Chapter

Role of Virus-Encoded microRNAs in Avian Viral Diseases

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Abstract

To survive in the host cells, viruses have to adapt various strategies, which include the modulation of microRNA (miRNA) pathway through virus-encoded miRNAs to modulate the host cellular environment. It has been shown that several avian viruses, mostly herpesviruses, encode a number of miRNAs. These include 26 miRNAs encoded by the highly oncogenic Marek's disease virus-1, 36 miRNAs encoded by avirulent Marek's disease virus-2, 28 miRNAs by herpesvirus of turkeys, 10 miRNAs by infectious laryngotracheitis virus, 41 miRNAs by duck enteritis virus, and 2 miRNAs by avian leukosis virus subgroup J. Although locations of some of the miRNAs are conserved within the repeat regions of the genomes among some of the antigenic and phylogenetic closely related herpesviruses, there are no sequence conservation of miRNAs encoded by different avian herpesviruses. Moreover, some of the virus-encoded miRNAs have the same seed sequence as host miRNAs serve as functional orthologs. For example, mdv1-miR-M4-5p, a functional ortholog of gga-miR-155, is critical for the Marek's disease virus in inducing tumors. In this review, we describe the advances in our understanding on the role of the herpesvirus-encoded miRNAs in avian diseases. Additionally, we also describe the potential association of avian leukosis virus subgroup J encoded E (XSR) miRNA in the induction of myeloid tumors in certain genetically distinct chicken lines.

Keywords: avian viruses, microRNAs, MDV, DEV, ILTV and ALV

1. Introduction

MicroRNAs (miRNAs) are small RNA molecules of ~22-nucleotide that profoundly affect gene expression by targeting the 3'UTR (untranslated region) of the targeted mRNA (messenger RNA). Ever since the first discovery of miRNA in *Caenorhabditis elegans* [1], the identification of miRNAs, of which some are evolutionarily conserved [2–4], has proceeded at a quick pace. It is well known now that miRNAs are key regulators of gene expression in many species including mammals, plants, flies, worms, as well as in a number of viruses. Out of 38,589 miRNAs identified so far, 530 are encoded by viruses (www.miRbase.org). Most of the viral miRNAs are encoded by DNA viruses, with members of the family *Herpesviridae* encoding for the vast majority suggesting the importance of miRNA-mediated gene regulation in the biology of herpesvirus infections. Long-term survival as latent infections in different cell types are characteristic feature of virus-host interactions in herpesviruses. This requires sophisticated survival strategy to avoid detection by the innate and adaptive immune mechanisms of the host. Herpesviruses achieve this using a variety of mechanisms through translational

control, epigenetic control of viral/host gene expression, and restricted gene expression [5, 6]. The miRNAs, with the small size along with the ability for specific repression of multiple transcript targets, make them ideal tools for herpesviruses to reshape the gene expression to favor viral replication in an infected cell. Hence, it is of no surprise that herpesviruses encode 91% of virus-encoded miRNAs identified so far. The number of miRNAs encoded by different herpesviruses varies, i.e., 5 miRNAs in Bovine herpesvirus 5 and 70 in Rhesus lymphocryptovirus (miRBase V22.1). In addition to herpesviruses, some of other virus families also encode miRNAs [7, 8] including miRNAs encoded by the human Torque Teno Virus [9]. Although retroviruses have not been widely documented to exploit the miRNA pathway [10], demonstration of E (XSR) miRNA encoded by avian leukosis virus subgroup J (ALV-J) using the canonical miRNA biogenesis pathway [11], and a conserved cluster of RNA polymerase III (pol III)-transcribed miRNAs from the bovine leukemia virus (BLV) genome [12, 13] showed that retroviruses also exploit the miRNA pathway.

2. miRNA-encoding avian viruses and associated diseases

Avian herpesviruses are a major group of pathogens affecting different species of poultry. All of the pathogenic avian herpesviruses belong to the subfamily *Alphaherpesvirinae*. These include infectious laryngotracheitis virus (ILTV, Gallid herpesvirus) in the *Iltovirus* genus, pathogenic Marek's disease virus-1 (MDV-1, Gallid herpesvirus 2) in *Mardivirus* genus, and unassigned duck enteritis virus (DEV). ILTV induces laryngotracheitis, a contagious respiratory tract infection that results in severe losses in egg production and high mortality in infected poultry flocks. Although the safety of vaccine strains has been questionable, live attenuated vaccines are used to control this disease [14]. *Mardivirus* genus is classified into three closely related, but distinct groups including the pathogenic Marek's disease virus-1 (MDV-1, Gallid herpesvirus 2), attenuated Marek's disease virus-2 (MDV-2, Gallid herpesvirus 3), and the antigenically related herpesvirus of turkey (HVT, Meleagrid herpesvirus 1). Based on their pathogenicity, MDV-1 is further grouped into different pathotypes named as virulent (vMDV), very virulent (vvMDV), and very virulent plus (vv + MDV) [12]. Marek's disease (MD) is an immunosuppressive, neurological, and oncogenic disease caused by highly contagious MDV-1. MD that is widespread in the poultry population around the world causes estimated economic losses of US$ 2000 million annually [15]. Although, controlled by the use of vaccines such as nonpathogenic HVT and MDV-2 vaccines or live attenuated MDV-1 strains [16], there is concern on the continued evolution of the virus toward greater virulence, challenging the sustainability of Marek's disease vaccination strategy [17, 18]. DEV induces acute disease in waterfowl species with high mortality [19, 20].

Avian leukosis viruses (ALVs) are members of *Alpharetrovirus* genus of the *Retroviridae* family. ALVs cause neoplastic diseases and other reproduction problems with enormous economic losses in the global poultry industry. According to their mode of transmission, the ALVs are classified as either endogenous (ALV-E) or exogenous viruses. Based on viral envelope interference, host range, and cross-neutralization patterns, exogenous ALVs from chickens have been further divided into different subgroups (A, B, C, D, and J) [21]. ALV-J was first described in the UK in the late 1980s [22] and has been primarily associated with myeloid leukosis in meat-type chickens. ALV-J caused more serious damage than all other subgroups worldwide. ALV-induced disease particularly by ALV-J is still widespread in poultry population in China and causes huge economic losses [23].

