Maternal Argonaute 2 Is Essential for Early Mouse Development at the Maternal-Zygotic Transition

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Activation of zygotic gene expression in the two-cell mouse embryo is associated with destruction of maternally inherited transcripts, an important process for embryogenesis about which little is understood. We asked whether the Argonaute (Ago)/RNA-induced silencing complex, providing the mRNA "slicer" activity in gene silencing, might contribute to this process. Here we show that Ago2, 3, and 4 transcripts are contributed to the embryo maternally. By systematic knockdown of maternal Ago2, 3, and 4, individually and in combination, we find that only Ago2 is required for development beyond the two-cell stage. Knockdown of Ago2 stabilizes one set of maternal mRNAs and reduces zygotic transcripts of another set of genes. Ago2 is localized in mRNA-degradation P-bodies analogous to those that function in RNAi-like mechanisms in other systems. Profiling the expression of microRNAs throughout preimplantation development identified several candidates that could potentially work with Ago2 to mediate degradation of specific mRNAs. However, their low abundance raises the possibility that other endogenous siRNAs may also participate. Together, our results demonstrate that maternal expression of Ago2 is essential for the earliest stages of mouse embryogenesis and are compatible with the notion that degradation of a proportion of maternal messages involves the RNAi-machinery.

INTRODUCTION

The transition from maternal to zygotic regulation is the first major transition that occurs after fertilization and comprises a dramatic reprogramming of gene expression that is essential for the development of most organisms. The timing of embryonic gene activation is species-specific (Manes, 1973; Camous et al., 1986; Braude et al., 1988; Crosby et al., 1988; Zernicka-Goetz, 1994; Hoffert et al., 1997). In mouse a major burst of zygotic gene expression occurs at the two-cell stage corresponding to the time at which mRNAs for the majority of maternal transcripts are degraded (Schultz, 1993). Global expression profiles have identified distinctive patterns of maternal mRNA degradation and zygotic genome activation in the mouse (Hamatani et al., 2004; Wang et al., 2004). However, little is known about the regulation of maternal mRNA stability and factors involved in mRNA degradation pathways at these early stages of mouse development. Because gene-silencing events mediated through the RNA-induced silencing complex (RISC) are proving to play key

developmental roles and work in part through regulating mRNA destruction, we considered whether this complex might contribute to the mechanism for degradation of maternal transcripts in the mouse embryo.

In most eukaryotes, repression by RISC can be mediated through several mechanisms in response to double-stranded RNA (dsRNA; for review see Denli and Hannon, 2003; Meister and Tuschl, 2004; Filipowicz, 2005; Sen and Blau, 2006; Valencia-Sanchez et al., 2006; Rana, 2007): by regulation of heterochromatic silencing and histone H3 K9 methylation (Volpe et al., 2002), inhibition of translation (Liu et al., 2004; Meister et al., 2004, Pillai et al., 2004), or sequence-specific cleavage of mRNA (Tuschl et al., 1999; Zamore et al., 2000; Hannon, 2002). On the other hand, activation by RISC has also been observed at both transcriptional (Li et al., 2006) and translational (Vasudevan and Steitz, 2007) levels. The trigger of these processes can be provided exogenously, for example, by interfering dsRNA, or endogenously by, for example, microRNAs (miRNAs) or other endogenous small RNAs. Within cells, either 21–24–nucleotide short interfering RNA (siRNA) fragments derived from longer dsRNA or miRNAs (Hammond et al., 2001; Hutvagner and Zamore, 2002; Mourelatos et al., 2002; Doi et al., 2003) can be incorporated into the RISC together with an Argonaute protein whose PAZ domain facilitates RNA recognition. Mammalian cells have several Argonaute-like genes, but only Ago2 has been shown to be required for RNA cleavage (Liu et al., 2004).

MiRNAs are short noncoding RNAs that inhibit translation and lead to mRNA instability (Roush and Slack, 2006). Although still little is known about their in vivo function, it has been demonstrated that miR-430 promotes deadenylation and clearance of maternal mRNAs in zebrafish embryos (Giraldez et al., 2006). Inhibited mRNAs as well as miRNAs may accumulate in cytoplasmic processing bodies (P-bodies) together with the enzymes required for mRNA degradation

(Liu *et al.,* 2005a; Pillai *et al.,* 2005; Sen and Blau, 2005). There are ~500–1000 miRNA genes in vertebrates and plants, and each miRNA is predicted to target hundreds of mRNAs (Bushati and Cohen, 2007).

RNA interference (RNAi) was first demonstrated to be effective in mammalian cells through experiments in which long dsRNAs were successfully used to silence gene activity in mouse oocytes and early preimplantation embryos (Svoboda *et al.,* 2000; Wianny and Zernicka-Goetz, 2000). This indicated that these cells ought to contain components of the enzymatic machinery required for the silencing process involving Argonaute proteins. It also raised the question of what the natural function of silencing might be at these early embryonic stages. Yet, eliminating zygotic expression of Ago2, the catalytic component of the RISC, indicated that there was no requirement for Ago2 until only after implantation (Liu *et al.,* 2004; Morita *et al.,* 2007) when it is involved in gastrulation and mesoderm formation (Alisch *et al.,* 2007). However, it remained possible that Ago2 is supplied maternally for functions in the early embryo (from Ago2+/− mothers). Alternatively, its function could be substituted by one of the other members of the Argonaute family. We addressed these questions by first examining which of the Argonaute family members are present in oocytes and preimplantation embryos and then by systematically depleting them in combination or individually. We found that both Ago2 and 3 are expressed throughout early mouse development. However, only specific elimination of Ago2 transcripts leads to developmental arrest at the two-cell stage. This is reflected in the stabilization of one set of maternal mRNAs. This also leads to the reduction in the appearance of zygotic transcripts of another set of genes. To explore a possible link between the observed mRNA stabilization of maternal transcripts by Ago2 and the endogenous miRNAs, we carried out miRNA screen and a bioinformatics search. This pointed to multiple miRNAs as potential candidates for the degradation of specific maternal messages in the two-cell embryo. However, this does not exclude the possible involvement of other natural endoegous siRNAs present in mouse oocytes and early embryos. Taken together, our results demonstrate an essential role for Ago2, a maternally expressed gene, at the earliest stages of mouse embryogenesis and indicate that Ago2, directly or indirectly, is required for degradation of a set of maternal transcripts to support development beyond two-cell-stage.

MATERIALS AND METHODS

Embryo Collection and Culture

F1 (C57BL/6xCBA) females were injected with 5 IU pregnant mare's serum gonadotrophin (PMSG; Folligon, Intervet, Cambridge, United Kingdom) and with 5 IU human chorionic gonadotrophin (hCG; Chorulon, Intervet) 48 h later to induce ovulation as described (Hogan *et al.,* 1994). Embryos were collected in M2 medium and cultured in drops of KSOM supplemented with amino acids and 4 mg/ml BSA, under paraffin oil in an atmosphere of 5% CO_2 in air at 37.5°C. Zygotes for RNA injections were collected from female mice 25 h after hCG. Two-, four-, and eight-cell embryos for in situ hybridization were collected 46 or 56 or 64 h after hCG, respectively. Morula and blastocysts were collected after 2.5 or 3 d, respectively. All animals were handled in accordance with Home Office legislation.

RNA In Situ Hybridization

Freshly collected embryos at various stages were fixed in 4% paraformaldehyde in PBS. In situ hybridizations were performed as described (Wilkinson and Nieto, 1993; Hogan *et al.,* 1994), except that the embryos were not dehydrated and the proteinase K treatment was omitted. Color visualization of the alkaline phosphatase was done using precipitating BM purple substrate according to manufacturer's instructions (Roche, Indianapolis, IN).

GFP and DsRed Constructs

GFP-Ago2 was constructed by amplifying full-length *Ago2* from a two-cell embryo using the Superscript One-Step RT-PCR for long templates (Invitrogen, Carlsbad, CA). Gene specific primers (Supplementary Table S1) were tagged with BamHI to amplify the full-length open reading frame (ORF) of *Ago2* to allow cloning in frame with the *GFP* gene. Reporter plasmid for DsRed protein expression (pDsREd2-N1, BD Bioscience Clontech, San Jose, CA) was constructed by amplifying the full-length ORF for *Dcp1a* with gene-specific primers (Supplementary Table S1) using EcoRI and KpnI sites and the subsequence subcloned the DsREd-Dcp1a into the pRN3 vector using EcoRI and NotI to replace the *GFP* gene in order to allow for T3 mMessage transcription (Ambion, Austin, TX).

