Chapter 2

RNAi and Cellular miRNAs in Infections by Mammalian Viruses

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Abstract

MicroRNAs (miRNAs) play an essential role in the regulation of eukaryotic gene expression. Recent studies demonstrate that miRNAs can also strongly affect the replication of pathogenic viruses. For example, cellular miRNAs can target and repress the expression of viral mRNAs, but there is also at least one example of a cellular miRNA that stimulates virus replication. Furthermore, viruses can encode their own miRNAs, trigger changes in cellular miRNA expression or encode RNA silencing suppressor factors that inhibit cellular miRNAs. These interactions together form a complex regulatory network that controls both viral and host gene expression, which ultimately determines the outcome of viral infection at the cellular level and disease progression in the host. Here, we summarize the literature data on such virus–cell interactions in mammals and discuss how miRNAs can be used as research tools or targets in the development of novel antiviral therapeutics.

Key words: Virus, microRNA, Regulation, RNA interference, RNA silencing suppressor, HIV-1, HCV, Adenovirus

1. Introduction

Early studies in plants showed that small RNA-induced gene silencing is a potent antiviral mechanism that plants need to survive viral infection (1). Since then, it has become clear that there are many more aspects to the interaction between the RNA silencing mechanism – in plants, insects and mammals – and viral infection. Since RNA silencing is a central regulatory mechanism in eukaryotic cell biology, it is involved in many different cellular processes. These processes not only include cell development, differentiation and proliferation, but also cell death, metabolism, transposon silencing and antiviral defences (2). Viruses, being strictly dependent on cellular resources for their

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replication, therefore interact with the RNA silencing machinery at multiple levels.

RNA silencing represents a general cellular phenomenon in which small RNA molecules of 20–30 nucleotides associate with Argonaute or Piwi proteins to trigger sequence-specific inhibition of gene expression (3). Currently, three classes of small RNAs that are involved in silencing have been identified in mammals, namely microRNAs (miRNAs), endogenous small interfering RNAs (endo-siRNAs) and piwi-associated RNAs (piRNAs). Endo-siRNAs and piRNAs are primarily involved in the repression of transposons, whereas miRNAs regulate cellular gene expression (4). In recent years, miRNA-mediated gene regulation has received much attention. So far, over 700 human miRNAs have been cloned, which are estimated to regulate the expression of at least 30% of human genes (5). This regulation involves miRNA-guided targeting of the multi-protein RNA-induced silencing complex (RISC) to partially complementary sequences in the 3′ untranslated region (3′UTR) of target mRNAs. Once RISC is bound to the target site, it triggers mRNA translational inhibition or destabilization (6).

Besides mediating regulation of gene expression, RNA silencing in plants, insects, nematodes and fungi plays an essential role in the antiviral "immune" response via virus-specific siRNAs (1, 7–9). Despite the fact that the RNA silencing or RNA interference (RNAi) mechanism and machinery is highly conserved in eukaryotes, researchers failed to detect virus-specific siRNAs in virus-infected mammalian cells (10). This initially suggested that RNAi does not have an antiviral role in mammals (11). Interestingly, recent studies used novel and very sensitive deep sequencing technology to show that small virus-derived RNAs do accumulate in virus-infected mammalian cells (12, 13). It currently remains unclear to what extent these RNA molecules contribute to the antiviral defence response.

An increasing number of studies have addressed novel aspects of the interaction between mammalian viruses and small RNA-induced silencing mechanisms. It has become clear that cellular miRNAs and other components of the miRNA pathway can interact with viruses at multiple levels to influence viral replication (Fig. 1). In this chapter we will focus on this multitude of possible interactions. For a good understanding of the way in which viruses interact with cellular miRNAs in mammals, we will first give a brief overview of different aspects of the miRNA and small interfering RNA (siRNA) pathways. Subsequently, we will discuss the effect of cellular miRNAs on viral gene expression, cellular miRNAs as determinants of the viral tropism for certain cell types, virus-encoded RNA silencing suppressors (RSS), and virus-induced changes in the cellular miRNA expression profile. Finally, we will discuss the use of miRNAs or miRNA-inactivating compounds in novel antiviral therapeutic strategies. Virus-encoded miRNAs, as

