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Epigenetic abnormalities of the mouse paternal zygotic genome associated with microinsemination of round spermatids

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Abstract

Although round spermatid injection can be used to create progeny for males who do not produce mature sperm, the rate of successful embryogenesis after such procedures is significantly lower than that for similar procedures using mature spermatozoa. The mechanisms underlying this difference are unknown. In this study, we demonstrate that, unlike the normal paternal genome, the paternal zygotic genome derived from a round spermatid is highly remethylated before first mitosis after demethylation. Genomes from elongated spermatids exhibited an intermediate level of DNA methylation, between those of round spermatids and mature spermatozoa, suggesting that the male germ cell acquires the ability to maintain its undermethylated state in the paternal zygotic genome during this phase of spermiogenesis. In addition, treatment of zygotes with trichostatin A led to a significant reduction in DNA methylation, specifically in the spermatid-derived paternal genome, except for the pericentromeric regions enriched by trimethylation of Lys9 of histone H3. These data provide insight into epigenetic errors that may be associated with the poor development of embryos generated from immature spermatozoa.

Keywords: Mouse; DNA methylation; Microinsemination; Round spermatid injection; Zygote

Introduction

Round spermatids are haploid cells of the spermatogenic line from which spermatozoa are subsequently formed through meiosis and a series of morphological and molecular modifications termed spermiogenesis (reviewed by de Kretser and Kerr, 1994). This process includes nuclear chromatin condensation, loss of excess cytoplasm, acrosome development, acquisition of oocyte-activating factor, and formation of a flagellum. In mice, the significantly lower success rate for offspring production following round spermatid injection (ROSI), as compared to intracytoplasmic sperm injection (ICSI) (Ogura et al., 1992; Kimura and Yanagimachi, 1995; Kishigami et al., 2004a; Yanagimachi, 2005), may arise from differences in spermatozoon and spermatid characteristics. In particular, differences in chromatin structure between spermatids and mature sperm (Kimmins and Sassone-Corsi, 2005) may directly affect the later epigenetic status of the paternal genome, such as DNA methylation in the zygote.

In addition to histone modification, DNA methylation, which occurs predominantly at CpG dinucleotides in mammals, serves as an epigenetic mark for chromatin and is involved in genomic functions including gene silencing, imprinting, X chromosome inactivation, and silencing of retrotransposons (reviewed by Bird and Wolffe, 1999; Li, 2002). Inactivation of the genes for three DNA cytosine methyltransferases (DNMTs) (Dnmt1, Dnmt3a, and Dnmt3b) leads to genome demethylation and lethality, indicating their essential roles during development (Li et al., 1992; Okano et al., 1999); these enzymes either maintain DNA methylation or catalyze de novo DNA methylation. Recent studies have identified protein partners for the DNMTs including histone deacetylases, histone methyltransferases, and transcription factors (reviewed by Robertson, 2002; Freitag and Selker, 2005). Consistent with interactions between DNMTs and histone H3 Lys9 methyltransferases, accumulating evidence suggests an evolutionary link between pathways for

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histone H3 Lys9 methylation and DNA methylation (Freitag and Selker, 2005).

In some mammalian species, including mouse and human, but not sheep or rabbit, the zygotic paternal genome is widely demethylated shortly after fertilization but before DNA replication, whereas the maternal genome remains highly methylated (Mayer et al., 2000; Oswald et al., 2000; Dean et al., 2001; Santos et al., 2002; Beaujean et al., 2004). This asymmetric patterning of DNA methylation in each parental genome at the one-cell embryo stage persists at least until the two-cell embryo (Barton et al., 2001). Although this asymmetric methylation state is believed to be responsible for functional differences between the parental genomes during development, its biological significance remains unclear.

In this study, we evaluated the DNA methylation status of zygotic paternal genomes derived from male germ cells during spermiogenesis. Our results indicate that (1) embryos formed from spermatid microinsemination start to develop with aberrant genome-wide DNA methylation states in their paternal genomes; (2) the male germ cell acquires the ability to maintain its undermethylated state in the paternal zygotic genome during spermiogenesis, independent of the acquisition of oocyte-activating ability; and (3) treatment of zygotes with trichostatin A (TSA) leads to a significant reduction in DNA methylation specific to spermatid-derived zygotic genomes, except in pericentromeric heterochromatin marked by H3 Lys9 trimethylation. These findings provide insight into the reprogramming of zygotic paternal genomes derived from immature spermatozoa.

Materials and methods

Animals

B6D2F1 mice (C57BL/6 × DBA/2 hybrids) were used to prepare spermatogenic cells and as oocyte donors. All animals were obtained from SLC (Shizuoka, Japan) and were maintained in accordance with the Animal Experiment Handbook at the Riken Centre for Developmental Biology.

