



## Differences in H3K4 trimethylation in *in vivo* and *in vitro* fertilization mouse preimplantation embryos

F.-R. Wu\*, Y. Liu\*, M.-B. Shang, X.-X. Yang, B. Ding, J.-G. Gao, R. Wang and W.-Y. Li

School of Life Science, Fuyang Teachers College,  
Key Laboratory of Embryo Development and Reproductive Regulation,  
Anhui Province, Fuyang, China

\*These authors contributed equally to this study.  
Corresponding author: W.-Y. Li  
E-mail: liwyfyu@yahoo.com.cn

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**ABSTRACT.** Trimethylation of lysine 4 at histone 3 (H3K4me<sub>3</sub>) is considered a marker of active transcription; it plays an important role in transcription reprogramming efficiency. We compared the levels of H3K4me<sub>3</sub> in mouse preimplantation embryos from MII stage oocytes produced by *in vivo* and *in vitro* fertilization (IVF) using immunofluorescence histochemistry. IVF embryos were further treated with trichostatin A (a histone deacetylase inhibitor) to investigate the effect of histone acetylation on H3K4me<sub>3</sub>. We found higher levels of H3K4me<sub>3</sub> in MII stage oocytes in metaphase chromosomes. The pattern of H3K4 trimethylation of *in vivo* embryos from zygote to blastocyst stages was similar to that of IVF embryos; however, the concentration of H3K4me<sub>3</sub> was significantly higher in the *in vivo* fertilization embryos. The levels of H3K4me<sub>3</sub> in the trichostatin A-treated groups were also significantly increased. We conclude that culture condition and environmental changes can cause histone modification and that the

effect of these environmental conditions on epigenetic changes should be taken into consideration.

**Key words:** Lysine 4 at histone 3; Trimethylation; Culture conditions; *In vivo* and IVF embryos; Mouse

## INTRODUCTION

DNA methylation and histone acetylation and methylation are important epigenetic modifications. Lysine methylation of histone was first reported by Murray (1964). Since then, greater attention has paid to the functional significance of this modification. Mammalian and yeast cells demonstrate that lysines 4, 36, and 79 of histone H3 are major methylation sites, the modification of which plays an important role in many biological processes of various species (Ruthenburg et al., 2007; Shilatifard, 2008). In general, the methylation of lysine 4 at histone H3 (H3K4), which is catalyzed by at least 6 H3K4 methyltransferases, is highly conserved through evolution. Four methylation states occur on H3K4: unmethylated and mono-, di-, and trimethylated (Flanagan et al., 2005; Shi et al., 2006; Wysocka et al., 2006). Previous studies have shown that H3K4 trimethylation (H3K4me3), together with acetylation of other residues (i.e., K9, K14, K18, K23, and K27) on the same H3 molecules, is localized on euchromatin and associated with the gene transcription activation (Nightingale et al., 2007). In mouse embryonic stem cells, the H3K4 methyltransferase, myeloid/lymphoid or mixed-lineage leukemia 2 is required for certain epigenetic decisions during differentiation and is related to DNA methylation of CpG islands on gene promoters (Glaser et al., 2009). Trichostatin A (TSA), an effective histone deacetylase (HDAC) inhibitor, has often been used to increase the level of histone acetylation (Baqir et al., 2002). Recently, it was found that TSA treatment improves the blastocyst rate but does not alter the acetylation levels of TSA (+) somatic cell nuclear transfer embryos or pig embryos fertilized via *in vitro* fertilization (IVF) at the pseudo-pronuclear and 2-cell stages (Yamanaka et al., 2009).

