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Transgenic RNAi-mediated reduction of MSY2 in mouse oocytes results in reduced fertility

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Abstract

MSY2 is implicated in regulating the stability and translation of maternal mRNAs during mouse oogenesis. We report here that by driving the expression of a transgene encoding an *Msy2* hairpin dsRNA in growing oocytes using the oocyte-specific *Zp3* promoter, the amount of MSY2 protein was reduced by at least 60% in fully grown oocytes. The decrease appeared specific because no decrease was observed in either non-targeted mRNAs or proteins. Fertility of transgenic females was severely reduced. Although transgenic eggs could be inseminated, the eggs did not exhibit the normal series of oscillations in intracellular Ca^{2+}, resume meiosis, undergo cortical granule exocytosis, or ZP2 cleavage to $ZP2_f$. Transgenic oocytes also displayed a higher incidence of both the non-surrounded nucleolus chromatin morphology, and abnormal meiotic spindle formation was observed following oocyte maturation. Transgenic oocytes contained less total mRNA (approximately 75–80% that of non-transgenic oocytes) and displayed a reduced level of protein synthesis. Moreover, several of the maturation-associated changes in protein synthesis failed to occur in the transgenic oocytes. These results support a role for MSY2 in stabilizing maternal mRNAs in growing oocytes, a process essential to generate meiotically and developmentally competent oocytes.

Keywords: *Msy2*; Transgenic RNAi; Protein synthesis; Oocyte maturation; Egg activation; mRNA stability

Introduction

The two interactive processes that occur during oogenesis, that is, oocyte growth and meiosis, result in forming a fully grown oocyte capable of meiotic maturation, fertilization, and development to term (Schultz, 1986). These processes, in particular oocyte maturation, depend on the timely recruitment of maternal mRNAs. Mammalian oocytes accumulate large quantities of maternal mRNAs during their prolonged growth phase (approximately 20 days in mouse); the fully grown mouse oocyte contains about 80 pg of total mRNA (Bachvarova et al., 1985). These maternal mRNAs are very stable in growing oocytes with a half-life of 8–12 days (Brower et al., 1981). Mechanisms that regulate the stability and translation of these maternal mRNAs, however, are not well understood due to the limited availability of biological material.

In non-mammalian oocytes, Y-box proteins are the major components that package maternal mRNAs into ribonucleoprotein particles (mRNPs), which suggests a role for these proteins in regulating the stability and translation of maternal mRNAs (Sommerville, 1999; Wolffe, 1994). MSY2 is a very abundant mouse germ cell-specific Y-box protein (approximately 2% of total oocyte protein) (Yu et al., 2001). Its accumulation during oocyte growth and degradation by the mid two-cell stage, which coincides with the massive degradation of maternal mRNAs, supports the hypothesis that MSY2 regulates the stability and translation of maternal mRNAs (Yu et al., 2001). Consistent with this is that recombinant MSY2 protein binds to mRNAs in vitro with relatively little sequence specificity, despite a preference for certain short RNA sequences ($U_{AC}C_ACAU_CCA_{CU}$) (Davies et al., 2000; Giorgini et al., 2001; Yu et al., 2002) and it can moderately repress the translation in vitro of mRNAs at a physiologically relevant protein/mRNA molar ratio (73:1) (Yu et al., 2002). Moreover, most of the MSY2

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is associated with an oocyte-specific Triton-insoluble fraction. This association, which requires the RNA-binding activity, could sequester maternal mRNAs and contribute to their stability (Yu et al., 2003). Although these data indicate that MSY2 likely plays a global role in the regulation of the stability and translation of maternal mRNAs during mouse oogenesis, the function of MSY2 in vivo remains unknown.

RNAi is a conserved cellular mechanism in which double-stranded RNA (dsRNA) is processed into 21- to 23-nt-long short interfering (si) duplex RNAs by a RNaseIII-like enzyme (Dicer). These siRNAs are then assembled into a large complex (RISC) that promotes the sequence-specific destruction of the targeted mRNA (Bosher and Labouesse, 2000; Hutvagner and Zamore, 2002). RNAi operates in different systems including mouse oocytes and early embryos (Svoboda et al., 2000; Wianny and Zernicka-Goetz, 2000). RNAi likely serves as a defense mechanism against viral infections, in transgene silencing, and in inhibiting transposon activity (Caplen et al., 2002; Fagard et al., 2000; Pal-Bhadra et al., 2002; Sijen and Plasterk, 2003; Tabara et al., 1999). Both long dsRNAs and siRNAs have been used to reduce mRNA expression as an efficient way to study gene function. Successful transgenic RNAi where the dsRNA is expressed from a transgene has been reported in *C. elegans*, *Drosophila*, and *Arabidopsis* (Chuang and Meyerowitz, 2000; Kennerdell and Carthew, 2000; Tavernarakis et al., 2000), and we recently demonstrated that transgenic RNAi can be used to study gene function in mouse oocytes (Fedoriw et al., 2004; Stein et al., 2003b).

We report here that the oocyte-specific *Zp3* promoter-mediated expression of *Msy2* hairpin dsRNA through transgenesis specifically reduces the amount of MSY2 protein by at least 60% in fully grown mouse oocytes. Decreased *Msy2* expression results in abnormal meiotic spindle formation and a low incidence of egg activation, which leads to a markedly decreased litter size. Moreover, this reduction in the amount of MSY2 protein results in a decrease in both protein synthesis and the total amount of mRNA in fully grown oocytes.

Materials and methods

Msy2 transgene construction, transgenic animal production, and transgenic mice genotyping

To prepare the vector for MSY2 transgene construction, the CMV promoter from pEGFP-N_2 (Clontech, Palo Alto, CA) was removed by digestion with *AseI* and *NheI*. The cohesive ends were filled in with Klenow fragment (New England Biolabs, Inc., Beverly, MA) before self-ligation. The *Bgl*II/*Nhe*I fragment containing the CMV promoter and the chemric intron and the *Not*I/*Bam*HI fragment containing the SV40 late polyadenylation signal from pRL-luc vector (Promega, Madison, WI) were then inserted into the *Bgl*II/*Bam*HI, and *Not*I/*Dra*III sites of the modified pEGFP-N_2 vector, respectively. To prepare the MSY2 hairpin, the *Msy2* cDNA coding for the entire cold-shock domain (CSD) and the C-terminal tail domain (approximately 0.85 kb) were first cloned into the *Eco*RI/*Eag*I sites of pET-28a (+) vector (Novagen, Madison, WI), and the *Eco*RI/*Spe*I sites of pXT-7 vector, which was a generous gift from Sergei Sokol, Harvard University. The *Bam*HI/*Eag*I *Msy2* cDNA fragment from pET-28a (+) vector was then inserted into the *Bam*HI/*Pst*I sites of pXT-7 vector that already contained *Msy2* cDNA between its *Eco*RI/*Spe*I sites. The *Eco*RI fragment containing the *Msy2* hairpin from the resulting pXT-7 vector was then cloned into the *Not*I site of pEGFP-N2 with the aforementioned modifications. Last, the CMV promoter was replaced with the *Zp3* promoter (*Bgl*II/*Pst*I), the generous gift of Jurrien Dean. The expression of *Msy2* hairpin dsRNA and its effect on *Msy2* mRNA level were confirmed by microinjecting the construct into the nucleus of meiotically incompetent oocytes followed by fluorescence microscopy to detect expression of the EGFP and RT-PCR analysis for endogenous *Msy2* after 24 h of culture.

The insert was separated from the vector by digestion with *Bgl*II/*Cla*I and purified as described in www.uphs.upenn.edu/genetics/core-facs/tcmf/. The DNA was resuspended to 5 ng/μl in 10 mM Tris, pH 7.4/0.1 mM EDTA, and submitted to the Transgenic and Chimeric Mouse Facility (TCMF) at University of Pennsylvania to produce transgenic B6SJLF1 mice. To generate F1 transgenic mice, wild-type CF1 females were mated to the founder B6SJLF1 transgenic male mice.

Southern blotting and PCR were used to identify the transgenic mice. Tail genomic DNA was isolated as described in the TCMF's protocol (www.uphs.upenn.edu/genetics/core-facs/tcmf/). About 5 μg genomic DNA from each tail sample was digested with *Pst*I overnight, and subjected to standard Southern blotting analysis using EGFP cDNA as a probe. For PCR screening, modified T7 primer (pRL-T7): 5′TAATACGACTCACTATAGGCT3′, and EGFP sequencing primer (EGFP-seqR): 5′CGTCGCCGTCCAGCTCGACCAG3′, were used, which would generate a 107-bp DNA fragment. About 400 ng of tail genomic DNA was used in a 25-μl PCR reaction. PCR was performed as follows: an initial denaturation step at 94°C for 5 min followed by 33 cycles of 94°C for 15 s, 55°C for 30 s, and 72°C for 30 s followed by 72°C for 5 min.

