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Global DNA Methylation of Porcine Embryos during Preimplantation Development

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ABSTRACT

DNA methylation at CpG sites, which is a epigenetic modification, is associated with gene expression without change of DNA sequences. During early mouse embryogenesis, dynamic changes of DNA methylation occur. In this study, DNA methylation patterns of porcine embryos produced *in vivo* and *in vitro* were examined at various developmental stages by the immunocytochemical staining method. Interestingly, active demethylation was not observed on the paternal pronucleus of porcine zygotes. However, differences were detected in the passive demethylation process between *in vivo* and *in vitro* embryos. There was no change in the DNA methylation state until the blastocyst stage of *in vivo* embryos, whereas partial demethylation was observed in several blastomeres from a 4 cell stage to a morula stage of *in vitro* embryos. The whole genome of inner cell mass (ICM) and trophectoderm (TE) cells in porcine blastocysts were evenly methylated without *de novo* methylation. Our findings demonstrate that genome-wide demethylation does not occur in pig embryos during preimplantation development unlike murine and bovine embryos. It indicates that the machinery regulating epigenetic reprogramming may be different between species.

(Key words : DNA methylation, 5-Methyl-cytosine, Immunostaining, Porcine embryos)

Ⅰ. INTRODUCTION

The developmental fate during embryogenesis is determined by both genetic and epigenetic regulations in mammals. Epigenetic modification such as DNA methylation and histone modification, which is no any change of DNA sequence, plays a important role in the tissue specific gene expression, global gene silencing and carcignogenesis. The disruption in the chromatin structure or epigenetic anomaly can lead to the dysregulation of developmental processes such as X chromosome inactivation, genomic imprinting and regulation of

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transposon genes. During mouse embryogenesis, the significant role of the epigenetic modification has been shown in mutations of Dnmt1, which encodes the protein for the maintenance methylation, or Dnmt3a and 3b which encode proteins required for *de novo* methylation. The mutations induce embryonic lethal at post-implantation stage due to possibly involving apoptosis (Jaenisch and Bird, 2003). In mouse early embryogenesis, there is an orchestrated change of DNA methylation. It starts with a wave of active demethylation on the paternal pronucleus prior to the first replication (Mayer et al., 2000; Oswald et al., 2000). After the completion of the first cell cycle, a genome-wide loss of DNA methylation goes on until morula stage because of the absence of Dnmt1 (Carlson et al., 1992; Rougier et al., 1998). The passive DNA demethylation, from zygotes to morulae, has been observed on various tissue-specific genes, housekeeping genes and repetitive sequences. At the blastocyst stage, *de novo* methylation appears dominantly on the genome of ICM (Jaenisch, 1997; Dean et al., 2001).

To monitor epigenetic reprogramming during preimplantation development in the pigs, in this study, DNA methylation patterns of *in vivo*- and *in vitro*-derived embryos have been investigated at various developmental stages by a immunocytochemical staining method using the 5-methyl cytosine antibody. In addition, it has been examined wether polyspemy affect DNA methylation in the porcine zygotes.

Ⅱ. MATERIALS AND METHODS

1. Culture Media

Unless otherwise mentioned, all chemicals used in this study were purchased from Sigma Chemical Co (St. Louis, MO). The medium used for porcine oocyte maturation was BSA-free North Carolina State University (NCSU) 23 medium (Petters and Wells, 1993) supplemented with 10% (v/v) porcine follicular fluid, 0.6 mM cysteine, 25 mg/ml gentamycin, 10 ng/ml EGF, 2.5 mM beta-mercaptoethanol, 10 IU/ml PMSG, and 10 IU/ml hCG. Porcine follicular fluid was collected from follicles of 3~6 mm in diameter, centrifuged 3 times at 2000×g for 30 min at 4℃, filtered through 0.8 μm syringe filters (Gelman Sciences, Ann Arbor, MI), and stored in aliquots at −20℃ until use. The basic medium used for IVF was essentially the same as described by Abeydeera and Day (1997). This IVF medium, designated and modified Tris-buffered medium (mTBM), consists of 113.1 mM NaCl, 3 mM KCl, 7.5 mM $CaCl_2 \cdot 2H_2O$, 20 mM Tris (crystallized free base; Fisher Scientific, Fair Lawn, NJ), 11 mM glucose, and 5 mM sodium pyruvate.