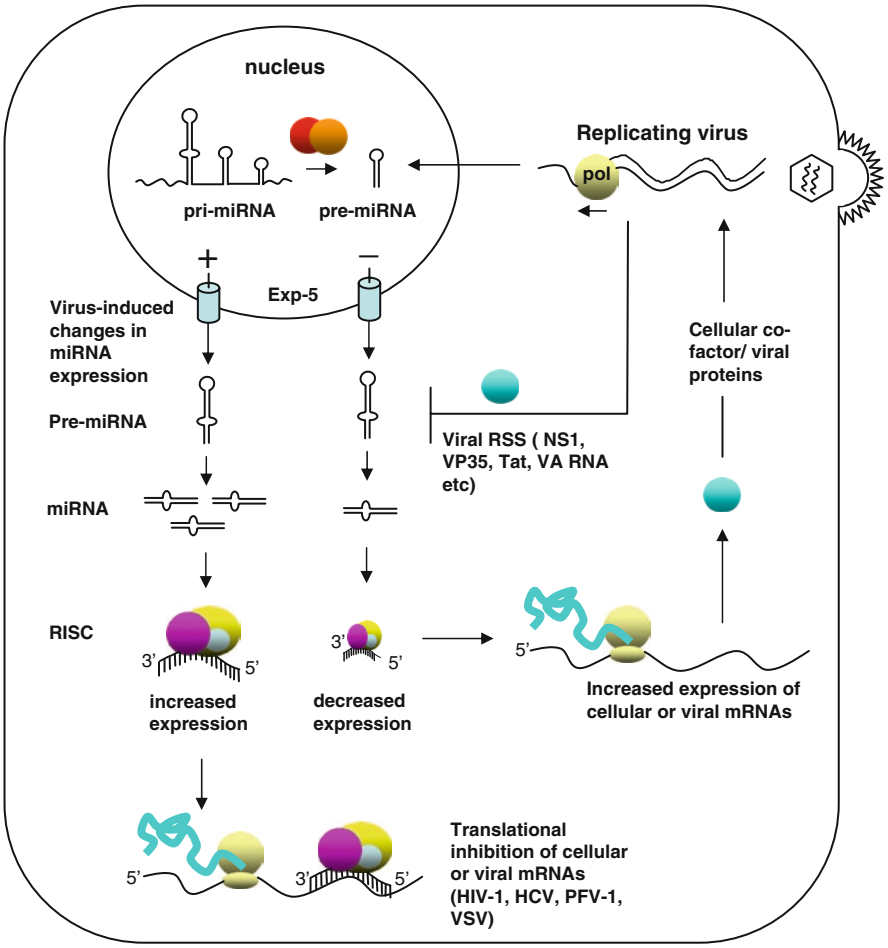

Fig. 1. Overview of the interactions between the cellular RNAi mechanism and an invading virus. After virus entry and start of replication the virus can induce (+) or inhibit (–) the expression of certain cellular miRNAs. These may influence signalling pathways and stress responses. Virus-encoded RSS factors may block specific RNAi actions. RSS factors like influenza virus NS1, Ebola virus VP35, and HIV-1 Tat can bind and sequester small RNAs/pre-miRNAs, whereas the adenovirus VA RNAs inhibit miRNA processing by saturating the miRNA pathway. Some viruses may depend on constitutively expressed miRNAs (HCV-miR-122). Increased expression of cellular miRNAs that target the viral RNA will result in inhibition of viral gene expression and decreased miRNA expression may increase virus replication. Changes in the miRNA profile may also affect cellular gene expression that may either stimulate or inhibit viral gene expression.

expressed by the herpes virus family and other DNA viruses (14), will be discussed in Chap. 3.

2. miRNA-Mediated Silencing in Mammals

Mature miRNAs are single-stranded RNAs of approximately 21–23 nucleotides that are processed from endogenously expressed primary transcripts (pri-miRNAs) (15). These transcripts are produced by polymerase II and contain several imperfectly

