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Cloning and Expression of Porcine Dicer and Argonaute-2

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CLONING AND EXPRESSION OF PORCINE DICER AND ARGONAUTE-2

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ABSTRACT

In vitro-produced embryos exhibit aberrations in development, but the reasons for these developmental problems are unknown. Recently, a class of small non-coding RNA called microRNA (miRNA) has been described and reported to have roles in normal mammalian embryonic development. These miRNAs are encoded in the genome, transcribed by RNA pol II and processed into fragments approximately 22 nt in length by ribonuclease enzymes, the final one being a protein called Dicer. miRNA work through the RNA-induced silencing complex (RISC), of which the argonaute gene family are key proteins. Argonaute-2 (Ago2) has been identified as the only member possessing endonuclease activity, which is responsible for the breakdown of the miRNA target message. The cDNA sequences for Dicer and Ago2 have yet to be identified in pigs. Our objective is to identify the cDNA sequence for porcine Dicer (pDicer) and Ago2 (pAgo2) and verify their expression in reproductive tissue. A PCR cloning strategy was implemented, using over-lapping primers generated to highly conserved nucleotide sequences of Dicer and Ago2 known from other species. tcRNA was isolated from porcine ovaries and subjected to endpoint RT-PCR. To generate PCR primers, the cDNA sequences for bovine, human, and mouse Dicer and Ago2 were aligned; primers were generated from highly conserved regions using the Vector NTI program. Eight primer sets were designed for overlapping fragments of the pDicer sequence, and five primer sets were designed for pAgo2. PCR reactions were visualized using slab gel electrophoresis, EtBr staining, and UV-light exposure; after which, they were subcloned into the pDrive Cloning Vector and subjected to dideoxy-sequencing at the Clemson

University Genomics Institute. Sequences were submitted to BLAST to verify each fragment as pDicer or pAgo2. Respective sequence fragments were assembled to generate the complete coding cDNA sequence for pDicer and pAgo2. Sequencing revealed two possible splice variants for pAgo2. Two additional primer sets were designed to confirm these splice variant sequences. The obtained pDicer sequence is 5,995 nt, contains the entire coding region, and exhibits a sequence identity of 91% to bovine, 90% to human, and 86% to mouse Dicer sequences. The obtained pAgo2 nucleotide sequence is 2,703 nt with a sequence identity of 94.2% to bovine, 92.2% to human, and 89.4% to mouse Dicer sequences. The sequencing data also indicate two possible splice variants of pAgo2, which could indicate the presence of as yet unidentified Ago2 variants in other species. Altogether, the data indicate that Dicer and Ago2 are present in porcine ovary and that the sequences are highly similar to those reported for other species. In addition, endpoint RT-PCR indicates that both Dicer and Ago2 are present in porcine embryos during early embryonic development, suggesting a role for miRNA in early embryonic development in pigs.

DEDICATION

This thesis is dedicated to my family: To Mom and Daddy, for their never-ending sacrifices and life-talks, and for their persistence and faith. To my husband, for his love and patience through everything. To my Mom- and Dad-in-law, for their support and continual encouragement. To my church family for always loving, always praying, and always being there. And, most importantly, to Abba, for walking with me every step of the way. I could not have done it without each one.

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CHAPTER ONE

LITERATURE REVIEW

Introduction

Assisted reproductive technologies (ART) have been useful tools in the livestock industry for decades; and have also been utilized in human medicine to help infertile couples. In spite of the usefulness of these tools, *in vitro*-produced embryos exhibit aberrations in development including decreased developmental rates and increased chromosomal abnormalities (McCauley *et al*, 2003; Hyttel *et al*, 2000). However, the cause/effect relationship of embryo culture to these developmental issues is unknown. Recently, a class of small non-coding RNA has been shown to be involved in development, including embryonic development, and may help explain altered embryonic development when using ART. These 22 nt RNA are called microRNA (miRNA). miRNA was shown to play a key role in embryo development when the knock-out of Dicer, an important protein in the production of miRNA, proved to be embryo lethal (Bernstein *et al*, 2003). By binding to messenger RNA (mRNA) and silencing or degrading that message, miRNA function to regulate translation and does so via another protein known as Argonaute. Argonaute is the main protein component of the RNA induced silencing complex (RISC) which carries miRNA to its target message RNA (mRNA). Four Argonaute proteins have been identified in humans (Sasaki *et al*, 2003), only one of which, Argonaute2 (Ago2), exhibits endonuclease activity to degrade the mRNA to which miRNA binds (Meister *et al*, 2004; Liu *et al*, 2004). Our objective is to understand the miRNA pathway in pigs, including: identifying miRNA expressed in

reproductive tissues (including ovary, oocytes, sperm, and embryos), describing the ontogeny of this pathway, and identifying the major proteins involved in the systhesis and action of miRNA. The nucleotide sequence for Dicer and Ago2, have yet to be identified in pigs. The objective of this study was to clone the cDNA for porcine Dicer and Ago2 (pDicer and pAgo2, respectively), as well as verify their expression in multiple reproductive tissues including ovary, oocytes, sperm, and developing embryos. We hypothesize that the Dicer and Ago2 sequences are highly conserved in pigs and that their messages are expressed in porcine embryos during the time of embryonic genome activation. Further, *in vitro* produced embryos could be compromised due to inadequate levels or abnormal timing of expression of Dicer and Ago2 during development.

Assisted Reproductive Technology

For over three decades, ART has been used to assist both human and livestock disciplines. In humans, these technologies are used to help overcome infertility; while in livestock, these technologies are useful in reducing generation intervals and increasing the number of offspring from genetically superior animals (Boerjan *et al*, 2000). ART has also become an invaluable tool in biomedical research; for example, the technology has facilitated the creation of transgenic disease models used in studying dozens of human diseases (as reviewed by Matsunari and Nagashima, 2009).

Since the birth of the first "test-tube baby" in 1978, reproductive technology has come a long way in its ability to overcome infertility (Sher *et al*, 2005). As defined by the Centers for Disease Control and Prevention (CDC), ART includes all treatments or

procedures involving the surgical removal of oocytes from a woman's ovaries and combining those oocytes with sperm in order to help a woman become pregnant (CDC, 2008). An ART cycle begins when a woman starts taking fertility drugs or has her ovaries monitored for follicular development. (CDC, 2008; Wright *et al*, 2006) The next step is egg-retrieval; after which, the eggs are combined with sperm in the laboratory. The subsequent embryos are evaluated for transfer to the woman. If the transfer is successful, implantation occurs and the cycle progresses to clinical pregnancy to be followed lastly by a live-birth delivery. An ART cycle could also begin when frozen embryos are thawed in preparation for transfer to a woman (CDC, 2008). These cycles do not involve egg retrieval, because the embryo has been fertilized from a previous cycle. At any step in this process the ART cycle can be disrupted, either for medical reasons or by the patient's choice. In 2006, 99,199 ART cycles were performed, resulting in 28,404 live births (of one or more infants) (CDC, 2008).

In human infertility clinics, common ART treatments include *in vitro* fertilization (IVF) and intracytoplasmic sperm injection (ICSI). *In vitro* fertilization (IVF) involves extracting a woman's oocytes, combining them with sperm, and then transferring the resulting embryo into the patient's uterus (reviewed by Wang and Sauer, 2006). A specialized technique known as intracytoplasmic sperm injection (ICSI) is sometimes used when there is a male infertility factor, such as low sperm motility or concentration (reviewed by Steirteghem *et al*, 2002). For this technique, the sperm is manually injected into the oocyte for fertilization to occur.

Even though ART provides advantageous tools for combating infertility, techniques have yet to be perfected. Table 1.1 is a clinic summary report from the Society for Assisted Reproductive Technology (SART) detailing the 2007 National Summary of ART clinics in the United States. The percentage of fresh embryo transfers resulting in live births was only about 46%. (SART, 2009).

Table 1.1: Society for Assisted Reproductive Technology 2007 National Summary of IVF Success Rates (www.sart.org) for fresh (not frozen) embryos from non-donor oocytes.

Fresh Embryos From Non-Donor Oocytes (for women under 35)	
Number of cycles	38,161
Percentage of cycles resulting in pregancies	45.8
Percentage of cycles resulting in live births	39.8
Percentage of retrievals resulting in live births	43.1
Percentage of transfers resulting in live births	46.1
Implantation rate	32.6

ART is also of great use to the livestock industry. With the applications of *in vitro* embryo production and embryo transfer, superior genetics can be cultivated rapidly. Also, interest is rising in producing large quantities of matured oocytes and embryos for biomedical research purposes, and given their physiological similarities to humans, pigs have become an increasingly important livestock species for xenotransplantation, disease models, and carrying cell marker genes (as reviewed by Matsunari and Nagashima, 2009).

Pigs have been recognized as excellent models for numerous diseases in a variety of areas, including: nutrition, toxicology, dermatology, diabetes, cancer, eye diseases, cardiovascular diseases, degenerative joint diseases, and skeletal growth (as reviewed by Matsunari and Nagashima, 2009). ART techniques such as ICSI and somatic cell nuclear transfer (SCNT) are used for creating these transgenic pig disease models. SCNT is the process used to create genetically identical individuals (reviewed by Wilmut *et al*, 2002). In this process, the nucleus is removed from a somatic cell and inserted into a de-nucleated oocyte. The re-nucleated oocyte is stimulated into dividing and proceeds into development. With SCNT, it is possible to create syngeneic, or genetically identical, individuals. Figure 1.1 illustrates a syngeneic donor-recipient system in pigs using SCNT technology. Syngeneic siblings are genetically modified to create disease models and individuals with fluorescent marker genes. Because they are syngeneic, they are capable of avoiding transplant rejection amongst themselves, making them valuable tools for stem cell therapy and transplantation research. (Matsunari and Nagashima, 2009)

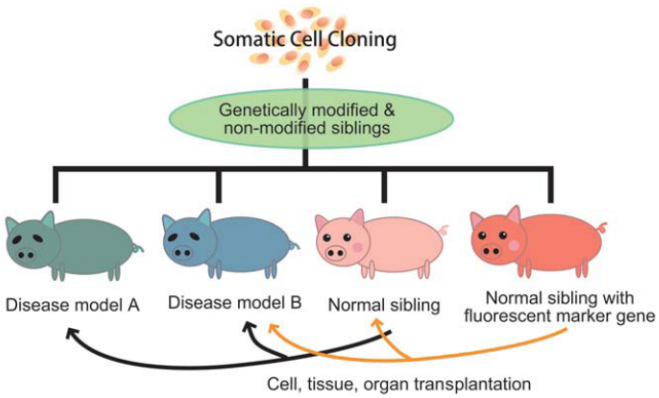

Figure 1.1: Cloned pig model for syngeneic background for translational research (Matsunari and Nagashima, 2009)

In vitro: Maturation, Fertilization, Culture

A study by Kikuchi in 2004 examined the development of porcine oocytes matured and fertilized *in vitro*; subsequent embryos were developed in either *in vivo* or *in vitro* conditions. For the *in vivo* conditions, oocytes were matured and fertilized *in vitro*, and transferred to the oviducts of recipients directly after fertilization. In contrast, for the *in vitro* blastocyst conditions, oocytes were matured and fertilized *in vitro* and then were either (1) cultured *in vitro* for 2 days and then transferred to recipient's oviducts or (2) cultured 6 days without transfer to the oviducts of recipients. Of the *in vivo* cultured blastocysts, 37% had developed to the blastocyst stage, with a mean cell number of 181.5 per blastocyst. For those cultured *in vitro* for 2 days or 6 days only 4.7% and 20.1%, respectively, developed to the blastocyst stage, most of which were still in their zona pellucida (indicating developmental delay); the mean cell numbers for the blastocysts were 58.2 and 38.4, respectively. A second experiment examined the effect of duration of *in vitro* culture (IVC) on the ability of the embryos to develop to the fetal stage or to term. Oocytes were matured and fertilized as in the first experiment, and were cultured in NCSU-37 media for 0, 24 or 48 hours. Development to fetuses, after transfer, of those cultured for 24 and 48 was significantly lower than those that were not cultured (1.7% and 2.0%, respectively, versus 6.7%). These results indicated that porcine IVM/IVF oocytes have high potential for developing to the blastocyst stage, but that the culturing conditions used were suboptimal for embryo development. (Kikuchi, 2004)

Wang and colleagues (1999) examined porcine embryos cultured *in vitro* versus those produced *in vivo* and evaluated morphology and actin filament organization. The

embryos cultured *in vitro* showed developmental delay, fragmentation, and a decrease in blastocyst nuclear number, all indicative of decreased developmental ability of embryos cultured *in vitro*. No embryo fragmentation was observed in the *in vivo* produced embryos. These researchers suggested the problems in development were most likely due to suboptimal culturing conditions.

In vitro culture conditions have higher oxygen concentrations than *in vivo* conditions, and studies have reported higher levels of reactive oxygen species (ROS) formation in these higher oxygen conditions (Goto *et al*, 1993). A study by Yang and colleagues evaluated the relationship between the presence of ROS and embryo fragmentation in embryos produced *in vitro* (1998). They found a direct correlation between embryo fragmentation and H_2O_2 concentration in the developing embryo.

Many studies have been conducted to examine how to improve oocyte *in vitro* maturation (IVM) conditions for subsequent development, including evaluating effects of gonadotropins (Sha *et al*, 2009), epidermal growth factors (Akaki *et al*, 2009), leptin and ghrelin (Suzuki *et al*, 2009) on porcine oocyte maturation. Sha and colleagues (2009) found that an increase of gonadotropins, including FSH, LH, and hCG, in the culture medium increased the percentage of oocytes that reached Metaphase II. They also noted higher levels of nuclear and cytoplasm maturation with higher concentrations of gonadotropins. Suzuki and colleagues, however, noted no improvement of maturation with the addition of ghrelin and leptin (2009). According to a review of *in vitro* production of oocytes and embryos by Abeydeera (2002), fetal calf serum and follicular fluid are often used successfully for the maturation of oocytes; however, with the use of

these substances, exact culture conditions are difficult to reproduce, creating variability among laboratories.

It is evident that *in vitro* produced embryos experience a high occurrence of abnormal embryo development. Consequently, though all of the assisted reproductive technologies are valuable tools in treating human infertility, aiding livestock production, and advancing biomedical research, the remaining inefficiencies render their full potential unmet.

Assessment of Embryo Quality

Embryo quality assessment has been largely based on attributes that can be observed, such as fragmentation, evenness of cellular cleavage, and cleavage rate. Livestock embryos are assigned grade score numbers based on the following criteria: regularity of shape, compactness of blastomeres, variation in cell size, color/texture of cytoplasm, diameter of embryo, extruded cells, regularity of zona pellucida, and presence of vesicles. Grade scores for cattle are as follows: 1 – excellent/good, 2 – fair, 3 – poor, 4 – dead/degenerating (Selk, 2009). In 1992, an embryo scoring technique was proposed by Steer and colleagues for human embryo assessment. This technique, known as the cumulative embryo score (CES) combines embryo morphology, cleavage rate, and number of embryos transferred into a single figure that represents their overall quality and is applied to embryo selection prior to transfer. These criteria helped increase pregnancy rates from 4% to 35% in older women (Visser and Fourie, 1993). A retrospective study of transferred human embryos indicated top quality embryos as:

absent of multinucleated blastomeres, containing four or five blastomeres on Day 2, seven or more cells on Day 3, and less than 20% fragmentation (Van Royen *et al*, 1999).

A review by Ebner and colleages (2003), however, suggests that embryo selection based on morphological criteria can be imprecise, which can lead to the transfer of embryos that are abnormal. For example, one study indicated that only 48% of embryos that would have been chosen at Day 3 for transfer or cryopreservation were chosen for such on Day 5/6 at the blastocyst stage (Graham *et al*, 2000). However, this is somewhat misleading, as further culture could be contributing to the degradation of the embryo, i.e. a viable embryo at Day 3 may no longer be viable after 2 to 3 days of *in vitro* culturing.

More recently, studies have been conducted to evaluate other non-invasive methods of determining embryo quality. For example, evaluating the culture media for specific protein levels associated with embryo quality would give researchers a biochemical means to more accurately predict and measure embryo quality without jeopardizing embryo viability. Such studies have evaluated embryonic platelet-activating factor (PAF), amino acid turnover, and soluble human leukocyte antigen-G (sHLA-G) (Roudebush *et al*, 2002; Brison *et al*, 2004, Booth *et al*, 2007; Warner *et al*, 2008). Embryonic PAF in the embryo culture media was measured and correlated with pregnancy outcomes. Patients receiving embryos producing low PAF levels had significantly reduced pregnancy rates (60%) than those from medium or high PAF levels (85% and 89%, respectively) (Roudebush *et al*, 2002). Assays of amino acid turnover in the culture media have also been evaluated as a possible means of selection (Brison *et al*,

2004, Booth *et al*, 2007). The turnover of three amino acids, Asn, Gly, and Leu, was significantly correlated with clinical pregnancy and live birth (Brison *et al*, 2004).

Gene expression studies have also been utilized to evaluate oocyte and subsequent embryo quality. A recent study by van Montfoort and colleagues (2008) used microarray analysis to evaluate embryo viability. Using early embryo cleavage (EEC) as a viability parameter, gene expression was analyzed for cumulus cells from oocytes that resulted in EEC and for those from oocytes that did not result in EEC. The most differentially expressed genes in the oocytes that did not result in EEC were genes that are involved in responding to hypoxic conditions or delayed oocyte maturation (van Montfoort *et al*, 2008). A similar study by Assou and colleages (2008) demonstrated that the expression of specific genes – such as Bcl-2-like protein 11 (BCL2L11), phosphoenolpyruvate carboxykinase 1 (PCK1), and nuclear factor-IB (NFIB) – in cumulus cells was significantly correlated with embryo potential and pregnancy outcome and proposed that these genes could be biomarkers for predicting pregnancy.

Because these gene expression studies have been conducted in human infertility clinics, however, researchers have been unable to compare *in vitro*-produced embryos with *in vivo*-produced embryos. Evaluating gene expression in *in vivo*-produced embryos versus *in vitro*-produced embryos would better elucidate genetic differences as well as the best biomarkers for predicting those *in vitro*-produced embryos with the best genetic potential. The flaws in differential expression studies detecting mRNA are that (1) protein expression should still be verified, and (2) it is now known that mRNA may be

present and not expressed due to post-transcriptional regulation by non-coding small RNA such as miRNA.

MicroRNA

MicroRNA (miRNA) has been hypothesized to have fundamental roles in mammalian embryonic development (Houbaviy, 2003). An important regulator of translation, miRNA has a key role in RNA interference (RNAi) in one of two ways: inhibiting translation when imperfectly paired with messenger RNA (mRNA), or degrading mRNA when perfect complimentarity binding occurs (Figure 1.2). miRNA are expressed during early embryonic development in all mammalian species examined to date (reviewed by Ouellet *et al*, 2006). Therefore, it is extremely likely that the post-transcriptional regulatory mechanism utilizing miRNA is present and active during early development.

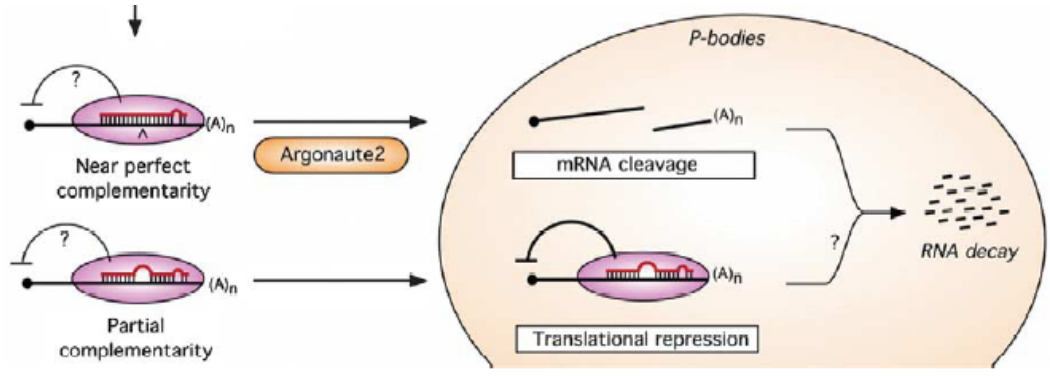

Figure 1.2: Illustration of miRNA functioning in RNAi by cleavage or repression (Wienholds and Plasterk, 2005).

History

In 1993, Lee and colleagues uncovered a novel regulatory mechanism in the discovery of the first miRNA, *lin-4*, in *C. elegans*. Previous studies revealed *lin-4* represses the translation of *lin-14*, a protein whose concentrations affect larval development. The *lin-14* transcript levels are constant throughout larval development; the LIN-14 protein levels, however, is abundant in late stage embryos but decreases dramatically by later larval stages (Wightman *et al*, 1993). This change in protein abundance without a change in transcript abundance indicates a post-transcriptional control mechanism.

Analysis of the *lin-4* genomic sequence elucidated that *lin-4* did not encode a protein; instead two transcripts were identified, approximately 22 and 61 nucleotides long (Lee *et al*, 1993). These RNA were found to be complimentary to the 3'UTR region of the *lin-14* transcript (Wightman *et al*, 1993), indicating that *lin-4* RNA regulated *lin-14* translation via an antisense mechanism, in which the miRNA binds to its complimentary target message RNA and subsequently blocks its translation.

In spite of this remarkable discovery, another miRNA was not discovered for many years, leaving the scientific community with the impression that *lin-4* was an oddity in *C. elegans*. However, the discovery of let-7 (Reinhart *et al*, 2000) gave rise to miRNA's establishment as a new class of regulatory molecules; though this molecule was discovered in *C. elegans*, it is conserved throughout metazoans (Pasquinelli *et al*, 2000). As of March 2009, there are 9,539 miRNA sequences reported in the miRBase: Sequence database Registry online (release 13.0), representing over 100 species. Also, the

miRBase current target database (v5) has predicted targets in over 500,000 transcripts for miRNAs in 24 species (Griffiths-Jones *et al*, 2008). Most of the mature miRNA sequences, for these species, have been experimentally verified (Table 1.2).

Table 1.2: The number of published mature miR sequences from select species; data from the 7.0 release in 2007 (Griffiths-Jones *et al*, 2008).

	Mature miR sequences	
	Distinct Forms	Experimentally verified
Homo sapiens	555	546 (98%)
Mus musculus	461	455 (99%)
Danio rerio	193	183 (95%)
Caenorhabditis elegans	135	135 (100%)
Drosophila melanogaster	88	85 (97%)
Arabidopsis thaliana	199	199 (100%)
Populus trichocarpa	215	55 (26%)

It has been estimated that mammalian genomes encode up to 1,000 miRNAs (Berezikov *et al*, 2005). However, in the 10.0 release of the miRBase, only 54 miRNA sequences were reported in pigs, and none of those predicted sequences had been experimentally validated (Griffiths-Jones *et al*, 2008; Kim *et al*, 2008). The first experimental validation of pig miRNA was published only last year, in which 25 porcine miRNAs were identified via sequence analysis of a cDNA library; 19 of those miRNA were previously unreported (Kim *et al*, 2008). Currently, 77 miRNA sequences are reported in the miRBase for pigs, 35 of which have yet to be experimentally verified (Griffiths-Jones *et al*, 2008). The number of predicted miRNA in pigs is likely to be so low due to the incomplete pig genome database; because of this, experimental approaches are best for identifying new porcine miRNA.

