide-strand will bind the 3'UTR of this transcript, which corresponds to the 5'LTR sequence. The binding recruits PCR2, which establishes H3K27me3 and may also interact with the secondary structures of the lncRNA. Higher order interactions may bring together the 3'end of the HIV-1 genome, recruiting CRCs and inducing heterochromatin. A binding site for the same siRNA strand remains in the DNA sense strand at the 3'LTR, which could potentially contribute to heterochromatin formation. HIV-1: Human immunodeficiency virus 1; DNMT3 A/B: DNA methyl-transferase A/B; HDAC: Histone deacetylase; MBD-protein: Methyl-CpG-binding protein; Cbx: Chromobox family; HKMT: Histone lysine (K) methyl-transferase; CRC: Chromatin remodelling complex.

Table 1 Canonical histone modifications implicated in TGS and TGA

Histone residue	Modification	Function	Writers	Erasers	Readers	Reviewed in
H3K4	Ac	Transcription activation				[228]
	me1 (enhancer sequences)	Transcription activation	SET1 (tri) ^[229] , SET7 (mono) ^[230] , MLL ^[231] , SMYD2 ^[232]	LSD1 (mono and di) ^[233] , JARID1A/KDM5A JARID1B/KDM5B (di and tri) ^[234]	CHD1 ^[235] , RAG2 ^[236] , TAF3 ^[237] , BPTF ^[238] , BHC80 ^[239] , ING FAMILY ^[240] , PYGO2 ^[241]	[166,242]
	me2/me3 (regulatory elements at the 5' end of active genes, and in poised genes)	Transcription activation, resolution of bivalency from poised genes				
H3T6	Phosphorylation	Transcription activation	PKC B		LSD1	[243]
H3K9	Ac	Transcription activation, histone deposition	GCN5/PCAF ^[244]	SIRT6 ^[245]	BRD4 ^[246]	[247]
	me1/me2					
	me3 (non-genic regions, centromeric heterochromatin, satellite sequences, long terminal repeats)	Transcriptional silencing, heterochromatin	SUV39H1/2 ^[143] , G9a ^[248] , SETDB1 ^[249]	JMJD1A/KDM3A ^[250] , JMJD1B/KDM3B ^[251] , JMJD1C/TRIP8, JMJD2A/KDM4A (B/C/D) ^[252]	HP1 ^[253] , EED 17406994), TDRD7 ^[254] , MPP8 ^[255] , UHRF1/2 ^[256] , GLP ^[248] , CDY FAMILY ^[257]	
H3K27	me1/me2/me3, heterochromatin and facultative heterochromatin	Transcriptional silencing, heterochromatin, poised genes	EZH2, EZH1 ^[258]	JMJD1A/KDM3A, JMJD1B/KDM3B, KDM6A/UTX, JMJD3/KDM68, JMJD3/KDM6B ^[259]	Cbx proteins ^[165] , EED ^[260]	[166,261]
H3K36	Ac	Transcription activation	GCN5, PCAF ^[244]			[262]
	me1/me2 (in the body and 3' end of genes)	Transcription elongation	NSD1, NSD2 ^[263] , SET2 ^[264] , SMYD2 ^[232] , MMSET ^[265]	ASH1 ^[266] , JHDM1 ^[267] , JHDM1A/KDM2A, JHDM1B/KDM2B ^[268]	ISW1B ^[269]	
	me2/me3 (gene bodies)					
H4K20	me1	Transcriptional silencing	PR-SET7/SET8 ^[270]	PHF8 ^[271]	L3MBTL1 ^[272]	[273]
	me2	silencing, heterochromatin, repression of proinflammatory genes	SUV420H1, SUV420H2 ^[274]	PHF2 ^[275]	PHF20 ^[276] , L3MBTL1 ^[277]	
	me3 (non-genic regions, centromeric heterochromatin, satellite sequences, long terminal repeats)		SUV420H2 ^[274] , SMYD5 ^[275]	PHF2 ^[275]	NcoR ^[275]	

