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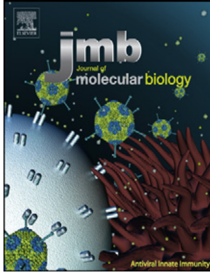

MicroRNAs and HIV-1 Infection: Antiviral Activities and Beyond

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Abstract

Cellular microRNAs (miRNAs) are an important class of small, non-coding RNAs that bind to host mRNAs based on sequence complementarity and regulate protein expression. They play important roles in controlling key cellular processes including cellular inception, differentiation and death. While several viruses have been shown to encode for viral miRNAs, controversy persists over the expression of a functional miRNA encoded in the human immunodeficiency virus type 1 (HIV-1) genome. However, it has been reported that HIV-1 infectivity is influenced by cellular miRNAs. Either through directly targeting the viral genome or by targeting host cellular proteins required for successful virus replication, multiple cellular miRNAs seem to modulate HIV-1 infection and replication. Perhaps as a survival strategy, HIV-1 may modulate proteins in the miRNA biogenesis pathway to subvert miRNA-induced antiviral effects. Global expression profiles of cellular miRNAs have also identified alterations of specific miRNAs post-HIV-1 infection both *in vitro* and *in vivo* (in various infected patient cohorts), suggesting potential roles for miRNAs in pathogenesis and disease progression. However, little attention has been devoted in understanding the roles played by these miRNAs at a cellular level. In this manuscript, we review past and current findings pertaining to the field of miRNA and HIV-1 interplay. In addition, we suggest strategies to exploit miRNAs therapeutically for curbing HIV-1 infectivity, replication and latency since they hold an untapped potential that deserves further investigation.
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Introduction

Human immunodeficiency virus type 1 (HIV-1), the causative agent of the acquired immunodeficiency syndrome (AIDS) pandemic, was discovered over three decades ago. Extensive research paved the way to the development of many anti-retroviral drugs targeting a limited number of steps on the virus life cycle, leading to combination anti-retroviral therapy (cART), which today results in controlled viral replication in many treated individuals. However, cART is not able to eradicate the virus due to the existence of viral reservoirs. In addition, these drugs are associated with frequent development of toxicities and side effects that may require changes in drug regimen or stopping treatment altogether. Furthermore, the continuous and perhaps increasing emergence of drug-resistant HIV-1 strains in infected patients, to some extent related with poor adherence to treatment, poses a threat to the effectiveness of the current cART regimens [1–3]. These compounding factors underscore the need to find new targets and to develop additional, complementary therapeutic approaches. Recently, much attention has been drawn to research pertaining to HIV–host interactions, in order to uncover novel drug targets.

To initiate infection, the HIV-1 envelope glycoprotein gp120 present in the surface of virions binds to its primary cellular receptor CD4, which is expressed on several immune and non-immune cells including T helper lymphocytes, macrophages, dendritic cells (DCs) and brain-resident microglia. CD4 binding triggers a conformational change that enables gp120 to bind to a co-receptor, mainly the G-protein-coupled chemokine receptors CCR5 and/or CXCR4, and the subsequent transmission of conformational alteration

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to the non-covalently bound gp41, a membrane glycoprotein that will mediate the fusion of the viral and cellular membranes and the release of the viral core into the cytoplasm of the host cell. The viral entry process has been suggested to determine the viral tropism [4,5]. Post-entry, the viral capsid undergoes rapid uncoating in the cytoplasm of infected cells while the genomic single-stranded RNA undergoes reverse transcription, forming a double-stranded cDNA that represents the complete viral genome [6,7] and is packed with additional viral proteins such as integrase to form the pre-integration complex (PIC). PICs are then imported into the nucleus, where the viral DNA is integrated into the host genome by integrase through recognition of ATT-rich sites within the two long terminal repeats (LTRs) in both ends of the viral cDNA [8–10]. After integration, multiple factors such as the integration site or the activation status of the cell will determine whether the viral genome remains latent [11,12] or is actively transcribed into viral mRNAs, a process that is modulated via activation of various host cellular transcription factors (including NF-κB, AP-1, C/EBPs, SP-1, NFATs, etc.) that can bind to multiple binding sites present in the viral LTR [13–17]. The viral mRNAs, now masquerading as host mRNAs, undergo the normal processing of host mRNAs (5′ capping, polyadenylation, splicing) and serve as templates for the synthesis of viral proteins in a tightly regulated process. Finally, full-length viral RNAs that will become the genomic RNA of the new virions assemble with the Gag polyprotein (containing the structural proteins) and the essential regulatory proteins leading to the formation of new virions, which will be released from the cellular membrane and undergo maturation, in a viral protease-mediated cleavage of Gag polyprotein that is required for infectivity [18–21].

Since the virus has evolved to carry such minimal machinery, it is not surprising that HIV-1 usurps a multitude of host cellular proteins for efficient completion of its life cycle. Several RNA interference (RNAi) screens have identified many host proteins—termed “HIV Dependency Factors” (HDFs)—and cellular pathways important for HIV-1 infection [22–30]. The essentiality of certain HDFs during different stages of the viral life cycle has been well studied. Thus, in addition to targeting HIV-1 proteins, therapeutic approaches directed toward HDFs have also become a potential strategy to combat HIV-1. Indeed, inhibiting these proteins by RNAi strategies such as small interfering RNA (siRNA)-mediated knockdown has been shown to affect HIV-1 replication, underscoring the importance of the targeted HDFs [31–33]. Therefore, manipulating the expression of such host proteins provides a novel avenue of reducing HIV-1 infectivity in otherwise susceptible cells.

Interestingly, eukaryotic cells possess endogenous RNAi mechanisms that have only been uncovered during the last two decades [34], with the so-called microRNAs (miRNAs) being an important family of small, non-coding RNAs (sncRNAs). Mammalian miRNAs are temporally and spatially regulated sncRNAs that are potent regulators of gene expression. In their mature form, miRNAs bind primarily to the 3′ untranslated region (UTR) of target mRNAs through base pair complementarity, leading to silencing or reduced protein levels either through translational repression or through mRNA degradation [35–37]. Cellular miRNAs play fundamental roles during basic cellular processes such as differentiation, development and death. In addition, studies involving miRNAs and their dysregulation have helped identify key concepts in infectious diseases, cancer and both generation and maintenance of adequate innate and adaptive immune responses [38–43].

Understanding the role of miRNAs in HIV-1 infection is an emerging area of interest. Few cellular miRNAs have been shown to directly bind to the viral RNA and inhibit HIV-1 replication. However, a growing number of cellular miRNAs can indirectly affect HIV-1 infection by targeting HDFs. As an ever-evolving strategy, HIV-1 seems to be able not only to modulate cellular miRNA profiles but also to interfere with the overall biogenesis of miRNAs. However, whether or not HIV-1 encodes viral miRNAs still remains a matter of debate. The importance of miRNAs in HIV-1 infection has been previously reviewed [44,45]. Here, we provide an overview of the current literature characterizing the complex interactions between HIV-1 and miRNAs and provide insights into potential therapeutic applications of miRNAs in the context of HIV-1 infection and eradication efforts.

miRNA Biogenesis and Interference of HIV-1 with the RNAi Pathway

The biogenesis of miRNAs and their mechanisms of post-transcriptional regulation have been reviewed elsewhere [46–48]. In mammalian cells, miRNAs are generated from multiple intron specific regions or can be transcribed from their own promoters. Similar to host mRNAs, miRNA biogenesis relies on specific transcription factors. RNA polymerase II or RNA polymerase III (for specific miRNAs such as the human chromosome 19 miRNA clusters that are interspersed in Alu-rich repeats [49]) generates large primary miRNA transcripts called pri-miRNAs. These molecules contain long stem–loop-like structures, which are important components for eventual recognition by miRNA regulatory proteins. Similar to host mRNAs, miRNAs are also processed to be capped and poly-adenylated. The miRNA nuclear microprocessor complex proteins are responsible for the alterations of pri-miRNA transcripts. Specifically, Drosha, a member of the RNase III nuclease super family, and DiGeorge syndrome critical region 8 (DGCR8), a dsRNA (*d*ouble-*s*tranded *RNA*) binding

protein, are directly responsible for the nuclear processing events. Drosha is the core protein that mediates the initiation step by cleaving the pri-miRNA transcript into pre-miRNA transcripts that are about 70 nt long. This cleavage results in a 3′-end dinucleotide overhang, which is an important recognition signal for nuclear export by Exportin 5. Drosha's endonuclease functionality requires continued interaction with DGCR8. In addition, DGCR8 enhances the protein stability of Drosha. A member of the karyopherin family of nuclear export binding proteins, Exportin 5, binds both the pre-miRNA and the active GTPase RAN-GTP in the nucleus, and this heterotrimeric complex is exported into the cytoplasm through the nuclear pore complex. Upon reaching the cytoplasm, a complex containing Dicer, a second RNase-III-like enzyme, cleaves the pre-miRNA in an event that is facilitated through Dicer's recognition of the hairpin-loop structure on the pre-miRNA, as well as the multidomain human TAR-RNA element binding protein (TRBP), generating a duplex that is the foundation of a functional miRNA but that requires additional processing to become a biologically active product.

