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Transcriptional and epigenetic status of protamine 1 and 2 genes following round spermatids injection into mouse oocytes

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Abstract

The use of round spermatids that are fully active at the transcriptional level to create zygotes (i.e. round spermatid injection; ROSI) raises the question regarding the downregulation of all specific genes that are transcribed from the paternal genome at fertilization. In this study, we show that protamine 1 and 2 mRNAs, which are specific to the round spermatid stage, are repressed at the two-pronuclei (6 h) and two-cell (30 h) stages postfertilization, respectively, in ROSI embryos, by distinct mechanisms. Both genes are fully methylated in round spermatids and sperm but unmethylated in oocytes. At 6 h postfertilization, the protamine 1 and 2 genes are actively demethylated, but the demethylation process happens more rapidly in ROSI than in sperm zygotes. Treatment of zygotes with trichostatin A, a histone deacetylase (HDAC) inhibitor, maintained the protamine 2 mRNAs expression up to 30 h postfertilization while the DNA methylation status of the gene is not affected. Thus, HDACs are involved in the clearance of protamine 2 mRNAs in ROSI two-cell embryos independently of the methylation status of the repressed gene. Contrastingly, HDACs are not directly involved in protamine 1 regulation since trichostatin A does not reverse the silencing of the gene in ROSI embryos at 6 h. The protamine 1 CpG island located in the coding region is actively demethylated in ROSI one-cell embryos where the gene is repressed and may contribute to the regulation of protamine 1 gene expression. The comparison with gene reprogramming occurring during nuclear transfer makes ROSI embryos an attractive model to study the mechanisms involved in gene silencing elicited by the oocyte.

Keywords: Round spermatid injection; Fertilization; Protamines 1 and 2; Methylation; TSA

Cells transmit information to the next generation *via* the genome and the epigenome. Whereas genetic inheritance is based on the DNA code, epigenetic information comprises modifications occurring directly on DNA or on the chromatin. The major type of DNA modification is methylation, whereas on the chromatin various modifications occur on specific residues of histones, including methylation, phosphorylation, acetylation, and ubiquitination [1,2]. The mammalian genome undergoes two major phases of epigenetic reprogramming, once in the primordial germ cells and once in the preimplantation embryos.

In mammalian fertilization, the paternal genome is delivered to the secondary oocyte by mature sperm, with protamine (Prm)- rather than histone-compacted DNA. In contrast, the maternal genome is arrested in meiotic metaphase II, with its 2C genome packaged with histones. Thus, at the beginning of fertilization, the two gametes have very different chromatin organization and epigenetic marks. Sperm DNA is globally hypermethylated compared with oocyte DNA, but both gametes are hypomethylated relative to somatic tissues [3].

In the newly fertilized egg, protamines in sperm chromatin are rapidly replaced with histones which are hyperacetylated [4,5]. Then, the male pronucleus DNA is rapidly and specifically demethylated by an active process in the absence of DNA replication [3,5,6]. In contrast, the maternal genome is gradually demethylated during the first cell cleavages [6]. High levels of methylation on histone H3-K9 in the female pronucleus may protect the maternal DNA against the rapid demethylation that occurs in the male pronucleus [5]. However, some regions of the male genome are not affected by this demethylating process, particularly the paternally imprinted genes [7] and the IAP sequences [8]. Both the mechanism and the function of paternal genome demethylation are unknown. Transcription initiates first in the male pronucleus, and the loss of paternal-specific

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methylation may simply reflect the early requirement for transcriptional derepression of paternal alleles [9]. The global demethylation of the male pronucleus has been observed in mouse, rat, pig, cow [5], and human [10] zygotes but not in sheep or rabbit embryos [10–12]. Histones incorporated into the male pronucleus are highly acetylated [4,5], but they are rapidly deacetylated and monomethylated [5,13,14]. The progressive histone modifications occurring on the paternal genome presumably leads to a chromatin state equivalent to that of the maternal genome.

Nuclear transfer in animal cloning results in the reprogramming of nuclei from a restricted somatic cell program to the totipotent pattern typical of early embryo. A key variable in the success of cloning is the resetting of the epigenetic status of the donor nucleus, including the pattern of DNA methylation [5,11,15]. Improper epigenetic reprogramming is observed in the majority of the cloned embryos produced, where aberrant patterns of DNA methylation [5,16,17] and gene expression occur [18–20]. The molecular nature of the reprogramming events taking place in nuclear transfer experiments remains unknown. Round spermatids are haploid cells with decondensed nuclei packaged with histones. They likely carry normal male imprinting marks [21]. Normally, prior to fertilization, the genomes of both sperm and metaphase II oocytes are transcriptionally inactive. In contrast, a round spermatid actively transcribes and stores a large amount of different mRNAs, some of which are specific to this stage (protamine 1 and 2 mRNAs), and its introduction into the oocyte requires the inactivation of several genes. We have previously shown that the expression pattern of round spermatids is reset by the oocyte cytoplasm [22]. Round spermatid injection (ROSI) appears to be a unique and valuable model to investigate the mechanisms involved in the inhibition of the transcriptional program of a host nucleus by the oocyte cytoplasm. In this study we examined two potentially effective epigenetic mechanisms usually involved in gene regulation, *i.e.,* histone acetylation and DNA methylation, and evaluated their implication in protamines 1 and 2 mRNAs repression in one-cell and two-cell ROSI zygotes.