RNA Synthesis and Microinjections

The templates used for dsRNA synthesis were PCR product. Oligonucleotides were linked with T7 promoter sites, and a 500–600–base pair PCR fragment was amplified. The primers used for generating dsRNA for Ago2, 3, and 4 are listed in Supplementary Table S1. Full-length *MmGFP* cDNA was done as previously described (Wianny and Zernicka-Goetz, 2000).

RNAs were synthesized using the T3 or T7 polymerase, using the Megascripts Kit (Ambion) for dsRNA and the mMessage for capped mRNA (Ambion). DNA templates were removed with DNase treatment. The RNA products were extracted with phenol/chloroform and ethanol-precipitated. To anneal sense and antisense RNAs, equimolar quantities of sense and antisense RNAs were mixed in the annealing buffer (10 mM Tris, pH 7.4, 0.1 mM EDTA) to a final concentration of 2 μM each, heated for 10 min at 68°C, and incubated at 37°C for 3–4 h. To avoid the presence of contaminating single-stranded RNA in the dsRNA samples, the preparations were treated with 2 μg ml−1 RNase T1 (Calbiochem, Nottingham, United Kingdom) and 1 μg ml−1 RNase A (Sigma, Poole, Dorset, United Kingdom) for 30 min at 37°C. The dsRNAs were then treated with 140 μg ml−1 proteinase K (Sigma), phenol/chloroform-extracted, and ethanol-precipitated. Formation of dsRNA was confirmed by migration on an agarose gel. RNAs were diluted in water, to a final concentration of ~1 mg/ml. siRNA SMART pool was obtained from Dharmacon; for Ago3 (siGenome SMARTpool M-054947-00-0005) and a nontargeting negative control pool (D-001206-13-05). The siRNA SMART pool was resuspended in 250 μl 1× siRNA buffer (20 mM KCl, 6 mM HEPES buffer (pH 7.5) and 200 μM $MgCl_2$) to give a final concentration of 20 μM according to the manufacturer's instructions.

The dsRNA or siRNA were microinjected, as described before, and each embryo was injected with ~10 pl dsRNA (Wianny and Zernicka-Goetz, 2000). Rhodamine-conjugated dextran (D-3308, Molecular Probes, Eugene, OR) was added to all RNA injections to identify embryos injected successfully. Embryos were observed by either transmitted light or fluorescent light microscopy using IP Lab software (Scanalytics, Billerica, MA).

RT-PCR Analysis

RT-PCR of RNA-injected embryos was performed using the Superscript One-Step RT-PCR with Platinum Tag (Invitrogen). The RT-PCR was done according to the manufacturer's instructions with some modifications:

Pools of four embryos were lysed in the RT buffer (1× reaction mix, 0.2 μM each of sequence specific primers) by heating to 65°C for 2 min and were left at room temperature for 2 min to allow annealing to the primers before left on ice before adding the Superscript/platinum tag. Oligo d(T) primed first-strand cDNA was synthesized as follows: 50°C for 30 min, 94°C for 2 min, and then 40 cycles of 94°C for 15 s, 55°C for 30 s, and 68°C for 30 s. with extension for 10 min at 72°C. PCR products were visualized on a 1.5% agarose gel containing ethidium bromide for UV visualization. All DNA oligonucleotides used for RT-PCR analysis are listed in Supplementary Table S1. All accession numbers for genes tested are listed in Supplementary Table S1.

cDNA Synthesis and Subtractive Hybridization (Representational Difference Analysis)

Construction of cDNA libraries from single cells and the representational difference analysis (RDA) were done as previously described (Hubank and Schatz, 1994; Dulac and Axel, 1995; Saitou *et al.,* 2002) with a few modifications. Briefly, the embryo was lysed at 65°C for 1 min in lysis buffer (0.5% NP40, 1.25 mM each of dATP, dCTP, dGTP, and dTTP, 1 μl of 50 OD/ml oligo d(T)$_{24}$, 0.4 U primeRNase inhibitor [Roche] and 0.38 U RNAguard [Amersham Biotechnology, Piscataway, NJ [in 1× MMLV (Moloney murine leukemia virus) buffer (Roche). The reaction was allowed to cool down at RT for 1 min and kept on ice before adding AMV-and MMLV-reverse transcriptases according to the manufacturer's instructions (Roche). cDNA libraries for subtractive hybridization were further treated with the tailing reaction using terminal deoxynucleotidyltransferase according the manufacturer's instructions (10533-065, Invitrogen) and subsequently amplified using the OL-1 primer to allow the insertion of restriction enzyme site. All oligonucleotides used in RT-PCR analysis for Argonaute 1-4 and control primers used in the subtractive hybridization are listed in Supplementary Table S1. Double-stranded cDNA from Ago2/3-depleted two-cell embryos were used as the

tester, and cDNA from untreated two-cell embryos was used as the driver using a ratio of 1:100 for the first round for selection and 1:800 for the two after rounds of selections. The cDNAs were used as tester and driver, respectively, for the forward subtraction and vice versa for reverse subtraction. Tester and driver cDNAs were hybridized, and the hybrid sequences were removed. Consequently, the nonhybridized cDNAs represent fragments corresponding to modulated or on/off-switched sequences. Differentially expressed genes were amplified from the subtracted cDNA using suppression PCR. A Gene-Amp 2700 (PE Biosystems, Warrington, United Kingdom) was used for thermal cycling. The conditions used were 94°C for 10 s, 68°C for 30 s, and 72°C for 90 s, for 20 cycles (the initial PCR), and 20 cycles (for the nested PCR). PCR amplicons from the tester, driver, and subtracted cDNA were electrophoresed on a 2% agarose gel, and prominent amplicons specific to the subtracted cDNA were excised from the gel. PCR analysis consists in the amplification of the housekeeping gene for β-actin, β-tubulin, and Oct4 cDNA in order to evaluate its expression in subtracted and unsubtracted cDNAs. All DNA oligonucleotides used for RT-PCR analysis are listed in Table S1 in Supplementary Material.

Micro-RNA Microarrays and Their Target Prediction

For miRNA microarray expression analysis total RNA was extracted from pools of 20 MII oocytes, 20 zygotes (25–26 h after hCG), 20 two-cell embryos (32–33 h after hCG), 20 two-cell embryos (47 h after hCG), 20 four-cell embryos (56–57 h after hCG), 20 eight-cell embryos (67–68 h after hCG), and 20 blastocysts (87–90 h after hCG) by adding 100 μl Trizol (Invitrogen). RNA labeling and hybridizations were carried out as described previously (Miska et al., 2004).

Microarrays used were custom-made and represented all human miRNAs according to miRBase release 8.0 (Griffiths-Jones, 2004; Griffiths-Jones et al., 2006). Probe design was carried out as described previously (Miska et al., 2004). For probe set and microarray data, see Supplementary Tables S2 and S3, respectively. Microarray slides were scanned using an Axon 4000B scanner (Molecular Devices, Sunnyvale, CA), and primary data were analyzed using the GenePix 6.0 (Molecular Devices) software. Each probe was represented by four spots on the microarray. Median signal intensities with local background subtraction for each spot were used for subsequent analyses. The median of the four spots for each probe was used to represent the signal for each probe. Interarray comparison was performed through array normalization through RNA spikes added before RNA labeling (Wienholds et al., 2005).

MiRNA target prediction was performed using the RNAfold program, which is part of the Vienna RNA Package (Mathews et al., 1999). The program was used to find the predicted binding between the miRNA and successive short sections of the mRNA sequence; thus scanning the mRNA for candidate binding sites. RNAfold yields a predicted fold structure and associated binding energy, in computer readable format, as output. RNAfold finds a minimum energy fold for a continuous RNA sequence, and this is exploited in this application to predict the binding of one sequence to another by computationally attaching the miRNA to each successive section of the mRNA in the correct orientation to with a flexible linker of Ns. We used mRNA sections 12 nucleotides longer than the miRNA, with a 12-mer N-linker and jumped three nucleotides between successive positions. We discarded all binding patterns that showed any loops in addition to a single "hairpin" between the mRNA section and the miRNA and tabulated the remaining binding sites for comparison between the miRNAs.

RESULTS

Ago2 Is Expressed in Preimplantation Embryos and Localizes to Cytoplasmic mRNA Decay P-Bodies

To examine a possible role of Argonaute proteins in the early mouse embryo, we first analyzed the expression of four Argonaute family members during preimplantation development, by performing gene-specific PCR on oligo d(T) first-strand cDNA (Figure 1A). We found that Ago2 and 3 mRNAs are expressed in oocytes and throughout all preimplantation stages, suggesting their gene products may be required maternally and zygotically. Ago4 mRNA was also present in oocytes and zygotes, but was down-regulated by the four-cell stage, suggesting a function restricted to oogenesis and the zygote. We were not able to detect a transcript for Ago1 throughout preimplantation development. Interestingly, mRNA for all four Argonaute genes was detected in embryonic stem cells (Figure 1A). Although we detected only one band of correct size for each PCR reaction, each PCR product was subjected to DNA sequencing to confirm its identity (data not shown), because of the high degree of similarity between the Argonaute members. For each RT-

PCR, control transcripts for β-actin and α-tubulin were included (Figure 1A).