Dicer plays an essential role in unwinding the duplex of the two pre-miRNA strands. The thermodynamic stability and base-pairing affinities between the two strands are important factors in determining the active miRNA that gets incorporated into the RNA-induced silencing complex (RISC). The strand of the duplex structure with the less stable base pair at the 3′-end is usually considered the active miRNA or the "guide" strand, while the other is called miRNA* or "passenger" strand. Although both strands can be functional, the role of the passenger strand or the miRNA* (miRNA star partner) remains to be fully elucidated. The active miRNA is then recruited into the multi-protein RISC, where it binds to the target mRNA to functionally regulate protein expression. The RISC complex contains members of the Argonaute (Ago) family of proteins as the catalytic subunits (Ago1–Ago4), which have been shown to have endonuclease activity. Among them, Ago2 plays a fundamental role in the cleavage of the target mRNAs, while TRBP, which can bind dsRNAs, plays an important role by binding to and recruiting Dicer and Ago2 to the RISC. When miRNAs bind to the target mRNA with perfect sequence complementarity, the result is endonucleolytic cleavage of the mRNA. However, imperfect complementarity results in rapid polyadenylation of the target mRNA, leading to repression of protein translation. Growing evidence suggests that most endogenously expressed miRNAs interact with host mRNAs by imperfect complementarity and therefore may preferentially regulate protein expression by translational repression.

Exceptions to the aforementioned canonical sequence of events are emerging. Dicer-independent processing and expression of functional miRNAs have been noted [50,51]. For instance, the requirement of Drosha's processing of pri-miRNA into pre-miRNA does not seem to be universal. Evidence suggests that Drosha-independent, functional miRNAs do exist, the so-called "Mirtrons". These are genes that encode for miRNAs in their introns. Post-splicing, the intron-derived miRNAs mimic the structural features of pre-miRNAs; thus, they can be exported to the cytoplasm and enter the miRNA-processing pathway without Drosha-mediated cleavage [52–54]. Although the existence of a few tens of mammalian Mirtrons has been characterized, further research is needed to understand the biological significance of these miRNA entities that are very likely to grow in number and potential biological relevance [55]. Finally, another non-canonical processing pathway has shown the existence of splicing-independent, Mirtron-like miRNAs called "Simtrons" that do not seem to require most of the proteins in the canonical miRNA pathway such as DGCR8, Exportin 5, Ago2 or Dicer, although they do require Drosha-mediated cleavage for efficient functionality [56]. While the miRNA biogenesis and mechanisms of regulation have been well studied, it is likely that our knowledge on the proteins that participate and regulate these pathways will continue to expand.

First discovered in plants and invertebrates and eventually identified in mammalian cells as well, miRNAs have been shown to play a crucial role in the innate antiviral defense [57–59]. Thus, it is not surprising to find evidence that multiple viruses have developed mechanisms by which they can subvert the cellular miRNA functionality, as a survival strategy to evade RNAi-mediated antiviral responses [60–63]. A classic example of the crucial importance played by cellular miRNAs during HIV-1 infection was demonstrated by Triboulet *et al.*; by knocking down two important miRNA processing proteins, Drosha and Dicer, they reported a significant enhancement of viral replication in peripheral blood mononuclear cells (PBMCs) from HIV-1-infected patients and in latently infected cells [64]. In addition, the same group has demonstrated that (i) HIV-1 mRNA binds to and co-localizes with proteins of the RISC complex such as Ago2 and that (ii) several proteins required for miRNA-mediated silencing (or miRNA effectors) negatively regulate HIV-1 gene expression by preventing viral mRNA association with polysomes [65]. Interestingly, they also showed that knockdown of some of these effectors led to virus reactivation in PBMCs isolated from HIV-1-infected patients undergoing suppressive cART. These studies underscore the importance of miRNAs in modulating HIV-1 infection. Not surprisingly, HIV-1 seems to have evolved several counteracting mechanisms that promote its survival (Fig. 1).

Thus, the viral protein Tat has been shown to physically interact with Dicer and to inhibit its activity. It was first shown by Bennasser et al. that HIV-1

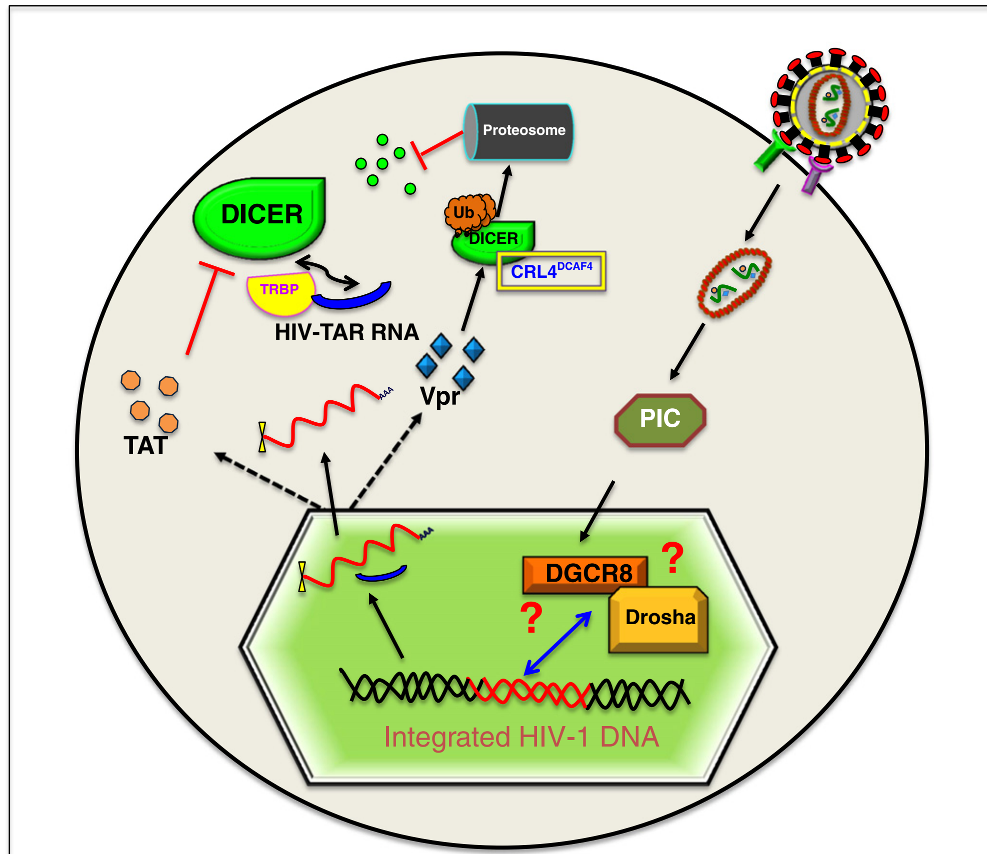

Fig. 1. Impact of HIV-1 on proteins involved in miRNA biogenesis pathway. Proteins in the RNAi machinery such as Dicer and Drosha seem to play a role in controlling HIV-1 replication. Infection of HIV-1 has not been reported to significantly alter the levels of nuclear microprocessor proteins DGCR8 and Drosha. Apart from regular translational activities, post-nuclear export of HIV mRNAs, the HIV-1 TAR RNA element is also expressed. TAR binds to the cytoplasmic RISC protein TRBP, which is essential for efficient cellular miRNA processing. Furthermore, upon transcriptional up-regulation, the cytoplasmic protein Dicer processes TAR element for efficient functioning. HIV-1 transactivating protein Tat inhibits Dicer expression, although the mechanism is not well characterized. HIV-1 Vpr has been shown to interact with the CRL4/DCAF4 E3 ubiquitin ligase complex, which results in poly-ubiquitination and degradation by the proteasome.

encodes for an siRNA-like small RNA and that Tat inhibited Dicer and had a “suppressor of RNA silencing” (SRS) (or “RNA silencing suppressor”, RSS) function [66]. Subsequently, they showed that HIV-1 Tat–Dicer interaction was dependent on the ability of Tat to bind host mRNAs and the helicase domain of Dicer but independent of its transactivation domain [67]. In addition, other studies have shown that the SRS/RSS function of Tat results in altered miRNA expression profiles in T cell lines and in primary PBMCs [68]. However, others have failed to replicate these effects since it has also been reported that Tat does not affect the global miRNA expression profiles in human cell lines or PBMCs and does not modify the expression of endogenous or exogenous miRNAs [69,70] and that exposure of human microglial cells to Tat does not directly affect the protein levels of Drosha and Dicer [71].