Results

Protamines expression

To follow the remodeling of the round spermatid genome after fertilization we analyzed the pattern of expression of protamines 1 and 2, which are exclusively expressed in round spermatid, at two key time points of the early ROSI embryo development: the two-pronuclei (2PN; 6 h) and the two-cell (30 h) stages. As shown in

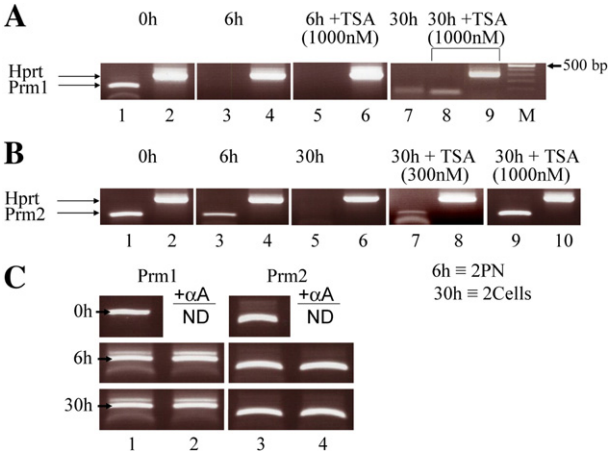
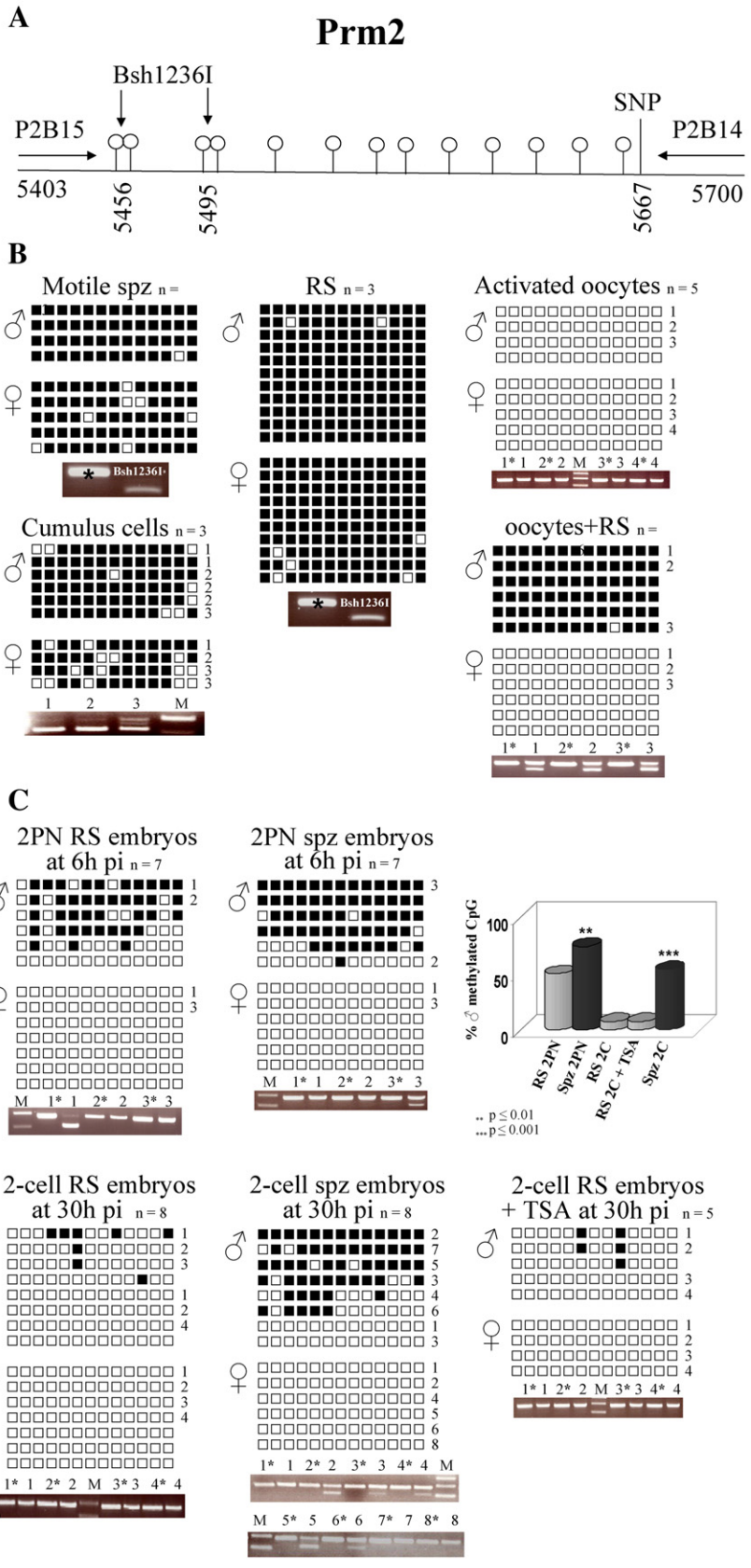


