Genome-wide and gene specific paternal demethylation in androgenetic embryos

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TABLE OF CONTENTS

1. ABSTRACT

In the present study, a genome-wide measure of demethylation employing the 5 methylcytidine antibody as well as a gene specific approach by bisulphite sequencing of a house keeping and imprinted genes was investigated to confirm if the active paternal demethylation was occurred correctly in mouse haploid, diploid androgenetic and triploid polyspermic embryos. The results indicated that the active demethylation of paternal genome in haploid, diploid, and triploid embryos occurred as similar as the normal ICSI embryos and completed the full demethylation within 6, 8, and 10 h after fertilization, respectively. The methylated CpG dinucleotides sites of alpha-*actin*, paternally expressed *Igf2*, paternal methylated *Gtl2* and *H19* gene loci were hypermethylated in mature sperm. After fertilization, methylated sites of two paternal methylated genes retained their methylation status whereas the paternal alleles of alpha-*actin* and *Igf2* rapidly underwent active demethylation in the diploid androgenetic embryos produced by two sperm injection into enucleated oocytes or pronuclear transplantation. These results indicated that no specificity of paternal specific active demethylation was found in androgenetic or triploid polyspermic embryos.

2. INTRODUCTION

DNA methylation is one crucial epigenetic modification for genome reprogramming and has been proposed essential for mammalian gametogenesis, embryogenesis (1), and cloned embryos/animals development (2, 3). During normal preimplantation development, the biparental genomes exhibit parent-specific epigenetic asymmetry (1). Upon fertilization, the sperm genome exchanges protamines for histones, undergoes a dramatic reprogramming that includes changes in DNA methylation and histone modifications (4-6). Active demethylation of paternal genome occurs within a few hours after fertilization and completes before DNA replication whereas the maternal genome is passively demethylated by a replication-dependent mechanism after the two-cell stage (7-10). The mechanism of active demethylation of paternal genome during the first few hours after fertilization remains unknown. Although one recent study has proposed that PGC7/Stella (DPPA3) might play an important role in protecting the maternal genome from active demethylation (11). Disturbances in the process of genome-wide demethylation might contribute to developmental failures and defects in mammals (12). Some factors, i.e. embryo culture or manipulation (13, 14),

quality of oocytes maturation (15), zona removal (16), even particulate in ambient air (17), would cause either abnormal genome-wide reprogramming or aberrant expression of imprinted genes during embryo development.

Among the process of natural fertilization, matured oocytes were occasional fertilized with more than one sperm. It was reported that two male (18), even up to five males pronuclei (8) in the same polyspermic oocyte were fully demethylated albeit at a reduced kinetic rate. Here we further demonstrated that the methylated CpG sites of paternally inherited loci were also parent-of-origin specific in imprinted genes while the housekeeping gene was completed fully demethylated in androgenetic embryos. No specific pattern of active demethylation was founded in abnormal fertilized embryos.

3. MATERIALS AND METHODS

3. 1. Animals
All studies adhered to procedures consistent with the National Institute of Biological Sciences Guide for the care and use of laboratory animals. Mice C57BL/6 and DBA/2 at 8 week of age were obtained from the Beijing Experimental Animal Center. Hybrid B6D2F1 mice were produced by natural mating of C57BL/6 and DBA2 mice in our animal facility. All animals were housed in the experimental animal center of NIBS under specified pathogen free standard conditions (22°C, 12 h light/12 h dark). All chemicals were obtained from Sigma Chemical Co. (St. Louis, MO), unless otherwise stated.

3.2. Preparation of Gametes
Sperm were collected from 8-week-old B6D2F1 males of proven fertility. In brief, the cauda epididymides from three males were dissected out and placed in HEPES-buffered CZB medium (HCZB) together to allow the sperm to escape into the medium. Soon after the sperm dispersed into the medium, drops of concentrated sperm suspension were incubated in 0.5 ml HCZB for 15 min at 37°C to allow the sperm to swim up. The sperm suspension was placed in liquid nitrogen directly without cryoprotectants. Prior to ICSI, six microliters of thawed sperm suspension was mixed with 12 μl of 12% (w/v) PVP-360 thoroughly. A small drop of this sperm/PVP mixture was kept under mineral oil in a plastic petri dish on the microscope stage for microinjection.

