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Comparison of histone modifications in *in vivo* and *in vitro* fertilization mouse embryos

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Abstract

Histone modifications are thought to play important roles in various cellular functions. In this article, the distribution patterns of acetylation on histone H4, methylation on histone H3 lysine 9, and phosphorylation on histone H3 serine 10 were examined in *in vivo* and *in vitro* fertilization (IVF) preimplantation mouse embryos by using indirect immunofluorescence and scanning confocal microscopy. We desired to know whether the IVF, which has been widely used as a routine assisted reproductive technology in animal and human, was safe at the epigenetic level. As results, we found that there was no difference in these histone modification patterns in *in vivo* and IVF mouse embryos from zygote to blastocyst stage. Moreover, these histone modifications had different distributions at all examined stages, but they were consistent with the mouse embryo developmental stages.

Keywords: Mouse; Histone; Acetylation; Methylation; Phosphorylation; IVF; Embryo

The nucleosome is the fundamental unit of chromatin, which is consisted of 147 bp of super helical DNA wrapped in 1.75 turns around an octamer of four core histone proteins (H2A, H2B, H3, and H4) [1]. The core histone proteins play important roles in chromatin structure and transcriptional regulation through modifications of the histone amino termini, such as acetylation, methylation, and phosphorylation.

In general, histone acetylation is thought to facilitate transcription, and deacetylation reverses this effect. The acetylation and deacetylation also provide specific binding surfaces for the recruitment of activators and repressors of gene activity [2]. Histone methylation can signal either activation or repression, depending on the sites of methylation [3]. Lysine 9 methylation of histone H3 (MeH3K9) has been correlated with repression of transcription and the formation of large constitutive heterochromatin as well as facultative heterochromatin of the inactive X chromosome [4,5]. Phosphorylation of histone H3 Serine 10 (PhH3S10) plays an important role in cell cycle-dependent chromosome condensation and segregation as well as for activating transcription, apoptosis, and DNA repair [6]. For instance, inhibition of the mitotic kinase Aurora B, which phosphorylates histone H3 Serine 10, causes retention of heterochromatin protein 1 (HP1) on mitotic chromosomes, suggesting that PhH3S10 is necessary for the dissociation of HP1 from chromatin in M phase [7,8].

Histone modifications are also closely correlated with each other. For example, PhH3S10 is reduced when lysine 9 is dimethylated, and histone H3 acetylation in lysine 9 or lysine 14 facilitates PhH3S10 [9]. Moreover, PhH3S10 facilitates the histone acetyltransferase (HAT) to acetylate lysine 14 [10]. In addition, histone modifications may function as epigenetic marks by which information about genomic function is transmitted from one generation of

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cells to the next, and the pattern of gene expression that defines a cell type is exactly maintained [11]. Therefore, the changes of histone modifications induced by manipulation or environmental influences will alter not only the cells initially subject to these influences but also the next.

Previous studies have shown that preimplantation embryos are sensitive to environment and manipulation that can affect developmental potential and fetal growth [12]. For example, the methylation pattern of imprinted genes has been shown to change in response to embryo culture conditions [13]. In addition, zona-free embryos that the zona pellucida are only removed between 1 and 8 h post-fertilization show a significant reduction in the DNA methylation level at two-cell and four-cell stages, but no difference in the histone H4 lysine 5 acetylation between zona-free and zona-intact embryos at all examined stages [14]. These results indicate that changes in environment such as artificial culture and manipulation can cause some epigenetic modification alterations. *In vitro* fertilization (IVF) is widely used as a routine assisted reproductive technology in animal and human clinic practice. However, in contrast to *in vivo* embryo, the IVF embryos are exposed to *in vitro* culture media and manipulation. This raises the question of whether the histone modification patterns of IVF embryos, which compare with those in *in vivo* embryos, will be altered.

In this study, the patterns of AcH4, MeH3K9, and PhH3S10 were compared between *in vivo* and IVF mouse embryos. We found that there was no difference in these histone modification patterns in *in vivo* and IVF mouse embryos from zygote to blastocyst stage.

