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Review
MicroRNA Regulation of RNA Virus Replication and Pathogenesis

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microRNAs (miRNAs) are non-coding RNAs that regulate many processes within a cell by manipulating protein levels through direct binding to mRNA and influencing translation efficiency, or mRNA abundance. Recent evidence demonstrates that miRNAs can also affect RNA virus replication and pathogenesis through direct binding to the RNA virus genome or through virus-mediated changes in the host transcriptome. Here, we review the current knowledge on the interaction between RNA viruses and cellular miRNAs. We also discuss how cell and tissue-specific expression of miRNAs can directly affect viral pathogenesis. Understanding the role of cellular miRNAs during viral infection may lead to the identification of novel mechanisms to block RNA virus replication or cell-specific regulation of viral vector targeting.

Introduction

RNAi evolved in plants and invertebrates as an antiviral mechanism, combating viral infections through degradation of viral RNA into siRNAs that bind viral nucleic acids and thus inhibit viral replication [1]. In vertebrates RNAi functions as an antiviral mechanism in non-differentiated embryonic stem cells only [2,3]. In differentiated vertebrate somatic cells, **type I interferon (IFN)** (see Glossary) and the antiviral effectors **IFN-stimulated genes (ISGs)** have evolved to mediate the majority of cellular antiviral responses [4]. The vertebrate RNAi system regulates the function of cellular mRNA through tissue- and cell-specific expression of host-cell-encoded **miRNAs** that bind mRNAs and alter their translation or abundance [5]. To date >5500 miRNAs have been predicted throughout the human genome [6] with many limited to specific tissues [5]. Invertebrates also use miRNAs to regulate mRNA translation and control protein abundance (reviewed in [7]). Recent evidence indicates that host miRNAs also bind to RNA virus genomes, regulating their translation and replication and altering viral pathogenesis. RNA virus infection can mediate changes in the expression of cellular miRNAs leading to downstream changes in the host transcriptome that can be advantageous to the virus. However, changes in miRNA expression can also lead to increases in antiviral effector activities resulting in decreased viral replication. This review focuses on recent advances in our understanding of RNA viruses and how they have evolved to use host miRNAs to evade antiviral immune responses, leading to enhanced viral replication and pathogenesis.

Factors Influencing miRNA–RNA Virus Interactions

miRNAs are small noncoding RNAs located primarily within the introns of both coding and noncoding host RNAs (Box 1). miRNAs bind to miRNA-binding sites within mRNAs and viral genomes to mediate miRNA function [8,9]. Naturally occurring miRNA-binding sites within viral genomes are generally located in the 5′ [10] and 3′ **non-translated regions (NTRs)** [11] but have recently been found in the coding regions of viral proteins [12,13]. Increasing the number of miRNA-binding sites in a viral 3′ NTR with perfect complementarity to the target miRNA has led

Trends

Some RNA viruses possess miRNA-binding sites in a range of locations within the viral genome, including the 5′ and 3′ non-translated regions.

Host cell miRNAs can bind to RNA virus genomes, enhancing genome stability, repressing translation of the viral genome, or altering free miRNA levels within the cell.

miRNAs contribute to viral pathogenesis by promoting evasion of the host antiviral immune response, enhancing viral replication, or, potentially, altering miRNA-mediated host gene regulation.

RNA virus infection can lead to widespread changes in the host transcriptome by modulating cell-specific miRNA levels.

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Box 1. Biochemistry of miRNA–RNA Virus Interactions

miRNA genes are transcribed by RNA polymerase II into primary miRNA (pri-miRNA) transcripts containing a stem–loop structure encompassing the miRNA of interest [82]. Within the cell nucleus, pri-miRNA is digested enzymatically by the Drosha protein into pre-miRNA containing a hairpin structure, which is exported from the nucleus into the cytoplasm (Figure 1) [83]. Subsequently, the Dicer protein cleaves the terminal loop structure of the hairpin leaving a small, 21–23-nucleotide miRNA duplex. The argonaute protein then associates with one strand of the miRNA duplex to form the RISC, mediating translational repression or mRNA destabilization [84,85]. Each strand in the miRNA duplex can function as an miRNA and be loaded into the RISC; however, the strand that is more thermodynamically unstable at the 5′ end is incorporated preferentially [86]. For a more detailed review of miRNA processing, see [82].

A **seed sequence** located at nucleotide positions 2–8 of the 5′ end of the miRNA binds complementary sequences within the 3′ NTR of either a host mRNA or the genome of an invading RNA virus [87]. A single miRNA can possess binding specificity for many different target mRNAs based on the cell type and the pattern of mRNA expression [88]. A perfect match between the miRNA and the entire mRNA target sequences leads to RNA cleavage, albeit rarely in mammals [87]. More commonly, an exact seed sequence match, – a canonical miRNA-binding site without complete complementarity in the remainder of the target mRNA – results in translation inhibition and, eventually, RNA destabilization and degradation [84,85]. Recently, noncanonical binding sites have also been described that have imperfect seed sequence complementarity. As an example, noncanonical miRNA-binding sites are thought to comprise up to 40% of the total miR-155 miRNA-binding sites in host mRNAs [89]. However, although noncanonical binding sites may mediate miRNA binding to the mRNA, these sites may not fully suppress mRNA translation [90]. If miRNAs can functionally inhibit RNA viruses through noncanonical interactions, the number of potential miRNA–RNA virus interactions would dramatically increase. Mutational analysis of known miRNA-binding sites into noncanonical target sequences will be required to determine whether such noncanonical interactions can mediate productive miRNA–RNA virus interactions.

to increased **translational repression** *in vitro* and greater attenuation in mice [14]. In similar studies, cooperativity between two or more miRNA-binding sites enhanced repression of mRNA translation via an unknown mechanism when sites were separated by 13–35 nucleotides [15]. Therefore, the location and number of miRNA-binding sites within a viral genome can influence miRNA function. Naturally occurring viral miRNA-binding sites within viral RNAs may not be uniformly distributed or have perfect complementary to the miRNA. Further experimental validation will be required to determine whether cooperativity between viral miRNA-binding sites leads to enhanced transcriptional repression. Furthermore, empirical studies are needed for all miRNA–viral interactions identified *in silico* – as computational methods might not fully recapitulate the cellular environment – to identify interactions that are not functional due to higher-order target RNA structures or other nucleic acid interactions.

