GENOMIC APPROACHES TO STUDY MOLECULAR AND CELLULAR

MECHANISMS OF HOST RESPONSE TO AVIAN INFLUENZA VIRUS

INFECTION IN CHICKENS

A Dissertation

by

YING WANG

December 2011

Major Subject: Poultry Science

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ABSTRACT

Genomic Approaches to Study Molecular and Cellular Mechanisms of Host Response to

Avian Influenza Virus Infection in Chickens. (December 2011)

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Avian influenza virus (AIV) is a type A virus of the family *Orthomyxoviridae* and its outbreaks not only cause economic losses in poultry, but also are worldwide threats to human health. The phenotypic changes in host cells induced by pathogens are always accompanied by remarkable changes in gene expression. Therefore understanding the gene expression profile of infected cells at the global level is important to get insights into interactions between hosts and viruses. Different genomic approaches have been utilized in the current study to investigate the host-AIV interactions in chickens.

The Ser to Asn mutation on position 631 in the chicken Mx1 protein was reported to result in a positive antiviral function *in vitro*. With AIV infection, the Mx1 mRNA expression levels in heterozygous birds were significantly up-regulated. Additional mutations on the chicken Mx1 coding region were identified by sequencing. The results showed that most identified mutations were co-segregated with S631N mutation except one insertion in the position of 1544bp in the heterozygous birds. We speculate this insertion might be related to the up-regulation of mRNA expression of heterozygous birds with AIV infection.

The miRNAs play critical roles in biological processes and are important effectors in host-pathogen interactions. The miRNA deep sequencing was used to profile miRNAs in AIV infected or non-infected chickens. Differentially expressed miRNAs identified have expanded our knowledge in the functions of these potential immune related chicken miRNAs regulating host response to AIV infection.

Both microarray and transcriptome analysis by RNA-Seq were used in the current study to investigate the global gene expression of host response to AIV infection. Through the comprehensive analysis, a list of strong candidate miRNAs such as miR-32 and their host target genes including Mx1 were identified for further elucidating the regulatory mechanism of host-AIV interaction.

In summary, we have identified many important candidate host genes and miRNAs which play important roles in the modulation of host response to AIV infection using genomic approaches. Further investigation of underline regulatory mechanisms of these genes, miRNAs or related pathways, followed by functional analysis, could lay solid foundation for understanding cellular and molecular mechanisms of the host-AIV interactions, thereby, pave a way for the development of novel protective strategies against AIV infection in chickens.

DEDICATION

To my parents, my husband and friends, who made all of these possible, for their endless

support, encouragement and patience.

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NOMENCLATURE

Ab, Antibody

AIV, Avian Influenza Virus

ANOVA, Analysis of Variance

APC, Antigen Presenting Cell

DC, Dendritic Cells

dsRNA, Double Stranded RNA

HA, Hemagglutinin

HP, High Pathogenic

HPAIV, Highly Pathogenic Avian Influenza Virus

IFN, Interferon

LP, Low Pathogenic

LPAIV, Pathogenic Avian Influenza Virus

M1, Matrix Protein

M2, Membrane Bound Ion Channel-like Protein

Mx1, Myxovirus Resistance Gene

NA, Neuraminidase

NEP, Nuclear Export Protein

NP, Nucleocapsid Protein

NS1, Non-Structural Protein 1

PAMP, Pathogen Associated Molecular Pattern

PFU, Plaque Forming Units

PRR, Pattern Recognition Receptor

RIG-I, Retinoic Acid-Inducible Gene I

RLR, Retinoic Acid-Inducible Gene I-like Recepto

RNP, Ribonucleoproteins

SPF, Specific Pathogen Free

Th cell, Helper T cells

TLR, Toll-Like Receptor

Treg, Regulatory T cells

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CHAPTER I

INTRODUCTION

Avain influenza virus

Gene coding assignment and protein function of influenza virus

Influenza viruses belong to *Orthomyxoviridae* family (Acheson, 2007). There are

five different genera in this family, including influenza A, B, C, Thogotovirus and

Isavirus (Sebbag, 1998). Influenza A viruses can infect avian and mammalian species

(Stephenson and Democratis, 2005).

Type A influenza virus genome consists of eight negative sense single-stranded

RNA segments. The structure of the influenza A virus includes a liquid membrane from

the host cell. Of the eleven viral proteins produced by type A viruses, nine are packaged

in virions (Knipe, 2007). Genome segments encode different viral proteins: three viral

polymerase subunits (PA, PB1 and PB2) and the non-structural pro-apoptotic protein

PB1-F2 generated by an alternative reading frame in PB1; the envelope glycoproteins

Hemaglutinin (HA) and Neuraminidase (NA); the nucleocapsid protein (NP); the matrix

protein (M1) and the membrane bound ion channel protein (M2); and the non-structural

protein (NS1) and nuclear export protein (NEP).

PA, PB1 and PB2 are the components of the RNA polymerase complex (Braam, et

al., 1983). They play distinct roles within the polymerase and are critical for viral

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This dissertation follows the style and format of *Poultry Science.*

transcription and replication (Deng, et al., 2005). Two glycoproteins HA and NA form projections on the surface of the viral particles and they are also major antigenic determinants recognized by the host adaptive immune system. The M1, the most abundant viral protein, lies just beneath the lipid envelope and is the inner layer of the viral envelope. The M2 proteins project from the surface of the virus serving as an ion-channel, which is essential for virus uncoating and maturation. Viral RNA segments, the polymerase complex and the nucleoprotein (NP) form ribonuceloproteins (RNP), is the core of the virus particle (Knipe, 2007). The viral RNA wraps around NP, which makes it accessible to the replication machinery (Zur Stadt, et al., 2005). NS1 is able to down-regulate host cell mRNA processing, sequester dsRNAs and reduce interferon response (Hale, et al., 2008). NEP (NS2) interacts with M1 protein and directs nuclear export of viral nucleocapids (O'Neill, et al., 1998). The viral RNA polymerase complex lacks proofreading mechanism, which makes the viral genome highly variable. Different mutations could occur in the HA and NA genes, which generate different virus subtypes (Brown, 2000).

Replication of influenza viruses

Influenza viruses bind to neuraminic acids (sialic acids) on the surface of the cells to initiate the replication. HA binds to sialic acid-containing receptors, and then they form a trimer in the virus envelope. Cleavage of HA by cellular proteases into two subunits activates its ability to carry out membrane fusion. Since having an envelope, influenza virus requires low pH to fuse with endosomal membranes. After binding to

cell receptors, virions enter the cell within an endosomal vesicle. Then HA undergoes a major conformational change into HA_1 and HA_2. HA_1 contains the sialic acid-binding domain. HA_2 is anchored in the virus envelope and contrains a hydrophobic fusion peptide created by protease cleavage. The cleavage of HA activates its ability to carry out fusion of viral and endosomal membranes.The HA-mediated fusion of the viral with endosomal membrane and the M2-mediated release of the RNP result in the appearance of free RNP complexes in the cytoplasm (Knipe, 2007). At this point, these RNP complexes are transported back to the nucleus and begin making mRNAs by copying its negative-strand RNA genome segments. Influenza viral RNAs are synthesized within the nucleus. Host cellular RNAs are used as primers for the initiation of viral mRNA synthesis. These vRNAs are exported through the nuclear pores into the cytoplasm where they are translated into viral proteins (Cros and Palese, 2003). Two viral proteins M1 and NEP (NS2) are involved in directing the nuclear export of RNPs (Cros and Palese, 2003). M1 makes contact with both vRNA and NP. NEP contains a nuclear export signal and it can also bind to the matrix protein. NEP is responsible for recruiting the export machinery and directing export of the complex. For influenza virus, HA, NA and M2 have all been shown localized to the apical surface of polarized cells (e.g. lung epithelial cells) (Rodriguezboulan and Sabatini, 1978). Following the assembly of viral components (HA, NA and M2), vRNAs are packaged by RNPs and then the replication is completed by budding from the plasma membrane. Finally, NA is able to reverse the HA binding by cleaving sialic acid bound from oligosaccharides and help virions reach

their target cells by releasing them from the surface of virus-producing cells or from mucoproteins that are abundant in the respiratory tract.

Ecology of avian influenza viruses

Influenza A can be divided into different subtypes, by sharing related nucleoprotein and matrix proteins but differing in hemagglutinin (HA) and/or neuraminidase (NA). Until now, 16 subtypes of HA (H1-H16) and 9 subtypes of NA (N1-N9) have been identified, some of which have been found in different combinations of HA and NA in various species. Waterfowl and seagulls are the natural reservoirs of AIVs and are hypothesized as the source of all mammalian influenza A viruses (Webster, et al., 1992; Knipe, 2007). Normally AIVs infect non-natural hosts such as chickens, pigs and humans without any clinical signs. However, genetic variability of AIVs is able to generate new virus strains that can cause pandemics (Acheson, 2007).

 Depending on the pathogenicity, AIVs are classified as low pathogenic (LP) or high pathogenic (HP) AIVs (Jackson, et al., 2009). HPAIV can cause clinical illness and disease with 100% mortality in some cases (Alexander, 2000). Only H5 and H7 subtype viruses have been classified as HPAIV, however not all of H5 and H7 subtypes belong to HPAIV (Alexander, 2000). LPAIV strains can cause asymptomatic to mild respiratory and enteric tract infections, followed by reduction in egg production or can lead to secondary bacterial infections (Acheson, 2007). LPAIV of H5 and H7 subtypes are important since they are capable of mutating to HPAIV (Acheson, 2007; Leijon, et

al., 2011). Although HPAI viruses have been intensively investigated, LPAI viruses have increasingly received more attention.

Host immune responses during avian influenza virus infection

AIVs are foreign organisms, which cause infection by invading host cells and replicating within host cells. During their life cycles, they have a relatively short extracellular period and a longer intracellular period during which they undergo replication. The host immune system has mechanisms that can attack against viruses in both phases of their life cycles, and that involve both innate and adaptive immune responses (Abbas A.K, 2007). Influenza viruses code the NS1 protein which is able to inhibit host innate immunity and essential for successful infection in host (Knipe, 2007). However, once the infection is initiated, the adaptive immune response is stimulated and cellular immunity is activated. Viral clearance can only be achieved through adaptive immunity (Yang, 2009).

Innate immunity

The first line of defense against AIVs is provided by the elements of innate immune system, such as mucus, phagocytes and natural killer (NK) cells (Yang, 2009). Proteins of mucus can kill, opsonize or inactivate microbial invaders (Sheehan JK, 2006). Macrophages have been shown to phagocytize cells that are infected by influenza viruses (Shiratsuchi, et al., 2000). Two natural cytotoxic receptors NKp44 and 46 expressed on the surface of NK cells are able to directly interact with the influenza virus

HA protein and activate NK cells, which result in killing of infected cells (Arnon, et al., 2004). The release of Type I IFNs from infected cells indicates the initiation of the immune response to AIV infection. Type I IFNs, including IFN-β and different IFN-α types, are produced in response to virus infection mainly by two mechanisms: Toll-like receptor (TLR) signaling and Retinoid acid inducible gene-I (RIG-1)-like receptors (RLR) (Pichlmair, 2006; Abbas A.K, 2007) .

Toll like receptors (TLRs)

Innate immunity is the first line of host defense by recognizing pathogen-associated molecular patterns (PAMPs) represented by conserved components of microorganisms. The pathogen can be detected by receptors expressed in immune cells. Members of the TLR family have been identified as a primary evolutionarily conserved sensor of PAMPs (Sonkoly, 2008). TLRs represent a set of pattern recognition receptors (PRRs), which recognize PAMPs of microbes (Abbas A.K, 2007). TLR3 binds double-stranded RNA (dsRNA), which is important for type I IFN release and activation of immunity to influenza virus infection (Heer, et al., 2007). TLR7 is activated by single-stranded RNA (ssRNA). The activation of TLR7 stimulates the production of type I IFN on plasmacytoid dendritic cells (pDCs). Influenza virus can induce pDCs and the induction has been proved to be an important mechanism for the IFN release during AIV infection (Yang, 2009).

RIG-I-like receptors (RLRs)

RLRs can sense the presence of viral infection by interacting with vRNA. The melanoma differentiation associated gene-5 (MDA-5) is specific for dsRNAs (Kato, et al., 2006). The other RLR, RIG-I is a cytoplasmic protein which is specifically activated by ssRNA products (Pichlmair, 2006). Upon binding dsRNA, RIG-I can activate transcription factors IRF-3 and IRF-7, and then activate the expression of IFN-α/β (Hiscott, et al., 2006a). A recent study showed that RIG-1 is present in ducks and apparently absent in chickens (Barber, et al., 2010). Since ducks generally do not develop disease with AIV infection, the absence of RIG-1 in chickens may account for the reason why ducks are more resistant and chickens are more susceptible to AIVs.

The Mx1 protein

Type I IFNs are secreted by virus-infected cells and bind to their receptors, which result in triggering the innate antiviral response. IFN-induced proteins such as Mx1 protein play important roles in the antiviral response. Mx1 proteins are interferon-inducible protein with an ability of hydrolyzing GTP, which belongs to the dynamin superfamily of guanosine triphosphatase (GTPase) involved in endocytosis and vesicle transportation (Martens and Howard, 2006). Expression of Mx1 gene interrupts viral replication through a dynamin-like force by wrapping around viral nucleocapsids then blocking the transcription of influenza viruses (Acheson, 2007). It also interferes with the transportation of influenza virus ribonucleoprotein complexes from the cytoplasm to

the nucleus, which leads to the termination of viral transcription and replication. Mx1 cDNA sequences were examined in many chicken breeds with surprisingly high polymorphism (Bernasconi, et al., 1995). An activity of a chicken Mx1 allele was first demonstrated in the White Leghorn breed in Germany (Bernasconi, et al., 1995). Avian cells expressing murine Mx1 protein showed resistance to three strains of influenza A, and three orders of magnitude reduction in influenza virus titers were observed (Garber, et al., 1991). Ko reported that there is an antiviral allele on Mx1 gene in some chicken breeds against influenza virus and vesicular stomatitis virus (VSV) (Ko,et al., 2002). The N631 allele was shown to confer antiviral activity independently of the alternative residues at any other polymorphic sites (Ko, 2004). Although this specific amino acid mutation was demonstrated to be the deciding factor in conferring the Mx1 antiviral activity against influenza (Ko, 2004), there are still some inconsistency on the among different studies (Benfield, 2008; Daviet, 2009). In these studies, no associations between the viral replication and the mutation were identified. Several other factors might be also involved in affecting the antiviral activity of the allele, such as genetic backgrounds or viral strains (Benfield, 2008; Dittmann, 2008). Based on these studies, the reported single amino acid substitution near the C-terminal (N631) (Ko, et al., 2002; Ko, 2004) may not be the only factor in conferring this antiviral phenotype and more studies need to be developed to confirm its antiviral activity.

Adaptive immunity

Both humoral and cell mediated the immunity play roles as specific effector mechanisms in antiviral immunity (Abbas A.K, 2007). The adaptive immunity is not only necessary for the clearance of the infection, but also provides long term immunological memory and confers the protection against re-infection of AIV. In mice, both CD4$^+$ and CD8$^+$ T cells help to clear the primary influenza infection (Moyron-Quiroz, et al., 2004). With AIV infection, B cells contribute to the control of the infection mainly through production of virus-specific antibodies (Abs) and CD4$^+$ T cells appear to promote the Ab response by improving the therapeutic activity of Ab-mediated effector mechanisms (Mozdzanowska, et al., 2005).

Effector CD8$^+$ cells are activated by recognizing (viral) antigens which have been synthesized within cell's cytosol. Specific antigenic peptides presented at the cell's surface as short peptides associated with self class I MHC molecules. CD8$^+$ cells receptors (TCR) recognize the specific antigenic peptide presented by MHC-1 molecules expressed on the surface of antigen presenting cells (APCs). Recognition of an APC by an antigen-specific CTL usually results in the destruction of the APC, through which virus infected epithelial cell in the lung or trachea will be eliminated (Topham, et al., 1997). The principal effector cells which are involved in clearing established viral infections are the virus specific CD8+ cytotoxic T lymphocytes (CTL) (Hikono, 2006).

CD4$^+$ T cells protect hosts from the primary influenza virus infection by enhancing CD8$^+$ T cells and B cell responses. A T helper 1 (Th1) type of CD4$^+$ T cell is required for the effective development of an anti-influenza virus immune response (Yang, 2009).

These cells secrete IFN-γ, which promotes the expansion and recruitment of T cells to the lung and contributes to the efficient clearance of virus infection (Turner, et al., 2007).

Specific antibodies are important and able to protect against viral infections. Mucosal IgA and systemic IgG neutralize virus growth and help to control the infection (Sangster, et al., 2003). The most effective type of antiviral antibody is "neutralizing" antibody, which binds to the viral envelope or capsid proteins and blocks the virus from binding and gaining entry to the host cell (Abbas A.K, 2007). Neutralizing antibodies against the HA protein limit the viral replication and anti-NA antibodies prevent viral release from infected cells to reduce the infection rate (Johansson, et al., 1989).

Circulating anti-influenza virus antibodies, CD8^{+} and CD4^{+} T cells can independently provide protection against secondary infection by influenza virus (Hikono, 2006). After the clearance of a primary influenza virus infection, substantial memory CD8^{+} T cells including both NP and PA specificities persist for a long time, 3-9 weeks post-inoculation, which help to control a secondary infection with the same or closely related virus (Singh, et al., 2010; Turner, et al., 2003). In chickens, the percentage of activated CD8^{+} T cells in lungs is related with the protective immunity against a variant AIV (Seo, et al., 1997).

Small RNA mediated gene expression regulation

Gene expression is a complex process that involves the transcription and translation for synthesizing a functional gene product by using the information from a gene (Nestler

and McClung, 2003; Schwanhausser, 2011). Products are proteins or functional RNAs including rRNAs, tRNAs, siRNAs or miRNAs (Eddy, 2001). By the stimulation of extracellular signals, gene expression regulation is a primary mechanism of development, homeostasis, and adaptation to the environment. The alteration of expression levels of specific genes by the modification of transcription factors is the ultimate step in many signal transductional pathways (Eric J. Nestler, 2002). Gene regulation can occur at the transcription of the gene known as transcriptional regulation, at the translation level known as translational regulation, and after the synthesis of gene products by either post-transcriptional or post-translational regulation (Nancy Jo Trun, 2003). Modifications of RNA, protein or non-coding RNA can play major roles in the biological function of gene products (Schwanhausser, 2011). As one of the non-coding RNAs, microRNAs (miRNAs) have been found to have significant roles in regulating gene expression at post-transcriptional and translational levels (Cui, et al., 2007).

miRNA biogenesis

miRNAs are short, ~22 nt regulatory RNAs, which comprise a large family of regulatory molecules found in all multi-cellular organisms (Bartel, 2004). Over 30% of genes are under the control of miRNAs, which prevent specific genes from being turned into protein and regulate many crucial processes (Slaby, et al., 2009).

The miRNAs are excised from long endogenous transcripts by the sequential action of two endonucleases Drasha and Dicer in different compartments in the cell (Fig. 1) (Sonkoly and Pivarcsi, 2009). The transcripts generated by miRNA genes are referred as

primary-miRNA (pri-miRNA), which can be several kilobases long, and consist of one or more hairpins. The pri-miRNAs is firstly cleaved by Drosha, producing segments 60 ~80 nt long hairpin-shaped precursor miRNA (pre-miRNA). Drosha is an RNase III domains and a dsRNA binding domain. DiGeorge syndrome critical region gene 8 (DGCR8)/Pasha of Drosha, is thought to be involved in recognizing the pri-miRNA (Singh, et al., 2008). The processing complex recognizes the stem-loop structure of the pri-miRNA and excises it by cutting at the base of the hairpin. Drosha cuts about two helical turns after the terminal loop of the hairpin, leaving a 5' phosphate and about a 2-nt 3' overhang, which is typical for RNase III enzymes. Each processed hairpin is now referred as a precursor miRNA (pre-miRNA). This process in animals is slightly different from plants since animals lack homologues to Drosha and DGCR8/Pasha (Rajesh K. Gaur, 2009). Then the pre-miRNA is exported from the nucleus by Exportin 5 in animals in a RanGTP-dependent manner (Beezhold, et al., 2010). Once in the cytoplasm, the pre-miRNAs are processed into mature miRNAs by Dicer. Dicer is a RNase III-like enzyme that has two RIIIDs, a dsRBD, a DEAD-box helicase domain, and a Piwi, Argonaute and Zwille (PAZ) domain (Rajesh K. Gaur, 2009). The PAZ domain binds 3' end of small RNAs and is found in members of the Dicer and Argonaute protein families. The result of Dicer activity is ~22 nt double strand RNA known as the miRNA:::miRNA complex. Which strand of the complex will be the mature miRNA is determined by the free energy of the ends of this complex (Rajesh K. Gaur, 2009). The strand whose 5' end is bound with the lower free energy becomes the guide strand, or mature miRNAs. The passenger strand does not target mRNAs and gets

degraded, although it is unclear how exactly the miRNA passenger strand is removed (Rajesh K. Gaur, 2009). Then the mature miRNA is loaded onto RNA induced silencing complex (RISC). Finally the RISC-complex miRNA binds to its target mRNA and inhibits the protein production. Perfect base pair matching between the RISC-bound miRNA and the target mRNA results in cleavage and degradation of the targets. However, imperfect complementary still can lead to translational repression of the targets (Taganov, et al., 2007).
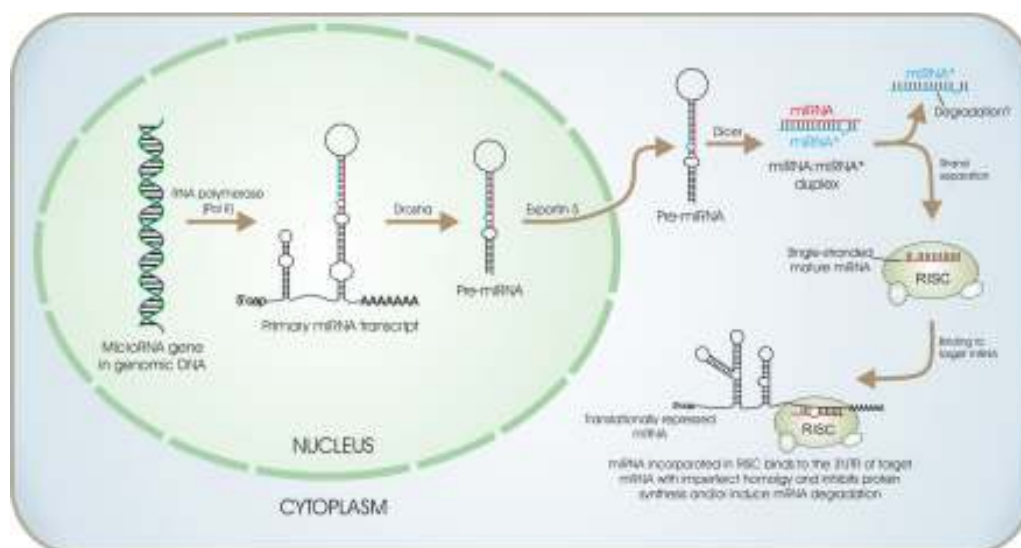
Figure 1 The biogenesis of miRNAs (Sonkoly and Pivarcsi, 2009). Note: Within the nucleus, miRNA gene generated primary miRNAs by the function of RNA polII. Then primary miRNAs will be cutted by Drosha into precursor miRNAs. Precursor miRNAs will be exported from the nucleus into the cytoplasm. By the function of Dicer, precursor miRNAs result in mature miRNAs. With the combination of mature miRNAs and RNA induced silencing complex (RISC), target mRNAs will be binded and translationally repressed.

Regulation of gene expression by miRNA

Upon the recognition of target genes, the manner in which miRNAs regulate expression of their target genes appears to be multiple mechanisms including miRNA-

mediated translational repression at initiation of protein synthesis, translation and at the

level of cap recognition; effects on mRNA stability, inhibition of target genes by

miRNA-mediated deadenylation and inhibiting target genes' elongation.

Target recognition

The 3' UTRs of regulated mRNAs contain multiple copies of sequences with

imperfect complementary to the miRNAs. These sites are necessary for miRNA-

mediated regulation and, accordingly, that target recognition is accomplished at least in

part by base-pairing interactions. The critical region of the miRNA is known as the seed

region, and "seed-pairing" forms the basis for popular target prediction algorithms. The

secondary structure can have profound effects on the ability of miRNAs to recognize

perfect seed matches. miRNAs can bind with perfect or imperfect complementary. It is

important to note that G: U base parings can be tolerated. More than one copy of one

miRNA or combinations of multiple miRNAs can bind to a single mRNA to regulate its

expression. miRNAs freely diffuse in the cytoplasm, and the recognition occurs

passively via random collision. While sometimes, the engagement of miRNA with target

mRNAs can be an active process, it is important to determine where in the cell and when

during the lifetime of an mRNA the recognition takes place. (Rajesh K. Gaur, 2009).

Mechanisms of miRNA-mediated gene regulation

miRNA-mediated translational repression

The miRNA-mediated translational repression can occur at several different levels

(Rajesh K. Gaur, 2009). First of all, miRNAs are involved in the initiation of protein

synthesis, a situation in which regulated mRNAs are prevented from engaging with ribosomes (Pillai, et al., 2007a). Sometimes, miRNAs might induce the degradation of protein even if it has already been synthesized. Several studies have found that miRNAs can inhibit protein synthesis at the level of initiation of translation (Maroney, et al., 2006; Standart and Jackson, 2007). miRNAs can also affect translation at the stage of cap recognition (Humphreys, et al., 2005; Valencia-Sanchez, 2006). Studies have shown that m^7G-capped mRNAs is repressed by miRNA let-7 (Pillai, et al., 2005). Similarly, miRNA-122 can mediate the repression of the amino-acid-starvation-induced release of endogenous cationic amino acid transporter 1 (CAT1) mRNA accompanied by a more effective recruitment of CAT1 mRNA to polysomes in human hepatoma cells (Bhattacharyya, et al., 2006). However, in some cases, miRNAs are able to regulate target gene expression by inhibiting translation without causing a significant, correlative reduction in target mRNA level (Valencia-Sanchez, 2006).

Effects on mRNA metabolism

Some miRNA-mediated mRNA degradation is not restricted in translation. With the exception of direct effects on translation and/or protein accumulation, miRNAs can also exert effects on other aspects of mRNA metabolism to promote mRNA degradation around the miRNA binding sites (Filipowicz, et al., 2010).

At the sites outside of their seed sequences, miRNAs can result in mRNA degradation possibly by directing exonucleases to the mRNAs (Filipowicz, et al., 2008). Researchers suggested that miRNAs can make target mRNAs into processing bodies, or

P bodies, and these cytoplasmic structures are the sites for mRNA degradation (Liu, et al., 2005). The P bodies have been reported to reduce the HIV-1 viral production and infectivity in humans (Nathans, et al., 2009). Knocking down of the miRNA specific Ago protein in *Drosophila* lead to up-regulation of a number of mRNAs that were strongly enriched at the miRNA target sites (Schmitter, et al., 2006). Researches also showed that miRNAs played a role in AU-rich element (ARE)-mediated mRNA instability (Jing, et al., 2005).

miRNA-mediated deadenylation

The miRNA-mediated repression can go through the deadenylation of target mRNAs (Beilharz, et al., 2009). The mRNA polyadenylation process has a versatile means to regulate gene expression, while miRNAs are able to disturb this process and then regultate target mRNA expression. By transfecting three constructs carrying imperfectly matching let-7 target sites in the 3' UTR into mammalian cells, a rapid target mRNA deadenylation was observed, which preceded the translational repression by let-7 miRNA (Beilharz, 2009). In mammalian cells, deadenylation not only contributes to mRNA decay, but also is the translational repression mediated by miRNAs (Beilharz, 2009). Accelerating deadenylation of target mRNAs by miRNAs has been observed in many species, which contributes to miRNA mediated destabilization of target genes (Izaurralde, et al., 2009).

Inhibition of translation elongation

After initiation, miRNAs can affect the translation elongation of target mRNAs. The miRNA-targeted mRNAs are loaded with ribosomes. The miRNAs may prevent protein production by blocking translation elongation or/and by promoting premature dissociation of ribosomes from completely reading the mRNA only in some cases (Eulalio, et al., 2008).

From the discussion of mechanisms of miRNA-mediated regulation, it is apparent that there is no single mechanism of action but multiple means by which miRNAs can exert their effects on regulating gene expression. Determining how and when these mechanisms of suppression are employed by individual miRNAs remains one of the greatest challenges in the field.

miRNAs and immunity

Effects of miRNAs on the host immune system

Studies, in which certain components in the miRNA biogenesis pathway have been knocked out, revealed that miRNA is critical for proper immune system development. Dicer is a key enzyme responsible for regulatory RNA biogenesis and it is responsible for the cleavage of long double-stranded RNAs and short-hairpin RNAs siRNAs and miRNAs (Ambros, 2003; Bartel, 2004). Dicer also plays important roles in the immune system. Two experimental systems to conditionally delete Dicer in mature $CD8^+$ T cells were used to study the function of Dicer in the immune system. Results demonstrated that Dicer is essential for the $CD8^+$ effector T cells response and it also controls $CD8^+$ T

cell activation, proliferation, migration and accumulation during acute infection (Zhang and Bevan, 2010). The function of dicer in the T cell instruction indicates that miRNAs regulate diverse aspects of T cell biology, including basic cellular processes such as proliferation and survival as well as cell-lineage decisions and cytokine production during T helper cell differentiation (Muljo, et al., 2005). B-cell integration cluster (Bic) was originally discovered as recurrent integration site of avian leukosis virus (ALV) in chicken lymphoma cells, and was recently observed as a primary miRNA precursor of miR-155 (Tam, 2001; van den Berg, et al., 2003). Increased expression levels of bic/miR-155 were observed in activated B cells, T cells, macrophages and dendritic cells. Over-expression of miR-155 has been reported in B cell lymphomas, solid tumors and Hodgkin's disease suggesting that the locus may also be linked to cancers (Kluiver, et al., 2006).

miRNAs effects on the development of hematopoietic cell lineages

There are distinct patterns of miRNA expression in different hematopoietic cell lineages by expression profiling studies (Chen, 2004). The miR-150, whose expression is rapidly decreased upon differentiation of naive T cells into Th1 or Th2 subtypes, was selectively expressed in mature resting B and T cells but not in their progenitors or other cell types (Monticelli, 2005). With the activation of CD4$^+$ and CD8$^+$ cells, expression of miR-150 was down-regulated (Wu, 2007). But the detailed function of miR-150 in lymphocytes is still not quite clear (Sonkoly, 2008).

miR-181a is involved in the development of both B and T lymphocytes (Sonkoly, 2008). Over-expression of miR-181a up-regulated the number of B cells. Therefore, miR-181a is a specific positive regulator for B lymphocyte differentiation in mouse bone marrow (Chen, 2004; Sonkoly, 2008). miR-181a also participates in the thymic T cell development. Knocking down of miR-181a impaired both positive and negative selection in thymic cell cultures (Davis, et al., 2007).

