ABSTRACT

HSIA, HUNG-CHING. Identification of Targets and Pathways Controlled by the Chicken MicroRNAs miR-10a and miR-143. (Under the direction of Dr. H. –C. Sunny Liu).

MicroRNAs are small non-coding RNAs that regulate gene expression at the post-transcriptional level. The importance of microRNAs in development, tumorogenesis, immune system function, and infectious diseases has gradually come to light. MicroRNAs are often expressed in a temporal and spatial manner and regulate specific gene sets to achieve phenotypic change. To understand the role of microRNAs in the chicken embryonic spleen, we have profiled microRNA expression at E15 and E20 chicken embryos. The objective of the current project is to identify the targets and pathways controlled by two microRNAs, miR-10a and miR-143. Target prediction was carried out by the algorithm miRanda. Eight predicted targets of each microRNA were subjected to validation using a reporter assay that incorporates synthetic or retroviral transduced microRNA. Validated targets for miR-143 known to have profound functions in apoptosis, T and B cell development and maturation, cancer, or adipocyte differentiation were identified with this approach; for miR-10a, validated targets involved in hematopoiesis, immune cells homing and migration and megakaryocyte differentiation were identified. Microarray analysis revealed that several targets not predicted by the algorithm were upregulated upon treatment with a specific microRNA inhibitor. This result suggests that both microRNAs may regulate genes that contribute to cancer formation. In addition, miR-10a may be involved in regulating the complement system, which is a crucial component of innate immunity; miR-143 may be involved in the PPAR/RXR pathway, which regulates glucose and lipid metabolism in cells. Taken together, our results suggest targets and pathways possibly regulated by miR-10a or

miR-143. The inferred functions of the targets and pathways identified in our study are mostly consistent with those from previous studies, thereby providing insights and directions for future research into the mechanisms by which miR-10a and miR-143 exert their function in the developing chick embryo. Furthermore, the RCAS system used in this project also proved the usefulness of engineered retrovirus for transducing microRNA. Further development of this tool may aid microRNA research and further contribute to understanding of the role of microRNAs in vertebrate development.

Identification of Targets and Pathways Controlled by the Chicken MicroRNAs miR-10a and miR-143

by
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BIOGRAPHY

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CHAPTER 1: LITERATURE REVIEW

The Discovery of RNA Interference

In eukaryotic cells, small RNAs serve important roles in the regulation of gene expression through a process called RNA interference (RNAi). Extensive study of RNAi and its related phenomena in the past two decades has contributed to our understanding of many different types of small RNAs in eukaryotic organisms and has led to the development of therapeutic applications for RNAi.

The initiation of RNAi study started with a serendipitous observation. In 1990, Napoli and colleagues attempted to overexpress the gene of chalcone synthase (CHS), a pigmented petunia enzyme that was essential for flavonoid biosynthesis and was involved in flower coloration. Overexpression of CHS was expected to intensify the flower color. Intriguingly, introduction of the transgene led to the production of completely or partially white flowers in transgenic plants. Examination of the CHS mRNA revealed that the mRNA level of both the transgenic and endogenous CHS was largely reduced. This phenomenon was named "cosuppression" to reflect the fact that the transgene suppressed the expression of itself and its endogenous homologue (Napoli *et al.* 1990). Shortly thereafter, similar observations were reported in other plant species (Smith *et al.* 1990; van der Krol *et al.* 1990; Romano and Macino 1992). Transgenes were also found to mediate virus resistance in plants. Transgenic tobacco plants expressing the coat protein gene of tobacco mosaic virus (TMV) were found to be resistant to TMV infection, showing no or delayed symptoms following TMV challenge (Abel *et al.* 1986). Similar research utilized transgenic plants expressing tobacco etch virus

(TEV) coat protein untranslatable transcript showed that the TEV resistance was mediated by the transgenic untranslatable RNA, indicating interaction between transgene RNA transcript and viral RNA by which the protection was exerted (Lindbo and Dougherty 1992). On the other hand, RNA-mediating gene silencing was also studied in animals. In *Caenorhabditis elegans*, injection of either antisense or sense RNAs could induce silencing of homologous genes (Guo and Kemphues 1995).

The molecular basis of these intriguing phenomena remained unclear until 1998. Then, Fire, Mello, and colleagues, using *C. elegans* as a model organism, demonstrated that double-stranded RNA (dsRNA) was the mediator of RNA-induced gene silencing, or RNA interference (RNAi) (Fire *et al.* 1998). Neither the ectopic mRNA (sense strand) nor the antisense RNA alone was capable of turning off gene expression of the corresponding gene– the gene was silenced only when both strands were present and formed dsRNA. They proposed that the silencing ability of sense RNA in Guo and Kemphues' experiments was a result of dsRNA contamination in the single-stranded RNA preparation. In addition, although dsRNAs targeting exons were potent inhibitors of gene expression, those targeting introns had a minimal effect, suggesting that RNAi reactions take place at the mRNA level.

Fire *et al.* also showed that although the silenced phenotype was dosage-dependent, the phenotype became evident at an extremely low concentration of dsRNA, and the silenced phenotype could be passed to the progeny (Fire *et al.* 1998). These results implied that some enzymatic reactions were involved in RNAi, amplifying the effect of the dsRNA, and that the RNAi mechanism might have additional characteristics that could epigenetically regulate gene expression at the DNA level. Collectively, these findings laid the foundation for RNAi

research. Fire and Mello were honored with Nobel Prizes in 2006 for their contribution to RNAi research.

This understanding of RNAi also helped unravel the mechanism of cosuppression. It is now clear that cosuppression is an RNAi-related process, because transgenes must be transcriptionally active to provoke cosuppression, and the decrease in the transgene mRNA level was a result of the reduced accumulation of steady-state mRNA, suggesting posttranscriptional mRNA degradation (Lindbo *et al.* 1993; Mette *et al.* 1999). Indeed, transgene integration tends to form complex repeat structures in the genome, and aberrant transcripts that are partially double-stranded may induce RNAi (Stam *et al.* 1998; Waterhouse *et al.* 1998; Stam *et al.* 2000). In addition, RNA-dependent RNA polymerases may use sense RNA as a template for producing dsRNA and are implicated in some cases of cosuppression (Cogoni and Macino 1999). Similarly, ectopic expression of viral genes could induce RNAi targeting viral genes, rendering the transgenic plants resistance to infections.

Mechanisms of RNAi

Not long after the initial discovery by Fire and Mello, two groups discovered that long dsRNAs were processed by RNase III-like enzymes into 21- to 23-nucleotide (nt) short-interference RNA (siRNA) duplexes with 2-nt 3′ overhangs. This type of RNA processing was the signature of RNase III. These siRNA duplexes appeared to guide the sequence-specific cleavage of target mRNA at 21- to 23-nt intervals (Zamore *et al.* 2000; Elbashir *et al.* 2001). Moreover, the addition of chemically synthesized siRNA duplexes to a *Drosophila* cell-free system or the transfection of such duplexes into cell-lines induced the desired

knock-down of target genes (Zamore *et al.* 2000; Elbashir *et al.* 2001). In other words, although the earlier experiments employed long dsRNAs to elicit RNAi responses, it was the resulting siRNA duplexes that mediated RNAi.

To generate siRNA duplexes, long dsRNAs are processed by Dicer, an RNase III endonuclease, in the cytoplasm (Bernstein *et al.* 2001; Ketting *et al.* 2001). The siRNA duplexes have the 2-nt 3′ overhang, which is the characteristic of RNase III processing. To carry out its functions, the siRNA duplex must be unwound and incorporated into the RNA-induced silencing complex (RISC). The guiding strand, which is antisense to the target mRNA and "guides" the RISC for silencing, remains in the RISC; the sense strand, also known as the "passenger strand," is cleaved by the enzyme Argonaute 2 and then discarded (Filipowicz 2005). The two strands of a siRNA duplex do not have an equal chance of being incorporated into RISC. The strand-selection preference is primarily based on the thermodynamic stability. The strand that has a higher free energy (that is, a weaker base-pairing) at its 5′-end will be preferentially retained in the RISC (Khvorova *et al.* 2003; Schwarz *et al.* 2003; Hutvagner 2005). It is believed that weak 5′ base-pairing favors the access of a putative helicase at the 5′ end, thereby facilitating the unwinding process, whereas the 3′ end is bound and oriented by the RNA-binding protein R2D2 (Preall and Sontheimer 2005).

Once RISC is loaded with the guide RNA, it is ready to execute the silencing missions. RISC comprises a group of proteins that include Argonaute 2, which serves as the principal mediator of silencing. The Argonaute family has several members and the number of members varies by species. All Argonaute proteins have a binding site for the 2-nt 3′-

overhang RNA duplex and are involved in different aspects of the RNAi pathway. Of all the mammalian Argonaute proteins, only Argonaute 2 has endonuclease activity and is the core player of the RNAi pathway (Liu *et al.* 2004; Hutvagner and Simard 2008). It is this endonuclease activity that confers Argonaute 2 with its ability to cleave target mRNA. Other proteins, such as fragile-X mental retardation protein and vasa intronic gene, have also been found to be associated with RISC, but their exact roles in RISC are as yet unclear (Caudy *et al.* 2002).

When RISC loaded with guiding RNA encounters and binds to complementary mRNA in the cytosol, Argonaute 2 cleaves the target mRNA at the position approximately 10 nt from the 5′ end of the guiding RNA, thereby silencing the expression of the gene at the posttranscriptional level (Elbashir *et al.* 2001). Argonaute 2 is therefore sometimes referred toas the "slicer" for its catalytic activity to cleave mRNA. RISC itself is a multi-turnover complex that can mediate multiple cycles of cleavage. An overview of the RNAi pathway is illustrated in Figure 1.1 (adapted from Sontheimer 2005).

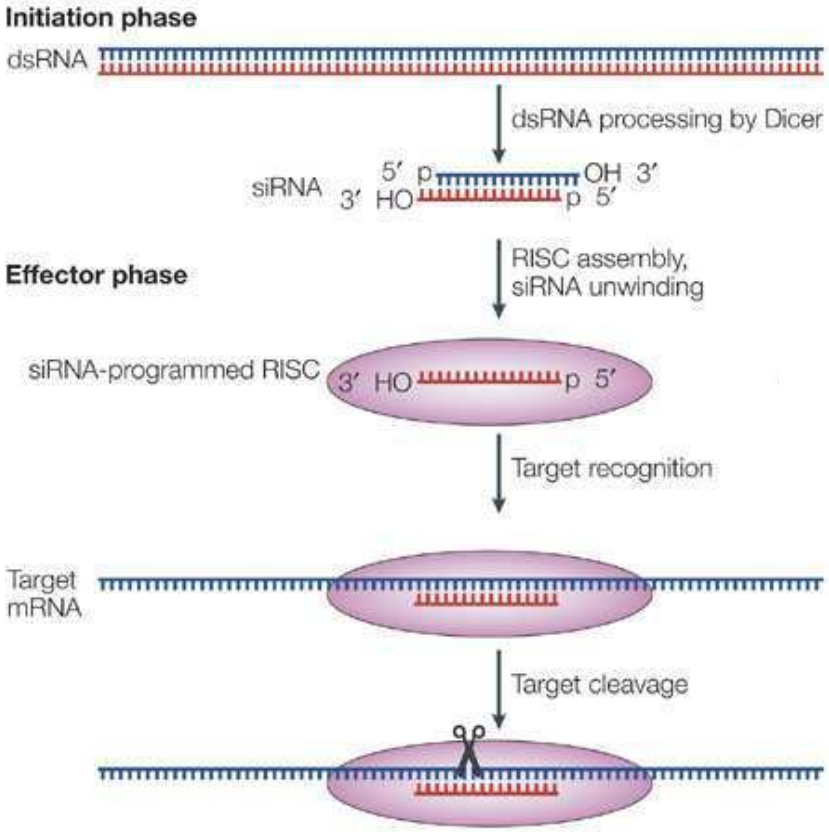

Figure 1.1 RNAi pathway. Double-stranded RNA from endogenous sources, viral replication intermediates, or experimental introduction is processed by Dicer in the cytoplasm to release the ~22nt short-interfering RNA duplex. The siRNA duplex is unwound and the guide strand is preferentially incorporated onto RNA-induced silencing complex (RISC). The siRNA-programmed RISC searches for and then cleaves the complementary mRNA, resulting in gene silencing. (Adapted from Sontheimer 2005)

Functions of RNAi

The RNAi pathway is evolutionarily conserved and has been proposed to serve some essential functions. Indeed, RNAi activity has been implicated in innate immunity against infection in some organisms. Furthermore, several newly discovered small regulatory RNA species, including microRNA and piwiRNA, employ the RNAi pathway to regulate gene expression and/or suppress transposable elements.

RNAi plays an important role in host defense in plants and invertebrates. Most RNA viruses produce long dsRNAs at some point during their replication cycle. Those dsRNAs are susceptible to the host RNAi machinery and can be processed into viral-origin siRNA, which leads to viral gene silencing. For example, virus-derived siRNAs have been detected in vesicular stomatitis virus (VSV) infected *C. elegans*, and RNAi pathway defective mutants showed an increased replication of VSV after infection (Schott *et al.* 2005; Wilkins *et al.* 2005). RNAi-defective *Arabidopsis* mutants also had increased susceptibility to cucumovirus infection (Mourrain *et al.* 2000). Conversely, many plant viruses encode RNAi suppressors to evade the host RNAi machinery (Roth *et al.* 2004). Taken together, the evidence that RNAi serves as part of the innate immunity against infection in plants and invertebrates is strong. However, whether the RNAi pathway is important in vertebrate innate immunity remains unclear.

The sequence-specific gene-silencing potential of siRNA has led to the development of siRNA-based therapeutics for infectious disease and even genetic diseases. In reality, however, this apparently promising approach has encountered many hurdles. The first problem is the off-target effect, which is due to complementarity between the siRNA and

bystander genes. With the aid of specific algorithms, the off-target effect can be minimized. Chemical modification of siRNA may also increase its specificity (de Fougerolles *et al.* 2007). Second, naked siRNAs are rapidly degraded, so the stability of the therapeutic siRNA should be taken into account to increase efficacy. Phosphorothioate (P=S) backbone and 2′-sugar modification render exonuclease and endonuclease resistance, respectively (de Fougerolles *et al.* 2007)

Many strategies have been developed to deliver siRNA effectively and specifically *in vivo*. For example, cholesterol-conjugated siRNA exploits the high level of cholesterol receptor expression in hepatocytes. Such conjugated siRNA can be delivered specifically to the liver after systematic administration (Soutschek *et al.* 2004). Thus far, only two siRNA-based drugs have been approved, but research and clinical trials to improve the design of siRNA-based drugs are ongoing. For example, T cell-specific siRNA delivery hinders HIV infection in humanized mice (Kumar *et al.* 2008). Our increasing understanding of the molecular mechanism of RNAi and its related processes should aid the advancement of siRNA-based therapies.

Discovery of microRNA

MicroRNA is a type of small regulatory RNA. The biogenesis of microRNA and the execution of its functions employ the RNAi pathway. In 1991, it was noticed that *lin-14*, a gene controlling the temporal sequence of *C. elegans* larval development, is subject to negative regulation during development. A region in the 3′-untranslated region (3′-UTR) of *lin-14* mRNA was found to be essential for this negative regulation (Wightman *et al.* 1991).

In support of this discovery, a non-protein-coding gene, *lin-4*, was found to produce small RNAs that are partially complementary to the critical region in the *lin-14* 3′-UTR (Lee *et al.* 1993). The *lin-4* null mutants were found to possess phenotypes similar to those of *lin-14* gain-of-function mutants, in which cell fate reiterated the larval 1 (L1) stage instead of entering the L2 stage normally (Chalfie *et al.* 1981). Expression of *lin-4* resulted in a reduction in the level of lin-14 protein without noticeably altering the mRNA level, suggesting translational repression (Wightman *et al.* 1993). In further investigations, the *lin-4* RNA transcript was predicted to form a stem-loop structure, and a small, single-stranded RNA released from the transcript was proposed to bind to multiple sites in the 3′-UTR of the *lin-4* mRNA and thus to inhibit translation through antisense RNA-RNA interactions (Lee *et al.* 1993). It is now clear that *lin-4* encodes microRNA that is essential for down regulating the level of lin-14 protein at the late L1 stage and that this decreased expression of lin-14 leads to entry into the L2 stage.

Biogenesis of MicroRNA

The characteristics and biogenesis of microRNA are similar to those of siRNA, but although it utilizes the RNAi machinery, the details are different. The distinct feature of microRNA is that the microRNA transcript can form an imperfect stem-loop structure in which the mature microRNA is localized solely within one arm. The whole transcript, also called primary microRNA (pri-miRNA), is transcribed by RNA polymerase II and is 5′-capped and 3′-poly(A)-tailed (Cai *et al.* 2004; Lee *et al.* 2004). MicroRNAs stem-loop structures may be located in non protein-coding transcripts or in the introns of coding

transcripts. Pri-miRNA is cropped in the nucleus by the RNase III enzyme Drosha and its cofactor (Pasha in fly or DGCR8 in human) to liberate the ~70-nt stem-loop portion (Lee *et al.* 2003; Denli *et al.* 2004; Gregory *et al.* 2004). The stem-looped RNA, also called precursor microRNA (pre-miRNA), is then transported out of the nucleus by exprotin-5 and the small GTPase Ran (Yi *et al.* 2003; Bohnsack *et al.* 2004; Lund *et al.* 2004). Once it enters the cytoplasm, another RNase III enzyme, Dicer-1, cleaves off the loop to release the ~21- to 25-nt RNA duplex with 2-nt 3' overhangs (Ketting *et al.* 2001; Knight and Bass 2001). The resultant RNA duplex is sometimes referred to as a miRNA:miRNA* duplex, where miRNA is the guiding strand that eventually ends up in the RISC, and miRNA* is the passenger strand that is degraded.

The strand selection mechanism of the microRNA duplex is similar to that of siRNA. In flies, Dicer-1 and Loquacious, a paralogue of the RNA-binding protein R2D2 in the RNAi pathway, orient the microRNA duplex for asymmetric RISC loading. Again, the strand that is less thermodynamically stable at its 5′ end will be preferentially retained in the RISC. Most of the time, only one strand of the microRNA duplex is loaded, whereas the other strand is cleaved by Argonaute 2 and then rapidly degraded; in some cases, however, the miRNA* sense strand can also be incorporated into RISC and is functional (Kim *et al.* 2008). The ratio between functional miRNA and miRNA* seems to be determined by the difference of free energy. When the base-pairing at one end is apparently weaker then the other end, one strand will predominate, but if the difference in free energy is small, both strands will have a chance to be loaded onto the RISC (Schwarz *et al.* 2003; Hutvagner 2005).

Recognition of MicroRNA Targets

It should be noted that the mechanism by which microRNAs carry out their function differs from that of canonical RNAi. In addition to the structural prerequisites of stem-loop structures in its pri-miRNA and pre-miRNA and the localization of the mature microRNA entirely within one of the two arms, the mature metazoan microRNA usually does not match perfectly with its target mRNA and does not directly lead to cleavage of its target mRNA. Instead, microRNAs usually induce translational inhibition without noticeably altering the target mRNA level (Wightman *et al.* 1993; Brennecke *et al.* 2003). Moreover, microRNA usually binds to the 3′-UTR of the mRNA, whereas the siRNA target may be anywhere in the mRNA.

The details of the microRNA mechanism have been gradually worked out. It has been elucidated that the second to seventh nucleotides at the 5′ end of the mature miRNA usually have perfect or near perfect complementarity to the target mRNA (Lai 2002). This region is known as the "seed region," reflecting its importance in microRNA target recognition, and is often conserved among homologous microRNAs across species (Lewis *et al.* 2003). In contrast, the 3′ ends of microRNAs are usually only partially complementary to their targets; mismatches and bulges can be tolerated. The thirteenth to sixteenth nucleotides of the microRNA are also somewhat important in determining the target specificity, especially when the seed region complementarity is not perfect (Grimson *et al.* 2007). Some other factors also contribute to microRNA target recognition, such as the position of the target site in the 3′-UTR and the mRNA secondary structure, which might affect the accessibility of RISC to the target site (Grimson *et al.* 2007). Contextual sequences in the 3′-UTR might also

affect target recognition in a case-dependent manner through a mechanism that remains largely obscure (Didiano and Hobert 2008).

Based on current knowledge of microRNA target recognition, many target prediction algorithms have been developed. PicTar, TargetScan, and miRanda are the most commonly used algorithms (John *et al.* 2004; Krek *et al.* 2005; Lewis *et al.* 2005). MiRbase (http://microrna.sanger.ac.uk), the official microRNA registry and database, also provides target prediction database based on miRanda (Betel *et al.* 2008).

Mechanisms of MicroRNA

MicroRNAs primarily down regulate gene expression through translational inhibition. Unlike siRNA, which exhibits perfect complementarity to and triggers the cleavage and degradation of its target mRNA, metazoan microRNA is usually only partially complementary to its target mRNA. In the rare cases that a microRNA is perfectly complementary to its target mRNA, the mRNA will be cleaved by Argonaute 2 as if it were siRNA. Of the animal microRNAs, miR-196 is the only one known to be a perfect match with its target. The *HOXB8* gene has a perfect miR-196 binding site in its 3′-UTR, and coexpression of miR-196 and *HOXB8* leads to the cleavage and degradation of *HOXB8* mRNA as well as a decrease in the protein level (Yekta *et al.* 2004). In most other circumstances where mismatches or bulges are present between the target and the miRNA, the common consequence of microRNA recognition is translational repression but not direct mRNA cleavage. The biogenesis and mechanism of microRNA is depicted in Figure 1.2 (adapted from He and Hannon 2004).