Synthesis

miRNA are short, non-coding, single-stranded, ribonucleic acids encoded in the genome. miRNA genes may contain their own promoters and enhancers, or be located within introns and exons (as reviewed by Zhao and Srivastava, 2007). As illustrated in Figure 1.3, RNA polymerase II transcribes miRNA genes into primary-miRNA (pri-miRNA) several kb long (Lee *et al*, 2002). These pri-miRNA are further processed in the nucleus by a Ribonuclease (RNase) III enzyme, Drosha, and its double-stranded RNA-binding domain (dsRBD) protein partner, Pasha, into pre-miRNA approximately 65-75 nucleotides (nt) in length (Lee *et al*, 2003; Denli *et al*, 2004; Gregory *et al*, 2004). This Drosha-Pasha Microprocessor complex leaves the pre-miRNA with a 2 nt 3' overhang, which is characteristic of dsRNA cleavage by RNase III. The pre-miRNA is then exported to the cytoplasm by Exportin-5 and Ran-GTP (Yi *et al*, 2003; Lund *et al*, 2004), which recognizes the characteristic 2 nt overhang. Once in the cytoplasm, the pre-miRNA is further processed by another RNase III enzyme, Dicer, and its dsRBD: Loquacious in *Drosophila* (Saito *et al*, 2005), or the *trans-activator RNA* (*tar*)-binding protein (TRBP) in mammals (Haase *et al*, 2005). Dicer also recognizes and binds the characteristic 2 nt overhang and cleaves the pre-miRNA into a double-stranded microRNA (ds-miRNA) approximately 21 nt in length. The ds-miRNA is then unwound and loaded into the effector complex known as the RNA-induced silencing complex (RISC), which will carry the miRNA to its target (Faller and Guo, 2008).

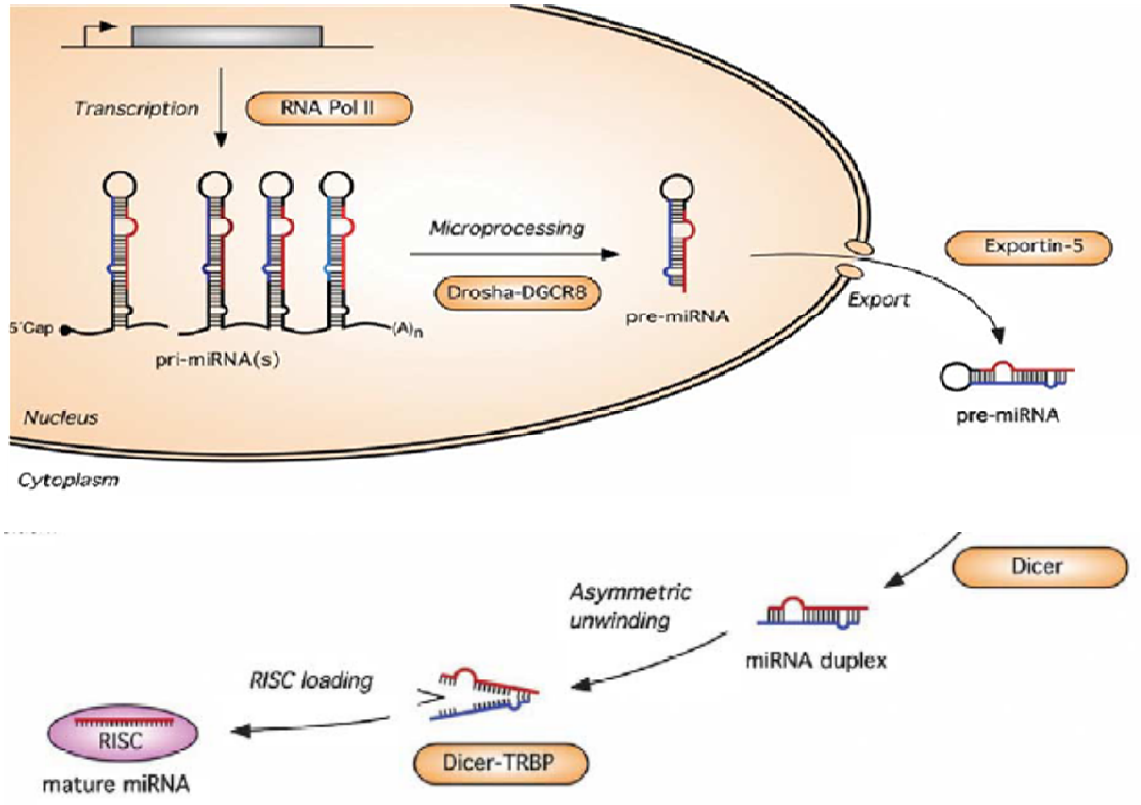

Figure 1.3: miRNA synthesis (Wienholds and Plasterk, 2005).

Function

As previously mentioned, miRNA are important regulators of translation. Their most noted roles have been through RNA interference (RNAi) pathways that result in either the silencing of the target mRNA or the breakdown of targeted mRNA. (For more detail, see the section "Mechanisms of Argonaute: RISC," page 34.) However, Vasudevan and colleagues (2007) demonstrated that miRNA also have a role in up-regulating translation. In their study, miR-369-3 up-regulated tumor necrosis factor-α (TNFα) mRNA during cell cycle arrest. They found that, upon cell cycle arrest, the AU-

rich elements (ARE) in the 3' untranslated region (UTR) of the TNFα message was transformed into a translation activation signal that recruited factors associated with the miRNA machinery, known as micro-ribonucleoproteins (microRNP). Two other miRNA, let-7 and a synthetic miRNA miRcxcr4, also activated translation of their respective messages. In proliferating cells, however, all of these miRNA repressed translation. This indicated that, dependent on the cell cycle, microRNP may in fact alternate between repression and activation of translation.

Regulation

A recent study by Bethke and colleagues (2009) revealed a hormone-mediated regulatory mechanism for miRNAs in *C. elegans*. The *C. elegans* nuclear receptor DAF-12 regulates developmental progression in response to the environment. In favorable environments, ligands bound to the DAF-12 receptor and development continues into the next larval stage; in unfavorable conditions, ligands were suppressed and DAF-12 induced arrest in a particular larval stage. This study demonstrated that, when in unfavorable conditions and unbound to its ligand, DAF-12 repressed miRNA expression and led to developmental arrest. On the other hand, in favorable environments, DAF-12 was bound to its ligand and activated miRNA *let-7* homologs which down-regulated their target, *hbl-l*, and allowed for development to the next larval stage; illustrated in Figure 1.4 (Bethke *et al*, 2009). It is known that DAF-12 is down-regulated by let-7 during later stages of development, suggesting both feed-forward and feed-back loops for initiating larval transitions (GroBhans *et al*, 2005).

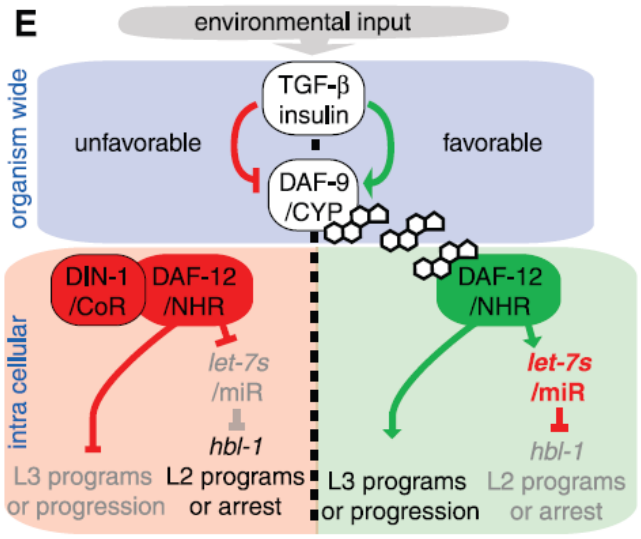

Figure 1.4: Illustration of miRNA regulation by the nuclear hormone receptor DAF-12 (Bethke *et al*, 2009).

Involvement in Embryonic Development

In 2003, Bernstein and others demonstrated that disruption of the *Dicer1* gene in mice was embryonic lethal, indicating an important role for miRNA in embryonic development. The *Dicer1* gene was functionally knocked out; afterward, chimeric mice were created which were able to transmit the *Dicer1* disruption through the germ line. Of the mice born from these crosses, none were homozygous mutants indicating this trait is embryonic lethal. In evaluating the *Dicer1*-deficient embryos at different developmental stages, it was elucidated that development is interrupted before gastrulation; embryos were arrested around day 7.5 (Bernstein *et al*, 2003). The results of this study strongly indicated that miRNA pathway components are required for vertebrate development.

Studies of individual miRNAs have revealed their involvement in specific developmental processes. For example, miR-1-1 and miR-1-2 are abundantly expressed

in the developing heart (reviewed by Zhao and Srivastava, 2007). Overexpression of miR-1 in the developing mouse results in decreased ventricular proliferation and expansion. miR-196 is involved in HOX gene regulation, which is important in developmental patterning. Table 1.3 lists other specific miRNAs, along with their targets and functions (as reviewed by Wienholds *et al*, 2005).

Table 1.3: Biological function of miRNA (Wienholds *et al*, 2005).

miRNA	***Function(s)***
Ceanorhabditis elegans	
lin-4	Early developmental timing
let-7	Late developmental timing
lsy-6	Left/right neuronal asymmetry
miR-273	Left/right neuronal asymmetry
Drosophila melanogaster	
bantam	Programmed cell death
miR-14	Programmed cell death and fat metabolism
miR-7	Notch signaling
Mus musculus	
miR-196	Developmental patterning
miR-181	Hematopoietic lineage differentiation
miR-1	Cardiomyocyte differentiation and proliferation
miR-375	Insulin secretion
Human and other vertebrate cell lines	
miR-16	AU-rich mediated mRNA instability
miR-32	Antiviral defense
miR-143	Adipocyte differentiation
SVmiRNAs	Susceptibility to cytotoxic T cells
Cancer in humans	
miR-15-miR-16	Downregulated in B-cell lymphocyte leukemia
miR-143, miR-145	Downregulated in colonic adenocarcinoma
miR-155/BIC	Upregulated in diffuse large B-cell lymphoma
let-7	Downregulated in lung cell carcinoma
miR-17-92	Upregulated in B-cell lymphoma

Houbaviy and colleagues (2003) identified possible embryonic stem (ES) cell specific miRNAs. Identified as part of a hairpin cluster and including miR-290, miR-291-as, miR-292-as, miR-293, miR-294, and miR-295, these miRNA were expressed in mouse ES cells, but were not detected in differentiated ES cells or adult tissues. A more recent study by Yu and colleagues (2006) elucidated that expression levels of miRNA targets are lower in all mouse and Drosophila tissues than in the respective embryos. These studies indicated a role for miRNAs in regulating pluripotency and early mammalian development.

Knockout of Dicer (and, therefore, knockout of miRNA) studies in zebrafish indicate that dicer mutant embryos develop normally until about Day 8 when maternal Dicer is no longer present. Further studies, however, indicated that even embryos lacking maternal Dicer are able to develop normally for the first 24 hours, indicating that miRNAs are not essential for early development in the first 24 hours. These embryos begin showing defects during gastrulation, brain formation, neural differentiation, somitogenesis, and heart development. In mice, however, Dicer mutant embryos die prior to axis formation and demonstrate ES cell loss. The difference between mice and zebra, in regards to development associated with miRNA loss, may be due to the developmental timing of each species (zebrafish develop much more rapidly than mice).

While miRNA involvement in developmental processes has been the focus of much research, less is known about the function of miRNA in fertilization. A study by Amanai and colleagues (2006) identified miRNA in mouse sperm. The levels of sperm

miRNA, however, were relatively low in comparison to unfertilized MII oocytes, and did not alter the miRNA profile after fertilization, indicating a limited role in fertilization.

miRNA have been identified in all tissues examined to date and have been shown to have important roles in developmental processes, as described above. Timing of miRNA expression is also very important in these processes. Given the important roles of Dicer and Argonaute2 in miRNA synthesis and function, the timing of their expression may be crucial to embryonic development.

Dicer

Exhibiting a molecular weight of approximately 200kDa, Dicer was first identified by Bernstein and colleagues in 2001 as the enzyme which produces the approximately 21 nt miRNA. Dicer is an RNase III enzyme, a class of enzymes that show specificity for dsRNA; this made it an obvious choice when looking at potential enzymes involved in the miRNA production pathway. Once identified, Bernstein and colleagues named this RNase III enzyme "Dicer" because of its ability to cleave RNA into consistently short 21 nt RNA.

Interestingly, in multicellular eukaryotes, the components of RNA-mediated silencing have significantly diversified, while in unicellular organisms there seems to have been a near complete loss of the RNA-silencing machinery. Thus far, Dicer has been identified in all species examined, except *Saccharomyces cerevisiae*, (reviewed by Jaskiewicz and Filipowicz, 2008) and is highly conserved across species. Plants encode four Dicer genes, fungus and insects encode two, while mammals encode only one

(reviewed by Ji, 2008). In plants, Dicer proteins seem to be localized to the nucleus (Xie *et al*, 2004; Hiraguri *et al*, 2005). In *Chlamydomonas reinhardtii*, a unicellular eukaryote that encodes three Dicer proteins, at least one Dicer (DCL1) is likely restricted to the nucleus (Casas-Mollano *et al*, 2008). Mammals, however, express one Dicer protein which seems to be only localized to the cytoplasm (Billy *et al*, 2001; Provost *et al*, 2002).

Structure

A large, multidomain protein, human Dicer (hDicer) is composed of six domains, including: helicase, a domain of unknown function (DUF 283), PAZ, two RNase III domains, and a double-stranded RNA-binding domain (dsRBD) (Figure 1.5). It is the characteristic of having two RNase III domains that classifies Dicer as an RNase III, class II enzyme as described by Jaskiewicz and Filipowicz (2008). Dicer functions as a monomeric protein, in which the two RNase III domains form a pseudo-dimer-type catalytic domain, as described below (Zhang et al, 2004; MacRae et al, 2006).

The PAZ domain, which is also found in Argonaute proteins, has been shown to contain an oligo-binding (OB)-like fold. OB-folds specifically recognize dsRNA ends that have a 2 nt overhang on the 3' end, as is characteristic of processing by an RNase III enzyme. In this way, the miRNA processing pathway has a recognition mechanism. Drosha processes pri-miRNA into pre-miRNA and leaves a 2 nt 3' overhang that will be recognized first by Exportin-5 for transporting the miRNA to the cytoplasm and then by Dicer which will further cleave the pre-miRNA (MacRae *et al*, 2006).

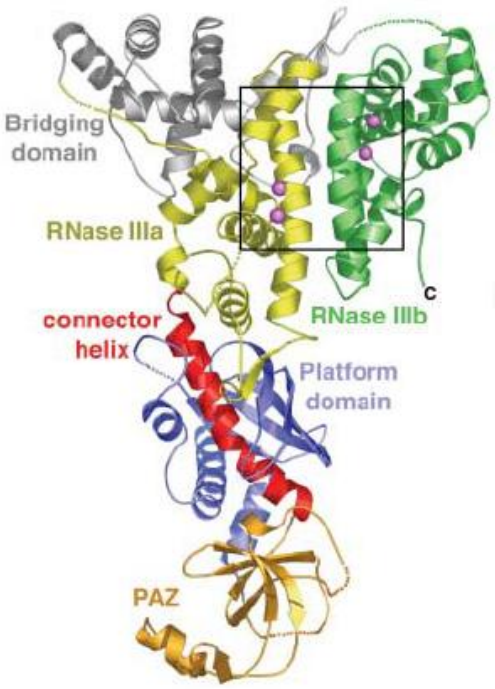

Figure 1.5: Structure of *Giardia* Dicer; box notes the two active sites formed between RNase IIIa domain and RNase IIIb domain (MacRae *et al*, 2006).

The two RNase III domains are only functional when they are dimerized. They fold together and two active sites are formed across the surface of the dimer, as shown in Figure 1.6. These active sites cleave the RNA. These active sites are offset from one another, indicating the mechanism by which this enzyme will leave a 2 nt overhang when the RNA is cleaved. Interestingly, when MacRae and his colleagues measured from the catalytic active sites to the 3' binding pocket in the PAZ domain, they discovered that it is 65 angstroms (Å), which is equivalent to about 25 nt. Consequently, not only is Dicer the scissors that cleave ds-miRNA, it is the molecular ruler that determines its final length, as illustrated in Figure 1.7. (MacRae *et al*, 2006)

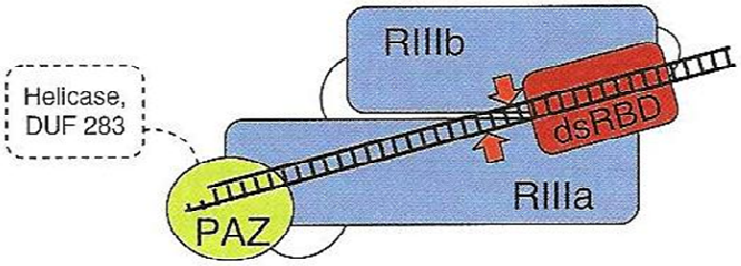

Figure 1.6: Cartoon depiction of the dimerized RNase III domains of Dicer; arrows indicate the two cleavage sites (Zhang *et al*, 2004).

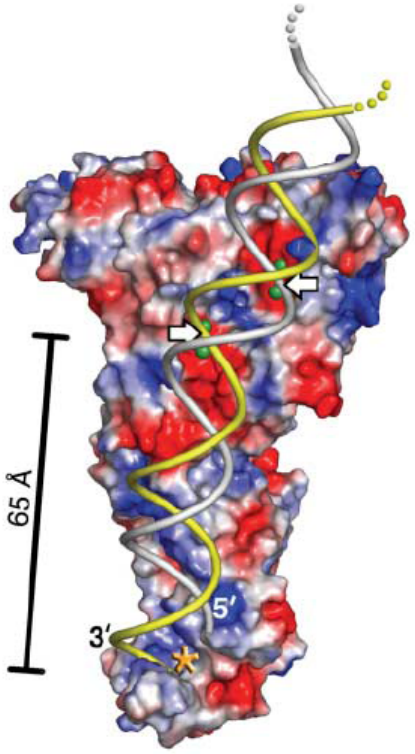

Figure 1.7: Model of Giardia Dicer bound to pre-miRNA. Asterisk indicates the 3' binding pocket of the PAZ domain; arrows indicate active sites at which cleavage occurs. The distance from the 3' binding pocket to the cleavage sites is measured at 65Å (MacRae *et al*, 2006).

Because only Giardia Dicer has been crystallized, which lacks the helicase domain, the DUF, and the dsRBD, these three domains have yet to be characterized. It has been noted, however, that though ATP stimulates the generation of siRNAs by the *C. elegans* Dicer and one of the two Drosophila Dicers, as is characteristic of an ATPase/helicase domain, the addition of ATP has no significant effect on the mammalian Dicer activity (Nyykanen *et al*, 2001; Ketting *et al*, 2001; Zhang *et al*, 2002; Liu *et al*, 2003).

Dicer Function: Plants and Drosophila

As previously mentioned, plants express four Dicer genes. These "Dicer like" (Dcl) proteins are numbered sequentially, Dcl1 – Dcl4, each having a distinct function. Dcl-1 processes miRNA precursors, including pri-miRNAs and pre-miRNAs (Kurihara and Watanabe, 2004). Dcl-2 generates siRNAs associated with antiviral defense. Dcl-3 generates siRNAs involved in chromatin modification and transcriptional silencing. Dcl-4 is required for trans-acting siRNAs (ta-siRNAs) biogeneisis and activity (Gasciolli *et al*, 2005; Xie *et al*, 2005). Because the small RNAs produced by each Dicer are involved in different processes, a mechanism must exist that mediates each one; Margis and colleagues (2006) suggest that the dsRBDs of Dicer fulfill this role.

Drosophila encode only two Dicer genes, Dicer-1 and Dicer-2, for which there are also distinct roles. Dicer-1 is involved in processing pre-miRNA for association with RISC, while Dicer-2 is involved in siRNA production for the RNAi pathway (Lee *et al*, 2004).

Protein Interaction

Dicer has been shown to have multiple interactions with other proteins; these include interactions with double-stranded (ds)-RNA binding domain (dsRBD) proteins and Argonaute proteins.

In most species examined to date, Dicer must be coupled with some sort of dsRBD in order to work properly. The first dsRBD protein required for Dicer activity was discovered in *C. elegans* by Tabara and colleagues in 1999, and was identified as Rde-4 by a genetic screen. Later, Grishok and colleagues (2000) showed that Rde-4 was required for the initiation step of RNAi in *C. elegans*, but is not required for miRNA processing.

As previously mentioned, Drosophila have two Dicer proteins with separate functions; each of these Dicer proteins partners with a different dsRBD. Dicer1 pairs with Loquacious (Loqs), while Dicer2 pairs with R2D2 (Satio *et al*, 2005; Liu *et al*, 2003). Loqs plays an important role in enhancing miRNA biogenesis, though it seems to unnecessary for assembly of miRNA-RISC (Liu *et al*, 2007). R2D2, on the other hand, does not assist in the production of siRNA but does help facilitate the assembly of the siRNA-RISC (Liu *et al*, 2003). R2D2 does this by forming a heterodimer with Dicer2 which senses the thermodynamic stability of the 5' ends of the siRNA; the more stable of the ends will bind to R2D2, while the less stable end binds Dicer. In this way, the less stable 5' end of the siRNA is incorporated into the RISC complex (Tomari *et al*, 2004).

In humans, two dsRBD proteins have been identified: TRBP (human immunodefinciency virus (HIV-1) transactivating-response (TAR) RNA-binding protein)

and PACT (PKR activator) (Haase *et al*, 2005; Lee *et al*, 2006). TRBP is required for optimal RNA silencing mediated by both siRNAs and endogenous miRNAs; it has also been shown, *in vivo*, to assist Dicer in the cleavage of pre-miRNA (Haase *et al*, 2005). Before it was identified as a Dicer binding partner, TRBP had already been assigned several functions. These include inhibition of protein kinase R (PKR), which is dsRNA-regulated and interferon-induced (Daher *et al*, 2001), and a role in modulating HIV-1 gene expression via TAR association (Dorin *et al*, 2003). The other mammalian dsRBD, PACT, is 42% identical to TRBP and also interacts with Dicer. Lee and colleagues (2006) isolated PACT in a complex with Dicer, TRBP, and hAgo2. It was then demonstrated that depletion of this protein causes an accumulation of mature miRNA in human cells, but does not affect pre-miRNA processing, indicating an important role in RISC assembly.

