

Differential gene expression in pre-implantation embryos from mouse oocytes injected with round spermatids or spermatozoa

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BACKGROUND: The use of immature male germ cells to fertilize human oocytes raises several questions. Spermatozoa are normally quiescent, but many genes are transcribed post-meiotically in round spermatids. This creates a novel situation for the oocyte. We have therefore explored the effects on early embryonic development of introducing a fully transcriptionally active round spermatid into the oocyte. **METHODS AND RESULTS:** Following the micro-injection of spermatozoa or spermatids into mouse oocytes we have analysed the expression, at various times, of six genes in the resulting embryo. Spermatozoa and spermatids produced similar fertilization rates. Hprt was expressed in all embryos at all stages tested. Hsp70.1 was found normally during the 2-cell stage and repressed by the 4-cell stage in embryos from both spermatozoa and round spermatids. However, the amplitude of the signal was greatly reduced in 2-cell embryos from round spermatids. Smcy also showed a disturbed pattern of expression in embryos from round spermatids. Protamine 2, which is normally restricted to the spermatid stage, was expressed following fertilization with round spermatids, but was already repressed at the two pronuclei stage. Ube1Y, which is normally expressed post-meiotically and not during the post-implantary development, was expressed up to the 2-cell stage in embryos from round spermatids only, and then repressed. Ube1X was also expressed up to the 2-cell stage, but in both embryo types. **CONCLUSIONS:** We therefore suspect that in embryos fertilized with round spermatids, regulatory mechanisms for inhibiting the inappropriate transcription of male post-meiotically expressed genes are activated following fertilization, permitting the zygotic genome activation to occur, though with some disturbances.

Key words: pre-implantation/round spermatids/spermatozoa

Introduction

Among azoospermic men with germinal failure, 40% have no recoverable spermatozoa, even after vigorous attempts at testicular sperm extraction. Before 1994 there was no hope of these men fathering their own children. Assisted reproduction by spermatid injection was first proposed in 1994 (Edwards *et al.*, 1994). The formation of round spermatids is the outcome of the second meiotic division. The nucleus of round spermatids contains a complete set of haploid chromosomes that are able to pair with those of the oocyte and to participate in syngamy (Ogura and Yanagimachi, 1993). The delivery of healthy, fertile offspring has been reported in the mouse and the rabbit when electrofusion or intracytoplasmic injection is used to introduce round spermatids into mature oocytes (Ogura *et al.*, 1994; Sofikitis *et al.*, 1994; Kimura and Yanagimachi, 1995a). In humans, Tesarik *et al.* (1995) achieved a successful birth using round spermatids (Tesarik *et al.* 1995); Fishel *et al.* (1995) used elongated spermatids (Fishel *et al.* 1995). Five years after the first human baby was born following spermatid

injection, the take-home baby rate remains very low (Al Hasani *et al.*, 1999) and there are still many concerns about the suitability and safety of this use of immature male gametes (Prapas *et al.*, 1999).

Spermatogenesis involves many profound morphological and functional changes. One unique aspect of the process is that gene expression continues in the haploid stage of spermatogenesis. Among the genes expressed in spermatids some, such as transition proteins 1 and 2 (Kleene, 1989) and protamines 1 and 2 (Kleene *et al.*, 1984), are unique to the testis, while others are testis-specific transcripts of unusual size or differently spliced variants of somatically expressed genes (Means *et al.*, 1991; Bolger *et al.*, 1996; Walensky *et al.*, 1998).

Mature spermatozoa, on the other hand, have lost most of their cytoplasm and it is generally acknowledged that they do not perform transcription (Bellvé *et al.*, 1988). Despite this apparently inert state, DNA polymerase activity has been described within the nucleus of mature sperm (Hecht, 1974),