To determine whether the localization of Ago2 or 3 transcripts show any spatial differences in preimplantation embryos, we carried out RNA in situ hybridization (Figure 1 in Supplementary Material). Detection of both Ago2 and 3 was weak at the early stages (two- to eight-cell embryos). As the inner cells form, expression appeared stronger and was enriched progressively within, but not restricted to, the inner cells and then the inner cell mass (ICM) of the blastocyst. In situ expression profiles of Ago2 and 3 transcripts were also shown to be similar at later postimplantation stages (Liu et al., 2004).
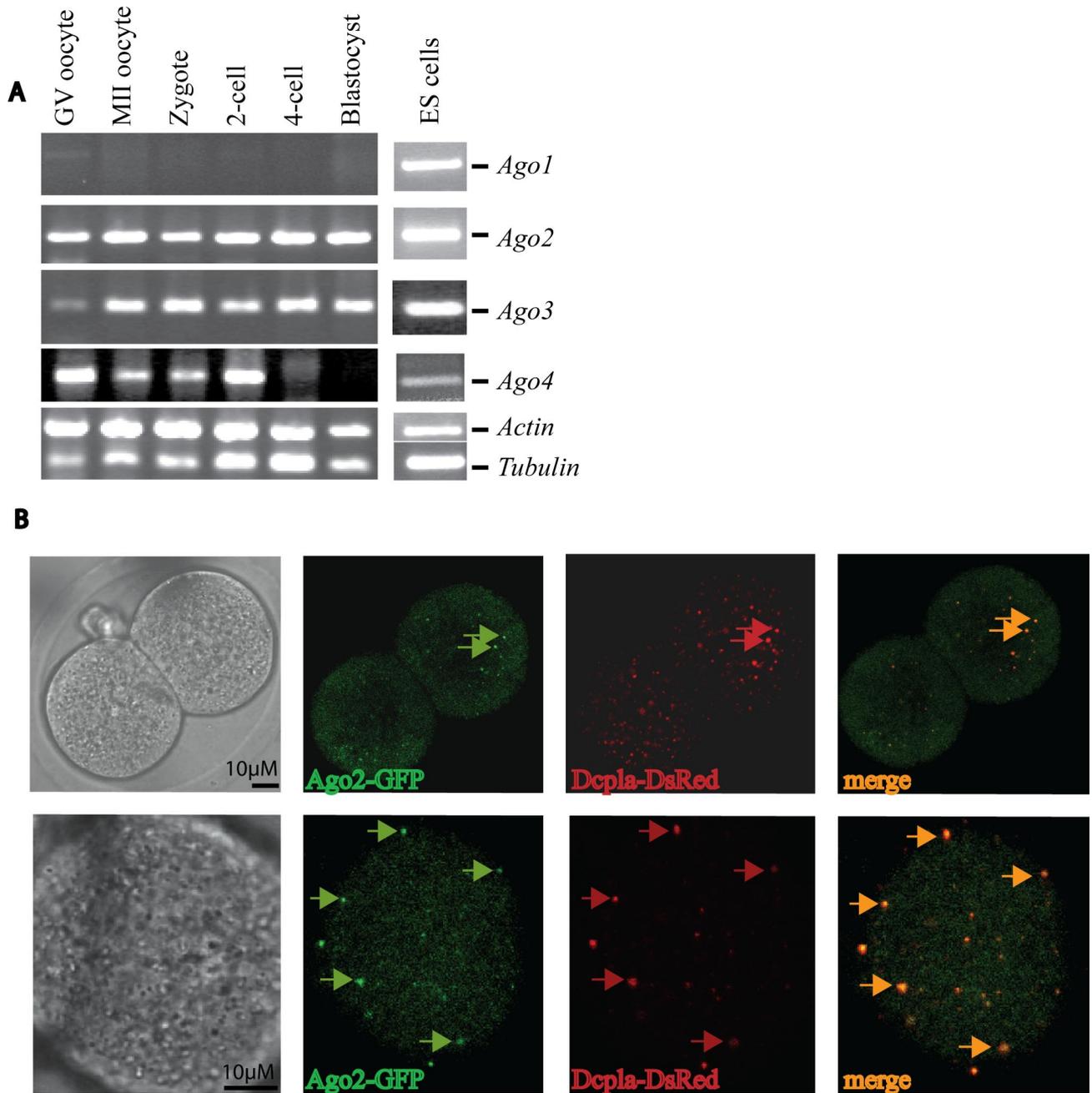
Because Ago2 protein has been shown to localize to P-bodies (Liu et al., 2005b; Sen and Blau, 2005) that serve as sites for mRNA destruction and decay (Sheth and Parker, 2003), we also wanted to examine its spatial distribution in the mouse embryo. To this end, we generated a construct encoding Ago2-green fluorescent protein (GFP) fusion protein and microinjected its synthetic message into zygotes and examined its distribution at the two-cell stage. This revealed that Ago2-GFP localizes to vesicle-like bodies that resembled the cytoplasmic P-bodies (Figure 1B). To assess whether the Ago2-containing bodies could be indeed P-bodies, we coinjected zygotes with synthetic, tagged mRNA for both Ago2-GFP and Dcp1a, a decapping protein that localizes to P-bodies and is required for functional miRNA RNAi (Behm-Ansmant et al., 2006) fused to DsRed (Dcp1a-DsRed). This showed that Ago2-GFP distribution was overlapping with that of the decapping enzyme Dcp1a-DsRed (Figure 1B).

Thus, these experiments indicate that there is a maternal contribution of Argonaute transcripts to early mouse embryos, and specifically Ago2 and 3 mRNAs are present throughout the preimplantation stages. Moreover, the spatial localization of Ago2 in the mouse embryo is similar to the localization of Ago2 in other cell types (Liu et al., 2005b; Sen and Blau, 2005).

Ago2 Knockdown Leads to Early Developmental Arrest

To assess the functional role of Argonaute proteins expressed in early mouse embryo, we used RNAi. We first showed that control embryos, both noninjected (Figure 2A) and injected as late zygotes with GFP dsRNA (Figure 2B), still expressed Ago2, 3, 4, β-actin, or the POU domain, class 5, transcription factor 1 (POU5F1, also known as Oct4) mRNAs 20–24 h after injection, as indicated for each gel. These control-injected embryos developed to the blastocyst stage as efficiently as noninjected embryos (Figure 2, A and B). We subsequently synthesized a 501-nucleotide dsRNA complementary to Ago3 transcript sequences. This sequence also had 74% identity to Ago2 and consistently when injected into mouse zygotes was able to down-regulate both Ago2 and 3 transcripts (Figure 2C) as revealed by RT-PCR. We found that the majority (80%: 42/54 embryos, five independent experiments) of such embryos arrested development at the two-cell stage and none developed to the blastocyst stage (Figure 2C). To determine whether this embryonic arrest was due to elimination of Ago2, 3, or both, we injected dsRNA targeted against specific sequences in the 5' end of the ORF of Ago2 mRNA. This resulted in 90% knockdown of Ago2 mRNA, but not Ago3 mRNA, and led to a similar phenotype of developmental arrest (76%: 29/38 embryos, three independent experiments) at the two-cell stage (Figure 2D).

