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Critical roles of non-coding RNAs in lifecycle and biology of Marek's disease herpesvirus

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Over the past two decades, numerous non-coding RNAs (ncRNAs) have been identified in different biological systems including virology, especially in large DNA viruses such as herpesviruses. As a representative oncogenic alphaherpesvirus, Marek's disease virus (MDV) causes an important immunosuppressive and rapid-onset neoplastic disease of poultry, namely Marek's disease (MD). Vaccinations can efficiently prevent the onset of MD lymphomas and other clinical disease, often heralded as the first successful example of vaccination-based control of cancer. MDV infection is also an excellent model for research into virally-induced tumorigenesis. Recently, great progress has been made in understanding the functions of ncRNAs in MD biology. Herein, we give a review of the discovery and identification of MDV-encoded viral miRNAs, focusing on the genomics, expression profiles, and emerging critical roles of MDV-1 miRNAs as oncogenic miRNAs (oncomiRs) or tumor suppressor genes involved in the induction of MD lymphomas. We also described the involvements of host cellular miRNAs, lincRNAs, and circRNAs participating in MDV life cycle, pathogenesis, and/or tumorigenesis. The prospects, strategies, and new techniques such as the CRISPR/Cas9-based gene editing applicable for further investigation into the ncRNA-mediated regulatory mechanisms in MDV pathogenesis/oncogenesis were also discussed, together with the possibilities of future studies on antiviral therapy and the development of new efficient MD vaccines.

herpesvirus, MDV, miRNA, lincRNA, circRNA, CRISPR, pathogenesis, oncogenesis

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Introduction

Marek's disease virus (MDV) is an important oncogenic avian herpesvirus that causes a highly contagious lymphoproliferative disorder and neoplastic disease in poultry, commonly known as Marek's disease (MD) (Nair et al., 2020). As one of the most important immunosuppressive and

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neoplastic diseases, MD has continuously caused huge economic losses of more than 1 billion US dollars annually to the poultry industry worldwide (Kennedy et al., 2017). Thus, for the control of the disease, more than 100 billion chickens worldwide need to be vaccinated before or immediately after hatching, using avirulent or attenuated MD vaccines to avoid early infection of MDV. Grouped within the genus Mardivirus, MD-associated avian herpesviruses include three types: MDV type 1 (MDV-1) or *Gallid alphaherpesvirus* 2 (GaAHV-2), MDV type 2 (MDV-2) or *Gallid alphaherpesvirus* 3 (GaAHV-3), and herpesvirus of turkey (HVT) or *Meleagrid alphaherpesvirus* 1 (MeAHV-1) (Gatherer et al., 2021). Among these, only the virulent MDV-1 strains are pathogenic and oncogenic to the hosts, while the MDV-2 and HVT are nonpathogenic. In the past 50 years, the virulence of MDV has persistently increased, thought to be due to multiple reasons, including continuous and high immune pressure from the large-scale application of MD vaccines in the poultry industry, the move toward intensive poultry production systems, and genetic selection of birds for increased productivity. Based on the increasing virulence resulting in higher mortality of infected birds, lesion frequency, and immune protection by the different commercial MD vaccines, MDV-1 isolates have been further grouped into four pathotypes: mild MDV (mMDV), virulent MDV (vMDV), very virulent MDV (vvMDV), and very virulent plus MDV (vv+MDV) (Witter, 1997). The emergence and prevalence of highly virulent epidemic strains such as vv+MDV and other variant MDVs in chicken flocks, accompanied with the coinfections of other oncogenic avian pathogens, provided new challenges for efficient disease control in the future (Deng et al., 2021; Song et al., 2022; Teng et al., 2022a; Zheng et al., 2022).

As one of the most virulent oncogenic herpesviruses known, the pathogenic MDV-1 strains can establish and maintain latent infection in its natural hosts to induce rapid-onset aggressive T-cell lymphomas. The induction of MD tumors can successfully be prevented by immunization using attenuated MDV-1 or nonpathogenic MDV-2 and/or HVT vaccines, representing the first example of efficient anticancer vaccines. Historically, MD has also been regarded as an excellent model for studying the biology of virally-induced cancers (Osterrieder et al., 2006). For a long time, revealing the underlying molecular mechanism of MD tumorigenesis has been an attractive topic for scientists. It is worth remembering that the first ever understanding of the causal relationship between tumor and virus came from another avian alpharetrovirus, Rous sarcoma virus (RSV), the first virus identified to cause solid tumors in animals (Coffin et al., 1997). Studies on RSV have opened up a new field to reveal the virally-induced tumorigenesis, which is important for understanding the causes of tumors and how normal cells are transformed into tumor cells. Retroviruses only encode a limited set of genes with a canonical viral RNA genome structure as 5′-R-U5-PBS-gag-pro-pol-env-PPT-U3-R-3′. However, following virus replication, the synthesized viral double-stranded DNA can integrate into host cell chromosomes to produce a provirus (Lesbats et al., 2016). Interestingly, some retroviruses, such as certain strains of RSV, may carry host cellular sequences inserted into the complete retrovirus genome, which play an important role as oncogenes in carcinogenesis (Coffin et al., 1997; Maeda et al., 2008). However, different from retroviruses, herpesviruses are large DNA viruses encoding hundreds of genes in their genomes, with some of these genes capable of functioning as oncogenes. MDV belongs to the subfamily of *Alphaherpesvirinae*, but its biological properties are similar to those of *Gammaherpesvirinae* such as Epstein-Barr virus (EBV), herpesvirus saimiri (HVS), and Kaposi's sarcoma herpesvirus (KSHV) (Osterrieder et al., 2006). Among the hundreds of genes in the viral genome, the MDV-1-specific gene *meq* (MDV EcoRI-Q) encodes a basic leucine zipper (bZIP) protein Meq. Because of its homology with oncoproteins known as the Fos/Jun family and the demonstration of loss of T-cell transformation ability in chicken hosts after its deletion, *meq* has been characterized as the major oncogene for MDV tumorigenesis (Lupiani et al., 2004; Nair, 2013). Further studies have also shown that other genes such as *pp38*, *vIL-8*, *ICP4*, and *vTR* may also play important roles in MDV replication, latent infection, and/or oncogenesis. In spite of these, the fundamental molecular mechanisms and determinants that trigger the development of MD lymphomas remain unknown.

The first two decades of 21st century saw the discovery of a series of new types of regulatory non-coding RNAs (ncRNAs), including microRNAs (miRNAs), long intergenic ncRNAs (lincRNAs), and circular RNAs (circRNAs) in mammals, vertebrates, plants, and even in viruses, which have revealed a novel regulatory mechanism with abundant RNAs exerting unimaginable functions in most aspects of cellular processes, such as cell growth, development, differentiation, apoptosis, epigenetic regulation, and most aspects of cancer biology (Chen and Xue, 2016; Liu et al., 2020; Lu et al., 2020; Saw et al., 2021; Xiong et al., 2021; Xue et al., 2020). Surprisingly, for viruses particularly those with large DNA genomes such as herpesviruses, hundreds of virus-encoded ncRNAs have been identified, and some of them have been demonstrated to play critical roles in virology, providing a new perspective for elucidating virus pathogenesis and oncogenesis (Boss et al., 2009; Sorel and Dewals, 2016; Tycowski et al., 2015). For the two types of MDV and HVT viruses, some small ncRNAs, e.g., miRNAs, were first identified in the viral genomes with crucial roles in regulating virus replication, latency, pathogenesis, and/or oncogenesis (Hicks and Liu, 2013; Luo et al., 2010). Recently, other ncRNAs, such as lincRNAs and circRNAs,

have also been found to participate in MD biology. For a better exploration in future work, we have presently reviewed the critical regulatory roles of both viral and cellular ncRNAs in MD pathogenesis/oncogenesis. The great achievements on ncRNA-mediated functions and molecular mechanisms have presented a new perspective to understand the pathogenesis and tumorigenesis of oncogenic avian herpesvirus.

Virus-encoded miRNAs in MDV biology

Genomic organization of MDV-encoded miRNAs

Since the first report on MDV-derived miRNAs in 2006 (Burnside et al., 2006), a large number of viral miRNAs have been identified in MDV and HVT genomes, including 26 MDV-1 miRNAs encoded from 14 precursors (pre-miRNA), 36 MDV-2 miRNAs from 18 pre-miRNAs, and 28 HVT miRNAs from 17 pre-miRNAs (Burnside et al., 2006; Burnside et al., 2008; Waidner et al., 2009; Yao et al., 2009; Yao et al., 2007; Yao et al., 2008). MDV genome is a linear double-strand DNA composed of a unique long region (U_L), a unique short region (U_S), and two inverted repeat regions on both sides of the U_L/U_S regions, known as terminal and internal repeat long regions (TR_L/IR_L) and terminal and internal repeat short regions (TR_S/IR_S) (Biggs and Nair, 2012). The genes in the unique U_L/U_S regions of herpesviruses are generally highly conserved. In contrast, the MDV-specific genes are mainly encoded in TR/IR. Interestingly, although the MDV miRNA sequences among all three types of viruses showed significant variations, their genomic loci are highly conserved within the TR/IR regions to form typical miRNA clusters, as reported previously (Luo et al., 2010).

Among all the three types of viruses, only MDV-1 is pathogenic and oncogenic; hence, the structure and function of MDV-1 miRNAs are of particular interest to the researchers. In MDV-1 viral genomes, as listed in Table 1, a total of 14 pre-miRNAs encodings 26 mature miRNAs are distributed in three clusters, named the Meq-cluster (Burnside et al., 2006), the Mid-cluster (Luo et al., 2010; Luo et al., 2011), and the LAT-cluster (Burnside et al., 2006), in sequence according to their related genomic locations (Figure 1), which are also designated as clusters 1, 2, and 3 in following studies (Yao and Nair, 2014; Zhao et al., 2011). The Meq-cluster, including pre-miRNAs mdv1-mir-M9 (abbreviated as mir-M9), mir-M5, mir-M12, mir-M3, mir-M2, and mir-M4, are antisense to the R-LORF8 transcript and adjacent to the upstream of MDV-1 oncogene *meq*. The LAT-cluster (mir-M8, mir-M13, mir-M6, mir-M7, and mir-M10) are mainly located in the large intron of the latency-associated transcript (LAT). The other three pre-miRNAs (mir-M11, mir-M31, and mir-M1) from the Mid-cluster are named from their middle location between the Meq and the LAT clusters.