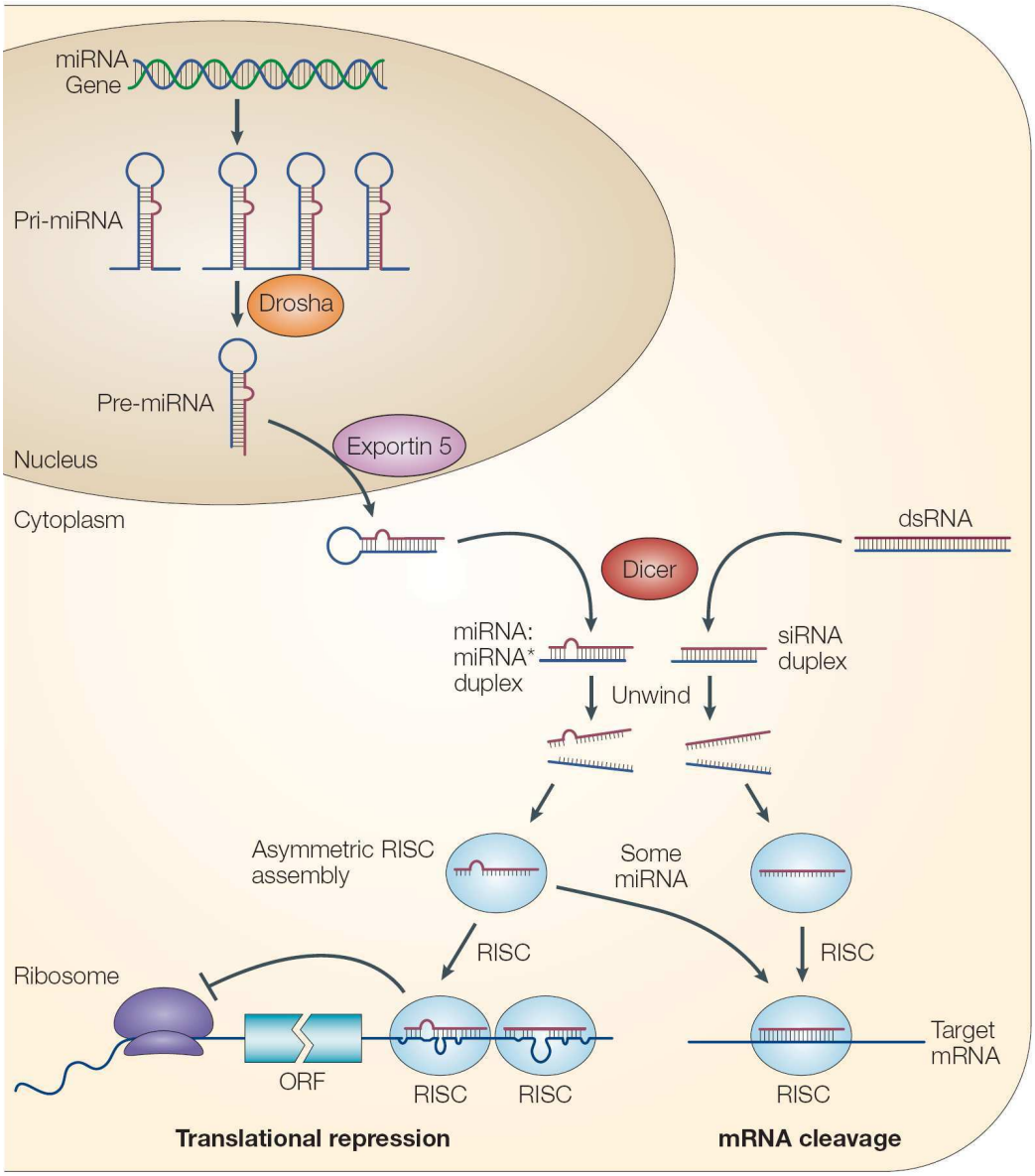

Figure 1.2 Biogenesis and mechanism of microRNA. Primary microRNA transcript is transcribed by RNA polymerase II. Primary microRNA is processed by Drosha and cofactors to release the stem-looped precursor microRNA, in which the mature microRNA locates in one of the arms. Precursor microRNA is transported to cytoplasm, where it is further processed by Dicer to release the microRNA:microRNA* duplex. The mature microRNA is preferentially loaded onto RISC and searches for its target mRNA. In rare cases where the microRNA has perfect complementary to mRNA, the mRNA will be cleaved as if it is recognized by siRNA. In general, microRNAs recognize the 3'-UTR of target mRNA through imperfect base-pairing, resulting in translational repression. (Adapted from He and Hannon 2004)

How microRNAs repress translation is still somewhat disputable. In general it has been accepted that microRNAs repress translation by targeting translation initiation and by sequestering mRNA in specific cellular locations. Pillai *et al.* found that ribosome loading is reduced on microRNA-targeted mRNA, but cap-independent translation is not affected, suggesting that microRNA represses translation by interfering with cap recognition in the initiation step (Pillai *et al.* 2005). Along with the same lines, microRNA-loaded RISC was able to repress loading of the 60S ribosomal subunit onto the mRNA (Wang *et al.* 2008). Furthermore, the 3′-poly(A) tail and the involvement of eIF4E and eIF4G were also required for microRNA-mediated translational repression (Humphreys *et al.* 2005; Pillai *et al.* 2005). Argonaute 2 was found to bind to the 5′-cap and to compete with eIF4E for cap binding, thus inhibiting the assembly of the initiation complex for translation (Kiriakidou *et al.* 2007).

Other than inhibiting translation initiation, the binding of a microRNA-guided RISC to an mRNA can cause the translocation of the mRNA to a processing-body (P-body) (Pillai *et al.* 2005). A P-body is a dynamic cytosolic granule comprising a variety of RNA metabolic enzymes, including decapping and deadenylase complexes and exonuclease. The P-body is considered to be a site for temporary RNA storage as well as for RNA degradation. Once the RISC-bound mRNA is translocated to the P-body, it is sequestered from the translational machinery, and microRNA-mediated translational inhibition is therefore further enhanced by compartmentalization.

The mRNA in the P-body may re-enter the active translational mRNA pool when proper stimuli exist and relieve the inhibition. For example, miR-122 targets cationic aminoacid transporter-1 (*CAT-1*) mRNA for translocation to the P-body. Upon amino acid

starvation, the *CAT-1* mRNA is released from the P-body and enters the cytosol for translation (Bhattacharyya *et al.* 2006). Nonetheless, whether all mRNAs sequestered in the P-body have a chance to re-enter the cytosol and how the recycling is achieved remain open issues. More commonly, mRNAs in the P-body are eventually decapped, deadenylated, and degraded (Peters and Meister 2007). Although there is no evidence to show that imperfect binding of microRNAs can trigger direct degradation of mRNA, a decrease of the mRNA level is sometimes observed. For example, Bagga et al. found that the mRNA levels of *let-7* targeted gene *lin-41* and *lin-4* targeted gene *lin-14* and *lin-28* are significantly decreased upon the expression of *let-7* or *lin-4*, respectively. All three targets possessed only partial complementary sites in 3'UTR to the microRNAs (Bagga *et al.* 2005; Behm-Ansmant *et al.* 2006; Wu *et al.* 2006). In line with this evidence, it was shown that microRNAs can promote decapping and deadenylation of targeted mRNAs in P-body, thereby facilitating the degradation of mRNAs (Behm-Ansmant *et al.* 2006; Wu *et al.* 2006). Therefore, degradation of mRNA could be an indirect consequence of imperfect microRNA binding, and microRNA may down regulate target gene expression through the combination of translational inhibition and facilitation of mRNA degradation. The change in the level of targeted mRNA allows the utilization of microarray technology to examine the mRNAs targeted by microRNA (Lim *et al.* 2005).

Though other factors are involved, microRNA target recognition is largely defined by the 6-nt region of complementarity between the microRNA seed region and mRNA. This characteristic renders flexibility to microRNA-based regulation of expression. Target prediction algorithms such as PicTar have suggested that one microRNA may have up to

several hundreds different mRNA targets (Krek *et al.* 2005). Lewis *et al.* estimated that 30% of the mammalian genome may be subject to microRNA regulation (Lewis *et al.* 2003). The capacity of a single microRNA to target a group of genes makes it more difficult to define and elucidate the exact roles of a particular microRNA. Moreover, it is common that multiple microRNA bindings are required for efficient translation repression. One mRNA may have multiple binding sites for one or several different microRNAs, and synergy among different microRNA might occur. The latter scenario also brings up the need for microRNA profiling in different cell types or conditions, as the combination of microRNAs can determine whether a gene is tightly regulated by the orchestration of multiple microRNAs, thereby affecting the phenotype of the cell in terms of proliferation, growth, cell death, and many other aspects.

MicroRNAs in Development

Several microRNAs have been shown to be important in developmental processes. In addition to the original discovery that *lin-4* dysregulation disrupts the normal developmental timing of *C. elegans* larvae. Another example is the association between microRNA and double muscling. Double muscling is often caused by the mutation and inactivation of myostatin, a muscle-specific protein that is a negative regulator of muscle development. Myostatin loss-of-function mutations result in increased muscle growth and exceptionally large volumes of muscle tissue. In some cases of double muscling in sheep, however, there are no mutations in the myostatin open reading frame; instead, a G→A transition occurs in the 3′-UTR of the myostatin gene. Further investigation revealed that this G→A transition

created an illegitimate binding site for miR-1 and miR-206, two muscle-specific microRNAs, which caused the down regulation of myostatin expression, inducing the double-muscling phenotype (Clop *et al.* 2006).

Another example of microRNA regulation in embryogenesis is the case of miR-430, which is expressed at the onset of zygotic transcription in zebrafish. Using a microarray-based approach, Giraldez *et al.* identified hundreds of miR-430 targets, the majority of which are maternally expressed mRNAs that accumulate in the absence of miR-430 (Giraldez *et al.* 2006). When present, miR-430 induced the deadenylation of targeted mRNAs and promoted their degradation, suggesting a critical role for miR-430 in facilitating clearance of the maternal mRNA in the maternal-to-zygotic transition. Interestingly, lack of miR-430 does not induce arrest of the embryo in maternal stage. Instead, the embryo developed into a mixed maternal-zygotic state, suggesting that miR-430 (and perhaps other microRNAs as well) does not function as an on-off switch but rather as a tool for sharpening the distinct transition stages by regulating a subset of genes (Giraldez *et al.* 2006).

MicroRNAs in Immunity and Infectious Diseases

The roles of microRNAs in the immune response have been studied extensively in mammalian systems. One of the most well-studied microRNAs is miR-155, which is upregulated in activated T and B cells, as well as in lymphomas (Eis *et al.* 2005; Kluiver *et al.* 2005). MiR-155 lies within the *BIC* locus, which was first identified as a hotspot for avian leukosis virus integration in chicken (Tam *et al.* 1997). The transforming potential of miR-155 has been confirmed by ectopic overexpression (O'Connell *et al.* 2008). Confirmed

targets of miR-155 include the genes for activation-induced cytidine deaminase (AID), which is a critical mediator in B-cell somatic hypermutation, and tumor protein 53-induced nuclear protein 1 (TP53INP1), which is a p53-induced proapototic gene (Gironella *et al.* 2007; Vigorito *et al.* 2007; Dorsett *et al.* 2008; Teng *et al.* 2008). Furthermore, expression of miR-155 can be induced following inflammatory stimuli in macrophages and activation of B-cell receptors and TLR4 signal transduction (O'Connell *et al.* 2007; Tili *et al.* 2007; Yin *et al.* 2008). Based on its confirmed targets and expression profile, it is tempting to hypothesize that miR-155 regulates a subset of genes that is usually down regulated in activated and proliferating immune cells.

Recently, it has been found that Kaposi's sarcoma-associated herpesvirus (KSHV) encodes a homologue of miR-155. The KSHV miR-155 homologue has a seed region identical to that of cellular miR-155. The target of this viral microRNA has been confirmed to overlap with a set of cellular miR-155 target genes, indicating that the virus might utilize the cellular miR-155 pathway for its own advantage (Gottwein *et al.* 2007; Skalsky *et al.* 2007). On the other hand, in B cells latently infected with Epstein-Barr virus (EBV), the host miR-155 level was elevated in response to the expression of the EBV protein LMP-1 (Motsch *et al.* 2007; Yin *et al.* 2008). Interestingly, another oncogenic herpesvirus, Marek's disease virus (MDV), also appears to encode a microRNA with a seed region sequence similar to that of host miR-155 (Gottwein and Cullen 2008). Whether the MDV miR-155 homologue is functional remains to be investigated. All three viruses cause lymphomas; KSHV and EBV induce B lymphomas and MDV induces a T lymphoma. Given our knowledge of the transforming capacity of miR-155, it is tempting to hypothesize that

viruses may either induce host microRNAs or encode their own microRNAs that mimic the host microRNAs to utilize the preexisting pathways and alter the host cell environment to their own advantages. In these cases, the viral microRNA may contribute to the oncogenicity of the virus.

Embryogenesis of Chicken Immune Organs

Bursa

The Bursa of Fabricius is a primary immune organ existing only in avian species. It originates from an epithelial bud of the cloacal wall during embryogenesis and is required for B cell proliferation and differentiation. In chicken embryo, the B cell progenitors migrate from spleen to bursa during E8–E14. After settling in the bursal follicles, the progenitors proliferate and start the gene conversion process to increase immunoglobulin variability. Unlike human and mouse immunoglobulin genes, which have many functional V, D, and J segments that can be combined in multiple ways to create the highly diversified immunoglobulin repertoire, the chicken immunoglobulin gene has a very limited number of V, D, and J segments. As a result, the rearrangement of V(D)J segments in chicken can only add limited diversity to the total immunoglobulin repertoire (Reynaud *et al.* 1985). Instead, the variability is generated primarily through gene conversion in the bursa. Chicken has multiple nonfunctional V pseudogene segments that act as donors, and small segments of the pseudogenes are transferred unidirectionally into the functional V segments (Thompson and Neiman 1987; Thompson 1992). The gene conversion process onsets at E15 and continues until after hatching.

Spleen

The spleen is the major secondary immune organ that hosts numerous T cells, B cells, red blood cells, and some other white blood cells. By definition, secondary immune organs are in charge of the late stage of cell differentiation and proliferation. The spleen is also where specific responses to pathogens are roused and amplified. Unlike its mammalian counterparts, the chicken spleen has a poorly developed smooth-muscle sheath, suggesting a lack of ability to squeeze out blood, and thus chicken spleen might not serve a critical role in temporary blood storage (Jeurissen 1991).

In chicken, splenic T cells are also formed in the thymus. As early as E6, thymocyte precursors are attracted to the thymus by free β2m, one of the components of MHC class I, secreted by the thymic epithelium. The thymocyte precursors enter the thymus in three waves (at E6, E12, and E18) and continue differentiation. The precursors in the first wave are mostly from the paraortic region, whereas those in the second and third waves come from the bone marrow. Once entering the thymus, T cells begin to express αβ or γδ T cell receptors and to proliferate. Accordingly, the thymocytes migrate to the periphery, including the spleen and intestine, in three waves approximately 10 days after colonizing the thymus. The first waves of αβ and γδ T cells arrive at the spleen at approximately E18–20 and E15–17, respectively.

Immune Responses in the Chicken Embryos

Although the ontogeny of the chicken immune system has been studied for decades, the chicken embryonic immune response has endured a lack of attention and knowledge.

Little is known about how embryos generate immune responses toward pathogens or stimuli. The only established fact is that chicken embryos can elicit functional responses against pathogens and retain this "memory" in their later life stages. The case of MDV illustrates this point. As an oncogenic chicken herpesvirus, MDV can cause extensive losses for the poultry industry. The MDV epidemic is now under control due to the development of MDV vaccines. These vaccines are usually made of attenuated viruses and are injected to chickens. Vaccination confers the protection to virus partially by modulating the cytokine expression profile. For example, vaccinated chickens express less IL-6, IL-10, and IL-18 after infection as compared to unvaccinated chickens (Abdul-Careem *et al.* 2007), and lower levels of IL-6 and IL-18 have been associated with genetic resistence to MDV (Kaiser *et al.* 2003). High viral loads in the organs of infected chickens are often associated with clinical symptoms, and vaccinated chickens have less viral loads in the internal organs (Tan *et al.* 2007).

Marek's disease vaccination may be performed in hatchlings or embryos, that is, *in ovo* vaccination. Compared to chicks vaccinated at hatching, chicks vaccinated at embryonic day 17 were better protected from MDV challenging as determined during the first four days after hatching (Zhang and Sharma 2001). In another study, chickens vaccinated at embryonic day 17.5-18.5 and at hatching showed comparable protection against MDV in terms of mortality, but the hatchability was lower in the *in ovo*-vaccinated chicks (Sarma *et al.* 1995). These results indicate that chicken embryos are capable of mounting a pre-hatch immune response to pathogens, and such protection may be maintained in later stage of life. The disadvantage of low hatchability in the *in ovo*-vaccinated chicks has been overcome by the introduction of the automatic injection system, INOVOJECT (Johnston *et al.* 1997). Therefore, *in ovo*

vaccination in chicken embryos has been widely adopted due to lower cost and the efficacy. These studies also indicate that even if the chicken embryos are not fully equipped with a mature immune system, the embryos can still generate immune response to some extent, but exactly how the embryonic immune response, if there is any, is generated and the difference from adult immune responses remains to be determined.

MicroRNA Profiling in Chicken Embryonic Spleen and Bursa

Intensive study has implicated microRNAs in many processes, including development, cell lineage determination, and immune cell activation. Artificial manipulation of microRNA expression may lead to an abnormal phenotype, and aberrant microRNA expression is often found in cancer cells.

To increase our understanding of the roles of microRNAs in the fine-tuning and regulation of immune system development and response in the late stage of embryogenesis, we first focused on microRNA expression profiling. In our previous work, we sought to profile the microRNA expression at embryonic day 11 (E11) chicken fibroblast and embryonic day 15 and 20 (E15 and E20) spleen and bursa (Hicks *et al.* 2008). The small-RNA fractions of E15 and E20 spleen and bursa were purified, and pyrosequencing was used to effectively sequence the entire population of small RNA species. Pyrosequencing allows simultaneous sequencing of multiple short sequences and is better suited to small-RNA library sequencing than are conventional sequencing methods. All resulting sequences homologous to any known protein coding sequences or rRNA were discarded, and the remaining small RNA sequences were compared with known microRNAs from chicken and

other species to identify putative microRNAs. Structural predictions were performed using the putative microRNA sequences to confirm that they met the structural prerequisites for pre-microRNA. Structural prediction was also carried out on the small RNAs showing no homology to any known RNA.

This pyrosequencing and hierarchical approach led to the discovery of many chicken microRNA homologues that had not been previously identified but were highly similar to known microRNAs of other species. Several novel RNAs were also found that showed no homology to any known microRNAs but met the structural criteria for microRNA. Because pyrosequencing is semi-quantitative in nature, the number of reads of a certain sequence reflects the abundance of that RNA species in a given RNA library, enabling us to establish a microRNA expression profile for chicken embryonic spleen and bursa. This profile contains information about the identity and relative abundance of the specific microRNAs being expressed in a given organ at a given time point. These microRNA expression profiles will lay the foundation for investigating how microRNAs contribute to modeling of the splenic microenvironment to provide suitable conditions for T cell homing at specific time-points, and how microRNAs affect subsequent cell survival, proliferation, or responses to stimulation.

Objectives of the project

The aim of the present project was to investigate the role of specific microRNAs expressed in chicken embryonic spleen. To achieve this goal, we sought to (1) determine the direct targets of specific microRNAs using algorithm prediction and *in vitro* reporter assays;

(2) carry out microarray analysis on cells transfected with synthetic microRNA; (3) combine the results to suggest pathways governed by the microRNA.

We selected two microRNAs, miR-10a and miR-143, from our previous profiling work for further investigation. MiR-10a was expressed only in E15 and E20 spleen but not in liver or bursa, whereas miR-143 was expressed in all three organs at both time-points (Hicks, 2007; Hicks, unpublished data). The miR-10a and miR-143 expression profiles suggested that miR-10a regulates some spleen-specific events and that miR-143 regulates some pathways essential to cell survival and proliferation. These results prompted us to further investigate their possible target genes and functions.

MiR-10a

In mouse, miR-10a is embedded in the *HOXB* cluster and lies upstream of the *HOXB4* gene. *HOXB4* and miR-10a have similar expression patterns in E10 mouse embryo and E4.5-E5 chicken embryo, suggesting a common regulatory mechanism that might be attributed to *cis*-regulatory elements (Mansfield *et al.* 2004). In human, bone marrow $CD34^+$ progenitors also express miR-10a. Garzon *et al.* showed that as the miR-10a level decreases dramatically during *in vitro* differentiation of human megakaryocytes, expression of *HOXA1* increases significantly. The finding that *HOXA1* is a direct target of miR-10a suggested a role for miR-10a in megakaryocytopoiesis in which it relieves the expression inhibition of crucial transcription factors (Garzon *et al.* 2006).

MiR-10a is also significantly upregulated in hepatitis C virus (HCV)-associated hepatocellular carcinoma and acute myeloid leukemia (AML) (Debernardi *et al.* 2007;

Ovcharenko *et al.* 2007; Varnholt *et al.* 2008). Again, *HOXB4* expression correlates with miR-10a, and *HOXB4* is also upregulated in AML patients (Debernardi *et al.* 2007; Ovcharenko *et al.* 2007). One of the distinctive features of AML is the high expression of *HOX* genes. The overexpression of *HOXB4* and the downregulation of microRNAs targeting other *HOX* genes might contribute to the general overexpression of *HOX* genes in AML. Therefore, it is possible that miR-10a is upregulated by the same mechanism that upregulates *HOXB4* and miR-10a as the disease progresses, rather than as a factor contributing to pathogenesis. The role of miR-10a in AML and HCV-associated hepatocellular carcinoma is therefore not yet clear.

More recently, miR-10a was found to serve a unique role in the regulation of translation. Most miRNAs negatively regulate translation by binding to the mRNA transcript and blocking translation. However, binding of miR-122, which is a microRNA highly expressed in liver, to the 5′ end of the HCV genome is required for HCV genome replication. Inhibition of miR-122 suppressed HCV replication in susceptible cell lines, and supplementation with exogenous miR-122 in non-permissive cells allowed HCV to replicate to some degree (Randall *et al.* 2007; Chang *et al.* 2008). MiR-122 was the first microRNA shown to determine virus tissue tropism by a single host miRNA.

MiR-10a is another exception that can act similarly to miR-122 to enhance translation. MiR-10a binds to the 5′-UTR of several ribosomal protein mRNAs and enhances the translation of those ribosomal proteins (Orom *et al.* 2008). As a result of increased ribosomal protein level, the global protein translation was also increased. This in turn favored rapid cell growth, one of the hallmarks of active cancer cells. Indeed, cells ectopically overexpressing

miR-10a exhibit increased rates of global protein synthesis and transformation (Orom *et al.* 2008). Although the details of the mechanism remain unknown, this translational enhancement characteristic of miR-10a may partially explain its overexpression in cancerous cells. This finding also broadens our expectations, suggesting that microRNA is more versatile than originally thought. Whether miR-10a plays a such role in chicken embryonic spleen remains unknown.

MiR-143

MiR-143 and miR-145 are expressed and processed from one non-coding RNA transcript in human and mouse, so they are usually coordinately expressed. The levels of miR-143 and miR-145 are decreased in colon cancer, cervical cancer, chronic lymphocytic leukemia (CLL), as well as in an EBV-transformed B-cell line (Michael *et al.* 2003; Akao *et al.* 2007; Lui *et al.* 2007), in which the miR-143 and miR-145 levels inversely correlate with cell growth rate (Akao *et al.* 2007). Thus, miR-143 and miR-145 may negatively regulate the expression of proto-oncogenes that induce cell growth and proliferation. One of the possible candidates, mitogen-activated protein kinase 7, also known as ERK5, has been validated as an miR-143 target and is upregulated in CLL (Akao *et al.* 2007).

MiR-143 also participates in adipocyte differentiation (Esau *et al.* 2004); differentiation is interrupted in the presence of miR-143 inhibitor. Interestingly, when pre-adipocytes were induced for differentiation in one study, the expression level of miR-143 did not change much at first, but by 10 days post-induction it had quadrupled (Esau *et al.* 2004). Expression of ERK5 again proved to be inversely correlated with the level of miR-143 in

adipocytes. However, although ERK5 is a true target of miR-143, whether ERK5 contributes to adipocyte differentiation remains unknown. The mechanism by which miR-143 regulates adipocyte differentiation is not yet clear. The upregulation of miR-143 in the late stage of adipocyte differentiation suggests that miR-143 fine-tunes and down regulates the expression of certain genes after the initial differentiation to maintain the cells in a differentiated stage or to prevent the overgrowth of cells. Therefore, by targeting some critical components in the pathway, it is possible that miR-143 helps turn off the signal transduction cascade after cells are induced and primed for differentiation.

Our previous studies revealed that miR-143 is expressed in embryonic spleen and bursa, suggesting an essential role for miR-143 in both organs at the E15 and E20 (Hicks, 2007; Hicks, unpublished data). Research performed in mammals has shown that miR-143 is involved in several different processes, including cell proliferation and differentiation, possibly by targeting different genes. Therefore, we are interested in studying the targets and possible functions of miR-143 in the context of chicken immune organ development.

CHAPTER 2: MATERIALS AND METHODS

Project Outlines and Objectives

As reviewed in chapter 1, microRNAs have important roles in development and cell lineage commitment. Our previous data (Hicks, 2007; Hicks, unpublished data) showed miR-10a is specifically expressed in embryonic spleen but not bursa or liver, indicating miR-10a may serve a unique function in spleen at that specific time-point. On the other hand, miR-143 is expressed in all organs tested (spleen, bursa, and liver), suggesting it may be involved in regulating some essential pathways, such as cell proliferation, metabolism, or cell death. It would be of great interest to elucidate their roles in chicken embryonic spleen by identifying the direct targets and pathways regulated by these microRNAs through gain- and loss-of-function studies.

