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Mouse Preimplantation Embryos Developed from Oocytes Injected with Round Spermatids or Spermatozoa Have Similar but Distinct Patterns of Early Messenger RNA Expression[1]

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ABSTRACT

Quantitative real-time polymerase chain reaction assay was used to compare the temporal transcriptional activation and mRNA removal for a number of genes in mouse embryos derived by round spermatid injection (ROSI) or intracytoplasmic sperm injection. A number of marker genes with widely different cellular functions were analyzed. Similar patterns of activation were found for the transcription factor Oct 4, the translation initiation factor eukaryotic initiation factor 1A, the L1 ribosomal protein, the chromatin modifying protein histone deacetylase 1, the enzyme hypoxanthine phosphoribosyl transferase, the murine endogenous retrovirus-like element, and the repetitive DNA LINE retrotransposons. Expression of the retrovirus-like mobile element intracisternal A particle, however, was markedly elevated from the two-cell to the blastocyst stages in ROSI embryos. Analyses performed for various paternal mRNAs introduced into the oocyte by the round spermatid, including protamines 1 and 2, transition protein 2, ropporin, and glyceraldehydes 3-phosphate dehydrogenase, revealed all were removed from the preimplantation embryos, albeit with distinct temporal patterns.

assisted reproductive technology, early development, embryo, gene regulation, sperm

INTRODUCTION

Among the available assisted reproduction technologies for humans, round spermatid injection (ROSI) into oocytes has produced disappointing clinical outcomes (reviewed in [1]). Compared with intracytoplasmic sperm injection (ICSI), ROSI produces more arrested embryos consistent with increased rates of developmental failure [2, 3]. Few ROSI embryos reach the blastocyst stage, and often these blastocysts do not implant [4]. Many factors may contribute to the lower efficiency of ROSI in humans, including 1) the difficulty associated with choosing a round spermatid from a limited number of small and difficult to identify round cells, 2) improper oocyte activation by the round spermatid, 3) a deficiency in the maturation of essential structures such as the centrosome in the round spermatid, and 4) the nonoptimal physiological state of the chosen round spermatid (ROSI often is performed with round spermatids obtained from azoospermic patients). Questions on possible differences between the functional state of the male genome in spermatids and spermatozoa have raised numerous concerns about the safety of this technique as a procedure for assisted human reproduction.

A more detailed base of knowledge of the molecular events that occur in early embryos derived by ROSI may alleviate some of the concerns raised by investigators for the application of ROSI to humans. The production of ROSI offspring in species such as the mouse and rabbit offers useful model systems for examining many of the parameters required for the successful utilization of this technique in humans [1]. Normal-appearing mice have been routinely produced by ROSI using round spermatids from both normal and infertile animals [5], indicating that in mice the postmeiotic differentiative events where spermatids mature to spermatozoa are not essential for normal embryonic development to term. Moreover, a sensitive technique for monitoring genomic differences, the single nucleotide primer extension assay, has demonstrated that the relative expression of maternal and paternal imprinted genes does not differ between ROSI embryos and control embryos [6]. Similarly, the deletion of spermiogenesis and prefertilization events in the male gamete did not lead to any significant deficiencies in fertility, learning, or behavior in fifth generation mice that were produced exclusively by ROSI [7]. By these criteria, evidence is accumulating that in the mouse system, ROSI can be a valuable assisted reproduction technique. However, even in laboratories with high levels of expertise, the efficiency of ROSI compared with that of ICSI is substantially reduced. Although this reduced efficiency may be in part due to technical difficulties, it may also be due to physiological differences in embryos resulting from differences in the activation patterns of the genomes of the round spermatid or spermatozoon. Alternatively, the round spermatid may be a more trouble-prone source of the paternal genome at fertilization because the introduction of the round spermatid into oocytes requires rapid inactivation and removal of many ''foreign'' mRNAs normally under precise temporal control in the round spermatid. The ectopic expression of the many proteins encoded by round spermatid mRNAs, such as the protamines, would be highly deleterious to normal cellular function.

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In this study, we used real-time reverse transcription polymerase chain reaction (RT-PCR), a quantitative method to measure mRNA levels, to compare the temporal activation pattern of a number of genes normally expressed in the preimplantation embryo in ICSI and ROSI embryos [8–12]. For many of the genes examined, the patterns of mRNA expression were similar in the ICSI or ROSI embryos. However, a marked increase in mRNA expression was found for the transposable element intracisternal A particle in ROSI embryos. In addition, we followed the fate of a number of abundant and normally highly stable paternal mRNAs from time of injection to their disappearance in the early embryo.