Fig. 1. Protamines 1 and 2 expression. (A) Expression pattern of Prm1 (221 bp) (lanes 1, 3, 5, 7, 8) and Hprt (lanes 2, 4, 6, 9) in ROSI zygotes at different times after fertilization: 0, 6, 30 h and 6, 30 h treated with trichostatin A (TSA). (B) Expression pattern of Prm2 (156 bp) (lanes 1, 3, 5, 7, 9) and Hprt (298bp) (lanes 2, 4, 6, 8, 10) in ROSI zygotes at different times after fertilization: 0 h (immediately after microinjection), 6 h (2PN stage), 30 h (two-cell stage), and 30 h treated with 300 or 1000 nM TSA. (C) Expression pattern of Prm1 (lanes 1, 2) and 2 (lanes 3, 4) in round spermatids cultured *in vitro* for 0, 6, or 30 h in presence (lanes 2, 4) or absence (lanes 1, 3) of 25 μM α-amanitin. These results are representative of at least three independent experiments.

Fig. 1A, protamine 1 transcripts disappear as early as 6 h after fertilization. Protamine 2 mRNAs were still present at the 2PN stage, 6 h postfertilization, but were removed from two-cell embryos at 30 h (Fig. 1B). Our detection of Prm2 at 6 h likely reflects a better availability of mRNAs due to an increased efficiency of the lysis protocol utilized in this work compared with our previous results [22]. No detectable transcripts for either Prm1 or Prm2 genes were observed in one-cell and two-cell control embryos generated from spermatozoa (data not shown and [22]). By contrast, in intact round spermatids, cultured with Sertoli-cell-conditioned medium to roughly restore their normal environment, Prm1 and Prm2 mRNAs were very stable: they were still present at 30 h even in presence of the transcriptional inhibitor α-amanitin (25 μM) (Fig. 1C). Deacetylated histones are usually associated with transcriptionally inactive chromatin. Therefore, deacetylation of histones in the early ROSI embryos may participate into the silencing of the round spermatid expressed genes. Treatment of the one-cell embryos with trichostatin A(TSA), an inhibitor of histone deacetylase (HDAC), did not thwart the rapid disappearance of Prm1 mRNAs (Fig. 1A). On the contrary, exposure of the embryos to TSA up to the two-cell stage maintained the expression of Prm2 mRNAs up to 30 h postfertilization, whereas they were undetectable at that time normally (Fig. 1B). The loss of

Fig. 2. Prm2 methylation profile. (A) Schematic representation of the studied fragment of Prm2 gene showing the 13 CpG sites. The two restriction sites of *Bsh*1236I (CG/CG) are shown with arrows. SNP, single-nucleotide polymorphism allowing differentiation between paternal and maternal alleles; P2B15 and P2B14, forward and reverse primers, respectively. Base numbering is according to GenBank Accession No. Z47352. Each circle represents a CpG site. (B and C) Methylation status of Prm2 in different cell types. Each row of squares represents a single cloned allele with 13 CpG sites. Open squares represent nonmethylated CpG sites and filled squares represent methylated CpG sites. RS, round spermatid; Spz, spermatozoa; activated oocytes were incubated with 8% ethanol in M16/bovine serum albumin for 3 min at 37 °C; 2PN, two-pronuclei stage, corresponding to 6 h postfertilization; 2C, two-cell stage, corresponding to 30 h postfertlization; pi, postinjection; ♀, maternal allele; ♂, paternal allele; n, number of independent PCR. The frequency of cytosine methylation found in different cell populations was compared using a χ^2 test; *** $p \leq 0.001$; ** $p \leq 0.01$. Each PCR amplified product used for cloning and sequencing was digested with *Bsh*1236I. For each cell type, examples of the digestion products are shown under the methylation profiles. Individual DNA strand methylation profiles and digestion profiles obtained from the same PCR amplifications are assigned the same number. * not cut; M, molecular weight marker.