base paired hairpin structures. In the nucleus the RNA hairpin structure is excised by the RNAse III-like enzyme Drosha and its co-factor DGCR8 to form the precursor miRNA (pre-miRNA), which represents an imperfect hairpin structure (16). There is a separate miRNA pathway that is independent of Drosha processing. In this pathway, introns containing the hairpin are termed mirtrons, which are spliced into pre-miRNA hairpins that access the miRNA pathway. First described in Drosophila and *Caenorhabditis elegans*, these mirtrons have recently also been reported in mammals (17). Both Drosha and the mirtron pathway generate an excised hairpin of approximately 60 nucleotides, the pre-miRNA. This pre-miRNA is exported to the cytoplasm by the nuclear RNA export factor Exportin 5 (Exp5), where it is further processed by the RNAse III-like enzyme Dicer in association with its co-factors TAR binding protein (TRBP) and PACT (18, 19). The Dicer-TRBP-PACT complex cleaves off the terminal loop resulting in the miRNA duplex with a two-nucleotide 3′ overhang on either side. Depending on the structure, miRNAs associate with one of the four Argonaute proteins to form the functional miRNA-loaded RISC complex. In *D. melanogaster* miRNAs with mismatches within the central part of the duplex are preferentially incorporated into AGO1. The miRNAs with more perfect duplexes associate with AGO2, which is the only member of the Argonaute protein family with slicing activity. Similar sorting mechanisms are conserved in C. elegans and mammals (20). Once loaded into RISC, the miRNA duplex is unwound into a guide strand and a complementary passenger strand that is subsequently degraded. RISC programmed by the guide miRNA subsequently targets mRNAs with complementary sequences within the 3′UTR. Pairing of the 5′ 7–8 nucleotides of the miRNA (seed region) to multiple sites within the 3′UTR of an mRNA is important for target recognition and translational inhibition (21). The targeted RNAs are translocated to cellular processing (P)-bodies where they are stored or de-adenylated, de-capped, and subsequently degraded (22). It was initially thought that miRNA targeting could result in cleavage of the mRNA target, but only in rare cases. However, at the recent 2010 Keystone meeting it was reported that, in contrast to general belief, miRNAs do frequently induce RNA destabilization instead of translational inhibition.

3. Virus-Derived Small Interfering RNAs

Unlike miRNAs, siRNAs are perfectly complementary duplexes of 21 nucleotides that originate from dsRNA precursors upon processing by Dicer. The siRNAs are loaded into AGO2-containing

RISC and, in case of sufficient complementarity to the target mRNA, guide RISC towards cleavage of the target. In plants, worms and insects, the dsRNA precursors can be long virus-derived dsRNAs, e.g. replication intermediates. Once incorporated in RISC, the processed virus-specific siRNAs trigger the sequence-specific cleavage of the homologous viral RNAs. Plant and insect viruses counter this RNA-based antiviral response through the production of specific RSS factors (23, 24). In mammals, perfectly base paired long dsRNAs trigger the interferon (IFN) response that signals cells to induce the innate antiviral IFN response, which involves the induction of many different genes (25). Despite serious attempts, virus-specific siRNAs have not been detected in virus-infected mammalian cells via conventional cloning and sequencing techniques (10, 26). Because mammals have the IFN response and virus-specific siRNAs could not be detected, there was little support for an antiviral function of RNAi in mammals. Therefore, studies on siRNAs in mammals mostly focused on the use of artificially introduced synthetic siRNAs or short hairpin RNA vectors as tools to induce sequence-specific knock-down of a gene of interest (27).

Recently however, new highly sensitive sequencing technology has become available that is being used to analyze low abundant species of regulatory RNAs including virus-derived siRNAs in mammalian cells. Watanabe was the first to use 454 sequencing to study small RNAs in mouse oocytes, where transposon-specific RNAs were found to accumulate (28). These so-called endo-siRNAs actively repress the expression of the corresponding transposable elements and certain protein-coding genes (28–30). The endo-siRNAs are processed from naturally occurring dsRNAs formed by hybridization of perfectly complementary transcripts that are made by bidirectional transcription of the genome segments.

Two other recent studies used deep sequencing technology to show that low amounts of small virus-specific RNAs accumulate during virus infection in mammalian cells. Parameswaran et al. reported small virus-derived RNAs in cells infected with dengue virus, vesicular stomatitis virus (VSV), poliovirus, hepatitis C virus (HCV) and West Nile virus (12). In addition, Yeung et al. reported the accumulation of small virus-derived RNAs in HIV-1 infected cells (13). In this study, several discrete small virus-specific RNA species were detected. One of the HIV-specific RNAs detected was a molecule of 18 nucleotides that was complementary to the HIV-1 primer binding site (PBS). This is the sequence element in the HIV-1 RNA genome to which $tRNA^{Lys3}$ binds to prime reversed transcription. This process is essential for the generation of the proviral DNA that integrates in the host genome and from which the viral proteins are subsequently expressed. Expression of the PBS-specific small RNA was found

to depend on Dicer. Furthermore, this small RNA was shown to associate with Ago2, suggesting a role in antiviral responses. However, recent studies indicate that cellular tRNA molecules can also be processed by Dicer into 18-nucleotide long fragments in uninfected cells (31). This raises the possibility that the PBS-specific small RNA is of cellular origin.