MATERIALS AND METHODS

Animals

B6D2F1 mice (C57BL/6 × DBA/2) were used as donors of spermatozoa and oocytes. Female and male donors were 3–4 and 3–6 mo old, respectively. Mature CD-1 females were used as surrogate mothers. All animals were kept in temperature- and light-controlled rooms. with 14L: 10D (lights-on 0700 h to 2100 h) in accordance with the guideline of the Laboratory Animal Services at the University of Hawaii and the *Guide for the Care and Use of Laboratory Animals* (National Academy of Sciences, 1996).

Reagents

Polyvinyl alcohol (PVA, cold water soluble, molecular weight about 10 000) and polyvinyl pyrrolidone (PVP, molecular weight about 360 000) were purchased from Sigma Chemical Co. (St. Louis, MO). Bovine testicular hyaluronidase (200 USP U/mg) was purchased from ICN Biochemicals (Costa Mesa, CA). BSA (fraction V) was purchased from Calbiochem (La Jolla, CA), and mineral oil was from Squibb and Sons (Princeton, NJ). All other reagents were obtained from Sigma unless specifically stated.

Media

CZB medium [13] supplemented with 5.56 mM glucose and 4 mg/ml BSA was used for the culture of mouse oocytes after microsurgery. The medium for collection of oocytes from oviducts and subsequent oocyte treatments, including micromanipulation, was a modified CZB (Hepes-CZB) [14] containing 20 mM Hepes-HCl, a reduced amount of $NaHCO_3$ (5 mM), and 0.1 mg/ml PVA instead of BSA. CZB was used under Hepes-$HC5O_2$ in air, and Hepes-CZB was used under air.

Preparation of Oocytes, Spermatozoa, and Spermatids

Oocytes, spermatozoa, and spermatids were collected and prepared for ICSI and ROSI according to the method of Kimura and Yanagimachi [14, 15]. Oocytes were collected from oviducts of superovulated females soon after ovulation, freed from cumulus cells by hyaluronidase treatment, and kept in CZB medium for <1 h. Spermatozoa from the cauda epididymis were allowed to disperse in Hepes-CBZ for 5–10 min before being suspended in Hepes-CZB containing 6% PVP. This sperm suspension was kept under mineral oil for up to 1 h. Round spermatids, characterized by a centrally located chromatin mass, were collected from the testes and kept in Hepes-CZB with 6% PVP for <1 h before being injected into oocytes.

ICSI, ROSI, and Assessment Of Fertilization

ICSI and ROSI were performed according to the method of Kimura and Yanagimachi [14, 15] except for being performed at room temperature (about 25°C). For ROSI, oocytes were activated first by 30-min treatment (at 37°C) with Ca-free CZB containing 10 mM $SrCl_2$.

Activated oocytes were rinsed and kept in CZB for 15 min (37°C) before injection of round spermatids. ICSI oocytes do not need to be activated because the spermatozoon itself has the ability to activate a mature oocyte. Oocyte activation by Sr^{2+} may not be quite normal, but Sr^{2+} is the only known parthenogenic agent that causes repetitive intracellular Ca^{2+} oscillations in oocytes during activation, as the native sperm factor does. ICSI and ROSI were performed at room temperature in Hepes-CZB within 30 min after spermatozoa and spermatids were suspended in PVP-containing Hepes-CZB. Between 5 and 6 h after ICSI or ROSI, the oocytes were examined under a dissecting microscope, and those with distinct signs of cytolysis were discarded. An oocyte with two distinct pronuclei and the second polar body was recorded as normally fertilized.

Embryo Development and Embryo Transfer to Surrogate Mothers

Normally fertilized oocytes were allowed to develop in CZB at 37°C. Those reaching specific developmental stages (from the one-cell stage to the blastocyst stage) were collected and frozen, and RNA was extracted. In a series of experiments, ICSI and ROSI embryos at the two-cell stage were transferred to the oviducts of pseudopregnant CD-1 females that had been mated during the previous night with vasectomized males of the same strain. Females were killed on Day 19.5 of pregnancy and examined for implantation sites. Live fetuses, if any, were raised by lactating CD-1 foster mothers.

Quantitative Real-Time RT-PCR

Total RNA was isolated from the ICSI and ROSI embryos using an RNA Microprep Kit (Stratagene, La Jolla, CA). To each sample, 0.03 pg of transcribed rabbit α-globin mRNA/embryo was added prior to RNA extraction to normalize for RNA extraction and RT efficiencies. The RNA preparations were incubated with RNase-free DNase for 15 min at 37°C, and RT was performed with the RETROscript Kit (Ambion, Austin, TX). The cDNA products were diluted to a final concentration of 1 μl/embryo in sterile water, and 1-μl aliquots, representing the amount of cDNA obtained from one oocyte or one embryo, and were subjected to quantitative real-time RT-PCR analysis.

