RNA viruses and microRNAs: challenging discoveries for the 21st century

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1Microbiology and Immunology Graduate Program, Drexel University College of Medicine, Philadelphia, Pennsylvania; 2Department of Microbiology and Immunology, Drexel University College of Medicine, Philadelphia, Pennsylvania; and 3Center for Molecular Virology and Translational Neuroscience, Institute for Molecular Medicine and Infectious Disease, Drexel University College of Medicine, Philadelphia, Pennsylvania

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Swaminathan G, Martin-Garcia J, Navas-Martin S. RNA viruses and microRNAs: challenging discoveries for the 21st century. Physiol Genomics 45: 1035–1048, 2013. First published September 17, 2013; doi:10.1152/physiolgenomics.00112.2013.—RNA viruses represent the predominant cause of many clinically relevant viral diseases in humans. Among several evolutionary advantages acquired by RNA viruses, the ability to usurp host cellular machinery and evade antiviral immune responses is imperative. During the past decade, RNA interference mechanisms, especially microRNA (miRNA)-mediated regulation of cellular protein expression, have revolutionized our understanding of host-viral interactions. Although it is well established that several DNA viruses express miRNAs that play crucial roles in their pathogenesis, expression of miRNAs by RNA viruses remains controversial. However, modulation of the miRNA machinery by RNA viruses may confer multiple benefits for enhanced viral replication and survival in host cells. In this review, we discuss the current literature on RNA viruses that may encode miRNAs and the varied advantages of engineering RNA viruses to express miRNAs as potential vectors for gene therapy. In addition, we review how different families of RNA viruses can alter miRNA machinery for productive replication, evasion of antiviral immune responses, and prolonged survival. We underscore the need to further explore the complex interactions of RNA viruses with host miRNAs to augment our understanding of host-virus interplay.

microRNA; RNA virus; pathogenesis

TWENTY YEARS AGO, TWO SEMINAL papers demonstrated for the first time the existence of a small 21-nucleotide-long RNA molecule that controlled developmental timing in Caenorhabditis elegans by posttranscriptional regulation of the heterochronic gene lin-14 (88, 171). Intriguingly, the small RNA (which was called lin-4) contained antisense sequences that were complementary to a sequence within the 3′-UTR (untranslated region) of the lin-14 mRNA and did not code for protein (88, 171). The discovery of tiny regulatory RNAs in invertebrates did not stimulate the enthusiasm of the scientific community until 2001 when three independent groups provided evidence on the abundance of tiny regulatory RNAs in invertebrates (88, 171). miRNAs are emerging as reliable biomarkers for a variety of diseases (24, 105, 112, 131); and 4) recently the development of miRNA inhibitors (“anti-oligo-miRs”) or overexpression of miRNAs (“mimics”) has paved the way for a new class of drugs for treating infectious diseases and cancer (61, 79, 96, 121).

Research in immunology and infectious diseases has increasingly intersected with the field of miRNAs. miRNAs have been shown to play a role in development of innate immune responses and orchestration of adaptive immune responses (30). Our understanding of microbial pathogenesis and the generation of immune responses has been greatly influenced by miRNAs influence the expression of >60% of all mammalian genes (20). MiRNAs can be incredibly potent in modulating cellular status owing to their pleotropic effects on the expression of multiple proteins (4). This is possible because one miRNA has the ability to bind multiple host mRNAs in their 8- to 10-nucleotide “seed” sequence region and thus downregulate protein expression (46). Conversely, multiple miRNAs can target the same protein owing to their redundancy in binding sites. Such miRNAs mostly belong to the same miRNA family and often converge in regulation of specific cellular signaling pathways (7). Since their discovery, miRNA studies have revolutionized many fields in the biological sciences. For instance, 1) miRNA research has influenced our fundamental understanding of basic biology in a laboratory setting; 2) studies on miRNAs have provided us with valuable molecular tools to manipulate in vitro and in vivo systems (108, 138); 3) miRNAs are emerging as reliable biomarkers for a variety of diseases (24, 105, 112, 131); and
the growing body of evidence characterizing miRNAs (32, 143, 154). Moreover, research on miRNAs is opening exciting avenues for understanding host-pathogen interactions and for developing therapeutic approaches to treatment of infectious diseases, viral infections in particular (32). Indeed, the breakthrough discovery of the role of the host miR-122 in the hepatitis C virus (HCV) life cycle paved the way for the first miRNA-targeting treatment to enter into human clinical trials, a specific inhibitor of miR-122 [miravirsen, a β-D-2-oxy-locked nucleic acid (LNA)-modified phosphorothioate antisense oligonucleotide; Santaris Pharma, Hørsholm, Denmark] for the treatment of HCV infection (63). While the contribution of miRNAs in DNA virus-host interactions has been studied and reviewed extensively, the interplay of miRNAs in the pathogenesis of RNA virus infections is less understood. Here we review the existing literature on RNA virus-encoded miRNAs, the use of RNA viruses as engineered vectors for delivery of miRNAs, the modulation of host miRNAs by RNA viruses, and the challenges in translational application of our knowledge of these interactions.