In mammals, the developmental potential and viability of preimplanted embryos are sensitive to the internal environment. Many studies have shown that changes in environment can alter epigenetic modification (Dey et al., 2004; Fleming et al., 2004). For example, changes in the expressions of some imprinted genes and the methylation state of CpG islands in preimplantation mouse embryos were found to correspond to the differential effects of culture conditions (Doherty et al., 2000). IVF is widely used as an assisted reproductive technology in mice owing to the easy manipulation of mouse ovulation and fertilization. In contrast to embryos fertilized *in vivo*, IVF embryos are exposed to *in vitro* culture medium and can be used to test various drug effects. *In vitro* conditions have important impacts on embryos, however, including alterations of epigenetic modification levels and the expression of development-related genes. Despite intensive research on the function and regulation of H3K4me3, no report details the differences in the H3K4me3 pattern in MII stage oocytes and *in vivo* and IVF embryos. To compare the levels of H3K4me3 in MII stage oocytes and *in vivo* and IVF embryos, indirect immunofluorescence staining was performed. Furthermore, to elucidate the relationship between the level of H3K4me3 and histone acetylation, IVF embryos were further treated with TSA. We found that the levels of H3K4me3 in *in vivo* embryos were higher than those in IVF embryos, and the pattern of H3K4me3 in the TSA-treated group was closer to that in *in vivo* embryos. Our results provide new information on the effects of environmental change on epigenetic modification.

## MATERIAL AND METHODS

### Animals

Kunming white mice were purchased from the Experiment Animal Center of Anhui Medical University and maintained in a 14-h light and 10-h dark photoperiod at 20-25°C for at least 2 weeks before use. All animals were maintained in accordance with the Animal Experiment Standard of Fuyang Teachers College.

### Collection and culture of oocytes and embryos

Female Kunming white mice, 40-45 days old, were superovulated with 5 IU pregnant mare serum gonadotropin (PMSG; Ningbo Second Hormone Factory, Zhejiang, China) followed by 5 IU human chorionic gonadotropin (hCG; Ningbo Second Hormone Factory) 48 h later. To obtain MII oocytes, superovulated oocytes were collected in M2 medium, and cumulus cells were removed with 0.1% hyaluronidase treatment for 3-5 min in the same solution. For *in vivo* embryo collection, one female was placed in a cage with one stud male after the administration of hCG, and embryos at different stages were collected as described elsewhere (Nagy et al., 2003). For IVF embryo collection, cumulus oocyte complexes from the ampullae of the oviducts were collected in modified CZB medium (a modification of BMOC2 medium, containing an increased lactate/pyruvate ratio, 0.1 mM EDTA, and 1 mM glutamine as a replacement for glucose) 14-15 h after hCG injection. Spermatozoa were collected from the caudal epididymis of adult Kunming white male mice and capacitated with preincubation in human tubal fluid medium for 1.5 h. Cumulus-oocyte complexes were inseminated with capacitated spermatozoa in a humidified atmosphere of 5% CO<sub>2</sub>, 95% air at 37°C. Five hours after insemination, the fertilized oocytes were washed and cultured in CZB medium (Nagy et al., 2003). Both *in vivo* and IVF embryos, zygotes, 2-cell embryos, 4-cell embryos, 8- to 16-cell embryos, morulae, and blastocysts were collected.

### TSA treatment

Briefly, TSA was dissolved in dimethyl sulfoxide, and the concentrated stock solution was stored at -20°C. The TSA stock solutions were added to the CZB culture media to a final concentration of 50 nM according to the experimental design. Four hours after fusion, the IVF zygotes were transferred to the media with TSA for 20 h and then transferred to the normal media. Two-cell, 4-cell, and 8- to 16-cell embryos, morulae, and blastocysts were collected. For control treatments, embryos at different stages were cultured in CZB containing 0.05% dimethyl sulfoxide.

### Indirect immunofluorescence histochemistry

Embryos at various developmental phases generated by *in vivo* fertilization and IVF were washed in 1X phosphate-buffered saline (PBS), fixed for 30 min in 4% paraformaldehyde in 1X 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% bovine serum albumin for 1 h at room temperature and incubated overnight at 4°C in a 1:100 dilution of primary

antibodies against H3K4me3 (Catalog No. A-4033-025, Epigentek Group Inc., USA). After extensive washing in PBS containing 0.1% Tween 20 and 0.01% Triton X-100, the embryos were labeled with secondary fluorescein isothiocyanate-conjugated antibody diluted 1:100 for 1 h at room temperature. The nuclear status of embryos was evaluated via staining with 10 µg/mL propidium iodide (Catalog No. P4170, Sigma, USA) for 5 min. After washing, samples were mounted on slides. In each experiment, samples without primary antibody were included as negative controls. Fluorescence was detected using a Carl Zeiss LSM710 nonlinear optic laser scanning confocal microscope according to the manufacturer instruction.

