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Identification and Function of MicroRNAs Encoded by Herpesviruses*

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Abstract

MicroRNAs (miRNAs) play important roles in eukaryotes, plants and some viruses. It is increasingly clear that miRNAs-encoded by viruses can affect the viral life cycle and host physiology. Viral miRNAs could repress the innate and adaptive host immunity, modulate cellular signaling pathways, and regulate the expression of cellular and viral genes. These functions facilitate viral acute and persistent infections, and have profound effects on the host cell survival and disease progression. Here, we discuss the miRNAs encoded by herpesviruses, and their regulatory roles involved in virus-host interactions.

Keywords

MicroRNA (miRNA); Herpesviruses; Latency and Replication

Introduction

MiRNAs are a class of small non-coding RNAs found in animals, plants, and viruses, and more recently in a single-cell eukaryote (3,23,65,100). As recent as October 2007, the combination of experimental methods with bioinformatics has led to the identification of up to 50,000 miRNAs loci from 58 species. Among this rapidly expanding family of small RNAs, over 500 of them have been identified in humans (42).

The biogenesis of miRNAs includes several processing events. Generally, it begins with a primary miRNA (pri-miRNA) which is transcribed by RNA polymerase II (Pol II) (14,60, 61), though some miRNAs may be processed by a RNA polymerase III (Pol III) (8,59), to produce a ~80 nt RNA hairpin. A ~60 nt stem loop is then released through cleavage of the pri-miRNA by a Drosha-DGCR8 complex (24,39,48,58,59). The processed RNA hairpin, termed precursor miRNA (pre-miRNA), is subsequently exported from the nucleus to the cytoplasm through the exportin-5 nuclear membrane trafficking machinery (63,98), and

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cleaved by the cytoplasmic enzyme Dicer to form the mature ~22 nt RNA duplex (5,43,53). One of the two strands of the RNA duplex, called mature miRNA, is loaded into the RNA-induced silencing complex (RISC) (54,66), whereas the unloaded strand is degraded (52,56, 82). The RISC complex directed by the mature miRNA to its targets mediates the specific cleavage of mRNAs or translation arrest of proteins, depending on the complementarities between the miRNA and its target mRNAs (47,54,99).

Over 100 miRNAs have been discovered in viruses so far. However, few target genes of viral miRNAs have been identified so far. Consequently, the functions of the majority of viral miRNAs remain unclear.

Herpesviruses are enveloped, double-stranded DNA viruses. There are three subfamilies: alpha, beta and gamma herpesviruses, based on their structure and biology (77). Herpesviruses have two distinct phases in their life cycle: latency and lytic replication. Interestingly, the majority of viral miRNAs identified are from herpesviruses, suggesting that viral miRNAs may play important roles in the unique life cycle of this family of viruses. In this review, we discuss the miRNAs encoded by herpesviruses and their regulatory roles in virus-host interactions.

Identification and Expression of miRNAs Encoded by Herpesviruses

Alpha herpesviruses

Herpes simplex virus 1 (HSV-1) is a neurotropic virus, belonging to genus *Simplexvirus*, one of the members of alpha herpesvirus subfamily. HSV-1 is commonly associated with herpes outbreaks of the face known as cold sores or fever blisters; it can also cause more severe infections in newborns or immunocomprised persons. As a well-studied virus, a number of animal infection models are available, which facilitates the study of HSV-1 pathogenesis (91). To identify miRNAs encoded by HSV-1, Cui *et al*. searched the entire viral genome for potential stem-loops using a computational approach, and predicted the existence of 13 miRNA precursors and 24 miRNA candidates in this herpesvirus. However, only one of them, miR-H1, was detected and confirmed by Northern-blot hybridization (22). miR-H1 is located upstream of the latency-associated transcript (LAT) thought to be a non-coding RNA. In contrast to the latent transcript LAT, miR-H1 and its precursor were expressed as late gene products.

