AIDS Rev. 2013;15:3-14

Micro-RNA: new players in HIV-pathogenesis, diagnosis, prognosis and antiviral therapy

Lauren Fowler and Nitin K. Saksena

Retroviral Genetics Division, Center for Virus Research, Westmead Millennium Institute, Westmead Hospital, University of Sydney, Sydney, Australia

Abstract

The role of small RNA (microRNA) as key regulators of gene and protein expression has been well established. Currently there is greater interest in these small RNA molecules because of their involvement in the regulation of a variety of animal and plant diseases, animal development, and physiology in addition to their critical role in a variety of cellular activities such as cell proliferation, apoptosis, morphogenesis, and differentiation. Overall, microRNA regulate gene and protein expression to control and guide decisions on the fate of cells. Given their stability* in vivo *and their importance in human diseases, microRNA are gaining increasing importance as new generation biomarkers for diagnostics and prognostics, along with becoming excellent therapeutic targets for treating human diseases. This review is focused largely on the role of microRNA in HIV infection. The main purpose of this review is to provide a comprehensive perspective on host microRNA in the context of infection by HIV and other viruses, their effect on viral pathogenesis, along with providing insights into virally encoded microRNA that participate in the infectious process. **(AIDS Rev. 2013;15:3-14)**

Corresponding author: Nitin K. Saksena, nitin.saksena@sydney.edu.au

Key words

MicroRNA. HIV-1. Biological markers. Virus latency. Gene expression profiling.

Introduction

According to the UNAIDS estimate, 34 million people around the world were living with HIV at the end of 2010, 17% more than in 2001[1]. It is estimated that 2.7 million people became infected in 2010, including close to 400,000 children. Having 3% of the global population, Sub-Saharan Africa still remains the region most affected by HIV, with an estimated 22.9 million people living with HIV in the region in 2010 – around two thirds of the global total. Although half the deaths in 2010 from AIDS-related causes occurred in southern Africa, the number of AIDS infections in other developing parts of the world is on the rise. In spite of the considerable amount of work being done on all fronts of HIV disease, antiretroviral therapy is currently the only option to control HIV, improve the quality of life of HIV patients, and reduce morbidity. There are no vaccines for HIV yet and the search for new-generation biomarkers for prognostics and diagnostics and new host targets for treating HIV has been intensified. Genomic medicine is becoming an increasingly important area, holding clues to treating a wide variety of human diseases, including HIV, with immense promise as a viable alternative to currently existing anti-HIV therapies. Further, genetic and proteomic expression and its regulation by microRNA (miRNA) has added another dimension to the understanding of HIV pathogenesis, but more work is needed to understand complex aspects of the human genome and its regulation by miRNA.

MicroRNA are small (21-22 nt), non-coding RNA fragments found in many organisms, from plants to humans, which function to negatively regulate gene

Correspondence to:
Nitin K. Saksena
Retroviral Genetics Division
Centre for Virus Research
Westmead Millennium Institute & Westmead Hospital
University of Sydney
Darcy Road, Westmead NSW 2145.
Sydney, Australia
E-mail: nitin.saksena@sydney.edu.au

expression. In humans, roles for miRNA have been identified in metabolism, growth, and development. Dicer, a protein critical for miRNA processing, has been associated with proper development of the limbs, lungs, and hair follicles and in T-cell differentiation[2]. Dysregulation of human miRNA has been implicated in the pathogenesis of many cancers[3]. As negative regulators of gene expression, miRNA can function as tumor suppressors that, if dysregulated, can increase the chance of cancer development[4]. For example, a chromosomal deletion associated with the development of chronic lymphocytic leukemia causes loss of intrinsic miRNA tumor suppressors[5]. Currently, over 800 miRNA with diverse functions have been identified in the human genome. MicroRNA function by binding to partially complementary sites in the messenger RNA (mRNA) of other genes, inhibiting the production of the proteins synthesized from these genes. A single miRNA can interact with hundreds and thousands of genes in regulating various physiological processes, thus adding to the complex regulation of the human genome.

Viral interactions with host cellular miRNA have also been analyzed and a number of viruses have been shown to encode their own miRNA. Virus-host interactions through host- and virus-encoded miRNA not only form a complex and novel layer of regulatory genetic interactions, but have also considerably expanded our appreciation of miRNA function, subversion, and dysregulation by a variety of viruses that cause human disease. Thus, a profound understanding of the genomic crosstalk between the host and virus miRNA would enhance our understanding of the molecular basis of viral pathogenesis, and lead to the development of new-generation therapeutics and strategies to combat viral infections.

Although the mechanisms by which miRNA regulate gene and protein expression remain unclear, further studies of these mechanisms will provide a clear view of these complex genomic interactions in human diseases and will pave the way for the development of new drugs and therapeutics to treat them. Thus, it is essential to understand this regulation process and its dysregulation in diverse human diseases. This review attempts to summarize the findings to date in the field of HIV disease to provide a comprehensive snapshot of where we stand and what more needs to be done in the context of miRNA-mediated regulation of HIV infection.