Reporter assay is a commonly used strategy to determine the direct target of microRNA in vitro (Clancy *et al.* 2007). The presumable target sequence is cloned into the immediate downstream region of the reporter gene open reading frame and serves as the 3'-UTR of the reporter gene transcript. After cotransfection of the synthetic microRNA and reporter plasmid into the desired cell culture system, the reporter gene expression is measured and normalized to a reference gene to determine the suppression caused by microRNA. This project utilized the psiCHECK2 vector (Promega), which encodes Renilla and firefly luciferases. The Renilla luciferase is used as a reporter gene whereas firefly luciferase is an internal transfection reference. The success of this strategy relies on the efficacy of plasmid DNA and small RNA cotransfection. However, the optimal transfection

conditions for plasmid DNA and small RNA are different, such that not all of the cells will be transfected with both RNA and luciferase reporter construct. The cell population harboring both molecules may be small, and the cells transfected with the reporter plasmid only will produce reporter activity that is not subject to regulation due to absence of coexistent small RNA, thereby compromising the sensitivity of the reporter assay. Moreover, the effect of transfection is transient. To get around this problem, a better approach is to either stably express the small RNA or the luciferase reporter gene and transfect the other. In our case, stable expression of miRNA rather than the reporter gene would be a better choice.

The use of retroviral vectors is a promising way to stably and efficiently express ectopic genes in many different systems. In chicken, Rous sarcoma virus (RSV), an avian retrovirus, derived vectors are commonly used. RSV is oncogenic because it contains the oncogene v-src. Like all retroviruses, RSV integrates its DNA into the host genome as a provirus. This characteristic has enabled retroviruses to be engineered into efficient foreign gene delivery and stable expression systems. Once the cells are infected by the engineered retrovirus, the foreign gene will be stably expressed. Of the RSV-derived vectors, the RCAS (Replication Competent with an ALV LTR and a Splice acceptor) vector, is commonly used. In the RCAS vector system, the RSV proviral sequence has been placed into an adaptor plasmid that can be propagated in *E. coli*. The plasmid DNA produced is considered to be an infectious clone. Additionally, the *v-src* oncogene of RSV has been deleted, and the gene of interest can be inserted instead. Once the engineered RCAS infectious clone is transfected into target cells, RCAS viruses carrying a gene of interest will be produced and infect more cells. The gene of interest is then expressed from a spliced mRNA driven by a promoter

embedded in the 5'LTR of RCAS genome (Hughes 2004). Because of its avian origin and infectivity to dividing cells, RCAS-mediated gene delivery has been widely applied for gain-of-function studies in chicken embryos and has greatly contributed to our understanding of vertebrate development. The comprehensive understanding of RCAS and its cellular receptor, tva, also enabled researchers to produce *tva* transgenic mice, further expanding the usefulness of RCAS into mammalian systems (Orsulic 2002).

Recently, the RCAS vector was modified to express shRNA driven by the RNA polymerase III promoter (Bromberg-White *et al.* 2004). Moreover, it is found that siRNA can be generated by modifying the mature miRNA sequence in the primary transcript, the so-called shRNA-mir (Silva *et al.* 2005; Zeng *et al.* 2005). The structural context of pri-miRNA renders a higher efficiency for excising mature siRNA. Chen and colleges utilized an RCAS vector to overexpress shRNA-mir in the context of miR-30a to elicit RNAi (Chen *et al.* 2007). In short, the chicken miR-30a gene was cloned into a Gateway entry vector. The stem region of miR-30a was replaced by the target sequence and its guide RNA sequence, which becomes incorporated into RISC. Upon Gateway recombination of entry vector and the RCAS vector, the engineered miR30a cassette was inserted into the RCAS provirus sequence to produce RCAS-shRNA-mir infectious clone. The resulting RCAS virus produces the shRNA in the context of miR-30a, which is processed by Drosha and Dicer as if it is endogenous miR-30a, eventually leading to the overexpression of the siRNA and knock down of the target gene.

Here we propose to employ the same system to overexpress our microRNA of interest and carry out the luciferase assay. The stem region of miR-30a will be replaced by another

mature microRNA sequence and its antisense sequence. However, after processing by Dicer, both strands of the short RNA duplex may have a chance to be loaded onto RISC. Based on current knowledge, the strand that has higher free energy and is less stable at the 5' end is usually preferentially loaded onto RISC (Schwarz *et al.* 2003). Therefore, the miRNA sequence must be placed at the correct orientation to favor its loading, or the bulge may have to be introduced into its 5' end. Furthermore, Northern blotting may be carried out after RCAS virus infection to confirm the overexpression of the microRNA of interest. To evaluate the efficiency and usefulness of the RCAS vector in microRNA research, synthetic microRNA (Dharmacon) was used for miR-10a luciferase assays and RCAS-miR143 virus was used for miR-143 luciferase assays.

On the contrary, loss-of-function study can be carried out by transfecting a microRNA inhibitor to block the endogeous microRNA function. The inhibitors function as degradation-resistant substrates of the RISC-microRNA complex. To explore the function of microRNA in spleen, we propose to inhibit the endogenous miR-10a and miR-143 in isolated spleen cells, and use microarrays to identify differentially expressed genes, which may be either direct targets of microRNA or the indirect targets involved in the pathways governed by microRNA. In our approach, a reference design was used, in which a portion of every RNA sample was pooled to make a "reference pool" and labeled with Cy5, and the remaining individual samples were labeled with Cy3. Cy3 labeled individual samples were then hybridized to Cy5 labeled reference on individual slides. Four replicates were performed for each condition. Moreover, due to the small size of embryonic spleen, multiple spleens have to be pooled to provide enough cells for downstream treatment. Pooling may decrease the

variation caused by individual difference. The microarray experimental design is illustrated in Figure 2.1.

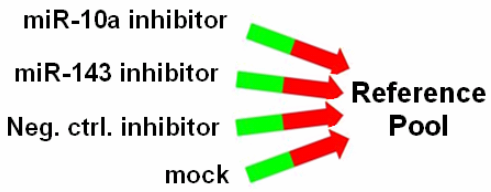

Figure 2.1 Microarray experimental design. Green color stands for Cy3 labeling, and red color stands for Cy5 labeling. Each arrow represents one hybridization. Four replicates were performed on four independently collected spleen pools.

The objectives of this project are:

1. To validate predicted target genes of miR-10a and miR-143 using reporter assays.
2. To explore the pathways governed by miR-10a and miR-143 in E15 spleen by microarray analysis.

MicroRNA Target Prediction

The chicken (*Gallus gallus*) Unigene database was downloaded from NCBI FTP. To predict possible miRNA targets, the algorithm miRanda version 3.0 (http://www.microrna.org/microrna/getDownloads.do) was used. Parameters were set as default except that the score threshold was set as >130 and the free energy threshold was set as <-18. Although the seed region has been shown to be crucial to target recognition, the complementarity between a seed region and its target mRNA sequence may not be perfect (Didiano and Hobert 2006), and other factors may be involved, such as the 13-16 nt complementarity and the binding site positioning in the 3'UTR (Grimson *et al.* 2007). To

narrow down the population of predicted targets and probably reduce the false positive predictions, miRanda results were further filtered by the following criteria: (1) 2-8 nt of miRNA match with the target (G:U base-pairing tolerated), or (2) 2-7 nt and 13-16 nt of miRNA match with the target (G:U base-pairing tolerated). Finally, the hits that lie within the open reading frame or that are not fully-annotated sequences were discarded. Only those possible miRNA binding sites lying in 3'UTR of a gene were retained and considered as putative targets.

Cloning of psiCHECK2 Constructs

Selected miRNA target sequences were obtained from GenBank database and primers were designed to clone the designated region containing the putative miRNA binding sites. XhoI and NotI restriction sites were included in forward and reverse primers, respectively. The complete primer sequences are provided in table 2.1 and 2.2.

DF1 genomic DNA was used as cloning template. To extract the DNA, confluent DF1 cells cultured on P150 plates were harvested by trypsinization and centrifugation. The cells were washed once with PBS pH 8.0 and resuspended in 6 ml of lysis buffer (10 mM Tris-Cl (pH 8.0), 0.1 M EDTA (pH 8.0), 0.5% (w/v) SDS, 20 ug/ml Dnase-free pancreatic RNase, 100 ug/ml Proteinase K) and incubated at 50℃ for 3 hours. The mixture was then cooled down to room temperature and an equal volume of phenol was added (equilibrated with 0.1 M Tris-Cl (pH 8.0)) and mixed gently for 10 minutes. Next, the mixture was centrifuged at 6500 rpm (SS-34 rotor) for 15 minutes at room temperature, and the supernatant was transferred into a new tube. The extraction was repeated once and supernatant portions were

combined, an equal volume of phenol:chloroform:IAA (25:24:1) was added, and the mixture was inverted gently followed by centrifugation at 6500 rpm for 15 minutes at room temperature. Again, the supernatant was transferred into a new tube, and DNA was precipitated with 0.2 volume of 10 M ammonium acetate and 2 volumes of 95-100% ethanol. The DNA precipitation was carefully transferred into a new tube by a pasteur pipette tip, washed twice with 70% ethanol, pelleted by centrifugation, and air-dried. The pellet was dissolved in 1 ml nuclease-free water at 4°C overnight and stored at 4°C.

For each 50 ul PCR reaction, 1 ug of genomic DNA template was added with 10 ul of 5X buffer, 2 ul of 20mM dNTP mix, 1 ul of 10 uM forward and reverse primers each, and 2.5 units of Taq polymerase (Promega). Magnesium was added to a final concentration of 3 mM and dH_2O was added to a final volume of 50 ul. The PCR reaction was carried out with the following parameters: Initial denaturation at 95°C for 3 minute, denaturation at 95°C for 1 minute, annealing at 55°C for 1 minute, elongation at 72°C for 1 minute. The cycle was repeated for 32 more times followed by a 5-minute 72°C incubation.

PCR products were purified with a PCR clean-up kit (Mo Bio) and then digested with NotI and XhoI (NEB). The psiCHECK2 vector (Promega) was also digested with NotI and XhoI. Digestion products were gel-purified (QAIGEN), ligated with T4 ligase (NEB), and transformed into DH5α host *E. coli*. Positive clones were identified by colony PCR and confirmed by restriction endonuclease digestion and DNA sequencing.

Cloning of RCAS-miR Constructs

Cloning of microRNA Entry Clones

The pENTR3C-miR-SphNgo plasmid was generously provided by Dr. Jerry Dodgson (Michigan State University). In this plasmid, the chicken primary miR-30a sequence was inserted into the cassette flanked by attR recombination sites. Sph and NgoMIV restriction sites were engineered to flank the precursor miR-30a, so that different precursor microRNA sequences can be cloned into this region to replace the stem region of pre-miR-30a while keeping the loop sequence identical to that of chicken miR-30a. To clone our microRNA of interest, a 76-nucleotide forward primer and a 68-nucleotide reverse primer were synthesized and PAGE purified (Invitrogen). The full primer list is provided in table 2.3. The primers were annealed to form short double-stranded fragments with sticky ends. To anneal the primers, forward and reverse primers were mixed in 10 ul dH_2O to final concentration of 1 uM, heated to 95℃ for 20 seconds, and cooled down to room temperature. The annealed product was confirmed by gel electrophoresis and ligated into the Sph and NgoMIV digested, gel purified pENTR3C-miR-SphNgo vector. The ligation product was transformed into DH5α *E.coli* and positive clones were selected by colony PCR and confirmed by restriction endonuclease digestion and sequencing.

Production of RCAS-miR Constructs and Viruses

The pENTR3C-miR-SphNgo microRNA entry clones were recombined with the pRCASBP(A)-YDV gateway destination vector to produce the recombinant RCAS-microRNA clones, which were then transfected into DF1 cells to produce the recombinant

RCAS viruses that expressed the microRNAs of interest. The vector maps and detailed recombination reaction flowchart were described in Figure 2.2.

The pRCASBP(A)-YDV gateway destination vector was generously provided by Dr. Jerry Dodgson at Michigan State University. To produce the RCASBP(A)-miR recombinant construct, 150ng of pENTR3C-miR-SphNgo entry clone and 150ng of RCASBP(A)-YDV vector were mixed with 2 ul of LR clonase mixture (Invitrogen), and added to TE buffer (pH 8.0) to 10 ul. The mixture was incubated at 25°C overnight and transformed into DH5 α *E.coli*. Plasmid DNA was purified from carbenicillin resistant colonies and digested to confirm that recombination had occurred.

To produce the RCASBP(A)-miR virus, RCASBP(A)-miR constructs were transfected into DF1 cells using FuGENE 6 (Promega). Briefly, 10^6 DF1 cells were seeded onto P35 one day before transfection. On the day of transfection, the media was changed to RPMI 1640 with 1% FBS but without antibiotics or fungicides. To prepare the transfection mixture, 9 ul of FuGENE 6 were diluted to 100 ul RPMI media without serum, antibiotics, or fungicides, and incubated at room temperature for 5 minutes. Two ug of plasmid DNA was then added to the diluted FuGENE 6 and mixed gently. The mixture was incubated at room temperature for 15 minutes, overlaid on the cells in a drop-wise manner, and swirled gently to mix. The cells were cultured at 37°C overnight and the culture media was changed on the next day. Cells were re-fed with fresh media or passed if necessary, and virus was harvested 6 days after transfection by collecting the supernatant. The supernatant was considered as virus stock, aliquoted into several tubes, and stored at -80°C.

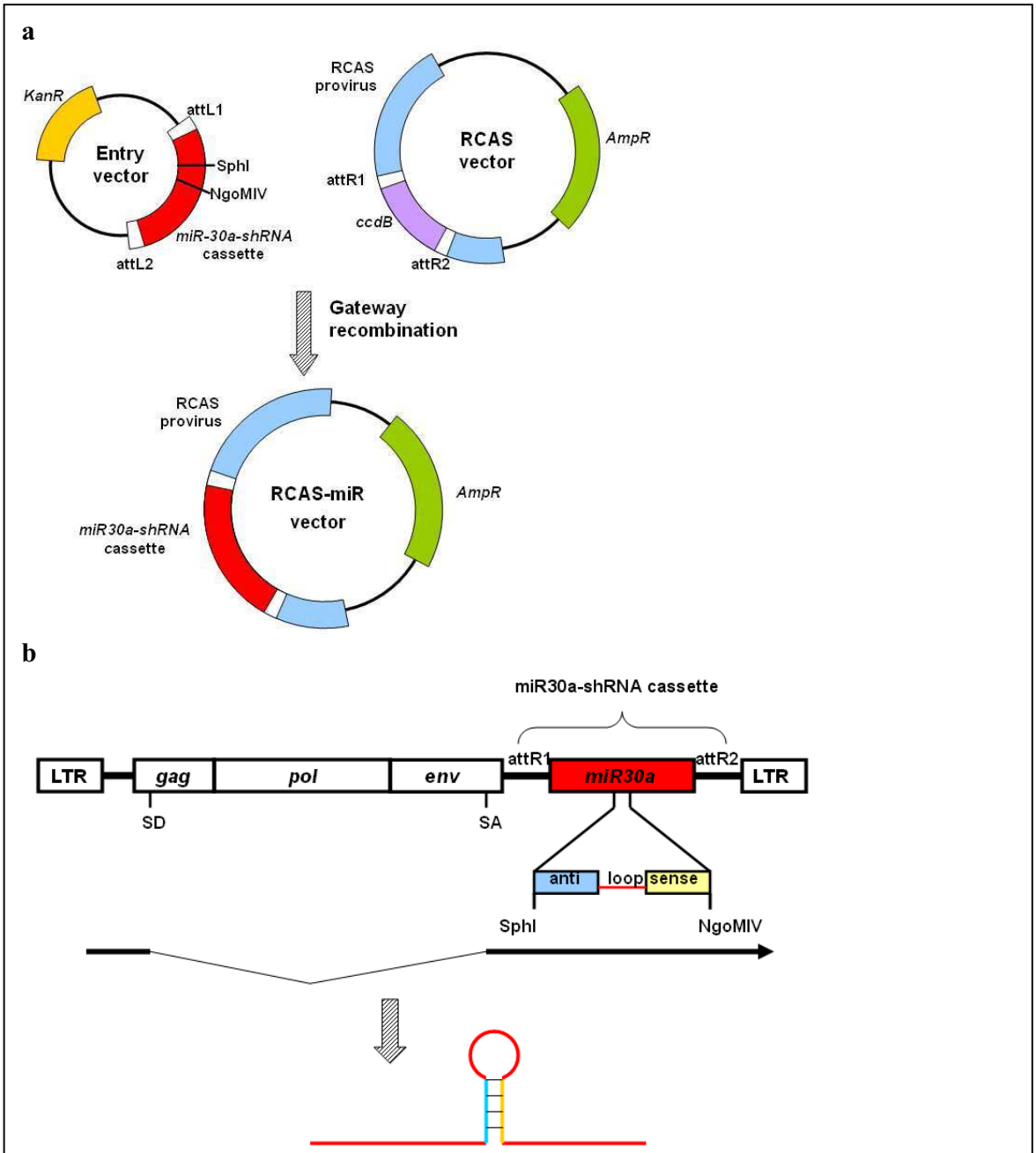

Figure 2.2 (a) Scheme of RCASBP(A)-miR construct cloning. The miR-30a precursor stem sequence in the entry clone is replaced by mature miR-143 and antisense sequences. The miR-30a-based cassette is recombined into RCASBP(A)-YDV vector to produce the RCASBP(A)-miR vector. (b) The recombinant RCAS proviral sequence. Spliced transcript will be produced from the LTR promoter. The red region in the transcript is original miR-30a sequence, and the yellow region is microRNA of interest.

Northern Blotting

To confirm that the RCASBP(A)-miR viruses express our microRNA of interest, Northern blotting was carried out. DF1 was transfected with the RCAS construct according to the procedure described above. After transfection, the cells were expanded every two to three days until reaching confluency in P150 plates. Total RNA was harvested by Tri reagent (Sigma) following the manufacturer's protocol with the following optimization to increase the yield of small RNA fraction: 2 ml of Tri reagent was used per P150 plate, and RNA was precipitated at -20℃ overnight, washed with 95-100% EtOH, and dissolved in a 40-80 ul of DEPC-treated water. RNA concentration was determined by Nanodrop.

Samples were separated on a 12.5% denaturing gel (urea, TBE, 40% acrylamide:bisacrylamide (19:1), APS, TEMED, and DEPC-treated water) which had been pre-electrophoresed with 1X TBE at 130V for 30 minutes. Twenty ug of RNA was loaded into each well and electrophoresed at 130V for ~1 hour. The gel was EtBr stained to confirm the quality and quantity of each lane before being transferred to a BrightStar-Plus membrane (Ambion) by a semi-dry method at 200Amp for 1 hour. The membrane was UV-cross-linked for 2 minutes and pre-hybridized in 5ml UltraHyb buffer (Ambion) for 1 hour at 42℃.

To label the probe, 1 ul of 10 uM primer was mixed with 1 ul polynucleotide kinase (Ambion), 4 ul [gamma-^{32}P]-ATP, 2ul 10X kinase buffer, and nuclease-free water was added to obtain a final volume of 20 ul. The reaction mixture was incubated at 37℃ for two hours. Probes were cleaned up with a QIAquick nucleotide removal kit (QIAGEN) following the manufacturer's protocol and dissolved in 40 ul nuclease-free water.

The probe was then added to the pre-hybridized membrane and hybridized at 40°C for 24 hours. Membrane was then exposed to BioMAX MS film (Kodak) and kept at -80°C for 72 hours before development.

Cell culture, Transfection, and RCAS-mir Virus Infection

RPMI 1640 with L-glutamine, penicillin (100 U/ml), streptomycin (100 ug/ml), fungizone (4 ug/ml), and 1% fetal bovine serum was used for propagating DF1 cells. Cells were maintained in a 37°C humidified incubator with a 5% CO_2 atmosphere.

To infect the cells with RCAS-mir virus, the virus stock solution was simply added into the cell culture plate. Infection was confirmed at 6 dpi by immunofluorescence staining with the mouse monoclonal 3C2 antibody against viral gag protein (Developmental Studies Hybridoma Bank at University of Iowa) and FITC-conjugated goat anti mouse IgG.

FuGENE (Promega) was used to transfect the cells with the psiCHECK2 construct. For each well in the 96-well plate, 0.3 ul of FuGENE6 was diluted with 5 ul of RPMI 1640 without additives, gently mixed, and left at room temperature for 5 minutes. Plasmid DNA (0.06 ug) was also diluted added to the diluted FuGENE 6. The mixture was left at room temperature for another 5 minutes and added to the cell culture gently. Medium was changed the next day.

Lipofectamine 2000 (Invitrogen) was used for DNA and RNA cotransfection. Transfection was carried out according to manufacturer's protocol. Cells were prepared in 96-well plates as mentioned above. For each well, 50 ng synthetic microRNA mimic or

microRNA mimic inhibitor and 40 ng of the psiCHECK2 construct were transfected. Medium was changed the next day.

Luciferase Assay

The luciferase assay was performed with a dual-luciferase reporter assay system (Promega) according to the manufacturer's manual. For miR-143, DF1 cells were cultured in 96-well plates and infected with RCAS-miR virus to overexpress the microRNA of interest. Six days after infection, cells were transfected with psiCHECK2 reporter constructs by FuGENE as mentioned above. For miR-10a, DF1 cells were cultured in 96-well plates and cotransfected with has-miR-10a mimic or mimic negative control-1 (Dharmacon) and psiCHECK2 reporter constructs by Lipofectamine 2000 as described. Forty-eight hours after transfection, the culture medium was removed, the cells were washed once with PBS (pH 8.0) and lysed in 30 ul of passive lysis buffer (Promega) per well. Then, 25 ul of luciferase assay substrate dissolved in luciferase assay buffer II was added and mixed. The firefly luciferase activity was determined using a VictorLight 1420 luminescence counter (PerkinElmer) for 10 seconds after adding the reagent. To measure the Renilla luciferase activity, 25 ul of Stop & Glo substrate was dissolved in Stop & Glo buffer and mixed. The Renilla luciferase activity was also determined 10 seconds after adding the reagent. The ratio of luminescence from the Renilla luciferase to luminescence from firefly luciferase was calculated to assess the impact on Renilla gene expression by microRNA overexpression. Each treatment was repeated in 3 replicates and statistical significance among treatments was determined using a two-tailed t-test.

Embryonic Spleen Isolation and Transfection

Spleens were collected from E15 chicken embryos (Charles River Inc.). Twenty spleens were pooled together, gently washed several times with RPMI 1640 medium, crushed into small pieces, and trypsinized for 30 minutes at 37°C with occasional pipetting to disperse the cells. Five independent spleen pools were prepared. The suspension was passed through sterile cheese cloth and centrifuged at 1000 rpm for 10 minutes at room temperature. The cells were resuspended in RPMI 1640 and counted. 9 x 10^6 cells were plated in one P60 plate with 6.5 ml media. Four plates were prepared from each pool. The cells were cultured in RPMI 1640 with 20% heat-inactivated FBS, L-glutamine, penicillin (100 U/ml), streptomycin (100 ug/ml), and fungizone (4 ug/ml), and followed by immediate transfection.

The synthetic microRNA inhibitor, hsa-mir-10a inhibitor, has-mir-143 inhibitor, and inhibitor negative control-1, were purchased from Dharmacon and dissolved in nuclease-free water to a final concentration of 20 uM. To transfect the cells with microRNA, 15 ul of siPORT Amine buffer (Ambion) was diluted into 285 ul RPMI medium and left at room temperature for 10 minutes. Thirty-two ul of 20 uM RNA was also diluted into 268 ul of RPMI medium and then added to the diluted Amine buffer and mixed gently. The mixture was left at room temperature for another 10 minutes before being transferred to cultures in a drop-wise manner. The final transfection concentration was 90 nM. Cells were maintained in 37°C humidified incubator with a 5% CO_2 in atmosphere until analysis.

RNA Extraction

Forty-eight hours after the spleen cells were transfected with microRNA inhibitor, cells were collected by centrifugation at 1000 rpm for 10 minutes, washed with PBS (pH 8.0) and pelleted again at 1000 rpm for 10 minutes. Total RNA was isolated with the RNeasy Mini Kit (QIAGEN). Six-hundred ul of buffer RLT was added to every 10^7 cells and the sample was homogenized by passing the lysate several times through a 20-gauge needle (0.9 mm diameter) fitted to an RNase-free syringe. Six-hundred ul of 70% ethanol was added to the homogenized lysate and mixed, and the samples were transferred to an RNeasy mini column, centrifuged for 15 seconds at 13000 rpm. The column was washed with 700 ul of buffer RW1 and centrifuged at 13000 rpm for 15 seconds. Next, the column was washed twice with 500 ul of buffer RPE and centrifuged at 13000 rpm for 15 seconds, and centrifuged for an extra 2 minutes to remove any residual buffers. Finally, 30 ul of RNase-free water was added to the column to elute the RNA. The amount of RNA recovered was determined by NanoDrop.

