

## Review

# Virus-Encoded microRNAs: Future Therapeutic Targets?

Peng Qi<sup>1</sup>, Jinxiang Han<sup>1,2,3</sup>, Yanqin Lu<sup>1,2</sup>, Chuanxi Wang<sup>1</sup> and Fanfeng Bu<sup>1</sup>

The discovery of microRNAs (miRNAs) is a remarkable breakthrough in the field of molecular genetics, as miRNAs are key actors which regulate gene expression in diverse cellular processes from unicellular yeast to human. The recent discovery of virus-encoded miRNAs indicates that viruses also use this fundamental mode of gene regulation. Research into viral miRNAs function demonstrates that some miRNAs play an important role in regulating both the viral life cycle and the interaction between viruses and their hosts. The first *in vivo* "antagomir" study provides an exciting first step towards miRNA therapy, and the potential for ultimately designing molecular medicines based on the modulation of miRNAs seems good. *Cellular & Molecular Immunology*. 2006;3(6):411-419.

**Key Words:** microRNA, gene expression, anti-miRNA oligonucleotide, therapeutic target, antiviral

## Introduction

### *Discovery of microRNAs as regulators of gene expression*

Until the early 1990s, the Central Dogma of genetic still persisted in the mono-mechanism that DNA was transcribed to mRNA, which was then translated into polypeptide or protein, resulting in a certain specific functions or phenotypes. For years, except a few exceptions such as the common infrastructural RNAs involved in protein synthesis, transport and splicing, ncRNAs were recognized as redundant debris and discarded after splicing. The discovery of microRNAs (miRNAs) is a remarkable breakthrough in the field of molecular genetics, as miRNAs are key actors which regulate gene expression in diverse cellular processes from unicellular yeast to human.

MiRNA was first observed in *C. elegans* as RNA molecules of 18-23 nt, which are partially complementary to several regions of the 3' untranslated regions (UTR) of the target transcripts, including the *lin-4* and *let-7* genes (1, 2). In subsequent studies, the other broad class of small regulatory RNAs with similar features to *lin-4* and *let-7* were identified

in diverse organisms, ranging from from *C. elegans* to *D. melanogaster*, and even in human (3-5). These small segments of RNA were named microRNAs according to their size, including exonic and intronic miRNAs. Thus far, over 3500 miRNAs have been identified, with more than 320 found in humans (MiRBase) (6, 7).

### *MicroRNA biogenesis and function*

Including virus-encoded miRNAs, miRNA genes are found as single or clustered transcription units (8, 9) and are expressed from intron regions of protein-coding or non-protein-coding genes, or in other cases, as independent transcription units (10, 11). Transcription of miRNA genes by RNA polymerase II or III results in the production of large primary miRNA transcripts (pri-miRNAs) that are capped, polyadenylated and spliced, containing numerous bulges and mismatches (Figure 1).

The first step in miRNA processing involves the recognition and nuclear cleavage of pri-miRNA by the microprocessor complex composed by Drosha and its associated binding partner, DGCR8 (DiGeorge-syndrome critical-region protein 8) in vertebrates (Figure 1) (12-14). This cleavage generates a short stem loop pre-miRNA, which is transported by a complex of the karyopherin exportin 5 and

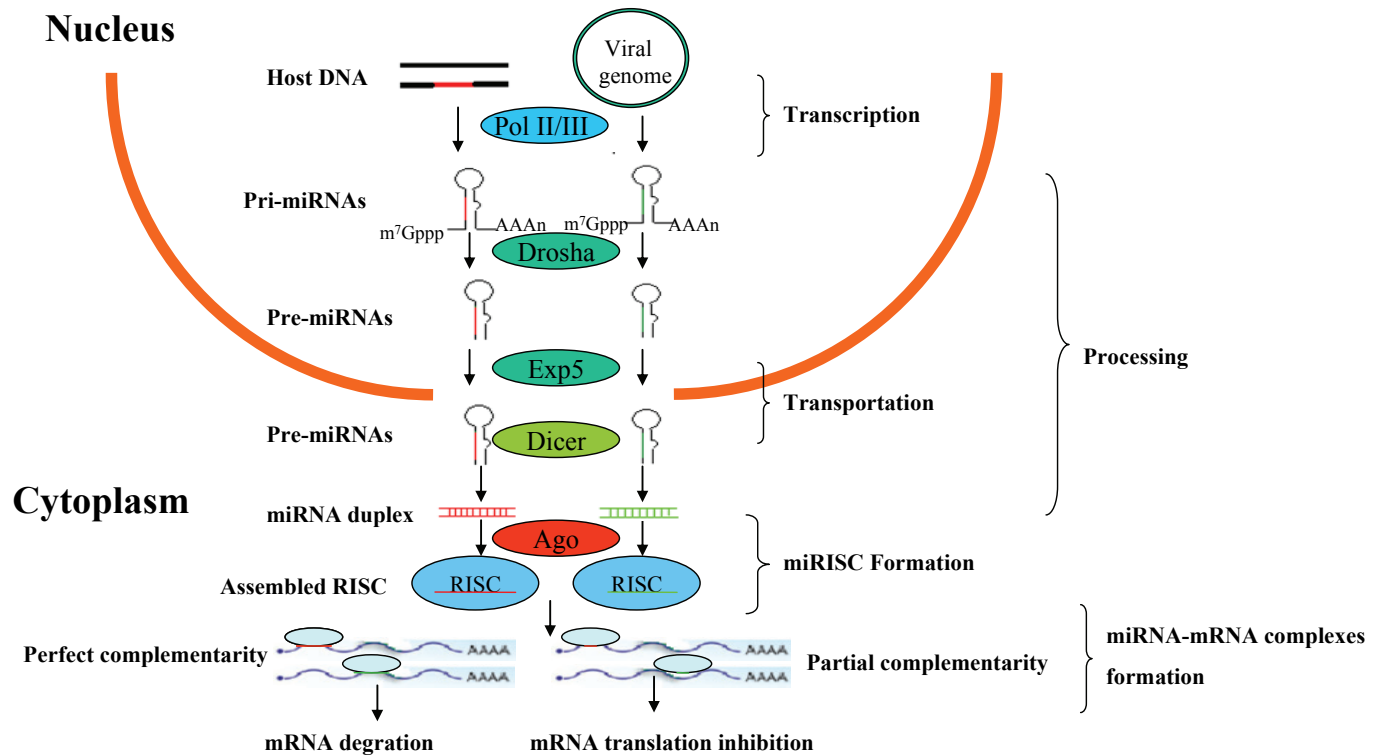
<sup>1</sup>Shandong Medicinal Biotechnology Center, Shandong Academy of Medical Science, Key Laboratory of Ministry of Health for Biotech-Drug, Jinan 250062, China;

<sup>2</sup>School of Medicine, Shandong University, Jinan 250012, China;

<sup>3</sup>Corresponding to: Dr. Jinxiang Han, Shandong Medicinal Biotechnology Center, Shandong Academy of Medical Science, Key laboratory of Ministry of Health for Biotech-Drug, Jinan 250062, China. Tel: +86-531-8291-9888, Fax: +86-531-8295-1586, E-mail: jinxianghan@sina.com.