2. *In Vitro* Maturation, Fertilization and Culture

Porcine ovaries were collected from a local slaughterhouse and transported to the laboratory at 25~30℃ in 0.9% (w/v) saline supplemented with 75 μg/ml potassium penicillin G and 50 μg/ml streptomycin sulphate. Cumulus-oocyte complexes (COCs) were obtained from follicles with a diameter of 3 to 6 mm using an 18-gauge needle connected to a 10 ml disposable syringe (Funahashi et al., 1994). The COCs were washed 3 times with Tyrode's lactate (TL)-Hepes medium. Approximately 50 COCs were cultured in 500 μl of maturation medium in the each well of a 4-well multidish at 39℃, in 5% CO_2 (Nunc, Roskilde, Denmark). After 22 hr of culture, oocytes were washed 3 times with TL-Hepes medium and further cultured in the maturation medium without hormone supplements (PMSG and hCG) for 22 hr. After the completion of IVM, cumulus cells of oocytes were removed by treatment with 0.1% (w/v) hyaluronidase in TL-Hepes medium. The denuded oocytes

were washed 3 times with the mTBM supplemented with 2.5 mM caffeine and 1 mg/ml BSA (A 6003) and placed into 48 μl of mTBM under paraffin oil. The semen was washed 3 times by centrifugation with DPBS supplemented with 1 mg/ml BSA (Fraction V, A 9647), 100 μg/ml penicillin, and 75 μg/ml streptomycin. At the end of washing, the spermatozoa were resuspended in mTBM. Two microliter of diluted spermatozoa was added to 48 μl of the fertilization medium containing oocytes to give a final sperm concentration of 1.2×10^5 cells /ml. The oocytes were co-incubated with spermatozoa for 6 hr at 39℃ in 5% CO_2. The fertilized eggs were cultured in 50 μl drops of NCSU 23 medium supplemented with 4 mg/ml BSA at 39℃ in 5% CO_2. Embryos were collected 20 hr, 48 hr, 72 hr , 96 hr, and 120 hr after IVF. In case of polyspermic embryos, the matured oocytes were fertilized with sperm at the concentration of 6.0×10^5 cells/ml and the resulting embryos were fixed at 10 hr, 12 hr and 17 hr after insemination.

3. *In Vivo*-Derived Embryos

Six prepubertal gilts (Landrace, approximately 6 months old and weighing 80 to 100 kg) were used to obtain *in vivo*-derived embryos. They were fed with 20 mg altrenogest daily for 9 days beginning on Day 16 of the estrus cycle, treatedwith PMSG (1500 IU, Folligon, Intervet, Boxmeer, Netherlands) at 24 to 30 hr after the last feeding of altrenogest and with hCG (750 IU, Pregnyl, Organon Inc., West Orange, NJ) after additional 78 hr. The gilts were hand-mated with boars 12 and 24 hr after hCG treatment. 1 cell stage, 2 cell stage, 4 cell stage, 8 cell stage, morua stage, and blastocyst stage embryos were surgically collected corresponded day after mating by flushing of the uterus with 50 ml PBS after cannulating the ovarian end of the uterine horn and then recovered under the dissecting microscope (Olympus).