Collection of oocytes and MII eggs

Meiotically incompetent oocytes (55 μm in diameter), fully grown oocytes (78 μm in diameter), and ovulated MII eggs were collected from the transgenic female mice (CF1x B6SJLF1) as previously described (Brower et al., 1981; Eppig, 1977). Oocytes were matured in vitro in CZB medium (Chatot et al., 1989). The *zona pellucida* (ZP)

was removed by briefly incubating oocytes in acidic Tyrode solution (pH 2.5).

Reverse transcription (RT)-PCR and real-time PCR

For semi-quantitative RT-PCR, RNA was extracted and reverse transcribed as previously described (Temeles et al., 1994). About 0.05 pg/oocyte globin mRNA was added before RNA extraction to normalize for RNA extraction and RT-PCR efficiency. The *Msy2* primers used were msy2F2: 5′ CAGCCTATAGCCGCAGAGAC 3′ and msy2R2: 5′ GGTGATGCCTCGGAACAATA 3′, which amplify a 302 bp sequence in the 3′end region of *Msy2*. The α-globin primers used were F: 5′ ACCACCAAGACCTACTTTCCT 3′, and R: 5′ GTCAGCACGGTGCTCACAGA 3′, which gives a 257-bp fragment. The PCR amplification conditions were the same for both *Msy2* and α-globin: an initial denaturation at 94°C for 5 min, followed by 32 or 33 cycles of 94°C for 15 s, 55°C for 30 s, and 72°C for 1 min, followed by 72°C for 5 min. The PCR products were run on a 1.5% agarose gel.

For real-time PCR of *Msy2* and *Msy4*, total RNA was isolated from 50 transgenic and control fully grown oocytes (three samples per group) with the Absolutely RNA Microprep Kit (Stratagene, La Jolla, CA), and reverse transcription was carried out with the RETROscript Kit (Ambion, Austin, TX). Rabbit globin mRNA (0.03 pg/oocyte) was added to each sample before RNA extraction to normalize for RNA extraction and reverse transcription efficiency. One oocyte equivalent of cDNA was used in each real-time PCR analysis, which was carried out with Sybr Green PCR Master Reagents and 7700 sequence detector (PE Applied Biosystems). Standard curves were constructed using cloned PCR product as templates. The primers used for both real-time PCR and template production were as follows: for *Msy2*, the forward primer was 5′CATCCTTATTGTTCCGAGGCA3′and the reverse primer was 5′GGAGGTATGAGCTGGCTGGTT3′; for Msy4, the forward primer was 5′GACCAAGGCAGGTGAAGCAC3′ and the reverse primer was 5′TCACTCGGCACTGCTCTGTT3′; and for α-globin, the forward primer was 5′GCCTCCCTGGACAAGTTCCT3′ and the reverse primer was 5′GGCTCCAGCTTAACGATATTTGG3′. PCR conditions were as follows: an initial denaturation step at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, and 60 °C for 60 s. Each sample was analyzed in duplicate. The relative expression level of each gene was calculated using the standard curve before normalization to globin mRNA. The specificity of primers was confirmed with gel electrophoresis.

Immunoblotting

Oocyte extracts were run on a SDS-PAGE gel (9% for MSY2, 12% for the oocyte-specific histone H1oo) and transferred to Immobilon P (Millipore, Bedford, MA) The membranes were processed and developed as previously described (Yu et al., 2001). The α-MSY2 was used in 1:2000 dilution (Yu et al., 2002); α-H1oo (a gift from Eli Adashi, University of Utah, Health Sciences Center, Salt Lake City, UT) was used at a 1:1000 dilution.

IVF and calcium oscillation

In vitro fertilization (IVF) of cumulus cell-free eggs was carried out as previously described (Moore et al., 1993). To monitor changes in intracellular Ca^{2+} following insemination, ZP-free eggs were incubated in Whitten's medium (Whitten, 1971) containing 0.01% PVA (average Molecular weight 30,000–70,000), 10 μM fura-2-AM (Molecular Probes Inc., Eugene, OR) and 0.025% Pluronic F-1 (Molecular Probes) at 37°C in an atmosphere of 5% CO_2 in a humidified chamber for 20 min. The fura-2 loaded eggs from both control and experimental groups were transferred to the same 10 μl drop of Whitten's medium containing no BSA and placed on a temperature-controlled microscope stage under laminar flow of 5% CO_2 in air. After the eggs settled, 10 μl of Whitten's medium containing 30 mg/ml BSA and 2×10^5 sperm was gently added to the egg drop. For Ca^{2+} imaging, the cells were illuminated using a 100-W xenon arc lamp; light output was passed through a Lambda 10–2 filter wheel (Sutter Instrument Co., Novato, CA) to alternate excitation wavelengths between 340 and 380 nm. Emitted light passed through a fura-2 bandpass filter cube and was recorded using a Princeton Instruments MicroMAX CCD camera (Roper Scientific, Trenton, NJ). The emitted fluorescence was averaged for each egg, and the 340/380 emission ratios were analyzed to determine alterations in intracellular Ca^{2+} using MetaFluor software (Universal Imaging Corp., West Chester, PA). Sperm from B6SJLF1 IVF males were used in all experiments.

ZP2 conversion

Following 2 h in vitro insemination, the *zona pellucida* (ZP) was isolated from both transgenic and control eggs as previously described (Kurasawa et al., 1989). The conversion of ZP2 to $ZP2_f$ in individual ZP was analyzed by a biotinylation-enhanced chemiluminescent assay (Moos et al., 1994).

Immunofluorescence and confocal microscopy

Immunofluorescence and confocal microscopy of ZP-free eggs were carried out as previously described (Yu et al., 2001). For tubulin staining, α-β tubulin (type I, mouse, 1:500, Sigma, St. Louis, MO), and Alexa 488 goat-anti mouse secondary antibody (1:500, Molecular probes) were used. For cortical granule (CG) staining, the eggs were first blocked with PBS/3 mg/ml BSA/0.1 M glycine for 15 min before permeabilization. The lectin, *Lens culinaris* aggluti-

nin (LCA) (1:500, Vector laboratories, Inc., Burlingames, CA) and Texas Red-avidin (1:500, Vector Laboratories) were used to stain the CG as previously described (Connors et al., 1998). For DAPI staining, cells were directly mounted in 0.5 μg/ml DAPI (Sigma)/Vector Shield (Vector Laboratories) solution.

Two-dimensional gel electrophoresis

Oocytes and MII eggs were cultured CZB (-glutamine, ± 0.2 mM 3-isobutyl-1-methylxanthine (IBMX)) containing [^{35}S]methionine [1 μCi/μl, 1500 Ci/mmol, Amersham) at 37°C in an atmosphere of 5% CO_2 for 3 h. Total oocyte and MII egg protein extracts containing the same number of oocytes and eggs were then subjected to two-dimensional gel electrophoresis using the Investigator 2-D Electrophoresis System (Millipore) according to the manufacturer's instructions. Radiolabeled proteins were detected by exposing the dried gels to the phosphorimager for 1 week, and scanned with Storm 860 system.

RiboGreen RNA quantification and relative rate of protein synthesis

Total poly(A) mRNAs were isolated from 100 transgenic or control fully grown oocytes with Dynabeads mRNA DIRECT™ kit according to the manufacturer's instructions (Dynal A.S., Olso, Norway). Quantification of RNA was carried out with RiboGreen kit(R-11490, Stratagene); *E. coli* 16S and 23S rRNA that is provided by the manufacturer was used to construct the standard curve.

Relative rates of protein synthesis were determined as previously described (Poueymirou and Schultz, 1987). The data were expressed as a ratio of the number of cpm in the acid-insoluble fraction/number of cpm in the acid-insoluble + acid-soluble fractions and the fraction obtained from the non-transgenic oocytes was set as 100%.

Results

Reduced MSY2 expression in transgenic oocytes

To study the function of *Msy2* in vivo during oocyte development, we employed a transgenic RNAi approach to reduce *Msy2* expression in growing oocytes. This approach has been successfully used to phenocopy the *Mos* null mutant and to study the function of maternal CTCF (Fedoriw et al., 2004) and its advantages described (Stein et al., 2003b). The transgene generates a hairpin dsRNA that is driven by the oocyte-specific *Zp3* promoter (Fig. 1A), which is activated at the onset of oocyte growth, and hence should target the endogenous *Msy2* mRNA once growth initiates. The function of the EGFP DNA is to separate the intron from the hairpin sequences because previous studies indicated that the RNAi effect is compromised when the intron is close to the hairpin sequence (Svoboda et al., 2001). To circumvent the potential of compromised infertility of transgenic female founder mice, transgenic transmitting male founders are mated to wild type females and oocytes derived from female transgenic offspring are analyzed; non-transgenic littermates serve as controls (Stein et al., 2003b).