Transcriptional, epigenetic, and environmental conditions drive miRNA gene transcription leading to temporal and cell-specific miRNA expression [16]. Common laboratory practices can also alter the miRNA environment within a cell. For example, culturing cells can reduce miRNA levels compared with primary tissues [17] and cells at a higher confluency can exhibit increased miRNA levels compared with less confluent cells due to increased cell–cell contact [18]. Compared with cells isolated from primary tissues, it is commonly known that cancer cell lines have different expression patterns of miRNAs that can contribute to carcinogenesis [19]. C6/36 cells derived from *Aedes albopictus* mosquitoes do not efficiently cleave viral RNA into siRNAs for use in the antiviral RNAi pathway but can still process miRNAs [20]. By contrast, Aag2 cells derived from *Aedes aegypti* mosquitoes have a functional double-stranded RNA processing pathway and can generate both siRNAs and miRNAs [21]. Furthermore, studies that globally inhibit the RNAi pathway in single cell types [22] are likely to fail in detecting cell/tissue-specific miRNA–viral interactions and can underestimate the impact of cell-specific miRNA–viral interactions occurring in infected hosts. Finally, *in vitro* models may not fully characterize how miRNA–viral interactions alter global cellular miRNA levels [23], which could possibly lead to important changes in paracrine or autocrine signaling in intact hosts [11]. Knowledge of the pathobiological features of a viral infection within a specific host (e.g., unexplained restriction in particular tissues) and using disease-relevant cell lines that express an miRNA of interest might also lead to the identification of cell-specific miRNAs that contribute to viral pathogenesis.

Glossary

Borna disease virus: non-segmented, negative-strand RNA virus that can establish persistent infection in human neuronal cells.

Bovine viral diarrhea virus (BVDV): a member of the *Pestivirus* genus that is an important pathogen of livestock due to its ability to affect reproduction.

Coronavirus: enveloped, positive-strand RNA virus that can infect humans and other animals and can cause the common cold and pneumonia in the elderly. Severe acute respiratory syndrome-related coronavirus (SARS-CoV) and Middle East respiratory syndrome virus (MERS) are related coronaviruses.

Enterovirus 71 (EV71): the genome of this virus contains a single polyprotein that encodes both structural and nonstructural proteins. EV71 causes hand–foot and mouth disease primarily in young children and elderly, but can also cause disease in adults.

Human T cell leukemia virus, type I (HTLV-1): a positive-sense RNA retrovirus that can cause adult T cell leukemia/lymphoma in humans.

Influenza PB1 protein: a component of the RNA-dependent RNA polymerase complex that is required for influenza virus replication.

Interferon-stimulated genes (ISGs): genes that are upregulated by IFN after recognition of viral infection by pattern-recognition receptors leading to innate immune responses.

miRNA: noncoding RNAs of 21–23 nucleotides that bind to cellular mRNAs and viral RNAs mediating translational repression or RNA degradation and thereby reducing protein levels within the cells.

Noncanonical miRNA target sites: contain at least one nucleotide mismatch between the miRNA seed sequence and the miRNA-binding site in the target mRNA.

Non-translated regions (NTRs): regions of mRNAs and viral genomes that do not encode proteins. These are typical locations within the mRNA and genomes for miRNA binding.

Pattern-recognition receptors: intra- and extracellular receptors that recognize pathogen-associated molecular patterns leading to the initiation of innate immune responses.

P bodies: structures within a cell that process mRNA. miRNA binding to

While miRNA–RNA viral genome interactions are governed by many of the same mechanisms governing miRNA–mRNA interactions, investigation of their role in viral pathogenesis should take into account cell specificity, the role of **noncanonical miRNA target sites**, the potential for mild alteration of viral activities at the cellular level leading to large effects in the host, and the potential for miRNA inhibition to alter cell–cell signaling in the complex environment of an infected animal. These ideas are explored in greater detail below.

miRNA Interactions with the RNA Viral Genome

Evidence is accumulating that host miRNAs can bind to a broad range of RNA viruses, directly regulating their pathogenesis (Table 1). A positive-strand RNA virus genome mimics cellular mRNAs allowing direct binding of the miRNA to the viral RNA, and potentially the regulation is analogous to that of host mRNAs. Two outcomes of these interactions have thus far been identified where viral replication is directly altered: (i) inhibition of translation of the viral genome preventing viral replication [11]; and (ii) stabilization of the virus RNA thereby enhancing replication (Figure 1, Key Figure) [10,24]. Both of these mechanisms reveal a novel method for regulating RNA virus replication within specific cells and/or tissues. In addition to expression of the virus receptor on the cell surface, miRNA-mediated virus repression may be another mechanism for determining cell permissiveness to infection. Another commonality of these two

target mRNAs can direct these mRNAs to P bodies for storage or decay through decapping mechanisms or 5′–3′ exonuclease activity mediating translational repression.

Primate foamy retrovirus type 1 (PFV-1): establishes lifelong persistent infection in humans and primates without causing signs of disease, which is unique to PFV.

Prodrome: the initial set of clinical symptoms that a viral infection elicits in an infected animal' may progress to different or more severe manifestations of infection.

Retinoic acid-inducible gene I (RIG-I): RNA helicase and pattern-recognition receptor that recognizes single-stranded RNA containing a 5′ triphosphate molecule leading to a signaling cascade initiating innate immune responses.

RNA-induced silencing complex (RISC): functional set of cellular proteins including argonaute proteins, which associate with the miRNA seed sequence to mediate binding of the RISC to complimentary sequences in target RNAs. The binding of the RISC to target RNAs leads to translational repression or destabilization of the target RNA.

Seed sequence: nucleotides 2–8 at the 5′ end of an miRNA that determine the target specificity of the miRNA by binding to complementary sequences in the target RNA. A single miRNA can bind to many different target RNAs based on the seed sequence.