A
Prm2
Bsh1236I
SNP
P2B15
P2B14
5403
5456
5495
5667
5700
B
Motile spz n =
RS n = 3
Activated oocytes n = 5
Bsh1236I
Cumulus cells n = 3
oocytes+RS n =
1 2 3 M
1* 1 2* 2 M 3* 3 4* 4
1* 1 2* 2 3* 3
C
2PN RS embryos at 6h pi n = 7
2PN spz embryos at 6h pi n = 7
% ♂ methylated CpG
RS 2PN
Spz 2PN
RS 2C
RS 2C + TSA
Spz 2C
.. p ≤ 0.01
... p ≤ 0.001
2-cell RS embryos at 30h pi n = 8
2-cell spz embryos at 30h pi n = 8
2-cell RS embryos + TSA at 30h pi n = 5
M 1* 1 2* 2 3* 3
1* 1 2* 2 M 3* 3 4* 4
1* 1 2* 2 3* 3 4* 4 M
M 5* 5 6* 6 7* 7 8* 8

repression of Prm2 expression by TSA was concentration dependent and attests to the efficacy of TSA treatment in the Prm1 experiment.

Methylation analysis of Prm1 and Prm2 genes

While histone acetylation plays a role in the regulation of Prm2 transcripts, the decrease in Prm1 mRNAs cannot be attributed solely to histone deacetylation. Therefore, we investigated the possible role of DNA methylation in this phenomenon. To assess the epigenetic status of the one- and two-cell ROSI embryos, we analyzed the methylation profile of a CpG dinucleotide-rich region at the 5′ portion of protamine 1 and 2 genes. Both genes are specifically expressed in round spermatids. A region comprising 13 CpG dinucleotides within exon 1 of protamine 2 was investigated using the bisulfite mutagenesis assay (Fig. 2A). This region carries a single-nucleotide polymorphism at base 5667 (GenBank Accession No. Z47352), being a cytosine in C57BL/6 mice and a guanine in DBA2 mice, which permits differentiation between the maternal and the paternal allele in C57BL/6 x DBA2 crosses. This region was heavily methylated in round spermatids where the gene is exclusively expressed, in motile sperm, and in cumulus cells (98.1, 93.2, and 82.4% of cytosine residues methylated, respectively) and 100% unmethylated in activated