Besides Tat, the viral protein R (Vpr) has been reported to also have SRS/RSS function by altering expression of Dicer. Coley et al. showed that Vpr (but not Tat, Nef or Env) is required to significantly reduce Dicer levels upon HIV-1 infection of macrophages [72]. Very recently, it was reported that HIV-1 Vpr reduces Dicer expression by interacting with the CRL4/DCAF1 ubiquitin ligase complex to target it for proteasomal degradation, and this in turn results in enhanced HIV-1 infectivity in macrophages [73]. Nonetheless, changes in miRNA expression profiles upon HIV-1 infection in lymphocytes had been shown to be independent of Vpr expression [68].

Discrepancies arising between some of the above-reported studies might be related with differences in the cell type being studied or in the expression levels of the effector proteins in the RNAi machinery among those cell types. For instance, Dicer expression is

inherently undetectable in primary monocytes (regulated by endogenous miR-106a) and its expression is significantly increased upon monocyte differentiation into macrophages [72]. Greater consistency in the strain of HIV-1 utilized for infection, the cell types under study and infection and activation status of the cells, needs to be achieved in order to better understand the specific changes in the levels and functionality of components of the RNAi machinery upon HIV-1 infection. Finally, the impact of HIV-1 on the nuclear proteins essential for successful expression of cellular miRNAs, such as Drosha and DGCR8, is not well characterized. Dicer-independent processing and expression of functional miRNAs have been well noted [50,51], while very few miRNAs (such as Mirtrons) have been shown to be functional without the requirement of Drosha and DGCR8. Even though Drosha and DGCR8 knockdown seems to enhance HIV-1 infection, the role of these nuclear miRNA-processing enzymes in HIV-1 infection is not completely understood. It will be interesting to explore if HIV-1 RNA or viral proteins such as Tat or Vpr can bind to and modulate Drosha and DGCR8 expression. Future experiments addressing these questions would shed light on the importance of Drosha in HIV-1 infection and would further enhance our current understanding of the interaction of HIV-1 with the host RNAi machinery. Overall, HIV-1-mediated modulation of cellular miRNAs, although not directly essential for basic replication stages, could play a role in pathogenesis and disease progression.

Does HIV-1 Express Functional Viral miRNAs? The Controversy Ensues!

Members of several families of viruses encode viral miRNAs or vmiRNAs. These vmiRNAs have crucial roles in controlling viral replication, evasion of immune responses and successful survival in the host [74,75]. The vmiRNAs have been better characterized in DNA viruses including members of the *Herpesviridae*, *Polyomaviridae* and *Adenoviridae* families [76–78]. On the other hand, encryption of miRNAs in their genomes by RNA viruses remains controversial. RNA viruses such as hepatitis C virus (HCV), yellow fever virus and HIV-1 have been shown not to encode for any functional miRNAs [79]. Various reasons have been suggested to explain why RNA viruses might not express miRNAs. The lack of nuclear localization by most RNA viruses, since replication takes place in the cytoplasm, prevents the interaction of the viral RNA with the nuclear microprocessor proteins such as Drosha and TRBP. However, RNA viruses that replicate in the nucleus such as influenza virus still do not seem to encode for a functional miRNA [80]. In addition, inclusion of a non-coding region for a functional miRNA might result in degradation of the entire viral RNA by traditional RISC-mediated mechanisms. Therefore, it has been generally accepted that RNA viruses do not encode vmiRNAs [81].

However, it was speculated that HIV-1 might encode for functional vmiRNAs because, as a retrovirus, the viral RNA is reverse transcribed into a double-stranded DNA form that is imported into the nucleus and integrated in the host genome. Therefore, HIV-1 should have access to both the nuclear and cytoplasmic RNAi machinery components, similar to host miRNAs. The oncogenic retrovirus bovine leukemia virus encodes an RNA pol III-transcribed miRNA cluster that is functionally similar to the human miR-29 and contributes to development of B cell tumors [82]. Computational algorithms have predicted that HIV-1 can encode five candidate pre-miRNAs [83]. In addition, the viral protein Nef has been reported to encode a vmiRNA (Nef-U3-miR-N367), which targets the Nef protein for degradation and decreases viral replication through various mechanisms [84,85]. Interestingly, synthetic dsRNAs constructed with mutated Nef genes derived from long-term non-progressors (LTNPs; HIV-1-infected patients who are naïve to cART but do not progress to AIDS [86]) seem to have a better ability to target homologous sequences of mRNA than the full-length, non-mutated Nef dsRNA, suggesting that they could interfere with HIV-1 transcription and play a role in the non-progression to AIDS of LTNPs [87]. However, whether this Nef-encoded vmiRNA could be responsible for, or at least contribute to, the reduced disease severity in LTNPs needs to be experimentally addressed. Also, if a Nef-encoded miRNA can inhibit viral replication, it might play a crucial role in establishment of HIV-1 latency.

Furthermore, Kaul et al. have discovered another HIV-1-encoded pre-miRNA sequence in the 3′-end of the viral genome, named hiv1-miR-H1, which has been reported to (i) target selectively and specifically the apoptosis antagonizing transcription factor that plays an important role in controlling cellular apoptosis; (ii) suppress important cellular proteins such as c-myc, Par-4, Bcl-2 and the RISC protein Dicer; and (iii) down-regulate the cellular miRNA miR-149 that can target the HIV-1 Vpr [88]. The authors proposed that this HIV-1-encoded vmiRNA seems to start an “epigenomic pathway” that leads mononuclear cells to initiate their apoptosis. In addition, while precursor miRNA sequences in non-coding regions of viral genomes seem usual, a unique recent publication by Holland et al. reported that HIV-1 Env and Gag-Pol protein coding regions can encode for several miRNA-like sequences [89]. Interestingly, the Env coding region of five HIV-1 genomes (viral strains of African origin) had near-perfect sequence similarity to the human cellular miR-195. Moreover, four other human miRNA-like sequences (similar to the human miR-30d, miR-30e, miR-374a and miR-424) were identified within the Env and Gag-Pol coding regions of several HIV-1 strains, albeit with lesser homology [89].

The cellular miR-195 is a member of the microRNA-15/16/195/424/497 family cluster that has been previously confirmed to have potent roles in cell cycle and apoptosis by regulating the expression of various proteins such as WEE1, CDK6, cyclin D1, E2F3 and Bcl-2 [90,91]. Interestingly, miR-195 is predicted to potentially target the RISC protein TRBP, the deadenylase CCR4-NOT's complex protein CNOT6L and the DNA helicase protein DDX3, as determined by using the sequence-complementarity-based, miRNA target prediction algorithm Targetscan[†] (Refs. [92–95]; personal observation). TRBP and CCR4-NOT play an important role in miRNA-mediated gene silencing pathway as previously noted. DDX3 has been shown to have an essential role in HIV-1 replication for efficient export of viral mRNAs [96]. Although the existence of miR-195-like sequences in the viral genome has been reported, it has not been experimentally confirmed whether or not they are expressed or whether they actually perform any biological function in the context of HIV-1 infection. However, it is important to highlight that, if this vmiRNA could function similarly to the host miR-195, it might have potent roles in regulating the above indicated proteins and modulating the cell cycle status of infected cells.