The presence of the *Msy2* transgene was identified by PCR analysis of the tail genomic DNA (Fig. 1B). Southern blot analysis revealed that multiple copies of the transgene were present in the transgenic founder mice (data not shown). From the forty-four founder mice, 15 transgenic females and nine transgenic males were identified, all of which appeared healthy. Two of the nine transgenic male founders transmitted the transgene. For one of these male founder males, little reduction in the amount of MSY2 protein, as detected by immunoblotting, was observed in oocytes isolated from transgenic female offspring (data not shown). When the other transgenic transmitting founder male was mated to a wild-type female, oocytes isolated from transgenic female offspring contained a reduced amount of *Msy2* mRNA and MSY2 protein (by at least 60%) when compared to non-transgenic littermates (Figs. 1C and D). The decrease in *Msy2* mRNA was specific because real-time PCR analysis revealed at least a 95% decrease for *Msy2* mRNA between transgenic and non-transgenic oocytes, whereas there was no detectable mRNA decrease for two other Y-box proteins, *Msy4* (Fig. 1E) or *Msy1* (data not shown). The lack of any apparent reduction in *Msy1* and *Msy4*, which contain the highly conserved cold-shock domain and are expressed in the oocyte (Giorgini et al., 2001; Paynton, 1998), was consistent with the high inherent specificity of RNAi (Hamada et al., 2002), and the absence of an RNA-dependent RNA polymerase amplification mechanism that would target related mRNAs (Schwarz et al., 2002; Stein et al., 2003a). In addition, there was no apparent decrease in the amount of the oocyte-specific histone H1oo protein in the transgenic oocytes when compared to that present in oocytes obtained from non-transgenic littermates (Fig. 1D). When F1 transgenic females were mated to wild-type males, oocytes obtained from transgenic female F2 offspring also displayed a similar 60% reduction in the amount of MSY2 protein (Fig. 1D). Similar results were also observed when F1 transgenic males were mated to wild-type females and the amount of MSY2 protein was measured in oocytes obtained from transgenic female F2 offspring (data not shown). Results of these experiments demonstrated that the transgenic RNAi approach resulted in a specific reduction of MSY2 protein by at least 60%.

Decreased fertility in transgenic female mice

We previously proposed that MSY2 is a global and positive modulator of oocyte mRNA stability (Yu et al., 2001, 2002, 2003). The decreased amount of *Msy2* mRNA

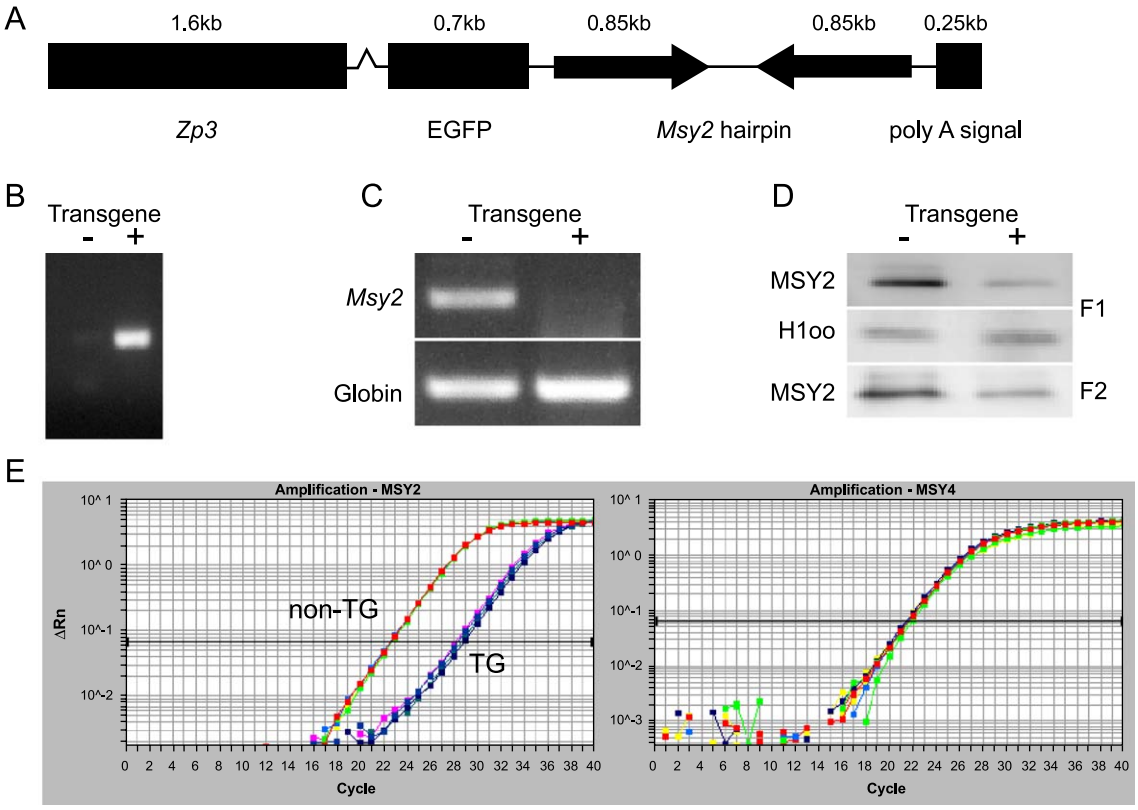

Fig. 1. Characterization and specificity of targeting of expression of *Msy2* hairpin-encoding transgene. (A) *Msy2* transgenic construct (4761 bp). The *Zp3* promoter is about 1.6 kb; the MSY2 hairpin has 1.7 kb with a 247-bp loop. The SV40 late polyadenylation signal was included at the end. (B) Tail sample screening by PCR using primers in the intron and the EGFP coding region. Shown is the 107-bp expected DNA fragment. (C) RT-PCR analysis of *Msy2* mRNA levels in control and transgenic oocytes. RT-PCR conditions were such that the amount of product generated is in the linear range of semi-log plots of the amount of product vs. cycle number. Globin mRNA was added to the samples before RNA extraction for normalization for both RNA extraction and RT-PCR efficiency (Temeles et al., 1994). (D) Immunoblot analysis of MSY2 protein level in control and transgenic oocytes. Control and transgenic oocyte protein extracts were made from F1 or F2 mice descended from F1 transgenic female mice (20 oocytes for MSY2; 39 oocytes for H1oo). (E) Real-time PCR analysis of *Msy2* and *Mys4*. TG; transgenic oocytes; non-TG, non-transgenic oocytes.

and protein present in oocytes derived from transgenic females could lead to perturbations in oocyte development, which in turn could result in females of compromised fertility. In fact, such was the case. When mated to wild-type males, the transgenic F1 females (derived by mating the transgenic male founder to wild-type females) gave birth to significantly smaller-sized litters with an average of 2.5, while their non-transgenic littermates had an average litter size of 12.5 (Table 1). The reduced litter size and other phenotype traits described below were unlikely due to the site of transgene integration because we noted that a founder transgenic female mice was totally infertile and oocytes recovered from this female revealed a >90% decrease in MSY2 protein when compared to non-transgenic littermates (data not shown).

The reduced fertility could be attributed to several factors, including failure of the oocytes to mature correctly, a lower incidence of fertilization, failure of inseminated eggs to undergo appropriate egg activation, or developmental arrest during either pre- or post-implantation development. Accordingly, we examined several of these factors. Ovulated transgenic and non-transgenic eggs were inseminated in vitro and development in vitro was monitored. Although there was no change in the incidence of insemination, as determined by DAPI staining, there was a significant decrease in the incidence of pronucleus (PN)

Table 1
Effect of reduced *Msy2* expression on ovulation, fertilization, and development

Transgene	Litter size	Fertilization (%)	PN formation (%)	2-cell formation (%)	No. of ovulated eggs
−	12.5 ± 0.5 (4)	87.4 ± 5.1 (52)	85.0 ± 6.9 (96)	78.9 ± 4.7 (96)	32.8 ± 4.3 (17)
+	2.5 ± 1 (4)	85.0 ± 9.5 (51)	29.2 ± 5.2 (58)	11.9 ± 4.1 (58)	16.4 ± 2.9 (16)

Four transgenic and four control mice were used to test litter size. Pronucleus (PN) formation and 2-cell formation were measured 8 and 24 h post-insemination. The numbers of cells/embryo analyzed are in parentheses. Seventeen control mice and sixteen transgenic mice (> 6 weeks) were superovulated (injected with PMSG and hCG with 48 h interval) and scored for the number of MII eggs ovulated. All the differences are significant ($P < 0.004$, t test) except for the incidence of fertilization ($P = 0.89$).

formation and cleavage to the 2-cell stage, which were scored at 8 and 24 h after insemination in vitro, respectively (Table 1). The 85% (11.9/78.9) reduction in the fraction of transgenic embryos developing to the 2-cell likely accounts for the 80% (2.5/12.5) reduction in litter size.