3. Identification of miRNAs encoded by avian viruses

The majority of viral miRNAs had initially been identified by a protocol involving size fractionation of small RNA, ligation of linkers, reverse transcription, concatamerization, cloning followed by Sanger sequencing [7]. The bioinformatics approaches to identify miRNA-encoding loci in viral genome based on commonalities in the predicated secondary structures of pre-miRNAs have also been developed. Exploring the small RNAs libraries with a higher degree of reliability and unprecedented depth has become possible with the advent of massively parallel sequencing technologies. We and others have reported the identification of miRNAs encoded by a number of avian herpesviruses. These include 14 miRNAs (26 mature sequences) encoded by MDV-1 [24, 25], 18 miRNAs (36 mature sequences) encoded by MDV-2 [26, 27], 17 miRNAs (28 mature sequences) encoded by HVT [27, 28], 7 miRNAs (10 mature sequences) encoded by ILTV [27, 29], 24 miRNAs (33 mature sequences) encoded by DEV [30], and 1 miRNA (2 mature sequences) encoded by ALV-J [11] (**Figure 1**).

3.1 MDV-1 miRNAs

High throughput sequencing of small RNA libraries from highly virulent RB1B strain infected chicken embryo fibroblast (CEF) has led to the identification of the first MDV-1-encoded miRNAs in 2006 [24]. Subsequently, more MDV-1 miRNAs were discovered by analyzing small RNA library of MSB-1, a MDV-1 cell line established from an MDV-induced spleen lymphoma [25, 31]. A total of 14 precursor sequences producing 26 mature miRNAs have been identified from the MDV-1 genome (www.miRbase.org; **Figure 1A**). The MDV-1 miRNAs are clustered in three separate genomic loci: cluster 1 (Meq-cluster), cluster 2 (Mid-cluster) flank the Meq oncogene, and cluster 3 (LAT-cluster) lies in the region encoding the latency-associated transcripts (LATs) [24, 25, 31] (**Figure 1A**). All three MDV-1 miRNA clusters are in the repeat regions of the viral genome. Although differing in virulence, the miRNA sequences are highly conserved among 23 different MDV-1 strains [31, 32]. Despite this, the expression level of Meq-cluster miRNAs is higher in vv+ MDV-induced lymphomas than vvMDV strain-induced lymphomas. One polymorphism in the miRNA promoter region has been proposed to be responsible for this differential expression. On the other hand, there is no difference on the level of the LAT-cluster miRNAs expression [31, 33], implying that miRNAs from Meq-cluster may play a more significant role in MD oncogenesis. Indeed, the significantly decreased oncogenicity of the virus with the deletion of the Meq-cluster miRNAs has proved this hypothesis [34, 35]. mdv1-miR-M4-5p, a functional ortholog of gga-miR-155 and a member of Meq-cluster miRNA, is the most highly expressed viral miRNA in tumors, representing over 70% of MDV miRNA sequencing reads [31]. Similar to the role of gga-miR-155 in lymphoid malignancies, mdv1-miR-M4-5p was shown to play a key role in MDV-1-induced oncogenesis [34, 35]. The promoter prmiRM9M4, corresponding to the 1300-bp immediately upstream from the first Meq-cluster miRNA mdv1-miR-M9, has been shown to drive the transcription of both Meq-cluster and Mid-cluster miRNAs with two distinct transcriptional models during different infection phases [33]. Indeed, this promoter has been shown to be active during MDV-1 latency by both DNA hypomethylation and active histone marks [36], confirming its transcriptional activity. In contrast, the transcription of LAT-clustered miRNAs is driven by a p53-dependent promoter, which contains at least two 60-bp tandem repeats harboring a p53-response element but has no consensus core promoter element [37].

Studies on correlation of miRNA expression signature with MDV-transformation have been undertaken. Microarray analysis has been carried

out to determine the global miRNA expression profiles in seven distinct MDV-transformed cell lines [38]. Compared to uninfected splenocytes and CD4+ T-cells or non-MDV avian viral transformed cell lines, these profiles revealed that the expression of a set of host miRNAs is altered in the MDV-transformed cell lines. The downregulated host miRNAs in the MDV-transformed cell lines include miR-26a, miR-150, miR-223, miR-155, and miR-451. The differential expression of these host miRNAs may contribute to MDV pathogenesis, considering the link of several mammalian homologs of these miRNAs with cancer development [39]. For example, miR-26a with tumor suppressor function is downregulated in a number of avian lymphoma cells. miR-26a has been shown to regulate interleukin 2 (IL-2) expression [40]. It is well known that IL-2 regulates T-cell proliferation, suggesting that downregulation of miR-26a and the upregulation of IL-2 expression could be a conserved mechanism in avian viral transformation. Taken together, analysis of the functional targets of the differentially expressed miRNAs would contribute toward better understanding of the molecular pathways of MD oncogenicity.

3.2 MDV-2 miRNAs

MSB-1 is a MDV-transformed lymphoblastoid cell line, which is co-infected with both BC-1 strain of MDV-1 and HPRS24 strain of MDV-2 [41]. About 17 novel MDV-2 miRNAs were identified by analyzing small RNA library from MSB-1 [26]. Of these, 16 are expressed in the same direction and clustered in a 4.2-kb long repeat region that encodes R-LORF2 to R-LORF5, suggesting that they may be derived from a common primary transcript. The single miRNA outside the cluster was located within the C-terminal region of the ICP4 homolog in the short repeat region. Despite lacking sequence homology, the relative genomic positions of miRNA clusters in MDV-1 and MDV-2 are conserved by locating within the repeat regions of the viral genome. Subsequently, an additional miRNA mdv2-miR-M32 has been reported by high throughput sequencing of small RNAs from MSB-1 cells [27]. Uniquely, all 18 MDV-2 encoded miRNA precursors give rise to 2 mature forms resulting in 36 mature MDV-2 miRNAs, representing both strands of the duplex (www.miRbase.org; **Figure 1A**). Two separate studies on sequencing of MSB-1 small RNA library showed that MDV-2 miRNAs accounted for 10 and 13% of the sequencing reads, respectively [25, 27].