HIV-1 encodes a transactivation RNA (TAR) element that contains the binding region for the viral protein Tat. In infected cells, the TAR element in nascent viral RNAs recruits Tat, which mediates transactivation of the viral promoter by recruiting the cyclin-dependent kinase CDK9 (a component of P-TEFb) and Cyclin T1 [97]. In addition, the TAR element has a structure similar to endogenous Dicer substrates with an imperfect 50-nt stem–loop region [98] and was computationally predicted to be one of the five regions in the HIV-1 genome that could interact with Dicer [83]. The HIV-1 TAR element's interaction with TRBP, a TAR-RNA binding protein (TRBP) and the human homologue of the *Drosophila*'s Loquacious protein, is important since TRBP binds to Dicer for optimal incorporation of mature miRNAs into RISC [99,100]. Interestingly, Klase et al. reported that HIV-1 TAR element is processed by Dicer and results in the formation of a virally encoded miRNA that participates in chromatin remodeling of the viral LTR [101], and they suggest that Dicer may be essential for successful HIV-1 infection since its levels are sub-optimal in monocytic cells, which are relatively resistant to productive HIV infection. The functional relevance of this interaction is supported by the fact that, when siRNA was used to knockdown TRBP in susceptible cells, HIV-1 replication and virus production were inhibited [102], and it has later been shown that TRBP promotes HIV-1 replication by down-regulating the dsRNA-dependent antiviral protein kinase PKR [103]. In addition, it has been reported that the TAR element goes through specific asymmetrical processing by Dicer and produces two strands of the TAR-miRNA, namely TAR-miR-5p and 3p, with miR-TAR-3p having been described to have greater silencing capacity [104,105]. Therefore, the TAR-miRNA would be an additional vmiRNA that might have important functions in HIV-1 replication, although the exact molecular mechanisms of this vmiRNA are not completely understood.

Finally, two recent studies provide further insight into this field. Schopman et al. have used the extremely sensitive SOLiD™ 3 Plus System to analyze viral RNA accumulation in infected T lymphocytes and reported the detection of numerous sncRNAs derived from the HIV-1 genome, which could correspond to both viral miRNAs and siRNAs with potentially functional relevance for viral production [106]. The other study by Althaus et al. used a novel, sequence-targeted enrichment strategy to capture, clone and sequence HIV-1-derived sncRNAs from HIV-1-infected primary $CD4^+$ T lymphocytes and macrophages, and it reported the finding of several hundred sncRNAs ranging in length between 16 and 89 nt [107]. However, their potential relevance is unknown.

Despite the evidence noted above, controversy over whether or not HIV-1 truly encodes functional vmiRNAs persists since (i) there is a lack of authoritative experimental demonstrations on the functional relevance of the vmiRNAs reported above and (ii) other evidence suggesting that HIV-1 does not encode for any functional miRNA has been reported. Contrary to the results presented above, Lin et al. showed that neither HIV-1 nor another member of the retroviral family, human T cell leukemia virus type 1, expresses any virally encoded siRNA-like or miRNAs in persistently infected T cells [69]. Of note, this report also dismissed the expression of HIV-1 TAR-miRNA in these infected cells. However, Harwig et al. have used the very sensitive SOLiD™ ultra-deep sequencing technology and confirmed the expression of a TAR-specific miRNA in HIV-1-infected cells [108]. Very recently, Whisnant et al. used deep sequencing in two infected cell lines and in primary PBMCs and macrophages and concluded that HIV-1 does not encode for any vmiRNAs in infected cells [109]. Even though they did detect that the HIV-1 genome contains binding sites for cellular miRNAs using photoactivatable, ribonucleoside-induced cross-linking and immunoprecipitation technology, they demonstrated that viral transcripts remained largely refractory to miRNA binding, probably due to their extensive secondary structure. There is therefore an urgent need in the field to standardize analyses and perhaps sharing of biological samples and expertise to try to clarify the discrepancies related to HIV-1-encoded vmiRNAs and their potentially functional relevance.

Meanwhile, Narayanan et al. very recently reported that the TAR-miRNA gets packaged in exosomes released from HIV-1-infected cells [110]. Exosomes are small, cell-derived vesicles that are produced as a natural mode of intercellular communication

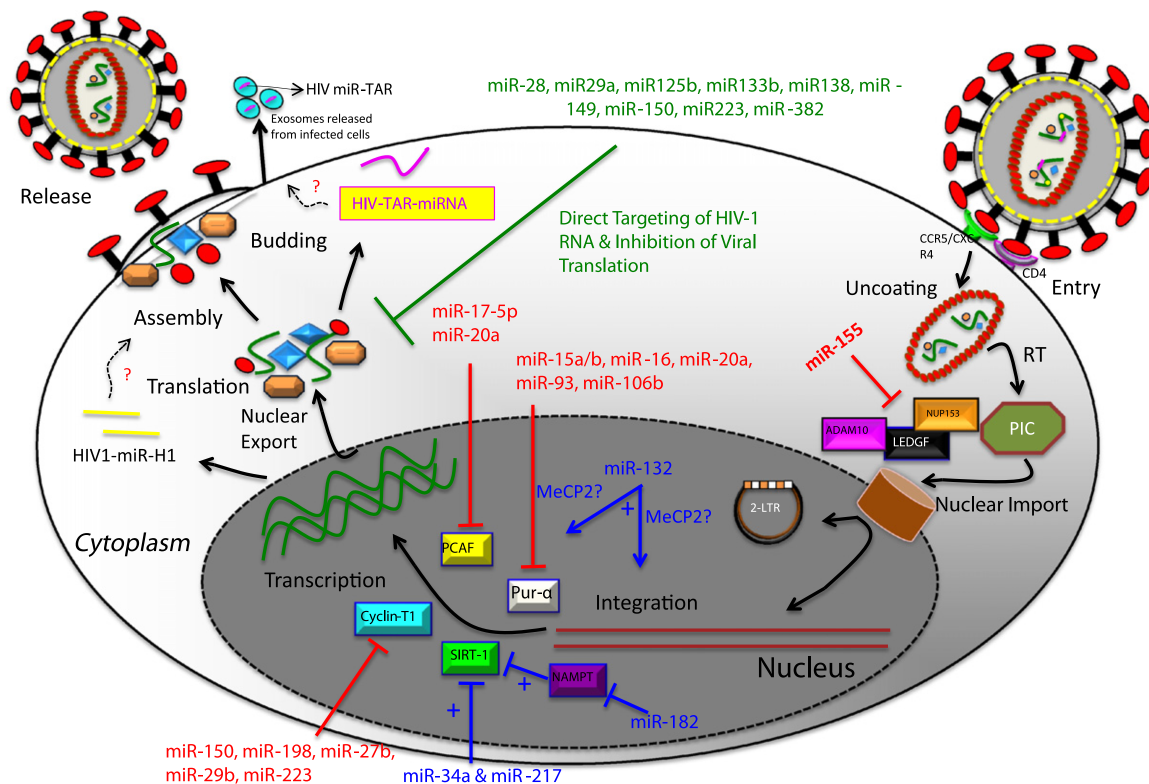

Fig. 2. Cellular miRNAs may modulate HIV-1 infectivity and replication. HIV-1 infection can be directly affected by cellular miRNAs that can target HIV-1 viral RNA through base pair complementarity (indicated in green). In addition, other miRNAs can inhibit host proteins that play an active role during productive HIV-1 infection and thereby indirectly inhibit HIV-1 replication (indicated in red). More recently, few miRNAs that can enhance HIV-1 infection have also been identified (indicated in blue). The mechanisms underlying these pro-HIV-1-miRNAs have not been well defined, but it is possible that these miRNAs can bind to and suppress negative regulators of HIV-1 infection. Finally, HIV-1-encoded vmiRNAs such as HIV1-miR-H1 and TAR-miRNA might also modulate viral infectivity. Further research is needed to elucidate the specific mechanisms by which these vmiRNAs and many cellular miRNAs might affect HIV-1 infectivity and replication.

[111,112]. Evidence suggests that exosomes can contain proteins and nucleic acids, including miRNAs, which can be taken up by other cells and result in a modulation of their functionality [113,114]. Therefore, TAR-miRNA-containing exosomes released from HIV-1-infected cells might be modulating uninfected cells, perhaps to increase their susceptibility to infection. Further research needs to be performed to confirm these findings and to clarify the potential biological significance of these TAR-miRNA-containing exosomes in HIV-1 infection and pathogenesis.