Translational repression: a functional outcome of miRNA binding to target RNA that results in reduced translation of the RNA leading to lower protein levels within the cell.

Type I interferon (IFN): a primary antiviral molecule that functions in both an autocrine manner – by upregulating ISGs leading to increased IFN production within the same cell – and a paracrine manner by priming naïve cells via the upregulation of antiviral ISGs.

Viral quasispecies: viruses that arise due to mutation of the virus genome; they compete for a fitness advantage within the host.

Table 1. Direct Binding of miRNAs to the RNA Virus Genome

Virus	miRNA	Phenotype	Refs
EEEV	miR-142-3p	↓ Viral translation and replication, myeloid cell replication, serum IFN-α/β, lymph node replication ↑ Virulence	[11]
PFV-1	miR-32	↓ Viral translation	[28]
HTLV-1	miR-28-3p	↓ Viral translation in T cells, transmission between humans	[29]
HIV	miR-28-5p, miR-150, miR-223, miR-382	↓ Viral translation ↑ Latency in T cells	[30]
	miR-29a	↑ Association with RISC and P bodies ↓ Viral replication	[31]
Influenza	miR-323, miR-491, miR-485, miR-654, miR-3145	↓ PB1 RNA levels	[12,33,34]
	let-7c	↓ Matrix protein	[35]
EV71	miR-296-5p	↓ Viral replication, VP1 and VP3 protein levels	[13]
	miR-23b	↓ Viral translation and replication	[32]
Infectious bursal disease virus	miR-21	↓ VP1 protein levels	[38]
PRRSV	miR-181, miR-206	↓ Viral translation and replication	[39]
	miR-23, miR-378, miR505	↓ Viral translation and replication ↑ IFN-α/β	[40]
HCV	miR-122	↑ RNA stability, viral replication, cell proteins repressed by miR-122 ↓ Free miR-122	[10,24,48]
BVDV	miR-17, let-7c	↑ RNA stability and viral translation, cell proteins repressed by miR-17 ↓ Free miR-17	[23]

↑, Increase; ↓ decrease.

Key Figure

Direct and Indirect Interactions between Cellular miRNAs and RNA Viruses
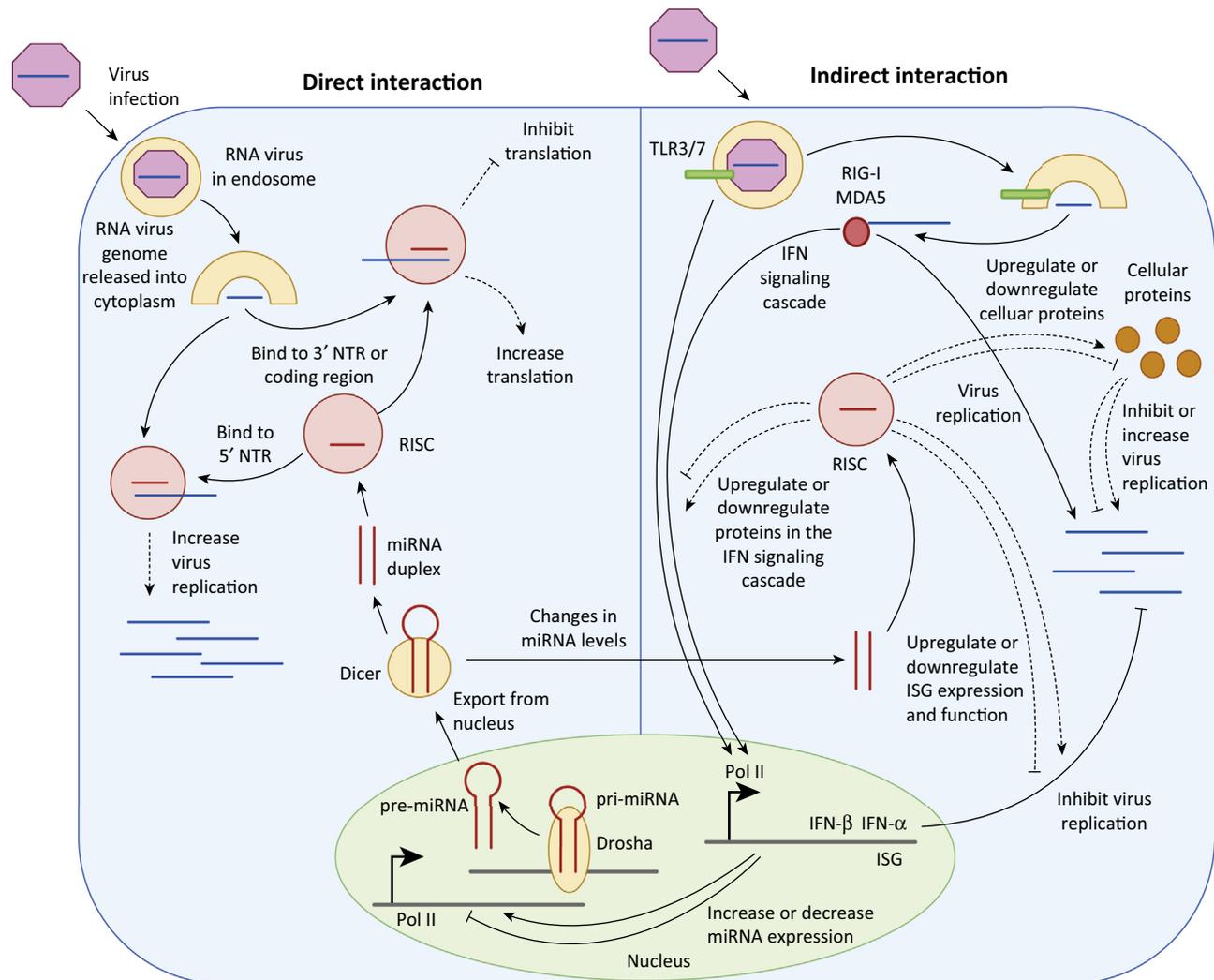
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Figure 1. miRNAs are transcribed in the nucleus by RNA polymerase II (Pol II) into a primary miRNA (pri-miRNA) that is cleaved by Drosha resulting in a pre-miRNA. The pre-miRNA is exported from the nucleus into the cytoplasm and is enzymatically cleaved by Dicer into the miRNA duplex. One strand of the miRNA duplex is recognized by the argonaute proteins to form the RNA-induced silencing complex (RISC). Direct interaction: After infection, the RNA virus genome is released into the cytoplasm where the miRNA-loaded RISC binds via the miRNA seed sequence to complementary nucleotides in the viral 5′ non-translated region (NTR), 3′ NTR, or coding regions. miRNA binding to the 5′ NTR leads to RNA stability and increased viral replication. miRNA binding to the 3′ NTR can lead to inhibition of viral translation or increased RNA stability and viral translation. Indirect interaction: Viral RNA is recognized by pattern-recognition receptors, Toll-like receptor (TLR)3, TLR7, and retinoic acid-inducible gene I (RIG-I) or melanoma differentiation-associated protein 5 (MDA5), leading to induction of the interferon (IFN) signaling cascade and upregulation of IFN-α, IFN-β, and other IFN-stimulated genes (ISGs) that inhibit viral replication. Viral infection and IFN and ISG induction can also lead to changes in miRNA expression within a cell. These upregulate or downregulate proteins involved in the IFN signaling cascade or ISG expression and function, as well as cellular proteins required for viral replication. Collectively, these virus-mediated changes in miRNA levels result in either a proviral or an antiviral environment.