In an attempt to specifically suppress Ago3 mRNA, we carried out RNAi using SMARTPool siRNA (Dharmacon),
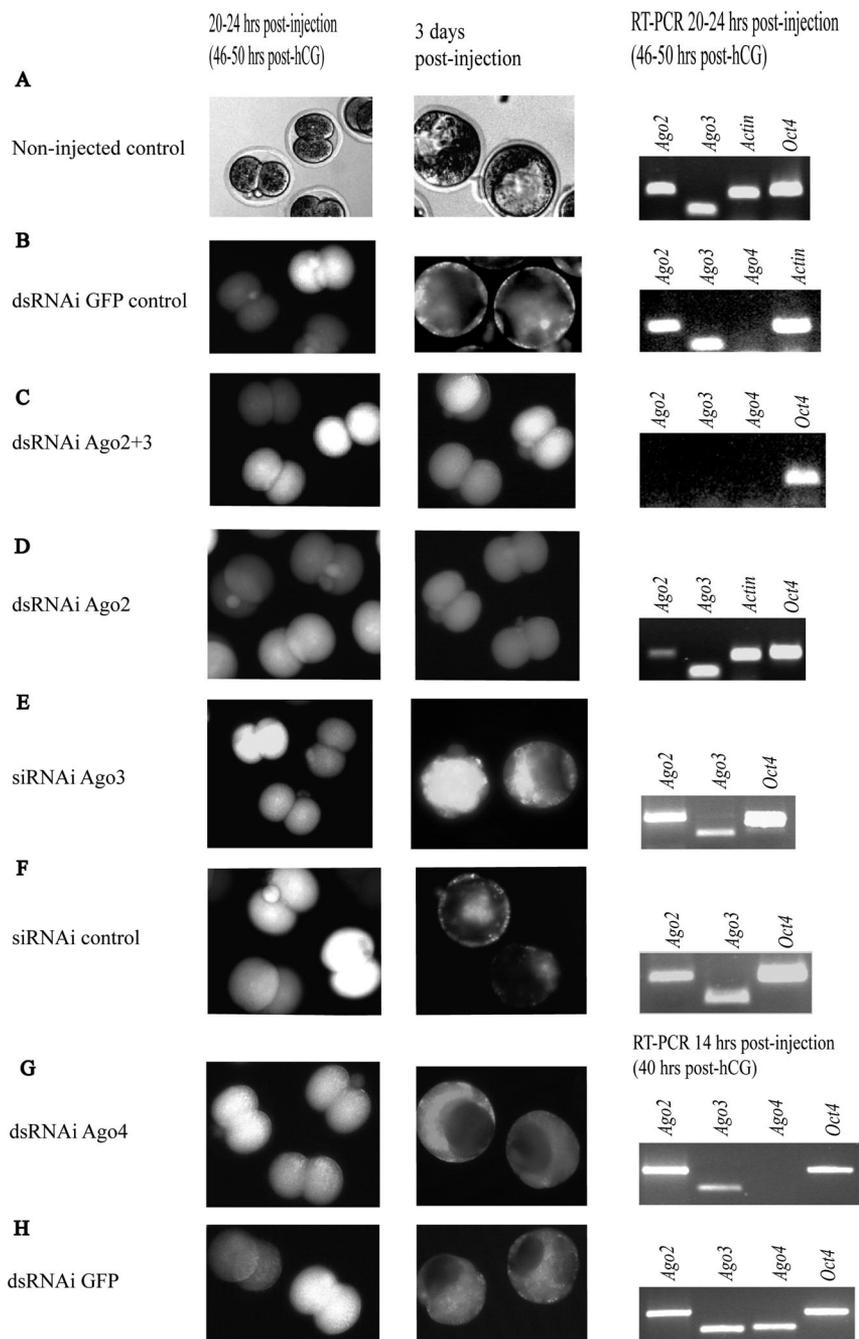
Figure 1. Ago2, 3, and 4 transcripts are expressed in oocytes and at early stages of development, with Ago2 and 3 transcripts in all preimplantation stages tested. (A) PCR analysis of *Ago1-4* gene family on preimplantation mouse first-strand cDNA from different stages. As a positive control for the PCR amplification, a band was successfully amplified for all Argonautes in ES cells. Actin and tubulin were used as control genes. (B) Localization of Ago2-GFP fusion protein in mRNA decay centers. Confocal laser microscopy images of two-cell embryos microinjected at zygote stage with tagged mRNA for Ago2-GFP and Dcp1a-DsRed. Top panel, vesicle-like localization of Ago2-GFP and Dcp1a-DsRed fusion proteins in a two-cell embryo (96 μM × 96 μM). Bottom panel, a higher magnification of one blastomeres in the two-cell embryo expressing Ago2-GFP and DsRed-Dcp1a (46 μM × 46 μM). Green, red, and orange arrowheads point to examples of vesicles showing localization of Ago2-GFP, DsRed-Dcpla, and Ago2-GFP+DsRed-Dcpla, respectively.

as we were unable to design long dsRNA to down-regulate expression of Ago3 mRNA only. Microinjection of siRNAs against Ago3 mRNA into zygotes revealed a reduction in the Ago3 transcript by the two-cell stage (Figure 2E), but the injected embryos were still capable of developing to the blastocyst stage. A siRNA control group showed normal expression of Ago2 and 3 mRNAs and also developed to the blastocyst stage (Figure 2F).

The expression of Ago4 mRNA declines by the four-cell stage, where the Ago4 transcript cannot be detected. Consistent with its temporal expression profile (Figure 1A), transcripts of Ago4 were not detectable when expression was analyzed 20–24 h after injection of dsRNA against GFP, Ago2 and 3 mRNAs (Figure 2, B and C). Therefore, in order to assess if Ago4 transcript might be important in two-cell embryos, we attempted to deplete Ago4 transcript from the

Figure 2. Ago2 is required for development beyond the two-cell stage. Early zygotes were injected with dsRNA for GFP or dsRNA or siRNA for combinations or individual Argonaute genes (as indicated) or were not injected. The embryos were cultured in vitro until the blastocyst stage. Images were taken using fluorescence light to identify embryos injected with both RNA and rhodamine-conjugated dextran (except the noninjected control group, shown as bright-field microscopy images). Embryos are shown at the two-cell stage (20–24 h after injection) and 3 d after injection when control embryos developed to blastocyst stage. Embryos of each experimental group were scored according to their developmental progress. The number of embryos in each experiment is indicated as follows: injected embryos (two-cell stage; IE2), arrested embryos (A), embryos developed to blastocyst stage (EB) and embryos fragmented or lysed (D). (A) noninjected control group (IE2 = 26, EB = 23, D = 3; (B) RNAi GFP: dsRNA for GFP control (IE2 = 39, EB = 30, D = 9); (C) RNAi Ago2 + Ago3 (IE2 = 54, A = 42, EB = 0, D = 12); (D) RNAi Ago2 (IE2 = 38, A = 29, EB = 0, D = 9); (E) siRNAi Ago3 (IE2 = 29, EB = 21, D = 8); and (F) siRNAi control (IE2 = 77, EB = 63, D = 14). The right side of each injection experiment shows RT-PCR analysis performed on two-cell embryos for Ago2, 3, 4, actin, or Oct4, as indicated. RT-PCR was done 20–24 h after injection, corresponding to 46–50 h after hCG, as indicated. (G) RNAi Ago4 (IE2 = 60, EB = 48, D = 12). (H) RNAi control (IE2 = 48, EB = 41, D = 7). RT-PCR analysis performed on Ago2, 3, 4, and Oct4, as indicated. RT-PCR was done 14 h after injection, corresponding to 40 h after hCG, as indicated.
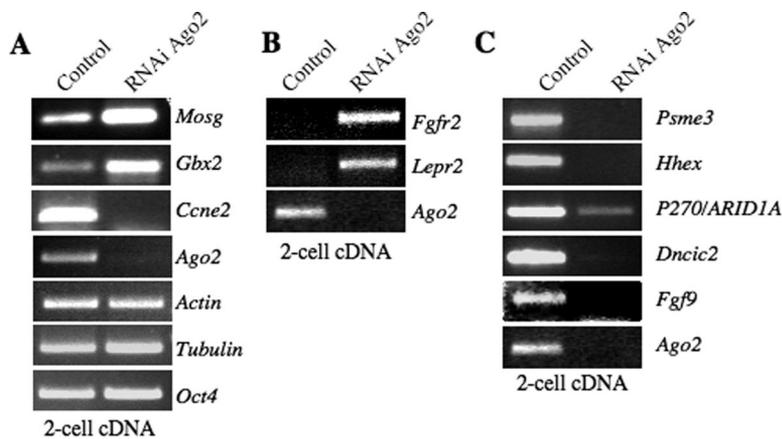
early two-cell stage (when the Ago4 transcript is still detectable in control embryos). This resulted in a depletion of the Ago4 transcript and also in an ~50% reduction of the Ago3 transcript, but still allowed embryos to develop to the blastocyst stage (80% developed to blastocyst: 48/60, two independent experiments; Figure 2G). The control RNAi embryos at 14 h after injection showed expression of Ago2, 3, and 4 transcripts (Figure 2H) and developed to blastocyst stage as expected (85% developed to blastocyst; 41/48, two independent experiments). For RNAi experiments directed specifically against Ago2, 3, and 4, we have also assessed the endogenous Oct4 mRNA. The expression of Oct4 mRNA remained unchanged, indicating the specificity of the effect. A summary of all RNAi experiments is schematically presented in Figure 2 in Supplementary Material.

Thus together these experiments suggest that maternally provided Ago2 mRNA is required to permit development of the mouse embryo beyond the two-cell stage.

