ABSTRACT

HICKS, JULIE ANN. MicroRNAs in the Spleen and Liver of the Developing Chick Embryo. (Under the direction of Dr. HC Sunny Liu.)

MicroRNAs are small (~19-24nt) non-coding RNAs that are involved in the regulation of gene expression. They are mainly expressed in development and many are expressed in a temporal as well as, a spatial manner. It is thought they may regulate up to 30% of all genes. Pyrosequencing using 454 Life Sciences Inc. (www.454.com) technology is becoming the preferred method for microRNA profiling and sequencing compared to the previous method of cloning and using traditional sequencing techniques. Use of 454 Life Sciences technology allows for a greater coverage of microRNAs and increases the chances of sequencing low abundance microRNAs. In the current project, we created four small RNA libraries from embryonic chick tissues, the spleen and liver at developmental time points E15 and E20. These libraries were then sequenced using 454 Life Sciences pyrosequencing. A total of 92,919 sequence reads were obtained, representing a total of 52,001 known chicken microRNAs. Of these 92,919 reads, 52,001 represented miRNAs matching the miRBase *G. gallus* database, and 3,472 were not found in the *G. gallus* database but were homologues of miRBase miRNAs from other species. Of these homologous reads 391 represented potential novel miRNAs. Other small RNAs, such as tRNA and rRNA, represented 24,672 of the reads, and 12,383 reads represented other types of sequences, such as degraded mRNA. More than one hundred different known miRNAs were identified in this study, and many were expressed in all four libraries. Common miRNAs that yielded multiple reads from all four libraries included *miR-125b* and *miR-21*, which are involved in general processes of cellular proliferation. Overall, the spleen libraries had a larger array of miRNAs than the liver

libraries. Much of spleen development occurs during the later stages of embryonic development, so we can reasonably expect that many gene expression changes occur during these stages. As a result of this study, we identified nine potential novel chicken miRNAs. These novel miRNAs appear to be tissue-specific. The potential novel miRNAs appeared to be expressed at lower levels than some of the known miRNAs, which could indicate that most of the highly-expressed chicken miRNAs have already been identified, whereas, for the most part, the miRNAs expressed at low levels remain to be discovered.

MicroRNAs in the Spleen and Liver of the Developing Chick Embryo

by

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CHAPTER 1: LITERATURE REVIEW

THE CENTRAL DOGMA

The study of genetics began in earnest in the mid-1800s with Gregor Mendel, who systematically studied inherited traits in pea plants. Based on the phenotypes of the parent plants, he was able to select for particular phenotypes of specific traits (such as color and shape) and to predict the probability that a plant would display a particular characteristic (Mendel 1866). After Mendel, research in genetics slowed until the early 1900s. In 1928, Frederick Griffith performed a now classic experiment in which he was able to transform a non-virulent strain of bacteria into a virulent strain by mixing it with heat-killed bacteria (Griffith 1928). Scientists then began to attempt to determine which component of the virulent strain, its protein or its DNA, was transferred to the non-virulent strain, although they would not find the answer for another 20 years.

In 1944, Oswald Avery and his colleagues showed that DNA, not protein, was responsible for the transformation of the non-virulent strain. When they treated a lysate of virulent bacteria with protease or ribonuclease (RNase), transformation still occurred, but treatment with deoxyribonuclease (DNase) prevented transformation, strongly suggesting that DNA was the transforming factor (Avery et al. 1944). Further evidence to support this hypothesis was produced by Hershey and Chase in 1952, who studied infection of bacteria by a simple virus (bacteriophage or phage). To determine which component (DNA or protein) of the phage infected the bacteria and caused production of more phage, Hershey and Chase labeled the protein with ^{35}S and the DNA with ^{32}P. When the bacteria were infected with the labeled phage, little of the ^{35}S, but much of the ^{32}P, was found inside of the bacteria, showing that the phage had transmitted its ^{32}P-labeled DNA to the bacteria (Hershey and Chase 1952).

This result provided strong evidence that DNA is the information-encoding molecule for an organism.

In the mid-1950s, Maurice Wilkins and Rosalind Franklin performed X-ray diffraction studies to examine the three-dimensional (3-D) structure of DNA (Wilkins et al. 1953). Using their work, together with that of Linus Pauling, who had determined the 3-D α-helical structure found in proteins (Pauling et al. 1951), James Watson and Francis Crick were able to determine the 3-D double-helical structure of DNA (Watson and Crick 1953). Their result inspired a surge in DNA research that continues to this day.

Research over several decades led to formation of the "central dogma," which states that DNA is transcribed into mRNA, which is then translated into protein, and that any DNA that is not transcribed and translated is disposable "junk." Recently, however, the central dogma has been shown to be inaccurate and incomplete. For example, some essential DNA is transcribed into small RNA molecules that are not translated into protein. These small regulatory RNA molecules were discovered in the mid-1990s, and since that time, many studies have shown that they play critical biological roles in regulation of gene expression and the antiviral immune response, and they have been linked to cancer.

One important class of small regulatory RNA is microRNA (miRNA), which has been shown to be important in developmental timing and cell differentiation and proliferation (Lee et al. 1993; Slack et al. 2000; Ambros et al. 2003; Brennecke et al. 2003; Wienholds and Plaster 2005). Gain- and loss-of-function studies of miRNAs have shown that deviant expression of miRNA causes improper development of the organism (Slack et al. 2000). A great deal of research examining the role of miRNA in development of model organisms, such as *Caenorhabditis elegans* and *Drosophila melanogaster*, has been performed.

RNA

Messenger RNA (mRNA), transfer RNA (tRNA), and ribosomal RNA (rRNA) are the major forms of RNA. As discussed above, mRNA is transcribed from DNA and then translated into protein. tRNA is a small molecule that transfers an amino acid to a growing peptide chain during protein synthesis, and rRNA, together with several proteins, forms ribosomes, the complexes that synthesize protein. Another class of RNA is ribozymes. Ribozymes are RNA molecules with catalytic activity and are involved in RNA cleavage as well as, being involved in ribosome aminotransferase function. Some small RNAs, such as small nuclear (sn) RNA, which is involved in mRNA splicing, and small nucleolar (sno) RNA, which is involved in chemical modification (such as methylation) of RNA, were also discovered years ago. The small regulatory RNAs, a very important group of RNAs, were not discovered until recently, however.

REGULATORY RNA

The small regulatory RNAs have recently been shown to control gene expression. In the last several years, many types of small regulatory RNAs have been discovered (Voinnet 2002), including miRNA and short interfering RNA (siRNA); the latter type can be subdivided into endogenous *trans*-acting siRNA (tasiRNA), repeat-associated siRNA (rasiRNA), and small scan RNA (scnRNA) (Kim 2005). Other small regulatory RNAs recently classified include PIWI-interacting RNA (Girard et al. 2006; Lau et al. 2006), tiny non-coding RNA (tncRNA) (Ambros et al. 2003), and small modulatory RNA (smRNA) (Kuwabara et al. 2004). The last two classifications are considered provisional, however, and little is known about these RNAs.

Trans-acting siRNAs are transcribed in either direction by RNA polymerase II to

produce double-stranded (ds) RNA. So far tasiRNAs have only been identified in plants. Three genes, *TAS1*, *TAS2*, and *TAS3*, have been found to encode tasiRNAs (Yoshikawa et al. 2005). Similar to what is seen in microRNAs tasiRNAs start out as larger transcripts and are then cleaved in 21nt increments to create multiple siRNAs (Yoshikawa et al. 2005). The dsRNA is cleaved by an RNase III Dicer-like endonuclease (DCL4), becomes single-stranded (*via* currently unknown mechanisms), and joins the RNA-induced silencing complex (RISC) that degrades mRNA (Vazquez et al. 2004). Although tasiRNAs play a role in mRNA degradation, their exact function remains unclear (Vazquez et al. 2004). However, it has been shown that in *Arabidopsis* mutants that lack the *TAS* genes and in particular *TAS3*, exhibit leaf morphology defects (Adenot et al. 2006). The hosting gene structures of most tasiRNAs are not known, with the exception that one set has been identified in an intron of a non-coding gene in *Arabidopsis* (Vazquez et al. 2004).

The rasiRNAs are thought to be derived from dsRNA transcribed in either direction from repetitive sequence elements. These siRNAs are mainly produced from the antisense strand (Klattenhoff et al. 2006). In *Drosophila* they have been found to produced in the germline in a Dicer-independent manner (Guanawardane et al. 2007). In *Drosophila*, ~178 rasiRNAs are expressed during development (Aravin et al. 2003). These RNAs are similar to transposable elements and satellite DNA. They are most highly expressed in the early embryo and are thought to regulate transposon activity and heterochromatin structure (Aravin et al. 2003). RasiRNAs are critical during development to suppress DNA damage (Kalttenhoff et al. 2006).

Small-scan RNA is found in ciliated protozoans, such as *Tetrahymena*, that have both a micronucleus and a polyploid macronucleus, creating an excess of DNA (Kim 2005). They

are only produced and accumulate during conjugation (Liu et al. 2004). More specifically they produced prior to chromosome rearrangement (Mochizuki et al. 2002) and the gene *PDD1* is required for scnRNA to be expressed. The macronucleus contains the functionally active DNA, whereas the DNA in the micronucleus is silent. The micronuclear dsRNA is thought to be transcribed in the micronucleus and then cleaved into scnRNA by a Dicer-like enzyme. The scnRNA then goes to the macronucleus, where it detects excess DNA (Mochizuki and Gorovsky 2004a), and joins the RNA-induced initiation of transcriptional gene silencing (RITS) complex, in particular they interact with Twi1p, a homolog to piwi (Mochizuki and Gorovky. 2004b). As a result, the histone H3 is methlyated at lysine 9, which is a hallmark of heterochromatin (Liu et al. 2004).

Another class of small regulatory RNAs has recently been discovered (Grivna et al. 2006a; Aravin et al. 2006). These small RNAs are called PIWI-interacting RNAs or piRNAs. They are slightly larger than the other types of small regulatory RNAs, with a size of 26–31 nts (Aravin et al. 2006). Currently piRNAs have only been identified in the testis (Grivna et al. 2006a; Grivna et al. 2006b; Aravin et al. 2006). These small RNAs were named piRNAs because they were found to interact with the PIWI subfamily of Argonute proteins (Girard et al. 2006). It has been shown that the mouse piwi homologue called miwi is required for spermatogenesis; $miwi^{null}$ male mice are sterile due to spermatogenic arrest (Deng and Lin 2002). Later, it was shown that MIWI interacts with a class of small RNAs (later named piRNAs) and also with translational machinery in polysomes and this is required for initiating spermiogenesis (Grivna et al. 2006b).

Piwi-interacting RNAs are different from siRNAs and miRNAs in that they do not come from a dsRNA precursor (Girard et al. 2006; Lau et al. 2006). In the genome, piRNAs

are located in large clusters that are many kilobases in length (Girard et al. 2006). These clusters are located in exons, introns, and intergenic regions, but are underrepresented in repeat regions (Grivna et al. 2006b). Some chromosomes appear to encode many piRNAs, while others encode only a few (Girard et al. 2006). The piRNAs also appear to exhibit strand asymmetry; in the piRNA gene clusters, almost all of the piRNAs are encoded by only one strand (Girard et al. 2006; Lau et al. 2006). Comparison of piRNA sequences has revealed little sequence conservation among them, except for a preference for a uridine at the 5′ end (Girard et al. 2006; Lau et al. 2006). piRNAs are expressed in the later stages of spermiogenesis and are expressed at very high levels; estimates are as high as 1 million piRNAs per spermatocyte (Aravin et al. 2006). The exact function/mechanism of piRNAs is unknown, but it is thought that they are involved in regulating timing during spermiogenesis, probably through transcriptional and/or translational repression (Aravin et al. 2006; Grivna et al. 2006b).

Not much is known about tiny non-coding RNA (tncRNA) and small modulartory RNA (smRNA). So far, 33 tiny non-coding RNAs have been identified in *C. elegans*. These RNAs are similar in length to microRNAs averaging ~20-21 nucleotides. Tiny non-coding RNAs may be developmentally regulated, but to not have to be. They are not processed from a hairpin precursor and do no exhibit phylogenetic conservation (Ambros et al. 2003). Small modulartory RNAs regulate gene expression during neuronal differentiation and have been found to regulate neuron specific gene expression (Kuwabra et al. 2004).

MicroRNAs are small (19–24 nt) non-coding RNAs that silence genes posttranscriptionally. In animals, gene silencing typically occurs *via* transcriptional repression rather than by degradation of mRNA (Bartel 2004). Lee et al. first discovered an

miRNA (*lin-4*) in 1993. This miRNA suppresses the expression of *lin-14*, a nuclear protein expressed during *C. elegans* development. MicroRNAs have since become an important area of study in functional genomics research.

MICRORNA

MicroRNAs are small regulatory RNAs involved in regulation of gene expression. They are typically found in either an intron of the coding region of a gene (for example *let-7c*) or in an exon in a non-coding RNA (for example *mir-21*) (Du and Zamore 2005). A computational approach looking for mRNAs with conserved microRNA complementarity sites across four genomes predicted that they control up to 30% of all protein-encoding genes (Lewis et al. 2005). This implies that microRNAs can either be under the control of a protein encoding gene's promoter or under the control of their own protein. A recent study comparing microRNA promoters to typical protein encoding gene promoters found that they had similar characteristics (Zhou et al. 2007). The DNA sequences encoding miRNAs are first transcribed into primary miRNAs (pri-miRNAs), which can range from several hundred to a few thousand nucleotides in length (Du and Zamore 2005). Pri-miRNAs are processed into pre-miRNAs, which are ~70-nt stem-loop RNAs. This processing is carried out by nuclear RNase III Drosha, as are the preceding steps. Next, the protein exportin transports the pre-miRNAs to the cytoplasm, where they are processed into mature miRNAs by RNase III Dicer (Du and Zamore 2005) (Figure 1.1).

Function of microRNA

MicroRNAs interact with their target mRNAs by base pairing of complementary sequences. In animals, this pairing silences protein expression in one of two ways. If the

complementarity between the miRNA and its target is perfect, then RNase III Dicer will degrade the mRNA. On the other hand, if the complementarity is imperfect, then the mRNA will not be degraded, ribosomes will be prevented from translating the mRNA, and the gene will be silenced (Bartel 2004). In plants, miRNA-paired mRNA is degraded even when the complementarity is imperfect (Bartel 2004). In yeast, plants, and possibly animals, miRNAs are thought to control gene expression using a third mechanism involving transcriptional silencing by histone methylation (Marzke et al. 2004).

Genes regulated by miRNA may have one or several binding sites for miRNA (Doench et al. 2003; Zeng and Cullen 2003). As might be expected, the sensitivity of a gene to miRNA control tends to increase as the number of miRNA binding sites increases, and the amount of translation that occurs from the transcript decreases as more miRNAs are bound (Zeng and Cullen 2003). This mechanism provides for complex and finely regulated control of gene expression, so that miRNA control is not an all-or-nothing mechanism.

MicroRNA and development

Regulation of gene expression during development is one of the most important functions of miRNA (Lee et al. 1993; Slack et al. 2000; Ambros et al. 2003; Brennecke et al. 2003; Weinholds and Plasterk 2005). An extensive amount of research into the role of miRNA in development has been performed in the model organism *C. elegans*.

Since the discovery of *lin-4* and *let-7*, expression of many other miRNAs has also been found to occur in a temporal- and tissue-specific manner during development (Weinholds and Plasterk 2005). The results of many loss- and gain-of-function studies have shown that alterations in the expression of miRNAs can disrupt the developmental sequence. For example, loss of *let-7* and/or gain of *lin-41* causes development to revert to an earlier state

(Slack et al. 2000).

MicroRNAs typically are not important in the early stages of development, but they are extremely important during the later stages, when they regulate cell differentiation and proliferation (Wienholds and Plaster 2005). When miRNAs *miR-124* and *miR-1*, which are preferentially expressed in brain and muscle, respectively, were introduced into HeLa cells, they caused the gene expression profiles to shift toward those of brain and muscle, respectively (Lim et al. 2005), suggesting that these miRNAs assist in controlling differentiation during development. Taken together, these studies indicate that miRNAs serve an important regulatory role during vertebrate development.

MicroRNA in eukaryotes

MicroRNAs have been found to be essential for proper development in many eukaryotic organisms, and defects in their regulation have been linked to various disorders, such as Fragile X mental retardation and DiGeorge syndrome (Ambros et al. 2003; Aboobaker et al. 2005; Biemar et al. 2005; Boehm and Slack 2005; Yoo and Greenwald 2005). They have also been linked to viral infections, such as those by herpesviruses and Hepatitis C virus, both in defensive roles and as targets of exploitation by viruses, and to carcinogenesis (Calin et al. 2004; Pfeffer et al. 2004; Cai et al. 2005; Couturier and Root-Bernstein 2005; Pfeffer et al. 2005; Sullivan et al. 2005).

MicroRNA in C. elegans

Much of what is currently known about miRNA and other small regulatory RNAs has come from studies in the nematode *C. elegans*, the organism in which they were initially discovered (Lee et al. 1993). Many *C. elegans* studies have validated the proposal that a

major function of miRNA is the control of developmental timing. Indeed, the first two miRNAs to be discovered, *lin-4* and *let-7,* are important in transitioning between larval stages during *C. elegans* development (Ambros et al. 2003). The *lin-4* miRNA, which targets the *lin-14* gene, is expressed during early development of *C. elegans*, and *let-7* is expressed during late development (Lee et al. 1993). Although the exact function of Lin-14 is unknown, blocking its expression allows cells to transition from the first larval stage into the second larval stage (Boehm and Slack 2005). In addition, *lin-4* was recently found to regulate the lifespan of *C. elegans* (Boehm and Slack 2005); when the level of *lin-4* is low in an adult worm, the lifespan of the worm is shortened, and its tissues age faster (Boehm and Slack 2005). Over expression of *lin-4* appears to increase lifespan.

