<Review>

Research Progress on MicroRNAs Involved in the Regulation of Chicken Diseases

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Running title: MicroRNAs in Chicken Diseases

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Abstract: MicroRNAs (miRNAs) are small, non-coding RNA molecules that inhibit protein translation from target mRNAs. Accumulating evidence suggests that miRNAs can regulate a broad range of biological pathways, including cell differentiation, apoptosis, and carcinogenesis. With the development of miRNAs, the investigation of miRNA functions has emerged as a hot research field. Due to the intensive farming in recent decades, chickens are easily influenced by various pathogen transmissions, and this has resulted in large economic losses. Recent reports have shown that miRNAs can play critical roles in the regulation of chicken diseases. Therefore, the aim of this review is to briefly discuss the current knowledge regarding the effects of miRNAs on chickens suffering from common viral diseases, mycoplasmosis, necrotic enteritis, and ovarian tumors. Additionally, the detailed targets of miRNAs and their possible functions are also summarized. This review intends to highlight the key role of miRNAs in regard to chickens and presents the possibility of improving chicken disease resistance through the regulation of miRNAs.

Keywords: chicken, miRNA, necrotic enteritis, ovarian tumor, virus disease
Introduction

Poultry rearing is an industry that is superior to other sectors in agriculture, and recently, this industry has undergone tremendous development (Uddin et al., 2010; Landoni and Albarello, 2015). Commercial poultry production is a very intensive animal agricultural system, and one poultry house or barn can contain as many as 100,000 commercial layers or broilers (Landoni and Albarellos, 2015). Given this, mortality of chickens due to various infectious and non-infectious diseases is emerging as the major constraint against profitable poultry production. For example, Marek’s disease (MD) and lymphoid leukosis (LL) are both viral diseases that affect chickens to cause tumors and high mortality (Kreager, 1998). Infectious bursal disease (IBD) is one of the most prevalent infectious virus diseases, causing bursal necrosis and immunosuppression that lead to severe damage to the immune system in chickens (Xu et al., 2019). Avian influenza (AI) is also a highly contagious viral disease that is responsible serious animal health crises that result in high fatality rates in poultry worldwide (Paul et al., 2019). Avian mycoplasmosis is another disease that can cause chronic respiratory disease (CRD) in chickens (Ley, 2008), and necrotic enteritis (NE) is a ubiquitous poultry disease caused by Clostridium perfringens that affects the intestines of chickens, resulting in major impacts on performance (Whelan et al., 2018). Given these examples and the prevalence of diseases affecting chickens, it is clear that disease control and prevention at all levels must be a major focus for the poultry industry.

MicroRNAs (miRNAs) are small, non-coding RNAs. In animals, miRNAs are approximately 22 nucleotides in length, and they play vital roles in almost all developmental and pathological pathways (Jansson and Lund, 2012). MiRNAs play an important role in mRNA degradation, where they can bind to the three prime untranslated regions (3’-UTR) of various mRNA transcripts to reduce translation (Bartel, 2004). It has been reported that within a given genome, 20%-30% of genes are
regulated by miRNA (Enright et al., 2003), and a single miRNA may target more than 100 mRNAs (Friedman et al., 2009). Therefore, identification of miRNA functions in different organisms is a critical step to facilitate our understanding of genome organization, genome biology, and evolution (Carrington and Ambros, 2003).

In the past ten years, miRNAs have been discovered to possess multiple functions in chickens, including the regulation of development and various growth processes (Bannister et al., 2011; Luo et al., 2014). Additionally, in recent decades researchers have reported that miRNAs play diverse roles in chicken diseases and immune system functions (Ahanda et al., 2009; Hicks et al., 2009; Dinh et al., 2014; Liu et al., 2016). The majority of these studies investigated the possible mRNAs that could be targeted by a given miRNA and the underlying mechanisms responsible for the observed effects. Thus, this review aims to introduce the functions of miRNAs in the context of chickens, and particular focus is placed on the advances related to the impact of miRNAs on various diseases that affect chickens. The identification of distinct miRNA expression patterns and the roles of those dysregulated miRNAs in the context of pathological conditions that affect chickens will broaden our understanding of how miRNAs regulate the chicken immune system and reveal potential therapeutic targets for the treatment of miRNA-related diseases.