Impact of Cellular miRNAs on HIV-1 Infection

Cellular RNAi machinery such as miRNA plays crucial roles in controlling viral infection in general, and HIV-1 may be no exception. The interactions between HIV-1 and the host cellular miRNAs have been studied, if not extensively, at least in some detail. However, it still remains a quite complex issue and it is likely that we may actually have only been scratching the surface of their relationship. A number of cellular miRNAs have been shown to play a role in modulating HIV-1 infection, either directly or indirectly. In this section, we review the reported impact of miRNAs on HIV-1 (Fig. 2) and discuss potential experimental avenues for future research.

miRNAs that directly target HIV-1

Cellular permissiveness to HIV-1 infection mainly depends on the expression of adequate levels of the primary receptor CD4 and one of the main co-receptors, the chemokine receptor CCR5 or CXCR4. However, the activation and differentiation status of the cells are known to influence their permissiveness to HIV-1 infection. For instance, monocytes are quiescent cells and do not generally support productive HIV-1 infection; however, they become highly susceptible upon differentiation into macrophages [115–118]. Similarly, resting CD4$^+$ T cells do not support productive HIV-1 replication

whereas the virus readily infects and replicates in activated CD4$^+$ T cells [119,120]. Although increases in cell surface expression of receptors CD4, CCR5 and/or CXCR4 have been implicated in enhanced susceptibility to infection, more intrinsic factors have also been reported to play a significant role in determining cell-type-specific HIV-1 restriction [121–125]. In this sense, in a ground-breaking study by Huang *et al*., a selected group of miRNAs were shown to play an active role in modulating HIV-1 infectivity of resting CD4$^+$ T lymphocytes [126]. They reported that a group of cellular miRNAs, including miR-28, miR-125b, miR-150, miR-223 and miR-382, had the ability to bind to the 3′ UTR of viral mRNAs through sequence complementarity and showed that activation of resting CD4$^+$ T cells resulted in down-regulation of these miRNAs, which correlated with enhanced HIV-1 susceptibility. Furthermore, this translational repression mediated by "anti-HIV-1 miRNAs" was proposed to be largely responsible for HIV-1 latency in resting CD4$^+$ T cells, since experiments in which all five of these miRNAs were inhibited in resting CD4$^+$ T cells from cART-treated individuals (with undetectable viremia) displayed enhanced HIV-1 production. The authors thus suggested that these novel anti-HIV-1 miRNAs could play a role in controlling HIV-1 latency, at least in these cells, and that their manipulation could potentially contribute to the purging of viral reservoirs [126]. In a similar study, Wang et al. showed that this group of anti-HIV-1 miRNAs were enriched in monocytes as compared to monocyte-derived macrophages [127] (macrophages differentiated from blood-derived monocytes in the presence of macrophage colony-stimulating factor in the cell culture medium). The authors reported that macrophages down-regulated these miRNAs upon differentiation and that this down-regulation was related with their ability to support productive HIV-1 infection. By combinatorial inhibition of these miRNAs, they also showed that monocytes had a limited increase in their susceptibility to HIV-1 infection [127]. However, it should be pointed out that a direct response to the original publication questioned that the magnitude of the changes observed in miRNA over-expression or inhibition experiments does not support the conclusions and that the authors did not consider the contribution of the modulation of the levels of receptors to the observed effects [128]. More recently, Sisk et al. published new experimental data and the results of a re-analysis of publicly available datasets suggesting a more complex scenario than that presented above with a uniform down-regulation of anti-HIV-1 miRNAs during the differentiation from monocytes to macrophages [129]. This study could only confirm that miR-223 is down-regulated in macrophages, while the rest of the miRNAs in question were either more abundant or unchanged in macrophages than in monocytes [129]. These discrepancies point again to the need for more standardized future studies that should ideally take into consideration the potential differences that may stem from the various available platforms for studying miRNAs, diverse experimental details in the monocyte isolation and/or in the monocyte-to-macrophage differentiation protocols, possible alterations in abundance of macrophage subpopulations and so on.

Modulation of these anti-HIV-1 miRNAs in response to various stimuli has also been reported to alter HIV-1 infectivity. Since drugs of abuse are intimately linked to the HIV-1 epidemic and are known to affect multiple aspects related to disease progression such as adherence to therapy, cognition, risk behaviors and so on, some studies have investigated the potential effects of drugs of abuse on expression of miRNAs that may be important for HIV-1 infection and replication. For instance, treatment of monocytes with morphine has been shown to lead to a reduction in expression of miR-28, miR-125b, miR-150 and miR-382, a subset of the anti-HIV-1 miRNAs reported in monocytes by the same group, and to enhance the susceptibility to infection of monocytes [130]. Furthermore, they also observed that PBMCs from uninfected, heroin-abusing individuals have lower levels of these miRNAs than those of healthy, non-abusing individuals, supporting their *in vitro* findings. Another commonly abused drug, cocaine, was also shown to inhibit one such anti-HIV-1 miRNA, miR-125b, in CD4$^+$ T cells, and subsequent experiments with a T cell line showed that miR-125b knockdown enhances HIV-1 replication, whereas its over-expression decreases HIV-1 replication [131]. Therefore, the authors suggested that miR-125b could be a key player for the cocaine-induced enhancement of HIV-1 replication in CD4$^+$ T cells. Other modulators of anti-HIV-1 miRNAs include cytokines and Toll-like receptor (TLR) ligands. For example, stimulation of TLR3 was shown to induce an anti-HIV-1 effect in primary macrophages, partially through up-regulation of the set of anti-HIV-1 miRNAs mentioned above [132]. Interestingly, up-regulation of these four anti-HIV-1 miRNAs was also reported in macrophages treated with type I interferons (IFN), IFN-α and IFN-β, and was shown to partially contribute to inhibition of HIV-1 infection in primary macrophages [133].

Another cellular miRNA that can directly target the 3′ UTR of viral mRNAs is miR-29. Binding of miR-29 to the HIV-1 mRNA has been shown to increase its association with proteins in the RISC complex and to augment its incorporation into processing bodies (or P bodies, cytoplasmic domains that contain proteins involved in post-transcriptional processes such as mRNA degradation, translational repression or RNA-mediated gene silencing), thereby inhibiting translation of viral proteins and viral replication [134]. Also, an independent study had shown that inhibition of miR-29a significantly enhanced HIV-1 infection,

presenting evidence as well that miR-29a down-regulates the expression of Nef protein [135].

miRNAs that affect HIV-1 infection by modulating the expression of HDFs

By utilizing genome-wide RNAi screens, various groups have identified many host factors that seem to be required for, or at least to participate to a certain extent in, one or more steps of the viral life cycle [22–30]. Although there was a limited overlap in the factors identified in the various screens, these and subsequent studies have certainly determined the role of some critical cellular factors and pathways during the HIV-1 life cycle, opening the door to new opportunities to curb productive HIV-1 infection through the targeting of the interactions of HIV-1 with these crucial HDFs [136]. Since miRNAs play such an important role in modulating cellular protein expression, it is not surprising that, during the last few years, several cellular miRNAs have been reported to regulate HIV-1 infection, indirectly, through the modulation of the levels of HDFs.

Cyclin T1 is an important component of the eukaryotic RNA polymerase II elongation complex through its association with CDK9. This heterodimer forms the positive transcription elongation factor B or p-TEFb [137,138]. The role of the p-TEFb complex in interacting with the HIV-1 TAR element and the viral protein Tat to facilitate viral transcription has been extensively characterized [139–141]. RNAi-mediated inhibition of this complex has been reported to inhibit HIV-1 replication [142], and relatively higher expression of Cyclin T1 in HIV-1 susceptible cells such as macrophages, as compared to monocytes, has also been reported [143]. Sung et al. identified a novel anti-HIV-1 role for miR-198 through down-regulation of Cyclin T1; they first observed an inverse correlation between the levels of Cyclin T1 and miR-198 in monocytes and macrophages, and subsequently, it was shown that miR-198 binds to the 3′ UTR of Cyclin T1 mRNA and reduces its protein levels [144]. In addition, the authors provided evidence that miR-198 over-expression inhibited HIV-1 replication in macrophages, supporting the concept that miRNAs exert cell-type-specific effects [144]. Chiang *et al.* have also reported that miR-198 is expressed at very low levels in resting $CD4^+$ T cells and is not modulated upon activation [145]. However, they identified another miRNA, miR-27b, which, in addition to other previously reported anti-HIV-1 miRNAs such as miR-29b, miR-150 and miR-223, inhibited the expression of Cyclin T1 in resting $CD4^+$ T cells. They reported that activation of $CD4^+$ T cells resulted in down-regulation of these miRNAs and a subsequent up-regulation of Cyclin T1, which correlated with enhanced HIV-1 susceptibility [145].

Interactions of the HIV-1 protein Tat with transcriptional co-activators such as cellular CREB binding protein p300 and p300-CREB binding protein associated factor (PCAF) have been shown to be important for Tat acetylation and eventual HIV-1 LTR-driven transcriptional up-regulation [146,147]. Interestingly, the cellular miRNA cluster miR-17/92 has been reported to target PCAF and to inhibit HIV-1 infection [64]. Specifically, miR-17-5p and miR-20a over-expression resulted in reduced mRNA levels and protein output of PCAF. Of note, HIV-1 infection actively suppressed these miRNAs to enhance its infectivity by usurping the p300/PCAF complex for efficient viral translation [64].