**BIОGENESIS OF miRNAs**

MiRNAs can be expressed in all cells of higher eukaryotic organisms. MiRNAs are encoded in the mammalian genome as independent transcription entities or within multiple intron-specific regions of protein-coding genes. A typical miRNA biogenesis begins in the nucleus by binding of a specific transcription factor, and this initial process is very similar to a protein-coding host mRNA transcription. The recruitment of RNA polymerase II (or in fewer cases RNA polymerase III) generates the primary miRNA transcripts (pri-miRNAs) containing long stem-loop-like structures (Fig. 1). The pri-miRNAs are ~1–2 Kb in length and are processed to form a 5′-cap structure and undergo splicing to remove introns and an addition of a 3′-poly A tail, akin to regular host mRNA transcripts (20). Nuclear processing of the pri-miRNA transcripts is carried out by a microprocessor complex of proteins consisting of a member of the RNase III nuclelease superfamily called Drosha and a double-stranded RNA (dsRNA)-binding protein called DGC8 (the DiGeorge syndrome critical region 8) (83, 91). Drosha/DGC8-mediated cleavage results in miRNA transcripts that have a dinucleotide 3′-UTR overhang, and this cleaved product is called the precursor miRNA (pre-miRNA). This 3′-UTR dinucleotide overhang serves as a recognition sequence for the nuclear export protein exportin-5, belonging to the karyopherin family. Exportin-5 cooperatively binds to the pre-miRNA in the nucleus and GTPase RAN in its active GTP-bound form and exports the pre-miRNA to the cytoplasm (95, 111, 168). Once in the cytoplasm, a complex consisting of another RNase III protein called Dicer (by recognizing the 3′-end of the hairpin loop structure) and a multidomain human TAR element binding protein (TRBP) acts upon the pre-miRNAs to generate a 20- to 25-base pair duplex, which contains the final miRNA and its reverse complement, termed miRNA* (78). Interestingly, exportin-5 (the karyopherin responsible for pre-miRNA export) interacts directly with Dicer...
mRNA as part of a regulatory mechanism. Inhibition of exportin-5 downregulated the expression of Dicer, the RNase III required for pre-miRNA maturation (8), suggesting a novel cross-regulatory mechanism between pre-miRNA or viral small RNAs and Dicer through exportin-5.

The less stable of the two strands in the miRNA duplex is usually considered the miRNA (the other is called the miRNA* or the “passenger” strand). The detailed role of the “star partner” of miRNAs remains incompletely understood (174). Even though both strands could be functional, typically the miRNA alone is recruited in the multiprotein RNA-induced silencing complex (RISC), where the miRNA binds to its target host mRNA and regulates protein expression. The catalytic subunits of the RISC complex are proteins of the Argonaute family (Ago 1–4). These proteins may encode endonuclease activity (Ago-2) and may directly cleave host mRNAs. However, Ago proteins are crucial for miRNA functionality in the RISC complex, in addition to Dicer and TRBP. The existence of miRNA-mRNA duplexes that are not stoichiometrically bound by Ago proteins has been recently suggested (62). Visualization of direct Ago binding to miRNA-mRNA duplexes in live cells using fluorescence lifetime imaging microscopy has demonstrated that in contrast to the consensus view that Ago proteins bind miRNA duplexes, they can bind and repress miRNA-mRNA duplexes (62). This new evidence may support a model of catalytic Ago function in translational repression.

In the RISC complex, miRNAs bind the host mRNAs typically by sequence complementarity in the 3′-UTR region. Perfect sequence complementarity with an mRNA usually results in endonucleolytic cleavage of the mRNA, whereas imperfect complementarity has been suggested to result in faster polyadenylation of the host mRNA, resulting in repression of the protein translation (4, 19). Novel partners in this complex process are being identified. For instance, it was recently shown that the deadenylase CCR4-NOT removes poly(A) from mRNAs destabilized by miRNAs in human cells (120). Interestingly, Bethune et al. (10) have found that translational repression is the dominant effect of miRNAs on newly synthesized targets. This step is followed by mRNA deadenylation and decay, which is the dominant effect at steady state in human cells (10).

Emerging evidence suggests the existence of noncanonical miRNA biogenesis pathways that can produce functional regulatory RNAs. Canonical miRNA biogenesis requires the microprocessor components, Drosha and Dicer, to generate premiRNA, and Dicer to form mature miRNA. However, the microprocessor is not required for processing of some miRNAs in which spliceosome-excised introns are direct Dicer substrates, such as “mirtrons.” In this regard, biogenesis of the splicing-independent mirtron-like miRNAs, termed “simtrons,” does not require the canonical miRNA biogenesis components, Dicer, exportin-5, or Argonaute 2; rather they are bound by Drosha and processed in vitro in a Drosha-dependent manner (50).

**IMPACT OF CELLULAR miRNAs ON RNA VIRUSES**

Cellular miRNAs control multiple cellular processes (40, 44, 48, 64, 135). Most RNA viruses, compared with DNA viruses, have significantly smaller genomes (35). This has evolutionarily made them adapt to usurp host cellular processes to perform their essential functions (active replication and successful infection). Because miRNAs can control the expression of proteins (4), they may influence cellular tropism of viruses, modulate viral infectivity, and play a crucial role in inducing appropriate antiviral immune responses (32, 71, 89, 97, 159, 175). It is therefore not surprising that RNA viruses may regulate the expression of specific miRNAs for efficient replication as an ever-evolving survival strategy. Here we review the interaction of RNA viruses with various cellular miRNAs. Among the plethora of RNA viruses that are known to be affected by their interactions with cellular miRNAs, we discuss examples from three different families of RNA viruses that have unique replication life cycles: retroviruses (RNA viruses that reverse transcribe and undergo a DNA intermediate and a nuclear component as part of their life cycle) (153), flaviviruses (RNA viruses with unsegmented genomes that replicate specifically in the cytoplasm of host cells) (49), and orthomyxoviruses (RNA viruses with segmented RNA genomes that have a nuclear component as part of their replication strategy) (110). Cellular miRNAs that affect viral replication by binding to viral RNA directly or target host factors to indirectly affect virus replication are summarized in Tables 1 and 2.

**Retroviridae**

**Human immunodeficiency virus.** Numerous reviews have extensively summarized recent findings in miRNA-mediated regulation of human immunodeficiency virus (HIV)-1 infection (76, 147, 150, 151). In a seminal paper, Yeung et al. (176) showed changes in miRNAs when the human HeLa cell line was transfected with an HIV-1 molecular clone, pNL4.3. Triboulet and colleagues (157) showed that silencing important proteins in the miRNA pathway, such as Drosha and Dicer, resulted in faster HIV-1 replication kinetics in peripheral blood mononuclear cells (PBMCs) from infected donors or in latently infected cells. In addition, HIV-1 suppressed the expression of the polycistronic miRNA cluster miR-17/92 through a mechanism that was dependent on P300/CBP-associated factor (PCAF), the histone acetyltransferase Tat cofactor that is

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**Table 1. Cellular miRNAs that directly bind viral RNA to modulate virus replication**