Thus, recent studies demonstrated that small RNAs do accumulate in virus-infected mammalian cells, but it is currently not clear whether these RNAs represent functional siRNAs that negatively affect virus replication. In any case, the highly sensitive deep sequencing technology will certainly help to further describe and classify this new class of regulatory RNAs.

4. Cellular miRNAs Targeting Viral Sequences

Although the role of antiviral siRNAs in mammalian cells is still unclear, there is evidence that cellular miRNAs are able to target and inhibit viral gene expression. At least four viruses have been reported to be subject to miRNA-mediated repression of gene expression. These include primate foamy virus type 1 (PFV-1), HIV-1, HCV and VSV (Table 1) (32–35). The first reported example of a cellular miRNA that targets a viral RNA genome is miR-32. This miRNA targets the retrovirus PFV-1 in a genome region that is present in the 3′UTR of all viral transcripts and open reading frame 2 that encodes both the viral Bet and EnvBet proteins. Targeting of the viral RNA by miR-32 results in reduced virus replication, and PFV-1 requires the RSS activity of its Tas protein to overcome this inhibition (33).

Another example of a virus that is restricted by cellular miRNAs is VSV. Ostsuka et al. showed that VSV transcripts encoding the viral large protein (L protein) and the phosphoprotein (P protein) are targeted by miR-24 and miR-93, respectively (34). Reduced expression of miR-24 and miR-93 in Dicer-deficient mice caused a strong increase of VSV replication leading to virus-induced lethality. Besides VSV, the authors also tested the susceptibility of Dicer-deficient macrophages for infection by other viruses such as encephalo-myocarditis virus, lymphocytic choriomeningitis virus, Coxsackievirus group B serotype 3, influenza A virus, herpes simplex virus type 1 (HSV-1) and vaccinia virus. Interestingly, only HSV-1 displayed increased virus production, suggesting that Dicer-mediated antiviral RNAi responses do not represent a general antiviral mechanism in mammals. As miRNAs are differentially expressed among different cell types and viruses likewise infect specific subsets of cells, one should thus be extremely careful in drawing general conclusions. The lack of an effect on virus replication in Dicer-deficient macrophages does

Table 1
Cellular miRNAs targeting viral genes

Virus	miRNA	Target	Effect	References
HIV-1	miR-28, miR-125b, miR-382, miR-150, miR-223	TAR, Env, NF-κb, Env, Nef	Induces viral latency	(32, 37)
	miR-29a	Nef	Inhibits translation/ replication	(39, 40)
PFV-1	miR-32	Bet and EnvBet proteins, 3′UTR	Inhibits translation/ replication (Tas protein counters this effect)	(33)
VSV	miR-24, miR-93	L and P protein	Inhibits translation/ replication	(34)
HCV	miR-122	5′UTR (IRES)	Stimulates translation/ replication (liver specific)	(42)
	miR-1, miR-30, miR-128, miR-196, miR-296, miR-351, miR-431, miR-448	C, NS5A (others not specified)	Inhibits translation/ replication (induced by IFN signalling)	(35)
	miR-199a	5′UTR (IRES)	Inhibits translation/ replication	(96)

not exclude the possibility that these viruses are affected by Dicer deficiency in the biologically more relevant cell types.

Besides inhibiting viral gene expression in an acute manner, the set of miRNAs expressed in a certain cell type may also determine the level of evolutionary freedom of the virus on a longer time scale. Certain nucleotide changes in the viral genome sequence will not be tolerated because they create more optimal miRNA target sequences that trigger repression by the miRNA machinery. This evolutionary pressure is illustrated by the observation that the artificial insertion of fully complementary miRNA target sites in viral genes results in severe attenuation of virus replication (36). This fine-tuning of the viral RNA to the miRNA composition of the host cell may also restrict the viral ability to adjust to a change in the environment, such as the invasion of a new host organism upon zoonotic transmission or the intra-host pressure when the immune system is induced. In addition to PFV-1 and VSV, cellular miRNAs have also been shown to target the RNA genome of HIV-1 and HCV. Because of the complex interaction of these viruses with miRNAs, these cases will be discussed separately.