Received Dec 14, 2006. Accepted Dec 25, 2006.

**Abbreviations:** miRNA, microRNA; pri-miRNA, primary miRNA transcript; miRISC, miRNA-containing RNA-induced silencing complex; EBV, Epstein-Barr virus; siRNA, small interfering RNA; KSHV, Kaposi's sarcoma-associated herpesvirus; HCMV, human cytomegalovirus; MDV, Marek's disease virus; CEF, chicken embryo fibroblasts; LAT, latency-associated transcript; HSV-1, herpes simplex virus type 1; SMAD3, mothers against decapentaplegic homologue; TGF- $\beta$ , transforming growth factor- $\beta$ ; VA RNA, virus-associated RNA; shRNA, short hairpin RNA; LTR, long terminal repeat; AMO, anti-miRNA oligonucleotide; rLCV, rhesus lymphocryptovirus; hAV, human adenovirus.



**Figure 1. MicroRNA biogenesis pathway.** Processing of both host miRNAs (red) and virus-encoded miRNAs (green) is thought to take place through the same pathway. Transcription of miRNA genes by RNA polymerase II or III results in the production of primary miRNA transcripts (pri-miRNAs). Two major processing events lead to the production of mature microRNAs (for simplicity, only catalytic components of the pathway are depicted in this diagram). Initially, within the nucleus, the long primary transcripts of miRNA (pri-miRNAs) containing the hairpin structure are processed by the nuclear RNase III Drosha to release the precursor of miRNA (pre-miRNA), and are then exported out of the nucleus by one of the nuclear transport receptors, Exportin-5 (Exp-5). Once in the cytoplasm, they are subjected to another processing step by another RNase III enzyme, Dicer. The ‘guide’ strand of the miRNA duplex then associate with RISC to target the specific host- or virus-encoded transcripts to regulate gene expression, resulting either cleavage or translational inhibition of its target mRNA, depending on the degree of complementarity between the RISC bound miRNA and target mRNA.

Ran-GTP. Upon arrival in the cytoplasm, GTP hydrolysis results in the release of the pre-miRNA, which is then bound by a second cellular RNase III enzyme Dicer, acting with a dsRNA-binding protein partner, the TRBP (transactivating region RNA-binding protein) in human or Logs (Loquacious) protein in flies (Figure 1) Dicer binds the 2-nt 3’ overhang at the base of the pre-miRNA hairpin and removes the terminal loop, leaving a second 2-nt 3’ overhang and generating the miRNA duplex (presumably transient). Only one of the two strands is the miRNA; the other counterpart is named miRNA\*.

Next, Dicer facilitates the assembly of the ‘guide’ strand of the miRNA duplex into a miRNA effector complex (also known as a miRNA-containing RNA-induced silencing complex, miRISC), while the ‘passenger’ strand is released and degraded (15, 16). The miRNA then acts as a guide to direct RISC to complementary mRNA chains (17), which were either cleaved or translationally inhibited, depending on the degree of complementarity between the RISC binding miRNA and the target mRNA (Figure 1). Perfect complementarity generally results in the mRNA cleavage and

degradation, while there is insufficient complementarity with the target, the miRNA-RISC represses translation of the mRNA transcripts.

Normally viruses utilize the connatural molecular mechanism to proliferate in host cell. It comes as no surprise that viruses also encode miRNAs in their smaller genomes (18), which may modulate both their own gene expression and that of their host cells. In this review, we describe the features of virus-encoded miRNAs and their potential involvement in the regulation of their own genes and in the evasion of cellular immune mechanisms. Our conclusions indicate that viral miRNAs may be applied as candidate therapeutic targets in the near future.

## Virus-encoded miRNAs

Because of the widespread expression and important regulatory functions of miRNAs in various organisms, it was easy to estimate that viruses also encode miRNAs (18). Viruses are minute infectious agents who are characterized

**Table 1.** Summary of known virus-encoded miRNAs

Virus family	Species	Num	miRNAs	Functions	Ref
Herpesvirus	EBV	23	miR-BHRF1-(1-3) miR-BART1-20	Modify BALF5 transcript? Target chemokines, cytokines, apoptotic and cell growth control genes?	18, 24
	KSHV	12	miR-K1-12	Regulate kaposin gene?	27
	HCMV	11	miR-UL22-1, miR-US5-1, miR-US5-2, miR-UL36-1, miR-UL112-1, miR-US25-1, miR-US25-2, miR-UL148D-1, miR-US33-1, miR-UL70-1, miR-US4-1	Control viral replication?	30, 31
	MDV	8	MDV-miR-(1-8)	Down-regulate RLORF8? Transform chicken T cells?	34
	HSV-1	1	miR-LAT	Antiapoptosis	26
	MHV68	9	miR-M1-(1-9)	Unknown	32, 42
	rLCV	16	miR-rL1-(1-16)	Unknown	24, 25
Polyomavirus	SV40	1	miR-S1	Down-regulate the expression of large T antigen	43
	mouse polyomavirus	1	Unnamed	Down-regulate the expression of the middle and large T antigens	44
	SA12 polyomavirus	1	Unnamed	Down-regulate the expression of large T antigen	45
Adenovirus	hAV	1	Unnamed	Just a byproduct?	46, 51
HIV	HIV-1	1?	miR-N367	Suppress both Nef function and HIV-1 virulence?	53