Under the same condition, expression of miR-146 was differentially up-regulated in the T helper cell 1 (Th1) subset and abolished in Th2 cells (Monticelli, 2005). The miR-146a is quite important in the regulatory T cell Regulatory (Treg) cell mediated control of Th1 responses (Lu, et al., 2010). High expression of mouse miR-155 in the spleen and bone marrow in a B cell specific manner showed that, accelerated rates of pre-B cell proliferation resulted in the development of a B cell lymphoproliferative disorder by 6 months (Calin and Croce, 2006).

miRNA regulation of signal transduction in immune cells

The miRNAs can regulate signaling pathways by targeting certain components of signal pathways. The regulations of miRNAs enable the multi-gene regulatory capacity or remodel the signaling landscape, and facilitate or reverse the transmission of information to downstream effectors (Tsang, et al., 2007). Emerging evidence suggests that miRNAs affect the response of host cells to signaling molecules such as TLRs or transforming growth factor-β (TGF-β) (Taganov, 2006, Hill and Schmierer, 2007). Serving as nodes of signaling networks, miRNAs affect homeostasis and regulate cancer,

metastasis, fibrosis and stem cell biology (Inui, et al., 2010). To regulate signaling

pathways, miRNAs are targeting gene promoters in default repression or activation of

these pathways and subsequently turn on or shut down the signaling cascades (Barolo

and Posakony, 2002; Halder, et al., 2006). The miRNAs are able to sharpen morphogen

gradient in TGFβ signaling (Martello, et al., 2007), attenuate RAS signaling (Nishino, et

al., 2008) and amplify signals in some cases (Volinia, et al., 2006; Thum, et al., 2008).

The miRNAs can also serve as mediators of crosstalk between signaling pathways and

confer signaling robustness by working as signaling balancers and buffers (Choi, et al.,

2007; Meng, et al., 2007; Yi, et al., 2008).

Several miRNAs including miR-146, miR-132, and miR-155 were significantly up-

regulated in response to lipopolysaccharide (LPS) in human monocytic cells (Taganov,

et al., 2007). In response to various microbial components, miR-146 expression level

was induced by certain members of the TLR family. The expression of miR-146 is also

weakly inducible by the proinflammatory cytokines, IL-1 and TNF, whose cognate

receptors share some of their signaling machinery with TLRs et al., 2007). The miR-146

was reported to participate in the regulation of TLR and cytokine signaling through a

negative feedback loop involving repression of IL-1 receptor-associated kinase 1

(IRAK1) and TNF receptor-associated factor 6 (TRAF6) at the translation level

(Taganov, 2006). The miR-132 has been recently shown to be a CREB-responsive gene

that regulates neuronal outgrowth in the rat by controlling the expression of the GPTase-

activating protein P250GAP (Vo, et al., 2005). Since the CREB family was reported to

be involved in LPS signaling, it may also mediate the up-regulation of miR-132

expression in response to endotoxin in monocytes or the activation goes through the classical inflammation mediator, NF-kappaB (Gilchrist, et al., 2006). The up-regulation of miR-155 is controlled by TLR3 and the IFNα/β receptor in mouse bone-marrow-derived macrophages (Baltimore, 2007). The miR-155 can be induced by both bacterial (i.e., LPS) and viral (i.e., double-stranded RNA) ligands, suggesting miR-155 has important roles in the regulation of antimicrobial defense. The miR-155 is also involved in the development of B cell malignancies. Therefore, miR-155 may provide a potential link between the inflammatory response and tumor (Taganov, et al., 2007).

miRNA and immune responses

Innate immune responses are initiated by binding of microbial ligands to membrane associated PRRPs, known as TLRs. With the treatment of LPS, the expression of miR-146a and miR-155 was significantly increased (Taganov, 2006). Further characterization revealed that miR-146a is induced by the ligands of TLR2, TLR4 and TLR5 recognizing bacterial constituents (Akira and Takeda, 2004). Expression of miR-146 can also be induced by the treatments of TNF-α and IL-1β through NFκB-dependant pathways by targeting on TRAF6 and IRAK1 (Taganov, 2006). In contrast to miR-146a that was stimulated by bacterial products, miR-155 was regulated by viral antigens such as TLR3 ligand poly (I:C) (Baltimore, 2007). The miR-155 expression can also be induced by antiviral response cytokines IFN-β, IFN-γ via TNF-α autocrine/paracrine signaling. The miR-155 is essential for proper function of T and B cells. Therefore, miR-155 has been

considered having complex roles in a broad range of inflammatory mediators for both bacterial and viral stimulations (Sonkoly, 2008).

A group of miRNAs, including miR-16, miR-21, miR-142-3p, miR-142-5p, miR-150, miR-15b and let-7f, have been reported to be associated with T cell activation and differentiation (Sonkoly, 2008). Most of these miRNAs were globally down-regulated with the differentiation of naïve $CD8^+$ T cells into effector cells, which lead to the dramatic up-regulation of the protein-coding genes in effector T cells (Wu, 2007). When T cells are activated by anti-CD3 antibodies *in vitro*, some miRNAs (miR-21, miR-22, miR-24, miR-103, miR-155 and miR-204) were significantly up regulated, while others (miR-16, miR-26, miR-30b,c, miR-150, miR-181) were repressed (Pillai, et al., 2007b). Further studies on these differentially expressed miRNAs are needed to reveal the roles of miRNAs in T cell activation and differentiation process.

miRNAs in antiviral immunity

The miRNAs have been proposed to contribute to the repertoire of host-pathogen interactions during viral infection. Host cellular miRNAs have effects on antiviral immunity by modulating the expression of various viral genes and impinging the viral life cycle, viral tropism and the pathogenesis of viral diseases (Cullen, 2006a). On the other hand, viruses encode viral miRNAs to protect themselves against cellular antiviral responses (Gupta, et al., 2006).

Host miRNAs play important roles in antiviral defense. With sequence-predicted targets within the hepatitis C virus (HCV) genomic RNA, interferon beta (IFN-β) is able

to induce several miRNAs such as miR-196, miR-296, miR-351, miR-431 and miR-448 (David, et al., 2007). Human miR-32 has a direct negative effect on the replication of retrovirus primate foamy virus type 1 (PFV-1), which is mediated through the down-regulation of replication-essential viral proteins encoded by open reading frame 2 (ORF2) (Lecellier, et al., 2005; Cullen, 2006b).

The miRNAs encoded in the viral genome potentially modulate the host cellular environment in order to maximize viral replication by specifically down-regulate host gene expression (Cullen, 2006b). Viral miRNAs might repress host genes to disturb processes in host immune responses such as antigen presentation or the interferon system (Cullen, 2006b). For instance, miR-LAT encoded by the herpes simplex virus-1 (HSV-1) inhibits apoptosis by targeting TGFβ and SMAD-3 of the TGFβ pathway (Gupta, et al., 2006). vRNAs are also capable of interfere with host antiviral immunity by targeting viral genes. The miRNAs encoded by Simian Virus 40 (SV40) reduced susceptibility of infected cells to cytotoxic T cells (Ganem, et al., 2005). A growing list of human viruses, including herpes viruses, polyomaviruses, and retroviruses, have been shown to encode miRNAs targeting a wide range of cellular genes, including cytokines and signaling proteins (Cullen, 2006b).

In some cases, hosts miRNAs and viruses have close interactions. A liver specific miRNA, miR-122, is essential for hepatitis C virus (HCV) replication. miR-122 was specifically detected in human Huh7 liver cells and HCV RNA constructs can only replicate in Huh7 cells. The expression of miR-122 was sequestered in human Huh7 cells stably expressing HCV replica. With the inactivation of miR-122, the level of HCV

viral replicon RNA was significantly reduce by 80 percent (Jopling, 2005). Meanwhile, miR-122 is also able to help host to clean up viruses. Over expression or inhibited expression of miR-122 demonstrated that miR-122 specifically inhibited borna disease virus (BDV) protein synthesis, viral replication and transcription in infected human oligodendroglial cells (Qian, et al., 2010).

Transcriptome analysis: from microarray to next generation sequencing

The responses of host cells to pathogenic microorganisms are the major focus of host-pathogen interactions. Pathogen induced phenotypic changes in host cells are always accompanied by remarkable changes in gene expression (Jenner and Young, 2005). Understanding the gene expression profiling at the global level is the key to get insights into cellular functions of interaction between hosts and viruses. There are thousands of genes targeted by viruses or other pathogens in many cell types that participate the mediation of inflammation, responding to interferon, activating or attenuating immune responses and activating lymphocytes (Jenner and Young, 2005). The profiles of genome-wide genes in terms of alterations in response to specific biological stimuli provide valuable insights for interpreting functional elements of the genome, revealing the molecular constituents of cells, and also understanding developmental and disease processes. Therefore, high throughput transcriptome profiling technologies are needed to carry out this type of studies.

Microarray analysis

Different types of technologies have been developed to interrogate transcriptome abundance. Hybridization-based microarray analysis have been the primary transcriptomic high-throughput tool for almost two decades, which has accelerated the study of transcriptome analysis by profiling thousands of genes simultaneously (Li, et al., 2008). Microarray technology can provide more comprehensive, relatively unbiased information of all gene networks including members of gene families, ligands, receptors and transcription factors.

While powerful, microarrays do have several limitations. (1) Microarray design requires a *priori* knowledge of the genome or genomic features, which affects the effectiveness if the genome information is incomplete, incorrect or the genome annotations are outdated; (2) Cross-hybridization problems between similar sequences. In this case, non-repetitive fraction of genomes and complicates analysis of related genes, alternatively spliced transcripts, allelic gene variants and SNPs are restricted (Okoniewski and Miller, 2006); (3) Microarray is an indirect quantification by hybridization-signal intensities (Cassone, et al., 2007). High signal to noise ratios and competitive hybridization on microarrays limit the dynamic range of high-confidence data. Low abundance transcripts are difficult to be detected; (4) The variety of available microarray formats, preparative methodologies and analytical approaches could limit the reproducibility of microarray data (Draghici, et al., 2006).

Digital gene profiling by next generation sequencing (NGS)

Digital expression profiling using next generation sequencing (NGS) promises to reduce or in some cases eliminate the weakness of microarrays. In addition, the massively-parallel sequencing platforms have improved the affordability of comprehensive genomic analysis (Hurd and Nelson, 2009).

Next generation sequencing

Next generation sequencing refers to a new class of instrumentation that combines rapid sample preparation and dramatically enhanced total throughput and promises to bring unprecedented capability in genomic research.

The first high-throughput sequencing platform is the 454 pyrosequencing by Roche, which is using emulsion PCR of DNA library fragments affixed to micro-beads. It has individual sequence lengths of up to 500 bp, and a single run can generate 500 Mb sequence. Having the longest reads of all of the NGSs, 454 sequencing is good at *de novo* genome assemblies. However, the inaccuracies in calling homopolymeric stretches of sequences are the major drawback (Janitz, 2008).

Applied Biosystems offers the next-generation SOLiD System sequencing technology, which is based on sequential ligation of dye-labeled oligonucleotides, enabling massively parallel sequencing of clonally amplified DNA fragments. Features inherent to this system, such as mate-paired analysis and two-base encoding, enable studies of complex genomes by providing a greater degree of accuracy. In addition, this system contains the necessary computing power and software to perform base calling on

a large scale without the need for additional computing hardware. A single run on the

SOLiD 3 has 35 bp read length with up to 400 million DNA tags, which yields nearly 15

Gb of total sequences. The throughput and scalability of the SOLiD System holds great

promise for large-scale re-sequencing, digital gene expression, hypothesis-free

chromatin immunoprecipitation (ChIP) and methylation studies (Janitz, 2008).

With the ability to sequence more than 60 million DNA fragments simultaneously,

the first generation of the Illumina Genome Analyzer (GA) had revolutionized the ability

to generate large volumes of sequence data in a short time at low cost. The massively

parallel nature of the Illumina GA system of sequencing millions of DNA fragments

facilitate the diverse application range and brings us significantly closer to understanding

the links between genotype and phenotype and in establishing the molecular basis of

many diseases. The updated version, the Genome Analyzer II, generates gigabases of

high-quality data per day with an uncomplicated process that requires just one operator

and less than 6 hours of hands-on time. The newly developed HiSeq sequencing system

offers unprecedented output, generating up to 600 Gb per run with highest yield of data.

Runs for HiSeq sequencing is quite easy to set up. One single run can process over 200

gene expression samples at a price less than microarrays. Unrivalled output and ease of

use provide the lowest overall operating cost (Goldfeder, et al., 2011).

Small RNA deep sequencing

Historically, identification of small non-coding RNA has been performed by

computational prediction, qPCR, and sequencing the bacterial cloning cDNA libraries

(Xu, et al., 2006; Gu, et al., 2007). With the development of the NGS, the massively parallel sequencing provides a superior sensitivity at high sequencing depth to discover especially those miRNAs with low abundance and novel miRNAs that are not able to be identified using traditional cloning approaches. Small RNA deep sequencing has been widely used to profile miRNAs, including both chicken miRNAs and Marek's disease virus miRNAs (Burnside, et al., 2008; Glazov, 2008; Wang, 2009a).

Transcriptome analysis by RNA-seq

RNA-Seq is a powerful sequencing-based method that enables to identify, profile and quantify RNA transcripts across the entire transcriptome. After poly (A) selection, mRNA is fragmented to small fragments and converted into a cDNA library, which provides a simple and more comprehensive way to measure transcriptome composition and to discover new genes by high-throughput sequencing without bacterial cloning of cDNA input (Mortazavi, et al., 2008). Studies using this technology have already changed our views regarding the extent and complexity of transcriptomes in an organism and improved our understanding of transcriptome.

The principle procedure of RNA-Seq is shown in Figure 2 (Mortazavi, et al., 2008). With two rounds of poly (A) selection, RNA is fragmented to an average length of 200-bp and then converted into cDNA by random priming. The cDNA is then converted into a molecular library for sequencing, and the resulting reads are mapped onto the genome. Transcriptome prevalence is normalized to reads per kilobase of exon per million mapped sequence reads (RPKM) (Mortazavi, 2008). RNA-Seq can analyze

novel transcripts, novel isoforms, alternative splice sites, rare transcripts and cSNPs in

one experiment (Wang, 2009b).
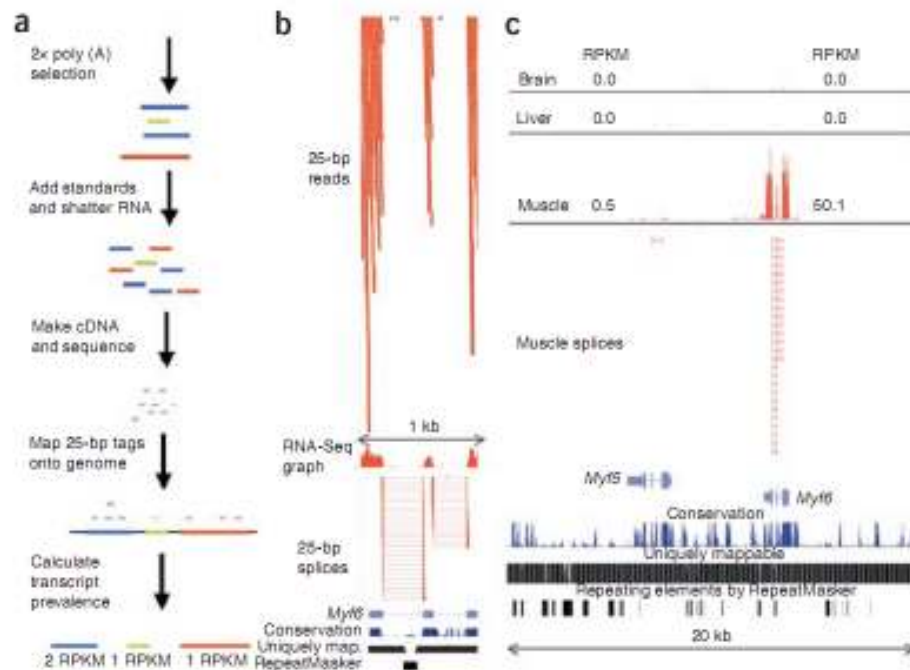
Figure 2. Outline of RNA-Seq procedure (Mortazavi, 2008). Note: A, With polyA selection, RNA is fragmented to small pieces and then converted into cDNA by random priming. The cDNA is then converted into a molecular library for sequencing, and the resulting reads are mapped onto the genome. Normalized transcript prevalence is calculated by statistical software. B, Primary data map uniquely in the genome to a 1-kb region, including reads that span introns. Each point represents the number of reads covering each nucleotide, per million mapped reads. C, It can also detect and quantify differential expression. For example, these two genes are muscle specific and only got mapped on muscle. The expression could be high or low.

Comparing with microarray analysis, RNA-Seq has several advantages including:

(1) RNA-Seq is not dependent on prior knowledge about the target sequences.

Knowledge of genome annotation is helpful but not required. (2) The digital nature of

RNA-Seq allows for much higher resolution and a large dynamic range and sensitivity of differential expression. (3) The survey of a transcriptome is more accurate in RNA-Seq, because the quantification of signal from sequence-based approaches is directly based on counting sequence tags rather than relative measures between samples. Therefore, RNA-Seq offers both single-base resolution for annotation and "digital" quantification at the RNA level, which allows the entire transcriptome to be analyzed in a high-throughput and quantitative manner (Wang, et al., 2010). However, the expense per sample for RNA- Seq is still a limiting factor in preventing researchers from sequencing multiple biological replicates per group, which are needed for statistically-significant analysis.

Next generation sequencing and microarrays are complementary technologies. Even NGSs have advantages over microarrays, microarray analysis may still be an option for gene expression profilings. Sample preparations for microarrays are easier and the image analysis is faster and more convenient. It will provide enough information by surveying thousands of genes on a single array, if gene expression changes are the specific interests. On the other hand, if large numbers of samples need to be tested, microarray analysis may be much useful as a screening tool (Hurd and Nelson, 2009). Analyzing microarray gene expression data is reaching a maturity with many good software tools readily available both commercially and with open-source software. Meanwhile bioinformatics for the data analysis of huge datasets brings us the bottle neck of the application of NGSs. The data analysis support for digital gene expression using sequencing is simply not as powerful as microarrays.

Currently there is a merger between microarrays and NGSs. For example, it makes sense to use microarrays to study a large population of samples, identify representative samples or outliers, and then apply digital gene expression to that smaller number of samples. Promising results from the NGS will generate new content which can be analyzed on microarray platforms. The parameters such as the cost, throughput, and data analysis would be major factors to be considered for choosing the technology for transcriptome analysis.

CHAPTER II

ASSOCIATIONS OF CHICKEN MX1 POLYMORPHISM WITH ANTIVIRAL

RESPONSES IN AVIAN INFLUENZA VIRUS INFECTED EMBRYOS AND

BROILERS

Introduction

Avian influenza virus (AIV) is a type A virus of the family *Orthomyxoviridae*. Mx1 proteins are interferon (IFN) induced GTPases and show antiviral activity in human and mice (Haller and Kochs, 2002; Haller, et al., 2007). The replication of influenza virus and other negative-strand viruses were affected through the interruption of the viral transcription by Mx expression (Acheson, 2007). Mx1 protein was reported to have intrinsic antiviral activity and be responsible for the influenza virus resistance in mammals (Arnheiter, et al., 1990). Chickens only have one Mx gene (Mx1) and are originally showed to lack antiviral activity (Bernasconi, et al., 1995). Chicken Mx1 protein is encoded by Mx1 gene and is composed of 705 amino acids in which a tripartite GTP-binding motif and a leucine zipper motif are conserved (Watanabe, 2007). There are a number of natural mutations in the chicken Mx1 gene. Interestingly the Ser (S) to Asn mutation at amino acid position 631(N) caused by a single nucleotide polymorphism at nucleotide postion 2,032 (G to A) of Mx1 cDNA had an antiviral activity *in vitro* on mouse 3T3 cell lines (Ko, et al., 2002; Ko, 2004). Mouse 3T3 cell lines expressing chicken Mx1 gene carrying N631 (NN genotype resistant allele) had significantly lower percentage of infected cells than those cell lines expressing S631 (SS

genotype susceptible allele) Mx1 mRNAs (Ko, 2004) . A skewed allele frequency distribution in the S631N mutation was observed in different chicken populations, in which the viral resistant amino acid N had a much higher frequency in Chinese native chicken breeds than in highly selected commercial lines (Li, et al., 2006). However, there is no consistent conclusion regarding antiviral activity of chicken Mx gene either *in vivo* or *in vitro* assays (Ewald, et al., 2011). Many studies in Mx antiviral function have been focused on *in vitro*. In order to fully understand antiviral activity of Mx1 protein, we sought evaluate whether the S to N mutation is associated with the Mx antiviral activity *in vivo* and *in ovo*.

Materials and methods

Experimental inoculation of embryos and chickens

Embryos and chickens were from the cross of Mx1 heterozygous (S631N) broiler parents with expected segregating ratio of 1:2:1 in the progeny. Total of 119 thirteen-day old embryonated chicken eggs were inoculated with 10^6EID$_{50}$ H5N9 AIV. Hemagglutination (HA) assay was used to evaluate virus replication in chicken embryos. Hemagglutinating units in allantoic fluid were determined at 48 hours post-inoculation for all infected embryos. DNA isolated from leg muscles was used for Mx1 genotyping.

For in vivo challenge study, birds were housed in negative pressure Horsfall-Bauer, temperature control isolation units and provided with water and commercial feed *ad libitum*. Chicken combs were collected to isolate DNA samples for Mx1 genotyping on day 1. Sixteen chickens from each genotype were used for experimental inoculations. At

one week of age, 8 chickens from each genotype were inoculated with 0.2 ml of

CK/TX/02/H5N3 virus containing 10^6 EID_{50}/ml while the remaining 8 chickens were

inoculated with PBS (mock treatment) by the intra-choanal cleft route. All birds were

sacrificed at 4 days post-infection (dpi) and chicken lung samples were collected for

RNA isolation. Virus replication at 4 dpi was determined through the lung total RNA

samples by real-time RT-PCR for influenza matrix gene (M protein) using AgPath-ID™

AIV- M kit (Ambion, Austin, TX) following the manufacturer's instructions. Control

RNA was extracted from serially diluted H5N3 virus ($10^{1.5}$–$10^{5.5}$ $\log_{10}$ EID_{50}/ml). A

standard curve was generated with control viral RNAsand the amount of viral RNA in

the samples was converted into $\log_{10}$ EID_{50}/ml by interpolation as described previously

(Lee and Suarez, 2004). The animal experiment was performed according to the

guidelines approved by the Institutional Animal Care and Use Committee, Texas A&M

University.

Genotyping and sequencing

Genomic DNA was extracted from the embryonic leg tissues or chick combs using

Wizard Genomic DNA purification kit (Promega, Madison, USA) following

manufacture's protocols. Genotyping of S631N was carried out by both PCR-RFLP. For

PCR-RFLP, the PCR primers (+MX1SER: 5' GCTCTCCTTGTAGGGAGCCAG 3';

+MX1ASN: 5' TAATAATAATAACCTCTCCTTGTAGGGAGCGAA 3' and -

MX1SERASN: 5' GTGACTAATTCTGCTGGTCAGTAAC 3') were designed to

amplify a fragment in the coding region of chicken Mx1 mRNA sequence (Accession

No. Z23168) including the substitution S631N. PCR conditions were 94 $^\circ$C 2 min, followed by 35 cycles at 94 $^\circ$C for 30 s, 55 $^\circ$C for 1 min, 72 $^\circ$C for 1 min, and a final extension at 72 $^\circ$C for 7 min. PCR products were then loaded on Synergel (0.7% agarose and 1.66% synergel) and run in a TAE buffer at 110 volts for 5 hours. Different genotypes were determined by the size of PCR products due to the different sizes of forward primers for alleles G and A.

In order to identifiy other Mx1 mutations in the coding region besides the S631N mutation (the mutation G2032A on the nucleotide sequences), total RNA isolated from chicken lung samples was reverse transcribed into cDNA by random hexamers using ThermoScript RT-PCR system (Invitrogen, Carlsbad, CA, USA) in a reaction volume of 20 µl. Using these cDNAs as template, the complete coding region of chicken Mx1 gene (Accession No. Z23168) was amplified by three pairs of primers (P1F1:5' GCTCGGTGCAGTACCTGCGG 3', P1R1: 5' TTCCCCACGGCCTCTCTGGC 3'; P2F2: 5' GCCAGAGAGGCCGTGGGGAA 3', P2R2: 5' CCCGTCCGCGGTACTGGTCT 3'; P3F3: 5' CCAGTACCGCGGACGGGAGT 3', P3R3: 5' GGTTGCTGCTAATGGAGGATTTTGC 3'). PCR conditions were 94 $^\circ$C 7 min, followed by 35 cycles at 94 $^\circ$C for 30 s, 55 $^\circ$C for 1 min, 72 $^\circ$C for 1 min, and a final extension at 72 $^\circ$C for 10 min. The PCR products were purified by PCR purification kit (Qiagen, Valencia, CA, USA). Purified PCR products were sent to the Gene Technologies Lab in Texas A&M University to do sequencing with Perkin Elmer ABI Big Dye reaction by ABI 3100 Automated Sequencer (Life Technologies, Carlsbad, CA, USA).

Gene expression

Primers (forward: 5' GCACACACCCAACTGTCAGCGA 3'; reverse: 5' CCCATGTCCGAAACTCTCTGCGG 3') were designed to examine chicken Mx1 gene expression by real-time PCR with an amplicon of 157 base pairs across both exons 10 and 11. cDNA from the previous reverse transcription reactions was used as templates. PCR reactions were performed in a 10 µl volume containing a $1\times$ SYBR Green Master Mix on an ABI Prism 7900HT sequence detection system (Applied Biosystems, Foster City, CA). The amplification condition were 50 °C for 2 min, 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 59 °C for 1 min, and a final soak at 4 °C. Chicken β-actin gene (forward: 5' ACG TCT CAC TGG ATT TCG AGC AGG 3'; reverse: 5' TGC ATC CTG TCA GCA ATG CCA G 3') was used for normalization by the same amplification condition. The expression levels of chicken Mx1 were measured in terms of threshold cycle value (CT) and normalized to β-actin using $2^{-\Delta CT}$(Schmittgen and Livak, 2008).

Statistical Analysis

Data were subjected to one way ANOVA of JMP 8.0 (SAS Institute, Cary, NC). Significance threshold of $P < 0.05$ was considered statistically significant.

Results and discussion

A PCR-RFLP method followed by Synergel gel separation was developed to identify three different Mx1 genotypes. Two forward primers (+MX1SER and

+MX1ASN) working with the same reverse primer (-MX1SERASN) generated PCR

products of different sizes based on which allele (A or G) was present in the sequence.

PCR products for genotypes NN (allele A resulting in amino acid N) were 211 bp long,

while for genotype SS were 199 bp (allele G resulting in amino acid S), and two bands

for the heterozygous NS genotype.

Chickens with NN genotype were considered resistant, while birds with SS

genotype were considered as the susceptible (Ko, et al., 2002; Ko, 2004).Virus titers

(Hemagglutinating units) for chicken embryos are shown in Figure 3. At 48 hours pi, SS

birds had a higher virus titer than NN genotype birds, although the difference was no

significant ($P > 0.05$). For young chicks, there was a similar tendency with SS genotype

birds having the highest virus titer (3.04) followed by NS and NN genotypes (Fig. 4).