Transcription and expression of MDV-encoded miRNAs

Very little is known about the transcription and expression patterns of MDV-2 and HVT miRNAs. However, due to the virulent and oncogenic properties of the MDV-1 virus, expression profiles of MDV-1 miRNAs are more attractive for the deep analysis of their biological functions. Many scientists have focused on both the *in vitro* and *in vivo* expression profiles and transcription patterns of MDV-1 miRNAs, which have provided important clues for further evaluating the potential roles of those miRNAs in the life cycle, pathogenesis and/or oncogenesis of MDV.

Transcription patterns of MDV-1 miRNAs

In the MDV genome, the Meq-cluster and Mid-cluster of MDV-1 miRNAs are focused within a 3.6-kb area in the IR_L/TR_L regions, spaced by the viral oncogene *meq*. The Meq-cluster miRNAs are located at 5′-end in the first intron of an unspliced transcript or the same origin of alternatively spliced transcripts covering the IR_L/TR_L regions, encompassing the *meq* and *vIL-8* genes. Interestingly, it has been demonstrated that the transcriptions of both Meq- and Mid-clusters are promoted under two distinct patterns: (i) during the latent phase, the prmiRM9M4 promoter drives transcription of the meq-Mid-cluster-vIL-8 genes and the Meq-cluster in the first intron of the corresponding transcripts and (ii) during the lytic phase it drives the transcription of the Meq-cluster only to generate unspliced mRNA, the meq-Mid-cluster-vIL-8 genes being transcribed principally from their promoters (Coupeau et al., 2012). The expression of viral miRNAs in the Meq-cluster and the Mid-cluster under two different transcriptional patterns during the latent and lytic phases of disease implies their potential differential roles in MD biology.

The LAT-cluster of MDV-1 miRNAs is focused within a 490-bp area near the 5′-end of a large intron in the LAT within the IR_S/TR_S regions. P53 promotes the primary transcription of the LAT-cluster through targeting the p53-responsive elements (RE), a variable number of 60-bp repeats existed in the transcription-inducing sequence near upstream of the LAT-cluster (Stik et al., 2010). No consensus core element was found in the promoter, but the minimal number of artificial functional RE is two. Interestingly, all the known virulent strains of MDV-1 possess at least two tandem repeats harboring the p53 RE. In contrast, all avirulent MDV-1 strains have been reported to lack such a regulatory sequence. *In vitro* experiments have shown that the p53 RE mutagenesis will abolish its transcriptional activity, while the induction of p53 enhances the expression of the LAT-clustered miRNAs (Stik et al., 2010).

Expression profiles of MDV-1 miRNAs

In several early reports (Burnside et al., 2006; Morgan et al.,

Table 1 MDV-1-encoded precursors and mature miRNAs

No.	Precursor [a]	Genomic location [b]	No.	miRNA [a]	Acc. No. [c]	miRNA sequences (5′–3′)	Length (nt)	Identification method [d]	Original signatures [Refs.]
01	mdv1-mir-M1	136,864–136,939 [+] 4,681–4,756 [−]	01	mdv1-miR-M1-5p	MIMAT0003920	UGCUUGUUCACU-GUGCGGCA	20	EX, CL, NB, SL	MDV-miR-1[e]; mdv1-miR-M1[f,g]
			02	mdv1-miR-M1-3p	MIMAT0005801	UGCUGCGCAUGAAA-GAGCGA	20	EX, CL, NB, SL	mdv1-miR-M1*[g]
02	mdv1-mir-M2	134,223–134,296 [+] 7,324–7,397 [−]	03	mdv1-miR-M2-5p	MIMAT0003921	GUUGUAUU-CUGCCCGGUAGUCCG	23	EX, CL, NB, SL	MDV-miR-2[e]; mdv1-miR-M2[f]; mdv1-miR-M2/5p[g]
			04	mdv1-miR-M2-3p	MIMAT0003922	CGGACUGCCGCA-GAAUAGCUU	21	EX, CL, NB, SL	MDV-miR-2*[e]; mdv1-miR-M2*[f]; mdv1-miR-M2*/3p[g]
03	mdv1-mir-M3	134,075–134,144 [+] 7,476–7,545 [−]	05	mdv1-miR-M3-5p	MIMAT0003923	AUGAAAAUGU-GAAACCUCUCCCGC	24	EX, CL, NB, SL	MDV-miR-3[e]; mdv1-miR-M3[f]
			06	mdv1-miR-M3-3p	MIMAT0009207	UGGGGGGUUCA-CAUUUUUAAGU	22	EX, SL	
04	mdv1-mir-M4	134,363–134,428 [+] 7,192–7,257 [−]	07	mdv1-miR-M4-5p	MIMAT0003924	UUAAUGCUGUAUCG-GAACCCUUC	23	EX, CL, NB, SL	MDV-miR-4[e]; mdv1-miR-M4[f,g]
			08	mdv1-miR-M4-3p	MIMAT0003925	AAUGGUUCUGACAG-CAUGACC	21	EX, CL, NB, SL	MDV-miR-4*[e]; mdv1-miR-M4*[f,g]
05	mdv1-mir-M5	133,601–133,675 [+] 7,945–8,019 [−]	09	mdv1-miR-M5-5p	MIMAT0005802	AACCGUAUGCGAU-CACAUUGAC	22	EX, CL, NB, SL	mdv1-miR-M5*[h]; mdv1-miR-M5*/5p[h]
			10	mdv1-miR-M5-3p	MIMAT0003926	UGUGUAUCGUGGUC-GUCUACUGU	23	EX, CL, NB, SL	MDV-miR-5[e]; mdv1-miR-M5[f]; mdv1-miR-M5/3p[g]
06	mdv1-mir-M6	142,321–142,440 [+] 176,004–176,123 [−]	11	mdv1-miR-M6-5p	MIMAT0005803	UCUGUUGUUCCGUA-GUGUUCUC	22	EX, CL, NB, SL	mdv1-miR-M6*[f]; mdv1-miR-M6*/5p[g]
			12	mdv1-miR-M6-3p	MIMAT0003927	GAGAUCCCUGC-GAAAUGACAGU	22	EX, CL, NB, SL	MDV-miR-6[e]; mdv1-miR-M6[f]; mdv1-miR-M6/3p[g]
07	mdv1-mir-M7	142,501–142,620 [+] 175,824–175,943 [−]	13	mdv1-miR-M7-5p	MIMAT0005804	UGUUAUCUCGGGGA-GAUCCCGAU	23	EX, CL, NB, SL	mdv1-miR-M7*/5p[g]
			14	mdv1-miR-M7-3p	MIMAT0003928	UCGAGAUCUCUAC-GAGAUUACAG	23	EX, CL, NB, SL	MDV-miR-7[e]; mdv1-miR-M7[f]; mdv1-miR-M7/3p[g]
08	mdv1-mir-M8	142,201–142,320 [+]	15	mdv1-miR-M8-5p	MIMAT0005805	UAUUGUUCUGUG-GUUGGUUUCG	22	EX, CL, NB, SL	mdv1-miR-M8*[f]; mdv1-miR-M8*/5p[g]
			16	mdv1-miR-M8-3p	MIMAT0003929	GUGACCUCUACG-GAACAAUAGU	22	EX, CL, NB, SL	MDV-miR-8[e]; mdv1-miR-M8[f]; mdv1-miR-M8/3p[g]
09	mdv1-mir-M9	133,369–133,438 [+] 8,182–8,251 [−]	17	mdv1-miR-M9-5p	MIMAT0005979	UUUUCUC-CUUCCCCCCGGAGUU	22	EX, CL, NB, SL	
			18	mdv1-miR-M9-3p	MIMAT0005980	AAACUCCGAGGG-CAGGAAAAAG	22	EX, CL, NB, SL	
10	mdv1-mir-M10	142,625–142,693 [+] 175,751–175,819 [−]	19	mdv1-miR-M10-5p	MIMAT0007294	GCGUUGUCUCGUA-GAGGUCCAG	22	EX, CL, SL	mdv1-miR-M10[f]
			20	mdv1-miR-M10-3p	MIMAT0005981	UCGAAAUCUCUAC-GAGAUAACA	22	EX, CL, NB, SL	mdv1-miR-M10*[f]; mdv1-miR-M10*/3p[g]
11	mdv1-mir-M11	136,050–136,114 [+] 5,506–5,570 [−]	21	mdv1-miR-M11-5p	MIMAT0005982	UUUUCCUUACCGU-GUAGCUUAGA	23	EX, CL, NB	
			22	mdv1-miR-M11-3p	MIMAT0005983	UGAGUUACAUGGU-CAGGGGAUU	22	EX, NB	
12	mdv1-mir-M12	133,888–133,949 [+] 7,671–7,732 [−]	23	mdv1-miR-M12-5p	MIMAT0009209	AGGCCCUCC-GUAUAAUGUAAAU-GU	24	EX, SL	
			24	mdv1-miR-M12-3p	MIMAT0005984	UGCAUAAUACG-GAGGGUUCU	20	EX, CL, NB, SL	mdv1-miR-M11[f]
13	mdv1-mir-M13	176,107–176,167 [−]	25	mdv1-miR-M13-3p	MIMAT0005985	GCAUGGAAACGUC-CUGGGAAA	21	EX, NB	
14	mdv1-mir-M31	136,539–136,607 [+] 5,013–5,081 [−]	26	mdv1-miR-M31-3p	MIMAT0007893	UGCUACAGUCGU-GAGCAGAUCAA	23	EX, NB, SL	mdv1-miR-M12[f]; mdv1-miR-M32[g]; mdv1-miR-M32-3p[i,j]

[a] Unified denotations of MDV-1 miRNAs and precursors are given according to the 36 iRbasee version 22.1 released in 2019. [b] Genomic loci of MDV-1 miRNAs based on the viral genome of Md5 (GenBank Acc No. AF243438). The sense and antisense strands of miRNA precursors are shown by plus or minus signs in square brackets, respectively. [c] Accession numbers dispatched to MDV-1 miRNAs in the database of 36 iRbasee v22.1, released in 2019 (http://www.mirbase.org/cgi-bin/mirna_summary.pl?org=mdv1). [d] EX, experimental method; CL, cloned; NB, Northern blot; SL, Solexa sequencing. [e] Burnside et al., 2006; [f] Burnside et al., 2008; [g] Morgan et al., 2008; [h] Yao et al., 2008; [i] Luo et al., 2010; [j] Luo et al., 2011.