Table I. PCR primer sequences

Gene	Primer sequences 5'-3' direction	Position	Annealing temperature (°C)	Product size (bp)		References
				cDNA	gDNA	
Hprt outer	CCTGCTGGATTACATTAAAGCACTG GTCAAGGGCATATCCAACAACAAAC	318–342 445–669	51	352	>352	Melton <i>et al.</i> , 1984
Hprt nested	TAGTGATAGATCCATTCTATGACTG GTCTGGAATTTCAAATCCAACAACACTG	351–376 620–642	50	292	>352	Melton <i>et al.</i> , 1984
Prm 2 outer	CGTACCGAATGAGGAGCCCAAGTG TTAGTGATGGTGCCTCCTACATTTC	106–130 503–528	60	318	423	Johnson <i>et al.</i> , 1988
Prm 2 nested	GGCCACCACCACACAGACACAGGCG GCATCTCCTCCTCCTTCGGGATCTTC	235–260 470–495	60	156	261	Johnson <i>et al.</i> , 1988
SmcY outer	CTGTCGTGGGATGAAGTCGATA GCACAGGCATGTTGAAGTAGTCAGCC	921–949 1171–1146	60	250	2500	Agulnik <i>et al.</i> , 1994
SmcY semi-nested	CTGTCGTGGGATGAAGTCGATA AAGTATACTCCTGTGTAGCCTG	921–949 1111–1090	60	191	2500	Agulnik <i>et al.</i> , 1994
Ube1Y/X outer	GCATCTGGATTATGAGATGGCTGCTGCC GCTCACAATCTCTGTCATCGGCTGATCC	429–456 1212–1185	60	784	>784	Mitchell <i>et al.</i> , 1991
Ube1Y nested	GGAGGGTCCGACACTGTGCTGTG GCTAAGTTGCAGTCCTTGCTC	484–507 1050–1030	60	567	>567	Mitchell <i>et al.</i> , 1991
Ube1X nested	GTCTTGAGGAGCTCAAAGCC GAGCTGTTGGTGCCCTTGAACCT	634–653 917–896	60	284	>284	Mitchell <i>et al.</i> , 1991
Hsp70.1 Outer	GGAGGTGCTGGACAAGTGC GCCAGCAGAGGCCTCTAATC	2509–2527 2748–2729	60	239	239	Hunt and Calderwood 1990
Hsp70.1 Semi-nested	GGAGGTGCTGGACAAGTGC CCTCCTCGATGGTGGGTCCTG	2509–2527 2726–2706	60	217	217	Hunt and Calderwood 1990

and specific RNA transcripts including *c myc* (Kumar *et al.*, 1993), HLA class I genes (Chiang *et al.*, 1994), *zfp59* (Passananti *et al.* 1995), β 1 integrin (Rohwedder *et al.*, 1996), protamines 1 and 2 and transition protein 2 (Wykes *et al.*, 1997) have been detected. Until now it has been unclear whether these mRNA transcripts are the product of active synthesis in mature spermatozoa or, more likely, synthesized during the final burst of transcription prior to the replacement of histones by protamines. However, the quantity of spermatozoa required for RT-PCR analysis of these transcripts suggest that their numbers per spermatozoon must be extremely small.

The injection of a spermatid into an oocyte represents the mass arrival of mRNA from various genes, some of which may be deleterious to the development of the embryo if translated. This is a situation that the oocyte has not been programmed to deal with. Available data do, in fact, indicate that inappropriate transcription of some genes during the period of nuclear reprogramming can have long-term detrimental effects on the embryo (Latham, 1999). The pattern of mRNA expression at various times following spermatid injection is therefore a key element in understanding how the oocyte reacts to restore the conditions for normal development.

We have developed a nested reverse transcription/polymerase chain reaction (RT-PCR) assay for the detection of mRNA from six genes at various times following intracytoplasmic injection of a spermatozoon or spermatid into a mouse oocyte. These are: Hprt which is normally expressed continuously in the embryo (Kratzer, 1983); protamine 2 (Prm2), the expression of which is strictly restricted to the spermatid stage (Kleene *et al.*, 1984); Hsp70.1 which is expressed early in the 2-cell stage (Christians *et al.*, 1995); and one X- and two Y-located genes which are expressed post-meiotically. These are: Ube1Y, which is not expressed before

post-implantatory development of the embryo (Odorisio *et al.*, 1996); its X homologue, Ube1X, which is expressed in both round spermatids and oocytes; and Smcy, which shows early zygotic expression (Agulnik *et al.*, 1994). This paper is the first report on gene expression in the early mouse embryo following intracytoplasmic injection of round spermatids.

Materials and methods

Media

The medium used for collecting and culturing oocytes was M16 (Sigma, St Louis, MO, USA). The medium used for culturing embryos after the 2-cell stage was CZB (Chatot *et al.*, 1989) supplemented with 5 mmol/l fructose. The medium for micromanipulation was M2 (Sigma) for oocytes and 10% M16-PVP (polyvinylpyrrolidone, Mr. 360 000; Sigma) for round spermatids and spermatozoa. All media were supplemented with 15 mg/ml bovine serum albumin (BSA fraction V, Sigma).

