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Time-lapse and retrospective analysis of DNA methylation in mouse preimplantation embryos by live cell imaging

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Abstract

Genome-wide change of DNA methylation in preimplantation embryos is known to be important for the nuclear reprogramming process. A synthetic RNA encoding enhanced green fluorescence protein fused to the methyl-CpG-binding domain and nuclear localization signal of human MBD1 was microinjected into metaphase II-arrested or fertilized oocytes, and the localization of methylated DNA was monitored by live cell imaging. Both the central part of decondensing sperm nucleus and the rim region of the nucleolus in the male pronucleus were highly DNA-methylated during pronuclear formation. The methylated paternal genome undergoing active DNA demethylation in the enlarging pronucleus was dispersed, assembled, and then migrated to the nucleolar rim. The female pronucleus contained methylated DNA predominantly in the nucleoplasm. When the localization of methylated DNA in preimplantation embryos was examined, a configurational change of methylated chromatin dramatically occurred during the transition of 2-cell to 4-cell embryos. Moreover, retrospective analysis demonstrated that a noticeable number of the oocytes reconstructed by round spermatid injection (ROSI) possess small, bright dots of methylated chromatin in the nucleoplasm of male pronucleus. These ROSI oocytes showed a significantly low rate of 2-cell formation, thus suggesting that the poor embryonic development of the ROSI oocytes may result from the abnormal localization of methylated chromatin.

Keywords: Live cell imaging; Retrospective analysis; DNA methylation; ICSI; ROSI; Preimplantation development

Introduction

Methylation of the cytosine residue in CpG dinucleotide plays a crucial role in the regulation of gene suppression such as X-chromosome inactivation, genomic imprinting, and inactivation of transposable elements (Ferguson-Smith and Surani, 2001; Jones and Takai, 2001; Jones et al., 1998; Li, 2002). The genome-wide change of DNA methylation is also implicated in the nuclear reprogramming during preimplantation development (Fulka et al., 2004; Reik et al., 2001). Following fertilization, methylated zygotic genomes undergo globally active and passive DNA demethylation in preimplantation development, and then the parental genomes acquire new methylation patterns specific for the cell types after the implantation of embryos. Embryos, which have been reconstructed by nuclear transfer of somatic cells, occasionally exhibit an aberrant pattern of DNA methylation at the preimplantation stages (Beaujean et al., 2004; Dean et al., 2001; Kang et al., 2001). In addition, embryos cultured under unusual conditions result in a developmental failure because of the disordered methylation pattern (Shi and Haaf, 2002). Thus, the precise regulation of DNA methylation in the preimplantation embryos is essential for the normal development.

Indirect immunostaining using anti-5-methylcytosine (5mC) antibody is usually utilized to examine the state of DNA methylation in fertilized oocytes and early embryos. However, there are several disadvantages for the use of the antibody. The intact chromatin structure is probably destroyed by an HCl treatment required for enhancement of the antibody accessibility to methylated DNA (Jorgensen et al., 2006). It is also difficult to observe living cells in real time due to the cell fixation. We (Yamagata et al., 2005) have recently established a monitoring system that allows visualization of methylated DNA in living oocytes and embryos previously microinjected with a synthetic RNA encoding enhanced green fluorescence protein

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(EGFP) fused to the methyl-CpG binding domain (MBD) and nuclear localization signal (NLS) of human methyl-CpG binding protein 1 (MBD1) (Fujita et al., 1999).

In this study, we have carried out time-lapse and retrospective analysis of DNA methylation in living mouse preimplantation embryos, using the above monitoring system. Time-lapse recording provides several new insights into the methylated chromatin dynamics in the (pro)nuclei of fertilized oocytes and early embryos: the migration and rearrangement of DNA-methylated sperm genome during pronuclear formation, the localization of methylated DNA in the male and female pronuclei, and the reconstitution of methylated chromatin during preimplantation development. Moreover, the comparison of DNA methylation states between the oocytes reconstructed by intracytoplasmic sperm injection (ICSI) and round spermatid injection (ROSI) shows the abnormal localization of methylated chromatin only in the male pronuclear nucleoplasm of the ROSI oocytes. This abnormality may explain the reason for a low rate of successful embryogenesis after ROSI.

