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Expression of microRNA processing machinery genes in rhesus monkey oocytes and embryos of different developmental potentials

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Abstract

MicroRNAs (miRNAs) are a class of small RNAs that silence gene expression. In animal cells, miRNAs bind to the 3′ untranslated regions of specific mRNAs and inhibit their translation. The correct regulation of mRNA expression by miRNAs is believed to be important for oocyte maturation, early development and implantation. We examined the expression of 25 mRNAs involved in the microRNA processing pathway in a non human primate oocyte and embryo model. We observed that mRNAs related to miRNA splicing are downregulated during oocyte maturation while those related to miRNA processing are upregulated, indicating that there may exist a temporal difference in their activities related to transcriptional activity in germinal vesicle stage oocytes. We also observed that the vast majority of mRNAs examined were insensitive to α-amanitin at the 8-16 cell stage. The expression data did not reveal a major impact of embryo culture, and hormonal stimulation protocol affected only a small number of mRNAs, suggesting that the components of the pathway may be accumulated in the oocyte during oogenesis and resistant to exogenous insults. In comparison to published mouse array data, we observed species differences and similarities in the temporal expression patterns of some genes, suggesting that miRNA processing may be regulated differently. These data extend our understanding of the potential roles of miRNA during primate embryogenesis.

Keywords

miRNA; embryo; oocyte; gene regulation; oocyte quality; in vitro maturation; cleavage; preimplantation embryo

INTRODUCTION

MicroRNAs (miRNAs) comprise a large family of short (17-25 nucleotides), noncoding endogenous RNAs that often repress the expression of complementary messenger RNAs, thus controlling many biological processes in development, differentiation, growth, and metabolism (Lagos-Quintana et al., 2001; Lau et al., 2001; Lee and Ambros, 2001; Bartel, 2004, He and Hannon, 2004; Bagga et al., 2005). In eukaryotes, miRNAs are derived from long primary transcripts (1°miRNAs) transcribed by RNA polymerase II (Lee et al., 2004; Kim, 2005), and then cropped into the hairpin-shaped pre-miRNAs by the nuclear RNase III Drosha into 70-nt precursors (Lee et al., 2003; Reviewed by Carmel and Hannon, 2004;

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Tomari and Zamore, 2005) (Fig 1). Drosha/DGCR8 cleaves near the base of the stem to liberate a 60-nucleotide pre-miRNA hairpin (Denli et al., 2004; Gregory et al., 2004; Landthaler et al., 2004; Han et al., 2004, 2006). The pre-miRNAs, are exported out of the nucleus by exportin-5 (XPO5) (Yi et al., 2003; Bohnsack et al., 2004; Lund et al., 2004), and then cleaved by the cytoplasmic RNase III nuclease DICER into 22-nt miRNA duplexes (Bernstein et al., 2001; Grishok et al., 2001; Hutvágner et al., 2001; Ketting et al., 2001; Knight and Bass, 2001). One strand of the duplex usually remains as a mature miRNA (Khvorova et al., 2003; Schwarz et al., 2003; Hammond, 2005; Valencia-Sanchez et al., 2006), which is incorporated into the silencing complex RISC.

DICER transcripts are expressed in germinal vesicle (GV) stage mouse oocytes, decline in abundance during development to the two-cell embryo stage, and remain stable during morula and blastocyst formation (Cuia et al., 2007). Mouse oocytes lacking DICER function display defects in meiotic progression and cleavage upon fertilization (Murchison et al., 2007, Tang et al., 2007). Loss of DICER function in the mouse embryo is embryonic lethal at embryonic day 7.5 (E7.5), and DICER-null embryos are deficient in stem cells(Bernstein et al., 2003).

Following fertilization of mouse eggs, the maternal-to-zygotic transition, which initiates during the one-cell stage, is clearly evident by the two-cell stage (Schultz, 1993; 1999). In macaque and human embryos, genome activation occurs at the six- to eight-cell stage (Tesarik, 1987; Tesarik et al., 1986a,b, 1988; Artley et al., 1992; Weston and Wolf, 1994; Schramm and Bavister, 1999). One outcome of this transition is that transcripts that are common to the oocyte and embryo are degraded during oocyte maturation following fertilization, and replaced with embryonic transcripts (e.g., actin) [Bachvarova et al., 1989]. In addition, a dramatic reprogramming of gene expression occurs during this transition (Latham et al., 1991; Zeng et al., 2004), and this reprogramming provides the molecular foundation for transforming the highly differentiated oocyte into the totipotent blastomeres of the early cleavage stage preimplantation embryo. More recent studies showed that loss of DICER activity leads to the enhanced expression of a large number of mRNAs that are siRNA targets fo regulation in mouse oocytes (Tam e al., 2008; Watanabe et al., 2008). The early embryonic lethal phenotype associated with DICER deficiency indicates that in mouse embryos miRNA-mediated gene silencing plays an important role in completing the maternal to zygotic transition and establishing totipotent blastomeres in the mouse. Whether this applies to primates has not been determined.

The rhesus monkey offers an ideal model system for understanding human embryogenesis. Rhesus monkey oocytes and embryos of diverse developmental potentials can be obtained through specific protocols (Schramm and Bavister, 1994). This provides an opportunity to determine whether the expression of particular genes is altered under these different circumstances, and thus may contribute to different developmental potencies. Moreover, the use of a non-human primate model permits research advances where legal and ethical restrictions inhibit the conduct of research on human embryos. For example, it is possible to employ highest quality non-human primate embryos for research, whereas a similar use of human embryos would raise significant ethical questions. However, non-human primate embryo research is quite costly, with individual oocytes and embryos representing substantial investments of resources and labor, and availability of material is limited by costs and seasonal restrictions. Because of this, the amount of molecular data that has been generated for non-human primate oocytes and embryos is lagging considerably behind other species. Thus, a system to permit molecular data to be obtained in a non-human primate species at little cost is helpful for advancing our understanding of human embryology. The Primate Embryo Gene Expression Resource (PREGER) has been established in order to permit such studies to be undertaken with little cost and effort (Zheng et al., 2004).

PREGER encompasses over 200 amplified cDNA libraries representing oocytes and embryos of many different stages obtained via diverse protocols, and permits comparisons between in vivo and in vitro development, different hormonal stimulation protocols, in vivo and in vitro maturation, and different culture systems. The PREGER resource was constructed to permit investigators to obtain expression data for genes of interest in a suitable non-human primate model species, without need for direct access to a non human primate colony, and without need to expend further resources. Using the PREGER resource, we have determined the temporal patterns of expression of mRNAs encoding components of the miRNA processing machinery, and investigated whether the expression of these genes is affected by culture system or by protocols that yield oocytes and embryos of different developmental potentials. We also compared these data to published expression for the mouse in order to evaluate potential differences in the regulation, production, and function of miRNA between the two species.

MATERIALS AND METHODS

The PREGER Resource

The studies undertaken here employed the Primate Embryo Gene Expression Resource (PREGER) (www.preger.org). The resource contains a collection of reverse transcribed and polymerase chain reaction (RT-PCR)-amplified cDNA libraries corresponding to more than 200 samples of rhesus monkey oocytes and preimplantation stage embryos. The PREGER sample collection was created using a well-established method for reverse transcription (RT) and exponential cDNA amplification that maintains the quantitative representation of mRNAs by amplifying the 3' terminal regions of the entire mRNA population (Brady and Iscove, 1993; Iscove et al., 2002). After amplification, aliquots of each sample library are spotted onto filters by dot blotting as described (Quantitative Amplification and Dot Blotting, QADB). It should be noted that, because the entire mRNA population is uniformly amplified during the RT-PCR procedure, the amount of input mRNA within the range of one to four embryos does not affect the quantitative representation of sequences within the amplified cDNA population. Once the dot blots are prepared, they are hybridized to mRNA-specific probes and the hybridization results analyzed.