<table>
<thead>
<tr>
<th>Virus</th>
<th>Family</th>
<th>miRNA</th>
<th>Effect</th>
<th>Study, Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human immunodeficiency virus (HIV-1)</td>
<td>Retroviridae</td>
<td>miR-28, miR-150, miR-223, and miR-382</td>
<td>inhibition</td>
<td>58</td>
</tr>
<tr>
<td></td>
<td></td>
<td>miR-29a</td>
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<td>1</td>
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<tr>
<td>Hepatitis C virus (HCV)</td>
<td>Flaviviridae</td>
<td>miR-122</td>
<td>enhancement</td>
<td>66, 97, 98</td>
</tr>
<tr>
<td></td>
<td></td>
<td>miR-199a</td>
<td></td>
<td>107</td>
</tr>
<tr>
<td>Influenza A virus (IAV)</td>
<td>Orthomyxovirida</td>
<td>miR-323, miR-491, and miR-654</td>
<td>inhibition</td>
<td>142</td>
</tr>
<tr>
<td></td>
<td></td>
<td>miR-let7c</td>
<td></td>
<td>104</td>
</tr>
</tbody>
</table>

miR or miRNA, microRNA.
required for HIV Tat transactivation. Overall, the authors suggested that Drosha and Dicer play key antiviral roles in human cells against HIV-1 (157). These studies were further extended to in vivo infections and led to the characterization of global miRNA changes in HIV-1-seropositive patients (57). By directly interacting with the HIV-1 RNA or by downregulating cellular factors that are essential for viral replication, cellular miRNAs could significantly inhibit HIV-1 replication. Interestingly, miRNAs can play a key role in restricting HIV-1 infection in monocytes and CD4+ T cells (27). Moreover, Wang et al. (169) have reported that a few “anti-HIV miRNAs,” including miR-28, miR-150, miR-223, and miR-382, that are present in monocytes but less abundant in macrophages directly target the 3’-UTR of the HIV-1 mRNA. The data might suggest that the lower susceptibility to HIV-1 infection of monocytes, compared with macrophages, correlates with the expression of these particular miRNAs. However, this specific alteration in the levels of miR-28, miR-150, miR-223, and miR-382 during monocyte differentiation into macrophages remains controversial, and thus the contribution of these miRNAs to monocyte/macrophage susceptibility to infection is still unclear (139). Enrichment of these miRNAs in resting (as compared with activated) CD4+ T cells (27). In addition, miR-29a was reported to reduce HIV-1 infection by directly binding the HIV-1 RNA or by downregulating viral miRNAs or by dramatically altering the level of cellular miRNA expression, these findings are controversial. Interestingly, comprehensive analysis of miRNA binding to the HIV-1 genome using the photoactivatable ribonucleoside-induced cross-linking and immunoprecipitation (PAR-CLIP) technique has revealed several binding sites for cellular miRNAs. Although a subset of these miRNAs were shown to be capable of mediating miRNA-mediated repression of HIV-1 gene expression, the extensive RNA secondary structure occluded the ability of cellular miRNAs to interact with viral miRNAs. These data suggest that HIV-1, rather than seeking to control miRNA function in infected cells, may have evolved a mechanism to become largely invisible to cellular miRNA effector mechanisms (170).

Much less is known about the importance of miRNA modulation in the context of HIV-1 and HCV coinfection. Thirty percent of HIV-1-infected individuals worldwide are estimated to be coinfected with HCV (150). To study the importance of miRNAs in this subset of HIV-infected patients, our group recently reviewed the potential implications of miRNAs in contributing to the interinfluential role of HIV-1 and HCV (47).

**Flaviviridae**

**HCV.** The dynamic interaction of HCV with the host miRNA pathway has been well noted. HCV infection results in modulation of multiple miRNAs that potentially aid in trafficking and/or nuclear import of the HIV-1 preintegration complexes (149). Uniquely, Chiang et al. (26) have reported that miR-132 enhances HIV-1 replication. They suggest that miR-132 targeting of MeCP2 (a cellular methyl CpG binding protein) may underlie this pro-HIV effect of miR-132, but the specific mechanism is yet to be defined (26). The significance of silencing particular miRNAs for reactivation and purging latent HIV-1 viral reservoirs has recently been reviewed (180). In contrast to activated CD4+ T cells and differentiated macrophages, resting CD4+ T cells and monocytes are nonpermissive for HIV-1 replication. We expect that the identification of miRNAs involved in T-cell activation and monocyte differentiation may help address the current challenge of how to activate latent viral reservoirs during or prior to antiretroviral therapy, with the goal of eliminating the reservoirs. Although some of the aforementioned data suggest that HIV-1 might subvert the function of the cellular miRNA machinery by expressing viral miRNAs or by dramatically altering the level of cellular miRNA expression, these findings are controversial.
replication, immune evasion, and survival strategies. Multiple recent publications have extensively reviewed the cellular miRNAs modulated by HCV infection (80, 117, 156). The interaction of HCV with the liver-enriched miRNA, miR-122, represents an exceptional example of how RNA viruses can usurp host cellular machinery for their benefit (65, 66). HCV encodes for two binding sites in its 5′-UTR region for miR-122 (66). Mechanistic studies have shown that binding of miR-122 to HCV RNA enhances RNA abundance, translation, and infectious virus production (126, 165). Mechanistically the role of miR-122 in the HCV life cycle is not fully understood. MiR-122 may promote cap-independent viral translation, enhance viral RNA stability, or facilitate de novo initiation of viral RNA synthesis. Recent studies show that miR-122 recruits Argonaute protein Ago-2 to the genomic RNA and mediates RNA stability and translation stimulation by slowing HCV RNA decay in infected cells (29). Li and colleagues (98) have investigated the decay pathways that mediate degradation of HCV viral RNA. Interestingly, they found that HCV RNA is degraded primarily by the cytoplasmic 5′-exonuclease Xrn1 in infected cells, whereas transfected HCV RNA is degraded by both the 5′-exonuclease Xrn1 and the 3′-exonuclease exosome complex. Although miR-122 acts to protect the viral RNA from Xrn1-mediated 5′-exonucleolytic decay, knockdown did not rescue replication of a viral mutant defective in miR-122 binding, indicating that miR-122 has additional as yet uncharacterized function(s) in the viral life cycle (29). Indeed, the same authors have suggested that miR-122 may function directly in viral RNA synthesis (97).