The observation that the transgenic eggs were inseminated but PN formation was markedly inhibited suggested that the inseminated transgenic eggs failed to undergo appropriate egg activation. In the mouse, fertilization results in a series of oscillations in the concentration of cytosolic-free Ca^{2+}, which is necessary and sufficient to cause the MII eggs to enter anaphase and complete meiosis (Schultz and Kopf, 1995). The increase in cytosolic-free Ca^{2++} also causes cortical granule (CG) exocytosis, which results in the cleavage of ZP2 (120 kd) to $ZP2_f$ (90 kd), and establishes the ZP block to polyspermy (Wassarman et al., 2001). Accordingly, each of these endpoints of egg activation was examined in inseminated transgenic eggs.

The majority of the inseminated transgenic eggs failed to undergo normal Ca^{2+} oscillations (Fig. 2C); we noted that approximately 45% failed to exhibit any Ca^{2+} oscillations and another 35% displayed a partial response, for

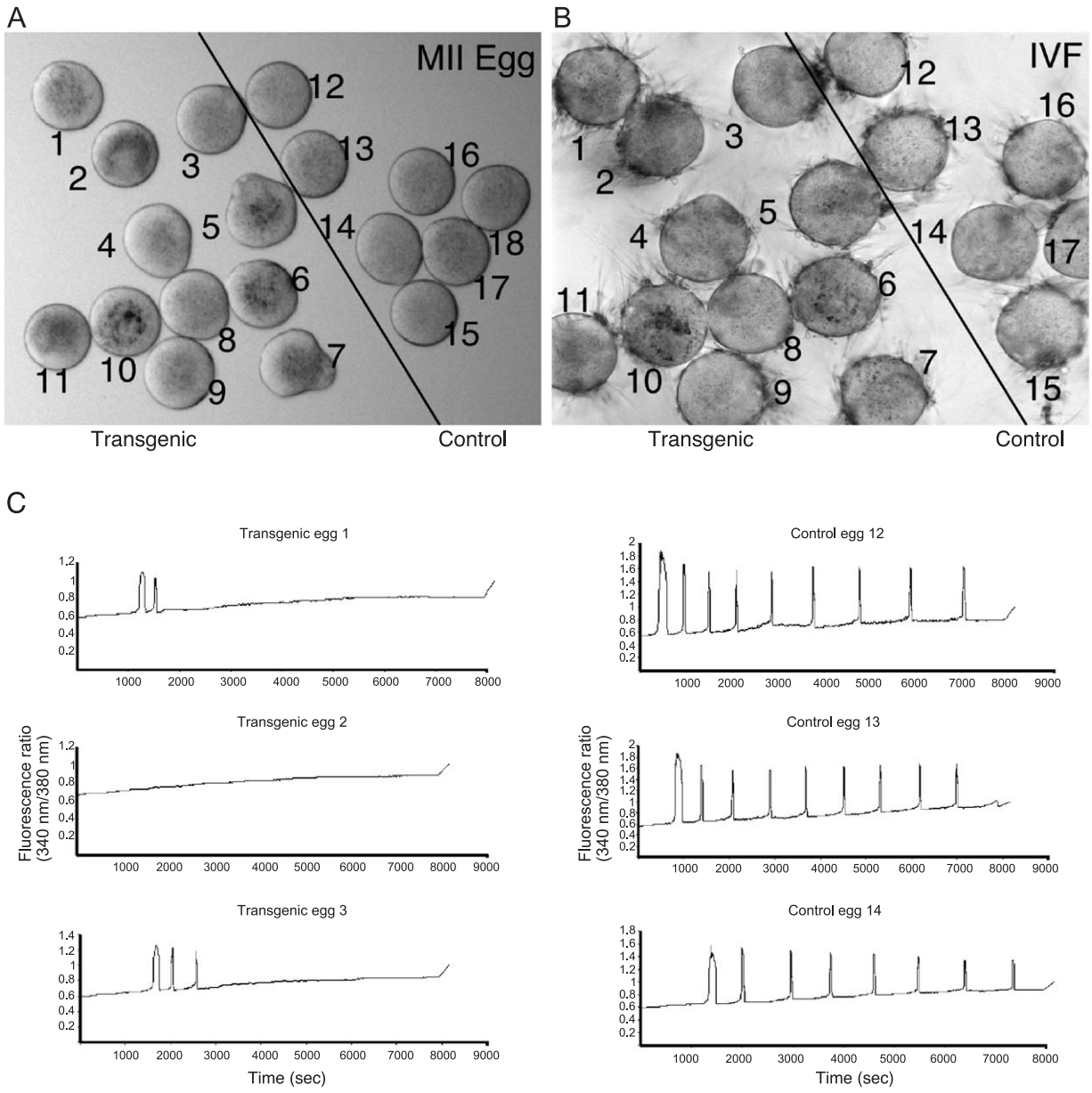

Fig. 2. Calcium oscillations following insemination of transgenic and non-transgenic eggs. MII-arrested eggs before (A) and post-insemination (B). The Metafluor system permits the analysis of calcium oscillations in all of the eggs present in the field. (C) Representative tracing for three transgenic and control eggs. The experiment was repeated three times and at least 10 eggs in each group were used for each experiment. Similar results were obtained for each experiment and representative calcium tracings from one experiment are shown.

example, transgenic eggs 1 and 3. The inability of the transgenic eggs to carry out Ca^{2+} oscillations correlated well with their aberrant morphology, that is, eggs that had dark granules had very few oscillations, while the eggs with relatively normal morphology had more oscillations. Consistent with this result, DAPI staining of the transgenic eggs 2 h after in vitro insemination showed that the majority of transgenic eggs still remained at MII, while the control eggs entered anaphase (Fig. 3A). The increased staining of CGs seen in fertilized transgenic eggs, when compared to inseminated non-transgenic eggs, indicated a failure of the transgenic eggs to undergo CG exocytosis, which was confirmed by monitoring the conversion of ZP2 to $ZP2_f$ (Fig. 3B). Following 2 h of in vitro insemination, ZP2 from the control eggs showed increased electrophoretic mobility, which indicated cleavage of this protein, while those from the transgenic eggs had the same mobility as that from MII eggs. The failure of transgenic eggs to undergo CG exocytosis and ZP2 conversion is the likely explanation for the polyspermy seen in the fertilized transgenic eggs (data not shown).

The reduced number of Ca^{2+} oscillations following insemination of transgenic eggs may be due to a reduced size of the intracellular Ca^{2+} pool. Thapsigargin inhibits endoplasmic and sarcoplasmic reticulum Ca^{2+}-ATPases, but not plasma membrane Ca^{2+}-ATPases and hence increases cytoplasmic Ca^{2+} by preventing Ca^{2+} reuptake (Lytton et al., 1991; Sagara and Inesi, 1991; Thastrup et al., 1990). Transgenic eggs treated with thapsigargin release less Ca^{2+} than their non-transgenic counterparts (Fig. 4A). Consistent with a markedly reduced store of intracellular Ca^{2+} is that transgenic eggs treated with Sr^{2+} resulted in only a single Ca^{2+} transient, whereas non-transgenic eggs displayed a normal series of Ca^{2+} oscillations (Figs. 4B and C).