MicroRNA and small interfering RNA

Small interfering RNA (siRNA) and miRNA originate similarly, have a similar structure, and perform the same function in cells. Hence, the difference between them is a regular source of confusion. MicroRNA originate from loci that are not used to encode other recognizable genes, where siRNA may be derived from DNA used for other coding purposes. For each miRNA hairpin precursor, only one miRNA duplex is formed. Small interfering RNA double-stranded precursors produce a multitude of siRNA duplexes from both strands. MicroRNA also form hairpin loop structures due to their internal complementarities, and a guide strand of the internal pairing is then chosen. Small interfering RNA do not form hairpin loop structures, and are processed from long bimolecular RNA duplexes or hairpins. MicroRNA are nearly always conserved between related organisms, unlike siRNA[6,7]. Recently the guidelines for naming and classifying small RNA have become more clear-cut, helping to minimize confusion[7].

The human microRNA pathway

MicroRNA are found in both introns and exons, and can often be found in clusters that would be transcribed together as polycistronic transcripts[8-10]. MicroRNA are transcribed from the germ line of a cell as primary RNA transcripts (pri-miRNA). They are processed in the nucleus by the enzyme Drosha and its cofactor DGCR8 to remove extraneous nucleotides, producing a hairpin loop structure; a pre-miRNA transcript of ~ 60 nt[11]. Pre-miRNA is exported from the nucleus through the exportin-5 complex, and is bound by the enzyme Dicer in the cytoplasm[12]. Dicer is responsible for cleaving the hairpin loop, producing a duplex of 18-23 bases[8].

The guide strand is selected as the strand of miRNA with the lowest stability at the 5' end of the double-stranded molecule[13], and is incorporated into the RNA-induced silencing complex (RISC). The other strand is designated the "passenger strand", and is degraded. The RISC includes the Argonaute proteins (Ago 1-4), along with TAR RNA-binding protein (TRBP) and other factors[2]. Ago2 cleaves mRNA showing complementarity to the loaded miRNA fragment[14,15].

The loaded RISC complex associates with cellular mRNA fragments and binds to the 3' untranslated region (3'UTR) of target mRNA exhibiting complementarity. The seed sequence of a miRNA is the sequence of 7-8 nt in the 3' end, which exhibits the greatest complementarity to its target, and may exist in multiples[16,17]. When a miRNA is of identical sequence to the mRNA transcript produced in a cell, it produces

transcriptional repression through the destruction of complementary mRNA transcripts[15,18]. Perfect complementarity is common in plant miRNA and some classes of eukaryotic organisms, but is far less common in humans[8].

When miRNA is of similar sequence to a mRNA transcript produced in a cell, translational repression results through inhibition of translation of target mRNA[15,18]. The RISC/mRNA complex is transported to P bodies in the cytoplasm, where RNA remodeling components are located[15,19]. The association of miRNA with the RNA-induced initiation of transcriptional silencing complex allows homing to regions of DNA and recruitment of histone-modifying enzymes to alter the chromatin structure and repress the transcription of genes complementary to the guide miRNA strand[20].

MicroRNA and their role in genomic and proteomic expression regulation

MicroRNA play an important role in the regulation of genomic and proteomic expression. Computational analysis of miRNA and their potential genomic targets has shown that miRNA may potentially regulate up to 30% of the protein coding mRNA in human cells[21]. Further, functional studies imply a significant role of miRNA in many cellular processes[2], including cell proliferation, apoptosis, stress response, neurodegeneration, hematopoiesis, and oncogenesis[17]. Recent work shows that over half of mammalian cellular messages are under selective pressure to remain complementary to and regulated by cellular miRNA[18,22]. Furthermore, specific expression profiles can be imposed upon genes when miRNA are located in their introns[6,17].

MicroRNA repression exhibits multiplicity and cooperativity; one miRNA can have binding sites in multiple targets, and one target can be repressed by multiple different miRNA[23]. Thus, each miRNA may regulate hundreds of targets. This suggests that miRNA may play some regulatory role in every biological process and/or pathway[17]. Furthermore, some miRNA are cell-specific. For example, miR-1 and miR-133 are muscle-specific miRNA, transcribed on the same polycistronic miRNA from the same chromosomal loci during development, functioning to control muscle gene expression and embryonic development[24]. Overexpression in skeletal muscle shows that miR-133 promotes proliferation, whilst miR-1 promotes myogenic differentiation. Furthermore, altered miRNA profiles lead to phenotypic variations in heart development[17,24].

Dysregulation of microRNA in human diseases

Dysregulation of cellular miRNA has been implicated in many forms of pathogenesis and human disease, most notably in cancer biogenesis. The genetic mutations that may generate a cancerous phenotype in protein-coding genes, such as rearrangements, deletions, and oxidative damage, can also occur in the DNA segments encoding miRNA. MicroRNA can act as tumor suppressors by suppressing oncogenic transcripts, or act as oncogenes themselves, if they repress the activity of a tumor suppressor transcript[25]. MicroRNA are commonly located in "fragile sites" – regions experiencing higher than usual rates of DNA mutation, and often regions affected by chromosomal alterations in cancer[3].

