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The roles of microRNAs in mammalian virus infection

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Summary

MicroRNAs (miRNAs) are post transcriptional regulators of gene expression that are important for the control of a multitude of critical processes in mammalian cells. Increasing evidence supports that miRNAs also have important functions in viral replication and may be used by host cells to control viral infection. Expression of miRNAs has been reported for various groups of viruses including herpesviruses, small DNA viruses and retroviruses. The recent identification of target genes regulated by some of these viral miRNAs suggests that they may function in the control of lytic and latent viral replication, in the limitation of antiviral responses, in the inhibition of apoptosis, and in the stimulation of cellular growth. In this review, we summarize in brief recent findings on the antiviral activities of cellular miRNAs and the viral counter-responses to the cell's RNAi restriction.

Keywords

microRNA; virus replication; innate immunity; post transcriptional regulation; viral gene expression; virus-host interaction

1. Introduction and approaches for analyzing viral and cellular microRNAs (miRNAs)

1.1 MiRNA biogenesis and function

MiRNAs have emerged as ubiquitous regulators of gene expression in metazoans, from plants to invertebrates to vertebrates. Increasingly, evidence supports that important cellular processes are regulated by miRNAs; these include cellular development, differentiation, proliferation, apoptosis, and metabolism [1], [2], [3]. Hence, it is not surprising that miRNAs may also play roles in the disease pathogenesis of cancers and infections [4], [5], [6], [7]. Here, we review in brief some recent findings on how the miRNA pathway is exploited by mammalian viruses and how host cell miRNAs may reciprocally influence viral replication.

Mature miRNAs are single-stranded oligoribonucleotides of 19–25nt in size that originate from larger RNA polymerase II (RNAP II) transcripts, termed primary miRNAs (pri-miRNA) [3], [8], [9]. Pri-miRNAs can reside in exons or introns of genes and contain a stem-loop structure

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with an imperfectly duplexed stem. During maturation, the stem-loop of a pri-miRNA is processed through the enzymatic activities of two type III RNAses, Drosha and Dicer. The processing starts in the nucleus where Drosha trims the pri-miRNA to a transcript of about 70nt, termed pre-miRNA, which is subsequently exported into the cytoplasm by exportin 5. In the cytoplasm, a second RNAse, Dicer processes the pre-miRNA into a ~22nt duplex mature miRNA. Thereafter, one strand of the mature miRNA (the "guide" strand) is captured into an Argonaute-containing effector complexes [RNA-induced silencing complex (RISC)] while the accompanying "passenger" strand is frequently degraded. The miRNA-guide strand in RISC serves for target recognition of protein-coding mRNAs via base complementarity with sequence elements, which mostly reside in their 3' untranslated regions (UTR). The number of targeted sequences and their extent of complementarity with the miRNA-guide stand determine the magnitude and mechanism of suppression. MiRNAs, that are fully complementary to their mRNA targets, can induce direct endonucleolytic cleavage within the base-paired region leading to rapid decay of the entire transcript [10],. On the other hand, in a setting of mRNA-miRNA base-pairings that are imperfectly matched, downregulation functions best if the two sequences can base pair through a 7-nt miRNA "seed" sequence, defined as positions 2–8 from the 5' end of the miRNA [11]. As yet, the suppression mechanism of protein production from mi-RISC-targeted mRNAs is not fully understood. Nonetheless, one plausible model for the mechanism by which miRNAs induce translational inhibition, deadenylation, and degradation is via the translocation of the targeted mRNA into P bodies, cytoplasmic structures, which do not contain ribosomes. Such translocation could be mediated by a P body component (GW182) binding to the Argonaute protein in the miRNA-RISC complex. Once within the P body, the targeted mRNA can be de-adenylated by resident de-adenylases, and then either decapped and degraded or held in stasis (i.e. spatially removed from the translational machinery) [11].

1.2 Identification of viral miRNAs and their targets

The most well-established method used to identify virus-encoded miRNAs is based on the experimental isolation of small RNAs from infected cells followed by cDNA cloning, sequencing and then *in silico* sequence analyses [9], [12], [13]. On the other hand, not infrequently, *de novo* computer-based miRNA gene prediction methods are used [14], [15] to initiate the identification of candidates miRNAs. Nevertheless, such candidate miRNAs need to be verified, if not by cloning, by direct detection with Northern blotting or real-time PCR.