mechanisms is that both lead to a fitness advantage for the virus at the level of the infected host, either directly by increasing replication – as with hepatitis C virus (HCV) [10,24] – or indirectly by suppressing innate immune responses and leading to increased overall host-level replication, as in the case of eastern equine encephalitis virus (EEEV) [11]. Considering the ability of RNA viruses to rapidly alter their genomes in the face of selective pressure [25], it may be that productive miRNA–RNA viral interactions are maintained only when a significant replicative advantage is conferred on the virus. Below, the outcomes of direct binding of miRNA to RNA viral genomes are discussed in detail.

Translational Inhibition of the RNA Viral Genome by Direct miRNA Binding

Alphaviruses. EEEV, an alphavirus, causes the most acutely virulent mosquito-borne viral disease in the USA, with a >30% mortality rate in humans and long-term neurological sequelae in surviving individuals [26]. In humans EEEV infection is characterized by a limited disease **prodrome** before the manifestation of encephalitis due to the inability of EEEV to replicate in hematopoietic cells and to elicit a strong innate immune response [11,27]. EEEV is a positive-sense RNA virus that encodes three canonical and one noncanonical miRNA-binding sites in the 3′ NTR that are specific for the hematopoietic cell-specific miRNA miR-142-3p [11]. miR-142-3p expression has been shown to prevent translation of the incoming EEEV genome and prevent virus replication in both human and murine macrophages as well as dendritic cells [11]. This inhibition of myeloid cell replication prevented IFN-α/β induction *in vivo*, directly contributing to the extreme virulence of EEEV that is seen in mice, and presumably in humans [11]. Mutation of the miR-142-3p-binding sites in the EEEV genome has been shown to allow virus translation and replication in both human and murine myeloid cells, which was shown to lead to systemic IFN-α/β production and virus attenuation in mice. Presumably, strong selective pressure has maintained these miR-142-3p-binding sites, since 17 of 23 North American EEEV strains isolated from nature have the same sequence and location of miR-142-3p binding sites within the 3′ NTR [11].

Retroviruses. Retroviruses are single-stranded, positive-sense RNA viruses with two different points in the virus life cycle where miRNAs can bind to virus RNA: the genomic RNA and the RNA transcribed from integrated DNA. For example, **primate foamy retrovirus type 1 (PFV-1)** encodes a binding site for miR-32 in the 3′ NTR of multiple protein transcripts that can inhibit PFV-1 replication. To evade miR-32 repression, PFV-1 encodes a transactivator protein, Tas, that nonspecifically suppresses the miRNA processing pathway and potentially inhibits the production of miRNAs that might limit virus replication, including miR-32 in infected mammalian cells [28]. **Human T cell leukemia virus, type I (HTLV-1)** translation is inhibited in resting human T cells by miR-28-3p. A single nucleotide variant in the miR-28-3p target sequence of the Japanese ATK-1 strain has been shown to confer miR-28-3p translational repression resistance, increasing virus replication as well as transmission between individuals, occurrences not normally seen with other HTLV-1 strains [29]. miRNA can also contribute to retroviral latency within T cells by preventing translation of the virus RNA and suppressing virus replication. For example, during HIV-1 infection of CD4$^+$ T cells *in vitro*, expression of miR-28-5p, miR-150, miR-223, and miR-382 has been found to mediate translational repression of HIV-1 RNA [30]. Another miRNA, miR-29a, functions through a different mechanism to restrict viral replication during HIV-1 infection by binding to the 3′ NTR leading to its increased association with the **RNA-induced silencing complex (RISC)** and **P bodies** and thus preventing virus translation [31].

Picornaviruses. Similar inhibition of virus translation and replication occurs following upregulation of miR-296-5p by the positive-sense picornavirus family member **enterovirus 71 (EV71)**. miR-296-5p negatively regulates virus replication in a human neuronal cell line due to the presence of two miR-295-5p-binding sites in the coding regions of virus proteins VP1 and VP3 [13]. Natural variants of EV71 bear mutations in the miR-296-5p-binding sites rendering this virus insensitive

to miR-296-5p restriction [13]. EV71 also contains a binding site for miR-23b; however, miR-23b is naturally downregulated during EV71 infection of monkey epithelial cells, preventing miR-23b binding to the virus genome and inhibiting virus replication [32].