4. Immunocytochemical Staining

Metaphase-arrested oocytes and preimplantation embryos were washed with PBS solution. The samples were fixed with fresh 4% formaldehyde in PBS for 1 hr at 4℃ and then washed for 1 hr with PBS containing 0.1% Tween-20. The oocytes and embryos were permeabilized with 0.5% Triton X-100 in PBS for 2 hr at room temperature. For the detection of 5-methylcytosine, the samples were treated with 2 M HCl at room temperature for 30 min and subsequently neutralized for 10 min with 100 mM Tris HCl buffer (pH 8.5) after permeabilization. The embryos were blocked with 1% BSA and 0.1% Tween-20 in PBS overnight at 4℃. The embryos were incubated with monoclonal antibody anti-5-methylcytosine (Eurogentec, Belgium) for 3 hr and washed with 0.1% Tween-20 in PBS for 3 hr. Secondary antibody, anti-mouse coupled with Cy3, was treated to the samples for 30 min at 4℃ and washed subsequently for 4 hr at room temperature. DNA was stained with the intercalating dye YOYO-1 iodide (Molecular Probes) at 100 nM and mounted on slides after air dry and observed with a fluorescent microscope (Olympus, Japan).

Ⅲ. RESULTS AND DISCUSSION

1. The DNA Methylation Pattern of *In Vivo*-derived Porcine Embryo

In order to study epigenetic fates during porcine embryogenesis, particularly preimplantation development, we observed the DNA methylation pattern of porcine embryos produced *in vivo* and *in vivo* using the immunocytochemistry staining method with the 5-methylcytosine antibody. The methyltion pattern of mouse embryos have been revealed by immunostaining (Dean et al., 2001). When the mouse zygote was stained with the 5-methylcytosine antibody, active demethylation was observed

on the paternal pronucleus as previously reported (Dean et al., 2001) (Fig. 1A). In metaphase-arrested porcine oocytes, the staining pattern of the 5-methylcytosine was co-localized with that of the YOYO (Fig. 1B). This result indicates that the genome of the metaphase-arrested oocyte is fully methylated. Next experiment was to examine the methylation patterns of *in vivo*-derived embryos from 1 cell to blastocyst stages. The antibody staining pattern of male and female pronuclei in the zygote was the same as YOYO staining (Fig. 2A). It was difficult to determine the sex between two pronuclei because of their similarity in size. In this study, however, active demethylation was not observed in the pig unlike the mouse. In the mouse,

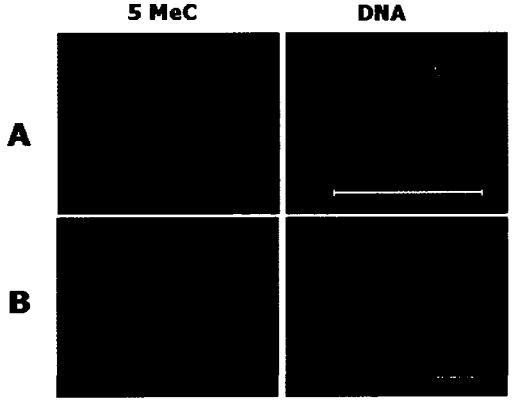

Fig. 1. Genomic DNA methylation status in the mouse zygote and the porcine oocyte. A. The mouse zygote. Maternal pronucleus is designated as 'm' and paternal pronucleus 'p'. The pb indicates polar body. B. Matured porcine oocyte. Porcine egg has a set of parental pronuclei. More than 20 eggs and oocytes were stained with the monoclonal antibody specific for 5-methylacytosine and then with Cy3-conjugated secondary antibody (red), respectively. DNA was stained with DAPI (blue) (A) or YOYO-1 (green) (B). Images were captured by using the Olympus Epifluorescent microscopy. Scale bar is 20 μm.

paternal genome of the zygote is selectively demethylated at 4 hr after fertilization and the maternal genome remain methylated (Santos et al., 2002), representing active demethylation. Active demethylation in mouse zygotes is referred to chromatin remodeling, which is began with protamine exclusion from sperm genome and histone addition to the paternal genome after fertilization.