Morphology, chromatin configuration, and meiotic spindle formation in transgenic oocytes

As described above, many of the ovulated transgenic eggs possessed dark granules (Figs. 2A and B). This led us to examine transgenic oocytes and eggs more carefully. Both fully grown oocytes and MII eggs isolated from the

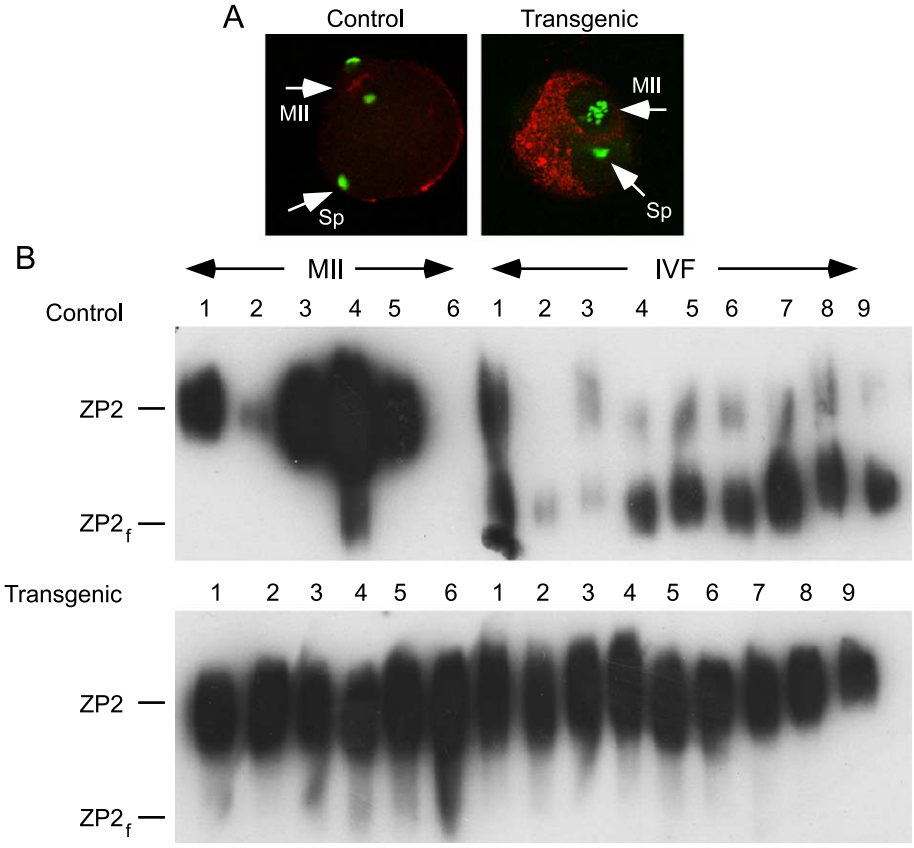

Fig. 3. Cell cycle resumption, cortical granule exocytosis, and ZP2 conversion in transgenic and non-transgenic eggs. (A) Fertilized transgenic eggs failed to enter anaphase and undergo cortical granule exocytosis. DNA staining (DAPI, green); cortical granule staining (red). The cells were processed following 2 h of in vitro insemination. The arrows point to either the sperm (Sp) or egg (MII) DNA. Note in the control eggs the separation of the egg chromosomes, indicating entry into anaphase, whereas in inseminated transgenic eggs the egg chromosomes remained condensed with no sign of entry into anaphase. The experiment was conducted three times and similar results were observed in each experiment; shown are representative images. (B) ZP2 conversion. ZPs were isolated from transgenic and control eggs following 2 h of in vitro insemination and then processed for ZP2 conversion. The lack of a signal in lane 6 likely reflects a failure of the biotinylation reaction for that sample.

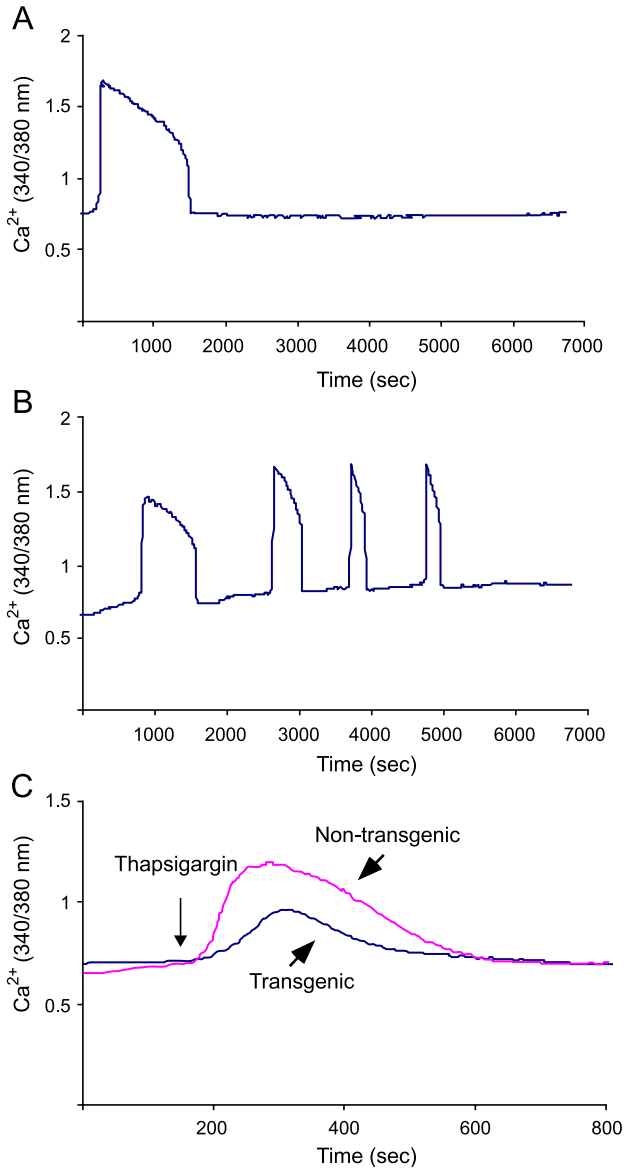

Fig. 4. Changes in intracellular Ca^{2+} concentration in transgenic and non-transgenic oocytes following either thapsigargin or Sr^{2+} treatment. Transgenic oocytes (A) and non-transgenic oocytes (B) were incubated in medium containing 10 mM Sr^{2+} to induce changes in intracellular Ca^{2+}. The experiment was repeated two times and at least 10 eggs in each group were used for each experiment. Similar results were obtained for each experiment and representative calcium tracings from one experiment are shown. (C) Transgenic and non-transgenic eggs were incubated in medium containing 50 μM thapsigargin and changes in intracellular Ca^{2+} concentration were measured. The experiment was repeated two times and at least 10 eggs in each group were used for each experiment. Similar results were obtained for each experiment and representative calcium tracings from one experiment are shown.

sexually mature transgenic females (older than 6 weeks) appeared morphologically abnormal (data not shown). Many of the transgenic oocytes looked darker than the controls, and this appeared even more pronounced in the transgenic MII eggs. The number and size of the oocytes isolated from the transgenic females were similar to those from the non-transgenic controls. The number of MII eggs collected from the superovulated transgenic females, however, was consistently lower than that from their non-transgenic littermate controls (Table 1).

Fully grown GV-intact oocytes exhibit two types of nuclear DNA configurations that differ by the presence or absence of a ring of condensed chromatin around the nucleolus. These configurations have been termed SN (surrounded nucleolus) and NSN (non-surrounded nucleolus) (Fig. 5) (Bouniol-Baly et al., 1999). While all the oocytes are of the NSN or partial NSN (pNSN) configurations in mice before 15 days after birth, the percentage of oocytes with the SN configuration increases with age and

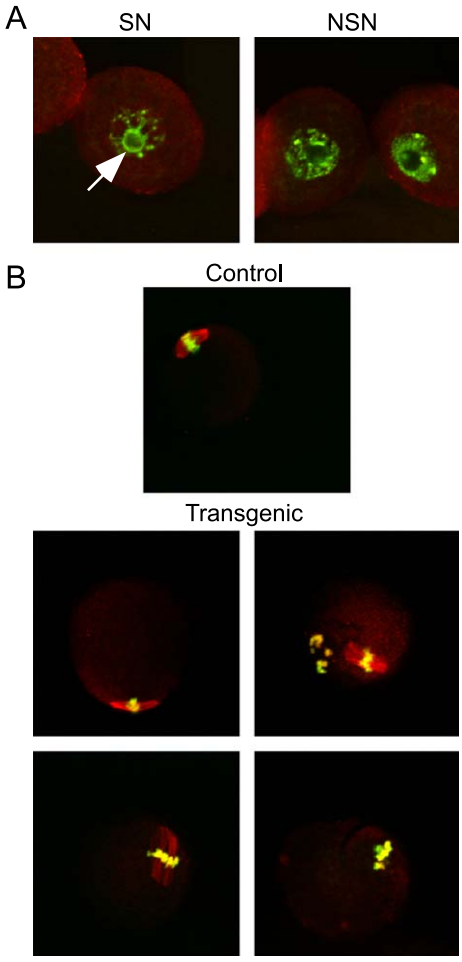

Fig. 5. DNA configuration and spindle formation in transgenic oocytes. (A) Examples of SN and NSN-type chromatin configuration in fully grown transgenic oocytes. DNA staining (DAPI, green); tubulin staining (red). The arrow points to the region of condensed chromatin that surrounds the nucleolus. (B) MII spindle formation following maturation in vitro for 18 h. The experiment was performed three times and similar results were obtained in each case; shown are representative images. For staining of GV-intact oocytes, 81 control and 93 transgenic oocytes were analyzed, respectively; the increased incidence of the NSN configuration is significant ($P < 0.02$). For staining of MII eggs, 65 control and 81 transgenic eggs were analyzed; the increased incidence of abnormal spindle formation is significant ($P < 0.005$).

increasing oocyte diameter. The SN-type oocytes display dramatically reduced levels of RNA polymerase I- and II-dependent transcription, while the NSN-type oocytes are actively transcribing (Bouniol-Baly et al., 1999; De La Fuente and Eppig, 2001). DAPI staining indicated a significantly higher percentage of NSN chromatin configurations in the fully grown transgenic oocytes. Most of the control oocytes (68%, $n = 81$) had a SN-type configuration, while only 35% of the transgenic oocytes ($n = 93$) displayed such a configuration (Fig. 5A). This suggests that the majority of the fully grown transgenic oocytes may not have developed properly and this would consequently impact negatively on their ability to undergo meiotic maturation and egg activation following fertilization.