PCR primer pairs and probes (see Table 1) were designed with Primer Express 1.5 software, which accompanies the Model 7700 sequence detector (PE Applied Biosystems, Foster City, CA). Probes were designed to overlap an intron, whenever possible. Before probes were synthesized, primer sets were tested to verify the amplification of a single band of the correct predicted size. TaqMan probes (PE Applied Biosystems) were labeled with 6-carboxy-fluorescein as a 5′ reporter dye and 6-carboxy-tetramethyl-rhodamine as a 3′ quencher.

Each target amplicon was cloned into a PCR-II TOPO cloning vector (Invitrogen, Carlsbad, CA), and these plasmids were used to generate a standard curve for each target gene. A 6-point standard curve for each target gene was prepared by serially diluting the plasmid DNA over 6 orders of magnitude (10 pg to 0.001 pg). Each point of the standard curve was run in triplicate at the same time that the experimental PCRs were completed in duplicate (duplicates of experimental samples were completed to conserve material). To normalize the data with respect to RNA extraction and RT efficiencies, all of the data were normalized to the exogenous reference rabbit α-globin mRNA added to each sample. The relative *x*-fold differences of gene expression among the developmental stages assayed in ICSI and ROSI samples was determined by setting the expression level of injected oocytes (collected immediately after injection) at the arbitrary value of 1, with the exception of eukaryotic initiation factor 1A (elF-lA) mRNA, which is not detectable until the middle of the two-cell embryonic stage; *x*-fold differences for eIF-1A were expressed relative to this stage.

RESULTS

Embryo Developmental Rates after ROSI and ICSI

Control injections were performed with spermatozoa and round spermatids (Table 2). Of the 183 oocytes injected with sperm, 164 (87.6%) survived the injection and 161 (98.2%) of these injected eggs were normally fertilized as assessed by the criterion of two equal-size pronuclei. One hundred sixty eggs (97.6%) reached the two-cell stage and 135 eggs (82.3%) became blastocysts. Of 181 oocytes injected with round spermatids, 157 (86.7%) survived the injections and 142 (90.4%) surviving oocytes appeared normally fertilized, as determined by the presence of two normal-size pronuclei and the second polar body. One hundred forty-two (90.4%) eggs developed to the two-cell stage, and 69 eggs (43.9%) developed to the blastocyst. Similar developmental survival rates were seen for four additional groups of ICSI and ROSI embryos that were analyzed.

TABLE 1. Primers and probes used for quantitave RT-PCR.

Gene (accession no.)	Primer/probe	Sequence	Nucleotide position
α-globin (J00658)	Forward primer	GCCTCCCTGGACAAGTTCCT	406–425
	Reverse primer	GGCTCCAGCTTAACGATATTTGG	452–474
	Probe	CCAACGTGAGCACCGTGCTGACC	428–450
Oct 4 (X52437)	Forward primer	CAACTCCCGAGGAGTCCCA	318–336
	Reverse primer	GCTTCAGCAGCTTGGCAAAC	369–389
	Probe	CATGAAAGCCCTGCAGAAGGAGCTAGAAC	340–368
HPRT (NM_013556)	Forward primer	AAGACTTGCTCGAGATGTCATGA	228–251
	Reverse primer	AAAGAACTTATAGCCCCCCTTGA	289–312
	Probe	CCATCACATTGTGGCCCTCTGTGTG	264–287
elF-1A (AF026481)	Forward primer	GGACGGTTGGAAGCAATGTG	1305–1324
	Reverse primer	ACCTTCTTTCTCAGCTTCCCTCTTAT	1353–1378
	Probe	ACGGTGTGAGGAGGCTGTGCCA	1330–1351
Ribosomal protein L1 (NM_053158)	Forward primer	CTGAATCTGGGTCCGTTTGTG	869–889
	Reverse primer	GCAATGAGTCAGTCTTCAGTAGTAAACC	923–950
	Probe	CCGTGCCTTCCTTCGTAGTTCAACCA	892–917
HDAC1 (NM_008228)	Forward primer	TGCTCGCTGCTGGACTTAC	941–959
	Reverse primer	GTAGGGCAGCTCATTAGGGATCT	985–1007
	Probe	AACAGCGGTGGCCCTGGACACA	962–983
IAP (NM_010490)	Forward primer	TGCTAATTTTACCTTGGTGCAGTTA	753–777
	Reverse primer	GTTTGCCAGTCAGCAGGAGTTA	800–821
	Probe	ACAGGCTCGCCGGCATGGC	779–797
ERV-L (Y12713)	Forward primer	GGAATGAAGGTATGGGTCAATCC	5680–5702
	Reverse primer	CCTTCACCTTCAGCCAGCAC	5731–5750
	Probe	CCAGGAAAAGAGCCAAGACCTGCTGA	5704–5729
LINE 1 (D84391)	Forward primer	ATGGACCATGTAGAGACTGCCA	6547–6568
	Reverse primer	CAATGGTGTCAGCGTTTGGA	6597–6616
	Probe	TCCAGGGATCCACCCCATAATCAGC	6571–6595
Protamine 2 (NM_008933)	Forward primer	GAATAGTCACCTGCCCAAGCA	477–497
	Reverse primer	GCAGCTCAGGGCTCAGACA	533–551
	Probe	AGGCCACACCACCATTCCATGTCG	508–531
TP2 (M60254)	Forward primer	CTGCCCCAAGAACAGGAAGA	1044–1063
	Reverse primer	CCGTTTCCGCCTCCTGA	1097–1113
	Probe	TTGAAGGGAAAGTGAGCAAGAGAAAGGCC	1067–1095
Gapds (NM_008085)	Forward primer	AGGACCAGGTGGTCTCCACG	1139–1158
	Reverse primer	GAGGGCAATTCCAGCCTTA	1197–1215
	Probe	ACGGCAATCCCCATTCTTCCATCTTTG	1166–1192
Protamine 1 (X14003)	Forward primer	AGGTGTAAAAAATACTAGATGCACAGAATAG	231–261
	Reverse primer	TTCAAGATGTGGCGAGATGCT	307–327
	Probe	AAACTCCTGCGTGAGAATTTTACCAGACTTCAA	273–305
Ropporin (AF178531)	Forward primer	AGGTCCTGCATTCTCGGGTT	218–237
	Reverse primer	AAGCGACCCACGTTCATCAC	307–338
	Probe	CTGGCAGACTGATCATCCACGCAGA	239–263