The first evidence that miRNA alterations and dysregulation may contribute to human disease occurred in 2002, when Calin, et al. discovered that two miRNA, miR-15a and miR-16a, are encoded at 13q14, a locus that is commonly (≅ 68%) deleted in B-cell chronic leukemia, the most common adult leukemia in the Western world[5]. Furthermore, the karyotype of patients predicts disease severity and prognosis[3]. These miRNA act as intrinsic tumor suppressors and when lost, enable cancer development. MicroRNA profiling of cancerous cells in tumors can be incredibly informative, and reflects the developmental lineage and differentiation state of a tumor. A general trend towards downregulation of cellular miRNA is seen in cancerous tissues compared with normal tissues[26]; however, both up- and down-regulation of specific miRNA may be seen in cancerous cells[25].

Dysregulated human microRNA in viral infections

Viruses are known to subvert and manipulate the human gene machinery and miRNA are no exception. Host miRNA are dysregulated by viruses, and both DNA and RNA viruses have been found to encode and express miRNA in the infected host, demonstrating the importance of the role of host- and virus-encoded miRNA in host-virus interactions. Although the role and functions of virally encoded miRNA are still unclear, recent analyses of target genes suggest their probable role in immune modulation and evasion by directly targeting proapoptotic genes and controlling viral latency, encouraging viral persistence in the host[27]. Nonetheless, virus-encoded miRNA bear complementarity with

host mRNA, as host-encoded miRNA bear complementarity with viral genes. This is an important process, which guides pathogenic events of host/virus interactions during viral infections and disease progression.

Evidence suggests that human miRNA expression patterns are affected by different viral infections in different ways, with different cellular miRNA acting as critical factors in each case. For example, when human cells are infected with primate foamy virus 1 (PFV-1, a retrovirus akin to HIV-1) a host miRNA, miR-32, limits viral replication and accumulation in cells through RNA interference and silencing[8,28]. As a counter-attack, PFV-1 produces Tas, a broadly effective suppressor of silencing. In hepatitis C, the virus (HCV) exploits the host cellular miRNA. MiR-122 binds selectively to the 5' non-coding end of the viral genome[8,29], encouraging viral accumulation in the hepatocytes. Infection of primary cultured B-cells with Epstein Barr virus (EBV) results in significant downregulation of cellular miRNA expression[30]. One native human miRNA, miR-146a, is downregulated on initial infection, but is significantly upregulated as the virus enters its lytic phase, inhibiting the viral lifecycle. These findings suggest that downregulation of tumor suppressors and antiviral miRNA provides advantage to EBV, an oncogenic virus associated with many forms of malignancy including lymphomas and carcinomas[31].

Computational analysis of the miRNA in the human genome shows that over 200 predicted miRNA exist that can potentially exert an antiviral effect on viral mRNA. Furthermore, the majority of the viral genomes exhibiting complementarity to these miRNA are single-stranded RNA viruses, suggesting the influence of miRNA-mediated antiviral defense in humans[32].

HIV infection affects and subverts host microRNA

The miRNA expression profile of cells is altered in both clinical peripheral blood mononuclear cell (PBMC) samples and cultured cells. The RNA silencing suppressor (RSS) Tat, encoded by HIV-1, alters the host miRNA expression profile to prevent inhibition of HIV-1 replication by host cells. Deactivating mutations in the Tat region of the HIV-1 genome reduces HIV-1 downregulation of 22 cellular miRNA in lymphocytes, indicating the role Tat plays in attenuating the cellular response to HIV infection[33]. Furthermore, Triboulet, et al. showed that HIV-1 infection leads to suppression of host PBMC miRNA miR-17-5p and miR-20. In the absence of this suppression, HIV-1 replication in the cells is enhanced[34]. Table 1 shows all the miRNA known in the functional context of HIV infection along with their targets and their effect on HIV infection.

Dysregulation of host microRNA in HIV Infection: Human microRNA correlating with plasma viremia and elite suppression

Cells transfected with an infectious HIV-1 strain have significantly altered miRNA profiles compared to control cells[35]. In 2008, Houzet, et al. showed that the vast majority of host miRNA are downregulated in HIV infection of PBMC. When individuals were separated into groups based on their T-cell counts and viral loads, specific miRNA "profiles" could be seen for each of the classes. Furthermore, many miRNA changes in host cells could not be accounted for by infection alone, suggesting a complex role of miRNA in host gene regulation[36].

In 2007, Huang, et al. showed overexpression of host miRNA in resting T-cells that target sequences in the 3' end of HIV-1 RNA, silencing viral mRNA and enforcing latency[37]. Furthermore, a 2012 study by Witwer, et al. has found that PBMC miRNA profiles can distinguish elite suppressors and uninfected controls from viremic HIV-1-infected patients[38]. The study found correlations between miRNA expression profiles and $CD4^+$ T-cell count and viral load; however, elite suppressors and viremic patients showed similar expression patterns for some miRNA. Some miRNA found to differ in expression have previously been shown to correlate to HIV-1 latency, including miR-29s, miR-125b and miR-150. Their analysis identifies several miRNA that have not been previously described in association with HIV infection, including miR-31, which distinguishes controls and elite suppressors from viremic individuals and regulates a protein with important implications for T-cell differentiation. Although this study has also shown that HIV-1-positive elite suppressors are characterized by a PBMC miRNA profile that in general resembles that of uninfected individuals, they also reiterate that the elite suppressors, on the basis of miRNA expression, are a heterogeneous group. This suggests that different mechanisms, shaped or marked by different miRNA expression patterns, underlie sustained and durable control in therapy naive HIV-infected individuals. Recently at the IAS meeting in Washington DC, Zhu, et al. showed a set of 18 differentially expressed miRNA, which could identify the outcome of HIV disease at the chronic stage more accurately, independent of currently available clinical prognostic factors: CD4 T-cell count and viral load. Six out of 18 miRNA were significantly related to a faster rate of CD4 decline[39].