Once a predicted miRNA is authenticated, understanding which mRNA(s) the miRNA targets is no simple feat. Bioinformatic computation is employed to search for miRNA-seed sequences in the 3' untranslated regions of potential target mRNAs [9], [16]. While certain targeting rules support that a "seed" sequence complementarity between miRNA-mRNA is critical to physiological interactions, experimental realities are that imperfect complementarities frequently suffice for silencing [17], and that one miRNA can potentially modulate the expression of up to 100 discrete mRNAs [18]. Because a miRNA can affect a large number of mRNAs, a useful methodology is to screen for potential targets using micro array –based analyses of mRNA down-regulation [19]. To this end, miRNA-encoding vectors expressing a single or multiple miRNAs can be introduced into cultured cells, and resulting mRNA profiles can be analyzed. Alternatively, miRNA targeted mRNAs can be isolated from P-bodies by immunopurification [20]. Array-based and P-body derived data can then be individually validated by confirming that a miRNA can indeed regulate a putative mRNA target. One way to perform the confirmation is to position the 3' UTR from the mRNA "targeted" by a miRNA into a chimeric transcript which contains a reporter gene. The ability of the miRNA when overexpressed to silence the reporter-containing transcript would be one criterion supportive of specific targeting.

1.3 Identification of cellular miRNAs and their viral targets

While it is well-accepted that mammalian viruses encode miRNAs that can regulate viral [12] [21] [22] [23] and cellular [24] [25] [26] functions, it remains debated whether cellular miRNAs physiologically regulate the replication of mammalian viruses [27]. Two methods have been used to shed light on this latter question. First, miRNA-processing enzymes such as Drosha and Dicer have been knocked down to reduce the processing of mature mammalian miRNAs [28], [29], [30]. When mammalian miRNAs levels were reduced, virus replication in such cells became more robust (see below). In a separate approach, when virus-encoded RNAi suppressors were overexpressed in mammalian cells, viral replication in such cells increased by 5- to 10- fold [31]. Taken together, these results suggest a mammalian miRNA/RNAi function that moderates the replication of viruses. Second, the role of individual cellular miRNA have been assessed using chemically modified antisense-oligoribonucleotides, or antagomirs, [32], [33]. This type of analysis is increasingly employed to inactivate individual cellular miRNAs in order to verify their roles in targeting viruses.

2. A selective overview of virus-encoded microRNAs

2.1 Herpesviruses

By far most of the extant viral miRNAs that have been identified come from herpesviruses. This is a family DNA viruses with large viral genomes approximating 150–230kb containing more than one hundred open reading frames. These viruses, besides their ability to lytically infect cells, can also establish latent infections in which viral gene expression is confined to a few specialized genes. Latency is closely related to the ability of these viruses to establish life-long persistence in their hosts. Many herpes miRNAs are expressed during latency, serving to allow the virus to control viral and cellular functions to minimize the exposure of viral proteins made in infected cells to the host's immune system. It should be noted that while cellular miRNAs are frequently conserved across species (e.g. between mouse and human), miRNAs encoded by different herpes viruses are mostly not conserved with each other or with their host miRNAs. This lack of conservation could indicate a rapid evolution of herpes miRNA-genes. Alternatively, in instances where herpes-miRNAs serve to target host mRNAs, this may reflect the genetic variability of the 3'UTR target sites in cellular transcripts. Because 3'UTRs are not used for protein coding, they may experience less selective pressure for conservation and thus could diverge more rapidly from species to species.

2.2 Herpes simplex virus (HSV)

HSV belongs to the alpha subgroup of herpesviruses, which can persist in neurons. Although several miRNAs are predicted for HSV1 and HSV2 [15], [34], one virally-encoded miRNA has been verified [34]. No viral miRNAs have been identified yet in the alpha herpes varicella zoster virus [15].

2.3 Human cytomegalovirus (HCMV)

HCMV is the prototype of beta herpesviruses. CMVs are viruses with the largest genome size; they persist in haematopoetic cells like granulocytes and their progenitors. Nine HCMV miRNAs were cloned from lytically infected primary cells. They originate from sequences distributed across the viral genome. Three of these are transcribed from the complementary strand of known ORFs, five miRNAs are located in intergenic regions, and one within an intron [15]. Murine cytomegalovirus (MCMV), a close relative of HCMV, provides the most frequently used animal model for HCMV, which does not replicate in mice. 17–18 miRNAs were found to be encoded by MCMV; all of them are expressed during lytic replication. None of the MCMV miRNAs has significant homologies with HCMV-miRNAs [35], [36]. As yet, no microRNAs have been found encoded by the beta herpesviruses HHV-6 and HHV7 [15].