A recent study of cell-cell interactions in vulva development has identified an miRNA (*mir-61*) that is important in controlling cell fate *via* a signaling pathway involving Lin-12 (Yoo and Greenwald 2005). In *C. elegans,* three vulval precursor cells, P5.p, P6.p, and P7.p, ultimately develop into one of two cell types (primary or secondary) in the adult vulva (Kornfeld 1997). Epidermal growth factor signals the precursor cells to become primary cells, and Lin-12 is involved in signaling them to become secondary cells. Lin-12 is thought to specify a secondary cell fate by targeting genes involved in turning off epidermal growth factor expression. Yoo and Greenwald (2005) found that one of the target genes of Lin-12 is the miRNA *mir-61*, which has as a target the gene *vav-1*, which encodes a Lin-12 antagonist. By repressing *vav-1*, *mir-61* assists Lin-12 in signaling the cell to take on a primary cell fate.

MicroRNA in Drosophila

The functions of miRNAs have also been studied in *Drosophila* (Brennecke et al. 2003;

Aboobaker et al. 2005; Biemar et al. 2005; Schuldt 2005; Sokol and Ambros et al. 2005). For example, the *bantam* gene encodes a ~21-nt miRNA that is up-regulated during the third instar larval stage, promotes cell proliferation and prevents apoptosis (Brennecke et al. 2003). An *in situ* hybridization study of miRNA expression during *Drosophila* development identified 38 expressed miRNAs (Aboobaker et al. 2005), which exhibited distinct patterns of expression in the embryo. For example, *mir-309* (polycistronic), *mir-10*, and *iab-4* are expressed in different regions along the anterior-posterior axis during early embryonic development, whereas *mir-1* and *mir-316* are expressed only in the mesoderm and are expressed slightly later during development. Another profiling study of miRNA expression during *Drosophila* embryonic development identified 62 expressed miRNAs that were shown by Northern blotting to have unique spatial profiles (Aravin et al. 2003).

MicroRNA in zebrafish

In addition to *C. elegans* and *Drosophila*, zebrafish has also been used as a model organism for studies of miRNAs and development (Berezikov and Plasterk 2005; Chen et al. 2005; Miska 2005; Weinholds and Plasterk 2005; Ying and Lin 2005). MicroRNA profiling in zebrafish has shown that many miRNAs are conserved among vertebrates (Weinholds and Plasterk 2005). This high level of conservation is evidence of the important functions of miRNAs.

MicroRNAs are involved in many aspects of zebrafish development. For example, a recent study found that 80% of miRNAs involved in zebrafish development are tissue-specific (Weinholds and Plasterk 2005). RNase III Dicer-deficient zebrafish embryos exhibit defects in gastrulation, brain formation, somatogenesis, and heart morphogenesis (Giraldez et

al. 2005). A recent miRNA profile of larval and adult zebrafish identified 139 miRNAs, 66 of which were novel (Kloosterman et al. 2006). *In situ* hybridization revealed that most of these RNAs are expressed later in development and are tissue-specific.

During the very earliest stages of vertebrate development, large amounts of maternal mRNA are present. Shortly after development begins, these mRNAs are lost through a poorly understood mechanism. A recent study suggests that the miRNA *miR-430* produced by the zygote is responsible for the loss of maternal mRNA (Giraldez et al. 2006).

MicroRNA in rodents

Mice have been used extensively in miRNA studies. In a recent study of lung morphogenesis during mouse fetal development, RNase III Dicer-null mouse embryos died prior to gastrulation, suggesting that miRNAs play an essential role during mouse embryonic development (Harris et al. 2006). When Dicer was inactivated at a later point in fetal development, *via* a Dicer conditional allele, the lungs developed improperly, failing to form the branches found in a normal lung.

In a study of muscle development, Chen et al. (2006) found that miRNAs are involved in both proliferation and differentiation of skeletal muscle. Two miRNAs in particular, *miR-1* and *miR-133*, play major roles in muscle development. The miRNA *miR-1* blocks expression of histone deacetylase 4 (HDAC4), a repressor of muscle gene expression, thus causing the muscle development genes to become active. By suppressing serum response factor, *miR-133* allows muscle cells to proliferate during development (Chen et al. 2006). In another study of muscle development, *miR-181* was found to be highly expressed during myoblast differentiation (Naguibneva et al. 2006). This miRNA targets Hox-A11, a suppressor of

differentiation.

MicroRNAs are also involved in skin morphogenesis during mouse embryonic development. MicroRNA profiling during skin development revealed the expression of more than 100 miRNAs (Yi et al. 2006). The different cells that constitute the skin, such as those that make up the epidermis and those that become hair follicles, were found to have different miRNA expression profiles, again indicating that miRNAs are important for differentiation during development.

MicroRNA in humans

The number of human miRNAs is estimated to be as high as 800 (Bentwich et al. 2005), and several hundred of these have been experimentally validated. These miRNAs are involved in the regulation of many different genes and processes. For example, a profile of human fetal liver identified 27 expressed miRNAs, of which five were novel (Fu et al. 2005), suggesting that miRNAs are crucial to hepatogenesis during embryonic development. They are also important in hematopoiesis. Two groups of miRNAs are involved in megakaryocytosis (Garzon et al. 2006). The miRNAs *miR-10a, -126, -106, -10b, -17,* and *R-20* are down-regulated, suggesting that they control genes involved in cellular differentiation. MicroRNAs *miR-101*, *-126*, *-99a*, *-135*, and *-20* were found to be up-regulated in megakaryoblastic leukemic cells, again suggesting that they control differentiation genes while they are up-regulated in cancerous cells; These cells exhibit less differentiation than their healthy counterparts (Garzon et al. 2006), suggesting that some miRNAs are involved in establishing the differentiation process while others are involved in maintaining it.

In addition to their roles in gene regulation, miRNAs have been linked to various

illnesses, including viral infections and cancer (Calin et al. 2004; Pfeffer et al. 2004; Cai et al. 2005; Couturier and Root-Bernstein 2005; Pfeffer et al. 2005; Sullivan et al. 2005). A recent study linked development of type II diabetes to miRNA *miR-375* (Poy et al. 2004), which is specifically expressed in the pancreas and is involved in the regulation of insulin exocytosis (Gauthier and Wollheim 2006). Glucose secretion is greatly reduced in cells overexpressing *miR-135*, and deletion of *miR-135* leads to the over-secretion of glucose (Poy et al. 2004), suggesting that defective regulation of *miR-135* expression might lead to the development of diabetes.

MicroRNA and chickens

Although miRNAs have been identified in the chicken, their roles in chicken development have not been a focus of miRNA research. A transcriptome analysis of almost 500,000 expressed-sequence tags (ESTs) in chicken identified 23 expressed miRNAs (Hubbard et al. 2005). The chicken orthologue of the *C. elegans* gene *lin-41* is expressed in chick limb development (Lancman et al. 2005); as in *C. elegans*, chicken *lin-41* has a binding site for the miRNAs *lin-4* and *let-7* in its 3′ untranslated region (UTR). These miRNAs are expressed during chick limb development, suggesting that they control the expression of chicken *lin-41*. Another miRNA, *miR-196*, is thought to be involved in specifying hindlimb development (Hornstein et al. 2005).

A recent study of somite development in the chick embryo has revealed that miRNAs, in particular *miR-124* and *miR-206*, are also involved in the regulation of the nervous system (Sweetman et al. 2006). Northern-blot analysis of the relative expression of these miRNAs between days 1.5 and 5 of development showed that they are more highly expressed during

the earliest days and the decline, reflecting the fact that the nervous system is one of the earliest developing systems during embryonic development. Northern-blot analysis also showed that *miR-124* is specifically involved in the development of the central nervous system, whereas *miR-206* is specifically expressed in developing somites. Its expression was first detected in stage HH14 of development around the time that the epaxial myotome emerges. In addition, *miR-206* expression is negatively regulated *via* FGF-mediated signaling. Based on the findings in this study, *miR-206* is most likely involved in myogenic differentiation (Sweetman et al. 2006).

Recently, high throughput *in situ* hybridization was employed to study microRNA expression in the early chick embryo (Darnell et al. 2006). In this work whole mount *in situ* hybridizations probing for various microRNAs were preformed on embryonic chicks ranging from days 0.5 -5 days of development. Overall 135 different microRNAs were found to be expressed and of these 75 showed differential expression over the different developmental time points. This study demonstrates that microRNAs are a very important gene regulatory mechanism in the chick development, similar to what has been found in other organisms.

MicroRNA and viruses

Recent studies have revealed that some viruses, including some retroviruses and herpesviruses, also encode miRNAs (Pfeffer et al. 2004; Cai et al. 2005; Couturier and Root-Bernstein 2005; Pfeffer et al. 2005; Sullivan et al. 2005; Burnside et al. 2006). Computational predictive studies and experimental profiling studies suggest that viral miRNAs are generally found in the large DNA viruses and are unlikely to be found in RNA viruses (Pfeffer et al. 2005). Unlike their plant and animal counterparts, viral miRNAs appear to be poorly

conserved (Pfeffer et al. 2005). This was determined by comparing microRNAs encoded by four herpesviruses, Kaposi sacroma-associated virus (KSHV), human cytomegalovirus (HCMV), mouse gamma herpes virus 68 (MHV68), and Epstein-Barr virus (EBV). When the microRNAs were compared between viruses no substantial sequence homology could be found.

Viral miRNAs control expression of both host and viral genes. They can suppress the expression of cytokines and their receptors and thus down-regulate the immune response to viral infection (Couturier and Root-Bernstein 2005). Certain host miRNAs are involved in the viral infection cycle or in viral replication, and others are thought to be important in anti-viral defenses (Chapman et al. 2004; Chen et al. 2004; Xie et al. 2004; Jopling et al. 2005; Omoto and Fuji 2005). For example, *miR-122* is a host miRNA that is required for replication of hepatitis C virus (HCV).

MicroRNA expression in herpesviruses has been studied quite extensively (Cai et al. 2005; Pfeffer et al. 2005; Cai et al. 2006; Grundhoff et al. 2006). Kaposi's sarcoma-associated herpesvirus (KSHV) is an approximately 140-kb DNA virus that is associated with B-cell-type lymphoma in immune-compromised individuals, such as those with HIV. The results of one miRNA profiling study of KSHV indicated that it encodes 10 miRNAs (Pfeffer et al. 2005); in another KSHV study, 11 miRNAs were found (Cai et al. 2005). In both studies, all of the viral miRNAs were found to in a ~4-kb region of the genome located near the *Kasposin* gene. Expression of KSHV miRNAs occurred in latently infected cells, suggesting that they may be important in helping the virus establish or maintain latency (Cai et al. 2005).

Another miRNA-encoding herpesvirus is Epstein-Barr virus (EBV) (Pfeffer et al.

2005), which encodes ~20 miRNAs (Cai et al. 2006; Grundhoff et al. 2006). As in KSHV, EBV miRNAs are located near each other on the genome, where they are split into two clusters, and they are highly expressed in latently infected cells (Cai et al. 2006). The two gene clusters exhibit distinct expression patterns; one cluster (located near the *BART* gene) is highly expressed in latently infected epithelial cells, whereas the other cluster (located near the *BHRF1* gene) is highly expressed in B cells during the later stages of latency (Cai et al. 2006).

An miRNA profile of chicken embryo fibroblast (CEF) cells infected with Marek's disease virus (MDV) (an alphaherpes virus) revealed eight MDV-specific miRNAs (Burnside et al. 2006). Five of these are located near the oncogene *Meq*, and the other three are found in genomic regions expressed during latency. Because the viral miRNAs are located in genomic regions that are expressed in latency it is thought that they may be somehow involved in helping the virus to transform latently infected T-cells (Burnside et al. 2006).

Some viruses have recently been shown to exploit host miRNAs to their own advantage (Jopling et al. 2005). For example, HCV uses the highly expressed, liver-specific, *mir-122* host miRNA (Esau et al. 2006). When *mir-122* was deleted from the livers of adult mice, cholesterol levels were reduced, production of fatty acids and cholesterol in the liver decreased, and fatty acid oxidation increased leading Esau et al. (2006) to suggest that *mir-122* is involved in regulating lipid metabolism. This miRNA is only expressed in cells that are permissive to infection by HCV. Mutational studies have shown that the 5′-noncoding region of the viral genome has a *mir-122* binding site that is conserved in all seven genotypes of the virus (Jopling et al. 2005). When *mir-122* expression in liver cells was suppressed using a 2′-methyl anti-sense oligonucleotide, HCV levels greatly decreased. Although the

role of *mir-122* in the viral life cycle has not yet been precisely defined, it probably affects replication and not translation.

MicroRNAs and cancer

Many studies have been performed to investigate the contribution of miRNAs to carcinogenesis. Originally, miRNAs were thought to be involved in carcinogenesis because many of them lie near fragile sites or breakpoints in DNA, which are often associated with cancer (Calin et al. 2004). In a recent study, Lu et al. (2005) profiled miRNA expression patterns of human cancers and found differential expression for each cancer type. They were even able to classify poorly differentiated tumors by their miRNA profiles. In addition, they also found that miRNA expression was generally down-regulated in tumors, consistent with a role in differentiation.

In another study, miRNAs were revealed to be frequently deleted or down-regulated in patients with chronic lymphocytic leukemia (CLL) (Calin et al. 2002). In particular, the miRNAs *mir-15* and *mir-16* are associated with this disease, and both are located at chromosome position 13q14, a site that has been linked to CLL (Calin et al. 2002). Because these two miRNAs normally suppress the anti-apoptotic gene *BCL-2* (Calin et al. 2005), their deletion increases *BCL*-2 activity, leading to cancer. Furthermore, because regulation of differentiation is one of the primary roles of miRNA, the deletion of an miRNA may cause a cell to divide rather than differentiate, leading to tumor formation (Couzin 2005).

Although cancer is most often associated with a decrease or loss of miRNAs, exceptions have been identified. For example, some miRNAs are up-regulated in papillary thyroid carcinoma, the most common type of thyroid cancer (He et al. 2005). Three miRNAs

in particular (*mir-221*, *-222*, and *-146*) are highly associated with this disease. He et al. (2005) found that tumor cells exhibiting high over-expression of these miRNAs also exhibited under-expression of the *Kit* oncogene, which encodes a tyrosine kinase receptor that is associated with a variety of cancers; this observation suggests that the over-expression of these three miRNAs disrupts regulation of the *Kit* gene, leading to the development of papillary thyroid carcinoma.

MicroRNA machinery and disease

Dysfunctions in miRNA regulation have often been linked to disease, *e.g.*, cancer; however, the protein components that make up miRNA machinery have also been recently linked to disease (Caudy et al. 2002; Ishizuka et al. 2002; Gregory et al. 2004; Jin et al. 2004; Landhaler et al. 2004). Fragile X mental retardation syndrome has been linked to excessive CGG repeats in the 5′ end of the *fragile x mental retardation 1* gene; these excessive repeats cause gene silencing, leading to the loss of the fragile X mental retardation protein. This protein had been shown to be a translational suppressor in neuron development, but little else about the protein was known (Laggerbauer et al. 2000). However, this protein has now been suggested to be a component of the miRNA pathway (Caudy et al. 2002; Ishizuka et al. 2002; Jin et al. 2004). Co-immunoprecipitation experiments showed that FMRP associates with Dicer, which processes mature pre-miRNAs into miRNAs using its endonuclease activity (Ishizuka et al. 2002; Jin et al. 2004). Additional co-immunoprecipitation experiments revealed that FMRP also interacts with the mammalian Argonute protein eIF2C2, further linking the FMRP to the miRNA pathway (Jin et al. 2004). These studies suggest that the Fragile X syndrome may be the result of dysfunctional regulation of the miRNA pathway during neuronal development.

Another inheritable genetic disorder that has recently been linked to miRNA machinery is DiGeorge Syndrome (Gregory et al. 2004; Landthaler et al. 2004). This syndrome has many symptoms, including congenital heart defects and immunodeficiency (Wurdak et al. 2006). A recent study of proteins that interact with Drosha (which is involved in processing pri-miRNAs to pre-miRNAs) revealed that Drosha forms a complex with DGCR8, a protein associated with DiGeorge Syndrome (Gregory et al. 2004). It was also demonstrated that in the absence of DGCR8, Drosha has non-specific RNase activity, suggesting that DGCR8 somehow leads to specificity in pri-miRNA cleavage (Gregory et al. 2004). Knock-down of DGCR8 expression *in vitro* leads to an accumulation of pri-miRNAs and a decrease in pre-miRNAs and mature miRNAs (Landthaler et al. 2004), further supporting the requirement of the DGCR8 protein for proper Drosha function.

THE CHICKEN EMBRYO AS A MODEL FOR STUDIES OF VERTEBRATE DEVELOPMENT

The chicken is one of the most important domestic animals. Billions of dollars are generated annually by the poultry industry worldwide. Chicken is not only an important food source, providing relatively inexpensive animal protein, but it is also an important tool in vertebrate research. The modern chicken is thought to be descended from red jungle fowl that were domesticated *circa* 5400 BCE in China (West and Zhou 1988). The results of a recent phylogenic study suggest that the domestic chicken had several different origins in Eurasia, southeast Asia and Japan, and southeast China (Liu et al. 2006).