The impact of targeting miR-122 as an anti-HCV treatment was initially supported by the remarkable results from a chimpansie study of HCV infection in which an LNA-modified oligonucleotide specifically targeting miR-122 significantly reduced viral RNA levels (84). This led to the development of miravirsen, an miR-122 inhibitor, by Santaris Pharma (63). Miravirsen is the first miRNA-targeting therapeutic agent to enter into human clinical trials for treating infectious diseases (101). In a phase 2a trial, miravirsen treatment resulted in significant HCV control in infected patients, with efficient safety profiles. Although it was previously noted that escape variants to miR-122 antagonism-based HCV therapy could potentially occur (99), miravirsen treatment did not seem to lead to any HCV escape mutations. Thus it shows potential as a long-lasting, interferon (IFN)-free treatment for HCV infection (63). In contrast to anti-miR-122-based monotherapy, the design and delivery of specific combinations of miRNA antagonists and mimics to cure HCV infection or to limit liver pathogenesis have recently been suggested (53).

Apart from miR-122, other miRNAs have been reported to affect HCV infection. Thus, the HCV genome contains binding sites for miR-199a in the HCV internal ribosome entry site. However, unlike miR-122, miR-199a binding to the viral genome has been shown to inhibit HCV replication by targeting the viral RNA to the RISC complex (107). The cellular miRNA let7b was shown to bind to the HCV nonstructural protein NS5B and to the 5′-UTR and to significantly suppress HCV infection, although the exact mechanism is incompletely understood (25). Other cellular miRNAs that affect HCV infection include miR-196 (through inhibition of host protein Bach1 and targeting of the viral 5′-UTR) and miR-29 (through an unknown mechanism) (6, 56). Apart from the role of some cellular miRNAs in the HCV life cycle, HCV may modulate the expression of host miRNAs for immune evasion. For instance, HCV infection of hepatoma cells enhances miR-130 levels, which in turn inhibits an IFN-induced antiviral protein, IFITM, and therefore aids in accelerated HCV replication (11). In addition, miR-21 was recently shown to be upregulated by HCV infection, which negatively regulates the IFN pathway by affecting innate immune adaptor molecules such as MyD88 and interleukin-1 receptor-associated kinase 1 (IRAK1) (23). HCV modulation of miRNA profiles has also been reported to contribute to HCV-induced pathologies including hepatocellular carcinoma (14, 136, 137, 182) and mixed cryoglobulinemia (39). Interestingly, an increase in the levels of circulating, extracellular miR-122 has been reported in the presence of liver damage. The serum level of miR-122 correlated with serum ALT activity and with necroinflammatory activity in patients with chronic HCV infection and elevated ALT levels, but not with fibrosis stage or functional capacity of the liver (13). In addition, miR-122 is a significant predictor of the presence of chronic HCV infection (161). Therefore, miR-122 has a significant role in the HCV life cycle and in liver disease in patients with chronic HCV infection. However, the contribution of miR-122 to hepatitis and cirrhosis is less well understood.

West Nile virus and Dengue virus. The RNA interference (RNAi) pathway plays a substantial role in West Nile virus (WNV) and Dengue virus (DV) replication and pathogenesis in both the mosquito vector and the human host. Enhanced replication of DV was observed in mosquitoes depleted for RNAi factors, underscoring the importance of RNAi-mediated antiviral mechanisms in restricting virus replication (130, 173). Moreover, suppression of the RNAi pathway in the mosquito midgut induced emergence of diverse viral mutants in replicating WNV (16), suggesting that positive selection of WNV within mosquitoes is stronger in regions highly targeted by the host RNAi response. Interestingly, WNV and DV were reported to encode for a subgenomic flavivirus RNA (sfRNA) in their 3′-UTRs, which suppresses miRNA-induced RNAi functionality. Specifically, WNV sfRNA inhibits cleavage of dsRNA by Dicer (132). WNV increased the expression of a cellular miRNA, Hs_154, which contributes to WNV-induced apoptosis by expressing multiple antia apoptotic factors (140). DV decreased the expression of many proteins in the RNAi pathway, including Dicer, Drosha, and Ago2, and the DV nonstructural protein NS4B was reported to contribute to this effect (67). The crucial role of RNAi in controlling the replication of DV was further characterized by studying the unique resistance of the Aedes aegypti mosquito (one of the vectors of DV and WNV transmission) to DV replication when the mosquito contained the endosymbiotic bacteria Wolbachia (166). Recent studies investigating the mechanism underlying this observation showed that Wolbachia mediated an increase in the mosquito miRNA aae-miR-2940, which targets the host DNA methyltransferase gene (AaDnmt2), an important protein for successful DV replication (179). Finally, infection of PBMCs by DV has been shown to result in a modulation of their miRNA profiles, which has been implicated in DV-induced cytokine storm and shock syndrome (123). In addition, a recent paper showed that DV increases miR-146a to evade IFN-mediated inhibition by suppressing the innate immune-adaptor...
protein tumor necrosis factor receptor-associated factor 6 (TRAF6) (170).