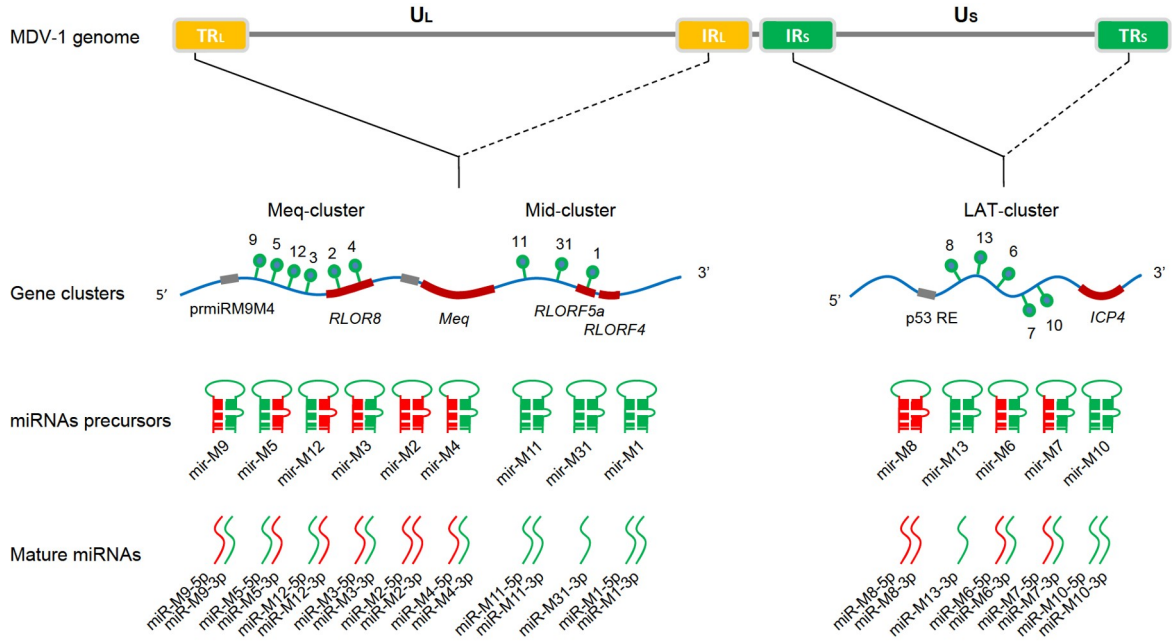

Figure 1 Genomic loci and distribution of MDV-1 miRNA and gene clusters in the viral genomes. The genome of MDV is composed of a U_L and a U_S segment, flanked by inverted repeats known as TR_L/IR_L and TR_S/IR_S. The relative locations of MDV-1 miRNAs at two identical genomic loci in inverted repeats TR_L/IR_L and IR_S/TR_S are shown by solid or dashed lines. The viral protein-coding genes, such as *RLORF8*, *meq*, and *ICP4*, are indicated in bold by red curves. The promoters prmiRM9M4, prMeq, and p53 RE are shown in bold by gray curves. The miRNA precursors are shown in green bonbons. Double strands of high or low expressed mature miRNAs are shown by red or green curves, and signatures of mature miRNAs are abbreviated, e.g., mdv1-miR-M4-5p is shortened as miR-M4-5p.

2008; Yao et al., 2008), the expression levels of some MDV-1 miRNAs were primarily determined in MDV-transformed MSB-1 cells, virus-infected chicken embryo fibroblasts (CEF) cells, and MD lymphomas using Northern blot analysis. Interestingly, various expression profiles were observed among the MDV-1 miRNAs, both *in vitro* and *in vivo*. An overall and detailed summary of the relative expression levels of MDV-1 miRNAs is shown in Table 2. Relatively, most of the mature miRNAs in the Meq-cluster include miR-M3-5p, miR-M4-5p, miR-M12-3p, miR-M2-5p/3p, and miR-M9-5p have displayed higher expression levels than those of the Mid-clustered miRNAs both *in vivo* and *in vitro*, especially for the miR-M11-5p/3p. Some Meq-clustered miRNAs are expressed at higher levels in MD lymphomas produced by a vv+MDV strain 615K than those caused by a vvMDV strain RB-1B, whereas the expression of LAT-clustered miRNAs remained similar (Morgan et al., 2008). The significant differentially expressed miRNA profiles in MD lymphomas induced by oncogenic virus strains with differing virulence imply that the Meq-cluster miRNAs may have a more critical role in MDV oncogenesis. Utilizing reverse transcription and real-time quantitative PCR (RT-qPCR), expression profiles of all MDV-1 miRNAs have been further systematically investigated in MDV-1-challenged birds during the virus life cycle at each phase of the disease development (Zhao et al., 2015). According to the *in vivo* dynamic expression profiles, MDV-1 miRNAs can be classified into two categories: early- or late-expressed miRNAs, accompanied by obvious tissue-specific and differential expression patterns (Luo et al., 2011). The viral miRNAs grouped may be involved in the same processes, such as the early cytolytic, latent, late cytolytic, and/or proliferative phases in the MDV's life cycle, providing meaningful clues for further studying the potential functions in MD biology.

Critical regulatory roles and functions of MDV-1 miRNAs

In the past decade, a series of reconstituted MDV-1 mutants with miRNA-deletions and revertant viruses have been constructed for evaluating the potential role in MDV pathogenesis and oncogenesis by using the bacterial artificial chromosomes (BAC) and Rec E/T homologous recombinant techniques. The deletion of the Meq-cluster miRNAs significantly decreased or almost abolished the pathogenicity and oncogenesis of vMDV and vvMDV strains (Teng et al., 2015; Yu et al., 2014; Zhao et al., 2011). However, it is surprising that viruses' pathogenicity and oncogenicity were significantly enhanced once the Mid-cluster miRNAs were omitted from the viral genomes (Teng et al., 2017). Different from the Meq and Mid-clusters, knocking out the LAT-cluster miRNAs resulted in more severe atrophy of lymphoid organs and reduced mean death time of the virus-challenged birds but did not affect the incidence of MDV-associated visceral tumors (Liao et al., 2020; Sun et al., 2021a). These results suggested that the viral miRNAs from distinct clusters may play various potential roles in MDV pathogenesis

Table 2 *In vivo* and *in vitro* relative expression levels of MDV-1 miRNAs determined by Northern blot analysis

miRNAs	*In vivo*									*In vitro*				
	Expression details in splenic tumors from birds infected with GX0101[a]				Expression levels of mature miRNAs in splenic tumors caused by distinct MDV-1 strains[b]					MSB-1[c,d]		RB1B-CEFs[c,d]		Relative expression levels[c,d]
	Pre-miR (Y/N)	Length (nt)	Mat-miR (Y/N)	Length (nt)	GX0101[e]	RB1B[c]	RB1B[d]	RB1B[f]	615K[f]	Pre-miR (Y/N)	Mat-miR (Y/N)	Pre-miR (Y/N)	Mat-miR (Y/N)	
(a) Meq-cluster														
miR-M3-5p	Y	65–70	Y	22–23	H	M	H	H	H	Y	Y	N	Y	H
miR-M4-5p	Y	65–70	Y	22–23	H	H	H	H	H	Y	Y	N	Y	H
miR-M12-3p	Y	60–65	Y	22–23	H	–	H	H	H	Y	Y	N	Y	H
miR-M2-5p	Y	65–70	Y	21–22	M	H	M	H	H	Y	Y	N	Y	H
miR-M2-3p	Y	65–70	Y	21–22	M	–	M	M	H	Y	Y	N	Y	H
miR-M9-5p	Y	60–65	Y	21–22	M	–	H	–	–	Y	Y	Y	Y	H
miR-M4-3p	Y	65–70	N	–	N	–	L	–	–	Y	Y	N	N	L
miR-M5-5p	Y	65–70	N	–	N	–	L	–	–	Y	Y	Y	N	L
miR-M5-3p	Y	65–70	N	–	N	M	H	M	H	Y	Y	N	N	H
miR-M9-3p	Y	60–65	N	–	N	–	L	–	–	Y	Y	Y	N	L
miR-M3-3p	–	–	–	–	–	–	–	–	–	–	–	–	–	–
miR-M12-5p	–	–	–	–	–	–	–	–	–	–	–	–	–	–
(b) Mid-cluster														
miR-M1-5p	Y	65–70	Y	22–23	L	L	–	H	H	Y	Y	Y	Y	L
miR-M31-3p	Y	60–65	Y	21–22	L	–	–	–	–	–	–	–	–	–
miR-M1-3p	Y	65–70	N	–	N	–	N	–	–	Y	Y	Y	N	L
miR-M11-5p	Y	65–68	N	–	N	–	N	–	–	Y	Y	Y	N	L
miR-M11-3p	Y	65–68	N	–	N	–	N	–	–	Y	Y	Y	N	L
(c) LAT-cluster														
miR-M7-5p	Y	68–70	Y	21–22	H	–	H	H	H	Y	Y	N	Y	H
miR-M8-3p	Y	65–68	Y	21–22	H	H	–	H	H	Y	Y	–	Y	H
miR-M6-5p	Y	110–120	Y	21–22	M	–	H	H	H	Y	Y	Y	Y	H
miR-M10-3p	Y	65–68	Y	21–22	M	–	N	–	–	Y	Y	N	N	L
miR-M6-3p	Y	110–120	N	–	N	L	–	L	L	Y	Y	–	Y	L
miR-M8-5p	Y	65–68	N	–	N	–	H	M	M	Y	Y	Y	Y	H
miR-M13-3p	Y	60–65	N	–	N	–	L	–	–	Y	Y	Y	N	L
miR-M7-3p	–	–	–	–	–	–	–	L	L	Y	N	–	Y	L

[a] The expressed or unexpressed precursor miRNAs (pre-miR) and mature miRNA (mat-miR) are shown as detectable (Yes, Y) or undetectable (No, N), respectively. [b] Descriptions of high (H), moderate (M) or low (L) are used to distinguish the relative expression levels of mature miRNAs that are compared to each other in independent reports, such as the refs. [c d e f] annotated on top-right corners. [c] Burnside et al., 2006; [d] Yao et al., 2008; [e] Luo et al., 2011; [f] Morgan et al., 2008; "–", data unavailable.

and tumorigenesis. For a better understanding of the possible role of MDV-1 miRNAs, a summary of viral miRNA targets, associated signaling pathways, and potential functions are listed in Table 3.