Female B6D2F1 mice were superovulated at 8~10 week of age by intraperitoneal injection of 5 IU pregnant mare serum gonadotropin followed by intraperitoneal injection of 5 IU human chorionic gonadotropin (hCG) 48 h later. Oocyte-cumulus complexes were collected 14-15 h after hCG injection and oocytes were released from the cumulus layers by treatment with 300 IU/ hyaluronidase in HCZB for 5 min. After washing with HCZB, oocytes with the obvious first polar body were cultured in CZB culture medium supplemented with 4 mg/ml BSA at 37°C under 5% CO_2 in air before use.

To obtain enucleated oocytes, the oocytes were rinsed in HCZB containing 5 μg/ml cytochalasin B (CB) and enucleated in the same medium using the Piezo manipulator (PMM-150FU, Prime Tech Ltd, Japan). After enucleation, the oocytes were washed and incubated in CZB culture medium for 0.5 to 1 h before sperm injection.

3.3. Intracytoplasmic Sperm Injection
ICSI was carried out as described by Szczygiel & Yanagimachi (19). Injections were performed in HCZB medium within 1-2 h of oocytes collection. Briefly, Thawed sperm head, separated from tail using Piezo pulse, was injected into the oocyte cytoplasm deeply to produce ICSI embryo. Normal diploid or triploid diandric embryos were produced from intact oocytes injected with one or two sperm heads followed by culture in KSOM medium without CB treatment. Haploid or diploid androgenetic embryos were produced from enucleated oocytes injected with one or two sperm heads followed by culture in CZB medium containing 5 μg of CB/ml for 2 h and then transferred into KSOM medium for culture until the pronuclei became visible.

3.4. Pronuclear Transplantation
Normal diploid ICSI embryos with two well-developed pronuclei were selected to produce diploid androgenetic embryos 6 h post-fertilization. Normal ICSI embryos were transferred into HCZB medium in the manipulation chamber and sucked the female karaplast and the second polar body out carefully. Male pronucleus from another embryo was introduced into the perivitelline space of the above embryo through the same slit on zona. After that, the couplet were induced to fusion in buffer comprising of 0.3 M mannitol, 0.05 mM $CaCl_2$, 0.1 mM $MgSO_4$, and 0.3 % fatty acid-free (FAF) BSA under the conditions of two DC pulses of 1.0 KV/cm for 10 μsec each. The fused embryos were well washed and transferred into KSOM medium for culture. Samples were collected 8 h post-fertilization for analysis.

3. 5. Indirect Immunofluorescence
Genome-wide demethylation was detected using monoclonal anti-5-MeC antibody (Calbiochem, NA81). Immunofluorescence was carried out as described in references (20, 21) with some modifications. More than 30 embryos were used for each test and all experiments were repeated at least three times. All reagents were prepared in phosphate-buffered saline (PBS) containing 0.05% Tween 20 and 0.1% polyvinyl alcohol (PBSTP). In brief, Well-washed embryos were fixed at 4°C in PBS + 4% paraformaldehyde overnight, and permeabilized with PBSTP + 0.2% Triton X-100 (Genview) for 15 min at room temperature. The permeabilized zygotes were treated with 2.0 mol/l HCl for 30 min at room temperature to denature the DNA and subsequently neutralized in 0.1 mmol/l Tris-HCl buffer (pH 8.0) for 10 min. Well-washed samples were blocked at 4°C in PBSTP block solution containing 1% BSA overnight followed by incubation in block solution supplemented with 1:600 diluted anti-5-MeC antibody at 37°C for 1 h. Well-washed samples were then incubated with 1:2000 diluted FITC conjugated goat anti-mouse IgG (Molecular Probes) in PBSTP for 1 h at room temperature.

Table 1. PCR primers used in bisulphite sequencing

Genes	Nested PCR	Forward (F) and reverse (R) primer sequences (5'-3')	Annealing temperature	References
Actin	1st	F: aagtagtgatttttggtttagtatagt; R: actcaataactttctttactaaatctccaaa	52°C	(38)
	2nd	F: ggggtagatagttggggatattttt; R: cctactactctaactctaccctaaata	52°C	
Igf2-1	1st	F: taattgaagttgtttgttttgtgga; R: taacctaaactcccatctaaatatc	46°C	---
	2nd	F: tgttttgtggaatttttaggtaggt; R: ccccaaatcaaaaaataaatctc	46°C	---
Igf2-2	1st	F: gggtgagataaagagattatttattttt; R: taatactacattacaaaattaccacataat	45°C	---
	2nd	F: gggtgagataaagagattatttattttt; R: aattaccacataatttaattcactaataat	44°C	---
H19	1st	F: gagtatttaggaggtataagaat; R: atcaaaaactaacataaacc	46.5°C	(39, 40)
	2nd	F: tttgtaaggagattatgtttattttttggat; R: ccctaacctcataaaacccataactataaa	50°C	
Gtl2	1st	F: ttaaggtatttttattgataaaataatgtagttt; R: cctactctataataccctatataattataccataa	47°C	(31)
	2nd	F: ttaggagttaaggaaaagaaagaaatagtatagt; R: tatacacaaaaatatatctatataacaccatacaa	48°C	