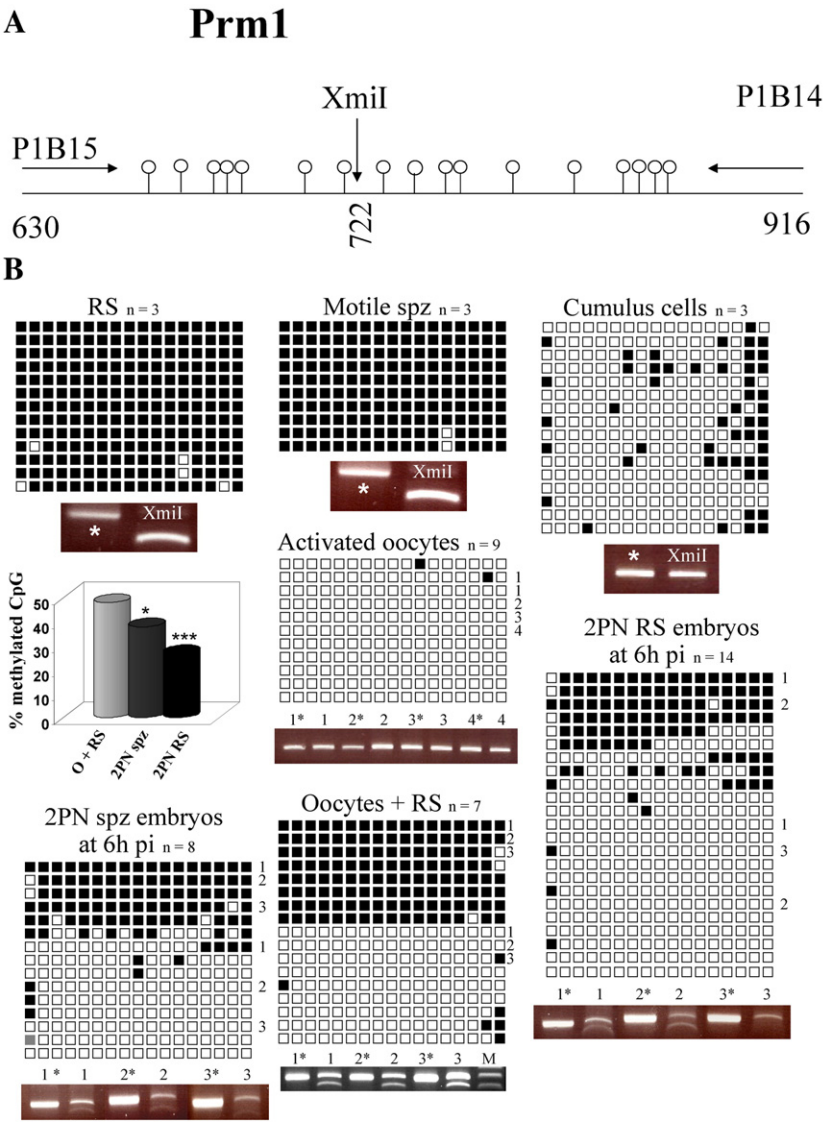


Fig. 3. Prm1 methylation profile. (A) Schematic representation of the studied fragment of Prm1 gene showing the 17 CpG sites. The restriction site of *Xmi*I (GT/CGAC) is shown with an arrow. P1B15 and P1B14, forward and reverse primers, respectively. Base numbering is according to GenBank Accession No. Z47352. Each circle represents a CpG site. (B) Methylation status of Prm1 in different cell types. Each row of squares represents a single cloned allele with 17 CpG sites. Open squares represent nonmethylated CpG sites and filled squares represent methylated CpG sites. RS, round spermatid; Spz, spermatozoa; activated oocytes were incubated with 8% ethanol in M16/bovine serum albumin for 3 min at 37 °C; 2PN, two pronuclei stage, corresponding to 6 h postfertilization; pi, postinjection, n, number of independent PCR. The frequency of cytosine methylation found in different cell populations was compared using a χ^2 test, * $p \leq 0.02$; *** $p \leq 0.001$. Each PCR-amplified product used for cloning and sequencing was digested with *Xmi*I. For each cell type, examples of the digestion products are shown under the methylation profiles. Individual DNA strand methylation profiles and digestion profiles obtained from the same PCR amplifications are assigned the same number. * not cut; M, molecular weight marker.

oocytes (Fig. 2B). Samples containing 20 activated oocytes plus 20 round spermatids were assayed to confirm that the primers used did not amplify preferentially either the methylated or the unmethylated form and that there was no cloning bias. Both the unmethylated maternal and the methylated paternal alleles were equally amplified. The analysis of ROSI one-cell embryos (2PN stage) collected at 6 h postinjection showed that the paternal allele underwent active demethylation (50% of paternal cytosine residues being unmethylated compared to 1.9% in round spermatids; $p<0.001$) and was nearly completely unmethyated in two-cell ROSI embryos at 30 h postinjection when expression is inhibited (92.3% unmethylated CpG versus 7.7% methylated CpG) (Fig. 2C). Embryos generated *in vivo* with spermatozoa also supported an active demethylation of Prm2 gene at the 2PN stage (34.4% of paternal cytosine residues were unmethylated compared to only 6.4% in spermatozoa; $p\leq0.01$) but they exhibited a higher pattern of methylation both at the one-cell stage (74.4% methylated cytosine residues) and at the two-cell stage (53.8% methylated cytosine residues) compared to ROSI embryos ($p\leq0.01$ and $p\leq0.001$, respectively). The culture of ROSI embryos in presence of 1 μM TSA, which inhibited histone deacetylation and maintained Prm2 expression, did not influence the demethylation process of the Prm2 gene observed at the two-cell stage (92.3% unmethylated CpG versus 7.7% methylated CpG).

Prm1 gene contains a small CpG island encompassing the two exons (length = 284 bp, 597–881; %GC = 64.4; ratio of observed to expected number of CpG = 0.653). Within this CpG island, the 278-bp fragment amplified, which contains 17 CpG (Fig. 3A), displayed a high methylation pattern in round spermatids and in motile spermatozoa (98% of cytosine residues methylated), whereas this region was unmethylated in activated oocytes and in cumulus cells where this gene is not expressed (Fig. 3B). According to Blake et al. [23], following bisulfite treatment, there was no workable polymorphism within the amplified region that could distinguish the maternal from the paternal allele. Particular care was taken to design primers that could equally amplify both unmethylated and methylated alleles. Both the paternal and the maternal alleles could be equally amplified when oocytes were mixed in a 1/1 ratio with round spermatids. Therefore, the significant hypomethylation observed in both spermatozoa-derived and ROSI embryos likely represents the active demethylation of the Prm1 sequence within the paternal pronucleus rather than a bias in cloning and sequencing. Furthermore, as observed for Prm2, the demethylating process at Prm1 locus occurs more rapidly in ROSI embryos than in *in vivo* embryos (27% total CpG methylated compared to 37.7%, respectively; $p<0.01$). Hypomethylation at Prm1 locus appears concomitant with the disappearance of Prm1 transcripts at 6 h postfertilization in ROSI embryos.