**Orthomyxoviridae**

**Influenza A virus.** Posttranscriptional regulation of gene expression by miRNAs has been shown to be involved in the influenza virus replication cycle. Loveday and colleagues (102) characterized the differential expression in miRNAs signatures induced by infection with a low-pathogenicity swine-origin influenza A pandemic H1N1 (2009) or with a highly pathogenic avian-origin influenza A H7N7 (2003); the study revealed temporal and strain-specific miRNA fingerprints during the viral life cycle that might be crucial for understanding the differential disease outcomes between two different subtypes of influenza A virus (IAV). Bioinformatic prediction analysis showed that various cellular miRNAs could bind directly to IAV RNA, and among those miRNAs, miR-323, miR-491, and miR-654 inhibited IAV replication through binding to the viral PB1 gene (142). In addition, the cellular miRNA let-7c was reported to inhibit the expression of viral protein M1 in IAV-infected lung epithelial cells (104). IAV modulated global miRNA profiles in infected human lung cell lines including miR-7, miR-132, miR-146a, miR-187, miR-200c, and miR-1275, which could potentially target important proteins in the innate immune pathway to downregulate antiviral cytokines and chemokines (17). Bakre and colleagues (5) have identified miRNA-mediated regulation of host kinase genes that play an important role in IAV replication. Among various miRNAs, miR-34c induced an increase in expression of the host kinase, PLK4, which enhanced IAV replication (5). MiRNA global profiling in human lung epithelial cells (A549) infected by two different subtypes of human IAVs (H1N1 and H3N2) has identified a common miRNA signature in response to infection by the two different strains. Among the five miRNAs that were commonly deregulated, miR-21, miR-29a, miR-29b, and miR-452 were decreased, whereas miR-146a was the only miRNA that was upregulated in response to IAV infection. Interestingly, the inhibition of miR-146a expression was associated with a significant increase in influenza viral production, suggesting the involvement of miR-146a in the antiviral response through an unidentified mechanism. The ability of IAV to induce apoptosis and cytopathic effects in infected cells is well known. Using miRNA array analysis of IAV-infected A549 cells, Guan et al. (43) identified several apoptosis-associated miRNAs that were stimulated by IAV infection. In particular, miR-29c was significantly upregulated and inhibited Bcl-2-like protein 2 (BCL2L2) protein expression, thereby inducing cellular apoptosis in A549 cells (43). The data discussed here suggest that the study of miRNA modulation upon influenza infection may lead to a better understanding of host-pathogen interactions and may help guide development of novel therapeutic approaches. Moreover, unique miRNA profiles that are identified in response to influenza infection could serve as reliable diagnostic tools. Indeed, a recent publication by Song et al. (141) characterized unique cellular miRNA expression profiles in PBMCs of critically ill patients infected with IAV and reported that miR-31, miR-29a, and miR-148a are significantly upregulated, suggesting their potential use as biomarkers for advanced disease upon IAV infection.

**VIRUSES THAT ENCODE miRNAs**

Multiple recent reports have characterized the existence of virally encoded miRNAs (vmiRNAs) in DNA viruses (reviewed in Refs. 41, 75). Most of these studies have focused on miRNAs encoded by members of the Herpesviridae family (94, 118, 125). These vmiRNAs were shown to regulate fundamental stages in the herpesvirus life cycle: latency and the switch from latent to lytic replication (reviewed in Refs. 15, 31). Moreover, miRNAs encoded by viruses from the Polyomaviridae family (145, 146) and Adenoviridae family (3, 103) have been identified. It was also noted that a double-stranded DNA (dsDNA) insect virus, *Heliothis virescens ascovirus*, encodes for a miRNA (59). The potency of virally encoded proteins in downregulating host antiviral responses is well known (reviewed in Ref. 106). Remarkably, vmiRNAs can also inhibit the expression of important proteins in the antiviral signaling cascades (144, 164). Therefore, the combination of protein-mediated and vmiRNA-mediated interference in the generation and functionality of the host defense system provides a strong immune evasion strategy for viruses to prolong their survival. Although many groups have characterized virally encoded miRNAs in DNA viruses, whether RNA viruses encode miRNAs remains controversial (Table 3). Parmesanwaran et al. (115) studied six RNA viruses in 41 susceptible and resistant hosts for virally encoded small RNAs. The authors found that all six RNA viruses could express virally encoded small RNAs that had the ability to modulate host cellular miRNA profiles. Global sequencing analyses exploring whether RNA viruses such as HCV, yellow fever virus, and IAV encode for miRNAs have resulted in no detection in cell culture experiments (115, 118). An miRNA-like small RNA called KUN-miR-1 (WNV’s Kunjin strain) that upregulated the cellular protein GATA-4 to facilitate enhanced WNV replication in mosquito cells has been reported (60). Many hypotheses have been postulated as to why RNA viruses do not encode functional miRNAs. Most RNA viruses listed here replicate primarily in the cytoplasm (with the exception of influenza viruses), so they may be unable to interact with the microprocessor complex involving Drosha/ DGC8 in the nucleus. All of the RNA viruses studied so far in the context of virally encoded miRNAs are single-stranded

<table>
<thead>
<tr>
<th>Virus</th>
<th>Family</th>
<th>miRNA</th>
<th>Type of RNA</th>
<th>Study, Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>WNV, Kunjin strain</td>
<td>Flaviviridae</td>
<td>KUN-miR-1</td>
<td>miRNA-like small RNA</td>
<td>60</td>
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<tr>
<td>HIV</td>
<td>Retrovirdae</td>
<td>HIV1-miR-H1</td>
<td>miRNA</td>
<td>69</td>
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<tr>
<td></td>
<td></td>
<td>encodes for sequences similar to human miR-195, miR-30e, miR-374a, miR-424</td>
<td>miRNA</td>
<td>54</td>
</tr>
<tr>
<td>Bovine leukemia virus (BLV)</td>
<td>Retrovirdae</td>
<td>cluster of five miRNAs (BLV-miR-B1 to B5); one of the BLV miRNAs, miR-B4, has partial homology to human miR-29</td>
<td>miRNA</td>
<td>74, 127</td>
</tr>
</tbody>
</table>

**Table 3. RNA viruses encoding miRNAs**
RNA viruses. Notably, most of these belong to the positive-sense single-stranded virus category, which undergo transcription of the entire genome to form a polyprotein complex and proteolytic cleavage to generate functional viral proteins. Because a majority of miRNAs are generated from endonucleolytic activity, encrypting a noncoding, functional miRNA from its own genome for unproductive cleavage may be disadvantageous to the virus, suggesting another reason why RNA viruses do not encode miRNAs (118, 160).