Finally, DNA was stained for 5 min with 5 μg/ml 4, 6-diamidino-2-phenylindole (DAPI, Molecular Probes) and the samples were placed in the small drop of PBS + 50% glycerol on slides which were pre-treated with ProlongR gold anti-fade reagent (Molecular Probes). Observations were performed and recorded with a confocal laser scanning Ultra view microscope (Zeiss LSM 510 Meta) using a Plan Neofluar 40 × Oil DIC objective and excitation wavelengths of 488 nm and 405 nm. All collected images were assembled using Adobe Photoshop software (Adobe Systems, San Jose, CA) without any adjustment of contrast and brightness to the images.

3.6. Bisulphite Analysis

The status of methylation sites of housekeeping gene alpha-*actin*, paternally expressed *Igf2*, maternally expressed *H19* and *Gtl2* were performed for sperm, diploid androgenetic embryos using bisulphite sequencing method described in reference (22) with some modifications. More than 30 embryos were used for each test and all experiments were repeated at least three times. All androgenetic embryos proven loss of global methylation at 8 h post sperm microinjection were obtained for use. Prior to bisulphite sequencing, all polar bodies (PB) of embryos were sucked off during the process of microinjection. In brief, well-washed sperm, or androgenetic embryos were lysed in 1 μl of buffer comprising of 10 mmol/l Tris-HCl, 1 mmol/l EDTA (TE), 0.03% SDS and 3.92 μg/ml Proteinase K for 30~90 min at 37°C followed by incubation for 15 min at 98°C. The above samples were denatured 37°C in 1 μl 2.0 mol/l NaOH for 15 min. Approximately 5 μl of above denatured DNA solution were mixed well at 37°C with 15 μl of molten 2% low melting points agarose solution. Agarose/DNA mixture was gently transferred into 500 μl cold mineral oil to form beads on ice at least 30 min. Five hundred microlitre of freshly prepared bisulphite solution were added to the bottom of beads/oil mixture with gentle inverting to bring the beads into the aqueous phase. The reaction tube was kept in foil bag and then incubated for 4-16 h at 50°C. Bisulphite reaction were stopped after 4 × 15 min washes using TE buffer (pH8.0) followed by desulphonation with two times washes using 0.2 mol/l NaOH, TE buffer, and H₂O in turn. Finally, the beads were used for PCR analysis directly or kept at -20°C until use.

The nested primer pairs used to amplify the products for each gene were shown in table 1. DNA fragment of alpha-*actin* (GenBank accession no. M12347; 13 CpG sites in nt 529 to 785 fragment), *H19* (GenBank accession no. U19619; 16 CpG sites in nt 1301 to 1732 fragment), and *Gtl2* gene loci (GenBank accession no. NT_166318; 33 CpG sites in nt 21749615 to 21750111 fragment) were amplified each. For *Igf2* gene loci, the methylation profile of 13 and 18 CpG sites (totally 31 sites) spanning two regions within the *Igf2* DMR2 imprinting element, namely *Igf2-1* and *Igf2-2*, were amplified separately. Each of above genes was amplified by nested PCR using the bisulphite-treated DNA samples in a 50-μl PCR reaction containing 34.7 μl ddH₂0, 5.0 μl 10 × buffer, 4.0 μl 2.5 mmol/l dNTPs, 0.5 μl 20 mmol/l forward primer, 0.5 μl 20 mmol/l reverse primer, 5.0 μl template and 1 U Hotstart Ex-Taq enzyme (Takara). First and second amplification conditions were as follows. 94°C, 5 min, 35 cycles of 94°C for 30 sec, optimal annealing temperature (table 1) for 30 sec, and 72°C for 30 sec, and final extension at 72°C for 6 min, then on ice. Five microlitre products from the first amplification were used for the second amplification. The resulting PCR products were gel-purified (Geneclean III), ligated into pMD18-T vector (Takara), and individual clones were sequenced using an ABI 3730 sequencer.