Preparation of oocytes

C57BL×CBA F1 female mice (6–10 weeks old; INRA, Jouy-en-Josas, France) were injected with 10 IU pregnant mare's serum gonadotrophin followed by 10 IU human chorionic gonadotrophin (HCG) 48 h later. Oocytes were collected from oviducts about 15 h after HCG injection. They were freed from the cumulus cells by 3 min incubation at 37°C with 0.1% bovine testicular hyaluronidase (type VI S; Sigma) in M16 medium. The oocytes were rinsed and kept in M16 medium at 37°C for 3 h under 5% CO₂ in air. Prior to round spermatid (RS) injection (or spermatozoa injection in preliminary experiments only), the oocytes were activated by 8% ethanol in M16 medium for 3 min at 37°C, then rinsed twice in M16 medium.

Preparation of spermatozoa and spermatids

Spermatogenic cells were obtained from 8–13 week hybrid F1 males (C57BL×CBA; INRA). Spermatozoa were collected from the

Table II. Fertilization of mouse oocytes by deferent duct spermatozoa and round spermatids, examined in living condition 5 h after injection

Type of cell injected	No. of experiments	Total no. of oocytes injected	No. (%) of oocytes survived	No. (%) of oocytes with two pronuclei ^a
Round spermatids	14	698	657 (94%)	293 (44.6%)
Deferent duct spermatozoa	8	317	286 (90%)	134 (46.8%)

^aOocytes with two distinct pronuclei and the second polar body were considered fertilized.

vas deferens. They were expressed into M16/BSA medium and incubated for 1.5 h at 37°C under 5% CO₂ in air to allow capacitation. They were finally resuspended in M16/BSA medium containing PVP to a final concentration of 8%.

Round spermatids were isolated from the testes by flow cytometry coupled to cell sorting as previously described (Ziyyat *et al.*, 1999) except that the discontinuous Percoll gradient step was omitted. The sorted spermatids were washed in M16/BSA and resuspended in M16/BSA containing PVP to a final concentration of 8%.

Injection of spermatozoa and spermatids into oocytes

The injection was performed in a cell chamber (POC chamber; Helmut Saur, Reutlingen, Germany) following the method of Kimura and Yanagimachi (1995a,b) (Kimura and Yanagimachi, 1995a,b), except that no piezo-assisted manipulator was used to facilitate zona pellucida penetration and that the entire procedure was performed at room temperature. The chamber was placed on the stage of an inverted microscope (Nikon Diaphot) equipped with Nomarski differential interference optics. Single spermatozoa were aspirated by the middle of their tails into the pipette, so forming them into a hairpin shape and minimizing the volume of medium (5 µm ID; Humagen, Charlottesville, VA, USA) introduced into the oocyte during the injection. Whole RS, each with its disintegrating plasma membrane, were injected into the oocytes.

Culture and examination of oocytes

After injection with either spermatozoa or RS, oocytes were stored at -80°C in batches of 40, or incubated in M16 medium at 37°C under mineral oil in a plastic dish under 5% CO₂ in air for 5 h, then examined using an inverted microscope with a X20 objective. Oocytes with two large pronuclei and one second polar body were considered 'fertilized'. They were cultured continuously in M16 medium for up to 24 h, corresponding to the 2-cell stage. The 2-cell stage embryos were transferred into CZB medium for a further 24 h culture taking them to the 4-cell stage. Embryos were cultured at a concentration of 10 embryos per 10 µl volume of medium. The entire culture plate was overlaid with heavy paraffin oil. At the end of the culture period, pools of 20 2-cell stage and 10 4-cell stage embryos were stored at -80°C in a minimal volume of medium.

Nested RT-PCR

Messenger RNA for Hprt, Smcy, Ube1y, Ube1X, Prm2 and Hsp70.1 was detected in whole oocyte/embryo homogenates using nested RT-PCR. To evaluate the dynamic of the genome and not only the carry-over of inherited transcripts, and considering that all cells are equivalent in a 2- and 4-cell embryo, the results were expressed per cell (i.e. per genome equivalent) rather than per embryo. Pools of 40 oocytes, 20 2-cell or 10 4-cell stage embryos in about 2 µl of medium were heated at 99°C for 1 min and then placed on ice. A mixture of 1 µl (10 IU) DNase I RNase-free (Boehringer Mannheim, Meylan, France), 1 µl 10×Gold Taq buffer (Perkin Elmer, Foster City, CA, USA) and 0.5 µl (20 IU) RNase inhibitor (Boehringer Mannheim)

was added to the lysate in a final volume of 10 µl. Samples were incubated at 37°C for 10 min followed by 15 min at 70°C to destroy DNase I.