However, noncanonical cytoplasmic processing of viral miRNAs has been reported. In particular, incorporation of a primary miRNA into a cytoplasmic virus resulted in the formation of a Dicer-dependent, DGCR8-independent, mature miRNA capable of conferring RNAi-like activity, suggesting the existence of a noncanonical, small-RNA-based activity capable of processing cytoplasmic hairpins and perhaps contributing to cellular antiviral responses (126). Remarkably, using a collection of miRNA-producing, recombinant Sindbis viruses, Shapiro et al. (133) demonstrated that processing of virus-derived cytoplasmic pri-miRNAs may not require nuclear involvement while maturation still relies on the canonical nuclear RNase III enzyme, Drosha. Interestingly, Drosha relocalization to the cytoplasm following viral infection did not affect the endogenous miRNA landscape during infection, although it allowed for robust synthesis of virus-derived miRNAs in the cytoplasm (133). This was the first report to describe a unique function for Drosha in the processing of highly structured cytoplasmic RNAs in the context of virus infection.

Members of the Retroviridae family represent a unique class of positive-sense single-stranded RNA viruses because they undergo reverse transcription to form a dsDNA intermediate that becomes integrated into the host genome (45). Localization of integration sites in the host genome usually determines the transcriptional activity of the integrated provirus and can lead to latency, although those latent proviruses may become active in response to changing cellular conditions. Researchers have investigated whether retroviruses such as HIV-1 may encode miRNAs. In silico analyses first showed that HIV-1 putatively encodes for five candidate pre-miRNAs (9). In addition, the HIV-1 viral protein Nef may encode for an miRNA termed Nef/U3 that is produced in HIV-1-infected cells. This miRNA was shown to suppress Nef protein’s function and overall HIV-1 virulence through the RNAi pathway (113, 114). Kaul et al. (69) showed that an HIV-1-encoded miRNA termed HIV1-miR-H1 downregulates the host cellular miRNA miR-149 that targets the viral accessory protein Vpr. Another recent study showed that the Env and Pol protein-coding regions of the HIV-1 genome encode several miRNA-like sequences that have homology to human miR-195, miR-30e, miR-374a, and miR-424 (54). Controversially, the existence of any HIV-1-encoded miRNAs has been disproven in other studies utilizing prediction-based computational algorithms, small RNA cloning strategies, and PAR-CLIP (100, 170). Therefore, although HIV-1 may encode for putative miRNA/siRNA sequences, whether it produces functionally active miRNAs is still highly contentious.

Although controversy continues regarding the existence of virus-encoded miRNAs in HIV-1 infection, Kincaid et al. (74) have shown that the bovine leukemia retrovirus (BLV) encodes a viral miRNA cluster that is transcribed by RNA polymerase III and that this viral miRNA shares sequence similarity to the host miRNA, miR-29, which is implicated in the pathogenesis of B-cell lymphomas. In addition, Rosewick et al. (127) confirmed that BLV encodes miRNAs and that these viral miRNAs result from the transcription of five independent transcriptional units located in particular proviral regions shown to be crucial for in vivo infectivity.

**ENGINEERING RNA VIRUSES TO ENCODE FOR miRNAs OR miRNA RECOGNITION SITES**

RNAi-mediated gene manipulation has achieved enormous potential as a therapeutic strategy for many diseases including viral infections (2, 33, 73, 124). Use of small interfering RNAs (siRNAs) and short-interfering RNAs (shRNAs) has provided some success; however, the use of viral vectors for delivery of miRNAs could be a more advantageous strategy. For example, using a recombinant adenovirus (rAd5), Chen et al. (21) reported successful delivery of shRNA against the foot-and-mouth disease virus that resulted in protection in vivo. Koppangi et al. (77) have reported potent inhibition of DV infection using a similar rAd5 strategy expressing shRNAs against all four serotypes of the virus. Although siRNAs and shRNAs can be specific and potent, manipulating miRNAs is an attractive strategy for gene therapy because one miRNA can modulate multiple target proteins that may participate in the same or different cellular pathways or processes. Two distinct strategies have been employed toward this end (Fig. 2): cloning miRNA target sites into viral genomes to restrict virus replication to specific cell types and/or modulate virus tropism, and engineering viruses to encode for miRNAs in order to manipulate host-cell functions or processes. Engineering of DNA viruses with miRNA recognition sites has been previously explored. By taking advantage of tissue-specific expression of miRNAs, Cawood et al. (18) (using a mouse model) and more recently Ylösmäki et al. (178) (using human liver tissues) have reported significant amelioration in oncolytic adenovirus-induced hepatotoxicity resulting from treatment with an rAd5 encoding for the liver-enriched miRNA-122 binding sites.

Although natural expression of miRNAs by RNA viruses remains controversial, RNA viruses are certainly capable of expressing miRNAs if they are artificially engineered (154) (Table 4). The existence of miRNAs that directly target viral RNAs and inhibit replication has been noted. For instance, it was reported that few “anti-HIV miRNAs” including miR-28, miR-150, miR-223, and miR-382 are present in monocytes that were infected with HIV-1. For example, engineering a perfect complementary target sequence could generate a functional antiviral miRNA, i.e., an artificially generated viral miRNA expressed upon infection that could inhibit its own replication owing to the traditional gene silencing effects of the RISC complex. In their seminal paper, Varble et al. (163) successfully demonstrated such a strategy. They showed that LAV, when cloned to incorporate a pre-miRNA sequence into its genome, can express a functional miRNA without any impact on viral growth (163). Rouha et al. (129) also demonstrated that tick-borne encephalitis virus, a cytoplasmic RNA virus belonging to the Flaviviridae family, was able to successfully express an engineered miRNA without impacting viral replication. These papers show that RNA viruses can express functional miRNAs, paving the way for
development of RNA viral vectors for expression of miRNAs of interest. Because several miRNAs have cell type-specific expression profiles, RNA viruses could be engineered to alter tropism, i.e., to inhibit or enable replication in specific cell types of interest. Multiple groups have utilized this approach with substantial success. Perez et al. (116) were the first to discover that a recombinant IAV that encodes for miR-93 binding sites (miR-93 is an miRNA expressed in all mammalian tissues) is self-restrictive in mice and generates potent immune responses. They proposed the feasibility of utilizing viruses that encode for miRNA recognition elements as recombinant vaccines (116). Using a similar strategy, the same group showed that IAV and DV could be engineered to have specific viral tropism by generating a recombinant virus containing miR-142 recognition elements (an miRNA expressed only in cells of hematopoietic origin) (85, 119). Moreover, Lee et al. (90) showed that DV could be engineered to exert the desired replication restriction effect by constructing a recombinant DV encoding for a miR-122 target sequence and thereby restricting its replication in the liver. Kelley et al. (72) were also able to attenuate vesicular stomatitis virus (VSV)-induced encephalitis in a mouse model by using VSV encoding for recognition sites for miR-125-MREs, miRNA response elements.

