Similar DNA Methylation and Histone H3 Lysine 9 Dimethylation Patterns in Tripronuclear and Corrected Bipronuclear Human Zygotes

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Abstract. After fertilization, male and female gametes undergo extensive reprogramming to restore totipotency. Both DNA methylation and histone modification are important epigenetic reprogramming events. Previous studies have reported that the paternal pronucleus of the human zygote is actively demethylated to some extent, while the maternal pronucleus remains methylated. However, to our knowledge, the relationship between DNA methylation and H3K9 dimethylation patterns in human embryos has not been reported. In this study, we examined the dynamic DNA methylation and H3K9 dimethylation patterns in triploid and bipronucleated zygotes and early developing embryos. We sought to gain further insight into the relationship between DNA methylation and H3K9 dimethylation and to investigate whether removing a pronucleus from triploid zygotes affects DNA methylation and H3K9 dimethylation patterns. We found that active DNA demethylation of the two male pronuclei occurred in tripronuclear human zygotes while the female pronucleus remained methylated at 20 h post-insemination. In tripronuclear human zygotes, H3K9 was hypomethylated in the two paternal pronuclei relative to the maternal pronucleus. Our data show that there are no differences in the DNA methylation and H3K9 dimethylation patterns between tripronuclear and corrected bipronuclear human zygotes. However, correction of 3PN human zygotes dispermic in origin could not improve subsequent embryo development. In conclusion, DNA methylation and H3K9 dimethylation patterns are well correlated in tripronuclear zygotes and embryos; early embryo development is not affected by removal of a male pronucleus. Our results imply that limited developmental potential of either 3PN or corrected 2PN embryos may not be caused by the abnormalities in DNA methylation or H3K9 dimethylation modification.

Key words: DNA methylation, Histone methylation, Male pronucleus, Reprogramming, Tripronucleate zygotes

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Materials and Methods

A total of 247 tripronuclear human zygotes with a visible second polar body were obtained 18–20 h post-insemination from patients undergoing conventional in vitro fertilization (IVF) after obtaining informed consent. The use of abnormally fertilized zygotes was approved by the ethics committee of Guangzhou Medical College. The average age of the women was 32 ± 4 years (ranging from 20 to 45 years), and the average age of the men was 34 ± 4 years (ranging from 22 to 48 years).

After retrieval, the oocytes were cultured in microdrops of IVF-30 medium (Vitrolife, Gothenburg, Sweden) supplemented with 6% human serum albumin and exposed to sperm overnight. The 3PN zygotes were kept in IVF-30 medium after checking for fertilization at 18–20 h post-insemination. All zygotes were identified with three PN and two polar bodies.

Pronucleus removal procedure: microtools and micromanipulation

For micromanipulation, 3PN zygotes were placed in a small (30 μl) drop of modified HTF medium (Irvine Scientific, Santa Ana, CA, USA) containing 7.5 μg/mL cytochalasin B (C6762, Sigma) covered with equilibrated mineral oil.

Zygotes were incubated for 10–15 min in the HTF medium and placed on the stage of an inverted microscope (TE2000, Nikon). A holding pipette (outer diameter, 100 μm; inner diameter, 30 μm) was used to hold and position the zygote. A pipette with an internal diameter of 12–15 μm was used for removal of supernumerary male pronuclei. Before pronucleus removal, 3PN zygotes were clumped against the holding pipette, rotated, and repositioned until we could identify all pronuclei and the second polar body. Then, the zygote was stabilized by applying negative pressure to the holding pipette. Three pronuclei were usually placed in a typical pyramidal position in the center of the ooplasm. The supernumerary male pronucleus was identified by its location distal from the second polar body [2, 5, 6]. For removal of the pronucleus, the micropipette was inserted through the zona pellucida (ZP) using a piezoelectric device (P150, PrimeTech) and gently positioned next to the targeted surface of the pronucleus. Negative pressure was then applied until the pronucleus was picked up. Pressure was maintained while the pipette holding the pronucleus was gently withdrawn from the syngamy area to the oolemma. Bipronuclear zygotes were then washed three times in G1.5 medium (Vitrolife, Gothenburg, Sweden) and cultured in 5% CO2 at 37 C for 5 to 10 h. Tripronuclear zygotes (controls) were similarly handled, but not microsurgically manipulated, and served as the 3PN control group.