RNA Amplification and Dye Labeling

RNA amplification was carried out with Amino Allyl MessageAmp II aRNA amplification kit (Ambion). A single round of amplification was performed with 2 ug of total RNA according to the manufacturer's protocol. Briefly, 1 ul of T7 Oligo(dT) primer was added to the RNA, and RNase-free water was added to 12 ul. The mixture was incubated at 70˚C for 10 minutes and placed on ice. Next, for each 20 ul reaction, 8 ul of reverse transcription master mix was prepared: 2 ul of 10X first strand buffer, 4 ul of dNTP mix, 1 ul

of RNase inhibitor, and 1 ul of ArrayScript were mixed and transferred to the tube containing RNA, mixed, and incubated at 42°C for 2 hours.

For the second strand cDNA synthesis, the master mix was prepared on ice. For every 100 ul reaction, 63 ul RNase-free water, 10 ul 10X second strand buffer, 4 ul dNTP mix, 2 ul DNA polymerase, and 1 ul of RNase H were mixed, transferred to the first strand sample, and incubated at 16°C for 2 hours.

The resulting cDNA was purified with a cDNA Filter Cartridge (Ambion) and used as the template of in vitro transcription. For a single 40 ul reaction, 3 ul of aaUTP (50mM), 12 ul of ATP, CTP, GTP mix (25mM), 3 ul of UTP (50mM), 4 ul of T7 10X reaction buffer, and 4 ul of T7 enzyme mix were mixed at room temperature and added to the cDNA template. The reaction was incubated at 37°C for 14 hours. The allyl-modified RNA (aRNA) was recovered by aRNA filter cartridge (Ambion), and the quality and quantity were determined by gel electrophoresis and NanoDrop.

To label the resulting aRNA with dyes, 20 ug of aRNA was concentrated by SpeedVac and resuspended in 9 ul coupling buffer. Eleven ul of DMSO was added to one tube of Cy3 or Cy5 (Ambion) in the dark, mixed, and transferred to the aRNA tube. The reaction was incubated in the dark at room temperature for 30 minutes. To quench the reaction, 4.5 ul of 4 M hydroxylamine was added, and the reaction was incubated in the dark at room temperature for another 15 minutes. Finally, 5.5 ul of nuclease-free water was added to each sample, and the labeled aRNA was purified by using an aRNA filter cartridge (Ambion). The resulting labeled aRNA was analyzed with the NanoDrop and dye incorporation rate was calculated.

Array Hybridization

The chicken 20.7k oligo microarrays were obtained from University of Arizona. The slides were first baked at 90˚C for one hour, and treated in pre-hybridization solution containing 5X SSC, 0.1% SDS, and 1% BSA at 42˚C for 45 minutes. The slides were then dipped into 2X SSC for 5 minutes, 0.2X SSC for another 5 minutes, and spin-dried at 1000 x g for 5 minutes.

To prepare the aRNA, 8 ug of labeled aRNA was incubated in 1X fragmentation buffer (Ambion) at 70˚C for 15 minutes. Stop solution was added and the reaction was dried down by SpeedVac to a minimal volume. Each sample was reconstituted in 30 ul of pre-warmed DIG Easy Hyb solution (Roche). Next, all reference samples were pooled together, aliquoted into 30 ul volumes, and mixed with an equal amount of individual treatment sample. Two and half ul of yeast tRNA (10 mg/ml) and 2.5 ul of salmon testes DNA (10mg/ml) (Sigma) were added to the target/reference mixture, incubated at 94˚C for 2 minutes, and cooled down to room temperature.

The samples were loaded onto the middle of a pre-treated slide held in a hybridization chamber (Corning) and overlaid with a LifterSlip (Erie Scientific). The hybridization chamber was incubated at 42˚C in a water bath overnight. The next day, the slides were washed with 1X SSC, 0.2% SDS, 0.5% DTT at 42˚C for 10 minutes, 0.1X SSC, 0.2% SDS, and 0.5% DTT for 5 minutes at room temperature, and 0.1X SSC, 0.5% DTT for 1 minute at room temperature. The slides were finally rinsed with dH_2O and dried by centrifugation and then stored in individual 50 ml tubes covered in aluminum foil until scanning.

Microarray Data Analysis

Microarrays were scanned with a ScanArray 4000 (PerkinElmer). Images were uploaded to ScanArray Express (PerkinElmer). The array Gal files were used as templates and associated with the image, and an adaptive threshold method was used to read the spots. Templates were also manually fine-adjusted to match each subarray and spots. Finally, the intensity readings were output as .csv files for further statistical analysis by JMP genomics (SAS).

In JMP Genomics, the experimental design file corresponding to the .csv files was created, and .csv files from ScanArray were input to generate the SAS data set. In the data set, $\log_2$ median intensity was first normalized within arrays then across arrays by Loess normalization. The normalization results were confirmed by a distribution plot and an MA plot before further analysis. To identify differentially expressed genes, one-way additive ANOVA with block as variable was used with the following settings: class variables: array, channel, and treatment; fixed effects: channel, treatment; random effect: array; LSMean effect: treatment. Cut-off was set at false positive rate = 0.05.

To investigate the pathway governed by the differential expressed genes in our experiment, the differential expressed genes were subject to Ingenuity Pathway Analysis (IPA). Because IPA only supports human pathway analysis, human homologs of our differentially expressed genes were used for analysis, and corresponding expression ratios between treatments were also uploaded. Analysis was carried out with the default setting, and the three highest scored function categories and two highest scored canonical pathways with statistical significance were of special interest.

Table 2.1 Primers used for miR-10a target cloning

Name	Sequence (5' → 3')	GeneID
HIF1AN For XhoI	GCCCTCGAGCTGGGTTTACATGTTGGTG	428952
HIF1AN Rev NotI	TGAGCGGCCGCCTGACATTTACCTATCTC	
HOXA1 For XhoI	CCCCTCGAGCCGCCAAGAGGTTTTCTTT	771094
HOXA1 Rev NotI	TTAGCGGCCGCTTTGGCCATCCGACACATT	
ITGB1 For XhoI	CACCTCGAGATTTAGGGCAAACTGCAGTG	374058
ITGB1 Rev NotI	TTCGCGGCCGCATGCTACATGAGCTTAA	
KLF11 For XhoI	GGGCTCGAGGGACACAGCACCTTCTTG	421934
KLF11 Rev NotI	TCTGCGGCCGCTCTGATACAGGTCAT	
PDPK1 For XhoI	GGGCTCGAGATCTGTATTTCATCAGGGAGC	416588
PDPK1 Rev NotI	TTCGCGGCCGCAGACATGTTGAAAATACCA	
RHOB For XhoI	TTTCTCGAGTTGTGCAAACTGGGGTAGTG	395734
RHOB Rev NotI	ATTGCGGCCGCGTAAGGAACAGTGTTTTC	
RHOBTB2 For XhoI	CGGCTCGAGCACAAAGCAGCAGCAGAA	419536
RHOBTB2 Rev NotI	TTTGCGGCCGCTTTTTTGGTGGTCTTC	
WDR1 For XhoI	GGGCTCGAGAATGTGCAGTTTCTTTGGAAG	422842
WDR1 Rev NotI	AAAGCGGCCGCAGTACAGAGAAGCAAAGCAA	

HIF1AN: hypoxia-inducible factor 1 alpha subunit inhibitor; HOXA1: homeobox A1; ITGB1: integrin beta 1; KLF11: kruppel-like factor 11; PDPK1: 3-phosphoinositide dependent protein kinase-1; RHOB: ras homolog gene family member B; RHOBTB2: rho-related BTB domain containing 2; WDR1: WD repeat domain 1.

Table 2.2 Primers used for miR-143 target cloning

Name	Sequence (5' → 3')	GeneID
CASP6 For XhoI	GCACTCGAGCTGGAATCAAACCTTAGTTTG	395477
CASP6 Rev NotI	AAAGCGGCCGCAAGTGTACTATCAGTTC	
KLF5 For XhoI	GAACTCGAGAGTTGTTGCCGAGGCTGTTC	418818
KLF5 Rev NotI	TCAGCGGCCGCCTGACAGATTACAATGCATG	
TARDBP For XhoI	TAGCTCGAGGTTCTCCTGTAGTACTTTACC	419453
TARDBP Rev NotI	AGAGCGGCCGCTAAAAATGGAACACCCACC	
UBE2E3 For XhoI	TGTCTCGAGGATACTGTTCCTGAAGTCAACC	424122
UBE2E3 Rev NotI	AAAGCGGCCGCAATCCACAGGTGTGCATTA	
MAP3K7 For XhoI	ATACTCGAGCCCTTATTATGACAACTCATGAG	421808
MAP3K7 Rev NotI	ACAGCGGCCGCAGTCAAAATGCAGGGCTAT	
MAPK6 For XhoI	GTTCTCGAGTCATCCATGCGTCCGTTG	415419
MAPK6 Rev NotI	ACCGCGGCCGCATTCATGCTTGCTTTGG	
TNFSF10 For XhoI	AGTCTCGAGAAAGGGAAAAGCCATCGG	378894
TNFSF10 Rev NotI L	TAAGCGGCCGCTACATTCACCATTCATCA	
TNFSF10 Rev NotI S	AAGGCGGCCGCTATTGTGAAGGTCTCCA	

CASP6: caspase 6; KLF5: kruppel-like factor 5; TARDBP: tar DNA binding protein; UBE2E3: ubiquitin-conjugating enzyme E2E3; MAP3K7: mitogen-activated protein kinase kinase kinase 7; MAPK6: mitogen-activated protein kinase 6; TNFSF10: tumor necrosis factor superfamily member 10.

Table 2.3 Primers for RCAS-miR cloning

Name	Sequence (5' → 3')
miR143 For	CGAAGCTACAGTGCTTCATCTCATAGTGAAGCAGCAGATGG TATGAGATGAAGCACTGTAGCTCTG
miR143 Rev	CCGGCAGAGCTACAGTGCTTCATCTCATACCATCTGCTGCTT CACTATGAGATGAAGCACTGTAGCTTCGCATG
miR143 SC For	CGACTTACAGCCAGTTCCTAGTATAGTGAAGCAGCAGATGG TATACTAGGAACTGGCTGTAAGCTG
miR143 SC Rev	CCGGCAGCTTACAGCCAGTTCCTAGTATACCATCTGCTGCTT CACTATACTAGGAACTGGCTGTAAGTCGCATG

*SC: scrambled control

CHAPTER 3: RESULTS AND DISCUSSION

Results

Algorithm MicroRNA Target Prediction

The algorithm miRanda predicts potential microRNA targets based on the microRNA:mRNA complementarity and free energy. A score is given for each potential target depending on the degree of complementarity, where a near-perfect complementarity results in a high score. Additionally, base-pairings between the seed region of microRNA and mRNA are weighted heavily, thereby favoring the potential targets that match the seed region of microRNA. On the other hand, a low free energy indicates a strong and stable interaction between microRNA and mRNA.

To predict potential targets of chicken miR-10a and miR-143 by miRanda, the chicken UniGene database was downloaded from the NCBI website: (ftp://ftp.ncbi.nih.gov/repository/UniGene/Gallus_gallus/Gga.seq.uniq.gz). Each UniGene entry is a set of transcripts that appears to come from the same transcription locus. In our screening, the unique (non-redundant) UniGene database was used, in which each gene was represented by the longest transcript sequence available. miRanda score and free energy thresholds were set at 130 and -16 kCal/mol, respectively. Evidence has shown that the seed region is important for determining microRNA targets, but other factors may come into play, such as the complementarity at the thirteenth to sixteenth nucleotides of each microRNA, especially when the seed region pairing is not perfect. Therefore, further screening was performed on the raw miRanda outputs. Only the potential targets sites that (1) base-paired

with the first to eighth nucleotides of microRNA (G:U base-pairing tolerated), or (2) base-paired with the second to seventh AND thirteenth to sixteenth nucleotides of the microRNA, were retained. Finally, the locations of the potential target sites were checked. Only those sites located within the 3'UTR were speculated to be potential targets, whereas other results were discarded. The prediction criteria and process are depicted in the figure 3.1 as a flowchart.

Based on the above screening process, 641 and 977 sequences were predicted to be targets of miR-10a and miR-143, respectively. For each microRNA, a luciferase assay was carried out on only eight of the predicted target genes because of their conservation across species, multiple target sites, or their implication in important cell functions or immune response. For miR-10a, the target genes were 3-phosphoinositide dependent protein kinase-1 (*PDPK1*), Kruppel-like factor 11 (*KLF11*), integrin β1 (*ITGB1*), WD repeat domain 1 (*WDR1*), homeobox A1 (*HOXA1*), hypoxia-inducible factor 1 alpha subunit inhibitor (*HIF1AN*), Rho-related BTB domain containing 2 (*RHOBTB2*), and Ras homolog b (*RHOB*). For miR-143, the target genes were caspase 6 (*CASP6*), mitogen-activated protein kinase 6 (*MAPK6*), Kruppel-like factor 5 (*KLF5*), tumor necrosis factor superfamily 10 (*TNFSF10*), mitogen-activated protein kinase kinase kinase 7 (*MAP3K7*), ubiquitin-conjugating enzyme E2E3 (*UBE2E3*), and Tar-DNA binding protein (*TARDBP*). The target sites and the base-pairing between target sites and microRNA are shown in figures 3.2 and 3.3.

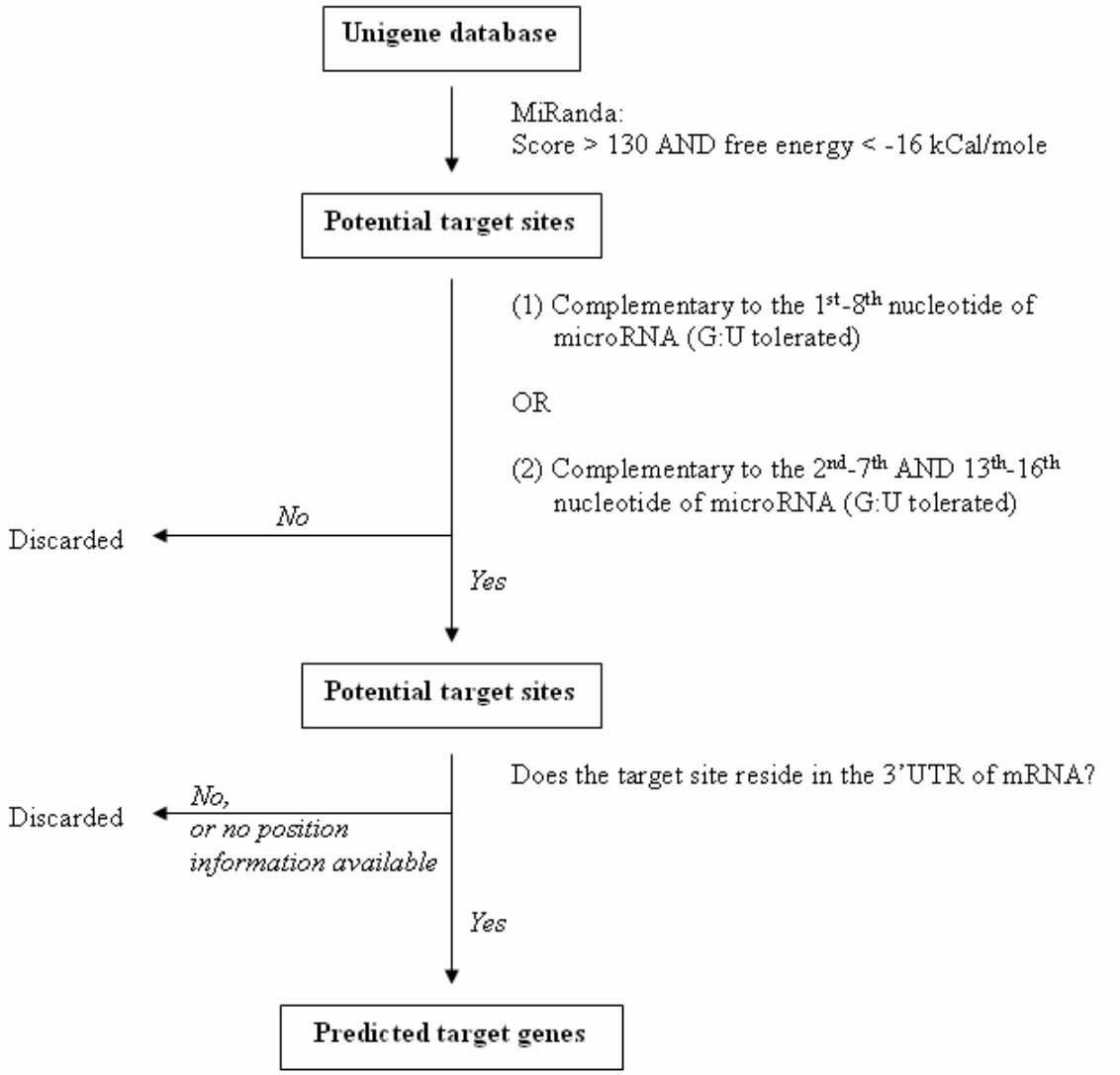

Figure 3.1 Flowchart of microRNA target prediction.

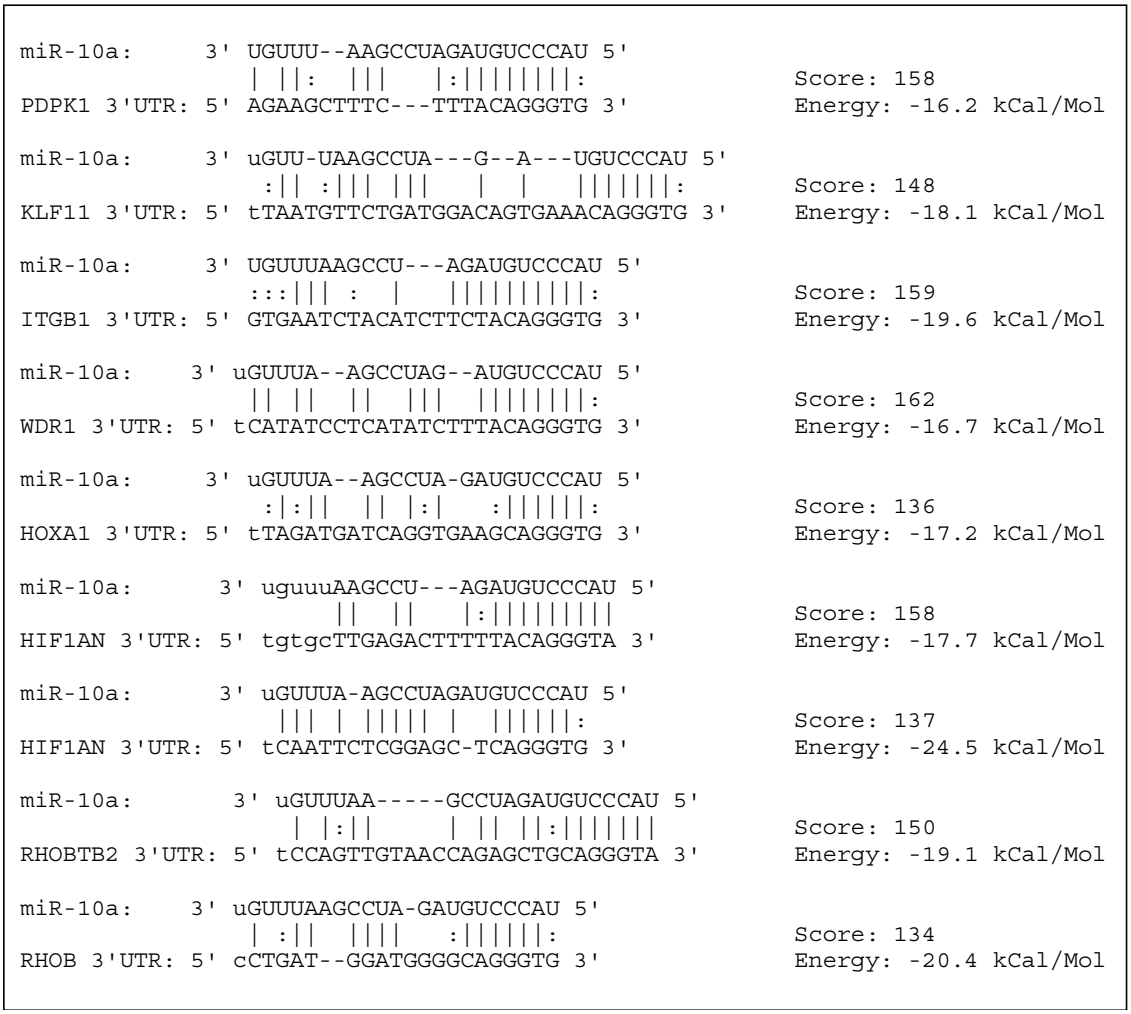

Figure 3.2 Predicted miR-10a targets. The base-pairing between miR-10a and the representative predicted targets are shown. Score and free energy of the base-pairing were calculated by miRanda.

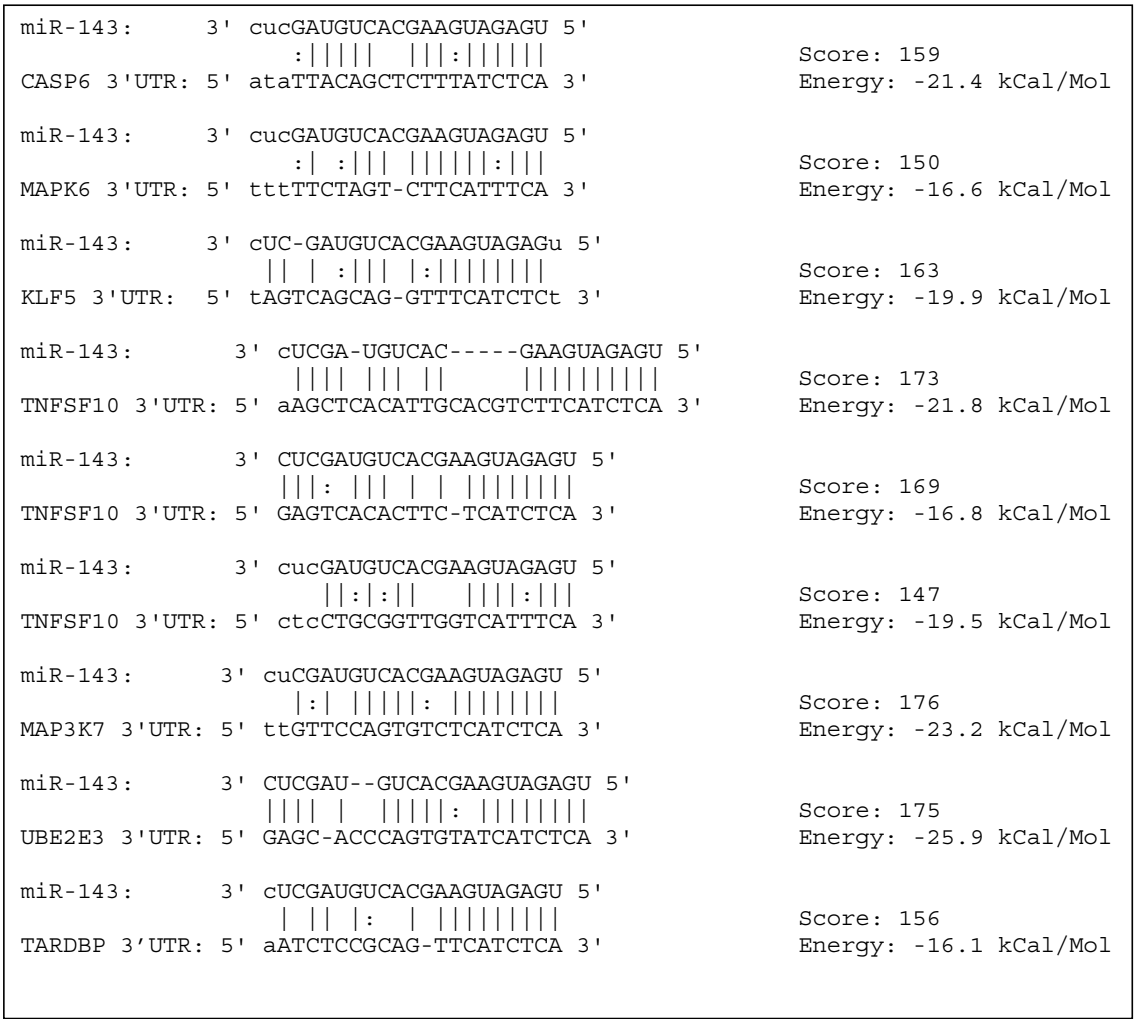

Figure 3.3 Predicted miR-143 targets. The base-pairing between miR-143 and the representative predicted targets are shown. Score and free energy of the base-pairing were calculated by miRanda.