Whole samples containing embryonic RNA were reverse-transcribed by incubating at 42°C for 1 h with 2 units MMLV reverse transcriptase (Life Technologies, Cergy Pontoise, France), 3 µmol/l random hexamer, 1 mmol/l each dNTP, 1 µl 10×Gold Taq buffer in a final volume of 20 µl. The reaction was terminated by heating at 95°C for 10 min and cooling to 5°C. A total of 5 µl of room temperature reaction volume was used for 35 cycles of first round PCR in a final PCR reaction volume of 50 µl containing 2.5 IU of Ampli Taq gold (Perkin Elmer), 1 mmol/l each dNTP and 10 pmol of each of the sense and antisense primers. A 2.5 µl aliquot of cDNA obtained from the first amplification served as template for a second DNA amplification reaction, using inner nested or semi-nested primers (Table I) (Melton *et al.*, 1984; Johnson *et al.*, 1988; Hunt and Calderwood, 1990; Mitchell *et al.*, 1991; Agulnik *et al.*, 1994). This second round PCR was run for 40 cycles. The PCR products (20 µl of each) were analysed on 2% agarose gels stained with ethidium bromide and molecular sizes were determined with the molecular weight marker φX 174 Hae Digest (Sigma).

Because RT-PCR analysis was applied to whole oocyte/embryo homogenates, care was taken after the treatment of samples with DNase to detect possible false-positive signals due to genomic DNA. Primers for Hprt, Ube1Y and X, Smcy and Prm2 were designed to span an intron such that the cDNA-derived products could be distinguished by size from possible amplified genomic products. Hsp70.1 has no intron; in this case, Hprt was used as negative internal control for genomic contamination. Simultaneous reactions in which reverse transcriptase was omitted prior to the PCR were also run (data not shown). RT-PCR analysis of RNA from whole testis served as positive control (data not shown). Hprt was used as a positive control for the efficiency of the synthesis of cDNA. For all amplifications, negative controls (water only) were included (data not shown). Primer sequences, annealing temperatures and sizes of PCR products are given in Table I. The mRNA for Smcy and Prm2 were detected in RS using the same RT-PCR procedure. The nested PCR products corresponding to Ube1Y/X and Prm2 were sequenced to confirm their identity.

Southern blot analysis

The cDNA probes used in this study were as follows: Smcy, a 251 bp PCR fragment (921 bp–1171 bp) (Mitchell *et al.*, 1991); Hsp70.1, a 223 bp PCR fragment (2528 bp–2750 bp) (Hunt and Calderwood, 1990). Fragments were verified by size and restriction enzyme mapping.

For Southern blot analyses, 20 µl of nested PCR products were run on 1.5% agarose gel and blotted to hybrid N⁺ membranes (Amersham, Uppsala, Sweden). Hybridization was carried out overnight using ³²P labelled probes and autoradiographic exposure was for 30 min at -80°C.

Table III. In-vitro development of mouse oocytes injected with deferent duct spermatozoa or round spermatids

Type of cell injected	No. of fertilized oocytes	No. (%) of 2-cell oocytes developed	No. of 2-cell oocytes	No. (%) of 4-cell oocytes developed from 2-cell oocytes
Round spermatids	229	212 (92.5)	128	106 (82.8)
Deferent duct spermatozoa	134	126 (94)	72	60 (83.3)