ROSI and ICSI Embryos Develop Normally

To assess whether the injected eggs are capable of normal development, two-cell embryos from ROSI and ICSI injections were transferred into oviducts (Table 3). For the ROSI injections, 163 two-cell embryos were transferred into 10 recipients over four replicates. Forty-nine (30.1%) embryos implanted, and 40 (21.5%) live pups were obtained. For the ICSI control injections, 143 two-cell embryos were transferred into nine recipients over three replicates. Sixty-nine (48.2%) embryos implanted, and 57 (39.8%) live pups were obtained.

Messenger RNA Activation in Preimplantation Embryos

To compare the activation of mRNA expression in ICSI and ROSI embryos, we isolated RNA from the one-cell to the blastula stages (see Table 4) and quantified the amount of transcript for each of the marker genes selected because they show known specific patterns of activation in mouse preimplantation embryos. The following group of marker genes with widely different cellular functions was chosen for analysis: Oct 4, a transcription factor with a known biphasic expression pattern during early development [16]; eIF-1A (previously named eIF-4C), a translation initiation factor whose transient expression initiates in early two-cell embryos [9]; histone deacetylase 1 (HDAC-1), a chromatin-modifying protein linked to the reprogramming of gene expression during preimplantation development [10]; the L1 ribosomal protein; and a constitutive enzyme, hypoxanthine phosphoribosyltransferase (HPRT), expressed in preimplantation embryos [12]. In addition, three endogeneous murine virus-like particles were analyzed: intracisternal A particle (IAP), an abundant transposable element [17]; ERV-L, an endogenous murine retrovirus [18]; and LINE-1, a repeti-

TABLE 2. In vitro development of ICSI and ROSI embryos.

Method	Oocytes (no. experiments)	No. (%) oocytes surviving injection	No. (%) oocytes normally fertilized	No. (%) embryos developed			
				2-cell	4-cell	Morula	Blastocyst
ICSI	183 (4)	164 (89.6)	161 (98.2)	160 (97.6)	160 (97.6)	148 (90.2)	135 (82.3)
ROSI	181 (5)	157 (86.7)	142 (90.4)	142 (90.4)	135 (86.0)	110 (70.0)	69 (43.9)

TABLE 3. Offspring from ICSI and ROSI embryos.

Method	No. recipients (No. experiments)	No. 2-cell embryos transferred into oviducts	No. (%) implants	No. (%) live pups
ICSI	9 (3)	143	69 (48.2)	57 (39.8)
ROSI	10 (4)	163	49 (30.1)	40 (21.5)

tive DNA family member found in all mammalian genomes [19].