H: Histone; K: Lysine residue; T: Threonine residue; me1: Monomethylation; me2: Di-methylation; m3: Tri-methylation; SET1: Su(var)3-9, Enhancer of Zeste, Trithorax protein 1; SMYD2: SET and MYND domain containing protein 2; MLL: Mixed lineage leukemia protein; PKC-β: Protein kinase C beta; GCN5: general control nonderepressible-5; PCAF: P300/CBP associated factor; SUV39H1/2: Suppressor of variegation 3-9 homolog 1 or 2; SETDB1: SET domain bifurcated 1; NSD1: Nuclear receptor binding SET domain protein 1; MMSET: Multiple myeloma SET domain; PR-SET7/SET8: SET domain containing lysine methyltransferase 8; LSD1: Lysine (K) specific demethylase 1; JARID1A/KMT5: Jumoni/ARID 1 domain containing protein 1A; SIRT6: Sirtuin 6; JMJD1A: Jumoni domain containing protein 1A; ASH1: Absent small or homeotic like 1; JHDM1: Jmjc domain containing histone demethylase 1; PHF8: PHD finger protein 8; CHD1: Chromodomain-helicase-DNA binding protein 1; RAG2: Recombination activating gene 2; TAF3: TATA box binding protein (TBP)-associated factor; BPTF: Bromodomain and PHD finger containing transcription factor; ING Family: Inhibitor of growth; PYGO2: Pygopus family PHD finger 2; BRD4: Bromodomain-containing protein 4; TDRD7: Tudor domain-containing protein 7; MPP8: Methyl-H3K9-binding protein 8; UHRF1/2: Ubiquitin-like containing PHD and RING finger domains 1 or 2; CDY Family: Chromodomain Y chromosome family; GLP: G9a-like protein; ISWI1B: Imitation switch protein 1B; L3MBTL1: Lethal (3) malignant brain tumor-like protein 1; NcoR: Nuclear receptor coRepressor.

further spreads silencing by repeating the loop^[151] (Figure 3A). In humans, DNMT3 establishes *de novo* methylation and is responsible for tissue-specific DNA methylation patterns^[153,154].

In the case of TGS, recent studies have shown that DNA methylation of CGIs is not required for siRNA-guided heterochromatin formation in fission yeast, as was initially described^[155]. Similarly, the signatures of TGS in mammals appear to be somewhat diverse and may require DNA methylation in some cases. Interestingly, there is an RNAi-directed DNA methylation process that

triggers TGS in plants^[156], which is reminiscent of a mechanism in mammalian cells: piRNAs are known to direct DNA methylation in the male germ line in order to repress expression of transposable elements, but a similar mechanism has not been described on somatic cells^[157]. However, there is some indirect evidence of a similar mechanism in mammalian somatic cells when transduced with lentiviral vectors. In fact, reduced expression of the introduced transgene was observed during differentiation in a murine model and silencing was found to be the result of DNA methylation of the promoter of the lentivirus driven

gene^[158]. Furthermore, it is well known that a considerable amount of integrated vectors become silent^[159], and this effect seems to be dependent on the promoter chosen to drive the ectopic expression of the gene^[160,161]. These observations could be related to ubiquitous RNA guided-DNA methylation pathway mechanism in mammalian cells aimed at controlling endogenous retroviruses. It is clear though, that *de novo* DNA methylation can provide stability for the inheritance of gene repression patterns through generations^[151]. In this instance, TGS involving DNA methylation is likely to characterize robust silencing of a gene.

The PcG defines a group of genes that play a fundamental role in development and whose deletion results in early embryonic lethality in mice^[162]. The PcG perform an antagonistic role to the trithorax group (TrxG) of proteins by inducing epigenetic gene repression. Both, PcG and TrxG, ensure the maintenance of proper expression patterns throughout the life span of a multicellular organism. There are two main repressive multi-subunit complexes formed by PcG: Polycomb-repressive complexes 1 and 2 (PCR1 and PCR2)^[163,164].

PCR1 efficiently compacts chromatin through a variety of subunits that either identify and bind to H3K27me₃, or mono-ubiquitilate Lys119 of histone 2 variant 2 (H2A), both of which promote nucleosome compaction. PCR1 is actually a group of functionally related but diverse protein complexes made up of different subunits that vary its function^[165]. In addition to its role in development, roles in senescence, self-renewal, cancer and even gene activation have been recently identified for PCR1^[165]. Interestingly, both complexes appear related, with PCR1 eventually acting downstream of PCR2 on certain loci.

PCR2 establishes the repressive epigenetic signature, H3K27me_{2/3} through its enhancer of zeste 1 and 2 subunits (EZH1, EZH2)^[164] and induces chromatin compaction. In addition to H3K27me₃, the activation mark H3K4me₃ is also established by PCR2. Characteristically, genes co-expressing both, H3K37me₃ and H3K4me₃ epigenetic marks, are poised for transcription in undifferentiated cells. This state of epigenetic bivalency is resolved by the exclusive expression of H3K4me₃ in transcriptionally active loci or H3K27me₃ in transcriptionally repressed loci^[166].

A direct link between PCR2 and TGS during regulation of granulopoiesis was elegantly demonstrated. Further this process was shown to be fundamental in driving progenitor lineage decisions at checkpoints of differentiation, in particular at the *NFI-A* gene. In this study, miRNA-223 directly bound to the *NFI-A* promoter region through its seed region and induced TGS of this gene through recruitment of the PcG proteins, YY1 and SUZ12, along with AGO-1 and Dicer^[65]. This evidence supports previous findings of a siRNA-directed TGS, involving AGO-1, recruitment of EZH2, induction of H3K9me₂ and the PTGS protein TRBP2^[114]. Furthermore, the primary miRNA-208b has recently been shown to interact with EZH2, a Polycomb-group protein associated with gene silencing through chromatin remodeling^[146]. Together, these studies clearly

show that not only siRNAs, but also endogenous promoter-targeted miRNAs are able to trigger TGS in mammalian cells through recruitment of PcG proteins.