LAT is an abundantly expressed RNA transcript in latently infected neurons (86), which had been shown to be nonessential for viral replication. However, LAT mutants had decreased frequencies of reactivation in an animal model (80). Recent data had demonstrated the important role of LAT in preventing cell death by blocking cell apoptosis (1,7,49,72). Because the multifunction of LAT and absence of any detectable protein product translated from this transcript, Umbach *et al.* attempted to identify potential miRNA precursor hairpins from LAT by cloning and expression of the corresponding genomic sequence in 293T cells. A total of 4 pre-miRNAs, that give rise to 6 mature miRNAs, were identified using a cDNA library deep sequencing method (46). To determine whether HSV-1 can encode more miRNAs, cDNA deprived from trigeminal ganglia of mice latently infected by HSV-1 was deep sequenced, which led to the identification of miR-H6. It is notable that miR-H6 is located in the opposite strand of miR-H1, which are largely complementary to each other (89). In contrast, miR-LAT also encoded by LAT described in an early report was not confirmed by Umbach *et al*. (45, 89).

Recently, a new viral miRNA encoded by herpes simplex virus 2 (HSV-2), which causes genital herpes, was reported (87). To identify putative miRNAs in the LAT region of HSV-2, Tang *et al*. used a similar strategy described by Umbach *et al*. to clone and express the entire HSV-2

LAT region resulting in the discovery of HSV-2 miR-I. This viral miRNA lies within LAT exon 2 and is complimentary to the neurvirolence factor ICP34.5. More interestingly, Tang *et al*. also predicted a homolog sequence to HSV-2 miR-I in the same region of HSV-1 LAT exon-2, which was identified by Umbach *et al.* as HSV-1 miR-H3 (89).

Marek's disease virus (MDV) is an avian herpesvirus, belonging to genus *Mardivirus*, another member of alpha herpesvirus subfamily. It includes two distinct MDV species, MDV-1 and MDV-2. MDV-1 consists of all oncogenic strains, while MDV-2 consists of non-oncogenic strains (6). Two studies identified a total of 13 pre-miRNAs in MDV-1 and 17 pre-miRNAs in MDV-2 (11,12,96,97). Burnside *et al*. reported the first identification of 8 pre-miRNAs in MDV-1, using a parallel sequencing approach, 5 of which were mapped near an important gene-meq while the other 3 were mapped to the LAT. The miRNAs flanking meq have similar expression pattern as meq (11). Further studies by Burnside *et al*. and Yao *et al* identified 5 additional pre-miRNAs in the MDV-1 genome (12,97). Analysis of the expression profiles by quantitative real-time reverse-transcription PCR showed that several MDV-1 miRNAs including miR-4, miR-8 and miR-12 are highly expressed in the tumor tissues compared to the non-tumor tissues (94).

Beta herpesviruses

Infection by beta herpesvirus human cytomegalovirus (HCMV) is common. Over 50% US individuals of ≥ six years old are infected by HCMV (85). CMV infection can lead to serious diseases in both immunocompetent and immunocompromised individuals as well as newborns (71,90). In 2005, a total of 13 pre-miRNAs were identified by three groups (27,40,73). Unlike gamma herpesviruses, HCMV miRNAs are not clustered but scattered over the entire viral genome. Pfeffer *et al*. cloned 9 of the 11 pre-miRNAs predicted by a bioinformatics approach; 5 mapped to the intergenic region, 3 derived from the antisense strand of known ORFs, and 1 mapped within the intron of UL36, which is dispensable for viral replication in cultured cells (70,73). A similar study by Grey *et al*. predicted 13 potential hairpin structures, which are conserved between chimpanzee CMV and HCMV, and have high scores using the MiRscan algorithm (40). Five of the 13 miRNAs were detected by Northern-blot hybridization, and 2 of which were newly discovered. Dunn *et al*. identified two new viral pre-miRNAs from two distinct cell lines infected by HCMV (27). These two miRNAs were not identified by the other two groups. The majority of HCMV miRNAs are expressed at the early time points of productive viral infection except UL70-1 and UL23-3p, which are expressed at the immediate-early (IE) time points of infection (27,40).