Materials and methods

Animal care and handling were conducted in accordance with policies on the care and use of animals promulgated by the Ethics Committee of the Institute of Zoology, Chinese Academy of Sciences. We used Kunming white mice as sperm and oocyte donors. Unless otherwise noted, all chemicals used in this study were purchased from Sigma (St. Louis, MO).

In vivo and IVF embryo collection. Female Kunming white mice, 4–6 weeks of age, were superovulated with 5 IU of pregnant mare's serum gonadotropin (Tianjin Animal Hormone Factory, Tianjin, China), followed 48 h later with 5 IU of human chorionic gonadotropin (hCG, Tianjin Animal Hormone Factory, Tianjin, China). For *in vivo* embryo collection, one female was placed in a cage with one stud male after the administration of hCG, and then embryos at different stages were collected as described previously [15]. For IVF embryo collection, cumulus-oocyte complexes were collected in Modified CZB medium (CZB-Hepes), from the ampullae of oviducts, 14–15 h after hCG injection. Spermatozoa were collected from the caudal epididymis of adult Kunming white male mice and capacitated by preincubation for 1.5 h in human tubal fluid (HTF) medium [16]. Cumulus-oocyte complexes were inseminated with capacitated spermatozoa in a humidified atmosphere of 5% CO_2, 95% air at 37 °C. Five hours after insemination, the fertilized oocytes were washed and cultured in CZB medium [17]. The interphase and mitosis of the subsequent developmental stages were examined in this study: Zygotes sampled at 6, 12, and 18 h after insemination, two-cell embryos, four-cell embryos, eight-cell embryos, morulae, and blastocysts were collected.

Indirect immunofluorescence and scanning confocal microscopy. Embryos at different developmental phases generated by *in vivo* and IVF were washed in phosphate-buffered saline (PBS), fixed for 30 min in 4% paraformaldehyde in PBS, and permeabilized with 0.2% Triton X-100 in PBS for 30 min at room temperature. The fixed embryos were blocked in PBS containing 1% BSA for 1 h at room temperature and incubated overnight at 4 °C in the appropriate primary antibodies diluted in 1% BSA-supplemented PBS. The anti-histone modification antibodies and the dilutions used in the study were as follows: Rabbit polyclonal anti-acetyl histone H4 (AcH4) antibody (1:300), anti-dimethyl lysine 9 in histone H3 (MeH3K9) antibody (1:300) and anti-phosphorylation serine 10 in Histone H3 (PhH3S10) antibody (1:300). All these antibodies were purchased from Upstate Biotechnology (Lake Placid, NY). After extensive washing in PBS containing 0.1% Tween 20 and 0.01% Triton X-100, the embryos were labeled with secondary FITC (fluorescein isothiocyanate) conjugated antibody diluted 1:100 for 1 h at room temperature. Nuclear status of embryos was evaluated by staining with 10 μg/ml PI (propidium iodide) for 5 min. Following extensive washing, samples were mounted on slides. The fluorescence was detected by a Confocal Laser-Scanning Microscope (Zeiss LSM 510 META, Germany). Instrument settings were kept constant for each replicate. Each developmental panel was repeated three times, and at least 20 embryos were evaluated each time.

The fluorescence images were analyzed by using the program Image-J from the National Institutes of Health (http://rsb.info.nih.gov/ij/) (USA). The relative intensity was calculated as described previously [18].

Statistical analysis. All data were analyzed by the χ^2 test and considered significant difference at $P < 0.01$. Lack of statistical significance was reported as no difference when all tests gave $P > 0.05$.

Results

Since fertilization *in vivo* occurs about 12 h after injection of hCG, we used this time point as the *in vivo* fertilization start point to synchronize with IVF start point for sampling. The embryos obtained by natural mating were asynchronous, so we only collected *in vivo* embryos that were synchronous with IVF embryos to compare with IVF embryos at the same time point. For presentation purposes, only certain stages were shown.