5. Cellular miRNAs and HIV-1 Latency

Huang et al. were the first to show that cellular miRNAs can target and potently repress HIV-1 gene expression (32). HIV-1 is a member of the lentivirus genus within the family of retroviruses. Its genome is encoded by a positive-stranded RNA molecule of 9.8 kb that is reverse transcribed into double-stranded DNA that integrates into the host cell genome. HIV-1 replicates in CD4-positive T cells and thus causes a direct attack on the immune system, which when left untreated will eventually result in the development of AIDS. It was shown that miR-28, miR-125b, miR-150, miR-223, and miR-382 are able to target sequences near the 3′ end of all HIV-1 transcripts and suppress the expression of viral mRNAs in resting $CD4^+$ T cells. The expression of this set of miRNAs is reduced in activated $CD4^+$ T cells, thus allowing active virus replication. This finding suggests that miRNAs play a role in the establishment of viral latency. HIV-1 latency occurs in resting $CD4^+$ T cells when the HIV-1 provirus is stably integrated into the host genome without producing new viral transcripts and proteins. HIV-1 latency may allow the virus to escape from the immune system and latency is a major problem in attempts to eradicate the virus in a patient by therapeutic intervention.

The latency-inducing miRNAs also appear to determine the susceptibility of peripheral blood monocytes for HIV-1 infection (37). These cells express all the essential receptors for HIV-1 entry, but become infrequently infected. New findings suggest that miRNA-mediated suppression protects the cells against productive HIV-1 infection. In other words, the cellular miRNAs form one of the determinants of the viral tropism for certain cell types. Several miRNAs including miR-29a were also predicted to target the HIV-1 RNA genome (38). The miR-29a recognizes a target within the viral *nef* gene to restrict expression of the Nef protein and overall virus replication (39). Nathans et al. subsequently showed that HIV-1 mRNAs that are targeted and inhibited by miR-29a are loaded into RISCs that associate with P bodies (40).

6. Cellular miRNAs Targeting HCV

Not all cellular miRNAs that target viral mRNAs have a negative effect on gene expression and viral replication. It was convincingly demonstrated that miR-122 is required for HCV replication (41, 42). This miRNA is highly expressed in human liver cells. The increased replication is at least in part caused by enhanced translation of the viral RNA via direct interaction of miR-122

with two target sites in the 5′UTR (43). This region in the HCV genome harbours the internal ribosomal entry site (IRES) that is instrumental for efficient HCV mRNA translation.

Besides the positive role of miR-122, a specific set of cellular miRNAs is involved in the restriction of HCV gene expression. These miRNAs (miR-1, miR-30, miR-128, miR-196, miR-296, miR-351, miR-431, and miR-448) were found to be up-regulated in response to IFN signalling that is triggered during HCV replication (35). The transfection of synthetic miRNA-mimics reproduced the antiviral effect of IFN-β on HCV replication. In addition, IFN treatment leads to a significant reduction in expression of the positive miR-122 co-factor. Therefore, the antiviral activity of IFN appears to be caused by up and down-regulation of specific cellular miRNAs. A recent follow-up study did however not reveal a correlation between the miR-122 expression level in HCV-infected individuals and the viral load (44).

7. Virus-Induced Changes in Cellular miRNA Expression

Virus infection triggers cells to mount an antiviral response that involves the expression of many antiviral and stress related genes. Similarly, virus-induced changes in the cellular miRNA expression profile may have a profound effect on the outcome of infection. In an early study, Yeung et al. determined the miRNA expression profile in HeLa cells transfected with HIV-1 DNA (45). The authors reported significant down-regulation of miR-93, miR-148b, miR-221, and miR-16. The miRNA profile in peripheral blood mononuclear cells (PBMCs) from HIV-1 infected patients showed even more dramatic changes compared to cells from uninfected controls (46). Depending on the disease stage of the patient, the T cell specific miR-223, miR-150, miR-146, miR-16, and miR-191 were down-regulated 3–9-fold. Changes in the cellular miRNA expression profile can affect the expression of protein co-factors that are required for viral replication. For example, Triloubet et al. reported increased expression of eleven miRNAs in HIV-1 infected cells, whereas expression of the polycistronic miRNA cluster miR17/92 was strongly decreased (47). This miR17/92 cluster comprises miR-17-(5p/3p), miR-18, miR-19a, miR-20a, miR-19b-1, and miR-92-1 and has been implicated in various types of cancer (48). Computer-assisted target prediction showed that the mRNA encoding histone acetylase PCAF has four target sequences in its 3′UTR for miR-17-5p and miR-20a. PCAF is an important co-factor of HIV-1 transcription in interaction with the viral Tat protein. Thus, HIV-induced down-regulation of miR-17/92 increases PCAF expression, which in turn results in further enhancement of HIV-1 replication.