Murine cytomegalovirus (MCMV) has long been used as an important animal model for HCMV. The discovery of HCMV miRNAs has also lead to the identification of miRNAs in MCMV. Buck *et al.* identified a number of MCMV miRNAs using a bioinformatics approach combined with the RNA cloning method (10). In addition to 2 other pre-miRNAs identified by Dolken *et al*. (25), a total of 18 miRNAs were identified and validated in MCMV. Unlike the scattered pattern of HCMV miRNAs, the MCMV miRNAs are distributed over the entire genome as small clusters. However, similar to the expression pattern of HCMV miRNAs, the MCMV miRNAs display both IE or early expression kinetics during viral infection (10,25).

Gamma herpesviruses

Epstein-Barr virus (EBV) is the first virus reported to encode viral miRNAs (74). EBV is a γ1-herpesvirus associated with several lymphoproliferative diseases, including post-transplant lymphoproliferative disorders, Hodgkin's disease, Burkitt's lymphoma and nasopharyngeal carcinoma (NPC) (76). Pfeffer *et al*. described the first cloning and identification 5 viral pre-miRNAs from a Burkitt's lymphoma cell line latently infected with EBV (74). In this milestone study, which influenced the subsequent discoveries of viral miRNAs in a number of

herpesviruses and other viruses, 5 pre-miRNAs were mapped to two regions in the EBV genome: *BHRF1* (Bam HI fragment H rightward ORF1) and *BART* (*Bam*H I-A region rightward transcript). Following this work, two studies by Cai *et al*. and Grundhoff *et al*. identified a panel of 17 additional novel pre-miRNAs encoded by EBV (16,44). Previous work by Cai *et al*. identified multiple Kaposi's sarcoma-associated herpesvirus (KSHV) miRNAs in a KSHV and EBV dually infected PEL cell line BC-1 (15,17). Extensive analysis of cDNA clones deprived from this cell line identified up to 14 novel EBV pre-miRNAs located within the *BART* region (16). A similar study by Grundhoff *et al*. identified 19 previously reported pre-miRNAs plus 4 novel EBV pre-miRNA that were mapped to the *BHRF1* and *BART* regions, respectively (44). Instead of using the common small RNA cloning method, the author utilized a new strategy to identify viral miRNAs based on a newly developed algorithm (virMir) combining with a microarray-based experimental approach. To address the expression profiling of EBV miRNAs, Cai *et al*. investigated a number of distinct cell lines latently infected with EBV as well as the NPC tumor C15 that was passaged in nude mice. The author indicated that the EBV miRNAs were differentially expressed in multiple latently infected cell lines with different types of viral latency (I, II, III), and the expression of several EBV miRNAs were increased after induction of lytic replication. Recently, a new report by Edwards *et al*. indicated that the pre-miRNAs within the first four introns of the *BART* transcript were processed from the primary transcript prior to its complicated splicing events (28). Despite these studies, the expression pattern of EBV miRNAs in clinical samples was also investigated (57,68,93).

Cai *et al*. inferred that the viral miRNAs should be conserved during long-term evolution if they were to play important role in viral life cycle. Rhesus lymphocryptovirus (rLCV), another γ1 herpesvirus, which has 65% sequence homology with EBV but is thought to be evolutionarily separated from EBV at least 13 million years ago (36), was investigated to validate the hypothesis. As expected, up to 16 pre-miRNAs were identified from the latently infected rhesus B cell line. A notable feature is that 8 miRNAs from 6 rLCV pre-miRNAs are highly conserved or almost completely conserved through sequence comparison between rLCV and EBV (16).