Argonaute proteins have also been shown to interact with Dicer. Tahbaz and colleagues (2004) demonstrated that part of the PIWI domain of Argonaute proteins, the PIWI-box, directly binds to the RNase III domain of Dicer. This interaction is dependent upon Hsp90 and inhibits the RNase activity of Dicer *in vitro*. They also showed that the interactions between Argonaute proteins and Dicer may occur in multiple cellular compartments.

Mechanisms of Dicer

Dicer mechanisms of pre-miRNA processing have been most extensively studied with human Dicer, which was over-expressed in insect cells and purified (Provost *et al*,

2002; Zhang *et al*, 2002). Mg^{2+} was required for the RNase activity of Dicer, but not for dsRNA binding; cleavage did not require ATP *in vitro* (Provost *et al*, 2002; Zhang *et al*, 2002). In fact, mutation of an essential amino acid in the ATPase domain of recombinant human Dicer had no effect on Dicer activity (Zhang *et al*, 2002). This may simply be due to lack of a fully functional recombinant Dicer protein. Zhang and colleagues (2002) proposed that if ATP did have a role in assisting Dicer in mammalian cells, it might be involved in product release from Dicer, aiding the multiple turnover of the enzyme.

By using substrates with modified ends, Zhang and colleagues (2002) also showed that Dicer process dsRNA from their termini. A few years later, they demonstrated that the processing of dsRNA and, specifically, that of pre-let-7 RNA occurs with a very low turnover rate. This is likely because the product remains associated with the enzyme. (Zhang *et al*, 2004)

Mutations in human Dicer indicated the following residues are essential for the cleavage activity of Dicer: Asp1320 and Glu1652 from the RNase IIIa domain, and Asp1709 and Glu1813 from the RNase IIIb domain. These cleavage sites generate products with 2 nt 3' overhangs. From these data, Zhang and colleagues (2004) proposed that Dicer functions via intramolecular dimerization of its two RNase III domains. The RNase IIIa domain processes the protruding 3'-OH-bearing RNA strand, and the RNase IIIb domain processes the opposite 5'-phosphate-containing strand.

These researchers went on to mutate the PAZ domain of Dicer, which greatly reduced Dicer's dsRNA-processing activity. As mentioned earlier, the PAZ domain recognizes the 3' overhangs of dsRNA; this is consistent with the finding that Dicer

cleaves dsRNA and pre-miRNA substrates containing 3' overhangs more efficiently than blunt ended dsRNA (Zhang *et al*, 2004).

With this information, Zhang and colleagues (2004) proposed a model for Dicer processing (Figure 1.8), which was later confirmed by MacRae and others (2006) when they obtained the crystal structure of the full-length Dicer from *G. intestinalis*. In this model, the 2 nt 3' overhang on the substrate is recognized by the PAZ domain and held in the binding pocket of the domain; Dicer formed an internal dimer between domains RNase IIIa and RNase IIIb, each domain processing one strand of the dsRNA, and leaving another 2 nt 3' overhang on the product.

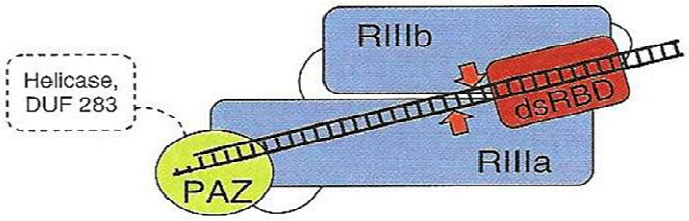

Figure 1.8: Cartoon depiction of the dimerized RNase III domains of Dicer; red arrows indicate the two cleavage sites (Zhang *et al*, 2004).

Involvement in RISC Formation

Though Dicer is essential to the processing of miRNA and siRNA, further research has revealed an important role for Dicer in the effector step of RNA silencing: the formation of the RNA-induced silencing complex (RISC). Thus far, the majority of research involving these intermediate steps has been conducted in *Drosophila*. Pham and

colleagues (2004) identified three separate intermediate complexes in *Drosophila*: R1, R2, and R3 (Figure 1.9). A precursor to R2 and R3, R1 is a complex of 360kDa consisting of Dicer2, R2D2, and possibly other unidentified proteins; its function is likely to process the long dsRNA and perhaps determine the guide/passenger strand asymmetry (Pham *et al*, 2004). The R2 complex likely functions in dsRNA unwinding; this may be initiated by the Dicer2-R2D2 complex, but requires Ago2 to proceed (Tomari *et al*, 2004). The R3 complex, also described as "holo-RISC," requires ATP and consists of: siRNAs, Dicer1, Dicer2, R2D2, Ago2, and other proteins. Pham and colleagues (2004) refer to the R3 complex as the "RNAi effector complex."

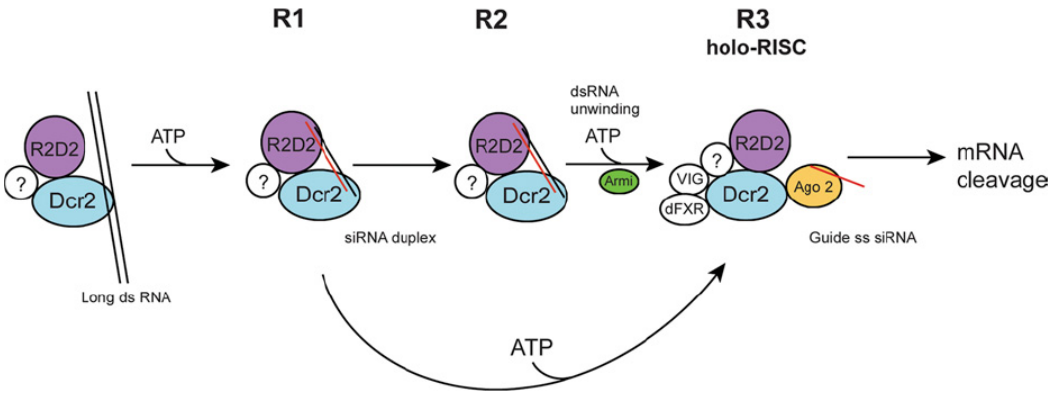

Figure 1.9: Model of RISC assembly in Drosophila (Jaronczyk *et al*, 2005)

Mammalian RISC assembly is less understood. In fact, some data indicate Dicer may not be required for RISC assembly in mammals. Cells depleted of Dicer were still capable of siRNA-mediated RISC activity (Martinez *et al*, 2002). However, multiple studies indicated Dicer does at least play a stimulatory role in the assembly and function

of RISC in that 30bp dsRNAs, which are processed by Dicer to induce RNAi, are more efficient at triggering an RNAi response than siRNAs (Kim *et al*, 2005; Rose *et al*, 2005; Siolas *et al*, 2005).

Expression

Many studies have been conducted to elucidate the consequences of loss of Dicer function using tissue-specific, conditional knockouts. These studies have shown that Dicer function, and therefore miRNA function, is essential for vertebrate development and required for the normal function of: T cells, B cells, chondrocytes, skin and hair follicles, brain, heart, skeletal muscle, lung epithelium, pancreatic islets, limb development, retina, spermatogenesis, spindle formation, corpus luteum angiogenesis, and general reproductive soundness (reviewed by Jaskiewicz and Filipowicz, 2008).

Argonaute2

Named after the squid-like phenotype of plants that lacked the functional protein (Bohmert *et al*, 1998), Argonaute proteins have been described as the "molecular scaffold" presenting small, guide RNA molecules of RNA silencing to their complementary targets (Parker and Barford, 2006). This includes Ago proteins' involvement in the RNA-induced silencing complex (RISC) as well as in the RNA-induced initiation of transcriptional gene silencing (RITS), which is involved in the assembly and function of heterochromatin (Verdel *et al*, 2004).

Argonuate proteins have a molecular weight of about 100 kDa (Cerutti, et al 2000) and have been studied in many organisms, including *Trypanosoma brucei* (*T. brucei*), *S. Pombe*, *Chlamydomonas reinhardtii* (*C. reinhardtii*), *Caenorhabditis elegans* (*C. elegans*), *Drosophila melanogaster* (*D. melanogaster*), and many mammals; the number of Argonaute genes varies across species, from one in *S. pombe* to 27 in *C. elegans* (Peters and Meister, 2007; Casas-Mollano *et al*, 2008; Shi *et al*, 2004). Eight Argonaute protein genes have been identified in humans; they are divided into two subfamilies: Ago and Piwi. The Piwi proteins, which total four, are expressed mainly in the testes. Of the Ago subfamily, there are four proteins, all of which are ubiquitously expressed in human tissues. These proteins contain the same protein motifs (the PAZ motif in the middle and the Piwi motif in the C-terminal region), which have been observed in the Ago family members of many non-human species as well, suggesting these may be important for the proteins' function (Sasaki *et al*, 2003).

A study conducted by Meister and colleagues (2005) showed that miRNA associate with all of these Ago proteins (1-4), with no preference between them. However, only one of these four Ago proteins, Argonaute-2 (Ago2), has slicing capabilities to degrade the mRNA to which miRNA binds (Meister *et al*, 2004; Liu *et al*, 2004). Also in 2004, Rand and colleagues isolated RISC and used mass spectrometry to identify proteins involved in RISC and concluded that only Ago2 composes the core RISC nuclease activity.

Structure

The crystallization of Ago2 confirmed it as the once unknown "Slicer" of miRNA-mediated mRNA degradation. Ago2 is one of many PAZ-Piwi Domain (PPD) proteins, which all have two characteristic domains: PAZ and Piwi (Figure 1.10).

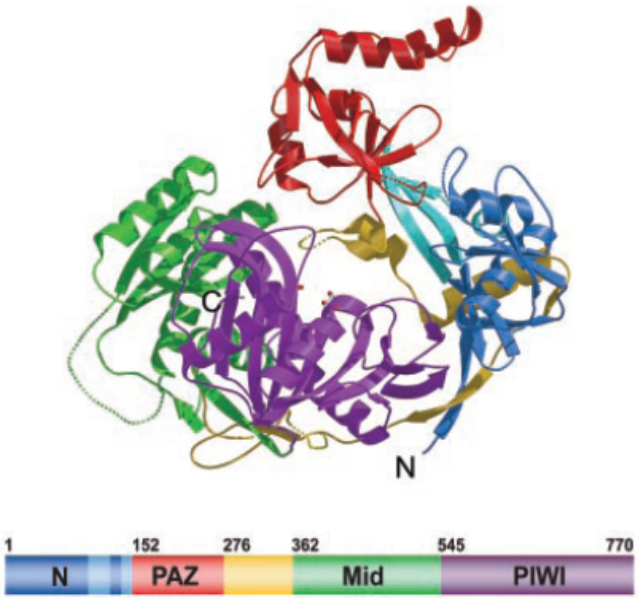

Figure 1.10: Structure of *P.furiosus* Argonaute (Song *et al*, 2004).

The PAZ domain (named after Piwi, Argonaute, and Zwilli) forms a module characteristic of nucleic-acid-binding molecules, an oligonucleotide/oligosaccharide-binding fold (OB fold), that specifically binds the 3' end of RNA (Figure 1.11). In fact, nucleic acid binding studies have shown that PAZ has a high affinity for single-stranded 3' ends and double-stranded ends with 2 nt 3' overhangs (Song *el al*, 2003; Lingel *et al*,

2003). These 2 nt 3' overhangs are characteristic of miRNA processing by the RNase III enzymes Drosha and Dicer.

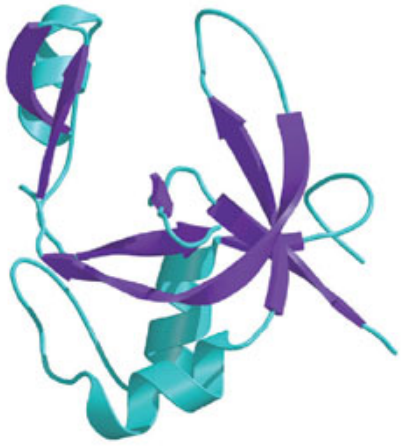

Figure 1.11: Ribbon structure of Drosophila Ago2 PAZ domain, forming an RNA-binding cleft (Song *et al*, 2003).

The crystallization of the Piwi domain immediately elucidated how Argonaute functions as "Slicer." The Piwi domain's tertiary structure matches that of the RNase H enzyme family (Figure 1.12) and is characteristic of enzymes with nuclease activities, therefore indicating the Piwi domain of Ago as the enzyme responsible for the mRNA cleavage characteristic of RNAi. In fact, in 2005, Rivas and colleagues demonstrated that Ago2 could recapitulate RISC activity.

Similar to RNase H, three residues within the Piwi domain form a catalytic triad. The human catalytic triad for Ago2 is D(597), D(669), and H(807), as identified by mutation studies (Rivas *et al*, 2005; Song *et al*, 2004). Ago2 has been identified as the only member of the human Ago subfamily with endonuclease activity (Liu *et al*, 2004; Meister *et al*, 2004). It is interesting to note that Ago3 is catalytically inactive, even

though the catalytic triad DDH is conserved (reviewed by Meister and Tuschl, 2004). This may indicate that posttranslational modifications or interactions with specific proteins may modify the activity of Ago proteins.

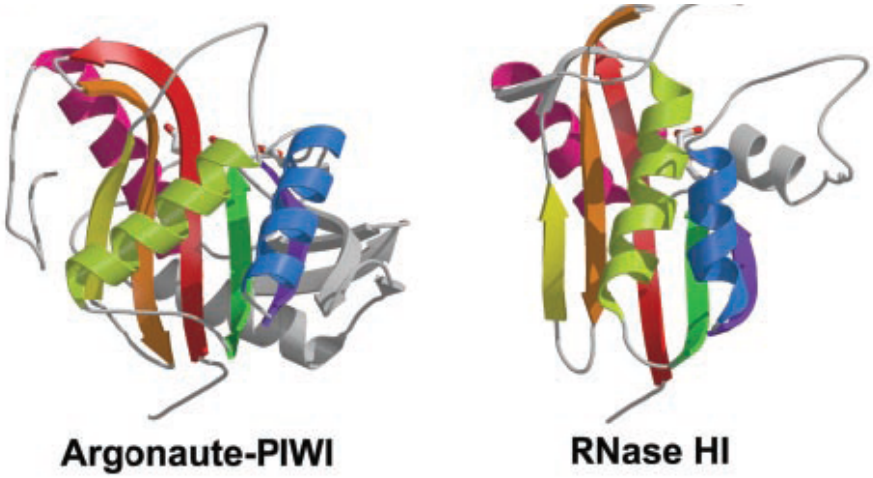

Figure 1.12: Ribbon structure of Drosophila Ago2 Piwi domain, depicting an RNase H-like structure (Song *et al*, 2004).

In a study of the AGO1 protein in the protozoan *T. brucei*, researchers uncovered an N-terminal domain with a high abundance of RGG repeats. Deletion of this domain blocked association of AGO1 with polyribosomes and severely affected mRNA cleavage. However, the mutant was still able to bind with siRNA. This N-terminal domain may be important for post-translational modifications in the RGG-rich sequences that are critical to the proper function of the protein. (Shi *et al*, 2004)

Another functionally important domain was discovered through x-ray crystallography of bacterial Argonaute proteins, located between the PAZ and PIWI

domain, and therefore dubbed the "MID domain" (Parker *et al*, 2005; Yuan *et al*, 2005). This MID domain [though one review by Parker and Barford (2006) refer to it as part of the PIWI domain] is the most conserved region in Argonaute. The region contains a highly basic pocket that binds the 5' phosphorylated nucleotide of the guide RNA strand. This 5' phosphate group is characteristic of mature miRNAs processed by Dicer. Rivas and colleagues showed that this 5' phosphate group is required for stability when in complex with hAgo2 and ensures "slicing fidelity" (2005). Interestingly, there are only nine conserved residues in the PIWI domain across divergent Argonaute sequences, four of which contact the 5' phosphate group of the guide strand (Parker and Barford, 2006). This highlights the importance of the binding pocket in this region. As illustrated in Figure 1.13, Argonaute proteins' PAZ domain binds the miRNA; at which point the miRNA binds its target, the Piwi domain of Argonaute cleaves the target message (Song *et al*, 2003; Song *et al*, 2004; Lingel and Izaurralde, 2004).

Thus far, members of the mammalian subfamily Piwi have yet to be analyzed for Slicer activity, although Drosophilia PIWI shows Slicer activity *in vitro* even though the catalytic triad is formed by DDK (Saito *et al*, 2006).

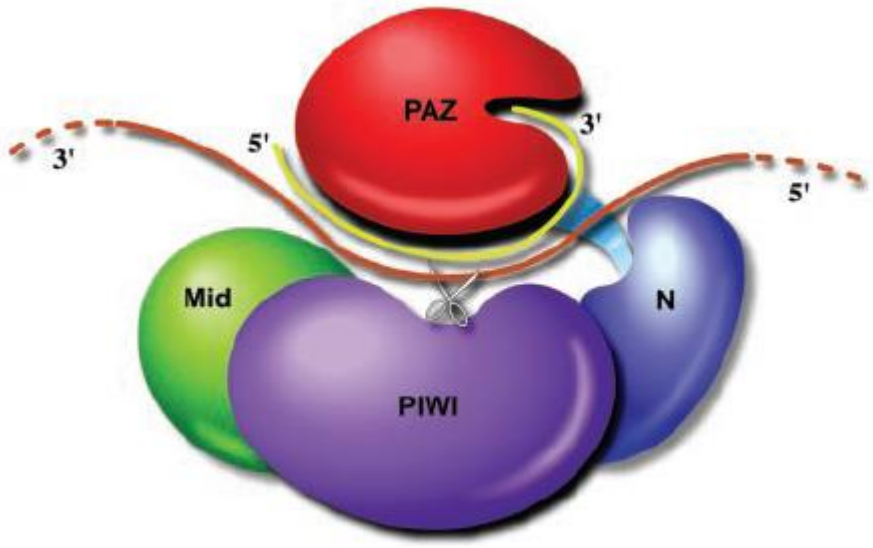

Figure 1.13: Cartoon depiction of the mechanism of Ago2. The PAZ domain binds the 3' end of the miRNA which binds its target mRNA and the Piwi domain cleaves the target message (Song *et al*, 2004).

Mechanisms of Argonaute: RISC

As reviewed above, Dicer processes miRNA precursors into approximately 21 nt long ds-miRNAs. Of this ds-miRNA, one strand is the referred to as the guide strand and will be loaded into the Ago protein; the other strand is the passenger strand and is discarded. Though it was thought that a helicase may assist with the unwinding process, Matranga and colleagues demonstrated that siRNAs are loaded into the Ago2 protein prior to release of the passenger strand after which Ago2 cleaves the passenger strand, facilitating its displacement. (Matranga *et al*, 2005)

It has been noted that for most miRNAs, only one strand from the ds-miRNA accumulates as the mature miRNA. This asymmetric loading is guided by the relative

thermodynamic stability of the 5' ends of the ds-miRNA. The less stably paired of the 5' ends in the ds-miRNA is the one preferentially incorporated into the Ago complex (Khvorova *et al*, 2003; Schwarz *et al*, 2003).

In Drosophila, R2D2 (the dsRNA binding domain (dsRBD) protein) forms a heterodimer with Dicer2 and binds the more stable end of the siRNA, thereby positioning the duplex to allow incorporation of the correct strand, as illustrated in Figure 1.14 (Tomari *et al*, 2004).

The human dsRBD proteins, as reviewed above, include TRBP and PACT. These reside independently in a complex with Ago2 and Dicer which is able to generate miRNAs from dsRNA precursors, transfer one strand to Ago2, and cleave complementary substrate RNAs. Inactivation of TRBP and PACT results in a loss of mature miRNAs, insinuating their roles in strand selection or Ago loading.

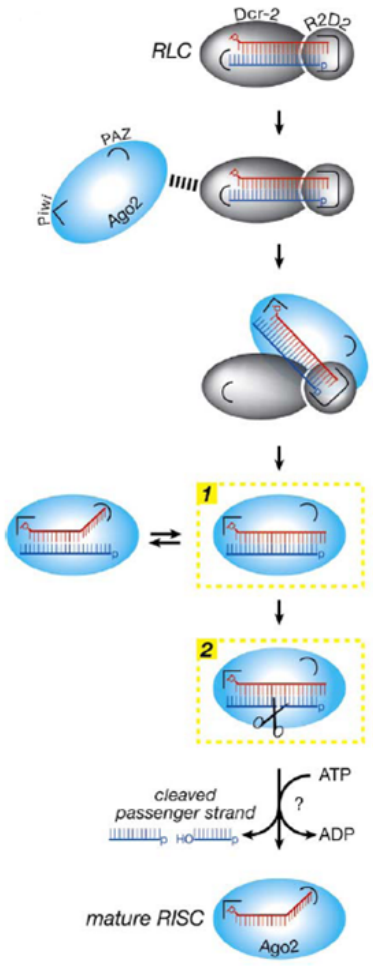

Figure 1.14: RISC assembly model for Drosophila (Matranga *et al*, 2005).

Argonaute proteins that lack slicing capabilities can still function in RNA silencing. If the miRNA guide-strand contains multiple mismatches to its target, the targeted mRNA is silenced, not sliced (Matranga *et al*, 2005). Parker and Barford propose that these Argonaute proteins may be involved in pathways that expose them to "mismatched precursors," such as miRNAs that would not fully compliment their target mRNA. These proteins include hAgo4 and Hiwi2.

The next step, after miRNA guide strand loading, is target recognition. Many studies have shown that all nucleotides in the miRNA sequence are not created equal. It appears that nucleotides 2 – 7 or 8 of the miRNA, as measured from the 5' end, compose the "seed" sequence. This seed sequence mediates the interaction with the target mRNA sequence (Lewis *et al*, 2003; Doench and Sharp, 2004; Lewis *et al*, 2005; Birmingham *et al*, 2006). It is thought that once the target strand binds the guide miRNA, the rest of the duplex continues to form onward toward the 3' end of the guide strand. Some studies indicate this may be coupled to a conformational change in Argonaute, in which the PAZ lobe of the protein retracts to make room for the duplex and position it in the active "Slicer" site of the PIWI domain, which lies under the PAZ lobe (Yuan *et al*, 2005).

Once the guide strand is bound to its target mRNA, the message then may be sliced or not (Figure 1.15). Whether or not the message is sliced is two-fold. Firstly, and simply, the bound Argonaute protein may not have an active catalytic triad, as discussed previously. Secondly, the guide strand may contain mismatches to its target sequence. Studies have shown that complementarity of the guide strand to its target is necessary for slicing, likely due to a necessary substrate geometry required in the active site that mismatches would disrupt (Haley and Zamore, 2004; Chiu and Rana, 2002; Hutvagner and Zamore, 2002).