Embryo developmental potential

Corrected bipronuclear (2PN) human zygotes and 3PN control zygotes were incubated until the blastocyst stage. Embryos were cultured in G1.5 medium (Vitrolife) until they reached the 8-cell stage. On the morning of day 3, the medium was replaced by G2.5 medium (Vitrolife). Embryonic development was checked every day. Development of 2PN and 3PN embryos was statistically compared using the χ2 test.

Immunofluorescence staining

Immunofluorescence staining was performed as described previously [29]. Firstly, tripronuclear human zygotes were fixed in PBS for 30 min with freshly prepared 2% paraformaldehyde at 20, 25 and 30 h post-insemination, and the corrected bipronucleated (2PN) zygotes were then fixed at 25 and 30 h post-insemination. After fixation, they were washed in 0.1% PVP/PBS twice and permeabilized with 0.5% Triton X-100 in PBS for 30 min. Then, they were treated with 2N HCl for 1 h at 37 C. Before being blocked for 1 h in 2% BSA in PBS, the embryos were rinsed three times. Double immunostaining of DNA and histone methylation was accomplished by sequential incubation with the relevant primary and secondary antibodies. To detect the DNA methylation patterns, the zygotes and embryos were incubated overnight at 4 C with anti-5-MeC antibody (Epigentek Group, Brooklyn, NY, USA, 1:200). To detect the patterns of H3K9 methylation, the zygotes and embryos were incubated overnight at 4 C with rabbit polyclonal anti-dimethylated K9 (H3-m2K9, 1:200) of histone H3 antibody (Upstate Biotechnology, Temecula, CA, USA). The zygotes and embryos were then rinsed and incubated with the goat anti-mouse IgG-Cy3 and goat anti-rabbit IgG FITC-conjugated secondary antibody (Santa Cruz Biotechnology).

Samples were mounted onto slides in DABCO antifade solution for observations. Ten images for every 0.5 μm of thickness were captured from each zygote or embryo. All different stages of embryos obtained from different patients were labeled in at least three independent experiments.

Confocal images were obtained using a Nikon C1SI (488 and 543 nm excitation lines) and an Olympus FV1000 (488 and 559 nm excitation lines). All images were recorded digitally with a high-resolution CCD camera.

Results

DNA and H3K9 methylation in tripronuclear human zygotes

We first examined DNA and H3K9 dimethylation of human tripronuclear zygotes at the mid-to-late pronuclear stages. In total, we double-labeled 35 tripronuclear zygotes with anti-5-MeC and anti-H3-m2K9 antibodies at approximately 20 h post-insemination to co-localize these two epigenetic marks.

Fig. 1A shows typical staining patterns of 5-methylcytosine (5-MeC) and dimethylated histone H3-lysine 9 (H3-m2K9). The two larger (paternal) pronuclei from the 30 human zygotes evaluated were actively demethylated to some extent, while the maternal pronucleus remained methylated. Also, the asymmetrical H3K9 dimethylation status of the maternal and paternal pronuclei was observed. The maternal pronucleus was intensely labeled with H3-m2K9, whereas two paternal pronuclei showed very weak labeling. Our results showed that there was apparent co-localization between H3-m2K9 and DNA demethylation.

The three pronuclei of the other three human zygotes showed roughly the same intensity of labeling (Fig. 1B).
Fig. 1. DNA and H3K9 methylation in tripronuclear human zygotes. A: The typical staining patterns of 5-methylcytosine (5-MeC) and dimethylated histone H3-lysine 9 (H3-m2K9) in tripronuclear human zygotes. B: The three pronuclei showing roughly the same intensity of labeling in a small proportion of tripronuclear zygotes. Scale bar=20 μm.

Fig. 2. Dynamic DNA and H3K9 methylation patterns in tripronuclear human zygotes and subsequent early cleavage. Tripronuclear human zygotes were collected at 25 (a–d) and 30 (b–f) h post-insemination and double-labeled with anti-5-MeC antibody and anti-H3-m2K9 antibody. (a) Pronucleus stage; (b,c) M phase; (d) 2-cell stage; (e) 3-cell stage; (f) 4-cell stage. Scale bar=20 μm.