Because it has many advantageous features, the chicken embryo has long been used as a model for studies of vertebrate embryonic development (Voropaeva et al. 1969; Drake et al. 2005). The embryo is easily accessible, readily obtainable, and can be manipulated *in vivo* (*in*

ovo) more easily than can mammalian embryos (Brown et al. 2003). A hen will typically lay many eggs a year, thus producing many more offspring than a typical mammal. In addition, chicken embryonic development from fertilization to hatching takes only 21 days, a much shorter developmental span than that of most mammals, allowing for faster accumulation and interpretation of data.

One very appealing feature of the egg for use in molecular research is the ease with which it can be modified or injected with various agents, ranging from vectors to cells. For example, quail cells can be added to the embryo to create a chimera for studies of cell fate, or a vector containing a particular gene can be inserted to determine the effects of over-expression of the gene. In addition, the recently developed RNA interference (RNAi) technique has opened up new avenues of research in which the egg is quite useful. In this technique, the role of a particular gene in a certain stage of development is evaluated by cloning an siRNA for that gene into a vector that is then placed into the egg, resulting in a "knock-down" of that gene. Conducting similar studies in mammals is much more difficult.

The mechanisms of vertebrate limb development have been studied extensively in chicken; in fact, our current understanding of vertebrate limb development has come largely from the chick model (Stark and Searls 1973; Wolpert et al. 1979; Amprino 1985; Schramm and Solursh 1990; Ros et al. 1994; Dudley et al. 2002; Vargesson 2003; Boulet et al. 2004; Bradley et al. 2005). Many of the cell-to-cell interactions and genes involved in limb formation were discovered using the chicken (Tickle 2004), and it has also been a productive model for studies of limb motor control, limb bud formation and growth, muscle formation, and the effects of stress and various agents on these processes (Wolpert et al. 1979; Amprino 1985; Schramm and Solursh 1990; Boulet et al. 2004; Bradley et al. 2005).

The chick embryo has also been useful in elucidating cell fate in other tissues (Douarin et al. 1991; Brown et al. 2003; Tickle 2004; Nagy et al. 2005). Chicken and quail cells exhibit different histological characteristics (quail nucleoli have heterochromatic DNA (Le Douarin et al. 1991), and therefore they can be readily distinguished. Combined with the ease of grafting in the chick embryo, this observation led to the creation of chicken-quail chimeras that have been used to study cell fate and the origins of tissues (Brown et al. 2003). A recent chick-quail chimera study demonstrated for the first time that peripheral blood fibroblasts exist in birds, and that these cells, together with macrophages, help to form the spleen (Nagy et al. 2005). The study of embryogenesis in the chicken has allowed the creation of many fate maps (Tickle 2004).

The chicken genome sequence has been determined (Wallis et al. 2004), greatly facilitating annotation of novel genes, mapping of chromosomes, and identification of orthologues. The complete sequencing of the chicken genome and the ease with which the chicken embryo can be manipulated make the chicken an excellent model for studies of vertebrate development.

The avian liver

The liver, the primary metabolic organ of vertebrates, functions in detoxification of the blood and is a major source of serum proteins (Suksaweag 2004). Originating from the ventral gut endoderm, it is a large organ comprised of various lobes of different shapes and sizes (Suksaweag 2004). Until the 1970s, studies of liver development were most often carried out in the chicken (Le Douarin 1975); since that time, however, most of our understanding of hepatogenesis has come from the mouse. Because avian and mammalian

livers develop similarly, research results obtained in mammalian liver can be applied to avian liver, and *vice versa*.

Using a mouse model, Vassy et al. (1988) showed that differentiation of precursor hepatoblasts into the hepatocytes that help form the liver takes several days. Hepatoblasts begin to differentiate at day ~12 of gestation and are not completely differentiated into hepatocytes until about day ~20. During this period, the differentiating cells assume more and more of the functions of normally functioning liver tissue, such as synthesis of secreted proteins and production of peroxisomes.

Development of the chick liver, unlike the spleen, begins early in embryogenesis (Seesawing 2004). The chick model has been used to elucidate the steps required for induction of liver development, as well as the creation of cell-fate maps (Yanai 2005). The chick model has also been used to show that induction of liver formation occurs in two steps. First, the ventral endoderm is induced to take on a hepatic fate (Le Douarin 1975). This induction occurs when the ventral endoderm comes into close contact with the precardiac splanchnic mesoderm. Second, the ventral endoderm is stimulated by the septum transversum meschyme, leading to the proliferation and differentiation of hepatocytes (Le Douarin 1975).

In chicken, induction of liver development occurs during days E2 and E3, when hepatocytes and bilary cells become the multipotent hepatoblasts that proliferate to form the hepatic cords (reviewd by Suksaweag 2004). Morphogenesis occurs during days E4 and E5, when the liver bud (E4) and the first lobe (E5) form. On day E6, the differentiation stage begins, and the second lobe is formed. Differentiation continues on day E7, and the third lobe is generated.

Metabolic switching

One important role of the liver during chick development is to control metabolic switching during the perihatching period. While it is developing, the chick embryo obtains most of its energy from the oxidation of fatty acids of yolk lipids. After hatching, the chick's metabolism must switch from catabolism of yolk lipids to catabolism of the carbohydrates and proteins present in feed (Cogburn et al. 2003). This metabolic switch is marked by a distinct change in the pattern of liver gene expression (Cogburn et al. 2003, 2004).

Microarray studies have identified 32 functionally related clusters of genes that are differentially expressed between embryonic stages and after hatching (Cogburn et al. 2003). The gene clusters involved in fatty acid metabolism are more highly expressed during the embryonic stage. These clusters include genes encoding the enzymes acetyl coenzyme A acetyltransferase (*ACAT2*) and pyruvate dehydrogenase kinase 4 (*PDK4*) (Cogburn et al. 2004). Genes involved in lipogenesis and energy metabolism, such as those encoding fatty acid synthase (FAS) and HMG coenzyme A synthase, are more highly expressed after hatching (Cogburn et al. 2004). Microarray studies of chick liver performed during the perihatching period have shown that many genes are differentially expressed during this stage of development. Since miRNAs are very important in regulating gene expression during development, the probability is very high that miRNAs play a role in controlling gene expression during the metabolic switch.

The avian spleen

Since miRNAs have been shown to be important in the regulation of developmental processes of vertebrate species, understanding their involvement in development is vital. The avian spleen has not been studied as extensively as its mammalian counterpart. In adult

chickens, the spleen is approximately 2 cm in width and is located in the abdomen near the gizzard (reviewed by John 1999). After hatching, the spleen increases in size until the bird reaches sexual maturity. The spleen is considered to be a secondary lymphoid organ, serving as a filter for the immune system. Blood is filtered through the spleen, which contains immune cells, to remove blood-borne pathogens.

The avian spleen and its mammalian counterpart are highly similar in structure and function (John 1994). The spleen is comprised of splenetic pulp, which consists of a matrix containing B and T cells, through which the central artery runs (Nagy 2005). The artery branches off into a network of capillaries that is surrounded by Schweigger-Seidel's sheath, also known as the ellipsoid. This sheath consists of supporting cells in which phagocytic cells are embedded (Nagy 2005). The ellipsoid is the splenic region that is first exposed to antigen. Once the phagocytic cells (predominantly macrophages) of the ellipsoid have phagocytosed an antigen, they leave the sheath and go to T-cell-dependent areas (Nagy 2005). Germinal centers consisting primarily of B cells are located near these areas and are the principal lymphoid structures of the spleen (Yassine 1989). These germinal centers are important to the avian immune system in that they are the sites of major antibody production (John 1994).

Development of the avian spleen occurs late during embryonic development and takes place rapidly once initiated. During most of the ~21-day period required for a chicken to hatch, the spleen does not exist as an organized structure but rather as a loose collection of cells (Yassine 1989). The first reticular fibers appear on day Ell; a specialized fiber network begins to form by day E15; and by day E18, the structure of the developing spleen is similar to that of an adult spleen. T and B lymphocytes first appear in the spleen on days E16 and E18, respectively (Yassine 1989). After the chick hatches, its spleen greatly increases in size

and weight, and germinal centers form in T cell-dependent areas (Yassine 1989).

Pyrosequencing

Pyrosequencing is a relatively new method for DNA sequencing. Originally developed in the late 1990s (Ronaghi et al. 1996), pyrosequencing is currently being used for applications such as genotyping and SNP analysis as well as EST sequencing (Amadian et al. 2006). It has several advantages over the traditional Sanger method, such as yielding much more sequencing data with a much shorter turn-around time. It also much more accurate at detecting small nucleotide variation, such as SNPs, than the Sanger method. However, the major drawback of pyrosequencing is the limited read length, which typically ranges from ~50 to 200 nts. However, when only a small sequence is required, such as sequencing a SNP, pyrosequencing is the method of choice.

Pyrosequencing is based on the idea of sequencing by synthesis, and can be carried out by two different methods. The first method uses three enzymes (Klenow DNA polymerase, ATP sulfurylase, and luciferase) and requires a wash step after each nucleotide addition to remove unincorporated nucleotides. The second method uses apyrase in addition to the three enzymes listed above. The apyrase is used to remove unincorporated nucleotides, thereby eliminating the wash steps and allowing the reaction to take place in a single tube (Ahmadian et al. 2006).

Because of its simpler design, the four-enzyme pyrosequencing method has become the most popular. This method is typically done by first PCR-amplifying the DNA that is to be sequenced (if the sequencing is *de novo* then adapters can be attached to the DNA) with one biotinylated primer and one non-biotinylated primer. The PCR product is then attached to streptavidin-coated beads *via* the incorporated biotin. The four enzymes are added to the

beads containing the DNA template(s), and the sequencing primer is annealed to the template. The four nucleotides are then added one at a time in cyclic manner. If a nucleotide is complementary to the next base in the template sequence, the DNA polymerase will incorporate it into the growing nucleotide chain, releasing an inorganic pyrophosphate molecule (PP_i). The ATP sulfurylase uses the PPi to produce ATP. This ATP is converted to light by the luciferase, and the light signal is detected by a CCD camera. The amount of light produced is proportional to the number of nucleotides incorporated, allowing for the detection of a string of the same nucleotides in a row. The apyrase then removes the unincorporated nucleotides (Ahmadian et al. 2006).

Previously miRNAs have been sequenced by isolating small RNA populations and ligating both 5′ and 3′ adaptors, followed by reverse transcription and PCR-amplification of the cDNA. Following PCR, the small-RNA cDNAs were either concatamerized and cloned into a vector or cloned without concatamerization and sequenced. However, this approach has the potential to be biased toward low abundance miRNAs, because small RNAs are lost at each step, and the read amounts that can be obtained by this approach are limited.

In recent miRNA profiling studies, pyrosequencing has been the method of choice for miRNA sequencing. The initial steps for miRNA sequencing are the same for both pyrosequencing and the cloning method, but in pyrosequencing, the PCR products obtained using the cDNAs from smalls RNAs is directly sequenced. Elimination of several steps needed for cloning thereby reduces the amount of small RNA that is lost. Pyrosequencing also yields ~100 times more reads than the cloning method, increasing the chance of finding low-abundance miRNAs.

PURPOSE OF THIS PROJECT

The purpose of this project is to develop microRNA profiles of the embryonic chick spleen and liver at two developmental time points, days E15 and E20. We want to determine what microRNAs are expressed in these two organs at these developmental time points and to uncover any previously unidentified microRNAs. The chick develops for 21 days in the egg before hatching, and during most of this time, the spleen is relatively small and does not play a major role. After hatching, the spleen greatly increases in size until the bird reaches sexual maturity. Therefore, many developmental changes must occur in the spleen later in chick development. Because so much of the spleen development occurs late in chick development it is likely that a wide variety of microRNAs are expressed in the E15 and E20 spleens. In the chicken, a metabolic switch occurs in the liver during the perihatching period; this event switches the chick's food source from the fatty acids in the yolk to the proteins and carbohydrates in feed. Therefore, there are many changes that occur in the liver between days E15 and E20 and it is likely that microRNAs are involved in these changes. To complete this project small RNA libraries will be generated for each organ at each developmental time point. The four libraries, spleen at E15 and E20 and liver at E15 and E20 will be sequenced using the 454 Life Sciences Inc. technology. 454 Life Sciences technology is based on pyrosequencing and yields 100 times more sequencing data than traditional sequencing, which will allow for the identification expressed microRNAs. From these microRNA profiles important developmental processes occurring in these organs at these developmental times points may be revealed.

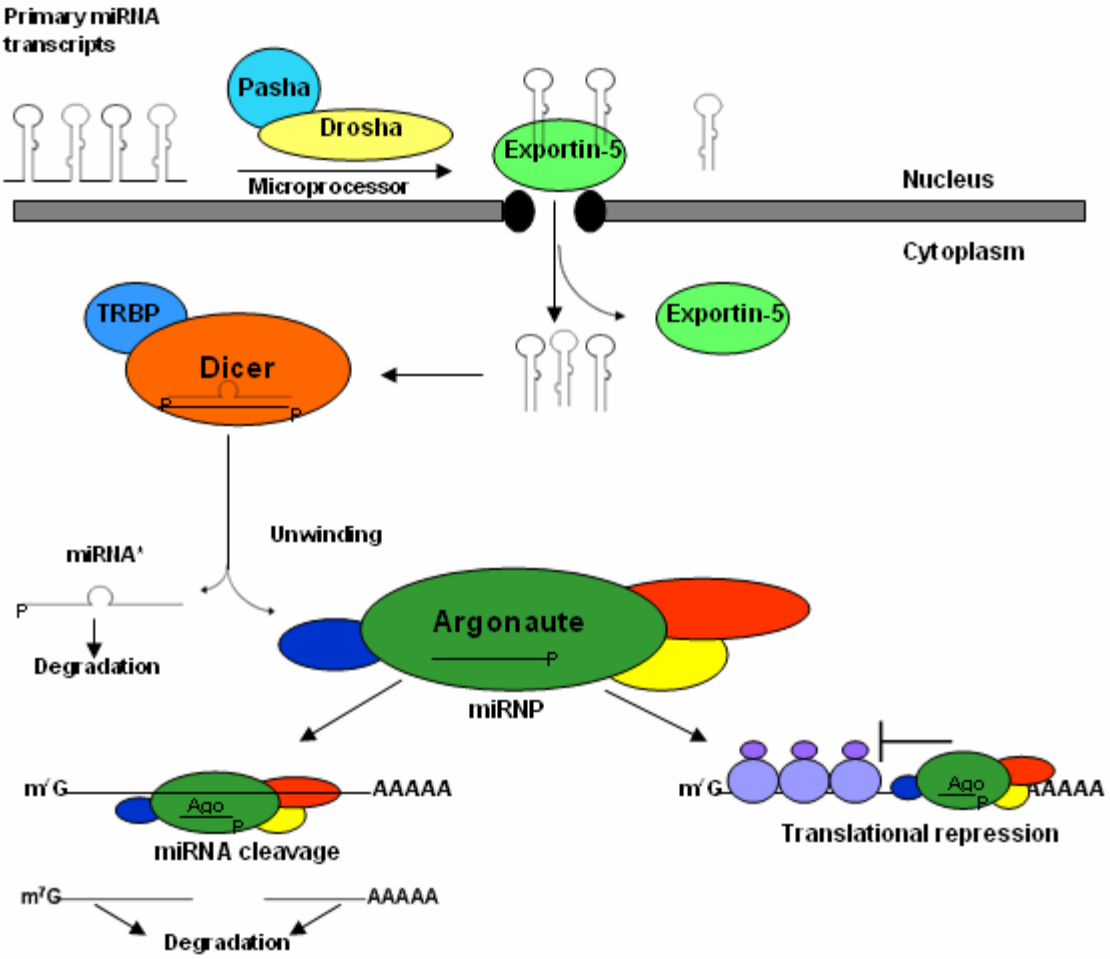

Figure 1.1. Processing and Action of mircoRNAs.

miRNAs are first synthesized in the nucleus as long primary transcripts. These transcripts are then processed by the Drosha complex into ~70nt stem loop precursor miRNAs (pre-miRNAs). pre-miRNAs are exported to the cytoplasm by exprotin-5. The Dicer complex cleaves the pre-miRNA into a dsRNA duplex. The strands are separated into the mature miRNA and the miRNA* strand. The mature microRNA then interacts with the Argonaute proteins to form a ribonucleoprotein complex. The miRNA guides the complex to its complementarily sequence in the target transcript. If there is perfect complementarity the transcript will be degraded, if there is only partial complementarity then translation will be blocked, but the transcript is not degraded.

Adapted from Chen and Meister (2005)

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CHAPTER 2: MATERIALS AND METHODS

INTRODUCTION

As described in the previous chapter, microRNAs are vital to proper development. A good deal of work has been done to study the role(s) of miRNA in the development of *C. elegans*, *Drosophila*, zebrafish, and mice, but its involvement in chicken development has not received much attention. In particular, miRNA profiling of chick development has not previously been reported, although determining miRNA function in chicken development should help bridge the gap in our knowledge between mammals and lower vertebrates.

The chick has long served as a model for several aspects of vertebrate development, including cell fate maps, limb formation, and the effects of environmental stress and agents (Brown et al. 2003; Bradley et al. 2005). Because many of the unique characteristics of chick development make the chick an appealing model, it has been used to study almost every aspect of development (LeDouarin et al. 1991; Bradley et al. 2005; Nagy et al. 2005).