To confirm that the cloning was not biased, we carried out restriction analysis of the mutagenized DNA with enzymes that cleave only the methylated templates. Cleavage, with either *Xmi*I that cleaved Prm1 amplicon or *Bsh*1236I that cleaved Prm2 amplicon, was performed on each PCR-amplified product that was used for cloning and sequencing. As shown in Figs. 2B and 3B, the results of restriction enzyme analysis were comparable to those obtained from sequencing, given that some products of restriction analysis are too small to visualize on agarose gels and that only a limited number of sites can be assayed with this technique.

Discussion

We previously demonstrated that mouse preimplantation embryos generated by ROSI expressed decreased levels of some zygotic genes whereas mRNAs corresponding to genes expressed specifically in round spermatids were degraded with different kinetics [22]. In the present study, we show that Prm1 mRNA disappearance is achieved within the first 6 h following fertilization, while Prm2 mRNA disappearance was effective only after the first cell division. These results are in agreement with those of Hayashi et al. [24]. We therefore postulate that Prm1 and Prm2 gene repression may make use of different pathways because the kinetics of disappearance were different. Within the testis, protamine mRNAs are very stable; they are stored in inactive ribonucleoprotein particles up to 8 days until they are translated in elongated spermatids [25]. We verified that up to 30 h in culture, either in M2 medium or in Sertoli-cell-conditioned medium which provided the spermatids with part of their natural environment, round spermatids expressed Prm1 and 2 mRNAs. We showed that they were expressed up to 30 h, even in the presence of an inhibitor of transcription. Therefore, the rapid disappearance of protamines mRNAs in ROSI zygotes must involve not only an arrest of transcription but also an active degradation of the preexisting mRNAs. In addition, both transcription and translation inhibitors were ineffective in maintaining Prm1 and Prm2 mRNAs in ROSI embryos (data not shown), demonstrating that their repression was exerted through factors already present in the oocyte cytoplasm.

The important epigenetic remodeling that occurs in the first hours of zygote life is now well documented. Upon decondensation, the sperm nucleus acquires acetylated histones and stains positively for monomethylated H3-K9 and K27 [5], while the DNA is actively and specifically demethylated up to 6 h postfertilization [3,5,6]. High levels of histone H3 di- and trimethylated K9 and K27 were observed in the female pronucleus, whereas they are absent from the sperm nucleus [5,26,27]. The existence of an epigenetic "conversation" between histones and DNA, involving cytosine methylation, histone deacetylation, and H3-K9 methylation, leading to transcriptional silencing is now well established (reviewed in [28]). In general, acetylation reduces DNA–nucleosomal interactions to facilitate transcription and deacetylases reverse this effect. The core histones are hyperacetylated in the whole spermatid nucleus [3]. Treatment of ROSI embryos with the histone deacetylase inhibitor TSA maintained Prm2 mRNA expression up to 30 h, without affecting the demethylating process, as Prm2 was unmethylated at 30 h even in presence of TSA. Thus, histone hyperacetylation, but not DNA methylation, is necessary for Prm2 mRNA expression and HDACs are involved in Prm2 repression. Inhibition of histone deacetylation has been already implicated in the derepression of other genes [29–31]. In contrast, Prm1 expression is not restored when histone deacetylation is inhibited. This discrepancy between Prm1 and Prm2 may reflect differences in their chromatin

organization. During meiosis, somatic histones are replaced by testis-specific histones, which later in haploid cells are replaced by transition proteins and finally by protamines. In humans and mice, the genes encoding protamines 1 and 2 and transition protein 2 are clustered in a small region [32] on chromosome 16 [33]. This cluster appears to contain a mixture of histones and protamines in human sperm [34]. But while Prm2 domain is enriched in histones, Prm1 domain is enriched in protamines.