The QADB method has been extensively validated by previous studies in both mouse and monkey embryos, and the sensitivity and reliability of the assay documented (reviewed, Wang and Latham, 2000; Wang et al., 2001; Zheng et al., 2004). The method is applicable to small amounts of material, even single or partial embryos, provides the ability to quantify expression of a large number of mRNAs, and can provide estimates of actual mRNA abundance. These properties make the QADB method ideal for examining mRNA abundances in rhesus monkey oocyte and embryos, which are of highly limited availability and costly to obtain. The QADB method is fully quantitative and produces hybridization signals that are linear over many orders of magnitude, extending to a very low mRNA abundance (at least 500 copies/mouse embryo), and with excellent reproducibility. The method maintains quantitative representation of sequences within the cDNA population during amplification by limiting the length of the first cDNA strand, and thus minimizing selection against mRNAs of long length (Brady and Iscove, 1993). Re-amplification of cDNA through additional rounds of PCR exerts a minimal effect on results (Domachenko et al., 1997). The RT-PCR method yields highly representative cDNA libraries (Brady and Iscove, 1993; Brady et al., 1995; Cano-Gauci et al., 1993;Weaver et al., 1999; Iscove et al., 2002). The method yields expected differences in gene expression, for example revealing effects of parental chromosome origin on the preimplantation expression of the imprinted *Ascl2* (*Mash2*), *U2afi-rs1*, *H19*, *Xist*, and *Snrpn* genes, including approximately two-fold greater levels of expression in the appropriate uniparental embryos as compared with control fertilized embryos (Latham et al., 1995; Mann et al., 1995; Rossant et al., 1998). The QADB

method first revealed that the *Xist* RNA is first transcribed at the two-cell stage in the mouse (Latham and Rambhatla, 1995), which has been confirmed (Hartshorn et al., 2002; Zuccotti et al., 2002). Estimates of mRNA copy number in mouse embryos based on the QADB method, and the temporal and quantitative aspects of the data are consistent with data obtained by other methods, (Rambhatla et al., 1995, Wang and Latham, 2000, Wang et al., 2001). The QADB method thus has allowed us to produce a resource for gene expression study in rhesus monkey oocytes and embryos that are exceedingly limited in availability. It should be noted that, because the entire mRNA population is uniformly amplified during the RT PCR procedure, the amount of input mRNA within the range of one to four embryos does not affect the quantitative representation of sequences within the amplified cDNA population. Once the dot blots are prepared, they are hybridized to mRNA-specific probes and the hybridization results analyzed.

Oocytes and embryos

The isolation and culture of the oocytes and embryos during the construction of the PREGER sample set has been described in detail (Zheng et al., 2004). Oocytes contained in the PREGER sample set were obtained from monkeys treated with follicle stimulating hormone (FSH) only, or FSH followed by human chorionic gonadotropin (hCG), and matured either in vitro or in vivo, respectively. The sample collection also contains oocytes obtained without hormonal stimulation (non stimulated, denoted as NS). Embryos were obtained from these three categories of ooctyes, and also by natural conception (morula/blastocysts) as described (Zheng et al., 2004). Between 3 and 13 samples of one to four oocytes or embryos were obtained for each stage. The embryos included in the PREGER sample set were all high quality and healthy in appearance. Samples of eight-cell and morula-stage embryos treated with the RNA polymerase II inhibitor α-amanitin from the pronucleate stage onward in HECM9 culture were included to evaluate transcriptional dependence of mRNA expression. Details concerning the array, diversity, and origin of samples, and the sensitivity and quantitative reliability of the quantitative amplification and dot blotting method have been described previously (Zheng et al., 2004, and in other references available at the PREGER website www.preger.org). All procedures employed to obtain oocytes and embryos were conducted according to recommendations of the Guide for the Care and Use of Laboratory Animals and the Animal Welfare Act, and its amendments.

Complementary DNA Probes and Hybridization

Complementary DNA probes were obtained by PCR, which was performed in 100 μl reactions containing 4 μl of plasmid DNA product (cDNA clones obtained from Open Biosystems Huntsville, AL, USA), 10 × PCR Buffer containing 15 mM $MgCl_2$ (Roche Diagnostics, Indianapolis, USA); 10 mM dNTPs (Roche Diagnostics); 10 μM for each of the forward and reverse primers (Table 1); and 5 U/μl TaqDNA polymerase (Roche Diagnostics). Reactions were run on a Techne PCR machine (Burlington, NJ, USA) at 94° for 5 min to denature, followed by 35 cycles of 94°C for 1 min, annealing at 55-60°C, for 1 min; 72°C for 2 min, final extension for 5 min at 72°C. PCR products were resolved on 1% agarose gels. Excised PCR products were purified using a QIAquick Gel Extraction Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol. Purified PCR products were then used for probe labeling.

Blot preparation, hybridization, and quantitative analyses were performed as described (Zheng et al., 2004). Gel-purified cDNA fragments were radiolabeled by the random primer method (Feinberg and Vogelstein, 1983). Labeled probes were hybridized to blots as described (Latham et al., 1999; Rambhatla et al., 1995). Hybridization results were visualized and quantified by storage phosphorimaging with background correction and normalization as described (Rambhatla et al., 1995). Data were expressed as the mean (±

standard error of the meanSEM), counts per minute (cpm) bound value for each stage/condition of oocytes and embryos included in the analysis. Probes that yielded weak or absent hybridization signals were repeated to confirm result. The statistical significance of differences was evaluated using the t-test ($P < 0.05$ considered significant). Where necessary a correction for multiple testing was applied, using a significance level of $P < 0.016$ as indicated.

Murine Gene Expression Data

Expression data for murine homologues were extracted from the microarray data available in the Gene Expression Omnibus repository byeng et al (2004, which were obtained originally using the Affymetrix MOE 430A and 430B chips. The stages represented were GV stage oocytes, and embryos at the one-cell, two-cell, eight-cell, and blastocyst stages. These data were expressed as the mean (+SEM) Affymetrix array hybridization signal.

RESULTS

We examined the patterns of expression of 25 genes involved in the miRNA processing pathway. The functions of these genes are described in Table 2 and Fig. 1. Of the 25 genes examined, we detected the expression of 21 in our sample set of rhesus monkey oocytes and embryos.

Expression of mRNAs encoding proteins involved in RNA splicing and editing during miRNA biogenesis

Once transcribed, the primary miRNAs are subjected to splicing and editing. Splicing is mediated by the survival of motor neuron (SMN) protein complex (Fig. 2). The SMN complex contains five proteins known as SIP1 (formerly GEMIN2; Liu et al., 1997), GEMIN3 (a DEAD-box putative RNA helicase; Charroux et al., 1999), GEMIN4 (Charroux et al., 2000), GEMIN5 (Meister et al., 2001;Gubitz et al., 2002), and GEMIN6 (Pellizzoni et al., 2002). The SMN complex plays important roles in the assembly/restructuring and function of diverse ribonucleoprotein (RNP) complexes, including spliceosomal small nuclear RNPs (snRNPs; Fischer et al., 1997;Pellizzoni et al., 1998;Meister et al., 2001), small nucleolar RNPs (snoRNPs; Jones et al., 2001;Pellizzoni et al., 2001a) heterogeneous nuclear RNPs (hnRNPs; Mourelatos et al., 2001), and transcriptosomes (Pellizzoni et al., 2001b). The YBX1 protein participates in different steps of mRNA biogenesis, including mRNA transcription, processing, and transport from the nucleus into the cytoplasm (Sommerville, 1999;Wilkinson and Shyu, 2001;Stickeler et al., 2001), binds to splice sites in pre-mRNA, and regulates splice site selection (Allemand et al., 2007). A total of six mRNAs encoding components of the SMN complex and other proteins involved in RNA splicing was analyzed (Table1, Fig. 2). The *SIP1*, *GEMIN4*, *GEMIN5*, *GEMIN6* and *GEMIN7* mRNAs were maternally expressed. *SIP1*, *GEMIN5*, and *GEMIN6* mRNA were highly expressed in GV oocytes and decreased after oocyte maturation, and were then expressed throughout development. Only the *GEMIN6* mRNA was significantly α-amanitin sensitive at the 8-16 cell stage. The *GEMIN7* and *YBX1* mRNAs increased in expression in the developing embryo, with highest signals in *YBX1* mRNA and both mRNAs upregulated at the morula stage.

After splicing, adenosine deaminases (ADARs) edit the primary miRNAs , converting adenosine to inosine in double-stranded (ds) RNA (Bass, 2002; Knight and Bass, 2002; Tonkin and Bass, 2003; Yang et al., 2005). ADARs edit specific adenosines at crucial positions such as the glutamine/arginine site in the GluR-B transcript, which can have major consequences for the encoded protein, as inosine is translated as guanosine, leading to incorporation of a different amino acid at the edited codon. ADARs are expressed in many

tissues and can bind and deaminate any dsRNA. Independent studies have shown that ADARs shuttle in and out of the nucleolus in living cells (Desterro et al., 2003; Sansam et al., 2003). Two mRNAs were analyzed in this category, *ADAR1* and *ADARB1* (Table 1, Fig. 3). The *ADARB1* mRNA was not detected. The *ADAR1* mRNA was maternally expressed and had significantly lower expression at the GV and MII oocyte stages than at the one- and two-cell stage ($P < 0.05$). Its expression decreased sharply thereafter, with no α-amanitin-sensitive expression evident at the 8-16 cell stage.