<table>
<thead>
<tr>
<th>Virus</th>
<th>Family</th>
<th>miRNA or miR-MREs</th>
<th>Study, Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>IAV</td>
<td>Orthomyxoviridae</td>
<td>miR-124</td>
<td>163</td>
</tr>
<tr>
<td>Tick-borne encephalitis virus (TBEV)</td>
<td>Flaviviridae</td>
<td>Epstein-Barr virus-specific miRNA, miR-BART2</td>
<td>129</td>
</tr>
<tr>
<td>IAV</td>
<td>Orthomyxoviridae</td>
<td>miR-93-MRE</td>
<td>116</td>
</tr>
<tr>
<td>DV</td>
<td>Flaviviridae</td>
<td>miR-142-MRE</td>
<td>85</td>
</tr>
<tr>
<td>Vesicular stomatitis virus (VSV)</td>
<td>Rhabdoviridae</td>
<td>miR-125-MRE</td>
<td>72</td>
</tr>
<tr>
<td>Chimeric tick-borne encephalitis/DV</td>
<td>Flaviviridae</td>
<td>MREs for miR-9, miR-124a, miR-128a, miR-218, or let-7c</td>
<td>51, 52</td>
</tr>
</tbody>
</table>

miR-MREs, miRNA response elements.

Table 4. RNA viruses engineered to express miRNAs or encode for miR-MREs

Fig. 2. Manipulation of RNA virus replication with miRNA. A: RNA virus tropism can be greatly altered, and viral replication can be restricted to specific cell type(s). This can be achieved by selection of an miRNA that is specifically expressed in a cell type of interest and cloning that miRNA’s recognition site into the noncoding region or protein-coding region of the viral genome. When such a recombinant virus enters the cells, the host cellular miRNA binds to and incorporates the viral RNA in the RISC and inhibits productive viral polyprotein expression via transcriptional repression or viral RNA degradation. B: RNA viruses can be engineered to overexpress miRNAs. RNA viruses that have a nuclear component in their life cycle (such as influenza A virus) can be engineered to encode for the pri-miRNA transcript that is acted upon by the nuclear microprocessor complex including Drosha and the DiGeorge syndrome critical region 8 protein (DGCR8). This then leads to the formation of a pre-miRNA that can be exported to the nucleus to be processed by cytoplasmic RNase III enzymes such as Dicer. For RNA viruses that do not have a nuclear component (such as flaviviruses), pre-miRNA sequences can be engineered in the viral genome to enable direct interaction with the Dicer complex and generation of a functional miRNA in the cytoplasm. This virally expressed artificial miRNA can then bind to and target the host miRNA and/or viral RNA to restrict or enhance infectivity. A similar strategy can used to engineer RNA viruses that express antisense sequences to inhibit cellular miRNAs.
for miR-125 (an miRNA expressed in the brain) and miR-206 (a muscle-specific miRNA). Finally, Heiss et al. (51) showed potent attenuation of neuroinflammation by a neurovirulent chimeric tick-borne encephalitis/DV that contained a single target site for each of the brain tissue-expressed miRNAs miR-9, miR-124a, miR-128a, miR-218, and let7c. These recombinant viruses were highly restricted in replication in primary neuronal cells, had limited access into the CNS of immunodeficient mice, and retained the ability to induce a strong humoral immune response in monkeys. In a mouse model of neurovirulence, the authors also characterized the escape viral mutants that arose and demonstrated that an increase in the number of miRNA target sites in the viral genome and the distance between the targets results in significant attenuation (51, 52).

Much of the work summarized above has dealt with engineering RNA viruses to encode for miRNA recognition sites to restrict virus replication and for generation of cell-specific immune responses or production of attenuated vaccine viruses. Another potential strategy for restricting viral infection (by using RNA viruses to encode for miRNAs) is to choose an RNA virus that is highly susceptible to a cellular antiviral mechanism such as type I IFNs. Viruses that are susceptible to type I IFNs include many RNA viruses such as enterovirus 71 (177) and VSV (158). Such an RNA virus could then be engineered to express an miRNA that targets a negative regulator of the type I IFN pathway. For instance, miR-146a enhances type I IFN production by targeting negative regulators such as IRF-5 and STAT-1 (152) and TRAF6, IRAK1, and IRAK2 (55). Another miRNA that has been shown to upregulate type I IFN expression is miR-155, through inhibition of the negative regulator SOCS-1 (167). Making recombinant viruses engineered to express such miRNAs would result in enhanced production of these antiviral cytokines and eventually result in inhibition of virus replication. Similarly, RNA viruses can be engineered to express antisense miRNAs or anti-oligo-miRs that would inhibit specific miRNAs or anti-oligo-miRs that would inhibit specific miRNAs that correlate with activation/inhibition of certain cellular pathways as well. Apart from utilizing this method for production of type IFN and eventual inhibition of virus replication, this strategy could also be used to modulate cellular pathways that are deregulated in various diseases. Overall, manipulation of RNA viruses to overexpress or inhibit miRNAs provides an exciting new avenue of therapy that requires substantial exploration and has attractive advantages for gene therapy.