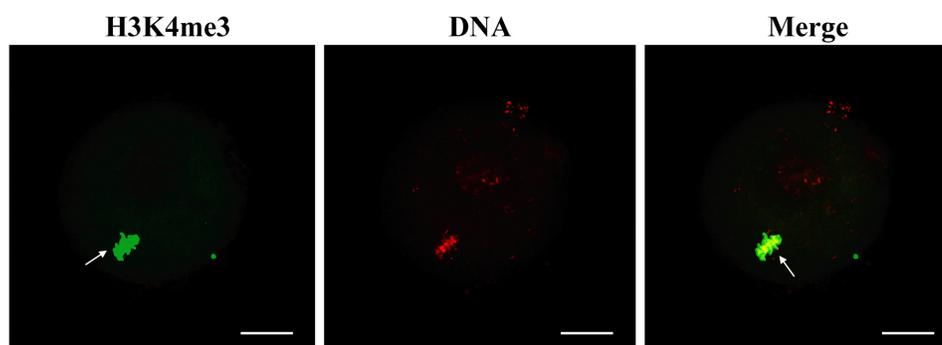
### Statistical analysis

Each developmental panel was repeated three times, and at least 20 oocytes or embryos were evaluated each time. The fluorescence images were analyzed using the program Image-J from the National Institutes of Health (<http://rsb.info.nih.gov/ij/>; USA). The relative intensity was calculated and analyzed as described elsewhere (Kim et al., 2002). The Kruskal-Wallis test was used to determine significant difference ( $P < 0.05$ ) with the GraphPad Prism 4 software (GraphPad Software, San Diego, CA, USA).

## RESULTS

### Levels of H3K4me3 in MII stage oocytes

The trimethylation levels of H3K4 were examined in MII stage oocytes. Indirect immunofluorescence with specific antibodies against H3K4me3 showed intense fluorescence signals in the metaphase chromosome of the examined oocytes (Figure 1).