Expression of genes involved in the RNA microprocessor complex

Three genes encoding components of the microprocessor complex were analyzed, *RNASEN* (homolog of Drosophila Drosha), *DGCR8*, (homolog of Drosophila Pasha) and *DICER1* (Table 1, Fig. 4). RNASEN is an RNase III enzyme responsible for initiating the processing of microRNA (miRNA). It cleaves long RNA primary transcripts (1° miRNAs) to produce stem-loop structures of about 70 base pairs long, known as pre-miRNAs (Han et al., 2004). RNASEN exists as part of the microprocessor complex, which also contains the double-stranded RNA binding protein. DGCR8 is essential for RNASEN activity, and binds single-stranded fragments of the 1° miRNA that are required for proper processing. DICER1 is a member of the RNase III family of nucleases that specifically cleave double-stranded RNAs (Bernstein et al., 2001). *RNASEN* mRNA expression was predominantly maternal, declining in abundance during development to the 8 to 16-cell stage. Its expression was significantly increased during maturation ($P < 0.001$). *DGCR8* mRNA expression was low at the GV stage and significantly increased during maturation ($P < 0.0001$). *DICER1* mRNA expression appeared very low, with low hybridization signals throughout development, but hybridization data indicated an apparent increase in signal upon oocyte maturation and again at the blastocyst stage (Fig. 4). None of these three mRNA's was α-amanitin sensitive at the 8- to 16-cell, indicating a predominantly maternal ooplasmic source of expression.

Expression of genes involved in transport of mRNA and miRNA from nucleus to cytoplasm

The traffic through the nuclear envelope is mediated by a protein family, which can be divided into exportins and importins. Binding of a molecule (a “cargo”) to exportins facilitates its export to the cytoplasm. Importins facilitate import into the nucleus. FMR1 protein is endowed with a nuclear localization and a nuclear export signal (Eberhart et al., 1996; Bardoni et al., 1997; Fridell et al., 1996), suggesting that it shuttles between nucleus and cytoplasm (Tamanini et al., 1999) and may participate in mRNA export from nucleus to cytoplasm. Exportin-5 (XPO5) mediates efficient nuclear export of short miRNA precursors (premiRNAs) and its depletion by RNA interference results in reduced miRNA levels (Lund et al., 2004). Five mRNAs involved in RNA export were analyzed, *FMR1, RANGAP1, XPO1, XPO4*, and *XPO5* (Table 1, Fig. 5). The *FMRI* mRNA was distributed throughout development and had a low expression level compared to other mRNA's in the group. *RANGAP1, XPO1, XPO4*, and *XPO5* mRNAs significantly decreased in abundance during maturation from GV-stage oocyte to the metaphase II (MII) stage ($P < 0.05$). The *RANGAP1* mRNA was transiently elevated at the morula and early blastocyst stages. The *XPO1* and *XPO5* mRNAs displayed somewhat uniform patterns of expression, and the *XPO4* mRNA increased gradually in expression over time. None of mRNAs in this group was α-amanitin sensitive at the 8-16 cell stage.

Expression of mRNAs encoding components of the RISC complex

ARGONAUTE family proteins are highly conserved and have been implicated in RNAi function and related phenomena in several organisms. In addition to roles in RNAi-like mechanisms, ARGONAUTE proteins influence development in *C. elegans* by the two small temporal RNAs (stRNAs) *lin-4* and *let-7* (Lee et al., 1993; Reinhart et al., 2000), and at least a subset are involved in stem cell fate determination (Brower-Toland, 2007; Carmel et al.,

2002; Lin, 2007). They contain two common domains named PAZ and PIWI domains (Cerutti et al., 2000; Song et al., 2003). The PAZ domain proteins include AGO proteins, also known as EIF2Cs in mammals (Lingel et al., 2004), and PIWI domain proteins include PIWIL proteins. Both domains are components of RNA-induced silencing complex (RISC) [Tahbaz et al., 2004]. The expression of 6 mRNAs encoding ARGONAUTE family proteins was examined (Table1, Fig. 6). Three PAZ domain mRNAs *EIF2C1, EIF2C2*, and *EIF2C3* were expressed at nearly constant levels throughout development. The *EIF2C4* mRNA had a predominantly embryonic expression pattern and yielded the strongest hybridization signal for the group. The *EIF2C3* mRNA produced weak hybridization signals in oocytes and embryos. None of the four PAZ domain mRNAs analyzed was α-amanitin sensitive at the 8-16 cell stage. Two PIWI domain mRNAs were analyzed, *PIWIL1* and *PIWIL2*. PIWI is essential for maintaining germline stem cells in adult *Drosophila* ovaries and testes (Cox et al., 1998). The *PIWIL2* mRNA displayed an overall low expression level and was detectable in oocytes, with increased expression from the eight-cell stage onward. Its pattern was similar to that of *EIF2C4* mRNA. The *PIWIL1* mRNA was not detected.

The TNRC6 family proteins are required for miRNA-guided gene silencing and are present in P bodies in *C. elegans* and *D. melanogaster* (Ding et al., 2005; Rehwinkel et al., 2005). TNRC6B and MOV10 are required for miRNA-guided mRNA cleavage in vivo and specifically associate with EIF2C proteins (Meister et al., 2005). The *TNRC6B* mRNA significantly decreased in abundance during maturation from the GV stage oocyte to the MII stage $P < 0.0001$ (Fig. 6), and was then expressed throughout development. The *TNRC6B* mRNA was not α-amanitin sensitive at 8-16 cell stage. The *MOV10* mRNA was not detected.

Analysis of Transcription Factor Binding Sites

To explore whether the 25 genes examined share possible aspects of gene regulation, we applied the oPOSSUM analysis software (http://www.cisreg.ca/cgibin/oPOSSUM/opossum) (Ho-Sui et al., 2005) to the list of genes. This software combines methods for detecting transcription factor binding sites (TFBS) documented in the JASPAR database, statistical methods for identifying over-represented TFBS amongst a group of genes, and a database of DNA regions that are conserved amongst moderately divergent organisms (i.e., phylogenetic footprinting). A combination of a Z score > 10 and Fisher P value < 0.01 provides minimal likelihood of false positive results. This analysis revealed one transcription factor-binding site (IRF2) that is significantly over-represented (found in *TNR6CB, RANGAP1, FMR1, GEMIN7*) (Table 3). One other transcription factor-binding site (STAT1) yielded borderline significance scores (found in *TNR6CB, RANGAP1, FMR1, GEMIN7, XPO5, MOV10, ADARB1*). The failure to detect expression of four of the miRNA pathway-associated mRNAs (*ADARB1, FMR1, MOV10, PIWIL1*) was reminiscent of the low or undetected expression of these mRNAs in the mouse (Zeng et al., 2004). We therefore also sought to determine whether these four genes might share any aspect of transcriptional regulation. This analysis revealed two transcription factor-binding sites with marginal significance for over-representation, PAX6 (found in *FMR1, MOV10*) and STAT1 (found in *ADARB1, FMR1, MOV10*). Further studies will be needed to ascertain whether these transcription factors indeed contribute to the regulation of this complex pathway in oocytes and embryos.

Effects of hormonal stimulation protocol and embryo culture on mRNA expression

We next wished to determine whether the expression of mRNAs related to the miRNA function pathway are affected by the hormonal stimulation and in vitro maturation conditions employed to produce the oocytes prior to in vitro fertilization. The PREGER sample set contains oocytes of high quality resulting from in vivo maturation (hCG oocytes), intermediate quality resulting from FSH stimulation in vivo followed by in vitro maturation

(FSH oocytes), and low quality resulting from in vitro maturation of oocytes from non-stimulated ovaries (NS oocytes) (Schramm and Bavister, 1994), along with cultured embryos derived from these three kinds of oocytes following in vitro fertilization. The resource also contains embryos flushed directly from the reproductive tract and lysed, which can be used to evaluate possible effects of embryo culture. We find that embryo culture had no significant effect on the expression of the mRNAs under study, however hormonal stimulation protocol and oocyte quality affected a small number of transcripts. In previous studies we observed that many maternal mRNAs are precociously eliminated by the two-cell stage with embryos from either FSH or NS oocytes (Zheng et al., 2005) as compared to embryos from FSH+hCG cycles. Of the 21 mRNAs for which expression was detected, 13 displayed values of 50 cpm bound or greater, and were selected for examining effects of hormonal stimulation protocol and IVM on expression. Of these 13, four (*ADAR, RANGAP1,SIP1, XPO5*) displayed significantly reduced ($p < 0.05$) expression, two of which (*RANGAP1, SIP1*) passed the more stringent level of $p < 0.016$, and were likewise reduced in embryos from FSH oocytes (Figure 7). One mRNA (*EIF2C4*) was elevated in NS embryos.