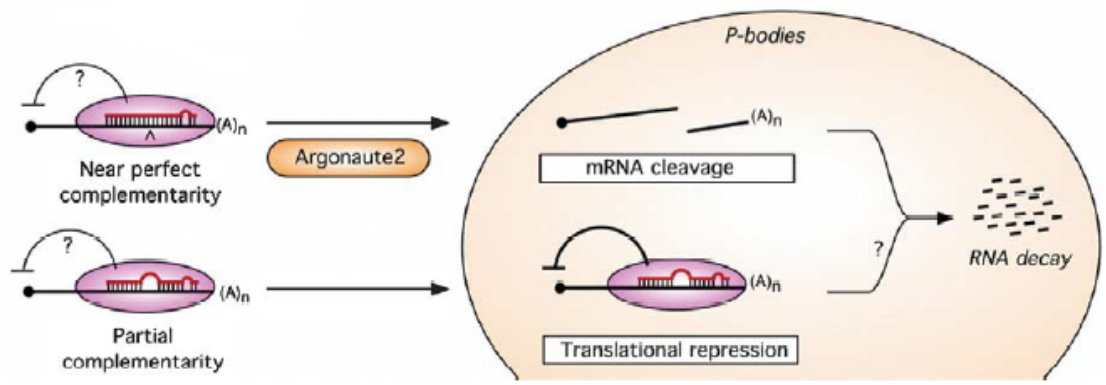

Figure 1.15: miRNA target recognition (Wienholds and Plasterk, 2005).

In studies of human RISCs containing human Argonaute1-4 (hAgo1-4), only hAgo2-RISC had any cleavage activity, even though, as previously mentioned, hAgo3 possesses a full catalytic triad and hAgo1 a partial triad (Meister *et al*, 2004; Liu *et al*, 2004). As noted by Parker and Barford (2006), these experiments may indicate the existence of other unidentified factors involved in slicing activity.

RISC has been shown to be a multiple-turnover enzyme, as indicated in a study by Hutvagner and Zamore in 2002. In another study, by Haley and Zamore (2004), the rate of multiple-turnover activity was increased by mismatches in the guide strand complementarity; the turnover rate was also increased with addition of ATP. The structure of the Ago protein in conducive to retaining the guide strand: containing a 3' binding pocket in the PAZ domain and a 5' binding pocket in the PIWI domain.

P-Bodies and Stress Granules

Localization studies have revealed that Ago proteins are enriched in distinct cytoplasmic foci, determined by colocalization studies to be P-bodies (Liu *et al*, 2005b;

Sen and Blau, 2005). P bodies are cellular sites where mRNA de-capping and degradation enzymes localize to ensure rapid and efficient mRNA turnover occurs (reviewed by Eulalio *et al*, 2007).

Depletion of P-body components, such as TNRC6A, TNRC6B, MOV-10, and Dcp1/2, inhibited miRNA-guided RISC activity (Jakymiw *et al*, 2005; Liu *et al*, 2005a; Meister *et al*, 2005; Rehwinkel *et al*, 2005). Mutated Ago proteins incapable of binding miRNA do not localize to P-bodies, suggesting that Ago proteins must first be incorporated into mRNA protein complexes (mRNPs) via miRNA-mRNA interactions in order to localize to P bodies.

Interestingly, there is a model for mRNA storage, implying that mRNA can re-localize to the cytoplasm and re-enter polyribosomes for translation. In fact, Bhattacharyya and colleagues (2006) showed that the CAT-1 mRNA, which is repressed by the liver-specific miR-122 and stored in P-bodies under normal conditions, is released upon cellular stress and is actively translated to produce CAT-1 protein (which is required for cellular stress response). Specifically, the mRNA is released from Ago by the RNA binding protein HuR, which is localized to the nucleus under normal conditions but is released into the cytoplasm during cellular stress. Here is an experimentally proven example that mRNA can be stored and released for future use.

In has been shown that Ago proteins do not only localize to P-bodies, however, but also to the diffuse cytoplasm and stress granules (SGs) (Leung *et al*, 2006). In fact, EGFP tagging of Ago2 revealed that only about 1.3% of EGFP-Ago2 localizes to P bodies, and further analysis revealed that the P-body pool of Ago proteins seemed to be

static whereas the SG-localized Ago proteins demonstrated more dynamic behavior. However, much is yet to be understood about the localization differences of Ago to SG or P-bodies.

Transcriptional Silencing

Although immunofluorescence experiments have been unable to locate Ago proteins in the nucleus, researchers suggest that it is becoming apparent that Ago proteins do indeed function in the nucleus. Nuclear functions for Ago proteins have been reported in many organisms including: *S. pombe*, plants, fungi, Drosophila, and *C. elegans* (reviewed by Matzke and Birchler, 2005). In mammals, the nuclear function of Ago has been a bit more elusive; however, a study by Janowski and colleagues in 2006 indicated that both Ago1 and Ago2 proteins associate with the progesterone receptor promoter. Also, with the depletion of human Ago1 and Ago2, no transcriptional silencing was observed, suggesting that Ago1 and Ago2 may be involved in transcriptional gene silencing as well as posttranscriptional gene silencing.

Piwi Subfamily and piRNAs

The human Piwi subfamily of Argonaute proteins was originally named after the Drosophila Piwi gene and includes: HIWI, HIWI2, HIWI3, and HILI. These proteins have been implicated in germ cell development, stem cell self-renewal, and retrotransposon silencing (Cox *et al*, 2000; Kalmykova *et al*, 2005). Members of the Piwi subfamily have been identified in many organisms, many of which seem to be germ-

cell specific (indicating functional conservation). Given all that has been uncovered about the Ago subfamily, however, the Piwi subfamily functions and properties have been less widely researched.

In the mouse, MILI and MIWI were shown to be essential for spermatogenesis (Deng and Lin, 2002; Kuramochi-Miyagawa *et al*, 2004). Interestingly, female mili knockout mice showed no abnormalities, while male mili knockouts had smaller testes and were sterile (Kuramochi-Miyagawa *et al*, 2004). Similar results were seen with miwi knockout mice, with the difference being that defects were seen at later stages of spermatogenesis (Deng and Lin, 2002).

A novel class of small RNA has been identified that interact specifically with members of the Piwi subfamily, and therefore have been dubbed piRNA. MIWI-associated piRNA are 29-31 nt, while MILI-associated piRNA are 26-28 nt. Many piRNA are not conserved from mouse to human on the sequence level. (Aravin *et al*, 2006; Girard *et al*, 2006; Grivna *et al*, 2006; Watanabe *et al*, 2006). The timing of piRNA expression and the phenotypes of Piwi knockouts indicate their role in sperm development. Also, piRNA seem to be abundantly expressed. According to Aravin and colleagues (2006), there are about 8000 copies of an individual piRNA and 1 million total piRNA per mouse spermatocyte. The function of piRNA, however, and whether or not they function similarly to siRNA or miRNA is yet unclear.

Expression

Liu and others published a study in Science in 2004 in which they disrupted the Ago2 gene in mice. Intercrosses of Ago2 heterozygotes produced only wildtype and heterozygous offspring, implicating that the disruption of Ago2 was embryonic lethal. Ago2-deficient embryo phenotypes included: defects in neural tubes, cardiac failure, and yolk sac and placental defects. They also used mouse embryo fibroblasts (MEFs) from embryos from Ago2 heterozygous intercrosses to explore whether embryos lacking Ago2 were resistant to experimental RNAi, using siRNA. These cells were unable to repress gene expression in response to siRNA, but were rescued by addition of a plasmid encoding for human Ago2. This indicates an important role for Ago2, and therefore miRNA function, in embryonic development (Liu *et al*, 2004).

Implications

Given the obvious importance of Dicer and Ago2 during embryonic development and the important role of each in the synthesis and function of miRNA, it can be concluded that miRNA is essential for proper embryonic development. Therefore, aberrant miRNA expression could be involved in impaired embryonic development. However, no information is currently available regarding the expression of Dicer and Ago2 in pigs. It is our goal to identify Dicer and Ago2 expression in porcine reproductive tissue and obtain their full length coding sequences.

CHAPTER TWO

CLONING AND EXPRESSION OF PORCINE DICER

Introduction

Recently, a class of small non-coding RNA called microRNA (miRNA) has been described and reported to have roles in normal mammalian embryonic development. These miRNAs are encoded in the genome, transcribed by RNA polymerase II and processed into fragments ~22 nt in length by Ribonuclease enzymes, the final one being a protein called Dicer. Dicer was first identified by Bernstein and colleagues in 2001 as the enzyme which produces the approximately 21 nt miRNA. Dicer is an RNase III enzyme, a class of enzymes that show specificity for dsRNA, and has been shown to process pre-miRNA from its 76 nt hairpin structure into mature ds-miRNA. Dicer is essential for miRNA production and Dicer knockouts have been shown to be embryonic lethal (Bernstein *et al*, 2001; Bernstein *et al*, 2003).

Our objective is to understand the miRNA pathway in pigs, including: identifying miRNA expressed in reproductive tissues, describing the ontogeny of this pathway, and identifying in this species the major proteins involved in the synthesis and function of miRNA. Dicer has yet to be identified in pigs. The objective of this study was to clone the cDNA for porcine Dicer (pDicer), as well as verify its expression in multiple reproductive tissues including oocytes and developing embryos. We hypothesize that the Dicer sequence is highly conserved in pigs and that its message is expressed in porcine embryos during the time of fetal genome activation.

Materials and Methods

RNA Isolation

Porcine ovaries were obtained from nearby abattoirs, frozen in liquid nitrogen, and stored at -80° C until further processed. Sections weighing approximately 200 mg were obtained from the frozen ovaries and used for total cellular RNA (tcRNA) isolation using the mirVana miRNA isolation kit (Ambion, Austin, TX).

RT-PCR/Primer Design

tcRNA was subjected to endpoint RT-PCR using SuperScript™ III First-Strand Synthesis Super Mix for reverse transcription (Invitrogen, Carlsbad, CA). The first strand reaction was utilized for PCR with GoTaq (Promega, Madison, WI). All PCR reactions were run with a total volume of 25 µl in an Eppendorf Mastercycler gradient thermocycler (Westbury, NY) according to the following program: Lid: 95.0°; (1) T=95.0°, 3:00min; (2) T=94.0°, 0:30min; (3) T=(annealing temperature specific to primer set), 0:30min; (4) T=72.0°, 0:30min; (5) GOTO 2 Rep 34; (6) T= 72.0°, 3:00min; (7) HOLD 4.0°. Specific annealing temperatures were obtained by running gradient programs for each primer set. PCR products were subjected to non-denaturing slab gel electrophoresis, using 1.2 to 2.0% TAE or TBE gels, and visualized using Ethidium Bromide (EtBr) staining and UV-light exposure.

To generate PCR primers, the cDNA sequences for bovine Dicer (bDicer), human Dicer (hDicer) and mouse Dicer (mDicer) (accession numbers AY386968, NM030621, and NM148948, respectively) were aligned. Primers were generated from highly

conserved regions by using Vector NTI (Invitrogen, Carlsbad, CA) or were designed by hand following Rybicki's guidelines in Molecular Biology Techniques Manual (2001). Eight primer sets were designed for eight overlapping fragments of the Dicer coding sequence, as illustrated in Figure 2.1. Primer sequences are listed in Table 2.1.

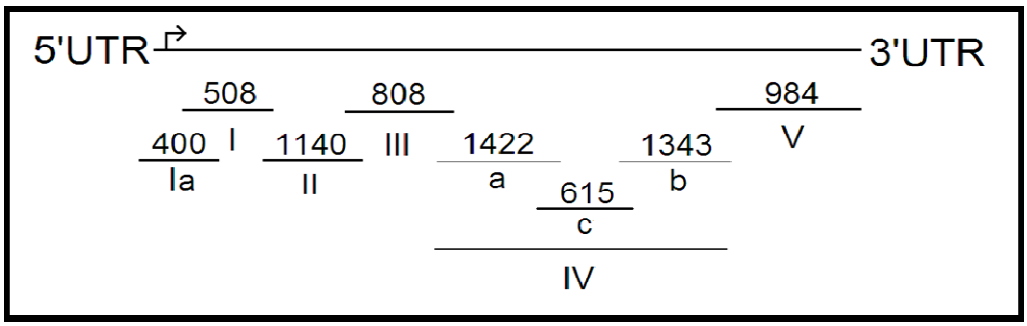

Figure 2.1: Dicer cloning strategy using RT-PCR. Based on bovine Dicer cDNA sequence (approximately 5796bp, accession: AY386968). Fragment sizes for each primer set shown.

Table 2.1: Primer sequences for pDicer fragments.

Fragment	Forward	Reverse
Ia	5'-TTGAAACACTGGATGAATGA	5'-TTCTAGGTTTGAGTATTCCC
I	5'-CATGACCCCTGCTTCCTCA	5'-GGGGTGGTCTAGGATTGCAAG
II	5'-GACGGTGTTCTTGTCAACTC	5'-GCTGATGTAAGCCAGCTCTG
III	5'-AGCGGCAGCAGTTYGAAAGY	5'-TCAGGCAACTCTCGGGTTCT
IVa	5'-ACAAAGCTATTGAAAAGATCTTGCG	5'-AAACGGCTTTTCTCCACAGT
IVb	5'-CCCAAGCCCAGCGATGAATG	5'-AGAAGGTAAGCCTTATTCTT
IVc	5'-CCAGCATCACTGTGGAGAAA	5'-GTTAGCATTTCCATCAAGGT
V	5'-TCAACTACCAGATTCAAGAATA	5'-GCAGAAGTGAGGAAAGAAGA

Subcloning/Sequencing

PCR products for each fragment were ligated into the pDrive Cloning Vector and transformed into competent *E.coli* cells using Qiagen PCR Cloning Kit (Qiagen, Valencia, CA). Transformed cells were plated onto agar plates which had been treated with 100 mg/mL ampicillin; prior to plating cells, all plates were coated with 75-100 µl IPTG (isopropyl-beta-D-thiogalactopyranoside) and 75-100 µl X-gal (5-bromo-4-chloro-3-indolyl-beta-D-galacto-pyranoside) for blue-white screening. Plates were incubated at 37° C overnight. Subsequent colonies were selected, streaked onto new agar/+amp plates, and grown in LB media at 37° C overnight. Plasmids were isolated from cultures using Qiagen QIAprep Spin Miniprep Kit (Qiagen, Valencia, CA). Isolated plasmids were digested with EcoR1 at 37° C, to confirm insertion of the gene of interest, and sent to Clemson University Genomics Institute (CUGI, Clemson, SC) for sequencing. Forward and reverse reactions using M13 primers were used for sequencing. Four clones were sequenced for each fragment to rule out possible GoTaq reading errors.

Sequences were analyzed in Vector NTI (Invitrogen) and aligned to bovine, human, and mouse sequences for which percent sequence identities were obtained.

Embryo Collection

Gilts were obtained from and housed individually at Starkey Swine Center, Clemson University. Estrus detection began twice daily when the average gilt weight was approximately 118 kg. Gilts were artificially inseminated at standing estrus and 12 hours post-standing estrus using Duroc semen obtained from Swine Genetics

International (Cambridge, Iowa). Inseminated gilts were slaughtered 3, 5, or 7 days post-insemination. Reproductive tracts were collected at the time of slaughter and immediately flushed to retrieve embryos. Tracts were flushed with approximately 100 mL solution of sterile PBS and BSA (4g/L), which was subsequently filtered to collect the embryos. Embryos were stored in RNAlater (Ambion, Austin, TX), snap-frozen in liquid nitrogen, and stored at -80° C until RNA Isolation.

Results

The nucleotide sequence obtained for porcine Dicer using our overlapping PCR strategy is 5,995 bp, with the coding sequence beginning at bp 17 and ending at bp 5762 (Table 2.2). Porcine Dicer has a sequence identity of 91% to bovine, 90% to human, and 86% to mouse Dicer sequences (Table 2.3) at the nucleotide level. The protein translation of porcine Dicer is 1,916 aa long (Tables 2.4 and 2.6), and has a sequence identity of 94% to bovine, 94% to human, and 92% to mouse.

Figure 2.2 illustrates the correct size product for each fragment: approximately 400 bp for Fragment Ia; 508 bp for Fragment I; 1,140 bp for Fragment II; 808 bp for Fragment III; 1,422 bp for Fragment IVa; 615 bp for Fragment IVc; 1,343 bp for Fragment IVb; and 984 bp for Fragment V.

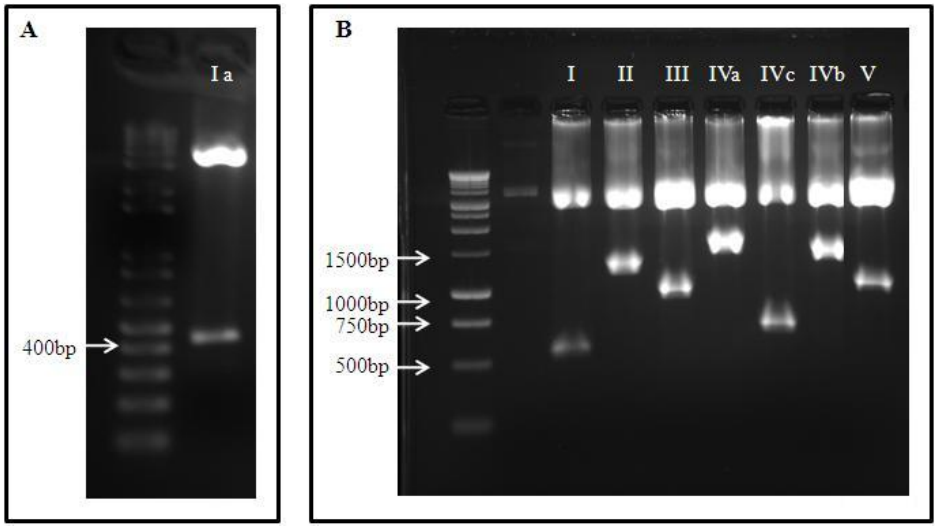

Figure 2.2: Plasmid digest of cloned Dicer fragments.