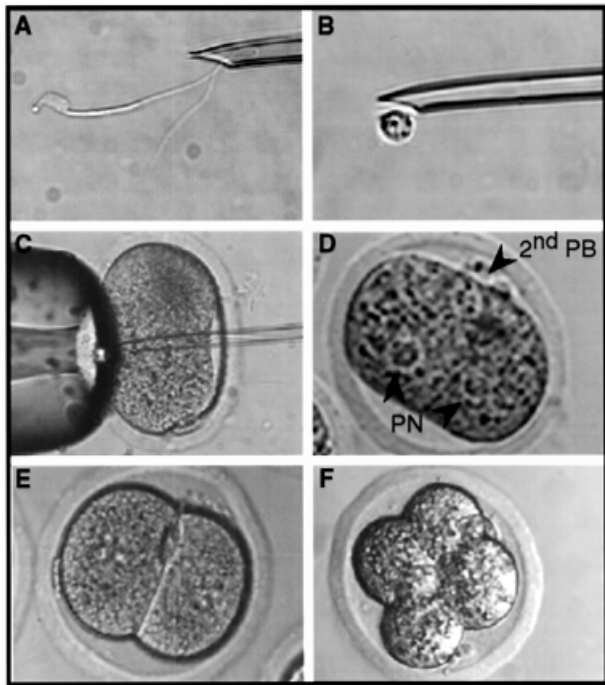


Figure 1. (A) a spermatozoon sucked by the middle of its tail into the injection pipette. (B) a sorted spermatid ready to be sucked into the injection pipette. (C) sperm injection pipette inserted deeply into an oocyte, the metaphase II spindle of the oocyte being at the 6 o'clock position (indicated by a hump in the oocyte cortex). (D) an oocyte 5 h after spermatid injection. Arrows indicate sperm and oocyte pronuclei. 2nPb: second polar body. (E) a 2-cell oocyte, 24 h after spermatid injection. (F) a 4-cell oocyte, 48 h after spermatid injection.

Results

Table II summarizes the results of experiments in which either a single spermatozoon or a RS was injected into an individual oocyte (see also Figure 1). Mature mouse spermatozoa are fully capable of activating oocytes to undergo normal embryonic development when injected into the ooplasm, so oocytes were not stimulated prior to ICSI (Kimura and Yanagimachi, 1995a). However, spermatids similarly injected do not activate oocytes (Kimura and Yanagimachi, 1995b); in this case oocytes were activated with alcohol prior to fertilization. Oocyte survival was similar whether injected with spermatozoa (286 out of 317 oocytes injected) or RS (657 out of 698 oocytes injected). The introduction of a spermatozoon, middle of tail first, inside the micro-injection pipette doubled the survival of oocytes following injection, probably because it reduced the volume of medium injected (Figure 1A). Oocytes containing two large

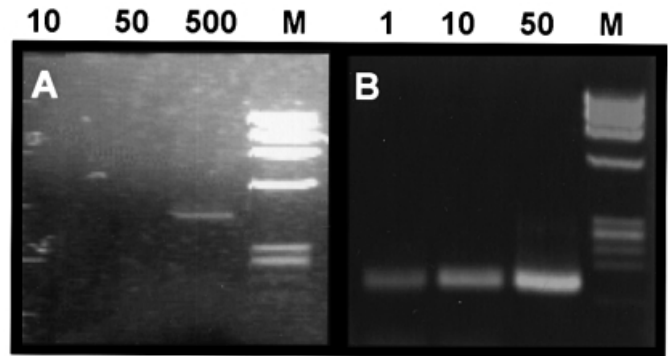


Figure 2. Expression pattern of mRNA transcripts. (A) Smcy in 10, 50 and 500 RS; molecular weight marker (M): puc 18 HaeIII digest. (B) Prm2 in 1, 10 or 50 round spermatids (RS); molecular weight marker: ϕ X174 Hae III digest. The experiment was performed four times. A typical result is shown.

pronuclei and one second polar body at 5 h post-injection were considered to be normally fertilized (Figure 1D).

RS selected through the flow cytometry/cell sorting procedure and spermatozoa were equally effective in fertilizing oocytes, the fertilization rates being 44.6% for RS and 46.8% for spermatozoa (Table II). Table III shows that most of the fertilized oocytes developed well into 2-cell embryos (92.5% for embryos from RS and 94% for those from spermatozoa). These 2-cell embryos (Figure 1E) developed to the 4-cell stage (Figure 1F) (82.8% for RS embryos and 83.3% for spermatozoa) as soon as they were moved to CZB medium supplemented with fructose; otherwise most of them remained blocked at the 2-cell stage (data not shown). There was no significant difference between RS and spermatozoa in their capacity to promote development up to the 4-cell stage.

To determine whether Hprt, Ube1Y, Ube1X, Smcy, Hsp70.1 and Prm2 were transcribed during the pre-implantation development of embryos from RS as well as spermatozoa we performed nested RT-PCR analyses on different embryo batches. cDNA was prepared from pooled embryos, as only half were expected to be male. As shown in Figure 2, Prm2 was strongly expressed in RS because a signal was detectable in a single RS using a nested RT-PCR assay, while Smcy was present at a low copy number as mRNA from 500 RS was necessary to obtain a signal. Results for Hprt, Ube1Y, Ube1X and Prm2 are shown in Figure 3. All assays were positive for Hprt, indicating the presence of cDNA after the reverse transcription reaction. Ube1Y was expressed immediately following fertilization by RS and up to the 2-cell stage, but it