CONCLUDING REMARKS AND PERSPECTIVES

miRNAs, the most important class of small noncoding RNAs, play a fundamental role in virus-host interactions. Expression of miRNAs by DNA viruses is well established. The expression of miRNAs by DNA viruses has been suggested to contribute to controlling of virus replication, maintenance of latency, and evasion of host immune responses. The ability of DNA viruses to express virally encoded miRNAs was less surprising owing to their large genome size and their ability to replicate in the nucleus and interact with many host proteins. In the last few years, a plethora of publications have analyzed the ability of RNA viruses to encode for and express short RNAs. While RNA viruses can express small RNAs, whether RNA viruses encode and produce miRNAs that function in a similar manner to host miRNAs remains controversial. Multiple hypotheses have been put forth to explain the inability of RNA viruses to encode miRNAs. The notion that cytoplasmic RNA viruses do not express miRNAs owing to the lack of nuclear localization is negated by the fact that influenza virus, a cytoplasmic RNA virus that replicates in the nucleus, also does not encode for its own miRNAs. However, these hypotheses need to be addressed experimentally for sufficient understanding in the future. In addition, several groups have demonstrated that it is possible to engineer RNA viruses to encode for miRNAs. This ability of RNA viruses to encode for engineered miRNAs reflects their capability to encode and express functional miRNAs in an infected cell without self-destructing their replicative life cycle. Despite this, why RNA viruses do not seem to encode their own miRNAs remains a mystery. One potential difficulty in studying these questions experimentally in RNA viruses is that each RNA virus carries out specific and different replicative life cycles in the host cells. This would make the generalization of common themes pertaining to the group of RNA viruses and their interaction with the miRNA machinery harder.

Numerous publications and reviews have characterized the use of viral vectors for vaccine development and gene therapy (162). An added “feather in the cap” of growing evidence in viral vector usage is the potential for engineering RNA viruses to encode for miRNAs. Expression of miRNAs by RNA viruses provides an exciting avenue to develop cell type-specific modulation of the host microenvironment. Patients who develop symptoms have specific biomarkers that are specific to a disease of interest. Whether it is a virus-infected cell or a cancerous cell, dysregulation of holistic cell integrity is breached at the level of protein expression. MiRNAs play an irreplaceable role in the regulation of protein expression profiles in all cells. Indeed, miRNAs have now been utilized as biomarkers in a variety of inflammatory diseases, metabolic diseases, viral infections, and cancers (38, 70, 128, 137). A relevant example of retrovirus-based gene therapy for a previously incurable disease is the development of chimeric antigen receptor (CAR) T cells for cancer therapy. The use of CAR-T cells to treat unique cancers such as acute lymphoid leukemia has shown much promise (42). The use of engineered retroviruses to reprogram important immune cells to express a specific antigen or to alter the endogenous expression of miRNAs also provides interesting avenues for future gene therapy. Because one miRNA can regulate multiple host proteins, processes, and/or signaling pathways, it would be exciting to attempt to modify multiple proteins that are deregulated in infectious diseases and cancer, by identifying one or more miRNAs that can specifically target them. However, the potential for “off-target” effects, whereby miRNAs affect the levels of unintended or unanticipated targets, must be carefully considered. Conversely, many investigators have studied specific miRNAs that are differentially expressed in disease states. Altering the levels of dysregulated miRNAs, either through overexpression (by engineering RNA viruses to encode for host pre-miRNA sequences) or through specific inhibition (by engineering RNA viruses to encode for complementary sequences to the miRNA of interest), might restore homeostasis and potentially serve as therapeutic intervention.

Viruses are usually cell-type specific. This is partially due to the limited expression of receptor proteins to which specific
viral envelope proteins bind for entry. However, few other RNA viruses have the ability to infect various cell types by binding to host receptors that are ubiquitously expressed in various cells. Cell type-specific expression of miRNAs can be used to our advantage for restricting viral replication to desired cell types. This can be achieved by producing RNA viruses that encode for binding sites for a host miRNA expressed in specific cell types. Additionally, viruses could be designed to infect immune cells only, by modifying env proteins for entry and cloning in miRNA binding sites (complementary to an miRNA expressed only in immune cells). This strategy will be particularly attractive for generation of protective neutralizing antibodies and potent virus-specific T-cell immunity (through effective antigen presentation by antigen-presenting cells, such as dendritic cells infected by these modified viruses). The main advantage of this approach will be to target immune cells only and yet ensure that viral infection is defective owing to replacement of the viral envelope. Perhaps additional attenuation of other essential genes in the viral genome, while ensuring expression of the viral immunodominant epitopes, will provide an effective strategy for an efficacious viral vaccine.

As potent as these strategies sound, use of these engineered RNA viruses to encode miRNAs or miRNA binding sites must be studied extensively to ensure prevention of escape mutants and consistent, long-lasting integrity of expression of these cloned-in miRNA-specific sequences. In addition, if these approaches reach a more practical stage, careful study of safety profiles will be required, including animal models and human clinical trials. Recently, investigators have reported that host innate immune receptors such as Toll-like receptors (TLRs) are able to recognize host miRNAs and aberrantly respond with the induction of inflammatory cytokines and chemokines (22, 36, 37, 92, 93). Therefore, engineering RNA viruses to express miRNAs may activate TLRs and other cytoplasmic RNA-sensing receptors and could be detrimental to the host cell owing to induction of overinflammation. Further well-controlled, thorough safety studies are warranted for developing these therapeutic concepts.

Apart from the exciting opportunities for “custom-making” these RNA viruses to manipulate host miRNA pathways, it is irrefutable that the natural course by which RNA viruses interact with host miRNAs plays a crucial role in the outcome of infection. As we have summarized, various RNA viruses interact with the cellular miRNA pathway in different ways (Fig. 3). This includes direct subversion of important proteins in the miRNA machinery and indirect regulation to reduce the expression of antiviral miRNAs. Another way by which RNA viruses manipulate host miRNA pathways is by upregulating miRNAs that target important innate immune receptors such as TLRs or adaptor proteins downstream of TLR pathways. In addition, RNA viruses can modulate miRNAs that target common positive or negative regulators of antiviral cytokines and chemokines to benefit their productive replication and survival.

Through multiple strategies, RNA viruses modulate the expression of cellular miRNAs, among other pathways, in their fight against the host. Even though what we know thus far in regards to RNA viruses and miRNAs is exciting, it is fairly limited and needs extensive exploration. Future research in this field will not only improve our overall understanding of virus-host interplay, it will also provide key preliminary information for the development of miRNA-mediated modulation of RNA viral infection and for using engineered RNA viruses in treating patients in a clinical setting.

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AUTHOR CONTRIBUTIONS

Author contributions: G.S., J.M.-G., and S.N.-M. conception and design of research; G.S., J.M.-G., and S.N.-M. analyzed data; G.S. and S.N.-M. prepared...

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