2.4 Epstein-Barr Virus (EBV)

EBV is a gamma herpesvirus. After a primary infection, EBV persists life-long in human B-lymphocytes [37]. The virus is capable of immortalizing normal human B-cells *in vitro* and is associated with several human malignant diseases including Hodgkin's lymphoma, endemic Burkitt's lymphoma and nasopharyngeal carcinoma. In malignant cells or in vitro transformed lymphoblastoid cells, a group of EBV latency-associated genes or subsets are expressed. The complete set of latency-associated genes contains latency associated membrane proteins (LMP) and Epstein-Barr virus associated nuclear antigens (EBNAs). The unspliced primary EBNA transcripts are the sources for the 3 miRNAs (BHRF1miRNAs) that are encoded in an intron [12], [38], [39]. In latent infection, EBV also expresses at least 14 BART miRNAs, which are encoded by a genomic region which also encodes the non-coding *BamH*I A rightward transcripts (BARTs) [12], [39], [40]. Seven of EBV's microRNAs are closely related to microRNAs of an EBV-related monkey virus (Rhesus lymphocryptovirus) and provides a first example of microRNA conservation within the herpesvirus family [41].

2.5 Kaposi's sarcoma-associated herpesvirus (KSHV *or* HHV8)

KSHV is another gamma herpesvirus linked to human malignancies such as Kaposi's sarcoma, primary effusion lymphoma (PEL), and Castleman's disease [42]. KSHV has 12 miRNAs within a 5-kb latency-associated region of the viral genome, which also encodes the transforming protein–coding Kaposin [15], [43]. The miRNAs are expressed in KSHV-positive primary effusion lymphoma-derived cells and endothelial cells. Rhesus Monkey Rhadinovirus (RRV), an animal virus model for KSHV, encodes several miRNAs in the same genomic location as the KSHV miRNAs. Nevertheless, KSHV and RRV miRNAs are unrelated in their primary sequences [44]. A related mouse gamma herpesvirus 68 (MHV68) expresses 9 miRNAs, which also cluster within a small (6-kb) region of the viral genome [15].

2.6 Small DNA Viruses

Viruses of the adenovirus and polyomavirus families have also been shown to express miRNAs. In the case of adenoviruses, the miRNA is processed from a non-coding cytoplasmic RNA polymerase III transcript, called virus-associated RNA (VAI). VAI confers resistance to cellular interferon-related defenses and contributes to viral replication. It was recently found that VAI RNA can be processed by Dicer into a miRNA that serves silencing function during adenovirus infection of cells [45]. Separately, a single miRNA (mirS1), which is derived from the 3' UTR of the viral late transcript, was characterized for SV40 [21].

2.7 Retroviruses

The human immunodeficiency virus (HIV-1) was predicted earlier by computational analyses to potentially encode five candidate miRNA precursors [14], [46]. With one exception [47], to date these putative miRNAs have not reportedly been cloned successfully [15], [13]; however, some predicted moieties from HIV envelope *Env* [33], *Nef* reading frame [48], [49] and the 5' non-coding TAR leader sequence [26] [50] have been detected and characterized by Northern blotting. The difficulties in cloning HIV-1 derived non-coding RNAs from infected cells [15] suggest that they exist in low abundance. Nonetheless, more recent in depth experiments accompanied by massively-parallel pyrosequencing have cloned and identified several HIV-1 small non-coding RNA (sncRNA) species (ML Yeung and KT Jeang, unpublished data). Additional studies are, however, needed to address the physiological relevance of these viral sncRNAs in HIV-1 infection [51].

3. Diverse functions provided by viral miRNAs

3.1 Regulation of viral transcripts

Frequently viral miRNAs are encoded by the opposite strand of a protein coding gene, which results in a perfect match of miRNA and the coding mRNA target. This complementarity holds obvious implications for the control of viral protein expression. Such control can affect the expression of regulatory proteins during the lytic or the latency/persistence phases of replication. For example, the latency associated membrane protein LMP-1 of EBV, a transforming protein, is under the control of viral miRNAs; a cluster of BART miRNAs targets the 3' UTR of the transcript and represses LMP1 protein expression. Functional consequences of this regulation affect LMP1-induced NF-κβ signaling and apoptosis resistance [52]. The regulation exerted by miRNAs could explain the discrepancy observed between LMP-1 protein expression and its mRNA expression, and the varying levels of LMP1 in nasopharyngeal carcinoma [52].