Distribution patterns of AcH4 in in vivo and IVF embryos

At the pronuclear stage, the pattern of AcH4 staining was always uniform in male and female pronuclei in *in vivo* and IVF zygotes (Fig. 1A and H). When chromosomes arranged on the metaphase plate, the AcH4 staining still displayed in a granular pattern co-localized with chromosomes (Fig. 1B, B′, I, and I′). With preimplantation embryo development, the pattern of AcH4 staining was the same as the first cell cycle, which kept the staining in both interphase and mitosis, and displayed staining in a homogeneous pattern in the interphase in *in vivo* (Fig. 1C–F) and IVF embryos (Fig. 1J–M). Intense staining was found on some regions of condensing chromosomes, generally at the periphery of the nucleolus (Fig. 1D). AcH4 frequently co-localized with chromatin rich regions. At the blastocyst stage (Fig. 1G and N), the staining of AcH4 signals was less intense in the inner cell mass (ICM) when compared to the trophectoderm cell (TE). As statistically compared, there was no difference at the distribution pattern of AcH4 between *in vivo* and IVF embryos from zygote to blastocyst stage.

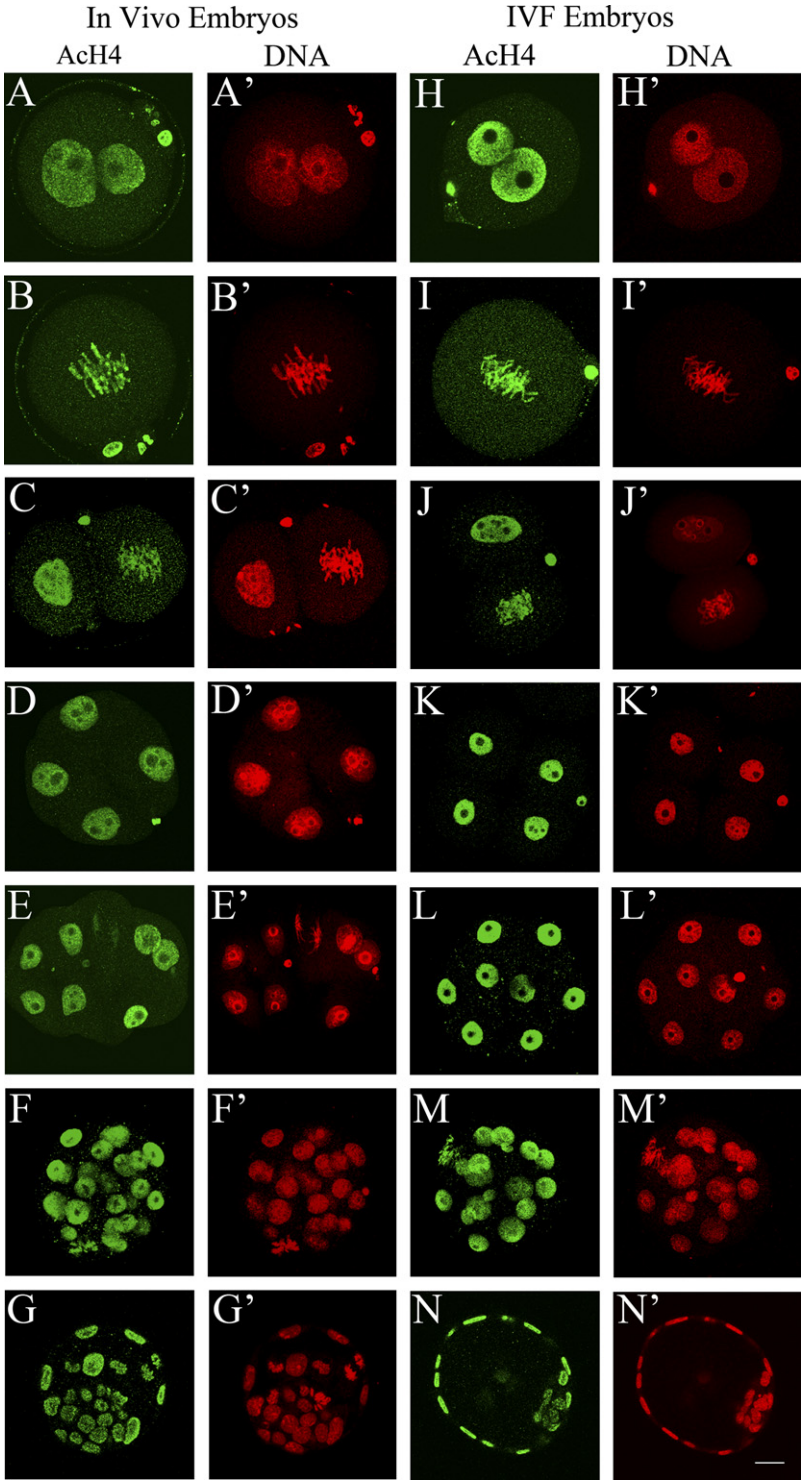

Fig. 1. Acetylation patterns of histone H4 (AcH4) in *in vivo* (A,A′–G,G′) and IVF mouse embryos (H,H′–N,N′). AcH4, green; DNA, red. The staining patterns in one-cell *in vivo* (A,A′) and IVF (H,H′) embryos at pronuclear stage, one-cell *in vivo* (B,B′) and IVF (I,I′) embryos at the first mitosis, two-cell *in vivo* (C,C′) and IVF (J,J′) embryos, four-cell *in vivo* (D,D′) and IVF (K,K′) embryos, eight-cell *in vivo* (E,E′) and IVF (L,L′) embryos, *in vivo* (F,F′) and IVF (M,M′) morulae, and *in vivo* (G,G′) and IVF (N,N′) blastocysts are shown. Bar, 20 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

Distribution patterns of MeH3K9 in in vivo and IVF embryos

At the pronuclear stage, MeH3K9 staining in *in vivo* and IVF zygotes was limited to the female pronucleus, which was intensely distributed at the nucleolar periphery, whereas the male pronucleus showed a complete lack of staining (Fig. 2A and H). This asymmetric methylation of MeH3K9 was maintained until the two-cell stage (Fig. 2C), in agreement with a previous study in IVF