Similar patterns of temporal mRNA expression were observed for ICSI and ROSI embryos for most of the genes assayed, including Oct 4, eIF-1A, the ribosomal protein L1, HDAC-1, HPRT, ERV-L, and LINE-1 (Fig. 1). However, the retrovirus-like mobile element, IAP, reproducibly showed an elevated pattern of mRNA activation in ROSI embryos. For IAP, increases in mRNA were seen in ROSI embryos starting at the mid two-cell stage and continuing to the blastocyst stage. The relative amounts of IAP in one representative experiment are presented in Figure 1. The differences for IAP in ROSI and ICSI embryos were significant (paired *t*-test, $P < 0.0005$). The relative expression levels of IAP mRNA in different stages of preimplantation embryos from three batches of ROSI and ICSI samples were normalized to the exogenous reference α-globin mRNA to exclude any effects due to RT efficiencies and input differences.

Pattern of Loss of Round Spermatid mRNA Differs for Each mRNA

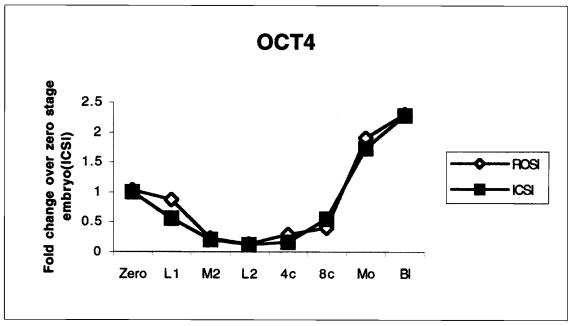
In addition to the mRNA upregulation of specific genes following fertilization, the introduction of round spermatids into oocytes necessitates a major inactivation of ''foreign'' mRNAs. The foreign mRNAs in the cytoplasm of the male gamete we examined are not normally expressed during embryogenesis. To examine the temporal pattern of the removal of such mRNAs, we quantified the amount of transcript for several mRNAs known to be at high levels in round spermatids following injection to the blastocyst stage. These transcripts included genes encoding structural proteins of the maturing spermatid and spermatozoon, including the transition proteins, the protamines, and two fibrous sheath proteins, glyceraldehydes 3-phosphate dehydrogenase (Gapds) and ropporin. Transition protein 2 and the protamines 1 and 2 are DNA-binding proteins whose mRNAs are normally stored for 3–7 days in the cytoplasm of maturing spermatids [20]. Gapds and ropporin are two components of the fibrous sheath whose mRNAs are also stored in the cytoplasm of spermatids before translation [21–23]. These ectopic mRNAs introduced into eggs by ROSI are removed from embryos following fertilization with similar but distinct temporal patterns of degradation (Fig. 2). The very abundant protamine 1 and transition protein 2 mRNAs were undetectable by the late two-cell stage, whereas the less abundant Gapds and ropporin mRNAs were still detectable at the late two-cell stage. Low levels of protamine 2 mRNA were detected in the ICSI embryos, revealing the sensitivity of the real-time PCR assays, in agreement with the very low levels of these mRNAs associated with spermatozoa [24].

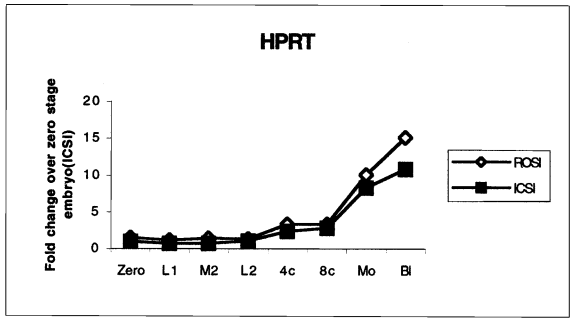
DISCUSSION

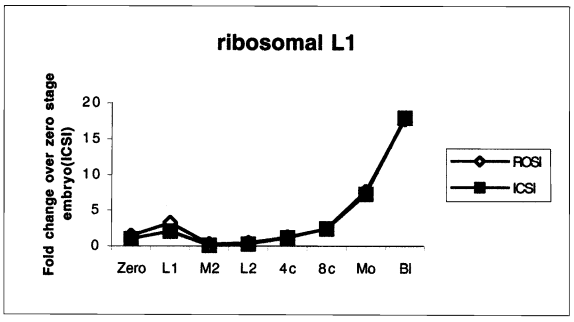
To gain insight into differences in genomic programming in preimplantation embryos derived by ROSI or ICSI that could influence the success rates for these procedures in assisted-reproduction laboratories, we quantitatively measured mRNA levels for a group of genes activated soon after fertilization. Our findings were both predictable and surprising. Consistent with the normal development of ROSI mice, the mRNA activation patterns of most of the genes we assayed, including the transcription factor Oct 4, the translation factor eIF-1A, the L1 ribosomal protein, HDAC-1, HPRT, EVR-L, and LINE-1, were similar in ROSI and ICSI embryos at least up to the blastocyst stage of development. The relative temporal patterns of mRNA expression we detected are in agreement with published reports of investigations using RT-PCR with embryos obtained by matings [9, 10, 12, 18]. IAP retrotransposon expression was substantially higher in ROSI embryos than in ICSI embryos. IAPs are endogenous retrovirus-like mobile elements that are present at about 1000 copies in the mouse genome [17]. They are differentially expressed in tissues and show stage-specific expression in normal mouse preimplantation embryos. IAPs are expressed at high levels in certain neoplastic tissues, and insertions of IAPs have created a number of coat color mutations at the agouti locus in mice [25]. Because these elements transpose in a replicative fashion by RT, their transposition is closely regulated by their transcription level [26]. Whether the upregulated