This modification does not occur on the maternal genome. The replacement of histone proteins with protamines immediately appears after insemination and is conducted prior to the first DNA replication (Nonchev and Tsanev, 1990; Santos et al., 2002). The chromatin remodeling may be required to stimulate the demethylation and the absence of the remodeling in maternal pronucleus may explain the persistence of methylation during the 1 cell stage (Santos et al., 2002). In the porcine embryos, however, the replacement of nuclear proteins by histone proteins and dissociation of protamines occur before the decondensation of sperm nuclei during *in vitro* fertilization. The two changes in the sperm nucleus appear at 4~5 hrs after insemination (Shimada et al., 2000; Nakazawa et al., 2002). Although the chromatin remodeling might occur in porcine embryos like mouse embryos, there was no change in DNA methylation of porcine embryos during preimplantation development (Fig. 2). It is probably suggested that the change of chromatin structure wouldn't be a prerequisite factor for active demethylation prior to the decondensation of a sperm nucleus in the 1 cell embryo. It is also speculated that unknown oocyte factors regulating DNA methylation may exist and may be dependent on species. At this time, why active demethylation does not occur in the porcine zygotes remains elusive.

From the 2-cell to blastocyst stages, the staining patterns of 5-methyl cytosine were also similar to YOYO staining (Fig. 2B~F). No differences were detected in the methylation states between ICM and

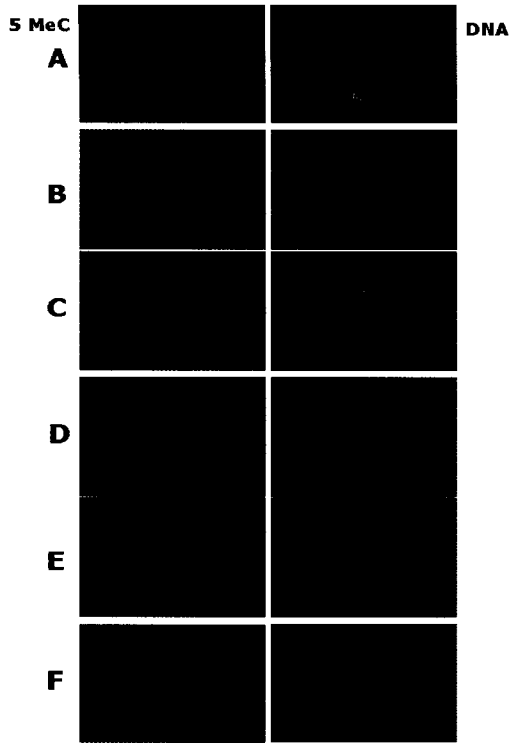

Fig. 2. DNA methylation patterns of *in vivo*-derived porcine embryos. Embryos were collected at the 1 cell (A), 2 cell (B), 4 cell (C), 8 cell (D), morula (E), and blastocyst stages (F). More than 20 eggs for each stage were stained with a monoclonal antibody specific for 5-methylacytosine and then with Cy3-conjugated secondary antibody (red). DNA was stained with YOYO-1 (green). Images were captured by using the Olympus Epifluorescent microscopy. Scale bar is 20 μm.

TE cells of porcine blastocysts (Fig. 2F). Therefore, our data demonstrated that passive demethylation until the morula stage and *de novo* methylation at the blastocyst stage were not observed in early porcine embryos. The result obtained from this study is a contrast to that of mouse embryos reported by Dean et al. (2001). In mouse embryos, passive demethylation occurs throughout 2 cell to morula stages and then ICM cells are dominantly *de novo* methylated at the blastocyst stage. The reprogramming of bovine embryos also follows the same pattern like the mouse embryos (Dean et al., 2001). The modifications in mouse embryos are associated with DNA methyltransferase family (Dnmt). The oocyte form of Dnmt may affect DNA methylation until the morula stage and Dnmt3b may determine the *de novo* methylation in the genome of ICM (Howell et al., 2001; Memili and First, 2000; Watanabe et al., 2002; Cardoso and Leonhardt, 1999). As shown in Fig. 2, however, the passive demethylation and differential methylation between ICM and TE cells were not detected in porcine embryos. Thus, our findings indicate that reprogramming of DNA methylation may differently occur among mammalian species.