**MiRNA Biogenesis**

The biogenesis of miRNA has been elaborately reviewed in the existing literature (Bushati and Cohen, 2007; Lin and Gregory, 2015). Briefly, miRNA begins from a primary transcript, termed the pri-miRNA, that is typically a long mRNA that is thousands of nucleotides in length and transcribed by RNA polymerase II (only a few miRNAs are transcribed by RNA polymerase III) (Magenta et al., 2013). Then, the pri-miRNA is processed by the nuclear RNase III Drosha-DGCR8 complex into an approximately 65 nucleotide hairpin precursor miRNA (pre-miRNA) (Denli et al., 2004). Next, the pre-miRNAs are exported from the nucleus into the cytoplasm by
exportin 5 and further processed by DICER1, an RNase III enzyme that measures from the 5’ and 3’ ends of the pre-miRNA to form the mature ~22 nucleotide miRNA:miRNA* (the complementary strand of miRNA) duplex (Lin and Gregory, 2015). The miRNA* is typically degraded, while the mature miRNA is incorporated into the RNA-induced silencing complex (RISC) (Bartel, 2004). MiRNAs play an important role in mRNA degradation, as they can bind to the 3’-UTR of mRNA transcripts to reduce translation (Bartel, 2004) (Figure 1).

MiRNAs Involved in Chicken Virus Diseases

The interactions among miRNAs and viruses are highly complicated. In some instances, viruses may encode their own miRNA to facilitate their pathogenesis (Muylkens et al., 2010). In other instances, however, host miRNAs can directly target viral RNAs during infections or they can regulate various immune responses to inhibit virus replication (Wang et al., 2010; Ingle et al., 2015; Wang et al., 2018).

MiRNA and infectious bursal disease

Infectious bursal disease (IBD) is an acute, highly contagious disease in young chickens (Pitcovski et al., 2003). Infectious bursal disease virus (IBDV), which destroys B-lymphocyte precursors and causes a high degree of immunosuppression, is considered to be one of the major threats to the poultry industry (Wang et al., 2018). IBDV contains two segments of double-stranded RNA (A and B) (Hirai and Shimakura, 1974, Azad et al., 1985). Segment A possesses two partially overlapping open reading frames (ORFs) (Kibenge et al., 1991), while segment B encodes an RNA-dependent RNA polymerase, viral protein 1 (VP1) (von Einem et al., 2004). Recent studies have revealed that miRNAs may suppress IBDV replication by targeting the viral genome segments and host cellular SOCSs (suppressor of cytokine signaling). SOCSs are considered to be key physiological regulators of the immune system and can negatively regulate type I interferon (IFN) (Fu et al., 2017) that allows host cells to recognize viral
dsRNA through the action of TLR3 or RIG-I molecules (Li et al., 2013) to combat viral infection. For example, according to Wang et al., gga-miR-155 could inhibit IBDV replication and enhance the expression of IFN via targeting SOCS1 and TANK (TNF receptor-associated factor family member-associated NF-κB activator) in response to IBDV infection (Table 1, Wang et al., 2018). Additionally, gga-miR-130b could suppress IBDV replication via targeting a specific sequence of IBDV segment A and enhancing the expression of IFN-β by binding to the host SOCS5 (Table 1, Fu et al., 2017). Similarly, gga-miR-454 was found to inhibit IBDV replication by binding to a specific sequence of IBDV segment B and enhancing the expression of IFN-β by targeting cellular SOCS6 (Table 1, Fu et al., 2018). Additionally, gga-miR-21 could target viral genomic RNA and inhibit VP1 translation to lower IBDV replication (Table 1, Wang et al., 2013a).

In contrast, gga-miR-9* negatively regulated the host antiviral innate immune response by suppressing type I IFN production by targeting IRF2 (interferon regulatory factor 2), an important factor that functions in IFN antiviral signal transduction pathways, to promote IBDV replication (Table 1, Ouyang et al., 2015). gga-miR-2127 overexpression also promoted IBDV replication and could down-regulate the expression of antiviral innate immunity genes by targeting p53 (Table 1, Ouyang et al., 2017). Additionally, gga-miR-142-5p targeted chMDA5 (chicken melanoma differentiation-associated gene 5), which encodes a protein that recognizes RNA viral infections and initiates an antiviral innate immune response, to promote IBDV replication in an IRF7-dependent pathway (Table 1, Ouyang et al., 2018).

**MiRNA and Marek’s disease**

Marek's disease (MD) is a type of chicken lymphoma that is caused by Marek's disease virus 1 (MDV1), an α-herpesvirus (Osterrieder et al., 2006), that results in immunosuppression, neurological disorders, and rapid-onset CD4+ T-cell lymphoma (Biggs, 1997). MD was the first tumor disease that could be prevented by vaccination,
and it provides an important animal model for the study of viral cancer development and immunity (Osterrieder et al., 2006).