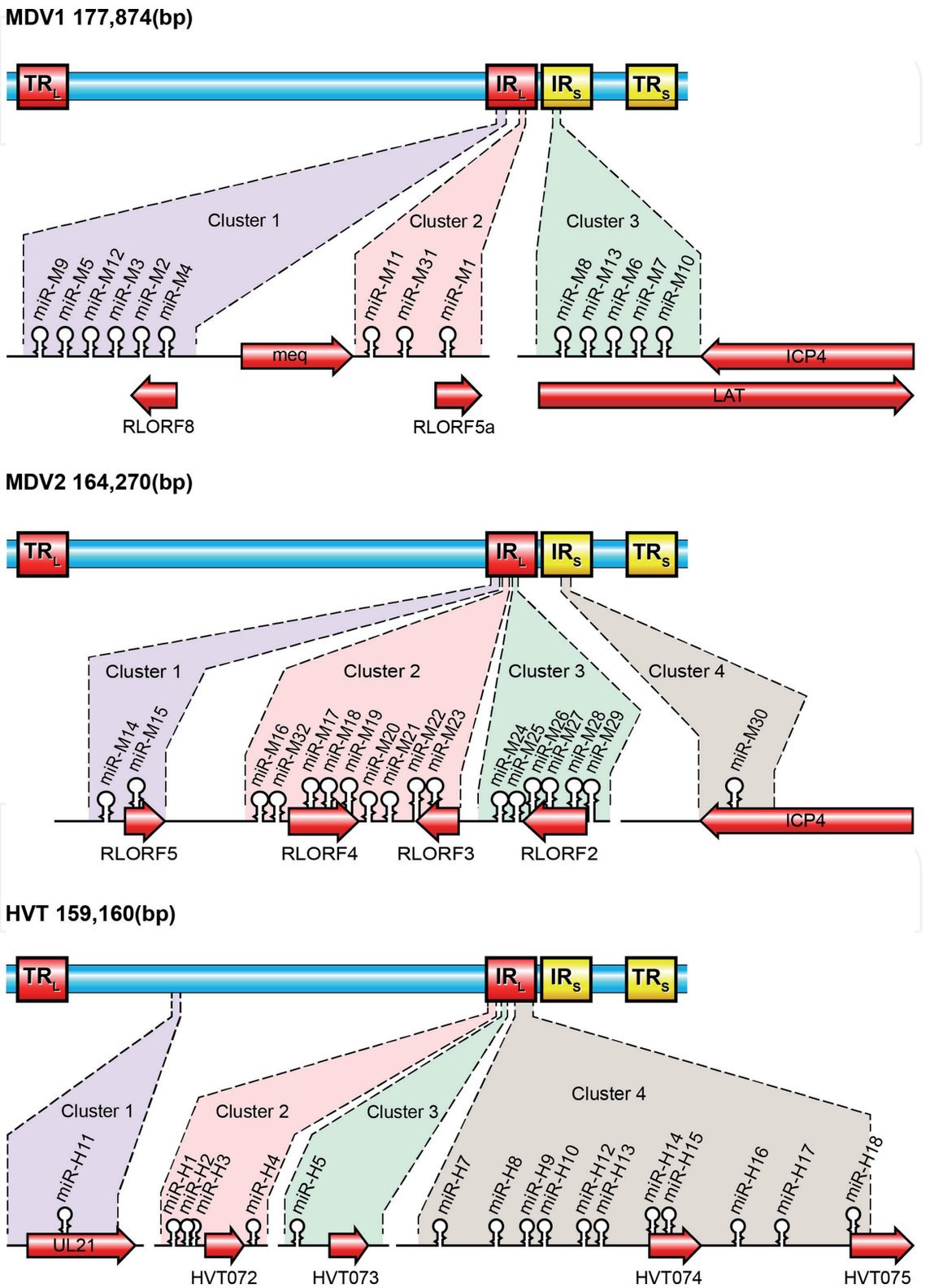
3.3 HVT miRNAs

HVT-encoded miRNAs were identified from HVT-infected CEF using both traditional cloning and sequencing of a small RNA library [28] and high throughput sequencing technology [27]. About 17 precursor miRNAs producing 28 mature miRNAs (www.miRbase.org; **Figure 1A**) have been identified in the HVT genome. Except for miR-H11, which is located within an intron of UL21 in the UL region, all other HVT miRNAs are located in the long-repeat regions, demonstrating some degree of positional conservation with MDV-1 and MDV-2. Interestingly, 10 HVT miRNAs were located in a region containing two tandem repeats, and small sequence variations were observed following multiple sequence alignment of the miRNA precursors, suggesting evolution by duplication. This represents the first example of evolution of virus-encoded miRNAs by duplication.

3.4 ILTV-miRNAs

A total of seven ILTV precursor miRNAs producing 10 mature miRNAs from ILTV-infected chicken embryo kidney (CEK) cells and leghorn male hepatoma

(LMH) cell line have been identified by deep sequencing of small RNA populations [27, 29] (www.miRbase.org; **Figure 1B**). Four of the miRNAs (iltv-miR-I1-I4), which are not associated with any annotated ORFs were located at the extreme terminus of the ILTV genome. miR-I5 and miR-I6, the two most highly expressed miRNAs, are located in the repeat regions within ICP4. The iltv-miR-I7 was mapped in the replication origin (oriL) of the palindrome stem loop sequence. Out of seven



(A)