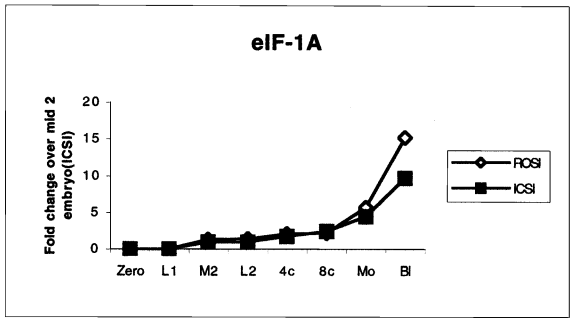
TABLE 4. Numbers of oocytes and embryos (2-cell to blastocysts) used for isolation of total RNA.

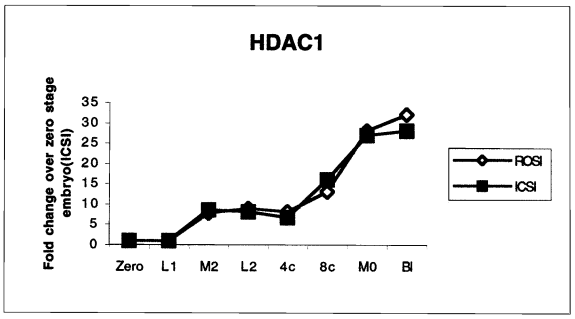
Method	Stage of embryo development	Time after injections (h)	No. injected oocytes	No. oocytes surviving injection	No. eggs fertilized	No. embryos developed				
						2-cell	4-cell	8-cell	Morula	Blastocyst
ICSI	Unfertilized	0	93	80						
	Late 1-cell	14	143	102	99					
	Mid 2-cell	31	110	94	93	92				
	Late 2-cell	37	143	96	92	89				
	4-cell	46	152	120	119		110			
	8-cell	57	135	122	117			97		
	Morula	70	56	46	46				41	
	Blastocyst	94	37	35	34					28
ROSI	Unfertilized	0	74	70						
	Late 1-cell	14	191	177	167					
	Mid 2-cell	31	162	147	138	138				
	Late 2-cell	37	186	153	138	137				
	4-cell	46	172	154	139		131			
	8-cell	57	181	156	145			130		
	Morula	70	58	53	53				30	
	Blastocyst	94	65	59	55					25