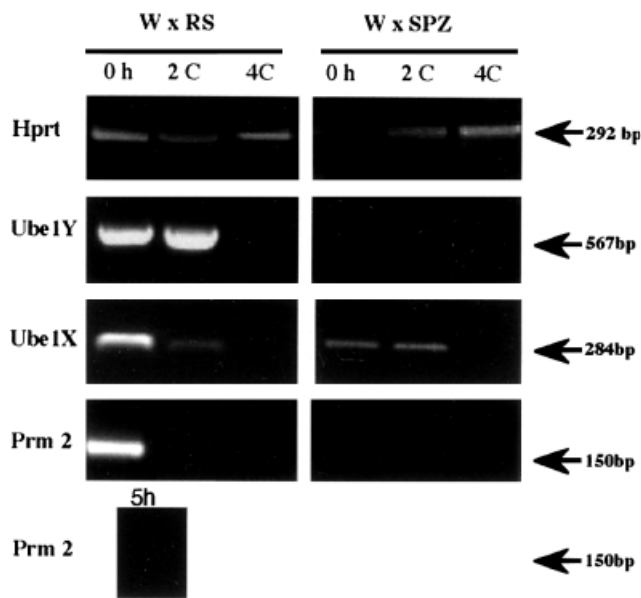


Figure 3. Expression pattern of mRNA transcripts for Hprt, Ube1y, Ube1X and Prm 2 in oocytes at 0 h post-injection (0 h), 5 h post-injection (for Prm 2 only) and 2-cell (2C) and 4-cell (4C) embryo stages. Oocytes (W) were injected with either RS or spermatozoa (SPZ). cDNA reverse transcribed from RNA of 10 embryo equivalents (0 h), five embryo equivalents (2C) or 2.5 embryo equivalents (4C) was used for RT-PCR analysis. The experiment was performed on four different batches of embryos. A typical result is shown.

was repressed by the 4-cell stage. By contrast, no PCR products for Ube1Y mRNA were detected in embryos from spermatozoa at any stages tested. Ube1X was detected in both embryos from RS and spermatozoa up to the 2-cell stage. In embryos from RS, Prm2 mRNA was detected only following fertilization. The disappearance of Prm2 transcripts took place as early as 5 h post-fertilization in embryos from RS. In preliminary experiments, we have activated oocytes with alcohol prior to sperm micro-injection. We saw no differences in the expression pattern of Hprt, Ube1Y and Prm2 up to the 2-cell stage in comparison with embryos from alcohol free oocytes (data not shown). Therefore, oocytes were not activated with alcohol prior to sperm micro-injection.

To increase the sensitivity of detection, RT-PCR products for Hsp70.1 and Smcy were analysed by Southern blotting. Figure 4 shows that Hsp70.1 was expressed in embryos from RS and spermatozoa at the 2-cell stage; a faint signal was obtained with embryos at the 4-cell stage. However, the signal given by the 2-cell embryos from spermatozoa was several orders of magnitude higher than that obtained with RS embryos at the same stage of development. After RS fertilization, embryo-coded Smcy showed less intense expression at 2-cell and more extended expression to 4-cell, in comparison with embryos from spermatozoa.

In order not to introduce a bias in interpreting the development of the embryos in terms of the maturity of the male gamete, only good quality oocyte batches with high surviving rates following micro-injection were used in this study. These

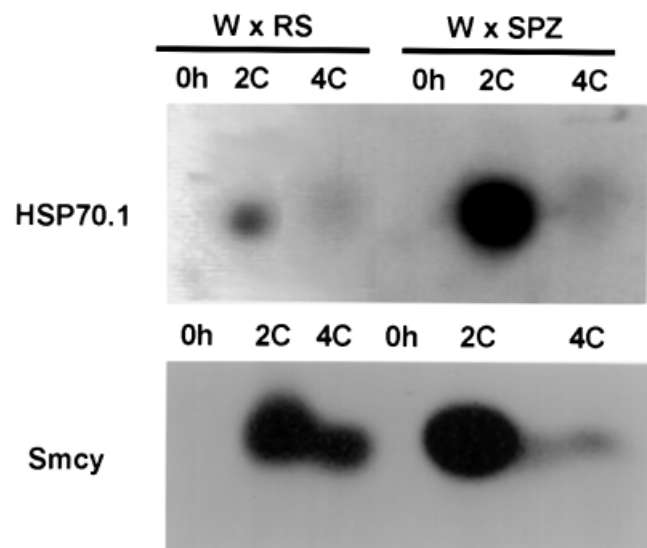


Figure 4. Representative expression pattern of mRNA transcript for Hsp70.1 and Smcy. Signal obtained following hybridization of RT-PCR products corresponds to 10 oocytes at 0 h post-injection (0 h), five 2-cell embryos (2C) and 2.5 4-cell embryos (4C). Oocytes (W) were injected with either RS or SPZ. Three repeated experiments resulted in similar patterns.

limits being granted, the expression patterns of the six genes analysed were similar from batch to batch of embryos, at any time tested.