Interestingly, genes that are repressed by PcG express short-RNAs (about 50-200 nts) that interact with PCR2 to promote silencing^[167]. However, no AGO proteins are involved in this case and the mechanism resembles that of X-chromosome inactivation (*Xi*) (explained in the next section), with SUZ12 subunit of PCR2 binding to a short RNA-stem loop from the *BSN* gene that mimics *Xist* A-Repeat (*RepA*) stem-loop. The important concept to highlight is that short RNAs can be transcribed from repressed loci and are used to guide repressor complexes to maintain these loci in a silent state.

Genomic imprinting is the mechanism by which parental-origin specific expression of imprinted genes is controlled in somatic cells (reviewed in^[168]). It requires the DNA methylation of a region within the imprinting control region (ICR) that lies in the cluster of imprinted genes. This ICR is only demethylated in the germ cells but is then specifically re-methylated during fertilization depending on whether the maternal or the paternal allele is to be expressed in the somatic cells^[169]. It is considered to be a very strong and stable silencing.

A well-studied case, that would be an example for the second TGS model, is *Xi*. During *Xi*, expression of the lncRNA *Xist* represses transcription from the paternal chromosome^[110]. However, *Xist* is further regulated by the antisense lncRNA *Tsix*. After transcription, lncRNA *Tsix* induces silencing of *Xist* by recruiting PCR2, establishing H3K27me₃ marks and enhancing *de novo* hyper methylation by DNMT3A^[170]. The crucial link between RNAi and genomic imprinting in *Xist* regulation seems to be in the cleavage of the *Xist-Tsix* duplex by Dicer, which generates siRNAs targeting *Xist* leading to heterochromatin formation. These siRNAs in turn silence *Xist* and in this system deletion of Dicer appears to abolish silencing^[145]. Currently, there is a dispute regarding the role of Dicer in this process and thus of RNAi in *Xi*, because Dicer knockout embryonic stem cells have shown contrasting results with either a defect in *Xi* (arguing in favor) or no defect at all (arguing against). A very detailed discussion about these contrasting results can be read in^[171]. It is worth noting that other nuclear endonucleases could potentially induce cleavage in the absence of Dicer. However, recent findings showed that depletion of Dicer in human female cells has no effect in the epigenetic silencing of *Xi*, but results in up-regulation of X-linked genes, indicating that Dicer may be important for dosage compensation of those genes in differentiated cells^[172].

Xi is just one of several examples of genomic imprinting during which specific DNA methylation and a lncRNA drive long-range epigenetic heterochromatic silencing through recruitment of PcG (Figure 2D). Because genomic imprinting involves recruitment of PcG proteins to an RNA intermediate, establishment of epigenetic repressive marks and short RNAs derived from the targeted genes, it supports the model of an RNA intermediate in sncRNA-directed TGS.