Table 2.2: Nucleotide sequence for pDicer with amino acid translation.

```
   1 - TTGAAACACTGGAUGAATGAAAAGCCCTGCTTTGCAACCCCTCAG  - 45
   1 -                M  K  S  P  A  L  Q  P  L  S   - 10

  46 - CATGGCAGGCCTGCAGCTCATGACCCCTGCTTCCTCACCAATGGG  - 90
  11 -  M  A  G  L  Q  L  M  T  P  A  S  S  P  M  G   - 25

  91 - TCCTTTCTTTGGACTGCCATGGCAACAAGAAGCAATTCATGATAA  - 135
  26 -  P  F  F  G  L  P  W  Q  Q  E  A  I  H  D  N   - 40

 136 - CATTTATACGCCAAGAAAATATCAGGTTGAACTGCTTGAAGCAGC  - 180
  41 -  I  Y  T  P  R  K  Y  Q  V  E  L  L  E  A  A   - 55

 181 - TCTGGATCATAATACCATAGTCTGTTTAAACACTGGCTCAGGGAA  - 225
  56 -  L  D  H  N  T  I  V  C  L  N  T  G  S  G  K   - 70

 226 - GACGTTTATTGCAGTACTACTCACTAAAGAGCTGTCCTATCAGAT  - 270
  71 -  T  F  I  A  V  L  L  T  K  E  L  S  Y  Q  I   - 85

 271 - CAGGGGAGACTTCAACAGAAATGGCAAAAGGACGGTGTTCTTGGT  - 315
  86 -  R  G  D  F  N  R  N  G  K  R  T  V  F  L  V   - 100

 316 - CAACTCTGCAAACCAGGTTGCTCAACAAGTGTCAGCTGTTAGGAC  - 360
 101 -  N  S  A  N  Q  V  A  Q  Q  V  S  A  V  R  T   - 115

 361 - TCACTCGGATCTCAAGGTTGGGGAATACTCGAACCTAGAAGTAAA  - 405
 116 -  H  S  D  L  K  V  G  E  Y  S  N  L  E  V  N   - 130

 406 - TGCATCTTGGACAAAAGAGAAATGGAACCAAGAGTTTACTAAGCA  - 450
 131 -  A  S  W  T  K  E  K  W  N  Q  E  F  T  K  H   - 145
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 451 - CCAGGTTCTTGTTATGACTTGCTCTGTCGCCTTGAATGTTTTGAA  - 495
 146 -  Q  V  L  V  M  T  C  S  V  A  L  N  V  L  K  - 160

 496 - AAATGGTTACTTAGCACTGTCAGACATTAACCTTTTGGTGTTCGA  - 540
 161 -  N  G  Y  L  A  L  S  D  I  N  L  L  V  F  D  - 175

 541 - TGAGTGTCATCTTGCAATCCTAGATCACCCCTACCGAGAGATTAT  - 585
 176 -  E  C  H  L  A  I  L  D  H  P  Y  R  E  I  M  - 190

 586 - GAAGCTCTGTGAAAATTGTCCATCATGTCCTCGTATTTTGGGGCT  - 630
 191 -  K  L  C  E  N  C  P  S  C  P  R  I  L  G  L  - 205

 631 - AACTGCTTCCATTTTAAATGGGAAATGTGATCCAGAGGAATTGGA  - 675
 206 -  T  A  S  I  L  N  G  K  C  D  P  E  E  L  E  - 220

 676 - AGAAAAGATACAGAAACTGGAGAAAATTCTTAAGAGTAATGCTGA  - 720
 221 -  E  K  I  Q  K  L  E  K  I  L  K  S  N  A  E  - 235

 721 - AACTGCAACTGACTTGGTGGTCTTAGACAGATATACTTCTCAGCC  - 765
 236 -  T  A  T  D  L  V  V  L  D  R  Y  T  S  Q  P  - 250

 766 - ATGTGAGATTGTGGTAGACTGTGGACCATTTACTGACAGAAGTGG  - 810
 251 -  C  E  I  V  V  D  C  G  P  F  T  D  R  S  G  - 265

 811 - GCTTTATGAAAGACTGCTGATGGAATTAGAAGAAGCTCTCAATTT  - 855
 266 -  L  Y  E  R  L  L  M  E  L  E  E  A  L  N  F  - 280

 856 - TATCAATGACTGTAACATAGCTGTACATTCAAAAGAAAGAGATTC  - 900
 281 -  I  N  D  C  N  I  A  V  H  S  K  E  R  D  S  - 295

 901 - TACTTTAATTTCCAAACAGATACTGTCAGACTGTCGTGCAGTATT  - 945
 296 -  T  L  I  S  K  Q  I  L  S  D  C  R  A  V  L  - 310

 946 - GGTAGTTCTGGGACCTTGGTGTGCAGATAAAGTAGCTGGAATGAT  - 990
 311 -  V  V  L  G  P  W  C  A  D  K  V  A  G  M  M  - 325

 991 - GGTAAGAGAACTACAGAAATATATCAAACATGAACAAGAGGAGCT  - 1035
 326 -  V  R  E  L  Q  K  Y  I  K  H  E  Q  E  E  L  - 340

1036 - GCACAGGAAATTTCTATTGTTTACAGACACTTTCCTGAGGAAAGT  - 1080
 341 -  H  R  K  F  L  L  F  T  D  T  F  L  R  K  V  - 355

1081 - ACACGCGCTGTGTGAAGGGCACTTCTCCCCTGCCGCGCTTGACCT  - 1125
 356 -  H  A  L  C  E  G  H  F  S  P  A  A  L  D  L  - 370

1126 - GAGATTTGTGACTCCTAAAGTCATAAAACTGCTCGAAATCTTACG  - 1170
 371 -  R  F  V  T  P  K  V  I  K  L  L  E  I  L  R  - 385

1171 - CAAGTACAAACCCTACGAGCGACAGCAGTTTGAAAGCGTTGAGTG  - 1215
 386 -  K  Y  K  P  Y  E  R  Q  Q  F  E  S  V  E  W  - 400

1216 - GTATAATAATAGGAATCAGGATAATTACGTGTCTTGGAGTGATTC  - 1260
 401 -  Y  N  N  R  N  Q  D  N  Y  V  S  W  S  D  S  - 415
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1261 - GGAGGATGATGAGGAGGACGAAGAAATTGAAGAAAAAGAAAAGCC  - 1305
 416 -  E  D  D  E  E  D  E  E  I  E  E  K  E  K  P  - 430

1306 - GGAGACGAATTTTCCTTCTCCATTTACCAATATTTTATGTGGAAT  - 1350
 431 -  E  T  N  F  P  S  P  F  T  N  I  L  C  G  I  - 445

1351 - TATTTTTGTGGAAAGAAGATACACGGCAGTTGTCTTAAACAGATT  - 1395
 446 -  I  F  V  E  R  R  Y  T  A  V  V  L  N  R  L  - 460

1396 - GATAAAGGAAGCTGGCAAACAAGATCCAGAGCTGGCTTACATCAG  - 1440
 461 -  I  K  E  A  G  K  Q  D  P  E  L  A  Y  I  S  - 475

1441 - CAGCAGCAATTTTATAACTGGACATGGCATTGGAAAGAATCAGCC  - 1485
 476 -  S  S  N  F  I  T  G  H  G  I  G  K  N  Q  P  - 490

1486 - TCGTAACAAACAGATGGAAGCAGAATTCAGAAAACAGGAAGAGGT  - 1530
 491 -  R  N  K  Q  M  E  A  E  F  R  K  Q  E  E  V  - 505

1531 - ACTTAGGAAATTTCGAGCTCACGAAACCAACCTGCTGATTGCCAC  - 1575
 506 -  L  R  K  F  R  A  H  E  T  N  L  L  I  A  T  - 520

1576 - GAGCATTGTGGAAGAGGGTGTTGATATACCAAAKTGCAACCTGGT  - 1620
 521 -  S  I  V  E  E  G  V  D  I  P  X  C  N  L  V  - 535

1621 - GGTTCGTTTCGATCTGCCCACAGAGTATCGATCCTACGTTCAGTC  - 1665
 536 -  V  R  F  D  L  P  T  E  Y  R  S  Y  V  Q  S  - 550

1666 - TAAGGGAAGAGCAAGGGCGCCAATCTCTAATTACGTCATGTTAGC  - 1710
 551 -  K  G  R  A  R  A  P  I  S  N  Y  V  M  L  A  - 565

1711 - AGATACGGACAAAATAAAGAGTTTTGAAGAAGACCTTAAAACATA  - 1755
 566 -  D  T  D  K  I  K  S  F  E  E  D  L  K  T  Y  - 580

1756 - CAAAGCTATTGAAAAGATCTTGAGAAACAAATGCTCCAAGTCCGT  - 1800
 581 -  K  A  I  E  K  I  L  R  N  K  C  S  K  S  V  - 595

1801 - TGAGAGTGGGGAGACCGACCTTGAGCCCGTGGTGGATGACGACGA  - 1845
 596 -  E  S  G  E  T  D  L  E  P  V  V  D  D  D  D  - 610

1846 - CATCTTCCCCCCCTACGTGCTGCGGCCCGACGATGGCGGTCCCCG  - 1890
 611 -  I  F  P  P  Y  V  L  R  P  D  D  G  G  P  R  - 625

1891 - GGTCACCATCAACACGGCCATTGGACACATCAACAGATACTGTGC  - 1935
 626 -  V  T  I  N  T  A  I  G  H  I  N  R  Y  C  A  - 640

1936 - TAGATTACCCAGTGACCCGTTTACTCATCTGGCTCCTAAGTGTAG  - 1980
 641 -  R  L  P  S  D  P  F  T  H  L  A  P  K  C  R  - 655

1981 - AACCCGAGAGTTGCCTGATGGTACATTTTATTCAACTCTTTATCT  - 2025
 656 -  T  R  E  L  P  D  G  T  F  Y  S  T  L  Y  L  - 670

2026 - GCCAATTAATTCACCTCTTCGAGCCTCCATTGTTGGCCCCCCAAT  - 2070
 671 -  P  I  N  S  P  L  R  A  S  I  V  G  P  P  M  - 685
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2071 - GAGCTGTATACGATTGGCTGAAAGAGTCGTGGCTCTCATTTGCTG  - 2115
 686 -  S  C  I  R  L  A  E  R  V  V  A  L  I  C  C  - 700

2116 - TGAAAAACTGCACAAAATTGGTGAACTGGATGACCATTTGATGCC  - 2160
 701 -  E  K  L  H  K  I  G  E  L  D  D  H  L  M  P  - 715

2161 - GGTTGGGAAAGAGACGGTTAAATACGAAGAGGAGCTTGATTTACA  - 2205
 716 -  V  G  K  E  T  V  K  Y  E  E  E  L  D  L  H  - 730

2206 - TGATGAGGAGGAGACCAGTGTTCCAGGAAGACCAGGCTCCACAAA  - 2250
 731 -  D  E  E  E  T  S  V  P  G  R  P  G  S  T  K  - 745

2251 - ACGAAGACAGTGCTACCCAAAAGCGATTCCAGAATGTTTGCGGGA  - 2295
 746 -  R  R  Q  C  Y  P  K  A  I  P  E  C  L  R  D  - 760

2296 - CAGCTACCCCAAGCCCGATCAGCCCTGTTACCTGTATGTGATAGG  - 2340
 761 -  S  Y  P  K  P  D  Q  P  C  Y  L  Y  V  I  G  - 775

2341 - GATGGTTCTGACAACACCTCTCCCCGATGAACTCAACTTTAGAAG  - 2385
 776 -  M  V  L  T  T  P  L  P  D  E  L  N  F  R  R  - 790

2386 - GCGGAAGCTCTATCCCCCCGAGGACACCACAAGATGCTTCGGAAT  - 2430
 791 -  R  K  L  Y  P  P  E  D  T  T  R  C  F  G  I  - 805

2431 - ACTGACAGCCAAACCCATACCTCAGATTCCTCACTTTCCTGTGTA  - 2475
 806 -  L  T  A  K  P  I  P  Q  I  P  H  F  P  V  Y  - 820

2476 - CACACGCTCTGGAGAGGTCACCATTTCCATTGAGTTGAAGAAGTC  - 2520
 821 -  T  R  S  G  E  V  T  I  S  I  E  L  K  K  S  - 835

2521 - TGGTTTCACGCTGTCTCTGCAAATGCTTGAGCTGATTACAAGACT  - 2565
 836 -  G  F  T  L  S  L  Q  M  L  E  L  I  T  R  L  - 850

2566 - TCACCAGTATATATTTTCACATATTCTTCGGCTTGAGAAACCTGC  - 2610
 851 -  H  Q  Y  I  F  S  H  I  L  R  L  E  K  P  A  - 865

2611 - ACTAGAGTTTAAACCCACCGACGCTGACTCAGCATACTGTGTTCT  - 2655
 866 -  L  E  F  K  P  T  D  A  D  S  A  Y  C  V  L  - 880

2656 - ACCTCTTAATGTCGTTAATGACTCCAGCACTTTGGACATTGACTT  - 2700
 881 -  P  L  N  V  V  N  D  S  S  T  L  D  I  D  F  - 895

2701 - TAAATTCATGGAAGACATCGAGAAATCAGAAGCTCGCATAGGCAT  - 2745
 896 -  K  F  M  E  D  I  E  K  S  E  A  R  I  G  I  - 910

2746 - TCCCAGTACAAAGTATTCAAAAGAAACACCTTTTGTTTTTAAATT  - 2790
 911 -  P  S  T  K  Y  S  K  E  T  P  F  V  F  K  L  - 925

2791 - AGAAGATTACCAAGATGCAGTTATCATTCCAAGGTATCGCAATTT  - 2835
 926 -  E  D  Y  Q  D  A  V  I  I  P  R  Y  R  N  F  - 940

2836 - TGATCAGCCTCATCGATTTTACGTAGCTGATGTGTACACTGATCT  - 2880
 941 -  D  Q  P  H  R  F  Y  V  A  D  V  Y  T  D  L  - 955
```

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2881 - TACCCCACTGAGTAAATTTCCTTCCCCTGAGTATGAAACTTTTGC  - 2925
 956 -  T  P  L  S  K  F  P  S  P  E  Y  E  T  F  A  - 970

2926 - AGAATATTATAAAACGAAGTATAACCTTGACCTGACCAATCTCAA  - 2970
 971 -  E  Y  Y  K  T  K  Y  N  L  D  L  T  N  L  N  - 985

2971 - CCAGCCGCTGCTGGATGTGGACCACACATCGTCAAGACTTAATCT  - 3015
 986 -  Q  P  L  L  D  V  D  H  T  S  S  R  L  N  L  - 1000

3016 - TTTGACACCTCGCCATTTGAATCAGAAGGGGAAAGCTCTTCCTCT  - 3060
1001 -  L  T  P  R  H  L  N  Q  K  G  K  A  L  P  L  - 1015

3061 - GAGCAGCGCTGAAAAGAGGAAAGCCAAATGGGAGAGTCTGCAGAA  - 3105
1016 -  S  S  A  E  K  R  K  A  K  W  E  S  L  Q  N  - 1030

3106 - CAAACAGATCCTGGTTCCGGAACTCTGTGCTATCCATCCAATTCC  - 3150
1031 -  K  Q  I  L  V  P  E  L  C  A  I  H  P  I  P  - 1045

3151 - AGCATCACTGTGGAGAAAAGCAGTCTGTCTCCCCAGCATCCTTTA  - 3195
1046 -  A  S  L  W  R  K  A  V  C  L  P  S  I  L  Y  - 1060

3196 - TCGCCTTCACTGCCTTCTGACCGCGGAGGAGCTAAGAGCCCAGAC  - 3240
1061 -  R  L  H  C  L  L  T  A  E  E  L  R  A  Q  T  - 1075

3241 - GGCCAGCGATGCTGGTGTGGGAGTCAGATCACTTCCCGTGGATTT  - 3285
1076 -  A  S  D  A  G  V  G  V  R  S  L  P  V  D  F  - 1090

3286 - TAGATACCCCAACTTAGACTTCGGGTGGAAAAAATCCATCGACAG  - 3330
1091 -  R  Y  P  N  L  D  F  G  W  K  K  S  I  D  S  - 1105

3331 - CAAATCTTTCATCTCAGTTGCTAACTCCTCTTCAGCTGAAAACGA  - 3375
1106 -  K  S  F  I  S  V  A  N  S  S  S  A  E  N  E  - 1120

3376 - GAACTACTGTAAGCACAGCCCCCTCGTCCCTGAACATGCTGCACA  - 3420
1121 -  N  Y  C  K  H  S  P  L  V  P  E  H  A  A  H  - 1135

3421 - TCGAGGTGCTAACCGACCCTCCGCTCTCGAAAATCACGGCCACAC  - 3465
1136 -  R  G  A  N  R  P  S  A  L  E  N  H  G  H  T  - 1150

3466 - GTCTGTGACCTGCCGAGCGCTCCTCAGCGAGTCCCCTGCTAAGCT  - 3510
1151 -  S  V  T  C  R  A  L  L  S  E  S  P  A  K  L  - 1165

3511 - CCCGATCGACGTTGCAACAGATCTGACAGCAGTGAACGGTCTTTC  - 3555
1166 -  P  I  D  V  A  T  D  L  T  A  V  N  G  L  S  - 1180

3556 - GTACAATAAAAATCTTGCCAATGGCAGTTACGACTTAGCTAACAG  - 3600
1181 -  Y  N  K  N  L  A  N  G  S  Y  D  L  A  N  R  - 1195

3601 - AGACTTTTGCCAAGGAAATCATCTGAGTTACTACAAGCAGGAAAT  - 3645
1196 -  D  F  C  Q  G  N  H  L  S  Y  Y  K  Q  E  I  - 1210

3646 - ACCTGTACAACCAACTACCTCATATCCCATTCAGAATTTATACAA  - 3690
1211 -  P  V  Q  P  T  T  S  Y  P  I  Q  N  L  Y  N  - 1225
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3691 - TTACGAGAACCAGCCCCAGCCCAGCGATGAATGTACTCTACTGAG  - 3735
1226 -  Y  E  N  Q  P  Q  P  S  D  E  C  T  L  L  S  - 1240

3736 - TAATAAATACCTTGATGGAAATGCTAACAAATCTACCTCAGAAGG  - 3780
1241 -  N  K  Y  L  D  G  N  A  N  K  S  T  S  E  G  - 1255

3781 - ACGTCCCACGATGCCTGGTACTACAGAGGCTGGTAAGGCGCTTTC  - 3825
1256 -  R  P  T  M  P  G  T  T  E  A  G  K  A  L  S  - 1270

3826 - GGAAAGGATGGCTTCTGCGCAGAGCCCTGCTCCGGGCTACTCCCC  - 3870
1271 -  E  R  M  A  S  A  Q  S  P  A  P  G  Y  S  P  - 1285

3871 - GAGGACTCCTGGCCCAAACCCTGGACTCATCCTTCAGGCTCTGAC  - 3915
1286 -  R  T  P  G  P  N  P  G  L  I  L  Q  A  L  T  - 1300

3916 - CCTTTCAAACGCTAGCGACGGATTTAACCTGGAGCGGCTCGAAAT  - 3960
1301 -  L  S  N  A  S  D  G  F  N  L  E  R  L  E  M  - 1315

3961 - GCTCGGTGACTCCTTCTTAAAGCACGCCATCACCACGTATCTCTT  - 4005
1316 -  L  G  D  S  F  L  K  H  A  I  T  T  Y  L  F  - 1330

4006 - TTGCACTTACCCTGATGCTCACGAGGGCCGCCTTTCGTATATGAG  - 4050
1331 -  C  T  Y  P  D  A  H  E  G  R  L  S  Y  M  R  - 1345

4051 - AAGCAAAAAGGTCAGCAACTGTAACCTGTATCGGCTTGGGAAGAA  - 4095
1346 -  S  K  K  V  S  N  C  N  L  Y  R  L  G  K  K  - 1360

4096 - GAAGGGCCTGCCCAGCCGCATGGTGGTGTCGATATTTGATCCCCC  - 4140
1361 -  K  G  L  P  S  R  M  V  V  S  I  F  D  P  P  - 1375

4141 - TGTGAACTGGCTTCCTCCTGGTTATGTAGTAAATCAAGACAAAAG  - 4185
1376 -  V  N  W  L  P  P  G  Y  V  V  N  Q  D  K  S  - 1390

4186 - TAACACAGACAAATGGGAAAAAGATGAAATGACAAAAGACTGCGT  - 4230
1391 -  N  T  D  K  W  E  K  D  E  M  T  K  D  C  V  - 1405

4231 - GCTGGCTAACGGCAGACTGGACGCCGACCTGGAGGAGGAGGACGC  - 4275
1406 -  L  A  N  G  R  L  D  A  D  L  E  E  E  D  A  - 1420

4276 - CGCCGCGCTCATGTGGAGGCCGCCCAGGGAGGAGGCCGAGGACGA  - 4320
1421 -  A  A  L  M  W  R  P  P  R  E  E  A  E  D  D  - 1435

4321 - CGAGGACCTCCTGGAGTACGACCAGGAGCACATCAGGTTCATAGA  - 4365
1436 -  E  D  L  L  E  Y  D  Q  E  H  I  R  F  I  D  - 1450

4366 - CAGCATGCTGATGGGGTCAGGAGCCTTCGTCAAGAAGATTGCTCT  - 4410
1451 -  S  M  L  M  G  S  G  A  F  V  K  K  I  A  L  - 1465

4411 - TGCTCCCTTCGCCGCCGCCGATCCTGCCTACGAATGGAAGATGCC  - 4455
1466 -  A  P  F  A  A  A  D  P  A  Y  E  W  K  M  P  - 1480

4456 - CAAAAAGGCCCCCCTGGGGAGCATGCCCTTTTCCGCAGATTTCGA  - 4500
1481 -  K  K  A  P  L  G  S  M  P  F  S  A  D  F  E  - 1495
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4501 - GGACTTTGACTACAGCTCGTGGGATGCCATGTGCTATCTGGACCC  - 4545
1496 -  D  F  D  Y  S  S  W  D  A  M  C  Y  L  D  P  - 1510

4546 - CAGCAAAGCCGTTGAGGAGGATGACTTTGTGGTGGGCTTCTGGAA  - 4590
1511 -  S  K  A  V  E  E  D  D  F  V  V  G  F  W  N  - 1525

4591 - TCCATCCGAAGAGAACTGTGGTGTGGACACAGGCAAACAGTCCAT  - 4635
1526 -  P  S  E  E  N  C  G  V  D  T  G  K  Q  S  I  - 1540

4636 - TTCTTACGACTTGCACACGGAGCAGTGCATCGCTGACAAAAGCAT  - 4680
1541 -  S  Y  D  L  H  T  E  Q  C  I  A  D  K  S  I  - 1555

4681 - CGCCGACTGTGTGGAAGCCCTGCTGGGCTGCTACTTGACCAGCTG  - 4725
1556 -  A  D  C  V  E  A  L  L  G  C  Y  L  T  S  C  - 1570

4726 - TGGCGAGCGGGCCGCTCAGCTCTTCCTCTGCTCGCTGGGCCTGAA  - 4770
1571 -  G  E  R  A  A  Q  L  F  L  C  S  L  G  L  K  - 1585

4771 - GGTGCTCCCGGCGGTGAAGAGGACCGATCGGGCACAGGCCGCCTG  - 4815
1586 -  V  L  P  A  V  K  R  T  D  R  A  Q  A  A  C  - 1600

4816 - CCCGGCCAGGGAGAGCTTCACCAGCCAACAAAAGACCCTTTCCGG  - 4860
1601 -  P  A  R  E  S  F  T  S  Q  Q  K  T  L  S  G  - 1615

4861 - GGGCCGGCCCGCCGCCGGCTCCCGCTCTTCCGGGTTGAAAGACTT  - 4905
1616 -  G  R  P  A  A  G  S  R  S  S  G  L  K  D  L  - 1630

4906 - GGAGTACGGCTGTTTGAAGATCCCACCGAGATGTATGTTTGATCA  - 4950
1631 -  E  Y  G  C  L  K  I  P  P  R  C  M  F  D  H  - 1645

4951 - CCCAGACGCAGACAGGACACTCAGTCACCTCATCTCGGGCTTTGA  - 4995
1646 -  P  D  A  D  R  T  L  S  H  L  I  S  G  F  E  - 1660

4996 - GAACTTCGAAAGGAAGATCAACTACAGCTTCAAGAATAAGGCTTA  - 5040
1661 -  N  F  E  R  K  I  N  Y  S  F  K  N  K  A  Y  - 1675

5041 - CCTTCTGCAGGCCTTCACCCACGCCTCCTACCACTACAACACCAT  - 5085
1676 -  L  L  Q  A  F  T  H  A  S  Y  H  Y  N  T  I  - 1690

5086 - CACCGATTGTTACCAGCGCCTGGAGTTCCTGGGAGATGCCATTCT  - 5130
1691 -  T  D  C  Y  Q  R  L  E  F  L  G  D  A  I  L  - 1705

5131 - GGACTACCTCATAACCAAGCACCTTTACGAAGACCCGCGGCAGCA  - 5175
1706 -  D  Y  L  I  T  K  H  L  Y  E  D  P  R  Q  H  - 1720

5176 - CTCCCCGGGGGTCCTGACCGACCTGCGCTCTGCTCTGGTCAACAA  - 5220
1721 -  S  P  G  V  L  T  D  L  R  S  A  L  V  N  N  - 1735

5221 - CACCATCTTCGCCTCGCTGGCCGTCAAGTACGACTACCACAAGTA  - 5265
1736 -  T  I  F  A  S  L  A  V  K  Y  D  Y  H  K  Y  - 1750

5266 - CTTCAAGGCCGTGTCGCCCGAGCTCTTCCACGTCATCGATGATTT  - 5310
1751 -  F  K  A  V  S  P  E  L  F  H  V  I  D  D  F  - 1765
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5311 - TGTGCAGTTTCAGCTTGAGAAGAACGAGATGCAGGGGATGGATTC  - 5355
1766 -  V  Q  F  Q  L  E  K  N  E  M  Q  G  M  D  S   - 1780

5356 - TGAGCTTAGGAGATCTGAGGAGGATGAAGAGAAAGAAGAGGATAT  - 5400
1781 -  E  L  R  R  S  E  E  D  E  E  K  E  E  D  I   - 1795

5401 - TGAAGTTCCGAAGGCCATGGGGGACATTTTTGAGTCGCTTGCTGG  - 5445
1796 -  E  V  P  K  A  M  G  D  I  F  E  S  L  A  G   - 1810

5446 - TGCCATTTACATGGATAGTGGAATGTCACTGGAGGTGGTTTGGCA  - 5490
1811 -  A  I  Y  M  D  S  G  M  S  L  E  V  V  W  Q   - 1825

5491 - GGTGTACTATCCGATGATGCGGCCGCTAATAGAAAAATTTTCTGC  - 5535
1826 -  V  Y  Y  P  M  M  R  P  L  I  E  K  F  S  A   - 1840

5536 - AAACGTGCCCCGTTCGCCTGTGCGAGAATTGCTTGAAATGGAACC  - 5580
1841 -  N  V  P  R  S  P  V  R  E  L  L  E  M  E  P   - 1855

5581 - AGAAACCGCCAAATTTAGCCCGGCTGAGAGAACTTACGATGGCAA  - 5625
1856 -  E  T  A  K  F  S  P  A  E  R  T  Y  D  G  K   - 1870

5626 - GGTCAGAGTCACCGTGGAAGTCGTAGGAAAGGGGAAATTCAAAGG  - 5670
1871 -  V  R  V  T  V  E  V  V  G  K  G  K  F  K  G   - 1885