Regulatory roles of MDV-1 miRNAs in MD oncogenesis

Based on the higher expression profiles in MD tumors and virus-transformed lymphoma cell lines, the Meq-cluster miRNAs have been regarded as the more important regulators in MD oncogenesis. The mdv1-miR-M4-5p (abbreviated as miR-M4-5p), one of the highest expressed MDV-1 miRNAs, has been characterized as a viral ortholog of the cellular oncogenic microRNA-155 (miR-155) and plays a critical role in the development of MD lymphomas (Zhao et al., 2009; Zhuang et al., 2017). The miR-M4-5p was separately removed from the viral genome of vMDV strain RB-1B and vvMDV strain GX0101. Compared to parental viruses, the RB-1B mutant completely lost the ability to induce MD lymphomas, and the incidence of tumors in GX0101 mutant virus-infected birds were also significantly decreased (Yu et al., 2014; Zhao et al., 2011). These data suggested that miR-M4-5p is not only the essential gene responsible for MDV oncogenesis but also a key regulator in the development of MD lymphomas. Up until now, we have known that miR-M4-5p targets and down-regulates both the viral and host genes, such as the viral protein-coding genes *UL28* and *UL32* involved in the cleavage and packaging of herpesvirus DNA, and the host genes *GPM6B*, *RREB1*, *c-Myb*, *MAP3-K7IP2*, *PU.1*, *C/EBP*, *LTBP1*, *hnRNPAB*, *RORA*, *WWOX*, etc (Chi et al., 2015; Dang et al., 2017; Ding et al., 2020; Muylkens et al., 2010; Zhu et al., 2021b). In addition to miR-M4-5p, deletions of other pre-miRNAs in Meq-cluster (i.e., mir-M2, mir-M3, mir-M5, mir-M9, and mir-M12) similarly reduced the pathogenicity and oncogenicity of MDV-1 in varying degrees (Teng et al., 2015). The miR-M3-5p, highly expressed viral miRNA in the Meq-cluster, was found to directly target and downregulate SMAD2 involved in several antiviral processes, including apoptosis, proactively creating a cellular environment beneficial for viral latency and oncogenesis (Xu et al., 2011). In a recent study (Zhu et al., 2020), the miR-M2-5p was demonstrated to target two tumor suppressors, *RBM24* and *MYOD1*, promoting cell proliferation by regulating RBM24-mediated p63 overexpression and MYOD1-mediated IGF2 signaling. It also suppresses cell apoptosis by targeting the MYOD1-mediated Caspase-3 signaling pathway.

MDV-1 miRNAs encoded in the Mid-cluster also play critical regulatory roles in MDV oncogenesis since knockout of the Mid-cluster miRNAs has shown dramatic changes in the biological features of MD. The absence of miR-M1-5p and miR-M11-5p/3p resulted in a significant increase in mortality and tumor occurrence, which implies a putative role of tumor suppressors in MD, primarily confirmed by the data that miR-M11-5p can target and downregulate the expression of MDV oncogene meq during virus infection (Teng et al., 2017). Different from the precursors mir-M1 and mir-M11, the deletion of mir-M31 from the Mid-cluster decreased the pathogenicity and oncogenicity of the virus. Previous works (Fornari et al., 2008; Galardi et al., 2007; Lambeth et al., 2009) have shown that the host cellular miRNAs, miR-221 and miR-222, target and suppress a key cell cycle inhibitory regulator protein $p27^{Kip1}$ to promote the growth and proliferation of tumor cells. The mir-M31-produced miR-M31-3p was found to share a conserved seed sequence with miR-221 (Morgan et al., 2008), which may be a good explanation for the potential role as an oncogene similar to the Meq-cluster miRNAs.

Involvements of MDV-1 miRNAs in MDV replication and latency

The LAT-cluster miRNAs located in the first intron of LAT genes were transcribed and processed together with LATs, which are abundantly expressed in the latent stage of virus infection, transformed cell lines, and tumors. Recently, in virus-infected chickens, it has been reported that deletion of the LAT-cluster miRNAs enhanced the early cytolytic replication and pathogenesis of MDV (Liao et al., 2020; Sun et al., 2021a). A previous study has revealed that the miR-M7-5p encoded in LAT-cluster was related to the establishment and maintenance of MDV latent infection by targeting and suppressing the expression of immediate early genes *ICP4* and *ICP27* (Strassheim et al., 2012). Distinct from miR-M7-5p, the other two miRNAs, miR-M6-5p and miR-M10-5p in the LAT-cluster, were reported to be dispensable for virus replication and pathogenesis in birds, but miR-M6-5p can suppress MDV replication in cell culture (Yang et al., 2021).

The other miRNAs in the Meq and the Mid-clusters may also be involved in virus replication and/or latency. Utilizing the clustered regularly interspaced short palindromic repeat (CRISPR) and CRISPR-associated protein 9 (Cas9)-based gene editing techniques, a series of miRNA-knockout mutants have been generated from the vvMDV strain RB-1B (Luo et al., 2020). Unexpectedly, the mutagenesis of targeted miRNAs from the Meq or the Mid-clusters conversely changes the *in vitro* virus growth kinetics. Compared to the parental RB-1B virus, the growth kinetics of CRISPR/Cas9-edited viruses in CEF cells have shown that knocking out of the Meq-cluster miRNAs significantly decreased the virus replication. In contrast, the deletion of the Mid-cluster miRNAs enhanced the virus replication (Luo et al., 2020). It is consistent with that of the *in vivo* virus proliferation patterns and the changed pathogenicity and oncogenicity of GX0101 mutants with deletions of the corresponding Meq or Mid-miRNAs, previously produced by BAC mutagenesis and Rec E/T homologous recombination techniques (Teng et al., 2015; Teng et al., 2017; Yu et al., 2014). Recently, MDV-

Table 3 Targets and potential functions of the viral and cellular miRNAs involved in MD biology

Category	miRNA cluster	Precursors	miRNAs	Targets [a]	Potential roles and functions	Refs.
Viral miRNAs	Meq-cluster	mir-M9	miR-M9-5p	UD	Promote tumorigenesis	Teng et al., 2015
			miR-M9-3p	UD		
		mir-M5	miR-M5-5p	UD	Promote tumorigenesis	Teng et al., 2015
			miR-M5-3p	*ICP22*		
		mir-M12	miR-M12-5p	UD	Promote tumorigenesis	Teng et al., 2015
			miR-M12-3p	UD		
		mir-M3	miR-M3-5p	*SMAD2*	Inhibit cisplatin-induced apoptosis; Promote tumorigenesis	Teng et al., 2015; Xu et al., 2011
			miR-M3-3p	UD		
		mir-M2	miR-M2-5p	*RBM24*, *MYOD1*	Promote cell proliferation and inhibit cell apoptosis to promote tumorigenesis	Teng et al., 2015; Zhu et al., 2020
			miR-M2-3p	UD		
		mir-M4	miR-M4-5p	*GPM6B*, *RREB1*, *c-Myb*, *MAP3-K7IP2*, *PU.1*, *C/EBP*, *LTBP1*, *hnRNPAB*, *ICP22*, *RORA*, *WWOX*	Promote cell proliferation and tumorigenesis	Chi et al., 2015; Dang et al., 2017; Ding et al., 2020; Muylkens et al., 2010; Zhao et al., 2009; Zhu et al., 2021b; Zhuang et al., 2017
			miR-M4-3p	*UL28*, *UL32*		Muylkens et al., 2010
	Mid-cluster	mir-M11	miR-M11-5p	*meq*	Downregulate the expression of meq and inhibit tumorigenesis	Teng et al., 2017
			miR-M11-3p	UD		
		mir-M31	miR-M31-3p	UD	Promote tumorigenesis	Morgan et al., 2008; Teng et al., 2017
		mir-M1	miR-M1-5p	*ICP22*	Inhibit virus replication and tumorigenesis	Boumart et al., 2018
			miR-M1-3p	UD		
	LAT-cluster	mir-M8	miR-M8-5p	UD	Dispensable for virus replication and pathogenesis	Yang et al., 2021
			miR-M8-3p	UD		
		mir-M13	miR-M13-3p	UD		
		mir-M6	miR-M6-5p	UD		
			miR-M6-3p	UD		
		mir-M7	miR-M7-5p	*ICP4*/*ICP27*	Repress virus replication and establish/maintain latency	Strassheim et al., 2012; Sun et al., 2021a
			miR-M7-3p	UD		
		mir-M10	miR-M10-5p	UD	Dispensable with replication and pathogenesis	Yang et al., 2021
			miR-M10-3p	UD		
Cellular miRNAs	NA	gga-miR-26a	gga-miR-26a-5p	*NEK6*	Inhibits lymphoma cell proliferation	Li et al., 2014
		gga-miR-181a	gga-miR-181a-5p	*MYBL1*, *ANP32A*	Inhibits lymphoma cell proliferation	Li et al., 2021; Lian et al., 2015a
		gga-mir-103	gga-miR-103-3p	*CCNE1*, *TFDP2*	Inhibits MSB1 cell migration	Han et al., 2016a
		gga-miR-130a	gga-miR-130a-3p	*HOXA3*, *MDFIC*	Inhibits lymphoma cell proliferation and migration	Han et al., 2016b
		gga-miR-219b	gga-miR-219b	*BCL11B*, *MMP2*, *MMP9*	Inhibits MSB1 cell proliferation, migration and invasion	Zhao et al., 2017
		gga-miR-130b	gga-miR-130b-3p	*MMP2*, *MMP9*	Inhibits MSB1 cell proliferation, migration and invasion	Zhao et al., 2018
		gga-mir-1a-2	gga-miR-1a-3p	*PAX3*	Induces cell apoptosis	Zhu et al., 2020
		gga-miR-206	gga-miR-206	*PAX3*	Induces cell apoptosis	Zhu et al., 2020
		gga-miR-223	gga-miR-223	*IGF2*	Inhibits cell proliferation	Zhu et al., 2020
		gga-miR-199	gga-miR-199-3p	UD	Inhibits MSB1 cell proliferation	Lian et al., 2015b
		gga-miR-140	gga-miR-140-3p	UD	Inhibits MSB1 cell proliferation	Lian et al., 2015b
		gga-miR-221	gga-miR-221-5p	UD	Inhibits MSB1 cell proliferation	Lian et al., 2015b
			gga-miR-221-3p	*p27Kip1*	Promote MSB1 cell proliferation	Lambeth et al., 2009
		gga-miR-155	gga-miR-155	*RORA*, *GATA4*	Increases MSB-1 proliferation, reduces cell apoptosis and increases invasiveness; mediates immune responses	Ding et al., 2020; Kang et al., 2015
		miR-222-2	gga-miR-222	*p27Kip1*	Promote MSB1 cell proliferation	Lambeth et al., 2009
		gga-miR-21	gga-miR-21-5p	*PDCD4*	Promotes tumor cell growth and apoptosis escape	Stik et al., 2013