In γ2 herpesviruses, there are several viruses that have been shown to encode multiple miRNAs in the genomes, including KSHV, rhesus rhadinovirus (RRV) and murine herpesvirus 68 (MHV68) (15,44,73,78,81). KSHV, a recently discovered virus recognized as a causative agent of Kaposi's sarcoma, is also linked to several other lymphoproliferative disorders including primary effusion lymphomas (PEL) and Multicentric Castleman's Disease (MCD) (2,18,34). As a prototype of the genus of *Rhadinoviruses*, whether this virus encodes miRNAs as EBV became an interesting question. Studies from four independent groups led to the identification of a total of 12 pre-miRNAs. Similar to the cluster pattern of EBV miRNAs, 10 of 12 pre-miRNAs are clustered in a 4-kb intronic region between ORF-K12 and ORF71. Shortly after the first miRNA work on EBV, Pfeffer *et al*. extended their interest to other members of herpesviruses including HCMV, MHV68 and KSHV (73). Using the same strategy in the EBV study, Pfeffer *et al*. cloned 10 KSHV pre-miRNAs, 5 of which were further confirmed by Northern-blot hybridization. In this study, KSHV-miR-K12-10 was found to be resided in ORF-K12 (kaposin) and has two variants with a single nucleotide difference at the position 2 of mature miRNA sequence, which might also affect the transcript of kaposin, but has no changes for the miRNA containing DNA fragments cloned from viral genome. The author reasoned that the single nucleotide conversion might be caused by a dsRNA-specific deaminase, which was partially confirmed by subsequent work of Candy *et al*. describing the existence of RNA editing in kaposin transcript (35). Another interesting finding of this special miRNA is that its expression level is increased more than 6 folds following induction of lytic replication, while the majority of KSHV miRNAs are not affected by lytic induction. Except for the work of Pfeffer *et al*., the two reports by Cai *et al*. and Samols *et al*. identified 10 and 9 pre-miRNAs, respectively (15,78). Subsequently, Groundhoff *et al*. reported the

identification of 12 KSHV miRNAs, of which one miRNA, miR-K12, was missed in previous investigations (44).

RRV is closely related to KSHV, and serves as an animal model for studying the infection and latency of gamma herpesviruses in a host closely related to human (69). Schafer *et al*. cloned and analyzed the miRNAs encoded by RRV. In this study, 7 pre-miRNAs were identified (81). In contrast to EBV and rLCV, while the genomic positions of RRV miRNAs are located in the similar region of KSHV, none of them has sequence homolog to KSHV miRNAs (81).

MHV68, the third γ2-herpesvirus found to encode miRNAs, is closely related to EBV and KSHV. MHV68 infection of mice provides a useful small animal model of gamma herpesviruses (84). As part of the work by Pfeffer *et al*., 9 pre-miRNAs were cloned from an infected B cell line. All these miRNAs are clustered within a 6-kb region, which also contains 8 tRNA genes. The genomic location pattern of the MHV68 miRNAs has suggested that these miRNAs might be transcribed by Pol III rather than Pol II as normally found for other cellular and viral miRNAs.

Functions of miRNAs Encoded by Herpesviruses

Viral miRNAs have been thought to play very important regulatory roles in viral life cycle. Viral miRNAs provide an attractive and economic strategy to facilitate viral growth by inhibiting host and viral genes without inducing immune responses commonly caused by viral proteins. Although more than 100 viral miRNAs have been identified, the functions of the majority of viral miRNAs remain unclear.

To date, the popular algorithms for cellular miRNA target prediction are based on the conservation of binding sites or seeds among different species, which is usually not present among viral miRNAs. Microarray approach has been used but can only compare the changes of gene expression profiles at the mRNA level. It seems that the cellular genes, in most cases, are not affected by cellular and viral miRNAs at the mRNA level, but rather, at translation stage. Because of the lack of accurate algorithm for target prediction of viral miRNAs, and the lack of efficient and high throughout methods for target validations, elucidation of the regulatory functions of viral miRNAs remains a daunting task. While microarray approach will undoubtedly miss the most of the targets, it is still the only reproducible way to simultaneously compare the expression profiles of more than 20,000 genes, which could provide information of the targets regardless at the mRNA or protein level when combined with other approaches such as pathway analysis and target confirmation.