MiRNAs have also been shown to be involved in the control of regulatory proteins expressed at the beginning of viral replication (Figure 1). For instance, the expression of the immediate early IE1 (UL123, IE72) protein of human cytomegalovirus (HCMV), a transcription factor required for the expression of many viral genes, is targeted by virus-encoded miRNA, miR-UL112-1. The IE1 3'UTR target site is necessary and sufficient to direct miR-UL112-1-specific inhibition of expression in transfected cells [22] [23]. Expression of early viral protein synthesis is also limited in SV40 by miRNA control. A microRNA expressed late in infection targets the 3’UTR of the early transcript and reduces the expression of T antigen. This repression of T antigen is advantageous for the virus since it reduces sensitivity of infected cells to cytotoxic T cells [21].

Similar to some herpesviruses, the role of viral miRNA in the life cycle of HIV remains incompletely clarified. The reported HIV-1 encoded small non-coding RNAs are capable, when over expressed, to reduce the levels of viral transcripts [33], [48], [26], and thus they could contribute to the maintenance and/or establishment of viral latency. Some of these viral sncRNAs lack classical targets in the 3’ UTR regions of genes and instead have complementarity with coding mRNA sequences. Thus, they may function by RNA destabilization, or as has been suggested for the Nef-miRNA and for the TAR-miRNA by suppression of long terminal repeat (LTR) transcription [48], [26].

3.2 Interference with host gene expression

Since miRNAs are not targets of the host’s immune response, they are ideal means for viruses to regulate the host cell during a latency phase. The 12 miRNAs encoded by KSHV are probably also part of its latency associated transcription program in transformed cells and in Kaposi’s sarcoma. Gene expression profiling in cells stably expressing all KSHV-encoded miRNAs identified eight genes which were down-regulated between 4- and 20-fold. One of these genes encodes thrombospondin 1 (THBS1) whose protein expression was decreased >10-fold [19]. THBS1 is a tumor suppressor and anti-angiogenic factor, which exerts its anti-angiogenic effect in part by activating the latent form of TGF-β (Figure 1). Thus KSHV-encoded miRNAs may contribute directly to pathogenesis by down-regulation of THBS1, a major regulator of cell adhesion, migration, and angiogenesis [19]. One KSHV miRNA, miR-K12-11, could in particular contribute to malignant transformation by the virus. It shares 100% seed sequence homology with the oncogenic hsa-miR-155, and accordingly is dubbed an ortholog of miR-155. MiR-155 has been shown to be up-regulated in lymphomas and to be critically important for B-cell development [53], [54]. Its overexpression in transgenic mice induces B -cell lymphoma [55]. Due to the homology in seed sequences, it is highly likely, that the viral miRNA is functionally equivalent to the oncogenic cellular microRNA. In support of this notion, miR-

K12-11 and miR-155 regulate a common set of cellular targets including the transcriptional repressor BACH-1 [24] [25].

4. Actions of cellular miRNAs in viral infections

4.1 Cellular miRNAs influence the replication of mammalian viruses

If viruses use virally encoded miRNAs to influence the cell's milieu, is there evidence that in return cellular miRNAs are employed by the host to reshape the course of viral infections? Several recent experimental findings are consistent with mammalian small non-coding RNAs acting to regulate the invading viruses. First, cellular short interfering RNAs (siRNAs) [56] and a separate class of small non-coding RNAs called piRNAs appear to work in germ and somatic cells to suppress the replication of mammalian endogenous retroviruses [57], [58]. Recently, a subset of Dicer-processed miRNAs has also been implicated in repressing retrotransposon activities in mouse ES cells [59]. Second, robust bioinformatics analyses coupled with experimental validation have revealed that discrete human miRNAs can indeed target various types of viruses including HIV-1 [60], [61], [62], [63], [64]. In most instances, the cellular miRNAs act to moderate viral replication in cells. Hence, human miR-32 has been reported to restrict the replication of primate foamy virus type 1 (PFV-1) [63], and the 3' portion of HIV-1 mRNAs was shown to be redundantly targeted for repression by a cluster of human miRNAs including miR-28, miR-125b, miR-150, miR-223 and miR-382 [62]. Interestingly, in a rare exception, human miR-122 targets the 5' UTR of HCV RNA; and rather than repressing replication, miR-122 appears to augment intracellular HCV production [65]. This latter phenomenon is incompletely understood since cells that lack miR-122 appear to support well HCV replication [66]. Third, three independent studies have documented that deliberate repression of mammalian Dicer (which reduces the overall processing of human miRNAs) increased the replication of HIV-1 [29], vesicular stomatitis virus (VSV) [30], [67], and influenza A virus [28]. One interpretation of these results is that the mammalian Dicer-miRNA pathway acts physiologically to censor the replication of viruses such as HIV-1, VSV, and influenza A.