The miR-198 inhibits the expression of Cyclin T1 in monocytes, which is required for HIV-1 transcription elongation (37). Once monocytes are activated to differentiate into macrophages, miR-198 expression is reduced, resulting in increased Cyclin T1 expression that facilitates HIV-1 replication. Although this is not a virus-induced change, it does indicate that miRNAs can be important determinants of viral replication by affecting the expression of essential host factors. In this manner, miRNAs can have an important role in determining the cell tropism of certain viruses.

A recent study analyzed the miRNA expression profile in bronchoalveolar stem cells upon infection by the SARS coronavirus (SARS-CoV) and the miRNA targets were identified. Intriguingly, the miRNAs that were up-regulated during infection could suppress virus replication. The authors describe this as "co-opting" by means of the viral miRNAs in order to minimize the expression of viral antigens to evade the immune system (49).

In an attempt to analyze the role of miRNAs in influenza virus pathogenicity, the miRNA expression profile was analyzed in mice infected with the 1918 pandemic influenza virus (50). A specific group of miRNAs was differentially expressed in mice infected with the pandemic 1918 virus compared to mice infected with a less pathogenic seasonal influenza strain. Interestingly, several of the corresponding target mRNAs encode proteins that are involved in the immune response. These results suggest that miRNAs and virus-induced changes in the miRNA expression profile may correlate with virus-induced pathogenicity (30). Wang et al. determined the differentially expressed miRNAs in chicken lung and trachea infected with the low pathogenic H5N3 avian influenza virus (51). It was reported that 73 and 36 miRNAs are differentially expressed in lungs and trachea, respectively, upon virus infection. This list includes miR-146a, which has been proposed to be involved in immune-related signalling pathways (52).

Finally, murine cytomegalovirus (MCMV) infection was shown to down-regulate the expression of miR-27a and b (53). This down-regulation does surprisingly not affect the other miRNAs within the same gene cluster. Therefore, the down-regulation was proposed to occur post-transcriptionally. Both miR-27a and b exhibit an antiviral effect, although it is as yet unclear how this works mechanistically.

8. Oncogenic Viruses and miRNAs

Changes in the cellular miRNA expression profile have been implicated in oncogenesis (54). Interestingly, oncolytic viruses also affect miRNA expression and this may contribute to the

multi-step oncogenic process. It is currently unclear whether these changes are part of an immune response, viral signalling, or other thus far unidentified mechanisms. Tomita et al. showed that the human T lymphotropic virus type I (HTLV-I) induces mir-146a expression, which in turn increases the growth characteristics of infected cells (55). In an earlier study miR-146a was shown to be increased in tumours (56). The T strain of the reticuloendotheliosis virus induces miR-155, which promotes cell survival (57). Marek's disease virus (MDV), which is a highly oncogenic herpes virus in poultry, triggers the up-regulation of miR-221 and miR-222 (58). These miRNAs have both been implicated in tumorigenesis and were found to be up-regulated in a number of cancers.

9. Virus-Encoded Suppressors of RNAi

Viral RSS factors were first described for plant viruses as multifunctional virulence or pathogenicity factors (24, 59). These RSSs were shown to inhibit RNA silencing via sequestration of antiviral siRNAs, protection of long virus-specific dsRNA from processing into siRNAs, or via direct blocking of specific components of the RNA silencing pathway. The identification of mammalian virus-encoded RSS functions provided one of the first indications that the RNAi mechanism is involved in antiviral responses in mammalian cells. So far, a total of 11 mammalian virus-encoded RSS factors have been described (60). These include the following proteins: influenza A virus NS1 (61, 62), vaccinia virus E3L (62), Ebola virus VP35 (63), HIV-1 Tat (63–65), PFV-1 Tas (33), and HCV coat and envelop protein (66–68). Most of these proteins display RSS activity when co-expressed together with a silenced reporter gene. Other RSS proteins prevent Dicer cleavage in vitro. An interesting way to determine RSS activity is by means of so-called trans-complementation assays in which putative RSS factor functionally replaces a known RSS in the viral context. RSS factors from plant or insect virus suppressors can sometimes be replaced by RSSs encoded by mammalian viruses. Another commonly used assay is to measure mammalian virus-encoded RSS activity in plants.