by a lack of independent metabolism and the inability to replicate outside living host cells. They would exploit the biosynthetic machinery of host cells to synthesize various components meanwhile inactivate the innate defence mechanisms of the host, such as the interferon and apoptosis responses. Although many large viruses achieve this by encoding proteins that specifically against host-cell defences, for instance, p19 encoded by tomato bushy stunt virus binds tightly to small interfering RNA molecules (siRNAs) duplexes and inhibits incorporation of siRNA into RISC (19-22), the viruses coding limited proteins make the tiny miRNAs a particularly efficient tool to turn off the expression of specific genes. These small RNAs occupy little genomic space, additionally, because of the regulation acting at the mRNA level, not the protein level, making miRNAs rapidly accumulate in infected cells, would be a powerful means of modulating viral and cellular gene expression. Indeed, recent studies show that some viruses do exploit this pathway by generating their own miRNAs (18). Up to date, 11 virus genomes encoding 84 novel viral miRNAs were confirmed (Table 1), which provides the opportunity to study the biogenesis and function in detail of miRNAs and the important regulatory functions for both virus gene expression and that of their host cells.

However, according to the miRNA biogenesis pathway, we can speculate that not all viruses have the potential to encode miRNAs. On the one hand, for most RNA viruses, as well as one group of DNA viruses, replicate and express their genome in the cytoplasm. As we know, the initial step in miRNA processing, cleavage of the pri-miRNA by Drosha,

occurs in the nucleus (12). This exposes a potential difficulty for these viruses to encode miRNAs. On the other hand, in the step of the pre-miRNA generation, only the pre-miRNA is then exported to the cytoplasm. That means if an RNA virus encodes a miRNA, the viral RNA genome (or the viral genome minus strand) would be subjected to the cleavage and destruction in the progress of entering the nucleus. Accordingly, it was thought that RNA viruses, as well as those cytoplasmic DNA virus family such as poxviruses, could not encode their own miRNAs. If the hypothesis is true, then whether or not others similar small RNA molecules such as HIV exogenous engineered short hairpin RNAs (shRNAs) (23), or other arcane ways make up the regulation of miRNAs still remains to be uncovered.

### Herpesviruses: the largest virus family

Among the various families of viruses, the herpesvirus family stands out in establishing long-standing latent infection as a major part of the viral life cycle. There are eight human herpesviruses, all of them linked to important disease syndromes in man. Epstein-Barr virus (EBV), a member of the  $\gamma$ -herpesvirus family, was the first demonstrated to encode miRNAs (18), and with holding the current record of 23. These miRNAs originated from two clusters in the EBV genome, however, these different viral miRNAs show no sequence similarity to each other or the miRNAs encoded by other viruses, with the exception of eight miRNAs that are conserved with the rhesus

lymphocryptovirus (18, 24, 25) who encoded 16 miRNAs. The first miRNA cluster, which consists of miR-BHRF1-1, 2 and 3, is adjacent to the BHRF1 gene, whose transcript is a homologue of the antiapoptotic protein Bcl-2. The others EBV miRNAs are located within the intron regions of BART gene. These 20 EBV miRNAs are consistent with the expression of BART in every stage of EBV infection, and could be detected during the lytic and the three latent stages, while the expressions of miR-BHRF1-1, 2 and 3 are related to the stage of latency. Interestingly, the miR-BHRF1 expression pattern also showed variations between cell lines. It was likely to classify these miRNAs based on the expression pattern. Although the functions of these miRNAs are unknown, the use of algorithms predictions suggest that some of these miRNAs may target chemokines, cytokines, and apoptotic and cell growth control genes such as p53, all transcripts that have profound potential to influence the pathogenicity of the virus. Notably, miR-BART2 is antisense to a region in a lytic mRNA (BALF5) which encodes the viral DNA polymerase. Because of this overlap, it was hypothesized that the miRNA might function as small interfering RNAs (siRNAs) against BALF5 mRNA, resulting in the DNA polymerase degradation. (siRNAs are a distinct class of 20–25-nucleotide RNA molecules that interfere with gene expression by destroying the transcripts after binding to complementary target sequences). Indeed, the 5.0-kb BALF5 mRNA has a 3.7-kb short form with a 3' terminus that maps to the predicted miR-BART2 cleavage site. This seems that viruses not only use miRNAs to regulate host specific genes but also use them as autoregulatory tools. Additionally, as mentioned above, the BHRF1 transcript is related to the antiapoptotic function. Whether the miR-BHRF1 plays a similar antiapoptotic function which HSV-1-encoded miRNA does (26), awaits further investigation.

The autoregulatory tools similar to miR-BART2 were also found in the studies of Kaposi's sarcoma-associated herpesvirus (KSHV), human cytomegalovirus (HCMV) and Marek's disease virus (MDV). For KSHV, 1 of the 12 miRNAs encoded by KSHV (27), miR-K12-10, is encoded in the open-reading frame of the kaposin gene. The products of kaposin A, B and C have important functions in cell growth and cytokine production (28, 29). As overexpression of either is deleterious to cells, the tight regulation may be performed by miR-K12-10 owing to the complementarity between the miRNA and the mRNA. As other herpesviruses, HCMV can establish lifelong latency in infected individuals. Otherwise, in contrast to EBV and KSHV miRNAs, HCMV miRNAs are spread across the viral genome (30, 31). Three of them are transcribed from the complementary strand of known ORFs, five are located in intergenic regions and one is located within an intron. Additionally, these miRNAs are produced during the lytic phase, whereas most of other viral miRNAs are expressed during the latent phase of viral life cycles. The three miRNAs complementary to protein-coding regions have the potential to function as siRNAs and regulate the level of viral proteins. For example, the miR-UL112-1 might target UL114, who is required for efficient viral DNA replication, resulting in the potential of miR-UL112-1 to

control viral replication. Curiously, though not yet confirmed by expression data, several of the miRNAs encoded by HCMV seem to be conserved with chimpanzee CMV (32). This observation suggests that some viral miRNAs have been subject to experiencing positive selection and, maybe, their expression confers a selective advantage.