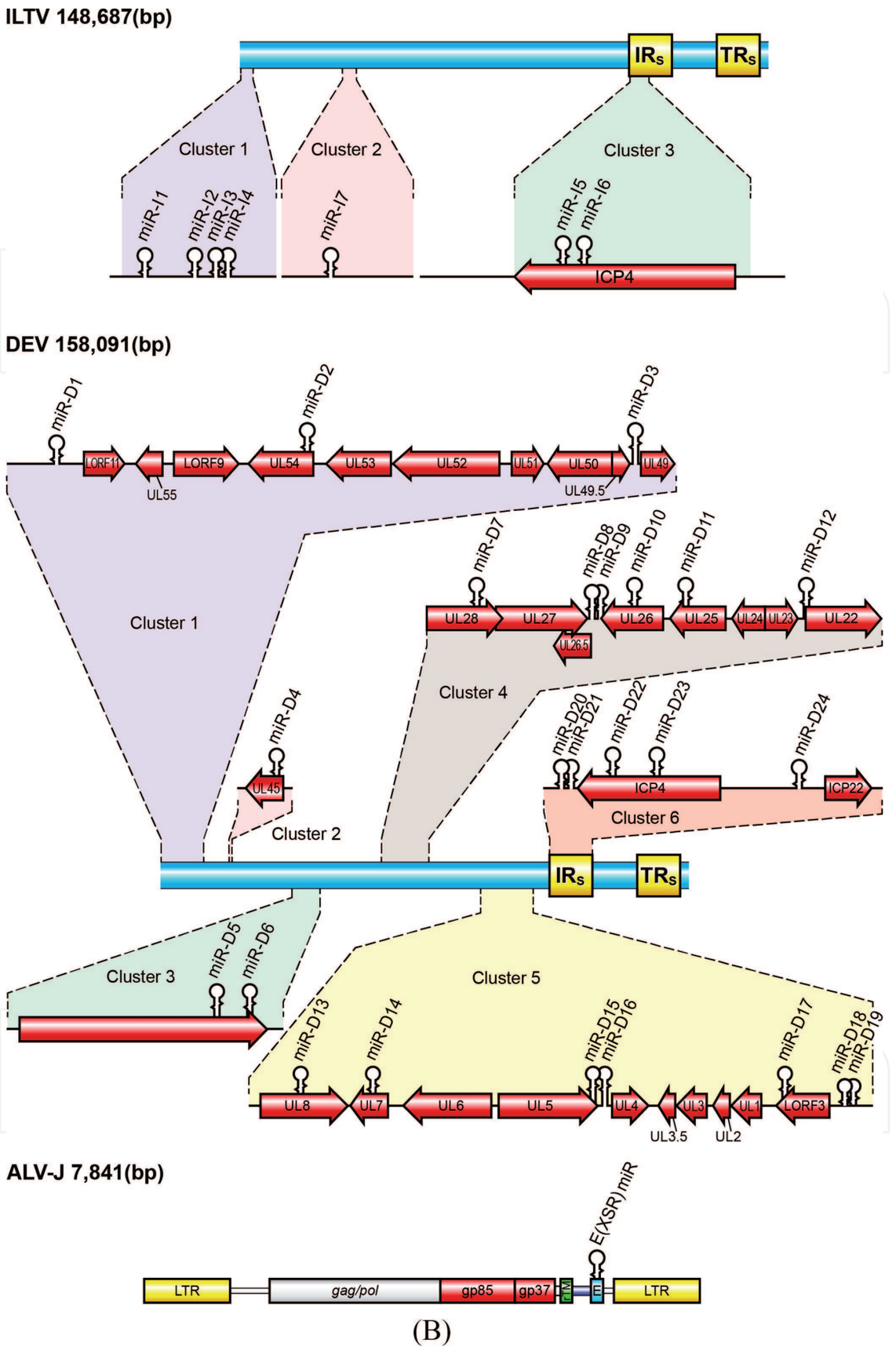


Figure 1.
Diagrammatic representation of the viral genomes showing the positions of miRNAs of MDV1, MDV2, HVT (A) and ILTV, DEV and ALV-J (B). Position and orientation of selected transcripts are shown. The genome size (base pairs) of viruses is shown on the top-left corner. Adapted from Yao et al., [92].

miRNAs, only three (iltv-miR-I3, -I5, -I6) have been confirmed by Northern hybridizations although the expression was confirmed for all by the end point PCR using small RNA libraries generated from ILTV-infected CEK.

3.5 DEV miRNAs

About 33 mature miRNAs derived from 24 pre-miRNA sequences within the DEV genome have been identified by deep sequencing of small RNAs from DEV vaccine strain-infected CEF cultures [30] (www.miRbase.org; **Figure 1B**). Compared to the location of most Mardivirus-encoded miRNAs which are at the repeat regions, genomic positions of DEV-encoded miRNAs are unique. Five out of six miRNA clusters from both the coding and non-coding regions of the 158,091-bp viral genome were encoded within the unique long region. Similar to miRNA-offset RNAs, two miRNA precursors, DEV miR-D18 and miR-D19, overlapped with each other although only the dev-miR-D18-3p was functional in reporter assays. In addition, 12 putative DEV miRNAs have been reported by computational approach [42], none of these miRNAs overlapped with the 24 DEV miRNAs described above [30]. In a recent report, 39 mature viral miRNAs have been identified from a Chinese virulent strain of DEV infected duck embryo fibroblast cells [43]. Of those, only 13 miRNA sequences and 22 "seed sequences" of miRNA were identical with the previously reported DEV-miRNAs encoded by vaccine strain [30]. About eight additional viral miRNAs were detected and confirmed by stem-loop RT-qPCR [43].