DISCUSSION

The correct regulation of mRNA expression by miRNAs is believed to be important for oocyte maturation and early development (Murchison et al., 2007; Tang et al., 2007; Bernstein et al., 2003). We provide here the first report of expression of genes that comprise this important pathway in a non human primate oocyte and embryo model species. Several striking features of the data can be noted. First, we observe that mRNAs related to miRNA splicing (*GEMIN5, GEMIN6, GEMIN7, SIP1*) are downregulated during oocyte maturation most likely via maternal mRNA degradation. Conversely one of the mRNAs related to miRNA processing (*DGCR8*) undergoes an apparent upregulation during maturation, most likely the result of increased mRNA polyadenylation. DICER also appeared to increase in hybridization signal during maturation, but the signal obtained was overall quite low. There may exist a temporal difference in maximal activities of these two portions of the pathway, splicing and processing, related, respectively, to transcriptional activity in the GV stage oocyte, and cytoplasmic processes upon maturation and after fertilization related to elimination of maternal mRNAs. This is further supported by the strongly maternal pattern of RNASEN mRNA expression. The *YBX1* and *GEMIN7* mRNAs were elevated at the morula stage, possibly related to a need for mRNA splicing at the time of embryonic genome activation. The *ADAR1* mRNA was most highly expressed as a maternal transcript, indicating a potentially important role for this protein in the transcriptionally active GV stage and early embryo.

The second striking result is that the vast majority of the mRNAs examined displayed insensitivity to α-amanitin at the 8-16 cell stage. Only *GEMIN6* was α-amanitin sensitive, *YBX1* and *GEMIN7* mRNAs displayed pronounced increases in expression at the morula stage, and *RANGAP1* and XPO4 mRNAs displayed a clear increase in expression after the morula stage. This obviously does not exclude active transcription after the morula stage, however, in contrast to many other mRNAs examined in earlier studies (Zheng et al.,2005, Mtango and Latham, 2007), this group of mRNAs appears notable for persistent contribution of maternally inherited transcripts throughout early and mid cleavage development. It is tempting to speculate that if correct regulation by miRNAs is important for early development, then one aspect of high oocyte quality could be a sufficient supply and persistence of maternal transcripts encoding these proteins.

Third, we do not observe a major impact of either embryo culture or hormonal stimulation/ oocyte maturation protocol on the expression of the mRNAs studied here. This suggests that

the components of the pathway may be accumulated in the oocyte early during oogenesis and be relatively refractory to exogenous insults. This may reflect the dynamic nature of oocyte-somatic cell interactions leading to an ever-changing ooplasmic state during oogenesis, with a significant role played by this pathway in preparing the oocyte for successful maturation, as strikingly demonstrated by the deficiency in meiotic progression of DICER deficient mouse oocytes (Murchison et al., 2007).

While we do not observe a major impact of either embryo culture or hormonal stimulation and oocyte maturation protocols on the expression of these mRNAs, we do observe that the *RANGAP1* mRNA is diminished in abundance in two-cell embryos from both FSH and NS oocytes, which have diminished developmental potential relative to hCG oocytes (Schramm and Bavister, 1994). RANGAP1 is a key regulator of bidirectional transport of proteins and ribonucleoproteins across the nuclear pore complex, and is also a component of the spindle (Bamba et al., 2002). Thus, in addition to its role in miRNA production, RANGAP1 regulates a myriad of processes at the level of nuclear-cytoplasmic exchange. Loss of the ubiquitin E2 ligase UBC9 results in a failure of RANGAP1 accumulation at the nuclear pore complex, and defects in blastocyst expansion and inner cell mass development leading to early postimplantation developmental arrest (DeGregori et al., 1994; Nacerddine et al., 2005). It is also interesting to note that in *Drosophila*, the Segregation Distorter locus encodes a mutant RanGap protein that mislocalizes to the nucleus, disrupting nuclear RAN pathway signaling, and leading to lethality, and that overexpression of RANGAP1 can also lead to segregation distortion via excessive localization to the nucleus (Merrill et al., 1999; Kusano et al., 2002). These observations indicate that the correct level of expression of RANGAP1 is essential for normal development. Deficient expression of *RANGAP1* mRNA, therefore, could contribute to reduced developmental potential in embryos derived from FSH and NS oocytes. Whereas in vivo matured oocytes (hCG treated females) can support approximately 30% blastocyst formation, FSH oocytes are much less able (4%) to do so, and NS oocytes do not support blastocyst formation following standard IVM and embryo culture (Schramm and Bavister, 1994).

Available data indicate that a lack of hormonal stimulation likely compromises correct transcriptional regulation during oogenesis, as GV stage oocytes from NS females do not display the expected nucleolar rimming, but FSH oocytes do undergo rimming and display enhanced ability to undergo IVM and to support embryogenesis (Schramm et al., 1993, Schramm and Bavister, 1994). Cumulus enclosed oocytes display superior characteristics after IVM as compared to denuded oocytes (Schramm and Bavister, 1994). Moreover, whilst NS oocyte culture with exogenous gonadotropins leads to enhanced meiotic maturation, activation, and early cleavage, but not enhanced developmental potential thereafter (Schramm and Bavister, 1995), co-culture of NS oocytes in vitro with granulosa cells from FSH-stimulated females leads to enhanced development (Schramm and Bavister, 1996). This is not seen with granulosa cells from NS females (Schramm and Bavister, 1996). In vitro matured rhesus monkey oocytes also display deficiencies in embryonic transcriptional activation (Schramm et al., 2003). Last, we reported previously that many maternal mRNAs may be precociously diminished in oocytes from NS females, leading to deficient expression after fertilization (Zheng et al., 2005). Collectively, these data indicate that hormonal stimulation operating in concert with signals from somatic cells in the follicle enhances both nuclear and cytoplasmic processes that later favor both meiosis and embryogenesis, and that interruption of these signals can disrupt the normal course of events during the period immediately preceding maturation and during meiotic progression, as well as during embryogenesis (Schramm and Bavister, 1999). Oocytes matured in vitro from FSH stimulated females display moderately reduced developmental potential, whilst those from NS females display severely reduced developmental potential, indicates that the beneficial effects of hormonal stimulation and possibly oocyte-somatic cell interactions may continue

even beyond the time when oocytes are typically removed for IVM. The identification of genes such as *RANGAP1* that display deficient expression in embryos from both FSH and NS females should help us to understand the normal course of events that establish high developmental potential, and the maternal components of the oocyte that may be affected when oocyte-somatic cell signaling is disrupted.

The availability of array data for gene expression in mouse embryos (Zeng et al., 2004; Fig. 8) provides the opportunity to evaluate the similarity and difference between the patterns of gene expression in rhesus monkey and a rodent model organism (Fig. 9). We observe some similarities between the two species for miRNA processing genes. Both species display predominantly maternal expression of *ADAR1*. Similar constitutive patterns are seen for *DGCR8, FMR1, XPO5,* and*EIF2C1*. In both species, the *XPO4* and *PIWIL2* mRNAs are upregulated over time. However, differences are also apparent. The *GEMIN* family of mRNAs displays quite different patterns of expression. The *RANGAP1* mRNA increases in abundance in both species around the time of transcriptional activation, but thereafter this mRNA declines in abundance to a greater degree in the monkey. Some mRNAs display more pronounced differences in expression pattern. The *Dicer* mRNA is more strongly expressed as a maternal transcript in mouse oocytes compared to mouse embryos. The *Tnrc6b* mRNA shows a comparatively larger decrease in expression in the later cleavage stages in the mouse than in the monkey. These observations indicate that the activity of some parts of this pathway likely follow a similar pattern between the two species, however differences in temporal patterns of expression of *DICER, GEMIN, TNRC6B, RANGAP1* and *RNASEN* mRNAs indicate that some processes may be regulated differently between the two species. Additional studies will be needed to ascertain whether the differences in DICER and RNASEN mRNA expression patterns between the two species reflect different temporal requirements for these gene products during oogenesis and embryogenesis, perhaps associated with different temporal kinetics of the maternal to embryonic transition and elimination of maternally inherited mRNAs. The mRNA expression data provided here will provide a useful foundation of information for designing detailed functional studies of miRNA production in rhesus monkey oocytes and embryos. Further detailed studies of the relevant proteins and their contributions to development will further advance the field. Moreover, the emerging ability to examine miRNA expression in early stage embryos (Tang et al., 2006) will provide the opportunity to compare between species the conservation of roles for individual miRNAs in oocytes and early embryos. Gene targeting studies in the mouse have revealed that a number of the genes examined here (*Eif2c2, Dgcr8, Dicer Rangap1, Sip1*) play essential roles in early embryo development (Morita et al., 2007; Wang et al., 2007; Bernestein et al., 2003; Deng and Lin., 2002; Carmel et al., 2007; Watanabe et al., 2008). *Sip1* (a.k.a. *Gemin2*) homozygous and heterozygous mutant and and wild type preimplantation embryos showed no morphological alterations (Jablonka et al., 2002). *Dgcr8* knockouts display abnormal development by embryonic day 6.5 (Wang et al., 2007). *Eif2c2* (a.k.a. *Ago2*), *Eif2c3* (*a.k.a. Ago3*), and *Dicer* mRNAs are detected by RT-PCR throughout oocyte growth and *Eif2c4* (a.k.a. *Ago4*) and *Mili* mRNAs at early stages of oocytes growth. Deficiency for *Eif2c2* mRNA leads to an elevation of many DICER target mRNAs (Watanabe et al., 2008) indicating the importance of AGO2 in regulating the maternal mRNA population. Further detailed studies of the expression and post-translational control of these proteins, and other functional studies in the rhesus monkey should provide additional insight into how the non human primate embryo correctly regulates this important class of molecules, the role of this pathway during embryogenesis, and how this pathway may be disrupted in response either to endogenous or exogenous factors.