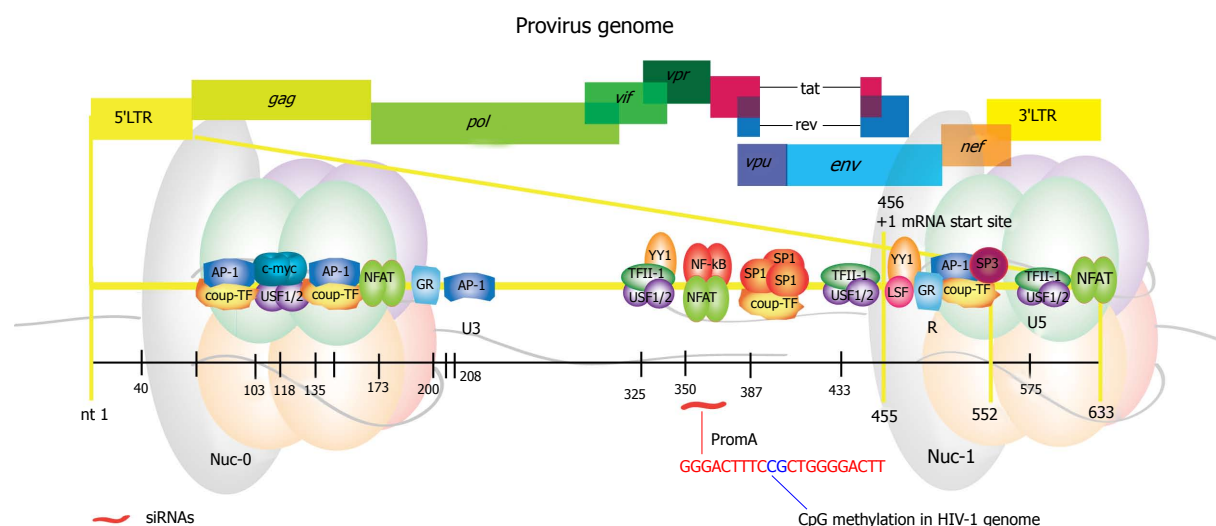


Figure 4 Map of the human immunodeficiency virus 1 genome showing in magnification the 5'LTR region with the location of transcription factor binding sites. The specific coordinates within the HIV-1 genome for each of the shown DNA regulatory elements is listed on Table 1. 5'LTR: 5' long terminal repeat; gag: Group specific antigen; pol: Polymerase; vif: Viral infectivity factor; vpr: Viral protein R; vpu: Viral protein unique; tat: Trans-activator of transcription; rev: RNA export element; env: Envelope; nef: Negative factor; 3'LTR: 3' long terminal repeat; AP-1: Activator protein 1; COUP-TF: Chicken ovalbumin upstream transcription factor; c-myc: V-myc avian myelocytomatosis viral oncogene homolog; USF 1/2: Upstream stimulatory factor 1 or 2; NFAT: Nuclear factor activated T cells; GR: Glucocorticoid receptor responsive element; YY1: Ying-yang 1; TFII-I: Transcription factor II-I; NF- κ B: Nuclear factor κ beta; SP: Specificity protein; LSF: Late SV40 factor; U3: Untranslated region 3; R: R region; U5: Untranslated region 5; Nuc-0: Nucleosome 0; Nuc-1: Nucleosome 1.

All these endogenous silencing mechanisms are an example of the different possibilities that may result when inducing TGS through sncRNAs (Figures 2 and 3). TGS is part of an enormous gene regulation network that involves a wide variety of mechanisms and protein interactions, whose combination yield diverse specific gene silencing outcomes. While we do not know yet how to induce each of these different epigenetic profiles, this mechanism has the power to silence the HIV-1 promoter in an inheritable, stable and permanent fashion, which we have reported through siRNA-induced TGS.

VIRUS: HIV

HIV establishes a long-term infection in dividing and non-dividing cells. The integrated proviral form is flanked by two long terminal repeats (LTRs) that originate from reverse transcription and are fundamental for viral replication^[173]. HIV provirus behaves like a cellular gene; it has its own promoter located in the 5' LTR and is rich in responsive elements for binding of several cellular transcription factors (Figure 4). It also has a 3' LTR, which ensures the viral mRNAs are polyadenylated and capped mimicking cellular transcripts^[174]. Of note, both LTRs have the same sequence and the 3'LTR is transcribed into the 3' UTR of the viral transcripts.

Upon integration, the provirus goes through an initial phase of abortive transcription. This phase is characterized by the presence of a non-processive RNA Pol II at the promoter region that is overcome upon expression of the viral trans-activating protein (Tat). Tat is imported back to the nucleus and binds the trans-activator response element (TAR), an RNA hairpin structure coded by the HIV

promoter, greatly enhancing transcription^[175]. Although most integrated proviruses are able to overcome abortive transcription, some become latent^[27].

HIV latency

HIV latency is an interesting model to study because it is likely to be the result of various endogenous TGS mechanisms. Studies have described a variety of epigenetic profiles at the HIV promoter some of which are associated with extremely robust silencing such that reactivation of HIV is resistant in the face of substantial cell activation.

Generally, H3K9me3 is considered to be mutually exclusive with H3K27me3, and are found in different loci. More specifically, H3K9me3 is associated with silencing of endogenous retroviruses and retro-transposons, and is also enriched in constitutive heterochromatin regions and pericentromeric heterochromatin^[176]. On the other hand, H3K27me3 is associated with a more dynamic silencing of varying strengths, which may depend on the presence of the H3K4me3 activation mark, as well as other undefined factors.

In HIV-1 infection, H3K27me3 has been found enriched in the 5'LTR promoter in cell line models of latent infection in which the virus reactivates upon stimulation^[177]. This is consistent with H3K27me3 being generally a more flexible epigenetic repressive mark and with the likelihood that most of the inducible latent provirus is silenced through pathways involving H3K27me3, rather than H3K9me3. H3K9me3 has only been found in a few HIV-1 latency studies and re-activation of latent provirus carrying this mark has either not been observed after strong stimulation (with Phorbol-Myristate-Acetate treatment) or has required