Luciferase Assay

Dual luciferase assays were performed as described in materials and methods. A two-tailed t-test was used to determine the statistical significance. Expression of miR-143 was confirmed by Northern blotting (Figure 3.4). No endogenous miR-143 expression was detected in uninfected DF1 cells, whereas moderate expression of miR-143 was detected in the RCAS-miR143 infected DF1.

Figure 3.4 Northern blotting of miR-143 in RCAS-miR-143 infected DF1 cells. DF1: uninfected DF1; miR-143: RCAS-miR143 infected DF1. Chicken 28s rRNA was used as loading control.

MiR-10a

Among all the target clones tested, *WDR1*, *ITGB1*, and *KLF11* showed significant reduction in Renilla luciferase activity in the presence of synthetic miR-10a mimic as compared to the negative control microRNA. *HOXA1* also showed a reduced luciferase activity but was not statistically significant. MAPK6, which has no binding site for miR-10a, was used as control. Results were shown in Figure 3.5.

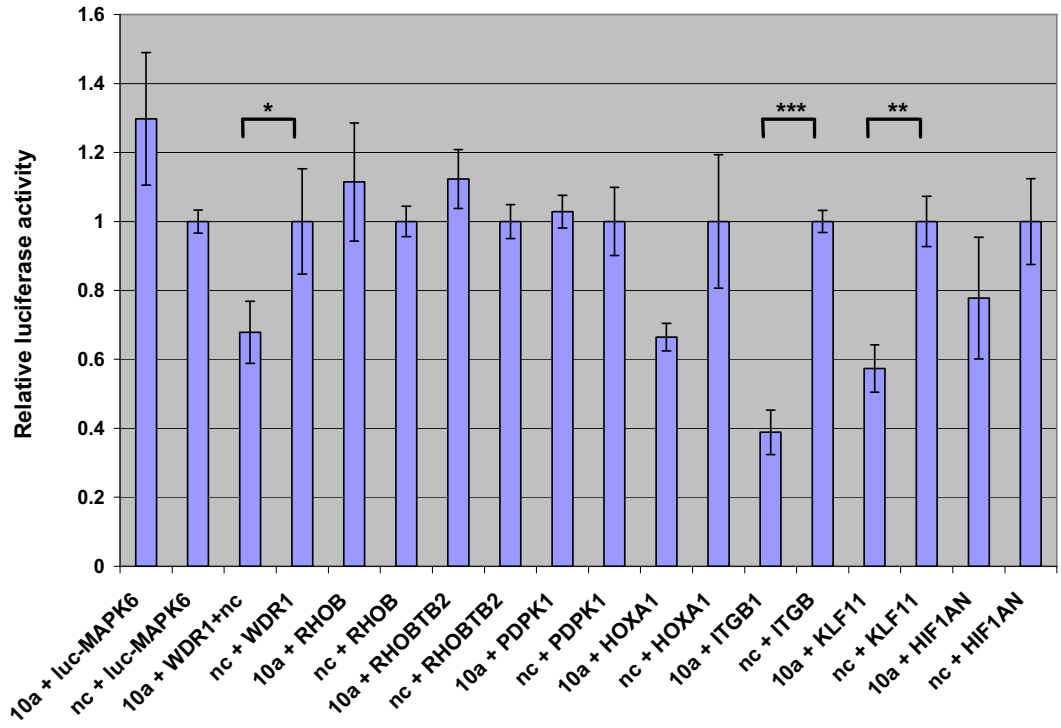

Figure 3.5 Luciferase assays for miR-10a target validation. Synthetic miR-10a mimic (10a) or negative control microRNA (nc) were cotransfected with predicted target 3'-UTR luciferase constructs. Relative Renilla luciferase activity is shown. *: $p<0.05$, **: $p<0.01$ ***: $p<0.001$.

MiR-143

TNFSF10-l and *TNFSF10-s* contain all three predicted binding sites or only two binding sites located at the 5' end of 3'-UTR, respectively. Among all constructs tested, *KLF5*, *MAP3K7*, *TNFSF10-l*, *TNFSF10-s*, *UBE2E3*, *TARDBP*, and *CASP6* all showed statistically significant decreased reporter activity when miR-143 was co-expressed. *MAPK6* reporter activity decreased slightly but was not statistically significant. In addition, the reporter activity of *KLF11*, which has no predicted binding site for miR-143, remained unchanged, suggesting the specificity of miR-143 targeting. Results were shown in figure 3.6.

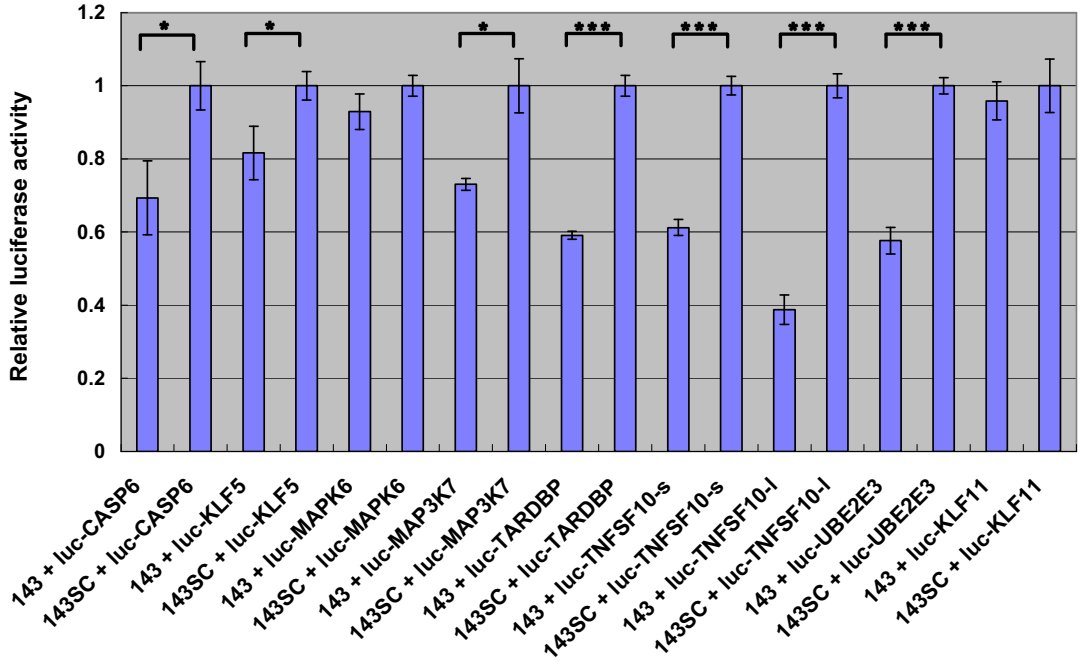

Figure 3.6 Luciferase assays for miR-143 target validation. MiR-143 (143) or miR-143 scrambled control (143SC) expressing RCAS infected DF1 cells were cotransfected with predicted target 3'-UTR luciferase constructs. Relative Renilla luciferase activity is shown. *: $p<0.05$, ***: $p<0.001$.

Microarray Analyses

Microarray analyses were carried out according to the procedure described in the materials and methods. In brief, microarray data were analyzed by JMP genomics. The readings were Loess normalized within and across slides. MiR-10a or miR-143 inhibitor treatments were compared to negative control inhibitor treatment. Normalized Cy3 density was analyzed with one-way ANOVA additive mode with block design. Differentially expressed genes between miR-10a or miR-143 inhibitor treatments and negative control inhibitor treatment were identified and used for Ingenuity Pathway Analysis.

One hundred and forty-seven genes were found to be differentially expressed between miR-10a inhibitor and negative control inhibitor treatments with $p<10^{-2.5}$, of which 93 were upregulated and 54 were down regulated in the miR-10a inhibitor treatment. Differentially expressed genes were subject to a miR-10a seed sequence match screen if their 3'UTR information was available. Ten out of ninety-three and three out of fifty-four genes were shown to have one or more 6-nt seed matches for miR-10a in their 3'UTRs (Figure 3.7a and table 3.1).

One hundred and fifty-three genes were found to be differentially expressed between miR-143 inhibitor and negative control inhibitor treatments with $p<10^{-2}$, of which 80 were upregulated and 72 were down regulated in the miR-143 inhibitor treatment. Differentially expressed genes were subject to a miR-143 seed sequence match screen if their 3'UTR information was available. Seven out of 80 upregulated genes and 7 out of 72 down regulated genes were shown to have one or more 6-nt seed matches for miR-143 in their 3'UTRs (Figure 3.7b and Table 3.2).

In the Ingenuity Pathway Analysis, the functions of the differentially expressed genes were categorized and ranked. For miR-10a, the top ranked functions were cancer, neurological disease and tumor, and cell cycle (Table 3.3). For miR-143, the top ranked functions were cell assembly and organization, cancer, and cell cycle (Table 3.4). The pathways involved were also suggested. Complement system and nucleotide excision repair were the top ranked pathways for miR-10a inhibitor treatment, and PPARα/RXRα activation and galactose metabolism were the most highly ranked for miR-143 inhibitor treatment, respectively (Table 3.5 and 3.6).

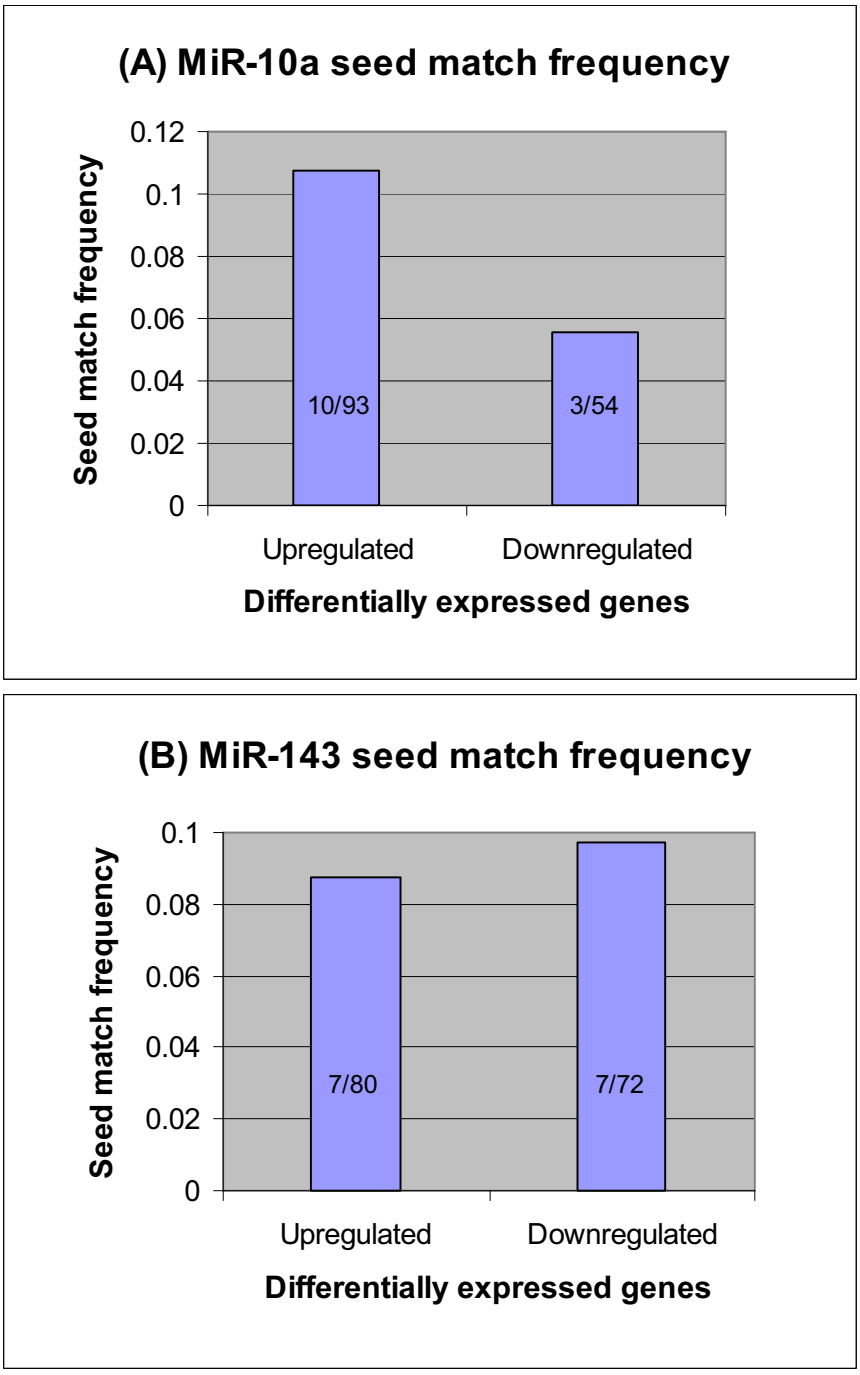

Figure 3.7 MicroRNA seed region match frequency for differentially expressed genes in microarray analyses of (A) miR-10a and (B) miR-143. Differentially expressed genes found in the microarray were subjected to a microRNA seed region complementary search if 3'UTR information was available. Six-nucleotide seed sequences were used in the search.

Table 3.1 Differentially expressed genes in a miR-10a inhibitor microarray with miR-10a 6-nt seed match sites in the 3'-UTR

Gene symbol	Entrez description	Gene ID	Fold change
NDRG1	N-myc downstream regulated gene 1	420321	1.62
ACSBG2	acyl-CoA synthetase bubblegum family member 2	420090	1.51
C10orf56	hypothetical LOC423722	423722	1.47
SDF2L1	stromal cell-derived factor 2-like 1	416770	1.44
PFKM	phosphofructokinase, muscle	374064	1.44
FNBP1L	formin binding protein 1-like	424495	1.39
C10orf118	CTCL tumor antigen L14-2	423905	1.38
N4BP1	Nedd4 binding protein 1	415739	1.37
CKAP4	cytoskeleton-associated protein 4	418073	1.36
YARS	tyrosyl-tRNA synthetase	419666	1.19
C16orf68	similar to CDNA sequence BC024814	425865	0.79
FAM102A	similar to RIKEN cDNA C230093N12	417228	0.77
BCL2L13	BCL2-like 13 (apoptosis facilitator)	418163	0.73

Table 3.2 Differentially expressed genes in the miR-143 inhibitor microarray with miR-143 6-nt seed match sites in the 3'-UTR

Gene symbol	Entrez description	Gene ID	Fold change
TERT	telomerase reverse transcriptase	420972	1.45
PYCR2	pyrroline-5-carboxylate reductase family, member 2	769203	1.39
GGNBP2	gametogenetin binding protein 2	425053	1.37
PSTPIP1	proline-serine-threonine phosphatase interacting protein 1	415349	1.37
ZNF800	zinc finger protein 800	417747	1.29
LOC422684	similar to KIAA1627 protein	422684	1.24
PDCD5	programmed cell death 5	415765	1.21
MED1	mediator complex subunit 1	420004	0.81
OBSCN	obscurin, cytoskeletal calmodulin and titin-interacting RhoGEF	420395	0.73
SECISBP2	SECIS binding protein 2	426815	0.72
ELMO1	engulfment and cell motility 1	420735	0.72
C9orf3	similar to hypothetical protein FLJ14675	427467	0.71
TMEM16A	transmembrane protein 16A	423144	0.69
NUAK1	NUAK family, SNF1-like kinase, 1	418074	0.66

Table 3.3 Top functions of differentially expressed genes in miR-10a inhibitor treatment

Functions involved	p-value	# of Molecules
Cell assembly and organization	$1.53*10^{-3} – 4.24*10^{-2}$	12
Cancer	$2.88*10^{-3} – 4.76*10^{-2}$	11
Cell cycle	$3.28*10^{-3} – 4.76*10^{-2}$	4

*# of molecules: number of differentially expressed genes involved in this function

Table 3.4 Top functions of differentially expressed genes in miR-143 inhibitor treatment

Functions involved	p-value	# of Molecules
Cancer	$9.40*10^{-5} – 4.98*10^{-2}$	13
Neurological disease, tumor	$9.40*10^{-5} – 4.53*10^{-2}$	7
Cell cycle	$2.08*10^{-4} – 4.98*10^{-2}$	5

Table 3.5 Top pathways of differentially expressed genes in miR-10a inhibitor treatment

Canonical pathway	p-value	# of Molecules
Complement system	$1.36*10^{-2}$	2
Nucleotide excision repair pathway	$1.52*10^{-2}$	2

Table 3.6 Top pathways of differentially expressed genes in miR-143 inhibitor treatment

Canonical pathway	p-value	# of Molecules
PPARα/RXRα activation	$7.34*10^{-3}$	4
Galactose metabolism	$2.07*10^{-2}$	2

Discussion

Luciferase Assay

MiR-10a

KLF11

Kruppel like factors (KLFs) are a family of zinc-finger containing transcription factors, named after the transcription factor Kruppel because of their homology to Kruppel. KLFs are characterized by three Cys2-His2 zinc fingers linked by highly conserved linker sequences. They recognize the CACCC element, and each member also recognizes similar GT-box sequences with differing affinities (Ghaleb *et al.* 2005).

KLF11 was first identified as fetal KLF for its restricted expression in human fetal liver but not in adult bone marrow. KLF11 activates the transcription of γ-hemoglobin, the fetal hemoglobin, and β-hemoglobin, the adult hemoglobin, to a lesser extent (Asano *et al.* 1999). In chicken, cells from two origins contribute to erythropoiesis. Primitive erythropoiesis starts from embryonic day 3 (E3), with erythrocytes originating from the yolk sac until E5. Definitive erythropoiesis begins around E6, with erythrocytes originating from embryonic mesenchyme and appearing in the blood and gradually dominating the erythrocyte population. No primitive erythrocytes occur after hatching (Lassila *et al.* 1982). The primitive erythrocytes mainly express ρ- and ε- hemoglobin, whereas definitive erythrocytes express β^H- and β^A-hemoglobin. Similar to their mammalian counterparts, chicken β- and ε-hemoglobin promoters also possess a CACCC element that contributes to active transcription of the locus (Jackson *et al.* 1989; Mason *et al.* 1996). The transcription factor(s) responsible for the CACCC-dependent transcriptional activation has not been identified in chicken, but it

is very likely to be KLF1, which activates β-hemoglobin transcription in human, and/or KLF11.

The KLF11 amino acid sequence is highly conserved between human and chicken , and chicken KLF11 is expressed in primitive (E5), transitional (E7), and definitive (E14) erythrocytes with little change in expression level (Basu *et al.* 2004). Furthermore, the miR-10a binding site in KLF11 3'UTR was predicted by both PicTar and TargetScan is also conserved among vertebrates. Our luciferase assay has demonstrated that this predicted binding site is genuine. In order to elucidate whether miR-10a is involved in chicken hematopoiesis by regulating KLF11 expression, a future research direction would be expression profiling of KLF11 and miR-10a in blood at multiple time-points in embryogenesis and in hatchlings.

WD repeat domain 1 (WDR1)

The WD repeat domain 1 protein contains 9 WD repeats, which are domains comprised of approximately 40 amino acids with tryptophan-aspartate at the C termini. WDR1 is also known as actin interacting protein 1 (AIP1) in human and is ubiquitously expressed. WDR1 binds to cofilin/actin complexes and induces actin filament disassembly in cooperation with cofilin (Okada *et al.* 1999; Ono *et al.* 2004). In mice, complete WDR1 loss of function is embryonic lethal, whereas hypomorphic mutation that results in partial loss of function of WDR1 protein causes massive infiltration of neutrophils, leading to autoinflammatory disease (Kile *et al.* 2007). A closer examination revealed that there was an aberrant cytoskeletal disassembly in response to chemokine stimulation and a reduction in

the migration rate of neutrophils. WDR1 mutant mice also display a decreased level of platelets due to impaired megakaryocyte maturation. The abnormal morphology of megakaryocytes and fragments of megakaryocyte cytoplasm in the spleen suggest defective megakaryocytopoiesis (Kile *et al.* 2007). Interestingly, miR-10a expression is dramatically down regulated during the process of human megakaryocytopoiesis. Although HOXA1 has been confirmed to be the direct target of miR-10a, the role of HOXA1 in megakaryocytopoiesis has not been studied.

Our validation of WDR1 as a target of miR-10a may help explain the importance of miR-10a downregulation in the course of megakaryocyte maturation, in which functional WDR1 protein is required. Although the miR-10a target site in WDR1 3'UTR is moderately conservered across species according to miRBase database, further experimentation in megakaryocyte cell-line or isolated megakaryocyte is still required to confirm this hypothesis.

ITGB1

Integrins are cell surface receptors that bind to specific ligands and then induce the transduction of signals from the extracellular matrix (ECM). Integrins are heterodimeric, comprised of two transmembrane subunits, α and β. The combination of 18 known α and 8 β subunits can give rise to 24 different receptors, each serving different functions but with some extent of redundancy. The β1 subunit is widely expressed. Several β1 integrins have been studied extensively, revealing their important roles in embryogenesis, angiogenesis, cell survival, activation, and migration. For example, integrin α4β1 binds to vascular cell adhesion molecule 1 (VCAM1) which is expressed on the surface of endothelial cells. This

interaction is crucial in many different settings where extravasation happens. When lymphocytes or monocytes are activated by chemokines, they adhere to endothelial cells tightly via the interaction between activated α4β1 integrin and VCAM1, allowing diapedesis and migration through the vascular wall to occur (Sixt *et al.* 2006). α4β1 is also involved in B cell retention in the splenic marginal zone and in immune synapse formation between naïve T cells and antigen presenting cells (dendritic cells and B cells) (Lu and Cyster 2002; Mittelbrunn *et al.* 2004).

On the other hand, collagen-binding integrins α1β1 and α2β1 are involved in the inflammatory response in colitis and arthritis by regulating T cell localization (de Fougerolles *et al.* 2000; Krieglstein *et al.* 2002). Endothelial cells also express β1 integrin in combination with different α subunits. Conditional knockout of β1 integrins in endothelial and a subset of hematopoietic cells causes defective vascular formation resulting from impaired migration and adhesion ability, increased apoptosis, and unresponsiveness to the ECM (Carlson *et al.* 2008). Because of its profound role in angiogenesis, cancer therapies targeting β1 integrin are also being investigated.

Taken together, β1 integrin is a versatile molecule that exerts profound functions in immune system regarding immune cell localization and activation. Additionally, *ITGB1* knockout mice results in early embryonic lethality, presumably due to severe vascular defects (Carlson *et al.* 2008). Our reporter assay demonstrated that chicken *ITGB1* is a target of miR-10a. The role of β1 integrin in chicken immune system is unclear, but it is highly possible that ITGB1 is involved in immune cells homing and/or retention in spleen or plays a role in immune response regulation as in mammals. Note worthily, the miR-10a binding site in

ITGB1 3'-UTR is not conserved. Given the importance of *ITGB1*, if this species-specific regulatory relationship can be proved valid *in vivo*, further investigation may be carried out to study whether this regulation contributes to any difference in chicken spleen development and maturation as compared to mammals.