Fig. 3. Dynamic DNA and H3K9 methylation patterns in corrected 2PN human zygotes and subsequent cleaved embryos. Corrected 2PN human zygotes were collected at 25 (A, B, C) and 30 (B, C, D) h post-insemination and double-labeled with anti-5-MeC antibody and anti-H3-m2K9 antibody. (A) Pronucleus stage; (B) M phase; (C) 2-cell stage; (D) 4-cell stage. Scale bar=20 μm.
Dynamic DNA and H3K9 methylation patterns of tripronuclear human zygotes in early development stages

At 25 h post-insemination, tripronuclear human zygotes progressed to various developmental stages and were categorized into groups as follows: group 1 contained zygotes in which the three pronuclei were still visible (46.15%, 18/39; Fig. 2a); group 2 contained zygotes with pronuclear membrane breakdown and chromosome condensation ready for mitosis (33.33%, 13/39; Figs. 2b and c); and group 3 contained embryos already cleaved and progressed to the 2-cell stage (20.15%, 8/39; Fig. 2d). Thus, the first cell division might occur at approximately 25 h post-insemination in tripronuclear human zygotes.

In group 1, the two larger (paternal) pronuclei of 15 human zygotes (83.33%, 15/18) were demethylated. While the maternal pronucleus remained methylated. Also, the maternal pronucleus was intensely stained with H3-m2K9, whereas two paternal pronuclei showed very weak staining (Fig. 2a).

In group 2 and group 3, both zygotes and embryos were stained indistinguishably for both 5-MeC and H3-m2K9. The results indicated restoration of the paternal 5-MeC and H3-m2K9 levels (Figs. 2b and c).

At 30 h post-insemination, tripronuclear human zygotes were also found at different stages and were categorized into groups as follows: group 1 contained zygotes with condensed chromosomes in preparation for mitosis (16.22%, 6/37; Fig. 2e), and group 2 contained embryos already cleaved and progressed to the 2-cell, 3-cell or 4-cell stages (83.78%, 31/37; Figs. 2f–i). In the two groups, both 5-MeC and H3-m2K9 were stained indistinguishably. Restoration of the paternal 5-MeC and H3-m2K9 levels was proven by the DNA and H3K9 methylation patterns at this time.

Our results indicate that 3PN human zygotes undergo active DNA demethylation and then de novo methylation during a narrow developmental window restricted to the pronucleus stage. H3K9 dimethylation is correlated with DNA methylation during the early embryo development.

Removal of a pronucleus does not affect the DNA and H3K9 methylation patterns of zygotes and early pre-implantation embryos

After removal of the supernumerary male pronucleus at about 20 h post-insemination, corrected bipronuclear (2PN) human zygotes were further cultured. They were then fixed at 25 and 30 h post-insemination and double-labeled with anti-5-MeC antibody and anti-H3-m2K9 antibody.

At 25 h post-insemination, 2PN zygotes developed to several different stages and were categorized into groups as follows: group 1 contained zygotes in which the two pronuclei were still visible (46.88%, 15/32; Fig. 3A); group 2 contained zygotes with condensed chromosomes or nuclei in preparation for mitosis (50%, 16/32; Fig. 3B); and group 3 contained embryos already cleaved and progressed to the 2-cell stage (3.13%, 1/32; Fig. 3C).

In group 1, the larger (paternal) pronuclei of twelve human zygotes (80%, 12/15) were still demethylated, while the maternal pronucleus remained methylated. Also, the maternal pronucleus was intensely stained with H3-m2K9, whereas paternal pronuclei showed very weak staining (Fig. 3A).

In group 2 and group 3, both zygotes and embryos were stained indistinguishably for both 5-MeC and H3-m2K9. The results indicated restoration of the paternal 5-MeC and H3-m2K9 levels (Figs. 3B and C).