3.6 ALV-J miRNAs

One of the distinct features of HPRS-103 sequence, the ALV-J prototype virus, is the presence of E (also called XSR) element, a distinct hairpin stem-loop structure in the 3′ untranslated region. Indeed, the expression of miRNA from this hairpin-like structure of E element has been demonstrated in ALV-J-infected/transformed cells [11]. E (XSR) miRNA, a small RNA population encoded from within the E (XSR) element, was identified using deep sequencing approach on ALV-J-transformed cell line IAH30 [11] (**Figure 1B**). E (XSR) miRNA is suggested to play a major role in ALV-J pathogenesis and neoplastic transformation as it accounted for a quarter of the sequences of the IAH30 "miRNAome." Although the E element per se is not absolutely essential for ALV-J induced tumor, the E element does contribute to the oncogenicity in certain genetic lines of chicken when the oncogenicity of viruses derived from the parental and E element deleted HPRS-103 viruses was compared [44]. The hypothesis of polymorphisms, particularly those in any potential E (XSR) miRNA target sites could account for differential susceptibility phenotypes among these lines can be investigated by comparison of the genomes of these lines.

4. Viral orthologs of host miRNAs

Two classes of virus-encoded miRNAs are grouped: viral specific miRNAs and orthologs of host miRNAs. Similar to some viral regulatory proteins, several viral miRNAs have evolved to mimic host effectors. The "seed" region of a miRNA (~nucleotides 2–8 at the 5′ end) plays a key role in directing RISC to its mRNA targets. Binding with perfect seed complementary to the target transcript takes ~60% of regulation by a particular miRNA [45]. Some of viral miRNAs share seed sequences with host miRNAs and at least three viruses: BLV, MDV-1, and Kaposi's Sarcoma-associated Herpesvirus (KSHV) have been shown to regulate transcripts via the same target docking sites as their counterpart host miRNAs [46–48]. Such viral miRNAs could potentially regulate hundreds of transcripts having target sites for a particular host miRNA. Such regulatory networks could affect specific functions such as inhibiting apoptosis.

KSHV and MDV-1 express two distinct miRNAs, which are functional ortholog of host miR-155, a conserved cellular miRNA that is required for the rapid expansion of B and T cells after antigenic stimulation and highly expressed in activated myeloid and lymphoid cells [31, 46–48]. Interestingly, in both MDV-1 and KSHV-induced tumors, there is downregulation of endogenous levels of miR-155 [38, 47, 49], although the mechanisms for such downregulation is not fully understood. It is intriguing to know why these two viruses chose to express their own miR-155 orthologs, while suppressing the host-encoded miRNA with potentially identical functions. The direct role of miR-155 on oncogenesis [50, 51] and induction of cancer [47, 52] have been shown by a number of studies. Furthermore, it has been shown that upregulation of miR-155 is associated with lymphocyte transformation by reticuloendotheliosis virus strain T (REV-T) [53, 54] and EBV [55]. It is therefore striking that mdv1-miR-M4-5p is highly expressed in MDV-1 transformed cells derived from MDV-1-induced T-cell lymphomas [31, 46]. Moreover, lymphoma induction in infected birds is greatly reduced with seed region mutagenesis or deletion of mdv1-miR-M4-5p, suggesting the importance of mdv1-miR-M4-5p in the induction of tumors [34, 35]. The fact that transformation ability of the miR-M4-deleted MDV-1 virus is partly restored by introduction of chicken miR-155, suggests that viral miRNAs can play a major role in enhancing the oncogenic potential of a herpesvirus *in vivo*. However, the oncogenicity of vvMDV strain GX0101 with mdv1-miR-M4 deletion has significantly decreased but was not totally abolished [35]. This report, coupled with the finding that HVT with mdv1-miR-M4 inserted in the genome failed to induce tumors [32], suggested that other factors are also required for viral transformation. Moreover, our recent studies have shown that mdv1-miR-M4 is not essential for maintaining the proliferation of transformed cell lines suggesting that it has probably a more significant role in the initiation of neoplastic transformation [56].

miR-H14-3p, one of the HVT-encoded miRNAs, showed close sequence identity with perfect match of the 21/23 nucleotides including identical seed sequence to the chicken gga-miR-221, suggesting that it is a virus-encoded ortholog [27]. Indeed, the proposed ortholog status of the two miRNAs are supported by the evidence that HVT-miR-H14-3p also modulates the expression of p27Kip1 (cyclin-dependent kinase inhibitor 1B), a known target of miR-221 [57]. Compared to other known viral miRNA orthologs, where only the seed sequences are identical, the full length mature miRNA sequences of miR-H14-3p and gga-miR-221 are almost identical, strongly suggesting that hvt-miR-H14-3p is most likely to have been acquired from the host genome. This is the first example of virus-encoded miRNA showing such close and extended sequence identity with a host miRNA. Furthermore, partial sequence conservation has been observed between the gga-miR-221 locus on chromosome 1 of the chicken genome and the downstream flanking region of hvt-miR-H14-3p in the HVT genome, suggesting hvt-miR-H14-3p is "pirated" by the virus from the host, despite of the fact that herpesviruses have frequently pirated and subverted host genes to their own purposes. Interestingly, p27Kip1, the target shared by the two miRNAs, is a regulator of the cell cycle G1 to S phase transition. MDV-1-induced tumorigenesis may also involve a similar mechanism as miR-221-mediated repression of p27 in cancer progression [58–60]. In order to support viral genome replication and to promote growth of infected cells for additional viral production, the downregulation of p27 could move the cell cycle to the S phase [32]. The sequence homology is also observed between mdv1-miR-M31 and miR-221 in the seed region [31], although it is limited to the minimal miRNA seed region at nucleotide positions 2–7. Although targeting of p27 by miR-M31-3p has not been experimentally validated, the finding that miR-M31 deleted virus reduced the mortality and gross tumor incidence of infected chickens significantly has implied that miR-M31 may act as potential oncogene by targeting p27 [61].

gga-miR-29 is known to function both as an oncogene and a tumor suppressor depending on the context [62]. Seed sequence homology has been observed between mdv2-miR-M21 and miR-29 suggesting the potential functional ortholog between the two miRNAs. In fact, blv-miR-B4, a BLV-encoded miRNA, has been shown to be a functional ortholog of host miR-29 [12]. Furthermore, RLCV-encoded miR-rL1-6-3p and EBV-encoded miR-BART1-3p also share seed sequence with miR-29. Thus, a picture is emerging wherein several virus-encoded miRNAs are likely to target host pathways [63].