2. The DNA Methylation of *In Vitro*-Derived Porcine Embryos

Both maternal and paternal pronuclei of IVF-derived zygotes were stained at similar density after staining with anti-5-methyl cytosine and YOYO (Fig. 3A), showing no active demethylation. This staining pattern remained that way until the 2-cell stage (Fig. 3B). However, uneven staining patterns were partially observed at some blastomeres of 4-cell to morular embryos. At the 4-cell stage, 93.7% (90/96) of IVF-derived embryos showed partial stained patterns after anti-5-methyl cytosine staining (Fig. 3C), whereas only some embryos (6.3%, 6/96) were evenly stained in all blastomeres (data not shown). From the 8-cell to morula stages, almost all embryos had a few stained blastomeres (Fig. 3D, 3E). The density of 5-methyl cytosine stain was less than that of YOYO stain in several blastomeres of an embryo under the same image. However, partial staining patterns were disappeared in the blastocyst stage (Fig. 3F), suggesting that *de novo* methylation may occur at the blastocyst stage even in the IVF- derived porcine embryos. Given

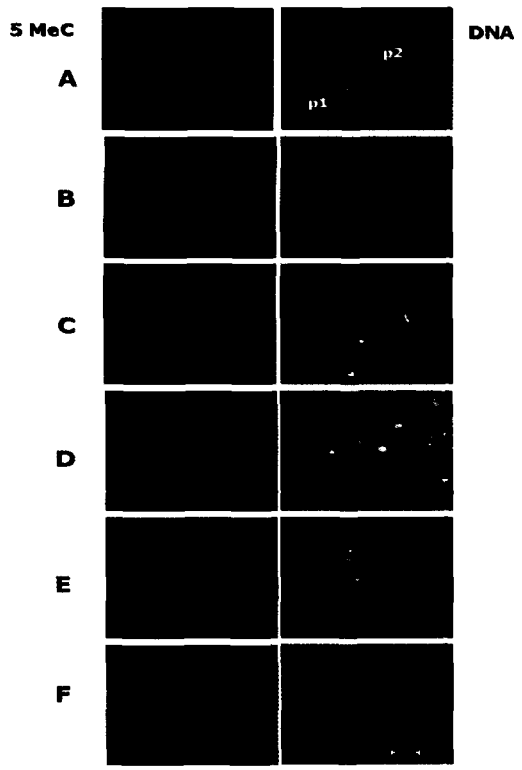

Fig. 3. The distribution of CpG methylation in IVF-derived embryos. At 1 cell (A), 2 cell (B), the pattern of immunostaining is overlapped with that of nuclear staining. In 4 cell (C), 8 cell (D), and morular (E) embryos, several blastomeres are partially stained with the monoclonal antibody specific for 5-methylacytosine. At the blastocyst stage, embryos are evenly methylated (F). Small triangles indicate partial stained blastomeres with the antibody. Scale bar is 20 μm.

the difference of methylation patterns between *in vivo*- and IVF-derived embryos during preimplantation development, the results indicate that the present IVM/IVF/IVC conditions may affect epigenetic reprogramming of porcine embryos. These differences may be induced by unknown environmental factors involved in IVF/IVC system (Shi and Haaf, 2002). In mouse embryos, environmental factors involved in superovulation and culture medium may interfere genome-wide methylation reprogramming and preimplantation development. Particularly, the culture of preimplantation embryos affect the regulation of various imprinted genes, leading to aberrant fetal growth and development (Feil, 2001).

3. Effect of Polyspermy on DNA Methylation

It was questioned whether the methylation difference of IVF-derived embryos might be derived from polyspermy. Polyspermic embryos with more than 3 pronuclei were determined by YOYO staining. At 10 hr after IVF, heads of sperm began to be decondense so that the size of male pronuclei increased although several sperm remained in the condensed state. All pronuclei of polyspermic zygotes were also evenly stained after anti-5-methyl cytosine (data not shown). At 12 hr to 17 hr after IVF, the pronuclei of polyspermic embryos became progressively large and did not represent any difference in the stain among pronuclei (data not shown). The results suggest that polyspermy does not affect reprogramming of DNA methylation at the zygote stage in the pig.

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