Table 2 Coordinates of transcription factor binding sites in the HIV-1 5'LTR

Name	Position ¹	Function	Cell type	Notes	Ref.
Nuc-0	About 40-200	Structural	Consistent across different cell types	Stable. Stability seems independent of transcription	[278]
AP-1/COUP-TF	About 103	Activation/Repression			[279]
c-myc/RBF-2 (USF1/2)	118-124	Repression/Activation	HeLa-CAT-CD4 and J-Lat J89 (Jurkat)	Binds the sequence CACTGAC in HIV promoter, but the canonical sequence is CACGTGAC Recruited by Sp1, can bind directly to the promoter to recruit HDAC1 RBF-2 can potentially bind to the CTGAC of this motif.	[280,281]
AP-1/COUP-TF NFAT	About 135 173	Repression/Activation Activation	Cell type variation Consistent across different cell lines	COUP-TF binds to the nuclear responsive element NFAT consensus sequence TGGAAA maps on antisense strand	[180,279] [282]
GRE-I	192-197	Repression/Activation	Cell type variation	GRE-like element AGAACA	[283-285]
AP-1	About 208			AP-1 recently found to be crucial for latency	[286]
YY1/RBF-2	About 336	Repression/Activation	Jurkat, HeLa	Putative E-box element RBEIII. Sequence overlaps YY1, RBF-2/TFII-I and AP-1 binding sites	[281,287,288]
NFAT/NF-κB	350	Activation/Repression	Consistent across different cell types	Two shared in-tandem binding sites for each transcription factor. NF-κB in the sense strand, NFAT in the antisense	[289-291]
COUP-TF / Sp1 / CTIP-2	About 388	Activation/Repression	Microglial, Oligodendrocytes, T lymphocytes	COUP-TF synergises and interacts with SP1 to activate, while CTIP2 directly binds to SP1 and represses transcription	[279,292,293]
Nuc-1	450-610	Structural	Consistent across different cell types	This nucleosome is remodelled to induce HIV latency or transcriptional gene silencing	[278]
RBF-2/AP-4	435-440	Activation/Repression	HEK293T, Jurkat	Both bind the E-box element CAGCTG, which has been named RBEI	[288,294-296]
GRE-II	450-455	Activation/Repression	Cell type variation	GRE-like element TGTACT	[283-285]
LSF/YY1	about 440-483	Repression	HeLa	LSF recruits YY1. This interaction recruits HDCA1 to initiate repression	[281,297,298]
GRE-III	471-476	Repression/Activation	Cell type variation	GRE-like element AGACCA	[283-285]
COUP-TF / AP-1 / SP3	About 485	Repression/Activation	Microglial	Synergises and interacts with SP3	[180,279]
RBF-2	About 576	Activation/Repression	Jurkat	Binds an atypical RBEIII element: ACTGCTGA	[288,294]
NFAT	618	Activation	Consistent across different cell lines	NFAT consensus sequence TGGAAA maps on sense strand	[291]

¹Genomic coordinates are based on the HBX2 numbering system. Nuc-0: Nucleosome 0; AP-1: Activator protein 1; COUP-TF: Chicken ovalbumin upstream transcription factor; c-myc: V-myc avian myelocytomatosis viral oncogene homolog; RBF-2: Ras-responsive binding factor 2; USF1/2: Upstream stimulatory factor 1 or 2; NFAT: Nuclear factor of activated T cells; GRE-I: Glucocorticoid responsive element I; YY1: Ying-yang 1; NF-κB: Nuclear factor kappa beta; Sp1: Specificity protein 1; CTIP-2: COUP-TF interacting protein 2; Nuc-1: Nucleosome 1; AP-4: Activator protein 4; GRE-II: Glucocorticoid responsive element II; SP3: Specificity protein 3.

silencing of HP1-γ or other factors through RNAi^[178,179]. This supports H3K9me3 as a more robust repressive epigenetic mark.

Similarly, Suv39H1, another HKMT responsible for H3K9me3, has been found to be recruited to latent HIV promoter in microglial cells^[180], while in a different T-cell latency model, G9a, another HKMT responsible for H3K9 methylation, was found to be a determinant of proviral latency^[181]. Moreover, the HKMT LSD1 is also recruited to the HIV promoter by the cofactor CTIP2 and establishes H3K9me3 to promote latency, rather than activation^[178]. Additionally, EZH2, one of the PCR2 subunits that establish H3K27me3, has been found to be present at the LTR of latent provirus. Knockdown of EZH2 resulted in higher transcriptional activation of the HIV promoter than when knocking down Suv39H1^[177], indicating that the former is associated with a more responsive epigenetic silencing.

Recently, a nuclear lncRNA expressed as an antisense transcript initiated from the viral 3'LTR, was found to modulate HIV-1 replication^[182]. This lncRNA was further

shown to exert epigenetic modulation of the 5'LTR HIV promoter by recruiting both DNMT3 and EZH2, resembling a genomic imprinting mechanism^[183]. These observations are consistent with HIV CpG islands being methylated in a latency model^[184]. It has been described that transcriptional silencing by *Xist* requires RepA, which is a short RNA transcript containing the A-repeat that forms an RNA secondary structure to which EZH2 and other PcG members bind, and whose deletion prevents silencing^[185]. Given the similarity of the HIV antisense lncRNA mechanism to that of *Xist*, the TAR RNA-loop secondary structure fundamental for HIV transcription could potentially be involved in an interaction with EZH2. While the latter statement is hypothetical, the evidence thus far points towards a robust silencing of HIV by this lncRNA. The scope of this discovery may be extrapolated to the barriers to achieving reactivation of latent provirus as a therapeutic approach. Reactivation strategies to purge the latent reservoir, such as the use of histone deacetylase inhibitors (HDACis) have not been successful, despite using a variety of agents like Vorinostat and Panabinstat, with

different potencies and specificities in inducing HIV specific chromatin relaxation^[32]. The mechanism by which this HIV antisense lncRNA maintains latency might explain in part this difficulty, because a very robust and deep silencing may be established in a great deal of latent proviruses that make up the reservoir. Moreover, it could be potentially harmful to aim at disrupting this HIV lncRNA silencing because strategies directed to it could have an impact on other genomic regions strongly repressed by similar mechanisms.