Another well-characterized HDF is the purine-rich element binding protein α (Pur-α), a sequence-specific DNA and RNA binding protein. It has been previously noted to bind to the HIV-1 TAR element and the viral Tat protein in the nucleus of infected cells to facilitate viral transcriptional up-regulation [148,149]. Recently, it was reported that expression of Pur-α was significantly lower in monocytes than in monocyte-derived DCs and that this difference contributed to the lower susceptibility to HIV-1 infection in monocytes, with the mechanism being attributed to higher expression levels of several cellular miRNAs (miR-15a, miR-15b, miR-16, miR-20a, miR-93 and miR-106b) that target Pur-α mRNA [150]. Accordingly, inhibition of these miRNAs in monocytes enhanced expression levels of Pur-α and also led to increased HIV-1 infection.

Unlike all the miRNAs reported above that target transcription, recent studies from our laboratory have resulted in the identification of a novel anti-HIV-1 role for the cellular miRNA miR-155, which seems to inhibit HIV-1 at a step prior to the integration of the viral cDNA into the host genome. miR-155 is also known as an onco-mir since it has been found altered in many cancers but is also crucial to establish appropriate immune responses (reviewed by O'Connell et al. [40]). For instance, miR-155 levels increase in the human monocytic cell line THP1 and in murine bone-marrow-derived macrophages in response to stimulation with LPS and poly(I:C), TLR4 and TLR3 ligands, respectively, and it seems to play a fundamental role in the macrophage inflammatory profile [151,152]. In addition, in DCs, miR-155 targets the transcription factor PU.1 and subsequently down-regulates the expression of DC-specific intercellular adhesion molecule-3 grabbing non-integrin (or DC-SIGN) [153], which binds to the HIV-1 envelope glycoprotein gp120 and is important in the process of trans-infection [154]. We identified that activation of TLR3 in primary human macrophages resulted in significant enhancement of miR-155 expression levels, which correlated with decreased HIV-1 infectivity [155]. Through over-expression studies, as well as inhibition of miR-155 in the context of TLR3 stimulation, we demonstrated that miR-155 inhibits HIV-1 at a post-entry, pre-integration step. Further analysis

showed that miR-155 targets the 3′ UTR of the mRNAs of at least three known HDFs, the cellular transcriptional co-activator lens epithelium-derived growth factor (LEDGF)/p75, ADAM10 (a member of *a d*isintegrin *a*nd *m*etalloprotease family of proteins) and the nucleoporin NUP153, and reduced the levels of protein expression of all three HDFs in primary macrophages [155]. Combinatorial inhibition of these three HDFs by miR-155 or by siRNA resulted in accumulation of late reverse transcripts and significantly decreased viral integration, in agreement with the roles of these HDFs in trafficking and/or nuclear import of HIV-1 PIC [155].

Finally, an attractive target among all known HDFs is the chemokine receptor CCR5 that serves as the major co-receptor for HIV-1 infection since it is known that individuals homozygous for a 32-base-pair deletion in CCR5 are resistant to infection by CCR5-using HIV-1 strains. In fact, treatment of $CD4^+$ T cells with zinc-finger protein nucleases specifically disrupting the CCR5 coding sequence induces resistance to CCR5-using HIV-1 infection *in vitro* and *in vivo* [156–158]. As an alternative to this genetic modification, cellular miRNAs that target CCR5 mRNA could potentially be used to reduce CCR5 levels and test experimentally whether it might have an impact on reducing HIV-1 entry and infection.

miRNAs that enhance HIV-1 infection

Very few studies have reported miRNA-mediated enhancement of HIV-1 infection. Recently, miR-132 was shown to enhance HIV-1 infection [159]. miR-132 expression was reported to be significantly higher in activated $CD4^+$ T cells than in resting cells. In addition, ectopic expression of miR-132 in Jurkat T cells was also shown to increase HIV-1 infection [159]. However, the mechanisms underlying this effect have not been uncovered. It would be interesting to determine the specific step on the viral life cycle that seems to be potentiated by miR-132. To this end, the authors showed preliminary data wherein miR-132 over-expression decreased the expression of a cellular protein MeCP2. MeCP2 is a methyl CpG binding cellular protein. The role of MeCP2 in regulating HIV-1 replication was shown by experiments in which siRNA-mediated inhibition of MeCP2 enhanced HIV-1 replication [160]; however, the specific role of miR-132 and MeCP2 in HIV-1 replication has not been identified. Recent evidence suggests that MeCP2 directly binds to LEDGF/p75 (a previously characterized HDF that interacts with the HIV-1 protein integrase and promotes viral nuclear import and integration) and significantly enhances MeCP2's transcriptional activity [160]. MeCP2 binds to LEDGF in its N-terminal region, whereas the integrase binding domain in LEDGF is located in the C-terminus [161]. Therefore, it is not likely that MeCP2 might be competitively binding to LEDGF in place of HIV-1 integrase. However, the N-terminus of LEDGF also contains the nuclear localization sequence, making it possible that the binding of MeCP2 might alter other functions of the protein that would indirectly affect the role of LEDGF in the HIV-1 life cycle. In addition, it would be interesting to determine if LEDGF is involved in the augmented HIV-1 replication observed in MeCP2-silenced cells.

The only other example in the literature relates to the sirtuin 1 (SIRT1)-mediated regulation of HIV-1 Tat transactivation. The viral protein Tat is active in its acetylated form (regulated by p300/PCAF complex), and SIRT1 is a class III deacetylase dependent on nicotinamide adenine dinucleotide that has been characterized to regulate HIV-1 transcription by deacetylating Tat [162]. Interestingly, miR-217 and miR-34a were recently shown to be significantly up-regulated upon Tat exposure and to have the ability to bind the 3′ UTR region of SIRT1 mRNA and inhibit its expression, thereby enhancing HIV-1 Tat-mediated transactivation [163,164]. The functional relevance of cellular miRNAs that target SIRT1 was shown by a recent report characterizing simian immunodeficiency virus (SIV) encephalitis. In this study, the authors demonstrated a significant up-regulation of miR-142 post-SIV infection that leads to down-regulation of SIRT1, which could potentially contribute to SIV replication and SIV-induced encephalitis [165]. In addition, SIRT1 is regulated by another cellular protein, nicotinamide phosphoribosyltransferase (NAMPT), which plays a role in maintaining the cellular levels of nicotinamide adenine dinucleotide. A recent study showed that miR-182 targets the 3′ UTR region of NAMPT mRNA and inhibits its expression [166]. Interestingly, Tat significantly up-regulated the levels of miR-182 expression, and down-regulation of NAMPT by miR-182 led to decreased expression of SIRT1 levels, which in turn enhanced HIV-1 Tat transactivation [166].

Overall, there are cellular miRNAs that have been reported to modulate HIV-1 infection, but this field promises to rapidly expand in the near future. As evidenced in this section, cell-type-specific expression of miRNAs and HDFs seems to contribute in determining HIV-1 susceptibility. It is also important to consider the activation and differentiation status of the cell when studying miRNA-mediated restriction or enhancement of HIV-1 infection. Furthermore, attention to the role of cellular restriction factors has grown exponentially in the recent past since they limit HIV-1 infection by multiple mechanisms and their expression may vary between different cell types, likely contributing to determine the cellular susceptibility to productive HIV-1 infection. As expected, HIV-1 seems to have evolved many specific mechanisms of evasion from restriction factor-mediated inhibition [167,168]. An interesting avenue for future research that has not yet been explored is to investigate if HIV-1

modulates the expression of cellular miRNAs that can alter the expression of restriction factors. Characterizing the potential for HIV-1-mediated enhancement of these miRNAs might help uncover important roles in enhanced viral replication, pathogenesis and survival of the virus.

miRNA Profiles in HIV-1-Infected Patient Cohorts: Potential Biomarkers?