Mark's disease (MD) is a lymphoid cell infiltration or lymphomatous tumor formation in the peripheral nerves and gonads result from infection of susceptible chickens with MDV (33). Productive infection occurs in epithelial cells and in B lymphocytes. Fully productive infection, which results in the release of infectious virus particles, occurs only in feather follicle epithelium. Burnside et al. tried to isolated small RNAs from MDV-infected chicken embryo fibroblasts (CEF) using the 454 Life Sciences sequencing technology, obtained eight miRNAs encoded by MDV (34). The first cluster consists of MDV-miR-1 to -5, which flank the meq oncogene, and the others (MDV-miR-6 to -8) map to the latency-associated transcript (LAT) region of the genome. The meq gene is unique to pathogenic serotypes of MDV and the Meq protein is the strongest candidate oncoprotein described so far for MDV, whose expression is important for the transformation of lymphoblastoid cells (35), Rat-2 cells (36), DF-1 cells (37, 38) and the formation of MDV oncogenesis (39). It seems that MDV-miR-1 to -5 may contribute to MDV-induced transformation of chicken T cells. In addition, MDV-miR-2 to -5 appear to be expressed as a single transcriptional unit in the same orientation as the meq gene, and this cluster of miRNAs is antisense to another MDV transcript, RLORF8, which encodes a potential ORF of 135 amino acids that has no homology with other known sequences. Up to date, no protein from this locus has been reported. If any, this cluster of miRNAs may carry out their conventional regulatory role on this protein. MDV-miR-6 to -8 map to a large intron in the 5' end of the latency-associated MSR transcript, which is presumably derived from a large 10-kb transcript that maps antisense to the ICP4 gene (40). Similarly, a recent astonishing discovery of an miRNA encoded by the  $\alpha$ -herpesvirus herpes simplex virus type 1 (HSV-1), named miR-LAT, also originated from the LAT (41). MiR-LAT could down-regulates transforming growth factor- $\beta$  (TGF- $\beta$ ) and SMAD3 (mothers against decapentaplegic homologue) mRNAs, who encode proteins that induce apoptosis in infected cells by activation of the TGF- $\beta$ -mediated signaling pathway. That means miR-LAT has a powerful antiapoptotic function that maintains latent viral infection. It was tempting to speculate that MDV-miR-6 to -8 might have the similar antiapoptotic function as miR-LAT.

Studies of MHV68 have revealed interesting and unexpected miRNA processing and expression patterns, providing further supporting the evidence that our current knowledge about the biogenesis and expression of miRNAs probably represents just the tip of the iceberg. At least nine MHV68 miRNAs, cloned from mouse B lymphoma S11 cells latently infected with MHV68, are located within a 6-kb region near the M1 terminus of the linear MHV68 genome, immediately downstream of predicted viral tRNA genes (32). This led to the speculation that MHV68 pre-miRNAs are

transcribed by Pol III from tRNA promoters (42). Indeed, primary-tRNA-pre-miRNA transcripts with unusual hairpin structures suggest that MHV68 miRNAs processing mechanism has distinct features compared with other miRNAs. This reminds us that the pathways to miRNAs may be more varied than presently assumed.

MiRNAs have also been computationally predicted in the genomes of other herpesviruses, and it demonstrated that there are no miRNAs encoded by the genome of the  $\alpha$ -herpesvirus HHV3 (also known as varicella-zoster virus), or the  $\beta$ -herpesviruses HHV6 and HHV7 (32), which also have a similar life cycle. If these viruses also encode miRNAs, it would be interesting to find out how they differ from the miRNAs found presently, and explore why they were not predicted computationally.

#### *Polyomaviruses-encoded miRNAs*

Polyomaviruses are a family of small double-stranded DNA viruses and oncogenic in various hosts including rodents, birds and human and non-human primates, most as latent infections. The SV40 miRNA is the first viral miRNA whose target is known with certainty. The SV40 genome is circular and the region that encodes the miRNAs on the late strand overlaps with, and is fully complementary to, viral early mRNAs encoding the viral T antigens (43). Unexpectedly, growth of the wild-type and mutant viruses was identical in tissue culture, in addition, cells infected with wild-type virus released less interferon- $\gamma$  than those infected with mutant virus, therefore, down-regulation of the T antigen, which is the main target of the cytotoxic T lymphocyte response, conveniently protects infected cells from elimination by the host immune system. Furthermore, a similar regulation of expression of the middle and large T antigens by a miRNA that emanates from a different region of the late pre-mRNA of the mouse polyomavirus has also been observed (44). A recent study demonstrated the expression of the pre-miRNA precursor and the mature miRNA in SA12 polyomavirus-infected BSC40 monkey kidney epithelial cells (45). The late DNA strand encodes the SA12 miRNA overlaps with the 3' end of large T antigen coding sequences, indicating a similar function of elimination by the host immune system played by this miRNA. Importantly, the pre-miRNA was computationally predicted to be conserved in other polyomaviruses, including JCV and BKV, which suggested a conserved function for the miRNAs generated among these viruses.

#### *Adenovirus encodes "byproduct" miRNAs*

Adenovirus encodes a ~160-nt noncoding RNA, called virus-associated RNAs (VA RNAs), that is transcribed in the nucleus by RNA polymerase III and expressed at very high levels (up to  $10^8$  copies per cell) during the late stage of its lytic replication cycle (46). It was noted that VA1 RNA also uses the nuclear-export receptor exportin 5 to exit the nucleus like pre-miRNA (47, 48), then the highly abundant VA1 RNA can inhibit the transport of pre-miRNAs from the nucleus to the cytoplasm by competing for exportin-5 (49), resulting the decrease of pre-miRNAs which can induce RNAi. Additionally, it has been reported that both VA1 and

VA2 RNA can act as functional competitive substrates for cytoplasmic RNase III enzyme Dicer (49, 50), which results the miRNAs processing involving the Dicer cleavage step very inefficient, with only ~1% of VA1 being processed into adenovirus-derived miRNAs (51). Because VA1 potently inhibited RNAi induced by shRNAs or human miRNA precursors, it seems that the adenoviral miRNA is simply a byproduct of inadvertent processing of VA1 by Dicer. However, because of the high levels of VA1, it would be predicted to give rise to up to  $10^6$  VA1-derived miRNAs in an adenovirus-infected cell, this number is probably higher than that of almost all endogenous miRNAs. Therefore, the role played by VA1-derived miRNAs awaits further study.