4.2 Interferon-mediated antiviral activity through miRNAs

The interferon (IFN) signaling pathway is well-recognized as a cellular antiviral defense. One prototypic way for eliciting IFN is through exposure of cells to long double-stranded (ds) RNAs. This stimulation holds some similarity to the use of short dsRNAs to elicit RNAi. Interestingly, there is evidence that suggests partial overlap between the IFN and the RNAi pathways. For example, RNA-binding proteins that suppress the IFN effector, protein kinase R (PKR) ([68], [69], [70], also appears to be capable of suppressing RNAi [33,71], and cellular proteins (e.g. PACT, TRBP) that act in IFN's antiviral mechanism [72], [73] are also important for miRNA processing and function [74], [75], [76], [77]. Recently, an even more direct link between IFN and RNAi has emerged. Interferon beta (IFNβ) was reported to modulate the expression of numerous cellular miRNAs. Intriguingly, eight of these IFNβ-induced miRNAs have sequence-predicted targets within the hepatitis C virus (HCV) genomic RNA. Moreover, the introduction of synthetic miRNA-mimics corresponding to these IFNβ-induced miRNAs recapitulated the antiviral effects of IFNβ on HCV replication and infection, while the neutralization of these IFNβ-induced miRNAs with antagomirs attenuated IFNβ's antiviral effects against HCV [78]. These new findings support a convergence in the use of miRNAs by mammalian cells in IFN-dependent and IFN-independent defenses against viral infection.

4.3 Viral counter-responses to the cell's RNAi restriction

Above, we have outlined evidence for miRNAs being used as a part of the cell's RNAi-restriction of virus replication. If cells thusly restrict viruses, how might the viruses respond? Viruses appear capable of responding in five different ways. These responses include

protection against restriction, suppression of RNAi, mutational escape from RNAi, modulation of the cell's miRNA/RNAi profile, and adaptation to restriction (Figure 2).

First, viruses can shield their genomes to protect them against the cell's RNAi restriction [79]. Second, viruses can transcribe small RNA-decoys that competitively occupy the cell's RNAi machinery [80], [81], [82]) or synthesize RNA-binding proteins [33], [71], [83], [84] that sequester and neutralizes the cell's antiviral siRNA/miRNA activities (Table 1). While RNA-binding proteins work well to suppress RNAi in plant cells, how well these proteins work in mammalian context has been debated [13]. Third, viruses can mutate their primary sequences or the secondary structure of their transcripts to evade complementarity driven siRNA/miRNA-restrictions [85], [86]. Finally, viruses may modulate the cell's miRNA expression profile [29], [87], in order to suppress noxious miRNAs and to elevate propitious counterparts, or alternatively, viruses may learn to adapt miRNA-restriction to benefit viral replication [65]. As alluded to above, human hepatitis C virus is currently the one rare example of a virus that has apparently evolved an adaptation paradigm [65].

5. Concluding remarks and future perspectives

About 8% of the human genome is composed of endogenous retroviruses (HERV) [88], [89]. HERVs contain repetitive genetic elements such as their long terminal repeat (LTR) sequences which in lower eukaryotic cells have been found to be processed into small repeat association short interfering RNAs (rasiRNAs; [90], [91]). While this brief review has primarily focused on mammalian cellular miRNAs, miRNAs encoded by mammalian viruses, and their relevant RNA-binding proteins, one should not lose sight of the fact that the universe of small RNA-based gene regulation is likely to be populated by other short non-coding RNAs including rasiRNA, piRNA [92], and additional yet discovered classes. Many mammalian viruses also encode perfectly matched sense and antisense transcripts [93], [94]. It remains to be explored how these RNAs form duplexes and whether these double stranded complexes are processed into small functional entities. In a relatively short period of time much has been achieved in characterizing small non-coding RNAs and their interactions with viruses; nevertheless, it is a good bet that much more remain to be discovered in the coming years.