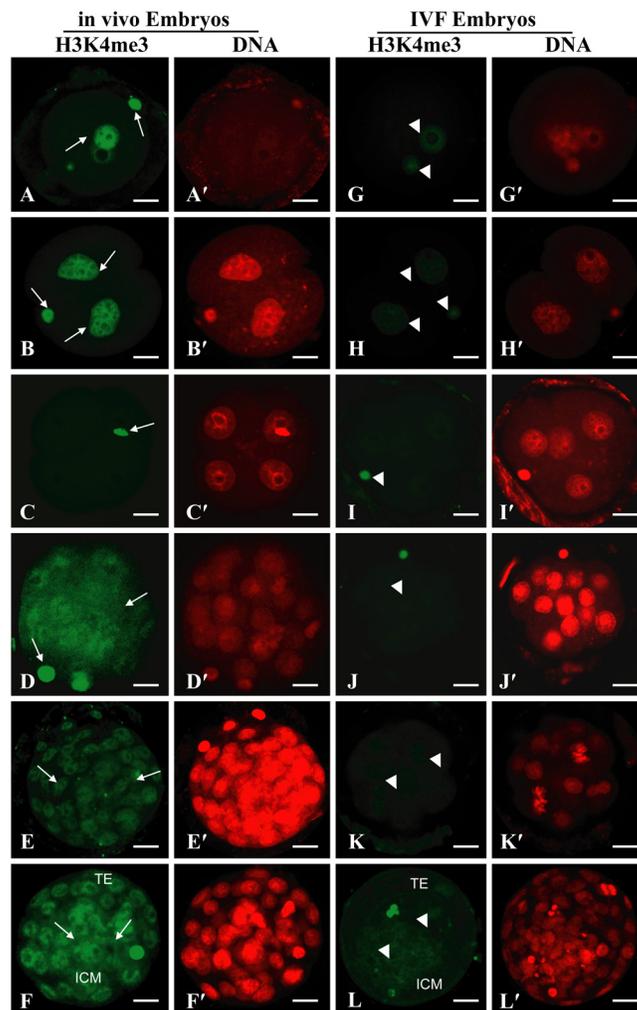


**Figure 1.** Trimethylation of lysine 4 at histone 3 at mouse MII oocytes. Oocytes were immunostained with specific antibodies against trimethylated lysine 4 at histone H3 (H3K4me3), in green; DNA, in red. Arrows indicate signals of H3K4me3 in MII oocytes. Scale bar = 20 µm.

### Pattern of H3K4me3 in *in vivo* and IVF embryos

At the pronuclear stage, H3K4me3-staining signals in *in vivo* and IVF zygotes were detected in the male pronucleus and polar body, showing an asymmetric pattern. Moreover,

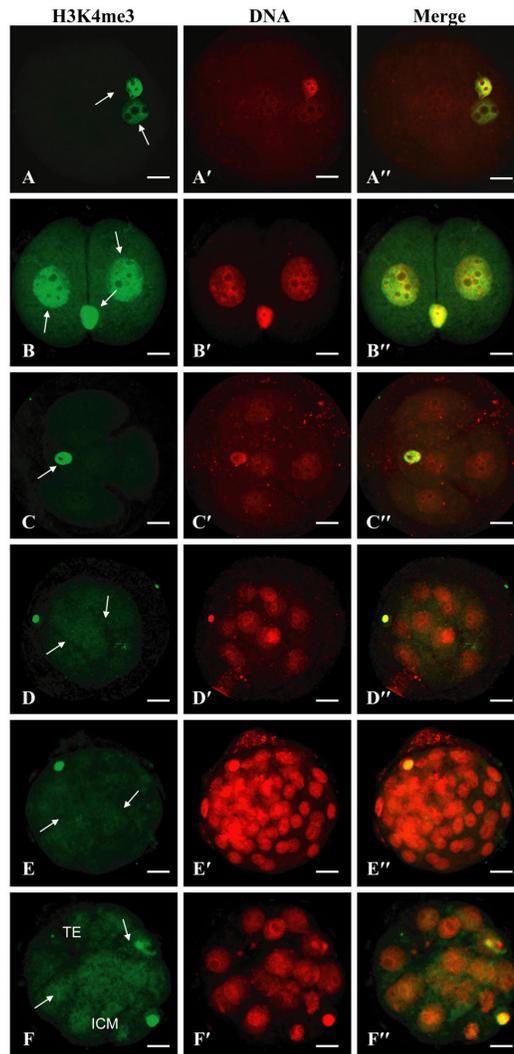
the signal in *in vivo* zygotes was stronger than that of IVF zygotes (Figure 2A and G). This asymmetric methylation of H3K4me3 was maintained until the 2-cell stage (Figure 2B and H). In 4-cell *in vivo* and IVF embryos, only a very weak signal was detected (Figure 2C and I). The levels of H3K4me3 increased between the 8- to 16-cell and blastocyst stages in both *in vivo* and IVF embryos (Figure 2D-F and J-L) and was distributed in the interphase nucleoplasm and mitotic chromosomes of the cells. At the blastocyst stage of *in vivo* and IVF embryos, the staining of H3K4me3 in the inner cell mass and trophoblast cells was similar (see Figure 2F and L). The results of the negative controls are not shown.