At 30 h post-insemination, 2PN human embryos developed to different stages and were categorized into groups as follows: group 1 contained zygotes with condensed chromosomes in preparation for mitosis (22.58%, 7/31; Fig. 3B), and group 2 contained embryos already cleaved and progressed to the 2-cell or 4-cell stages (77.42%, 24/31; Figs. 3C and D). In the two groups, both 5-MeC and H3-m2K9 were stained indistinguishably. Thus, the first cell division might occur at approximately 25 to 30 h post-insemination in corrected bipronuclear (2PN) human zygotes.

Our results show that pronucleus removal does not affect the DNA and H3K9 methylation patterns of zygotes and embryos. Similar to 3PN zygotes, the corrected 2PN zygotes also underwent active DNA demethylation and then remethylation during a narrow developmental window restricted to the pronucleus stage. H3K9 dimethylation is correlated with DNA methylation during early embryo development.

Embryo development of corrected bipronuclear (2PN) human zygotes

Twenty-three of 28 3PN human zygotes (82.14%) survived after the microsurgical manipulation (Table 1), of which 21 diploidized embryos (91.30%) cleaved after 24 h of culture and 71.42% (15/21) reached the 6- to 8-cell stage on day 3 of development (Table 1). On day 5, one manipulated human zygote (4.76%) progressed to the blastocyst stage (Table 1).

The in vitro development ability of diploidized embryos was comparable to that of control embryos (71.42% vs. 75.56% at the 8-cell stage; 4.76% vs. 6.67% at the blastocyst stage; P>0.05; Table 1).

### Table 1. In vitro development of corrected bipronuclear zygotes and tripronuclear zygotes

<table>
<thead>
<tr>
<th>Number of embryos</th>
<th>Embryo development (%)</th>
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<td></td>
<td>Initial Cultured</td>
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<td></td>
<td>Cleavage 8-cell Morula</td>
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<tr>
<td>Experimental group</td>
<td>28 23</td>
</tr>
<tr>
<td>Controls</td>
<td>45 45</td>
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* The same superscripts in the same column indicate no difference (P>0.05).
Discussion

Human oocytes and embryos are difficult to obtain for study because of the limited resources. About 7% of human oocytes fertilized in vitro are 3PN zygotes. With patient consent, these 3PN zygotes can be a valuable source for diagnosis [30] and therapy [5, 6], as well as for obtaining information on embryo development [5, 31].

In the present study, we compared the dynamic changes of DNA methylation and histone modification in early embryo development between tripronuclear zygotes and corrected bipronuclear (2PN) zygotes. Because the 3PN human zygotes used in this study were obtained from conventional IVF and the second polar bodies were identified, the additional pronucleus observed in these 3PN zygotes arose from supernumerary sperm that penetrated the oocyte [32]. Our data show that there are no differences in the DNA and H3K9 methylation patterns between tripronuclear human zygotes and corrected bipronuclear (2PN) human zygotes. Removal of a male pronucleus does not affect the DNA and H3K9 methylation patterns of zygotes and embryos. To our knowledge, this is the first report to investigate the relationship between histone modification pattern and the DNA methylation pattern in human zygotes.

Both male and female gametes are highly specialized and terminally differentiated cells. After fertilization, they undergo extensive reprogramming to restore totipotency. Despite the similar genetic content of the gametes, the paternal and maternal genomes are reprogrammed in different ways. Epigenetic modification is an integral process in the reprogramming [19].

One of the important epigenetic modifications is DNA methylation, which is associated with gene silencing and the formation of heterochromatin [8]. Interestingly, there are several different opinions on DNA methylation reprogramming patterns during fertilization and early embryogenesis. Previous research results show that the DNA methylation reprogramming patterns do not seem to be conserved among mammalian species [17, 33]. Although active demethylation of the male pronucleus has been observed in mouse and human zygotes, it does not appear in rabbit and sheep zygotes [17]. However, a recent study shows that DNA demethylation of the paternal chromosomes in the rabbit occurs at slightly advanced pronuclear stages [34]. As a result, the authors speculated that mechanisms of active DNA demethylation are apparently conserved among mammalian species [34]. In this study, we found that the two male pronuclei are selectively demethylated in 3PN human zygotes, while the female pronucleus remains methylated at 20 h post-insemination. Our results showing active paternal pronuclei demethylation are similar to the results of previous work [17, 27, 28]. It is striking that 3PN human zygotes undergo active DNA demethylation and then de novo methylation during a narrow developmental window restricted to the pronuclear stage, which is different from the finding in previous work [27].