Our results show that chickens of NN genotype had a tendency for lower virus titers than

SS birds, although the differences were not significant ($P > 0.05$).
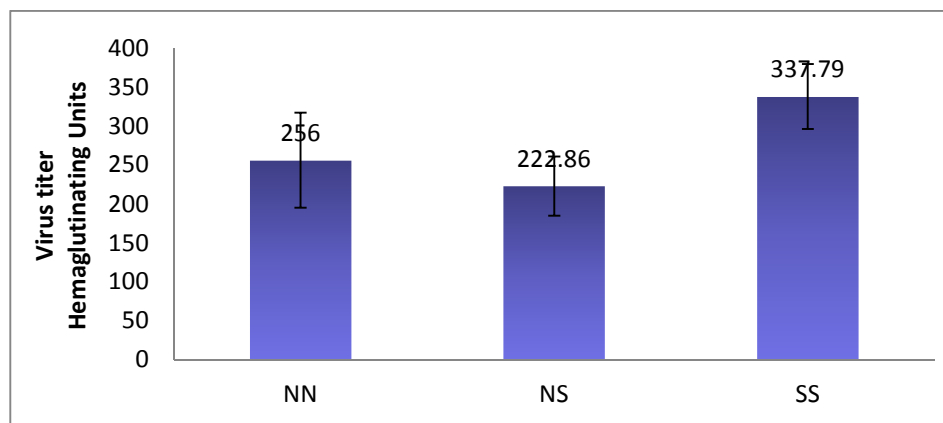
Figure 3. Virus titers of different Mx1 genotypes in chicken embryos. Note: Virus titers were determined by HA tests 48 hours post inoculation. Embryos with NN genotype had a lower virus titer (Hemaglutinating Units) than SS, although the difference was not significant ($P > 0.05$).
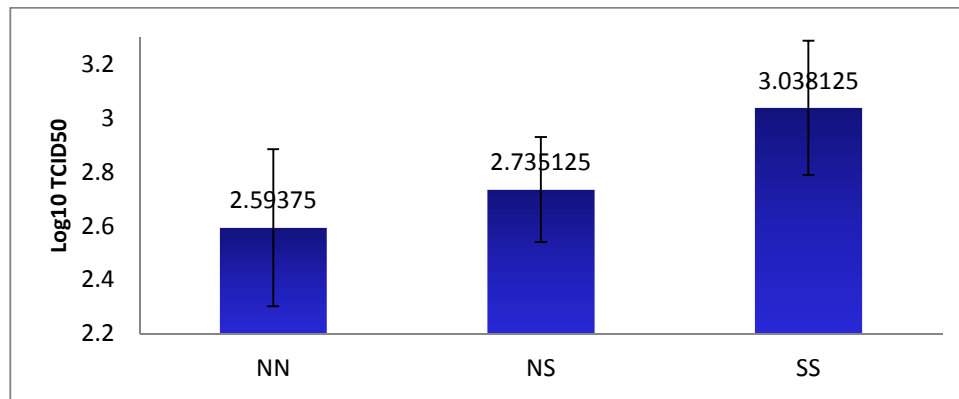
Figure 4. Virus titers of different Mx1 genotypes in chickens. Note: Virus titers were determined by the real-time PCR 4 days post inoculation. Chickens with NN genotype had a lower virus titer than SS birds, although the difference was not significant ($P > 0.05$).

Genetic resistance to avian viruses has been studied for a long time and differences in genetic susceptibility or resistance are known to exist for major viral pathogens such as avian leukosis and Marek's disease viruses in poultry (Bumstead, 1998). Genetic resistance is a quantitative trait with multiple genes contributing toward resistance (Ewald, et al., 2011). Significant associations between AIV titers and genotypes of the amino acid 631 mutation on chicken Mx1 protein have been reported for both embryos and chicks (Ko, 2004; Li, et al., 2006; Yin, et al., 2010b). However, such an association has not been confirmed in the current study. We speculate that other coding region mutations besides amino acid 631 might contribute AIV resistance. Three pairs of primers were designed to amplify the entire coding region of chicken Mx1 gene and PCR products were sequenced to screen for additional muations. Besides the substitution at amino acid 631 (nucleotide G2032A), thirteen additional point mutations were identified within the chicken Mx1 coding region and genotypes of these mutations are presented in Table 1. More mutations (7 out of 13) cosegregated with S631N (G2032A)

except one insertion at the nucleotide postion of 1544 bp on the chicken Mx1. It was

very interesting that at the beginning of the coding region (nucleotide 60-300) NS and

SS genotypes had the same genotype which was different from NN chickens, suggesting

that a recombination event might have taken place around this region. Therefore, more

mutations should be identified to increase the marker density and haplotype analysis

should be preformed to validate the antiviral activity of chicken Mx1 gene.

Table 1. Genotypes of identified sigle nucleotide polymorphisms in the coding region of chicken Mx1 cDNA sequences

S631N (G2032A)	C12T	A61G	G121C	C124T	A155T	A280G	T792C	A813G	G922A	A1015G	1544 bp	A1545G	A1747G
NN	CC	GG	CC	TT	TT	GG	TT	AA	GG	AA		AA	AA
NN	CC	GG	CC	TT	TT	GG	TT	AA	GG	AA		AA	AA
NN	CC	GG	CC	TT	TT	GG	TT	AA	GG	AA		AA	AA
NN	CC	GG	CC	TT	TT	GG	TT	AA	GG	AA		AA	AA
NN	CC	GG	CC	TT	TT	GG	TT	AA	GG	AA		AA	AA
NN	CC	GG	CC	TT	TT	GG	TT	AA	GG	AA		AA	AA
NS	TT	AA	GG	CC	AA	AA	TC	AG	GA	AG	AA	GG	AG
NS	TT	AA	GG	CC	AA	AA	TC	AG	GA	AG	AA	GG	AG
NS	CT	AA	GG	CC	AA	AA	TC	AG	GA	AG	AA	GG	AG
NS	CT	AA	GG	CC	AA	AA	TC	AG	GA	AG	AA	GG	AG
NS	TT	AA	GG	CC	AA	AA	TC	AG	GA	AG	AA	GG	AG
NS	CT	AA	GG	CC	AA	AA	TC	AG	GA	AG	AA	GG	AG
SS	TT	AA	GG	CC	AA	AA	CC	GG	AA	GG		GG	GG
SS	TT	AA	GG	CC	AA	AA	CC	GG	AA	GG		GG	GG
SS	TT	AA	GG	CC	AA	AA	CC	GG	AA	GG		GG	GG
SS	TT	AA	GG	CC	AA	AA	CC	GG	AA	GG		GG	GG
SS	TT	AA	GG	CC	AA	AA	CC	GG	AA	GG		GG	GG
SS	TT	AA	GG	CC	AA	AA	CC	GG	AA	GG		GG	GG

Mx1 mRNA expression increases in chicken embryo fibroblasts (CEF) by the treatment with poly I: C (Yin, et al., 2010a). Using a chicken 44K gene expression microarray we have previously shown that , the expression level of Mx1 gene was highly up-regulated (11.46 folds) after AIV infection (Data not published). In the current study, we examined chicken Mx1 gene expression levels in the three Mx1 genotypes by real-time RT-PCR. The Mx1 gene expression levels in AIV infected and non-infected chickens are shown in Figure 5. For AIV infected and non-infected chickens, Mx1 mRNA expression was greater in NN genotype birds than SS birds, although the difference was not statistically significant ($P > 0.05$). This result was consistent with a previous study, in which chickens of the NN genotype had greater Mx1 expression levels than the SS genotype in both Beijing-You and White Leghorn lines. (Yin, et al., 2010b). After AIV infection, Mx1 mRNA expression in chickens of the NS genotype was significantly up-regulated compared to the non-infected ones ($P<0.05$). It is important to mention that there was a nuleotide insertion (A) at nucleotide position 1544 of the chicken Mx1 cDNA, which was only presented in the heterozygous chickens. Whether the insertion is related with the up-regulation of Mx1 mRNA expression in chickens of the NS genotype after AIV infection warrants further investigation.
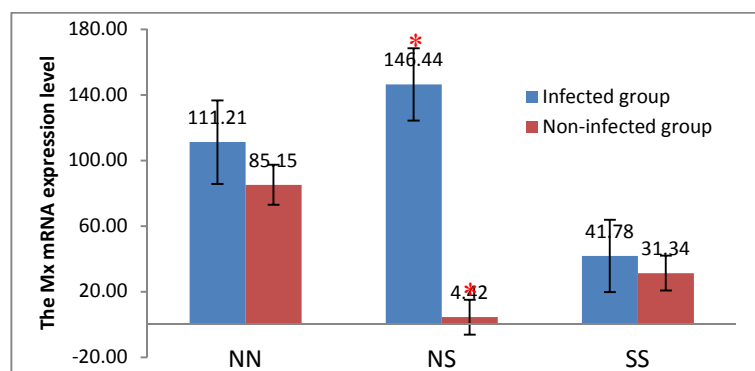
Figure 5. The Mx1 mRNA expressions of infected and non-infected birds with different genotypes. Note: The mRNA expression of chicken Mx gene was detected by real-time PCR. For AIV infected and non-infected chickens, Mx1 mRNA expression was greater in NN genotype birds than SS birds, although the difference was not statistically significant ($P > 0.05$); Mx1 mRNA expression in chickens of the NS genotype was significantly up-regulated compared to the non-infected heterozygous birds ($P < 0.05$).

The sample size can affect significance level in a given experiment. We used 119 chicken embryos and 48 chicks in the current study. The relatively small population sizes, especially for the chick animal trial, plus standard variation among individual chickens need be taken into account for the reason why the differences were not significant.

Meanwhile, there has been a discussion of whether the S631N mutation in the chicken Mx1 protein plays an important antiviral role in AIV infection (Benfield, et al., 2008; Daviet, et al., 2009). Different anti-viral responses to HPAIV infections were found among different genetic lines and limited resistance to LPAIV was observed in a recent study (Sironi, et al., 2011). They concluded that no significant association between anti-AIV response and polymorphisms of Mx1 gene in a genome-wide (60K SNP array) analysis existed (Sironi, et al., 2011). Another report showed that the

antiviral effect of type I IFN in chicken CEF cells were not dependent on Mx1 protein, suggesting that chicken Mx1 probably does not play a critical role in the inhibition of AIV replication in chickens (Hartle, et al., 2011).

In addition, genetic background might play a significant role in the anti-AIV response in the chicken. In 2007, Mx1 polymorphisms of 294 samples from 37 strains of 17 chicken breeds were examined. White Leghornhad a higher frequency of the resistance allele (N) on Mx1 proteins, broilers had a higher frequency of the susceptible allele(S) (Watanabe, 2007). In another study, the positively antiviral variant was not able to inhibit influenza virus replication in primary chicken embryo fiboblasts from a commercial broiler population (Benfield, et al., 2008). Broilers and layers have developed different characteristics of their immune systems. Broilers are specialized in the production of a short term humoral response, while layers have a long-term humoral response in combination with a strong cellular mediated response (Koenen, et al., 2002). These might cause the distinct Mx1 activities after AIV infection. Since broilers were used in the current study, it might not easy to identify significant antiviral activities.

The antiviral activity of chicken Mx1 gene might also depends on different strains of influenza viruses. A range of influenza A virus strains were tested for murine and human Mx1 proteins and remarkable differences among them were found (Dittmann, 2008). This was also confirmed in chicken studies. The Mx1 N631 variant alleles had effects on reductions in morbidity, early mortality, viral shedding and cytokine responses in chicken infected withHPAIV (H5N2), while the results were not reproduced when a LPAIV (H5N9) was used (Ewald, 2011). LPAIV strains (H5N9 and H5N3) were used in

our studies, which might determine that no significant effects were identified in the different Mx1 genotypes.

In summary, we developed a very efficient PCR-RFLP approach using a single PCR reaction to screen the genotypes of S631N of the chicken Mx1 gene. Our results suggest that embryos or chickens carrying the resistant N631 had lower virus titers and greater Mx1 gene expression levels than those chickens carrying the susceptible S631, although there were no statistically significant differences among them observed. However, a comprehensive analysis including the association of chicken Mx1 S631N variant alleles with virus titers, Mx1 mRNA expression in different genotypes and identification of additional Mx1 point mutations, has expanded our knowledge in the potential role of chicken Mx1 protein on the genetic resistance to AIV in chickens and its potential application in the poultry breeding industry.

CHAPTER III

IDENTIFICATION OF DIFFERENTIALLY EXPRESSED MIRNAS IN CHICKEN

LUNG AND TRACHEA WITH AVIAN INFLUENZA VIRUS INFECTION BY A

DEEP SEQUENCING APPROACH*

Introduction

Avian influenza virus (AIV) is a type A virus of the family *Orthomyxovirida.*
Although wild aquatic birds such as waterfowl and seagulls are their natural reservoir
(Webster, 1992), land-based birds including chickens may also be infected, which causes
significant economic losses to the poultry industry, and raises a great public health threat
due to potential host jump from animals to humans (Webby and Webster, 2003).

miRNAs are non-coding, single-stranded RNAs of 19~23 nucleotides which
represent a novel class of gene regulators and play important roles in a variety of
biological processes in both plants and animals (Ambros, 2003; Carrington and Ambros,
2003; Bartel, 2004). miRNAs modulate gene expression largely at the post-
transcriptional level by different mechanisms including direct cleavage of targeted
mRNAs (Bartel, 2004), inhibition of translation (Zhang, 2006) or even up-regulation of
translation (Vasudevan, et al., 2007). miRNAs are involved in different biological
activities such as development, differentiation, growth and metabolism (Lee, et al.,
1993a; Guo, 2005; Hatfield, 2005; Lindsay, 2008). Recently, in mammals, miRNAs

have been reported to participate in the regulation of immunity, including development and differentiation of lymphocytes, monocytes and neotrophils, and modulation of inflammation (Lindsay, 2008). miR-150 expresses in mature B and T cells derived from mouse hematopoietic stem cells, and is able to block early B cell development when expressed prematurely (Zhou, et al., 2007). miR-181a is an intrinsic modulator of T cell sensitivity and selection in mice (Li, et al., 2007a). After exposure of THP-1 (human acute monocytic leukemia cell line) cells to lipopolysaccharides (LPS), miR-146 is identified as an inhibitor of signalling proteins of the innate immune responses by NF-kappaB (Taganov, 2006). miRNAs have also been found to be critical effectors in the regulation of viral pathogenesis. Two human encoded miRNAs (miR-136 and miR-507), have been shown to have potential binding sites for the genes that code for the polymerase basic 2 (PB2) and hemmagglutinin (HA) proteins and are reported to be involved in the pathogenesis of H5N1 AIV (Scaria, et al., 2006). All of these evidences suggest that certain miRNAs might be important in the modulation of AIV infections in chickens.

In order to effectively control AIV infection in poultry, it is essential to elucidate the mechanisms of virus pathogenesis in chickens. However, how host cells interact with AIVs during infection in poultry remains poorly understood. Identification of differentially expressed miRNAs in AIV infected chickens will pave a novel avenue to understand host-virus interaction. With the development of next generation sequencing, massively parallel sequencing holds great promise for expression profiling (Torres, et al., 2008) and it can provide a superior sensitivity at high sequencing depth to discover

especially those miRNAs with low abundance and novel miRNAs that are not able to be identified using traditional cloning approaches. Deep sequencing has been previously used to profile both chicken miRNAs and Marek's disease virus miRNAs (Burnside, 2008; Glazov, 2008). In the current study, a Solexa Sequencer was used to deep sequence differentially regulated chicken miRNAs in H5N3 infected and non-infected SPF chickens. Our results will expand the list of miRNAs which might be related to the host immune responses in animals.

Materials and methods

Sample collection and RNA isolation

One week old commercial Leghorn SPF chickens were randomly divided into two groups (4 chickens per group), housed in a negative pressure Horsfall-Bauer, temperature control isolation unit, and provided with water and commercial feed *ad libitum*. At three weeks of age, one group was inoculated with 0.2 ml H5N3 virus containing 10^6 EID_{50}/ml, while the other group was inoculated with PBS by the intra-choanal cleft route. Based on the pilot study at 4 dpi, depression and severely congested lungs and trachea were observed. Therefore, all chickens were euthanized at 4 days post-inoculation, and lung and trachea epithelial layers were collected for RNA isolation. The animal experiment was performed according to the guidelines approved by the Institutional Animal Care and Use Committee, Texas A&M University.

Two pools of total RNA samples (two random chickens per pool) of each tissue from each group were generated. Total RNAs were isolated using Trizol (Invitrogen,

Carlsbad, CA) following the manufacturer's protocol. Dnase I (Ambion, Austin, TX)

digestion was carried out after RNA isolation according to manufacturer's instructions.

The RNA concentration and purity were determined by measuring absorbance at 260 nm

and A260/A280 ratio using a NanoDrop ND-1000 spectrophotometer (Nanodrop

Technologies, Wilmington, DE). RNA samples were stored at -80 ºC until further use.

Viral Titration

Virus replication at 4 dpi was determined by real-time RT-PCR for influenza matrix

gene using AgPath-ID™ AIV- M kit (Ambion, Austin, TX) following the

manufacturer's instructions. Control RNA was extracted from serially diluted H5N3

virus ($10^{1.5}$–$10^{5.5}$ $\log_{10}$ EID_{50}/ml). Standard curve was generated with control viral

RNAs. The amount of RNA in the samples was converted into $\log_{10}$ EID50/ml by

interpolation as described previously (Lee and Suarez, 2004).

Small RNA sequencing and analysis

For small RNA library construction, RNA samples were prepared using the DGE-

Small RNA Sample Prep Kit (Illumina, San Diego, CA). In brief, RNA was purified by

polyacrylamide gel electrophoresis (Pages, et al.), to enrich for molecules in the range of

18-30 nt, and ligated with proprietary adapters to both 5' and 3' termini of the RNA.

Ligated samples were used as templates for cDNA synthesis and then amplified with 15

PCR cycles to produce sequencing libraries. A total of eight Solexa-ready small RNA

templates were prepared through two gel purification steps to eliminate concatenated

adaptors without inserts. Purified cDNAs were quantified using the Quant-iT PicoGreen dsDNA Kit (Invitrogen, Carlsbad, CA) and diluted to 10 nM for sequencing on an Illumina 1G Genome Analyzer at the Genome Sequencing Center of Baylor College of Medicine. Cluster generation was performed and clusters were sequenced.

For each sample, sequences were first passed through an adaptor filter that searched for sequences that were followed by at least 6 nucleotides of the 3' adaptor. Out of the total reads, any reads without a perfect 10-nt linker subsequence were directly discarded adjoining the insert, yielding of length 10 nt or longer that were subject to further processing. All full-length, exact sequence matches to *E. coli* (k12, o157:h7, o157:h7 edl933, cft073) were discarded to eliminate possible sequence artifacts arising from the amplification process. All unique sequence reads with a minimum read count of 10 were aligned with precursor chicken miRNA sequences from miRBase version 13.0 (Ambros, et al., 2003; Griffiths-Jones, 2004; Griffiths-Jones, et al., 2006; Griffiths-Jones, et al., 2008a). Reads of each miRNA were the sum of exact and loose matches (± 4 bp) to known miRNAs. For each sample, counts were normalized to the total number of small RNA sequences, and then for each miRNA, the normalized number of counts was compared between groups or between tissues.

Fisher's Exact test was used to identify differentially expressed miRNAs at a 5% false discovery rate. False discovery rate (FDR) (Q values) was calculated by R program according to Benjamin and Hochberg's method (Benjamini, 1995). Ratios were calculated as the ratio of normalized reads of infected over non-infected group or lung over trachea.

Statistics related to over representation of functional categories were performed using DAVID, which is based upon a Fisher Exact statistic methodology similar to that described by Al-Shahrour et al (Al-Shahrour, et al., 2004). A $P < 0.05$ was considered as significant.

Novel miRNAs from both lung and trachea libraries were identified using the method by Creighton et al. 2009 (Creighton, et al., 2008). In brief, the first step is to take the sequence reads that did not map to known miRNA precursors, mapped them to the chicken genome, and got an exact sequence match along with 100 bases flanking either side. About 220-bp sequences were then tested for miRNA-like hairpin structure, and folded with the Vienna package (Hofacker, et al., 1994). The miRNA hairpin structures that meet the Ambros (Ambros, et al., 2003) criteria were identified. Specifically, the putative miRNA must lie on one arm of a single-loop hairpin with minimum free energy less than -25 kcal/mol. The sequence reads that were appropriately placed in these miRNA-like hairpins were considered as 'putative mature miRNAs' (pmms). Then we examined the pmms for cross-species conservation of the hairpin structure. The sequence reads with strong conservation of the mature miRNA, significant conservation of the hairpin arm opposite the mature miRNA, and little or no conservation of the hairpin loops were considered as novel miRNAs.

Confirmation by TaqMan MicroRNA Assay

To determine the expression of miRNAs by quantitative RT-PCR (qRT-PCR), TaqMan microRNA assay was preformed. The specific stem-loop RT primers of miR-1a,

miR125b, miR-146a and U6 were obtained commercially from Applied Biosystems (Foster City, Calif., USA). In brief, cDNA was synthesized from total RNA by using the gene specific primers according to the protocol of TaqMan Micro RNA Assays (Applied Biosystems, CA, USA). Reverse transcriptase reactions contained 10 ng of RNA samples, 3 μl stem loop RT primer and reagents from a TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems, CA, USA). The 15 μl reactions were incubated for 30 min at 16 °C, 30 min at 42 °C and 5 min at 85 °C, and then held at 4 °C.

Real-time PCR was performed by using gene specific probes and a pair of primers (TaqMan MicroRNA Assays, Applied Biosystems) and reagents of TaqMan 2* Universal PCR Master Mix (No AmpErase UNG) (Applied Biosystems, CA, USA). The 20 μl PCR reactions included 1.33 μl RT-PCR product, 10 μl PCR master mix, and 1μl 20* TaqMan MicroRNA Assay mix (Applied Biosystems, CA, USA). These reactions were incubated at 95 °C for 10 min, followed by 40 cycles at 95 °C for 10 s, 60 °C for 40 s and 72 °C for 1 s by ABI 7900 Realtime PCR instrument (Applied Biosystems, CA, USA). All reactions were run in triplicates. The threshold cycle was defined as the fractional cycle number at which the fluorescence passes the fixed threshold. The expression levels of miR-1a, miR-125b and miR-146a in each sample were measured in terms of threshold cycle value and normalized to U6 using $2^{-\Delta\Delta CT}$ (Schmittgen, 2008). U6 was used as an internal control.

Results

Virus titration

Virus replication was examined by real-time RT-PCR for influenza matrix gene from total RNAs of lung and trachea at 4 dpi. The titer of infected samples was 12.29 $\log_{10}$ EID_{50}/ml in lung, and 3.89 $\log_{10}$ EID_{50}/ml in trachea. Both non-infected lung and trachea samples were negative.

Small RNA libraries

A total of 278,398 and 340,726 filtered high quality reads were obtained from chicken lungs and trachea, respectively (Table 2). In the libraries of chicken lungs, 98,849 and 179,549 reads were obtained from infected and non-infected lungs, respectively. Out of these reads, 52,363 of these high quality reads were exact matches while another 9,357 reads were loose matches to known chicken miRNAs. All reads with a perfect match to mature miRNA sequences from chicken deposited in miRBase (http://microrna.sanger.ac.uk/) with insertions or deletions of 1-4 nucleotides was considered as a loose match to represent dicer-processing products from each of the chicken miRNA precursors. An example from gga-mir-181a is shown in Figure 6. Here we saw that the sample had 77 copies of a sequence that was identical to that of the mature gga-mir-181a (denoted by a '*'). In addition we observed significant alternate processing at the 3'-end that was characteristic of miRNAs in various copy numbers. Loose matches were defined by sequence reads that aligned with chicken miRNA consensus sequence with 1-4 mismatches. These may represent sequencing errors (when

occurring in low copy numbers), mutations and/or RNA editing events. In the libraries of chicken trachea, 250,460 reads were obtained from infected trachea and 90,266 were obtained from non-infected trachea. Out of these reads, 44,243 of these high quality reads were exact matches and another 4,178 reads were loose matches to known chicken miRNAs. The sum of exact and loose match reads was used as the total number of reads for each miRNA.

Table 2. Number of reads of miRNAs from AIV infected and non-infected chicken lungs and trachea

	Infected lungs	Non-infected lungs	Infected trachea	Non-infected trachea
High quality /both adapter	98849	179549	250460	90266
Exact match to known chicken miRNAs	10939	41424	36405	7838
Loose match to known chicken miRNAs	1441	7916	2926	1252

```
gga-mir-181a
GCCATCTTTGGATAGCTTCAGTGAACATTCAACGCTGTCGGTGAGTTTGAGAACTAAGAAAAACCATCGACCGTTGACTGTACCTTGAGGT
.(((....)))...(((((((.(.(((.((((((..(((((((.((((...........))))))))))))))))))).))).).))))))) Reads
*********************AACATTCAACGCTGTCGGTGAGT****************************************************
.....................AACATTCAACGCTGTCGGTGAGT................................................... 71*
.....................AACATTCAACGCTGTCGGTGAGTTT................................................. 66
.....................AACATTCAACGCTGTCGGTGAGTT.................................................. 45
.....................AACATTCAACGCTGTCGGTGAG.................................................... 40
.....................AACATTCAACGCTGTCGGTGA..................................................... 24
.....................AACATTCAACGCTGTCGGTG...................................................... 11
.....................AACATTCAACGCTGTCGGT....................................................... 1
.....................................................................AACCATCGACCGTTGACTGTA..........  1
......................................................................ACCATCGACCGTTGACTGTAC.........  1
```

Figure 6. Reads aligning with of gga-mir-181a. Notes: Sequence of gga-mir-181a hairpin is described in the top line. The brackets below denote the secondary structure. Reads aligning with the mature gga-mir-181a sequence as found in miRBase is denoted by a '*' sequence. The +/- 4 nt matches representing reads that perfectly matched the gga-mir-181a precursor with insertions or deletion of 1-4 nucleotides from the 5' and 3' ends are shown below the mature miRNA exact matches. The number of reads corresponding to each sequence is shown at the right hand end of the dotted line.

To display the distribution of miRNAs at each library, reads of individual miRNA within each group were transformed to $\log_{10}$. The plots of distributions of transformed reads for each miRNA from each group are shown in Figure 7. Most miRNAs had around 10-100 reads in these four groups. There was no significant difference in the medians of the four libraries ($P > 0.05$) indicating they had similar distributions of the miRNA reads.
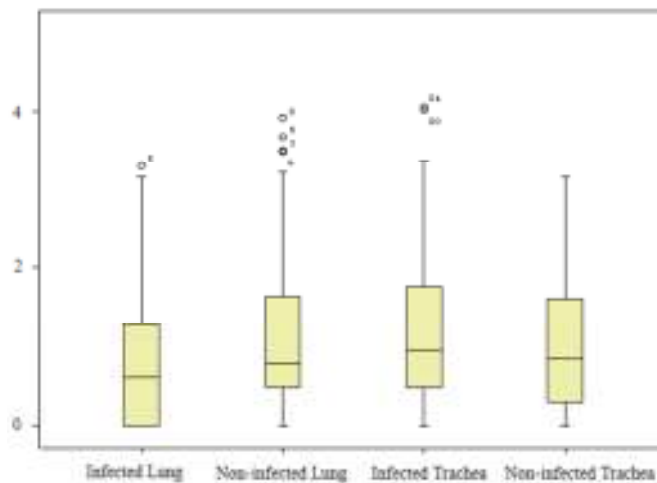
Figure 7. Distributions of reads from each group. Notes: All the reads in each group have been transformed by Log_{10}.

Of the 475 distinct Gallus gallus miRNA entries in miRBase (Ambros, et al., 2003; Griffiths-Jones, 2004; Griffiths-Jones, et al., 2006; Griffiths-Jones, et al., 2008a), 377 miRNAs were identified in chicken lungs and 149 miRNAs in chicken trachea. In addition, we identified 87 potential novel miRNA sequences, in which 84 novel

miRNAs were identified in chicken lung and 3 miRNAs were identified in chicken trachea.

miRNAs expression profiling analysis

In the present study there were four different groups: two tissues (lung and trachea) and two states per tissue (infected and non-infected states). Two comparisons were made between infected *vs.* non-infected within tissue and another two comparisons between lung and trachea at either infected or non-infected state. Differentially expressed miRNAs in each comparison were identified ($P < 0.05$, $Q < 0.05$ and fold change > 2). Within tissues, 73 and 36 miRNAs were differentially expressed between infected and non-infected groups in lungs and trachea, respectively (Tables 3 and 4). When between tissues were compared, 57 and 78 miRNAs were differentially expressed at infected and non-infected states, respectively (Tables 3 and 4).

More miRNAs (60 out of 73 miRNAs in lungs and 27 out of 36 miRNAs in trachea) were down-regulated than up-regulated with AIV infection in both lungs and trachea. When infected *vs.* non-infected was compared, 5 miRNAs (miR-106, miR-1729, miR-1798, miR-429 and miR-1711) were only expressed in the non-infected lungs, while 2 miRNAs (miR-1576 and miR-1636) were only expressed in infected lungs (Table 3). Between infected and non-infected trachea, two (miR-1612 and miR-1458) out of 27 down-regulated miRNAs were expressed only in non-infected trachea (Table 4).