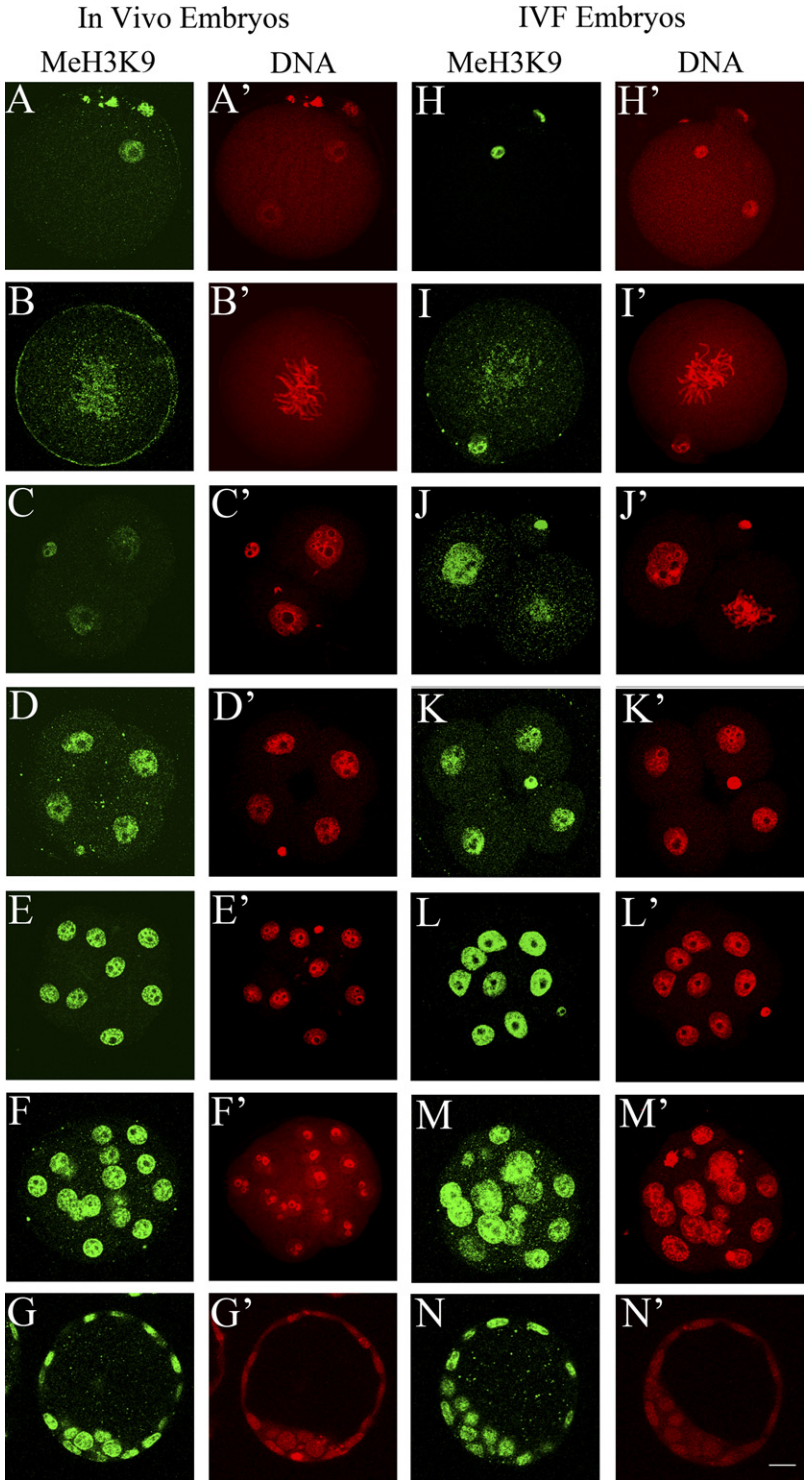

Fig. 2. Methylation patterns of histone H3 Lysine 9 (MeH3K9) in *in vivo* (A,A′–G,G′) and IVF mouse embryos (H,H′–N,N′). MeH3K9, green; DNA, red. The staining patterns in one-cell *in vivo* (A,A′) and IVF (H,H′) embryos at pronuclear stage, one-cell *in vivo* (B,B′) and IVF (I,I′) embryos at the first mitosis, two-cell *in vivo* (C,C′) and IVF (J,J′) embryos, four-cell *in vivo* (D,D′) and IVF (K,K′) embryos, eight-cell *in vivo* (E,E′) and IVF (L,L′) embryos, *in vivo* (F,F′) and IVF (M,M′) morulae, and *in vivo* (G,G′) and IVF (N,N′) blastocysts are shown. Bar, 20 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

embryos [19]. The staining was displayed in interphase nucleoplasm and mitotic chromosome at following developmental stages, although the staining was less intense at mitosis than that in interphase in both *in vivo* (Fig. 2C–F) and IVF (Fig. 2J–M) embryos. The level of MeH3K9 was increasing from the four-cell stage and