Discussion

We have shown that RS isolated by the flow cytometry/cell sorting procedure (Ziyyat *et al.*, 1999) were as effective as spermatozoa in fertilizing oocytes and in promoting embryonic development up to the 4-cell stage. In this respect our results differ from those of Kimura and Yanagimachi (1995b). In fact, the exact stage of the RS used by Kimura and Yanagimachi was not defined. The method of selection that we used yields a homogenous population of RS at a late stage of development: step 6–7 of the cap phase, just before the elongating process starts (Ziyyat *et al.*, 1999). Sofikitis *et al.* (1997) have shown that rabbit RS at later stages have a greater fertilization capacity than RS at earlier stages (Sofikitis *et al.*, 1997). The fact that no piezo-assisted micromanipulator system was used in that study might explain the overall low fertilization rate observed with respect to the results of Kimura and Yanagimachi (1995a,b). On the other hand, using a conventional pipette, the oocytes survival rates reported in this study were higher than those reported by Yanagimachi (Kimura and Yanagimachi, 1995b; Suzuki and Yanagimachi, 1995).

In the mouse embryo, a major reprogramming of the pattern of gene expression occurs during the 2-cell stage. While some of these changes may be due to maternal mRNA, many are attributable to the transcription of zygotically expressed genes (Latham *et al.*, 1991). This reprogramming of gene expression is likely to be critical for further embryonic development. The effects on early embryonic development of the presence in the oocyte of mRNA from genes expressed post-meiotically in RS

are a matter of speculation. Does the activation of the zygotic genome occur normally in this new context, and what happens to the expression of round spermatid specific genes? To answer the first question, we looked for the expression of Hsp70.1 and Smcy, both known to be activated during the 2-cell stage. To answer the second, we followed the expression of Prm2 and Ube1Y, both of which are expressed specifically in round spermatids. Christians *et al.* (1995) have shown that the onset of zygotic genome activity is marked by the constitutive expression of Hsp70.1, the major inducible heat shock gene. Our results, which show the expression of Hsp70.1 early in the 2-cell stage, are consistent with those of Christians *et al.* (1995). Hsp70.1 followed the same temporal pattern of expression whether the embryo came from the injection of RS or spermatozoa; however, the level of expression obtained in embryos from spermatozoa was several orders of magnitude higher than that obtained in embryos from RS.

Smcy codes for an epitope of the male specific transplantation antigen H-Y; in mouse development, Smcy expression has been detected as early as the 2-cell stage (Agulnik *et al.*, 1994). In our work, Smcy expression followed the same temporal pattern as that of HSP70.1; it was expressed during the 2-cell stage but repressed by the 4-cell stage. Smcy was expressed in spermatids, but only at a relatively low level because mRNA from 500 round spermatids was necessary to get a signal following a nested RT-PCR assay. This low expression in RS probably explained the absence of a signal following injection in embryos from RS, and it argues for zygotic expression of Smcy in 2-cell embryos. Embryos from spermatozoa expressed Smcy with the same temporal pattern of expression than that observed for Hsp70.1, while RS embryos showed less intense expression at 2-cell and more extended expression at 4-cell. Considering the expression patterns of Hsp70.1 and Smcy in embryos from RS, it would appear that immature male gametes are less efficient at activating the embryonic genome. The expression status of the round spermatid greatly differs from that of the spermatozoon, and this might influence the onset of the zygotic genome activation in different ways: it might delay the first wave of activation; this would explain the expression profile of Smcy in RS embryos, with a retarded peak beyond the 2-cell; or/and it might affect the intensity of the activation leading to low expression of Hsp70.1.