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Figure 1.

Summary of miRNA processing machinery. In the nucleus miRNA are transcribed by RNA polymerase and are termed primary miRNAs (1°miRNAs). The dsRNA-specific ribonuclease RNASEN (Drosha) digests the 1°miRNAs in the nuclease to release hairpins, precursor miRNA (pre-miRNA) which are transported from the nucleus to the cytoplasm by exportins. Once in the cytoplasm, DICER cleaves the pre-miRNA approximately 19 bp from the Drosha cut site. The resulting double-stranded RNA has 1–4 nt 3′ overhangs at either end. Only one of the two strands is the mature miRNA. To control the translation of target mRNAs, the double-stranded RNA produced by DICER must strand separate, and the single-stranded mature miRNA must associate with the RISC. Post- transcriptional modifications such as splicing and editing play a part in miRNA processing.

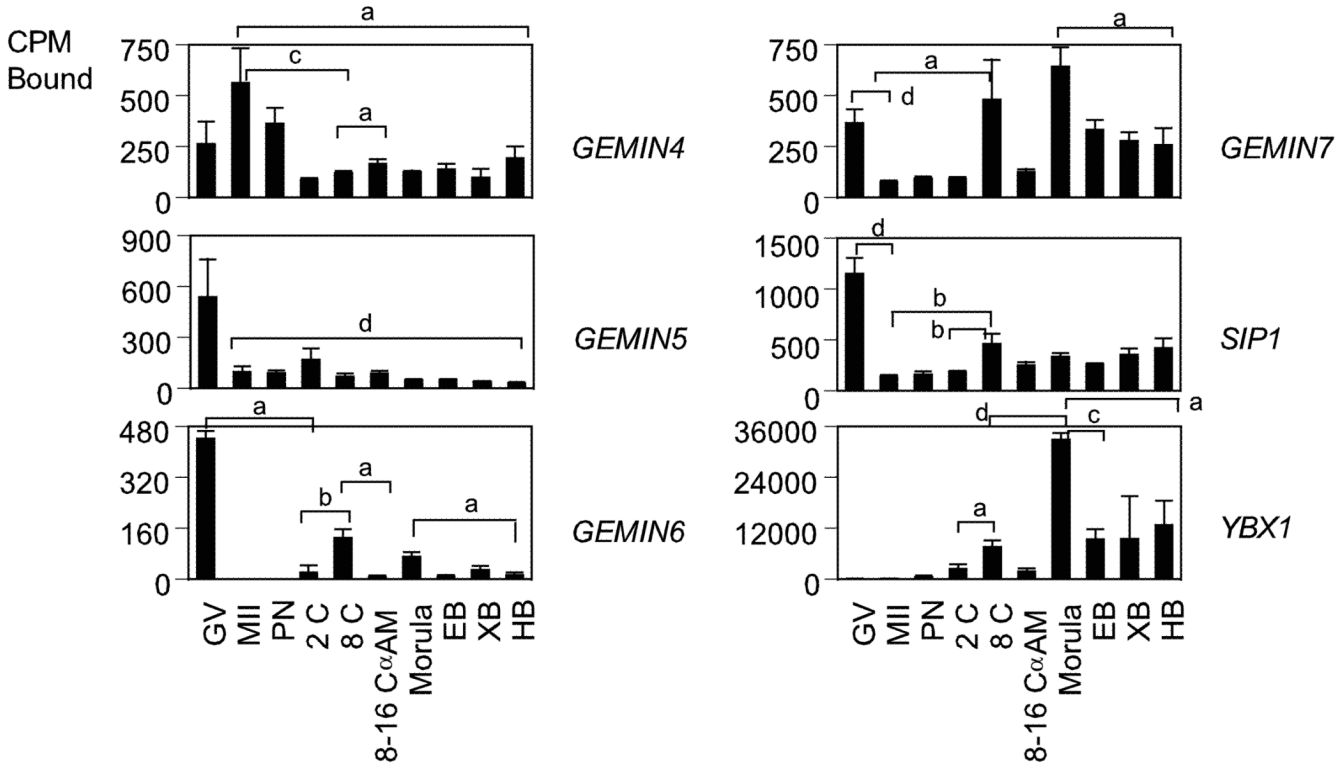

Figure 2.

Temporal expression patterns of mRNAs encoding proteins involved in RNA splicing in rhesus monkey oocytes and embryos. Graphs show the relative levels of expression for GV and MII stage oocytes and pronucleate through hatched blastocyst stage embryos produced by in vitro fertilization of oocytes from hCG-stimulated females, and then cultured in vitro in HECM9. GV, germinal vesicle stage oocyte; MII, MII-stage oocyte; PN, pronucleate 1-cell stage embryo; 2C, 2-cell stage embryo; 8C, 8-cell stage embryo; 8–16C αAm, 8- to 16-cell stage cultured in α-amanitin; EB, early blastocyst; XB, expanded blastocyst; HB, hatched blastocyst. Expression data for the mRNAs encoding the indicated proteins are expressed as the mean CPM bound, and the standard error of mean (SEM) is indicated. Statistically significant differences in gene expression corresponding to some of the major increases or decreases in expression are denoted by the brackets (for comparisons between stages at the ends of the brackets). Letters a through d indicate $P < 0.05$, 0.01, 0.001, and 0.0001, respectively.

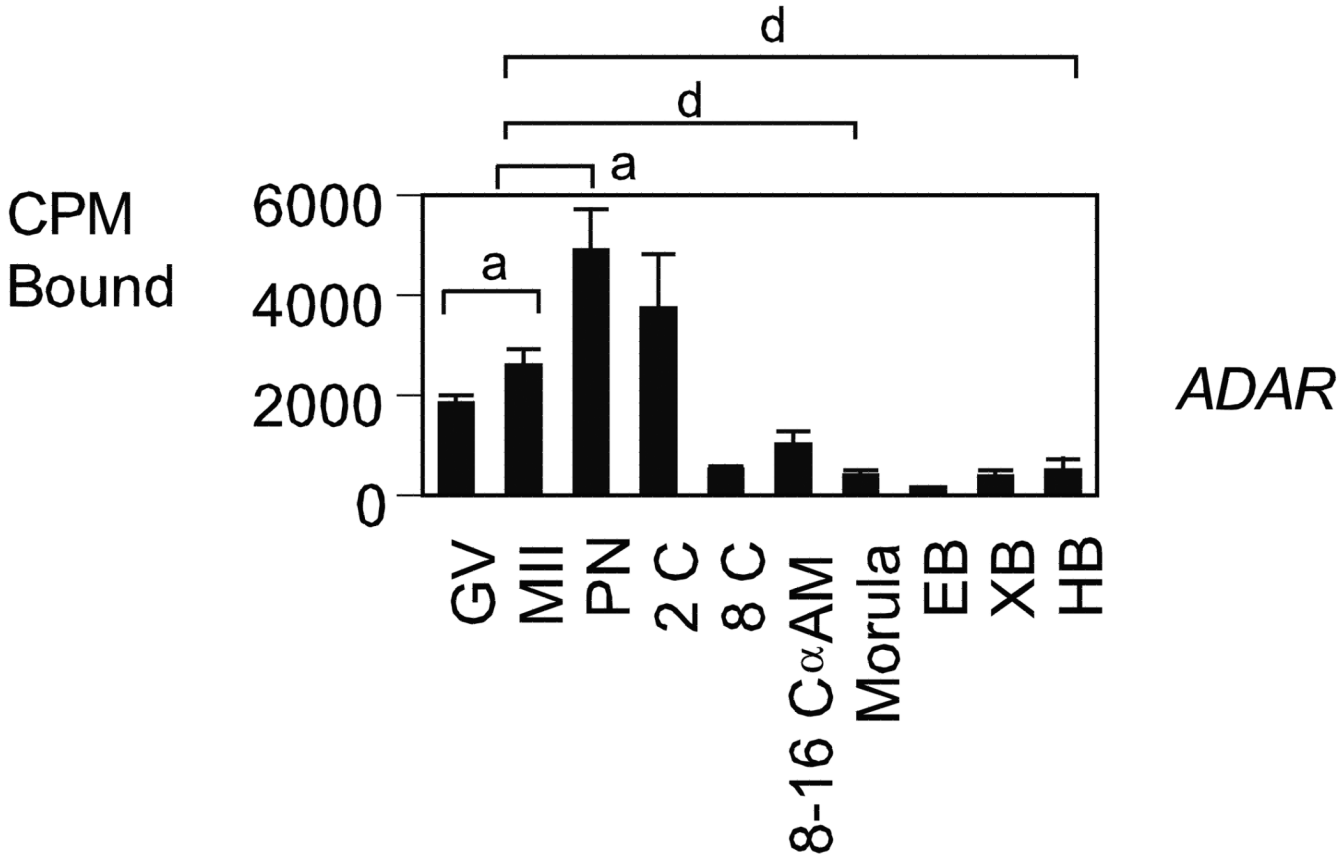

Figure 3.
Temporal expression patterns of mRNAs encoding proteins related to miRNA editing. Data are presented as in Fig.2.