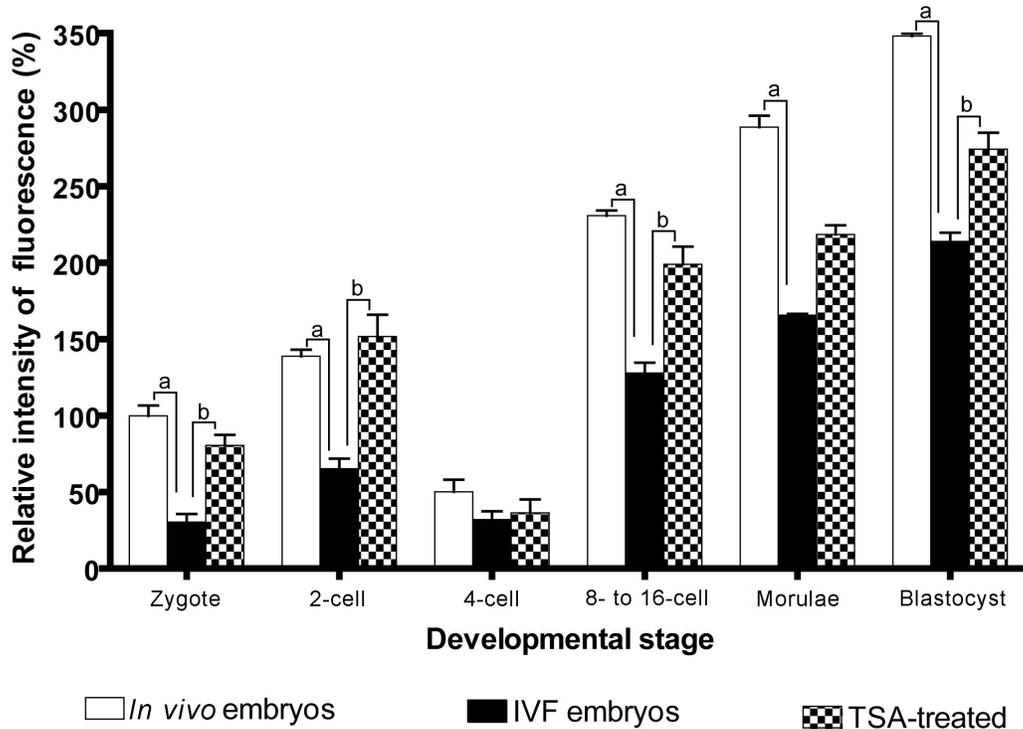
**Figure 2.** Patterns of H3K4 trimethylation in *in vivo* (A, A'-F, F') and IVF (G, G'-L, L') mouse embryos. The H3K4me3 patterns in 1-cell at pronuclear stage, 2-cell, 4-cell, 8- to 16-cell, morulae, and blastocysts *in vivo* (A, A'-F, F') and IVF (G, G'-L, L') embryos are shown. Arrows and arrowheads indicate signals of H3K4me3 in *in vivo* and IVF mouse embryos, respectively. ICM = inner cell mass; TE = trophoblast cell. Bar = 20  $\mu$ m.

### Effect of TSA on the levels of H3K4me3

To study the effect of histone acetylation on the levels of H3K4me3, zygotes were cultured with TSA for 20 h. Compared with untreated IVF embryos, TSA-treated IVF embryos displayed increased levels of H3K4me3 (Figure 3A-F). The patterns of H3K4me3 in TSA-treated and control groups (data not shown) were similar to those in *in vivo* and IVF embryos (see Figures 2A-L and 4). Furthermore, the signals in IVF embryos were weaker than those in *in vivo* embryos (Figure 4).



**Figure 3.** Effects of TSA on H3K4me3 in IVF mouse embryos. The H3K4me3 patterns in 1-cell at pronuclear stage (A, A', A''), 2-cell (B, B', B''), 4-cell (C, C', C''), 8- to 16-cell (D, D', D''), morulae (E, E', E''), and blastocysts (F, F', F'') in IVF embryos are shown. Arrows indicate signals of H3K4me3 in TSA-treated IVF mouse embryos. Bar = 20  $\mu$ m.



**Figure 4.** Semi-quantification of trimethylated histone H3K4 of the *in vivo*, IVF and TSA-treated mouse embryos. Data were collected from three independent experiments, and at least 20 oocytes or embryos were evaluated each time. The columns and bars represent means  $\pm$  SEM; a and b indicate statistical significance at  $P < 0.05$  (a, *in vivo* versus IVF; b, IVF versus TSA-treated embryos).