#### *Human immunodeficiency virus-encoded 'controversial' miRNAs*

Extensive studies across many families of RNA viruses have failed to identify virus own miRNAs, including HCV and yellow-fever virus (32). Retroviruses are small, enveloped RNA viruses that have the ability to establish persistent infection similar to the herpesviruses family. The integrated provirus serves as the template for viral gene expression *via* host Pol II-mediated transcription. These make retroviruses the likeliest of all RNA viruses to encode miRNAs, since all retroviral transcription emanates from host machinery similar to that directing expression of cellular miRNAs. Up to date, however, human immunodeficiency virus 1 (HIV-1) encodes 'controversial' miRNAs. The initial studies have come up empty handed (32). Bannasser et al. uncovered a few potential candidates in the regions of the HIV genome for pre-miRNA-like stem-loop structures, and proposed that they can function as siRNAs (52), but these findings could not be verified in an independent study. In another study that used HIV-1-infected MT-4 T cells, a novel miRNA named miR-N367, was isolated within the nef region of the genome, and proposed a role in suppressing both Nef function and HIV-1 virulence through the long terminal repeats (LTR) U3 region negative-response element (53). At present, it is not clear why the above-mentioned HIV studies have different outcomes, whether or not experimental designs influenced the outcomes of these studies, and maybe HIV-1 can also encode functionally important miRNAs.

## **MiRNAs editing**

Though it is likely that many others viruses have the potential to encode their own miRNAs, such as human papilloma viruses, which are nuclear DNA viruses that establish long-term persistent infections, these miRNAs do not share homologies with each other or with the host miRNAs with a few exceptions as mentioned above. A recent study demonstrated that BART-1, BHRF1-1 and BHRF1-2 miRNAs of EBV seem to be conserved in a Cercopithecine herpesvirus 1, and miR-K12-11, which is encoded by KSHV, shares the first eight nucleotides with hsa-miR-155 though these miRNAs do not seem to be related (54). Even though, the lack of conservation of miRNAs between different human

viruses could be viewed as evidence that these miRNAs do not have an important role in virus replication. But as we know, inhibition of target mRNA expression may require only the complementarity between the 'seed match' region in the mRNA and the 'seed' region in the miRNA (55). Moreover, miRNAs are only ~22 nt in length, they could rapidly target new mRNA just to meet the requirement of 'editing' only a few nucleotides. This 'mutation' phenomenon was found indeed. Recently, adenosine deaminase (ADAR) editing of specific pri-miRNAs has been reported. This A-I editing event leads to decreased processing of the miRNA by Drosha and increases turnover by the Tudor-SN nuclease, a component of RISC and also a ribonuclease specific to inosine-containing dsRNAs (56). Notably, the viral miRNA, KSHV-miR-K12-10, with a single adenosine residue substituted by guanosine (miR-K12-10b) is frequently detected among cDNA isolates identified by the small-RNA cloning method. This indicates that editing of this particular site does not, in fact, inhibit pri-miR-K12-10 RNA processing, but leads to expression of mature miRNA with the edited sequence (32). In addition, the human and mouse pre-miRNAs of miR-22 are also edited at several positions, including sites in the mature miRNA (57). Although the extent of A-to-I editing was low (less than 5% across all adenosines analyzed), targeted adenosines were at positions predicted to influence the biogenesis and function of miR-22. Importantly, editing may influence miRNA strand choice because they would weaken the 5' end of the pre-miRNA dicer product (58, 59), resulting in directing RISC to target genes different from those silenced by the unedited miRNAs. This indicates that the miRNAs originated from the same pre-miRNA may target more corresponding complementary mRNA, making the fine-tuning of the network more complicated.

### MiRNA: a new therapeutic target

The development of any new therapeutics begins with the identification and early (pre-clinical) validation of novel biological targets, a process often termed target discovery (60). Novel targets are identified through a variety of avenues, including clinical observations, animal studies and molecular approaches such as genomics, proteomics and genetic association, as well as transgenic/knockout animals. Conventional therapeutic targets are proteins that interact with the proteins or with small molecules. However, such targets are susceptible to inhibitors that disrupt their functional interaction. Genomics has changed the way biological discoveries are being made, and has enabled the identification of new therapeutics targets for unmet medical needs. MiRNAs have been implicated in important regulatory roles in various biological processes, such as cell cycle control, apoptosis (61), haematopoiesis (62), adipocyte differentiation (63), insulin secretion (64) and tumorigenesis (65), which opens a new avenue for our understanding of gene expression and will become one of the most widely applied techniques in biomedical research, playing a major

#### MiR-122

3' U-G-U-U-U-G-U-G-G-U-A-A-C-A-G-U-G-U-G-A-G-G-U 5'

#### Antagomir-122

5' A--C--A-A-A-C-A-C-C-A-U-U-G-U-C-A-C-A-C-U--C--A--Chol 3'

**Figure 2. The sequence of miR-122 and the structure of its inhibitor antagomir-122.** MiR-122 has standard RNA bases while the RNA backbone of antagomir-122 was modified, at each nucleotide, by an O-methyl moiety at the 2'-ribose position. Short dashes (-) indicate phosphodiester linkage, and long dashes (--) indicate phosphorothioate linkage. In addition, a cholesteryl functionality at the 3' end of the nucleic acid.

role in the molecular investigation of disease pathogenesis. Determination of the applicable miRNAs at the molecular level is already beginning to inform the design of new therapeutic strategies. Although miRNAs do not encode proteins, they have significant potential as therapeutic targets. The Watson-Crick base-pair complementarity between miRNAs and mRNAs is essential for the function of miRNAs, therefore, the most logical inhibitor of such interactions is an oligonucleotide that is antisense to the miRNA. Specific removal of the target miRNAs by anti-miRNA oligonucleotides (AMOs) can correct the aberrant activity of any miRNA-mRNA interaction, which contributes causally to a disease, such as cancer (66), neurological diseases (67) and most recently also with viral (34) and metabolic diseases (64).