Regulation of host genes by viral miRNAs

It is conceivable the viral miRNAs regulate the components of host immune system to avoid the immune surveillance and subsequent immune clearance to virus. Thus, viral miRNAs provide a novel immune-evasion mechanism for virus survival in the host. For most of algorithms developed for target prediction of mammalian miRNAs, the most important feature is the consideration of the evolutionary conservation of the binding sites in different species. But for targets of viral miRNAs, it does not seem to be the case because of the strict host range limitation for most of the known viruses. Stern-Ginossar *et al*. developed an algorithm for miRNA target prediction, which is based on the observation that the more miRNA binding sites exist within the 3′UTR of a potential target mRNA, the more likely this candidate is the real target. Although this limited algorithm will undoubtedly miss a considerable number of actual targets with only one binding site, an immune-related gene, MICB was successfully predicted to be the target gene of HCMV-miR-UL112 with a high-ranking score. MICB is a ligand for the NKG2D type II receptor. Binding of the ligand activates the cytolytic response of natural killer (NK) cells, CD8 alphabeta T cells, and gammadelta T cells which express the

receptor (37,75). Stern-Ginossar *et al*. transduced a number of tumor cell lines that endogenously express MICB with a lentivirus-based HCMV-miR-UL112 expression construct. The author found the protein level of MICB was reduced in these tested tumor cell lines without significant change at the mRNA level, which indicated the specific regulation of MICB by the HCMV-miR-UL112. Additional experiments confirmed that HCMV-miR-UL112-mediated reduction of MICB indeed affected its interaction with NKG2D receptor, leading to less efficient killing by the natural killer cells. Subsequent functional binding site studies of MICB 3′UTR provided strong evidences that MICB is directly targeted by the HCMV-miR-UL112. Previous studies have shown that the UL16 protein encoded by HCMV can bind to MICB resulting in intracellular sequestration of NKG2D ligands and reduction of the cell susceptibility to natural killer cells (20,26). All these findings suggest a complicated and cooperative mechanism by which a virus evades the attack of the host immune system (92).

EBV encodes multiple miRNAs differentially expressed in distinct cell lines and clinical samples. The complicated expression patterns of EBV miRNAs implicate the complexity of their functions. *CXCCL-11/I-TAC*, the interferon-inducible T-cell attracting chemokine, has been recently shown to be a potent antitumor actor (50,95). It was also predicted to be a putative cellular target gene of BHRF1-3 miRNA based on the published data that show *CXCCL-11/I-TAC* contains a 100% complimentary sequence to the BHRF1-3 miRNA sequence in its 3′ UTR. To prove the prediction, Xia *et al*. tested the expression level of *CXCCL-11/I-TAC* in two different cell lines with significant difference in BHRF1-3 miRNA level, and found that the expression level of *CXCCL-11/I-TAC* inversely correlates with the level of the BHRF1-3 miRNA (93). Further experiments using BHRF1-3 miRNA sense and anti-sense oligos showed significant changes of *CXCCL-11/I-TAC* expression in a dose-dependent fashion. Despite these observations, the failure to identify the mRNA binding sites renders difficult to judge whether *CXCCL-11/I-TAC* is indeed the direct target of the BHRF1-3 miRNA. Nevertheless, the BHRF1-3 miRNA-mediated suppression of *CXCCL-11/I-TAC* may still be regarded as a potential immune evasion mechanism in EBV-infected lymphomas.

Like EBV, KSHV also encodes a number of miRNAs clustered in an intergenic region in the genome. Recently, several studies reported that KSHV miRNAs could target cellular genes (38,79,83). These findings shed light on how KSHV miRNAs may regulate cellular genes and contribute to KSHV-induced malignancies. Samons *et al*. attempted to identify cellular genes targeted by KSHV miRNAs. Briefly, a cluster of 10 KSHV miRNAs were cloned and stably expressed in 293T cells. A microarray analysis was then performed to detect differential expression profiles as a result of overexpression of the viral miRNAs. Several potential cellular targets with significant changes at mRNA level were identified, and one of them, thrombospondin 1 (THBS1), was confirmed (79). THBS1, an extracellular glycoprotein, has antiproliferative, antiangiogenic, and immune modulatory activities (9). THBS1 is poorly expressed in KS lesions and KSHV-infected cells, and known to inhibit KS cells and HIV-1 Tat-induced angiogenesis (88). Samons *et al*. observed a significant decrease of THBS1 protein in 293T cells expressing the KSHV miRNA cluster. The reduction of THBS1 could be translated into reduced TGF-beta activity. Further evidences indicated that THBS1 is targeted by multiple KSHV miRNAs.