Thompson *et al.* (1995) demonstrated that the regulation of Hsp70.1 during early mouse development is dependent on the maturation of chromatin structure (Thompson *et al.* 1995). In fact, the most important event determining the nuclear status of sperm cells is the replacement of histones by protamines, the basic nuclear proteins of mature spermatozoa. To accommodate the cessation of transcription several days before the completion of spermiogenesis, mRNA encoding the protamines is synthesized in round spermatids (steps 7–9), stored as cytoplasmic ribonucleoprotein particles for up to a week (steps 7–12), and finally translated in elongated spermatids (steps 12–16) (Kleene *et al.*, 1984). Therefore, the round spermatids that were injected into the ooplasm were able to express their protamine genes. Because paternal pronuclei are more effective than maternal pronuclei

in transcribing genes, it has been suggested that important early events in fertilized embryos may be predominantly under paternal control (Henery *et al.*, 1995). Within the first 8 h following fertilization, the paternal nucleus normally undergoes profound changes; in particular, histones replace protamines during pronucleus formation (McClay and Clark, 1997). For the normal development of RS embryos it therefore appears crucial to prevent protamine expression from occurring. Our data clearly demonstrate that Prm2 expression was down-regulated in embryos from RS: if Prm2 mRNA was present in oocytes just after RS injection, it was undetectable in 1-cell embryos as early as 5 h following fertilization. As expected, at none of the stages tested were transcripts detected in embryos from spermatozoa.

By contrast, Ube1Y, which is not normally transcribed during pre-implantation development (Odorisio *et al.*, 1996), was expressed in embryos from round spermatids from fertilization through to the 2-cell stage. Even though there is no direct evidence of continued transcription, the level of the message is high and apparently equivalent in 1-cell and 2-cell embryos, independently of the fact that the number of embryos (but not of cells) was halved. Therefore, the absence of detectable Ube1Y message in the 4-cell is rather the expression of down-regulation than dilution of the paternal mRNA. No transcripts were detected in embryos from spermatozoa at any of the stages tested. Ube1Y, a Y-linked gene, is transcribed in the testis together with its ubiquitously expressed homologue on the X chromosome (Ube1X). It encodes ubiquitin-activating enzyme E1, an enzyme essential for eukaryotic cell proliferation. X and Y transcripts are present in spermatogonia A, almost undetectable in pachytene spermatocytes, and then return to high levels in round spermatids (Odorisio *et al.*, 1996). Ube1X was expressed in embryos from both RS and spermatozoa up to the 2-cell stage. Ube1X is not only maternally-inherited, but also actively transcribed in the spermatid, while it is not expressed in the preimplantation embryo. If maternally inherited transcripts are diluted from 1-cell to 4-cell, because we are halving the number of embryos (but not of cells) at each stage, the absence of detectable signal in 4-cell embryos must also reflect the arrest of transcription from the paternal copy of the gene. The complete amino acid sequence of Y- and X-encoded proteins indicates that they probably have the same function; this could explain why Ube1Y is not rapidly repressed following fertilization with RS.

Immediately after micro-injection, the expression status of the embryo is obviously the picture of both paternal and maternal contributions: spermatids expressed Ube1Y and Prm2 while spermatozoa which are transcriptionally quiescent have undetectable levels of Ube1Y and Prm2 mRNA in extracts from 100 spermatozoa (A.Z. and A.F., personal observation) and, similarly, embryos from RS expressed both Ube1Y and Prm2 while embryos from spermatozoa did not. Besides, Ube1X followed the same pattern of expression in embryos from RS or spermatozoa, whether the oocytes are activated with alcohol or not. Therefore, the differences between sperm-derived and spermatid-derived

embryos are likely to reflect the differences in the expression status observed between spermatozoa and spermatid.

In summary, our results suggest that, in the mouse, regulatory mechanisms are activated soon after fertilization with RS to inhibit the inappropriate transcription of male post-meiotically expressed genes. The kinetics of this inhibition was dependent on the gene to be switched off: the protamine 2 gene was rapidly repressed following fertilization, while Ube1Y expression persisted up to the 2-cell stage. On the other hand, mRNA species of RS origin that persist in the early embryo permit the expression of Hsp70.1 and Smcy in a narrow window during the first phase of the activation of the zygotic genome. Even though embryos from RS developed up to the 4-cell stage more or less normally after a short period for reprogramming of the male genome, it does not foresee their future. More work will be necessary to explain the failure of RS embryos, particularly in humans, to support full embryonic development. This should include following-up embryos beyond the 4-cell stage, and an analysis of gene expression at later stages. In interpreting the results we have to bear in mind that the embryos we analysed were from RS of normally fertile mice, and that the findings are not necessarily applicable to embryos from the RS of infertile men. Particularly, RS from patients with complete spermiogenesis failure had significantly higher frequencies of apoptosis-specific DNA damage in comparison with patients with incomplete spermiogenesis failure (Tesarik *et al.*, 1998a). They also failed to support the special form of Ca²⁺ oscillations that is the sign of normal activation of the oocyte (Tesarik *et al.*, 1998b). These observations might explain the low success rates of spermatid conception in these cases.

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