Another important epigenetic modification to the chromatin is histone modification, which widely regulates gene transcription and silencing [20, 21]. Previous findings suggest that histone H3K9 methylation is associated with the formation of constitutive heterochromatin [23] and gene silencing [22]. In particular, H3K9 methylation has been suggested to be correlated with DNA methylation. In the present study, our results show that 3PN human zygotes tend to have an asymmetric dimethylated H3K9 distribution between parental pronuclei, which co-localized with the asymmetric distribution of DNA methylation. Coincident with the pattern of asymmetric DNA methylation in parental genomes, H3K9 methylation exhibited asymmetric modifications in the parental pronuclei, which is similar to the results of recent studies [35, 36]. In zygotes, histone H3K9 is highly dimethylated in the maternal pronucleus, while the paternal pronucleus exhibits a low level of dimethylation. These results show that the limited developmental potential of both 3PN and corrected 2PN embryos may not be caused by the abnormalities in DNA methylation or H3K9 dimethylation modification. Therefore, the presence of H3K9 in the female pronucleus may protect the maternally derived DNA from active demethylation, while the absence of dimethylation on H3K9 in the male pronucleus may allow active DNA demethylation to occur [35].

The mechanisms and factors responsible for the post-fertilization demethylation of paternal DNA and modifications of the histones remain unclear. Paternal chromatin decondenses in the ooplasm to permit protamine-histone exchange, and this decondensation provides a unique opportunity for demethylating enzyme(s) and/or other protein factors to bind to the exposed male chromatin. Our results show that the two male pronuclei are selectively demethylated in most 3PN human zygotes (Fig. 1A). A previous study [15] has also demonstrated that the mouse ooplasm is able to demethylate up to five supernumerary male pronuclei. As a result, the factors involved in the selective demethylation events are present in more than adequate concentrations in the ooplasm.

Removal of extra pronuclei from tripronuclear human zygotes has proved to be feasible [2–7]. Correction of 3PN human embryos has been used in clinical assisted reproductive technology (ART) [6]. Such observations provide further support that 3PN zygotes may behave normally. However, 3PN human zygotes have limited in vitro development potential [5, 31, 37]. A possible reason for this is that embryo development is severely influenced by both ploidy and parental inheritance. In this study, we found that correction of 3PN human zygotes, dispermic in origin, could not improve subsequent embryo development (Table 1). Both 3PN and corrected 2PN zygotes had similar DNA and H3K9 methylation patterns. Thus, both 3PN discarded from IVF cycles and corrected 2PN zygotes can be an alternative source of embryos for stem cell research.

Significantly, several 3PN and corrected 2PN human zygotes did not display obvious male pronuclei demethylation, and this has also been found in human zygotes observed by other researchers [27, 28]. The reason for this may be that those zygotes came from infertile patients. There is increasing evidence that genetic factors in infertile couples as well as environmental factors (hormones and culture media) can have adverse effects on epigenetic reprogramming [38–40]. Even in mouse zygotes that are fertilized normally, approximately 10% show an aberrant pattern of labeling [41]. Considering the complexity of the epigenetic reprogramming processes, faults in clearing the differentiation-associated epigenetic traces from a donor cell and then resetting the donor epigenome after somatic cell nuclear transfer may result in delayed and incom-
plete reprogramming of methylation patterns. These epigenetic anomalies are most likely responsible for low efficiency of cloning [42], low implantation and high intrauterine fetal demise and abnormality rates [43].

In summary, this is the first report to compare the dynamic changes of DNA and H3K9 methylation patterns between tripronuclear zygotes and corrected bipronuclear (2PN) zygotes and their subsequent early cleaved embryos. Our data show that DNA methylation and H3K9 dimethylation patterns are well correlated and that embryos and zygotes are not affected by removal of a male pronucleus. However, diploidization does not improve embryo development potential. Our results imply that the limited developmental potential of either 3PN or corrected 2PN embryos may not be caused by the abnormalities in DNA methylation or H3K9 dimethylation modification.

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