In recent decades, numerous studies have been performed to investigate the involvement of miRNAs (both host and MDV miRNAs) in MD tumorigenesis. Through the use of microarrays and qRT-PCR analysis, two clades of chicken miRNAs were found to be increased in splenic tumors and non-tumorous spleen tissues derived from MDV1-infected chickens (Li et al., 2014a). Additionally, chicken lines 63 and 72 are two highly inbred lines of specific-pathogen-free white leghorn chickens that are resistant or susceptible, respectively, to MD tumors. According to Tian et al., gga-miR-15b, which targets ATF2 (activating transcription factor 2), was reduced in MDV-infected susceptible chicken splenic tumors. To further understand the potential functions of miRNAs in MDV resistance and susceptibility, the expression of ATF2, a protein that can interact with MDV oncogene Meq, was determined. The results of this study revealed that the protein level of ATF2 was significantly increased in infected line 72 chickens compared to that of MDV-free chickens, while its expression was stable in line 63 chicken before and after MDV challenge. Chicken ATF2 was observed to form a heterodimer with c-Jun, the MDV oncogene Meq, and other b-ZIP proteins (Huguier et al., 1998; Levy et al., 2003; Osterrieder et al., 2006). It has been reported that ATF2 exerts tumor suppressor or oncogene activities in different cancers by cooperation with other tumor suppressors or oncogenes (Bhoumik et al., 2004; Bhoumik and Ronai, 2008). As the MDV oncogene Meq was highly expressed in line 72 after MDV infection, ATF2 likely formed a heterodimer with Meq to promote MD development, and in chicken line 63, due to the absence of Meq after MDV exposure, ATF2 may cooperate with c-Jun to facilitate distinct functions, similar to observations in line 72. Taken together, the results of this study suggested that gga-miR-15b may target ATF2 to regulate MD resistance/susceptibility (Tian et al., 2012). Recently, gga-miR-219b was found to be decreased in MDV-induced lymphoma. Additionally, gga-miR-219b was
demonstrated to target BCL11B (B-cell chronic lymphocytic/lymphoma 11B), a gene that not only plays an important role in thymocyte development but has also been implicated in lymphoproliferative diseases (Grabarczyk et al., 2007; Gutierrez et al., 2011). The knockdown of BCL11B also reduced the proliferation, migration, and invasion of the Marek’s disease tumor cell MSB1. The inhibition of gga-miR-219b was further found to down-regulate the expression of the MDV oncogene Meq; however, BCL11B knockdown significantly induced Meq expression (Table 1, Zhao et al., 2017a).

In other studies, gga-miR-26a, -181a, and -130a were similarly reported to inhibit the proliferation of MD lymphoid cells by NEK6 (never in mitosis gene A-related kinase 6), MYBL1 (v-myb myeloblastosis viral oncogene homolog-like 1), and HOXA3 (homeobox A3), respectively (Table 1, Li et al., 2014b; Lian et al., 2015; Han et al., 2016). Additionally, it is known that miRNAs can be packaged into exosomes, a subset of extracellular vesicles that are of 30-150 nm in diameter and secreted by various cell types. Thus, exosome-mediated transport of miRNAs may provide a novel mechanism of intercellular gene regulation. According to Nath Neerukonda et al., serum exosomes were isolated from MDV-infected chickens that were either vaccinated against MD or unvaccinated and bearing MDV-induced tumors. When detecting the expression of various miRNAs within exosomes, researchers observed that exosomes from vaccinated chickens exhibited greater expression of tumor suppressor miRNA (gga-miR-146b) and less expression of oncomiR (gga-miR-21) compared to those obtained from unvaccinated controls. Thus, gga-miR-146b and -21 may serve as serum exosome biomarkers for vaccine-induced protection and for MD tumors, respectively (Nath Neerukonda et al., 2019).