MiRNA orthologs are commonly seen among different metazoan eukaryotes, but for viral miRNAs, it has rarely happened. Two recent studies all showed that KSHV-miR-K12-1 not only shares the same seed sequence with the cellular miR-155, but also shares a set of target genes, indicating that miR-K12-11 is an ortholog of human miR-155 (38,83). miR-155 is derived from the non-coding transcript of the BIC gene (29) and is thought to be an oncomir due to an observation that overexpression of this miRNA led to cancer in a transgenic mice model (21). The finding that KSHV miR-K12-11 is an ortholog of miR-155 suggests that miR-

K12-11 may play an important role in the development of KSHV-associated lymphomas by hijacking the same targets of miR-155. Interestingly, with the exception of BACH-1, no overlap of cellular targets of miR-K12-11 was identified by microarray analysis in these studies, possibly because of the use of different experimental cell lines. Gottwein *et al*. also presented a list of potential cellular orthologs of other viral miRNA, which remains to be further confirmed.

Regulation of viral genes by viral miRNAs

The life cycle of all herpesviruses have two distinct phases, latency and lytic replication. In latent phase, only a few of viral genes are expressed; while in lytic phase, almost all the viral genes are expressed (77). Viral miRNAs encoded by herpesviruses may directly target viral transcripts to regulate viral life cycle.

Umbach *et al*. recently found several viral miRNAs in or near the LAT region of HSV-1. miR-H2, miR-H3 and miR-H4 were mapped to the complimentary strand of several ICP transcripts indicating that these miRNAs might regulate the expression of ICP transcripts. ICP0 is an activator of viral genes, and is required for efficient initiation of lytic infection and reactivation from latency (31). The expression of ICP0 is sufficient to increase lysis of HSV infected cells by natural killer cells (19). Since miR-H2 is antisense to ICP0 transcripts, it is conceivable that HSV-1 may inhibit the expression of ICP0 via miR-H2 to maintain latent infection, and evade the immune responses. To test this hypothesis, an ICP0 expression plasmid and a miR-H2-3p expression plasmid were co-transfected into 293T cells, resulting in a significant reduction of ICP0 protein expression. The sequence of mature miR-H2 does not perfectly match those in the ICP0 transcripts, inferring the absence of siRNA cleavage of the target mRNAs. In indeed, the ICP0 transcript was almost unaffected in the study. miR-H3 and miR-H4 are transcribed antisense to ICP34.5, but the possible effect of miR-H3 and miR-H4 on ICP34.5 has not been investigated. A recent study by Umbach *et al*. showed that miR-H6, with partial complementarity to ICP4 transcript, acts as a suppressor of the ICP4 protein expression without cleaving of its transcript (89). A similar study by Tang *et al*. showed that HSV-2 miR-I, transcribed antisense to ICP 34.5, efficiently inhibits the expression of ICP34.5 through the siRNA-mediated suppression mechanism (87). Together, results from the HSV-1 and HSV-2 studies indicate that, despite viral miRNAs are 100% complementary to the transcripts, they might not inhibit the expression of the genes on the opposite strand in the same fashion.