Modified synthetic AMOs are useful tools in specifically inhibiting individual miRNAs. Most of the studies employed 2'-O-methyl (2'-O-Me) substituted RNA oligonucleotides. There were also reports using DNA (68, 69) or a mixed locked nucleic acid (LNA)-DNA AMO (70) and two studies have used 2'-O-methoxyethyl (2'-O-MOE) substituted RNA oligonucleotides (63, 71), another used 2'-sugar and phosphodiester backbone AMO (72). In a recently published study, KrCtzfeldt et al. were able to ablate the function of miR-122 in living mice upon administering specially developed antisense agents (73). These so-called antagomirs are synthesized single-stranded 23-nt RNA molecules complementary to the targeted miR-122. The RNA backbone was modified, at each nucleotide, by an O-methyl moiety at the 2'-ribose position (Figure 2). The terminal nucleotides at both ends were also modified with a phosphorothioate linkage, in contrast to the standard phosphodiester linkage in RNA and DNA (Figure 2). Such modifications can prevent nuclease degradation in the culture media leading a drastic increase in the biological half-life of the substances. Another modification was cholesteryl functionality at the 3' end of the nucleic acid (Figure 2). This improved the stability and half-life in serum by binding to serum proteins and also enhanced cellular uptake. Injection of these antagomirs into the tail veins of the mice led to a degradation of the corresponding miR-122 but not other species of miRNAs from the liver, even after more than 20 days, indicating that

the antagomir had a durable effect. A closer biochemical examination of the mice treated with antagomir-122 showed that above all, levels of mRNAs containing recognition sequences for the corresponding miR-122 in their 3' UTRs (ca. 360 mRNAs) were increased by a factor of at least 1.4. Interestingly, in addition to the aforementioned over-expression of mRNAs, a down-regulation of nearly as many mRNAs occurred. At least 11 genes involved in cholesterol biosynthesis, including the rate-limiting enzyme 3-hydroxy-3-methylglutaryl-coenzyme A-reductase, were among the genes that were down-regulated by antagomir-122. The authors speculate that through a degradation of miR-122, an upregulation of repressor proteins might occur, leading to inhibition of the expression of the corresponding effector genes and help maintain the adult-liver phenotype by suppressing 'nonliver' genes.

Curiously, in another study, Esau et al. developed 2'-O-methoxyethyl phosphorothioate-modified antisense oligonucleotides that were complementary to miR-122, this resulted in a reduction in cholesterol levels, accompanied by a considerable decrease in hepatic steatosis (74, 75). As we know, the successful survival of viruses crucially depends on their ability to exploit the biosynthetic machinery of host cells, it seems that the cholesterol-biosynthesis pathway modulates HCV RNA replication by regulating the cellular levels of geranylgeranyl diphosphate, most probably affecting the activity of a geranylgeranylated host protein involved in HCV RNA replication. Thus, antagomirs have the potential to become powerful tools in interfering with miRNA pathways thereby contributing to the dissection of their functions and their putative role in human disease processes.

### Concluding remarks

It has been less than 2 years since the discovery of the first viral-encoded miRNAs (18), and it is likely that many others await discovery. The first *in vivo* study provides an exciting first step towards miRNA therapy, and the potential for ultimately designing molecular medicines based on the modulation of miRNAs seems good. The effects of the antagomirs on the protein level, however, were only examined for a few enzymes connected with cholesterol metabolism in the antagomir study (73). The physiological effect of a complete and prolonged degradation of miR-122 is in the end relatively small. Moreover, the modified antagomirs remain difficult to target the brain. The ability to efficiently, stably produce and deliver sufficient amounts of antagomir into the proper target cell without overt toxicity requires fine tuning of the technology before it can be tried clinically. In addition, unwanted cellular responses to such therapeutic agents may occur over the long term.

Recent patent applications in miRNAs has advanced rapidly (76). The miRNA biogenesis and their importance function in regulating gene expression in various organisms including virus are rapidly being elucidated. These scientific advances are swiftly being translated into therapeutic

approaches to delivering antagomirs into cells to tackle a variety of diseases. Because certain virus-encoded miRNAs can help viruses escape detection by antiviral innate immune responses (26, 43), related antagomirs may influence viral tropism and the outcome of viral replication. Such data would contribute to basic science research in the field of virus biology and to identification of potential therapeutic targets. Notably, the exciting observation that the inhibition of the expression of the corresponding effector genes may be induced, raises the possibility of using repressor proteins as antiviral agents indirectly. As miRNA-based therapies begin to be evaluated in clinical studies, the next few years will test the promise of relevant drugs, allowing us to combine the specificity of siRNA and the mismatch tolerance of miRNA. It will likely bring many more surprises.

### Acknowledgements

The research in the author's laboratory was supported by the National Natural Science Foundation of China, No. 30371328, the Key Project of Natural Science Foundation of Shandong Province, No.Z2002C01 and the Key Project of Shandong Academy of Medical Sciences, No.2005007.

### References

1. Lee RC, Feinbaum RL, Ambros V. The *C. elegans* heterochronic gene *lin-4* encodes small RNAs with antisense complementarity to *lin-14*. *Cell*. 1993;75:843-854.
2. Lau NC, Lim LP, Weinstein EG, Bartel DP. An abundant class of tiny RNAs with probable regulatory roles in *Caenorhabditis elegans*. *Science*. 2001;294:858-862.
3. Lee RC, Ambros V. An extensive class of small RNAs in *Caenorhabditis elegans*. *Science*. 2001;294:862-864.
4. Lai EC, Tomancak P, Williams RW, Rubin GM. Computational identification of *Drosophila* microRNA genes. *Genome Biol*. 2003;4:R42.
5. Lagos-Quintana M, Rauhut R, Meyer J, Borkhardt A, Tuschl T. New microRNAs from mouse and human. *RNA*. 2003;9:175-179.
6. Griffiths-Jones S. The microRNA registry. *Nucleic Acids Res*. 2004;32:D109-D111.
7. Griffiths-Jones S, Grocock RJ, van Dongen S, et al. miRBase: microRNA sequences, targets and gene nomenclature. *Nucleic Acids Res*. 2006;34:D140-D144.
8. Lagos-Quintana M, Rauhut R, Lendeckel W, Tuschl T. Identification of novel genes coding for small expressed RNAs. *Science*. 2001;294:853-858.
9. Lee Y, Jeon K, Lee JT, Kim S, Kim VN. MicroRNA maturation: stepwise processing and subcellular localization. *EMBO J*. 2002;21:4663-4467.
10. Rodriguez A, Griffiths-Jones S, Ashurst JL, Bradley A. Identification of mammalian microRNA host genes and transcription units. *Genome Res*. 2004;14:1902-1910.
11. Kim VN. MicroRNA biogenesis: coordinated cropping and dicing. *Nature Rev Mol Cell Biol*. 2005;6:376-385.
12. Lee Y, Ahn C, Han J, et al. The nuclear RNase III *drosha* initiates microRNA processing. *Nature*. 2003;425:415-419.
13. Denli AM, Tops BBJ, Plasterk RHA, Ketting RF, Hannon GJ. Processing of primary microRNAs by the microprocessor