5671 - TGTTGGCCGAAGTTACAGGATTGCCAAATCTGCAGCAGCACGACG  - 5715
1886 -  V  G  R  S  Y  R  I  A  K  S  A  A  A  R  R   - 1900

5716 - AGCCCTGCGAAGCCTCAAAGCTAATCAACCTCAGGTTCCCAACAG  - 5760
1901 -  A  L  R  S  L  K  A  N  Q  P  Q  V  P  N  S   - 1915

5761 - CTGAAACCCCTTTTTAAAATAACGAAAAGAAGCAGAGTTAAGGTG  - 5805
1916 -  *

5806 - GAAAATATTTAAGTGGAAAAGGATGATTTAAAATTGGCAGTGAGT  - 5850
1916 -

5851 - GGAATGAATTGAAGGCAGAAGTTAAAGTTTGATAACAAGCTAGAT  - 5895
1916 -

5896 - TGCAGAATAAAACATTTAACATATGTATAAAACCTTTGGAACTAA  - 5940
1916 -

5941 - TTGTAGTTTTAGTTTTTTGCGCAAACACAATCTTGTCTTCTTTCC  - 5985
1916 -

5986 - TCACTTCTGC                                     - 5995
1916
```

Table 2.3: Protein translation of pDicer cDNA aligned with bovine and human Dicer protein sequences. Color-coded regions indicate known domains, as reported for bovine Dicer (accession AY386968.1). Red = DEXDc; Purple = Helicase domain; Orange = dsRNA binding domain; Green = PAZ domain; Blue = RNase III domains

```
Bovine   MKSPALQPLSMAGLQLMTPASSPMGPFFGLPWQQEAIHDNIYTPRKYQVELLEAALDHNT 60
Human    MKSPALQPLSMAGLQLMTPASSPMGPFFGLPWQQEAIHDNIYTPRKYQVELLEAALDHNT 60
Porcine  MKSPALQPLSMAGLQLMTPASSPMGPFFGLPWQQEAIHDNIYTPRKYQVELLEAALDHNT 60
         ************************************************************

Bovine   IVCLNTGSGKTFIAVLLTKELSYQIRGDFNRNGKRTVFLVNSANQVAQQVSAVRTHSDLK 120
Human    IVCLNTGSGKTFIAVLLTKELSYQIRGDFSRNGKRTVFLVNSANQVAQQVSAVRTHSDLK 120
Porcine  IVCLNTGSGKTFIAVLLTKELSYQIRGDFNRNGKRTVFLVNSANQVAQQVSAVRTHSDLK 120
         ****************************.*****************************

Bovine   VGEYSNLEVSASWTKEKWNQEFTKHQVLIMTCYVALNVLKNGYLSLSDINLLVFDECHLA 180
Human    VGEYSNLEVNASWTKERWNQEFTKHQVLIMTCYVALNVLKNGYLSLSDINLLVFDECHLA 180
Porcine  VGEYSNLEVNASWTKEKWNQEFTKHQVLVMTCSVALNVLKNGYLALSDINLLVFDECHLA 180
         *********.******:***********:*** ***********:***************

Bovine   ILDHPYREIMKLCENCPSCPRILGLTASILNGKCDPEELEEKIQKLEKILKSNAETATDL 240
Human    ILDHPYREIMKLCENCPSCPRILGLTASILNGKCDPEELEEKIQKLEKILKSNAETATDL 240
Porcine  ILDHPYREIMKLCENCPSCPRILGLTASILNGKCDPEELEEKIQKLEKILKSNAETATDL 240
         ************************************************************

Bovine   VVLDRYTSQPCEIVVDCGPFTDRSGLYERLLMELEEALNFINDCNISVHSKERDSTLISK 300
Human    VVLDRYTSQPCEIVVDCGPFTDRSGLYERLLMELEEALNFINDCNISVHSKERDSTLISK 300
Porcine  VVLDRYTSQPCEIVVDCGPFTDRSGLYERLLMELEEALNFINDCNIAVHSKERDSTLISK 300
         ************************************************:************

Bovine   QILSDCRAVLVVLGPWCADKVAGMMVRELQKHIKHEQEELHRKFLLFTDTFLRKIHALCE 360
Human    QILSDCRAVLVVLGPWCADKVAGMMVRELQKYIKHEQEELHRKFLLFTDTFLRKIHALCE 360
Porcine  QILSDCRAVLVVLGPWCADKVAGMMVRELQKYIKHEQEELHRKFLLFTDTFLRKVHALCE 360
         ********************************:**********************:*****

Bovine   EHFSPASLDLKFVTPKVIKLLEILRKYKPYERQQFESVEWYNNRNQDNYVSWSDSEDDEE 420
Human    EHFSPASLDLKFVTPKVIKLLEILRKYKPYERQQFESVEWYNNRNQDNYVSWSDSEDDDE 420
Porcine  GHFSPAALDLRFVTPKVIKLLEILRKYKPYERQQFESVEWYNNRNQDNYVSWSDSEDDEE 420
         *****:***:************************************************:*

Bovine   DEEIEEKEKPETNFPSPFTNILCGIIFVERRYTAVVLNRLIKEAGKQDPELAYISS-NFI 479
Human    DEEIEEKEKPETNFPSPFTNILCGIIFVERRYTAVVLNRLIKEAGKQDPELAYISS-NFI 479
Porcine  DEEIEEKEKPETNFPSPFTNILCGIIFVERRYTAVVLNRLIKEAGKQDPELAYISSSNFI 480
         ********************************************************* ***

Bovine   TGHGIGKNQPRNKQMEAEFRKQEEVLRKFRAHETNLLIATSIVEEGVDIPKCNLVVRFDL 539
Human    TGHGIGKNQPRNKQMEAEFRKQEEVLRKFRAHETNLLIATSIVEEGVDIPKCNLVVRFDL 539
Porcine  TGHGIGKNQPRNKQMEAEFRKQEEVLRKFRAHETNLLIATSIVEEGVDIPXCNLVVRFDL 540
         ************************************************ *********
```

```
Bovine   PTEYRSYVQSKGRARAPISNYVMLADTDKIKSFEEDLKTYKAIEKILRNKCSKSVDTGEA 599
Human    PTEYRSYVQSKGRARAPISNYIMLADTDKIKSFEEDLKTYKAIEKILRNKCSKSVDTGET 599
Porcine  PTEYRSYVQSKGRARAPISNYVMLADTDKIKSFEEDLKTYKAIEKILRNKCSKSVESGET 600
         **********************:**********************************::**:

Bovine   DTEPVVDDDDVFPPYVLRPEDG-PRVTINTAIGHVNRYCARLPSDPFTHLAPKCRTRELP 658
Human    DIDPVMDDDDVFPPYVLRPDDGGPRVTINTAIGHINRYCARLPSDPFTHLAPKCRTRELP 659
Porcine  DLEPVVDDDDIFPPYVLRPDDGGPRVTINTAIGHINRYCARLPSDPFTHLAPKCRTRELP 660
         * :**:****:********:** **********:************************

Bovine   DGTFYSTLYLPINSPLRASIVGPPMSCIRLAERVVALICCEKLHKIGELDDHLMPVGKET 718
Human    DGTFYSTLYLPINSPLRASIVGPPMSCVRLAERVVALICCEKLHKIGELDDHLMPVGKET 719
Porcine  DGTFYSTLYLPINSPLRASIVGPPMSCIRLAERVVALICCEKLHKIGELDDHLMPVGKET 720
         ***************************:********************************

Bovine   VKYEEELDLHDEEETSVPGRPGSTKRRQCYPKAIPECLRESYPRPGQPCYLYVIGMVLTT 778
Human    VKYEEELDLHDEEETSVPGRPGSTKRRQCYPKAIPECLRDSYPRPDQPCYLYVIGMVLTT 779
Porcine  VKYEEELDLHDEEETSVPGRPGSTKRRQCYPKAIPECLRDSYPKPDQPCYLYVIGMVLTT 780
         ***************************************:***:*.**************

Bovine   PLPDELNFRRRKLYPPEDTTRCFGILTAKPIPQIPHFPVYTRSGEVTISIELKKSGFTLS 838
Human    PLPDELNFRRRKLYPPEDTTRCFGILTAKPIPQIPHFPVYTRSGEVTISIELKKSGFMLS 839
Porcine  PLPDELNFRRRKLYPPEDTTRCFGILTAKPIPQIPHFPVYTRSGEVTISIELKKSGFTLS 840
         ************************************************************ **

Bovine   LQMLELITRLHQYIFSHILRLEKPALEFKPTDADSAYCVLPLNVVNDSSTLDIDFKFMED 898
Human    LQMLELITRLHQYIFSHILRLEKPALEFKPTDADSAYCVLPLNVVNDSSTLDIDFKFMED 899
Porcine  LQMLELITRLHQYIFSHILRLEKPALEFKPTDADSAYCVLPLNVVNDSSTLDIDFKFMED 900
         ************************************************************

Bovine   IEKSEARIGIPSTKYSKETPFVFKLEDYQDAVIIPRYRNFDQPHRFYVADVYTDLTPLSK 958
Human    IEKSEARIGIPSTKYTKETPFVFKLEDYQDAVIIPRYRNFDQPHRFYVADVYTDLTPLSK 959
Porcine  IEKSEARIGIPSTKYSKETPFVFKLEDYQDAVIIPRYRNFDQPHRFYVADVYTDLTPLSK 960
         ***************:********************************************

Bovine   FPSPEYETFAEYYKTKYNLDLTNLNQPLLDVDHTSSRLNLLTPRHLNQKGKALPLSSAEK 1018
Human    FPSPEYETFAEYYKTKYNLDLTNLNQPLLDVDHTSSRLNLLTPRHLNQKGKALPLSSAEK 1019
Porcine  FPSPEYETFAEYYKTKYNLDLTNLNQPLLDVDHTSSRLNLLTPRHLNQKGKALPLSSAEK 1020
         ************************************************************

Bovine   RKAKWESLQNKQILVPELCAIHPIPASLWRKAVCLPSILYRLHCLLTAEELRAQTASDAG 1078
Human    RKAKWESLQNKQILVPELCAIHPIPASLWRKAVCLPSILYRLHCLLTAEELRAQTASDAG 1079
Porcine  RKAKWESLQNKQILVPELCAIHPIPASLWRKAVCLPSILYRLHCLLTAEELRAQTASDAG 1080
         ************************************************************

Bovine   VGVRSLPVDFRYPNLDFGWKKSIDSKSFISIANSSSAENENYCKHSTIVVPENAAHQGAN 1138
Human    VGVRSLPADFRYPNLDFGWKKSIDSKSFISISNSSSAENDNYCKHSTIVP-ENAAHQGAN 1138
Porcine  VGVRSLPVDFRYPNLDFGWKKSIDSKSFISVANSSSAENENYCKHSPLVP-EHAAHRGAN 1139
         *******.**********************::*******:******.:*  *:***:***

Bovine   RTSPLENHDQMSVNCRTLFSESPGKLQIEVSTDLTAINGLSYNKSLANGSYDLANRDFCQ 1198
Human    RTSSLENHDQMSVNCRTLLSESPGKLHVEVSADLTAINGLSYNQNLANGSYDLANRDFCQ 1198
Porcine  RPSALENHGHTSVTCRALLSESPAKLPIDVATDLTAVNGLSYNKNLANGSYDLANRDFCQ 1199
         *.*.****.: **.**:*:****.** ::*::****:******:.***************
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```
Bovine   GNHLNYYKQEIPVQPTTSYPIQNLYNYENQPKPSDECTLLSNKYLDGNANTSTSDGSPVT 1258
Human    GNQLNYYKQEIPVQPTTSYSIQNLYSYENQPQPSDECTLLSNKYLDGNANKSTSDGSPVM 1258
Porcine  GNHLSYYKQEIPVQPTTSYPIQNLYNYENQPQPSDECTLLSNKYLDGNANKSTSEGRP-- 1257
         **:*.**************.*****.*****:******************.***:* *

Bovine   AAVPGTTETGEAPPDRTASEQSPSPGYSSRTLGPNPGLILQALTLSNASDGFNLERLEML 1318
Human    AVMPGTTDTIQVLKGRMDSEQSPSIGYSSRTLGPNPGLILQALTLSNASDGFNLERLEML 1318
Porcine  -TMPGTTEAGKALSERMASAQSPAPGYSPRTPGPNPGLILQALTLSNASDGFNLERLEML 1316
         .:****:: :.   *  * ***: ***.** ***************************

Bovine   GDSFLKHAITTVSLSALILDAHEGRLSYMRSKKVSNCNLYRLGKKKGLPSRMVVSIFDPP 1378
Human    GDSFLKHAITTY-LFCTYPDAHEGRLSYMRSKKVSNCNLYRLGKKKGLPSRMVVSIFDPP 1377
Porcine  GDSFLKHAITTY-LFCTYPDAHEGRLSYMRSKKVSNCNLYRLGKKKGLPSRMVVSIFDPP 1375
         ***********  * .   ****************************************

Bovine   VNGLPPGYVVNQDKSNTEKWEKDEMTKDCMLANGKLDDDFEEEEEEEDLMWRAHKEDAD 1438
Human    VNWLPPGYVVNQDKSNTDKWEKDEMTKDCMLANGKLDEDYEEEDEEEESLMWRAPKEEAD 1437
Porcine  VNWLPPGYVVNQDKSNTDKWEKDEMTKDCVLANGRLDADLEEED--AAALMWRPPREEAE 1433
         ** **************:***********:****:** * ***:     ****. :*:*:

Bovine   DEDDFLEYDQEHIKFIDNMLMGSGAFVKKISLSPFSATDSAYEWKMPKKSSLGSLPFSSD 1498
Human    YEDDFLEYDQEHIRFIDNMLMGSGAFVKKISLSPFSTTDSAYEWKMPKKSSLGSMPFSSD 1497
Porcine  DDEDLLEYDQEHIRFIDSMLMGSGAFVKKIALAPFAAADPAYEWKMPKKAPLGSMPFSAD 1493
         ::*:********:***.************:*:**:::*.*********:.***:***:*

Bovine   FEDFDYSSWDAMCYLDPSKAVEEDDFVVGFWNPSEENCGVDTGKQSISYDLHTEQCIADK 1558
Human    FEDFDYSSWDAMCYLDPSKAVEEDDFVVGFWNPSEENCGVDTGKQSISYDLHTEQCIADK 1557
Porcine  FEDFDYSSWDAMCYLDPSKAVEEDDFVVGFWNPSEENCGVDTGKQSISYDLHTEQCIADK 1553
         ************************************************************

Bovine   SIADCVEALLGCYLTSCGERAAQLFLCSLGLKVLPVIKRTDREKAMCPTRENFTSQQKNL 1618
Human    SIADCVEALLGCYLTSCGERAAQLFLCSLGLKVLPVIKRTDREKALCPTRENFNSQQKNL 1617
Porcine  SIADCVEALLGCYLTSCGERAAQLFLCSLGLKVLPAVKRTDRAQAACPARESFTSQQKTL 1613
         ***********************************.***** :* **:**.*.****.*

Bovine   SGSRAAASGAGYRASVLKDLEYGCLKIPPRCMFDHPEADRTLRHLISGFENFEKKINYRF 1678
Human    SVSCAAASVASSRSSVLKDSEYGCLKIPPRCMFDHPDADKTLNHLISGFENFEKKINYRF 1677
Porcine  SGGRPAA---GSRSSGLKDLEYGCLKIPPRCMFDHPDADRTLSHLISGFENFERKINYSF 1670
         * . .**    . *:* *** ****************:**:** **********:**** *

Bovine   KNKAYLLQAFTHASYHYNTITDCYQRLEFLGDAILDYLITKHLYEDPRQHSPGVLTDLRS 1738
Human    KNKAYLLQAFTHASYHYNTITDCYQRLEFLGDAILDYLITKHLYEDPRQHSPGVLTDLRS 1737
Porcine  KNKAYLLQAFTHASYHYNTITDCYQRLEFLGDAILDYLITKHLYEDPRQHSPGVLTDLRS 1730
         ************************************************************

Bovine   ALVNNTIFASLAVKYDYHKYFKAVSPELFHVIDDFVQFQLEKNEMQGMDSELRRSEEDEE 1798
Human    ALVNNTIFASLAVKYDYHKYFKAVSPELFHVIDDFVQFQLEKNEMQGMDSELRRSEEDEE 1797
Porcine  ALVNNTIFASLAVKYDYHKYFKAVSPELFHVIDDFVQFQLEKNEMQGMDSELRRSEEDEE 1790
         ************************************************************

Bovine   KEEDIEVPKAMGDIFESLAGAIYMDSGMSLETVWQVYYPMMRPLIEKFSANVPRSPVREL 1858
Human    KEEDIEVPKAMGDIFESLAGAIYMDSGMSLETVWQVYYPMMRPLIEKFSANVPRSPVREL 1857
Porcine  KEEDIEVPKAMGDIFESLAGAIYMDSGMSLEVVWQVYYPMMRPLIEKFSANVPRSPVREL 1850
         ******************************.*****************************
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Bovine   LEMEPEITKFSPAERTYDGKVRVTVEVVGKGKFKGVGRSYRIAKSAAARRALRSLKANQP 1918
Human    LEMEPETAKFSPAERTYDGKVRVTVEVVGKGKFKGVGRSYRIAKSAAARRALRSLKANQP 1917
Porcine  LEMEPETAKFSPAERTYDGKVRVTVEVVGKGKFKGVGRSYRIAKSAAARRALRSLKANQP 1910
         ****** :**************************************************