The first reported mammalian RSS factors were E3L and NS1. Both proteins are able to rescue the replication in insects of a flock house virus (FHV) variant in which the RSS B2 gene is replaced by GFP. The FHV B2 protein inhibits RNAi by sequestration of long viral dsRNAs and siRNAs. These results indicate that E3L and NS1 are the functional equivalents of the B2 protein (62). In addition, the influenza virus NS1 protein was shown to suppress RNA silencing in plants and to increase the virulence of potato virus X in *Nicotiana Benthamiana* (61).

There are a number of reports on RSS activity of the HIV-1 Tat protein. The Tat protein stimulates transcription from the HIV-1 long terminal repeat (LTR) promoter by interacting with the transactivation response (TAR) element, a stable RNA stem-loop structure present at the 5′ terminus of each viral transcript (69). Bennasser et al. were the first to show Tat-mediated RSS suppression in luciferase-based reporter assay (64). They showed that Tat-mediated RSS activity is independent of the transcriptional transactivation activity of the protein. We could confirm these results using an HIV-1 variant that is independent of Tat-mediated transcription activation (63). Only Tat mutants or heterologous factors that exhibit RSS activity could restore virus production of a mutant virus lacking Tat RSS activity. Another study showed that the HIV-1 Tat RSS function can be functionally replaced by the RSS protein P19 of tomato bushy stunt virus and that the Tat protein exhibits RSS activity in *Nicotiana Benthamiana* protoplasts (65). We also showed that the RSS protein NS3 of rice hoja blanca virus (RHBV), a plant virus, is able to complement the RSS function of HIV-1 Tat (70). The NS3 protein of RHBV is an RSS that exclusively binds small dsRNA molecules such as miRNAs and siRNAs. An NS3 mutant that is deficient in dsRNA binding and RSS activity is unable to rescue production of the Tat-negative HIV-1 variant. This result suggests that HIV-1 replication is inhibited either by siRNAs or miRNAs and that Tat RSS activity is required to counter this inhibition. Similarly, the Tas transcriptional transactivator protein of PFV-1 was shown to exhibit RSS activity (33). The Tas protein RSS function represses miR-32 accumulation, thus allowing efficient virus replication. PFV-1 Tas also shows considerable RSS activity in Arabidopsis plants (33).

Most of the mammalian virus-encoded RSS factors are proteins. However, the human adenovirus encodes an RSS function that follows a different strategy. During the late phase of infection, the virus expresses high levels of two non-coding but highly structured virus-associated (VA) RNAs, RNAI and RNAII. Previously, these RNAs were shown to block antiviral PKR activity (71). The structured nature of the VA molecules shows similarity with pre-miRNAs, raising the possibility that the VA RNAs are Dicer substrates. It was demonstrated that the VA RNAs are recognized and processed by Dicer into virus-derived si/miRNAs that are functionally incorporated into RISC (72–76). Because of their extremely high expression level of up to 1×10^8 copies per cell, the VA RNAs cause saturation of the RNAi pathway. Thus, the VA RNAs act as suppressor by saturation of the RNAi machinery. It remains possible that the VA RNAs also represent a virus-encoded miRNA, but their extremely high copy number perhaps argues against this possibility (76).

The majority of mammalian virus-encoded RSS factors described above have also been reported to have IFN-antagonistic properties. For example, the Ebola virus VP35, influenza virus NS1, and vaccinia virus E3L proteins also act as antagonists of the cellular dsRNA sensors RNA helicases retinoic acid-inducible gene I (RIG-I), dsRNA-dependent protein kinase (PKR), or 2′–5′ oligo adenylate synthetase enzymes (OAS) (77–85). In mammals the IFN pathway is activated upon virus infection when viral nucleic acids are sensed by pattern-recognition receptors on the host cell. This activation leads to inhibition of viral replication and cell proliferation, apoptosis, and destruction of virus-infected cells by natural killer cells. The double function of many viral RSS/IFN-antagonistic proteins is perhaps not that surprising considering that both the RNAi and IFN pathways are induced by the same virus-derived molecule, dsRNA. Possibly, the RNAi and IFN pathways co-operate in the innate defence response against invading viruses. The antiviral responses against HCV provide a good example on how the IFN and RNAi mechanisms may co-operate to optimally combat virus infections (35).