[a] *ICP22*, infected cell protein 22; *SMAD2*, SMAD family member 2; *RBM24*, RNA-binding protein 24; *MYOD1*, myogenic differentiation 1; *GPM6B*, glycoprotein M6B; *RREB1*, ras responsive element binding protein 1; *c-Myb*, MYB proto-oncogene; *MAP3-K7IP2*, TGF-beta activated kinase 1 (MAP3-K7) binding protein 2; *PU.1*, Spi-1 proto-oncogene; *LTBP1*, latent transforming growth factor beta-binding protein 1; *C/EBP*, CCAAT/enhancer binding protein; *hnRNPAB*, heterogeneous nuclear ribonucleoprotein A/B; *RORA*, retinoid acid receptor-related orphan receptor alpha; *WWOX*, host WW domain containing oxidoreductase; *SOCS1*, suppressor of cytokine signaling 1; *UL28*, unique long gene 28; *UL32*, unique long gene 32; *meq*, MDV EcoRI-Q; *ICP4*, infected cell protein 4; *ICP27*, infected cell protein 27; *NEK6*, Never In Mitosis Gene A (NIMA)-related kinase 6; *MYBL1*, v-myb myeloblastosis viral oncogene homolog-like 1; *ANP32A*, acidic nuclear phosphoprotein 32A; *CCNE1*, cyclin E1; *TFDP2*, transcription factor Dp-2; *HOXA3*, homeobox A3; *MDFIC*, MyoD family inhibitor domain containing; *BCL11B*, B-cell chronic lymphocytic/lymphoma 11B; *MMP2*, matrix metallopeptidase 2; *MMP9*, matrix metallopeptidase 9; *PAX3*, paired box 3; *IGF2*, Insulin-like growth factor-2; *GATA4*, GATA binding protein 4; *p27Kip1*, cyclin-dependent kinase (cdk) inhibitor $p27^{Kip1}$; *PDCD4*, programmed death cell 4; UD, undefined; NA, not applicable.

encoded immediate early gene ICP22 is shown to be down-regulated by miR-M5-3p and miR-M1-5p during latency but upregulated by miR-M4-5p, which is necessary for the lytic replication of the virus (Boumart et al., 2018). Thus, it is likely that viral miRNA can affect MDV's pathogenicity and oncogenicity by self-regulation of viral protein-coding genes and affect the microenvironment for virus replication and survival through regulating host cellular targets, which together can contribute to MDV pathogenesis.

Regulatory network mediated by MDV-1 miRNAs

In recent years, several target genes regulated by MDV-1 miRNAs have been identified and the associated signaling pathways potentially involved in MD pathogenesis, and tumorigenesis have been revealed. As early as 2011, scientists have found that miR-M3-5p encoded in the Meq-cluster targets the signal molecule SMAD2 in the TGF-β signal pathway to protect infected cells from cisplatin-induced apoptosis and to provide sufficient space for the replication and survival of MDV-1 (Xu et al., 2011). Interestingly, the subsequent research found that miR-M4-5p also targets the TGF-β signaling pathway by down-regulating the LTBP1 expression to suppress the maturation and secretion of TGF-β1 and activate the expression of host oncogene c-Myc, triggering the development of MD lymphoma (Chi et al., 2015). It seems that the TGF-β signaling, as demonstrated in Figure 2, is a possible core pathway involved in herpesvirus oncogenesis. Previous studies have shown that among the miRNAs associated with cancers, the host cell-encoded miR-155 and its functional viral orthologs, e.g., miR-M4-5p encoded by MDV-1 and miR-K12-11 encoded by KSHV (Kaposi's sarcoma-associated herpesvirus), are critical and widely involved in multiple tumors (Bondada et al., 2019; Due et al., 2016; Narayan et al., 2018; Seddiki et al., 2014). Like MDV-1, the human tumorigenic γ-herpesvirus EBV and KSHV also use the miR-155 pathway to regulate the transformation process. KSHV-miR-K12-11, a functional KSHV-encoded miR-155 ortholog of KSHV, suppresses TGF-β signaling by down-regulating the expression of SMAD5 (Liu et al., 2012) and thrombospondin 1 (THBS1) (Samols et al., 2007), a crucial tumor suppressor and anti-angiogenic factor that exerts anti-angiogenic effects in part by activating the latent TGF-β. Interestingly, EBV has not been found to encode its viral miR-155 homolog, but has adapted to upregulate the host cellular miR-155 expression, promoting cell proliferation and neoplastic transformation (Linnstaedt et al., 2010; Wood et al., 2018). Thus, the TGF-β signaling pathway appears vitally important for pathogenesis and neoplastic transformation by oncogenic herpesviruses. Still, it is possibly only the tip of the iceberg of regulatory networks mediated by miR-155 and its functional viral analogs in virally-induced cancers.

Except for the involvement in TGF-β signaling, miR-M4-5p has also been found to target and inhibit cell apoptosis by down-regulating the expression of the host tumor suppressor WWOX to potentially promote MD tumor occurrence (Zhu et al., 2021b). Furthermore, it also regulates an important host transcription factor, hnRNPAB, which is closely involved in cell proliferation and neural development, suggesting that miR-M4-5p may contribute to nervous system damage, a feature also associated with MDV infection (Dang et al., 2017). Recent research has also reported that the miR-M2-5p, another important viral miRNA in the same cluster, plays a dual role in contributing to MDV oncogenesis by down-regulating the expression of two tumor suppressors, RBM24 and MYOD1, through two distinct pathways. On one hand, it leads to the overexpression of p63 and IGF2 to promote cell proliferation. On the other hand, it inhibits cell apoptosis by targeting the caspase-3 signaling pathway mediated by MYOD1 (Zhu et al., 2020). Undoubtedly, the data obtained from the Meq-cluster miRNAs have displayed that MDV-induced T-cell lymphomas can be simultaneously triggered by both the viral miRNA-promoted cell proliferation and inhibition of apoptosis.

However, compared to the Meq-cluster miRNAs promoting oncogenesis, rapid-onset MD lymphomas are also somewhat inhibited by the other viral miRNAs in both the Mid and LAT clusters through the regulation of virus replication and latency and also by the downregulation of viral oncogene expression. During MDV-1 infection, the major viral oncogene *meq* is directly targeted and suppressed by the Mid-cluster miRNA miR-M11-5p (Teng et al., 2017), which may block the formation of oncoprotein Meq/c-MYC heterodimers that trigger the induction of MD lymphomagenesis. Further study conducted through the CRISPR/Cas9-based gene editing and knockout of the pre-miRNA mir-M11 from the viral genome demonstrated significantly enhanced virus replication *in vitro* and *in vivo* (Luo et al., 2020; Teng et al., 2017). The miR-M11-5p/3p appears to be important regulators responsible for MDV latency and lymphomagenesis considering the overexpression of mir-M11 miRNAs in latent MSB-1 cells and its low-level expression during the cytolytic stages of virus replication. Previously, it has been shown that viral miRNA in the LAT-cluster, such as miR-M7-5p, targets and suppresses the expression of MDV protein-coding genes *ICP4* and *ICP27* to contribute to the establishment and maintenance of latency (Strassheim et al., 2012; Sun et al., 2021a). Recently, more viral miRNA self-regulations have been reported on miR-M5-3p and miR-M1-5p to downregulate *ICP22* expression during latency. Still, unexpectedly, miR-M4-5p was found to upregulate the expression of *ICP22*, which is necessary for the lytic replication of the virus (Boumart et al., 2018).