In addition to animals, herpesviruses also encode miRNAs. Early in 2006, Burnside and colleagues found that eight miRNAs were encoded by MDV. Five of these miRNAs flank the Meq oncogene, and three of them map to the latency-associated transcript (LAT) region of the virus genome (Burnside et al., 2006). Later, they also identified an
additional seven miRNAs and ‘*’ strands by deep sequencing of MDV1 infected CEF cells (Burnside et al., 2008). Currently, twenty-six MDV-1-encoded miRNAs processed from fourteen pre-miRNAs (MDV1-miR-M31 and MDV1-miR-M1 to MDV1-miR-M13) have been identified (http://microrna.sanger.ac.uk). All of the MDV-1-encoded miRNAs are focused within three gene clusters, including the Meq cluster, the LAT cluster, and the Mid cluster. The first cluster of MDV1-miRNAs, including MDV1-miR-M2, -M3, -M4, -M5, -M9 and -M12, is antisense to the MDV-1 gene R-LORF8 and located adjacent to the Meq oncogene (Luo et al., 2010). MDV1-miR-M4 is a functional ortholog of miR-155 (Zhao et al., 2009). Recently, reports have indicated that MDV1-miR-M4-5p could induce the over-expression of the oncogene c-Myc by targeting LTBP 1 (latent TGF-β binding protein 1) and inactivating the TGF-β signaling pathway during MDV1-infection (Table 1, Chi et al., 2015). Further study revealed that MDV1-miR-M4-5p could also target chicken hnRNPAB (heterogeneous nuclear ribonucleoprotein AB) to promote proliferation of both primary CEF and transformed chicken fibroblast DF-1 cells (Table 1, Dang et al., 2017). Additionally, using viruses modified by reverse genetics of the infectious bacterial artificial chromosome (BAC) clone of the oncogenic RB-1B strain of MDV, Zhao and colleagues demonstrated that the deletion of the six-miRNA cluster 1 from the viral genome abolished the oncogenicity of MDA. This loss of oncogenicity may be due to MDV1-miR-M4, as its deletion or a 2-nucleotide mutation within its seed region was sufficient to inhibit the induction of lymphomas. This role was further confirmed through the rescue of oncogenic phenotype by revertant viruses that expressed either the miR-M4 or the cellular homolog gga-miR-155 (Zhao et al., 2011). Using luciferase reporter assays, Muylkens and colleagues found that MDV1-miR-M4-5p could target the 3’UTR of GPM6B (glycoprotein M6-b), RREB1 (Ras-responsive element-binding protein 1), c-Myb, MAP3-k7ip2 (mitogen-activated protein kinase kinase kinase 7-interacting protein 2), PU.1, and C/EBP (CCAAT-enhancer-binding proteins).
Additionally, MDV1-miR-M4-5p specifically inhibited the translation of two viral proteins (UL28 and UL32) that are involved in the cleavage and packaging of herpesvirus DNA (Table 1, Muylkens et al., 2010).

**MiRNA and avian influenza**

Avian influenza, also known as “Bird Flu”, is one of the most severe respiratory viral infectious diseases of this decade (Koparde and Singh, 2011). Avian influenza virus (AIV) is a type A virus of the family Orthomyxoviridae. Although wetland birds such as wild ducks, gulls, and shore-birds are the natural hosts of AIV, this virus can also infect poultry birds, resulting in significant economic losses and an increased threat to public health due to the potential for host jumping from animals to humans (Webby and Webster, 2003). MiRNAs have been found to regulate AIV replication during infection. In chickens, when the miRNA expressions between H5N3 infected and non-infected commercial SPF layer chickens were compared at 4 days post-infection, 73 and 36 miRNAs were differentially expressed in lung and trachea, respectively, and there were more miRNAs highly expressed in non-infected tissues (Wang et al., 2009). In subsequent experiments examining broilers, however, a greater number of miRNAs were highly expressed in infected lungs than in non-infected lungs, indicating that the regulatory mechanism of the host response to AIV infection mediated by miRNAs may be different between layers and broilers (Wang et al., 2012). In another study, 1004 miRNAs were obtained from H9N2-infected and non-infected chicken embryo fibroblasts, and 48 miRNAs were expressed differently between the two groups. These miRNAs were predicted to target immune response-related genes (Peng et al., 2015). In recent years, experiments using mammalian cells have demonstrated that host gga-miR-1249 targets the H5N1 and H1N1 gene PB2 that encodes an RNA polymerase required for viral replication to suppress virus replication (Table 1, Wang et al., 2017). Similarly, gga-miR-485 was found to bind to the H5N1 gene PBI to inhibit H5N1 replication, and gga-miR-485 could also target the host gene RIG-I (retinoic
acid-inducible gene I). Basal amounts of RIG-I protein detect viral nucleic acids in the cytosol and induce type I IFNs. In turn, these IFNs increase the expression of IFN-stimulatory genes, including RIG-I. Thus, gga-miR-485 could suppress the RIG-I-dependent expression of type I and type III IFNs in H5N1-infected cells and dampen the RIG-I-dependent inflammatory response (Table 1, Ingle et al., 2015).