It is estimated that ~8% of avian virus-encoded miRNAs possess identical heptameric seed sequence with host miRNAs, thus could potentially act as functional orthologs [7]. However, based on low abundance, untested biogenesis, and unknown functional relevance, it is not clear, whether all of the currently annotated viral or host miRNAs are bona fide miRNAs, thus some seed matches between host and viral miRNAs could arise by chance. Therefore, any proposed functional orthologs between the viral and host miRNAs require further experimental validation.

5. Target identification of avian herpesvirus miRNAs

Although over 500 virus-encoded miRNAs have been identified, an in depth functional study is lacking for most. However, it is clear that virus-encoded miRNAs can target both cellular and viral mRNAs and this has been confirmed by several studies analyzing the mRNA targetome of the virus-infected cells [64]. The best characterized viral miRNA functions in supporting viral replication include regulating the latent-lytic switch; evading the immune response; promoting cell survival, proliferation, and/or differentiation. All of these functions should be particularly important during persistent infections. As viral miRNAs and proteins work synergistically to promote a cellular environment favorable to the completion of the viral life cycle, modulation of the host cell environment is achieved by multiple and partly redundant mechanisms. The identified targets of avian herpesvirus-encoded miRNAs are summarized in **Table 1**.

5.1 Viral targets of viral miRNAs

Compared to identification of cellular targets, identifying viral targets of viral miRNAs is more straightforward as viral genomes encode fewer candidate mRNAs. Known examples of viral targets include transcripts, which are antisense to the viral miRNA precursor and transcripts elsewhere with imperfect matches. Perhaps, the former one is the most straightforward examples of determining viral miRNA function as the perfect matching between the miRNA and its target would be predicted to result in a siRNA-like cleavage of the target mRNA if both are co-expressed. For example, iltv-miR-I5 that is antisense to ICP4 cleaves ICP4 mRNA [65]. ICP4, an immediate early viral transactivator, plays a key role in the induction of lytic replication. The ICP4 targeting by viral miRNAs is thought to mediate lytic/latent switch and render the latent state more robust [66]. Apart from iltv-miR-I5, iltv-miR-I6 also maps antisense to the ICP4 gene. However, reporter assay on inhibition of luciferase activity by iltv-miR-I6 was not significant [65]. It has been proposed that this is due to the blockage of accessibility to the binding region following in silico folding of RNA containing the targets for iltv-miR-I5 and iltv-miR-I6. This is consistent with the previous finding that target RNA folding is essential for the efficacy of designed siRNAs [67, 68]. Sequences antisense to known miRNA stem-loop structures may fold into stem-loop structures themselves at an increased propensity.

Virus	miRNAs	Targets	Proposed function
MDV-1	mdv1-mir-M3	Smad2	Anti-apoptotic [32]
	mdv1-mir-M4-5p	Pu.1, CEBPβ, HIVEP2, BCL2L13, PDCD6, GPM6B, RREB1, c-Myb, MAP3K7IP2, C1orf103, CSNK1A1, LATS2, MAP3K14, NR1D2, RORA, RPS6KA3, WEE1, FCHSD2, JARID2, PBEF1, RAP2A	Mimics cellular mir-155 [33-35] in regulating apoptosis, proliferation, immune tolerance and tumorigenesis.
		LTBP1	Anti-apoptotic [36]
		hnRNPAB	Promote proliferation [37]
		UL28	Impair MDV morphogenesis/maintain latency [34]
	mdv1-mir-M4-3p	UL32	Impair MDV morphogenesis/maintain latency [34]
	mdv1-mir-M7-5p	ICP4 and ICP27	Establish and/or maintain latency [38]
	mdv1-mir-M11	Meq	Tumor suppressor [39]
	mdv1-mir-M2 mdv1-mir-M3 mdv1-mir-M4 mdv1-mir-M12	R-LORF8	Lymphocyte growth [35]
	MDV1-miR-M2-3p MDV1-miR-M9-5p MDV2-miR-M18-5p MDV2-miR-M18-5p MDV2-miR-M26-5p MDV2-miR-M28-5p MDV2-miR-M30-3p	IL-18	Restricting innate antiviral immunity [35]
MDV-2	mdv2-mir-M24 mdv2-mir-M25 mdv2-mir-M26 mdv2-mir-M27 mdv2-mir-M28 mdv2-mir-M29	R-LORF2	Lymphocyte growth [35]
ILTV	iltv-mir-I5	ICP4	Establish and/or maintain latency [40]
ALV-J	E(XSR)miRNA		Potential role in oncogenesis

Table 1.
Avian virus encoded miRNAs and proposed functions highlighted in this review.

This strategy could allow a virus to express both the viral gene and encode miRNAs antisense to mRNA transcripts lying on the other strand.

Analysis of miRNA binding clusters on the MDV-1 genome by PAR-CLIP identified very strong binding clusters near the 5′ end of the genome. These clusters essentially are antisense to the MDV-1 miRNAs miR-M2, miR-M3, miR-M4, and miR-M12, which are members of Meq-cluster, adjacent to each other, highly expressed and all located antisense to an MDV-1 mRNA encoding the viral R-LORF8 protein [64]. Analysis of indicator constructs in co-transfected 293 T cells or transduced MSB-1 cells confirmed that R-LORF8 is indeed targeted by all four miRNAs. Interestingly, expression of R-LORF8 gene in a form lacking the viral miRNA binding sites caused a substantial reduction in MSB-1 cell growth, suggesting that dysregulated expression of R-LORF8 can exert a deleterious effect in MDV-1-transformed T cells. The molecular basis for this effect is not clear as the function of R-LORF8 in the viral life cycle is currently unknown. The same pattern is observed in MDV-2, where viral miRNAs (miR-M24, M25, M26, M27, M28, and M29) are transcribed from the DNA strand located antisense to R-LORF2. In addition, there are also some other miRNAs encoded by MDV-1, MDV-2, HVT, and DEV, which are antisense to certain viral transcripts [24–28], the possible regulatory roles of those miRNAs against their antisense mRNA transcripts require to be verified individually.