Table 1. Host microRNA targeting viral genes

MiRNA name	Target	Mechanism of action against HIV-1	Cell types examined
miR-29a[2,45,46]	Nef 3'LTR/UTR	Downregulates Nef protein expression and interferes with HIV-1 replication. Inhibits viral production and infectivity. Binds Nef 3'LTR at position 420, or NL4-3 HIV strain at 9206	HIV-infected human T lymphocytes, human PBMC and infected CD4+ T-cells *in vitro*.
miR-28*†[37,41,42,46] miR-125-b*[37,42,46] miR-150*†[37,41,42,46] miR-223†[37,41,46] miR-382*†[37,41,42,46]	3'UTR	Enriched in resting CD4+ T-cells, enforces latency, reduces protein translation and virus production. *Reduced expression in the PBMC of opioid dependent individuals. †Aid in resisting infection in monocytes and macrophages. miR-150 binds Nef 3'LTR at position 89, 773 or NL4-3 HIV strain at 8875, 9559. miR-223 binds Nef 3'LTR at position 408, or NL4-3 HIV strain at 9194.	Peripheral blood monocytes, resting primary CD4+ T-cells and infected CD4+ T-cells *in vitro*.
miR-17-5p[34,89] miR-20a[34,89]	3'UTR of PCAF acetyltransferase	Indirect – inhibits HIV-1 infection.	PBMC from infected donors and latently infected cells.
miR-198[90]	Cyclin T1	Indirect – inhibits HIV-1 replication in monocytes by repressing cyclin T1 expression.	Monocytic cell line.
miR-29b[46]	Nef 3'-LTR	Binds Nef 3'LTR at position 420, or NL4-3 HIV strain at 9206.	Infected CD4+ T-cells *in vitro*.
miR-29c[46]	Nef 3'-LTR	Binds Nef 3'LTR at position 420, or NL4-3 HIV strain at 9206.	Infected CD4+ T-cells *in vitro*.
miR-24[46]	Nef 3'-LTR	Binds Nef 3'LTR at position 766, 444, 503, 68 or NL4-3 HIV strain at 9552, 9230, 9289, 8854.	Infected CD4+ T-cells *in vitro*.
miR-15a[46]	Nef 3'-LTR	Binds Nef 3'LTR at position 724, 156 or NL4-3 HIV strain at 9510, 8942.	Infected CD4+ T-cells *in vitro*.
miR-15b[46]	Nef 3'-LTR	Binds Nef 3'LTR at position 724,156 or NL4-3 HIV strain at 9510, 8942.	Infected CD4+ T-cells *in vitro*.
miR-16[46]	Nef 3'-LTR	Binds Nef 3'LTR at position 724, 156 or NL4-3 HIV strain at 9510, 8942.	Infected CD4+ T-cells *in vitro*.
miR-1224-3p[46]	Nef 3'-LTR	Binds Nef 3'LTR at position 193, or NL4-3 HIV strain at 8979.	Infected CD4+ T-cells *in vitro*.

miRNA miR-122, miR-370, miR-373 and miR-297 are also upregulated in infection but their purpose is unknown thus far[34].
*Reduced expression in the PBMC of opioid dependent individuals. †Aid in resisting infection in monocytes and macrophages.
miRNA: micro RNA; LTR: long terminal repeat; UTR: untranslated region; PBMC: peripheral blood mononuclear cell.

Studies of larger cohorts of individuals are needed to address miRNA specific to different stages of HIV disease and explain the underlying genomic basis of natural control of HIV in therapy naive elite suppressors.

Peripheral blood monocytes express anti-HIV microRNA

Although host miRNA are dysregulated by HIV infection, they also play a significant role in countering viral infections. Even though both monocytes and macrophages have essential components for HIV-1 entry, peripheral blood monocytes are refractory to HIV infection both *in vivo* and *in vitro*. In contrast, tissue macrophages and monocyte-derived macrophages *in vitro* show high susceptibility to HIV infection with HIV-1 R5 tropic strains. Peripheral blood monocytes are infrequently infected by HIV-1 and rarely harbor virus as a latent reservoir[40]. This significant trait of monocytes is explained by the presence of significantly higher levels of anti-HIV-1 microRNA (miR-28, miR-150, miR-223, and miR-382) in freshly isolated monocytes from peripheral blood than their monocyte-derived macrophage counterparts. The suppression of these anti-HIV-1 miRNA in