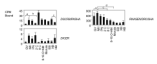

Figure 4.
Temporal expression patterns of mRNAs encoding proteins involved in the RNA microprocessor complex. Data are presented as in Fig.2.

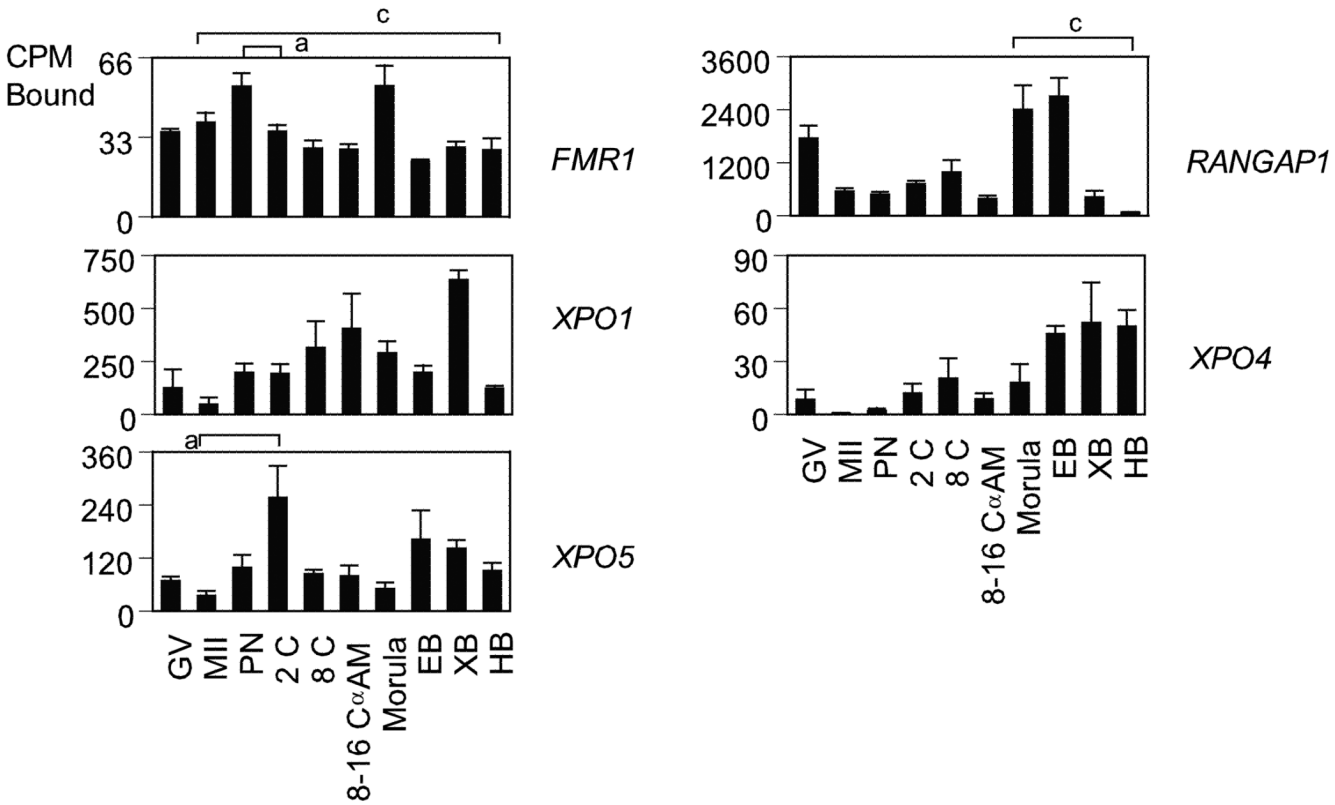

Figure 5.
Temporal expression patterns of mRNAs encoding proteins involved in the transport of mRNA and miRNA from nucleus to cytoplasm. Data are presented as in Fig.2.

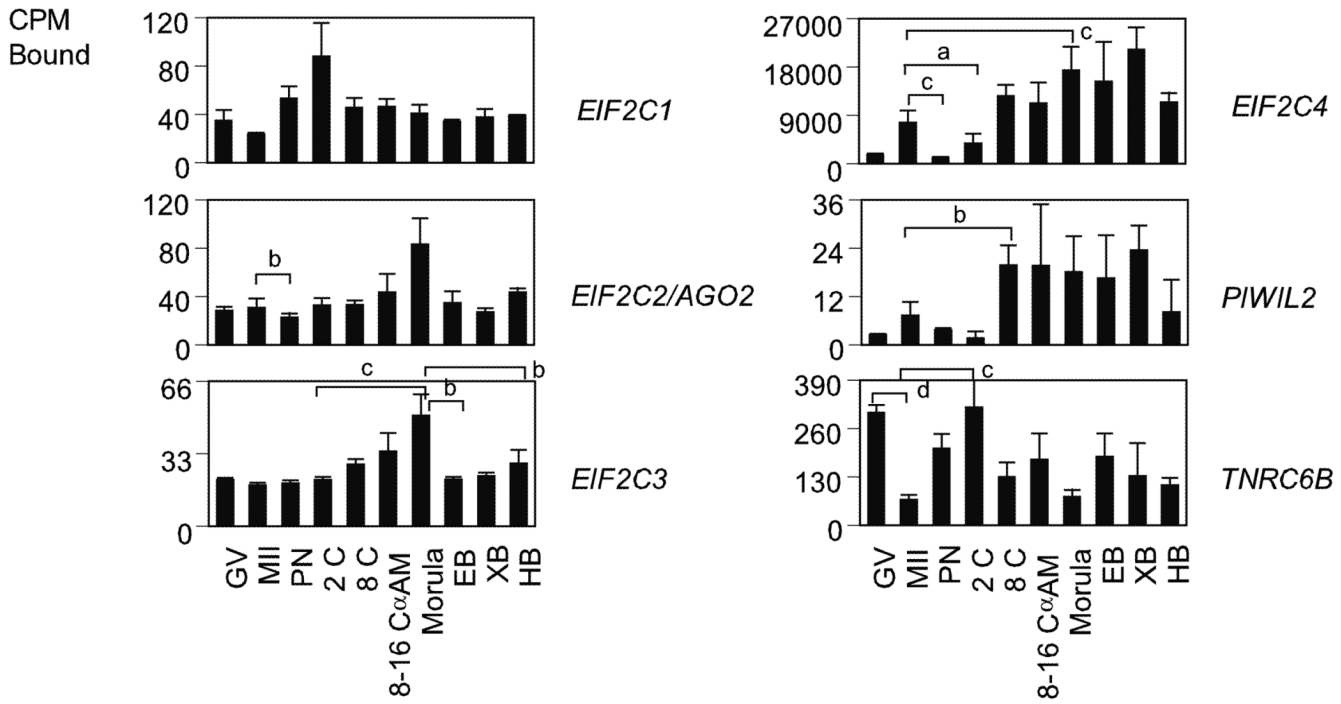

Figure 6.
Temporal expression patterns of mRNAs related to components of the RISC complex. Data are presented as in Fig. 2.