**MiRNA and subgroup J avian leukosis virus**

Avian leukosis viruses (ALVs) are a group of avian retroviruses that induce tumors in chickens (Lin et al., 2011). Chicken ALVs are classified into six subgroups (A-E and J). ALV-J, which primarily induces myeloid leukemia, causes more serious damage than that caused by the other virus subgroups (Gao et al., 2012). To broaden our understanding of the molecular mechanisms that occur during ALV-J infection, efforts have been made to examine the miRNA expression patterns in chickens that are infected with ALV-J. Through the use of chicken dendritic cells (DC), ALV-J was found to induce apoptosis during the early infection stages. MiRNA sequencing data from ALV-J-infected and -uninfected DCs revealed 122 differentially expressed miRNAs (115 that were up-regulated and 7 exhibiting down-regulation after injection). Through GO analysis, the target genes of these miRNAs, including gga-miR-204, -211, -221 and -6651, were found to be involved in the processes indicated by the ‘antigen processing and presentation of exogenous peptides’ and ‘apoptosis’ GO terms (Liu et al., 2016). Using miRNA microarray analysis, Li and colleagues found that in 10-week-old chickens, 12 miRNAs were differentially expressed within the livers of ALV-J-uninfected and -infected chickens. The expression levels of gga-mir-221, -222, -1456, -1704, -1777, -1790, and -2127 were up-regulated by ALV-J infection, while the expression levels of gga-let-7b, -let-7i, -125b, -375, and -458 were significantly down-regulated (Li et al., 2012). Among these differentially expressed miRNAs, gga-miR-375 overexpression was observed to decrease DF-1 cell proliferation and promote DF-1 apoptosis. Through prediction and dual luciferase reporter assays,
gga-miR-375 was confirmed to target \textit{YAP1} (yes-associated protein 1), an effector of Hippo signaling, that restrains cell proliferation and promotes apoptosis to influence normal cell fate and tumorigenesis (Harvey and Tapon, 2007; Saucedo and Edgar, 2007). Additionally, \textit{in vivo} assays further indicated that gga-miR-375 was decreased in chicken livers 20 days after ALV-J infection. Simultaneously, the expression levels of \textit{cyclin E} and \textit{DIAP1} (drosophila inhibitor of apoptosis protein 1) were up-regulated after infection. DIAP1 functions in the early embryo to inhibit apoptosis (Yoo \textit{et al.}, 2000). Cyclin E, an important regulator of cell cycle progression, was reported to be increased in the majority of breast tumor tissues and in most leukemia solid tumors (Keyomarsi \textit{et al.}, 1994). Thus, the above findings indicate that gga-miR-375 functions as a tumor suppressor and plays an important role in inhibiting ALV-J tumorigenesis (Table 1, Li \textit{et al.}, 2014c). In contrast, in their later study researchers discovered that gga-miR-221 and -222 may be tumor formation-relevant genes that could contribute to tumorigenesis during ALV-J infection by targeting \textit{BMF} (BCL-2 modifying factor), a gene that exhibits pro-apoptotic function (Table 1, Dai \textit{et al.}, 2015).

In addition to control tumorigenesis, chicken miRNAs can also regulate ALV replication directly. The genome of ALV-J is composed of three genes, including \textit{gag}, \textit{pol}, and \textit{env} (Li \textit{et al.}, 2015). Wang and colleagues revealed that gga-miR-1650 could target the 5’UTR of ALV-J, and gga-miR-1650 overexpression could decrease gag protein expression and inhibit ALV-J replication (Table 1, Wang \textit{et al.}, 2013b). The gp85 protein is a viral envelope polypeptide that is encoded by the \textit{env} gene within the ALV-J genome. As reported, gga-miR-23b expression was up-regulated in ALV-infected chicken spleens and could target \textit{IRF1} (interferon regulatory factor 1), a critical regulatory protein of the inflammatory response that functions as a tumor suppressor and is involved in cell cycle progression and apoptosis (Hong \textit{et al.}, 2013). Additionally, gga-miR-23b overexpression resulted in increased gp85 expression, while \textit{IRF1} overexpression caused a decrease in gp85 level. These results indicate that
gga-miR-23b promoted ALV-J replication by targeting IRF1 and by affecting gp85 expression (Table 1, Li et al., 2015). gga-miR-34b-5p could also target MDA5 (melanoma differentiation-associated gene 5), a gene that encodes a protein that can detect ALV-J infection and trigger the MDA5 signaling pathway, to promote ALV-J-infected cell proliferation. Additionally, MDA5 overexpression significantly decreased env levels and ALV-J virion secretion, while gga-miR-34b-5p overexpression elevated env protein expression after ALV-J infection to induce ALV-J replication (Table 1, Li et al., 2017).