monocytes possibly facilitates HIV-1 infectivity, and an increase of the anti-HIV-1 miRNA expression in macrophages inhibits HIV-1 replication. These findings provide evidence at the molecular level to explain the refractory nature of monocytes to HIV-1 infection and to support the notion that intracellular anti-HIV-1 miRNA-mediated innate immunity may have a key role in protecting monocytes/macrophages from HIV-1 infection[41]. However, the same analysis could not demonstrate that low-level expression of both CCR5 and CD4 is not the more significant factor in explaining their relative resistance to infection, despite higher levels of anti-HIV miRNA in monocytes. Overall, these studies imply that the action of host anti-HIV-1 miRNA may produce some protection from HIV-1 infection. Supporting these findings, opioid treatment of monocytes has been shown to reduce their intrinsic anti-HIV-1 miRNA levels. This mirrors the finding that heroin-dependent HIV-infected individuals have lower levels of anti-HIV miRNA in their peripheral blood monocytes, increasing susceptibility of the cells to HIV infection[42]. These observations attest the intrinsic role of miRNA in antiviral control. Recently, Swaminathan, et al.[43] have commented on the study of Wang, et al.[41], which describes a for and against view on anti-HIV miRNA in monocytes.

Human microRNA show complementarity to HIV genes

Hariharan, et al. showed that host miRNA expressed in human T-cells show complementarity to the mRNA of HIV-1 accessory proteins in highly conserved regions[44]. Using a consensus scoring approach and four well-established target predication softwares, high-scoring miRNA-target pairs were selected. Hsa-mir-29a and 29b target the nef gene, hsa-mir-149 targets the vpr gene, hsa-mir-378 targets env, and hsa-mir-324-5p targets the vif gene, with the target regions being highly conserved across the various clades of HIV-1. Effective inhibition by these miRNA would affect viral infectivity, integration of the provirus into the genome, viral gene expression, cell cycle arrest and apoptosis, and viral particle production[44]. Nef, whose mRNA is inhibited by miR-29a and miR-29b, is critical in HIV-1 disease progression, and functions early in the HIV-1 lifecycle to repress CD4, promote virion release, and establish long-term infection. HIV-1 virus with Nef deletion produces a delay in the appearance of AIDS symptoms and in some cases, even after 10 years of infection, patients show no symptoms. Overexpression of miR-29a reduces viral replication and Nef levels[45]. Recently Sun, et al. have shown, using miRNA array analyses of *in vitro* HIV-1-infected $CD4^+$ cells, that the miR-223 levels were significantly enriched in HIV-1-infected $CD4^+CD8^-$ PBMC and that miR-29a/b, miR-155, and miR-21 levels were significantly reduced. They suggest that based on the potential for miRNA binding sites in a conserved sequence of the Nef-3'-long terminal repeat (LTR), several host miRNA potentially could affect HIV-1 gene expression. Among those miRNA, the miR-29 family has seed complementarity in the HIV-1 3'UTR, but the potential suppressive effect of miR-29 on HIV-1 is severely blocked by the secondary structure of the target region. Their data provides support for a possible regulatory circuit at the peak of HIV-1 replication, which involves downregulation of miR-29, expression of Nef, the apoptosis of host CD4 cells, and upregulation of miR-223[46].

The 3' ends of HIV-1 mRNA are targeted by a group of cellular miRNA including miR-28, miR-125b, miR-150, miR-223, and miR-382. All of these are enriched in resting T-cells compared to active T-cells and act to enforce latency and inhibit viral replication. Specifically inhibiting the action of these miRNA alters viral replication[37]. MiR-29a is highly abundant in HIV-1-infected human T-cells, and specifically targets the HIV-1 3'UTR region. When miR-29a is inhibited, viral replication is enhanced, but when miRNA is enhanced, viral replication is suppressed[47]. Recently, Gana, et al., at the recently concluded IAS Meeting in Washington DC, concurred with these findings. Their work showed that the cellular miRNA, namely hsa-miR-29a, hsa-miR-20a, hsa-miR-330-5p, and hsa-miR-191, suppressed HIV-1 replication, while hsa-miR-511 enhanced viral antigen production. Immunoblotting analysis suggests that hsa-miR-29a directly binds to HIV-1 Nef mRNA, while hsa-miR-20a, hsa-miR-330-5p, hsa-miR-191, and hsa-miR-511 bind to cellular factors that indirectly influence HIV-1 replication[48]. This further goes to show the role of miRNA in latency, and that targeting the dysregulated miRNA is a possible tool for eradicating HIV.

Overall, this complementarity between host miRNA and viruses, including HIV, is of considerable evolutionary significance, suggesting that such interactions between host and virus have evolved in concert over time and, therefore, are intrinsically related.

Does HIV-1 encode its own microRNA and a suppressor of RNA silencing? A for and against perspective

The question whether HIV encodes its own miRNA and is potentially involved in host pathogenesis is intriguing

and a hotly debated issue, particularly given the documented participation of host miRNA in the replicative cycles of a variety of mammalian viruses.