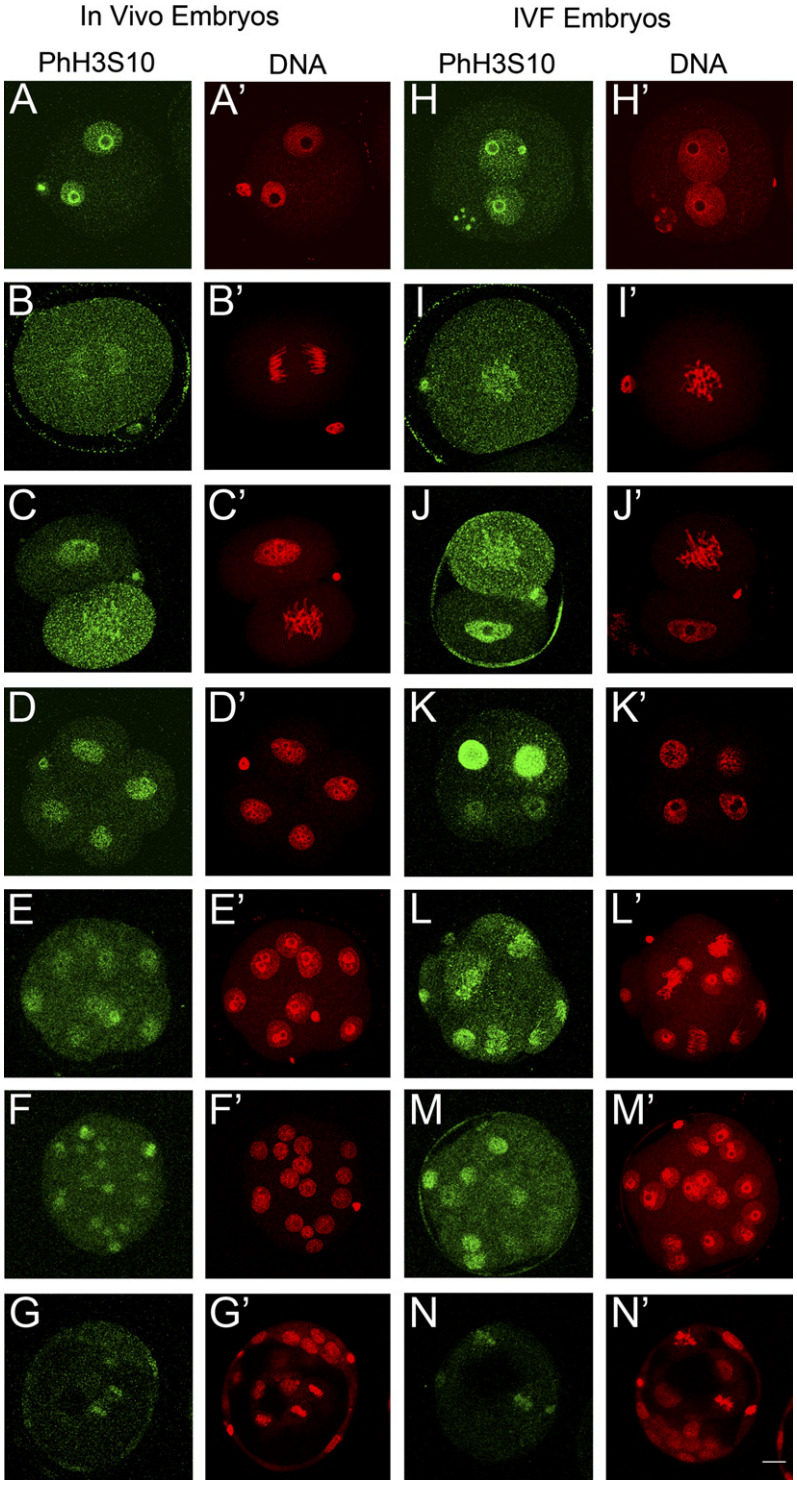

Fig. 3. Phosphorylation patterns of histone H3 Serine 10 (PhH3S10) in *in vivo* (A,A′–G,G′) and IVF mouse embryos (H,H′–N,N′). PhH3S10, green; DNA, red. The staining patterns in one-cell *in vivo* (A,A′) and IVF (H,H′) embryos at pronuclear stage, one-cell *in vivo* (B,B′) and IVF (I,I′) embryos at the first mitosis, two-cell *in vivo* (C,C′) and IVF (J,J′) embryos, four-cell *in vivo* (D,D′) and IVF (K,K′) embryos, eight-cell *in vivo* (E,E′) and IVF (L,L′) embryos, *in vivo* (F,F′) and IVF (M,M′) morulae, and *in vivo* (G,G′) and IVF (N,N′) blastocysts are shown. Bar, 20 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

uniform fluorescence was observed. At the blastocyst stage (Fig. 2G and N), the staining of MeH3K9 was homogeneous, without difference between the ICM and the TE, which was consistent with that reported by a recent study in *in vivo* embryos [20].

Distribution patterns of PhH3S10 in in vivo and IVF embryos

The previous study has shown that heterochromatin is predominantly localized at the perinucleolar region and

intranuclear patches in the zygote [21]. At the pronuclear stage, the strong staining of PhH3S10 at the perinucleolar region was observed except weak staining interspersed throughout the nucleoplasm in *in vivo* (Fig. 3A) and IVF (Fig. 3H) zygotes. During the period of the first mitosis, the increased staining kept on the condensed chromosomes (Fig. 3I), and then gradually decreased at anaphase (Fig. 3B). Like in the first mitosis, the staining of PhH3S10, compared with that in interphase, increased during the following mitosis, and weakly scattered throughout the blastomere except the strong staining co-localized with the chromosome (Fig. 3C, J, K, and L). In the following interphase, the staining was obvious in the nucleoplasm from two-cell to morula stage (Fig. 3C–F, J–M), but undetected at blastocyst stage (Fig. 3G and N). As compared, there was no difference at the distribution pattern of PhH3S10 between *in vivo* and IVF embryos at all examined stages.