- complex. *Nature*. 2004;432:231-235.
14. Han J, Lee Y, Yeom KH, et al. Molecular basis for the recognition of primary microRNAs by the Drosha-DGCR8 complex. *Cell*. 2006;125:887-901.
  15. Maniatakis E, Mourelatos Z. A human, ATP independent, RISC assembly machine fueled by premiRNA. *Genes Dev*. 2005;19:2979-2990.
  16. Hammond SM, Bernstein E, Beach D, Hannon GJ. An RNA-directed nuclease mediates posttranscriptional gene silencing in *Drosophila* cells. *Nature*. 2000;404:293-296.
  17. Schwarz DS, Hutvagner G, Haley B, Zamore PD. Evidence that siRNAs function as guides, not primers, in the *Drosophila* and human RNAi pathways. *Mol Cell*. 2002;10:537-548.
  18. Pfeffer S, Zavolan M, Grasser FA, et al. Identification of virus-encoded microRNAs. *Science*. 2004;304:734-736.
  19. Lakatos L, Szittyá G, Silhavy D, Burgyan J. Molecular mechanism of RNA silencing suppression mediated by p19 protein of tombusviruses. *EMBO J*. 2004;23:876-884.
  20. Baulcombe DC, Molnar A. Crystal structure of p19—a universal suppressor of RNA silencing. *Trends Biochem Sci*. 2004;29:279-281.
  21. Silhavy D, Molnar A, Luciola A, et al. A viral protein suppresses RNA silencing and binds silencing-generated, 21- to 25-nucleotide doublestranded RNAs. *EMBO J*. 2002;21:3070-3080.
  22. Ye K, Malinina L, Patel DJ. Recognition of small interfering RNA by a viral suppressor of RNA silencing. *Nature*. 2003;426:874-878.
  23. Bannasser Y, Le SY, Yeung ML, Jeang KT. HIV-1 encoded candidate micro-RNAs and their cellular targets. *Retrovirology*. 2004;1:43.
  24. Cai X, Schafer A, Lu S, et al. Epstein-Barr virus microRNAs are evolutionarily conserved and differentially expressed. *PLoS Pathog*. 2006;2:e23.
  25. Grundhoff A, Sullivan CS, Ganem D. A combined computational and microarraybased approach identifies novel microRNAs encoded by human  $\gamma$ -herpesviruses. *RNA*. 2006;12:1-18.
  26. Gupta A, Gartner JJ, Sethupathy P, Hatzigeorgiou AG, Fraser NW. Anti-apoptotic function of a microRNA encoded by the HSV-1 latency-associated transcript. *Nature*. 2006;442:82-85.
  27. Cai X, Cullen BR. Transcriptional origin of Kaposi's sarcoma-associated herpesvirus microRNAs. *J Virol*. 2006;80:2234-2242.
  28. Kliche S, Nagel W, Kremmer E, et al. Signaling by human herpesvirus 8 kaposin A through direct membrane recruitment of cytohesin-1. *Mol Cell*. 2001;7:833-843.
  29. McCormick C, Ganem D. The kaposin B protein of KSHV activates the p38/MK2 pathway and stabilizes cytokine mRNAs. *Science*. 2005;307:739-741.
  30. Grey F, Antoniewicz A, Allen E, et al. Identification and characterization of human cytomegalovirus-encoded microRNAs. *J Virol*. 2005;79:12095-12099.
  31. Dunn W, Trang P, Zhong Q, Yang E, van Belle C, Liu F. Human cytomegalovirus expresses novel microRNAs during productive viral infection. *Cell Microbiol*. 2005;7:1684-1695.
  32. Pfeffer S, Sewer A, Lagos-Quintana M, et al. Identification of microRNAs of the herpesvirus family. *Nat Methods*. 2005;2:269-276.
  33. Witter RL, K Schat. Marek's disease. In: Saif YM, ed. *Diseases of poultry*, 11th ed. Iowa State University Press: Ames; 2003; 407-464.
  34. Burnside J, Bernberg E, Anderson A, et al. Marek's disease virus encodes microRNAs that map to meq and the latency-associated transcript. *J Virol*. 2006;80:8778-8786.
  35. Xie Q, Anderson A, Morgan R. Marek's disease virus (MDV) ICP4, pp38, and meq genes are involved in the maintenance of transformation of MDCC-MSB1 MDV-transformed lymphoblastoid cells. *J Virol*. 1996;70:1125-1131.
  36. Liu JL, Ye Y, Lee LF, Kung HJ. Transforming potential of the herpesvirus oncoprotein MEQ: morphological transformation, serum-independent growth, and inhibition of apoptosis. *J Virol*. 1998;72:388-395.
  37. Himly M, Foster DN, Bottoli I, Iacovoni JS, Vogt PK. The DF-1 chicken fibroblast cell line: transformation induced by diverse oncogenes and cell death resulting from infection by avian leukosis viruses. *Virology*. 1998;248:295-304.
  38. Levy AM, Gilad O, Xia L, et al. Marek's disease virus Meq transforms chicken cells *via* the v-Jun transcriptional cascade: a converging transforming pathway for avian oncoviruses. *Proc Natl Acad Sci U S A*. 2005;102:14831-14836.
  39. Lupiani B, Lee LF, Cui X, et al. Marek's disease virus-encoded Meq gene is involved in transformation of lymphocytes but is dispensable for replication. *Proc Natl Acad Sci U S A*. 2004;101:11815-11820.
  40. Cantello JL, Parcels MS, Anderson AS, Morgan RW. Marek's disease virus latency-associated transcripts belong to a family of spliced RNAs that are antisense to the ICP4 homolog gene. *J Virol*. 1997;71:1353-1361.
  41. Gupta A, Gartner JJ, Sethupathy P, Hatzigeorgiou AG, Fraser NW. Anti-apoptotic function of a microRNA encoded by the HSV-1 latency-associated transcript. *Nature*. 2006;442:82-85.
  42. Bowden RJ, Simas JP, Davis AJ, Efstathiou S. Murine  $\gamma$ herpesvirus 68 encodes tRNA-like sequences which are expressed during latency. *J Gen Virol*. 1997;78:1675-1687.
  43. Sullivan CS, Grundhoff AT, Tevethia S, Pipas JM, Ganem D. SV40-encoded microRNAs regulate viral gene expression and reduce susceptibility to cytotoxic T cells. *Nature*. 2005;435:682-686.
  44. Sullivan CS, Ganem D. MicroRNAs and viral infection. *Mol Cell*. 2005;20:3-7.
  45. Cantalupo P, Doering A, Sullivan CS, et al. Complete nucleotide sequence of polyomavirus SA12. *J Virol*. 2005;79:13094-13104.
  46. Mathews MB, Shenk T. Adenovirus virus-associated RNA and translation control. *J Virol*. 1991;65:5657-5662.
  47. Bohnsack MT, Czaplinski K, Gorlich D. Exportin 5 is a RanGTP-dependent dsRNA-binding protein that mediates nuclear export of pre-miRNAs. *RNA*. 2004;10:185-191.
  48. Gwizdek C, Ossareh-Nazari B, Brownawell AM, et al. Exportin-5 mediates nuclear export of minihelix-containing RNAs. *J Biol Chem*. 2003;278:5505-5508.
  49. Lu S, Cullen BR. Adenovirus VA1 noncoding RNA can inhibit small interfering RNA and microRNA biogenesis. *J Virol*. 2004;78:12868-12876.
  50. Andersson MG, Haasnoot PC, Xu N, Berenjian S, Berkhout B, Akusjarvi G. Suppression of RNA interference by adenovirus virus-associated RNA. *J Virol*. 2005;79:9556-9565.
  51. Sano M, Kato Y, Taira K. Sequence-specific interference by small RNAs derived from adenovirus VA1 RNA. *FEBS Lett*. 2006;580:1553-1564.
  52. Bannasser Y, Le SY, Benkirane M, Jeang KT. Evidence that HIV-1 encodes an siRNA and a suppressor of RNA silencing. *Immunity*. 2005;22:607-619.
  53. Omoto S, Fujii YR. Regulation of human immunodeficiency virus 1 transcription by nef microRNA. *J Gen Virol*. 2005;86:751-755.
  54. Nair V, Zavolan M. Virus-encoded microRNAs: novel