As described above, mdv1-miR-M4-5p is a functional ortholog of cellular miR-155. mdv1-miR-M4-5p is also the first avian herpesvirus miRNA known to target both viral and cellular mRNAs. In addition to the cellular targets shared with miR-155, mdv1-miR-M4-5p and mdv1-miR-M4-3p also target viral UL28 and UL32 proteins, respectively. This provided the first evidence of late viral gene targeting by herpesviral miRNA [69]. Rather than 3′UTR, both target sequences are located in the coding region. UL28 and UL32 homologs, which are required for the cleavage and packaging of virion DNA in human herpesvirus 1 (HHV-1) have been found in all subfamilies of mammalian and avian herpesviruses. However, the role of UL28 and UL32 in MDV-1 packaging has not been demonstrated. As UL28 and UL32 are involved in the later stages of MDV replication, it is possible that mdv1-miR-M4 contributes in maintaining MDV-1 latency by downregulating the production of UL28 and UL32 and impairing late MDV morphogenesis and reactivation.

Following the observation of the increased viral pathogenicity or oncogenicity of infected chickens when mdv1-miR-M11 is deleted from Mid-cluster, the potential mechanisms mediated by miR-M11 has been investigated [61]. Meq gene has been predicated as candidate target gene by bioinformatics approach with RNAhybrid. Indeed, Meq as miR-M11 target has been confirmed by dual luciferase assay and qRT-PCR showing downregulation of Meq transcript level following virus infection. It has been suggested that the viral miRNAs in the Meq- or Mid-clusters cooperate with each other for establishing, maintaining latency, and/or triggering tumorigenesis.

ICP4 and ICP27, two MDV immediate-early (IE) genes, have been identified as potential targets for mdv1-miR-M7-5p by both bioinformatics prediction and subsequent experimental validation [70]. Indeed, this is reflected by a negative correlation between an increase in ICP27 expression and the decreased expression of mdv1-miR-M7-5p during virus reactivation. This is consistent with the early finding that miR-M7-5p is at extremely low levels in MDV-infected CEF but highly expressed in MSB-1 cells [25]. MDV miRNAs produced from LAT-cluster may contribute to the latency by targeting two IE genes. These findings further support the view that herpesvirus miRNAs play a key role in controlling the lytic/latent switch during infection [71, 72].

5.2 Cellular targets of viral miRNAs

Viruses such as herpesviruses with latent infection need to keep the host cells alive long enough. Thus, viral miRNAs can promote virus replication through prolonging cell survival and evading immune recognition. Among avian virus-encoded miRNAs, the targets of mdv1-miR-M4-5p are most extensively studied due to its critical role in virus induced oncogenesis. As viral orthologs of miR-155, both kshv-miR-K12-11 and mdv1-miR-M4 potentially target the same group of transcription factors as gga-miR-155 including Pu.1, CEBPβ, HIVEP2, BCL2L13, PDCD6, MAP3K7IP2, GPM6B, RREB1, and c-Myb [46, 69]. Subsequent analysis of >1000 cellular mRNAs targeted by MDV-1 miRNAs in MSB-1 cells using photoactivatable ribonucleoside-enhanced cross-linking and immunoprecipitation (PAR-CLIP) resulted in the identification of 73 mRNA 3′UTR targets for miR-M4-5p, of which nine (C1orf103, CSNK1A1, LATS2, MAP3K14, MYB, NR1D2, RORA, RPS6KA3, and WEE1) had previously been identified as targets for miR-155 or kshv-miR-K12-11 in EBV-transformed human B cells and KSHV-transformed human B cells, respectively [64]. Indicator analysis of these 3′ UTRs gave data consistent with the hypothesis that these mRNAs are indeed targets for both miR-M4 in MSB1 and miR-155 in human B-cell lymphomas. Four additional mRNA targets (*FCHSD2, JARID2, PBEF1*, and RAP2A) were also conserved between miR-M4 in MSB-1 and

miR-155 in EBV-transformed B cells. Among these mRNA targets, JARID2, a cell cycle regulator and part of a histone methyltransferase complex, has been shown to promote apoptosis and decrease cell survival when ectopically expressed [53]. *WEE1* encodes a kinase that blocks cell-cycle progression and has been associated with inflammation and cancer [73]. MYB, a transcription factor, is involved in the regulation of hematopoiesis and tumorigenesis. Another interesting shared gene target for miR-155/miR-M4/miR-K12-11 is large tumor suppressor (LATS), which inhibits cell proliferation and promotes apoptosis by inhibiting YAP transcriptional activity through Hippo pathway. Being evolutionarily conserved from avian to mammal, loss of LATS expression is observed in many human cancers, including acute lymphoblastic leukemia [74]. These observations suggest a potential selective advantage for MDV-1 in inducing downregulation of LATS. Taken together, these observations provide additional evidence for the impact of miR-155 and its orthologs on pathways regulating lymphocyte activation, differentiation, apoptosis, proliferation, immune tolerance, and tumorigenesis [7].

In addition to the candidate mRNA targets described above, latent TGF-β binding protein 1 (*LTBP1*) has also been identified as bona fide host mRNA targets for miR-M4 [75]. Inhibition of *LTBP1* expression by miR-M4-5p induced a significant decrease of TGF-β1 secretion and activation, with suppression of TGF- β signaling and upregulation of c-Myc expression. Interestingly, miR-155 has been shown to suppress TGF-β signaling through targeting SMAD2 and SMAD5 in human diseases [76, 77]. The KSHV-encoded miR-K12-11 also inhibits TGF-β signaling through downregulation of SMAD5 [78]. Thus, these findings indicate that dysregulation of the TGF-β signaling pathway by miR-155 and its viral orthologs may be a common feature shared by oncogenic herpesviruses. Interestingly, MDV1-encoded miRNA miR-M3 also suppress TGF-β signaling through targeting host gene SMAD2, and has been shown to suppress drug-induced apoptosis in cell culture [79], thus adding another layer of confidence to the conclusion that suppression of TGF-β signaling pathway is indeed involved in MDV-induced oncogenesis.