Mir-143

Caspase 6 (CASP6)

Caspase 6 is a cysteine protease involving in the apoptosis pathway. Like all other caspases, pro-caspase 6 is activated by proteolytic cleavage. Caspase 6 can be processed by caspase 7, 8 and 10 and is categorized as an effector caspase (Stennicke and Salvesen 1998). The predicted miR-143 binding site in the caspase 6 3'UTR is not conserved across species, raising the possibility of a false positive. However, reporter activity was indeed reduced upon miR-143 expression. Caspases are relatively short half-lived proteins. Therefore, inhibition of caspase translation by may lead to profound implication by reducing the caspase cellular concentration rapidly.

Kruppel like factor 5 (KLF5)

KLF5, or intestine-enriched KLF (IKLF), is considered to be a key player in the cell cycle, tumorgenesis, adipogenesis (Oishi *et al.* 2005), and in cardiovascular remodeling . In the intestines KLF5 is predominantly expressed in the nuclei of active proliferating epithelial cells in the base of crypt, but is absent in the terminally differentiated cells in the villi (Ghaleb and Yang 2008). Ectopic expression of KLF5 in several epithelial cell-lines results

in increased proliferation rates (Chanchevalap *et al.* 2004; Nandan *et al.* 2004; Goldstein *et al.* 2007). In addition, KLF5 is induced by the MEK/ERK pathway, and the oncogenic potential of H-Ras activation is mediated by KLF5 induction via MEK/ERK signaling. KLF5 expression in turn activates the transcription of cell cycle promoting genes (Nandan *et al.* 2004; Nandan *et al.* 2005). Taken together, KLF5 is indeed a positive regulator of cell proliferation.

The role of KLF5 in tumorgenesis remains controversial. Ectopic expression of KLF5 in a mouse esophageal cancer cell-line results in increased apoptosis through Bax, a pro-apoptotic factor (Yang *et al.* 2005). In addition, gene deletion or protein degradation of KLF5 was observed in several types of cancer, suggesting its role as a potential tumor suppressor (Chen *et al.* 2002; Chen *et al.* 2003; Bateman *et al.* 2004). On the contrary, overexpression of KLF5 in a human bladder cancer cell line increases the proliferation rate, and higher KLF5 expression has been associated with lower survival rate of sporadic breast cancer patients (Chen *et al.* 2006; Tong *et al.* 2006). In these cases, KLF5 seems to serve as an oncogene. So far, no conclusive statement can be made on the role of KLF5 in tumorgenesis. These contradictory observations imply the opposite influence of KLF5 in tumorgenesis by serving as either a tumor suppressor or as an oncogene, depending on the cell type and the cellular context.

KLF5 is also an indispensable mediator of adipogenesis. KLF5+/- heterozygous mice show reduced body white fat, and expression of dominant negative KLF5 in epithelial cells results in decreased adipogenesis, whereas KLF5 overexpression leads to increased body fat formation. KLF5 expression is elevated at the early stage of adipogenesis and exerts its

function by cooperating with C/EBPδ to transactivate the transcription of PPARγ and C/BEPβ (Oishi *et al.* 2005).

Chicken KLF5 was predicted to have one potential miR-143 binding site. Luciferase assays also showed a mild reduction of relative reporter luciferase activity ($p<0.05$). MiR-143 is down regulated in a variety of tumors, including B cell lymphoma, chronic lymphocytic leukemia, and colorectal and cervical cancers (Michael *et al.* 2003; Akao *et al.* 2007; Lui *et al.* 2007; Slaby *et al.* 2007). Given the transforming potential of KLF5 in Ras-mutated colorectal cancers, downregulation of miR-143 may contribute to upregulation of KLF5 and favor tumor formation. Interestingly, as this miR-143 binding site is only moderately conserved across species, TargetScan predicted a highly conserved miR-145 binding site in the KLF5 3'UTR. Since miR-143 and miR-145 are coordinately expressed from the same transcript and show correlated downregulation levels in many cancerous cell microRNA profiling studies, it is not surprising that some genes may have binding sites for both microRNAs and are subject to synergetic regulation. Thus, it would be worth investigating the contribution of miR-145 in controlling KLF5 expression in the intestine and in cancers.

On the other hand, miR-143 is indispensable for adipocyte differentiation. Although ERK5 has been confirmed to be targeted by miR-143, whether it plays a role in adipogenesis and how it may do so remains undefined. Furthermore, miR-143 is constitutively expressed in pre-adipocytes throughout the process of adipogenesis, but its expression level remains mostly unchanged until the late stage of differentiation, when the level of miR-143 increases by 4-fold (Esau *et al.* 2004). In line with these data, KLF5 expression peaks at 3 hours post

induction of adipogenesis and declines over time. These findings suggest that miR-143 may tune down KLF5 expression at the late stage of adipogenesis to avoid excess adipocyte formation. Nonetheless, this microRNA-target gene relationship cannot explain the fact that miR-143 is required for adipogenesis and inhibition of miR-143 blocked adipogenesis (Esau *et al.* 2004). It is likely that miR-143 targets other genes involved in adipocyte differentiation.

Mitogen-activated protein kinase kinase kinase 7 (MAP3K7)

MAP3K7, or TGF-β activated kinase 1 (TAK1), belongs to the family of mitogen-activated protein kinase kinase kinases (MAPKKK). Its role in mediating TGF-β signal transduction was first discovered in the budding yeast *Saccharomyces cerevisiae* by means of the yeast two-hybrid system (Yamaguchi *et al.* 1995). TAK1 serves as a critical mediator for NF-κB and Jnk activation in response to IL-1β receptor and Toll-like receptor activation as well as to T cell receptor and B cell receptor cross-linking (Ninomiya-Tsuji *et al.* 1999; Takaesu *et al.* 2000; Sato *et al.* 2005; Shim *et al.* 2005; Safina *et al.* 2008). These various stimuli lead to the recruitment of the adapter proteins for TAK, TAB1 and TAB2 (Takaesu *et al.* 2000; Takaesu *et al.* 2001; Adhikari *et al.* 2007). Following binding to the TAB1/TAB2 complex, active TAK1 phosphorylates IκB kinase (IKK), and IKK phosphorylates IκB, the inhibitor of NF-κB, to promote its proteasomal degradation, thereby liberating NF-κB to enter the nucleus and activate transcription of downstream genes (Takaesu *et al.* 2003; Adhikari *et al.* 2007). Active TAK1 complex is also responsible for transducing TGF-β signal to p38, a member of MAPK family, via activation of mitogen-activated kinase kinase 6 (MAPKK6) (Hanafusa *et al.* 1999).

Studies in mice have revealed the critical roles of TAK1 in aspects of development, inflammation, and immunity. TAK1 knock-out mice are embryonic lethal (Sato *et al.* 2005; Shim *et al.* 2005). Conditional TAK1 knock-out mice develop extensive apoptosis in liver and bone marrow, leading to liver failure and pancytopenia, a shortage of all types of blood cells (Tang *et al.* 2008). B cell-specific TAK1 knock-out mice showed relative normal B cell development except for having less peritoneal B-1 cells. However, TAK1 negative B cells fail to activate NF-κB and Jnk in response to IL-1β and TLR3, TLR4, and TLR9 ligand stimuli, and show impaired enhancement of cell survival and proliferation after BCR cross-linking. Moreover, TAK1 deficient B cells are unable to produce IgG3 after being challenged by T cell independent antigen, indicating impaired isotype-switching (Sato *et al.* 2005).

T cell-specific TAK1 knock-out mice also demonstrate the importance of TAK1 in T cell development and activation. Conditional knock-out mice have a reduced peripheral T cell population which is likely due to extensive apoptosis of thymocytes at the single-positive stage. The proliferation capacity of T cells in response to TCR-cross-linking and TNF stimulation is also impaired (Liu *et al.* 2006; Sato *et al.* 2006). In addition, T cell-specific TAK1 knock out mice develop colitis in later age and show only activated or memory T cells in their periphery, probably caused by the absence of $CD4^{+}CD25^{+}Foxp3^{+}$ regulatory T cells (Sato *et al.* 2006).

Taken together, TAK1 is pivotal player in T and B cell development, innate and adaptive immunity, as well as in cell survival and proliferation in various tissues. TAK1 is evolutionarily conserved and has one conserved miR-143 binding site across species according to TargetScan and PicTar. Although the algorithm may give false positive

predictions, sequence conservation suggests a conserved and important biological function in vertebrate species. Indeed, reporter luciferase activity was repressed by miR-143 for more than 20% in our study ($p<0.05$). Based on the work done in mammals, we may speculate that TAK1 also have critical functions in chicken development and immune response. In this regard, microRNA regulation may fine tune gene expression to meet the specific requirements at a given time point and tissue type. Additionally, TAK1 was found to be indispensable for hematopoietic cells survival rather than the development of bone marrow microenvironment (Tang *et al.* 2008). In order to define the exact role of TAK1 and how the interplay between miR-143 and TAK1 modulate immune cell development and survival in chicken, it would be of interest to investigate the TAK1 expressing cell types in chicken spleen and bursa.

Ubiquitin-conjugating enzyme E2E3 (UBE2E3)

The ubiquitin (Ub) conjugating machinery is comprised of three components, the Ub priming enzyme E1, Ub carrier enzyme E2, and Ub ligase E3. E1 binds to Ub covalently via an ATP-dependent process and transfers Ub to the E2 enzyme. E2 and E3 usually form a complex that determines the substrate specificity and transfers Ub to the substrate. UBE2E3 was identified as a class III E2, which is characterized by a conserved UBC domain that is essential for E2 function and that has an extended N terminus (Ito *et al.* 1999). UBE2E3 is one of the E2 partners of E3 ligases Nedd4 and Nedd4-2 (Debonneville and Staub 2004; Fotia *et al.* 2006). The functions of UBE2E3 were poorly understood, but recently it was demonstrated that UBE2E3 is required for retinal pigment epithelial cell proliferation, and its

expression level is transcriptionally down regulated in the course of maturation (Plafker *et al.* 2008). UBE2E3 possesses one conserved miR-143 binding site and was down regulated more than 40% in our luciferase assay ($P<0.001$). Due to an incomplete understanding of UBE2E3 function, the biological consequence of this regulation remains to be defined.

TAR-DNA binding protein (TARDBP)

TARDBP, or TAR-DNA binding protein-43kD (TDP-43) was first found to be an ubiquitously expressed RNA binding protein that binds to the HIV TAR element and inhibits TAR-dependent transcription activation (Ou *et al.* 1995). TDP-43 also has two fully functional RNA binding domains, by which TDP-43 may bind to the UG repeats near exon 9 in the cystic fibrosis transmembrane conductance regulator (CFTR) pre-mRNA and regulates the splicing process (Buratti and Baralle 2001; Buratti *et al.* 2001). Recently, TDP-43 was found to be the major component in the protein aggregates of neurodegenerative diseases (Arai *et al.* 2006; Neumann *et al.* 2006; Mackenzie *et al.* 2007). Its ubiquitous expression of TDP-43 suggests that it may be involved in some essential function in normal cells, but the exact role remains to be defined. The miR-143 binding site in TARDBP is conserved based on PicTar and TargetScan prediction. In our reporter assay, this single binding site rendered 40% reduction in reporter activity ($p<0.001$). MiR-143 also has a rather ubiquitous expression profile, and is down regulated in several types of cancer. Therefore, it would be worth investigating the interaction between miR-143 and TDP-43 in the context of normal cells.

Tumor necrosis factor superfamily 10 (TNFSF10)

Tumor necrosis factor superfamily 10, or tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), has been extensively studied in regard to its pro-apoptotic capacity, and has been recognized as a potential direction for cancer therapy because of its ability to induce apoptosis in various tumor types while sparing normal cells. TRAIL exists as a transmembrane protein or in a soluble form if cleaved by cysteine proteases. Although its function is most well understood in cancer cells and immune cells, TRAIL is actually widely expressed in a variety of tissues. TRAIL has five receptors, either membrane-bound or soluble, and it is believed that the expression array of these receptors determines the different outcomes of TRAIL activation in different cell types. TRAIL induces apoptosis through receptors TRAIL-R1 and TRAIL-R2. Ligand binding and trimerization of TRAIL-R1 or TRAIL-R2 transduce the signal through the adaptor FADD and activate caspase 8 to initiate apoptosis (Baker and Reddy 1998; White-Gilbertson *et al.* 2008). TRAIL knock out mice show a higher incidence of developing tumors and autoimmunity, suggesting its crucial role in tumor surveillance and control of activated immune cells (Lamhamedi-Cherradi *et al.* 2003; Zerafa *et al.* 2005).

Other than its pro-apoptotic function, TRAIL has been found to influence hematopoiesis. TRAIL negatively regulates erythroid maturation through an ERK-dependent pathway (Secchiero *et al.* 2004). Conversely, TRAIL promotes the maturation of megakaryoblasts (Melloni *et al.* 2005).

Given the wide expression of TRAIL and its complex of receptor arrays, it is not surprising that TRAIL serves multiple functions and is subject to microRNA regulation. We

predicted three miR-143 binding sites in the chicken TRAIL 3'-UTR. To validate these putative binding sites, we cloned either two of the binding sites or all three binding sites into the luciferase transcript 3' UTR. Indeed, the presence of these predicted binding sites exerts negative effect on reporter gene expression. The luciferase activity decreased 40% when two binding sites were present, and 60% when all three binding sites were present, indicating efficient and synergic inhibition by multiple miR-143 binding sites. Apoptosis is required at several stages of normal immune system development, including elimination of self-reactive T cells and B cells failing to produce immunoglobin after gene rearrangement, but this process must be carefully regulated. Because of its ability to induce apoptosis, it is not surprising that TRAIL is under tight regulation, including being controlled by microRNA. MiR-143 also has a wide distribution in different tissues, so we may speculate that in the cell types where TRAIL and miR-143 are co-expressed, TRAIL is subject to significant regulation by miR-143, and the expression of TRAIL in turn modulates the microenvironment and contributes to the survival and differentiation of adjacent cells. Surprisingly, the miR-143 target sites predicted in chicken TRAIL 3'UTR are not conserved in other species. The importance of this chicken specific regulation remains to be investigated.

Taken together, our reporter assay suggests that *KLF5*, *MAP3K7*, *TNFSF10*, *UBE2E3*, *TARDBP*, *and CASP6* are all targets of miR-143. On contrary, MAPK6 may not be a target of miR-143. We also found that binding site sequence conservation among species may be a good indicator of a genuine target, because the conserved binding sites tend to render a given gene to a greater susceptibility to miRNA regulation, as the cases in *MAP3K7*, *UBE2E3*, *and*

TRADBP. In the case of *TNFSF10,* we also proved that multiple binding sites can result in a more significant inhibition by microRNA. In addition, *KLF11*, which has no miR-143 predicted binding sites, showed unaffected expression level under miR-143 regulation, confirming the specificity of microRNA regulation. We also demonstrated the usefulness of RCAS vector in expressing microRNA. However, it should be noted that for valid microRNA-target interaction *in vivo*, microRNA and the target transcript must co-exist at a given time point in the same cell. Also, the extent of inhibition by a single microRNA in our luciferase assay may not represent the condition *in vivo*, because the level of microRNA and reporter transcript in our in vitro experiment may differ from the physiological level. In this regard, microarray analysis from embryonic culture upon microRNA and microRNA inhibitor treatment may be useful to elucidate the global gene expression controlled by a single microRNA.

Pathways Regulated by miR-10a and miR-143

In order to identify the biological pathways regulated by miR-10a and miR-143, we have employed microarray approach in our studies. Microarray analysis is a screening technique that can detect the change of gene expression at the transcription level. As mentioned, microRNAs regulate gene expression by translational inhibition and/or facilitating mRNA degradation. For cases in which translational inhibition is the major regulatory mechanism, microarray analysis may not be an ideal approach for determining the target genes because some of the target genes that change significantly in protein level may only show minimal differences at the mRNA level. However, microarray analysis is still a

powerful tool for revealing some of the target genes and indicating pathways that may be controlled by microRNA.

Unlike other researchers who overexpressed microRNAs in their studies, we chose to knock down the endogenous miR-10a and miR-143 in E15 embryonic spleen cells by transfecting miR-10a or miR-143 specific inhibitors. The mRNA level of microRNA target genes may increase due to the relief of microRNA regulation, and we expected to see more potential targets in the upregulated gene population in microarray. To test this hypothesis, the 3'-UTRs of differentially expressed genes were searched for matches to the 6-nucleotide microRNA seed region. Upregulated genes in miR-10a inhibitor treatment indeed showed a higher incidence of miR-10a seed matches in their 3'UTRs. In the miR-143 experiment, however, no such trend was observed. This is not unexpected because differentially expressed genes may be direct targets of microRNA or the downstream genes controlled by the direct targets. It is likely that the majority of differentially expressed genes are indirect targets of microRNA.

In conventional microarray analysis the threshold for a gene to be considered as differentially expressed is usually two-fold in addition to statistical significance. This approach was not adapted in our analysis. As mentioned above, microRNAs usually fine-tune gene expression. A two-fold threshold imposes a considerably high stringency and may result in false negative assignments. Indeed, as seen by others, and as we observed in our microarray analysis, very few, if any, genes change their expression level by more than two-fold (Gottwein *et al.* 2007; Skalsky *et al.* 2007). Therefore only the p-value was used for determining the statistical significance of any observed changes.

In both the miR-10a and miR-143 microarray analyses, the predicted target genes confirmed by luciferase assays were not observed to be differentially expressed. This result could be due to (1) these genes were not co-expressed in the spleen at the time-point we investigated, or (2) regulation of these genes by miR-10a or miR-143 was through translational inhibition, therefore the transcript level did not increase when microRNA repression is relieved. However, there are several upregulated genes worth noting, including the N-myc downstream regulated gene 1 *(NDGR1*) in the miR-10a microarray and telomere reverse transcriptase (*TERT*) in miR-143 microarray. Both genes have miR-10 or miR-143 target sites in their 3'UTRs, respectively.

NDRG1 is a tumor suppressor that is commonly down regulated in breast, colorectal, and pancreatic cancer (Kovacevic and Richardson 2006). It was first identified as a gene that was upregulated at the late stage of colon epithelial cell differentiation, but down regulated in colorectal neoplasm (van Belzen *et al.* 1997). Recent research also suggests that downregulation of *NDRG1* results in tumor metastasis. Because miR-10a is overexpressed in cancer including HCV-associated hepatoceullar carcinoma and acute myeloid leukemia, it is rational to speculate that miR-10a may target some tumor suppressor genes such as *NDRG1*. However, this targeting remains to be validated.

Telomerase is a reverse transcriptase that uses RNA as template to elongate telomeres and prevent telomere loss, which is the major factor directly contributing to replicative cell senescence. Telomerase is highly expressed in embryonic stem cells. In the adult telomerase expression is mostly restricted to cells that divide regularly, including immune cells. For example, during the course of T cell development, telomerase activity decreases over time:

all subsets of thymocytes express telomerase; immature CD4 CD8 double-positive or double-negative T cells express high level of telomerase, while mature single positive T cells express limited levels of telomerase. In the peripheral tissues, resting mature T cells express little or no telomerase, but the expression can be induced rapidly after TCR activation (Weng 2008). Similar reduction of telomerase expression or activity occurs during the course of B cell maturation. Mature naïve or memory B cells express low levels of telomerase, but the expression can be induced by proper stimulation. Additionally, germinal center B cells, which undergo constant cell division, express high levels of telomerase (Weng 2008). Abnormal expression of telomerase may favor tumorgenesis by helping the cells escape the replicative cell senescence that occurs as a result of the loss of telomeric sequences.

Detection of telomerase expression was not unexpected for embryonic cells. However, the significant upregulation of telomerase expression in response to miR-143 loss-of-function is worth noting. Mir-143 is often found to be down regulated in cancers, and our finding provides an insight regarding how miR-143 plays a role as tumor suppressor. Although one miR-143 seed match in chicken *TERT* 3'-UTR was predicted, this feature is not conserved in vertebrates and still awaits validation. It is possible that telomerase upregulation is due to an indirect interaction in which telomerase expression is controlled by miR-143 direct target(s). In this scenario, the pathway between miR-143 and telomerase may be elusive, but the importance of miR-143 in telomerase regulation may be elucidated by similar experimentation in other vertebrates to determine whether the telomerase expression shows a similar trend in response to miR-143 loss-of function. It would also be interesting to investigate telomerase expression in cancer cells in which down regulation of miR-143

occurs, as well as how the telomerase expression changes when miR-143 is expressed ectopically.

The pathways involved in miR-10a and miR-143 identified from our work are components of the complement system and PPAR/RAR activation mechanisms, respectively. Complement component C1q B- and C-chain are the two of the most down regulated genes that occur during miR-10a inhibitor treatment (fold change: 0.39 and 0.51, respectively). C1q forms a complex with C1r and C1s. This complex either binds to the pathogen surface directly, or binds to the Fc portion of the antibody that binds to the pathogen surface, and initiates the classical pathway of complement system activation. Why the C1q B- and C-chain is down regulated in response to miR-10a inhibitor is unclear, but this observation suggests that miR-10a may be required for proper maintenance and/or induction of the complement system activity in embryonic spleen.

Peroxisome proliferators-activated receptor α (PPARα) is a transcription factor that heterodimerizes with the retinoid X receptor (RXR) and binds to the peroxisome proliferators-responsive element (PPRE) to activate target gene transcription. PPARα plays a profound role in lipid and glucose metabolism and homeostasis by activating the transcription of fatty acid oxidation enzymes in adipocyte and other tissue types (Mazzucotelli *et al.* 2007; Yang and Li 2007). PPARα also exerts anti-inflammatory effects by inhibiting macrophage activation and production of inflammatory cytokines, evidenced by prolonged inflammation in PPARα knockout mice (Zandbergen and Plutzky 2007). Four of the differentially expressed genes in miR-143 inhibitor treatment are associated with PPARα/RXRα activation. How miR-143 contributes to the regulation of PPARα/RXRα

activation is unclear. Investigation of PPARα/RXRα may be a promising direction to reveal the mechanism of miR-143 in adipocyte differentiation and inflammatory response.

Conclusion

In this project, we have used a luciferase assay to confirm selected direct targets of chicken microRNAs miR-10a and miR-143. We have also demonstrated the usefulness of the RCAS vector in ectopic microRNA expression. Such design may facilitate future microRNA research in chicken because of the well-established RCAS platform. Additionally, a similar approach can be applied in mammalian research because of the availability of transgenic mice that express the RCAS receptor tva.

Validated miR-10a targets in this study included *KLF11*, *WDR1*, and *ITGB1*. *KLF11* and *WDR1* have implications in hematopoiesis and megakaryocytopoiesis. *WDR1* also modulates cytoskeletons. *ITGB1* plays multiple functions in the immune system. The results supported the importance of miR-10a in megakaryocytopoiesis and shed lights on the importance of miR-10a in the immune system. Microarray data suggest miR-10a may control cell assembly and organization and cancer. This finding may partially explain the dysexpression of miR-10a in cancers and the observation that human miR-10b, which only differs in one nucleotide compared to human miR-10a, contributes to tumor metastasis in breast cancer (Ma *et al.* 2007). Taken together, these results suggest that miR-10a participates in several aspects of immune system development, tumorgenesis, and cell organization.

For miR-143, we have confirmed that caspase 6, *KLF5*, *TNFSF10*, and *MAP3K7* are target genes of miR-143. The products of these genes have profound functions in apoptosis, cell proliferation, T and B cell maturation, as well as in adipogenesis. Microarray analysis also suggest the involvement of miR-143 in cancer and PPARα/RXRα activation, and telomerase may be a downstream gene either directly or indirectly controlled by miR-143. All these observations are in line with previous studies showing that miR-143 is a tumor suppressor and is essential in adipogenesis.

In summary, we have successfully applied bioinformatics tools and biological experimentation to characterize chicken microRNAs. The results of our studies have provided directions for future studies of miR-10a and miR-143 in the areas of tumorgenesis and immune responses.

REFERENCES

Abdul-Careem, M. F., B. D. Hunter, P. Parvizi, H. R. Haghighi, N. Thanthrige-Don and S. Sharif (2007). "Cytokine gene expression patterns associated with immunization against Marek's disease in chickens." Vaccine **25**(3): 424-32.

Abel, P. P., R. S. Nelson, B. De, N. Hoffmann, S. G. Rogers, R. T. Fraley and R. N. Beachy (1986). "Delay of disease development in transgenic plants that express the tobacco mosaic virus coat protein gene." Science **232**(4751): 738-43.