10. miRNAs in Novel Antiviral Approaches

Therapeutic RNAi via delivery of synthetic siRNA or intracellular shRNA expression is currently being developed as a novel approach to treat various genetic diseases as well as pathogenic virus infections (27). Indeed, direct targeting of the RNAi mechanism towards viral mRNAs can potently inhibit virus replication. RNAi-mediated inhibition of host factors that are required for virus replication is also being considered in RNAi-based antiviral strategies. The newly discovered interactions between cellular miRNAs and pathogenic viruses likewise provide new possibilities for antiviral drug design. In fact, the therapeutic use of such miRNA co-factors is similar in concept to antiviral silencing of protein co-factors that are required for virus replication. Targeting of the set of cellular miRNAs that have been implicated in HIV-1 latency has been suggested as a strategy to block or reduce HIV-1 latency (32, 86). HIV-1 latency poses a serious problem for the treatment of HIV-1 infected individuals with antiviral drugs. Antiviral drugs can strongly reduce the level of replicating virus, but HIV-1 will rapidly re-emerge from the latent reservoir after stopping therapy. Antisense inhibitors of the latency-inducing miRNAs could counter latency and induce active virus production. Activation of the latent reservoir may eventually allow for purging of the viral reservoir, such that one can eradicate the virus in an infected individual and cure the patient.

Another example of a promising new target in antiviral drug design is miR-122. This miRNA is required for HCV translation and replication (41, 42). Inhibition of miR-122 by antisense oligonucleotides indeed results in a strong decrease of HCV replication in vitro (42, 87–89). Interestingly, Lanford et al. recently demonstrated that treatment of chronically HCV-infected chimpanzees with a locked nucleic acid (LNA)-modified oligonucleotide that targets miR-122 leads to long-lasting suppression of HCV viremia (90).

Besides direct targeting of specific cellular miRNAs to block viruses, researchers have also recognized the use of miRNAs as a novel tool in the design of viral vectors (36). Insertion of miRNA targets in vectors can be used to alter the cellular tropism of the vector or to generate attenuated virus variants that may be used for vaccine development. In this strategy, the inserted targets are usually fully complementary to the cellular miRNAs, thus resulting in cleavage of the viral/vector RNA. For example, insertion of miR-142-3p targets in a lentiviral vector restricted transgene expression in the hematopoietic cell lineage where this miRNA is highly expressed, whereas optimal transgene expression was maintained in non-hematopoietic cells (91). Insertion of the liver-specific miR-122 target in adenovirus severely attenuated virus replication in hepatocytes, but allowed normal replication in other cells (92). Insertion of targets for the muscle specific miR-133 and miR-206 in the 3′UTR of an oncolytic picornavirus, Coxsackie virus A21, strongly attenuated viral pathogenicity (93). Another example is VSV, which has been proposed as possible recombinant vaccine platform, but which causes encephalomyelitis in rodents and primates. By insertion of targets for neuron-specific miRNAs, virus replication in neurons could be inhibited without compromising replication in other tissues (94). A similar approach was used to construct an attenuated influenza A virus vaccine. Perez et al. inserted miRNA response elements into the open reading frame of the viral nucleoprotein. The resulting virus displayed a 2 log reduction in mortality and elicited a diverse antibody response, illustrating the potency of miRNA-mediated control of live-attenuated virus vaccines (95).

Finally, in addition to inhibition, overexpression of specific cellular miRNAs may also be used in therapeutic antiviral approaches. This strategy could be employed when the expression of a certain miRNA is down-regulated upon virus infection in order to increase the expression of a required host co-factor. Overexpression of this miRNA should inhibit the accumulation of the host protein and thus inhibit virus replication. So far, this approach has not been realized, but miR-198 and miR-20 form attractive candidates for such an anti-HIV approach (37, 47). A potential danger of this strategy is that miRNA overexpression affects the expression of many cellular genes. In the optimal scenario, miRNA expression is restored to the level of the uninfected cell.

11. Conclusions

In recent years much has been learned about miRNA biogenesis and function. However, because relatively few miRNA targets have been identified thus far, little is known about miRNA regulatory networks. Even more so, the way in which viruses interact with these systems remains largely unknown. It has gradually become apparent that an interaction between viruses and the host miRNA pathway can take place at multiple levels. To complicate matters further, cellular miRNAs can stimulate or inhibit virus replication, and sometimes a complex dual effect is observed. These RNAi-virus interactions vary significantly depending on the particular virus strain and the host cell type used for the infection, thus causing much variation among experimental systems. Many mechanistic details on how these processes interact and what the consequences are for viral replication, the host cell and virus-induced pathogenesis remains to be determined. Novel technology for high-throughput analysis of small virus-derived RNAs will help to answer some of these questions. It is likely that an increased understanding of these processes will ultimately lead to the identification of new targets for the development of antiviral therapeutics.

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