Collection and culture of oocytes

Collection and culture of oocytes were performed according to described protocols (Kishigami et al., 2004a). Briefly, oocytes were collected from oviducts approximately 16 h after hCG injection and placed in CZB medium. The oocytes were then placed in KSOM culture medium (Specialty Media, Phillipsburg, NJ, USA) and incubated at 37°C under 5% CO_2. For aphidicolin- or 5-azacytidine treatment of fertilized oocytes, oocytes microinjected with sperm or spermatids were transferred into KSOM medium containing 2 mg/ml aphidicolin (Sigma Chemical Co., St. Louis, MO, USA), 10 mM 5-azacytidine (Sigma), or 500 nM TSA (Sigma) within 2 h after ICSI or ROSI. For demecolcine treatment, oocytes microinjected with sperm or spermatids were transferred into KSOM medium including 20 ng/ml of demecolcine (Wako; Osaka, Japan) 8 h after ICSI or ROSI and cultured for an additional 12 h.

Microinsemination with spermatozoa (ICSI) and round spermatids (ROSI)

Collection of spermatogenic cells and injections were performed according to previously described methods (Kishigami et al., 2004a). For ICSI, the head of each spermatozoon was separated from the tail by applying pulses to the head–tail junction by means of a Piezo-driven pipette (PrimeTech; Ibaraki, Japan). Only the sperm head was injected into each oocyte. For ROSI, oocytes were activated by incubation in Ca^{2+}-free CZB medium containing 5 mM $SrCl_2$ for 20 min. Forty to 80 min after activation, the oocytes were injected with the nuclear region of a round spermatid (characterized by a ~10-μm diameter and a centrally located, distinct nucleolus) (Kishigami et al., 2004b). For heat-inactivated sperm injection, sperm were incubated at 56°C for 30 min before ICSI (Cozzi et al., 2001), which was performed following oocyte activation as described above for ROSI.

Indirect immunofluorescence

Zygotes generated by ICSI or ROSI were fixed overnight at 4°C in phosphate-buffered saline (PBS) containing 4% paraformaldehyde. The fixed zygotes were washed in PBS containing 0.1% polyvinyl alcohol (PBS/PVA) and blocked in PBS containing 3% bovine serum albumin (BSA) and 0.2% Triton X-100 overnight at 4°C. All subsequent steps were carried out at room temperature.

Primary antibody incubations were carried out in PBS/PVA for 2–3 h. Primary antibodies against trimethyl lysine 9 on histone H3, acetyl-histone H3, and acetyl-histone H4 (Upstate Biotechnology, Waltham, MA, USA; catalogue numbers 07-523, 06-599, 06-598, respectively) were used. After extensive washing, oocytes were stained with Alexa Fluor-488 secondary antibodies (Molecular Probes, Eugene, OR, USA). Zygotes were then mounted on a drop of Vectashield (Vector Laboratories) containing 4′,6′-diamidino-2-phenylindole (DAPI). DNA was stained with 5 μg/ml DAPI for 30 min and mounted in Vectashield. Specimens were examined using an Olympus BX51 microscope (Olympus; Tokyo, Japan). All images were captured with a DP70 Olympus digital camera using Olympus Analysis software to apply color to DAPI-stained DNA (red) and merged images.

For 5-methyl-cytosine detection, fixed zygotes were permeabilized with 0.2% Triton X-100 in PBS for 15 min at room temperature. They were then treated with 2 N HCl at room temperature for 50 min, neutralized for 20 min in 100 mM Tris–HCl buffer (pH 8.0) (Dean et al., 2001), and extensively washed with PBS containing 1% BSA. Subsequent steps followed the standard protocol for indirect immunofluorescence, as described above. An anti-5-methylcytidine antibody (Eurogentec; Seraing, Belgium; catalogue number MMS-900S-B) was used.

For quantitative analysis of pronuclear DNA methylation levels, fluorescence images were subjected to densitometric analysis using the program Image-J from the National Institutes of Health (http://rsb.info.nih.gov/ij/) (USA). For each pronucleus, the relative intensity of methylation (RIM) of the paternal genome was calculated as a percentage of the fluorescence intensity of the maternal genome.

Histone H3 Lys9 trimethylation in spermatozoa and round spermatids in seminiferous tubules was detected by indirect immunofluorescence. Freshly collected seminiferous tubules were mounted on slides and fixed in absolute ethanol for 10 min (Parvinen and Hecht, 1981). They were then immunostained for trimethylation of H3 Lys9.

Statistical analysis

Data were analyzed by Scheffe tests for multiple mean comparisons using the statistical program SPSS version 12.0 (SPSS Inc., Chicago, IL, USA). All percentile data were subjected to arcsine transformation before statistical analysis.

Results

DNA methylation after ICSI and ROSI

Methylation states of paternal genomes after ICSI and ROSI were examined at various times after injection. Four hours after injection, spermatid-derived paternal genomes exhibited methylation similar to or greater than that of sperm-derived