4. RESULTS

4. 1. Conservation of Paternal Genome Demethylation in Androgenetic Embryos

Genome-wide demethylation can be examined by immunostaining with the antibody against 5-methylcytosine (7, 20, 21). The DNA methylation pattern and degree in natural, IVF and ICSI-produced mouse embryos have been shown similarly (23). Here, we firstly used immunostaining to detect the global DNA methylation status of paternal genomes in triploid polyspermic and diploid androgenetic embryos produced by microinjection of two sperm into intact or enucleated oocytes. As a control, the paternal demethylation of one sperm injected into a intact oocyte (diploid) was kept less methylation status at 6 h (Figure 1 c) and completed within 8 hours after injection (Figure 1 d), whereas the maternal genome kept high methylation level. The similar demethylation results
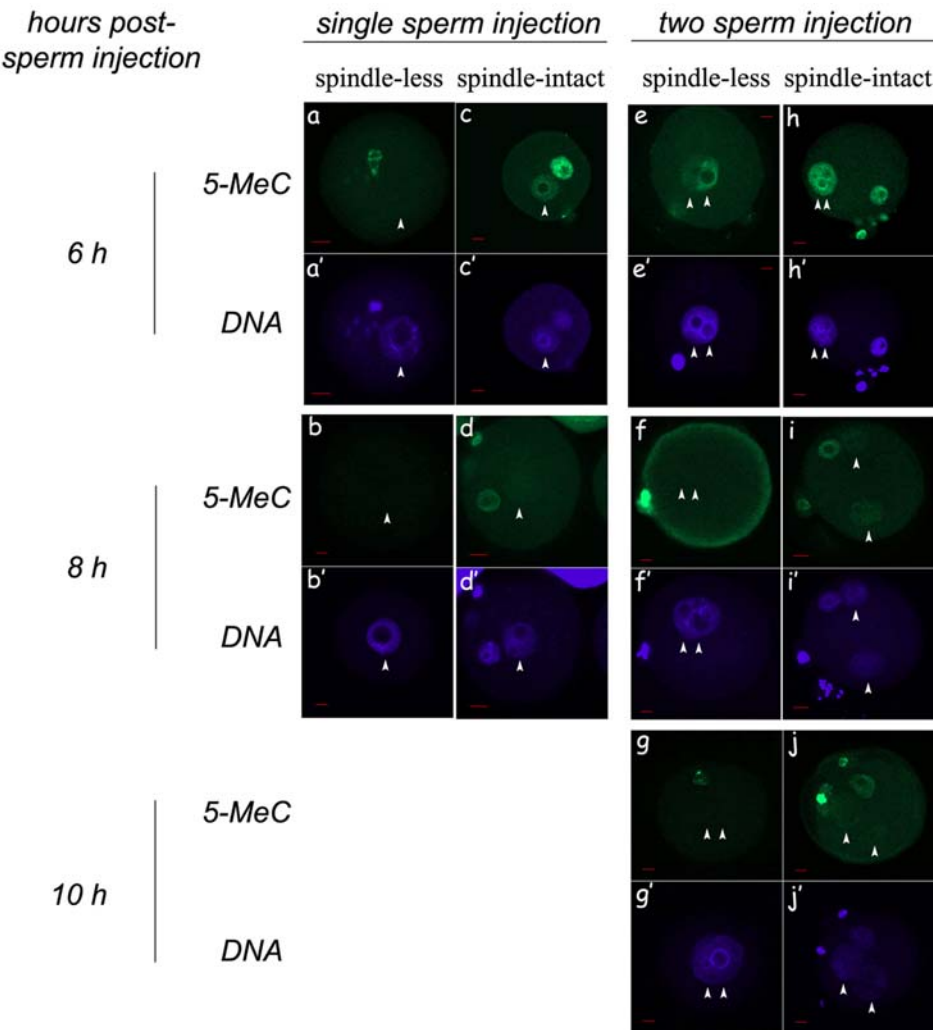
Figure 1. Global methylation patterns of paternal genome in normal ICSI and androgenetic mouse embryos. Indirect immunofluorescence with the use of monoclonal antibody to 5-MeC (in green) shows that the loss of methylation of single- (c, d) and two-copy (h~j) paternal genome(s) in intact oocytes is within 8 (d) and 10 h (j), respectively, whereas completely demethylation of single (a, b) and two (e~g) sperm in enucleated oocytes is occurred within 6 h (a) and 8 h (f) after sperm injection. DNA staining is in blue (a'~j'). Arrow heads indicate male pronuclei. Scale bar represents 10 μm.

were obtained in the groups of androgenetic embryos. Rapidly paternal demethylation of single (haploid, Figure 1, a-b) or two (diploid, Figure 1, e-f) sperm was also occurred completely and independently in enucleated oocytes within 6 and 8 hours, respectively. But for triploid polyspermic embryos (two sperm in intact oocyte), it needed 10 hours to complete the fully paternal demethylation (Figure 1, h~j). These results indicated that the genome-wide demethylation of paternal genomes in diploid androgenetic and polyspermic embryos showed the similar patterns of normal ICSI embryos.