To find out whether meiotic maturation occurs normally in transgenic oocytes, in vitro maturation (IVM) was carried out. Although all of the transgenic eggs contained condensed chromosomes, following 18 h of IVM 69% of them contained either an abnormal spindle, or a spindle that displayed markedly reduced tubulin staining. In contrast, only 2% of the control eggs displayed an abnormal spindle (Fig. 5B). The spindle effect was even more pronounced in the transgenic female founder that was analyzed, in which virtually all of the matured oocytes displayed condensed chromosomes but no visible spindle (data not shown); the increased severity of this phenotype was consistent with the greater reduction of MSY2 protein in these oocytes.

Protein synthesis and mRNA content in transgenic oocytes

We have proposed *Msy2* to be a global regulator of maternal mRNA stability (Yu et al., 2001, 2002, 2003). If such were the case, a reduction in the amount MSY2 protein would result in a decrease in both the amount of mRNA and protein synthesis in the fully grown oocyte. Quantification of total isolated poly(A) mRNA using RiboGreen indicated a decrease of approximately 25% in the transgenic fully grown oocytes when compared to non-transgenic oocytes (62.03 ± 4.46 pg/oocyte vs. 80 ± 5.69 pg/oocyte, where measurements were made on 10 transgenic and 8 non-transgenic samples; $P < 0.05$, t test). The value of 80 pg of poly (A) mRNA/oocyte we obtained for non-transgenic oocytes using this method is in excellent agreement with the value of 80 pg/oocyte previously reported (Bachvarova et al., 1985).

Transgenic oocytes and metaphase II-arrested eggs also displayed reduced levels of protein synthesis as evidenced by the decrease in the overall staining intensity following two-dimensional gel electrophoresis, consistent with the global reduction in the amount of mRNA (Fig. 6). Consistent with this finding is that incorporation of [^{35}S]methi-

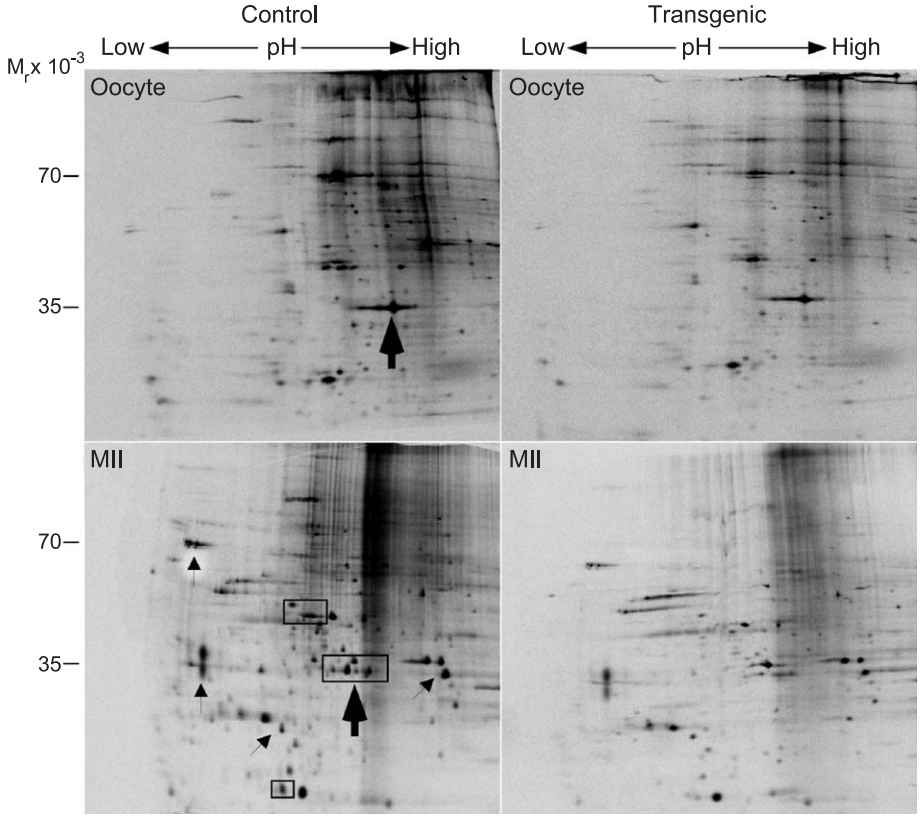

Fig. 6. Two-dimensional gel analysis of protein synthesis in control and *Msy2* transgenic oocytes and MII eggs. The arrows point to representative regions containing polypeptides that display the maturation-associated changes in both control and transgenic oocytes. The polypeptide(s) in the rectangles is representative of maturation-associated changes that do not occur in the transgenic oocytes. The thick arrow points to the region of the gel containing spindlin and reveals that the maturation-associated changes in spindlin do not occur in the transgenic oocytes.

nione into acid-insoluble material was reduced by approximately 55% in the transgenic oocytes, when compared to non-transgenic oocytes. Although the relative rate of synthesis of many polypeptides was reduced, that of others appeared unaffected in both oocytes and eggs. In addition, many of the maturation-associated changes in protein synthesis did not occur during maturation of transgenic oocytes, including spindlin (Oh et al., 1997). This failure may account for the reduced fertilization potential of the transgenic eggs.

Discussion

We report here that reducing the amount of MSY2 protein expression in oocytes by a transgenic RNAi approach results in a marked reduction in fertility that is likely attributable to the production of "low quality" eggs. Although these eggs can be inseminated, they do not undergo appropriate egg activation responses (e.g., intracellular Ca^{2+} oscillations, cell cycle resumption, cortical granule exocytosis, and ZP modifications) and hence arrest predominantly at the 1-cell stage. Consistent with our previous proposal that *Msy2* is a global regulator of maternal mRNA stability the amount of total mRNA is reduced approximately 20–25% in fully grown transgenic oocytes. This global reduction in maternal mRNA presumably is responsible for the altered morphological appearance of the oocytes, and the widespread decrease in protein synthesis and maturation-associated defects, for example, absence of several of the maturation-associated changes in protein synthesis and defective spindle formation. Of interest is that the maturation-associated changes in spindlin are not observed in the transgenic oocytes. Spindlin, which is a substrate for MAP kinase (Oh et al., 1998), associates with the spindle (Oh et al., 1997), and the failure of these maturation-associated changes in spindlin synthesis to occur may account, at least in part, for the abnormal spindles that develop during maturation of the transgenic oocytes.

We have previously used a transgenic RNAi approach to target *Mos* to demonstrate the feasibility of this approach and observed that we could phenocopy the *Mos* null mutant, that is, the eggs did not arrest at metaphase II but undergo spontaneous activation at a high frequency due to a failure to activate MAP kinase (Stein et al., 2003b). One reservation about the generality of this approach is that *Mos* mRNA is not translated until meiosis resumes in the fully grown oocyte (Gebauer and Richter, 1997). Thus, the RNAi mechanism would have the entire period of oocyte growth to target the endogenous *Mos* mRNA. The results presented here and elsewhere (Fedoriw et al., 2004) strongly suggest that this transgenic RNAi approach should be suitable to study the function of any gene in the oocyte, even genes that encode for abundantly expressed transcripts. MSY2 is one of the most abundant proteins in the oocyte, constituting approximately 2% of total oocyte protein (Yu et al., 2001). *Msy2* mRNA is likely to be a very abundant transcript because there is in general an excellent correspondence between amount of protein and mRNA abundance. In addition, MSY2 is synthesized throughout oocyte growth and its concentration remains essentially constant (Yu et al., 2001).