Ago2 Depletion Results in Stabilization of Some Maternal mRNAs in the Two-Cell Embryo

Maternal factors are known to control development before the activation of the embryonic genome at the two-cell stage (Paynton and Bachvarova, 1988; Bashirullah et al., 2001; Oh et al., 2000). We considered that one possible explanation for the developmental arrest of Ago2-depleted embryos was that RISC-mediated silencing might be required to destroy a subset of maternal RNAs to facilitate further development. If this were the case, it would suggest that after knockdown of

Figure 3. Transcripts identified with significant different expression in Ago2-depleted two-cell embryos. RNAi GFP and RNAi Ago2 embryos were tested for differential gene expression using gene-specific primers on first-strand cDNA. (A) Nested PCR for Mosg, Gbx2, and Ccne2 on oligo d(T) first-strand cDNA. mRNAs for Mosg and Gbx2 maintained their expression in RNAi Ago2 embryos, in contrast to RNAi GFP embryos Ccne2 mRNA was not detected in RNAi Ago2 embryos, in contrast to RNAi GFP embryos (B) Nested PCR on oligo d(T) first-strand cDNA for Fgfr2 and Lepr2 mRNA shows expression in RNAi Ago2 in contrast to RNAi GFP embryos, where no expression could be detected. (C) Nested PCR on oligo d(T) first-strand cDNA for Psme3, Hhex, p27/ARID1A, Dncic2, Fgf9, and Ago2 mRNAs showed a down-regulation in Ago2-depleted embryos. All transcripts could be detected in RNAi GFP embryos.

Ago2 mRNA we might see the stabilization of some maternal transcripts and a failure of other transcripts to appear. To address this we performed a subtractive hybridization between wild-type control and Ago2-depleted two-cell stage embryos and also examined the expression of other genes previously shown by microarray analysis to be expressed around the time of this developmental transition (Wang *et al.*, 2004).

Our subtractive screen revealed genes that showed strong changes in expression pattern after Ago2 RNAi as shown by nested PCR on oligo d(T) first-strand cDNA on two-cell control and Ago2-depleted embryos. This showed that both the mouse oocyte-specific gene (Mosg) and the gastrulation brain homeobox gene 2 (Gbx2) transcripts appeared to be stabilized in the Ago2 RNAi two-cell embryos, whereas in control embryos they undergo substantial degradation during the two-cell stage (Figure 3A). This suggests that Ago2 function might be required to degrade these maternal mRNAs. We have also seen the decreased expression of Cyclin E2 (Ccne2) mRNA upon Ago2 RNAi (Figure 3A). Levels of expression of control genes such as β-actin, α-tubulin, and Oct4 were consistent (Figure 3A). These experiments were also carried out in parallel using a gene-specific RT-PCR approach on two-cell control and Ago2-depleted embryos, which showed consistent results ensuring that no artifacts were introduced during generation of oligo d(T) cDNA libraries of two-cell stage embryos (see Supplementary Material, Figure S3).

As a complementary approach we also interrogated our previous microarray gene profiling data (Wang *et al.*, 2004) to identify maternal mRNAs known to be down-regulated at the maternal to zygotic transition and selected 16 of these for detailed analysis as a possible candidates for regulation by the RISC/Ago2 machinery. The genes selected in this way were chosen at random to avoid bias toward any particular biological pathway or specific functions in the maternal to zygotic transition. Seven of the 16 genes analyzed displayed altered transcript level after Ago2 RNAi (Figure 3, B and C). We found stabilization of fibroblast growth factor receptor 2 (Fgfr2) and leptin receptor 2 (Lepr2) transcripts (Figure 3B), suggesting that Ago2 might normally be required to degrade these maternal mRNAs. In contrast we found that transcripts for proteasome subunit 3 (Psme3), hematopoietically expressed homeobox (Hhex), p27/ARID1A (subunit of the SWI/SNF complex), dynein, cytoplasmic, IC 2 (Dncic2), and fibroblast growth factor 9 (Fgf9) were down-regulated upon Ago2 depletion, in contrast to the control group, where all transcripts were detected (Figure 3C). On the other hand, a number of other gene transcripts (bone
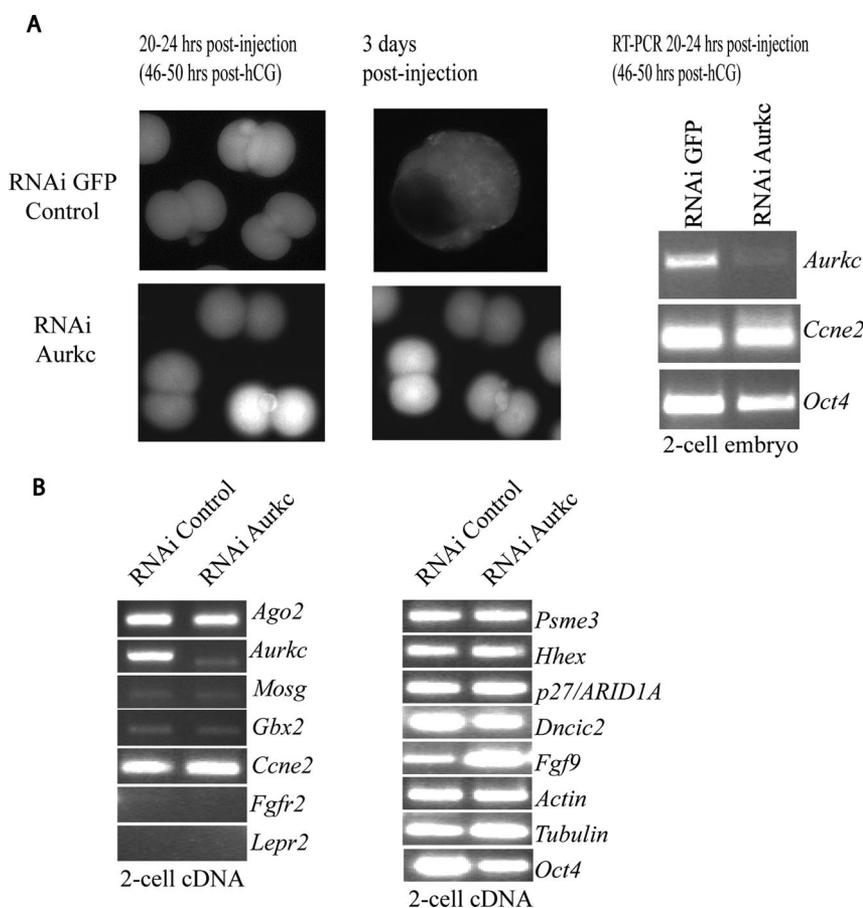
morphogenetic protein 15 (Bmp15), Oct4, β-actin, α-tubulin, and a mitogen-activated protein binding protein interacting protein (MAPBPIP) showed no significant difference between the control and Ago2 dsRNA treated embryos (data not shown).

To test whether the two-cell arrest phenotype caused by Ago2 depletion could indeed be linked to the specific regulation of the switch from maternal to zygotic expression, we chose to compare the effects of knocking down aurora kinase C, a maternally contributed protein that is required for cell cycle progression (Petersen *et al.*, 2001). When dsRNA against aurora kinase C was microinjected into zygotes, it resulted in ~90% of reduction of aurora kinase C and also led to a two-cell arrest as a result of failure of cell division (Figure 4A). RNAi control embryos showed no developmental arrest and developed to blastocyst stages (Figure 4A). We then examined whether genes showing altered expression in the RNAi Ago2 two-cell embryos were similarly affected after aurora kinase C RNAi. As before, we assessed the expression of these genes by nested PCR on first-strand cDNA generated from control and aurora kinase C RNAi two-cell embryos (Figure 4B, three independent experiments). This showed that mRNA levels of the maternally expressed genes Mosg, Gbx2, Fgfr2, and Lepr2 were neither stabilized nor up-regulated after aurora kinase C depletion as they were after Ago2 RNAi (compare Figure 3, A–C, with 4B). Moreover, transcripts which were strongly down-regulated in the Ago2-depleted embryos (psme3, Hhex, p27/ARID1A, Dncic2, Fgf9; Figure 3C), showed no reduction in expression in either control or aurora kinase C RNAi embryos (Figure 4B). This indicates that these changes in gene expression specifically require functional Ago2 in two-cell embryos.