Table 3. Differentially expressed miRNAs between infected and non-infected lungs (P < 0.05, Q < 0.05 and Ratio > 2)

miRNA	Position on chromosomes	Reads in infected	Reads in non-infected	Ratio infected/non-infected (Normalized)
gga-mir-1576	chr13:18532632-18532726	8	0	-[1]
gga-mir-1636	chr 15: 4729959-4730046	6	0	-
gga-mir-206	chr3: 110390439-110390514	101	9	20.38
gga-mir-1793	chr9: 25115521-25115617	25	5	8.36
gga-mir-1599	chr7: 25926968-25927029	48	13	6.71
gga-mir-1701	chr4: 82234261-82234337	27	9	5.45
gga-mir-449	chrZ: 16040613-16040698	38	14	4.93
gga-miR-140	chr11: 21030641-21030735	1438	803	3.25
gga-mir-1416	chrZ: 34596479-34596567	17	10	3.09
gga-mir-1458	chr9: 11743528-11743637	25	15	3.03
gga-mir-1612	chr9: 6031748-6031831	39	31	2.29
gga-mir-1a-1	chr20: 8107831- 8107901	551	471	2.12
gga-mir-1a-2	chr2: 105673483- 105673567	593	523	2.06
gga-mir-103-2	chr4: 91906889-91906971	49	148	0.49
gga-mir-99a	chr1: 102424333-102424413	23	92	0.45
gga-mir-456	chr3: 32679710-32679821	30	122	0.45
gga-let-7c	chr1: 102425086-102425169	735	3059	0.44
gga-mir-181b-2	chr17: 10220137- 10220221	20	89	0.41
gga-let-7j	chr26: 1442697-1442779	648	2938	0.40
gga-mir-15b	chr9: 23742966- 23743056	9	41	0.40
gga-let-7a-1	chr12: 6302911-6303000	658	3020	0.40
gga-let-7a-2	chr24: 3380993-3381064	671	3113	0.39
gga-let-7g	chr12: 2809078-2809160	342	1623	0.38
gga-mir-181a-2	chr17: 10218497-10218587	57	283	0.37
gga-mir-200b	chr21: 2585642-2585726	14	72	0.35
gga-let-7a-3	chr1: 73421272-73421347	892	4699	0.34
gga-mir-100	chr21: 3372894-3372973	15	79	0.34
gga-mir-30d	chr2: 148337263-148337326	60	334	0.33
gga-mir-30e	chr23: 5248414-5248509	20	112	0.32
gga-mir-181a-1	chr8: 2001561-2001664	65	366	0.32
gga-mir-181b-1	chr8: 2001750-2001838	18	106	0.31
gga-let-7k	chr26: 1442897-1442979	53	327	0.29
gga-mir-125b	chr1: 102457647-102457736	19	119	0.29
gga-mir-1b	chr23: 4663912-4663975	10	63	0.29
gga-mir-146b	chr6: 24570060-24570164	102	691	0.27
gga-mir-10a	chrun_random: 379304-379377	86	598	0.26
gga-mir-101-2	chr8: 29051918-29051993	18	136	0.24
gga-mir-27b	chrZ: 41157642-41157738	5	41	0.22
gga-mir-144	chr19: 5824123-5824207	11	94	0.21
gga-let-7f	chr12: 6302497-6302583	947	8228	0.21
gga-mir-33-1	chr1: 51372282-51372350	2	19	0.19
gga-mir-126	chr17: 8431742-8431825	25	250	0.18
gga-mir-1456	chrZ: 44167486-44167595	2	20	0.18
gga-mir-22	chr19: 5352096- 5352195	4	43	0.17
gga-mir-16c	chr4: 4048689- 4048759	6	65	0.17

Table 3 continued

miRNA	Position on chromosomes	Reads in infected	Reads in non-infected	Ratio infected/non-infected (Normalized)
gga-mir-101	chrZ: 28037874- 28037952	19	219	0.16
gga-mir-30a-5p	chr3: 85102239- 85102310	68	785	0.16
gga-mir-30c-1	chr23: 5249637-5249725	3	37	0.15
gga-mir-146c	chr4: 92169271- 92169399	18	243	0.13
gga-mir-26a	chr2: 4467516- 4467592	98	1326	0.13
gga-mir-451	chr19: 5823968-5824036	93	1287	0.13
gga-mir-146a	chr13: 7555593- 7555691	7	105	0.12
gga-mir-21	chr19: 7322072-7322168	46	771	0.11
gga-mir-24	chrZ: 41158175-41158242	18	344	0.10
gga-mir-17-3p	chr1: 152248781-152248865	2	44	0.08
gga-mir-20a	chr1: 152248306-152248403	1	23	0.08
gga-mir-23b	chrZ: 41157406-41157491	12	285	0.08
gga-mir-142-3p	chr19: 496983-497070	2	49	0.07
gga-mir-142-5p	chr19: 496983-497070	2	49	0.07
gga-mir-17-5p	chr1: 152248781-152248865	2	55	0.07
gga-mir-19b	chr1: 152248183-152248269	1	31	0.06
gga-mir-30c-2	chr3: 85126853-85126924	1	34	0.05
gga-mir-739		7	241	0.05
gga-mir-15c	chr4: 4049055-4049130	1	46	0.04
gga-mir-15a	chr1: 173700493-173700575	2	102	0.04
gga-mir-16-2	chr9: 23742791-23742884	1	67	0.03
gga-mir-16-1	chr1: 173700351-173700434	1	107	0.02
gga-mir-106	chr4: 3970359-3970439	0	27	+[2]
gga-mir-1729	chr15: 769596-769666	0	24	+
gga-mir-1798	chr20: 9654914-9655009	0	24	+
gga-mir-429	chr21: 2580812-2580895	0	22	+
gga-mir-1711	chr12: 17010140-17010207	0	18	+

Note: [1] Specifically expressed in infected lungs. [2] Specifically expressed in non-infected lungs.

Table 4. Differentially expressed miRNAs between infected and non-infected trachea (P < 0.05, Q < 0.05 and Ratio > 2)

miRNA	Position on chromosomes	Reads in infected	Reads in non-infected	Ratio infected/non-infected (Normalized)
gga-mir-1a-2	chr2: 105673483- 10567356	11423	441	9.34
gga-mir-1a-1	chr20: 8107831- 8107901	10438	405	9.29
gga-mir-455-3p	chr17:6021890-6021975	29	2	5.23
gga-mir-455-5p	chr17:6021890-6021975	29	2	5.23
gga-mir-34b	chr24: 5684900-5684983	870	82	3.82
gga-mir-499	chr20: 2599334-2599424	154	15	3.70
gga-mir-34c	chr24: 5685637-5685710	540	64	3.04
gga-mir-140	chr11: 21030641-21030735	2334	383	2.20
gga-mir-1b	chr23: 4663912-4663975	66	11	2.16
gga-mir-456	chr3: 32679710-32679821	37	28	0.48
gga-mir-125b	chr1: 102457647-102457736	31	24	0.47
gga-mir-148a	chr2: 32053543-32053610	116	94	0.44
gga-let-7b	chr1: 73422101-73422185	1761	1460	0.43
gga-mir-181a-1	chr8: 2001561-2001664	59	49	0.43
gga-mir-146c	chr4: 92169271- 92169399	42	36	0.42
gga-mir-181a-2	chr17: 10218497-10218587	51	45	0.41
gga-let-7g	chr12: 2809078-2809160	413	373	0.40
gga-mir-206	chr3: 110390439-110390514	95	88	0.39
gga-mir-222	chr1: 114216027-114216124; chr1: 114218422-114218519	14	13	0.39
gga-let-7k	chr26: 1442897-1442979	58	54	0.39
gga-mir-181b-2	chr17: 10220137- 10220221	20	21	0.34
gga-let-7i	chr1: 34895687-34895770	123	136	0.33
gga-mir-126	chr17: 8431742-8431825	17	19	0.32
gga-mir-99a	chr1: 102424333-102424413	14	16	0.32
gga-mir-30a-3p	chr3: 85102239- 85102310	66	76	0.31
gga-mir-30a-5p	chr3: 85102239- 85102310	74	87	0.31
gga-mir-146b	chr6: 24570060-24570164	78	107	0.26
gga-mir-181b-1	chr8: 2001750-2001838	18	25	0.26
gga-mir-30d	chr2: 148337263-148337326	46	68	0.24
gga-mir-100	chr21: 3372894-3372973	25	41	0.22
gga-mir-92	chr1: 152248070-152248070	24	40	0.22
gga-mir-15a	chr1: 173700493-173700575	3	9	0.12
gga-mir-451	chr19: 5823968-5824036	14	89	0.06
gga-mir-10a	chrun_random: 379304-379377	11	139	0.03
gga-mir-1612	chr9: 6031748-6031831	0	9	-[1]
gga-mir-1458	chr9: 11743528-11743637	0	7	-

Note: [1] Specifically expressed in non-infected trachea.

In the comparisons between tissues, only few miRNAs (6 out of 57 miRNAs in the infected state and 1 out of 78 miRNAs in the non-infected state) were highly expressed in trachea compared to lungs (Tables 5 and 6). Under the infected state, 28 miRNAs were specifically expressed in lungs and 23 miRNAs were expressed at higher levels in lungs than in trachea. In the non-infected state, 11 miRNAs specifically expressed in lungs and 66 miRNAs were expressed at higher levels in lungs than trachea. Of particular interest, miR-1a, miR-140, and miR-449, which were highly expressed in infected tracheas than the non-infected ones, and also were differentially expressed between infected tissues (higher expression levels in infected trachea than infected lungs). In the tissue comparison under the non-infected state, miR-206 was the only miRNA that had higher expression level in trachea than in lungs. In general, those highly abundant miRNAs were observed across all four groups examined (Table 7).

Table 5. Differentially expressed miRNAs between infected lungs and trachea (P < 0.05, Q < 0.05 and Ratio > 2)

miRNA	Position on chromosomes	Reads in lungs	Reads in trachea	Ratio lung/trachea (Normalized)
gga-mir-1599	chr7: 25926968-25927029	48	0	-[1]
gga-mir-1612	chr9: 6031748-6031831	39	0	-
gga-mir-1701	chr4: 82234261-82234337	27	0	-
gga-mir-1458	chr9: 11743528-11743637	25	0	-
gga-mir-1793	chr9: 25115521-25115617	23	0	-
gga-mir-181b-2	chr17: 10220137- 10220221	20	0	-
gga-mir-1416	chrZ: 34596479-34596567	17	0	-
gga-mir-7-1	chrZ: 8107831-8107901	16	0	-
gga-mir-7b	chr1: 73422101-73422185	15	0	-
gga-mir-1638	chr5: 58712377- 58712463	13	0	-
gga-mir-144	chr19: 5824123-5824207	11	0	-
gga-mir-1761	chr8: 17523212-17523292	9	0	-

Table 5 continued

miRNA	Position on chromosomes	Reads in lungs	Reads in trachea	Ratio lung/trachea (Normalized)
gga-mir-1576	chr13:18532632-18532726	8	0	-
gga-mir-1814	chr4: 61722590-61722663	8	0	-
gga-mir-1452	chrZ: 8107831-8107901	7	0	-
gga-mir-1815	chr6: 29566734-29566810	7	0	-
gga-mir-122-1	chrZ: 649337-649413	6	0	-
gga-mir-122-2	chrurn_random: 12066796-12066872	6	0	-
gga-mir-1636	chr15: 4729959-4730046	6	0	-
gga-mir-1659	chr7: 14764187-14764287	6	0	-
gga-mir-1786	chr14: 7801714-7801822	6	0	-
gga-mir-218-1	chr4: 77774698-77774806	5	0	-
gga-mir-218-3		5	0	-
gga-mir-7-2	chr10: 14823525-14823623	5	0	-
gga-mir-1467	chr2: 141373919-141374028	4	0	-
gga-mir-1630	chr9: 1883593-1883649	4	0	-
gga-mir-1816	chr2: 90603851-90603955	4	0	-
gga-mir-7-3	chr28: 4436025-4436119	4	0	-
gga-mir-10a	chrun_random: 379304-379377	86	11	19.81
gga-mir-451	chr19: 5823968-5824036	93	14	16.83
gga-mir-184	chr10: 22146245-22146318	11	3	9.29
gga-mir-193b	chr14: 759453-759535	11	3	9.29
gga-mir-181a-2	chr17: 10218497-10218587	57	18	8.02
gga-mir-205a	chr26: 2896047-2896142	6	2	7.60
gga-mir-92	chr1: 152248070-152248070	42	24	4.43
gga-mir-99a	chr1: 102424333-102424413	23	14	4.16
gga-mir-126	chr17: 8431742-8431825	25	17	3.73
gga-let-7i	chr1: 34895687-34895770	177	123	3.65
gga-mir-146b	chr6: 24570060-24570164	102	78	3.31
gga-mir-30d	chr2: 148337263-148337326	60	46	3.30
gga-mir-181a-1	chr8: 2001561-2001664	65	51	3.23
gga-let-7b	chr1: 73422101-73422185	2008	1761	2.89
gga-mir-206	chr3: 110390439-110390514	101	95	2.69
gga-mir-148a	chr2: 32053543-32053610	114	116	2.49
gga-mir-30a-5p	chr3: 85102239- 85102310	68	74	2.33
gga-let-7k	chr26: 1442897-1442979	53	58	2.32
gga-mir-30a-3p	chr3: 85102239- 85102310	58	66	2.23
gga-mir-30e	chr23: 5248414-5248509	20	23	2.20
gga-let-7g	chr12: 2809078-2809160	342	413	2.10
gga-mir-456	chr3: 32679710-32679821	30	37	2.05
gga-mir-103-2	chr4: 91906889-91906971	65	82	2.01
gga-mir-1b	chr23: 4663912-4663975	10	66	0.38
gga-mir-34b	chr24: 5684900-5684983	112	870	0.37
gga-mir-34c	chr24: 5685637-5685710	78	540	0.37
gga-mir-499	chr20: 2599334-2599424	13	154	0.21
gga-mir-1a-1	chr20: 8107831- 8107901	551	10438	0.13
gga-mir-1a-2	chr2: 105673483- 10567356	593	11423	0.13

Note: [1] Specifically expressed in infected lungs.

Table 6. Differentially expressed miRNAs between non-infected lungs and trachea (P < 0.05, Q < 0.05 and Ratio > 2)

miRNA	Position on chromosomes	Reads in lungs	Reads in trachea	Ratio lung/trachea (Normalized)
gga-mir-30c-2	chr3: 85126853-85126924	34	0	-[1]
gga-mir-19b	chr1: 152248183-152248269	31	0	-
gga-mir-1798	chr20: 9654914-9655009	24	0	-
gga-mir-1456	chrZ: 44167486-44167595	20	0	-
gga-mir-1711	chr12: 17010140-17010207	18	0	-
gga-mir-122-1	chrZ: 649337-649413	17	0	-
gga-mir-203	chr:5: 53206814-53206911	16	0	-
gga-mir-122-2	chrurn_random: 12066796-12066872	14	0	-
gga-mir-1599	chr7: 25926968-25927029	13	0	-
gga-mir-1638	chr5: 58712377- 58712463	12	0	-
gga-mir-1761	chr8: 17523212-17523292	12	0	-
gga-mir-144	chr19: 5824123-5824207	94	2	23.63
gga-mir-146a	chr13: 7555593- 7555691	105	3	17.60
gga-mir-739		241	7	17.31
gga-mir-106	chr4: 3970359-3970439	27	1	13.57
gga-mir-16-1	chr1: 173700351-173700434	107	4	13.45
gga-mir-193a	chr18: 6423770-6423846	26	1	13.07
gga-mir-142-3p	chr19: 496983-497070	49	2	12.32
gga-mir-142-5p	chr19: 496983-497070	49	2	12.32
gga-mir-1729	chr15: 769596-769666	24	1	12.07
gga-mir-20a	chr1: 152248306-152248403	23	1	11.56
gga-mir-21	chr19: 7322072-7322168	771	40	9.69
gga-mir-17-5p	chr1: 152248781-152248865	55	3	9.22
gga-mir-16-2	chr9: 23742791-23742884	67	4	8.42
gga-mir-24	chrZ: 41158175-41158242	344	21	8.24
gga-mir-30e	chr23: 5248414-5248509	112	7	8.04
gga-mir-15c	chr4: 4049055-4049130	46	3	7.71
gga-mir-223	chr:4: 232949-233048	15	1	7.54
gga-mir-29a	chr1: 3236329-3236417	15	1	7.54
gga-mir-29c	chr26: 2511658-2511746	15	1	7.54
gga-mir-17-3p	chr1: 152248781-152248865	55	3	7.37
gga-mir-451	chr19: 5823968-5824036	1287	89	7.27
gga-mir-101	chrZ: 28037874- 28037952	219	16	6.88
gga-mir-126	chr17: 8431742-8431825	250	19	6.61
gga-mir-26a	chr2: 4467516- 4467592	1326	103	6.47
gga-mir-130c	chr19: 7145027-7145120	25	2	6.28
gga-mir-23b	chrZ: 41157406-41157491	285	23	6.23
gga-mir-30c-1	chr23: 5249637-5249725	37	3	6.20
gga-mir-193b	chr14: 759453-759535	23	2	5.78
gga-mir-101-2	chr8: 29051918-29051993	136	12	5.70
gga-mir-15a	chr1: 173700493-173700575	102	9	5.70
gga-let-7f	chr12: 6302497-6302583	8228	781	5.30
gga-mir-27b	chrZ: 41157642-41157738	41	4	5.15
gga-mir-30a-5p	chr3: 85102239- 85102310	785	87	4.54
gga-mir-30a-3p	chr3: 85102239- 85102310	656	76	4.34
gga-mir-16c	chr4: 4048689- 4048759	65	8	4.08

Table 6 continued

miRNA	Position on chromosomes	Reads in lungs	Reads in trachea	Ratio lung/trachea (Normalized)
gga-mir-181a-1	chr8: 2001561-2001664	366	49	3.76
gga-mir-200b	chr21: 2585642-2585726	72	10	3.62
gga-mir-22	chr19: 5352096- 5352195	43	6	3.60
gga-let-7a-3	chr1: 73421272-73421347	3193	468	3.43
gga-mir-146c	chr4: 92169271- 92169399	243	36	3.39
gga-mir-146b	chr6: 24570060-24570164	691	107	3.25
gga-mir-181a-2	chr17: 10218497-10218587	283	45	3.16
gga-let-7k	chr26: 1442897-1442979	327	54	3.04
gga-mir-15b	chr9: 23742966- 23743056	41	7	2.94
gga-let-7a-2	chr24: 3380993-3381064	3113	540	2.89
gga-mir-99a	chr1: 102424333-102424413	92	16	2.89
gga-mir-1b	chr23: 4663912-4663975	63	11	2.88
gga-mir-199-1	chr17: 5667150-5667243	541	95	2.86
gga-mir-199-2	chr8: 4732773-4732880	541	95	2.86
gga-let-7a-1	chr12: 6302911-6303000	3020	532	2.85
gga-let-7a-3	chr1: 73421272-73421347	1506	266	2.85
gga-let-7j	chr26: 1442697-1442779	2938	522	2.83
gga-mir-221	chr1: 114218926-114219024	129	23	2.82
gga-mir-128-1	chr7: 3222815032228231	43	8	2.70
gga-mir-125b	chr1: 102457647-102457736	119	24	2.49
gga-mir-30d	chr2: 148337263-148337326	344	68	2.47
gga-mir-103-2	chr4: 91906889-91906971	243	51	2.40
gga-let-7i	chr1: 34895687-34895770	633	136	2.34
gga-mir-107	chr6: 20487964-20488044	77	17	2.28
gga-let-7c	chr1: 102425086-102425169	3059	679	2.26
gga-mir-456	chr3: 32679710-32679821	122	28	2.19
gga-let-7g	chr12: 2809078-2809160	1623	373	2.19
gga-mir-10a	chrun_random: 379304-379377	598	139	2.16
gga-mir-181b-1	chr8: 2001750-2001838	106	25	2.13
gga-mir-181b-2	chr17: 10220137- 10220221	89	21	2.13
gga-mir-34b	chr24: 5684900-5684983	337	82	2.07
gga-mir-206	chr3: 110390439-110390514	9	88	0.05

Note: [1] Specifically expressed in non-infected lungs.

Table 7. High abundant miRNAs in all four libraries

Name	Uninfected lung (179,549)[1]	Infected lung (98,849)	Uninfected trachea (90,266)	Infected trachea (250,460)
gga-let-7b	4,732	2,008	1,460	1,761
gga-let-7f	8,228	947	781	1,949
gga-let-7a-3	3,193	562	468	917
gga-let-7c	3,059	735	679	1,327
gga-mir-140	803	1,438	383	2,334
gga-mir-1a-2	523	593	441	11,423
gga-mir-1a-1	471	551	405	10,438
gga-let-7a-2	1,633	341	276	549
gga-let-7g	1,623	342	373	413
gga-let-7a-1	1,510	329	266	532

Note: [1] Total number of reads for each group

Confirmation of differentially expressed miRNA

TaqMan miRNA assays were used to confirm the expression pattern of

differentially expressed miRNAs in lungs. There were general consistency between

TaqMan assay and deep sequence analysis in three miRNAs (miR-1a, miR-125b and

miR-146a) in terms of directions of regulation and significance. Specifically, there was a

1.16 fold up-regulation (2.12 folds in deep sequencing analysis) in miR-1a, 2.13 fold

down-regulation (8.33 fold in deep sequencing analysis) in miR-125b, and 3.03 fold

down-regulation (3.45 fold in deep sequencing analysis) in miR-146a ($P < 0.05$).

Clustering of chicken miRNAs

Chromosomal positions of differentially expressed miRNAs revealed that some of

them were very close to each other. According to a previous report (Gu, et al., 2007),

miRNAs can be grouped as one cluster if they are less than 1,000 bp apart on the same

chromosome. Based on the miRBase 13.0 (Ambros, et al., 2003; Griffiths-Jones, 2004; Griffiths-Jones, et al., 2008a), there are 20 miRNA clusters in the chicken genome according to the criteria above. Eighteen of these clusters were detected in lungs and 12 clusters in trachea, respectively (Table 8). Each cluster contained at least two miRNAs, and total of 47 miRNAs were included in these clusters. Within these clusters, the mir-92-mir-19b-mir-20a-mir-19a-mir-18a-mir-17, which is equivalent to the mammalian mir-17-92 cluster, and mir-302b-mir-302c-mir-1811-mir-302a-mir-302d-mir-367 cluster were the biggest clusters containing six miRNAs. Both of them were detected in lungs. There were only seven clusters differentially expressed (all miRNAs within the cluster differentially expressed in the comparison of infection vs. non-infection or between tissues). Clusters mir-16-1-mir-15a, let-7f-let-7a-1, mir-181a-1-mir-181b-1, let-7j-let-7k, mir-23b-mir-27b-mir-24, and mir-16-2-mir-15b were down-regulated in lungs and mir-181a-1-mir-181b-1 was also down-regulated in trachea with AIV infection. Cluster mir-34b-mir-34c was up-regulated in trachea.

Table 8. Identified miRNA clusters

| Cluster	Chromosome locations	Expressed in lungs	Expressed in trachea
let-7a-3-let-7b	Chr1: 73421272-73421347; 73422101-73422185	Y[1]	Y
mir-222-mir-221	Chr1: 114218422- 114218519; 11421 8926-114219024	Y	Y
mir-92-mir-19b-mir-20a-mir-19a-mir-18a-mir-17	Chr1: 152248070-152248147; 152248 183-152248269; 152248306- 1522484 03; 152248492 -152248572; 1522486 26-152248718; 152248781-152248865	Y	N[2]
mir-16-1-mir-15a	Chr1: 173700351-173700434; 173700 493-173700575	Y	Y
mir-20b-mir-18b	Chr4: 3970047-3970131; 3970228-3970311	Y	N
mir-302b-mir-302c-mir-1811-mir-302a-mir-302d-mir-367	Chr4: 58651314-58651385; 58651576-58651640; 58651698-58651778; 5865 1879-58651945; 58652214-58652282; 58652350-58652422	Y	N
mir-1547-mir-204-2	Chr10: 6651001-6651074; 6651274-6651374	Y	N
mir-1720-mir-7-2	Chr10: 14823390-14823454; 14823525 -14823623	Y	Y
let-7f-let-7a-1	Chr12: 6302497-6302583; 6302911-6303000	Y	Y
mir-1763-mir-1564	Chr14: 12895655-12895720; 12896507-12896577	Y	N
mir-34b-mir-34c	Chr24: 5684900-5684983; 5685637-5685710	Y	Y
let-7j-let-7k	Chr26: 1442697-1442779; 1442897-1442979	Y	Y
mir-29c-mir-29b-2	Chr26: 2511658-2511746; 2512569-2512648	Y	N
mir-181a-1-mir-181b-1	Chr8: 2001561-2001664; 2001750-2001838	Y	Y
mir-1b-mir-133c	Chr23: 4663912-4663975; 4664051-4664129	N	Y
mir-449-mir-449b	ChrZ: 16040613-16040698; 16040763 -16040856	Y	Y
mir-216b-mir-1461	Chr3: 288214-288302; 288216-288301	N	N
mir-23b-mir-27b-mir-24	ChrZ: 41157406-41157491; 41157642-41157738; 41158175-41158242	Y	Y
mir-194-mir-215	Chr3: 19924487-19924561; 19924793 -19924897	Y	N
mir-16-2-mir-15b	Chr9: 23742791-23742884; 23742966 -23743056	Y	Y

Note: [1] Identified in the library; [2] Not identified in the library

Gene ontology analysis

Potential target genes of differentially expressed miRNAs in each comparison were predicted by miRanda (John, et al., 2004). In brief, each differentially expressed miRNA was submitted to miRanda individually and all of its targets predicted in miRanda were used for the following gene ontology (GO) analysis. For each comparison, target genes of induced and repressed miRNAs were separately analyzed. All targets of induced miRNAs in each comparison were submitted to DAVID program (Dennis, et al., 2003) and so were the targets of repressed miRNAs. Functional category enrichment based on the GO terms was evaluated on the targets of these differentially expressed miRNAs. Immune related GO terms of each comparison are presented in Figures 8, 9, 10 and 11.
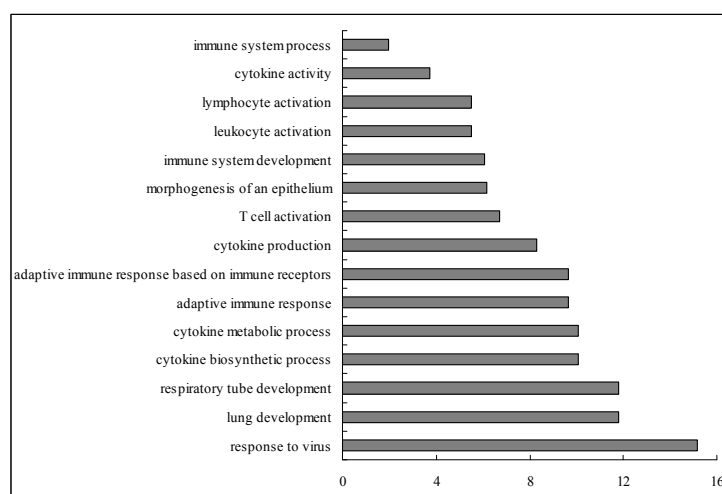
Figure 8. Enriched immune related GO terms of target genes of repressed differentially expressed miRNAs in the comparison of infected vs. non-infected lungs. Notes: Fold enrichment is a ratio obtained by dividing user's percentage by the percentage of each category of the whole genome.
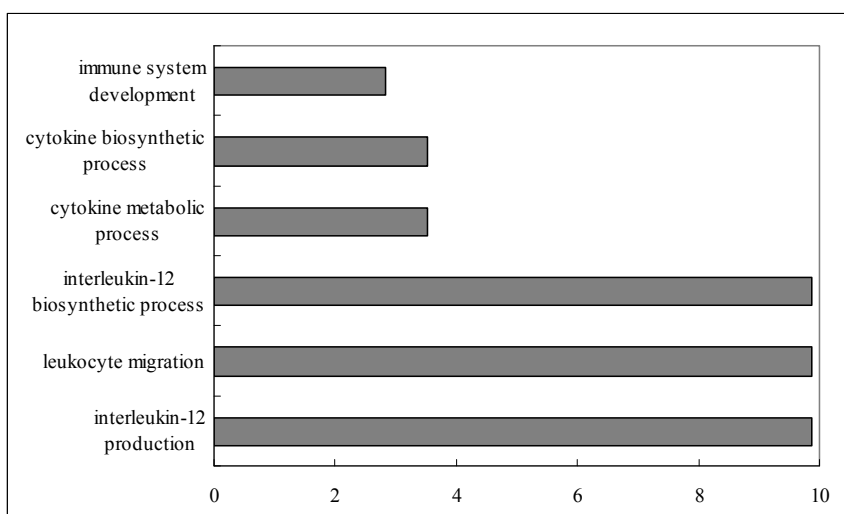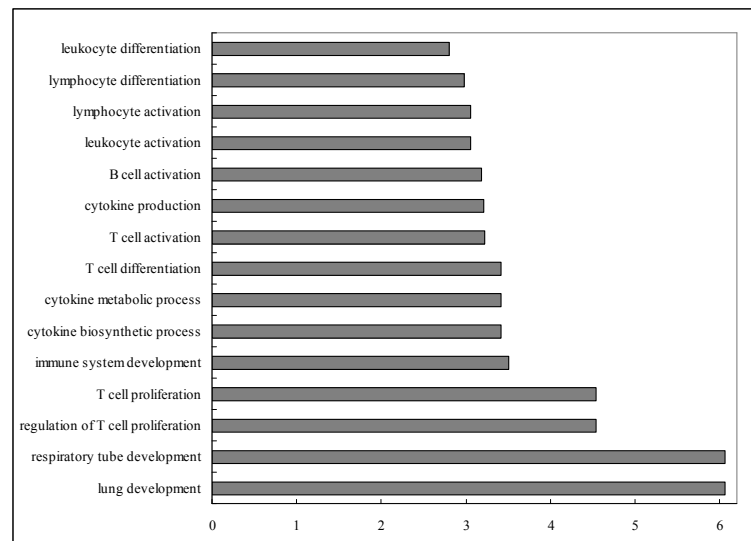
Figure 9. Enriched immune related GO terms of target genes of repressed differentially expressed miRNAs in comparison of infected vs. non-infected trachea. Notes: Fold enrichment is a ratio obtained by dividing user's percentage by the percentage of each category of the whole genome.
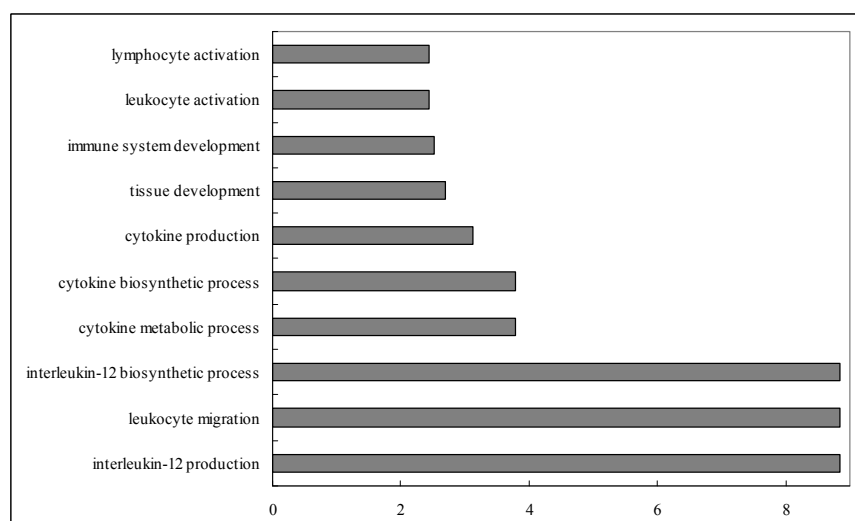
Figure 10. Enriched immune related GO terms of target genes of induced differentially expressed miRNAs in comparison of infected lungs and trachea. Notes: Fold enrichment is a ratio obtained by dividing user's percentage by the percentage of each category of the whole genome.