Pan-HDACis have been developed that target more than one class of HDACs and the development of HDACis with isozyme specificity are on the scope^[186]. However, HDACis will not specifically target only HIV, instead these drugs induce general chromatin relaxation on cellular genes and so have effects that are no HIV-specific. In addition, given the evident epigenetic complexity of HIV latency, more than one type of enzyme involved in epigenetic silencing will be needed to fully disrupt the latent provirus.

Collectively, the characteristic heterogeneity observed in the studies describing either HIV latency or on those aimed at re-activation of the latent provirus may be explained by the considerable density of binding sites for cellular transcription factors within the 5'LTR (Figure 4 and Table 2), in conjunction with the modulation executed by the HIV antisense lncRNA. Thus, it is possible that inducing TGS through siRNAs/shRNAs that target different regions within these DNA binding elements could result in the establishment of varied epigenetic profiles.

RNAI FOR HIV

PTGS for HIV

Initial applications of RNAi to HIV were designed to target viral mRNA transcripts through the PTGS pathway^[187]. These first attempts used transfection of one siRNA directed against important viral transcripts such as *gag*^[187], *env*^[188] and *rev*^[189], and also cellular genes important for HIV-1 replication cycle, such as CD4^[190] and CCR5 or CXCR4 chemokine receptors^[191]. Suppression was not sustained whenever only viral mRNAs were targeted due to the emergence of resistant variants^[192-194]. It became clear that a combinatorial RNAi against HIV would provide better protection and this correlated with delayed viral escape^[195]. Further analysis of resistant viruses was useful to guide the design of more effective shRNAs^[194]. Indeed, escape-proof shRNAs were identified that exerted potent and prolonged HIV suppression^[196]. However, this approach was not completely robust as escape was observed from combinatorial shRNAs despite these being specifically designed to target previously characterized resistant viral variants^[197]. Since then, multiple design approaches have been developed using a variety of strategies in search of the best combination of siRNA/shRNAs molecules that might prevent viral escape^[198,199].

Following these findings, shRNAs targeting both conserved viral genes and host cellular genes required for viral replication became the preferred way to

overcome this problem. Indeed, targeting only cellular genes such as CD4^[190] and CXCR4 and particularly the CCR5 chemokine receptor dramatically reduced the emergence of resistant viruses^[200]. Currently, PTGS is not envisaged as a stand-alone strategy for treating HIV. Rather its putative use is in combination with other types of gene therapy technologies, which we will discuss in the section for alternative gene therapy approaches.

TGS for HIV

The field of sncRNA-induced TGS for HIV therapeutics is less developed and has been hampered by the doubts regarding the existence of the pathway in mammalian nuclei. Nonetheless, siRNA and shRNA approaches have been efficiently developed that achieve long-term *in vitro* suppression of HIV replication, accompanied by epigenetic profiles which resemble those described in studies of the latent form of HIV-1.

We designed a siRNA, designated PromA, directed to the tandem repeat of NF- κ B binding sites found in the HIV promoter (Figure 4). It can induce prolonged suppression of active HIV-1 infection *in vitro* and induces methylation of the CpG dinucleotide that maps to the sequence linking NF- κ B tandem sites^[201]. This HIV suppression was associated with recruitment of AGO-1 and HDAC1, and increased presence of H3K9me2 at the HIV promoter and involved nucleosome remodeling^[202]. Later, long-term suppression (about 90 d) in conjunction with enrichment of H3K27me3 was observed when using stable expression of a shRNA targeting the same region^[203]. H3K9me2 and H3K9me3 were also enriched but at much lower levels (H3K27me3 >>> H3K9me2 > H3K9me3). Suppression was then proved to be specific, as mutations in the shRNA sequence impaired virus suppression^[204]. Interestingly, we identified F-actin as a key player in nuclear transportation of promoter-targeted siRNAs in mammalian cells, using the same siRNA constructs^[205]. Results from this study are consistent with selective transport of promoter-targeted sncRNAs, which has also been shown for AGO-1 by other groups^[135], as mentioned earlier.