Chick embryos are relatively inexpensive and easy to maintain in a laboratory setting as compared with embryos of other vertebrates. Unlike other higher vertebrate systems, such as mouse ova, fertilized eggs can be sustained in a simple incubator, with need for upkeep or any manipulation of the mother; therefore expenditures of time and money for feeding and cage cleaning are unnecessary. Chicks are also ideal for developmental studies because the embryo is easily accessible and relatively easy to manipulate. Penetration of the egg and access to the developing embryo requires much less time and effort than in other vertebrates. In mammals, access to the embryo cannot be acquired without manipulation of the mother,

which is generally an invasive procedure that is stressful and potentially dangerous for both the mother and the developing embryo.

As discussed in the previous chapter, miRNAs are important in developmental timing and in cell differentiation and proliferation (Lee 1993; Slack et al. 2000; Ambros et al. 2003; Brennecke et al. 2003; Wienholds and Plaster 2005). Gain- and loss-of-function studies of miRNAs have shown that when miRNA expression deviates from its wild-type expression patterns, the organism develops improperly (Slack et al. 2000). Studies in organisms such as *C. elegans and Drosophila* have shown that miRNAs constitute a complex regulatory system during development. They are involved in various cellular pathways dictating cell fate, patterning, and/or proliferation, etc. (Ambros et al. 2003; Brennecke et al. 2003; Yoo and Greenwald 2005), as illustrated by the first two discovered miRNAs, *lin-4* and *let-7*.

The *lin-4* and *let-7* miRNAs are important in developmental timing in *C. elegans* (Lee et al. 1993). Targeting of *lin-41* (whose specific function is as yet unknown) by *lin-4* allows cells to enter the second larval stage (Boehm and Slack 2005). When expression of *let-7* is repressed, *C. elegans* cells revert back to an earlier stage of development, suggesting that this particular miRNA is important for maintaining cells in later stages of development (Slack et al. 2000).

A well-characterized *Drosophila* miRNA, *bantam*, promotes cell proliferation and prevents apoptosis during the third instar larval stage (Brennecke et al. 2003). A recent mouse developmental study has shown that Dicer (the endonuclease involved in processing pre-miRNAs) -null mouse embryos fail to develop past gastrulation, providing evidence that miRNAs are essential during embryonic development (Harris et al. 2006). Several miRNAs

have been found to be involved in embryonic limb development in the chick, suggesting that miRNAs are also vital to proper embryonic development in the chick (Lancman et al. 2005; Hornstein et al. 2005). In addition to playing roles in development, miRNAs also have been associated with viral infections (Pfeffer et al. 2005; Cai et al. 2005; Cai et al. 2006; Grundhoff et al. 2006) and have also been implicated in cancer development (Calin et al. 2004; He et al. 2005; Lu et al. 2005).

Two important vertebrate organs are the spleen and the liver. The spleen is a secondary lymphoid organ important in the immune system and the liver is considered the center of metabolism. Understanding spleen development will further our understanding of how the immune system develops as well as of how it functions. This knowledge is essential for development of treatments not only for infectious agents, but also for autoimmune disorders. The liver is critical for vertebrate survival, processing or producing many of the various substances that are needed to maintain an organism. Understanding its development will allow for better understanding of metabolism and of metabolic disorders.

Pyrosequencing, a sequence-by-synthesis method developed in the late 1990s (Ronaghi et al. 1996), is ideal when only a short sequencing read is needed. Pyrosequencing yields much more data in less time than the traditional Sanger method, making it an excellent choice for miRNA profiling studies. Increasing the number of sequencing reads obtained optimizes the chances of finding low-abundance miRNAs. The pyrosequencing procedure is based on the following principles: (1) the DNA to be sequenced serves as a template for DNA polymerase, and incorporation of a base into the growing chain produces an inorganic pyrophosphate (PPi); (2) the PP_i is used by ATP sulfurylase to make ATP; (3) the ATP is

then used by luciferase to produce light which is detected by a CCD camera. The amount of light produced is proportional to the number of nucleotides incorporated (Ahmadian et al. 2006).

SPECIFIC AIMS

Because avian species serve as a link between lower and higher vertebrates, and because they have a important economic role, it is vital that we understand their development. Since miRNAs play a major role in the development of an organism, an understanding of chick development requires a strong understanding of miRNA functions. The first step in understanding what involvement miRNAs play in any organism is to produce an miRNA profile. Because expression of miRNAs is both temporally and spatially specific, different tissues produce unique miRNA profiles that vary at different stages of development. A complete profile therefore requires profiling of each organ at critical developmental stages.

To begin miRNA profiling of chick development, we can begin with the spleen, which is an important immunological organ, and the liver, which is the center of metabolism. The chick develops for 21 days in the egg before hatching, and during most of this time, the spleen is relatively small and does not play a major role. After hatching, the spleen greatly increases in size until the bird reaches sexual maturity. Therefore, many developmental changes must occur in the spleen later in chick development. The miRNAs that are important in spleen development should be detectable by profiling and comparing miRNA expression in the spleen at a middle stage of development and during the perihatching period, when the spleen is rapidly developing.

In the chicken, a metabolic switch occurs in the liver during the perihatching period; this event switches the chick's food source from the fatty acids in the yolk to the proteins and carbohydrates in feed. Liver miRNA profiling during a developmental stage when the chick is using the yolk for an energy source and during a stage when the chick is preparing to use feed nutrients should reveal the miRNAs involved in the metabolic switch.

OBJECTIVES

(1) To determine miRNA profiles of the chick liver and spleen at days E15 and E20.

(2) To determine the sequence and identity of miRNA differentially expressed in these two developmental stages.

MATERIALS, METHODS, AND DESIGN

Sample Collection

Specific-pathogen-free broiler eggs from flock m173 were purchased from Charles River Laboratories (USA) and incubated at 37 °C for either 15 or 20 days. The spleen and the liver were collected from chick embryos at days E15 and E20. To isolate these tissues, the embryo was removed from the egg with forceps, and scissors and forceps were used to remove the liver and spleen, which were then placed into cryogenic tubes.

Tissues were immediately frozen in liquid nitrogen and then stored at -80 °C until analysis.

Total RNA Isolation

Total RNA was isolated from the tissues using Tri reagent (Sigma). Briefly, the stored liver or spleen samples were immediately placed in liquid nitrogen and then broken into

small pieces using a tissue pulverizer (manual) that had been cooled in liquid nitrogen. The tissue pieces were ground into a fine powder in a mortar and pestle that had also been cooled in liquid nitrogen. Tri reagent was added to the powder in multiple 500-µl aliquots, and grinding was continued until a paste formed; the paste was washed from the mortar with multiple 500-µl aliquots of Tri reagent; and 1-ml aliquots of the paste were placed into 1.5-ml tubes. In total, 1 ml of Tri reagent was used per 50–100 mg of tissue, and 400–500 mg of frozen tissue was used per RNA isolation.

The homogenized tissue aliquots were incubated at room temperature for 5 min, and then 0.2 ml of chloroform (Sigma) were added for each 1 ml of Tri reagent in the tube. The tubes were inverted for several minutes and then incubated at room temperature for 10 min. They were centrifuged at 13,000 rpm at 4 °C for 15 min in a tabletop microcentrifuge (AccuSpin Micro R, Fisher Scientific, USA) to separate the RNA-containing aqueous phase from the DNA-containing interphase and the protein-containing organic phase. The upper, aqueous phase containing the RNA was removed by pipetting and placed into a clean 1.5-ml tube. Next, 0.5 ml of isopropanol (#32727; Acros Organics) was added to the tube for each 1 ml of Tri reagent in the tube. The tubes were inverted several times to mix and incubated at –20 °C overnight. The precipitated RNA was collected by centrifugation at 13,000 rpm at 4 °C for 10 min in a tabletop microcentrifuge and air-dried.

The pellet was dissolved in 50–100 µl of DEPC-treated water and placed in a 55 °C water bath for 5–10 min until the RNA was solubilized and the pellet was no longer visible; while in the water bath, the tube was flicked every 2 min. The solubilized RNA concentration was determined using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies)

and 600–700 µg of RNA were combined into one tube along with an equal volume of isopropanol. The precipitated RNA was stored overnight at -20 °C.

MicroRNA Isolation And Analysis

A procedural overview of microRNA isolation and sequencing is shown in figure 2.1. Once 600–700 µg of total RNA was obtained, it was separated on a 15% denaturing PAGE gel (1× TBE, 15% urea, 40% acrylamide, with distilled water, TEMED, and ammonium persulfate) that had been pre-electrophoresed for 15 min in 1× TBE. Samples were electrophoresed at 150 V for 2 h along with standard 19- and 24-mer markers, and the gel region containing RNAs of 19–24 bp was excised. The small RNA was purified from the gel by cutting the gel piece into small chunks and vortexing overnight at room temperature in 5 vol of an elution solution consisting of 20 mM Tris (pH 8), 1 mM EDTA, 0.4 M ammonium acetate, and 0.5% SDS. The mixture was then spun through a Nanosep MF centrifugal column with a GHP Membrane (Pall Life Sciences) to remove the acrylamide, and the small RNA was eluted from the column. The RNA was precipitated by adding 1 µl of 20 µg/ml glycogen and 3 vol of 100% ethanol and incubating at –20 °C overnight. The small RNA was collected by centrifugation at 13,000 rpm at 4 °C for 10 min, washed 3 times with 70% ethanol, air-dried, and dissolved in DEPC-treated water.

For cloning, the modban 3′ adaptor (5′-AMP-CTGTAGGCACCATCAAT-ddC-3′; miRNA CloningLinker1; Integrated DNA Technologies) was ligated to the small RNAs using T4 RNA ligase (Fermentas) and T4 RNA ligase buffer without ATP (to prevent self-ligation of small RNAs) overnight at 37 °C. After incubation, the samples were again

fractionated by 15% denaturing PAGE, as described above, with 37- and 44-mer markers. (The 17-bp adaptor increases the size of the small RNAs.) The gel region between 27 and 44 bp was excised, and the RNA was purified as described previously. Next, a 5′ adaptor (5′-ATCGTr(AGGCACCUGAAA)-3′; Integrated DNA Technologies) was ligated to the 3′-adaptor-ligated small RNA for overnight at 37 °C. In this ligation reaction, the buffer did contain ATP, which facilitates the ligation reaction (Fig 2.1).

Next, the ligation reaction was extracted by addition of an equal volume of phenol/chloroform (1:1), mixing by shaking, and centrifugation for 3 min at 13,000 rpm in a microcentrifuge. The aqueous phase was removed by pipetting and placed into a new 1.5-ml tube. An equal volume of chloroform was added to the aqueous phase to remove any traces of phenol. The sample was centrifuged again as described above, and the aqueous phase was removed to a new 1.5-ml tube. The ligated product was precipitated as described above.

The small RNA with both 3′ and 5′ adapters was reverse-transcribed using 5 µM Ban Rev primer (5′-ATTGATGGTGCCTACAG-3′), which was designed for the 3′ adaptor, and the Superscript RT kit (Invitrogen). The reaction was carried out by adding 5 µM Ban Rev primer, 10 mM dNTP mix (supplied with the kit), and DEPC-treated water to a final volume of 10 µl. The sample was incubated at 65 °C for 5 min to start the reaction and then placed on ice. Next, a cDNA synthesis mix was prepared, and 10 µl was added to each sample from the previous reaction. For each reaction, the cDNA synthesis mix was prepared using the following components of the kit: 2 µl of 10× RT Buffer, 4 µl of 25 mM $MgCl_2$, 2 µl of 0.1 M DTT, 1 µl of RNaseOUT, and 1 µl SuperScript III Reverse Transcriptase. The reaction was

incubated at 50 °C for 50 minutes and then at 85 °C for 5 min; following incubation, the reaction was terminated by placing it on ice.

The cDNA was immediately used to perform PCR using the Ban For primer (5′-ATCGTAGGCACCTGAAA-3′), which is designed for the 5′ adaptor, and the Ban Rev primer, which is designed for the 3′ adaptor and is the same primer used in the reverse transcription reaction. PCR was preformed using only 25 cycles instead of the standard 35 cycles to prevent the amplification of background. The PCR cycle parameters consisted of an initial denaturation step (95 °C for 1 min); 25 cycles of denaturation (94 °C for 10 sec), annealing (50 °C for 1 min), and elongation (72 °C for 20 sec); and a final elongation step (72 °C for 5 min). The final PCR products were expected to be 52–60 bp in length.

The PCR products were used as templates for PCR using fusion primers designed for pyrosequencing *via* the method developed by 454 Life Sciences Inc. (Branford, CT, USA). Primers were designed such that the 5′ end contained the primer sequence used by 454 Life Sciences, Inc. for PCR and sequencing, followed by a unique 4-nt “tag” for each tissue at each developmental time point and a primer sequence for each adapter at the 3′ end. The primers used are shown in Table 2.1. Because each sequence obtained can be assigned to a particular sample by its unique tag, the tags allow for the pooling of all samples into a single tube for sequencing in the same reaction.

For each sample, 5 ng of the previously generated PCR product were amplified by high-fidelity *Taq* polymerase using the fusion primers designed for that tissue. The PCR cycle parameters consisted of an initial denaturation step (94 °C for 3 min); 29 cycles of

denaturation (94 °C for 30 sec), annealing (57 °C for 45 sec), and elongation (72 °C for 1 min); and a final elongation step (72 °C for 2 min). PCR products were assessed on a 1% agarose gel stained with ethidium bromide. The expected product size was ~100 bp. Samples were then pooled and sent to 454 Life Sciences, Inc. for pyrosequencing.

Sequence Analysis

A flow chart of the steps involved in sequence analysis is given in figure 2.2. The first step in analysis of the sequences was to group the raw sequences according to the type of sample; for example, all of the sequences tagged with TCGT belonged to the E15 liver sample. To identify possible known and/or predicted miRNAs, the small RNA sequences were next checked against the miRBase database (http://microrna.sanger.ac.uk/sequences/) using the database's default settings. MiRBase serves as the primary miRNA database available to the public (Griffith-Jones et al. 2006) and also serves as a source for naming novel miRNAs, so that a uniform naming system is maintained. MiRBase contains hundreds of predicted and validated microRNAs from 40 different species and six viruses (http://microrna.sanger.ac.uk). Currently, miRBase contains 144 chicken miRNAs, of which many remain to be experimentally validated.

The remaining sequences were checked against the Ensembl *Gallus gallus* database (http://www.ensembl.org/Gallus_gallus/blastview) using default parameters, with the exception of near-match (oligo) for search sensitivity. They were also checked against the complete NCBI chicken genome database (http://www.ncbi.nlm.nih.gov/genome/seq/BlastGen/BlastGen.cgi?taxid=9031) using the BLASTn program and the default parameters to determine the location of the best alignment

to the chicken genome. Once the genomic location of the sequence was determined, the 300 bases flanking the sequence on either side were checked against the Rfam database of RNA families (http://www.sanger.ac.uk/Software/Rfam/search.shtml) using default parameters to determine if the sequence was another type of small RNA, such as tRNA, rRNA, or snoRNA.

If no matches were found in the Rfam database, the sequence and its 300-bp upstream and downstream flanking sequences were entered into Mfold (http://bioweb.pasteur.fr/seqanal/interfaces/mfold-simple.html), with default settings, a program designed to predict secondary structure of RNA, using default parameters. This step served to determine whether the sequence was part of a stem-loop structure, which is the secondary structure adopted by precursor miRNA. If the sequence of the small RNA was able to form a stem-loop, then it was considered to be a potential novel miRNA.

In 2003 the leaders in small RNA research aome together to determine the criteria to annotate novel microRNA (Ambros et al, 2003). These criteria include both expression and biogenesis criteria. To fulfill the expression criteria of a novel microRNA the sequence must be ~22nt long and have been found in a cDNA library of size fractionated RNA and secondly they must be detected at the proper size range when hybridized to size fractionated RNA, this is typically done by Northern blotting. For the biogenesis criteria the sequence must be mapped back to the genome to identify its surrounding sequence. This surrounding sequence should be able to fold in to a hairpin structure and the entire small RNA sequence should lie in one of the arms. If the hairpin structure shows phylogenetic conservation and has a decreased transcripts in Dicer defective organisms, this can also help fulfill the biogenesis criteria.

MicroRNAs have certain conserved identifying characteristics. Specifically, they have a “seed sequence” consisting of nucleotides 2–7 of the miRNA; this sequence perfectly complements a sequence known as the “seed match” in the 3′-untranslated region of the target mRNA. Another possible identifying characteristic is that the first nucleotide of an miRNA is typically, but not necessarily, a uridine. If the identified potential miRNAs exhibited these characteristics and were able to form a stem-loop structure, we concluded that they were probable novel miRNAs.

To determine the potential target mRNAs for the novel chicken microRNAs the Miranda (http://www.microrna.org/miranda.html) algorithm was used. The Miranda algorithm was designed to identify genomic targets of microRNA. The microRNAs were uploaded into the program in one file and the 3’UTRs, which were obtained from the Ensembl databases, were uploaded into a second file. The Miranda algorithm will check each microRNA against each 3’UTR. It will then determine the local alignment of the microRNA and the 3’UTR, and it will also determine the thermodynamic stability of the RNA duplex. In animals there is usually no perfect complementarity between the miRNA and its target mRNA, however as mentioned above there is a “seed sequence” match between them. Therefore if the binding of the miRNA and the 3’UTR was above both the alginment and thermodynamic thresholds in Miranda and there was a “seed” match then that mRNA was classified as a potential target for the miRNA.