Adhikari, A., M. Xu and Z. J. Chen (2007). "Ubiquitin-mediated activation of TAK1 and IKK." Oncogene **26**(22): 3214-26.

Akao, Y., Y. Nakagawa, Y. Kitade, T. Kinoshita and T. Naoe (2007). "Downregulation of microRNAs-143 and -145 in B-cell malignancies." Cancer Sci **98**(12): 1914-20.

Arai, T., M. Hasegawa, H. Akiyama, K. Ikeda, T. Nonaka, H. Mori, D. Mann, K. Tsuchiya, M. Yoshida, Y. Hashizume and T. Oda (2006). "TDP-43 is a component of ubiquitin-positive tau-negative inclusions in frontotemporal lobar degeneration and amyotrophic lateral sclerosis." Biochem Biophys Res Commun **351**(3): 602-11.

Asano, H., X. S. Li and G. Stamatoyannopoulos (1999). "FKLF, a novel Kruppel-like factor that activates human embryonic and fetal beta-like globin genes." Mol Cell Biol **19**(5): 3571-9.

Bagga, S., J. Bracht, S. Hunter, K. Massirer, J. Holtz, R. Eachus and A. E. Pasquinelli (2005). "Regulation by let-7 and lin-4 miRNAs results in target mRNA degradation." Cell **122**(4): 553-63.

Baker, S. J. and E. P. Reddy (1998). "Modulation of life and death by the TNF receptor superfamily." Oncogene **17**(25): 3261-70.

Basu, P., T. G. Sargent, L. C. Redmond, J. C. Aisenberg, E. P. Kransdorf, S. Z. Wang, G. D. Ginder and J. A. Lloyd (2004). "Evolutionary conservation of KLF transcription factors and functional conservation of human gamma-globin gene regulation in chicken." Genomics **84**(2): 311-9.

Bateman, N. W., D. Tan, R. G. Pestell, J. D. Black and A. R. Black (2004). "Intestinal tumor progression is associated with altered function of KLF5." J Biol Chem **279**(13): 12093-101.

Behm-Ansmant, I., J. Rehwinkel, T. Doerks, A. Stark, P. Bork and E. Izaurralde (2006). "mRNA degradation by miRNAs and GW182 requires both CCR4:NOT deadenylase and DCP1:DCP2 decapping complexes." Genes Dev **20**(14): 1885-98.

Bernstein, E., A. A. Caudy, S. M. Hammond and G. J. Hannon (2001). "Role for a bidentate ribonuclease in the initiation step of RNA interference." Nature **409**(6818): 363-6.

Betel, D., M. Wilson, A. Gabow, D. S. Marks and C. Sander (2008). "The microRNA.org resource: targets and expression." Nucleic Acids Res **36**(Database issue): D149-53.

Bhattacharyya, S. N., R. Habermacher, U. Martine, E. I. Closs and W. Filipowicz (2006). "Relief of microRNA-mediated translational repression in human cells subjected to stress." Cell **125**(6): 1111-24.

Bohnsack, M. T., K. Czaplinski and D. Gorlich (2004). "Exportin 5 is a RanGTP-dependent dsRNA-binding protein that mediates nuclear export of pre-miRNAs." Rna **10**(2): 185-91.

Brennecke, J., D. R. Hipfner, A. Stark, R. B. Russell and S. M. Cohen (2003). "bantam encodes a developmentally regulated microRNA that controls cell proliferation and regulates the proapoptotic gene hid in Drosophila." Cell **113**(1): 25-36.

Bromberg-White, J. L., C. P. Webb, V. S. Patacsil, C. K. Miranti, B. O. Williams and S. L. Holmen (2004). "Delivery of short hairpin RNA sequences by using a replication-competent avian retroviral vector." J Virol **78**(9): 4914-6.

Buratti, E. and F. E. Baralle (2001). "Characterization and functional implications of the RNA binding properties of nuclear factor TDP-43, a novel splicing regulator of CFTR exon 9." J Biol Chem **276**(39): 36337-43.

Buratti, E., T. Dork, E. Zuccato, F. Pagani, M. Romano and F. E. Baralle (2001). "Nuclear factor TDP-43 and SR proteins promote in vitro and in vivo CFTR exon 9 skipping." Embo J **20**(7): 1774-84.

Cai, X., C. H. Hagedorn and B. R. Cullen (2004). "Human microRNAs are processed from capped, polyadenylated transcripts that can also function as mRNAs." Rna **10**(12): 1957-66.

Carlson, T. R., H. Hu, R. Braren, Y. H. Kim and R. A. Wang (2008). "Cell-autonomous requirement for beta1 integrin in endothelial cell adhesion, migration and survival during angiogenesis in mice." Development **135**(12): 2193-202.

Caudy, A. A., M. Myers, G. J. Hannon and S. M. Hammond (2002). "Fragile X-related protein and VIG associate with the RNA interference machinery." Genes Dev **16**(19): 2491-6.

Chalfie, M., H. R. Horvitz and J. E. Sulston (1981). "Mutations that lead to reiterations in the cell lineages of C. elegans." Cell **24**(1): 59-69.

Chanchevalap, S., M. O. Nandan, D. Merlin and V. W. Yang (2004). "All-trans retinoic acid inhibits proliferation of intestinal epithelial cells by inhibiting expression of the gene encoding Kruppel-like factor 5." FEBS Lett **578**(1-2): 99-105.

Chang, J., J. T. Guo, D. Jiang, H. Guo, J. M. Taylor and T. M. Block (2008). "Liver Specific microRNA, miR-122, Enhances the Replication of Hepatitis C Virus in Non-hepatic Cells." J Virol.

Chen, C., M. S. Benjamin, X. Sun, K. B. Otto, P. Guo, X. Y. Dong, Y. Bao, Z. Zhou, X. Cheng, J. W. Simons and J. T. Dong (2006). "KLF5 promotes cell proliferation and tumorigenesis through gene regulation and the TSU-Pr1 human bladder cancer cell line." Int J Cancer **118**(6): 1346-55.

Chen, C., H. V. Bhalala, H. Qiao and J. T. Dong (2002). "A possible tumor suppressor role of the KLF5 transcription factor in human breast cancer." Oncogene **21**(43): 6567-72.

Chen, C., H. V. Bhalala, R. L. Vessella and J. T. Dong (2003). "KLF5 is frequently deleted and down-regulated but rarely mutated in prostate cancer." Prostate **55**(2): 81-8.

Chen, M., A. J. Granger, M. W. Vanbrocklin, W. S. Payne, H. Hunt, H. Zhang, J. B. Dodgson and S. L. Holmen (2007). "Inhibition of avian leukosis virus replication by vector-based RNA interference." Virology **365**(2): 464-72.

Clancy, J. L., M. Nousch, D. T. Humphreys, B. J. Westman, T. H. Beilharz and T. Preiss (2007). "Methods to analyze microRNA-mediated control of mRNA translation." Methods Enzymol **431**: 83-111.

Clop, A., F. Marcq, H. Takeda, D. Pirottin, X. Tordoir, B. Bibe, J. Bouix, F. Caiment, J. M. Elsen, F. Eychenne, C. Larzul, E. Laville, F. Meish, D. Milenkovic, J. Tobin, C. Charlier and M. Georges (2006). "A mutation creating a potential illegitimate microRNA target site in the myostatin gene affects muscularity in sheep." Nat Genet **38**(7): 813-8.

Cogoni, C. and G. Macino (1999). "Gene silencing in Neurospora crassa requires a protein homologous to RNA-dependent RNA polymerase." Nature **399**(6732): 166-9.

de Fougerolles, A., H. P. Vornlocher, J. Maraganore and J. Lieberman (2007). "Interfering with disease: a progress report on siRNA-based therapeutics." Nat Rev Drug Discov **6**(6): 443-53.

de Fougerolles, A. R., A. G. Sprague, C. L. Nickerson-Nutter, G. Chi-Rosso, P. D. Rennert, H. Gardner, P. J. Gotwals, R. R. Lobb and V. E. Koteliansky (2000). "Regulation of inflammation by collagen-binding integrins alpha1beta1 and alpha2beta1 in models of hypersensitivity and arthritis." J Clin Invest **105**(6): 721-9.

Debernardi, S., S. Skoulakis, G. Molloy, T. Chaplin, A. Dixon-McIver and B. D. Young (2007). "MicroRNA miR-181a correlates with morphological sub-class of acute myeloid leukaemia and the expression of its target genes in global genome-wide analysis." Leukemia **21**(5): 912-6.

Debonneville, C. and O. Staub (2004). "Participation of the Ubiquitin-Conjugating Enzyme UBE2E3 in Nedd4-2-Dependent Regulation of the Epithelial Na+ Channel." Mol. Cell. Biol. **24**(6): 2397-2409.

Denli, A. M., B. B. Tops, R. H. Plasterk, R. F. Ketting and G. J. Hannon (2004). "Processing of primary microRNAs by the Microprocessor complex." Nature **432**(7014): 231-5.

Didiano, D. and O. Hobert (2006). "Perfect seed pairing is not a generally reliable predictor for miRNA-target interactions." Nat Struct Mol Biol **13**(9): 849-51.

Didiano, D. and O. Hobert (2008). "Molecular architecture of a miRNA-regulated 3' UTR." Rna **14**(7): 1297-317.

Dorsett, Y., K. M. McBride, M. Jankovic, A. Gazumyan, T. H. Thai, D. F. Robbiani, M. Di Virgilio, B. R. San-Martin, G. Heidkamp, T. A. Schwickert, T. Eisenreich, K. Rajewsky and M. C. Nussenzweig (2008). "MicroRNA-155 suppresses activation-induced cytidine deaminase-mediated Myc-Igh translocation." Immunity **28**(5): 630-8.

Eis, P. S., W. Tam, L. Sun, A. Chadburn, Z. Li, M. F. Gomez, E. Lund and J. E. Dahlberg (2005). "Accumulation of miR-155 and BIC RNA in human B cell lymphomas." Proc Natl Acad Sci U S A **102**(10): 3627-32.

Elbashir, S. M., J. Harborth, W. Lendeckel, A. Yalcin, K. Weber and T. Tuschl (2001). "Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells." Nature **411**(6836): 494-8.

Elbashir, S. M., J. Martinez, A. Patkaniowska, W. Lendeckel and T. Tuschl (2001). "Functional anatomy of siRNAs for mediating efficient RNAi in Drosophila melanogaster embryo lysate." Embo J **20**(23): 6877-88.

Esau, C., X. Kang, E. Peralta, E. Hanson, E. G. Marcusson, L. V. Ravichandran, Y. Sun, S. Koo, R. J. Perera, R. Jain, N. M. Dean, S. M. Freier, C. F. Bennett, B. Lollo and R. Griffey (2004). "MicroRNA-143 regulates adipocyte differentiation." J Biol Chem **279**(50): 52361-5.

Filipowicz, W. (2005). "RNAi: the nuts and bolts of the RISC machine." Cell **122**(1): 17-20.

Fire, A., S. Xu, M. K. Montgomery, S. A. Kostas, S. E. Driver and C. C. Mello (1998). "Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans." Nature **391**(6669): 806-11.

Fotia, A. B., D. I. Cook and S. Kumar (2006). "The ubiquitin-protein ligases Nedd4 and Nedd4-2 show similar ubiquitin-conjugating enzyme specificities." The International Journal of Biochemistry & Cell Biology **38**(3): 472-479.

Garzon, R., F. Pichiorri, T. Palumbo, R. Iuliano, A. Cimmino, R. Aqeilan, S. Volinia, D. Bhatt, H. Alder, G. Marcucci, G. A. Calin, C. G. Liu, C. D. Bloomfield, M. Andreeff and C. M. Croce (2006). "MicroRNA fingerprints during human megakaryocytopoiesis." Proc Natl Acad Sci U S A **103**(13): 5078-83.

Ghaleb, A. M., M. O. Nandan, S. Chanchevalap, W. B. Dalton, I. M. Hisamuddin and V. W. Yang (2005). "Kruppel-like factors 4 and 5: the yin and yang regulators of cellular proliferation." Cell Res **15**(2): 92-6.

Ghaleb, A. M. and V. W. Yang (2008). "The Pathobiology of Kruppel-like Factors in Colorectal Cancer." Curr Colorectal Cancer Rep **4**(2): 59-64.

Giraldez, A. J., Y. Mishima, J. Rihel, R. J. Grocock, S. Van Dongen, K. Inoue, A. J. Enright and A. F. Schier (2006). "Zebrafish MiR-430 promotes deadenylation and clearance of maternal mRNAs." Science **312**(5770): 75-9.

Gironella, M., M. Seux, M. J. Xie, C. Cano, R. Tomasini, J. Gommeaux, S. Garcia, J. Nowak, M. L. Yeung, K. T. Jeang, A. Chaix, L. Fazli, Y. Motoo, Q. Wang, P. Rocchi, A. Russo, M. Gleave, J. C. Dagorn, J. L. Iovanna, A. Carrier, M. J. Pebusque and N. J. Dusetti (2007). "Tumor protein 53-induced nuclear protein 1 expression is repressed by miR-155, and its restoration inhibits pancreatic tumor development." Proc Natl Acad Sci U S A **104**(41): 16170-5.

Goldstein, B. G., H. H. Chao, Y. Yang, Y. A. Yermolina, J. W. Tobias and J. P. Katz (2007). "Overexpression of Kruppel-like factor 5 in esophageal epithelia in vivo leads to increased proliferation in basal but not suprabasal cells." Am J Physiol Gastrointest Liver Physiol **292**(6): G1784-92.

Gottwein, E. and B. R. Cullen (2008). "Viral and cellular microRNAs as determinants of viral pathogenesis and immunity." Cell Host Microbe **3**(6): 375-87.

Gottwein, E., N. Mukherjee, C. Sachse, C. Frenzel, W. H. Majoros, J. T. Chi, R. Braich, M. Manoharan, J. Soutschek, U. Ohler and B. R. Cullen (2007). "A viral microRNA functions as an orthologue of cellular miR-155." Nature **450**(7172): 1096-9.

Gregory, R. I., K. P. Yan, G. Amuthan, T. Chendrimada, B. Doratotaj, N. Cooch and R. Shiekhattar (2004). "The Microprocessor complex mediates the genesis of microRNAs." Nature **432**(7014): 235-40.

Grimson, A., K. K. Farh, W. K. Johnston, P. Garrett-Engele, L. P. Lim and D. P. Bartel (2007). "MicroRNA targeting specificity in mammals: determinants beyond seed pairing." Mol Cell **27**(1): 91-105.

Guo, S. and K. J. Kemphues (1995). "par-1, a gene required for establishing polarity in C. elegans embryos, encodes a putative Ser/Thr kinase that is asymmetrically distributed." Cell **81**(4): 611-20.

Hanafusa, H., J. Ninomiya-Tsuji, N. Masuyama, M. Nishita, J. Fujisawa, H. Shibuya, K. Matsumoto and E. Nishida (1999). "Involvement of the p38 mitogen-activated protein kinase pathway in transforming growth factor-beta-induced gene expression." J Biol Chem **274**(38): 27161-7.

He, L. and G. J. Hannon (2004). "MicroRNAs: small RNAs with a big role in gene regulation." Nat Rev Genet **5**(7): 522-31.

Hicks, J. A. (2007). "MicroRNAs in the spleen and liver of the developing chick embryo." MS thesis. North Carolina State University.

Hicks, J. A., P. Tembhurne and H. C. Liu (2008). "MicroRNA Expression in Chicken Embryos." Poult Sci **87**(11): 2335-43.

Hughes, S. H. (2004). "The RCAS vector system." Folia Biol (Praha) **50**(3-4): 107-19.

Humphreys, D. T., B. J. Westman, D. I. Martin and T. Preiss (2005). "MicroRNAs control translation initiation by inhibiting eukaryotic initiation factor 4E/cap and poly(A) tail function." Proc Natl Acad Sci U S A **102**(47): 16961-6.

Hutvagner, G. (2005). "Small RNA asymmetry in RNAi: function in RISC assembly and gene regulation." FEBS Lett **579**(26): 5850-7.

Hutvagner, G. and M. J. Simard (2008). "Argonaute proteins: key players in RNA silencing." Nat Rev Mol Cell Biol **9**(1): 22-32.

Ito, K., S. Kato, Y. Matsuda, M. Kimura and Y. Okano (1999). "cDNA cloning, characterization, and chromosome mapping of UBE2E3 (alias UbcH9), encoding an N-terminally extended human ubiquitin-conjugating enzyme." Cytogenet Cell Genet **84**(1-2): 99-104.

Jackson, P. D., T. Evans, J. M. Nickol and G. Felsenfeld (1989). "Developmental modulation of protein binding to beta-globin gene regulatory sites within chicken erythrocyte nuclei." Genes Dev **3**(12A): 1860-73.

Jeurissen, S. H. (1991). "Structure and function of the chicken spleen." Res Immunol **142**(4): 352-5.

John, B., A. J. Enright, A. Aravin, T. Tuschl, C. Sander and D. S. Marks (2004). "Human MicroRNA targets." PLoS Biol **2**(11): e363.

Johnston, P. A., H. Liu, T. O'Connell, P. Phelps, M. Bland, J. Tyczkowski, A. Kemper, T. Harding, A. Avakian, E. Haddad, C. Whitfill, R. Gildersleeve and C. A. Ricks (1997). "Applications in in ovo technology." Poult Sci **76**(1): 165-78.

Kaiser, P., G. Underwood and F. Davison (2003). "Differential cytokine responses following Marek's disease virus infection of chickens differing in resistance to Marek's disease." J Virol **77**(1): 762-8.

Ketting, R. F., S. E. Fischer, E. Bernstein, T. Sijen, G. J. Hannon and R. H. Plasterk (2001). "Dicer functions in RNA interference and in synthesis of small RNA involved in developmental timing in C. elegans." Genes Dev **15**(20): 2654-9.

Khvorova, A., A. Reynolds and S. D. Jayasena (2003). "Functional siRNAs and miRNAs exhibit strand bias." Cell **115**(2): 209-16.

Kile, B. T., A. D. Panopoulos, R. A. Stirzaker, D. F. Hacking, L. H. Tahtamouni, T. A. Willson, L. A. Mielke, K. J. Henley, J. G. Zhang, I. P. Wicks, W. S. Stevenson, P. Nurden, S. S. Watowich and M. J. Justice (2007). "Mutations in the cofilin partner Aip1/Wdr1 cause autoinflammatory disease and macrothrombocytopenia." Blood **110**(7): 2371-80.

Kim, S., U. J. Lee, M. N. Kim, E. J. Lee, J. Y. Kim, M. Y. Lee, S. Choung, Y. J. Kim and Y. C. Choi (2008). "MicroRNA miR-199a* Regulates the MET Proto-oncogene and the Downstream Extracellular Signal-regulated Kinase 2 (ERK2)." J Biol Chem **283**(26): 18158-66.

Kiriakidou, M., G. S. Tan, S. Lamprinaki, M. De Planell-Saguer, P. T. Nelson and Z. Mourelatos (2007). "An mRNA m7G cap binding-like motif within human Ago2 represses translation." Cell **129**(6): 1141-51.

Kluiver, J., S. Poppema, D. de Jong, T. Blokzijl, G. Harms, S. Jacobs, B. J. Kroesen and A. van den Berg (2005). "BIC and miR-155 are highly expressed in Hodgkin, primary mediastinal and diffuse large B cell lymphomas." J Pathol **207**(2): 243-9.

Knight, S. W. and B. L. Bass (2001). "A role for the RNase III enzyme DCR-1 in RNA interference and germ line development in Caenorhabditis elegans." Science **293**(5538): 2269-71.

Kovacevic, Z. and D. R. Richardson (2006). "The metastasis suppressor, Ndrg-1: a new ally in the fight against cancer." Carcinogenesis **27**(12): 2355-66.

Krek, A., D. Grun, M. N. Poy, R. Wolf, L. Rosenberg, E. J. Epstein, P. MacMenamin, I. da Piedade, K. C. Gunsalus, M. Stoffel and N. Rajewsky (2005). "Combinatorial microRNA target predictions." Nat Genet **37**(5): 495-500.

Krieglstein, C. F., W. H. Cerwinka, A. G. Sprague, F. S. Laroux, M. B. Grisham, V. E. Koteliansky, N. Senninger, D. N. Granger and A. R. de Fougerolles (2002). "Collagen-binding integrin alpha1beta1 regulates intestinal inflammation in experimental colitis." J Clin Invest **110**(12): 1773-82.

Kumar, P., H. S. Ban, S. S. Kim, H. Wu, T. Pearson, D. L. Greiner, A. Laouar, J. Yao, V. Haridas, K. Habiro, Y. G. Yang, J. H. Jeong, K. Y. Lee, Y. H. Kim, S. W. Kim, M. Peipp, G. H. Fey, N. Manjunath, L. D. Shultz, S. K. Lee and P. Shankar (2008). "T cell-specific siRNA delivery suppresses HIV-1 infection in humanized mice." Cell **134**(4): 577-86.

Lai, E. C. (2002). "Micro RNAs are complementary to 3' UTR sequence motifs that mediate negative post-transcriptional regulation." Nat Genet **30**(4): 363-4.

Lamhamedi-Cherradi, S. E., S. J. Zheng, K. A. Maguschak, J. Peschon and Y. H. Chen (2003). "Defective thymocyte apoptosis and accelerated autoimmune diseases in TRAIL-/- mice." Nat Immunol **4**(3): 255-60.

Lassila, O., C. Martin, P. Toivanen and F. Dieterlen-Lievre (1982). "Erythropoiesis and lymphopoiesis in the chick yolk-sac-embryo chimeras: contribution of yolk sac and intraembryonic stem cells." Blood **59**(2): 377-81.

Lee, R. C., R. L. Feinbaum and V. Ambros (1993). "The C. elegans heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14." Cell **75**(5): 843-54.

Lee, Y., C. Ahn, J. Han, H. Choi, J. Kim, J. Yim, J. Lee, P. Provost, O. Radmark, S. Kim and V. N. Kim (2003). "The nuclear RNase III Drosha initiates microRNA processing." Nature **425**(6956): 415-9.

Lee, Y., M. Kim, J. Han, K. H. Yeom, S. Lee, S. H. Baek and V. N. Kim (2004). "MicroRNA genes are transcribed by RNA polymerase II." Embo J **23**(20): 4051-60.

Lewis, B. P., C. B. Burge and D. P. Bartel (2005). "Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets." Cell **120**(1): 15-20.

Lewis, B. P., I. H. Shih, M. W. Jones-Rhoades, D. P. Bartel and C. B. Burge (2003). "Prediction of mammalian microRNA targets." Cell **115**(7): 787-98.

Lim, L. P., N. C. Lau, P. Garrett-Engele, A. Grimson, J. M. Schelter, J. Castle, D. P. Bartel, P. S. Linsley and J. M. Johnson (2005). "Microarray analysis shows that some microRNAs downregulate large numbers of target mRNAs." Nature **433**(7027): 769-73.

Lindbo, J. A. and W. G. Dougherty (1992). "Untranslatable transcripts of the tobacco etch virus coat protein gene sequence can interfere with tobacco etch virus replication in transgenic plants and protoplasts." Virology **189**(2): 725-33.

Lindbo, J. A., L. Silva-Rosales, W. M. Proebsting and W. G. Dougherty (1993). "Induction of a Highly Specific Antiviral State in Transgenic Plants: Implications for Regulation of Gene Expression and Virus Resistance." Plant Cell **5**(12): 1749-1759.

Liu, H. H., M. Xie, M. D. Schneider and Z. J. Chen (2006). "Essential role of TAK1 in thymocyte development and activation." Proc Natl Acad Sci U S A **103**(31): 11677-82.

Liu, J., M. A. Carmell, F. V. Rivas, C. G. Marsden, J. M. Thomson, J. J. Song, S. M. Hammond, L. Joshua-Tor and G. J. Hannon (2004). "Argonaute2 is the catalytic engine of mammalian RNAi." Science **305**(5689): 1437-41.

Lu, T. T. and J. G. Cyster (2002). "Integrin-mediated long-term B cell retention in the splenic marginal zone." Science **297**(5580): 409-12.