Most tissue-specific genes are hypomethylated in their expressing tissue. Thus, the 5′ region of the transition protein 1 is partially methylated in somatic tissues and undergoes hypomethylation as spermatogenesis proceeds [35]. As demonstrated by restriction enzyme analysis, the two protamines became progressively more methylated during spermatogenesis [35] and are fully methylated when they are expressed in round spermatids. Using the bisulfite mutagenesis technique, we confirm that in the 5′ coding region, both Prm1 and 2 were fully methylated in round spermatids and in motile spermatozoa, while transcription had stopped. Surprisingly, both genes were completely unmethylated in oocytes. However, Prm1 was also hypomethylated in cumulus cells while Prm2 was highly methylated in these cells. Contrary to Prm2, the Prm1 gene exhibits a small CpG island in its coding region (length=284 bp; %GC=64.4; ratio of the observed to expected number of CpG=0.653), according to the definition of Gardiner-Garden and Frommer [36]. Generally, gene expression is associated with unmethylated CpG islands within the promoter region [for review, see [37]]. A recent article demonstrated that the methylation of the coding region of Tact1, a spermatid-specific gene, was responsible for its repression in somatic cells, whereas it was hypomethylated and expressed in the adult testis [38]. The case of Prm1 appears rather singular since it is methylated in round spermatids where it is expressed and hypomethylated in oocytes and cumulus cells where it is repressed. DNA methylation may play a role in the expression of Prm1, even though the gene is methylated and repressed in spermatozoa. The demethylation of Prm1 might not be necessary in spermatozoa for its repression, because the compaction of the chromatin by the protamines at the Prm1 locus could prohibit the attachment of transcription factors and thus induce silencing of the gene.

After fertilization, Prm1 and Prm2 genes were actively demethylated. They were both approximately 50% methylated at 6 h postfertilization, although they did not exhibit the same pattern of expression. For both genes, demethylation occurred more rapidly in ROSI embryos than in sperm embryos. This delay in demethylation in sperm embryos may be due to the need for the sperm nucleus to acquire acetylated histones. Demethylation of Prm1 might participate in its silencing in ROSI embryos. However, with regard to Prm2, its pattern of methylation is not correlated with expression in ROSI embryos treated with TSA; demethylation of the paternal allele may simply be necessary to bring paternal and maternal alleles in phase to undergo somatic marking in the zygote.

Our results show not only that ROSI embryos exhibit different patterns of expression as we [22] and others [24] have previously described but also that the epigenome remodeling is not identical to that of embryos obtained from sperm, as observed by Kishigami et al. [39] for global methylation.

In conclusion, our study is the first attempt to characterize the mechanisms involved in gene silencing in the early ROSI embryos. These mechanisms are probably similar to those solicited for the management of nucleus transfer. This hypothesis makes ROSI an attractive model for studies on genome reprogramming governed by the oocyte. On the other hand, ROSI is no longer considered an attractive option to restore male fertility in ART centers; from 1995 until now, only about 12 children were born from the injection of a round spermatid. The disparity in the development between ROSI and ICSI embryos observed in several groups, using different approaches, may account for this poor efficiency.

Materials and methods

Animals

B6D2F1 mice (C57BL/6 x DBA2 hybrids) were usually used to prepare spermatogenic cells, oocyte donors, and cumulus cells. For analysis of methylation patterns of Prm2 in one-cell and two-cell embryos, fertilizations were performed with spermatozoa and round spermatids obtained from DBA2 males, while oocytes were prepared from C57BL/6 females, taking advantage of a single-basepair polymorphism that distinguishes the maternal from the paternal allele. All animals were obtained from Charles Rivers France.

Collection of oocytes

Mature oocytes were collected from 8- to 10-week old females that were induced to superovulate with 5 IU PMSG followed by 5 IU of hCG 48 h later. Oocytes were collected from the oviducts 12 to 14 h after hCG injection. They were denuded of cumulus cells by hyaluronidase treatment as previously described [22], placed in M16 medium with 4 mg/ml bovine serum albumin (BSA) and stored at 37 °C (5% CO_2/95% air). For methylation analysis, control oocytes were activated as detailed below.

Preparation of round spermatids and spermatozoa

Spermatogenic cells were obtained from 9- to 11-week-old males. Spermatozoa were collected from vas deferens as previously described [22] and allowed to capacitate for at least 1.5 h at 37 °C under 5% CO_2. Round spermatids were prepared from testis by elutriation according to the procedure of Meistrich [40].

Fertilization and embryo collection

For *in vivo* fertilization, animals were mated immediately following hCG injection to females. Microinjection of spermatozoa and round spermatids was done according to Kimura and Yanagimachi [41], modified by Ziyyat and Lefèvre [22]. Prior to injection, oocytes were activated with 8% ethanol in M16/BSA for 3 min at 37 °C. Oocytes with two decondensed pronuclei and a second polar body were considered fertilized. One-cell embryos were collected 6 h postinjection or 18 h post-hCG for *in vivo* fertilization. Two-cell embryos were collected 30 h postinjection or 42 h post-hCG for *in vivo* fertilization. Prior to collection, embryos were treated with hyaluronidase (1 mg/ml in M16, at 37 °C for 3 min) to ensure removal of any contaminating cumulus cells and rinsed three times in a large volume of phosphate-buffered-saline. When particular treatments were required, the drug (α-amanitin, cyclohexemide, or TSA; for concentrations see the appropriate figure legends) was added to the injection medium (M2; Sigma France) and to the zygote incubation medium (M16).