In 2004, Pfeffer, et al. discovered virally derived miRNA in human cells latently infected with EBV[49], and then went on to identify other pathogenic miRNA in virally infected cells using gene prediction and small-RNA cloning[50]. Since then, miRNA have been found in large DNA viruses and smaller RNA retroviruses, including HIV. However, it is still being debated whether HIV encodes its own miRNA. This is because retroviral transcription emanates from host machinery similar to that directing cellular miRNA expression. Retroviruses (small, enveloped RNA viruses, replicating by reverse transcription to integrate a provirus into the host genome) are considered the most likely of all RNA viruses to encode miRNA; however, a study by Tuschl, et al. did not identify any virally derived miRNA from HIV-infected cells by molecular cloning techniques, even after screening over 1,500 amplicons[50]. In contrast, Fuji, et al. have reported evidence for a miRNA within the nef region of the genome, as judged by cloning and Northern blot analysis, and proposed a role for it in downregulation of viral transcription based on preliminary studies with reporter genes[51,52]. But the mechanism that could account for such a phenotype is unclear, as is the reason for the disparity between the two different cloning studies. The recent evidence of miRNA encoding sequences in the HIV-1 genome, and the discovery of functional virus-derived miRNA, further suggests a role for RNA interference (RNAi) in the regulation of HIV-1 gene expression[53].

Another study also reported that HIV encodes small RNA derived from hairpin structures composed of a 19 base pair perfectly complimentary stem and a small loop – a structure somewhat similar to endogenous miRNA but most reminiscent of exogenous engineered small hairpin RNA (shRNA)[54]. Bennasser, et al. found five potential pre-miRNA in the HIV-1 genome; near TAR, in the capsid Gag, near the Gag-Pol frameshift, in the Nef gene, and in the 3'LTR[55]. The 10 miRNA that could be produced could then potentially target large numbers of cell transcripts and account for the gene landscape changes seen in HIV-1 infection. As mentioned previously, a strong correlation exists between regions of the HIV-1 genome and the sequence of proteins expressed by $CD4^+$ T-cells and macrophages, including CD28, CTLA-4 and some interleukins[56], further suggesting the translational repression potential of HIV-1 miRNA.

According to Pfeffer, et al., the probability of virus families other than large DNA viruses encoding miRNA was predicted to be low[50]. According to findings of Cai, et al., EBV (a herpesvirus, with a double-stranded DNA genome) expresses 17 miRNA in infected cells[57]. In EBV, induction of lytic phase replication enhances expression of many viral miRNA, whose expression has differential regulation, suggesting roles for these miRNA in the infection of different human tissues. In Kaposi's sarcoma-associated herpesvirus (KSHV), another pathogenic human herpesvirus, 11 distinct miRNA are expressed at detectable levels in latently infected cells, suggesting the role of these miRNA for the establishment and maintenance of KSHV latent infection[58]. Contradictory to these findings of miRNA in DNA viruses, research by Cai, et al. shows that cells infected with the double-stranded DNA (dsDNA) virus human papillomavirus 31 (HPV31) do not show evidence of the production of viral miRNA, and suggests that human papillomaviruses in general do not express viral miRNA[59].

In agreement with the observations and hypothesis of Pfeffer, et al., miRNA cloning from cells infected with HIV-1 and human T-cell leukemia virus-1 (both RNA retroviruses) failed to identify any viral miRNA[60]. Pfeffer's research with HCV and yellow fever virus, both of which are positive strand RNA viruses, also did not result in the identification of any viral miRNA, which is intriguing.

Speculation also exists regarding the activity of Tat as a suppressor of RNA silencing (SRS). HIV infection triggers antiviral miRNA defense in human cells. To combat this, HIV's Tat protein possesses an SRS function, whereby it subverts the cell's RNA-silencing defenses by inhibiting the activity of Dicer[34,54,61]. This limits the ability of the cell to process precursor dsRNA into siRNA capable of inhibiting viral replication. When the affects of Tat are negated, HIV-1 replication is far more significantly repressed by cellular miRNA[15]. Vpr and Nef have also recently been demonstrated as additional HIV-1 proteins that act to suppress Dicer[15,62].

In summary, although others have reported the successful detection of HIV-1 miRNA[52,63], the question of whether HIV-1 miRNA do exist remains an open area of investigation. The following is what is currently known of possible HIV-encoded miRNA.

TAR microRNA

The TAR miRNA is a 50 nt hairpin structure encoded at the 5' end of the viral mRNA. TAR miRNA is capable

of binding both TRBP and Dicer. Loss of TRBP leads to loss of RNA silencing capability[64]. The TAR miRNA has been shown to downregulate viral gene expression and affect the host cell cycle. It also downregulates host cell machinery involved in replication, receptor signaling, repair, apoptosis, and mitochondrial function, protecting the infected cell from stress-induced cell death and prolonging its lifespan[65,66].

Nef microRNA

Nef is an accessory gene, highly conserved in HIV-1, HIV-2, and SIV. It is encoded at the 3' end overlapping the 3' LTR, is highly expressed in early infection, and is required for maintenance of high viral loads during the course of persistent infection of the host[67]. Nef miRNA has already been shown in HIV-infected cells, and functions to downregulate HIV-1 transcription and Nef protein expression[51,52,68], contributing to long-term nonprogressor status clinically.

MiR-H1

MiR-H1 is an 81 nt stem loop downstream of two NFKB sites in the HIV-1 LTR. MiR-H1 has been demonstrated to degrade the product of apoptosis antagonizing transcription factor, resulting in decreased cell viability, and acting antagonistically to the antiapoptotic effects of TAR miRNA[69]. Furthermore, as a result of HIV's error prone reverse transcriptase, individual miRNA sequences may correlate with other AIDS-related conditions, including AIDS-related lymphoma and HIV-1-associated dementia[70].