Oocytes (and preimplantation embryos) are ideally suited for the transgenic RNAi approach using long dsRNA. Although oocytes and preimplantation embryos possess the apoptotic machinery (Brison and Schultz, 1997; Jurisicova et al., 1998), long dsRNA does not elicit the apoptotic response observed in somatic cells due to inhibiting global protein synthesis (Svoboda et al., 2000). Nevertheless, developing transgenic oocytes are exposed to dsRNA for longer periods of time than previously used. Hence, it is formally possible that the *Msy2* dsRNA activated PKR, which in turn phosphorylated and activated eIF2α. dsRNA can also stimulate 2′–5′oligoA synthetase to produce a series of short 2′, 5′-oligoadenylates that activates RNase L which then cleaves single-stranded RNA (Clemens and Elia, 1997; Stark et al., 1998). Either or both of these processes would result in a decrease in protein synthesis and total mRNA abundance and lead to the observed deficiencies in oocyte maturation and fertilization. Several lines of evidence argue against this scenario. First, transgenic oocytes expressing *Mos* dsRNA or *Ctcf* dsRNA appear morphologically normal and are able to mature successfully to metaphase II (Fedoriw et al., 2004; Stein et al., 2003b). Results of immunoblotting experiments reveal that the concentration of PKR in oocytes (and blastocysts) is at most 3% that of somatic cells (Stein and Schultz, unpublished observations). Moreover, the concentration of eIF2α in oocytes is approximately 4% that of somatic cells and the same fraction of the active phosphorylated form of eIF2α is present in both transgenic and non-transgenic oocytes (Yu, Hecht, and Schultz, unpublished observations). Last, in the transgenic oocytes, we do not observe any substantial decrease in either *Msy1* or *Msy4*, implying that the RNase L-mediated mRNA degradation pathway is not activated. Likewise, in transgenic oocytes expressing either *Mos* or *Ctcf* dsRNA, there was no decrease in non-targeted mRNAs (Fedoriw et al., 2004; Stein et al., 2003b).

We obtained the same number of oocytes from transgenic and non-transgenic females and these oocytes were of similar diameter. Nevertheless, significantly fewer ovulated transgenic eggs were obtained. It is unlikely that faulty maturation of transgenic oocytes (e.g., high incidence of correct spindle formation and absence of many of the maturation-associated changes in protein synthesis) is the cause for the lower extent of ovulation. In *Cdc25b* knockout mice, oocytes fail to resume meiosis, but hormone stimulation results in similar numbers of ovulated GV-intact oocytes (Lincoln et al., 2002). Likewise, inhibiting the oocyte-specific cyclic nucleotide phosphodiesterase PDE-3 results in ovulation of a similar number of GV-intact oocytes (Wiersma et al., 1998). Defects in follicle

cell–oocyte communication, however, could be responsible. A growing body of evidence indicates that the oocyte and follicle cells communicate bi-directionally via both gap junctions and secretory mechanisms (Matzuk et al., 2002). Compromised oocyte development in the transgenic oocytes could result in follicles with a diminished response to hormonal stimulation.

The increased incidence of the NSN DNA chromatin configuration in transgenic oocytes may also reflect perturbations in oocyte–follicle cell bidirectional signaling. The SN DNA configuration is not observed when denuded oocytes isolated from 12-day-old mice are cultured for 4 days (De La Fuente and Eppig, 2001). In contrast, then oocyte–granulosa cell complex isolated from 12-day-old mice are cultured for 4 days, the SN staining pattern develops. Moreover, transcription remains elevated in the cultured denuded oocytes, whereas it displays a pronounced decrease when the oocytes are associated with the granulosa cells. If there is a direct linkage between formation of the SN DNA staining pattern and decreased transcription, transcription may continue at elevated levels in the transgenic oocytes. Such dysregulated transcription could contribute to the formation of fully grown oocytes with reduced meiotic and developmental competence. Last, the increased incidence of the NSN DNA configuration in the transgenic oocytes that is associated with a decreased ability to mature to metaphase II and then activate following insemination is similar to the reduced ability of fully grown oocytes that exhibit the NSN phenotype to complete maturation and become activated following Sr^{2+} treatment (Liu and Aoki, 2002). Current studies are addressing follicle cell–oocyte communication in the transgenic females.

The abundance of MSY2 protein to oocyte mRNA (a calculated molar ratio of about 70:1, (Yu et al., 2001)) and the concatameric properties of MSY2 (Yu et al., 2002) suggest that many of the oocyte mRNAs are bound by multimers of MSY2. The reduction of MSY2 protein levels by our transgenic RNAi approach creates a deficiency in MSY2 protein that drastically reduces fertility, while modestly reducing the total mRNA level. This reduction should lead to mRNAs lacking or with reduced amounts of MSY2 protein. To date, little is known of the MSY2 protein/mRNA ratios needed for individual mRNAs to maintain their stability. This reduction likely differentially affects mRNAs because similar but not identical patterns of protein synthesis are seen in control and transgenic eggs.

In summary, the results described here provide further support for *Msy2* as a global modulator of maternal mRNA stability and highlight the robustness of the transgenic RNAi approach to study gene function in oocyte development. Further analyses of knockout and additional knockdown lines of Msy2-deficient mice will allow a more detailed understanding of *Msy2* function during oocyte development.

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References

Bachvarova, R., De Leon, V., Johnson, A., Kaplan, G., Paynton, B.V., 1985. Changes in total RNA, polyadenylated RNA, and actin mRNA during meiotic maturation of mouse oocytes. Dev. Biol. 108, 325–331.

Bosher, J.M., Labouesse, M., 2000. RNA interference: genetic wand and genetic watchdog. Nat. Cell Biol. 2, E31–E36.

Bouniol-Baly, C., Hamraoui, L., Guibert, J., Beaujean, N., Szöllösi, M.S., Debey, P., 1999. Differential transcriptional activity associated with chromatin configuration in fully grown mouse germinal vesicle oocytes. Biol. Reprod. 60, 580–587.

Brison, D.R., Schultz, R.M., 1997. Apoptosis during mouse blastocyst formation: evidence for a role for survival factors including transforming growth factor α. Biol. Reprod. 56, 1088–1096.

Brower, P.T., Gizang, E., Boreen, S.M., Schultz, R.M., 1981. Biochemical studies of mammalian oogenesis: synthesis and stability of various classes of RNA during growth of the mouse oocyte in vitro. Dev. Biol. 86, 373–383.

Caplen, N.J., Zheng, Z., Falgout, B., Morgan, R.A., 2002. Inhibition of viral gene expression and replication in mosquito cells by dsRNA-triggered RNA interference. Molec. Ther. 6, 243–251.

Chatot, C.L., Ziomek, C.A., Bavister, B.D., Lewis, J.L., Torres, I., 1989. An improved culture medium supports development of random-bred 1-cell mouse embryos in vitro. J. Reprod. Fertil. 86, 679–688.

Chuang, C.F., Meyerowitz, E.M., 2000. Specific and heritable genetic interference by double-stranded RNA in Arabidopsis thaliana. Proc. Natl. Acad. Sci. U. S. A. 97, 4985–4990.

Clemens, M.J., Elia, A., 1997. The double-stranded RNA-dependent protein kinase PKR: structure and function. J. Interferon Cytokine Res. 17, 503–524.

Connors, S.A., Kanatsu-Shinohara, M., Schultz, R.M., Kopf, G.S., 1998. Involvement of the cytoskeleton in the movement of cortical granules during oocyte maturation, and cortical granule anchoring in mouse eggs. Dev. Biol. 200, 103–115.

Davies, H.G., Giorgini, F., Fajardo, M.A., Braun, R.E., 2000. A sequence-specific RNA binding complex expressed in murine germ cells contains MSY2 and MSY4. Dev. Biol. 221, 87–100.

De La Fuente, R., Eppig, J.J., 2001. Transcriptional activity of the mouse oocyte genome: companion granulosa cells modulate transcription and chromatin remodeling. Dev. Biol. 229, 224–236.

Eppig, J.J., 1977. Analysis of mouse oogenesis in vitro: oocyte isolation and utilization of exogenous energy sources by growing oocytes. J. Exp. Zool. 198, 375–382.

Fagard, M., Boutet, S., Morel, J.B., Bellini, C., Vaucheret, H., 2000. AGO1, QDE-2, and RDE-1 are related proteins required for post-transcriptional gene silencing in plants, quelling in fungi, and RNA interference in animals. Proc. Natl. Acad. Sci. U. S. A. 97, 11650–11654.

Fedoriw, A.M., Stein, P., Svoboda, P., Schultz, R.M., Bartolomei, M.S., 2004. Transgenic RNAi reveals essential function for CTCF in H19 gene imprinting. Science 303, 238–240.