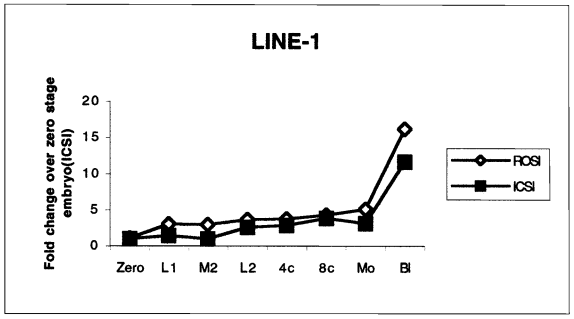


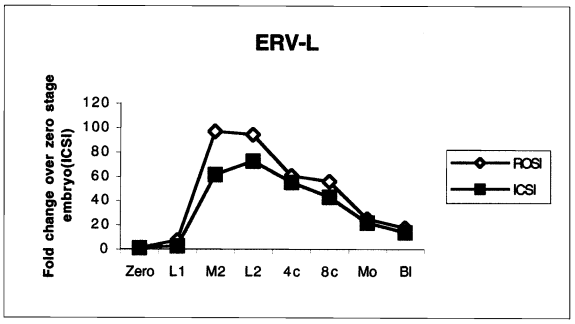


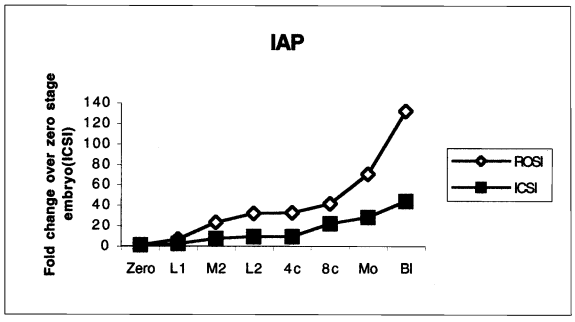












	Zero	L1	M2	L2	4C	8C	Mo	Bl
ROSI	1.6	6.6	23.0	32.3	32.9	41.1	70.3	131.9
ICSI	1	2.5	7.6	9.2	9.6	21.9	28.1	44.0

FIG. 1. Quantitation by real-time PCR of upregulated mRNAs in ICSI and ROSI embryos. Relative *x*-fold differences are shown following fertilization by setting the mRNA value of the zero-time oocyte at an arbitrary value of 1. The curves are representative of at least three assays of samples prepared from three different injections.

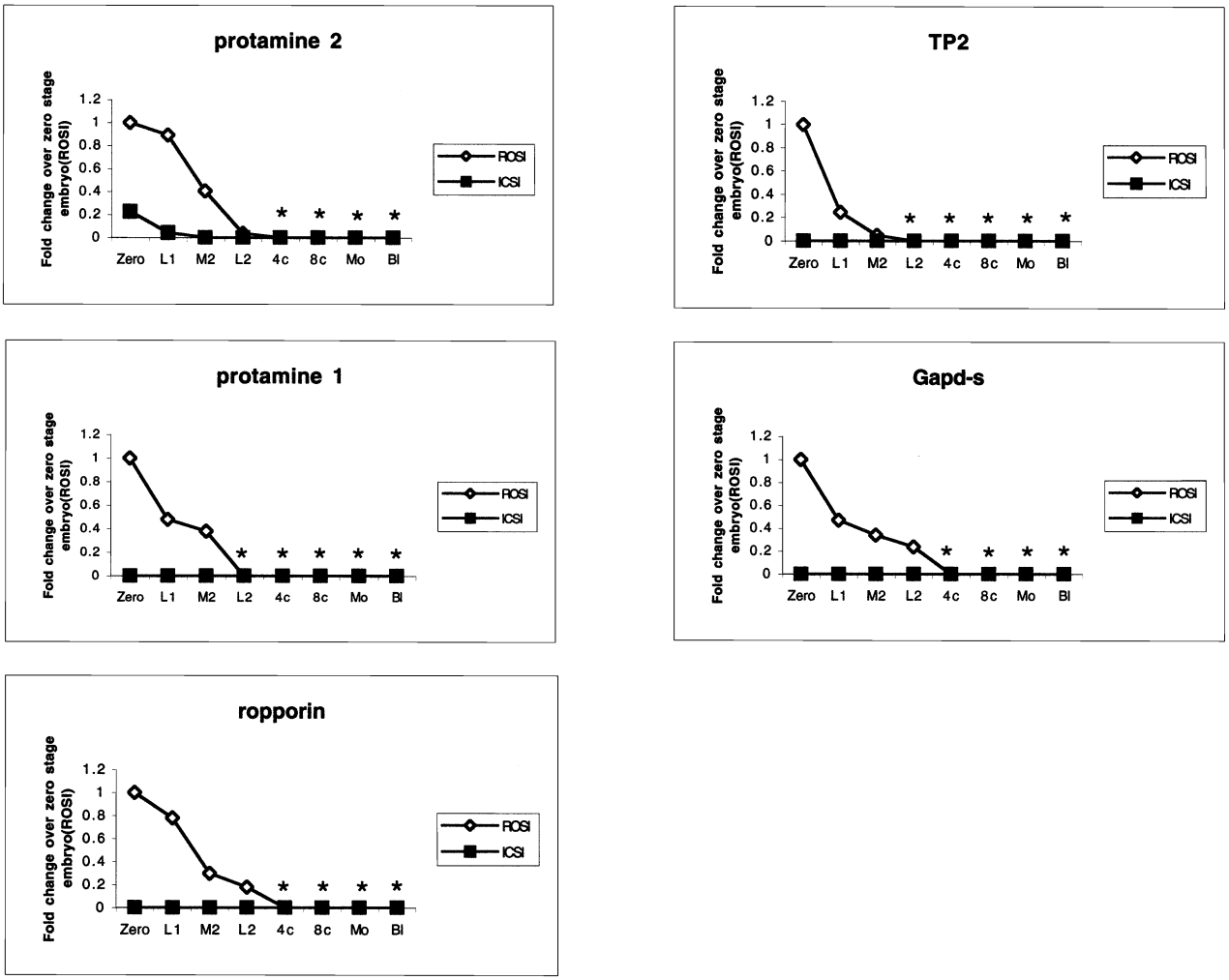


FIG. 2. Quantitation by real-time PCR of round spermatid mRNA levels in ICSI and ROSI embryos. The relative changes are presented as described in Figure 1. Asterisks denote nondetectable levels.