Figure 11. Enriched immune related GO terms of target genes of induced differentially expressed miRNAs in comparison of non-infected lungs and trachea. Notes: Fold enrichment is a ratio obtained by dividing user's percentage by the percentage of each category of the whole genome.

In the comparison of infected *vs.* non-infected lungs, 15 immune related GO terms in biological process were significantly enriched ($P < 0.05$) (Fig. 8) from the targets of down-regulated miRNAs. Response to virus was the GO term with the highest fold enrichment (15 folds). Other functional terms including immune system response, lymphocytes, and lung development were also identified.

In the comparison of infected *vs.* non-infected trachea, six immune-related GO terms in biological process were significantly enriched ($P < 0.05$) (Fig. 9) from the targets of down regulated miRNAs, in which interleukin-12 production and interleukin-12 biosynthetic process had the highest fold enrichment (9.8 folds). For the targets of up-regulated miRNAs between the infected *vs.* non-infected both in lung and trachea

comparisons, only two immune related GO terms: immunoglobulin I set and immunoglobulin subtype 2 in INTERPRO category were significantly enriched.

For the tissue comparison under the infected state, targets of induced miRNAs in lungs were associated with ten immune related GO terms in biological process including tissue development, interleukin-12, lymphocytes cytokines and immune system development ($P < 0.05$) (Fig. 10). Targets of repressed miRNAs in lungs were associated with immune system development and immunoglobulin subtype 2 in the INTERPRO category. Under the non-infected state, 15 immune related terms were significantly enriched in biological process in the targets of miRNAs highly expressed in lungs. These GO terms were related to lung development and host immune system. The GO term NF-kappaB binding was also enriched in molecular function ($P < 0.05$) (Fig. 11).

Discussion

The impact of miRNAs expression on the understanding of molecular mechanisms in gene regulations has been remarkable. Although thousands of small RNAs have been identified over the last decade, the challenge remains to fully identify all small nuclear RNAs, especially very low abundant ones and to determine their individual functions. The majority of known miRNAs have been identified through traditional cloning method, which is both time consuming and labor intensive. The advantages of next-generation sequencing technologies have provided an innovative tool to look into the genome with unprecedented depth of coverage. Solexa deep sequencing is one of these high throughput technologies, by which miRNAs can be detected in any organism without

prior sequence or secondary structure information. This technology has been used in many species including human, mice and birds (Burnside, 2008; Friedlander, et al., 2008; Glazov, et al., 2008; Hafner, et al., 2008; t Hoen, et al., 2008). Expression of miRNAs varies in different developmental stages (Dhanasekaran, 2004; Darnell, et al., 2006; Goldsmith, et al., 2006). Chicken miRNAs identified in the present study provided novel information in the profiling of miRNAs not only in AIV infected chickens, but also the two tissues (lung and trachea) that have not been previously examined for miRNA profiling in chickens. To our knowledge, this is the first study to profile chicken miRNAs in AIV infected chickens by deep sequencing approach. There are 475 chicken miRNAs predicted in miRBase 13.0 (Ambros, et al., 2003; Griffiths-Jones, 2004; Griffiths-Jones, et al., 2008b). The deep sequencing results in the current study experimentally confirmed 377 miRNAs in chicken lungs and 149 miRNAs in chicken trachea, and the approach is more powerful than other conventional technologies previously used in birds (Xu, et al., 2006). The identification of these chicken miRNAs will be very useful in further investigating the functions and regulatory mechanisms of miRNAs in the chicken.

Growing evidence has suggested a relationship between differential miRNA expression and human diseases (Jiang, et al., 2008; Jones, et al., 2008). miRNAs can regulate many aspects of the immune response, including the development and differentiation of B and T cells, proliferation of monocytes and neutrophils, antibody switching and the release of inflammatory mediators by regulating basic component of host immune system (Sonkoly, et al., 2008; Turner and Vigorito, 2008; Sonkoly and

Pivarcsi, 2009). miR-155 has been reported by several groups to play important roles in both innate and adaptive immune responses in mammals (O'Connell, et al., 2006; Lindsay, 2008; Romania, et al., 2008). miR-155 deficient mice lacked the capability to generate defensive immune responses and to develop lymphocytes, especially B-cell, and antigen-presenting cell functions (Thai, et al., 2007). However, miR-155 showed very low abundance in both lungs and trachea and no significant differential expression was observed in the present study. Over expression of miR-181a in mature mouse T cells can augment the sensitivity to peptide antigens, while suppressing miR-181a expression can reduce sensitivity and impair both positive and negative selection (Li, et al., 2007a). Selective expressions of miR-181a in the thymus and miR-223 in the bone marrow have been shown to be involved in the differentiation of pluripotent hematopoietic stem cells into the various blood cells lineages including B and T cells (Chen, et al., 2004; Cobb, et al., 2006). In the present study, miR-223 was not significantly regulated while miR-181a was down-regulated in both infected lungs and trachea. In addition, miR-181a had a higher expression level in lungs than in trachea under both infected and non-infected states. The expression levels of miR-181a, 181a* and 181b were investigated in LPS activated and CD40-lignad activated macrophages of chickens, respectively (Ahanda, et al., 2009). Only miR-181b was expressed in the macrophage cell line HD11 as well in the spleen adherent cells and that its expression increased after activation by LPS or CD40-ligand (Ahanda, et al., 2009). In the current study, miR-181b had same expression pattern with miR-181a in both lung and trachea comparisons. These results suggest that miR-181a and miR-181b may be strong miRNA candidates that regulate host response

to AIV infection, and warrant further investigation of their targets and regulation mechanism in chickens.

Although the interaction between miRNA expression and virus infection remains to be elucidated, we speculated that miRNA might target immune related genes or modulate virus replication. Sequencing of chicken miRNAs in Marek's disease virus (MDV) infected and non-infected chicken embryo fibroblast (CEF) indicated that more miRNAs were up-regulated in MDV infected cells (Burnside, 2008). These results differ from the current study in which most differentially expressed miRNAs (55 out of 73 in lungs and 27 out of 36 in trachea) were down-regulated in AIV infected tissues. These results indicate that the mechanisms of miRNA regulation of the host response to different types of virus in chickens are different. Chicken miR-221 and miR-222, the most abundant miRNAs in the CEF small RNA libraries, had significantly higher reads in MDV infected than non-infected CEF (Burnside, 2008). While both miR-221 and 222 had relatively lower abundance in the present study. These results demonstrate that miRNA expression can be tissue-specific with high abundance of miR-221 and 222 in the CEF libraries and low abundances in lungs and trachea. It can also be speculated that host miRNAs expression may be suppressed by AIV replication based on the miRNA expression patterns observed in the current study.

Some miRNAs have been shown to be directly involved in virus replication. A liver specific miRNA (miR-122) was shown to be required for Hepatitis C virus (HCV) replication in humans (Jopling, et al., 2005). MiR-122 can positively affect the viral replication and has become a therapeutic target for the treatment of HCV infection (Pan,

et al., 2007). In the current study, miR-122 specifically expressed in chicken lungs compared to trachea under both infected and non-infected states. These data suggest miR-122 might play a more important role in tissue distribution than the responses to AIV infection in chickens. Another two human miRNAs miR-507 and miR-136 have potential target binding sites in polymerase basic 2 (PB2) and hemagglutinin (HA) genes of AIV, respectively (Scaria, et al., 2006). Unfortunately, these two miRNAs are absent in the chicken genome, which might indicate different infectivity and lethality of the virus between chickens and humans.

Although in the present study most differentially expressed miRNA were down-regulated during AIV infection, some miRNAs were also up-regulated. miR-1a, miR-140 and miR-449 were significantly up-regulated in both tissues, while miR-455, miR-34b and miR-34c were only up-regulated with AIV infection in trachea. This suggests different miRNA regulation mechanisms might exist on host response to virus infection. These up-regulated miRNAs might inhibit gene expression of their target genes; therefore down-regulation of these target genes might help the host to inhibit virus replication.

Different tissues serve different biological functions in animals and the expression patterns of miRNAs can vary in different tissues (Gu, et al., 2007; Xu, et al., 2006). miRNAs in bursa and spleen of developing chicken embryo have been recently identified, and diverse expression patterns of these miRNAs between different immune organs were observed, suggesting that miRNAs may function as dynamic regulators of the vertebrate immune system (Hicks, et al., 2009). Some miRNAs show tissue-specific

distribution in mouse, suggesting specific functions within these tissues (Lagos-Quintana, et al., 2002). In the current study, chicken lung and trachea were examined, as they are both part of the respiratory system and important sites for AIV replication. There was a significant difference in miRNA expression between lung and trachea with more miRNAs expressed in lungs (377 miRNAs identified) than trachea (149 miRNAs identified), although only small percentage of miRNAs (19% in lung and 24% in trachea) were significantly differentially expressed in AIV infected samples.

When tissues in the state of virus infection were compared, 28 and 23 miRNAs were specifically and highly expressed in lungs, respectively, and only 6 miRNAs (miR-1a-1 and 2, miR-1b, miR-34b, 34c and miR-449) were highly expressed in trachea. When tissues were compared under the non-infected state, all differentially expressed miRNAs were expressed at higher levels in lungs than trachea with the only exception of miR-206, which showed a higher expression level in non-infected trachea than lung. More interestingly, miR-206 was up-regulated in virus infected *vs.* non-infected lungs and was down-regulated in infected *vs.* non-infected trachea. We can conclude that miR-206 has an opposite regulatory role in lungs and trachea or might have different targets in different tissues and therefore play different roles in host-virus interactions. MiR-1458 and miR-1612 were up-regulated in AIV infected chicken lungs, while they were specifically expressed in non-infected trachea not the infected one. The different regulation of miR-1458 and miR-1612 between lung and trachea suggests they may also have different mechanisms in response to AIV infection between tissues.

We hypothesize that miR-34b, miR-34c, miR-206, miR-1458 and miR-1612 might be some of the most important miRNAs associated with AIV infection. Significantly different miRNA expression pattern between lung and trachea suggests the regulatory mechanism of miRNAs on host response to the AIV infection between lung and trachea is distinct. However, similar regulatory mechanism might also exist in these two tissues. Within the down-regulated miRNAs in infected *vs.* non-infected lungs and trachea, there were 18 miRNAs which overlapped in both tissues. This suggests that these 18 miRNAs might have common modulation mechanisms with the AIV infection in chickens.

GO term enrichment analysis has been widely used in functional analysis and allows the identification of important categories associated with functions of interests. GO terms enriched by the target genes of differentially expressed miRNAs in the current study can provide useful information for the follow-up study to elucidate the regulatory mechanism of miRNAs in host immune response to AIV infection. During AIV infection, the host immune system is stimulated to develop a defensive mechanism, which might be the reason why genes involved in immune system development were enriched in all comparisons.

With virus infection, more immune related GO terms were enriched by the targets of repressed miRNAs in lungs than in trachea (15 terms in lung comparison and 6 terms in trachea comparison) (Figures 8 and 9). Response to virus was identified as the most enriched term (15 fold enrichment) in lung comparison, confirming that genes related to virus infection were regulated by miRNAs. The hyperinduction of proinflammatory cytokines such as TNF-α and IFN-β in human macrophages and respiratory epithelial

cells by the highly pathogenic AIV H5N1 was believed to contribute to its high pathogenecity (Hui, et al., 2009). Lymphocytes were also reported to be suppressed by AIV (Wiwanitkit, 2008). Enrichments of T-cell and leukocytes activation and cytokines activities terms identified in the comparison of infected vs. non-infected lungs might be an indication of host immune system response against virus infection. Meanwhile, GO terms involved in lung development and epithelium morphogenesis were enriched, suggesting the genes associated with lung epithelium development in lungs may be important for the recovery from AIV infection in chickens.

It was interesting that two GO terms, interleuklin-12 production and biosynthetic process, were enriched in the infected tissue comparison, which were not included in the non-infected comparison. These two terms were also enriched in the comparison of infected vs. non-infected trachea instead comparison of lung. IL-12 plays a pivotal regulatory role in the anti-viral response due to its induction of IFN-γ, an anti-viral cytokine (Thomas, et al., 2008). These may suggest that a different defensive mechanism against virus infection might occur in trachea compared to lungs.

The two terms, response to virus and T-cell activation were also enriched by immune related genes differentially expressed in the early immune responses to H9N2 infection in tracheal organ cultures (TOC) (Reemers, et al., 2009). Host immune response, showed as adaptive immune responses in the current study, was enriched by differentially expressed genes in H5N1 infected chicken embryo fibroblasts (CEF) as well (Sarmento, et al., 2008). Influenza virus triggered a cascade of both innate and specific immune responses. Then both immune related genes and miRNAs who might

regulate these genes maybe involved in similar biological processes with the same GO terms.

Of special note, NF-KappaB binding was also enriched in the comparison between lung and trachea under non-infected state. A similar GO term, regulation of NF-KappaB, was enriched in the previous TOC model with the infection of AIV H9N2 (Reemers, et al., 2009). Activation of NF-KappaB pathway is an essential immediate early step of immune activation. Many viruses have developed strategies to manipulate NF-KappaB signalling through the use of multifunctional viral proteins that target the host innate immune response pathways (Hiscott, et al., 2006b). Enrichment of GO term NF-KappaB binding suggests these two tissues might utilize this signal pathway differently.

Post-transcriptional gene activity can be regulated through the interaction of regulatory RNA-binding proteins and small non-coding RNAs such as miRNAs (Bartel, 2004; Keene, 2007). miRNAs can modulate protein activities by altering mRNA stability, translational efficiency or localization (Chabanon, et al., 2004; Keene, 2007). The 3' untranslated regions (3' UTR) are widely accepted as important post-transcriptional regulatory regions of mRNAs, which are particularly rich in cis-acting regulatory elements (Chen, et al., 2006). miRNAs can regulate their target genes through the cis-acting regulatory elements (Xie, et al., 2005). miRNAs within the same cluster might share the same cis-regulatory elements (Gu, et al., 2007), and therefore, might have the same regulatory mechanism for their target genes. Out of the 18 miRNA clusters identified in lungs and 12 miRNA clusters identified in trachea, there were 7 miRNA clusters differentially expressed in different comparisons. The miRNAs from five of

these clusters (mir-16-1-mir-15a, mir-16-2-mir-15b, let-7f-let-7a-1, let-7j-let-7k and mir-23b-mir-27b-mir-24) identified in both lungs and trachea were significantly down-regulated in infected lungs compared to non-infected lungs and also had higher expression levels in non-infected lungs than non-infected trachea. The mir-181a-1-mir181b-1 cluster was significantly down-regulated in both infected lungs and trachea. And the mir-34b-mir-34c cluster was the only significantly up-regulated cluster in the AIV infected trachea. Different miRNA clusters had different regulation direction in AIV infected tissues in the present study. This illustrates that, during AIV infection, different modulation mechanisms among different miRNA clusters might coexist in both lungs and trachea.

It is interesting to note that when considering the miRNA clusters that were most active in chicken lung and trachea, mir-17-92 cluster (consisting of six miRNAs) and mir-302b-mir-302c-mir-1811-mir-302a-mir-302d-mir-367 cluster are highly associated with cell proliferation and self-renewal of stem cells and cancer cells (Aguda, et al., 2008; He, et al., 2005; Mendell, 2008; Wang and Lee, 2009). In addition the miRNAs clusters that were significantly down-regulated miR-15/16 and let-7 are typically down-regulated in stem cells and cancer (Cimmino, et al., 2005; Cho, 2007; Nimmo and Slack, 2009). These results suggest that AIV infection in chickens may instigate cell proliferation and self-renewal like behaviour in chicken lung epithelium and the newly recruited T lymphocytes.

Modulation of target genes by miRNA is one of most critical steps for gene expression regulation. The targeted genes for some differentially expressed miRNAs in

the current study were predicted using miRanda (Enright, et al., 2003; John, et al., 2004). Interestingly, many of the target genes were involved in the host immune system. The potential target genes for miR-1a and miR-1b are the T-cell immuno-modulatory protein. MiR-34b and miR34c, whose target genes are B-cell CLL-pymphoma 2 & 11, might be involved in the B-cell differentiation. Target genes for miR-206 were associated with monocyte macrophage differentiation, suggesting they maybe associated with antigen presentation. Based on other immune related miRNA studies in mammals (Chen, et al., 2007; Lindsay, 2008), differentially expressed miRNAs of their mammalian homologs and their targets are presented in Table 9. MiR-15a, miR-21 and miR-181a have important functions in lymphocytes development and modulations while miR-122 and miR-24 are related to virus infection and miR-146a, induced by macrophages, can activate Toll like receptor (TLR) and expose antigens to interleukin-1 beta. Although the exact functions of these miRNAs in the AIV infected chickens remains to be determined, candidate miRNAs and their potential targets identified in the current study provide strong evidence of their roles and warrant further investigation. Whether these chicken miRNAs have the same function as mammals or not need to be validated in the future studies. On-going efforts in the author's laboratory focusing on gene expressions of these target genes and determination of target genes for these differentially expressed miRNAs will provide new insights of miRNA regulations on AIV infection in chickens.

Table 9. miRNAs involvement in immune responses (Lindsay, 2008)

miRNA	Functions	Targets
miR-15a	Decreased expression in chronic lymphocytic leukaemia	Bcl-2
miR-16	Binds to UA rich elements in 3' UTR and induces TNF alpha mRNA degradation	TNFα
miR-21	Increased expression in B-cell lymphoma and chronic lymphocytic leukaemia	
miR-17-5p	Inhibits monocyte proliferation, differentiation and maturation	AML-1
miR-20a	Inhibits monocyte proliferation, differentiation and maturation	AML-1
miR-106a	Inhibits monocyte proliferation, differentiation and maturation	AML-1
miR-24	Inhibits replication of vesicular stomatitis virus	
miR-29a	Down-regulated in B-cell chronic lymphocytic leukemia	Tcl-1
miR-122	Required for hepatitis C proliferation in liver	
miR-125b	Expression downregulated by LPS and oscillations in expression after exposure to TNF alpha	TNFα
miR-146a	Expression induced in macropahges and epithilial following activation of TLR or exposure to TNF alpha and IL-1beta	IRAK1, TRAF6
miR-146b	LPS induced expression induced in macrophages	IRAK1, TRAF6
let-7i	Regulates TLR-4 and contributes to cholangiocyte immune responses	
miR-181a	Positive regulator of B-cell development and CD4^{+} T-cell selection, activation and sensitivity	SHP-2, PTPN22, DUSP5, DUSP6

miRNAs have recently been implicated in the intricate cross-talk between host and pathogen in viral infections and are critical in viral pathogenesis. In AIV infected tissues, expression patterns of some host miRNAs were significantly differentially regulated in the current study, supporting the hypothesis that certain miRNAs are essential in the host-pathogen interactions. Once the role of these miRNAs in the regulation of host-AIV interaction has been determined, it will improve the protective strategies of AIV infection in poultry.

CHAPTER IV

INTEGRATED ANALYSIS OF MIRNA EXPRESSION AND MRNA

TRANSCRIPTOME IN LUNGS OF AVIAN INFLUENZA VIRUS INFECTED

BROILERS

Introduction

Avian influenza virus (AIV) infection is a world-wide threat to both human and avian species. AIV causes an infection of the respiratory tract of the host, triggering a cascade of innate and adaptive immune responses. Efforts have been made to develop new intervention strategies to control AIV infections. Poultry can be infected with influenza viruses. Therefore, understanding the pathogenesis of AIV infection and chicken-virus interaction is not only essential to the poultry industry, but also provides key insights into the prophylactic and therapeutic protection for other influenza hosts including humans.

miRNAs are short, 17-24 nt RNAs, which comprise a large family of regulatory molecules found in almost all multi-cellular organisms (Bartel, 2004). These small RNAs have been demonstrated to have important functions in a variety of biological processes and have been implicated in many diseases including influenza, hepatitis and cancer (Lee, et al., 1993b; Guo, et al., 2005; Hatfield, et al., 2005; Jopling, et al., 2008; Lindsay, 2008; Sassen, et al., 2008; Song, et al., 2010). miRNAs are capable of regulating mammalian immune cell differentiation, the outcome of immune responses to infection, and the development of diseases of immunological origin (Baltimore, et al.,

2008). There are multiple mechanisms of miRNA-mediated regulation of gene expression including translational repression, disruption of mRNA stability, miRNA-mediated deadenylation and inhibition of polypeptide elongation (Appasani, 2008). Determining how and when miRNA suppression of target mRNA gene expression remains one of the greatest challenges in the field.

Through recognition of sequence-complementary target elements, miRNAs can either translationally suppress or catalytically degrade both cellular and viral RNA (Bartel, 2004; Sullivan, et al., 2005). Host miRNAs are able to impinge on viral life cycles, viral tropism, and the pathogenesis of viral diseases (Cullen, 2006b). miRNAs can potentially regulate different steps of a virus life cycle and abrogate toxicities of replication-competent viruses (Lecellier, et al., 2005; Otsuka M, 2007; Kelly and Russell, 2009; Nathans, et al., 2009). For example, human miR-32 represses the replication of the primate foamy virus type 1 (PFV-1), a retrovirus, through the down-regulation of replication-essential viral proteins encoded by open reading frame 2 (ORF2) (Lecellier, et al., 2005). Based on computational prediction, human miR-136 and miR-507 have potential binding sites at the polymerase basic 2 (PB2) and hemmagglutinin (HA) proteins of H5N1 AIV, and those two miRNAs may modulate AIV infection in humans (Scaria, et al., 2006).

Next generation sequencing (NGS, deep sequencing) has provided a powerful tool to identify differentially expressed miRNAs especially low abundance ones, under conditions of physiological perturbation. We previously used a Solexas Sequencer to identify differentially expressed chicken miRNAs in AIV infected lungs and trachea of

layer type birds (Wang, et al., 2009). Genetics play a significant role in host response to viral infection. We hypothesize that gene expression of host cellular miRNAs following virus infection could be different between different chicken genetic lines. There are two major types of chickens: broilers (meat type chicken) and layers (egg type chickens). In general, there are significant genetic differences between these two types of chickens.. In the current study, a deep sequencing approach was employed to identify differentially expressed miRNAs after AIV infection of broilers. Identification of associated host miRNAs is just the first step towards understanding miRNA regulation of host-virus interactions. Dissection of miRNA modulation of both host and viral mRNA expression will provide insight in the cellular mechanisms of host-virus interaction. A powerful symbiosis between microarrays and NGS technologies has been witnessed (Hurd and Nelson, 2009). Therefore, global gene expression (mRNA) profiling of host response to AIV infection was conducted using a chicken 44K Agilent microarray. Host mRNA profiling and miRNA profiling were integrated and compared between infected and non-infected birds. Our results suggested that gga-miR-34a, gga-miR-106, gga-miR-146a, gga-miR-155 and gga-miR-206 were strong candidate miRNAs involved in regulating the host response to AIV infection in the lungs of broiler chickens.

Materials and methods

Sample collection and RNA isolation:

Day old broilers (Cobb-Vantress, Inc.) were randomly divided into two groups (4 chickens per group), housed in negative pressure Horsfall-Bauer, temperature control

isolation units and provided with water and commercial feed *ad libitum*. At one week of age, one group was inoculated with 0.1 ml of CK/TX/02/H5N3 virus containing $10^{7.5}$ EID_{50}/ml and the remaining chickens were inoculated with PBS (mock treatment) by the intra-choanal cleft route. At 4 days post-inoculation (dpi), depression and severely congested lungs were observed in the treated chickens. Therefore, all chickens were humanely euthanized at 4 dpi, and lungs were collected for RNA isolation. The animal experiment was performed according to the guidelines approved by the Institutional Animal Care and Use Committee, Texas A&M University.

Two pools of total RNA samples (2 random chickens per pool) were generated from the infected and non-infected group. Total RNAs were isolated using Trizol (Invitrogen, Carlsbad, CA) following the manufacturer's protocol. Dnase I (Ambion, Austin, TX) digestion was carried out after RNA isolation according to manufacturer's instructions. RNA concentration and purity were determined by measuring absorbance at 260 nm and A260/A280 ratio using a NanoDrop ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE). RNA samples were stored at -80 ºC until further use.

Viral Titration

Virus titers in lungs of inoculated chickens were determined at 4 dpi by real-time RT-PCR of influenza virus matrix gene using AgPath-ID™ AIV- M kit (Ambion, Austin, TX) following the manufacturer's instructions. For quantitation of virus load, RNA was extracted from serially diluted H5N3 virus stock ($10^{1.5}$–$10^{5.5}$ $\log_{10}$ EID_{50}/ml) and used to generate a standard curve. The amount of RNA in the samples was

converted into $\log_{10}$ EID_{50}/ml by interpolation as described previously (Lee and Suarez, 2004).

Small RNA sequencing and analysis

For small RNA library construction, total RNA samples from lungs of infected and non-infected broiler chickens were prepared using the DGE-Small RNA Sample Prep Kit (Illumina, San Diego, CA) as previously described (Wang, et al., 2009). A total of two Solexa-ready small RNA templates were analyzed on an Illumina 1G Genome Analyzer at the University of Houston. Cluster generation was performed and clusters were sequenced. Initial sequence process and analysis was done as previously described (Wang, et al., 2009a). All unique sequence reads with a minimum read count of 5 were aligned with precursor chicken miRNA sequences from miRBase version 16 (Griffiths-Jones, 2004; Griffiths-Jones, et al., 2006; Griffiths-Jones, et al., 2008b). Reads of each miRNA were the sum of exact and loose matches ($\pm$ 4 bp) to known miRNAs. For each sample, counts were normalized to the total number of small RNA sequences, and then for each miRNA, the normalized number of counts was compared between groups. False discovery rate (FDR) (Q values) was calculated by R program according to Benjamin's method (Benjamini, et al., 2001). Fisher's exact test was used to identify differentially expressed miRNAs at a 5% false discovery rate. Ratios were calculated as the ratio of normalized reads of infected over non-infected group. Statistics related to over representation of functional categories were performed using DAVID (Dennis, et al., 1993; Huang da W, 2009). A $P < 0.05$ was considered significant. Novel miRNAs were

identified using the methods of Creighton et al. and Wang et al. (Creighton, et al., 2008; Wang, et al., 2009a).

Confirmation of miRNA expression by Northern-blot

Expression of two potential novel miRNAs was confirmed by Northern blot analysis using the same total RNA samples as those used for small RNA library construction. Total RNA of infected and non-infected lung samples (15 µg each) were separated on a 15% denaturing acrylamide gel and transferred onto a GeneScreen Plus nylon membrane (GE Healthcare, Piscataway, NJ). Membranes were fixed by UV cross-linking at 1200 µJ and baking at 80 °C for 1 hour. DNA probes (antisense to two mature miRNA sequences) were end-labeled with [γ-^{32}P] ATP (GE Healthcare, Piscataway, NJ) using a mirVana Probe & Marker Kit (Ambion, Austin, TX). Pre-hybridization, hybridization and washes were carried out at 42°C using ULTRAhyb-Oligo hybridization buffer according to the manufacturer's instructions (Ambion, Austin, TX). Chicken U6 small nuclear RNA was used as an internal control to account for loading differences between samples.

Confirmation of differentially expressed miRNAs by TaqMan miRNA Assay

To determine the expression of miRNAs by quantitative RT-PCR (qRT-PCR), TaqMan miRNA assays were performed. The specific stem-loop RT primers for miR-206, miR-451 and U6 were obtained commercially from Applied Biosystems (Foster City, CA). In brief, cDNA was synthesized from total RNA by using the miRNA

specific primers according to the protocol of TaqMan Micro RNA Assays (Applied Biosystems, CA). Reverse transcriptase reactions contained 10 ng of RNA samples, 3 μl of 50 nM stem loop RT primer and reagents from the TaqMan MiRNA Reverse Transcription Kit (Applied Biosystems, CA). The 15 μl reactions were incubated for 30 min at 16 °C, 30 min at 42 °C and 5 min at 85 °C, and then held at 4 °C. Real-time PCR was performed using gene specific probes and a pair of primers and TaqMan 2X Universal PCR Master Mix (No AmpErase UNG) (Applied Biosystems, CA). The 20 μl PCR reactions included 1.33 μl cDNA product, 10 μl PCR master mix, and 1μl 20X TaqMan MiRNA Assay mix (Applied Biosystems, CA). These reactions were incubated at 95 °C for 10 min, followed by 40 cycles at 95 °C for 10 s, 60 °C for 40 s and 72 °C for 1 s using an ABI 7900 Realtime PCR instrument (Applied Biosystems, CA). All reactions were run in triplicate. The threshold cycle was defined as the fractional cycle number at which the fluorescence passes the fixed threshold. The expression levels of miR-206 and miR-451 in each sample were measured in terms of threshold cycle value and normalized to U6 expression using $2^{-\Delta\Delta CT}$ (Schmittgen and Livak, 2008).

miRNA prediction and validation

Target prediction

The chicken (*Gallus gallus*) Unigene database (NCBI) and the miRNA target prediction algorithm miRanda 3.1 (http://www.microran.org/microrna/getDownloads.do) were employed to predict potential targets of all the differentially expressed miRNAs. For miRanda, default parameters were used with the following exceptions: the score was

set to ≥ 130 and the free energy was set to ≤ -16 kCal/mol. The predicted targets were

further filtered using more stringent criteria in which they must contain either (1) a

match between nucleotides 2-8 of the miRNA with the target sequence or (2) a match

between nucleotides 2-7 and 13-16 of the miRNA with the target sequence (G:U base-

pairing was tolerated). A set of target genes containing miR-146a binding sites within

their 3´UTRs were selected for further analysis using a dual luciferase reporter assay.