Using a TGS-based gene therapy for treating HIV infection has several advantages over other therapies. First, TGS acts directly at the HIV promoter giving the virus virtually no opportunity to develop resistance; Second, it is likely able to act on latent provirus, whereby it potentially locks the virus in the latent state impeding future re-activation; Third, small doses of the effector molecules are sufficient to induce silencing since integrated provirus in a clinical setting is limited to less than 2 to 3 copies per cell^[206]; And fourth, the silencing could potentially be inherited, though this remains to be definitely demonstrated.

Furthermore, an interesting point to note is that since the 5'LTR promoter contains the same sequence as the 3'LTR, a siRNA/shRNA designed to target the promoter region will also have a second target in the proviral 3'LTR. This could be potentially beneficial, as

heterochromatin could be induced from both ends of the provirus (Figure 3B). Other potential targets are the 3'UTRs of viral mRNAs, whose targeting mainly depends on the efficiency of a siRNA to induce PTGS, or both PTGS and TGS simultaneously. In the latter case, PTGS would function until TGS is established, impeding transcription of viral mRNAs. However, an efficient siRNA/shRNA targeting both PTGS and TGS pathways has not yet been identified. Indeed, our siRNA PromA targeting the NF- κ B did not show a significant PTGS effect on viral mRNAs^[202] when we measured the effect in a setting mimicking an active HIV transcription owing to its clinical relevance, rather than using a weak promoter. In addition, the 1-LTR and 2-LTR circle intermediates of abortive HIV integration, which reside within the nucleus, may be targeted as well. While transcription and translation of viral genes from these unintegrated DNA forms has been observed, the contribution of these to actual infection is not clear^[207]. And lastly, the linear DNA intermediate, that is synthesized in the cytoplasm by the RT enzyme and will become integrated as provirus, also contains the two viral LTRs, and several host proteins are known to interact with it^[207]. While PTGS acts only post-HIV integration on viral mRNAs, rather than on incoming viral RNA genomes^[208], the effect of promoter-targeted siRNAs in the incoming reverse-transcribed HIV genome and other unintegrated DNA forms has not been investigated.

Essentially, if sequence complementarity and/or sequence features of the promoter-targeted siRNA are the main determinant for target binding, then an activated RITS complex could potentially bind to any type of molecule containing the target sequence.

OTHER GENE THERAPY STRATEGIES FOR HIV

Hope for an HIV cure re-emerged after the successful bone marrow transplantation of Timothy Ray Brown - the leukemia patient known as the Berlin patient - with stem cells homozygous for the $\Delta 32$ deletion in the CCR5 gene (CCR5 $\Delta 32$)^[209]. This gene encodes an important co-receptor used by the virus to enter the host cells and individuals carrying the homozygous mutation have proven resistant to HIV infection by CCR5-tropic viruses^[210]. Timothy was cured from both leukemia and HIV. Years after the transplant, he remains virus-free even when no longer under cART^[211]. Since then, researchers have been developing various strategies to transform hematopoietic stem cells (CD34+) into HIV resistant cells, with the aim of reproducing this outcome.

Consequently, CCR5 has become the favorite cellular factor to target, especially since HIV CCR5-tropic strains are predominantly present during early stages of the disease and often persist into later stages^[212,213]. Moreover, individuals with this mutation appear to be otherwise healthy apart from an as yet unconfirmed increase in susceptibility to West Nile infection^[214] and hepatitis B virus

infection^[215]. These statements have raised the concern of whether CCR5 is implicated in immune system-related diseases^[216]. An interesting discussion in this topic can be read in^[217]. Thus, the effect of knocking down CCR5 could result in unpredicted effects.

Presently, different genetic therapy technologies are being tested for their *in vivo* ability to generate HIV resistant cells. From combined PTGS approaches, to genome editing with Zinc finger nucleases (ZFN), transcription activator-like effector nucleases (TALENs) or clustered regularly interspaced short palindromic repeats elements (CRISPR) associated caspase 9 (Cas9).

The most recent strategies involving PTGS use triple combination vectors. For example, a viral vector expressing shRNA against CCR5, an shRNA against TRIM5 α isoform and a TAR-decoy against HIV^[218] was successfully tested in a humanized NOD-RAG^{-/-}IL2 γ ^{-/-} knockout mouse model. Similarly, a strategy using a viral vector expressing an shRNA against HIV *tat/rev*, a TAR-decoy element and ribozyme against CCR5^[219] was initially tested using modified autologous CD4+ T cells in HIV positive patients who had failed therapy (NCT01153646), and is now being tested as an adjunct therapy using modified CD34+ T cells in patients with acquired-immune deficiency syndrome (AIDS)-related non-Hodgkin Lymphoma (NHL) (NCT01961063) and in patients with AIDS-related NHL requiring stem cell transplantation (NCT00569985). Importantly, long-term expression of the effector molecules from this construct has been detected in multiple cell lineages from treated patients, in which a combination of transduced and untransduced CD34+ cells were used^[220].