**MiRNA Involved in Avian Mycoplasmosis**

*Mycoplasma*, an important prokaryote, can infect humans and a wide range of economically important livestock species (Hu et al., 2016; Nicholas and Ayling, 2016). *Mycoplasma gallisepticum* (MG) is one of the most important mycoplasmas that is frequently associated with avian CRD (Ley, 2008), which causes severe inflammation in the tracheas and lungs of chickens and turkeys (Davidson et al., 1982; Stipkovits et al., 2012). Recently, several studies have suggested that miRNAs play a key role in MG infection in chicken. By deep sequencing, Zhao and colleagues found that miRNAs are associated with MG infection in chicken lungs at 3 and 10 days post-infection. They found 45 and 68 differentially expressed miRNAs at 3 and 10 days, and these miRNAs targeted 6290 and 7181 genes, respectively. GO, KEGG, miRNA-GO-network, path-net, and gene-net analyses determined that the altered miRNAs regulated a large number of genes that function throughout various signaling pathways including the MAPK pathway, the focal adhesion regulatory pathways, the Wnt pathway, and the JAK/STAT pathway (Zhao et al., 2017b). gga-miR-101-3p was found to be up-regulated in the lungs of MG-infected chicken embryos, and gga-miR-101-3p could target and inhibit the host EZH2 (enhancer of zeste homolog 2), a growth suppressor gene involved in the regulation of cell cycle progression and proliferation, to suppress the proliferation of chicken embryonic fibroblasts by blocking the G1-to-S phase transition (Chen et al.,
Additionally, SMARCA5 (SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily A, member 5) is a member of the SWI/SNF family that exhibits ATPase and helicase activities. It has also been reported that SMARCA5 plays an important role in promoting cell proliferation both in vitro and in vivo (Stopka et al., 2000; Gigek et al., 2011). According to a study by Zhao et al., gga-miR-99a was significantly down-regulated in the lungs of MG-infected chicken embryos and could target SMARCA5 to repress the proliferation of DF-1 cells by inhibiting the transition from the G1 phase to the S and G2 phases (Zhao et al., 2017c). Additionally, gga-miR-19a, which targeted the host ZMYND11 (zinc-finger protein, MYND-type containing 11), was found to be up-regulated both in chicken embryonic lungs and in MG-infected DF-1 cells to activate the NF-κB signaling pathway, ultimately promoting TNF-α expression and enhancing cell cycle progression and proliferation to defend against MG infection (Table 1, Hu et al., 2016). gga-miR-130b-3p was also markedly up-regulated in MG-infected DF-1 cells. Luciferase reporter assays confirmed that PTEN (phosphatase and tensin homolog deleted on chromosome ten) was a direct target of gga-miR-130b-3p. As one of the most important tumor suppressors, PTEN is a phosphatase that exhibits both protein and lipid activities, and this protein plays a central role in various cellular functions and in the immune response (Di Cristofano and Pandolfi, 2000). Studies also demonstrated that PTEN exerted its inhibitory effects against cell proliferation by blocking cell cycle progression at the G1 phase by facilitating the down-regulation of cyclins and CDKs proteins (Moon et al., 2004). Thus, the overexpression of gga-miR-130b-3p remarkably promoted MG-infected DF-1 proliferation by binding to PTEN (Table 1, Yuan et al., 2018). Matrix metalloproteins (MMPs), a family of zinc-dependent endopeptidases, are important for a number pathological process such as inflammation, cardiovascular disease, and cancer (Huang et al., 2017; Mali et al., 2017). A recent study revealed that gga-miR-146c was up-regulated in response to MG infection, and MMP16 was validated as the target gene.
of gga-miR-146c (Zhang et al., 2019). Additionally, using loss- and gain-of-function approaches, Zhang and colleagues also found that gga-miR-146c participated in the activation of the TLR6/MyD88/NF-κB pathway. It was also observed that gga-miR-146c could decrease cell apoptosis and stimulate DF-1 proliferation after MG infection. Taken together, these findings indicated that gga-miR-146c defended against host MG infection by inhibiting \( \text{MMP16} \) expression, activating the TLR6/MyD88/NF-κB signaling pathway, and promoting cell proliferation (Table1, Zhang et al., 2019).