Gebauer, F., Richter, J.D., 1997. Synthesis and function of Mos: the control switch of vertebrate oocyte meiosis. BioEssays 19, 23–28.

Giorgini, F., Davies, H.G., Braun, R.E., 2001. MSY2 and MSY4 bind a conserved sequence in the 3′untranslated region of protamine 1 mRNA in vitro and in vivo. Mol. Cell. Biol. 21, 7010–7019.

Hamada, M., Ohtsuka, T., Kawaida, R., Koizumi, M., Morita, K.,

Furukawa, H., Imanishi, T., Miyagishi, M., Taira, K., 2002. Effects on RNA interference in gene expression (RNAi) in cultured mammalian cells of mismatches and the introduction of chemical modifications at the 3′-ends of siRNAs. Antisense Nucleic Acid Drug Dev. 12, 301–309.

Hutvagner, G., Zamore, P.D., 2002. RNAi: nature abhors a double-strand. Curr. Opin. Genet. Dev. 12, 225–232.

Jurisicova, A., Latham, K.E., Casper, R.F., Varmuza, S.L., 1998. Expression and regulation of genes associated with cell death during murine preimplantation embryo development. Mol. Reprod. Dev. 51, 243–253.

Kennerdell, J.R., Carthew, R.W., 2000. Heritable gene silencing in *Drosophila* using double-stranded RNA. Nat. Biotechnol. 18, 896–898.

Kurasawa, S., Schultz, R.M., Kopf, G.S., 1989. Egg-induced modifications of the *Zona pellucida* of mouse eggs: effects of microinjected inositol 1,4,5-trisphosphate. Dev. Biol. 133, 295–304.

Lincoln, A.J., Wickramasinghe, D., Stein, P., Schultz, R.M., Palko, M.E., De Miguel, M.P., Tessarollo, L., Donovan, P.J., 2002. Cdc25b phosphatase is required for resumption of meiosis during oocyte maturation. Nat. Genet. 30, 446–449.

Liu, H., Aoki, F., 2002. Transcriptional activity associated with meiotic competence in fully grown mouse GV oocytes. Zygote 10, 327–332.

Lytton, J., Westlin, M., Hanley, M.R., 1991. Thapsigargin inhibits the sarcoplasmic or endoplasmic reticulum Ca-ATPase family of calcium pumps. J. Biol. Chem. 266, 17067–17071.

Matzuk, M.M., Burns, K.H., Viveiros, M.M., Eppig, J.J., 2002. Intercellular communication in the mammalian ovary: oocytes carry the conversation. Science 296, 2178–2180.

Moore, G.D., Kopf, G.S., Schultz, R.M., 1993. Complete mouse egg activation in the absence of sperm by stimulation of an exogenous G protein-coupled receptor. Dev. Biol. 159, 669–678.

Moos, J., Kalab, P., Kopf, G.S., Schultz, R.M., 1994. Rapid, nonradioactive, and quantitative method to analyze zona pellucida modifications in single mouse eggs. Mol. Reprod. Dev. 38, 91–93.

Oh, B., Hwang, S.-Y., Solter, D., Knowles, B.B., 1997. Spindlin, a major maternal transcript expressed in the mouse during the transition from oocyte to embryo. Development 124, 493–503.

Oh, B., Hampl, A., Eppig, J.J., Solter, D., Knowles, B.B., 1998. SPIN, a substrate in the MAP kinase pathway in mouse oocytes. Mol. Reprod. Dev. 50, 240–249.

Pal-Bhadra, M., Bhadra, U., Birchler, J.A., 2002. RNAi related mechanisms affect both transcriptional and posttranscriptional transgene silencing in Drosophila. Mol. Cell 9, 315–327.

Paynton, B.V., 1998. RNA-binding proteins in mouse oocytes and embryos: expression of genes encoding Y box, DEAD box RNA helicase, and polyA binding proteins. Dev. Genet. 23, 285–298.

Poueymirou, W.T., Schultz, R.M., 1987. Differential effects of activators of cAMP-dependent protein kinase and protein kinase C on cleavage of one-cell mouse embryos and protein synthesis and phosphorylation in one- and two-cell embryos. Dev. Biol. 121, 489–498.

Sagara, Y., Inesi, G., 1991. Inhibition of the sarcoplasmic reticulum Ca2+ transport ATPase by thapsigargin at subnanomolar concentrations. J. Biol. Chem. 266, 13503–13506.

Schultz, R.M., 1986. Oogenesis and the control of meiotic maturation. In: Pedersen, R., Rossant, J. (Eds.), Experimental Approaches to Mammalian Embryonic Development. Cambridge Univ. Press, New York, pp. 195–237.

Schultz, R.M., Kopf, G.S., 1995. Molecular basis of mammalian egg activation. Curr. Top. Dev. Biol. 30, 21–62.

Schwarz, D.S., Hutvágner, G., Haley, B., Zamore, P.D., 2002. Evidence that siRNAs function as guides, not primers, in the Drosophila and human RNAi pathways. Mol. Cell 10, 537–548.

Sijen, T., Plasterk, R.H.A., 2003. Transposon silencing in the *Caenorhabditis elegans* germ line by natural RNAi. Nature 426, 310–314.

Sommerville, J., 1999. Activities of cold-shock domain proteins in translation control. Bioessays 21, 319–325.

Stark, G.R., Kerr, I.M., Williams, B.R., Silverman, R.H., Schreiber, R.D., 1998. How cells respond to interferons. Annu. Rev. Biochem. 67, 227–264.

Stein, P., Svoboda, P., Anger, M., Schultz, R.M., 2003a. RNAi: mammalian oocytes do it without RNA-dependent RNA polymerase. RNA 9, 187–192.

Stein, P., Svoboda, P., Schultz, R.M., 2003b. Transgenic RNAi in mouse oocytes: a simple and fast approach to study gene function. Dev. Biol. 256, 187–193.

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

Svoboda, P., Stein, P., Schultz, R.M., 2001. RNAi in mouse oocytes and preimplantation embryos: effectiveness of hairpin dsRNA. Biochem. Biophys. Res. Commun. 287, 1099–1104.

Tabara, H., Sarkissian, M., Kelly, W.G., Fleenor, J., Grishok, A., Timmons, L., Fire, A., Mello, C.C., 1999. The rde-1 gene, RNA interference, and transposon silencing in *C. elegans*. Cell 99, 123–132.

Tavernarakis, N., Wang, S.L., Dorovkov, M., Ryazanov, A., Driscoll, M., 2000. Heritable and inducible genetic interference by double-stranded RNA encoded by transgenes. Nat. Cell Biol. 2, 180–183.

Temeles, G.L., Ram, P.T., Rothstein, J.L., Schultz, R.M., 1994. Expression patterns of novel genes during mouse preimplantation embryogenesis. Mol. Reprod. Dev. 37, 121–129.

Thastrup, O., Cullen, P.J., Drobak, B.K., Hanley, M.R., Dawson, A.P., 1990. Thapsigargin, a tumor promoter, discharges intracellular Ca^{2+} stores by specific inhibition of the endoplasmic reticulum $Ca^{2(+)}$-ATPase. Proc. Natl. Acad. Sci. U. S. A. 87, 2466–2470.

Wassarman, P.M., Jovine, L., Litscher, E.S., 2001. A profile of fertilization in mammals. Nat. Cell Biol. 3, E59–E64.

Whitten, W.K., 1971. Nutrient requirements for the culture of preimplantation mouse embryo in vitro. Adv. Biosci. 6, 129–139.

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

Wiersma, A., Hirsch, B., Tsafriri, A., Hanssen, R.G., Van de Kant, M., Kloosterboer, H.J., Conti, M., Hsueh, A.J., 1998. Phosphodiesterase 3 inhibitors suppress oocyte maturation and consequent pregnancy without affecting ovulation and cyclicity in rodents. J. Clin. Invest. 102, 532–537.

Wolffe, A.P., 1994. Structural and functional properties of the evolutionarily ancient Y-box family of nucleic acid binding proteins. Bioessays 16, 245–251.

Yu, J., Hecht, N.B., Schultz, R.M., 2001. Expression of MSY2 in mouse oocytes and preimplantation embryos. Biol. Reprod. 65, 1260–1270.

Yu, J., Hecht, N.B., Schultz, R.M., 2002. RNA-binding properties and translation repression in vitro by germ cell-specific MSY2 protein. Biol. Reprod. 67, 1093–1098.

Yu, J., Hecht, N.B., Schultz, R.M., 2003. Requirement for RNA-binding activity of MSY2 for cytoplasmic localization and retention in mouse oocytes. Dev. Biol. 255, 249–262.