ZFN strategies predominantly target CCR5. Recently, a phase I clinical trial (NCT00842634) testing the transduction of CCR5 ZFN-modified autologous CD4+ T cells into HIV positive patients^[221] showed that the procedure was feasible and safe. During an anti viral therapy treatment interruption the modified cells had a higher survival over non-modified cells. Also, patients showed decreased HIV DNA levels in blood. Currently, the effect of repeated doses of the ZFN-modified CD4+ T cells is being tested (NCT02225665). Although, these clinical trials use modified CD4+ T cells rather than CD34+ cells, recent studies in a humanized mice model showed low engraftment, but proper multi-lineage differentiation of the CCR5-ZFN CD34+ cells^[222].

TALENs and CRISPR have not yet been trialed in humans. However, the results from *in vitro* studies are very promising^[223], with CRISPR editing able to excise the provirus from infected cells, and thus able to target latent proviruses^[224]. ZFNs have also been used to target the provirus, using lentivirus to achieve stable expression of the nucleases^[225]. However, the above-mentioned ZFN-related clinical trials used adenovirus vectors. Generally, genome-editing approaches use non-integrative adenoviral vectors. Adenoviral vectors are diluted after each cell division and direct transient expression of the editing nuclease. Transient expression has been the choice for genome-editing approaches on

the grounds that a continuous expression of a selected editing nuclease could be potentially risky as it may result in off-target genome editing. To date, it remains to be addressed if ZFN/TALEN/CRISPR genetically modified CD34+ are safe to use in humans and whether they are feasible approaches towards a functional cure.

CONCLUSION

Presently, a variety of strategies are being tested in order to breakthrough this highly challenging treatment barrier. There are still several large hurdles to be surmounted. Currently there is a lack of adequate delivery systems for targeting cells with HIV infection and the latent reservoir. Further TGS/PTGS approaches require stable expression from vectors, such as lentiviral vectors but this must be combined with high transduction and engraftment rates, for therapy to be effective. In the same way, genome-editing approaches rely on vectors that drive transient expression of the editing enzyme, but get diluted after each cell division. Thus, achieving high genome editing efficiency is one of the limitations.

Importantly, TGS and CRISPR genome editing have the potential to target proviruses directly, and therefore could be effective in targeting latent provirus. Yet this strength may also be an inherent weakness and thus a careful selection of the targeted sequences of HIV-1 is fundamental. Unfortunately, 5'LTR sequences from proven replication competent proviruses are the least represented in curated databases in comparison to other HIV genomic regions. Nonetheless, combinatorial strategies are also an option within these therapies, and may be designed to target an additional host factor as well.

Gene therapy technologies that target only CCR5 may be unable to target latent provirus that is already present. In addition, they may select HIV-1 viruses with tropism for the CXCR4 co-receptor, allowing escape and potentially more rapid disease progression. This evolution is more likely if latent provirus remains in untargeted compartments.

The combinatorial strategies from PTGS, which target the virus and a host factor such as CCR5, provide an additional mechanism that directly restricts the virus and could possibly delay or impede viral evolution. In this regard, it could potentially provide some protection from CXCR4-tropic emerging viruses or re-activating from latent proviruses.

Basically, with present technologies none of the effector molecules for these therapies can be directly administered to an infected patient. Rather, autologous cells are obtained, genetically modified, and then transferred back to the patient. Generally, these therapies aim at modifying CD34+ cells in order to develop multi-lineage HIV resistance and thus long-term protection to the infection. Indeed, the limitation of most of these therapies relies on the efficiency of several steps throughout the complete intervention process. For instance, the efficiency or success to which the autologous cells are first, modified *ex vivo*; Second, re-mobilized or transplanted;

third, engrafted within the bone marrow; and fourth, either achieve a sustained and prolonged multi-lineage expression of the modified trait/gene or achieve a certain percentage of modified cells from all the lineages enough to provide protection. Furthermore, the engrafted modified cells will share a niche with the wild-type cells, unless ablation of the immune system is performed before. Therefore, understanding the interactions and signaling between these two populations sharing a niche could give us a better prediction of the long-term success of these therapies. Factors such as symmetric and asymmetric cell division^[226], unidentified endogenous mechanisms of genomic mosaicism detection in stem cells^[227] and other cellular and molecular pathways may play an important role. For instance, if it is confirmed that Piwi proteins are expressed in hematopoietic stem cells, this could potentially have an impact in those therapies that rely on integrative gene therapy vectors.

Finally, other concerns remain such as the worldwide implementation of these gene-therapy strategies and their cost, particularly in developing countries. Consequently, the development of delivery methods that facilitate the clinical application of these therapies is an important quest.

The various RNAi strategies to target HIV reviewed here provide a potential alternate approach to combating HIV infection and the latent reservoir, with the results of current and future RNAi therapeutic trials poised to reveal whether this approach represents a possible pathway towards a functional HIV cure.

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P- Reviewer: Arriagada GL, Zou C S- Editor: Tian YL
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