Materials and methods

Plasmid construction

Expression plasmids encoding fusion proteins of EGFP or monomeric DsRed (mRFP, Clontech, Mountain View, CA) with MBD and NLS of human MBD1, and of EGFP with human centromeric protein B (CENPB) were constructed as described (Shelby et al., 1996; Yamagata et al., 2005). Briefly, DNA fragments encoding MBD, NLS, and CENPB were PCR-amplified from a first-strand cDNA library of HeLa cells using following primer sets: 5′-CCAAGCTTCATGGCTGAGGACTGGCTGGAC-3′ and 5′-AAGCGGCCGCTTACGGGGCCTCCTTCCTGACCT-3′ for MBD; 5′-CCAAGCTTCTGCTATCCAGCCCCCAAGGC-3′ and 5′-AAGCGGCCGCTTACGGGGCCTCCTTCCTGACCT-3′ for NLS; 5′-AGAATTCGCCACCATGGGCCCCAAGAGGCGACAG-3′, and 5′-GGAAGCTTGAGCTCGAGATCAGTGCTCCCGCCACTGCCCT-3′ for CENPB. The amplified fragments were digested by appropriate restriction enzymes, and ligated to a pcDNA 3.1–polyA vector (Yamagata et al., 2005) together with a DNA fragment of EGFP or mRFP. Two MBD mutants (Fujita et al., 2000), R30A and D32A, containing Ala residues instead of Arg and Asp at positions 30 and 32 in MBD, were prepared by site-directed mutagenesis using the following primer sets: 5′-CCACCTGTGGAGCCTCAGACACCTATTAC-3′ and 5′-GTAATAGGTGTCTGAGGCTCCACAGGTGG-3′ for R30A, and 5′-GGACGCTCAGCCACCTATTACC-3′ and 5′-GGTAATAGGTGGCTGAGCGTCC-3′ for D32A. For the expression of EGFP–MBD–NLS plasmids in *Escherichia coli*, the MBD fragments were amplified by using 5′-CCAAGCTTCATGGCTGAGGACTGGCTGGAC-3′ and 5′-AAGCGGCCGCCGGGGCCTCCTTCCT-3′, digested by restriction enzymes, and then introduced into a pET23d (Novagen, Madison, WI) vector with a fragment of EGFP from pEGFP-C2 (Clontech, Mountain View, CA). The plasmids were expressed in *E. coli* BL21 (DE3), and recombinant proteins produced were purified by using an Ni-NTA His Bind resin (Novagen, Madison, WI). Expression plasmids encoding 386- and 42-residue *Spiroplasma monobiae* CpG methyltransferase SssI fused to the gal4 binding domain (GAL4BD) at the N-terminus were prepared as described (Yamagata et al., 2005). GAL4BD was used for the transport of SssI to the cell nucleus.

Dot blot assays

A CpG-methylated DNA was prepared by treatment of pUC19 with SssI methyltransferase (New England Biolabs, Ipswich, MA) according to the manufacture's instruction. The methylated plasmid was recovered using a Wizard DNA Clean-up kit (Promega, Madison, WI), extracted with phenol–chloroform, and precipitated by ethanol. The methylated pUC19 DNA was spotted on Hybond-N^+ nylon membrane (GE Healthcare Bio-Sciences Corp., Piscataway, NJ), fixed with 0.4 M NaOH, washed, blocked with 5% skim milk, mixed with recombinant proteins (8 μg/ml) or anti-5mC antibody (Eurogentec, Seraing, Belgium, catalogue number MMS-900P-A), and incubated for 60 min at room temperature in phosphate-buffered saline (PBS). Fluorescence was measured by a Molecular Imager FX fluorescence scanner (Bio-Rad Laboratories, Hercules, CA).