Cellular microRNA in HIV latency

The latency of HIV-1 in resting primary CD4+ T-cells is the biggest impediment for the eradication of the virus in patients receiving antiretroviral therapy. It is known that several restriction factors that act upon different stages of the viral lifecycle are able to contribute to HIV latency. Huang, et al. have shown that cellular miRNA potently inhibit HIV-1 production in resting primary CD4+ T-cells[37]. They found that the 3' ends of HIV-1 mRNA were targeted by a cluster of cellular miRNA, including miR-28, miR-125b, miR150, miR-223 and miR-382, which were enriched in resting CD4+ T-cells as compared to activated CD4+ T-cells. Specific inhibitors of these miRNA substantially counteracted their effects on the target mRNA, measured either as HIV-1 protein translation in resting CD4+ T-cells transfected with HIV-1 infectious clones, or as HIV-1 virus production from resting CD4+ T-cells isolated from HIV-1-infected individuals on suppressive HAART. Their data indicate that cellular miRNA are pivotal in HIV-1 latency and suggest that manipulation of cellular miRNA could be one of the novel approaches that may provide avenues for purging latent HIV-1 reservoirs.

MicroRNA and antiviral therapy

The siRNA, shRNA and miRNA structures have all been shown capable of inducing the RNAi pathway in human cells[71] and as such RNAi treatment using any of these constructs is of great interest in the potential future genetic treatment of HIV infection. Inhibition of HIV-1 replication in primary CD4+ T-cells through RNAi has been reported, interestingly including the inhibition of HIV-1 prior to reverse transcription[72]. Studies have shown that siRNA against HIV proteins Gag, Pol, Int, Vpu[73], Tat, Rev[74], Vif and Nef[75] amongst others, can all limit target expression and inhibit viral replication in human cell lines, including T-cells and primary lymphocytes. Provision of RNAi against Tat through a human miRNA backbone is 80% more effective at reducing HIV-1 p24 antigen in human cells than straight shRNA delivery[76].

The most effective way to inhibit HIV-1 replication through RNAi appears to be through the targeting of multiple sites in the HIV-1 genome at lower concentration[72] or through the collaborative use of different types of therapies. For example, immature cells vector transduced with a lentiviral vector containing shRNA targeting Tat and Rev, a transactivation response (TAR) decoy and a CCR5 ribozyme give rise to phenotypically normal, transgenic T-cells that resist HIV-1 infection *in vitro*[77]. In another study, the complementary use of antibody and siRNA allowed specific targeting of T-cells and prevented loss of CD4+ T-cells in a humanized mouse model of HIV-1, with no evidence of toxicity[78,79].

Furthermore, it is possible that a potential antiviral therapy might also be achieved by targeting host miRNA involved in the infection and propagation process directly using antagonists or inhibitors. In HCV infection, host miRNA miR-122 in the liver binds selectively to the 5' end of the HCV genome, encouraging viral accumulation and survival in the hepatocytes[29]. Miravirsen, a specific inhibitor of miR-122, recognizes and sequesters miR-122, rendering it unavailable to the virus, thereby decreasing HCV viremia[80]. In chronically

infected chimpanzees, trials have showed the effectiveness of miravirsen as a anti-miR-122, which was well tolerated and lead to long-lasting suppression of viremia with no evidence of viral resistance[81,82].

In human phase I and IIa trials, administration of miravirsen led to dose-dependent antiviral activity, which was maintained for more than four weeks after the conclusion of treatment, with some patients becoming HCV RNA undetectable during the study[83]. Miravirsen is the first miRNA-targeted drug to enter human clinical trials, and this proof of concept provides hope that targeting dysregulated miRNA as targets may be achievable against HIV, given further examination[81].

Various pharmaceutical companies have started studies on creating and finding viable therapeutic candidates that target miRNA through appropriate inhibitors and miRNA mimetics to treat diverse diseases such as viral infections, neurological disorders, cancers, and cardiovascular/metabolic disorders[84]. Thus, miRNA are making their way as the most attractive therapeutic, diagnostic, and prognostic targets. Furthermore, a number of pre-clinical and clinical trials are being currently conducted, but only two in the area of viral infections (miR-122 for HCV) and an unspecified miRNA therapy for HIV by Rosetta Genomics, Columbia University-CUMC, USA, whereas all others are in the area of cancers (leukemia, lymphoma, carcinoma melanoma, etc.) and metabolic disorders (cardiovascular diseases, hypercholesterolemia, hypertension, etc.)[85].

Possible control of HIV through a cell surface protein, human leukocyte antigen-C: A complex regulation by microRNA and new insights into HIV control by microRNA

HIV disease progression to AIDS is largely influenced by the host genetic make up, as is evident in examination of HIV-infected individuals who display slow, rapid, and non-progression of HIV disease. Variation in the HLA class I loci of the genome appears to have a stronger influence on the outcome of HIV disease than any other locus in the host genome. The human leukocyte antigen C (HLA-C) locus has been shown to be associated with HIV disease progression. HIV-positive individuals with high expression of HLA-C loci appear to progress slower to AIDS accompanied by better control of viremia than HIV-positive individuals with low expression of HLA-C loci[86,87].