Discussion

When immunofluorescence localization and statistic analysis of AcH4, MeH3K9, and PhH3S10 were performed, no significant differences were seen between *in vivo* and IVF embryos from zygote to blastocyst stage. These histone modifications have different distribution patterns at all examined stages, but they are consistent with the developmental stages.

In all developmental stages examined, homogeneous staining pattern of AcH4 was kept in *in vivo* and IVF embryos (Fig. 1).The maintenance of acetylation through replication and mitosis would provide a mechanism by which transcription could be reactivated in next cell cycle. Moreover, at blastocyst stage, we observed that the staining in the TE was more intense than that in the ICM. Coincidentally, DNA methylation is in a lower level in the TE than that in the ICM at this stage [22]. In general, histone acetylation is thought to facilitate transcription, but DNA methylation and MeH3K9, which has different at advanced stages [23] although MeH3K9 is no different between TE and ICM at this stage, reverses this effect. The epigenetic asymmetry established in the blastocyst is important for development.

Our data indicated that H3S10 was phosphorylated at mitosis from zygote to blastocyst stage. In the interphase, the staining of PhH3S10 was less intense than that at mitosis from zygote to morula stage, but strong in heterochromatin (Fig. 3A and H). At blastocyst stage, however, no staining of PhH3s10 was observed in the interphase. These might correlate with different transcription statuses of embryo developmental stages. PhH3S10 is involved in both transcription and cell division. In interphase, PhH3S10 correlates with chromatin relaxation and gene expression, whereas in mitosis it correlates with chromosome condensation and segregation [6].

To our knowledge, this study was the first report to compare the histone modification patterns between *in vivo* and IVF embryos. Our results indicated that there was no difference in AcH4, MeH3K9, and PhH3S10 between *in vivo* and IVF embryos from zygote to blastocyst stage. The histone modifications as epigenetic marks that control the developmental pattern of the preimplantation mouse embryo are impacted by many factors, such as the status of sperm, oocyte quality, and environmental influence (*in vitro* culture, manipulation). For instance, a recent report [24] demonstrates that the paternal zygotic genomes derived from spermatids are distinguishable from normal zygotic paternal genomes in terms of epigenetic modifications. This might be one of the most important reasons that there is a significantly lower success rate for offspring produced by round spermatid injection, compared to sperm injection [25,26]. Moreover, the zygotes obtained from *in vitro* matured oocytes have a reduced competence in histone H4 acetylation in comparison with that in zygotes from *in vivo* matured oocytes [27]. In addition, *in vitro* culture and manipulation of early embryos also affect epigenetic modifications, although their responses are different. Khosla et al. have shown that the addition of fetal calf serum to M16 medium can decrease *H19* and *Igf2* expression, but embryos cultured in M16 medium without fetal calf serum are no different from those in the control [28]. In our study, mature spermatozoa and *in vivo* matured oocytes used in IVF were the same as those in *in vivo* embryos; embryos cultured in CZB, which is an optimal medium; others were the same as those in *in vivo* embryos. These were parts of the most important reasons that this widely used optimal IVF procedure *per se* did not alter histone acetylation patterns at all tested stages. These suggested that the IVF procedure *per se*, to some extent, was relatively safe. Therefore, we should not be surprised to see the fact that the enormous majority of the offspring conceived by IVF are healthy [29].

However, it must be noted that we cannot exclude that other abnormalities, originating from the aberrance of individual genes and resulting in long-term defects, may arise from IVF. *In vitro* culture and embryo manipulation have been associated with aberrant fetal growth. In mice and human, there seems to be a reduction in birth weight, whereas in cattle and sheep which is referred to as the large offspring syndrome. It has been suggested that at least some of the problems may result from an accumulation of epigenetic alterations during embryo culture [30,31]. This has raised the questions that need further study: to which extent epigenetic abnormalities are caused by those factors such as *in vitro* culture and embryo manipulation, and how these factors induce epigenetic alterations.

In summary, we conclude that there was no difference in the distribution patterns of the histone modifications between *in vivo* and IVF embryos from zygote to blastocyst stage. This suggests that the widely used optimal IVF procedure *per se* may not alter the histone modification patterns during the preimplantation embryo development.

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