4. 2. Methylation Status of Specific Gene Loci in Androgenetic Embryos

To investigate the DNA demethylation specificity of individual gene loci in androgenetic embryos, we investigated some methylated CpG sites of four endogenous genes in androgenetic embryos by bisulphite

genomic sequencing. Three imprinted genes, one that ordinarily undergoes demethylation (*Igf2*) and two maternally methylated genes (*Gtl2* and *H19*) were that served as controls. In mature sperm, nearly all assayed CpG sites in alpha-*actin*, *Igf2*, *Gtl2* and *H19* gene loci were highly methylated (Figure 2). Eight hours after fertilization, the methylation level of the sperm-derived epigenetic marks of imprinted gene *Igf2* dropped significantly in both androgenetic embryos. Thirty-one CpG sites of *Igf2* were examined and among them 16 (52%) sites in the group of sperm injection and 20 (65%) sites in the group of pronuclear transplantation have been found demethylated. In contrast, the paternally methylated genes, *Gtl2* and *H19*, in the current study, were not affected by this reprogramming process and remained highly methylated in both androgenetic embryos 8 h after fertilization (Figure 2), similarly as described in (24, 25). But for housekeeping alpha-*actin* gene loci, all assayed methylated CpG sites
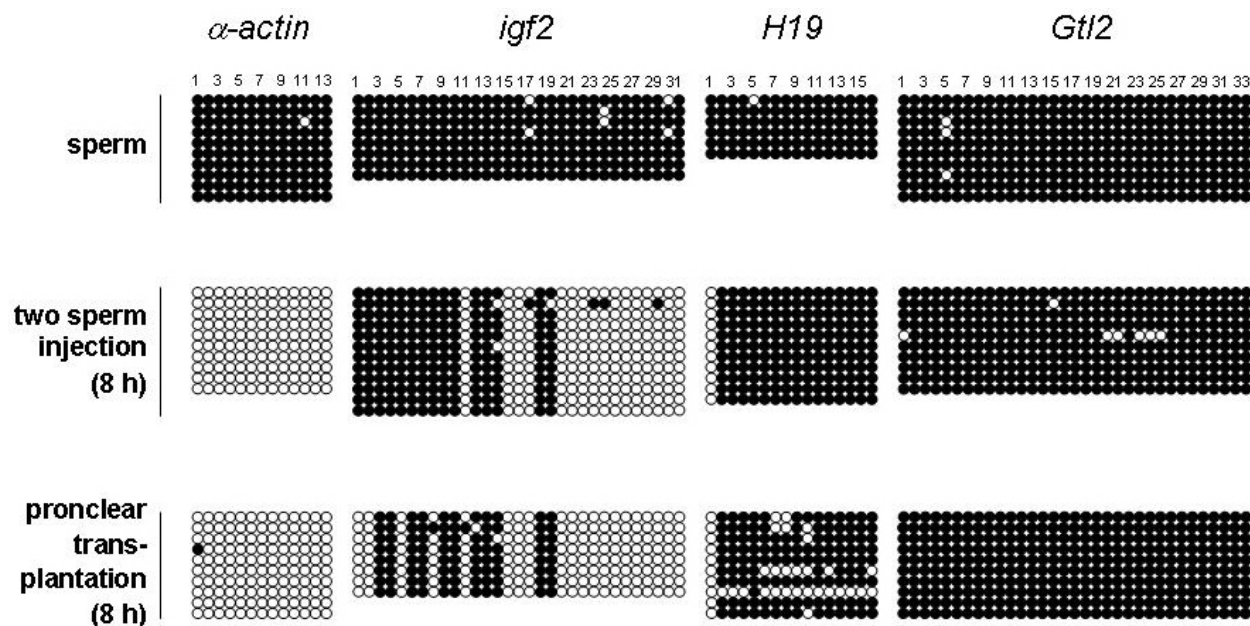
Figure 2. Methylation status of both housekeeping and imprinted genes in sperm and androgenetic embryos. The genomic DNA from mouse sperm, diploid androgenetic embryos produced from enucleated oocyte injection with either two sperm or pronuclear transplantation underwent bisulfite treatment followed by nested PCR amplification and sequencing. Individual lines, clones sequenced; circles, CpG sites within the regions analyzed; filled circles, methylated cytosines; open circles, unmethylated cytosines. Most CpG sites of four genes are hypermethylated in mature sperm. Eight hours after sperm injection, housekeeping gene alpha-*actin* is completely demethylation, whereas paternally methylated genes *H19* and *Gtl2* retain their methylation status. Among 31 CpG sites of paternally expressed gene *Igf2*, 16 (52%) sites in the group of two sperm injection, and 20 (65%) sites in the group of pronuclear transplantation are demethylated.