Possible Involvement of Small Endogenous RNAs in Destruction of Specific Maternal Messages in the Two-Cell Embryo

Degradation of mRNAs by Ago2 with the RISC complex generally requires that these mRNAs must contain target sites for one or more miRNAs or other endogenous siRNAs. We therefore performed a miRNA-microarray screen, asking which mouse counterparts of an array of human miRNA sequences could be detected at developmental stages from MII oocytes to blastocysts (Supplementary Tables S2 and S3). From the resultant data we identified 24 miRNAs that were present in either the early or late two-cell embryos that could therefore be candidates to mediate degradation of maternal transcripts. We then used the sequence of the

A

20-24 hrs post-injection
(46-50 hrs post-hCG)

3 days
post-injection

RT-PCR 20-24 hrs post-injection
(46-50 hrs post-hCG)

RNAi GFP
Control

RNAi
Aurkc

B

RNAi Control RNAi Aurkc

Ago2
Aurkc
Mosg
Gbx2
Ccne2
Fgfr2
Lepr2

2-cell cDNA

RNAi Control RNAi Aurkc

Psme3
Hhex
p27/ARID1A
Dncic2
Fgf9
Actin
Tubulin
Oct4

2-cell cDNA

Figure 4. Down-regulation of aurora kinase C leads to the two-cell arrest, but does not affect the degradation of maternal mRNAs or zygotic activation of selected genes. Early zygotes were injected with dsRNA for GFP or aurora kinase C. Embryos were cultured in vitro until the blastocyst stage. Images were taken using fluorescence light to identify embryos injected with both dsRNA and rhodamine-conjugated dextran. Embryos of each experimental group were scored according to their developmental progress. The number of embryos in each experiment is indicated as injected embryos (two-cell stage; IE2), arrested embryos (A), embryos that developed to blastocyst (EB), and the number of embryos fragmented or dead (D). (A) RNAi GFP-injected control group (IE2 = 18, EB = 15, D = 3) and RNAi aurora kinase C (Aurkc; IE2 = 20, A = 16, D = 4). RT-PCR analysis was performed 24 h after injection, as shown on the right panel. Aurkc, aurora kinase C. This shows that aurora kinase C mRNA is greatly down-regulated, whereas Ccne2 and Oct4 mRNA remains constant in both experiments. Nested PCR on first-strand cDNA generated from either control or RNAi aurora kinase C two-cell embryos. (B) Mosg, Gbx2, Ccne2, Fgfr2, Lepr2, Psme3, Hhex, p270/ARID1A, Dncic2, Fgf9, and control genes Ago2, Aurkc, actin, tubulin, and Oct4. All genes tested showed similar expression levels of the transcripts as the control, except aurora kinase C, which was greatly reduced in the aurora kinase C-depleted embryos.

corresponding mouse miRNAs to predict computationally whether any of these miRNAs were likely to target the mRNAs for Mosg, Gbx2, Fgfr2, and Lepr-2 (Supplementary Table S4).

Although the precise mechanism of miRNA-mediated degradation of mRNAs is not clear, some features are widely accepted and form the basis for most computational methods for miRNA target prediction. MiRNA target prediction relies largely on determining the strength and pattern of binding of the miRNA to the target mRNA in its 3' UTR region (Rajewsky, 2006). Therefore, we scanned all currently known mouse miRNAs, extracted from miRBase (http://microrna.sanger.ac.uk/sequences/index.shtml; Griffiths-Jones, 2004; Griffiths-Jones et al., 2006), looking for the best binding positions in the 3' UTR, using the RNAfold program from the Vienna RNA package (http://www.tbi.univie.ac.at/RNA). We used two indicators for strength of miRNA/mRNA target prediction: 1) the predicted binding energy of the miRNA-mRNA duplex, and 2) the presence of "seed binding" over most of the first eight bases of the miRNA (Rajewsky, 2006; Sethupathy et al., 2006). We considered an mRNA a likely miRNA target if the predicted binding energy was better than −16 kCal/mol or had at least seven Watson/Crick bonds in the miRNA seed region and a predicted binding energy better than −8 kCal/mol.

Supplementary Table S4 lists the miRNAs that were present at either the early or late two-cell embryos and that are predicted by these criteria to have the potential to target the mRNAs of Mosg, Gbx2, Fgfr2, and Lepr-2. All four of these mRNAs gave reasonable target predictions with two or more miRNAs, making it impossible to tell which of these

specific miRNA would target which mRNA, and indeed it could be that degradation is achieved by the cumulative effect of more than one miRNA (Krek et al., 2005b; Saetrom et al., 2007). In addition, some uncertainty remains, as choice of the length of the mRNA section to test can influence the binding patterns and energies. Finally, we would like to note that it is also possible that other natural siRNAs, for example, those derived from pseudogenes (Kawamura et al., 2008; Tam et al., 2008; Watanabe et al., 2008), might participate in the RISC complex as partners of Ago2 in the early mouse embryo.

DISCUSSION

We have found that maternally provided Ago2, best known as the active endonuclease component of the RISC, is required for successful development of the early mouse embryos and appears to be involved in the maternal to zygotic transition. Of the four Argonaute family members, we show that both Ago2 and 3 transcripts are expressed in oocytes and throughout the preimplantation development. But only depletion of Ago2 mRNA leads to the developmental arrest at the two-cell stage, at the time of the maternal-to-zygotic transition. GFP-tagged Ago2 colocalizes with the decapping enzyme, Dcp1a, to cytoplasmic P-bodies, supporting the hypothesis that Ago2/RISC could be involved in maternal mRNA degradation. Indeed we have found a subset of specific individual maternally degraded mRNAs that could be under the control of this mRNA degradation system as well as zygotic transcripts which expression fails upon depletion of Ago2 mRNA.

The arrest of Ago2-RNAi embryos, depleted of maternal Ago2 transcripts from the zygote stage, at the two-cell stage contrasts to the postimplantation lethality of the Ago2−/− embryos, in which zygotic expression of Ago2 is eliminated. This implies either that Ago2 function is first critical for progression through these very early developmental stages but subsequently not until much later or alternatively that a significant maternal contribution of Ago2 permits development through a large part of embryogenesis. The timing of the observed phenotype after Ago2 knockdown correlates with the timing of degradation of many maternal mRNAs and activation of embryonic transcription. It is thus a pivotal point for changes in patterns in gene expression. Depletion of Ago2 or aurora kinase C, an Ago2/RISC-unrelated gene, causes mouse embryos to arrest development at the two-cell stage. However, the consequences on gene expression are quite different in these two cases. Down-regulation of Ago2, but not aurora kinase C, causes the stabilization of some maternal transcripts that would normally be degraded by the late two-cell stage. Conversely, whereas Ago2 down-regulation prevents the onset of expression of a number of zygotic transcripts, mRNAs for these genes accumulate in the two-cell stage arrest after aurora kinase C depletion. Thus, changes in gene expression we describe here appear specifically to reflect involvement of Ago2 in a gene silencing process. Intriguingly, not all transcripts tested displayed altered gene expression upon Ago2 RNAi; for example, levels of bone morphogenetic protein 15 (Bmp15), Oct4, β-actin, α-tubulin, and a MAPBPIP remained unchanged, indicating that the Ago2 would not be involved in regulating all transcripts. Ago2 has been shown to be biochemically distinct from other Argonaute proteins as it is the only one that is able to catalyze the siRNA-directed mRNA cleavage (Liu *et al.*, 2004; Okamura *et al.*, 2004). The requirement for Ago2 during the maternal-zygotic transition and its presence in cytoplasmic P-bodies therefore provides a hypothesis for a link between Ago2 and siRNA-induced mRNA instability in the mouse maternal-to-zygotic transition.

Our results point to a possible involvement of Ago2, a factor best known as a key component of RISC, in degradation of a set of maternal transcripts in the mouse embryo. This could be a prerequisite for the onset of a major wave of zygotic gene transcription at the two-cell stage. The efficient way in which RNAi can achieve knockdown of Ago2 mRNA levels and a developmental phenotype within 24 h suggests the Ago2 maternal contribution is in the form of both protein and mRNA. Thus, one can hypothesized that a pool of maternal Ago2 protein would be able to form a RISC complex with the exogenous interfering RNA, whereas the mRNA pool would be destroyed by this complex and so would be unable later to supply functional Ago2 protein to mediate destruction of other maternal mRNAs. This implies that in normal development there must be a mechanism for temporal regulation of the translation of maternal Ago2 mRNA at this specific stage. Once translated Ago2 would then mediate destruction of some other maternal transcripts, and this would appear necessary to allow zygotic development to continue. There is some evidence from a study in *Drosophila* (Edgar and Datar, 1996) and in porcine embryos (Anderson *et al.*, 2001) that degradation of many maternal mRNAs is indeed necessary to permit zygotically synthesized transcripts and proteins to take control of development at the maternal-zygotic transition (Bashirullah *et al.*, 2001).