Insertion of target sequences into psiCHECK-2

For each potential target gene, the region of 3´UTR flanking the miR-146a binding

sites was PCR amplified from Red Jungle Fowl genomic DNA using gene specific

primers. Each PCR product was cloned into the 3´UTR of the *Renilla* reporter gene in

the psiCHECK-2 vector (Promega, WI) using NotI and XhoI restriction sites from the

multicloning site.

Construction of RCAS viruses expressing chicken miR-146a

The previous described RCASBP(A)-miR vector (Chen, et al., 2008) was used to

ectopically express miR-146a. In order to produce RCAS viruses expressing chicken

miR-146a, an entry vector was constructed using PAGE purified 76-nt forward and 68-nt

reverse oligos (Invitrogen) (Supplemental Table1). Restriction sites for SphI and

NgoMIV were introduced at the 5´- and 3´-ends, respectively. Forward and reverse

oligos were mixed at a final concentration of 1 μM, denatured at 95 °C for 20 sec and

annealed at RT to generate a short double-stranded DNA fragment. The fragment was

then cloned into the pENTR3C-miR-SphNgo vector at the SphI and NgoMIV restriction sites. The RCASBP(A)- miR-146a vector was generated via a recombination between the pENTR3C- miR-146a entry vector and RCASBP(A)-YDV gateway destination vector using a LR clonase kit (Invitrogen, CA). To produce miR-146a expressing viruses (RCAS- miR-146a), the RCASBP (A)-miR146a plasmid vector was transfected into DF-1 cells, a chicken embryo fibroblast continuous cell line, using FuGENE 6 (Promega, WI). Virus stock was harvested at day 6 post transfection and titer was determined using immunofluorescence staining with the monoclonal 3C2 antibody against the RSV/ALV gag protein (Developmental Studies Hybridoma Bank, University of Iowa) and FITC-conjugated goat anti mouse IgG (Invitrogen, CA). In addition, RCAS viruses (RCAS-*SC*) expressing a scrambled control sequences were produced to serve as a negative control. Ectopic expression of the miR-146a was validated using a miScript Reverse Transcription kit and a miScript SYBR Green PCR kit (Qiagen, CA).

Dual luciferase reporter assay

DF1 cells were infected with either RCAS-miR-146a or RCAS-*SC* at a multiplicity of infection of 1 and maintained for 6 days in a 96-well plate in RPMI 1640 medium supplemented with 1 % heat-inactivated FBS, L-glutamine, penicillin (100 U/ml), streptomycin (100 µg/ml), and fungizone (4 µg/ml), at 37 °C with 5% CO_2. The psiCHECK-2 construct (100 ng) for each potential target gene, as well as the scramble control, were then transfected into both RCAS-*miR-146a* or RCAS-*SC* infected DF-1 cells using FuGENE 6 (Promega, WI). Forty-eight hours post-transfection, cells were

washed with PBS and lysed in Passive Lysis Buffer (Promega, WI). For each

transfection, firefly and Renilla luciferase activities were determined using the Dual-

Luciferase Reporter Assay System (Promega, WI) and a VictorLight 1420 luminescence

counter (PerkinElmer, MA). The Renilla luciferase signal was normalized to the firefly

luciferase signal. The normalized Renilla luciferase activity was compared between the

RCAS-miR-146a and the RCAS-SC using student's t-test ($P < 0.05$). Triplicates for

each target construct were performed and the assay was repeated to confirm the results.

Microarray analysis

Microarray experiment design: Four biological replicates from infected and non-

infected groups were used to do microarray analysis. Dye swap were used to to prevent

dye-bias during sample labeling.

Labeling and hybridization: The integrity of total RNA samples was confirmed

using Agilent Bioanalyzer 2100 Lab-on-chip system (Agilent Technologies, Palo Alto,

CA). Four hundred nano-grams (ng) of total RNA were reverse-transcribed to cDNA

during which a T7 promoter sequence was introduced into the cDNA. T7 RNA

polymerase-driven RNA synthesis was used for preparation and labeling of RNA with

Cy3 (or Cy5) dye. Fluorescent cRNA probes were purified using Qiagen RNeasy Mini

Kit (Qiagen, Valencia, CA), and an equal amount (825 ng) of Cy3 and Cy5 labeled

cRNA probes were hybridized to a 44 K chicken Agilent array (GEO accession:

GSE9416). The hybridized slides were washed using a commercial kit package (Agilent

Technol-ogies, CA) and then scanned using a Genepix 4100A scanner (Molecular Devices Corporation, Sunnyvale, CA) with a tolerance of saturation setting of 0.005%. Microarray data collection and analysis: For each channel, the median of the signal intensity and local background values were used. A Locally Weighted Linear Regression (LOWESS) normalization was applied to remove signal intensity-dependent dye bias for each array using R program. The Student's t test was used to identify differentially expressed genes. $P < 0.05$ was considered significant.

Gene ontology

Functional annotations for differentially expressed genes were performed through the use of the Database for Annotation, Visualization and Integrated Discovery (DAVID) (Dennis, et al., 2003; Huang, et al., 1993; Huang da W, 2009). Statistics related to over representation of functional categories was based upon a Fisher Exact statistic methodology similar to that described by Al-Shahrour et al (Al-Shahrour, et al., 2004). A $P < 0.05$ was considered as significant.

Results

Virus titers in lungs

Virus replication in lungs of infected chickens was examined by real-time RT-PCR of influenza virus matrix gene from total RNAs at 4 days post inoculation (dpi). Virus titers in the four infected chicken, determined by extrapolation of real-time RT-PCR data,

were 1.69, 3.41, 3.81, and 4.52 $\log_{10}$ EID_{50}/ml. Lung samples from all 4 non-infected chickens were negative.

miRNA sequences from small RNA libraries

A total of 2,672,582 and 3,318,307 filtered high quality reads were obtained from infected and non-infected chicken, respectively (Table 10). In the library from infected chicken lungs, 2,314,793 of these reads were exact matches and another 357,789 reads were loose matches to known chicken miRNAs. In the library of non-infected chicken lungs, 2,875,366 of these reads were exact matches and another 442,941 reads were loose matches to known chicken miRNAs. All reads with a perfect match to mature miRNA sequences from chicken deposited in miRBase version 16.0 (http://microrna.sanger.ac.uk/) (Griffiths-Jones, 2004; Griffiths-Jones, et al., 2006; Griffiths-Jones, et al., 2008b) with insertions or deletions of 1- 4 nucleotides at the 5' and 3' ends of miRNAs were considered to represent Dicer-processing products from each of the chicken miRNA precursors. The loose match reads were defined as no more than 4 nt differences comparing to the known chicken miRNA sequences determined as we previously described (Wang, et al., 2009). The sum of exact and loose match reads was used as the total number of reads for each miRNA.

Table 10. Number of reads of miRNAs from lungs of AIV infected and non-infected chickens

	Infected lung	Non-infected lung
High quality/both adapter	2,672,582	3,318,307
Exact match to known chicken miRNAs	2,314,793	2,875,366
Loose match to known chicken miRNAs	357,789	442,941

Of the 499 distinct *Gallus gallus* miRNA entries in miRBase (Griffiths-Jones, 2004; Griffiths-Jones, et al., 2006; Griffiths-Jones, et al., 2008b), 271 miRNAs were identified in the current chicken lung small RNA library. Also, there was one potential novel miRNA. Expressions of the novel miRNA "N1" and chicken miRNA miR-1711 were confirmed by Northern blotting (Fig. 12).
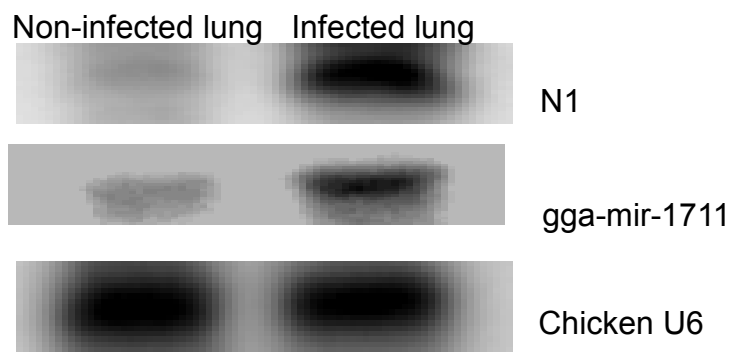
Figure 12. Confirmation of miRNAs. Northern blot analysis was performed to confirm the presence of a novel miRNA (N1) and another known chicken miRNA (gga-miR-1711) in infected and non-infected chicken lungs. U6 probe was used as a control.

miRNAs expression profiling analysis

miRNA expression profiles of infected and non-infected chicken lungs were compared. Differentially expressed miRNAs were identified (P < 0.05, Q < 0.05 and fold change > 2). Between infected and non-infected lungs, 121 miRNAs were differentially expressed. Of those, 43 miRNAs were unique to infected lungs and 8 miRNAs were unique to uninfected lung. Sixty-five miRNAs were more highly expressed in infected lungs, while 5 miRNAs were more highly expressed in non-infected lungs (Table 11).

Table 11. Differentially expressed miRNAs between lungs of infected and non-infected chickens (P<0.05, Q<0.05 and Ratio>2)

miRNA	Position on chromosomes	Reads in infected	Reads in non-infected	Ratio infected/non-infected (Normalized)
gga-miR-1719	chr12:842924-843012	109	0	-[1]
gga-miR-1585	chr19:8800028-8800118	65	0	-
gga-miR-1777	chr28:2555498-2555592	24	0	-
gga-miR-460b-5p	chr4:26873962687485	23	0	-
gga-miR-1716	chr5:60283968-60284072	22	0	-
gga-miR-3537	chr6:18024717-18024793	19	0	-
gga-miR-1718	chr5:33777662-33777741	18	0	-
gga-miR-1354	chr4: 3970359-3970439	18	0	-
gga-miR-1792	chr3:7712006-7712104	16	0	-
gga-miR-3535	chr9:16372628-16372709	15	0	-
gga-miR-1610	chr8:12398260-12398347	15	0	-
gga-miR-1631	chrZ:15789429-15789502	13	0	-
gga-miR-1805-5p	chr1:135141607-135141690	12	0	-
gga-miR-1604	chr1:312691-312787	12	0	-
gga-miR-153	chr2:8765687-8765773	12	0	-
gga-miR-1593	chr1:61691788-61691877	12	0	-

Table 11. continued

miRNA	Position on chromosomes	Reads in infected	Reads in non-infected	Ratio infected/non-infected (Normalized)
gga-miR-3538-1	chrUn:44040736-44040810	10	0	-
gga-miR-3538-2	chr1:52608155-52608229	10	0	-
gga-miR-1723	chr2:41377973-41378078	10	0	-
gga-miR-1584	chrZ:18238506-18238570	10	0	-
gga-miR-1754	chr9:25014275-25014342	9	0	-
gga-miR-3528	chr17:8404342-8404438	9	0	-
gga-miR-1644	chr14:8284308-8284393	8	0	-
gga-miR-1745-1	chr24:5271413-5271449	8	0	-
gga-miR-1770	chr2:151547087-151547183	7	0	-
gga-miR-1809	chr8:23417849-23417956	7	0	-
gga-miR-34a	chr21:3251514-3251622	7	0	-
gga-miR-1681	chr2:96361604-96361703	6	0	-
gga-miR-1692	chr9:23692587-23692675	6	0	-
gga-miR-1805-3p	chr1:135141607-135141690	6	0	-
gga-miR-1463	chr5:11171642-1171751	6	0	-
gga-miR-1560	chr11:20587431-20587444	6	0	-
gga-miR-1700	chr1:140966218-140966317	6	0	-
gga-miR-1712	chr3:81937337-81937409	6	0	-
gga-miR-1772	chr6:11560478-11560546	6	0	-
gga-miR-1713	chr7:17384289-17384387	6	0	-
gga-miR-1781	chr14:3330762-3330854	6	0	-
gga-miR-1551	chr14:5233361-5233450	5	0	-
gga-miR-2127	chr1:170154815-170154918	5	0	-
gga-miR-3527	chrMT:8673-8781	5	0	-
gga-miR-3533	chrUn:20438961-20439044	5	0	-
gga-miR-3536	chr25:1478485-1478562	5	0	-
gga-miR-1785	chr11:20641236-20641337	5	0	-
gga-miR-1594	chrZ:75709-75799	473	17	34.54
gga-miR-1599	chr7: 25926968-25927029	184	7	32.64
gga-miR-1767	chr3:44732913-44732971	71	7	12.59
gga-miR-1662	chr2:1721334-1721406	140	17	10.23
gga-miR-202	chr6:22813068-22813156	40	5	9.93
gga-miR-122-1	chrZ: 649337-649413	1952	279	8.69
gga-miR-1766-1	chr2:77319215-77319307	42	6	8.69
gga-miR-122-2	chrUn:12066796-12066872	1685	258	8.11
gga-miR-32	chr2:86506451-86506520	45	7	7.98

Table 11. continued

miRNA	Position on chromosomes	Reads in infected	Reads in non-infected	Ratio infected/non-infected (Normalized)
gga-miR-204-2	chr10:6651274-6651374	12	2	7.45
gga-miR-211	chr28:1784394-1784467	12	2	7.45
gga-miR-451	chr19: 5823968-5824036	207487	35518	7.25
gga-miR-19b	chr1: 152248183-152248269	955	181	6.55
gga-miR-1694	chr7:5419755-5419852	26	5	6.46
gga-miR-1729	chr15:769596-769666	4292	843	6.32
gga-miR-1611	chr10:16350472-16350560	167	38	5.46
gga-miR-2188	chr22:2684926-2685094	7045	1800	4.86
gga-miR-18a	chr1:152248626-152248718	88	23	4.75
gga-miR-1581	chr1:51158137-51158222	18	5	4.67
gga-miR-193b	chr14: 759453-759535	159	47	4.20
gga-miR-1451	chr3:78710207-78710207	129	42	3.81
gga-miR-1587	chr19:1782806-1782901	15	5	3.72
gga-miR-1572	chr12:9668820-9668820	182	61	3.70
gga-miR-3523	chr13:8968882-8969047	65	22	3.67
gga-miR-18b	chr4:3970228-3970311	70	24	3.62
gga-miR-155	chr1:105930213-105930275	40	14	3.55
gga-miR-454	chr15:399833-399953	31	11	3.50
gga-miR-15a	chr1: 173700493-173700575	2413	861	3.48
gga-miR-144	chr19: 5824123-5824207	13216	4727	3.47
gga-miR-551	chr9:21966405-21966517	25	9	3.45
gga-miR-218-1	chr4:77774698-77774806	11	4	3.41
gga-miR-218-2	chr13:4322806-4322954	11	4	3.41
gga-miR-193a	chr18: 6423770-6423846	1230	461	3.31
gga-miR-223	chr4: 232949-233048	1842	717	3.19
gga-miR-30b	chr2:148331598-148331684	165	67	3.06
gga-miR-214	chr8:4739550-4739659	74	32	2.87
gga-miR-142-3p	chr19: 496983-497070	1055	461	2.84
gga-miR-142-5p	chr19: 496983-497070	1100	481	2.84
gga-miR-106	chr4: 3970359-3970439	897	394	2.83
gga-miR-16-2	chr9:23742791-23742884	1952	856	2.83
gga-miR-16-1	chr1: 173700351-173700434	2724	1206	2.80
gga-miR-1579	chr6:3677284-3677350	164	73	2.79
gga-miR-20a	chr1: 152248306-152248403	426	190	2.78
gga-miR-1416	chrZ: 34596479-34596567	25	12	2.59
gga-miR-146a	chr13: 7555593- 7555691	1331	639	2.59

Table 11. continued

miRNA	Position on chromosomes	Reads in infected	Reads in non-infected	Ratio infected/non-infected (Normalized)
gga-miR-1798	chr20:9654914-9655009	36	18	2.48
gga-miR-3531	chr23:417154-417240	22	11	2.48
gga-miR-20b	chr4:3970047-3970131	734	378	2.41
gga-miR-1434	chr28:1055204-1055280	89	46	2.40
gga-miR-29a	chr1: 3236329-3236417	205	108	2.36
gga-miR-29c	chr26: 2511658-2511746	205	108	2.36
gga-miR-24	chrZ:41158175-41158242	10052	5343	2.34
gga-miR-7b	chrUn:38163821-38163930	251	134	2.33
gga-miR-17-5p	chr1:152248781-152248865	1831	982	2.32
gga-miR-15c	chr4:4049055-4049130	1002	538	2.31
gga-miR-1763	chr14:12895655-12895720	147	80	2.28
gga-miR-23b	chrZ:41157406-41157491	15783	8718	2.25
gga-miR-147-1	chr1:12334922-12334991	92	52	2.20
gga-miR-17-3p	chr1:152248781-152248865	1480	853	2.15
gga-miR-1800	chr5:47604931-47605006	98	58	2.10
gga-miR-458	chr13:8034158-8034273	69	41	2.09
gga-miR-92	chr1:152248070-152248417	13819	8291	2.07
gga-miR-7-1	chrZ:39554766-39554874	81	49	2.05
gga-miR-1705	chr17:9510405-9510494	26	16	2.02
gga-miR-7-2	chr10:14823525-14823623	71	44	2.00
gga-miR-1306	chr15:1296916-1296984	20	62	0.40
gga-miR-206	chr3: 110390439-110390514	28	98	0.35
gga-miR-301	chr15:406313-406405	5	19	0.33
gga-miR-1638	chr5:58712377-58712463	5	23	0.27
gga-miR-187	chr2:85892470-85892555	6	33	0.23
gga-miR-449b	chrZ: 16040763-16040856	0	23	0^2
gga-miR-460a	chr2:3583690-3583779	0	11	0
gga-miR-1765	chr18:5840573-5840677	0	9	0
gga-miR-216c	chr3:288216-288301	0	7	0
gga-miR-1607	chr2: 45452355-45452433	0	6	0
gga-miR-1555	chr1:149148336-149148421	0	6	0
gga-miR-1c	chr7:36625855-36625928	0	6	0
gga-miR-3529	chr10:14823529-14823619	0	6	0

Note: [1] Specifically expressed in infected lungs; [2] Specifically expressed in non-infected lungs.

TaqMan miRNA assays were used to confirm the expression pattern of two differentially expressed miRNAs. There was general consistency between the TaqMan assays and deep sequence analysis of miR-451 and miR-206 in terms of direction of regulation and statistical significance. Specifically, there was a 2.05 fold up-regulation (7.25 fold in deep sequencing analysis) in miR-451, and 4.71 fold down regulation (2.86 fold in deep sequencing analysis) in miR-206 with AIV infection in lungs ($P < 0.05$).

miRNA target identification and validation

Potential targets of differentially expressed miRNAs were predicted by the miRNA target prediction algorithm miRanda 3.1 (John, et al., 2004). One hundred and seventy one immune related genes were predicted to be targets of 35 differentially expressed miRNAs. Some miRNAs have several immune related targets, while some immune related genes each had several predicted miRNA binding sites. For example, IL-17 receptor D (Accession No.: AY278204), has predicted binding sites for seven differentially expressed miRNAs,: gga-miR-30b, 34a, 142-5p, 202, 449b, 460a, and 460b-5p. Additionally, some immune related gens were targeted by both up and down regulated miRNAs following AIV infection. For instance, IL-17 receptor D was a target of gga-miR-202 (which was up-regulated 9.93 folds with AIV infection), and gga-miR-460a (which was specifically expressed in non-infected lungs).

gga-miR-146a is one of differentially expressed miRNAs that was associated with virus infection in chickens (Wang, et al., 2009). Seven potential target genes (Table 12) of gga-miR-146a were selected for the validation by a dual luciferase reporter assay. The

results are shown in Figure 13. The 3' UTR of five (ARL11, CHMP2B, POU1F1, PDHB

and HIF1AN) out of the seven genes targeted by miR-146a showed significant

suppression of *Renilla* luciferase activity in RCAS-miR-146a infected cells relative to

those infected with RCAS-SC ($P < 0.05$). Inhibition of the luciferase activity of

significant targets varied between 65-85% amongst target sites.

Table 12. Potential gga-miR-146a targets

Symbol/ GI	gga-miR-146a:mRNA(3'UTR) interaction	miRanda score/ energy (kcal/mol)	Binding site	Insert location*

HIF1AN/ 118092762	`3' UUGGGUACCUUAAGUCAAGAGU 5'`
	`   |:|::  |||    ||  ||||||||`
	`5' AGCTTCTGG--TTGAGTTCTCA 3'`
		167/-18.0	2261-2280	1959-2417

PDHB/ 118097022	`3' UUGGG--UACCUUAAGUCAAGAGU 5'`
	`   |:||:   |  ||  |  ||||||||||`
	`5' AGCCTAAAAGGCA-TCAGTTCTCA 3'`
		168/-22.3	3732-3754	3365-3864

LATS1/ 118088356	`3' UUG--GGUACCUUAA----GUCAAGAGU 5'`
	`   |:|   |::|||||| |    :|||||||||`
	`5' AGCTGCTGTGGAAATGGCATAGTTCTCA 3'`
		167/-20.5	4243- 4270	4067-4381

POU1F1/ 45383513	`3' UUGGGUACCUUAAGUCAAGAGU 5'`
	`   |  :|:  |  |:  | |||||||`
	`5' ACTCTCTTCAGGT-AGTTCTCA 3'`
		150/-16.5	2979-2999	2327-3057

CHMP2B/ 71896762	`3' UUGGGUAC-CUUAAGUCAAGAGU 5'`
	`   || :|  ||  ||||  ||||||||`
	`5' AAGTC-TGAGAATGCAGTTCTCA 3'`
		174/-21.1	1893-1914	1778-2027

ARL11/ 118084874	`3' UUGG-GUA---CCUUAAGUCAAGAGU 5'`
	`   :||| |||   |||    ||||||||`
	`5' GACCGCATATAGGA----AGTTCTCA 3'`
		157/-19.1	1579-1600	1424-1694

MAP3K3/ 118102843	`3' UUGGGU----A-CCUUAAG----UCA-AGAGU 5'`
	`   ::|||||      |  |||||  :    |||  |||||`
	`5' GGCCCAAGAGTGGGAATGTAAGAAGTGTCTCA 3'`
		127/-19.6	2235-2266	2057-2320

Note: * The 3'UTR predicted target genes containing gga-miR-146a binding sites were cloned into the 3'UTR of the psiCHECK-2 vector (Promega).
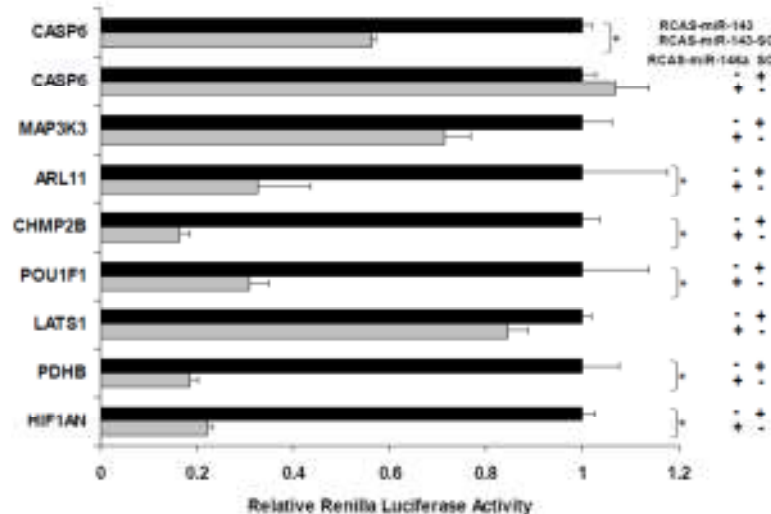
Figure 13. Validation of *miR-146a* target genes in the *Renilla* luciferase reporter system. Notes: Seven potential miR-146a target genes predicted by the miRanda algorithm were chosen for validation. For each predicted target gene a luciferase reporter vector was constructed in which the predicted miR-146a binding site was cloned into the 3' UTR of a Renilla luciferase reporter gene. The Renilla luciferase activities were normalized to Firefly luciferase activities (under the control of an independent promoter). The relative expression of each Renilla luciferase target construct was compared between cells expressing miR-146a and those expressing the scrambled control sequence (SC) using a t-test for statistical significance (p<0.05). Error bars indicate standard deviation. CASP6, a gene containing no binding site for miR-146a but predicted to contain a miR-143 target site was used as negative control.

Recent evidence indicates that cellular miRNAs can also target viral genes (Lecellier, Dunoyer, Arar, Lehmann-Che, Eyquem, Himber, Saib and Voinnet, 2005). Potential viral genes targeted by differentially expressed miRNAs were predicted using Vita program (Hsu, et al., 2007). All of the AIV genes were predicted to be targeted by at least one of up or down regulated miRNAs (Table 13) and 28 differentially expressed miRNAs were predicted to target AIV gene products. gga-miR-34a, which was only expressed in infected chicken lungs, not only had 14 immune related target genes in the host transcriptome, but also targeted the AIV HA, NA, PA, PB1 and PB2 genes. In

general, more AIV genes were targeted by induced host miRNAs than repressed

miRNAs (8.33 times higher). Some miRNAs had only two viral targets, such as gga-mir-

32 (targeting HA and NS genes) and gga-miR-30b (targeting M and NA genes). Some

other differentially expressed miRNAs had multiple predicted viral targets, such as gga-

miR-202 which is predicted to target all of the 9 AIV genes.

Table 13. AIV viral targets of differentially expressed miRNAs between lungs of infected and non-infected chicken (P<0.05, Q<0.05 and Ratio>2).

miRNA	Ratio of Infected /Non-infected (Normalized)	AIV RNA segments
gga-miR-153	only in infected lung	HA, NA, PA, PB1 and PB2
gga-miR-34a	only in infected lung	HA, NA, PA, PB1 and PB2
gga-miR-202	+9.93	HA, M, NA, NP, NS, PA, PB1 and PB2
gga-miR-32	+7.98	HA and NS
gga-miR-211	+7.45	HA, M, NA, NP, NS, PA, PB1 and PB2
gga-miR-19b	+6.55	HA, NS, PA and PB1
gga-miR-18a	+4.75	HA, M, NA, PB1 and PB2
gga-miR-18b	+3.62	HA, M, NA, PB1 and PB2
gga-miR-155	+3.55	HA, NA, NP, NS and PB1
gga-miR-15a	+3.48	HA, M NP, NS and PB2
gga-miR-223	+3.19	HA, NA, PB1 and PB2
gga-miR-30b	+3.06	M and NA
gga-miR-142-3p	+2.84	HA, NA, PA, PB1 and PB2
gga-miR-106	+2.83	HA, NA, PA, PB1 and PB2
gga-miR-20a	+2.78	HA, NA, NP, PB1 and PB2
gga-miR-146a	+2.59	HA, M, NA, NP, NS, PA, PB1 and PB2
gga-miR-20b	+2.41	HA, M, NA, NP, NS, PB1 and PB2
gga-miR-29a	+2.36	HA, M, NA, NP, PA, PB1 and PB2
gga-miR-29c	+2.36	HA, M, NA, NP, PA and PB1
gga-miR-24	+2.34	HA, M, NA, NP, NS, PA, PB1 and PB2
gga-miR-7b	+2.33	HA, M, NA, PA, PB1
gga-miR-17-5p	+2.32	HA, M, NA, NP, PA, PB1 and PB2
gga-miR-23b	+2.25	HA, M, PA and PB1
gga-miR-17-3p	+2.15	HA, M, NA, NP, PA and PB2
gga-miR-92	+2.07	HA, M, NP, NS, PB2
gga-miR-206	-2.86	HA, NA, NP, PB1 and PB2
gga-miR-301	-3.03	HA, NA, PB1 and PB2
gga-miR-187	-4.35	NA, NP, PB1 and PB2

Note: + Up-regulated with AIV infection; - Down-regulated with AIV infection.

Host mRNA profile analysis

    The genome-wide expression profiling of host response to AIV infection was carried out by using chicken 44K Agilent microarray. There were 1,303 genes differentially expressed (303 up-regulated vs. 1000 down-regulated) between AIV infected vs. non-infected chickens (P<0.05, Fold-change>1.2). The fold-change of gene expression between infected and non-infected group ranged from 34.33 to -10.10 (Fig. 14).
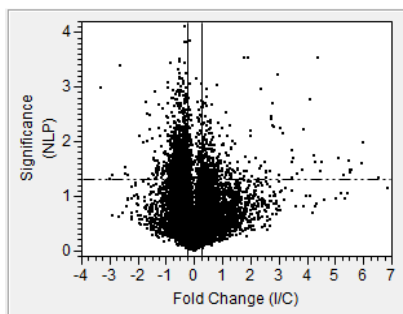
Figure 14. Differentially expressed host genes between lungs of AIV infected and non-infected chicken (P<0.05, Fold-change>1.2). Notes: Volcano plots of differentially expressed genes between AIV infected and non-infected chicken. NLP represents negative $\log_{10}$ of P-value. Fold change was $\log_2$ transformed. Positive values means gene expression is higher in infected group than non-infected one.