Bovine   QVPNS 1923
Human    QVPNS 1922
Porcine  QVPNS 1915
         *****
```

Table 2.4: Percent identity of the pDicer nucleotide and amino acid sequences in comparison to bovine, human, and mouse.

	Porcine	
	Nucleotide	**Amino Acid**
Bovine	91	94
Human	90	94
Mouse	86	92

Expression Data

Preliminary expression data of pDicer in sperm, oocytes, and embryos indicated that pDicer is expressed in porcine MII oocytes and hatched blastocysts. The data also indicated that pDicer is not expressed, in detectable quantity, in sperm or 8-cell embryos (Figure 2.3).

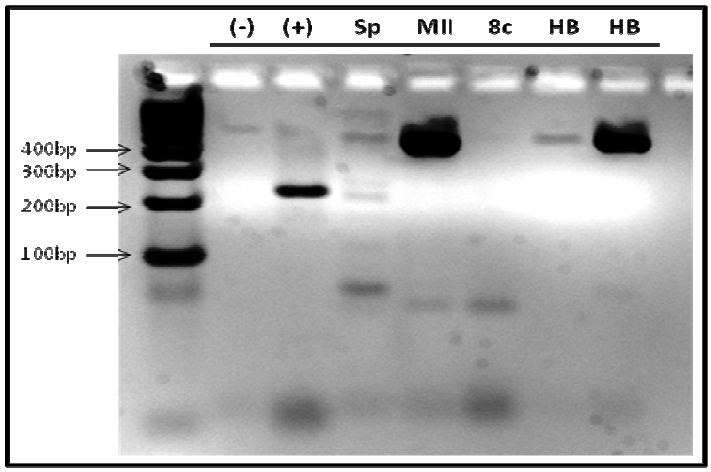

Figure 2.3: Agarose check-gel for Dicer endpoint PCR in sperm, oocytes, and embryos. Lanes: molecular weight ladder; (-) negative control: H2O blank; (+) positive control: Ago2 in MII oocytes; Sp: Sperm; MII: MII oocytes; 8c: 8 cell embryo; HB: hatched blastocyst.

Discussion

Cloning and sequencing of pDicer indicate that Dicer is highly conserved among species. Our data confirm the presence of Dicer in porcine ovary, oocytes, 8-cell and blastocyst embryos. The data indicate that the pDicer message of maternal origin is lost or drastically decreased at the 8-cell stage but rebounds by the Day 7 blastocyst stage. These data support other observations identifying miRNA pathways present during embryonic development and the possible impact miRNA has on porcine embryonic development. At the hatched blastocyst stage, the embryo is likely producing its own Dicer protein. Many studies have shown that the knock-out of Dicer leads to abnormalities in the early embryo. This is consistent with our preliminary data in pigs, which indicate that Dicer is produced by the embryo very early in development. Further

work is needed to elucidate Dicer expression in other early stage embryos (i.e. 16-cell, morula, expanded blastocyst, etc.) and to determine if the ontogeny or level of Dicer expression can impact embryo development and differs between *in vivo* and *in vitro* produced embryos.

Research has clearly indicated that Dicer is required for the processing of miRNA and essential for normal development. Characterizing Dicer ontogeny throughout porcine embryonic development will begin elucidating miRNA involvement during early development in this species. Furthermore, once normal Dicer expression during porcine embryonic development has been described, studies can be done to evaluate aberrations in Dicer expression that may occur during in developmentally compromised embryos. Ultimately, characterizing the miRNA pathway during porcine embryonic development may offer valuable insight into potential causes of aberrant embryonic development.

CHAPTER THREE

CLONING AND EXPRESSION OF PORCINE ARGONAUTE-2

Introduction

Recently, a class of small non-coding RNA has been shown to be involved in embryonic development, and may help explain altered embryonic development when using ART techniques. These 22nt RNA are called microRNA (miRNA). miRNA was shown to play a key role in embryo development when the knock-out of Dicer, an important protein in the production of miRNA, proved to be embryo lethal (Bernstein *et al*, 2003). By binding to messenger RNA (mRNA) and silencing or degrading the message, miRNA function to regulate translation and does so via another class of proteins known as Argonaute. An Argonaute protein is the main protein component of the RNA induced silencing complex (RISC) which carries miRNA to its target. Four Argonaute proteins have been identified in humans (Sasaki *et al*, 2003), only one of which, Argonaute-2 (Ago2), exhibits endonuclease activity to degrade the mRNA to which miRNA binds (Meister *et al*, 2004; Liu *et al*, 2004).

Our objective is to understand the miRNA pathway in large animals, including: identifying miRNA expressed in reproductive tissues, describing the ontogeny of this pathway, and identifying in these animals the major proteins involved in the synthesis and function of miRNA. Ago2 has yet to be identified in pigs. The objective of this study was to clone the cDNA for porcine Ago2 (pAgo2), as well as verify its expression in multiple reproductive tissues including oocytes and developing embryos. We

hypothesize that the Ago2 sequence is highly conserved in pigs and that its message is expressed in porcine embryos during the time of fetal genome activation.

Materials and Methods

RNA Isolation

Porcine ovaries were obtained from nearby abattoirs, frozen in liquid nitrogen, and stored at -80° C until further processed. Sections weighing approximately 200 mg were obtained from the frozen ovaries and used for total cellular RNA (tcRNA) isolation using the mirVana miRNA isolation kit (Ambion, Austin, TX).

For verification of possible deletions, two additional porcine ovaries were collected from separate gilts and two samples were taken from each ovary. tcRNA was isolated from all four samples and subjected to endpoint PCR.

RT-PCR/Primer Design

RNA was subjected to endpoint RT-PCR using SuperScript™ III First-Strand Synthesis Super Mix for reverse transcription (Invitrogen, Carlsbad, CA). The first strand reaction was utilized for PCR with GoTaq (Promega, Madison, WI). All PCR reactions were run with a total volume of 25µl in an Eppendorf Mastercycler gradient thermocycler (Westbury, NY) according to the following program: Lid: 95.0°; (1) T=95.0°, 3:00min; (2) T=94.0°, 0:30min; (3) T=(annealing temperature specific to primer set), 0:30min; (4) T=72.0°, 0:30min; (5) GOTO 2 Rep 34; (6) T= 72.0°, 3:00min; (7) HOLD 4.0°. Specific annealing temperatures were obtained by running gradient programs for each primer set.

PCR products were subjected to non-denaturing slab gel electrophoresis, using 1.2 to 2.0% TAE or TBE gels, and visualized using Ethidium Bromide (EtBr) staining and UV-light exposure.

To generate PCR primers, the cDNA sequences for bovine, human and mouse Ago2 (BC151491, NM012154, and NM153178, respectively) were aligned. Primers were generated from highly conserved regions for each respective cDNA by using the Vector NTI program (Invitrogen, Carlsbad, CA) or were designed by hand following Rybicki's guidelines in Molecular Biology Techniques Manual (2001). Given the length of the coding sequence for Ago2, four primer sets were designed for four overlapping fragments, as illustrated in Figure 3.1. Primer sequences are listed in Table 3.1. Further primer sets were designed for Ago2 to confirm possible deletions. These primers were designed to the porcine sequences obtained and were designated D2 and D3 (for deletion 2 and deletion 3). The following primer sets and corresponding fragments were used to generate the complete coding sequence for pAgo2: Ia, II, D2, and IV (Figure 3.1).

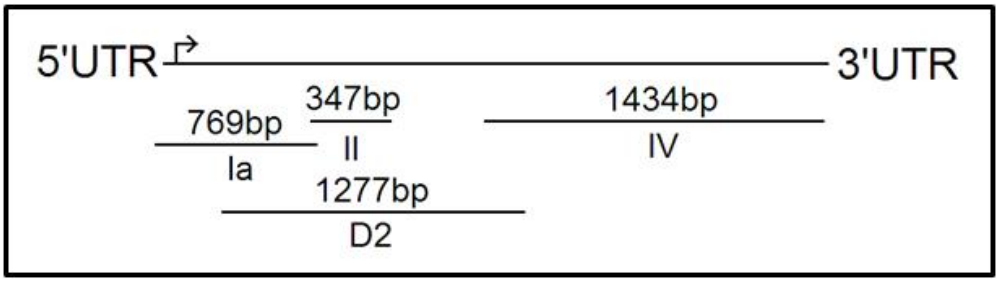

Figure 3.1: pAgo2 cloning strategy using RT-PCR; based on bovine Ago2 cDNA sequence (approximately 3579bp; accession: BC151491). Fragment sizes shown.

Table 3.1: Primer sequences for pAgo2 fragments and deletions.

Fragment	Forward	Reverse
Ia	5'-CGGCGGCGCCACCATGTACT	5'-GAGGTTTCTGTTGTTCTTCAATACT
II	5'-TGGTTTGGCTTCCATCAGTC	5'-TCCTTGAAGTACTGGGCCAC
III	5'-CAAGGATATGCCTTCAAGCC	5'-ATGACCACCACCAGCTGCAG
IV	5'-CCTCTACGGGGGCAGGAATAA	5'-TCATGTTCGATGCTGGCTGTC
D2	5'-TTGGGGATCGGAAACCAGTG	5'-CGTACGTGTTCTTCAGGTGC
D3	5'-TGTGTAGCCATGCTGGCATCCA	5'-CCTTCGGCACTATCATGTTC

Subcloning/Sequencing

PCR products for each fragment were ligated into the pDrive Cloning Vector and transformed into competent *E.coli* cells using Qiagen PCR Cloning Kit (Qiagen, Valencia, CA). Transformed cells were plated onto agar plates which had been treated with 100 mg/mL ampicillin; prior to plating cells, all plates were coated with 75-100 µl IPTG (isopropyl-beta-D-thiogalactopyranoside) and 75-100 µl X-gal (5-bromo-4-chloro-3-indolyl-beta-D-galacto-pyranoside) for blue-white screening. Plates were incubated at 37° C overnight. Subsequent colonies were selected, streaked onto new agar/+amp plates, and grown in LB media at 37° C overnight. Plasmids were isolated from cultures using Qiagen QIAprep Spin Miniprep Kit (Qiagen, Valencia, CA). Isolated plasmids were digested with EcoR1 to confirm insertion of the gene of interest, and sent to Clemson University Genomics Institute (CUGI, Clemson, SC) for sequencing. Forward and reverse reactions using M13 primers were used for sequencing. Four clones were sequenced for each fragment to rule out any GoTaq reading errors.

Sequences were analyzed in Vector NTI (Invitrogen) and aligned to bovine, human, and mouse sequences, from which percent identities were obtained.

Embryo Collection

Gilts were obtained from and housed individually at Starkey Swine Center, Clemson University. Estrus detection began twice daily when the average gilt weight was approximately 118 kg. Gilts were teased with a boar and artificially inseminated at standing estrus and 12 hours post-standing estrus using "mixed dark" Duroc semen obtained from Swine Genetics International (Cambridge, Iowa). Inseminated gilts were slaughtered 3, 5, or 7 days post-insemination. Reproductive tracts were collected at the time of slaughter and immediately (within 2 hours) flushed to retrieve embryos. Tracts were flushed with approximately 100 mL solution of sterile PBS and BSA (4 g/L), which was subsequently filtered to collect the embryos. Embryos were stored in RNAlater (Ambion, Austin, TX), snap-frozen in liquid nitrogen, and stored at -80° C until used for RNA Isolation.

Results

Sequencing

The nucleotide sequence obtained for pAgo2 using our overlapping PCR strategy is 2,703 bp, with the coding sequence beginning at bp 14 and ending at bp 2,596 (Table 3.2). The translation of the pAgo2 cDNA sequence is 860 amino acids (Table 3.3) and has a sequence identity of 99.6% to bovine, 99.5% to human, and 99.2% to mouse (Table 3.4). For the coding sequence, alignments of pAgo2 with bovine, human, and mouse Ago2 sequences showed a sequence identity of 94.2%, 92.2%, and 89.4% respectively (Table 3.4).

Table 3.2: Nucleotide sequence for pAgo2 with amino acid translation.

```
  1 - CGGCGGCGCCACCATGTACTCGGGAGCCGGCCCCGTGCTCGCGCC  - 45
  1 -             M  Y  S  G  A  G  P  V  L  A  P   - 11

 46 - TCCTGCACCGCCACGGCCGCCCATCCAAGGATATGCCTTCAAGCC  - 90
 12 -  P  A  P  P  R  P  P  I  Q  G  Y  A  F  K  P   - 26

 91 - TCCACCTAGACCCGACTTCGGGACCTCCGGGAGAACAATCAAGTT  - 135
 27 -  P  P  R  P  D  F  G  T  S  G  R  T  I  K  L   - 41

136 - ACAGGCCAACTTCTTTGAAATGGACATTCCAAAAATTGACATCTA  - 180
 42 -  Q  A  N  F  F  E  M  D  I  P  K  I  D  I  Y   - 56

181 - TCATTATGAGTTGGATATCAAGCCAGAGAAATGCCCAAGGAGAGT  - 225
 57 -  H  Y  E  L  D  I  K  P  E  K  C  P  R  R  V   - 71

226 - TAACAGGGAAGTAGTGGAACATATGGTTCAGCACTTTAAAACACA  - 270
 72 -  N  R  E  V  V  E  H  M  V  Q  H  F  K  T  Q   - 86

271 - GATCTTTGGGGATCGGAAACCAGTGTTTGATGGAAGGAAGAATCT  - 315
 87 -  I  F  G  D  R  K  P  V  F  D  G  R  K  N  L   - 101

316 - GTACACAGCGATGCCGCTTCCCATCGGGAGGGATAAGGTGGAGCT  - 360
102 -  Y  T  A  M  P  L  P  I  G  R  D  K  V  E  L   - 116

361 - GGAGGTCACACTGCCCGGAGAGGGGAAGGACCGCATCTTCAAGGT  - 405
117 -  E  V  T  L  P  G  E  G  K  D  R  I  F  K  V   - 131

406 - GTCCATCAAGTGGGTGTCCTGCGTGAGCTTACAGGCGTTACACGA  - 450
132 -  S  I  K  W  V  S  C  V  S  L  Q  A  L  H  D   - 146

451 - TGCACTTTCGGGGCGGCTGCCCAGCGTCCCCTTCGAGACGATCCA  - 495
147 -  A  L  S  G  R  L  P  S  V  P  F  E  T  I  Q   - 161

496 - GGCCCTGGATGTGGTCATGAGGCATTTGCCGTCCATGAGGTACAC  - 540
162 -  A  L  D  V  V  M  R  H  L  P  S  M  R  Y  T   - 176

541 - CCCTGTGGGCCGCTCCTTCTTCACGGCGTCTGAGGGCTGCTCCAA  - 585
177 -  P  V  G  R  S  F  F  T  A  S  E  G  C  S  N   - 191

586 - CCCCCTGGGCGGGGGCCGAGAAGTGTGGTTCGGCTTCCATCAGTC  - 630
192 -  P  L  G  G  G  R  E  V  W  F  G  F  H  Q  S   - 206

631 - GGTGCGGCCTTCCCTCTGGAAGATGATGCTGAACATTGACGTCTC  - 675
207 -  V  R  P  S  L  W  K  M  M  L  N  I  D  V  S   - 221

676 - GGCAACAGCGTTTTATAAGGCACAGCCAGTCATCGAGTTTGTGTG  - 720
222 -  A  T  A  F  Y  K  A  Q  P  V  I  E  F  V  C   - 236
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 721 - TGAAGTCTTGGATTTTAAAAGTATTGAAGAACAACAAAAACCTCT  - 765
 237 -  E  V  L  D  F  K  S  I  E  E  Q  Q  K  P  L  - 251

 766 - GACAGATTCCCAAAGGGTAAAGTTTACCAAAGAAATCAAAGGTCT  - 810
 252 -  T  D  S  Q  R  V  K  F  T  K  E  I  K  G  L  - 266

 811 - CAAGGTGGAAATAACGCACTGCGGGCAGATGAAGAGGAAGTACCG  - 855
 267 -  K  V  E  I  T  H  C  G  Q  M  K  R  K  Y  R  - 281

 856 - CGTCTGCAATGTGACCCGGCGGCCCGCCAGTCACCAAACGTTCCC  - 900
 282 -  V  C  N  V  T  R  R  P  A  S  H  Q  T  F  P  - 296

 901 - GCTGCAGCAGGAGAGCGGGCAGACGGTCGAATGCACGGTGGCCCA  - 945
 297 -  L  Q  Q  E  S  G  Q  T  V  E  C  T  V  A  Q  - 311

 946 - GTACTTCAAGGACAGGCACAAGCTGGTTCTGCGCTACCCCCACCT  - 990
 312 -  Y  F  K  D  R  H  K  L  V  L  R  Y  P  H  L  - 326

 991 - CCCATGTTTACAAGTTGGACAGGAGCAGAAACACACCTACCTTCC  - 1035
 327 -  P  C  L  Q  V  G  Q  E  Q  K  H  T  Y  L  P  - 341

1036 - CCTCGAGGTCTGTAACATAGTGGCGGGACAGAGATGTATAAAAAA  - 1080
 342 -  L  E  V  C  N  I  V  A  G  Q  R  C  I  K  K  - 356

1081 - GCTGACCGACAATCAGACCTCAACCATGATCAGAGCCACAGCCAG  - 1125
 357 -  L  T  D  N  Q  T  S  T  M  I  R  A  T  A  R  - 371

1126 - GTCAGCCCCTGATCGGCAGGAAGAGATTAGCAAACTGATGAGAAG  - 1170
 372 -  S  A  P  D  R  Q  E  E  I  S  K  L  M  R  S  - 386

1171 - TGCCAGTTTCAACACAGACCCATATGTTCGTGAATTTGGAATCAT  - 1215
 387 -  A  S  F  N  T  D  P  Y  V  R  E  F  G  I  M  - 401

1216 - GGTCAAAGACGAGATGACAGATGTGACCGGCCGGGTCCTCCAGCC  - 1260
 402 -  V  K  D  E  M  T  D  V  T  G  R  V  L  Q  P  - 416

1261 - GCCCTCCATCCTCTACGGGGGCAGGAATAAAGCGATCGCCACCCC  - 1305
 417 -  P  S  I  L  Y  G  G  R  N  K  A  I  A  T  P  - 431

1306 - AGTCCAGGGCGTCTGGGACATGAGGAACAAGCAGTTCCACACGGG  - 1350
 432 -  V  Q  G  V  W  D  M  R  N  K  Q  F  H  T  G  - 446

1351 - CATCGAGATCAAGGTGTGGGCCATCGCGTGCTTCGCCCCCCAGCG  - 1395
 447 -  I  E  I  K  V  W  A  I  A  C  F  A  P  Q  R  - 461

1396 - CCAGTGCACGGAGGTGCACCTCAAGTCCTTCACGGAGCAGCTCAG  - 1440
 462 -  Q  C  T  E  V  H  L  K  S  F  T  E  Q  L  R  - 476

1441 - AAAGATCTCGAGAGACGCGGGAATGCCAATCCAGGGCCAGCCGTG  - 1485
 477 -  K  I  S  R  D  A  G  M  P  I  Q  G  Q  P  C  - 491

1486 - CTTCTGTAAATACGCCCAGGGGGCGGACAGCGTGGAGCCCATGTT  - 1530
 492 -  F  C  K  Y  A  Q  G  A  D  S  V  E  P  M  F  - 506
```

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1531 - CAGGCACCTGAAGAACACGTACGCCGGCCTGCAGCTGGTCGTGGT  - 1575
 507 -  R  H  L  K  N  T  Y  A  G  L  Q  L  V  V  V  - 521

1576 - CATCCTGCCCGGGAAAACCCCCGTTTACGCCGAGGTCAAGCGTGT  - 1620
 522 -  I  L  P  G  K  T  P  V  Y  A  E  V  K  R  V  - 536

1621 - GGGAGACACGGTGCTGGGCATGGCCACGCAGTGCGTGCAGATGAA  - 1665
 537 -  G  D  T  V  L  G  M  A  T  Q  C  V  Q  M  K  - 551

1666 - GAACGTGCAGAGGACCACGCCGCAGACCCTGTCCAACCTCTGCCT  - 1710
 552 -  N  V  Q  R  T  T  P  Q  T  L  S  N  L  C  L  - 566

1711 - GAAGATCAACGTCAAACTGGGGGGCGTCAACAACATCCTGCTGCC  - 1755
 567 -  K  I  N  V  K  L  G  G  V  N  N  I  L  L  P  - 581

1756 - GCAGGGCAGGCCTCCAGTGTTCCAGCAGCCCGTCATCTTTCTGGG  - 1800
 582 -  Q  G  R  P  P  V  F  Q  Q  P  V  I  F  L  G  - 596

1801 - AGCGGATGTCACTCACCCACCCGCCGGGGACGGCAAGAAGCCTTC  - 1845
 597 -  A  D  V  T  H  P  P  A  G  D  G  K  K  P  S  - 611

1846 - CATCGCCGCCGTTGTGGGCAGCATGGACGCCCACCCCAACCGCTA  - 1890
 612 -  I  A  A  V  V  G  S  M  D  A  H  P  N  R  Y  - 626

1891 - CTGCGCCACCGTCCGTGTCCAGCAGCACCGGCAGGAGATCATCCA  - 1935
 627 -  C  A  T  V  R  V  Q  Q  H  R  Q  E  I  I  Q  - 641

1936 - GGACCTGGCGGCCATGGTGCGCGAGCTGCTCATCCAGTTCTACAA  - 1980
 642 -  D  L  A  A  M  V  R  E  L  L  I  Q  F  Y  K  - 656

1981 - GTCCACGCGCTTCAAGCCCACGCGCATCATCTTCTACCGCGACGG  - 2025
 657 -  S  T  R  F  K  P  T  R  I  I  F  Y  R  D  G  - 671

2026 - CGTCTCCGAGGGCCAGTTCCAGCAGGTCCTTCACCACGAGTTGCT  - 2070
 672 -  V  S  E  G  Q  F  Q  Q  V  L  H  H  E  L  L  - 686

2071 - GGCCATCCGCGAGGCGTGCATCAAGCTAGAGAAGGACTACCAGCC  - 2115
 687 -  A  I  R  E  A  C  I  K  L  E  K  D  Y  Q  P  - 701

2116 - GGGGATCACGTTCATCGTGGTCCAGAAGAGGCACCACACGCGGCT  - 2160
 702 -  G  I  T  F  I  V  V  Q  K  R  H  H  T  R  L  - 716

2161 - CTTCTGCACGGACAAGAACGAGCGGGTTGGCAAAAGCGGAAACAT  - 2205
 717 -  F  C  T  D  K  N  E  R  V  G  K  S  G  N  I  - 731

2206 - TCCAGCAGGCACAACCGTGGACACGAAAATCACCCACCCCACGGA  - 2250
 732 -  P  A  G  T  T  V  D  T  K  I  T  H  P  T  E  - 746

2251 - GTTTGACTTCTACCTGTGTAGCCATGCTGGCATCCAGGGAACAAG  - 2295
 747 -  F  D  F  Y  L  C  S  H  A  G  I  Q  G  T  S  - 761

2296 - CAGGCCTTCCCACTATCACGTGCTTTGGGATGACAATCGTTTCTC  - 2340
 762 -  R  P  S  H  Y  H  V  L  W  D  D  N  R  F  S  - 776
```

```
2341 - TTCCGATGAGCTGCAGATTCTCACCTACCAGCTGTGTCACACGTA  - 2385
 777 -  S  D  E  L  Q  I  L  T  Y  Q  L  C  H  T  Y  - 791

2386 - TGTGCGCTGTACGCGCTCCGTGTCCATCCCGGCGCCAGCCTACTA  - 2430
 792 -  V  R  C  T  R  S  V  S  I  P  A  P  A  Y  Y  - 806

2431 - TGCTCACCTGGTGGCCTTCCGGGCCAGGTACCACCTGGTGGATAA  - 2475
 807 -  A  H  L  V  A  F  R  A  R  Y  H  L  V  D  K  - 821

2476 - AGAACATGATAGTGCCGAAGGAAGCCATACCTCCGGGCAGAGCAA  - 2520
 822 -  E  H  D  S  A  E  G  S  H  T  S  G  Q  S  N  - 836

2521 - TGGACGCGACCATCAGGCCTTGGCCAAGGCCGTGCAGGTCCACCA  - 2565
 837 -  G  R  D  H  Q  A  L  A  K  A  V  Q  V  H  Q  - 851

2566 - GGACACGCTGCGCACCATGTACTTTGCGTGACAAGTTTCAGTGTT  - 2610
 852 -  D  T  L  R  T  M  Y  F  A  *                 - 860

2611 - TACGCTTGTGTACCGAGGTGGATTCACACGAGACCAGCTACACTC  - 2655
 860 -

2656 - AGACCAACAAATGCCCAGCCCTTCCATGACAGCCAGCATCGAACA  - 2700
 860 -

2701 - TGA                                          - 2703
 860 -
```

Table 3.3: Protein translation of pAgo2 cDNA aligned with bovine and human Ago2 protein sequences. Orange and blue fonts indicate sequence deletions (orange = D2; blue = D3). Domain regions highlighted: yellow = DUF; green = PAZ domain; pink = Piwi domain.

```
Bovine   MYSGAGPALAPPAPPPPPIQGYAFKPPPRPDFGTSGRTIKLQANFFEMDIPKIDIYHYEL 60
Human    MYSGAGPALAPPAPPPP-IQGYAFKPPPRPDFGTSGRTIKLQANFFEMDIPKIDIYHYEL 59
Porcine  MYSGAGPVLAPPAPPRPPIQGYAFKPPPRPDFGTSGRTIKLQANFFEMDIPKIDIYHYEL 60
         *******.******* * ****************************************

Bovine   DIKPEKCPRRVNREIVEHMVQHFKTQIFGDRKPVFDGRKNLYTAMPLPIGRDKVELEVTL 120
Human    DIKPEKCPRRVNREIVEHMVQHFKTQIFGDRKPVFDGRKNLYTAMPLPIGRDKVELEVTL 119
Porcine  DIKPEKCPRRVNREVVEHMVQHFKTQIFGDRKPVFDGRKNLYTAMPLPIGRDKVELEVTL 120
         **************:****************************************

Bovine   PGEGKDRIFKVSIKWVSCVSLQALHDALSGRLPSVPFETIQALDVVMRHLPSMRYTPVGR 180
Human    PGEGKDRIFKVSIKWVSCVSLQALHDALSGRLPSVPFETIQALDVVMRHLPSMRYTPVGR 179
Porcine  PGEGKDRIFKVSIKWVSCVSLQALHDALSGRLPSVPFETIQALDVVMRHLPSMRYTPVGR 180
         ************************************************************

Bovine   SFFTASEGCSNPLGGGREVWFGFHQSVRPSLWKMMLNIDVSATAFYKAQPVIEFVCEVLD 240
Human    SFFTASEGCSNPLGGGREVWFGFHQSVRPSLWKMMLNIDVSATAFYKAQPVIEFVCEVLD 239
Porcine  SFFTASEGCSNPLGGGREVWFGFHQSVRPSLWKMMLNIDVSATAFYKAQPVIEFVCEVLD 240
         ************************************************************
```

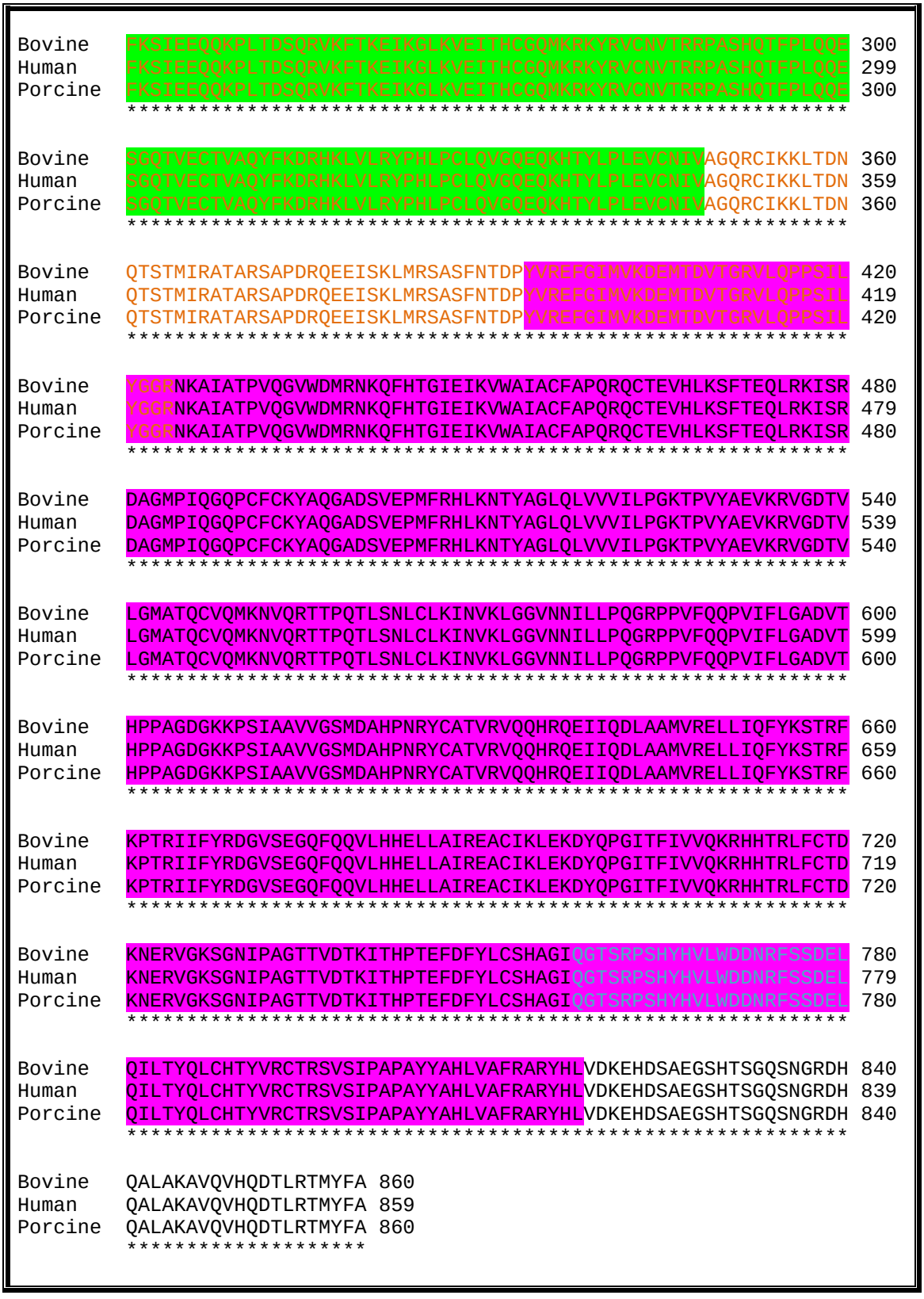

Table 3.4: Percent identity of the pAgo2 nucleotide and amino acid sequences in comparison to bovine, human, and mouse.