During the course of our study examining possible roles of Argonaute proteins in the early mouse embryos, it was demonstrated that the RNAi machinery is also used in the zebrafish embryo to degrade stores of maternal mRNAs (Giraldez *et al.*, 2006). Analogous to these author's findings of the involvement of miR-430 in the degradation of maternal transcripts we have found that the depletion of Ago2 protein does not arrest the embryos in a "maternal state," but it appears to be a combination of a maternal and zygotic state arrest. This might suggest a more universal mechanism for the Ago2 protein at this specific stage in development in which Ago2 might not only function to permit degradation of maternal mRNAs, but also to promote activation of transcription. Indeed such a role for Ago2 has been postulated in human cell lines (Li *et al.*, 2006). Finally, we would like to note that Ago2 might also have a slicer-independent role in the early mouse embryos. Such a role for Ago2 has been recently demonstrated in hematopoiesis in the regulation of miRNA homeostasis (O'Carroll *et al.*, 2007; Martinez and Busslinger, 2007).

The Ago2/RISC factors are essential components of RNAi pathway. However, the RNAi pathway includes also other crucial factors such as the conserved ribonuclease Dicer and the short noncoding small RNAs (Bartel, 2004; He and Hannon, 2004; Bagga *et al.*, 2005; Kawamura *et al.*, 2008; Tam *et al.*, 2008; Watanabe *et al.*, 2008). In agreement with our findings, Dicer and miRNAs have also been recently found to be essential for mouse female germ-line meiosis and early development. It has been shown that depletion of Dicer from oocytes results in many miRNAs being lost from these cells (Tang *et al.*, 2007). Moreover, Dicer appears crucial for completion of meiosis in female germ-line (Murchison *et al.*, 2007). This suggests that Ago2 might also function during the process of oocyte maturation, a developmental stage not examine by experiments reported here. Indeed it could be postulated that miRNAs may be involved in the turnover of maternal transcripts essential for successful oocyte maturation.

Our own miRNA screen in conjunction with bioinformatics analysis identified several miRNAs expressed at these early stages of development as potential candidates to work with RISC to degrade maternal mRNAs. Of these, several miRNAs could possibly target and regulate stability of the Mosg, Gbx2, Fgfr2, and Lepr-2 transcripts. However, it is also possible that other dsRNAs could be involved such as the natural siRNAs derived from pseudogenes (Sasidharan and Gerstein, 2008). Thus, while our study points to an important role for Ago2 at the earliest stages of mouse embryogenesis, it remains of considerable future interest to identify precisely whether and how specific small endogenous dsRNAs might work in conjunction with Ago2 to regulate mRNA stability at this crucial developmental stage.

REFERENCES

Anderson, J. E., Matteri, R. L., Abeydeera, L. R., Day, B. N., and Prather, R. S. (2001). Degradation of maternal cdc25c during the maternal to zygotic transition is dependent upon embryonic transcription. Reprod. Dev. *60*, 181–188.

Alisch, R. S., Jin, P., Epstein, M., Caspary, T., and Warren, S. T. (2007). Argonaute2 is essential for mammalian gastrulation and proper mesoderm formation. PLoS Genet. *3*, e227.

Bagga, S., Bracht, J., Hunter, S., Massirer, K., Holtz, J., Eachus, R., and Pasquinelli, A. E. (2005). Regulation by let-7 and lin-4 miRNAs results in target mRNA degradation. Cell *122*, 553–563.

Bartel, D. P. (2004). MicroRNAs: genomics, biogenesis, mechanism, and function. Cell *116*, 281–297.

Bashirullah, A., Cooperstock, R. L., and Lipshitz, H. D. (2001). Spatial and temporal control of RNA stability. Proc. Natl. Acad. Sci. USA *98*, 7025–7028.

Behm-Ansmant, I., Rehwinkel, J., Doerks, T., Stark, A., Bork, P., and Izaurralde, E. (2006). mRNA degradation by miRNAs and GW182 requires both CCR4, NOT deadenylase and DCP1, DCP2 decapping complexes. Genes Dev. *20*, 1885–1898.

Braude, P., Bolton, V., and Moore, S. (1988). Human gene expression first occurs between the four- and eight-cell stages of preimplantation development. Nature *332*, 459–461.

Bushati, N., and Cohen, S. M. (2007). microRNA functions. Annu. Rev. Cell. Dev. Biol. *23*, 175–205.

Camous, S., Kopecny, V., and Flechon, J. E. (1986). Autoradiographic detection of the earliest stage of [3H]-uridine incorporation into the cow embryo. Biol. Cell *58*, 195–200.

Crosby, I. M., Gandolfi, F., and Moor, R. M. (1988). Control of protein synthesis during early cleavage of sheep embryos. J. Reprod. Fertil. *82*, 769–775.

Denli, A. M., and Hannon, G. J. (2003). RNAi: an ever-growing puzzle. Trends Biochem. Sci. *10*, 196–201.

Doi, N., Zenno, S., Ueda, R., Ohki-Hamazaki, H., Ui-Tei, K., and Saigo, K. (2003). Short-interfering-RNA-mediated gene silencing in mammalian cells requires Dicer and eIF2C translation initiation factors. Curr. Biol. *13*, 41–46.

Dulac, C., and Axel, R. (1995). A novel family of genes encoding putative pheromone receptors in mammals. Cell *83*, 195–206.

Edgar, B. A., and Datar, S. A. (1996). Zygotic degradation of two maternal Cdc25 mRNAs terminates *Drosophila*'s early cell cycle program. Genes Dev. *10*, 1966–1977.

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

Giraldez, A. J., Mishima, Y., Rihel, J., Grocock, R. J., Van Dongen, S., Inoue, K., Enright, A. J., and Schier, A. F. (2006). Zebrafish MiR-430 promotes deadenylation and clearance of maternal mRNAs. Science *312*, 75–79.

Griffiths-Jones, S. (2004). The microRNA Registry. Nucleic Acid Res. *32*, D109–D111.

Griffiths-Jones, S., Grocock, R. J., van Dongen, S., Bateman, A., and Enright, A. J. (2006). miRBase: microRNA sequences, targets and gene nomenclature. Nucleic Acid Res. *34*, D140–D144.

Hamatani, T., Carter, M. G., Sharov, A. A., and Ko, M.S.H. (2004). Dynamics of global gene expression changes during mouse preimplantation development. Dev Cell *6*, 117–131.

Hammond, S. M., Boettcher, S., Caudy, A. A., Kobayashi, R., and Hannon, G. J. (2001). Argonaute2, a link between genetic and biochemical analyses of RNAi. Science *293*, 1146–1150.

Hannon, G. J. (2002). RNA interference. Nature *418*, 244–251.

He, L., and Hannon, G. J. (2004). MicroRNAs: small RNAs with a big role in gene regulation. Nat. Rev. Genet. *5*, 522–531.

Hoffert, K. A., Anderson, G. B., Wildt, D. E., and Roth, T. L. (1997). Transition from maternal to embryonic control of development in IVM/IVF domestic cat embryos. Reprod. Dev. *48*, 208–215.

Hogan, B., Beddington, R., Costantini, F., and Lacy, E. (1994). Manipulating the Mouse Embryo: A Laboratory Manual, Cold Spring Harbor, NY: Cold Spring Harbor Press.

Hubank, M., and Schatz, D. G. (1994). Identifying differences in mRNA expression by representational difference analysis of cDNA. Nucleic Acids Res. 5640–5648.

Hutvagner, G., and Zamore, P. D. (2002). A microRNA in a multiple-turnover RNAi enzyme complex. Science *297*, 2056–2060.

Combinatorial microRNA target predictions. Nat. Genet. *37*, 495–500.

Kawamura, Y., Saito, K., Kin, T., Ono, Y., Asai, K., Sunohara, T., Okada, T. N., Siomi, M. C., and Siomi, H. (2008). *Drosophila* endogenous small RNAs bind to Argonaute 2 in somatic cells. Nature *453*, 793–797.

Krek, A., *et al.* (2005). Combinatorial microRNA target predictions. Nat. Genet. *5*, 495–500.

Li, L. C., Okino, S. T., Zhao, H., Pookot, D., Place, R. F., Urakami, S., Enokida, H., and Dahiya, R. (2006). Small dsRNAs induce transcriptional activation in human cells. Proc. Natl. Acad. Sci. USA *103*, 17337–17342.

Liu, J., Carmell, M. A., Rivas, F. V., Marsden, C. G., Thomson, J. M., Song, J.-J., Hammond, S. M., Joshua-Tor, L., and Hannon, G. J. (2004). Argonaute2 is the catalytic engine of mammalian RNAi. Science *395*, 1437–1441.

Liu, J., Rivas, F. V., Wohlschlegel, J., Yates, J. R., Parker, R., and Hannon, G. J. (2005a). A role for the P-body component GW182 in microRNA function. Nat. Cell Biol. *7*, 1161–1166.