    Seventeen immune related host genes were significantly up or down regulated with AIV infection. Six genes were significantly up-regulated, while the rest were significantly down-regulated (Table 14). Chicken MX1 gene, which was reported to be associated with influenza virus resistance (Ko, et al., 2002), had the highest fold-change (11.46 fold) followed by interleukin 8 (11.03 fold) and interferon regulatory factory 7

(2.11 fold). Tumor necrosis factor receptor superfamily member 19 was down-regulated

(1.85 fold).

Table 14. Differentially expressed immune related host mRNAs between lungs of infected and non-infected chickens (P<0.05 and Fold-change>1.2)

Gene description	Gene Accession	Infected vs. Non-infected (Fold-change)	miRNA[1] (fold change)
MX1 myxovirus (influenza virus) resistance 1	Z23168	+11.46	gga-miR-155(+3.55) gga-miR-206(-2.86)
Interleukin 8 (IL8)	M16199	+11.03	gga-miR-32(+7.98)
Interferon regulatory factor 7 (IRF7)	U20338	+2.11	gga-miR-142-5p(+2.84)
Interleukin1receptor-like1, transcript variant1	AB041738	+1.65	gga-miR-460 (only expressed in infected lungs)
Ficolin 2	CR406783	+1.38	NA[2]
Phosphoinositide-3-kinase catalytic, α polypeptide	AF001076	+1.31	gga-miR-451(+7.25)
TNF receptor superfamily, member 19	BX931334	-1.85	gga-miR-187(-4.35)
Ripartite motif-containing 7.1	BX934475	-1.81	NA
RAC serine/threonine-protein kinase3 (ATK3)	BX950472	-1.65	NA
C-fringe-1	U97157	-1.52	NA
Cell division cycle 42	CR385975	-1.52	NA
CKLF-like MARVEL transmembrane domain 3	BX935400	-1.49	gga-miR-7b(+2.33)
Integrin beta 1 binding protein 3	CR352634	-1.42	gga-miR-24(+2.34)
Mitogen-activated protein kinase kinase 5	AJ721122	-1.27	gga-miR-451(+7.25)
TGF beta-inducible nuclear protein 1	CR523694	-1.26	gga-miR-451(+7.25) gga-miR-34a (only expressed in infected lungs)

Note: [1]miRNAs targeting on differentially expressed immune related genes; [2] No miRNAs targeting on the gene; +: Up-regulated with AIV infection; –: Down-regulated with AIV infection.

Gene ontology (GO) analysis

The significantly enriched functional terms in biological processes from

differentially expressed host genes and predicted target genes of differentially expressed

miRNAs with AIV infection, respectively are presented in Figure 15. Immune response (3.68 fold) was the only term enriched by induced host genes. There were no immune related GO terms for targets of repressed miRNAs. GO terms enriched in repressed genes included four clusters: cytoskeleton-dependent intracellular transport; DNA unwinding during replication and DNA repair; microtubule-based movement; and response to DNA damage stimulus and response to endogenous stimulus. Five GO terms were enriched in targets of induced miRNAs. These included calcium ion transport; hemophilic cell adhesion; T cell activation; gamma-aminobutyric acid signaling pathway; and Wnt receptor signaling pathway.
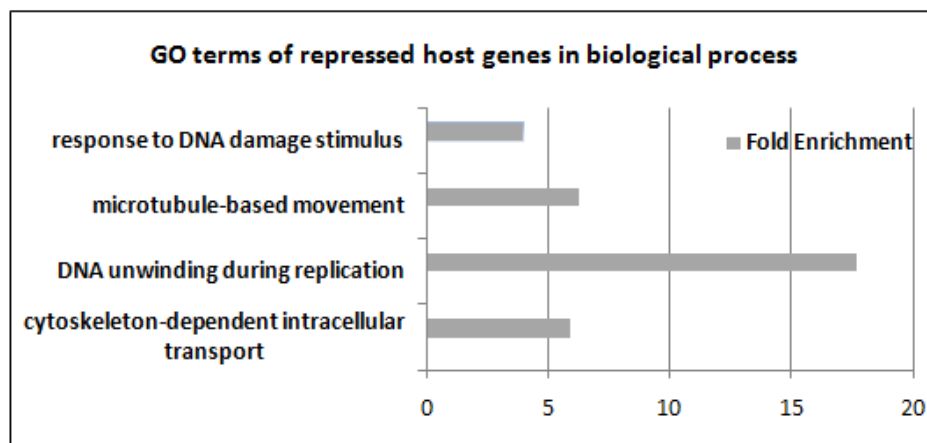
A

Figure 15. Gene ontology (GO) annotation of differentially expressed genes (A) and target genes of differentially expressed miRNAs (B) between lungs of AIV infected and non-infected chicken in biological process category (P<0.05). Notes: Fold enrichment is a ratio obtained by dividing user's percentage in certain categories by the percentage of each category of the whole genome.
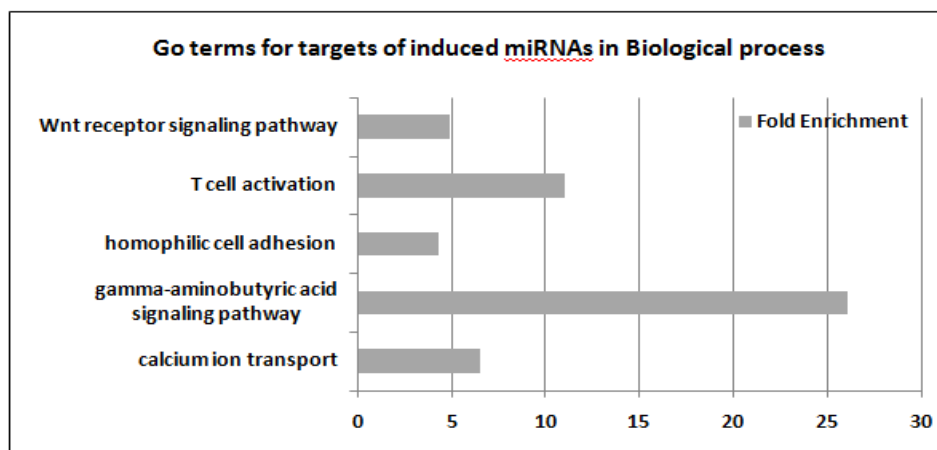
B

Figure 15. continued.

Discussion

miRNAs are evolutionarily conserved RNA molecules that regulate and integrate hundreds of genes within and across multiple signaling pathways. The discovered miRNA functions are currently revolutionizing both basic biomedical research and drug discovery (Appasani, 2008). Global profiling of miRNAs expression may enhance our understanding of regulatory mechanisms in many biological processes. In the present study with broiler chickens, there were more miRNAs up-regulated than down-regulated with virus infection, which was the opposite of our previous miRNA profiling in AIV infected layers (more down-regulated than up-regulated) (Wang, et al., 2009). Table 15 lists differentially expressed miRNAs in both current broiler (2nd deep sequencing) and previous layer studies (1st deep sequencing). Only two (mir-1599 and mir-1416) of eighteen miRNAs had consistent directions of regulation following AIV infection. These findings suggest that these two miRNAs are conserved responses to AIV infection in

chickens across diverse genetic lines. The major discrepancies between two studies might be due to: broilers and layers being genetically distinct chicken breeds with long-term diverse selection targeting on growth and egg production, respectively. Genetics play an important role in the regulation of miRNAs expression in animals (Bueno, et al., 2008). The genetic differences between broilers and layers might contribute to miRNAs expression differences. Broilers have rapid growth rate and larger size and percentage of meat than layers (Jones, et al., 1986). miR-206 was reported promoting muscle differentiation in mouse myoblasts (Kim, et al., 2006). In our results, gga-miR-206 was highly up-regulated in layers while down-regulated in broilers. With the down-regultion of miR-206 in broilers, muscle differentiation might be inhibited which made broilers hardly keep a high growth rate with AIV infection. Also, broilers and layers have developed different characteristics of their immune systems. Broilers are specialized in the production of a short term humoral response, while layers have a long-term humoral response in combination with a strong cellular mediated response (Koenen, et al., 2002). With AIV infection, it was expected that a different host response between broilers and layers would be observed. The differential expression of miRNAs between chicken breeds reflects their different responses to virus infection. Differential expression of miR-142-3p in conventional $CD4^+$ T cells and $CD25^+$ T_{REG} cells in mice is able to control the functions of both effector and suppressor cells. In our study, gga-miR-142-3p was down-regulated in layers and up-regulated in broilers, which indicated that regulation of host immune system is different between genetic lines. 3) Different development stages might contribute to miRNA expression differences (Hicks, et al.,

2008; Hicks, Tembhurne and Liu, 2009). The broilers used in the present study were challenged on day 7 of age and layer chickens used previously were challenged on day 21, which represents different developmental stages. These results suggest that genetic backgrounds and/or age at time of challenge play a vital role in the regulation of miRNAs during AIV infection in chickens.

Table 15. Comparison between two deep sequencing results (P<0.05, Q<0.05 and Ratio>2).

	miRNAs	3 week old layers			1 week old broilers		
		Infected	Non-infected	Ratio (Normalized)	Infected	Non-infected	Ratio (Normalized)
Inconsistent	gga-mir-106	0	27	+	897	394	2.83
	gga-mir-142-3p	2	49	0.07	1055	461	2.84
	gga-mir-142-5p	2	49	0.07	1100	481	2.84
	gga-mir-144	111	94	0.21	13216	4727	3.47
	gga-mir-146a	7	105	0.12	1331	639	2.59
	gga-mir-15a	2	102	0.04	2431	861	3.48
	gga-mir-16-1	1	107	0.02	2724	1206	2.80
	gga-mir-1729	0	24	+	4292	843	6.32
	gga-mir-19b	1	31	0.06	955	181	6.55
	gga-mir-193a	5	26	0.35	1230	461	3.31
	gga-mir-206	101	9	20.38	28	98	0.35
	gga-mir-20a	1	23	0.08	426	190	2.78
	gga-mir-20b	0	10	+	734	378	2.41
	gga-mir-223	1	15	0.12	1842	717	3.19
	gga-mir-29a	2	15	0.24	205	108	2.36
	gga-mir-451	93	1287	0.13	207487	35518	7.25
Consistent	gga-mir-1599	48	13	6.17	184	7	32.64
	gga-mir-1416	17	10	3.09	25	12	2.59

Note: [+] Specifically expressed in non-infected lungs.

miRNAs regulate gene expression mainly by binding to their target mRNAs in one of two places: the coding sequence or 3' UTR. It is clear that the major role of miRNAs

is to down-regulate the protein production by targeting mRNAs (Cannell, et al., 2008). Mammals encode two isoforms of miR-146 (a and b). Both miR-146a and miR-146b respond to LPS in lymphocyte cell lines, but only the expression of miR-146a was induced (Taganov, et al., 2007). miR-146a is predicted to have an antiviral role by targeting Primate Foamy Virus type 1 (PFV-1) virus, Dengue virus, Hepatitis C virus, influenza virus and several other viruses (Hsu, et al., 2007). It was also reported to be able to inhibit a group of interferon-responsive genes in an Epstein-Barr virus (EBV) latency type III cell line, which suggested that miR-146a functions in a negative feedback loop to modulate the intensity and/or duration of the interferon effects (Cameron, et al., 2008). Chicken miR-146 has three isoforms miR-146a, miR-146b and miR-146c. It will be very interesting to examine if miR-146 also has targets on AIV genes. In the current study, of the 3 isoforms, only miR-146a was significantly up-regulated with AIV infection and targeted on Toll-like receptor 3 (TLR3) by computational target prediction. TLR3 is a part of host innate immunity, which is capable of recognize dsRNA and trigger antiviral and inflammatory responses to viral infection and has antiviral role against AIV (Wong, et al., 2009). In addition, miR-146a was also differentially expressed in the previous AIV study although the direction of regulation was opposite (Wang, et al., 2009). Therefore, we decided to examine the predicted targeted genes of miR-146a in order to understand underlying regulatory mechanism of AIV infection. Although five predicted target genes were confirmed by Dual luciferase reporter assay, unfortunately, none of them were significantly differentially regulated at the mRNA level (P > 0.05) by microarray analysis. miRNAs

fine tune target gene expression, this might be one of the reason that no dramatic

changes on these target genes were identified. In addition, the regulatory mechanism for

gga-miR-146a may primarily regulate the protein rather than the mRNA levels of these

five target genes.

miR-155 has been reported to play important roles in both innate and adaptive

immunity in mammals (O'Connell, 2007; Lindsay, 2008; Romania, et al., 2008). miR-

155 knock-out mice are not capable of generating defensive immune responses,

developing lymphocytes, or antigen-presenting cell functions (Thai, et al., 2007). miR-

155 is up-regulated with poly (I:C) and IFNβ stimulation in mouse bone-marrow derived

macrophages (O'Connell, 2007). Poly(I:C) is a ligand for TLR3, which in turn induced

the expression of miR-155 directly and immediately, while the induction of miR-155 by

IFN is indirect and requires TNFα autocrine and paracrine signaling (Taganov, et al.,

2007). These studies suggest an important role of miR-155 in the regulation of viral

infection. In the current study, gga-miR-155 was significantly induced by AIV infection,

which was consistent with other study in which miR-155 was up-regulated by a variety

of pathogen stimuli (Taganov, et al., 2006). Based on target prediction, miR-155 could

target the chicken anti-influenza gene Mx1, therefore playing a role in host and AIV

interactions in chickens. The activation of c-Jun NH_2-terminal kinases (JNK) pathway

can eliminate virus-infected cells by apoptosis. The inhibition of JNK pathway blocks

the expression of miR-155 in murine macrophages (Taganov, et al., 2006; O'Connell,

2007). Based on host mRNA profiles by microarray, TNFRSF19 (TNF receptor

superfamily member 19), one of the genes in the JNK pathway, was significantly down

regulated with AIV infection, which indicate that antiviral activities through JNK pathway might be inhibited. Therefore, up-regulated miR-155 in the current study might also activate JNK pathway, and subsequently induce apoptosis to eliminate virus-infected cells.

miRNAs play vital roles in host-virus interactions. Host cellular machinery determines virus survival and propagation, and cellular miRNAs and their targets have been shown to be involved in the regulation of host-pathogen interactions (Wang, et al., 2009). Many cellular miRNAs have been found to directly regulate virus replication. Human miR-32 had a direct negative effect on the replication of retrovirus Primate Foamy Virus type 1 (PFV-1) by down regulating replication-essential viral proteins encoded by open reading frame 2 (ORF2), causing translation inhibition (Lecellier, et al., 2005). miR-32 was significantly up-regulated (7.98 folds) with AIV infection in this study, which might indicate a similar host defensive mechanism in order to block viral infection. miR-32 was predicted to target several chicken immune related genes such as TNF receptor-associated factor 3, NFKB-1 and IL8 as well as HA and NS genes of AIV (Table 13). NS1 protein has been shown to block the synthesis of cellular mRNAs and also inhibits activation of PKR (dsRNA-dependent serine/threonine protein kinase R) induced by interferon, which initiate the interferon and cellular responses to viral infection (Knipe, 2007). We speculate that the induction of chicken miR-32 during AIV infection could lead the degradation of NS1mRNA, improve the integrity of host mRNAs, and promote the PKR pathway that inhibits viral replication.

Microarray analysis provides a powerful tool to identify differentially expressed genes in a global level. In the current study, 17 immune related host genes were differentially regulated (Table 14). As expected, the Mx1 gene, originally discovered as an interferon induced protein with the ability to block replication of influenza viruses (Knipe, 2007), was highly expressed during AIV infection (11.46 fold up-regulation). Antiviral genes such as interleukin 8, interferon regulatory factor (IRF)-1(P=0.08 with fold-change of 2.78), and IRF-7 were also highly up-regulated during AIV infection. IRF-1, a transcriptional activator of interferon α and β, has been shown to be essential for host response to virus infection (Yu-Lee, et al., 1990). This indicates the possibility that virus infection initiates host anti-viral defense response (Mogensen and Paludan, 2001). On the contrary, tumor necrosis factor superfamily member 19 (TNFRSF19), which is capable of inducing apoptosis (Hu, et al., 1999), was significantly down regulated; so were mitogen-activated protein kinase 5 (MAP2K5) and TGF beta-inducible nuclear protein 1 (Ramachandran, et al., 2005). All three genes are associated with cell signaling, differentiation and apoptosis.

Both Mx1 gene (11.46 fold up-regulation) and TGF beta-inducible nuclear protein 1 (TGFβINP1) (1.26 fold down-regulation) were predicted to be targeted by two differentially expressed miRNAs (miR-155 and miR-206 for Mx1, and miR-451 and miR-34a for TGFβINP1, respectively). From miRNA deep sequencing results in the current study, miR-155 was up-regulated, while miR-206 was down-regulated with AIV infection. Based on negative theoretical regulation of miRNA on mRNA expression and the high fold up-regulation of Mx1 observed we suggest miR-206 plays a major

regulatory role on Mx1 expression. On the other hand, both miR-34a and miR-451 were up-regulated with AIV infection, suggesting that down-regulation of TGFβINP1 at the mRNA level might be due to suppressive effects of both miRNAs.

Cellular miRNAs can regulate viral genome transcription and translation. Human miR-122 has been reported to play an essential role in HCV virus replication by interacting with HCV genome at both 3' and 5' non-coding regions (Jopling, et al., 2005; Jopling, et al., 2008). Of particular interest, miR-122-1 and miR-122-2 were up-regulated during AIV infection (fold-changes of 8.11-8.69, respectively) in the current study. Although no viral targets for miR-122-1 and miR-122-2 were identified, it is noteworthy to further examine if and how miR-122 might regulate AIV virus replication. On the other hand, 28 differentially expressed miRNAs had multiple AIV gene targets (Table 13). Some miRNAs (miR-153, miR-34a, miR-202, miR-211, miR-142-3p, miR-106, miR-146a, miR-29a, miR-24 and miR-17-5p) specifically expressed in infected lungs or were up regulated during AIV infection. They target AIV polymerase basic 1 (PB1), polymerase basic 2 (PB2) and polymerase acid (PA) mRNAs. PB1, PB2 and PA are components of influenza virus RNA dependent RNA polymerase complex and are essential for virus replication (Knipe, 2007). We speculate that up-regulation of these miRNAs may inhibit AIV replication, and experiments to examine the effects of these miRNAs on AIV replication are underway. Most of these up-regulated miRNAs were predicted to target the hemaglutinin (HA) and neuraminidase (NA) mRNAs such as miR-34a and miR-155. Both HA and NA are major surface glycoproteins. HA is responsible for receptor binding and virus fusion (Knipe, 2007), while NA is responsible

for receptor destruction and virion release (Knipe, 2007). Therefore, induction of these miRNAs might affect virus attachment and release and therefore the formation of new infectious viral particles. Further more, three down-regulated miRNAs (miR-206, miR-301 and miR-187) also were predicated to target the AIV genome. The first line of evidence from this analysis strongly indicates several candidate miRNAs including miR-34a, 146a, 155 and 206, basing on both the differential expression with AIV infection and their host and viral targets, warrant further investigation to understand the mechanisms of miRNA regulation of AIV infection in chickens.

In summary, by integrating both cellular miRNA and mRNA expression during AIV infection in broiler chickens, this comprehensive analysis has provided several lines of new evidence on how host miRNA might regulate host response to AIV replication in broilers. Specifically, this study generated a list of strong candidate miRNAs including miR-32, 34a, 146a, 155, 187, 206, and 451 for our on-going effort to further elucidate regulatory mechanisms of miRNAs on AIV infection in chickens. In addition, several candidate genes including MX1, IL-8, IRF-1, 7, TNFRS19, MAPK5 have been identified to be associated with AIV infection in broilers. Finally, comparison with our previous report on layer miRNA expression profile, this study strongly indicates that genetic background is a critical factor in determining miRNA abundance and regulation during AIV infection.

CHAPTER V

EVALUATION OF THE COVERAGE AND DEPTH OF TRANSCRIPTOME BY RNA-

SEQ IN CHICKENS*

Introduction

The transcriptome catalogues the complete set of transcripts in a cell.

Transcriptomic regulation is critical to all physiological, developmental and pathological

processes (Blencowe, et al., 2009), and mRNA expression profiles can represent the

characteristics of a cell at a specific state and help to govern its present and future

activities (Mortazavi, et al., 2008). The profiles of a transcriptome in terms of alterations

in response to specific biological stimuli provides valuable insights for interpreting

functional elements of the genome, revealing the molecular constituents of cells, and

also understanding developmental and disease processes.

Different types of technologies have been developed to interrogate transcript

abundance, including hybridization-based and sequencing-based approaches.

Hybridization-based microarrays have been the primary transcriptomic high-throughput

tool for almost two decades, which has accelerated the study of transcriptome analysis

by profiling thousands of genes simultaneously (Bhattacharjee, et al., 2001; Jurata, et al.,

2004; Li, et al., 2008). However, microarray technology has several limitations

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including: indirect quantification by hybridization-signal intensities (Cassone, et al., 2007), background and cross-hybridization problems (Okoniewski and Miller, 2006) and reproducibility issues (Draghici, et al., 2006). The development of next generation sequencing with improved qualitative and quantitative measurements holds great promise in transcriptome analysis.

RNA-Seq is a recently developed approach to map and quantify transcriptomes by digitally recording how frequently each transcript is represented in a sequence sample. After poly (A) selection, RNA is fragmented to small fragments and converted into a cDNA library, which provides a simple and more comprehensive way to measure transcriptome composition and to discover new genes by high-throughput sequencing without bacterial cloning of cDNA input (Mortazavi, et al., 2008). Studies using this technology have already altered our views regarding the extent and complexity of transcriptomes in an organism and dramatically improved our understanding of transcriptome. RNA-Seq has several advantages over micorarrays including: 1) RNA-Seq is not dependent on prior knowledge about the target sequence; 2) It has a large dynamic range and sensitivity due to its digital nature, which is especially important for highly abundant and extremely low abundant genes; 3) The survey of a transcriptome is more accurate because the quantification of each transcript is directly based on digital counts of the transcript. Therefore, RNA-Seq offers both single-base resolution for annotation and "digital" quantification at the RNA level, which allows the entire transcriptome to be analyzed in a high-throughput and quantitative manner (Wang, et al., 2010). However, the expense per sample for RNA- Seq is still a limiting factor in

preventing researchers from sequencing multiple biological replicates per group, which are needed for statistically-significant analysis. It is common to adopt a pooling strategy to reduce the cost for RNA-Seq studies (Liu, et al., 2011). With the continued enhancement of sequencing output and the development of multiplex labelling techniques, the cost per sample could be significantly reduced if several samples are multiplexed and sequenced in the same lane, given sufficient transcriptome coverage per sample. Therefore, it is imperative to address the trade-off between the depth of RNA-Seq and the coverage of the transcriptome in an organism. The objective of this study was to evaluate what coverage or sequencing depth of transcriptome would be sufficient to interrogate gene expression profiling in the chicken by RNA-Seq.

Materials and methods

RNA preparation

Total RNA was isolated from four chicken lungs from two genetic chicken lines leghorn and fayoumi (two samples per line) by Trizol (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. Two RNA samples from the same line were pooled to generate totals of two pooled RNA samples (Sample1 and Sample2). DNase I (Ambion, Austin, TX) digestion was carried out after RNA isolation and the RNA concentration and purity were determined by measuring absorbance at 260 nm and A260/A280 ratio using a NanoDrop ND-1000 spectrophotometer (Nano-drop Technologies, Wilmington, DE). RNA samples were stored at -80 ºC until further use.

cDNA library preparation and sequencing by RNA-Seq

Total RNA (7 µg) was subjected to two rounds of hybridization to oligo (dT) beads (Invitrogen, Carlsbad, CA) to enrich mRNA. Ribosomal RNA contamination was evaluated by RNA pico chip using a BioAnalyzer (Agilent, Santa Clara, CA). The resulting mRNA was then used to prepare cDNA libraries using the RNA sequencing sample preparation kit (Illumina, San Diego, CA). Sample1 and Sample2 were sequenced by Illumina Genome Analyzer and then Genome Analyzer II, which generated four datasets: S1-R1, S2-R1, and S1-R2, S2-R2, respectively.

Data filtering, mapping reads and identifying transcriptome contents

The sequences generated went through a filtering process first. Any reads that contained numerous interspersed Ns in their sequences, or had relatively short reads (<17 bp), were removed for the following analysis. Sequence reads obtained after quality control with filtering were analyzed using CLC Genomics Workbench 4 (CLC bio, Cambridge, MD). After mapping, the gene expression level was quantified by simply dividing the number of reads mapped to each gene by the size of its transcripts, commonly known as the number of reads per kilobase of exon per million mapped reads (RPKM) (Mortazavi, et al., 2008), for all 15,742 annotated chicken genes in the database. The gene expression level was then $\log_2$ transformed.

Random sampling of S1-R2 and S2-R2

We have obtained RNA-Seq data in three different levels of depth: 1.6 M, 4.9 M, and about 30 M reads. Clearly, there was a big gap between 4.9 M and 30 M reads. In order to identify the appropriate depth of transcriptome per sample that is sufficient for whole genome transcriptome profiling, it is important to generate additional datasets at different levels of depth. It would be very costly to re-sequence each sample to generate RNA-Seq data at different levels of sequencing depth. Random sampling from the current dataset might provide a cost-effective approach for this purpose. This procedure synthetically created samples from the originally sequenced samples. Thus, for samples S1-R2 and S2-R2 data sets, by drawing without replacement a fixed number of reads from the overall data set, we randomly selected 10 M, 15 M and finally 20 M reads. These random selections were repeated 4 times, resulting in total of 24 technical replicates with different transcriptome depth. Each one of the reads in the FASTQ format of the input files, which were used in the sampling, was selected equally likely. A program in Perl was written to serve this purpose. Then, the resulting replicate datasets were uploaded into CLC Genomics Workbench in the FASTQ format for the analysis individually. The correlation coefficients of gene expression (RPKMs) between replicates of each sequencing depth were calculated by JMP (SAS, Cary, NC). The average RPKMs of transcripts identified by each sequencing depth (10 M, 15 M and 20 M) were calculated to represent the gene expression values for further analysis.

Correlation coefficients between different sequencing depths from the same sample

In order to evaluate how reliable the sequence data is at each level of sequencing depth, correlation coefficients between lower and high depth sequence data for each sample were calculated by JMP (SAS, Cary, NC). Any genes with no gene expression at either sequence dataset were excluded from the correlation coefficient computation. The depth of sequencing is highly correlated with the abundance of gene expression, therefore, genes were divided into the four quartile groups based on expression levels for each dataset, from the bottom 25% (1st quartile) to the top 25% (4th quartile). Subsequently, correlation coefficients between lower-depth sequence data and the data with 28.7- 29.6 M reads within each of the four quartile groups were calculated.

Results

RNA-Seq for cDNA libraries

The two chicken cDNA libraries (Sample1 and Sample2) were sequenced by the Illumina Genome Analyzer, which generated 4.9 M (60 bp) reads (S1-R1) and 1.6 M (60 bp) reads (S2-R1), respectively. Then, two technical replicate cDNA libraries from the same RNA samples were re-sequenced using the Genome Analyzer II, which generated 29.6 M (75 bp) reads (S1-R2) and 28.7 M (75 bp) reads (S2-R2).

Random sampling of the S1-R2 and S2-R2

The datasets of S1-R2 (29.6 M) and S2-R2 (28.7 M) were each randomly re-sampled into 10 M, 15 M, and 20 M reads with four replicates each. The correlation

coefficients between every two replicates for each re-sampled level (10 M, 15 M and 20

M) within each sample (S1-R2 and S2-R2) were all greater than 0.98, which

demonstrated that the sampling procedure is consistent and accurate. Averages of gene

expression from the four replicates at each re-sampled level for each sample were used

for further analysis.

Effects of sequence depth on the distributions of transcripts

The distributions of transcript abundance at different levels of sequence depth from

Sample1 and Sample2 are presented in Figures 16 and 17, respectively. In general, the

median and 75% percentile were similar across five different levels of depth, while the

95% percentile showed a slight increase; especially from 20 M to 30 M. Significant

decreases at the 25% and 5% percentile were observed, especially from 20 M to 30 M.

In addition, a significant decrease was also observed from 1.6 M to 10 M in Sample2.
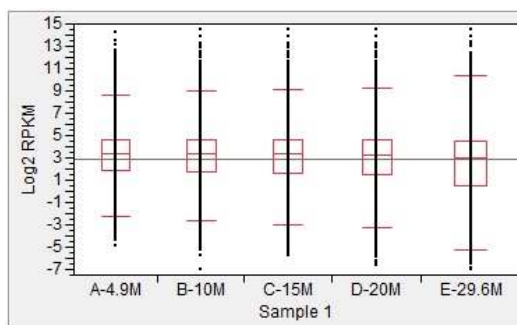
**Figure 16.** Distributions of log2 transformed reads of transcripts at different sequencing depths for Sample1.
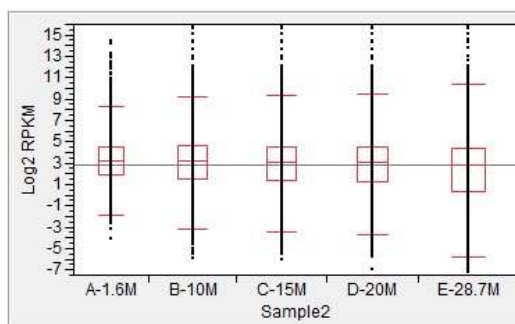
Figure 17. Distributions of log2 transformed reads of transcripts at different sequencing depths for Sample2.

Coverage of annotated chicken genes

There are about 15,742 annotated chicken genes in the NCBI database. Number of detected chicken genes at different levels of sequencing depth from Samples 1 and 2 are presented in Table 16. There were 14,336 genes detected in S1-R2 (29.6 M) and 14,212 genes in S2-R2 (28.7 M), which accounted for 91.07% and 90.28% of all 15,742 annotated chicken genes in the database, respectively. With the reduction of sequencing depth, the number of detectable genes also decreased from 91% to 78% in Sample1 (Fig. 18A), and from 90% to 68% in Sample2 (Fig.18B). Two significant drops of transcriptome coverage were observed: from 30 M to 20 M, and 10 M to 1.6 M.