Lui, W. O., N. Pourmand, B. K. Patterson and A. Fire (2007). "Patterns of known and novel small RNAs in human cervical cancer." Cancer Res **67**(13): 6031-43.

Lund, E., S. Guttinger, A. Calado, J. E. Dahlberg and U. Kutay (2004). "Nuclear export of microRNA precursors." Science **303**(5654): 95-8.

Ma, L., J. Teruya-Feldstein and R. A. Weinberg (2007). "Tumour invasion and metastasis initiated by microRNA-10b in breast cancer." Nature **449**(7163): 682-8.

Mackenzie, I. R., E. H. Bigio, P. G. Ince, F. Geser, M. Neumann, N. J. Cairns, L. K. Kwong, M. S. Forman, J. Ravits, H. Stewart, A. Eisen, L. McClusky, H. A. Kretzschmar, C. M. Monoranu, J. R. Highley, J. Kirby, T. Siddique, P. J. Shaw, V. M. Lee and J. Q. Trojanowski (2007). "Pathological TDP-43 distinguishes sporadic amyotrophic lateral sclerosis from amyotrophic lateral sclerosis with SOD1 mutations." Ann Neurol **61**(5): 427-34.

Mansfield, J. H., B. D. Harfe, R. Nissen, J. Obenauer, J. Srineel, A. Chaudhuri, R. Farzan-Kashani, M. Zuker, A. E. Pasquinelli, G. Ruvkun, P. A. Sharp, C. J. Tabin and M. T. McManus (2004). "MicroRNA-responsive 'sensor' transgenes uncover Hox-like and other developmentally regulated patterns of vertebrate microRNA expression." Nat Genet **36**(10): 1079-83.

Mason, M. M., J. A. Grasso, O. Gavrilova and M. Reitman (1996). "Identification of functional elements of the chicken epsilon-globin promoter involved in stage-specific interaction with the beta/epsilon enhancer." J Biol Chem **271**(41): 25459-67.

Mazzucotelli, A., N. Viguerie, C. Tiraby, J. S. Annicotte, A. Mairal, E. Klimcakova, E. Lepin, P. Delmar, S. Dejean, G. Tavernier, C. Lefort, J. Hidalgo, T. Pineau, L. Fajas, K. Clement and D. Langin (2007). "The transcriptional coactivator peroxisome proliferator activated receptor (PPAR)gamma coactivator-1 alpha and the nuclear receptor PPAR alpha control the expression of glycerol kinase and metabolism genes independently of PPAR gamma activation in human white adipocytes." Diabetes **56**(10): 2467-75.

Melloni, E., P. Secchiero, C. Celeghini, D. Campioni, V. Grill, L. Guidotti and G. Zauli (2005). "Functional expression of TRAIL and TRAIL-R2 during human megakaryocytic development." J Cell Physiol **204**(3): 975-82.

Mette, M. F., J. van der Winden, M. A. Matzke and A. J. Matzke (1999). "Production of aberrant promoter transcripts contributes to methylation and silencing of unlinked homologous promoters in trans." Embo J **18**(1): 241-8.

Michael, M. Z., O. C. SM, N. G. van Holst Pellekaan, G. P. Young and R. J. James (2003). "Reduced accumulation of specific microRNAs in colorectal neoplasia." Mol Cancer Res **1**(12): 882-91.

Mittelbrunn, M., A. Molina, M. M. Escribese, M. Yanez-Mo, E. Escudero, A. Ursa, R. Tejedor, F. Mampaso and F. Sanchez-Madrid (2004). "VLA-4 integrin concentrates at the peripheral supramolecular activation complex of the immune synapse and drives T helper 1 responses." Proc Natl Acad Sci U S A **101**(30): 11058-63.

Motsch, N., T. Pfuhl, J. Mrazek, S. Barth and F. A. Grasser (2007). "Epstein-Barr virus-encoded latent membrane protein 1 (LMP1) induces the expression of the cellular microRNA miR-146a." RNA Biol **4**(3): 131-7.

Mourrain, P., C. Beclin, T. Elmayan, F. Feuerbach, C. Godon, J. B. Morel, D. Jouette, A. M. Lacombe, S. Nikic, N. Picault, K. Remoue, M. Sanial, T. A. Vo and H. Vaucheret (2000). "Arabidopsis SGS2 and SGS3 genes are required for posttranscriptional gene silencing and natural virus resistance." Cell **101**(5): 533-42.

Nandan, M. O., S. Chanchevalap, W. B. Dalton and V. W. Yang (2005). "Kruppel-like factor 5 promotes mitosis by activating the cyclin B1/Cdc2 complex during oncogenic Ras-mediated transformation." FEBS Lett **579**(21): 4757-62.

Nandan, M. O., H. S. Yoon, W. Zhao, L. A. Ouko, S. Chanchevalap and V. W. Yang (2004). "Kruppel-like factor 5 mediates the transforming activity of oncogenic H-Ras." Oncogene **23**(19): 3404-13.

Napoli, C., C. Lemieux and R. Jorgensen (1990). "Introduction of a Chimeric Chalcone Synthase Gene into Petunia Results in Reversible Co-Suppression of Homologous Genes in trans." Plant Cell **2**(4): 279-289.

Neumann, M., D. M. Sampathu, L. K. Kwong, A. C. Truax, M. C. Micsenyi, T. T. Chou, J. Bruce, T. Schuck, M. Grossman, C. M. Clark, L. F. McCluskey, B. L. Miller, E. Masliah, I. R. Mackenzie, H. Feldman, W. Feiden, H. A. Kretzschmar, J. Q. Trojanowski and V. M. Lee (2006). "Ubiquitinated TDP-43 in frontotemporal lobar degeneration and amyotrophic lateral sclerosis." Science **314**(5796): 130-3.

Ninomiya-Tsuji, J., K. Kishimoto, A. Hiyama, J. Inoue, Z. Cao and K. Matsumoto (1999). "The kinase TAK1 can activate the NIK-I kappaB as well as the MAP kinase cascade in the IL-1 signalling pathway." Nature **398**(6724): 252-6.

O'Connell, R. M., D. S. Rao, A. A. Chaudhuri, M. P. Boldin, K. D. Taganov, J. Nicoll, R. L. Paquette and D. Baltimore (2008). "Sustained expression of microRNA-155 in hematopoietic stem cells causes a myeloproliferative disorder." J Exp Med **205**(3): 585-94.

O'Connell, R. M., K. D. Taganov, M. P. Boldin, G. Cheng and D. Baltimore (2007). "MicroRNA-155 is induced during the macrophage inflammatory response." Proc Natl Acad Sci U S A **104**(5): 1604-9.

Oishi, Y., I. Manabe, K. Tobe, K. Tsushima, T. Shindo, K. Fujiu, G. Nishimura, K. Maemura, T. Yamauchi, N. Kubota, R. Suzuki, T. Kitamura, S. Akira, T. Kadowaki and R. Nagai (2005). "Kruppel-like transcription factor KLF5 is a key regulator of adipocyte differentiation." Cell Metab **1**(1): 27-39.

Okada, K., T. Obinata and H. Abe (1999). "XAIP1: a Xenopus homologue of yeast actin interacting protein 1 (AIP1), which induces disassembly of actin filaments cooperatively with ADF/cofilin family proteins." J Cell Sci **112 (Pt 10)**: 1553-65.

Ono, S., K. Mohri and K. Ono (2004). "Microscopic evidence that actin-interacting protein 1 actively disassembles actin-depolymerizing factor/Cofilin-bound actin filaments." J Biol Chem **279**(14): 14207-12.

Orom, U. A., F. C. Nielsen and A. H. Lund (2008). "MicroRNA-10a binds the 5'UTR of ribosomal protein mRNAs and enhances their translation." Mol Cell **30**(4): 460-71.

Orsulic, S. (2002). "An RCAS-TVA-based approach to designer mouse models." Mamm Genome **13**(10): 543-7.

Ou, S. H., F. Wu, D. Harrich, L. F. Garcia-Martinez and R. B. Gaynor (1995). "Cloning and characterization of a novel cellular protein, TDP-43, that binds to human immunodeficiency virus type 1 TAR DNA sequence motifs." J Virol **69**(6): 3584-96.

Ovcharenko, D., K. Kelnar, C. Johnson, N. Leng and D. Brown (2007). "Genome-scale microRNA and small interfering RNA screens identify small RNA modulators of TRAIL-induced apoptosis pathway." Cancer Res **67**(22): 10782-8.

Peters, L. and G. Meister (2007). "Argonaute proteins: mediators of RNA silencing." Mol Cell **26**(5): 611-23.

Pillai, R. S., S. N. Bhattacharyya, C. G. Artus, T. Zoller, N. Cougot, E. Basyuk, E. Bertrand and W. Filipowicz (2005). "Inhibition of translational initiation by Let-7 MicroRNA in human cells." Science **309**(5740): 1573-6.

Plafker, K. S., K. M. Farjo, A. F. Wiechmann and S. M. Plafker (2008). "The human ubiquitin conjugating enzyme, UBE2E3, is required for proliferation of retinal pigment epithelial cells." Invest Ophthalmol Vis Sci.

Preall, J. B. and E. J. Sontheimer (2005). "RNAi: RISC gets loaded." Cell **123**(4): 543-5.

Randall, G., M. Panis, J. D. Cooper, T. L. Tellinghuisen, K. E. Sukhodolets, S. Pfeffer, M. Landthaler, P. Landgraf, S. Kan, B. D. Lindenbach, M. Chien, D. B. Weir, J. J. Russo, J. Ju, M. J. Brownstein, R. Sheridan, C. Sander, M. Zavolan, T. Tuschl and C. M. Rice (2007). "Cellular cofactors affecting hepatitis C virus infection and replication." Proc Natl Acad Sci U S A **104**(31): 12884-9.

Reynaud, C. A., V. Anquez, A. Dahan and J. C. Weill (1985). "A single rearrangement event generates most of the chicken immunoglobulin light chain diversity." Cell **40**(2): 283-91.

Romano, N. and G. Macino (1992). "Quelling: transient inactivation of gene expression in Neurospora crassa by transformation with homologous sequences." Mol Microbiol **6**(22): 3343-53.

Roth, B. M., G. J. Pruss and V. B. Vance (2004). "Plant viral suppressors of RNA silencing." Virus Res **102**(1): 97-108.

Safina, A., M. Q. Ren, E. Vandette and A. V. Bakin (2008). "TAK1 is required for TGF-beta 1-mediated regulation of matrix metalloproteinase-9 and metastasis." Oncogene **27**(9): 1198-207.

Sarma, G., W. Greer, R. P. Gildersleeve, D. L. Murray and A. M. Miles (1995). "Field safety and efficacy of in ovo administration of HVT + SB-1 bivalent Marek's disease vaccine in commercial broilers." Avian Dis **39**(2): 211-7.

Sato, S., H. Sanjo, K. Takeda, J. Ninomiya-Tsuji, M. Yamamoto, T. Kawai, K. Matsumoto, O. Takeuchi and S. Akira (2005). "Essential function for the kinase TAK1 in innate and adaptive immune responses." Nat Immunol **6**(11): 1087-95.

Sato, S., H. Sanjo, T. Tsujimura, J. Ninomiya-Tsuji, M. Yamamoto, T. Kawai, O. Takeuchi and S. Akira (2006). "TAK1 is indispensable for development of T cells and prevention of colitis by the generation of regulatory T cells." Int Immunol **18**(10): 1405-11.

Schott, D. H., D. K. Cureton, S. P. Whelan and C. P. Hunter (2005). "An antiviral role for the RNA interference machinery in Caenorhabditis elegans." Proc Natl Acad Sci U S A **102**(51): 18420-4.

Schwarz, D. S., G. Hutvagner, T. Du, Z. Xu, N. Aronin and P. D. Zamore (2003). "Asymmetry in the assembly of the RNAi enzyme complex." Cell **115**(2): 199-208.

Secchiero, P., E. Melloni, M. Heikinheimo, S. Mannisto, R. Di Pietro, A. Iacone and G. Zauli (2004). "TRAIL regulates normal erythroid maturation through an ERK-dependent pathway." Blood **103**(2): 517-22.

Shim, J. H., C. Xiao, A. E. Paschal, S. T. Bailey, P. Rao, M. S. Hayden, K. Y. Lee, C. Bussey, M. Steckel, N. Tanaka, G. Yamada, S. Akira, K. Matsumoto and S. Ghosh (2005). "TAK1, but not TAB1 or TAB2, plays an essential role in multiple signaling pathways in vivo." Genes Dev **19**(22): 2668-81.

Silva, J. M., M. Z. Li, K. Chang, W. Ge, M. C. Golding, R. J. Rickles, D. Siolas, G. Hu, P. J. Paddison, M. R. Schlabach, N. Sheth, J. Bradshaw, J. Burchard, A. Kulkarni, G. Cavet, R. Sachidanandam, W. R. McCombie, M. A. Cleary, S. J. Elledge and G. J. Hannon (2005).

"Second-generation shRNA libraries covering the mouse and human genomes." Nat Genet **37**(11): 1281-8.

Sixt, M., M. Bauer, T. Lammermann and R. Fassler (2006). "Beta1 integrins: zip codes and signaling relay for blood cells." Curr Opin Cell Biol **18**(5): 482-90.

Skalsky, R. L., M. A. Samols, K. B. Plaisance, I. W. Boss, A. Riva, M. C. Lopez, H. V. Baker and R. Renne (2007). "Kaposi's sarcoma-associated herpesvirus encodes an ortholog of miR-155." J Virol **81**(23): 12836-45.

Slaby, O., M. Svoboda, P. Fabian, T. Smerdova, D. Knoflickova, M. Bednarikova, R. Nenutil and R. Vyzula (2007). "Altered expression of miR-21, miR-31, miR-143 and miR-145 is related to clinicopathologic features of colorectal cancer." Oncology **72**(5-6): 397-402.

Smith, C. J., C. F. Watson, C. R. Bird, J. Ray, W. Schuch and D. Grierson (1990). "Expression of a truncated tomato polygalacturonase gene inhibits expression of the endogenous gene in transgenic plants." Mol Gen Genet **224**(3): 477-81.

Sontheimer, E. J. (2005). "Assembly and function of RNA silencing complexes." Nat Rev Mol Cell Biol **6**(2): 127-38.

Soutschek, J., A. Akinc, B. Bramlage, K. Charisse, R. Constien, M. Donoghue, S. Elbashir, A. Geick, P. Hadwiger, J. Harborth, M. John, V. Kesavan, G. Lavine, R. K. Pandey, T. Racie, K. G. Rajeev, I. Rohl, I. Toudjarska, G. Wang, S. Wuschko, D. Bumcrot, V. Koteliansky, S. Limmer, M. Manoharan and H. P. Vornlocher (2004). "Therapeutic silencing of an endogenous gene by systemic administration of modified siRNAs." Nature **432**(7014): 173-8.

Stam, M., R. de Bruin, R. van Blokland, R. A. van der Hoorn, J. N. Mol and J. M. Kooter (2000). "Distinct features of post-transcriptional gene silencing by antisense transgenes in single copy and inverted T-DNA repeat loci." Plant J **21**(1): 27-42.

Stam, M., A. Viterbo, J. N. Mol and J. M. Kooter (1998). "Position-dependent methylation and transcriptional silencing of transgenes in inverted T-DNA repeats: implications for posttranscriptional silencing of homologous host genes in plants." Mol Cell Biol **18**(11): 6165-77.

Stennicke, H. R. and G. S. Salvesen (1998). "Properties of the caspases." Biochim Biophys Acta **1387**(1-2): 17-31.

Takaesu, G., S. Kishida, A. Hiyama, K. Yamaguchi, H. Shibuya, K. Irie, J. Ninomiya-Tsuji and K. Matsumoto (2000). "TAB2, a novel adaptor protein, mediates activation of TAK1 MAPKKK by linking TAK1 to TRAF6 in the IL-1 signal transduction pathway." Mol Cell **5**(4): 649-58.

Takaesu, G., J. Ninomiya-Tsuji, S. Kishida, X. Li, G. R. Stark and K. Matsumoto (2001). "Interleukin-1 (IL-1) receptor-associated kinase leads to activation of TAK1 by inducing TAB2 translocation in the IL-1 signaling pathway." Mol Cell Biol **21**(7): 2475-84.

Takaesu, G., R. M. Surabhi, K. J. Park, J. Ninomiya-Tsuji, K. Matsumoto and R. B. Gaynor (2003). "TAK1 is critical for IkappaB kinase-mediated activation of the NF-kappaB pathway." J Mol Biol **326**(1): 105-15.

Tam, W., D. Ben-Yehuda and W. S. Hayward (1997). "bic, a novel gene activated by proviral insertions in avian leukosis virus-induced lymphomas, is likely to function through its noncoding RNA." Mol Cell Biol **17**(3): 1490-502.

Tan, J., J. Cooke, N. Clarke and G. A. Tannock (2007). "Molecular evaluation of responses to vaccination and challenge by Marek's disease viruses." Avian Pathol **36**(5): 351-9.

Tang, M., X. Wei, Y. Guo, P. Breslin, S. Zhang, S. Zhang, W. Wei, Z. Xia, M. Diaz, S. Akira and J. Zhang (2008). "TAK1 is required for the survival of hematopoietic cells and hepatocytes in mice." J Exp Med **205**(7): 1611-9.

Teng, G., P. Hakimpour, P. Landgraf, A. Rice, T. Tuschl, R. Casellas and F. N. Papavasiliou (2008). "MicroRNA-155 is a negative regulator of activation-induced cytidine deaminase." Immunity **28**(5): 621-9.

Thompson, C. B. (1992). "Creation of immunoglobulin diversity by intrachromosomal gene conversion." Trends Genet **8**(12): 416-22.

Thompson, C. B. and P. E. Neiman (1987). "Somatic diversification of the chicken immunoglobulin light chain gene is limited to the rearranged variable gene segment." Cell **48**(3): 369-78.

Tili, E., J. J. Michaille, A. Cimino, S. Costinean, C. D. Dumitru, B. Adair, M. Fabbri, H. Alder, C. G. Liu, G. A. Calin and C. M. Croce (2007). "Modulation of miR-155 and miR-125b levels following lipopolysaccharide/TNF-alpha stimulation and their possible roles in regulating the response to endotoxin shock." J Immunol **179**(8): 5082-9.

Tong, D., K. Czerwenka, G. Heinze, M. Ryffel, E. Schuster, A. Witt, S. Leodolter and R. Zeillinger (2006). "Expression of KLF5 is a prognostic factor for disease-free survival and overall survival in patients with breast cancer." Clin Cancer Res **12**(8): 2442-8.

van Belzen, N., W. N. Dinjens, M. P. Diesveld, N. A. Groen, A. C. van der Made, Y. Nozawa, R. Vlietstra, J. Trapman and F. T. Bosman (1997). "A novel gene which is up-

regulated during colon epithelial cell differentiation and down-regulated in colorectal neoplasms." Lab Invest **77**(1): 85-92.

van der Krol, A. R., L. A. Mur, M. Beld, J. N. Mol and A. R. Stuitje (1990). "Flavonoid genes in petunia: addition of a limited number of gene copies may lead to a suppression of gene expression." Plant Cell **2**(4): 291-9.

Varnholt, H., U. Drebber, F. Schulze, I. Wedemeyer, P. Schirmacher, H. P. Dienes and M. Odenthal (2008). "MicroRNA gene expression profile of hepatitis C virus-associated hepatocellular carcinoma." Hepatology **47**(4): 1223-32.

Vigorito, E., K. L. Perks, C. Abreu-Goodger, S. Bunting, Z. Xiang, S. Kohlhaas, P. P. Das, E. A. Miska, A. Rodriguez, A. Bradley, K. G. Smith, C. Rada, A. J. Enright, K. M. Toellner, I. C. Maclennan and M. Turner (2007). "microRNA-155 regulates the generation of immunoglobulin class-switched plasma cells." Immunity **27**(6): 847-59.

Wang, B., A. Yanez and C. D. Novina (2008). "MicroRNA-repressed mRNAs contain 40S but not 60S components." Proc Natl Acad Sci U S A **105**(14): 5343-8.

Waterhouse, P. M., M. W. Graham and M. B. Wang (1998). "Virus resistance and gene silencing in plants can be induced by simultaneous expression of sense and antisense RNA." Proc Natl Acad Sci U S A **95**(23): 13959-64.

Weng, N. P. (2008). "Telomere and adaptive immunity." Mech Ageing Dev **129**(1-2): 60-6.

White-Gilbertson, S., S. Rubinchik and C. Voelkel-Johnson (2008). "Transformation, translation and TRAIL: an unexpected intersection." Cytokine Growth Factor Rev **19**(2): 167-72.

Wightman, B., T. R. Burglin, J. Gatto, P. Arasu and G. Ruvkun (1991). "Negative regulatory sequences in the lin-14 3'-untranslated region are necessary to generate a temporal switch during Caenorhabditis elegans development." Genes Dev **5**(10): 1813-24.

Wightman, B., I. Ha and G. Ruvkun (1993). "Posttranscriptional regulation of the heterochronic gene lin-14 by lin-4 mediates temporal pattern formation in C. elegans." Cell **75**(5): 855-62.

Wilkins, C., R. Dishongh, S. C. Moore, M. A. Whitt, M. Chow and K. Machaca (2005). "RNA interference is an antiviral defence mechanism in Caenorhabditis elegans." Nature **436**(7053): 1044-7.

Wu, L., J. Fan and J. G. Belasco (2006). "MicroRNAs direct rapid deadenylation of mRNA." Proc Natl Acad Sci U S A **103**(11): 4034-9.

Yamaguchi, K., K. Shirakabe, H. Shibuya, K. Irie, I. Oishi, N. Ueno, T. Taniguchi, E. Nishida and K. Matsumoto (1995). "Identification of a member of the MAPKKK family as a potential mediator of TGF-beta signal transduction." Science **270**(5244): 2008-11.

Yang, Q. and Y. Li (2007). "Roles of PPARs on regulating myocardial energy and lipid homeostasis." J Mol Med **85**(7): 697-706.

Yang, Y., B. G. Goldstein, H. H. Chao and J. P. Katz (2005). "KLF4 and KLF5 regulate proliferation, apoptosis and invasion in esophageal cancer cells." Cancer Biol Ther **4**(11): 1216-21.

Yekta, S., I. H. Shih and D. P. Bartel (2004). "MicroRNA-directed cleavage of HOXB8 mRNA." Science **304**(5670): 594-6.

Yi, R., Y. Qin, I. G. Macara and B. R. Cullen (2003). "Exportin-5 mediates the nuclear export of pre-microRNAs and short hairpin RNAs." Genes Dev **17**(24): 3011-6.

Yin, Q., J. McBride, C. Fewell, M. Lacey, X. Wang, Z. Lin, J. Cameron and E. K. Flemington (2008). "MicroRNA-155 is an Epstein-Barr virus-induced gene that modulates Epstein-Barr virus-regulated gene expression pathways." J Virol **82**(11): 5295-306.

Yin, Q., X. Wang, J. McBride, C. Fewell and E. Flemington (2008). "B-cell receptor activation induces BIC/miR-155 expression through a conserved AP-1 element." J Biol Chem **283**(5): 2654-62.

Zamore, P. D., T. Tuschl, P. A. Sharp and D. P. Bartel (2000). "RNAi: double-stranded RNA directs the ATP-dependent cleavage of mRNA at 21 to 23 nucleotide intervals." Cell **101**(1): 25-33.

Zandbergen, F. and J. Plutzky (2007). "PPARalpha in atherosclerosis and inflammation." Biochim Biophys Acta **1771**(8): 972-82.

Zeng, Y., X. Cai and B. R. Cullen (2005). "Use of RNA polymerase II to transcribe artificial microRNAs." Methods Enzymol **392**: 371-80.

Zerafa, N., J. A. Westwood, E. Cretney, S. Mitchell, P. Waring, M. Iezzi and M. J. Smyth (2005). "Cutting edge: TRAIL deficiency accelerates hematological malignancies." J Immunol **175**(9): 5586-90.

Zhang, Y. and J. M. Sharma (2001). "Early posthatch protection against Marek's disease in chickens vaccinated in ovo with a CVI988 serotype 1 vaccine." Avian Dis **45**(3): 639-45.