Our profile should reveal which miRNAs are differentially expressed between two developmental time points (E15 and E20), as well as between two tissue types (liver *vs.* spleen). If an miRNA is highly expressed, its sequence will be amplified more frequently.

For example, if one particular miRNA in the E15 liver constitutes 100 out of 1,000 reads, it may constitute only one or two out of 1,000 reads at day E20. Such a result would indirectly suggest that this miRNA performs a particular function during liver development around E15 but not later in development. Therefore, we should be able to compare miRNA read numbers between the samples to reveal miRNAs that are important at particular temporal time points in particular tissues.

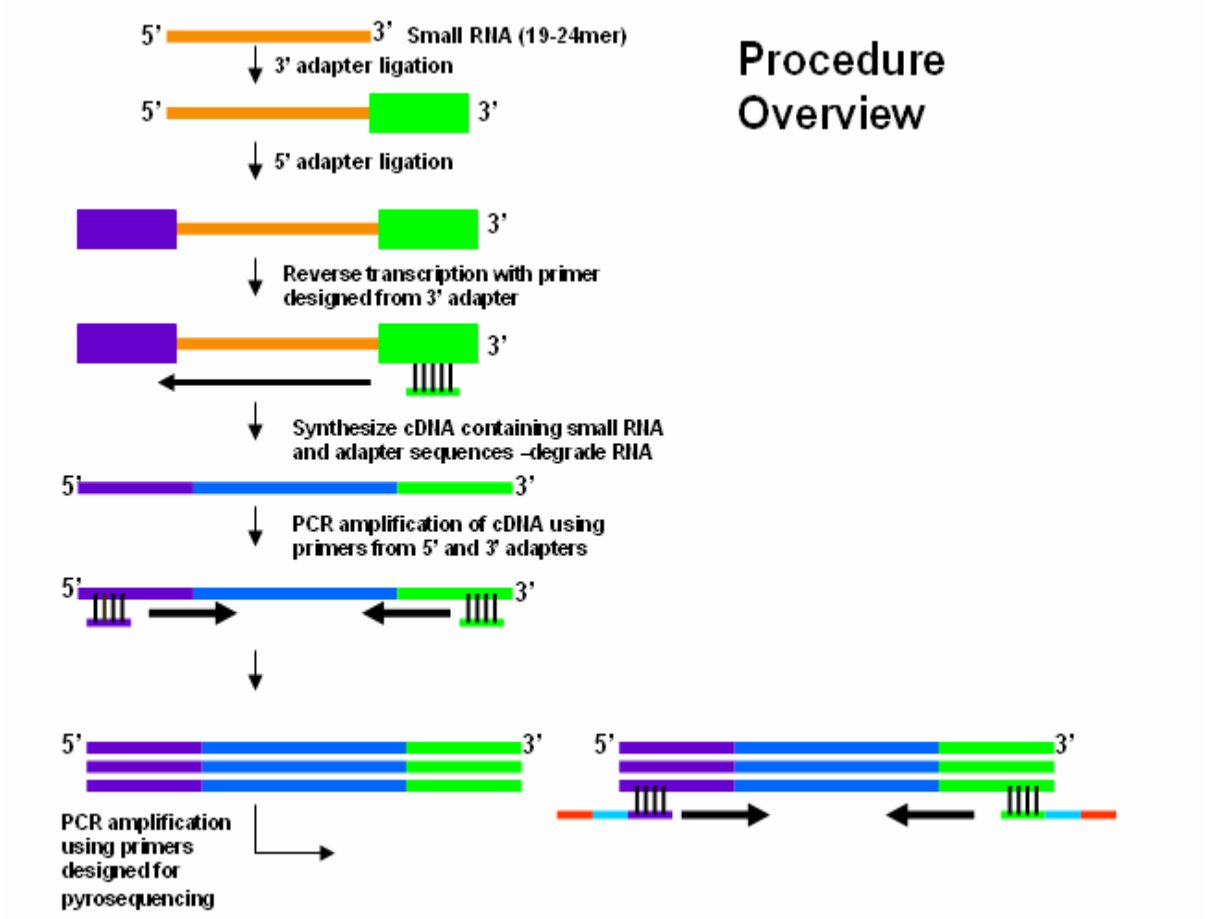

Figure 2.1 Procedure Overview.

The small RNA (19-24mers) fraction is isolated from total RNA. Then a 3' adapter is ligated to the 3' end of the small RNAs, this is followed the ligation of a 5' adapter onto the 5' end of the small RNA. Reverse transcription is performed using a primer designed from the 3' adapter to produce cDNA. This cDNA is used as a template for PCR using primers designed from the 5' and 3' adapters. 5ng of PCR product is used to perform another PCR using primers designed for pyosequencing (primers contain 5' and 3' adapter sequences a 4nt "tag" and sequences requested by 454 Life Sciences).

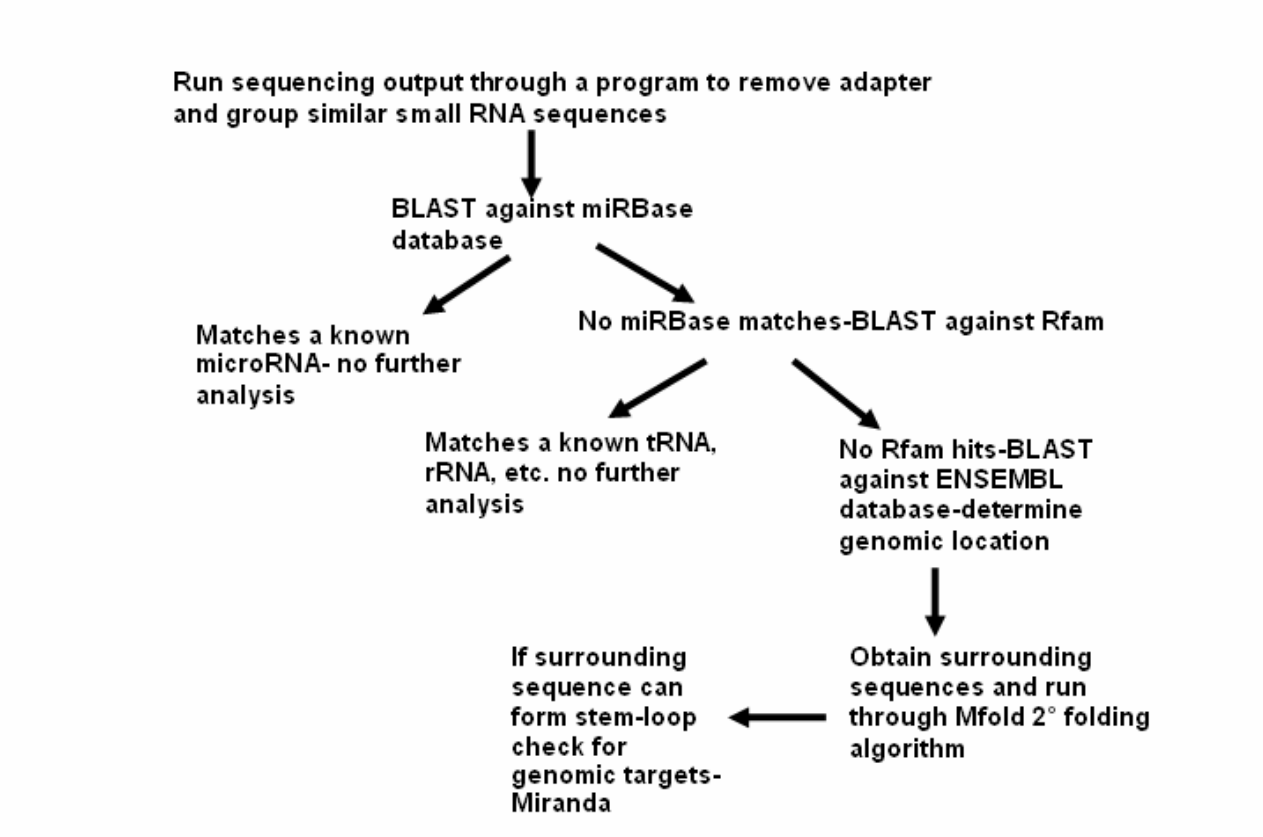

Figure 2.2. Flow Chart of Sequence Analysis.

The small RNA sequences are isolated from the surrounding sequence and sequences representing the same small RNA sequence are grouped. The sequences are blasted against miRBase to determine if they represent known microRNAs. If there are no hits to miRBase they are then blasted against the Rfam database to determine if they represent some other type of RNA, such as tRNA, rRNA, etc.). If they do not represent a known type of RNA they are then blasted against the chicken genome in ENSEMBL to determine genomic location and to obtain the surrounding sequence. The small RNA and the surrounding sequence are then ran through the Mfold program to determine their 2° structure, If the secondary structure can form a stem loop this sequence could represent a novel microRNA. Potential targets of the sequences are determined using the Miranda algorithm.

Table 2.1. Fusion primers for pyrosequencing by 454 Life Sciences, Inc. [a, b]

Forward Primers (shown 5′ → 3′)	
Liver E15	*GCCTCCCTCGCGCCATCAG***TCGT**ATCGTAGGCACCTGAAA
Liver E20	*GCCTCCCTCGCGCCATCAG***AGTC**ATCGTAGGCACCTGAAA
Spleen E15	*GCCTCCCTCGCGCCATCAG***CTAG**ATCGTAGGCACCTGAAA
Spleen E20	*GCCTCCCTCGCGCCATCAG***CATA**ATCGTAGGCACCTGAAA
Reverse Primers (shown 5′ → 3′)	
Liver E15	*GCCTTGCCAGCCCGCTCAG***TCGT**ATTGATGGTGCCTACAG
Liver E20	*GCCTTGCCAGCCCGCTCAG***AGTC**ATTGATGGTGCCTACAG
Spleen E15	*GCCTTGCCAGCCCGCTCAG***CTAG**ATTGATGGTGCCTACAG
Spleen E20	*GCCTTGCCAGCCCGCTCAG***CATA**ATTGATGGTGCCTACAG

[a] Bold text indicates the “tag” for each sample.

[b] Italicized text indicates sequences included per 454 Life Sciences, Inc. protocol.

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CHAPTER 3: RESULTS & DISCUSSION

RESULTS

Pyrosequencing Read Characteristics of Chick Embryonic Samples

A total of 92,919 small-RNA sequence reads were obtained from chick E15 and E20 spleen and liver tissues. Of these 92,919 reads, 52,001 represented miRNAs matching the miRBase *G. gallus* database, and 3,472 were not found in the *G. gallus* database but were homologues of miRBase miRNAs from other species. Of these homologous reads, 391 represented potential novel miRNAs. Other small RNAs, such as tRNA and rRNA, represented 24,672 of the reads, and 12,383 reads represented other types of sequences, such as degraded mRNA (Table 3.1).

MicroRNA Profile of the Chick Spleen at Day E15

Of the 52,001 reads representing miRNA sequences present in the *G. gallus* miRBase database, 13,620 reads representing 109 different miRNAs were obtained from the E15 spleen library. The most frequently sequenced miRNA was gga-*miR-126*, representing 1,218 sequences, followed by gga-*miR-142-3p* (1,077 sequences), gga-*miR-223* (936 sequences), and gga-*miR-125b* (637 sequences). A list of all E15 spleen matches to the miRBase is shown in Table 3.2.

Of the 3,472 reads matching miRBase sequences from species other than chicken, 1,290 were from the spleen E15 library (Table 3.3). The most frequently sequenced (483 times) of the 1,290 reads was a homologue of *miR-143*. The *G. gallus* sequences and their counterparts obtained from the miRBase database were highly homologous, with an average of 93% identity between the predicted *G. gallus* sequences and their homologues. The

location of these mircoRNA in the chicken genome as while as the location of potential target genes is given in Table.3.3.

Of the 391 reads representing potential novel miRNAs, 138 were obtained from the E15 spleen library. These 138 reads represent a total of three potential novel miRNAs (Table 3.4), which are classified as such because they do not match any miRNAs currently present in miRBase; they map to non-coding regions of the genome, such as intergenic regions and introns; and their flanking regions form a predicted stem-loop structure (Fig. 3.1). This structure is characteristic of miRNA precursors (pre-miRNAs). Table.3.4 also lists the genomic location of these targets as well as, the location of potential target genes.

MicroRNA Profile of the Chick Liver at Day E15

Of the 52,001 reads matching miRNA sequences present in the *G. gallus* miRBase database, 14,607 reads representing 94 different miRNAs were obtained from the E15 liver sample. Of these miRNAs, the one most frequently sequenced was gga-*miR-122a*, which was sequenced a total of 8,365 times. The next most frequently sequenced miRNAs were gga-*miR-126* (458 reads), gga-*miR-17-5p* (458 reads), and gga-*miR-19b* (301 reads). The 94 different miRNAs are listed in Table 3.5.

Of the 3,472 total reads matching miRBase sequences from species other than chicken, 593 were from the E15 liver sample (Table 3.6). The most frequently sequenced (227 times) of these potential miRNAs was homologous to *miR-143* of other species, followed by *miR-454-3p*. The identity between the sequenced chick miRNAs and their homologues averaged 95%. Genomic locations of these microRNA and their potential target genes are shown in Table3.6.

Forty-two sequences potentially representing two novel miRNAs were found in the chick liver at day 15 of gestation (Table 3.7). These sequences were classified as potential novel miRNAs because they fit the predetermined criteria discussed above. Their stem-loop precursor sequences are shown in Figure 3.2 and there genomic locations and target genes are shown in Table 3.7.

MicroRNA Profile of the Chick Spleen at Day E20

Of the 52,001 reads representing miRNA sequences present in the *G. gallus* miRBase database, 8,991 reads representing 98 different miRNAs were obtained from the E20 spleen sample (Table 3.8). The most frequently sequenced miRNA was gga-*miR-142-3p*, constituting 923 reads, followed by gga-*miR-126* (699 reads), gga-*miR-223* (569 reads), and gga-*miR-125b* (456 reads).

Of the 3,472 total reads matching miRBase sequences from species other than chicken, 1,102 were from the spleen E20 sample (Table 3.9). The genomic location of these microRNAs and their targets are also given in Table3.9. The *miR-143* homologue comprised 415 of these reads. As in the other samples, the *G. gallus* sequences and their counterparts obtained from the miRBase database were highly homologous (average of 90% identity).

Eighteen sequences obtained from the E20 spleen sample were classified as potential novel miRNAs (Table 3.10) according to the above-described criteria. Their genomic locations and their potential targets are also shown in Table3.10. The predicted stem-loop structure is presented in Figure 3.3.

MicroRNA Profile of Chick Liver at Day E20

From the embryonic chick liver at day E20, we obtained 80 different sequences that represented miRNAs in the *G. gallus* miRBase database (Table 3.11); these 80 miRNAs were read a total of 14,783 times. The most frequently read (8,337 times) miRNA was gga-*miR-122a*, followed by gga-*miR-126* (770 times), gga-*miR-451* (495 times), and gga-*miR-125b* (481 times).

Of the miRBase sequences from species other than chicken, 487 homologous reads were found in the E20 liver sample (Table 3.12). Genomic locations of these microRNAs and that of their targets are given in Table3.12. Of these sequences, *miR-143* was most frequently read (236 times), followed by *miR-148b*. The homology between the sequenced chick miRNAs and their homologues averaged 92%.

Three potential novel miRNAs were identified in the chick E20 liver (Table 3.13); their predicted stem-loop structures are shown in Figure 3.4 and their genomic locations and as well as the location of their poteintal target genes are given in Table3.13. These novel miRNA constituted a total of 92 reads. As with the other samples, these three miRNAs were classified as potentially novel because they met the criteria: they do not match any miRNAs currently present in miRBase; they map to non-coding regions of the genome, such as intergenic regions and introns; and their flanking regions are predicted to form stem-loop structures.

Table 3.1. Chicken microRNA profiling statistics.

	# of Reads	% of Reads
Matches to sequences in *G. gallus* miRBase database	52001	56%
Matches to miRBase sequences from species other than *G. gallus*	3472	4%
Potential novel microRNAs	391	0.4%
tRNAs, rRNAs, mtRNAs, and snRNAs	24672	27%
Other sequences	12383	13%
Total Reads	92,919	
E15 spleen total reads	27,271	
E15 liver total reads	24,260	
E20 spleen total reads	15,711	
E20 liver total reads	25,677	
Total Reads	92,919	

Table 3.2. E15 spleen matches to microRNAs found in the miRBase *G. gallus* database.

Name	Sequence (5′ → 3′)	Reads (#)
miR-16	uagcagcacguaaauauuggug	591
miR-9	ucuuugguuaucuagcuguauga	2
miR-21	uagcuuaucagacugauguuga	429
miR-24	uggcucaguucagcaggaacag	81
miR-32	uauugcacauuacuaaguugc	7
miR-33	gugcauuguaguugcauug	3
miR-92	uauugcacuugucccggccug	97
miR-100	aacccguagauccgaacuugug	34
miR-101	uacaguacugugauaacugaag	119
miR-103	agcagcauuguacagggcuauga	108
miR-106	aaaagugcuuacagugcaggua	149
miR-107	agcagcauuguacagggcuauca	27
miR-126	ucguaccgugaguaauaaugcgc	1218
miR-128	ucacagugaaccggucucuuu	34
miR-138	agcugguguugugaauc	51
miR-140	agugguuuuacccuaugguag	56
miR-144	cuacaguauagaugauguacuc	151

Table 3.2 continued.