Orthomyxoviruses. miRNAs can also bind to negative-strand RNA viruses after transcription of the negative-strand RNA genome into the positive-sense RNA intermediate needed for virus replication. For instance, miR-323, miR-491, miR-485, miR-654, and miR-3145 have been shown to bind to the coding region of the **influenza PB1** gene segment leading to RNA degradation in infected human and canine epithelial cell lines *in vitro* [12,33,34]. Similar degradation of virus RNA was found when the miRNA let-7c bound to the 3′ NTR of the influenza matrix protein in human alveolar epithelial A549 cells [35]. Other potential miRNA-binding sites in the influenza RNA genome have been computationally predicted; however, they have yet to be experimentally validated [36,37]. Although an miRNA may restrict influenza virus replication in cell culture, it may not be constitutively expressed in infected cells *in vivo* and therefore may not necessarily affect viral pathogenesis *in vivo* [33].

Other Viruses. Finally, interactions between miRNAs and RNA viruses also occur with nonhuman viruses. In chickens infectious bursal disease virus replication is inhibited by miR-21 due to miR-21-binding sites in the coding region of the VP1 protein [38]. In pigs naturally minimally permissive cells can express multiple miRNAs that inhibit replication of porcine reproductive and respiratory syndrome virus (PRRSV) [39,40]. Treatment with a miR-181 mimic limits PRRSV replication *in vivo* leading to extended infected-pig survival times, thus demonstrating the therapeutic potential of this miRNA during an RNA virus infection [39]. While these studies suggest widespread negative regulation of RNA virus replication by host cell miRNAs, the capacity for RNA viruses to mutate rapidly to eliminate miRNA binding [9,41,42] indicates that direct antiviral activities of miRNAs may be limited to particular cells or to a subset of **viral quasispecies** present at any given time within the host. Therefore, any antiviral effects at the cellular level should be considered in the context of the overall effect of tissue-specific viral inhibition on viral quasispecies fitness in the intact host and on transmission between hosts.

miRNA-Mediated Stabilization of RNA Virus Genomes: A Focus on Flaviviruses

A noncanonical interaction between miRNAs and RNA viruses has been identified that leads to increased stability of the viral RNA. The most studied interaction of this type was first identified in 2005 and occurs between liver-specific miR-122 and HCV. Rather than binding to the 3′NTR, miR-122 bound to the 5′ NTR of HCV [10] preventing 5′ host exonuclease activity and RNA degradation in the infected human hepatocarcinoma-derived cell line Huh-7 [24]. Moreover, removal of miR-122 from Huh-7 cells could reduce HCV replication [10]. Thus, miR-122 is currently an active target for antiviral therapeutics in the treatment of chronic HCV infection [10,43]. Recently, miRNAs were also found to increase RNA stability through interactions with the 3′ NTR; miR-17 and let-7c both bound to the 3′ NTR of the **bovine viral diarrhea virus (BVDV)** genome increasing virus translation and RNA stability rather than causing translational repression [23]. Interestingly, let-7 sites are present within several RNA viruses, including alphaviruses [23]. The ability of two diverse members of the *Flaviviridae* family, HCV and BVDV, to utilize host miRNAs to help stabilize virus RNA through two different sites within the virus genome suggests that this interaction might have evolved independently. It will be interesting to determine whether other virus families use miRNAs in a similar manner to help stabilize viral RNA.

Modulation of Host miRNA Levels during Viral Infections

Recognition of viral infection by **pattern-recognition receptors** initiates the innate immune response signaling cascade leading to transcription factor activation and changes in the cell transcriptome, including changes in miRNA expression (Figure 1) [44]. It has been proposed that a critical role for miRNAs during a viral infection is in the modulation of cytokine responses, either

by augmenting productive responses or by suppressing potentially damaging responses [45]. Specific miRNA expression patterns have been associated with pathogenic versus attenuated influenza virus [46] and hantavirus (Hantaan) [47] infections. Viruses may also alter global miRNA activity by binding and sequestering free miRNA. For example, miR-122 binding to HCV has been shown to sequester and reduce free miR-122 inside Huh-7 cells leading to changes in expression of the cellular proteins that are normally repressed by miR-122 [48]. Although both miR-17 and let-7c can bind the BVDV genome, only miR-17 sequestration has been reported to lead to changes in the host transcriptome, demonstrating that not all miRNA–RNA interactions lead to similar results [23]. Such changes in the host transcriptome due to sequestration may positively or negatively affect viral replication and pathogenesis and may vary depending on the cell and tissue expression of each miRNA. Finally, RNA viruses can encode proteins that modulate the miRNA or RNAi pathway, especially in invertebrates (Box 2). Moreover, as discussed below, RNA virus infection can lead to many changes in miRNA expression (Table 2) influencing viral replication as well as host immune responses.

miRNA-Mediated Changes in Protein Expression That Alter Host Responses to Infection

In addition to tissue-specific changes in miRNA expression during viral infections (Box 3), other important changes in miRNA-mediated protein expression can occur. For instance, IFN-α/β is a key cytokine induced by viral infection that leads to the downstream upregulation of antiviral ISGs. Viruses have evolved multiple mechanisms to evade the IFN system to establish a productive infection. Typically, during an infection viral proteins inhibit either pattern-recognition receptor recognition or downstream signaling cascades to prevent ISG induction [49]. However, a role for virus-mediated regulation of the IFN signaling cascade through changes in host miRNA levels has recently been proposed. For example, EV71 [50], dengue virus (DENV) [51], and Japanese encephalitis virus (JEV) [52] induction of miR-146a in infected cells has been shown to negatively regulate the IFN signaling protein tumor necrosis factor receptor-associated protein 6 (TRAF6), thus preventing the induction of IFN-α/β. In addition, downregulation of miR-432 by JEV infection has been reported to increase expression of the negative regulator of cytokine signaling suppressor of cytokine signaling 5 (SOCS5) in infected human microglial cells, thereby dampening the antiviral response [53]. Induction of miR-29c, miR-451, and miR-485 in influenza-infected cells has also been found to limit cytokine and chemokine responses through inhibition of various antiviral proteins (Table 2) and to prevent overactive IFN and inflammatory responses [34,54,55]. HCV infection can upregulate miR-758 [56], miR-130a [57], and miR-373 [58], inhibiting various components of IFN signaling to decrease IFN-α/β production (Table 2). Another example is avian leukosis virus subgroup J infection, which in chicken spleen was shown to lead to miR-23b expression and downregulation of both IFN regulatory factor 1 (IRF1)