**MiRNAs Involved in Chicken Necrotic Enteritis**

Necrotic enteritis (NE), commonly caused by *Clostridium perfringens*, is an acute clostridial disease that causes weight depression, loss of appetite, and sudden death (Hermans and Morgan, 2007). NE has re-emerged as a significant problem as a result of restrictions on the use of antibiotics in the poultry industry (Williams, 2005). In 2014, Dinh and colleagues investigated the effects of miRNAs on NE induced by *Eimeria maxima* and *Clostridium perfringens* in two genetically disparate chicken lines (Marek’s disease resistant line 63 and Marek’s disease-susceptible line 72). Their results demonstrated that miR-215, -217, -194, -200a, -200b, -216a, -216b, and -429 were highly expressed in intestine tissues derived from line 72 and that miR-1782 and -499 were down-regulated in these tissues. In spleen tissues, miR-34b and -1684 were the most up-regulated miRNAs in line 63. Additionally, the immune-related target genes (\( \text{CXCR5} \), \( \text{BCL2} \), \( \text{GJA1} \), \( \text{TCF12} \) and \( \text{TAB3} \)) of these miRNAs were differentially expressed between the two chicken lines, and they were suppressed in the Marek’s disease-susceptible chicken line (Dinh et al., 2014). Later, using the same chicken lines, Truong and colleagues explored the effects of TGF-β and miRNAs on NE. Members of the TGF-β family that is composed of TGF-β, BMPs, and SMADs function in the immune response (Escalona et al., 2012). According to their results, TGF-β1-3, BMP1-7, and SMAD1-9 were differentially expressed between the two chicken lines.
relative to their respective controls. Also, six miRNAs (gga-let-7c, -199, -200a, -200b, -429, and -499) were predicted and validated as the targets of BMP7 (Truong et al., 2017a), indicating that the modulation of the TGF-β signaling pathway through BMP7 and miRNAs may play a role in the complex regulation of signaling pathways involved in the spleens of two NE-induced chicken lines. Additionally, the JAK-STAT signaling pathway is activated by over 40 cytokines and growth factors. This pathway is involved in cell differentiation, proliferation, apoptosis, and the progression of several diseases through its ability to regulate gene expression. In the spleens of chickens from line 63 and 72, 116 JAK-STAT pathway genes were found to be differentially expressed, and 63 mature miRNAs that variably target JAK-STAT pathway genes were also differentially expressed in the spleens of chickens from both lines (Truong et al., 2017b).

**MiRNAs Involved in Chicken Ovarian Tumor**

The laying hen is the only non-human animal that spontaneously develops ovarian cancer at a high prevalence rate, and this animal can be used as a model for ovary development and tumorigenesis (Johnson and Giles, 2013). Kang and colleagues found that in ovaries isolated from sexually mature (162-d) and sexually immature (42-d) chickens, 93 miRNAs were significantly differentially expressed, and these included gga-miR-1a, 21, 26a, 137, and 375 (Kang et al., 2013). Additionally, in recent years, miRNA has been found to regulate ovarian tumorigenesis. Avian beta-defensin (AvBD) proteins are members of beta-defensin subfamily and can protect against various microorganisms (van Dijk et al., 2008). In a study by Lim et al., gga-miR-1615 could influence AvBD-11 expression via its 3’-UTR, and AvBD-11 was most abundant in the glandular epithelium of endometrioid-type ovarian tumors, but not normal ovaries of laying hens, suggesting that AvBD-11 and gga-miR-1615 may be useful as biomarkers for the diagnosis of ovarian cancer (Table 1, Lim et al., 2013).
Conclusions

Our knowledge regarding the functions of miRNAs in chickens has expanded in the past several decades. Current studies provide promising evidence concerning the ability of miRNAs to regulate immune function and diseases in chickens. Compared to the number of human studies, however, very few studies have been performed in farm animal to determine the mechanisms underlying the function of miRNAs. In these studies, many were limited to the identification of new and differentially expressed miRNAs or to the prediction of possible targets, and only a small number of these studies further validated the function of these miRNAs and the predicted targets experimentally. Recently, new mechanisms of miRNA action have been discovered, including the ability of these molecules to spread throughout the body when transported within exosomes and to act as protein ligands. Thus, continued mechanistic studies are required to more fully assess the impact of these miRNAs on the biological processes in chickens. Progress in the study of the roles and mechanisms of miRNAs in chickens will provide new ideas and targets for the improvement of chicken growth and the treatment of chicken diseases.