	Porcine	
	Nucleotide	**Amino Acid**
Bovine	94.2	99.6
Human	92.2	99.5
Mouse	89.4	99.2

The original primer sets for pAgo2 fragments included Ia, II, III, and IV. Fragment sizes for each primer set are illustrated in Figure 3.2 and are as follows: 769 bp for Fragment Ia; 347 bp for Fragment II; 1,507 bp for Fragment III; and 1,434 bp for Fragment IV. PCR product of Fragment III revealed two other products at 877 bp and 575 bp. These products were sequenced and determined by BLAST and sequence alignments to be shorter fragments of Ago2, with deletions occurring in the center of the fragment. Sequence alignments for the smaller products (at 877 bp and 575 bp) showed deletions of 603 bp and 932 bp, respectively, occurring in the middle of the sequence (illustrated in Figure 3.3). Sequence alignments of Fragment IV revealed a third deletion in the pAgo2 sequence, this one much smaller at 70 bp (Figure 3.3).

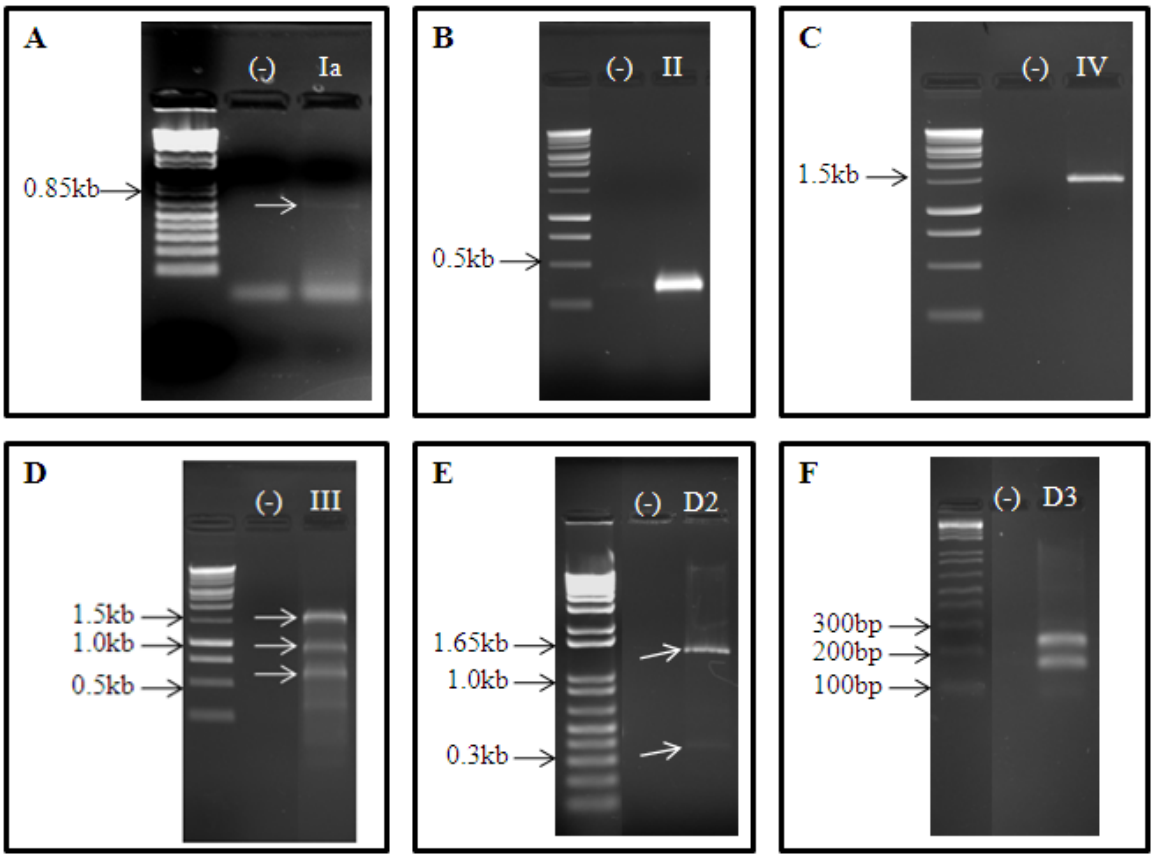

Figure 3.2: PCR products of primer sets used for sequencing pAgo2. (A) Fragment Ia, 769 bp. (B) Fragment II, 347 bp. (C) Fragment IV, 1,277 bp. (D) Fragment III, three products at 1,507 bp, 877 bp, and 575 bp. (E) Fragment D2, two products at 1,277 bp and 345 bp. (F) Fragment D3, two products at 232 bp and 162 bp.

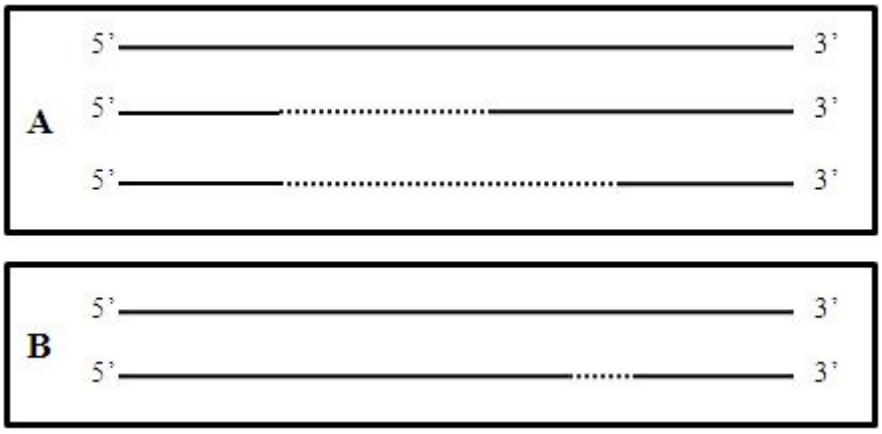

Figure 3.3: Illustration of pAgo2 sequence deletions. The top line is the complete sequence for each fragment. (A) Fragment III sequences: 1,507 bp, 877 bp, and 575 bp respectively. (B) Fragment IV 70 bp deletion. The solid strands indicate where the sequences align with the top strand; the dotted sections represent deleted sequence in the alignment.

Primer sets D2 and D3 were designed to these deleted sections (Figure 3.4) in order to confirm the presence of these deletions in the pAgo2 sequence. Presuming the deletions are present, the primer sets would amplify three products for D2 at 1,277 bp, 962 bp, and 345 bp and two products for D3 at 232 bp and 162 bp. We were only able to reproducibly amplify and clone two products for D2, which were 1,277 bp and 345 bp (Figure 3.2). Both D3 products were visible upon re-amplification of the PCR product (Figure 3.2). Sequencing and BLAST confirmed all of these products as Ago2. Deleted segments are noted on the pAgo2 translation alignment in Table 3.3.

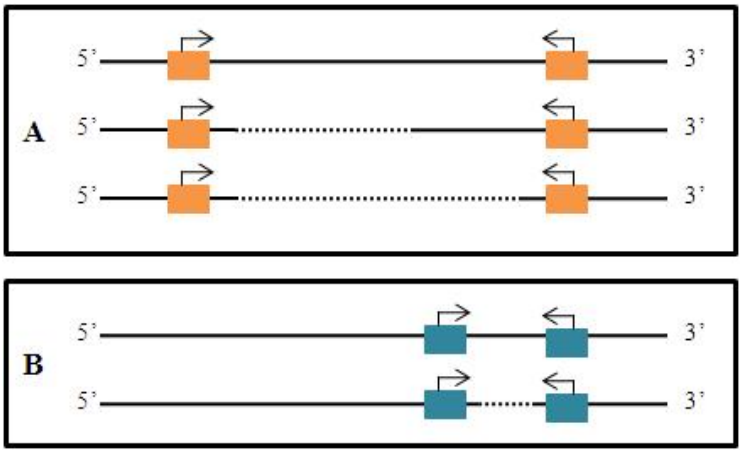

Figure 3.4: Illustration of primers designed for pAgo2 sequence deletions. (A) D2 primers should give three products. (B) D3 primers should give two products.

Expression Data

Preliminary expression data of Ago2 in sperm, oocytes, and embryos indicated that Ago2 is expressed in porcine MII oocytes and hatched blastocysts. The data also indicated that Ago2 is not expressed, in detectable quantity, in sperm or 8-cell embryos (Figure 3.5).

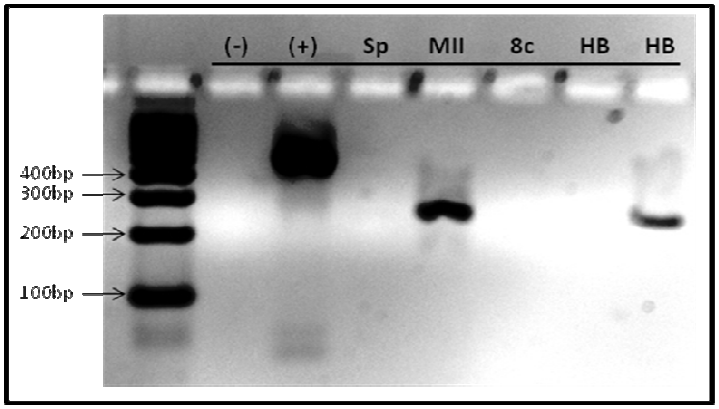

Figure 3.5: Ago2 endpoint PCR agarose check-gel in porcine sperm, oocytes, and embryos. Lanes: molecular weight ladder; (-) negative control: H2O blank; (+) positive control: Dicer in MII oocytes; Sp: Sperm; MII: MII oocytes; 8c: 8 cell embryo; HB: hatched blastocysts.

Discussion

Cloning and sequencing of pAgo2 indicate that Ago2 is highly conserved among species. Sequencing also revealed two possible deletions, in the pAgo2 coding sequence, that have not previously been reported in the literature. This is not entirely surprising, however, since many reported sequences are computer-generated predictions that are not often verified experimentally. Furthermore, given that Ago2 is highly conserved among species, it is predicted that these variants are likely expressed in other species as well.

The first deletion (Variant 1) occurs in the 5' region, encompassing a domain of unknown function and the PAZ domain (illustrated in Figure 3.6). As a nucleic acid binding domain, the PAZ domain is responsible for binding the 3' end of miRNA. Without this domain, the Ago2 protein would, theoretically, be unable to bind miRNA. The implications of having this type of Ago2 variant could be of great interest. For instance, if during certain stages of development the general up-regulation of mRNA is necessary, splicing of the Ago2 message into a variant incapable of binding miRNA would act much like an inhibitor to the inhibitor (miRNA). That is, Ago2 would be unable to carry miRNA to its target, and therefore the mRNA would not be degraded (illustrated in Figure 3.7).

The shorter deletion (Variant 2) in the 3' region occurs in the Piwi domain (Figure 3.6). The Piwi domain of Ago2 is the nuclease responsible for the mRNA cleavage that is characteristic of RNAi. Similar to RNase H, three residues within the Piwi domain form a catalytic triad. The human catalytic triad for Ago2 is D(597), D(669), and H(807) (Rivas *et al*, 2005; Song *et al*, 2004). The deletion in this region of pAgo2 does not

encompass these active sites, and therefore may have little effect on the nuclease activity of this domain. However, Ago3 is catalytically inactive even though the catalytic triad is conserved (reviewed by Meister and Tuschl, 2004). This may indicate that posttranslational modifications or interactions with specific proteins may modify the activity of Ago proteins. The implications of this type of Ago2 variant are also of interest. If the Piwi domain of pAgo2 Variant 2 is inactive, this protein would retain its ability to bind miRNA (unlike Variant 1) but would not be able to degrade the target mRNA. In this case the Ago2 variant might function to sequester the target mRNA, which could then be released at a later time (illustrated in Figure 3.7).

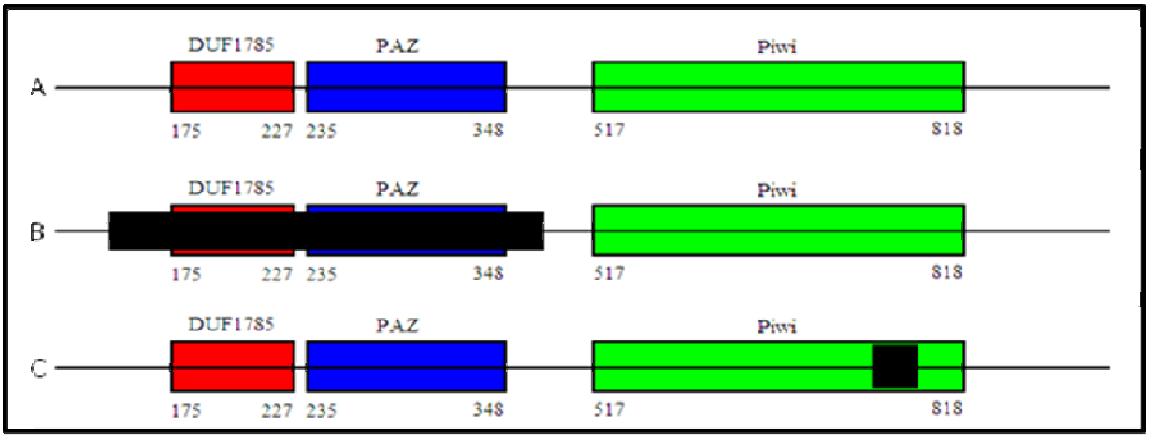

Figure 3.6: Schematic of pAgo2 sequence deletions. (A) Full length pAgo2, (B) 5' deletion, (C) 3' deletion.

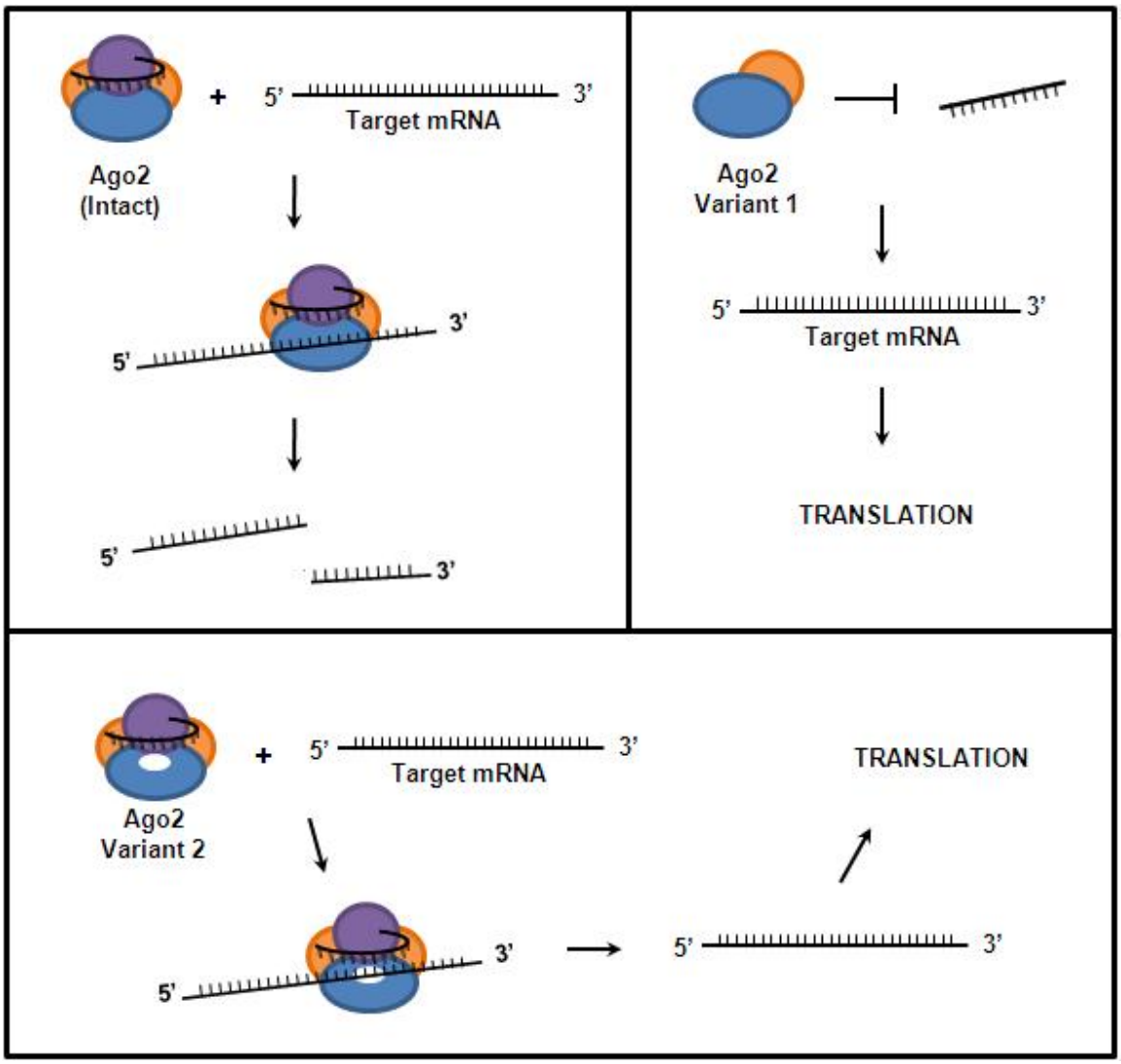

Figure 3.7: Proposed mechanisms for Ago2 Variants 1 and 2.

Our expression data indicated the presence of Ago2 in porcine ovary, oocytes, and blastocyst embryos. These data indicated that the pAgo2 message of maternal origin is lost or drastically decreased at the 8-cell stage but rebounds by the Day 7 blastocyst stage. These data support other observations identifying miRNA pathways present during embryonic development and the possible impact miRNA has on porcine

embryonic development. This indicated that maternal Ago2 is present in oocytes, but is depleted by the time the embryo reaches the 8-cell stage. At the hatched blastocyst stage, the embryo is likely producing its own Ago2 protein. Further work is needed to elucidate pAgo2 expression in other early stage embryos (i.e. 16-cell, morula, expanded blastocyst), as well as to determine if the ontogeny or level of pAgo2 expression can impact embryo development and differs between *in vivo* and *in vitro* produced embryos.

Research has indicated that Ago2 is required for the miRNA-mediated cleavage of mRNA and is essential for normal development. Characterizing Ago2 ontogeny throughout porcine embryonic development will begin elucidating miRNA involvement during early development in this species. Furthermore, once normal pAgo2 expression during embryonic development has been described, studies can be done to evaluate aberrations in Ago2 expression that may occur in developmentally compromised embryos. Ultimately, characterizing the miRNA pathway during porcine embryonic development may offer valuable insight into potential causes of aberrant embryonic development.

APPENDICES

Appendix A

Various Methods

Southern Blotting and Detection

For southern blotting procedures, protocols outlined in *Current Protocols in Molecular Biology* (1999) 2.9.1-2.9.20 were used; details and changes are as follows. PCR product was subjected to slab gel electrophoresis using a 1.2 to 2.0% TBE agarose gel; products less than 300 bp were run on a 2% gel, all others were run on a 1.2 to 1.5% gel. Gels were blotted overnight onto Sigma Nylon BioBond Membranes (St. Louis, MO) using a Whatman wick setup. Membranes were UV cross-linked twice, left to dry, and stored until detection.

Amersham Biosciences' Gene Images AlkPhos Direct Labelling and Detection System (Piscataway, NJ), used in conjunction with CDP-*Star* chemiluminescent detection reagents, were used for southern blot detection.

Appendix B

Various Results

Table A.1: Protein properties of pDicer.

Analysis	Entire Protein
Length	1,916 aa
Molecular Weight	216,844.06 m.w.
1 microgram =	4.612 pMoles
Molar Extinction coefficient	199080
1 A[280] corr. to	1.09 mg/ml
A[280] of 1 mg/ml	0.92 AU
Isoelectric Point	5.66
Charge at pH 7	-37.52

Table A.2: Protein properties of pAgo2.

Analysis	Entire Protein
Length	860 aa
Molecular Weight	97,378.32 m.w.
1 microgram =	
Molar Extinction coefficient	
1 A[280] corr. to	
A[280] of 1 mg/ml	
Isoelectric Point	9.35
Charge at pH 7	

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