Box 2. Viral Proteins That Inhibit the RNAi Machinery

RNA viruses can also encode proteins that inhibit the miRNA processing pathway. These proteins lead to changes in host miRNA and protein levels that modulate innate immune and other cell stress responses, causing more rapid and extensive viral replication during acute infections, decreased virus clearance, and enhanced immunopathology. Although miRNAs do not appear to have a host-protective direct antiviral role against RNA virus infection in vertebrates [2,3], some animal virus proteins have the capacity to suppress the miRNA pathway. The Ebola virus VP35 [91] and VP30 proteins [92] and the HCV core protein [93] interact with Dicer or Dicer-interacting proteins to prevent final processing into miRNAs. The HIV-1 Tat [94] and influenza NS1 [95] proteins have also been proposed to suppress the RNAi pathway through inhibition of Dicer and binding to double-stranded RNA, respectively; however, other reports have failed to support this hypothesis [96,97].

In invertebrates many viruses encode proteins that inhibit the RNAi machinery at multiple steps, all leading to suppression of the antiviral RNAi pathway and increased viral replication. These steps include preventing the processing of RNAs into siRNAs by Dicer proteins, inhibiting the function of argonaute proteins, and inhibiting the final formation of the RISC (reviewed in [98]). Evidently, further studies will be required to determine the exact role of RNAi-suppressing viral proteins in animal virus pathogenesis.

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Table 2. Changes in Cellular miRNA Expression after RNA Virus Infection

Virus	miRNA	Protein target	Phenotype	Refs
EV71	↑ miR-146a	↓ TRAF6, IRAK1, SOS1	↓ IFN-α/β ↑ Apoptosis	[50] [107]
	↑ miR-141	↓ eIF4E	↓ Host translation	[70]
	↓ miR-370	↑ GADD45β	↑ Apoptosis	[107]
	↑ miR-526a	↓ CYLD	↑ IFN-α/β ↓ Viral replication	[66]
DENV	↑ miR-146a	↓ TRAF6	↓ IFN-α/β	[51]
	↑ miR-30e*	↓ IκBα ↑ IFN-β, OAS1, MxA, IFITM1	↓ Viral replication	[61]
JEV	↑ miR-146a	↓ TRAF6	↓ IFN-α/β	[52]
	↓ miR-432	↑ SOCS5	↓ IFN-α/β	[53]
	↑ miR-155	↓ SHIP1	↑ IFN-β, proinflammatory cytokines	[99]
	↑ miR-15b	↓ RNF125	↑ RIG-I	[101]
	↑ miR-29b	↓ TNFAIP3	↑ Proinflammatory cytokines	[100]
Influenza virus	↓ miR-24	↑ Furin	↑ Hemagglutinin cleavage	[68]
	↓ miR-548an	↑ NS1-binding protein	↓ Apoptosis	[69]
	↓ miR-4276	↑ COX6C	↑ Caspase-9 ↓ Viral replication	[109]
	↑ miR-485	↓ RIG-I	↓ IFN-α/β	[34]
	↑ miR-29c	↑ TNFAIP3	↓ Proinflammatory cytokines	[54]
	↑ miR-451	↓ YWHAZ/14-3-3ζ	↓ IL-6, TNF, CCL5, CCL3	[55]
HCV	↑ miR-758	↓ TLR3/7	↓ IFN-α/β	[56]
	↑ miR-373	↓ JAK1, IRF9	↓ IFN-α/β	[58]
	↑ miR-130a	↑ ISG15, USP18, MxA ↓ miR-122 ↓ IFITM1	↑ IFN-α/β ↓ Viral replication ↑ Viral replication	[60] [57]
	↑ miR-146a-5p	N.D.	↑ Viral assembly and egress	[71]
Hendra virus	↑ miR-146a	↓ RNF11	↑ NF-κB ↑ Viral replication	[110]
WNV	↑ miR-532-5p	↓ SESTD1, TAB3	↓ Viral replication	[62]
	↑ miR-6124	↓ CTCF, ECOP	↑ Apoptosis	[108]
Coxsackie B3 virus	↑ miR-19b	↓ GJA1	↑ Pathogenesis, myocarditis	[105]
	↑ miR-1	↓ GJA1, KCNJ2	↑ Pathogenesis, myocarditis	[105,106]
RSV	↓ miR-221	↑ NGF, TrKA	↓ Apoptosis ↑ Viral replication	[72]
	↑ let-7f	↓ SOCS3, ELF4, DYRK2, CCND1	↑ Viral replication	[67]
	↑ let-7b, let-7i, miR-30b	N.D.	↑ IFN-β ↓ Viral replication	[65]
Coronavirus OC43	↓ miR-9	↑ NFKB1	↑ NF-κB	[64]

Table 2. (continued)

Virus	miRNA	Protein target	Phenotype	Refs
Avian leukosis virus J	↑ miR-23b	↓ IRF1	↓ IFN mRNA ↑ Viral replication	[59]
Borna disease virus	↓ miR-155	↑ SOCS3	↓ IFN-β ↑ Viral replication	[63]

N.D., not determined; ↑ increase; ↓ decrease.
IRAK1, interleukin 1 receptor-associated kinase 1; SOS1, SOS Ras/Rac guanine nucleotide exchange factor 1; eIF4E, eukaryotic translation initiation factor 4E; GADD45β, growth arrest and DNA damage-inducible protein 45β; CYLD, CYLD lysine 63 deubiquitinase; OAS1, 2′-5′-oligoadenylate synthetase 1; MxA, MX dynamin-like GTPase 1; SHIP1, Src homology 2-containing inositol phosphatase 1; RNF125, ring-finger protein 125; E3 ubiquitin protein ligase; TNFAIP3, TNF alpha-induced protein 3; COX6C, cytochrome c oxidase subunit 6C; YWHAZ/14-3-3ζ, tyrosine 3-monoxygenase/tryptophan 5-monoxygenase activation protein; CCL, C-C motif chemokine ligand; TLR, Toll-like receptor; JAK1, Janus kinase 1; ISG15, ISG15 ubiquitin-like modifier; USP18, ubiquitin-specific peptidase 18; RNF11, ring-finger protein 11; CTCF, CCCTC-binding factor; ECOP, epidermal growth factor receptor-coamplified and overexpressed protein; GJA1, gap junction protein alpha 1; KCNJ2, potassium voltage-gated channel subfamily J member 2; NGF, nerve growth factor; TrKA, tyrosine receptor kinase A; ELF4, E74-like ETS transcription factor 4; DYRK2, dual-specificity tyrosine phosphorylation-regulated kinase 2; CCND1, cyclin D1; NFKB1, nuclear factor kappa B subunit 1.