As demonstrated in Figure 3, MDV-1 miRNAs contribute greatly to the virus life cycle and the development of MD tumors, some acting as oncogenes and some acting as pu-

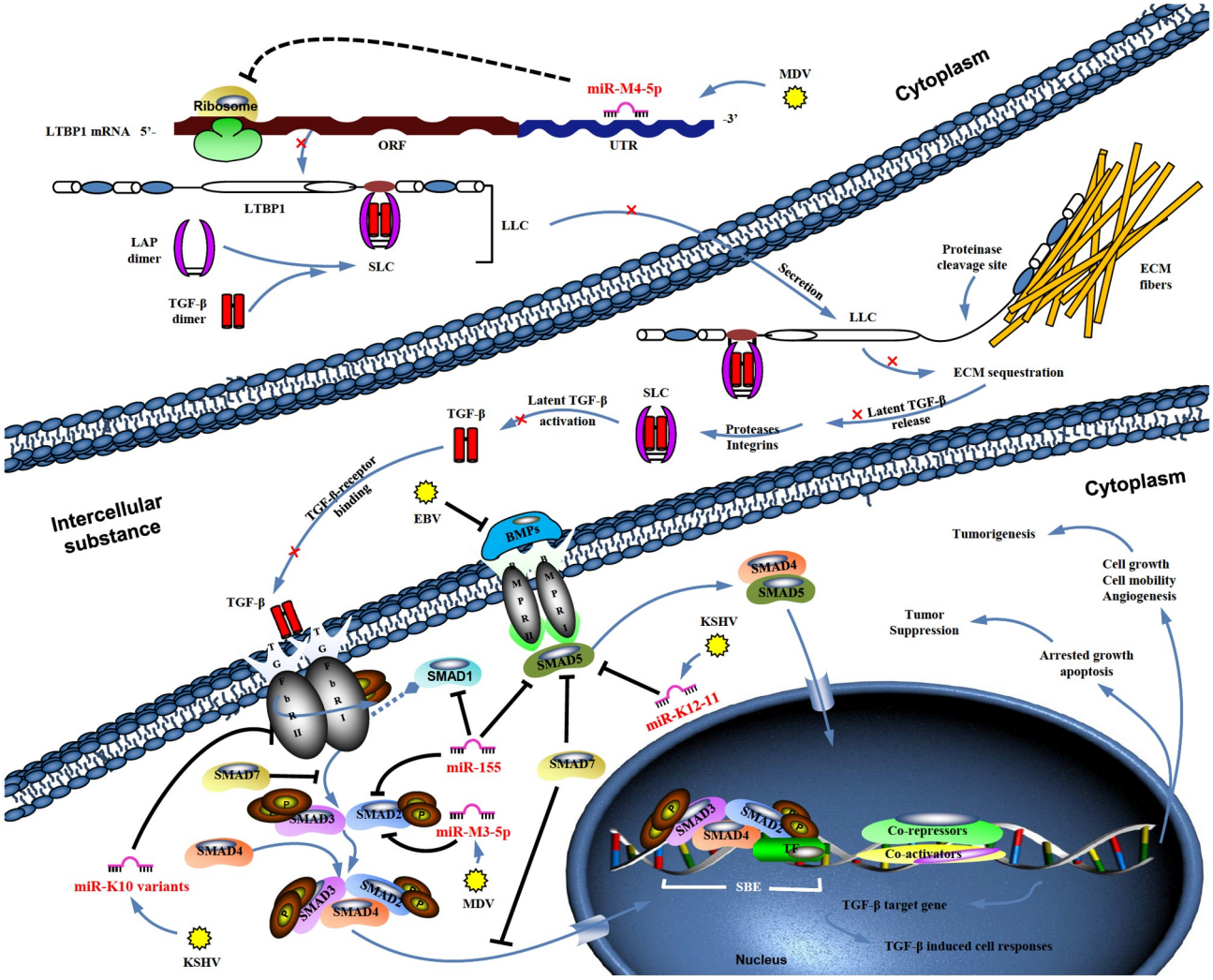

Figure 2 Schematics of TGF-β signaling targeted and regulated by the cellular miR-155 and its viral orthologs. miR-155, cellular miRNA-155; miR-M4-5p, MDV-1-encoded viral ortholog of miR-155; miR-K12-11, KSHV-encoded viral ortholog of miR-155; miR-K10 variants, KSHV-encoded miR-K10 variants; TGF-β, transforming growth factor beta; LTBP1, latent TGF-β binding protein 1; ORF, open reading frame; UTR, untranslated region; LAP, latency-associated peptide; SLC, small latent complex; LLC, large latent complex; ECM, extracellular matrix; TGFBRI, type I receptor of TGF-β; TGFBRII, type II receptor of TGF-β; BMPs, bone morphogenetic proteins; BMPRI, bone morphogenetic protein receptor I; BMPRII, bone morphogenetic protein receptor II; SMAD, small mothers against decapentaplegic; TF, transcription factor; SBE, Smad binding element. Arrows and T-shaped lines indicate the *cis*-acting regulatory or suppression effects respectively. Red crosses show the suppressed signaling mediated by miR-M4-5p.

tative tumor suppressors to keep a relative balance in MD biology. The balance is finally broken and results in cancers by some underlying mechanisms hiding in the regulatory networks, which deserves further study.

Host-encoded miRNAs in MDV biology

The host cell-encoded miRNAs, as shown in Table 3, have also been involved in modulating virus replication, antiviral immune response, viral latency, and pathogenesis. As a successful pathogen, MDV has also developed multiple mechanisms to avoid being targeted by cellular miRNAs. MDV mediates canonical and noncanonical interactions with cellular miRNAs to downregulate specific targets to promote viral genome stability, translation, and/or RNA accumulation. Small RNA-seq analysis of the bursa from MDV-infected resistant and susceptible chicken lines has demonstrated 54 novel differentially expressed miRNAs, suggesting a critical role in facilitating resistance or mediating susceptibility to MD (Heidari et al., 2020).

Inhibitory roles of cellular miRNAs in MDV oncogenesis

Research into the pathogenesis of MDV infection has unraveled some of the host miRNAs potentially participating in the suppression of MD tumorigenesis. Two miRNAs, gga-miR-26a and gga-miR-181a, have shown to suppress the proliferation of MDV-transformed lymphoid cell line, e.g., MSB-1 cells, by targeting the host genes Never In

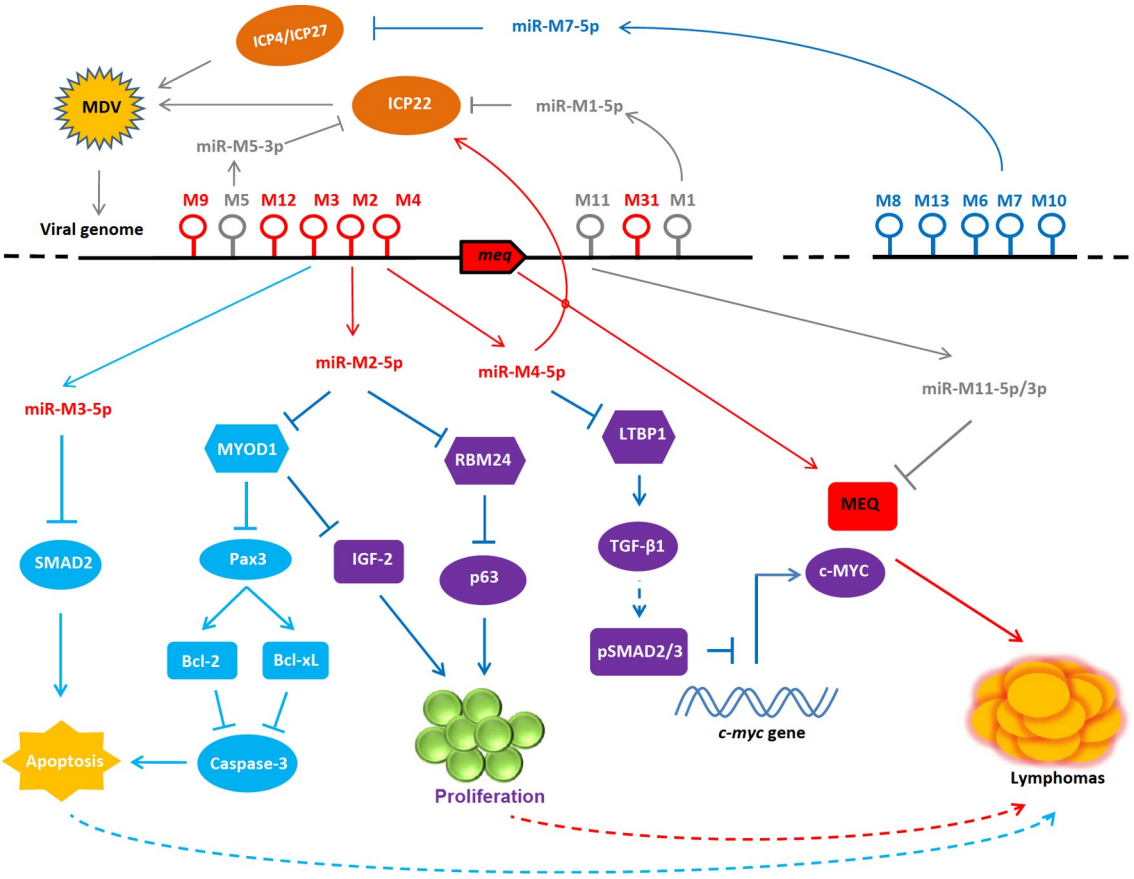

Figure 3 Regulatory mechanisms and network mediated by viral miRNAs in MDV pathogenesis and tumorigenesis. The miRNA precursors distributed in three gene clusters are shown by colored bonbons and are marked with abbreviated signatures, e.g., mdv1-mir-M9 is shortened as M9. The confirmed or suggested regulatory mechanisms of each miRNA are shown in different colors. Arrows and T-shaped lines indicate the *cis*-acting regulatory or suppression effects, respectively.

Mitosis Gene A (*NIMA*)-related kinase 6 (*NEK6*), proto-oncogene like 1 (*MYBL1*) and acidic nuclear phosphoprotein 32A (*ANP32A*), respectively (Li et al., 2014; Li et al., 2021; Lian et al., 2015a). However, several host miRNAs were found to suppress the proliferation, migration, and invasion of MSB-1 cells, such as the gga-miR-103-3p targeting host genes cyclin E1 (*CCNE1*) and transcription factor Dp-2 (*TFDP2*), gga-miR-130a targeting homeobox A3 (*HOXA3*) and myogenic differentiation (*MyoD*) family inhibitor domain containing (*MDFIC*), and the gga-miR-219b targeting B-cell chronic lymphocytic/lymphoma 11B (*BCL11B*) (Han et al., 2016a; Han et al., 2016b; Zhao et al., 2017). The suppressive effect of gga-miR-219b and gga-miR-130b-3p on MSB-1 cells was also found to be mediated by inhibiting the expressions of two cell invasion-related genes matrix metallopeptidase 2 (*MMP2*) and matrix metallopeptidase 9 (*MMP9*) (Zhao et al., 2018; Zhao et al., 2017). In the process of MDV infection, the host gga-miR-1, gga-miR-206, and gga-miR-223 are all down-regulated in the MYOD-1-associated signaling regulated by the viral miRNA miR-M2-5p to promote cell proliferation or inhibit apoptosis, which implies that they are closely related to tumor suppression (Zhu et al., 2020). Additionally, the host gga-miR-199-3p, gga-miR-140-3p, and gga-miR-221-5p were found to be down-regulated since the early stage of MD tumor transformation and displayed inhibitory effects on the proliferation of MSB-1 cells, suggesting a putative role as MD tumor suppressors, but the regulatory mechanism remains unclear (Lian et al., 2015b).