Name	Sequence (5′ → 3′)	Reads (#)
miR-147	gugugcggaaaugcuucugc	8
miR-153	uugcauagucacaaaaguga	6
miR-155	uuaaugcuaaucgugauagggg	1
miR-183	uauggcacugguagaauucacug	3
miR-190	ugauauguuugauauauuaggu	15
miR-193	aacuggcccacaaagucccgcuuu	4
miR-196	uagguaguuucauguuguugg	2
miR-199	cccaguguucagacuaccuguuc	438
miR-203	gugaaauguuuaggaccacuug	5
miR-204	uucccuuugucauccuaugccu	1
miR-206	uggaauguaaggaagugugugg	1
miR-215	augaccuaugaauugacagac	3
miR-218	uugugcuugaucuaaccaugu	51
miR-219	ugauuguccaaacgcaauucu	3
miR-221	agcuacauugucugcuggguuuc	100
miR-222	agcuacaucuggcuacugggucuc	53
miR-223	ugucaguuugucaaauacccc	936
miR-301	cagugcaauaauauugucaaagcau	66

Table 3.2 continued.

Name	Sequence (5′ → 3′)	Reads (#)
miR-375	uuuguucguucggcucgcguua	2
miR-383	agaucagaaggugauuguggcu	28
miR-451	aaaccguuaccauuacugaguuu	437
miR-455	uaugugcccuuggacuacaucg	18
miR-456	caggcugguuagaugguuguca	44
miR-460	ccugcauuguacacacugugug	9
miR-499	uuaagacuuguagugauguuuag	2
miR-10b	uacccuguagaaccgaauuugu	2
miR-122a	uggagugugacaaugguguuugu	73
miR-124a	uuaaggcacgcggugaaugcca	3
miR-125b	ucccugagacccuaacuuguga	637
*miR-126**	cauuauuacuuuugguacgcg	402
miR-130a	cagugcaauauuaaaagggcau	166
miR-130b	cagugcaauaaugaaagggcgu	66
miR-133a	uuggucccuucaaccagcugu	7
miR-135a	uauggcuuuuuauuccuauguga	1
miR-140	agugguuuuacccuaugguag	9
*miR-140**	ccacaggguagaaccacggac	51

Table 3.2 continued.

Name	Sequence (5′ → 3′)	Reads (#)
miR-142-5p	cccauaaaguagaaagcacuac	259
miR-142-3p	uguaguguuuccuacuuuaugg	1077
miR-146a	ugagaacugaauuccauggguu	94
miR-146b	ugagaacugaauuccauaggcg	5
miR-148a	ucagugcacuacagaacuuugu	165
miR-153	uugcauagucacaaaaguga	16
miR-15a	uagcagcacauaaugguuugu	160
miR-15b	uagcagcacaucaugguuugca	398
miR-17-3p	acugcagugaaggcacuugu	15
miR-17-5p	caaagugcuuacagugcagguagu	506
miR-181a	aacauucaacgcugucggugagu	51
miR-181b	aacauucauugcugucgguggg	52
miR-18a	uaaggugcaucuagugcagaua	322
miR-18b	uaaggugcaucuagugcaguua	170
*miR199**	uacaguagucugcacauugg	414
miR-19a	ugugcaaaucuaugcaaaacuga	263
miR-19b	ugugcaaauccaugcaaaacuga	621
miR200b	uaauacugccugguaaugaugau	5

Table 3.2 continued.

Name	Sequence (5′ → 3′)	Reads (#)
*miR-202**	uuuccuaugcauauacuucuuu	1
miR-205a	uccuucauuccaccggagucug	2
miR-205b	cccuucauuccaccggaaucug	2
miR-20a	uaaagugcuuauagugcagguag	238
miR-20b	caaagugcucauagugcagguag	188
miR-218	uugugcuugaucuaaccaugu	4
miR-219	ugauuguccaaacgcaauucu	4
miR-23b	aucacauugccagggauuacc	96
miR-26a	uucaaguaauccaggauaggc	272
miR-27b	uucacaguggcuaaguucugc	32
miR-29a	uagcaccauuugaaaucgguu	30
miR-29b	uagcaccauuugaaaucaguguu	15
miR-30a-3p	cuuucagucggauguuugcagc	7
miR-30a-5p	uguaaacauccucgacuggaag	113
miR-30b	uguaaacauccuacacucagcu	53
miR-30c	uguaaacauccuacacucucagcu	96
miR-30d	uguaaacaucccgacuggaag	24
miR-30e	uguaaacauccuugacugg	1

Table 3.2 continued.

Name	Sequence (5′ → 3′)	Reads (#)
miR-34a	uggcagugucuuagcugguuguu	10
miR-499	uuaagacuuguagugauguuuag	1
miR-92	uauugcacuugucccggccug	13
miR99a	aacccguagauccgaucuugug	58
let-7a	ugagguaguagguuguauaguu	290
let-7b	ugagguaguagguuguguggguu	91
let-7c	ugagguaguagguuguaugguu	127
let-7d	agagguaguggguugcauagu	1
let-7f	ugagguaguagauuguauaguu	201
let-7g	ugagguaguaguuuguacagu	93
let-7i	ugagguaguaguuugugcugu	117
let-7k	ugagguaguagauugaauaguu	37

Table 3.3. E15 spleen homologues to miRBase microRNA sequences from species other than *G. gallus.*

Name	Sequence (5′ → 3′)	Reads (#)	Chromosome # (base position)	Potential Target Gene Location	Target Gene Description
mir-143	ugagaugaagcacuguagcuc	483	15 (473851–473882)	1(21761267-21800005)	ERK5
miR-10a	uacccuguagauccgaauuugu	191	Un_random (379349–370)	20(10543-32881)	Zinc finger protein 532
miR-214	uacagcaggcacagacaggcagu	93	10 (1395871–889)	28(223-13540)	Not annotated
miR-363	aauugcacgguauccaucugu	91	4 (3968812–832)	20(10543-32881)	Not annotated
miR-27a	uucacaguggcuaaguuccgc	87	Z_random (14203495–515)	5(14562-20495)	Not annotated
miR-148b	ucagugcaucacagaacuuggu	85	Z (67248694–712)	13(10446-11040)	POU transcription factor
miR-338	uccagcaucagugauuuuguuga	67	1 (715566062–715566083)	10(11999-13592)	Not annotated
miR-145	guccaguuuucccaggaaucccu	56	6 (1874242–262)	6(18764-20643)	Not annotated
miR-454-3p	uagugcaauauugcuuauagggu	49	15 (399860–882)	22(5798-14233)	Bifunctional methylenetetrahydroflate dehydrogenase
miR-210	cugugcgugugacagcggcuaa	46	3 (4147887–902)	5(14562-20495)	Not annotated
miR-22	aagcugccaguugaagaacugu	15	19 (5352113–134)	7(45813-79162)	PAR3L

Table 3.3 continued.

Name	Sequence (5′ → 3′)	Reads (#)	Chromosome # (base position)	Potential Target Gene Location	Target Gene Description
miR-191	caacggaaucccaaaagcagcug	9	3 (103440627-643)	28(27057-28246)	Not annotated
miR-551b	gcgacccauacuugguuuucagu	8	9 (21966435–452)	13(8515-9573)	POU transcription factor
miR-212	uaacagucuacagucauggcu	3	8 (4976180–198)	13(10446-11040)	POU transcription factor
miR-458	auagcucuuugaaugguacug	3	13 (8034187–208)	5(14562-20495)	Not annotated
miR-132	uaacagucuacagccauggucg	2	3 (4173161–177)	13(10446-11040)	POU transcription factor
miR-720	ucaaaucucggugggaccucca	1	Un_random (7073116–135)	13(8515-9573)	POU transcription factor
miR-677	uguucaugaugauuaguucuac	1	8 (7733664–685)	20(70858-73707)	Not annotated

Table 3.4. Potential novel microRNAs identified in E15 chick spleen.

Name	Sequence (5′ → 3′)	Reads (#)	Chromosome (Base Position)	Potential Target Gene Location	Target Gene Description
miR-S15Y	aagguccaaccucacauguccu	121	22 (2685020–041)	25(1420938-1442110)	Nuclear pore membrane glycoprotein
miR-S15ii	ucgcacaggagcaaguuaccgc	64	3 (78710232–253)	13(10446-11040)	POU transcription factor
miR-S15a	cgcgaccucaggucagacu	56	1 (1044837–834)	20(44609-46941)	Not annotated

Table 3.5. Chicken E15 liver matches to microRNAs found in the miRBase *G. gallus* database.

Name	**Sequence (5′ → 3′)**	**Reads (#)**
miR-16	uagcagcacguaaauauuggug	238
miR-7	uggaagacuagugauuuuguug	8
miR-21	uagcuuaucagacugauguuga	81
miR-24	uggcucaguucagcaggaacag	23
miR-32	uauugcacauuacuaaguugc	3
miR-92	uauugcacuugucccggccug	57
miR-100	aacccguagauccgaacuugug	144
miR-101	uacaguacugugauaacugaag	133
miR-103	agcagcauuguacagggcuauga	59
miR-106	aaaagugcuuacagugcaggua	58
miR-107	agcagcauuguacagggcuauca	67
miR-126	ucguaccgugaguaauaaugcgc	458
miR-128	ucacagugaaccggucucuuu	21
miR-140	agugguuuuacccuaugguag	54
miR-144	cuacaguauagaugauguacuc	23
miR-147	gugugcggaaaugcuucugc	1

Table 3.5. continued.

Name	**Sequence (5′ → 3′)**	**Reads (#)**
miR-153	uugcauagucacaaaaguga	1
miR-365	uaaugccccuaaaaauccuuau	2
miR-190	ugauauguuugauauauuaggu	15
miR-193	aacuggcccacaaagucccgcuuu	8
miR-196	uagguaguuucauguuguugg	1
miR-199	cccaguguucagacuaccuguuc	101
miR-203	gugaaauguuuaggaccacuug	18
miR-204	uucccuuugucauccuaugccu	1
miR-429	uaauacugucugguaaugccgu	12
miR-218	uugugcuugaucuaaccaugu	7
miR-221	agcuacauugucugcuggguuuc	113
miR-222	agcuacaucuggcuacugggucuc	9
miR-223	ugucaguuugucaaauacccc	17
miR-301	cagugcaauaauauugucaaagcau	5
miR-375	uuuguucguucggcucgcguua	43
miR-200a	uaacacugucugguaacgaugu	3
miR-451	aaaccguuaccauuacugaguuu	286

Tabłe 3.5. continued.

Name	**Sequence (5′ → 3′)**	**Reads (#)**
miR-455	uaugugcccuuggacuacaucg	2
miR-456	caggcugguuagaugguuguca	14
miR-460	ccugcauuguacacacugugug	17
miR-499	uuaagacuuguagugauguuuag	2
miR-10b	uacccuguagaaccgaauuugu	9
miR-122a	uggagugugacaaugguguuugu	8365
miR-124a	uuaaggcacgcggugaaugcca	1
miR-125b	ucccugagacccuaacuuguga	286
*miR-126**	cauuauuacuuuugguacgcg	158
miR-130a	cagugcaauauuaaaagggcau	173
miR-130b	cagugcaauaaugaaagggcgu	121
miR-133a	uuggucccccuucaaccagcugu	1
miR-135a	uauggcuuuuuauuccuauguga	1
miR-221	agcuacauugucugcuggguuuc	9
miR-222	agcuacaucuggcuacugggucuc	75
*miR-140**	ccacaggguagaaccacggac	50
miR-142-5p	cccauaaaguagaaagcacuac	12

Tabłe 3.5. continued.

Name	**Sequence (5′ → 3′)**	**Reads (#)**
miR-142-3p	uguaguguuuccuacuuuaugg	38
miR-146a	ugagaacugaauuccauggguu	81
miR-148a	ucagugcacuacagaacuuugu	265
miR-15a	uagcagcacauaaugguuugu	82
miR-15b	uagcagcacaucaugguuugca	268
miR-17-3p	acugcagugaaggcacuugu	14
miR-17-5p	caaagugcuuacagugcagguagu	312
miR-181a	aacauucaacgcugucggugagu	34
miR-181b	aacauucauugcugucgguggg	16
miR-18a	uaaggugcaucuagugcagaua	87
miR-18b	uaaggugcaucuagugcaguua	27
*miR199**	uacaguagucugcacauugg	115
miR-19a	ugugcaaaucuaugcaaaacuga	81
miR-19b	ugugcaaauccaugcaaaacuga	301
miR200b	uaauacugccugguaaugaugau	20
*miR-202**	uuuccuaugcauauacuucuuu	8
miR-20a	uaaagugcuuauagugcagguag	134

Tabłe 3.5. continued.

Name	**Sequence (5′ → 3′)**	**Reads (#)**
miR-20b	caaagugcucauagugcagguag	97
miR-23b	aucacauugccagggauuacc	59
miR-26a	uucaaguaauccaggauaggc	195
miR-27b	uucacaguggcuaaguucugc	67
miR-30a-3p	cuuucagucggauguuugcagc	22
miR-30a-5p	uguaaacauccucgacuggaag	121
miR-30b	uguaaacauccuacacucagcu	48
miR-30c	uguaaacauccuacacucucagcu	212
miR-30d	uguaaacaucccgacuggaag	90
miR-34a	uggcagugucuuagcugguuguu	50
miR99a	aacccguagauccgaucuugug	30
let-7a	ugagguaguagguuguauaguu	172
let-7b	ugagguaguagguuguguggguu	10
let-7c	ugagguaguagguuguaugguu	15
let-7d	agagguaguggguugcauagu	8
let-7f	ugagguaguagauuguauaguu	92
let-7g	ugagguaguaguuuguacagu	33

Tabłe 3.5. continued.

Name	**Sequence (5′ → 3′)**	**Reads (#)**
let-7i	ugagguaguaguuugugcugu	34
let-7k	ugagguaguagauugaauaguu	3

Table3.6. E15 liver homologues to miRBase microRNA sequences from species other than *G. gallus*.

Name	Sequence (5′ → 3′)	Reads (#)	Chromosome # (base position)	Potential Target Gene Location	Target Gene Description
miR-139	ucuacagugcacgugucuccagu	7	18 (5449346–361)	5(21864-22497)	Not annotated
miR-143	ugagaugaagcacuguagcuc	227	15 (473851–473882)	1(21761267-21800005)	ERK5
miR-145	guccaguuuucccaggaaucccu	42	6 (1874242–262)	6(18764-20643)	Not annotated
miR-150	ucucccaaccccuguaccagu	26	5 (30632216-30632232)	16(41921-90500)	Not annotated
miR-152	ucagugcacuaacagaacuuuguu	3	10 (638130-638146)	4(87571-100995)	hyaluronidase
miR-189	ucccguggaucgccccagcu	28	1 (104452210–229)	13(8515-9573)	POU transcription factor
miR-191	caacggaaucccaaaagcagcug	15	3 (103440627–643)	28(27057-28246)	Not annotated
miR-210	cugugcgugugacagcggcuaa	8	3 (4147887–902)	5(14562-20495)	Not annotated
miR-214	uacagcaggcacagacaggcagu	21	10 (1395871–889)	28(223-13540)	Not annotated
miR-22	aagcugccaguugaagaacugu	26	19 (5352113–134)	7(45813-79162)	PAR3L
miR-275	uaggcaccugaaguaggcacca	23	1 (74837579–594)	7(45813-79162)	PAR3L
miR-338	uccagcaucagugauuuuguuga	9	1 (715566062-715566083)	10(11999-13592)	Not annotated

Table 3.6. continued.

Name	Sequence (5′ → 3′)	Reads (#)	Chromosome # (base position)	Potential Target Gene Location	Target Gene Description
miR-363	aauugcacgguauccaucugu	43	4 (3968812–832)	20(10543-32881)	Not annotated
miR-454-3p	uagugcaauauugcuuauaggguc	91	15 (399860–882)	22(5798-14233)	Bifunctional methylenetetrahydrofolate dehydrogenase
miR-551b	gcgacccauacuugguuuucagu	9	9 (21966435–452)	13(8515-9573)	POU transcription factor
miR-720	ucaaaucucgugggaccucca	15	Un_random (7073116–135)	13(8515-9573)	POU transcription factor

Table 3.7. Potential novel microRNAs identified in E15 chick liver.

Name	Sequence (5′ → 3′)	Reads (#)	Chromosome (Base Position)	Potential Target Gene Location	Target Gene Description
miR-L15b	augccugggcagggcgaa	30	1 (104484394–411)	16(115271-120779)	MHC B locus
miR-L15rrr	augcagaagugcacggaaacagc	12	Z (68816781–803)	20(70858-73707)	Not annotated

Table 3.8. E20 spleen matches to microRNAs found in the miRBase *G. gallus* database.

Name	**Sequence (5′ → 3′)**	**Reads (#)**
miR-16	uagcagcacguaaauauuggug	332
miR-9	ucuuugguuaucuagcuguauga	5
miR-21	uagcuuaucagacugauguuga	427
miR-24	uggcucaguucagcaggaacag	62
miR-26a	uucaaguaauccaggauaggcu	258
miR-32	uauugcacauuacuaaguugc	13
miR-33	gugcauuguaguugcauug	3
miR-92	uauugcacuugucccggccug	47
miR-100	aacccguagauccgaacuugug	46
miR-101	uacaguacugugauaacugaag	69
miR-103	agcagcauuguacagggcuauga	68
miR-106	aaaagugcuuacagugcaggua	28
miR-107	agcagcauuguacagggcuauca	17
miR-126	ucguaccgugaguaauaaugcgc	699
miR-128	ucacagugaaccggucucuuu	36
miR-138	agcugguguugugaauc	9

Table 3.8. continued.