Liu, J., Valencia-Sanchez, M. A., Hannon, G. J., and Parker, R. (2005b). MicroRNA-dependent localization of targeted mRNAs to mammalian P-bodies. Nat. Cell Biol. *7*, 719–723.

Manes, C. (1973). The participation of the embryonic genome during early cleavage in the rabbit. Dev. Biol. *32*, 453–459.

Martinez. J., and Busslinger, M. (2007). Life beyond cleavage: the case of Ago2 and hematopoiesis. Genes Dev. *21*, 1983–1988.

Mathews, D. H., Sabina, J., Zuker, M., and Turner, D. H. (1999). Expanded sequence dependence of thermodynamic parameters improves prediction of RNA secondary structure. J. Mol. Biol. *288*, 911–940.

Meister, D., and Tuschl, T. (2004). Mechanisms of gene silencing by double-stranded RNA. Nature *431*, 343–349.

Meister, G., Landthaler, M., Patkaniowska, A., Dorsett, Y., Teng, G., and Tuschl, T. (2004). Human Argonaute2 mediates RNA cleavage targeted by miRNAs and siRNAs. Mol. Cell *15*, 185–197.

Miska, E. A., Alvarez-Saavedra, E., Townsend, M., Yoshii, A., Sestan, N., Rakic, P., Constantine-Paton, M., and Horvitz, H. R. (2004). Microarray analysis of microRNA expression in the developing mammalian brain. Genome Biol. *5*, R68.

Morita, S., Horii, T., Kimura, M., Goto, Y., Ochiya, T., and Hatada, I. (2007). One Argonaute family member, Eif2c2 (Ago2), is essential for development and appears not to be involved in DNA methylation. Genomics *89*, 687–696.

Mourelatos, Z., Dostie, J., Paushkin, S., Sharma, A., Charroux, B., Abel, L., Rappsilber, J., Mann, M., and Dreyfuss, G. (2002). miRNPs: a novel class of ribonucleoproteins containing numerous microRNAs. Genes Dev. *16*, 720–728.

Murchison, E. P., Stein, P., Xuan, Z., Pan, H., Zhang, M. Q., Schultz, R. M., and Hannon, G. J. (2007). Critical roles for Dicer in the female germline. Genes Dev. *21*, 682–693.

Oh, B., Hwang, S., McLaughlin, J., Solter, D., and Knowles, B. B. (2000). Timely translation during the mouse oocyte-to-embryo transition. Development *127*, 3795–3803.

O'Carroll, D., Mecklenbrauker, I., Das, P. P., Santana, A., Koenig, U., Enright, A. J., Miska, E. A., and Tarakhovsky, A. (2007). A Slicer-independent role for Argonaute 2 in hematopoiesis and the microRNA pathway. Genes Dev. *21*, 1999–2004.

Okamura, K., Ishizuka, A., Siomi, H., and Siomi, M. C. (2004). Distinct roles for Argonaute proteins in small RNA-directed RNA cleavage pathways. Genes Dev. *14*,1655–1666.

Paynton, B. V., and Bachvarova, R. R. (1988). Changes in state of adenylation and time course of degradation of maternal mRNAs during oocyte maturation and early embryonic development in the mouse. Dev. Biol. *129*, 304–314.

Petersen, J., Paris, J., Willer, M., Philippe, M., and Hagan, I. (2001). The *S. pombe* aurora-related kinase Ark1 associates with mitotic structures in a stage dependent manner and is required for chromosome segregation J. Cell Sci. 4371–4384.

Pillai, R. S., Artus, C. G., and Filipowicz, W. (2004). Tethering of human Ago proteins to mRNA mimics the miRNA-mediated repression of protein synthesis. RNA *10*, 1518–1525.

Pillai, R. S., Bhattacharyya, S. N., Artus, C. G., T., Z., Cougot, N., Basyuk, E., Bertrand, E., and Filipowicz, W. (2005). Inhibition of translational initiation by Let-7 MicroRNA in human cells. Science *309*, 1573–1576.

Rajewsky, N. (2006). microRNA target predictions in animals. Nat. Genet. *38*, S8–13.

Rana, T. M. (2007). Illuminating the silence: understanding the structure and function of small RNAs. Nat. Rev. Mol. Cell Biol. *8*, 23–36.

Roush, S. F., and Slack, F. J. (2006). Micromanagement: a role for microRNAs in mRNA stability. ACS Chem. Biol. *1*, 132–134.

Saetrom, P., Heale, B. S., Snøve, O. J., Aagaard, L., Alluin, J., and Rossi, J. J. (2007). Distance constraints between microRNA target sites dictate efficacy and cooperativity. Nucleic Acids Res. *7*, 2333–2342.

Saitou, M., Barton, S. C., and Surani, M. A. (2002). A molecular programme for the specification of germ cell fate in mice. Nature *418*, 293–300.

Schultz, R. M. (1993). Regulation of zygotic gene activation in the mouse. BioEssays *15*, 531–538.

Sen, G. L., and Blau, H. M. (2005). Argonaute 2/RISC resides in sites of mammalian mRNA decay known as cytoplasmic bodies. Nat. Cell Biol. 7, 633–636.

Sen, G. L., and Blau, H. M. (2006). A brief history of RNAi: the silence of the genes. FASEB J. *20,* 1293–1299.

Sethupathy, P., Megraw, M., and Hatzigeorgiou, A. G. (2006). A guide through present computational approaches for the identification of mammalian microRNA targets. Nat. Methods *11,* 881–886.

Sheth, U., and Parker, R. (2003). Decapping and decay of messenger RNA occur in cytoplasmic processing bodies. Science *300,* 805–808.

Svoboda, P., Stein, P., Hayashi, H., and Schultz, R. M. (2000). Selective reduction of dormant maternal mRNAs in mouse oocytes by RNA interference. Development *127,* 4147–4156.

Tam, O. H., *et al.* (2008). Pseudogene-derived small interfering RNAs regulate gene expression in mouse oocytes. Nature *453,* 534–538.

Sasidharan, R., and Gerstein, M. (2008). Genomics: protein fossils live on as RNA. Nature *453,* 729–731.

Tang, F., Kaneda, M., O'Carroll, D., Hajkova, P., Barton, S. C., Sun, Y. A., Lee, C., Tarakhovsky, A., Lao, K., and Surani, M. A. (2007). Maternal microRNAs are essential for mouse zygotic development. Genes Dev. *21,* 644–648.

Tuschl, T., Zamore, P. D., Lehmann, R., Bartel, D. P., and Sharp, P. A. (1999). Targeted mRNA degradation by double-stranded RNA in vitro. Genes Dev. *13,* 3191–3197.

Valencia-Sanchez, M. A., Liu, J., Hannon, G. J., and Parker, R. (2006). Control of translation and mRNA degradation by miRNAs and siRNAs. Genes Dev. *20,* 515–524.

Vasudevan, S., and Steitz, J. A. (2007). AU-rich-element-mediated upregulation of translation by FXR1 and Argonaute 2. Cell *128,* 1105–1118.

Volpe, T. A., Kidner, C., Hall, I. M., Teng, G., Grewal, S. I., and Martienssen, R. A. (2002). Regulation of heterochromatic silencing and histone H3 lysine-9 methylation by RNAi. Science *297,* 1833–1837.

Wang, Q. T., Piotrowska, K., Ciemerych, M. A., Milenkovic, L., Scott, M. P., Davis, R. W., and Zernicka-Goetz, M. (2004). A genome-wide study of gene activity reveals developmental signalling pathways active in mammalian oocytes and pre-implantation embryos. Dev. Cell *6,* 133–144.

Watanabe, T., *et al.* (2008). Endogenous siRNAs from naturally formed dsRNAs regulate transcripts in mouse oocytes. Nature *453,* 539–543.

Wianny, F., and Zernicka-Goetz, M. (2000). Specific interference with gene function by double-stranded RNA in early mouse development. Nat. Cell Biol. *2,* 70–75.

Wienholds, E., Kloosterman, W. P., Miska, E., Alvarez-Saavedra, E., Berezikov, E., de Bruijn, E., Horvitz, H. R., Kauppinen, S., and Plasterk, R. H. (2005). MicroRNA expression in zebrafish embryonic development. Science *309,* 310–311.

Wilkinson, D. G., and Nieto, M. A. (1993). Detection of messenger RNA by in situ hybridization to tissue sections and whole mounts. Methods Enzymol. *225,* 361–373.

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

Zernicka-Goetz, M. (1994). Activation of embryonic genes during preimplantation rat development. Reprod. Dev. *38,* 30–35.