Cell culture

NIH3T3 cells were cultured in Dulbecco's modified essential medium (Gibco BRL, Gaithersburg, MD) supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 0.1 mg/ml streptomycin, and 0.5 μM DNA methyltransferase inhibitor 5-aza-2′-deoxycytidine (5-AzaC, ICN Biomedicals, Inc., Costa Mesa, CA) at 37 °C under 5% CO_2 in air for 4 days. EGFP–MBD–NLS plasmid was co-transfected with mRFP–NLS plasmid into 5-AzaC-treated NIH3T3 cells, using a PerFectin transfection reagent (Gene Therapy System, San Diego, CA). Cells were further cultured in the above medium for 2 days, and then observed under an Olympus IX-70 fluorescent microscope (Tokyo, Japan).

Fig. 1. Specific binding of EGFP–MBD–NLS fusion protein to methylated DNA. (A) Production of recombinant fusion proteins. His-tagged recombinant proteins of EGFP–MBD–NLS, EGFP–R30A–NLS, and EGFP–D32A–NLS (arrow) were produced in *E. coli*, and purified by using Ni-NTA His Bind resin. After SDS–PAGE, proteins on gels were stained with Coomassie brilliant blue. (B) Binding of EGFP–MBD–NLS to methylated pUC19 DNA in vitro. The CpG-methylated pUC19 was prepared by treatment with SssI methyltransferase. The methylated (+) and unmethylated (−) DNAs (0–0.4 μg) were spotted onto Hybond-N^+ nylon membranes, incubated with recombinant proteins (8 μg/ml) or anti-5mC antibody (5mC), washed, and observed by a fluorescence scanner. Data are expressed as means ± S.E., where $n = 3$. (C) Localization of EGFP–MBD–NLS fluorescence in pronuclear-stage oocytes. Metaphase II-arrested oocytes were injected with RNA encoding each of four EGFP fusion proteins (16, 16, 14, and 10 oocytes for EGFP–MBD–NLS, EGFP–R30A–NLS, EGFP–D32A–NLS, and EGFP–NLS, respectively), inseminated, incubated for 15 h, and observed under a fluorescent microscope. Dotted signals (arrows) are present only in the EGFP–MBD–NLS-expressing female pronucleus. Scale bar: 20 μm. (D) Binding of EGFP–MBD–NLS to methylated pUC19 DNA in vivo. EGFP–MBD–NLS RNA was co-injected with methylated and unmethylated pUC19 (250 ng/μl) into the cytoplasm of fertilized oocytes (34 and 30 cells, respectively), incubated for 12 h, and observed. Arrows indicate the localization of EGFP–MBD–NLS in the nucleus (left panel) or cytoplasm (right panel) of the oocytes. (E) Fluorescent patterns of EGFP–MBD–NLS in pronuclei of fertilized oocytes containing enzymatically active or inactive CpG methyltransferase SssI. EGFP–MBD–NLS RNA was co-injected into fertilized oocytes (51 and 32 oocytes) with RNA encoding 386-residue (active) and 42-residue (inactive) SssI fused to GAL4BD, respectively. The male and female pronuclei were observed 12–15 h after insemination. Scale bar: 20 μm. (F) Fluorescent patterns of NIH3T3 cells treated with DNA methylation inhibitor 5-AzaC. Cells were co-transfected with EGFP–MBD–NLS and mRFP–NLS plasmid DNAs, cultured in the presence (+) and absence (−) of 5-AzaC (AzaC), and observed under a fluorescent microscope. Untransfected cells were co-probed with anti-5mC (5mC) and anti-histone H2B antibodies. The EGFP fluorescence of 14 and 15 cells treated with and without 5-AzaC was measured (20 and 25 cells for anti-5mC antibody), respectively. The fluorescent intensities of the signals detected by anti-5mC antibody and EGFP–MBD–NLS were normalized to those by anti-histone H2B antibody and mRFP–NLS, respectively. Scale bar: 20 μm. (G) Fluorescent patterns of EGFP–MBD–NLS at the metaphase stage of NIH3T3 cells treated with 5-AzaC. Cells expressing EGFP–MBD–NLS were cultured in the presence (+) and absence (−) of 5-AzaC. Following counterstaining with Hoechst 33342 (Hoechst), 24 and 10 cells treated with and without 5-AzaC, respectively, were observed. The images merged are represented as green and red colors for EGFP–MBD–NLS and Hoechst, respectively. Scale bar: 20 μm.