To study whether HCMV miRNAs target its viral genes, Grey *et al*. (41) applied a computational approach to predict the potential binding sites within the 3′UTR of viral transcripts. Since there was no existing HCMV 3′UTR database available due to the lack of experimental data, the author had to establish a putative 3′UTR database of HCMV transcripts. The 3′UTR of viral transcripts was defined as the sequence from the 3′ end of each annotated ORF to the first typical poly (A) signal, which inevitably excluded some of the complicated splicing events of viral transcripts. Following the prediction and comparison with another closely related chimpanzee cytomegalovirus (CCMV), 14 3′UTR were predicted to contain putative binding sites for miR-UL112-1 in both HCMV and CCMV. All 14 putative 3′UTR were then inserted into a luciferase reporter and tested for the inhibitory effect of miR-UL112-1, leading to the identification of 3 positive targets, IE72 (UL123, IE1), UL120/121 and UL112/113. IE72 is an HCMV IE locus product required to execute the viral transcriptional program (64). Additional studies confirmed that the IE72 protein could be specially repressed by miR-UL112-1 at the translation stage without affecting its RNA level. Previous data showed that when the IE72 expression level is high during the early HCMV infection phase, miR-UL112-1 expression is low (40). Grey *et al*. further examined whether this repressing effect could affect the viral life cycle by transfecting a synthetic miR-UL112-1 RNA duplex into cells prior to viral infection. He found that the levels of both IE72 expression and viral DNA

replication was reduced (41). Similar results were reported in another study, in which Murphy *et al*. (67) used the Monte Carlo simulation to predict the potential viral target for HCMV miRNAs. The IE72 was further examined because of its important function in HCMV life cycle. By labeling the proteins with ^{35}S, Murphy *et al*. found that the newly synthesized IE72 protein had longer half-life in HCMV-infected cells. In this study, the author also made prediction of targets for miRNAs of other herpesviruses. Among some of these putative targets of viral miRNAs are BZLF1 and BRLF1 for EBV, and ORF-K8 and RTA (ORF50) for KSHV.

EBV is the first virus known to encode miRNAs, but how EBV miRNAs regulate its own viral transcripts had been unclear until two recent reports examined their targets (4,62). EBV-encoded LMP1 is an important factor for EBV-related malignancies. LMP-1 induces cell growth and transformation at low expression level; however when it is expressed at high level, it causes growth inhibition (30). Bioinformatics analysis by Lo *et al*. showed that the 3′UTR of LMP1 contains binding sites to several BART miRNAs. Reporter assay with luciferase plasmids embedded with multiple target sequence identified 3 BART miRNAs, including BART16, 17-5p and 1-5p, which target LMP1. In this study, the entire BART cluster 1 miRNAs cloned in an expression vector or individual synthetic BART miRNAs were co-transfected with a LMP1 expression vector containing the 3′UTR of LMP1. The results showed a significant reduction of LMP1 protein. High level of NF-κB activity, which is the major pathway activated by LMP1, is believed to be essential for the survival and growth of EBV-transformed cells (13,32,51,55). Results of this study indicate that low level of LMP1 was sufficient to activate the NF-κB pathway, but increased level of LMP1 would actually reduce the NF-κB activity. Thus, the BART cluster 1 miRNAs appear to fine-tune the NF-κB activity through LMP1. Together, this delicate interaction might serve as parts of the immunomodulatory mechanism to maintain viral latency.

The EBV-encoded BALF5 is a viral DNA polymerase, which is essential for viral DNA replication (33). miR-BART2 transcribed antisense to the BALF5 transcript has perfect complementary match to the BALF5 3′ UTR, and thus may target this mRNA for degradation (74). Barth *et al*. confirmed that miR-BART2 indeed could inhibit the activity of a luciferase reporter containing the BALF5 3′UTR, and reduce the endogenous BALF5 protein level in EBV-infected cells. The expression levels of miR-BART2 and BALF5 were inversely correlated. While miR-BART2 is expressed in latency, BALF5 is not present during latent infection. In contrast, an increase in the miR-BART2 level in EBV-infected cells undergoing lytic replication resulted in a ~50% reduction in the BLAF5 protein level and ~20% reduction of virus production. These results clearly illustrate a strategy by which the virus inhibits the expression of viral lytic genes through viral miRNAs to maintain latency.

Conclusion

Recent studies on the viral miRNAs have revealed many functions in regulating cellular and viral activities, including the cell proliferation, differentiation, cell apoptosis, viral gene expression and virus replication. However, it is still early to construct the entire networks of functions performed by viral miRNAs. Further development of better target prediction algorithms combined with high throughput validation methods should promise better understanding of the roles of these newly discovered fascinating small molecules.

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