Another identified cellular target of miR-M4 is chicken heterogeneous nuclear ribonucleoprotein AB (hnRNPAB). HnRNPAB, a member of the hnRNP family proteins, plays important roles in both normal biological processes and cancer development. Downregulation of hnRNPAB expression promotes proliferation of both CEF and chicken fibroblast cell line DF-1 [80]. Hence, downregulation of hnRNPAB by miR-M4-5p may be one of the important strategies for MDV-1 to trigger the development of MD lymphomas.

PAR-CLIP identified a list of 1104 cellular mRNAs targeted by MDV-1 miRNAs and a list of 1183 mRNAs targeted by MDV-2 miRNAs in MSB1, of which 419 mRNA targets were shared. Analysis of those cellular mRNAs identified several that were targeted by five or more different viral miRNAs in their 3′ UTRs, suggestive of a possibly important role in restricting innate antiviral immunity. IL-18, a proinflammatory cytokine induced upon infection by several different viruses, is able to stimulate IFN-γ production from T cells [81]. Surprisingly, chicken IL-18 gene contains seven viral miRNA target sites in its 3′ UTR [64]. Indeed, MSB1 cell growth is highly sensitive to inhibition by ectopic IL-18 expression. Whether this is due to induction of chicken IFN-γ expression remains to be determined. Thus, in addition to express viral interleukin-8 (vIL-8), which is required for disease progression and tumor development, this data suggest a second way in which MDV manipulates the host cell immune response.

Taken together, all of the data above suggests that latent/oncogenic viruses may proactively create a cellular environment beneficial to viral latency and oncogenesis through viral miRNA targeting cellular factors involved in antiviral processes including apoptosis. Surely, more targets of miRNAs encoded by avian viruses will

be identified with the advances in high-throughput technologies. We should be able to understand the role played by these small and highly effective modulators of gene expression once more targets of viral miRNAs are discovered and an integrated approach of demonstrating the functions and molecular pathways is developed.

6. Role of viral miRNAs in pathogenesis

Despite, several possible cancer-related genes have been identified as their targets of viral miRNAs, the direct *in vivo* role of those miRNAs such as KSHV-encoded miRNAs in neoplastic diseases is difficult to demonstrate in the absence of suitable models. However, excellent disease models of infection by MDV in natural avian host allow examination of the oncogenic potential of virus-encoded miRNAs. As described above, it has been shown that the deletion of the Meq-clustered miRNAs from the viral genome by BAC mutagenesis abolished the oncogenicity of the virus. miR-M4 is essential for the virus in inducing tumor as the mutant virus with miR-M4 deletion or seed sequence mutation almost lost its oncogenicity, providing evidence for a direct *in vivo* role of a viral miRNA in tumor induction [34]. In a separate study, deletions of the Meq-cluster or miR-M4 alone from the viral genome of vvMDV strain GX0101 strongly decreased its oncogenicity [35]. Subsequent work demonstrated that except for mdv1-miR-M4, most of the other Meq-clustered miRNAs also play critical roles in MDV oncogenesis as both mortality and gross tumor incidence of birds infected with the mutant viruses have reduced significantly for the corresponding virus with single miRNA deletion [82]. These studies provide further information for understanding the molecular determinants that trigger the development of tumors by oncogenic MDVs.

Having demonstrated the critical roles of Meq-clustered miRNAs in MD pathogenesis and/or tumorigenesis, the role of miRNAs in Mid-cluster, which is transcribed by the same promoter as the Meq-cluster in the latent phase of the infection has been investigated by infection of miRNA deleted virus in animal model [61]. The role of miR-M31-3p acting as oncogene and miR-M11-5p acting as tumor suppressor in MD lymphomagenesis have been proposed based on the decreased mortality and gross tumor incidence by miR-M31 deletion mutant and increased viral pathogenicity or oncogenicity of infected chickens when miR-M11 is deleted.

7. Conclusions

Recent advances in sequencing technology have led to the identification of a number of miRNAs encoded by avian viruses. Given their ability to target cellular and viral transcripts, and the lack of immune response, miRNAs represent an ideal mechanism of gene regulation during viral infection, latency, and persistence. Similar to host miRNAs, the determination of biologically relevant activities of the reported viral miRNAs is the next step forward. Although the function of most viral miRNAs is to be determined, evidence so far does support the view that viral miRNAs are key regulators in virus biology. By targeting key viral lytic genes directly or modulating cellular regulatory pathways indirectly, the virus-encoded miRNAs could contribute significantly toward switching between lytic and latent infections, thereby regulating viral pathogenesis *in vivo*. Whether a small or large number of critical miRNA-target interactions have functional significance is a key question to be answered.

It is crucial to define the miRNA targets for both understanding observed phenotypes and providing clues to their functional role. Genome wide biochemical screening are becoming more powerful approaches for the generation of reliable

and accurate target information of viral miRNAs although target identification using target prediction software provided an initial strategy. Several techniques such as RIP-CHIP (RNA-binding protein immunoprecipitation microarray), HITS-CLIP (high-throughput sequencing cross-linking and immunoprecipitation), PAR-CLIP and proteomics analysis have all contributed large amount of data on potential targets of virus-encoded miRNAs [47, 83–89] although only PAR-CLIP has been used for avian herpesvirus miRNA targetome identification [64]. Cross-linking, ligation and sequencing of hybrids (CLASH) technology, another biochemical screen for miRNA targets, promises to generate the most accurate target information to date, leading the way in the generation of high confidence target datasets which will be invaluable for future studies [90, 91]. These new technologies allow unprecedented and largely unbiased views into miRNAs-mediated regulation of gene expression in virus-infected cells. Undoubtedly, further studies using different approaches and technologies are required toward the clear definition of miRNAs targetome and their functional relevance in viral infection, latency, reactivation, and pathogenesis.

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Conflict of interest

The authors declare no conflict of interest.

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