As pointed out by Kerrigan in her commentary[87] to the article by Kulkarni, et al.[86], biological systems are inherently complex. Generally, the actions of various biological components are functionally intertwined and interdependent, such that the effects of manipulating one component may impinge on the functionality of other components. In fact, deciphering this kind of regulation is crucial for identifying the best therapeutic targets. This is exactly what the study by Kulkarni, et al. has shown as an example of such complexity, which can be utilized in the control of HIV/AIDS.

Kulkarni, et al. studied how miRNA regulate HLA-C expression. By sequencing and analyzing many different variants of the HLA-C locus they found that a particular miRNA (miR-148a) does not bind to its target site in some variants, resulting in high HLA-C expression. Further, using different naturally occurring and artificially created molecular variant constructs of the HLA-C mRNA at the miR-148a binding site and nearby regions, they were able to demonstrate that there is differentially regulated HLA-C expression attributed to nucleotide variations in the region. This team of researchers found the first evidence in favor of miR-148/HLA-C binding in direct determination of expression of HLA-C. As stated by Kerrigan, a clear understanding of these intricate details will help in developing new strategies for targeted drug development for treating AIDS.

Challenges confronting microRNA-based therapies

There are hurdles and challenges in any drug development process, and the process of designing miRNA-based therapies is no exception. This is largely because we know very little about their function and mechanism of action. Nonetheless, it is the fastest developing field in the area of genomic medicine and a number of miRNA candidates in early stage clinical trials show a great potential and promise for miRNA in diagnosis, prognosis, and treatment of a variety of diseases and infections.

One of the unique aspects of miRNA is gene regulation, which they carry out through targeting. Single miRNA can target hundreds and thousands of genes and one of the challenges will be to clearly delineate this language of functional interaction between miRNA and mRNA.

In the context of viral infections, including HIV, the use of RNAi therapies is potentially problematic for several reasons. If not selected properly, siRNA can

enhance rather than inhibit viral activity by increasing reverse transcriptase activity to generate escape mutants[73]. Furthermore, long-term RNAi targeting of Nef using a lentiviral vector with suppressive shRNA has been shown to select for HIV escape mutants that can escape RNA interference[88]. Taken together, it remains to be established whether successful inhibition of miRNA will yield clinically meaningful results.

Despite various ongoing clinical trials for miRNA-based therapies, a number of factors that will impinge on the success and outcome of these trials have not been properly addressed. One of the risks associated with off-target or non-specific effects of miRNA-based therapy is the fundamental one. Second, the precise biochemical role of factors (LOQS, PACT, HEN1, SE, etc.) associated with the miRNA biogenesis remains to be elucidated, which is critical to understand for effective drug targeting. The miRNA undergo enzymologic modifications through processes such uridylation, adenylation, and methylation, and the significance of these processes needs urgent attention as they may play a significant part in drug targeting and prove vital in designing of miRNA inhibitors. Third, drug delivery to the right anatomical area without side effects and technologies that can modulate sustained miRNA expression *in vivo* still remains to be determined. Even in the most successful trials in cell culture, only a maximum of a few days successful suppression occurs[72]. Converting results in a laboratory setting in cell culture to effective viral suppression *in vivo*, then, is a significant challenge for the future use of miRNA as a therapy for the treatment of HIV infection. Targeting and delivery of such treatments to the necessary cells of the body also remains a challenge, and the reader is referred to the literature for an examination of some of the techniques that have been and are being attempted[81].

In the case that dysregulated miRNA are targeted for antiviral treatment, it is essential that the potential cross-interactions of these treatments be thoroughly examined. As previously mentioned, miRNA function exhibits multiplicity and cooperativeness[23], and thus it will be essential to examine the many possible effects of altering the regulation of miRNA such that the administration of treatment for one condition does not cause the development of severe or irreversible side-effects. Nonetheless, as the master regulators of gene and protein expression, the miRNA hold a great therapeutic promise in the control of viral infections and many human diseases. Once the *in vivo* delivery and non-specificity of miRNA is worked out, they will not only be excellent targets for therapeutics, but will open the doors for new generations of biomarkers in the diagnosis and prognosis of a whole gamut of human and animal diseases.

Conclusion and future outlook

Viral miRNA regulation is an emerging component of the complex relationship that controls host-virus interactions. From miRNA targets identified based on sequence specificity with host mRNA, it is apparent that viral miRNA play a significant role not only in the viral lifecycle, but in determining disease course through host-virus interactions and subversion and manipulation of the host genome. This may have important implications in immune evasion by inhibiting immune surveillance and extending the life of the infected host cell. Thus, determination of such targets and their interaction during the disease course may provide a clear view of this host-virus interaction and may lead to new therapeutic strategies. Similarly, the identification of dysregulated miRNA during HIV infection may yield attractive targets for anti-HIV therapy. In this context, it is important to mention that because of the stability of miRNA in human tissues and fluids, they are emerging as a distinct class of biomarkers in diagnostics and prognostics. The role of these molecules in immune evasion is a critical factor not to be overlooked in the search for an HIV vaccine or genome-based treatment methods. Therefore, studying this novel class of viral post-transcriptional regulators has a great potential in both diagnosing and treating a range of human diseases and infections, including HIV.

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