## DISCUSSION

We investigated and compared the patterns of H3K4me3 in *in vivo* and IVF embryos. The results showed that levels of H3K4me3 in *in vivo* embryos were higher than those in IVF embryos. Furthermore, raising the levels of histone acetylation synchronously raised the levels of H3K4me3 in IVF mouse embryos.

H3K4me3 is a marker associated with active transcription, and it has a close relationship with histone acetylation and deacetylation (Eissenberg and Shilatifard, 2010; Guillemette et al., 2011). A previous study has shown that histone H3 is deacetylated globally in the MII oocytes of 3-week-old mice, and the acetylation at all of the lysine residues of histone H3 was undetectable or negligible in the oocytes during meiosis (Kim et al., 2003). When the nuclei of mammalian somatic cells are transplanted into amphibian oocytes, H3K4me3 is important for efficient reprogramming of pluripotency genes (Murata et al., 2010). Furthermore, the acetylation levels of lysine 14 at histone H3 in mouse oocytes are gradually increased during postovulatory aging (Huang et al., 2007a). The oocytes in our study were harvested earlier than those used in previous studies; hence, the levels of H3K4me3 were more intensive. This finding is consistent with previous results. Our results also indicated that H3K4me3 might

activate a series of key maternal transcription factors and is thus required to establish a new program after fertilization.

We found that the levels of H3K4me3 of *in vivo* and IVF embryos are dissimilar in the zygote and blastocyst stages, but the patterns of H3K4me3 in *in vivo* embryos are similar to those of IVF embryos. The difference can be attributed to the higher levels of H3K4me3 in *in vivo* embryos compared to those in IVF embryos. We know that the development of preimplantation embryos in mice is a complex process that involves several maternal-effect growth factors, cytokines, and hormones (Li et al., 2010). For example, transcription from newly formed zygotic and embryonic genomes occurs before cleavage and at the 2-cell stage, respectively (Hamatani et al., 2004). Unlike in *in vivo* embryo development, *in vitro* culture and manipulation cause environmental changes during IVF embryo development. Many local gonadal factors and maternal hormones cannot participate in the developmental process. Culture and environmental changes might be among the most important influences of changes in epigenetic modification.

In mice, an important estrogen, 17 $\beta$ -estradiol, can increase the acetylation of histone H3 by increasing HDAC1 expression and reducing HDAC2 expression (Zhao et al., 2010). With this consideration in mind, we added TSA to the culture medium to inhibit the activity of HDAC. Interestingly, H3K4me3 levels in TSA-treated groups were increased compared to those in IVF embryos, similar to the levels of H3K4me3 in *in vivo* embryos. Furthermore, normal embryonic development to the blastocyst stage within the reproductive tract (from the oviduct to the uterine horn) requires the presence of ovarian estrogen (McLaren, 1971; Dey et al., 2004). These findings, together with our results, suggest that H3K4me3 is regulated by the acetylation of H3 and by the maternal environment, and that local factors play important roles in epigenetic modification and the establishment of nuclear totipotency during later stages of development.

Although IVF is a widely used technology in the fields of developmental and reproductive research, we must note that abnormalities can be caused by *in vitro* manipulation and the absence of maternal-effect genes, hormones, and some growth-promoting factors. For example, a lack of estrogen-synthesizing capacity in mouse embryos results in unfavorable implantation at later stages (Strömstedt et al., 1996). Compared to those of normal fetuses, the birth weights of fetuses derived from IVF mouse embryos were reduced (Young and Fairburn, 2000). The patterns of H4 acetylation and H3K9me3, however, were similar between *in vivo* and IVF embryos from the zygote to the blastocyst stage in mice (Huang et al., 2007b). We do not know whether the environment of *in vitro* cultures selectively induces epigenetic alterations of only histone H3.

Taken together, our results showed that the levels of H3K4me3 in *in vivo* embryos were much higher than those in IVF embryos from the zygote to the blastocyst stage. These differences might be derived from the culture conditions and environmental changes that accompany assisted reproductive technology. Systematic study of the involvement of culture conditions in mouse preimplantation embryo development and the epigenetic risks related to assisted reproductive technologies is warranted.

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