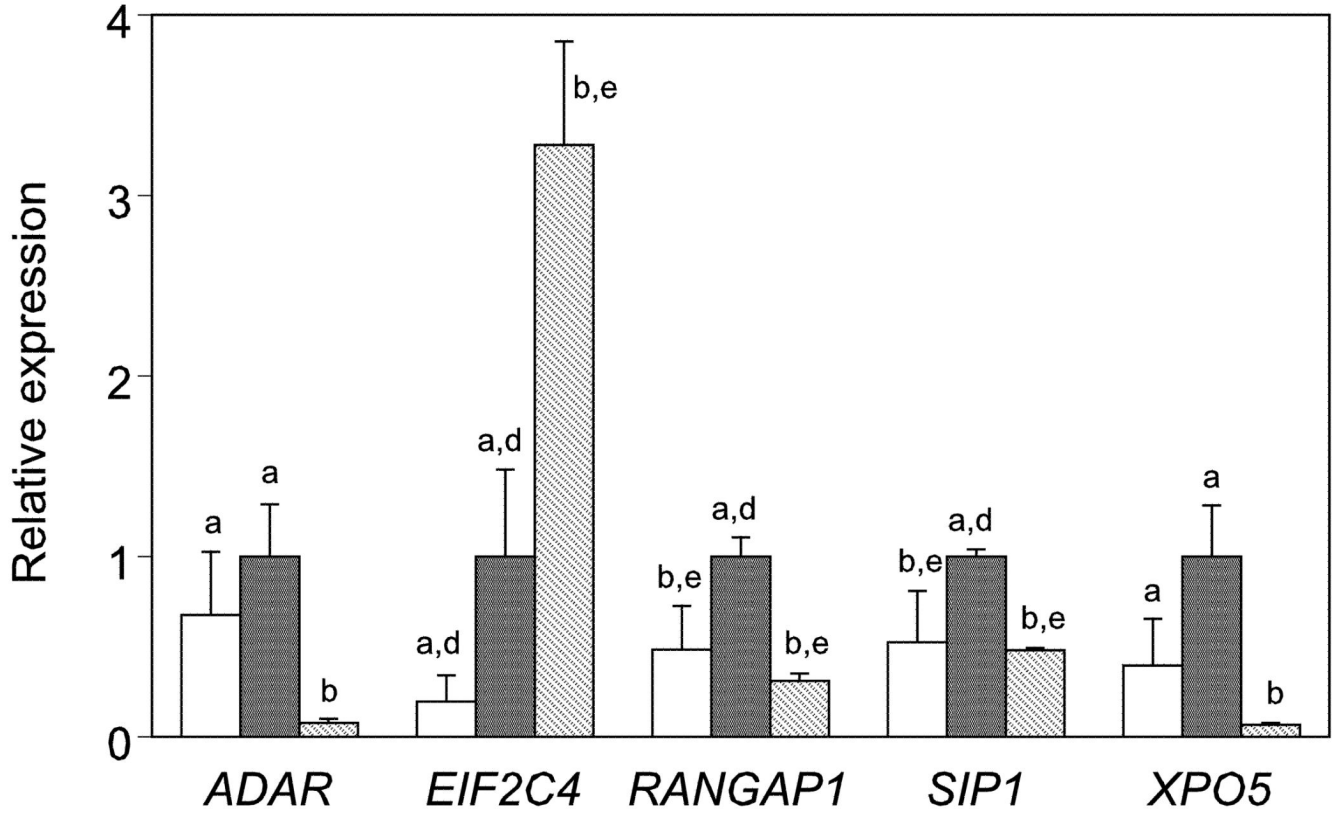

Figure 7.

Effect of hormonal stimulation in two-cell stage embryos produced by in vitro fertilization. Stimulation protocols: white bar, FSH, females given FSH injection only, fully grown oocytes from large antral follicles matured in vitro; black bar, hCG, females injected with FSH and hCG, fully grown oocytes from large antral follicles matured in vivo; stippled bar, NS, females received no stimulation, fully grown oocytes from small antral follicles matured in vitro. Genes are listed alphabetically. Values for average expression and S.E.M. are normalized to the average hCG value (assigned a value of 1.0) for each mRNA. Different letters (a-c) denote statistically significant differences within stage $P < 0.05$; d-f denotes $P < 0.016$.

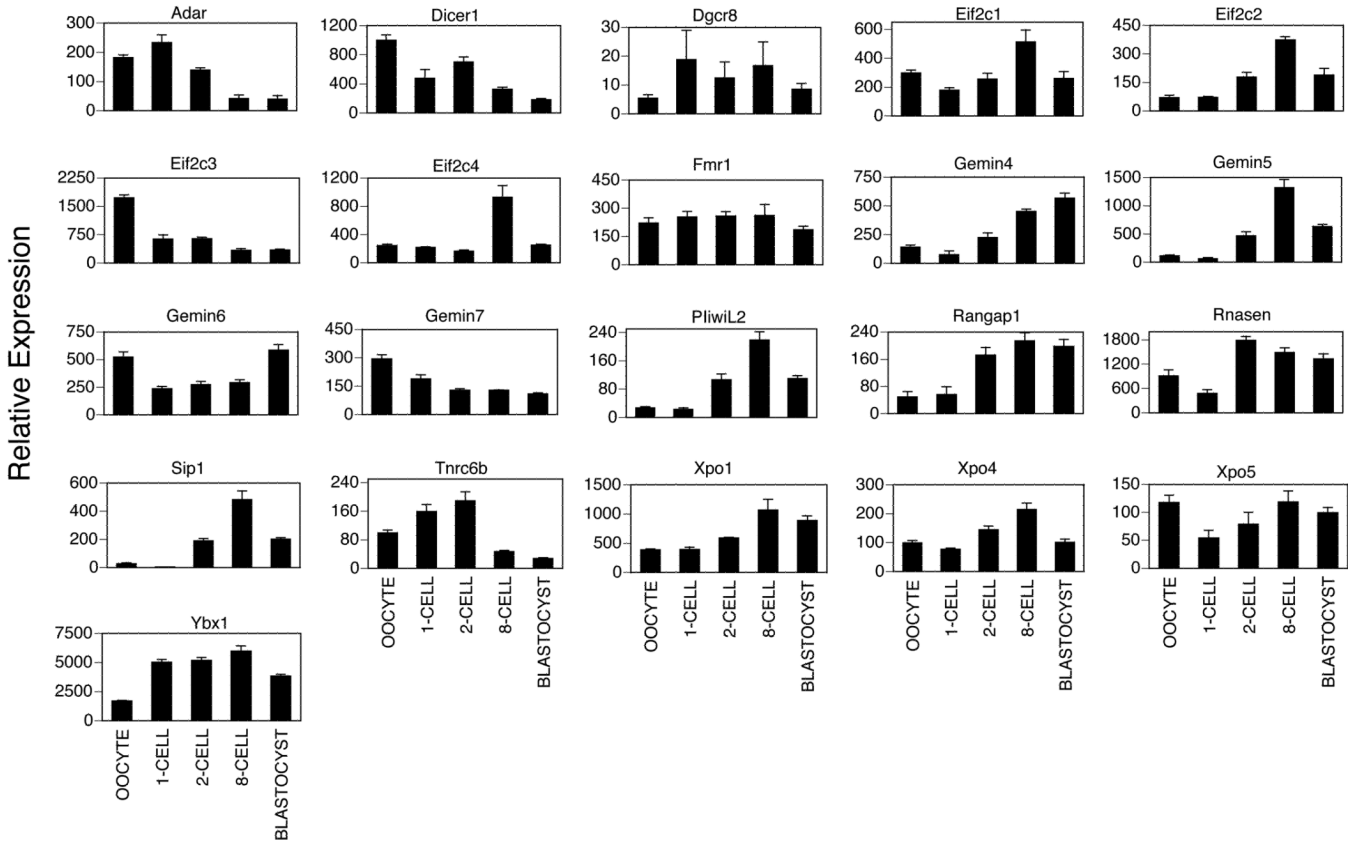

Figure 8.

Expression patterns of microRNA processing genes in mouse oocytes and embryos. Expression data for murine homologues were extracted from the microarray data deposited in the Gene Expression Omnibus repository by Zeng et al (2004), which were obtained originally using the Affymetrix MOE 430A and 430B chip. The stages represented were GV stage oocytes, and embryos at the one-cell, two-cell, 8-cell, and blastocyst stages. These data were expressed as the mean (±SEM) Affymetrix array hybridization signal.