Oncogenic roles of cellular miRNAs in MD biology

Some cellular miRNAs are found to be upregulated post-MDV infection and possibly promote tumorigenesis by targeting the genes that participate in antiviral responses. The cellular miR-155 is a conserved multifunctional miRNA involved in the modulation of immune responses. In recent years, there has been ongoing attention to the significant role of miR-155 and its viral orthologs in neoplastic transformation by avian oncogenic viruses (Bondada et al., 2019). The gga-miR-155 can lead to increased MSB-1 proliferation, reduced cell apoptosis rates, and increased invasiveness by targeting host *RORA*. However, it is down-regulated in MDV-infected birds or lymphocyte cell lines (Ding et al.,

2020). Further studies have shown that gga-miR-155 interacts with circGTDC1 and circMYO1B and targets immune-related genes, such as *GATA4*, which indicates the role of non-coding RNAs played in the regulation of immune responsive genes (Kang et al., 2015; Wang et al., 2020). In a previous study (Lambeth et al., 2009), the host-encoded miR-221 and miR-222 were found to be upregulated in MDV-infected cells and target the cell cycle regulatory molecule p27 (Kip1) to promote MSB-1 proliferation. The gga-miR-21, an oncogenic miRNA (oncomiR-21) transactivated by the viral oncoprotein Meq, was demonstrated to be over-expressed during MD lymphomagenesis and to promote tumor cell growth and escape apoptosis by targeting programmed cell death 4 (PDCD4) (Stik et al., 2013).

LincRNAs in MDV biology

The lincRNAs, a group of ncRNAs associated with many cancers and other diseases, have also been identified in MD biology (Table 4). Previously, a number of reports had demonstrated that lincRNAs function as powerful *cis*- and *trans*-regulators of gene activity (Qi et al., 2013; Saw et al., 2021), which can not only modulate gene expression in *cis* by acting as scaffolds for the recruitment of chromatin-modifying complexes or by altering the chromatin structure of target genes but also regulate gene expression in *trans* by directing the chromatin localization of protein binding partners or by suppressing mRNA translation. Two types of ncRNAs, the LATs and viral telomerase RNA (vTR), have been discovered in the MDV-1 genome and suggested to be involved in MD biology (Nair, 2013). The LATs, earliest studied MDV-1 lincRNAs in the IRs/TRs of the MDV-1 genome, are antisense to ICP4 and abundantly expressed in lymphomas and lymphoblastoid cell lines, possibly contributing to the maintenance of latency and/or cell transformation (Gennart et al., 2015). While, vTR has shown to share a structure and function similar to telomerase RNA and contributes to MDV-induced lymphomagenesis (Kaufer et al., 2010). Recently, characterization of the deferentially expressed lincRNAs in bursa or spleens from MD-resistant and susceptible chicken lines has further highlighted their potential importance in MD resistance, pathogenesis and/or tumorigenesis (He et al., 2015; You et al., 2019). Another viral lincRNA, the ERL lncRNA, has shown to function as a natural antisense transcript to MDV carcinogen during the lytic and latent phases of infection and virus reactivation. Still, it could be disrupted by the hyper editing mediated by ADAR1 (Figueroa et al., 2016). Interestingly, in the same research, it has been shown that the viral miRNA miR-M4-5p upregulates the expression of ADAR1 through suppressing JAK/STAT IFN-response pathway by targeting and repressing expression of suppressor of cytokine signaling 1 (SOCS1), which sequentially disrupted the natural antisense transcription activity of ERL lncRNA.

MDV also hijacks the host cellular lincRNAs to promote disease progression. The linc-satb1 was the first lincRNA identified to play a crucial role in MD immune responses, which induces tumor cell multiplication and metastasis through positive regulation of nearby protein-coding gene *SATB1* (He et al., 2015). The linc-GALMD3 has been demonstrated to not only indirectly regulate MD lymphomagenesis by positively regulating the expression of gga-miR-223 that was considered to be associated with MD tumorigenesis and neurologic lesions but also negatively regulate the expressions of some mitochondrial structure genes to affect mitochondrial dysfunction and cause the disease (Han et al., 2017). The linc-GALMD1 was significantly down-regulated in an MD-resistant line of chickens during MDV infection and suppressed tumorigenesis by coordinating the expression of MDV genes, tumor-related genes and regulating immune responses to MDV infection (He et al., 2019). Based on the lincRNA identification pipeline and construction of the co-expression network, five differentially expressed lncRNAs (lincRNA-MSTRG.6754.1, lincRNA-MSTRG.15539.1, lincRNA-MSTRG.7747.5, lincRNA-MSTRG.6725.1, and lincRNA-MSTRG.360.1) were strongly correlated with MD-resistant candidate genes (i.e., *IGF-I*, *CTLA4*, *HDAC9*, *SWAP70*, *CD72*, *JCHAIN*, *CXCL12*, and *CD8B*) and were identified and suggested that host cellular lincRNAs may affect MD resistance and tumorigenesis through the targeted genes (You et al., 2019). Furthermore, a recent study has shown that the reversible N^6-methyladenosine (m^6A) modifications occurring in lincRNAs following MDV infection may play important regulatory roles in virus replication (Sun et al., 2021b). Still, the molecular mechanisms linking m^6A-modified lincRNAs with MDV pathogenesis and/or tumorigenesis need further investigation.

Although a rapidly increasing number of reports on lincRNA expression across MDV-infected cells and tissues indicated that aberrant lincRNA expression plays a major role in virally-induced tumorigenesis, they are still formidable to comprehensively identify and characterize. As it has been drawn in a previous review (Zhang et al., 2021), the interactions between lincRNAs and viral or host miRNAs may co-contribute to MD pathogenesis and oncogenesis; lincRNAs could drive their *cis*- and *trans*-regulation to influence the expression of viral/host miRNAs but inversely the viral/host miRNAs and their target genes might also be associated with the transcription and hyper editing of lincRNAs. It will be intriguing to orient future research to focus on the regulatory networks of circRNA/lincRNA-miRNA-mRNA along with the discovery of other ncRNA functions.

Table 4 Targets and potential functions of the long non-coding RNAs involved in MD biology

Category	ncRNA signatures	Viral/host targets	Potential roles and functions	Refs.
LincRNAs	LATs	miR-M8-M10	Maintain latency and/or cell transformation	Gennart et al., 2015
	vTR	*TERT*, *RPL22*	Promote tumorigenesis	Kaufer et al., 2010
	ERL lncRNA	miR-M1-5p, miR-M4-5p	Function as a natural antisense transcript to MDV carcinogen	Figueroa et al., 2016
	linc-satb1	*SATB1*	Mediates immune responses	He et al., 2015
	linc-GALMD3	gga-miR-223	Influences mitochondrial structure and cell cycle processes	Han et al., 2017
	linc-GALMD1	*meq*	Inhibits tumorigenesis and regulates immune responses	He et al., 2019
	lincRNA-MSTRG.6754.1	*IGF-I*, *CD72*, *CXCL12*, *WISP1*	Modulate host immunity, regulate the Wnt signaling pathway	You et al., 2019
	lincRNA-MSTRG.15539.1	*HDAC9*, *SWAP70*, *JCHAIN*, *CD72*	Modulate host immunity	You et al., 2019
	lincRNA-MSTRG.7747.5	*HDAC9*, *CD8B*, *CD72*, *IGF-I*, *TOP2A*	Modulate host immunity, regulate cell cycle	You et al., 2019
	lincRNA-MSTRG.6725.1	*CTLA4*, *JCHAIN*	Modulate host immunity	You et al., 2019
	lincRNA-MSTRG.360.1	*TNFRSF6B*, *HDAC9*, *CTLA4*, *CXCL12*, *SWAP70*, *JCHAIN*, *WISP1*	Modulate host immunity, regulate the Wnt signaling pathway	You et al., 2019
CircRNAs	circZMYM3	gga-miR-214, gga-miR-429-3p, gga-miR-200b-3p	Mediates immune responsive genes	Wang et al., 2020
	circGTDC1	gga-miR-155	Mediates immune responsive genes	Wang et al., 2020
	circMYO1B	gga-miR-155	Mediates immune responsive genes	Wang et al., 2020

CircRNAs in MDV biology

New ncRNAs involved in MDV pathogenesis, such as circRNAs, have also been recently identified using bioinformatics strategy. CircRNAs are a recently discovered family of functional ncRNAs processed by back-splicing machinery and have been demonstrated to mainly act as miRNA or RNA-binding protein sponge (Wang et al., 2018; Zhu et al., 2021a), interact with proteins to regulate gene expression (Du et al., 2016), and regulating transcription and splicing (Ashwal-Fluss et al., 2014; Li et al., 2015). In a recent study (Wang et al., 2020), the transcriptomes and features of circRNA and miRNA in spleens from MDV-infected birds were analyzed and extensive competing endogenous RNA networks involving circRNA, lincRNA, miRNA, and mRNA were predicted to be involved in MD tumorigenesis. As listed in Table 4, an intronic circRNA (i.e., circZMYM3), possibly interacts with seven miRNAs targeting host immune genes *SWAP70* and *CCL4*, and the circGTDC1 and circMYO1B may serve as miRNA sponges to interact with gga-miR-155 that is specifically down-regulated in MDV-transformed tumor cells, indicating the roles of circRNAs in regulating host immune responses and contributing to MD biology. Interestingly, the circWWOX is produced from the exons of parental gene *WWOX* that has been recently confirmed to be down-regulated by miR-M4-5p, which appears to be involved in the pathogenesis of MDV (Zhu et al., 2021b). Moreover, studies have reported that some circRNAs processing coding ability and the peptide or protein products exhibit important regulatory roles in cell development, making the competing endogenous RNAs network associated with MD biology more complicated. In addition, the regulation of m^6A modification of circRNAs in infected chickens has shown more possibilities for host circRNAs to participate in the processes of MDV infection, such as regulating the insulin signaling pathway (Sun et al., 2021c). Future work deserves further studies on the underlying molecular mechanism of how virus affects the m^6A modification of circRNAs and the regulatory role of m^6A-modified circRNAs in MDV biology.