transcription of IAP is deleterious to the developing embryos and could contribute to the reduced number of offspring obtained by ROSI needs to be established because human embryos also express endogenous retroviruses [27]. The apparent upregulation of IAP transcription appears specific to IAP; both ROSI and ICSI embryos expressed similar levels of the endogenous retrovirus-like particle ERV-L and the LINE 1 transposable elements.

The introduction of round spermatids into oocytes requires inactivation of a number of male germ cell mRNAs in the developing embryos. In contrast to spermatozoa, round spermatids are actively transcribing and storing large amounts of different mRNAs, including mRNAs encoding the male germ cell-specific chromatin proteins, transition proteins, protamines 1 and 2, and components of the fibrous sheath such as Gapds and ropporin. These mRNAs are abundant, with estimates of up to 20 000 copies/round spermatid for the protamine 1 mRNA [28]. Thus, in contrast to ICSI embryos where the spermatozoon contributes at most small amounts of select mRNAs to the embryo, ROSI introduces a large number of transcripts whose translation is normally repressed and temporally regulated during spermiogenesis. As has been reported previously for Hsp 70.1, Smcy, protamine 2, Ube1Y, and Ube 1X [29], the round spermatid mRNAs we examined are not stabilized in the embryos but are rapidly lost. Nested RT-PCR revealed that protamine 2 transcripts disappear as early as 5 h after fertilization [29]. Similarly, protamine 2 mRNA is rapidly removed from embryos. Our detection of protamine 2 mRNA up to the mid two-cell stage embryos likely reflects the increased sensitivity of mRNA detection in our experiments. The temporal degradation patterns differ among the five mRNAs we examined. The protamine 2 and transition protein 2 mRNAs disappeared before the Gapds and rapporin mRNAs. These different patterns of mRNA removal likely reflect sequence and secondary structure differences of these ectopic mRNAs rather than abundance because quantitation of mRNA copy number using serially diluted plasmids indicates the protamine mRNAs are 3- to 10-fold more abundant than the Gapds and rapporin mRNAs.

The normal stability of the transition protein 2 and protamine 1 and 2 mRNAs is 3–7 days in the cytoplasm of the differentiating spermatids in the testis; however, these mRNAs are degraded rapidly in embryos. In the cytoplasm of round spermatids, many mRNAs undergo translation delays; transcription ceases in mid-spermiogenesis but protein synthesis is essential throughout spermiogenesis [20]. Most likely, the egg and embryo cytoplasm lack the equivalent translation repressing and/or stabilizing factors used by spermatids to regulate germ cell-specific mRNAs. Such factors probably are not normally expressed in somatic cells or during embryogenesis. For instance, the protamine

mRNAs need to be quickly removed from the embryo to avoid their translation, because they encode ''dangerous'' highly basic germ cell-specific DNA-binding proteins, e.g., precocious expression of protamine 1 in male germ cells leads to spermatogenic arrest [30]. Any delays in the removal of round spermatid mRNAs could allow their translation, and these ectopically expressed proteins may contribute to the developmental arrest seen frequently with ROSI. The removal of foreign mRNAs in the embryo may be an active process, and physiologically suboptimal oocytes may lack the ability to rapidly remove such mRNAs from their cytoplasm.

ROSI and ICSI embryos show similar qualitative and quantitative patterns for mRNA upregulation and for a number of essential early embryonic genes. These data are derived from surviving embryos and are likely to be markedly different in embryos whose development is arrested. ROSI oocytes rapidly degrade the ectopic mRNAs introduced by the round spermatids, albeit with different kinetics. Considering the success of ROSI and ICSI, these findings are expected. However, the overexpression of an endogeneous retrovirus, the IAP, in ROSI embryos starting at the two-cell stage and continuing to the blastocyst is puzzling and difficult to interpret. The biological effects of IAPs expressed in normal mouse embryos or of similar widely expressed RNA intermediate retroelements in other eukaryotes such as humans are unknown. The high levels of expression of IAPs in certain neoplastic tissues and the capacity of IAPs to create mutation by genomic insertion suggest that more detailed analyses of parameters such as mutation rates in ROSI embryos would be worthwhile.

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