In spite of the experimental evidence characterizing the importance of cellular miRNAs on HIV-1 infection in *in vitro* and *ex vivo* studies, only a limited number of reports have attempted to explore global miRNA profiles in HIV-1-infected patients. Houzet et al. published one of the first reports profiling miRNA expression in HIV-1 seropositive individuals [169]. By grouping patients into four categories based on differential $CD4^+$ T cell counts and viral load, the authors compared miRNA expression profiles in PBMCs and found that, of the 63 miRNAs that were commonly altered, 59 miRNAs were down-regulated and the rest were up-regulated in seropositive individuals when compared to uninfected, healthy controls [169]. Furthermore, they explored miRNA changes in specific subsets of HIV-1 susceptible cells and concluded that several T-cell-specific miRNAs were down-regulated in all seropositive individuals; these miRNAs included miR-150, miR-191, miR-223, miR-16 and miR-146b [169]. Of note, miR-150 and miR-223 are signature anti-HIV-1 miRNAs that have been reported to directly inhibit HIV-1 transcription [126]. In addition, the authors noted that the vast down-regulation of several cellular miRNAs might be due to the down-regulation of essential proteins in the miRNA machinery such as Dicer and Drosha. As mentioned earlier, Triboulet et al. showed that knockdown of Dicer and Drosha enhanced HIV-1 infection of T cell lines [64]. Therefore, it is possible that reduced levels of Dicer and Drosha in PBMCs of HIV-1 seropositive individuals may lead to reduced global miRNA levels, even though the study by Houzet et al. was limited due to the small number of patients included [169].

Elite suppressors (ES; also known as elite controllers) are a group of HIV-1-infected patients that are able to maintain extremely low to undetectable viral loads in blood ($<$50 copies/ml, below the limit of detection of commercial assays) for many years without cART and generally do not show any clinical signs of disease progression. Various innate and adaptive immune factors in ES have been previously reported [170–172]. Therefore, it is important to understand the specific mechanisms behind the differential evolution of HIV-1 infection in ES *versus* the rest of HIV-1-infected individuals and potentially to exploit these mechanisms in the field of HIV-1 vaccine development. In this regard, Witwer et al. have characterized global miRNA expression profiles in PBMCs from HIV-1-infected viremic patients and ES [173], to determine if differential miRNA expression profiles in ES might shed light on their resistance to virus-induced disease progression. To increase reliability, the authors employed three different miRNA amplification platforms and were able to identify specific miRNAs that were uniquely expressed in PBMCs from ES when compared to viremic patients and uninfected controls. Remarkably, signature anti-HIV-1 miRNAs miR-125b, miR-150 and miR-29 were down-regulated in both ES and viremic patients. Another important miRNA that has been reported by our group to inhibit HIV-1 infection in macrophages, miR-155 [155], was significantly higher only in viremic patients as compared to ES or healthy controls. Other miRNAs that were only up-regulated in viremic patients include miR-9, miR-34a and miR-181. Interestingly, miR-181 (miR-181abc and miR-181d) has been predicted to bind to and potentially inhibit the HIV-1 restriction factor SAMHD1 (personal observation). Increase in miR-34a could possibly be explained by the recent evidence suggesting that miR-34a is up-regulated by the viral protein Tat, leading to down-regulation of SIRT1 and to enhanced viral translation [163]. However, miR-31 and miR-31*, which to date have not been reported to play a role in HIV-1 replication, were found to be significantly reduced only in viremic patients, with respect to ES and uninfected controls, perhaps suggesting a potential functional relevance. In addition, the authors found correlations between miRNA changes and plasma viral load and $CD4^+$ T cell counts in these patients. Overall, this study brought in the concept of using differentially expressed miRNAs as biomarkers to predict the risk of disease progression [173], although further studies are needed to achieve this goal.

Similar to, but different from ES, LTNP are HIV-1 seropositive individuals that naturally control viremia, but to detectable although low levels (10,000 copies of RNA/ml of blood) without cART, have normal $CD4^+$ T cell counts and do not experience the typical disease progression of HIV-1-infected individuals [86,174]. Additionally, multiply exposed uninfected individuals (MEU) are those rare individuals who have been potentially exposed to HIV-1 in multiple occasions but remain HIV-1 seronegative. Mechanisms underlying resistance to or efficient control of HIV-1 infection in these patient cohorts have not been well characterized. To this end, Bignami et al. evaluated miRNA expression profiles in $CD4^+$ T cells from LTNP, naïve healthy individuals and MEU individuals [175]. The authors found that 23 miRNAs were differentially expressed between MEU and naïve healthy individuals. For example, resting $CD4^+$ T cells from MEU individuals had significantly lower levels of miR-28-5p, miR-125b and miR-223. Interestingly, only one miRNA, miR-155, had significantly higher levels in LTNP than in MEU. However, a response to this publication

by Seddiki et al. argued that the increase in miR-155 levels might be largely contributed to by effector/memory regulatory T cell subsets (Tregs) [176] and suggested that the changes in the prevalence of $CD4^+$ T cell subsets in response to HIV-1 infection might be the major factor responsible for differential miRNA expression profiles in these patient cohorts. Furthermore, Bignami et al. showed that the miRNA biogenesis proteins such as Dicer and Drosha are significantly down-regulated in $CD4^+$ T cells derived from MEU individuals [175], although the mechanisms are not well understood. Finally, in another response to this study, Witwer et al. re-analyzed the miRNA expression profiles and raised questions regarding the validity of the conclusions since their new analysis revealed that several previously characterized miRNAs in HIV-1-infected individuals such as miR-125b, miR-31, miR-146b and miR-29a are actually lower in HIV-1-infected, cART naïve patients [177].

Previous reports have demonstrated that an increase in the levels of the cytokine IL-10 post-HIV-1 infection plays a detrimental role by inhibiting HIV-1-specific T cell responses [178]. A recent study reported that IL-10 mRNA is directly targeted by the Let-7 family of miRNAs and showed that enhancement of IL-10 levels after *in vitro* infection with HIV-1 correlated with a decrease in Let-7 miRNA levels [179]. In addition, it was shown that levels of Let-7 miRNAs were significantly lower in $CD4^+$ T cells from chronically HIV-1-infected patients as compared to uninfected individuals and LTNP. Therefore, they proposed that the decrease in Let-7 miRNAs might be directly responsible for enhanced IL-10 production in HIV-1-infected cells [179]. Similarly, Seddiki et al. have reported that the expression of B lymphocyte-induced maturation protein-1 (BLIMP-1) is significantly increased, while levels of miR-9 expression are significantly reduced, in $CD4^+$ T cells from chronically infected HIV-1 patients as compared to uninfected healthy individuals or LTNP [180]. They showed that BLIMP-1 suppresses IL-2 (an important cytokine required for T cell growth and survival) and implied that increased BLIMP-1 expression in HIV-1-infected individuals might contribute to virus-induced T cell dysregulation through alterations in IL-2 levels. Also, the authors demonstrated that cellular miR-9 directly binds to and inhibits BLIMP-1, thereby uncovering a potential role for miR-9 in HIV-1-infected patients [180].

Very recently, Duskova et al. have published a global miRNA-mRNA expression profile study, comparing PBMCs from chronically HIV-1-infected patients and uninfected healthy controls. They demonstrated that several miRNAs including miR-19b, miR-146a, miR-615-3p, miR-382, miR-34a, miR-144 and miR-155 were significantly increased in infected patients as compared to healthy controls [181] and also showed alterations in the levels of pro-inflammatory cytokines and chemokines, although the significance of such findings is yet unclear.

Finally, a word of caution: the majority of the studies mentioned above have characterized the miRNA expression profiles in various groups of patients and controls using PBMCs or purified $CD4^+$ T cells, but only few miRNAs seem to be consistently modulated. It needs to be considered that the cellular isolation procedures might affect the outcome of these analyses because miRNAs are tightly modulated in specific cell types based on activation stimuli, differentiation states or other changes in the cellular environment. Thus, profiling miRNAs in undefined, mixed cell populations such as PBMCs (that may contain quite different frequencies of specific cell types) will reduce the likelihood of identifying potentially biologically significant changes that may occur in individual cell types. This is in agreement with a recent report that profiled miRNAs and mRNAs in nine human immune cell subsets derived from healthy donors and found that cellular miRNA and mRNA expression patterns can be highly specific to each of the cell subsets investigated [182]. In addition to considering the potential variability between cell types, miRNA expression analyses in patient cohorts should utilize stringent criteria for data analyses and reliable expression profiling techniques to ensure consistency between multiple studies and patient cohorts. Lastly, it might be beneficial if a consortium of basic investigators and clinicians interested in profiling miRNA expression in HIV-1-infected patients could be established in the future, to facilitate open discussion and sharing of analysis tools, patient samples and gene expression profiling information.

miRNAs: Potential Therapeutic Applications

HIV-1 infection of activated $CD4^+$ T cells results in rapid apoptotic death of infected cells and uninfected bystander cells [183,184]. On the other hand, HIV-1-infected macrophages, as innate immune cells, are relatively resistant to cytopathic effects induced by the virus. As resident innate immune sentinels, macrophages are located in various tissues including the brain and serve as HIV-1 reservoirs [185,186]. In addition, HIV-1 infection of macrophages provides a niche for the virus to undergo ample virus replication masking host antiviral responses and anti-retroviral drugs [115,187–190]. While infected macrophages and DCs support active replication and play a role as a reservoir throughout the body, the major latent reservoir in infected patients is likely the resting $CD4^+$ T cells [11,14,116,191–195]. Latent reservoirs of HIV-1 can be defined as cells that contain successfully integrated HIV-1 genomes yet do not

have active virus replication and do not produce progeny virions. The lack of active viral replication in an infected patient might be due to multitude of reasons including cART, host restriction factors, host antiviral immune responses and so on. Although an infected patient on cART might have remarkably low or even undetectable levels of virus in blood, the presence of latent reservoirs enables the virus to be "reactivated" upon certain conditions such as treatment interruption or lack of adherence to the prescribed cART and changes in cellular activation states. While anti-retroviral drugs inhibit active viral replication, they do not affect the integrated pro-viruses, which have the potential to be reactivated to produce newly infectious viruses when a more favorable cellular environment arises [11,192,193]; therefore, there is a need to develop new strategies that purge latent reservoirs and will bring us one step closer to the real goal: a cure for HIV-1 infection [2,196–198].