Table 16. Numbers of detected annotated chicken genes at different levels of sequence depth. Note: [1] percentage of identified known chicken genes across all genes in the database.

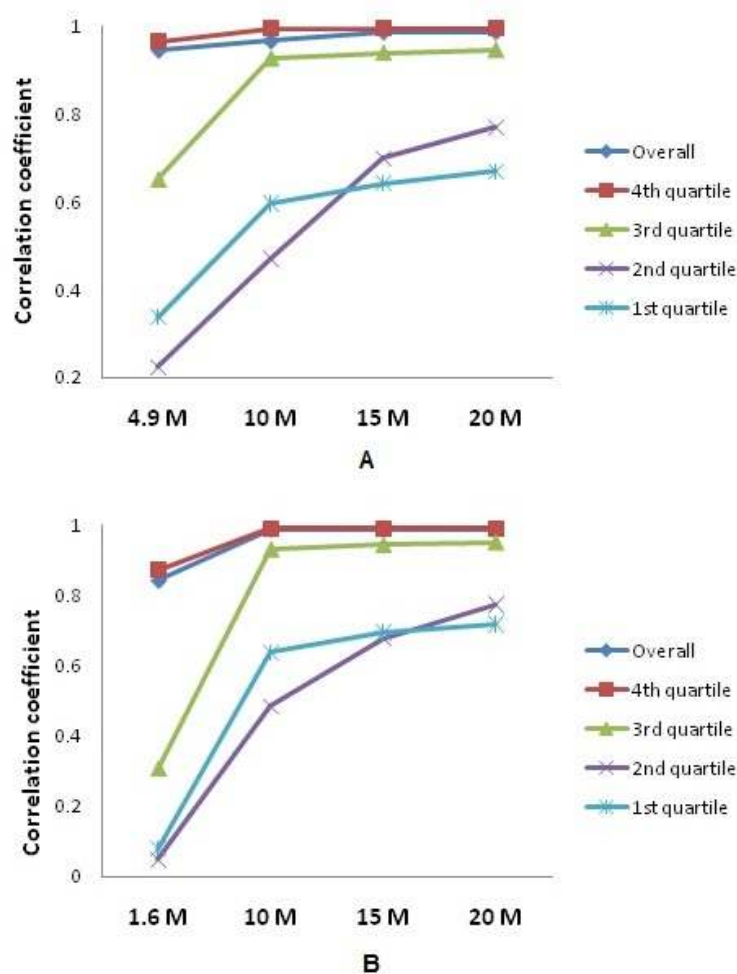|	Sample 1			Sample 2	
	No.of genes	%[1]		No. of genes	%[1]
29.6M	14336	91.07	28.7M	14212	90.28
20M	13011	82.65	20M	12895	81.91
15M	12822	81.45	15M	12690	80.61
10M	12515	79.50	10M	12406	78.81
4.9M	12276	77.98	1.6M	10664	67.74
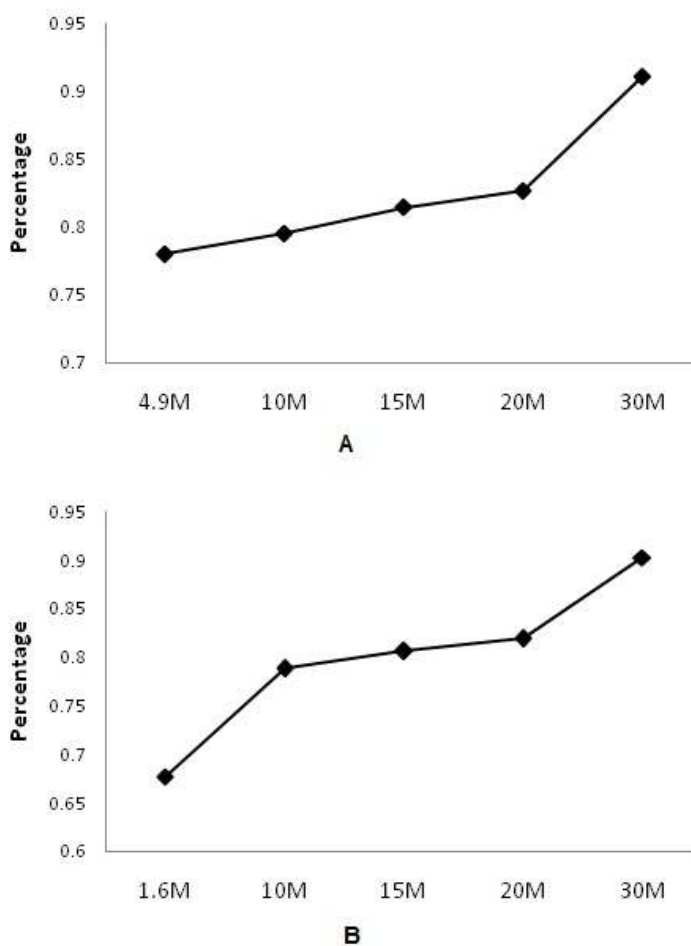
Figure 18. Percentages of detected chicken genes at different levels of sequence depth across all annotated chicken genes. A: Sample1; B: Sample2.

Correlation coefficients between different sequencing depths

To evaluate the appropriate depth of sequence that is needed for transcriptome profiling, correlation coefficients between different levels of sequencing depth and the most abundant reads for each sample were calculated. For Sample1, overall correlation

coefficients at four different levels of depth were greater than 0.95. When we examined the four quartile groups based on expression level (Fig. 19A), correlation coefficients ranged from 0.34 to 0.67 for the 1st quartile, 0.22 to 0.77 for the 2nd quartile, 0.65 to 0.95 for the 3rd quartile, and 0.97 to 1.0 for the 4th quartile. A similar pattern in terms of correlation coefficient change was observed for the 1st, 2nd, and 3rd quartiles; a significantly increased correlation coefficient from 4.9 M to 10 M, and kept relative flat from 10 M to 20 M. For the 4th quartile, correlation coefficients at four different levels of depth were greater than 0.95. From the 1st to the 4th quartiles, there were significant increases for correlation coefficients between every two quartile groups ($p<0.01$). For Sample2, overall correlation coefficients at four different levels of depth were greater than 0.98, except for 1.6 M at 0.84. Correlation coefficients ranged from 0.08 to 0.72 for the 1st quartile, 0.05 to 0.78 for the 2nd quartile, 0.31 to 0.95 for the 3rd quartile, and 0.87 to 1.0 for the 4th quartile (Fig. 19B). The same pattern in terms of correlation coefficients changes at different levels of depth between Sample1 and Sample2 was also observed.

Figure 19. Correlation coefficients between 30 M reads and four different levels of sequence depth at different quartiles. A: Sample1; B: Sample2.

Discussion

In the current study, RNA-Seq was performed twice using two chicken cDNA samples. The first run of RNA-Seq had fewer numbers of reads and larger variation in terms of total number of reads between the two samples, while the second run had greater number of reads and very small variation between the two samples. The first run was performed at the very early stage of the sequencing technology when it was still in

the testing phase. The lower reads and larger variation in the first run may be coming from two major sources of technical error: the purification of cDNA templates during the library preparation, and the loading of libraries onto flow cells (RNA-Seq technical guide and personal communications, Illumina technical support staff). These potential sources of errors were corrected during the second RNA-Seq analysis, which provided improved sequencing depth with greater number of reads. The first RNA-Seq datasets were directly derived from actual experiment, which made the results more informative than replicating datasets by random sampling. Therefore, we chose to include these two early datasets in the analysis in the current study. Furthermore, all of the reads from each sample were normalized by the RPKM and the datasets can serve as a reference for random sampling at different sequencing depths from the exact same samples.

The capacity of sequencing length of 60 bp for the first run was increased to 75 bp at the second RNA-Seq analysis. Longer reads should reduce estimation error and mapping uncertainty, and read lengths have consistently increase with improving Illumina massively parallel sequencing technology. However, people have noted that the number of reads is more important than the read length once reaching a minimum read length of 25 bp (Li, et al., 2010a; Nicolae, et al., 2011). The read lengths (60 bp and 75 bp) in the current study were larger than 25 bp; therefore, the read length will not affect the overall conclusions drawn.

As a powerful new technology for transcriptome analysis, RNA-Seq provides a more comprehensive view of the transcriptome than earlier technologies. Besides its ability to detect splicing variation, RNA editing and discovery of new transcripts

(Wilhelm, et al., 2011), RNA-Seq can also function in the role of a conventional microarray in measuring gene expression due to its accurate measurements. In order to detect less abundant transcripts, appropriate sequencing depth is needed. The transcriptome coverage or sequencing depth needed for a given study can be affected by several factors such as genome size, transcriptome complexity and objectives of the study. In general, the more complex the transcriptome, the more sequencing depth is required for adequate coverage (Cloonan, et al., 2008). Depending on the purpose of transcriptome analysis, the requirement of sequencing depth varies. In most transcriptome studies, quantifying mRNA abundance is one of the major objectives. There is a certain sequencing depth that is sufficient in simple transcriptomes. For example, in the yeast genome, a 29.9 M (35 bp) reads dataset was generated by RNA-Seq which was able to get 100% transcriptome coverage (Nagalakshmi, et al., 2008). The number of transcripts detected by RNA-Seq in the yeast dataset was able to reach 80% transcriptome coverage at 4M mapped reads, and even though the sequencing depth doubled as 8M reads, the transcriptome coverage only increased 10% (Nagalakshmi, et al., 2008; Wang, et al., 2009b). These results suggest that the improvement of sequencing depth or transcriptome coverage after reaching a certain sequencing depth had relatively less impact on detecting low abundant genes (Wilhelm, et al., 2008). In addition, the cost per sample per lane by RNA-Seq is still not affordable for most laboratories. Recent development in multiplex labelling using bar-coded libraries by Illumina and continued increase in sequence output have made it possible to sequence multiple samples per lane without extra cost or running time (Stiller, et al., 2009).

Therefore, it is imperative to examine the correlation between sequencing depth and transcriptome coverage; in other words, what sequencing depth might be sufficient in reaching a certain level of transcriptome coverage and reliable measurement for RNA-Seq. In order to accomplish this objective, two approaches could be applied: experimental or simulation methods. Both methods have been applied in this study. High correlation among replicates within each sequencing depth, gradual increase in correlation coefficients from 10 M to 20 M, and consistent patterns observed between Samples1 and 2 (Fig. 19) have demonstrated that random sampling was an effective and reliable method in reaching the goals of this study.

Transcriptome coverage is one of the most important parameters in profiling global gene expression. The number and level of transcript isoforms is not always known and transcription activity varies across the genome (Wang, Gerstein and Snyder, 2009b). This was confirmed in a study by using the number of unique transcription start sites as a measure of coverage in mouse cells (Wilhelm, Marguerat, Watt, Schubert, Wood, Goodhead, Penkett, Rogers and Bahler, 2008). In the current study, we took a more practical approach using all annotated genes in the chicken genome. Because the chicken genome is far under-annotated, we assume that the 15,742 annotated chicken genes in the database would well represent different levels of expression abundance in the chicken genome, which is essential for the analysis of transcriptome coverage in this study. Since gene expression depends on tissue and time of biological process (Wilhelm, et al., 2008), it is impossible for any single tissue to have all genes in the genome expressed. Ninety percent of all annotated genes (Fig. 18) detected at about 30 M (75 bp)

reads might represent a saturated detection of the whole genome. The analysis results indicated significant improvements of transcriptome coverage occurred from 1.6 M to 4.9 M and from 20 M to 30 M. Depending on the purpose of transcriptome analysis, the current study suggested that 10 M (75 bp) reads could have 80% of transcriptome coverage, while 30 M (75 bp) reads could reach 90% of transcriptome coverage.

When we analyzed overall correlation coefficients at different levels of sequencing depth regardless of gene expression level, we observed very high correlation coefficients between each level of sequencing depth compared with 30 M, except for 1.6 M. One might draw a conclusion that there is no significant difference among different levels of sequencing depth. But as we see in Figure 19, this might be true in the case of highly abundant genes (the 4th quartile group), but not in the case of the 1st to 3rd quartile groups, especially the first two quartile groups (i.e. expression below the median). High abundant genes will be less affected by sequencing depth than low abundant genes, because high abundant genes are more likely to be captured even when the sequencing depth varies (Ramskold, et al., 2009). This is also confirmed by our analysis. Collectively, the following conclusions can be inferred: 1) Sequencing depth below 20 M (75 bp) reads had a significant effect on detecting transcript levels of medium and low abundant transcripts; 2) Sequencing depth at both 1.6 M and 4.9 M would result in unreliable mRNA expression on all genes except highly abundant transcripts; 3) There was no significant improvement in correlation coefficients when sequencing depth doubled from 10 M to 20 M. Based on these analysis, the results suggested: 1) 5 M reads might be sufficient in obtaining reliable mRNA expression measurement on highly

abundant transcripts; 2) When sequencing depth is beyond 10 M reads, a relatively reliable measurement of mRNA expression is expected, especially for abundant transcripts; 3) It seems that 30 M of reads is needed to achieve reliable measurement of mRNA expression across all genes in the chicken genome. To our knowledge, this is the first study evaluating the appropriate sequencing depth using RNA-Seq in farm animals and will provide the first reference for similar studies. The knowledge generated from this study has laid a solid foundation for applying this analysis to other species.

CHAPTER VI

SUMMARY AND DISCUSSION

Influenza A viruses are important pathogens causing contagious respiratory diseases. In most cases, influenza infections are self-limiting but sometimes they cause substantial morbidity and mortality worldwide (Katz, 2003). As a negative stranded RNA virus, influenza viruses show high mutation rates especially in the antigenic regions such as HA and NA segments. Due to the small sizes of gene segments, reassortments of influenza viral genes during mixed infections with two or more virus subtypes, even between species, can make pandemic influenza A viruses (Reid, et al., 2001). Since the late 1990s, interspecies transmission of avian influenza viruses to humans has occurred on a global scale and has heightened concerns of AIVs impact on the public health (Katz, 2003). Understanding molecular mechanisms of host AIV interactions not only benefit poultry industry, but also provide valuable knowledge for human influenza prevention and treatment.

The main characteristic of pathogenic microorganisms is their abilities to cause tissue damage and consequently disease in the hosts. Various methods have been utilized to study how pathogens interact with the hosts and the mechanisms by which hosts protect themselves from pathogenic microorganisms. The generated results from these studies have been valuable for the development of safe and effective diagnostics, therapeutics and vaccines (Small, et al., 2001). Currently there are several genomics approaches available to thoroughly study the host-pathogen interactions from the host

aspect. These methods include genetic association analysis, DNA methylation, gene expression profiling, and proteomics analysis. The current dissertation demonstrated the applications of different genomics approaches for studying the host-AIV interactions in chickens.

Disease susceptibility is, in general, inheritable, although the heritability of disease resistance is usually low (Knight and Kwiatkowski, 1999). Therefore, genetic selection of birds with more disease resistance has offered a promising approach for the disease control (Wigley, 2004). The diversity of host immune systems during pathogen infections may result in genetic differences in disease susceptibility among different genetic lines (Hilleman, 2004). Inbred lines have provided a unique model to study genetic resistance to disease resistance. Several inbred chicken lines have been shown different degree of susceptibility to viral infections such as genetic resistance to Marek's disease (MD) in line 6 and line 7 where line 6 is resistant to MD, and line 7 is susceptible to MD (Bacon, et al., 2000). For AIV studies, two highly inbred chicken lines (Leghorn and Fayoumi) with distinct genetic characteristics (Zhou and Lamont, 1999) were used to examine genetic resistance to AIV infection. Interestingly, AIV titers in trachea at 4 days post infection in the two lines were significantly different (Fig. 20). Leghorn birds had higher viral titer (relatively susceptible) and Fayoumi birds had lower viral titer (relatively resistant) to AIV infection. Therefore, these two genetic lines will be good models to investigate genetic resistance to AIV infection in the chicken.

Disease resistance is a quantitative trait. Both linkage analyses such as quantitative trait loci (QTL) mapping and associations studies can be used to identify markers

associated with disease resistance trait. In the chicken, several genes have been demonstrated to be associated with pathogen infections such as chicken TLR4 (Wigley, 2004), CD28 and MD-2 genes (Malek, et al., 2004) for *Salmonella* resistance; growth hormone gene for MD (Liu, et al., 2001) resistance. Chicken Mx1 gene is widely reported having anti-influenza activities *in vitro* (Ko, et al., 2002; Ko, 2004) and *in vivo* (Yin, et al., 2010b). While the antiviral capabilities among different chicken lines varied with different viral strains (Watanabe, 2007). In the current study, with AIV infection in both embryos and birds, low virus titers were observed in embryos or birds carrying Mx1 N631 allele (resistant allele) compared to S631 allele (susceptible allele), although there was no significant difference between them. In terms of Mx1 gene expression for three different genotypes: NN, NS, and SS, the expression level was significantly up-regulated with AIV infection only in the NS genotype birds (P < 0.05). The NN genotype birds had higher Mx1 mRNA expression levels than SS birds under both infected or non-infection status, while the differences were not statistically significant (P > 0.05). This was the first study that integrated Mx1 polymorphism association with AIV titers and Mx1 mRNA level among different genotypes with AIV infection. Further study is warranted to focus on the regulatory role of the Mx1 mutation in the AIV infection in chickens.

AIV titers in the infected Leghorn and Fayoumi birds
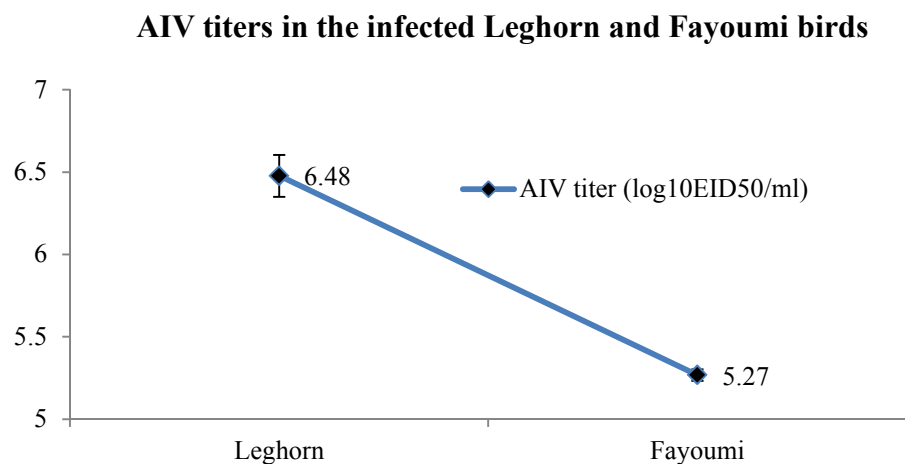
Figure 20. AIV titers in the two highly inbred genetic chicken lines. Virus titers were determine through chicken lung total RNA samples by real-time PCR.

The other approach at the DNA level for the host-pathogen interaction study is DNA methylation. DNA methylation is a stable epigenetic modification found in both plants and animals and it plays a critical role in many biological processes such as gene expression regulation (Bird, 2002). Chicken genome-wide DNA methylation map was developed in the Red Jungle fowl and broilers (Li, et al., 2011). Methylations of cytosines within CpG dinucleotides at promoter regions are important for gene silencing and genome integrity (Jones, et al., 1999). Methylations of gene coding regions are related with post transcriptional gene silencing (PTGS) in plants (VanHoudt, et al., 1997) and they also play roles in controlling gene expression in animals (Bhakat and Mitra, 2003). DNA methylation is usually served as a defensive mechanism to protect host against viral infection (Yoder, et al., 1997). During host-pathogen interactions, the DNA adenine methylation (Dam) regulates virulence genes by reparing mismatches and

transcriptional regulation (Marinus and Casadesus, 2009). Retroviruses are known to integrate in host cell genome as proviruses, and the down-regulation of proviruses is mediated by DNA methylation (Svoboda, et al., 2000). However, the Epstein-Barr Virus (EBV) relies heavily on DNA methylation to regulate its promoter usage (Robertson, 2000), which is highly associated with viral immortalization into host cells. By utilizing DNA methylation, EBV is able to maximize latency and escape from the host immune detection (Robertson and Tao, 2003). Clearly, DNA methylation is important in regulating host-pathogen interactions. A study focusing on DNA methylation related to AIV infection in the chicken using next generation sequencing is currently undergoing in our laboratory.

Gene expression profiling is one of the most popular approaches for studying the alteration of host mRNA or miRNA expression with pathogen infection. Activities of thousands of genes or miRNAs are measured simultaneously, which provide a global view of cellular function in responding to pathogen infection. The cDNA microarray and next generation sequencing (NGS) are two most popular techniques for mRNA and miRNA expression profiling to study the alteration of host mRNA gene and miRNA expression.

Microarray technology has been widely applied for host-pathogen interaction analysis in chickens. It not only provides us the relative mRNA expression and differentially expressed genes on a global level, but also offers an important tool to infer gene networks and to identify highly conserved gene pathways (Li, et al., 2008). For the host-pathogen interaction studies, the cDNA microarray has been used to identify

differentially expressed genes in *Campylobacter jejuni* colonization and infectivity (Li, et al., 2010b; Zhang, et al., 2006) and *Salmonella enteritidis* infections on chicken heterophils (Chiang, et al., 2008). With the infection of MD in chicken embryo fibroblasts, inductions of several host immune genes including interferon-inducible protein, MHC class I and II were found using a cDNA microarray (Morgan, et al., 2001). In an influenza A virus-infected human lung epithelial cells study, a significant induction of genes involved in the IFN pathway was identified (Geiss, et al., 2002). Host cellular gene expression response to infection with five different human and avian influenza A virus strains in a human lung epithelial cell line was investigated and about 300 differentially expressed genes were identified between infected and non-infected cells (Josset, et al., 2010). These common differentially expressed genes among different viral strains can be used as a global gene expression signature for its use to examine drugs effectiveness on all influenza A viruses (Josset, et al., 2010). A list of differentially expressed genes in the current study were identified including IL8, IRF-1 and IRF-7, which were associated with the initiation of host immune responses (Hu, et al., 1999), and tumor necrosis factor superfamily member 19 (TNFRSF19), mitogen-activated protein kinase 5 (MAP2K5), and TGF beta-inducible nuclear protein 1 (TINP1), which were associated the inhibition of inducing apoptosis (Mogensen and Paludan, 2001; Ramachandran, et al., 2005; Yu-Lee, et al., 1990). These results have laid the foundation for further investigation of host immune response to AIV infection in the chicken.

Although microarray technology has been widely applied for gene profiling analysis, recent development in digital expression profiling using next generation

sequencing (NGS) such as RNA-Seq has become one of more powerful high-throughput sequencing technologies for transcriptome profiling in organisms, which has many advantages over microarray technology especially for low abundant transcripts. Due to relative high cost per lane, RNA-Seq has not been widely utilized in gene profiling analysis. With newly developed multiplex labeling technology in Illumina, multiple samples running in the same lane becomes possible. Thereby, genome coverage would be critical to determine how many samples can be multiplexed into the same lane. Our preliminary analysis has demonstrated that 30 M (75 bp) reads is sufficient to detect all annotated genes in the chicken, and RNA-Seq at this depth can serve as an alternative transcriptome profiling technology. Furthermore, our results indicated that the depth of sequencing had a more significant impact on measuring gene expression of low abundant genes.

Another important aspect for host-pathogen interaction studies is the profiling of regulatory miRNAs. Approaches for miRNA discovery and profiling have been improved in the past few years. Initially, forward genetics methods were instrumental in identifying the first two miRNAs lin-4 and let-7 (Lee, et al., 1993a). But only a few miRNAs were discovered by this method. Meanwhile, since the special secondary structure and highly phylogenetic conservation of both sequences and structures, a set of distinctive miRNAs were identified by computational prediction (Bentwich, 2005; Lim, et al., 2003). Later on, cloned small RNA cDNA libraries became the preferred approach to identify miRNAs. Most of the known miRNAs have been successfully identified through these small RNA libraries. However, this method has its limitation in finding

miRNAs with low expression levels or in rare cell types (Berezikov, et al., 2006). Massively parallel sequencing by NGSs such as small RNA deep sequencing boosts the discovery of small RNAs and results in identification of more novel miRNAs.

Small RNA deep sequencing was used to profile chicken miRNA expression in chicken embryo and embryo fibroblasts with MDV infection in the past few years (Burnside, 2008; Hicks, et al., 2008). Differentially expressed miRNAs between developmental stages or between immune organs were identified (Hicks, et al., 2009). Two runs of miRNA deep sequencing, for layers and broilers, respectively, were conducted in our study. miRNAs, such as miR-1599 and miR-1416, had the same regulatory direction in both layers and broilers might be important candidates for further functional study. Chicken miRNA profiling in both lung and treacheae were compared in the layer miRNA deep sequencing. There was a significant difference in miRNA expression profiles between lung and trachea. For those ubiquitous miRNAs or tissue specific miRNAs, there is a promising application in which they can be applied to attenuate AIVs to make vaccination more safety (Perez, et al., 2009).

Differentially expressed miRNAs identified by the deep sequencing approach indeed provided some insights of the regulatory mechanism in host-pathogen interactions. In addition, these immune related chicken miRNAs can be involved in different phases of the host immune response. Some of them might have effects on the components of immune systems. For example, miR-142-3p differentially regulated between broilers and layers, which is participating functions of both effector and suppressor cells. miR-181a is related to lymphocyte development with AIV infection in

chickens. Some other miRNAs might take part in the signal transduction in immune cells. With the infection of AIV, miR-146a was significantly up-regulated in broilers which could inhibit the TLR3 production, then weakened host antiviral and inflammatory responses to viral infection. Up-regulation of gga-miR-155 in the current study might rescue the function of the JNK signaling pathway, and subsequently induce apoptosis to eliminate virus infected cells.

The miRNAs are a set of gene regulators that can modulate cellular gene expression at different levels. Having both the host cellular gene expression profiles by microarray or RNA-Seq and miRNA profiles by miRNA microarray or small RNA deep sequencing, we are able to integrate both mRNA and miRNA data to understand how host miRNAs regulate cellular gene expression with AIV infection. Both Mx1 gene (up regulated with AIV infection) and TINP1 (down regulated with AIV infection) were predicted to be targeted by two differentially expressed miRNAs (miR-155 and miR-206 for Mx1, and miR-451 and miR-34a for TINP1, respectively). The miR-155 was up-regulated, while miR-206 was down-regulated with AIV infection. Based on negative regulation of miRNA on mRNA expression and the observed high fold up-regulation of Mx1expression, it suggested that miR-206 plays a major regulatory role on Mx1 expression. On the other hand, both miR-34a and miR-451 were up-regulated with AIV infection, suggesting that down-regulation of TINP1 at the mRNA level might be due to suppressive effects of both miRNAs. Basing on the target regulation mechanisms, miRNAs can have several target genes; one target gene is able to be targeted by several

miRNAs. miRNAs seem to be responsible for fine regulation of gene expression, tuning the cellular phenotype during delicate processes (Sevignani, et al., 2006).

Alternative splicing of mRNAs generate more distinct proteins than the number of original mRNAs (Maniatis and Tasic, 2002). Additionally, during the posttranslational stage, cells could make modifications to proteins (Kirkpatrick, et al., 2005). Therefore, mRNAs are able to sever as the basis for lots of potential proteins leading to various biological functions (Sinchaikul, et al., 2008). Having the information in cellular protein production will be more important than knowing how much mRNA is generated from each gene (Wilhelm, et al., 2007). Proteomics analysis will provide indispensable insights into host-pathogen interactions. The proteome is the entire complement of proteins (Wilkins, et al., 1996), and generally proteomics deals with the large-scale determination of gene and cellular function directly at the protein level (Aebersold and Mann, 2003). Mass spectrometry (MS) is one of the most popular approaches for proteomic analysis because of its capability for analyzing complex protein samples (Aebersold and Mann, 2003). Liquid chromatography (LC) coupled to tandem MS, called LC-MS/MS, is a powerful tool for the analysis of peptides and proteins. Biological materials are efficiently separated by LC, then identification and quantification of each individual protein have been carried out by MS. Thousands of proteins even with complicated structure can be directly analyzed (Mann, et al., 2001). A proteomic analysis using the LC-MS/MS method is the underway to analyze differentially expressed proteins with AIV infection in both Leghorn and Fayoumi lines. Over 2000 proteins have been identified with more proteins having higher expression

levels in Fayoumi than Leghorn birds pre-inoculation. However, more proteins were highly expressed in Leghorn birds than in Fayoumi birds post infection. Within genetic lines, more proteins were down-regulated in Fayoumi and more were up-regulated in Leghorn birds with AIV infection. This has demonstrated different defensive mechanisms between Leghorn and Fayoumi lines with AIV infection, given the nature of genetic resistance difference between these two lines (Leghorn is more susceptible, and Fayoumi is more resistant). Further data mining is necessary to discover crucial proteins involved in chicken and AIV interactions.

In conclusion, different genomic approaches can be utilized to elucidate molecular and cellular mechanisms of host-pathogen interactions at different regulatory levels. Specifically, studies in the dissertation generated a list of strong candidate miRNAs including miR-1, 32, 34a, 146a, 155, 181a, 187, 206, and 451 for our on-going effort to further elucidate regulatory mechanisms of miRNAs on AIV infection in chickens. In addition, several candidate genes including Mx1, IL-8, IRF-1, 7, TNFRS19, MAPK5 have been identified to be associated with AIV infection in broilers. Collectively, the results generated in this study have laid solid foundation not only in improving the prevention and treatment strategies of AIV in poultry industry, but also helping to control influenza infections as well as pandemic influenza viruses in humans.

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