Name	**Sequence (5′ → 3′)**	**Reads (#)**
miR-140	agugguuuuacccuaugguag	48
miR-144	cuacaguauagaugauguacuc	24
miR-147	gugugcggaaaugcuucugc	2
miR-153	uugcauagucacaaaaguga	16
miR-155	uuaaugcuaaucgugauagggg	1
miR-183	uauggcacugguagaauucacug	1
miR-184	uggacggagaacugauaagggu	1
miR-190	ugauauguuugauauauuaggu	8
miR-193	aacuggcccacaaagucccgcuuu	9
miR-196	uagguaguuucauguuguugg	1
miR-199	cccaguguucagacuaccuguuc	277
miR-203	gugaaauguuuaggaccacuug	1
miR-204	uucccuuugucauccuaugccu	1
miR-148a	ucagugcacuacagaacuuugu	1
miR-218	uugugcuugaucuaaccaugu	32
miR-219	ugauuguccaaacgcaauucu	2
miR-221	agcuacauugucugcuggguuuc	69

Table 3.8. continued.

Name	**Sequence (5′ → 3′)**	**Reads (#)**
miR-202	agaggcauagagcaugggaaaa	1
miR-223	ugucaguuugucaaauacccc	569
miR-301	cagugcaauaauauugucaaagcau	22
miR-217	uacugcaucaggaacugauuggau	2
miR-383	agaucagaaggugauuguggcu	10
miR-451	aaaccguuaccauuacugaguuu	178
miR-455	uaugugcccuuggacuacaucg	19
miR-456	caggcugguuagaugguuguca	14
miR-460	ccugcauuguacacacugugug	5
miR-365	uaaugccccuaaaaauccuuau	2
miR-10b	uacccuguagaaccgaauuugu	52
miR-122a	uggagugugacaaugguguuugu	84
miR-124a	uuaaggcacgcggugaaugcca	2
miR-125b	ucccugagacccuaacuuguga	456
*miR-126**	cauuauuacuuuugguacgcg	206
miR-130a	cagugcaauauuaaaagggcau	115
miR-130b	cagugcaauaaugaaagggcgu	46

Table 3.8. continued.

Name	**Sequence (5′ → 3′)**	**Reads (#)**
miR-429	uaauacugucugguaaugccgu	2
miR-449	uggcaguguauguuagcuggu	8
miR-200a	uaacacugucugguaacgaugu	12
*miR-140**	ccacaggguagaaccacggac	34
miR-142-3p	uguaguguuuccuacuuuaugg	923
miR-142-5p	cccauaaaguagaaagcacuac	201
miR-302c	uaagugcuuccauguuucagugg	14
miR-146a	ugagaacugaauuccauggguu	43
miR-146b	ugagaacugaauuccauaggcg	4
miR-148a	ucagugcacuacagaacuuugu	128
miR-15a	uagcagcacauaaugguuugu	168
miR-15b	uagcagcacaucaugguuugca	289
miR-17-3p	acugcagugaaggcacuugu	8
miR-17-5p	caaagugcuuacagugcagguagu	314
miR-181a	aacauucaacgcugucggugagu	48
miR-181b	aacauucauugcugucgguggg	20
miR-18a	uaaggugcaucuagugcagaua	133

Table 3.8. continued.

Name	**Sequence (5′ → 3′)**	**Reads (#)**
miR-18b	uaaggugcaucuagugcaguua	81
*miR199**	uacaguagucugcacauugg	287
miR-19a	ugugcaaaucuaugcaaaacuga	130
miR-19b	ugugcaaauccaugcaaaacuga	278
miR200b	uaauacugccugguaaugaugau	16
*miR-202**	uuuccuaugcauauacuucuuu	10
miR-205a	uccuucauuccaccggagucug	5
miR-205b	cccuucauuccaccggaaucug	2
miR-20a	uaaagugcuuauagugcagguag	189
miR-20b	caaagugcucauagugcagguag	118
miR-23b	aucacauugccagggauuacc	76
miR-26a	uucaaguaauccaggauaggc	259
miR-27b	uucacaguggcuaaguucugc	15
miR-29a	uagcaccauuugaaaucgguu	15
miR-29b	uagcaccauuugaaaucaguguu	11
miR-30a-3p	cuuucagucggauguuugcagc	11
miR-30a-5p	uguaaacauccucgacuggaag	64

Table 3.8. continued.

Name	**Sequence (5′ → 3′)**	**Reads (#)**
miR-30b	uguaaacauccuacacucagcu	29
miR-30c	uguaaacauccuacacucucagcu	69
miR-30d	uguaaacaucccgacuggaag	12
miR-30e	uguaaacauccuugacugg	3
miR99a	aacccguagauccgaucuugug	43
let-7a	ugagguaguagguuguauaguu	264
let-7b	ugagguaguagguuguggguu	89
let-7c	ugagguaguagguuguaugguu	70
let-7d	agagguaguggguugcauagu	3
let-7f	ugagguaguagauuguauaguu	167
let-7g	ugagguaguaguuuguacagu	76
let-7i	ugagguaguaguuugugcugu	71
let-7j	ugagguaguagguuguauaguu	2
let-7k	ugagguaguagauugaauaguu	39

Table 3.9. E20 spleen homologues to miRBase microRNA sequences from species other than *G. gallus*.

Name	Sequence (5′ → 3′)	Reads (#)	Chromosome # (base position)	Potential Target Gene Location	Target Gene Description
miR-139	ucuacagugcacgugucuccagu	17	18 (5449346–361)	5(21864-22497)	Not annotated
miR-143	ugagaugaagcacuguagcu	415	15 (473851–473882)	1(21761267-21800005)	ERK5
miR-145	guccaguuuucccaggaaucccu	59	6 (1874242–262)	6(18764-20643)	Not annotated
miR-148b	ucagugcaucacagaacuuggu	28	Z (67248694–712)	13(10446-11040)	POU transcription factor
miR-150	acccacuccugguacca	25	5 (30632216–30632232)	16(41921-90500)	Not annotated
miR-189	augcagaagugcacggaaacagcu	21	1 (104452210–229)	13(8515-9573)	POU transcription factor
miR-191	caacggaaucccaaaagcagcug	7	3 (103440627–643)	28(27057-28246)	Not annotated
miR-192	caacggaaucccaaaagcagcugu	1	24 (4993982–998)	28(27057-28246)	Not annotated
miR-210	cugugcgugugacagcggcuaa	31	3 (4147887–902)	5(14562-20495)	Not annotated
miR-214	uacagcaggcacagacaggcagu	47	10 (1395871–889)	28(223-13540)	Not annotated
miR-22	aagcugccaguugaagaacugu	14	19 (5352113–134)	7(45813-79162)	PAR3L
miR-25	agugcaaugaaggcagg	1	3 (27752946–962)	20(10645-32881)	zinc finger protein 532

Table 3.9. continued.

Name	Sequence (5′ → 3′)	Reads (#)	Chromosome # (base position)	Potential Target Gene Location	Target Gene Description
miR-27a	uucacaguggcuaaguuccgc	48	Z_random (14203495–515)	5(14562-20495)	Not annotated
miR-338	uccagcaucagugauuuuguuga	60	1 (715566062–715566083)	10(11999-13592)	Not annotated
miR-363	aauugcacgguauccaucugu	67	4 (3968812–832)	20(10543-32881)	Not annotated
miR-551b	gcgacccauacuugguuucag	2	9 (21966435–452)	13(8515-9573)	POU transcription factor
miR-592	ccacaucaucccauacuaaca	1	Z (72653268–285)	16(180632-187797)	Zinc finger protein 692

Table 3.10. Potential novel microRNA identified in E20 chick spleen.

Name	Sequence (5′ → 3′)	Reads (#)	Chromosome (Base Position)	Potential Target Gene Location	Target Gene Description
miR-S20iiiii	gcucugacuuuauugcacuacu	18	19 (7144791–812)	13(10446-11040)	POU transcription factor

Table. 3.11. E20 liver matches to microRNAs found in the miRBase *G. gallus* database.

Name	Sequence (5′ → 3′)	Reads (#)
miR-16	uagcagcacguaaauauuggug	180
miR-34a	uggcagugucuuagcugguuguu	2
miR-21	uagcuuaucagacugauguuga	291
miR-24	uggcucaguucagcaggaacag	26
miR-32	uauugcacauuacuaaguugc	1
miR-92	uauugcacuugucccggccug	72
miR-100	aacccguagauccgaacuugug	174
miR-101	uacaguacugugauaacugaag	151
miR-103	agcagcauuguacagggcuauga	30
miR-106	aaaagugcuuacagugcaggua	43
miR-107	agcagcauuguacagggcuauca	53
miR-126	ucguaccgugaguaauaaugcgc	770
miR-128	ucacagugaaccggucucuuu	22
miR-140	agugguuuuacccuaugguag	41
miR-144	cuacaguauagaugauguacuc	22
miR-187	ucgugucuuguguugcagcc	2

Table 3.11. continued.

Name	Sequence (5′ → 3′)	Reads (#)
miR-219	ugauuguccaaacgcaauucu	1
miR-365	uaaugccccuaaaaauccuuau	20
miR-190	ugauauguuugauauauuaggu	5
miR-193	aacuggcccacaaagucccgcuuu	16
miR-196	uagguaguuucauguuguugg	1
miR-199	cccaguguucagacuaccuguuc	126
miR-205a	uccuucauuccaccggagucug	3
miR-204	uucccuuugucauccuaugccu	1
miR-429	uaauacugucugguaaugccgu	6
miR-218	uugugcuugaucuaaccaugu	5
miR-221	agcuacauugucugcuggguuuc	56
miR-222	agcuacaucuggcuacugggucuc	44
miR-223	ugucaguuugucaaauacccc	58
miR-301	cagugcaauaauauugucaaagcau	25
miR-375	uuuguucguucggcucgcguua	28
miR-200a	uaacacugucugguaacgaugu	3
miR-451	aaaccguuaccauuacugaguuu	495

Table 3.11. continued.

Name	Sequence (5′ → 3′)	Reads (#)
miR-455	uaugugcccuuggacuacaucg	2
miR-456	caggcugguuagaugguuguca	18
miR-460	ccugcauuguacacacugugug	20
miR-10b	uacccuguagaaccgaauuugu	2
miR-122a	uggagugugacaaugguguuugu	8337
miR-124a	uuaaggcacgcggugaaugcca	1
miR-125b	ucccugagacccuaacuuguga	481
*miR-126**	cauuauuacuuuugguacgcg	256
miR-130a	cagugcaauauuaaaagggcau	164
miR-130b	cagugcaauaaugaaagggcgu	143
miR-29a	uagcaccauuugaaaucgguu	6
miR-29b	uagcaccauuugaaaucaguguu	3
*miR-140**	ccacaggguagaaccacggac	55
miR-142-5p	cccauaaaguagaaagcacuac	11
miR-142-3p	uguaguguuuccuacuuuaugg	48
miR-146a	ugagaacugaauuccauggguu	59
miR-148a	ucagugcacuacagaacuuugu	209

Table 3.11. continued.

Name	Sequence (5′ → 3′)	Reads (#)
miR-15a	uagcagcacauaaugguuugu	79
miR-15b	uagcagcacaucaugguuugca	251
miR-17-3p	acugcagugaaggcacuugu	4
miR-17-5p	caaagugcuuacagugcagguagu	106
miR-181a	aacauucaacgcugucggugagu	17
miR-181b	aacauucauugcugucgguggg	18
miR-18a	uaaggugcaucuagugcagaua	59
miR-18b	uaaggugcaucuagugcaguua	21
*miR199**	uacaguagucugcacauugg	81
miR-19a	ugugcaaaucuaugcaaaacuga	31
miR-19b	ugugcaaauccaugcaaaacuga	146
miR200b	uaauacugccugguaaugaugau	27
miR-20a	uaaagugcuuauagugcagguag	54
miR-20b	caaagugcucauagugcagguag	25
miR-23b	aucacauugccagggauuacc	29
miR-26a	uucaaguaauccaggauaggc	206
miR-27b	uucacaguggcuaaguucugc	19

Table 3.11. continued.

Name	Sequence (5′ → 3′)	Reads (#)
miR-30a-3p	cuuucagucggauguuugcagc	15
miR-30a-5p	uguaaacauccucgacuggaag	69
miR-30b	uguaaacauccuacacucagcu	64
miR-30c	uguaaacauccuacacucucagcu	141
miR-30d	uguaaacaucccgacuggaag	58
miR-34a	uggcagugucuuagcuggguuguu	79
miR99a	aacccguagauccgaucuugug	49
let-7a	ugagguaguagguuguauaguu	230
let-7b	ugagguaguagguuguguggguu	19
let-7c	ugagguaguagguuguaugguu	39
let-7f	ugagguaguagauuguauaguu	152
let-7g	ugagguaguaguuuguacagu	69
let-7i	ugagguaguaguuugugcugu	38

Table 3.12. E20 liver homologues to miRBase microRNA sequences from species other than *G. gallus*.

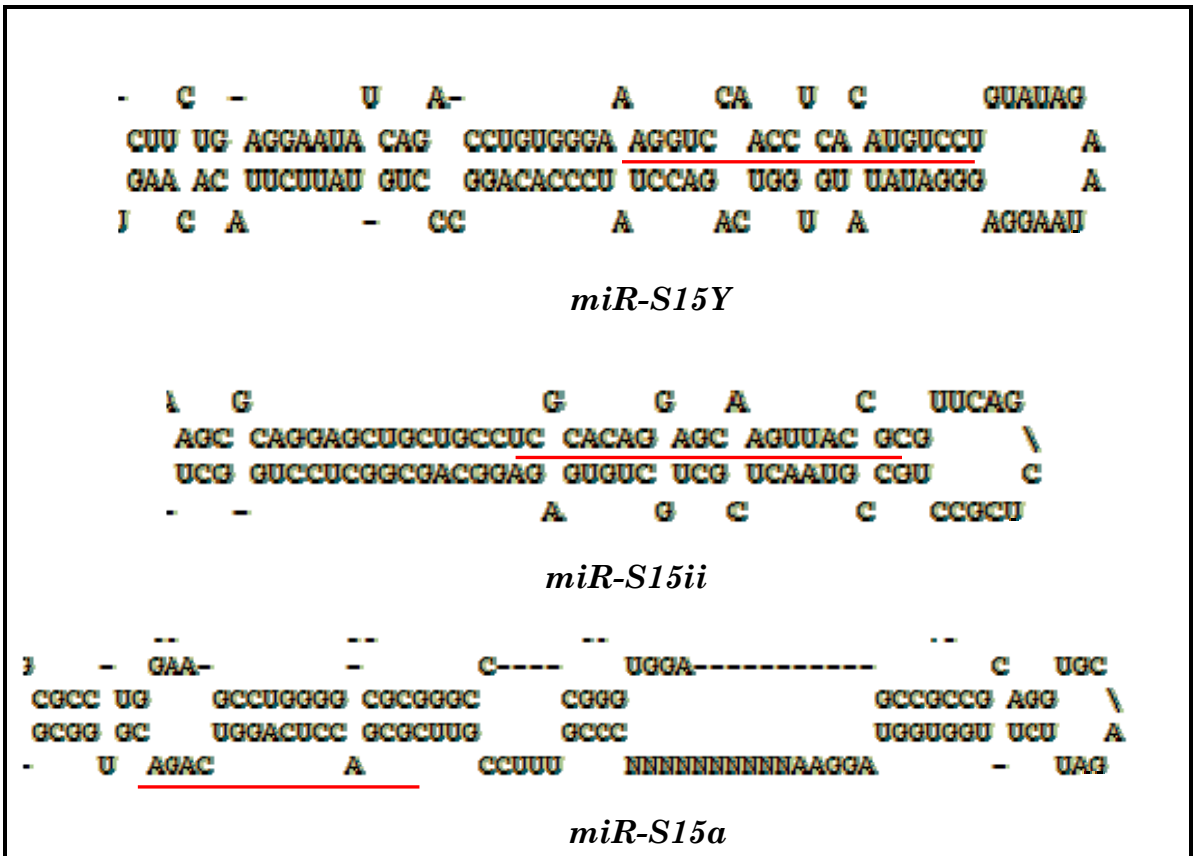
Name	Sequence (5′ → 3′)	Reads (#)	Chromosome # (base position)	Potential Target Gene Location	Target Gene Description
miR-10a	uacccuguagauccgaauuugu	12	Un_random (379349–370)	20(10543-32881)	Zinc finger protein 532
miR-139	ucuacagugcacgugucuccag	1	18 (5449346–361)	5(21864-22497)	No annotated
miR-143	ugagaugaagcacuguagcuc	236	15 (473851-473882)	1(21761267-21800005)	ERK5
miR-145	guccaguuuucccaggaaucccu	21	6 (1874242–262)	6(18764-20643)	Not annotated
miR-148b	ucagugcaucacagaacuuggu	47	Z (67248694–712)	13(10446-11040)	POU transcription factor
miR-150	ucucccaaccccuguaccaguga	12	5 (30632216–30632232)	16(41921-90500)	Not annotated
miR-191	caacggaaucccaaaagcagcu	10	3 (103440627–643)	28(27057-28246)	Not annotated
miR-212	uaacagucuacagucauggcuac	1	8 (4976180–198)	13(10446-11040)	POU transcription factor
miR-214	acagcaggcacagacaggcagu	25	10 (1395871–889)	28(223-13540)	Not annotated
miR-22	aagcugccaguugaagaacugu	41	19 (5352113–134)	7(45813-79162)	PAR3L
miR-27a	uucacaguggcuaaguuccgc	13	Z_random (14203495–515)	5(14562-20495)	Not annotated

Table 3.12. continued.