Box 3. Tissue-Specific Changes in miRNA Expression during Viral Infection

Tissue-specific changes in miRNA expression can also contribute to virus-induced pathology in mice. For instance, JEV and WNV infection of the central nervous system [99–102] and influenza virus infection of the lung [103,104] can lead to changes in multiple miRNAs implicated in the regulation of proinflammatory responses. Upregulation of miR-19b and miR-1 by Coxsackie B$_3$ virus infection was found to downregulate the expression of gap junction proteins – required for cell-to-cell contact – in human and mouse cardiomyocytes, leading to the development of viral myocarditis [105,106]. Another study documented that induction of miR-146a and downregulation of miR-370 following EV71 infection and miR-6124 expression after WNV infection led to increased cellular apoptosis and cell death through the regulation of apoptotic and antiapoptotic proteins [107,108]. Obviously, changes in miRNA levels associated with viral infection can contribute to pathological and/or protective responses. However, as with antiviral activities, the role of these effects in the replication and disease process should be considered carefully with respect to the effects on single cells or tissues and the overall pathogenesis of the virus in a given host, as well as host–host transmission mechanisms.

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levels and innate immune induction [59]. It is likely that future work will identify more miRNAs that are involved in downregulating the innate immune response during RNA virus infections.

Viral infection may also increase the levels of miRNAs leading to the upregulation of antiviral molecules that are detrimental to viral replication. For example, while miR-130a can downregulate IFN-induced transmembrane protein 1 (IFITM1) protein levels after HCV infection to increase virus replication [57], miR-130a induction can also lead to antiviral ISG expression, increased IFN production, and reduced virus replication in Huh-7.5.1 hepatocarcinoma cells [60]. These results demonstrate that the same miRNA can have pleiotropic effects during virus infection, which may be due to two different mechanisms: direct targeting of a cellular protein (IFITM1) [57] or targeting of an unknown master regulator that controls the expression of many ISGs [60]. Interestingly, upregulation of miR-130a following HCV infection can also downregulate the expression of miR-122, providing a second mechanism to limit HCV replication [60]. Following DENV infection of a human monocyte cell line, miR-30e* induction has been shown to suppress virus replication by downregulating nuclear factor kappa B (NF-κB) inhibitor alpha (NFKBIA or IκBα) leading to continual NF-κB activation and upregulation of ISGs and IFN-α/β production [61]. Finally, rather than modulating ISG levels, miR-532-5p upregulation during West Nile virus (WNV) infection has been shown to lead to downregulation of SEC14 and of spectrin domain-containing 1 (SESTD1) and TGF-beta-activated kinase 1/MAP3K7-binding protein 3 (TAB3), two proteins required for virus replication in infected human embryonic kidney (HEK) 293 cells [62].

With some viruses, virus-encoded proteins can inhibit the upregulation of miRNAs that are triggered after viral infection to suppress the innate immune response. For example, the **borna**

disease virus phosphoprotein was shown to inhibit the expression of a known regulator of innate immune responses, miR-155, limiting the induction of IFN-α/β during infection in human oligodendroglioma cells [63]. Also, the **coronavirus** OC43 nucleocapsid protein has been found to bind the NF-κB negative regulator miR-9 in HEK293T cells, allowing continual NF-κB translation; however, the role of this activity during coronavirus infection remains unclear [64]. The respiratory syncytial virus (RSV) NS1 and NS2 proteins can antagonize let-7i and miR-30b induction through the inhibition of innate immune signaling proteins in human bronchial epithelial cells [65]. EV71 3C protein can block the upregulation of miR-526a by degrading IRF7 and preventing enhancement of **retinoic acid-inducible gene I (RIG-I)** signaling [66]. Finally, virus protein expression can also lead to the upregulation of miRNAs, as in the case of the RSV G protein, which can increase let-7f expression, negatively regulating IFN production and delaying virus clearance in human alveolar epithelial A549 cells [67]. Potentially, viral protein-mediated changes in miRNA expression may occur through an indirect mechanism governed by viral inhibition of the innate immune response rather than a direct interaction between the viral protein and the transcription factors that regulate miRNA expression, but further experiments would be required to elucidate this point.

miRNA-Mediated Changes in Protein Expression That Promote Viral Replication

As described above, miRNA sequestration and/or alteration of expression levels by RNA viruses can lead to cellular transcriptome changes that promote favorable conditions for viral replication. For example, influenza virus infection has been shown to downregulate miR-24 in A549 cells leading to increased furin protease levels and, thus, increased cleavage of the influenza hemagglutinin protein, leading to increased numbers of infectious influenza particles [68]. Also in A549 cells, influenza virus downregulation of miR-548an was shown to lead to increased NS1-binding protein levels and increased RNA stability by decreasing cellular apoptosis [69]. In another study EV71 infection increased miR-141 levels in human rhabdomyosarcoma cells, leading to the downregulation of eukaryotic translation initiation factor 4E protein and thus contributing to the switch from cap-dependent translation of cellular mRNAs to cap-independent translation of virus RNAs [70]. Furthermore, miR-146a-5p upregulation after HCV infection of human hepatocytes and in liver tissues from HCV-infected patients has been shown to promote the assembly of virus particles and egress from an infected cell, increasing HCV infection, through an unknown mechanism [71]. In another example RSV infection of human bronchial epithelial cells resulted in the downregulation of miR-221 levels, preventing infected cell apoptosis and leading to increased virus replication [72]. Finally, transfection of miRNAs into cells before virus infection has led to the identification of novel proteins involved in virus replication, such as heterogeneous nuclear ribonucleoprotein C (C1/C2) (hnRNP C) and leukemia inhibitor factor (LIF) in poliovirus [73] and influenza infections, respectively [74]. The use of miRNA expression screens to identify novel cellular proteins involved in RNA virus replication will be more common in the future. However, confirmation of the role of these miRNAs *in vivo* will be needed to determine their specific functional contribution to viral replication and pathogenesis.