Nested RT-PCR

RT-PCR analysis was done on pools of 20 one-cell- or 10 two-cell-stage embryos. Following lysis and DNase treatment, embryos were subjected to an

RT-PCR protocol in one tube, as previously described [22], except that embryos were lysed by 3 cycles of thawing and freezing. The equivalent of 9.5-cell cDNA for protamines analysis and 1-cell cDNA for Hprt control were subjected to nested PCR; the cDNAs originated from the reverse transcription of total DNA from the same pool of embryos. Analysis for Prm1, Prm2, and Hprt was done on the same initial pool of embryos. Primers were the following: Prm1 forward: 5′-cacctgctcacaggttgg-3′, reverse: 5′-gtggcattgttccttagcagg-3′; inner forward: 5′-ggccagataccgatgctgccg-3′, inner reverse: 5′-cgagatgctcttgaagtctgg-3′; Prm2: forward: 5′-cgctaccgaatgaggagccccagtg-3′, reverse: 5′-ttagtgatggtgcctcctacatttcc-3′; inner forward: 5′-ggccaccaccaccacagacacaggcg-3′, inner reverse: 5′-gcatctcctcctccttcgggatcttc-3′; Hprt forward: 5′-gcgatgatgaaccaggttatgacc-3′, reverse: 5′-agtctggcctgtatccaacacttc-3′; inner forward: 5′-atgggaggccatcacattgtgg-3′, inner reverse: 5′-tggggctgtactgcttaaccag-3′. The first PCR was run for 35 cycles and the second PCR for 39 cycles (95 °C for 45 s, 60 °C for 1 min, 72 °C for 1 min). Nested PCR generated a 221-bp product for Prm1 and a 156-bp product for Prm2; both were cloned and sequenced to confirm their idendity. For Prm1 and Prm2: Genbank Accession No. Z47352. Each experiment was repeated independently at least three times.

DNA preparation and bisulfite modification

DNA was isolated from 20 cells or embryos and subjected to sodium bisulfite modification, PCR amplification, cloning, and sequencing as described [42]. The primers were designed to amplify mutagenized DNA at the one-cell level. Two rounds of PCR were done with nested primer pairs. They were as follows: Prm1 forward: 5′-gttaagttagtattatggttagatatygatg-3′, reverse: 5′-craaatactcttaaaatctaataaaattctc-3′; inner forward: 5′-gttaagttagtattatggttagatatygatg-3′, inner reverse: 5′-actattctatacatctaatatttttttacacc-3′; Prm2 forward: 5′-aatgaggagtttttagtgagggttygtatt-3′, reverse: 5′-aatacctcctacatttcctacacctacatc-3′; inner forward: 5′-ttygtattagggggtttggataagatta-3′, inner reverse: taaataaacaacaacaacacaaataacca-3′. Each PCR was run for 39 cycles (Prm1 first round: 95 °C for 45 s, 61 °C for 1 min, 72 °C for 1 min; second round: 95 °C for 45 s, 58 °C for 1 min, 72 °C for 1 min; Prm2 first round: 95 °C for 45 s, 52 °C for 1 min, 72 °C for 1 min; second round: 95 °C for 45 s, 61 °C for 1 min, 72 °C for 1 min). As shown by us [42] and others [43], the determination of the DNA methylation status of genes is reliable when performed on limited samples (down to the one-cell level) if the results are properly analyzed: amplification of a given pattern among a few DNA molecules is random, and it is impossible to determine whether two identical patterns are derived from two original alleles exhibiting identical methylation patterns or whether they both result from the amplification of the same allele. Unless the amplification is done on a sample of several thousand oocytes, the statistical validity of the data obtained from a single sample with a single amplification is questionable. One way to overcome this difficulty is to multiply the number of independent amplifications from independent samples and sequence only two to four clones per experiment. We have considered that different methylation profiles obtained from the same amplification and the same methylation profile obtained from independent amplifications obviously originated from an independent allele. The frequency of cytosine methylation found in different cell populations was compared using a χ^2 test. The sequencing results were validated by enzyme restriction analysis of the same PCR product used for cloning, with enzymes that cut only the methylated templates. Prm1 products were cut with *Xmi*I (GT/CGAC) and Prm2 products with *Bsh*12361 (CG/CG). *Xmi*I digestion of Prm1 PCR product generated two fragments of 194 and 83 bp. *Bsh*1236l has two restriction sites within the PCR fragment amplified with the Prm2 primers and generated 244- and 33-bp fragments when the first site only is cut, 205- and 72-bp fragments when the second site only is cut, and 205-, 39-, and 33-bp fragments when both sites are cut.

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