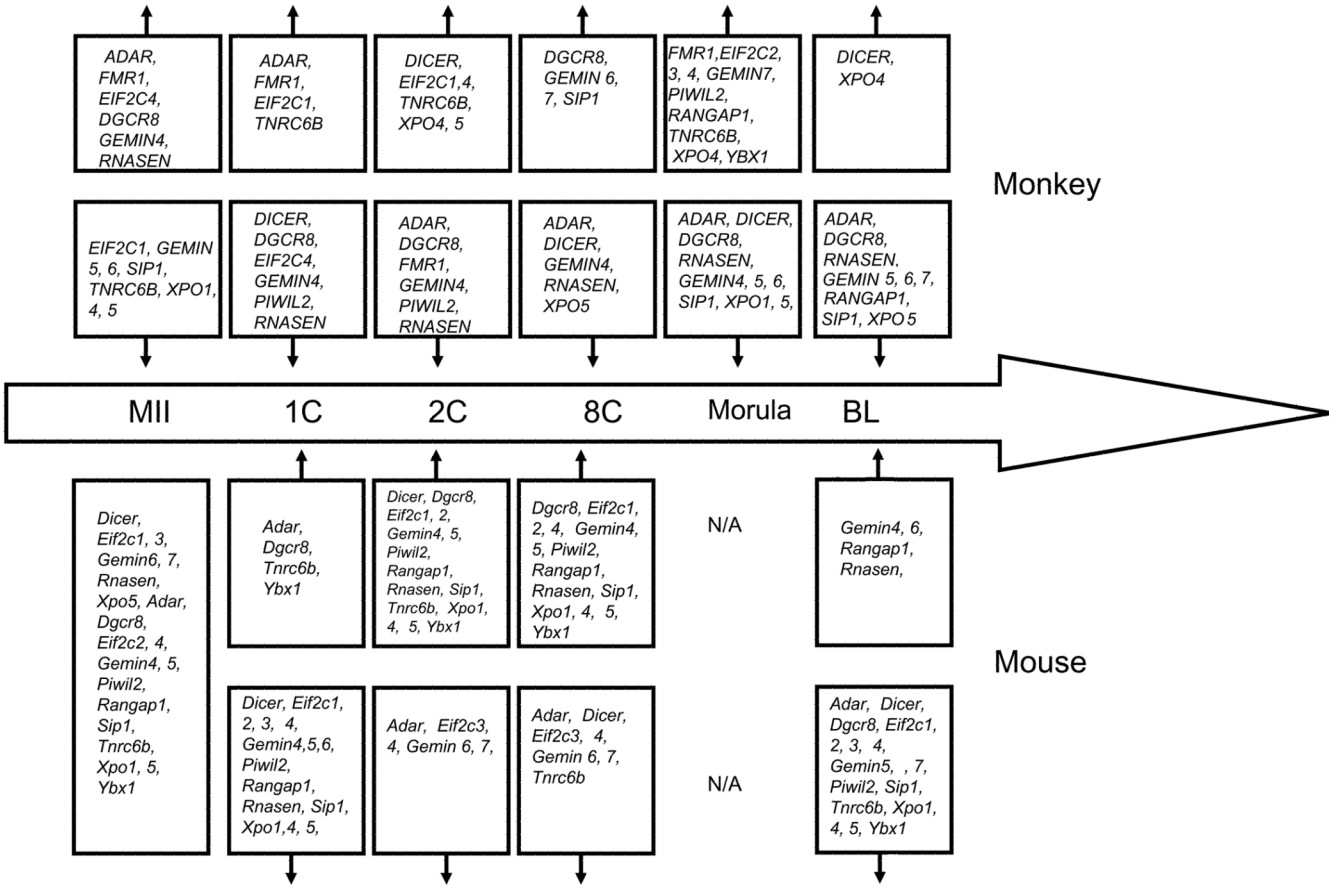

Figure 9.

Developmental transitions in expression of miRNA pathway genes in rhesus monkey and mouse (from array data of Zeng et al., 2004) oocytes and embryos. Genes initially expressed at MII stage in mouse are indicated. The stages at which individual genes undergo increases or decreases in mRNA expression are indicated by upward and downward pointing vertical arrows, respectively.

Table 1

Primers employed for obtaining cDNA probes

Gene symbol	GeneBank Accession number	Upper primer 5′-3′	Lower primer 5′-3-	Product size (bp)
ADAR	BC038227	ACAGGACAGAGGAGGCAGAG	GACTGCAGCCATCATCACAG	308
ADARB1	BC065545	GACCAGTTCTCACTCACGCC	TTGAAAGCAGTCACTGGACG	303
DICER1	BC068591	GAGCTTAGGGCTTTGCATTG	CAGGGGATCACAGAATGCTT	334
DGCR8/PASHA	BC078147	CTTGCTTCTGTCTTGCCTCC	TCAGACAATCAAGGCTCGC	340
EIF2C1/AGO1	BC063275	CATGACAAGTCTCGGAGCTG	AACTCATCAGGCATGGGAAG	312
EIF2C2/AGO2	BC018727	GTGGCTCACCCAAGTTCCT	TCACCACAGACCCGTATTCTT	300
EIF2C3	BC066888	GCCAGTTAGGAGGCTTTTCA	TGGATGCTCCTCCTCCTATG	343
EIF2C4	BC016012	TTGCAAGGTCTCTGGGTAAAA	ATCTTGGCTCACTGCAACCT	303
FMR1	BC086957	ACCATTGGAAATTTCACGTC	AAAAACATCATGACTTTGAACTGA	302
GEMIN4	BC020062	TGAGCAAGACCAACCCTTCT	TTGCCATGAAGTTTTGTGGA	304
GEMIN5	BC036894	GCCAAGATTACCAGTGTGGC	TGCCTTAGGTTGAGAGAGCC	300
GEMIN6	BC018195	AGGGGACCATAGAGTGAGGG	CACACTTCCTGGCCTCTCAT	302
GEMIN7	BC007793	TGAGCTCCCTTGGAATTTTG	CTGGTTCTTTAGCAGGAGGG	300
MOV10	BC009312	CTCTACCTCAGGGCCCCAC	GTTTTCTTCCTTCCCCTTCC	305
PIWIL1/HIWI	BC028581	CTTTCAGGAGTGAGCAAGTCC	CATGTCCCAGTTCCAAAACA	356
PIWIL2/MILI	BC111751	CCATCAGAGTTCCAGCTCCT	CAAGGCCATTTTCAGCTGTT	318
RANGAP1	BC041396	AGATTGTCCAGGAGCAGGAG	CACACACAGAGCACAAAGGG	302
/SCPEP1/ RISC	BC010078	GTCTCTGGAGGCAATTTGGA	TTGACATGAAAAGGTGTTTCAGA	355
RNASEN/DROSHA	BC054003	CCCAGATCATCATGAAGGACA	TCACCAAAGTCAAGTTTTCCG	305
SIP1/GEMIN2	BC104968	GGCAGCCTAACTCTGAGGAA	AGGCCATCCATTGTCTTTTG	374
TNRC6B	BC028626	CAGAACCAGTCAGATCCCGT	TTAGTGCTGCTGCTGTTCCA	303
XPO1	BC032847	GTGACAGGGCTTTTCAGCTT	TTGGTCGACAAATACCCACA	301
XPO4	BC113573	TTGCAAAAGCACAACACAGA	CAGTGCACTTTGCAGAAAGG	318
XPO5	BC062635	AGCTGTAATGGAGCAAATCCC	AGGATGCCCAAAAGCTTGAT	300
YBX1	BC015208	CCGGCTTACCATCTCTACCA	CAGGTGCTTGCAGTTTGTTG	376

Table 2

Descriptions of miRNA processing genes functions.

Gene	**Known (or potential) function**
ADAR	
ADARB1	Binds to short interfering RNAs (siRNA) without editing them and suppresses siRNA-mediated RNA interference
DICER1	Involved in cleaving double-stranded RNA in the RNA interference (RNAi) pathway. It produces 21 to 23 bp dsRNAs (siRNAs) which target the selective destruction of homologous RNAs
DGCR8	Critical for the processing of pri-miRNA into pre-miRNA
EIF2C1	
EIF2C2/AGO2	
EIF2C3	Provides endonuclease activity to RNA-induced silencing complexes (RISC). Cleaves siRNA/mRNA heteroduplexes bound to RISC.
EIF2C4	Essential for embryonic development as well as RNA-mediated gene silencing (RNAi)
FMRI	RNA-binding protein. Associated with polysomes and might be involved in the transport of mRNA from the nucleus to the cytoplasm
GEMIN4	
GEMIN5	
GEMIN6	
GEMIN7 *SIP1/GEMIN2*	The survival of motor neuron (SMN) complex plays an essential role in spliceosomal snRNP assembly in the cytoplasm and is required for pre-mRNA splicing in the nucleus
MOV10	Required to mediate microRNA-guided mRNA cleavage
PIWIL1 *PIWIL2*	Probable component of some RISC complex (RNA-induced silencing), which plays a role in spermatogenesis and in germ cell production, proliferation and maintenance. Inhibits endoribonuclease DICER1 activity.
RANGAP1	Involved in mRNA processing and transport
RISC/SCPEP1	
RNASEN/DROSHA	Executes the initial step of microRNA (miRNA) processing in the nucleus that is cleavage of pri-miRNA to release pre-miRNA. Involved in pre-rRNA processing. Cleaves double-strand RNA and does not cleave single-strand RNA
TNRC6B	Required for miRNA-guided gene silencing and are present in P bodies
XPO	
XPO4	Mediates the nuclear export of micro-RNA precursors, which form short hairpins. Also mediates the nuclear export of synthetic short hairpin
XPO5	RNAs used for RNA interference, and adenovirus VA1 dsRNA.
YBX1	Binds to splice sites in pre-mRNA and regulates splice site selection. Binds and stabilizes cytoplasmic mRNA. Contributes to the regulation of translation by modulating the interaction between the mRNA and eukaryotic initiation factors

Table 3

Over-Represented Transcription Factor Binding Site Analysis

Transcription Factor	No. Target Genes	Z-score	Fisher P
Total gene set			
IRF2	4	13.5	0.00396
STAT1	7	6.93	0.00427
Non-expressed genes			
PAX6	2	10.2	0.0251
STAT1	3	8.94	0.00105