Maintenance of miRNA-Binding Sites in the RNA Virus Genome

RNA viruses can mutate rapidly due to the lack of proofreading activity in the RNA polymerase [75]. Therefore, positive selection, or at least the absence of negative selection, is likely to be required to maintain the miRNA-binding sites within the virus genome. For example, EEEV replication in a non-miR-142-3p-expressing mammalian cell line resulted in a 238-nucleotide deletion in the 3′ NTR encompassing all of the miR-142-3p-binding sites [41]. Similarly, artificially inserted miRNA-binding sites in the 3′ NTR of DENV [42] and tick-borne encephalitis virus (TBEV) [14] were deleted *in vivo* during infection in mice. These results demonstrate that miRNA-binding sites in the 3′ NTR without a positive influence on virus replication can be rapidly deleted *in vivo*. Therefore, it is unclear how sites that negatively impact replication might be retained in virus populations. With EEEV, replication in the mosquito vector appears to provide positive selection

Box 4. The Clinician's Corner

miRNAs play a role in many different processes within a cell by regulating the production of cellular proteins. miRNA regulation of the innate immune response modulates the production of antiviral molecules by enhancing their response during the initiation of innate immunity, but also by downregulating this response to prevent aberrant physiological responses and/or immunopathology in a host.

Cell-specific miRNA expression directly contributes to the pathogenesis of medically important viruses either by promoting avoidance of the innate immune response (e.g., EEEV) or by stabilizing the viral genome and enhancing viral replication (e.g., HCV).

Other miRNAs can be regulated via viral infection to establish a pro- or antiviral environment. miRNA expression patterns can be used to identify a viral infection and help to determine whether a viral strain is pathogenic or attenuated.

In the future miRNAs might be used as antiviral therapeutics to prevent certain viral infections. Furthermore, the incorporation of tissue-specific miRNAs into viral vaccine and gene therapy vectors might enhance the specific targeting of viral proteins and/or, hopefully, limit adverse reactions due to infection.

Outstanding Questions

For arthropod-borne viruses, what role do miRNAs play in determining host, reservoir, or vector range during the viral transmission cycle?

Is location within the genome or the number of miRNA-binding sites the key factor determining translational repression and, consequently, the suppression of viral replication?

Do viral noncanonical miRNA-binding sites mediate miRNA binding and translational repression?

How does viral infection mediate changes in miRNA levels within the cell and does this affect cellular gene regulation?

Can knowledge regarding the role of miRNAs during RNA virus infection lead to the development of miRNA therapeutics or targeted viral expression vectors?

for retention of miR-142-3p-binding sites, as deletion of the sites decreased virus replication in mosquito cell cultures and greatly suppressed acquisition by mosquito vectors [11]. By contrast, site removal has no impact on replication in mammalian cells that do not express the miRNA. Mosquitoes do not express miR-142-3p [76], suggesting that another, unknown mechanism underlies the requirement for these sites for efficient replication.

The presence of miRNA-binding sites within many different RNA viruses may suggest a positive selective pressure for retention of these sites within the genome occurring at the cellular or organismal level or during virus transmission between hosts, particularly in instances where replication is inhibited at the single-cell level. Consequently, miRNA repression of viral replication may exert powerful effects on the composition of RNA virus quasispecies during different phases of virus residency within a host. One potential selective pressure to maintain miRNA-binding sites may result from tempering of viral replication in certain cells or tissues that enables efficient virus dissemination without severe detrimental effects to the host. However, it is clear that more research is needed to fully understand how the replication-inhibiting miR-142-3p-binding sites in EEEV, and specific sites in other viruses, can be maintained throughout the virus replication/transmission cycle.

Concluding Remarks

miRNAs regulate many processes within cells, including RNA virus infections. Direct binding of miRNAs to viral genomes that leads to an antiviral effect may be rare due to the high mutation rate of RNA viruses, unless there is a strong selective pressure to maintain the miRNA-binding sites within the viral genome. It may be more common for RNA viruses to manipulate the levels of particular miRNAs within the cell to establish a proviral environment that enhances viral replication and dissemination within and between hosts. In addition, changes in miRNA levels after viral infection are clearly an important component of the host response to infection, sculpting both the initiation and the resolution phases of the antiviral response.

Over the past few years, there has been a large expansion in the number of studies identifying miRNAs as modulating factors during RNA virus infections. Future work (see Outstanding Questions and Box 4) will help to define the role of miRNAs during these processes. However, knowledge regarding the role of miRNAs has already been applied to the development of antiviral therapeutics [43] and gene therapy vectors (reviewed in [77]). An miRNA therapeutic for Ebola virus was used during the recent outbreak in Africa; however, testing was ultimately suspended as the drug did not demonstrate a significant role in patient recovery [78]. Also, insertion of tissue-specific miRNA-binding sites has been used as an effective mechanism to prevent virus

replication in particular tissues as an attenuation mechanism for vaccines, helping to limit adverse events [79]. Furthermore, recent evidence suggests the presence of virus-derived small RNAs during infection of both mammalian and arthropod cells [80,81]. While controversial, their presence and function during animal infections will need to be further examined. Viral interactions with the miRNA system can affect cellular miRNA and transcriptome levels as well as viral replication within a cell expressing a specific miRNA; these may indirectly modulate cytokine expression and tissue responses throughout an infected host. Consequently, future approaches tackling these questions are likely to emerge from this exciting area of investigation.

Acknowledgments

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