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| miRNA   | immune function                       | Tissue/ cell | Targets                     | Reference       |
|---------|---------------------------------------|--------------|-----------------------------|-----------------|
| gga-miR-155 | inhibits IBDV replication             | DF-1 cell    | SOCS1, TANK                 | Wang et al., 2018 |
|         |                                       |              | SOCS5, the specific         |                 |
| gga-miR-130b | inhibits IBDV replication             | DF-1 cell    | sequence of IBDV segment A  | Fu et al., 2017  |
|         |                                       |              | SOCS6, the specific         |                 |
| gga-miR-454 | inhibits IBDV replication             | DF-1 cell    | sequence of IBDV segment B  | Fu et al., 2018  |
| gga-miR-21  | inhibits IBDV replication             | DF-1 cell    | VP1 of IBDV genome          | Wang et al., 2013a |
| gga-miR-9*  | promotes IBDV replication             | DF-1 cell    | IRF2                        | Ouyang et al., 2015 |
| gga-miR-2127 | promotes IBDV replication             | DF-1 cell    | p53                         | Ouyang et al., 2017 |
| gga-miR-142-5p | promotes IBDV replication           | DT40 cell    | chMDA5                      | Ouyang et al., 2018 |
| gga-miR-15b  | regulates MDV1-induced tumorigenesis  | spleen       | ATF2                        | Tian et al., 2012  |
gga-miR-26a suppresses MD lymphoma cell proliferation
MSB-1 cell  \textit{NEK6}  \textit{Li et al., 2014b}

gga-miR-181a suppresses MD lymphoma cell proliferation
MSB-1 cell  \textit{MYBL1}  \textit{Lian et al., 2015}

gg-miR-130a suppresses MD lymphoma cell proliferation
MSB-1 cell  \textit{HOXA3}  \textit{Han et al., 2016}

gga-miR-219b suppresses MD lymphoma cell proliferation, migration and invasion
MSB-1 cell  \textit{BCL11B}  \textit{Zhao et al., 2017a}

MDV1-miR-M4-5p promotes MDV1-induced tumorigenesis
CEF and DF-1 cells  \textit{LTBP1, hnRNPAB}  \textit{Chi et al., 2015; Dang et al., 2017}

MDV1-miR-M4-5p modulates immune response to MDV infection
DF-1, MDCC-MSB-1 and MDCC-54-O cell  \textit{GPM6B, RREB1, c-Myb, MAP3-K7IP2, PU.1, C/EBP}  \textit{Muylkens et al., 2010}

inhibits H5N1 replication and dampens RIG-I-dependent inflammatory response
HEK 293T cell, A549 cell, MCF7 cell  \textit{PB1} of H5N1 and \textit{RIG-I} of host  \textit{Ingle et al., 2015}

gga-miR-485 suppresses MD lymphoma cell proliferation
MSB-1 cell  \textit{PB1} of H5N1 and \textit{RIG-I} of host  \textit{Ingle et al., 2015}
gga-miR-1249 inhibits H1N1 replication in HEK 293T cell, A549 cell, MDCK cell, and PB2 of H1N1. Wang et al., 2017

gga-miR-375 suppresses proliferation and promotes apoptosis of cells infected with ALV-J in DF-1 cell. YAP1. Li et al., 2014c

gga-miR-221 and -222 promote proliferation and inhibit apoptosis of cells infected with ALV-J in DF-1 cell. BMF. Dai et al., 2015

gga-miR-1650 inhibits ALV-J replication and infection in DF-1 cell and ALV-J genome. Wang et al., 2013b

gga-miR-23b inhibits ALV-J replication and infection in DF-1 cell and IRF1. Li et al., 2015

gga-miR-34b-5p promotes ALV-J-infected cell proliferation and ALV-J replication in DF-1 cell. MDA5. Li et al., 2017

gga-miR-101-3p suppresses the proliferation of chicken embryonic fibroblast infected with DF-1 cell. EZH2. Chen et al., 2015
| miRNA     | Function                                                                 | Cell Line | Target Gene | Publication                  |
|-----------|--------------------------------------------------------------------------|-----------|-------------|-----------------------------|
| gga-miR-99a | Suppresses proliferation of chicken embryonic fibroblast infected with MG | DF-1 cell | SMARCA5     | Zhao et al., 2017c          |
| gga-miR-19a | Activates NF-κB signaling pathway, promotes TNF-α expression and enhances cell cycle progression and proliferation of cells infected with MG | DF-1 cell | ZMYND11     | Hu et al., 2016             |
| gga-miR-130b-3p | Promotes MG-infected cell proliferation | DF-1 cell | PTEN        | Yuan et al., 2018           |
| gga-miR-146c | Activates TLR6/MyD88/NF-κB signaling pathway and promotes cell proliferation by inhibiting apoptosis of cells infected with MG | DF-1 cell | MMP16       | Zhang et al., 2019          |
| gga-miR-1615 | Regulates ovarian tumorigenesis in chicken ovary | Chicken ovary | AvBD-11    | Lim et al., 2013           |