Name	Sequence (5′ → 3′)	Reads (#)	Chromosome # (base position)	Potential Target Gene Location	Target Gene Description
miR-338	uccagcaucagugauuuuguuga	6	1 (715566062–715566083)	10(11999-13592)	Not annotated
miR-347	ucgugucccaucugggucgcca	6	Un_random (454883350–454883367)	28(75493-85598)	Not annotated
miR-363	aauugcacgguauccaucugu	26	4 (3968812–832)	20(10543-32881)	Not annotated
miR-551b	gcgacccauacuugguuuca	6	9 (21966435–452)	13(8515-9573)	POU transciption factor

Table 3.13. Potential novel microRNAs identified in E20 chick liver.

Name	Sequence (5′ → 3′)	Reads (#)	Chromosome (Base Position)	Potential Target Gene Location	Target Gene Description
miR-L20ii	cggcugggagccgcgagcu	33	Z (53415195–213)	16(170473-171233)	MHC type antigen B-G
miR-L20kk	ggcugggagccacgauucaga	32	4 (6334646–66)	6(10707-17646)	Not annotated
miR-L20ss	uccccauuccacuccucctuu	25	14 (3904434–53)	16(322516-335176)	C-type lectin

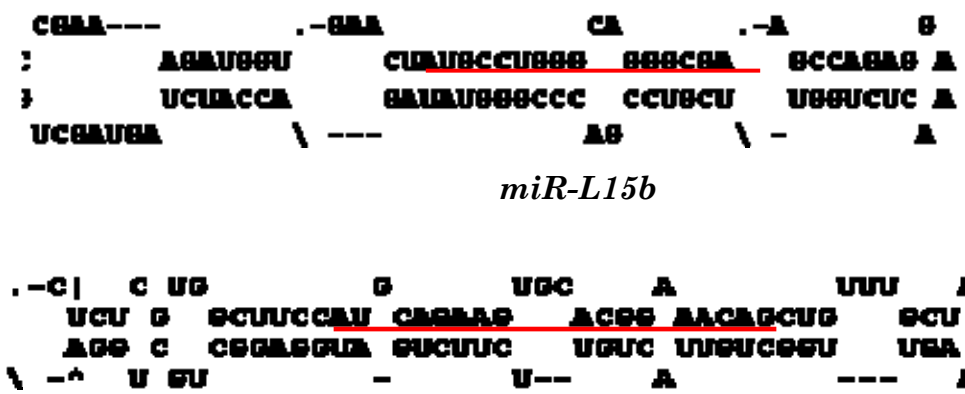


miR-S15Y

miR-S15ii

miR-S15a

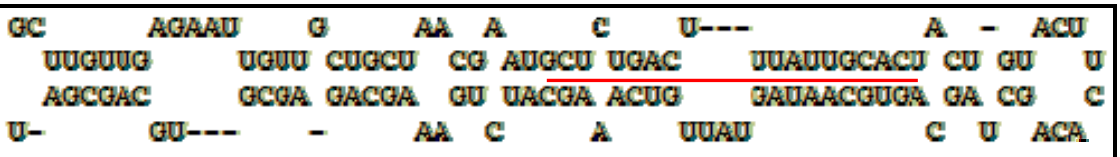
Figure 3.1. Hairpin loop precursors for potential novel microRNAs from the chick E15 spleen small RNA library. Red underlining indicates the mature sequence.



miR-L15b

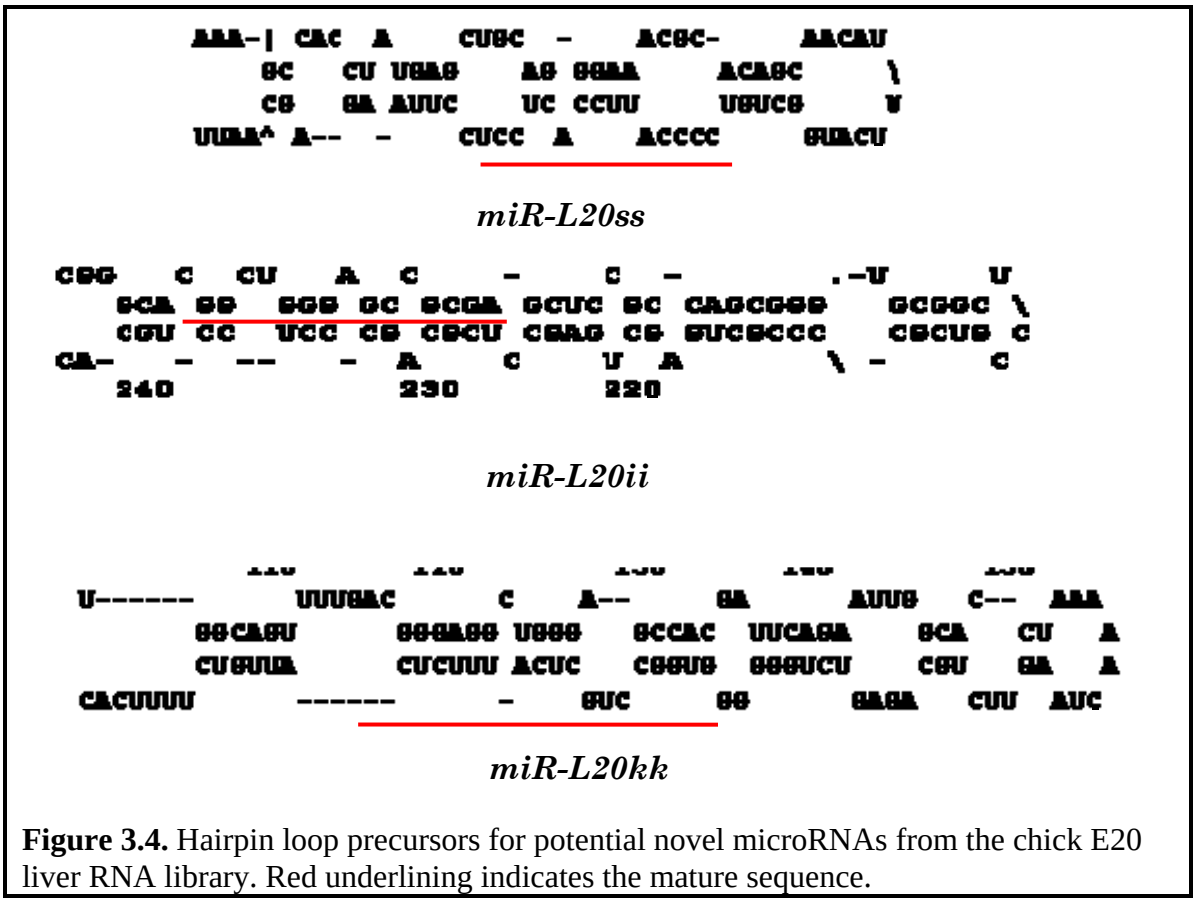
miR-L15rrr

Figure 3.2. Hairpin loop precursors for potential novel microRNAs from the chick E15 liver RNA library. Red underlining indicates the mature sequence.



miR-S20iiiiii

Figure 3.3. Hairpin loop precursors for a potential novel microRNA from the chick E20 spleen small RNA library. Red underlining indicates the mature sequence.



miR-L20ss

miR-L20ii

miR-L20kk

Figure 3.4. Hairpin loop precursors for potential novel microRNAs from the chick E20 liver RNA library. Red underlining indicates the mature sequence.

DISCUSSION

We obtained a total of 92,919 sequence reads from small-RNA libraries of embryonic chick spleen and liver from days E15 and E20. Of these reads, 52,001 represented known chicken miRNAs; 3,472 represented miRNAs that had been annotated in other species, but had not been previously identified in chicken; and 500 reads represented potential novel miRNAs not previously identified in any species. More than one hundred different known miRNAs were identified in this study, and many were expressed in all four libraries. For example, many reads of *miR-126* were obtained from all four libraries, suggesting that this miRNA is likely to be involved in regulating a general developmental process and to function in different cell types. Other common miRNAs that yielded multiple reads from all four libraries included *miR-125b* and *miR-21*, which are involved in general processes of cellular proliferation (Chan et al. 2005; Lee et al. 2005). For example, depletion *of miR-125b* from various differentiated cell lines using 2′-O-methyl antisense oligonucleotides showed that it is involved in the proliferation of differentiated cells (Lee et al. 2005), and *miR-21* targets apoptosis-inducing genes, indicating that it is an antiapoptotic factor that contributes to cell proliferation (Chan et al. 2005; Cheng et al. 2005).

Of the chicken sequences that were identified as homologues of miRNAs from other species but had not been previously identified in the chicken, *miR-143* was highly abundant, yielding several-hundred reads for each of the four libraries. This miRNA is thought to be involved in regulating adipocyte differentiation (Esau et al. 2004). Its high abundance at E15 and E20 may indicate that adipocyte differentiation occurs in the

spleen and liver during this stage of development. Alternatively, *miR-143* may have target genes in chicken that have not been previously identified in other species, which would suggest that it regulates some other type of developmental process. In both the E15 and E20 liver samples, *miR-122a* represented ~30% of the total reads, consistent with other studies showing that *miR-122a* is the most highly expressed miRNA in the liver (Chang et al. 2004; Jopling et al. 2005). This miRNA is highly conserved across vertebrates and is expressed at very high levels in the embryonic liver, but at low levels in the adult liver (Chang et al. 2004).

Although many miRNAs were found in all four libraries, some were sequenced far more frequently in one or two of the libraries than in the other libraries. For example, *miR-142-3p*, *miR-223*, and *miR-17-5p* reads were obtained much more often from the spleen libraries than from the liver libraries, suggesting that these miRNAs are important in regulating gene expression during spleen development. Furthermore, several miRNAs, such *miR-451* and *miR-15b*, were found to be expressed in spleen but not at all in liver. Both *miR-451* and *miR-15b* yielded many reads from the spleen, but not the liver, libraries, demonstrating that expression of many miRNAs is tissue-specific. Few miRNAs have been found to be expressed across a wide range of different tissues.

Some of the miRNAs also exhibited differential expression between the two developmental time points. For example, *miR-20a*, *-221*, *-20b*, *-19a*, and *-27b* were sequenced more frequently from the E15 liver library than from the E20 liver library, and *miR-203* and *miR-7* were sequenced multiple times from the E15 liver library but not at all from the E20 liver library. These results suggest that these miRNAs are involved in

regulating gene expression earlier in liver development and are not very important in the later stages.

Other miRNAs, including *let-7g*, *miR-221*, *miR-222*, and *miR-223*, had opposite characteristics; they were sequenced much more frequently from the E20 library than from the E15 library. Of these miRNAs, *miR-221* and *miR-222* belong to the same miRNA family and have been shown to target the Kit mRNA (Felli et al. 2005). Since loss of the Kit mRNA leads to erythroblast proliferation, this finding suggests that at E15 a substantial amount of cell proliferation is still occurring in the spleen and liver, and that this cell expansion phase ends by E20 because the increase in *miR-221* and *miR-222* blocks that proliferation.

The miRNA *miR-144* was sequenced 151 times from the E15 spleen library, but was only sequenced about 20 times from each of the other three libraries. This result suggests that *miR-144* plays an important function in spleen development at day E15 but has less involvement in later stages of development and in liver development. Since inhibition of *miR-144* expression in HeLa cells leads to a decline in cell growth (Cheng et al. 2005), *miR-144* is probably involved in regulating cell proliferation. In fact, many different miRNAs known to be involved in cell proliferation were sequenced multiple times from the E15 spleen library, which would be consistent with proliferation of different cell types during this stage of spleen development.

Some miRNAs are involved in inhibiting cell growth. For example, a microarray screen for targets of *miR-16* revealed that this miRNA negatively regulates the expression of genes involved in cell cycle progression, and that *miR-16* expression leads to a decrease in cell growth and cell cycle arrest at the G_0G_1 stage (Linsley et al. 2007). In our

study, each of the four libraries yielded several hundred reads of *miR-16*. Determining the gene targets of this miRNA in chicken, as well as the cell type(s) involved, could be interesting because these cells may be important during the earliest stages of development but not during later stages of development, and their growth is inhibited by *miR-16*.

Overall, the spleen libraries had a larger array of miRNAs than the liver libraries. Much of spleen development occurs during the later stages of embryonic development, so we can reasonably expect that many gene expression changes occur during these stages. The abundant miRNA present in the spleen during these stages might serve to regulate these changes.

As a result of this study, we identified nine potential novel chicken miRNAs. These novel miRNAs appear to be tissue-specific. For example, the novel miRNA *miR-S15y* yielded multiple reads from both spleen libraries but yielded no reads from either of the liver libraries. Also, the novel miRNA *miR-L15b* was sequenced multiple times from both liver libraries, but was not found in the spleen libraries.

The potential novel miRNAs appeared to be expressed at lower levels than some of the known miRNAs, which could indicate that most of the highly-expressed chicken miRNAs have already been identified, whereas, for the most part, the miRNAs expressed at low levels remain to be discovered. Because of their low abundance in the small-RNA library, these potential novel miRNAs probably would not have been identified if we had chosen to use the cloning approach for miRNA profiling; however, by utilizing the 454 Life Sciences technology, we were able to obtain thousands of reads for each library and were therefore able to identify low-expression miRNAs. Despite their low abundance,

these miRNAs could play very important roles in development and might be useful in discovery of previously unidentified developmental pathways.

Since the putative novel miRNAs appear to be tissue-specific, they may have functions that are specific to the spleen or liver only. Some may function in the regulation of cell proliferation in the spleen, since the spleen increases in size during the later stages of development. We also identified putative novel miRNAs in the liver at two different metabolic stages; at E15, the liver is utilizing the yolk for energy production, whereas at E20, it is preparing to switch to the use of feed as an energy source. Therefore, some of these miRNAs may be involved in that switch, although confirmation of this suggestion will require further investigation.

A recent study employing a computational approach to predict miRNAs targets in a variety of mouse tissue identified many transcription factors as potential target genes (Inaoka et la. 2007). In our study we also identified several transcription factors as being potential miRNA targets. For example, we identified the microRNAs *miR-148b*, *miR-212*, *miR-132*, *miR-S15ii*, and *miR-20iiiiii* as all potentially targeting the same transcription factor. We also identified *miR-189*, *miR-551b*, and *miR-720* as also targeting a transcription factor, which is in the same family of transcription factors as the one targeted by *miR-148b*, *miR-212*, *miR-132*, *miR-S15ii*, and *miR-20iiiiii*. Two of our potential novel microRNAs, one in the E15 spleen library (*miR-S15ii*) and one in the E20 spleen library (*miR-S20iiiiii*) could potentially target the same transcription factor. Perhaps this transcription factor is involved in turning on a pathway that is not needed or could even be detrimental during spleen development at this stage and so to make sure that this pathway is turned off, multiple miRNAs target the same gene. We also

identified a couple of different zinc finger proteins as being potential microRNA targets. For example, the microRNAs *miR-25* and *miR-10a* were identified as potentially targeting the gene encoding the zinc finger protein 532 and the microRNA *miR-592* could target the gene encoding the zinc finger protein 692. In proteins zinc finger domains are sites of DNA binding and are often found on transcription factors and other regulators of gene expression. In our study we have identified several genes that are involved in regulation gene expression as potential microRNA targets. This would suggest that during organogenesis that the repression and/or activation of different pathways is critical and that microRNAs could play a major role in this regulation.

A microarray profile of the chick liver in the perihatching period in which the metabolic switch occurs identified various immune genes as being differentially expressed (Cogburn et al. 2003). This is interesting because our profile of liver development also occurred during the time of the metabolic switch and several of our novel liver specific microRNAs were identified as potentially targeting immune genes. For example, the microRNAs *miR-L15b* and *miR-L20ii* were both found to possibly target genes at the MHC B locus. The microRNA *miR-L20ss* was identified as potentially targeting a gene encoding a C-type lectin. C-type lectins have been identified in as having many different immune functions such as, being involved in inflammation and to serve as receptors on natural killer cells, among others (Weis et al. 1998). Since these three microRNAs were found to potentially target immune genes it is interesting to speculate that they may be involved in the regulation of the metabolic switch, though this will need to be further validated.

The next step in this research will be to confirm that the potential novel miRNAs are expressed, using Northern blotting. We also need to identify their target gene(s), which can be done using the MiRanda algorithm. Once these targets have been identified computationally, experimental validation will be necessary. This validation could be achieved by first cloning the 3′ UTR of the target gene into a reporter vector containing a reporter gene such as luciferase, transfecting this vector into a cell line such as DF1 along with the novel miRNA, and quantifying the resulting decrease (if any) in reporter gene (luciferase) activity.

DNA microarray screening is another possible approach to validation of target genes. After expression of the novel miRNAs is blocked using 2′-O-methyl antisense oligonucleotides, a microarray screen could be carried out to determine which genes are up-regulated; these genes would be considered potential targets of the novel miRNA. We also need to examine the expression of the putative novel miRNAs across different chick tissues, either by Northern blotting or by *in situ* hybridization, to determine if they are expressed in any other tissues or if they are tissue-specific miRNAs.

In general, we found that each of the four libraries contained a large variety of miRNAs. This result suggests that the spleen and liver at days E15 and E20 are actively undergoing many different developmental processes with dynamic gene expression and that microRNAs play a major role in the regulation of these processes. In our study we have identified hundreds of different miRNAs as being expressed in spleen and liver development of the embryonic chick; including novel chicken miRNAs as well as, some brand new miRNAs that have not previously been identified in any organism.

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