In addition, through the use of specific miRNA mimics and inhibitors [199–204], the modulation of miRNAs that regulate expression of HDFs could be an attractive avenue for future research. If the HDFs targeted by particular miRNAs are involved in the pre-integration stages of the viral life cycle, they would also have the advantage of resulting in inhibition or reduction of integration, which would suppress, or at least reduce, the pool of cells with integrated pro-viruses that increase the size of the viral reservoir and potentially the number of latently infected cells that resist elimination during cART. Furthermore, a single miRNA may be able to target more than one HDF or several miRNAs with different targets may be combined so that various proteins involved in different steps of the viral life cycle can be regulated, enabling a combinatorial, multi-target approach that could complement current cART for the inhibition of HIV-1 infection. One such example is cellular miR-155, which binds to three HDFs, namely, LEDGF, ADAM10 and NUP153, resulting in either inhibition or large reduction in HIV-1 integration in primary macrophages [155]. Up-regulating the expression of some miRNAs such as miR-155 could also provide an additional benefit since it has been well characterized that it inhibits the cellular protein suppressor of cytokine signaling-1 (SOCS-1), a negative regulator of the activity of type I IFNs. Therefore, inhibiting this negative regulator of antiviral cytokines would likely result in an augmented antiviral state that could make the cells more refractory to infection [205–207]. Furthermore, inhibition of selected miRNAs can also be used to our advantage. For instance, inhibition of miRNAs that target common cellular antiviral proteins or pathways might enhance the antiviral responses triggered by HIV-1 entry or infection, which could result in an increased resistance to productive infection. Finally, miRNAs that may modulate the levels of known restriction factors, such as APOBEC3G, TRIM5α or SAMHD1, could be of interest as well. It is plausible that HIV-1-infected cells might secrete soluble factors such as type I IFNs, which might down-regulate miRNAs that target these restriction factors. Therefore, inhibition of these miRNAs could enhance the basal expression of the restriction factors in susceptible cells, potentially decreasing their susceptibility to productive HIV-1 infection.

For a number of years, several drugs such as prostratin (a plant-based protein kinase C activator) and other phorbols have been used to activate cellular transcription factors that lead to the initiation of HIV-1 transcription from latent pro-viruses [208–212]. In addition, multiple histone deacetylase (HDAC) inhibitors have been tested as re-activators of latent reservoirs [213,214]. The concept behind the usage of these drugs is to re-activate latent reservoirs (i.e., to restore viral protein production and new virion generation) and, subsequently, to use cART that will inhibit the re-awakened virus preventing the establishment of new viral reservoirs. This "reactivate and purge" strategy has been met with definite success in recent clinical trials; however, the long-term effects and safety profiles of using HDAC inhibitors have not been well characterized [215–217]. Since a role for cellular anti-HIV-1 miRNAs (miR-28, miR-125b, miR-150, miR-223 and miR-382) in imparting a transcriptional block to HIV-1 in resting $CD4^+$ T cells has been reported, it might be worth exploring the potential utility of a combinatorial inhibition of these miRNAs that directly target the viral mRNA. Therefore, if successful, this strategy could be a complementary approach to the use of HDAC inhibitors or protein kinase C activators that are not specific to HIV-1 transcriptional regulation, perhaps providing a safer, potent option in combination with cART.

From a therapeutic standpoint, the use of RNAi strategies to inhibit HIV-1 infection is already been explored. Both miRNAs and short hairpin RNAs (shRNAs) targeting fully complementary and partially complementary sequences on the HIV-1 genome have been shown to successfully inhibit HIV-1 in *in vitro* experiments [218], with miRNAs being more efficient in suppressing imperfect targets, which may be important in the context of a viral genome that is prone to generate escape mutants. However, several miRNA-based strategies have been shown to be more advantageous than siRNA- or shRNA-based anti-HIV-1 strategies. Thus, the use of polycistronic miRNA constructs expressing multiple HIV-1 genome targeting siRNAs or miRNAs [219] or constructs that express multiple HIV-1 targeting siRNAs in combination with nucleolar RNAs [220,221] has been shown to be more efficacious than individual anti-HIV-1 siRNAs or shRNAs and could be a promising therapeutic approach.

As potent as the idea of manipulating cellular miRNAs seem, extreme caution needs to be employed

to understand the short-term and long-term potential side effects. Since miRNAs play crucial roles in specific, time-dependent and tightly regulated expression of host proteins, they are important for maintenance of cellular homeostasis. In addition, each miRNA has the potential to modulate the expression of multiple targets, potentially complicating even more the safety of their use. However, there is a prominent example of modulating cellular miRNAs to control virus replication, as evidenced by miR-122 inhibition for the treatment of HCV infection. The liver-specific miR-122 is a pro-HCV miRNA that has been shown to enhance HCV-RNA stability and translation, resulting in an overall increase in virus replication [222–229]. Therefore, a specific antagonist or anti-oligomiR against miR-122 has been developed and has shown efficient virus inhibition *in vitro*, *ex vivo* and in studies with animal models [230–232]. Very recently, results from human clinical trial phase 2a utilizing the anti-miR-122 inhibitor for treating HCV-infected patients showed potent suppression of HCV viremia, absence of generation of viral escape mutants and excellent safety profiles [233]. Although there are some specific considerations that apply to the miR-122-HCV success story, at least to date, that may not apply to HIV-1 and other viral infections, these results are encouraging and provide a promising platform for the potential development of future miRNA-related therapeutics to complement cART for the treatment of HIV-1 infection.

Conclusions

HIV-1 infection continues to remain an area of global health burden despite successful cART. Based on cellular differentiation or activation states, differential expression of miRNAs seems to alter susceptibility to productive HIV-1 infection. It has been shown that some miRNAs can directly target the viral mRNAs, and indirect inhibition by cellular miRNAs targeting the mRNAs of HDFs has also been noted. As an ever-evolving strategy to infect and persist, HIV-1 seems to down-regulate some of these cellular miRNAs that have anti-HIV-1 effects. Although not completely understood, there is evidence suggesting that HIV-1 inhibits the proteins involved in biogenesis and maturation of cellular miRNAs, and global expression profiles of miRNAs seem to be modulated in HIV-1-infected cells and in patients. It may be conceivable that, in the future, miRNAs could potentially be used as biomarkers to estimate or predict disease progression rates in infected patients, but further studies exploring miRNA modulation in patient cohorts and greater consistency in the miRNAs identified will be needed before this strategy could be implemented. Further research and more collaborative studies are also needed to clarify whether functional vmiRNAs are produced by HIV-1 and whether miRNA modulation could be an appropriate therapeutic approach to complement and reinforce cART with the goal of HIV-1 eradication.

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Abbreviations used:
miRNA, microRNA; HIV-1, human immunodeficiency virus type 1; cART, combination anti-retroviral therapy; PIC, pre-integration complex; LTR, long terminal repeat; RNAi, RNA interference; HDF, HIV dependency factor; siRNA, small interfering RNA; sncRNA, small, non-coding RNA; UTR, untranslated region; RISC, RNA-induced silencing complex; PBMC, peripheral blood mononuclear cell; SRS, suppressor of RNA silencing; RSS, RNA silencing suppressor; Vpr, viral protein R; HCV, hepatitis C virus; LTNP, long-term non-progressor; TLR, Toll-like receptor; DC, dendritic cell; LEDGF, lens epithelium-derived growth factor; SIV, simian immunodeficiency virus; shRNA, short hairpin RNA.

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