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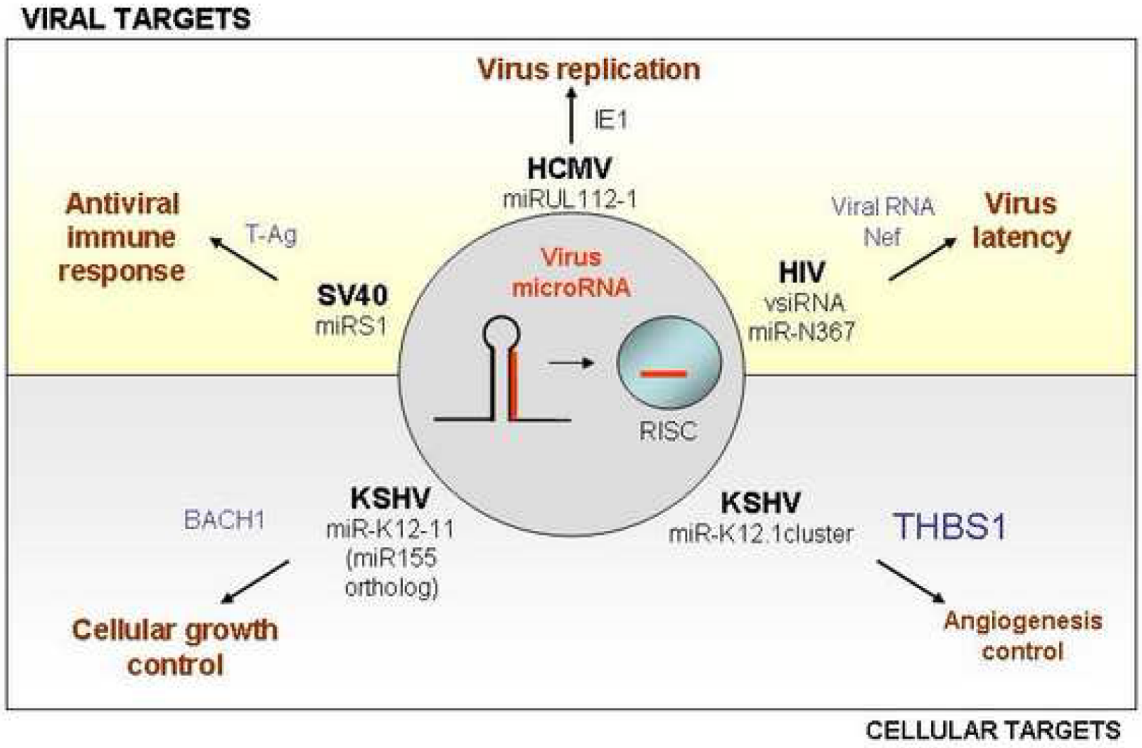

Figure 1. Potential functions of viral miRNAs

Like their cellular counterparts, viral miRNAs (red) are produced from hairpin-shaped RNA progenitors and are incorporated into the RNA-induced silencing complex (RISC). Viral miRNAs can act on both, the control of viral genes (top) or on cellular genes (bottom) by repressing their expression. The figure shows examples of miRNAs from different viruses, their targets (blue), and the proposed effects on antiviral immune response, viral replication and persistence, apoptosis and growth control (brown).