were rapidly erased fully in both androgenetic zygotes, before the first round of DNA replication initiated. These results provided the direct evidence that the paternal methylation on specific gene loci was not showed aberrant in androgenetic embryos.

5. DISCUSSION

Among mammalian species, the paternal genome exhibits species- and temporal-special demethylation patterns (10, 20, 26). It was reported that complete demethylation of single-copy paternal genome in mouse IVF-produced embryos was rapidly occurred within 4-6 h, and even five-copy sperm genomes within 10 h post-IVF (8). Although other study revealed that it needed at least 7 h for paternal genome to complete demethylation in natural embryos (27). In the present study, loss of paternal methylation completely in haploid, diploid, and triploid embryos was occurred at 6-, 8- and 10 h, respectively. It suggested that the multiple paternal genomes introduced into zygotes by a variety of methods (i.e. normal fertilization, ICSI, and pronuclear transplantation) all undergo rapid, global demethylation, irrespective of ploidy, as measured by 5MeC immunohistochemistry. It also suggests that the time to complete paternal demethylation might decide by the copies of whole genomes regardless its parent-of-origin. The demethylation factors, distributed in oocyte cytoplasm, could re-allocate into male pronucleus asymmetrical whereas a portion of them could diffuse, and enrich into female pronucleus during fertilization. Although

maternal genome is protected from this reprogramming and remains relatively high methylation after fertilization(10, 28, 29). For multi-copy genomes in oocyte cytoplasm, the amount of reprogramming factors and/or histone chromatin proteins (cytoplasm) per nucleus is decreased to cause demethylation delayed or incompletely. Similarly, loss of any cytoplasm (demethylation potency, etc) in oocytes might be resulted in its further developmental depression in mouse embryos (30).

It has been reported that highly methylated imprinted genes in sperm are rapidly demethylated in the zygotes before the first round of DNA replication commences (9). All four endogenous genes loci investigated in the current study were hypermethylated in mature sperm (Figure 2), as similarly described in previous studies (9, 31, 32). In case of *Igf2* region, its CpG sites were found fully methylated in sperm and 52~65% of all methylated CpG sites were demethylated in the androgenetic embryos 8 h after ICSI, as similarly as described by Oswald *et al* (9). In their study, the paternal *Igf2* CpG island lost most of its methylation in normal zygotes after fertilization, whereas androgenetic embryos retain half of the CpG methylation (9). By contrast, paternal methylated *H19* and *Gtl2* alleles were largely exempt from this reprogramming and retained their methylation established during spermiogenesis (33-35) whereas all CpG sites of alpha-*actin* gene loci were demethylated completely within 8 h after fertilization. It suggested that the active DNA demethylation activity in

androgenetic embryos only could demethylate the specific genes from paternal origin and there is no aberrant expression of both housekeeping and imprinted genes. Some studies further investigated the expression of imprinted genes in uniparental embryos on D9.5. *Igf2* expression in androgenetic embryos was identical (36) or 2.5-fold (37) of that in the controls and not detected in the parthenogenetic fetuses. Whereas *H19* and *Gtl2* expression was not detected in androgenetic embryos but in the parthenogenetic fetuses, it was 1.5- and 9.0-fold of that in the controls (37). Taken together the results of methylation changes of imprinted genes and genome-wide paternal demethylation in androgenetic embryos here, suggested that the active DNA demethylation activity present in androgenetic embryos is as similar as that of normal embryos.

6. ACKNOWLEDGMENT

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Abbreviations: IGF2: insulin-like growth factor; ICSI: Intracytoplasmic Sperm Injection; IVF: *in vitro* fertilization; DMR: differentially methylated region; 5MeC: 5-methyl Cytidine

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