Recently, virus-encoded circRNAs have been identified in the genomes of more than 10 different viruses with various genome types (Zhang et al., 2022). The most widely studied viral circRNAs encoded by DNA viruses are related to the disease pathogenesis, which acts as a miRNA sponge to regulate the expression of mRNA by a competing endogenous RNAs (ceRNAs) mechanism (Abere et al., 2020; Qiao et al., 2019; Yang et al., 2022). In the latest study (Chasseur et al., 2022), a genome-wide and infection stage-dependent analysis of viral circRNA expression in MDV infections has been performed, and it has demonstrated that numerous noncanonical viral circRNAs were processed by the U2-independent splicing machinery, coexisting with the canonical viral circRNAs. Interestingly, the MDV-encoded circRNAs have been demonstrated to correspond to the major MDV virulence factors such as oncogene meq and LATs, providing the first evidence of viral circRNAs expressing at key steps of the MDV life cycle. Undoubtedly,

the potentially critical role of viral circRNAs involved in MD biology deserves to be studied.

Conclusion and future prospects

In conclusion, we know that the avian oncogenic herpesvirus encodes not only viral oncoprotein Meq but also many ncRNAs, such as miRNAs, lincRNAs and circRNAs. The oncoprotein Meq can directly induce cell transformation, but the virus-encoded ncRNAs are also involved in regulating the occurrence and development of MD tumors. Interestingly, the virus is smart to encode many ncRNA genes, such as viral miRNAs distributed in different genomic loci and located in clusters which are expressed, processed, and maturated using the same or independent promoters at different phases of disease and thus having different dynamic expression patterns. These small ncRNAs play crucial roles as oncomiRNAs, tumor suppressors, or other regulators in the processes of virus self-regulation, mutual checks, and balances, as demonstrated in Figure 3. This consequently resulted in MD tumorigenesis or delaying the rapid-onset tumor formation to provide the virus with a longer infection period and a larger survival space rather than killing the chicken hosts quickly. Simultaneously, viral ncRNAs can also regulate host genes and related signaling pathways to meet the needs of the virus life cycle and pathogenesis/oncogenesis, whereas the cellular ncRNAs also participate in antagonizing the virus, some of which are even utilized by the virus. More underlying regulatory mechanisms mediated by these ncRNAs need to be further studied, especially for the newly discovered viral/cellular lincRNAs and circRNAs potentially involved in MDV pathogenesis/oncogenesis. Based on the elucidation of ncRNAs encoded by herpesvirus, represented by MDV, it has displayed a new field in which extracellular organisms such as viruses exert gene regulation through ncRNAs. Compared to RNA viruses such as the oncogenic RSV, it may provide a more complex, interesting, and comprehensive understanding of the pathogenic and/or tumorigenic mechanisms of oncogenic DNA viruses.

As one of the most important immunosuppressive and neoplastic diseases in poultry, MD has been historically regarded as an excellent biomedical model for studying virally-induced lymphomagenesis. Revealing the underlying regulatory functions of both viral and host ncRNAs involved in MD biology will be helpful for further understanding the pathogenesis of herpesviruses. Although many progress signs on MDV-1 miRNAs playing important roles in MDV pathogenesis have been gleaned, details of the currently available regulatory mechanisms and networks are still incomplete. In particular, the regulatory functions of the Mid and LAT-cluster members had not been deeply studied. However, some researchers have confirmed their important roles in the MDV infection process. The mir-M6 in the LAT-cluster is the first mirtron described in MDV that can affect the expression of other miRNAs from its cluster and the host transcript, but the mechanism remains unclear (Rasschaert et al., 2016). Recently, exosome-transferred functional miRNAs have been widely recognized as a functional form of cell-to-cell communication, especially in cancer signaling and regulations (Nath Neerukonda et al., 2019; Neerukonda et al., 2019; Wang et al., 2022), which will provide new ideas for the functional study of MDV-1 miRNAs. Most of the current studies on viral miRNAs have tended to explore their target and regulatory mechanism, while the expression pattern and regulatory factors of viral miRNA in MDV pathogenic processes remain obscure. Whether viral miRNA interacts with or is regulated by other types of ncRNAs should be focused on for future research on the function of MDV-1 miRNAs.

Due to the pathogenic and oncogenic features of the virus, the MDV-1 miRNAs have attracted most of the attention in early studies. However, the functions of MDV-2 and HVT miRNAs were poorly explored. Both MDV-2 or HVT miRNAs are clustered in a similar orientation to that of MDV-1 miRNAs and are almost located in the TR_L/IR_L regions, forming an obvious miRNA gene cluster. The genomic loci of these miRNAs in highly variable and evolving regions indicate an important role in MD biology. Compared to that of the HVT-vaccinated birds, the expression of HVT miRNAs was affected in spleens of the birds co-infected with MDV-1 strain Md5, and the differentially expressed viral miRNAs are predicted to be involved in regulating several cellular processes (Goher et al., 2013). Further study has shown that HVT may protect cellular transformation and tumor progression by regulating cellular miRNAs in HVT-vaccinated chickens that are subsequently infected with an oncogenic MDV-1 strain (Hicks and Liu, 2019). In conclusion, more vigorous research on MDV-2 and HVT miRNAs will help us understand MDV's pathogenic mechanism and evolutionary characteristics and provide new ideas for better control of MD.

With the development of new generations of high-throughput sequencing and bioinformatics technologies in recent years, more and more ncRNAs have been identified and manifested to play key regulatory roles in many important biological processes, including the progression of diseases/cancers (Saw et al., 2021; Xue et al., 2020; Zhang et al., 2022). It is now clear that the lincRNAs and circRNAs can both serve as miRNA sponges to regulate the expression of target mRNAs by a ceRNA mechanism, but the miRNAs might inversely regulate their own transcription and target mRNA genes (Figueroa et al., 2016; Mehta et al., 2021; Militello et al., 2017). Furthermore, the involvement of RNA-binding proteins, *cis*-regulatory elements, *trans*-acting factors, and m^6A modifications, as we know presently, may

further increase the complexity of ncRNA-mediated networks (Liu and Chen, 2022; Wang et al., 2021; Zhang et al., 2019a). For herpesviruses, especially the oncogenic MDV-1 strains, the viruses not only encode their ncRNAs but also hijack host cellular ncRNAs to facilitate disease progression. As for the novel types of viral ncRNAs newly discovered in virus biology, the following questions are needed to be further addressed: why do viruses produce so many ncRNAs and derive mechanisms from hijacking and regulating host cellular ncRNAs? Are these ncRNAs induced under certain circumstances and perform specific functions in virus infection and the different stages of the disease? How do viruses smartly balance the distinct regulatory roles of various ncRNAs or mRNA genes in their life cycle?

The role and regulatory mechanisms of ncRNAs in MDV-1-triggered tumorigenesis have been studied a lot, but the ncRNAs involved in other host responses to MDV-1 infection, such as immunosuppression and neurological symptoms, have not been well studied. Previously, the miR-M4-5p was involved in the regulation of host innate immune molecules to promote viral replication, in which the lincRNA and circRNA were also involved (Hu et al., 2016). The important role of ncRNAs in MDV infection may provide new ideas for the development of new efficient MD vaccines, such as the elimination of functional ncRNAs from the viral genome to improve the immune response ability of vaccine strain, relieve the inhibition of the natural immune response of the host, and accelerate the early host immune response to vaccines. In a previous report (Fang et al., 2017), miRNA sponges specifically binding to the Meq-cluster miRNAs (miR-M2-3p, miR-M3-5p, miR-M5-3p, miR-M9-5p, and miR-M12-3p) have been designed to investigate the effect of miRNA sponges on MDV tumorigenicity. The results showed that miRNA sponges not only knocked down the expression levels of viral miRNAs, inhibited the proliferation/invasion/tumorigenic ability, and enhanced apoptosis of MDV-1 transformed MSB-1 cells *in vitro*, but also alleviated the growth inhibition of MSB-1 cells *in vivo* and reduced the mortality for birds during a 60-days animal experiment. It implies that miRNA sponges targeting specific viral ncRNAs may be an effective anti-tumor strategy for the gene therapy of virally-induced cancers. Furthermore, various host ncRNAs targeted by viruses may also be used as potential targets for the future treatment and therapy of MD, providing more possibilities for the efficient control of MD. It will be a major and hard work to validate further and reveal more regulatory networks mediated by the circRNAs/lincRNAs-miRNAs-mRNAs interactions for illustrating the detailed molecular regulatory mechanisms involved in MD biology, using the previously developed computational/experimental methods and the newly developed technologies. During the past decade, the CRISPR/Cas9-based gene editing techniques have been successfully developed and widely applied in virology research, including exploring the functions of MDV protein-coding genes and ncRNAs (Teng et al., 2021; Teng et al., 2022b; Zhang et al., 2019b; Zhang et al., 2019c). Recently, it has been demonstrated that using the CRISPR/Cas9 system with the designed multiple guide RNAs (gRNAs) specifically targeting essential viral genes (e.g., *UL6*, *UL19*, *UL30*, *UL49* and *ICP4*) completely abrogated MDV replication, providing a future tool for protecting chickens against MDV infection (Hagag et al., 2020). It also means that the CRISPR/Cas9-based gene editing technology may be further applied as a screening tool to identify critical viral or cellular ncRNAs responsible for MDV pathogenesis/oncogenesis. With the application of newly developed technologies and bioinformatics strategies, undoubtedly, the functions of both viral and host ncRNAs involved in MD biology will be better understood soon in the coming future.

Compliance and ethics *The author(s) declare that they have no conflict of interest.*

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