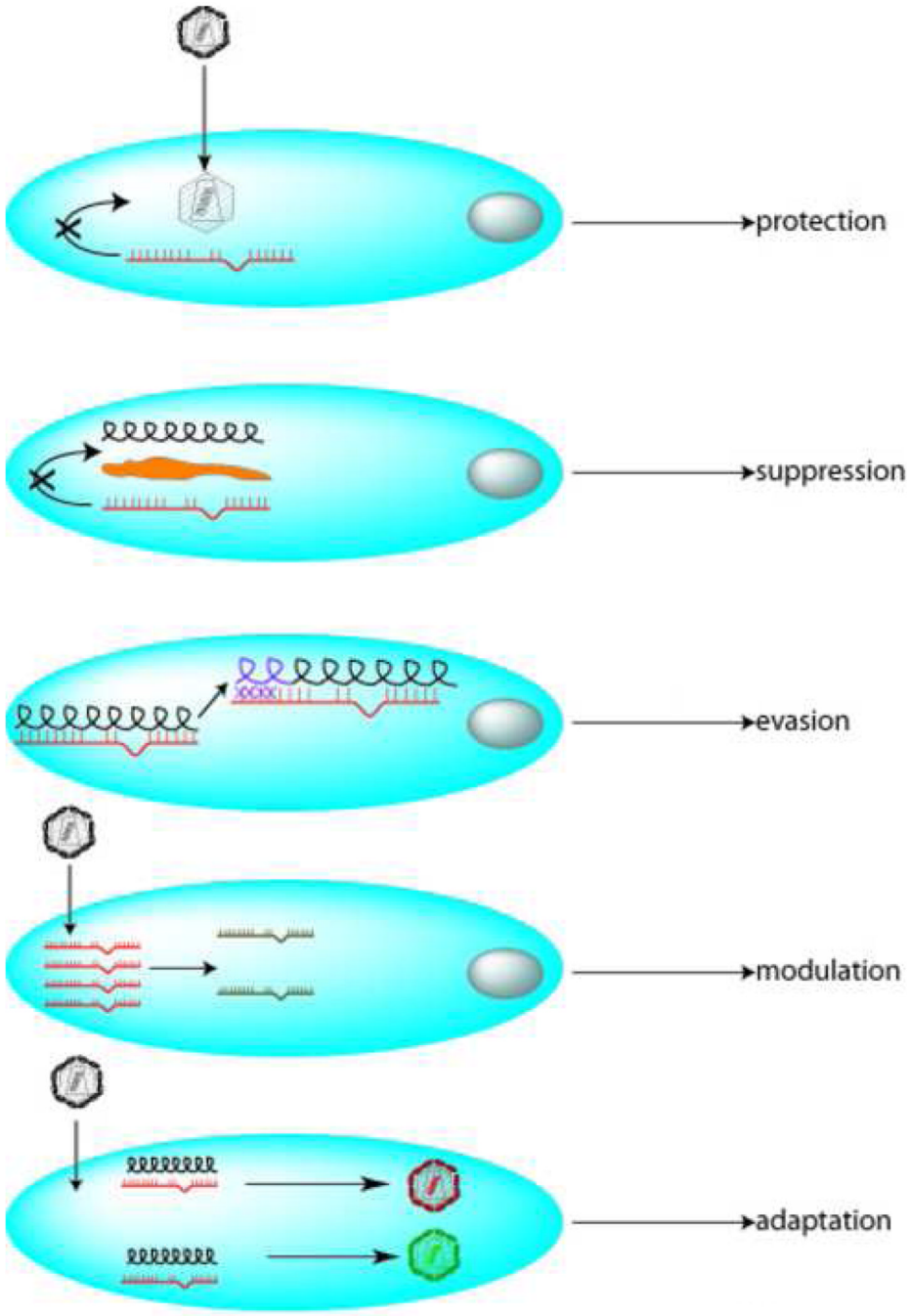

Figure 2. Five potential measures used by viruses to counter the cell's RNAi restriction

A virus particle is shown to infect a cell. In the first "protection" measure, viral RNAs could be sheathed by proteins so as to be protected against access by cellular RNAi. Second, viruses could encode RNA-binding proteins or RNA-decoys that sequester the cell's RNAi effectors leading to "suppression" of interference. In a third way, viruses can mutate their nucleotide sequences so that previous complementarities present in si-/mi-RNA sequences are not longer valid. The mutations then allow for "evasion" by the viruses from RNAi. Fourth, the virus can change the expression profile of cellular miRNAs producing a "modulation" of miRNA levels. Finally, viruses (red) initially sensitive to restriction by a miRNA could evolve to a form (green)

that can beneficially utilize the miRNA for its replication. HCV is a putative example of this "adaptation".

Table 1.

Selected examples of virus-encoded RNAi counter measures

Virus	Viral Effectors	References
Adenovirus	VA RNA	[80]; [81]; [95]
HCV	Core protein	[96]; [97]
HIV	Tat protein, TAR RNA	[33]; [71]; [82]; [98]
Ebola	VP35 protein	[71]
Influenza A virus	NS1 protein	[69]; [99]; [100]; [71]
Nodamura Virus	NoV B2 protein	[84]
Primate Foamy Virus	Tas protein	[63]