- regulators of gene expression. *Trends Microbiol.* 2006;14:169-175.
55. Bartel DP. MicroRNAs: Genomics, biogenesis, mechanism, and function. *Cell.* 2004;116:281-297.
  56. Yang W, Chendrimada TP, Wang Q, et al. Modulation of microRNA processing and expression through RNA editing by ADAR deaminases. *Nat Struct Mol Biol.* 2006;13:13-21.
  57. Luciano DJ, Mirsky H, Vendetti NJ, Maas S. RNA editing of a miRNA precursor. *RNA.* 2004;10:1174-1177.
  58. Khvorova A, Reynolds A, Jayasena SD. Functional siRNAs and miRNAs exhibit strand bias. *Cell.* 2003;115:209-216.
  59. Schwarz DS, Hutvagner G, Du T, Xu Z, Aronin N, Zamore PD. Asymmetry in the assembly of the RNAi enzyme complex. *Cell.* 2003;115:199-208.
  60. Lindsay MA. Target discovery. *Nat Rev Drug Discov.* 2003;2:831-838.
  61. Brennecke J, Hipfner DR, Stark A, Russell RB, Cohen SM. bantam encodes a developmentally regulated microRNA that controls cell proliferation and regulates the proapoptotic gene hid in *Drosophila*. *Cell.* 2003;113:25-36.
  62. Chen CZ, Li L, Lodish HF, Bartel DP. MicroRNAs modulate hematopoietic lineage differentiation. *Science.* 2004;303:83-86.
  63. Esau C, Kang X, Peralta E, et al. MicroRNA-143 regulates adipocyte differentiation. *J Biol Chem.* 2004;279:52361-52365.
  64. Poy MN, Eliasson L, Krutzfeldt J, et al. A pancreatic islet-specific microRNA regulates insulin secretion. *Nature.* 2004;432:226-230.
  65. Volinia S, Calin GA, Liu CG, et al. A microRNA expression signature of human solid tumors defines cancer gene targets. *Proc Natl Acad Sci U S A.* 2006;103:2257-2261.
  66. Gregory RI, Shiekhattar R. MicroRNA biogenesis and cancer. *Cancer Res.* 2005;65:3509-3512.
  67. Dostie J, Mourelatos Z, Yang M, Sharma A, Dreyfuss G. Numerous microRNPs in neuronal cells containing novel microRNAs. *RNA.* 2003;9:180-186.
  68. Boutla A, Delidakis C, Tabler M. Developmental defects by antisense-mediated inactivation of micro-RNAs 2 and 13 in *Drosophila* and the identification of putative target genes. *Nucleic Acids Res.* 2003;31:4973-4980.
  69. Hutvagner G, Simard MJ, Mello CC, Zamore PD. Sequencespecific inhibition of small RNA function. *PLoS Biol.* 2004;2:E98.
  70. Chan JA, Krichevsky AM, Kosik KS. MicroRNA-21 is an antiapoptotic factor in human glioblastoma cells. *Cancer Res.* 2005;65:6029-6033.
  71. Esau C, Davis S, Murray SF, et al. miR-122 regulation of lipid metabolism revealed by *in vivo* antisense targeting. *Cell Metab.* 2006;3:87-98.
  72. Davis S, Lollo B, Freier S, Esau C. Improved targeting of miRNA with antisense oligonucleotides. *Nucleic Acids Res.* 2006;34:2294-2304.
  73. Krutzfeldt J, Rajewsky N, Braich R, et al. Silencing of microRNAs *in vivo* with 'antagomirs'. *Nature.* 2005;438:685-689.
  74. Kapadia SB, Chisari FV. Hepatitis C virus RNA replication is regulated by host geranylgeranylation and fatty acids. *Proc Natl Acad Sci U S A.* 2005;102:2561-2566.
  75. Ye J, Wang C, Sumpter R Jr, Brown MS, Goldstein JL, Gale M Jr. Disruption of hepatitis C virus RNA replication through inhibition of host protein geranylgeranylation. *Proc Natl Acad Sci U S A.* 2003;100:15865-15870.
  76. Recent patent applications in microRNAs. *Nat Biotechnol.* 2006;24:44.