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Epigenetic changes of histone deacetylation in murine oocytes matured *in vitro* versus *in vivo*

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Abstract. – OBJECTIVE: In this study, we studied changes in histone acetylation during mouse oocytes meiosis. The aim was to investigate HDAC1 expression patterns in the mouse oocytes and effects of *in-vitro* maturation on epigenetic modifications during meiosis.

MATERIALS AND METHODS: Immature and mature oocytes were collected from female Kunming white mice of 4-6 weeks in age. Dynamic changes of histone H3K9, H4K12 acetylation were explored. HDAC1 spatial and temporal expression patterns during meiosis and their expression changes in *in-vitro* maturation were determined.

RESULTS: It was found that histone H3K9 and H4K12 acetylations were gradually disappeared during the meiotic maturation of mouse oocytes. HDAC1 proteins were localized mainly throughout the nucleoplasm in GV-intact oocyte, and colocalized with chromosomes at metaphase II (MII). The acetylated H3K9 and H4K12 were absent in oocytes matured *in vivo*, while the elevated acetylation of H3K9 and H4K12 was detected in oocytes matured *in vitro*. When cultured *in vitro*, the decrease of HDAC1 protein level and mRNA level were observed compared with oocytes matured *in vivo*.

CONCLUSIONS: the acetylation of H3K9, H4K12 decreased gradually to undetectable during oocyte meiosis. The histone deacetylation in oocytes was inadequate during *in vitro* maturation, and the *in vitro* maturation might lead to reduced HDAC1 expression in oocytes.

Key Words:
In vitro maturation, Oocytes, Histone deacetylase, HDAC1.

Introduction

The human oocytes used for IVF and ICSI are typically matured *in vivo* by various hormonal treatments[1]. IVM of human oocytes would greatly enhance the pool of available oocytes for *in vitro* procedures, while simultaneously minimizing undesirable side effects of conventional IVF protocols associated with the intensive hormonal treatment[2]. Also, assisted reproductive technologies are associated with an increased *occurrence* of epigenetic aberrations. In this study, we used the murine oocyte as a model to determine putative epigenetic changes caused by the type of maturation, either *in vitro* maturation (IVM) in Tissue Culture Medium 199, or *in vivo* maturation. We investigated the global levels of these two histone modifications, acetylation of H3K9 and H4K12. We also investigated the expression of histone deacetylase1 (HDAC1) as well as histone deacetylase2 (HDAC2) in murine oocyte maturation. We found that global levels of Ac-H3K9 and Ac-H4K12 decreased during maturation. The expression levels of Hdac correlated well with these findings.

Materials and Methods

Animals

Animal care and handling were conducted in accordance with policies by the Ethics Committee of the First Affiliated Hospital, Sun Yat-Sen University. We used Kunming white mice as oocyte donors.

In Vivo Oocyte Collection

Female Kunming white mice of 4-6 weeks age were superovulated with 10 IU of pregnant mare's serum gonadotropin (Ningbo Pharmacy Factory, China), followed by 10 IU of human chorionic gonadotropin (Lizhu Pharmacy, China) treatment after 48h. For *in vivo* matured oocyte collection, cumulus-oocyte complexes were collected from the ampullae of oviducts, 12-14 h after hCG injection. Cumulus-oocyte complexes were wa-

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shed in HEPES. The "naked" oocytes were used for further examinations.

IVM

For IVM group, mice were superovulated by 10 IU PMSG by 48 h before being sacrificed by cervical dislocation. The ovaries were excised, and antral follicles were punctured with needles in tissue culture medium 199 (TCM 199; Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% serum protein substitute (SAGE In-Vitro Fertilization, Inc., Trumbull, CT, USA). The cumulus-enclosed oocytes at GV stage were selected. Immature oocytes were cultured in TCM-199 in a humidified atmosphere of 5% CO_2 in air at 37°C for 14-16 hours. Oocytes were observed under microscopy, and the disappearance of germ vesicle and the extrusion of the first polar body were used as the criteria of the maturation of oocytes.

Immunofluorescence Staining

Oocytes were fixed for 20 min in 2% formaldehyde in phosphate buffered saline (PBS; pH 7.4) at 37C°. They were permeabilized with 0.2% Triton X-100 for 1 h at room temperature (RT), then blocked for 1 h in 1% bovine serum albumin (BSA)-supplemented PBS. Oocytes were incubated overnight at 4C° in the primary antibodies diluted in blocking solution, followed by the second antibody conjugated with Alexa Fluor for 1 h at RT, while chromatin was stained with 4',6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich, St. Louis, MO, USA). The antibodies and the dilutions used in the study were as follows: H3K9ac (1:100, Abcam, Cambridge, UK); H4K12ac (1:1000, CST, USA); HDAC1 (1:200, Abcam UK); α-tubulin (1:1000, Abcam UK); Alexa Fluor 488, goat anti-mouse (1:1000, Molecular Probes, Eugene, OR, USA); Alexa Fluor 546, goat anti-rabbit (1:1000, Molecular Probes, Eugene, OR, USA). Labeled oocytes were then washed and mounted to a slide with Mounting Medium (R&D System, Minneapolis, MN, USA) and examined under a laser scanning confocal microscope (Olympus, FV1000, Tokyo, Japan). Negative controls were run in the absence of primary antibodies.

For each H3K9 (or H4K12), the laser power and the interval of the Z series were kept constant so that all images were scanned at the same laser power.

Real-time Quantitative PCR

Every 30 "naked" oocytes were loaded into each PCR tube. RNA extraction, cDNA synthesis and qRT-PCR analysis were performed using the TagMan Gene Expression Cells-to-CT Kit (Applied Biosystems, Foster City, CA, USA) and TaqMan Gene Expression Assay (Applied Biosystems, Foster City, CA, USA) for HDAC1 (Hs02621185_s1), and ACTB (Hs99999903_m1) according to manufacturer's instructions. ACTB served as an endogenous control. Real-time qPCR was performed using the ABI 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Relative mRNA expression levels of target genes were calculated using the comparative ΔCT method. ACTB served as an endogenous reference gene. In addition, results of RNA from sample oocytes were normalized to the GV oocyte.

Western Blotting

Protein from mouse oocytes was isolated via cell lysis buffer and was separated on 10% SDS-polyacrylamide gel before transferring onto a nitrocellulose membrane. Each membrane was washed with tris buffered saline-tween (TBS-T) and blocked with 5% skim milk for 1 h at room temperature prior to incubation with the primary antibody for 1 h (HDAC1 diluted as 1:500; H3K9ac diluted as 1:500; H4K12ac diluted as 1:500; α-tubulin diluted as 1:1000). The membranes were then washed in TBS-T and probed with the corresponding HRP-conjugated secondary antibody (1:5000) for 1 h. Bands were visualized using an enhanced chemiluminescence kit (Amersham Pharmacia Biotech, Buckinghamshire, UK).

Statistical Analysis

Statistical comparisons between groups were performed with analysis of one-way ANOVA in SPSS 13.0 (SPSS Inc., Chicago, IL, USA). LSD test was used for post hoc test. The difference of $p<0.05$ was considered as statistically significant.

Results

Histone Acetylation Patterns in Mouse Oocytes

As shown in Figure 1, the nucleus of GV-stage oocytes was positively stained with the ac-H3K9 antibody (Figure A1-A3), but the chromosomes of MII oocytes showed no staining for ac-H3K9 (Figure B1-B3). The staining of ac-H4K12 showed the same pattern as ac-

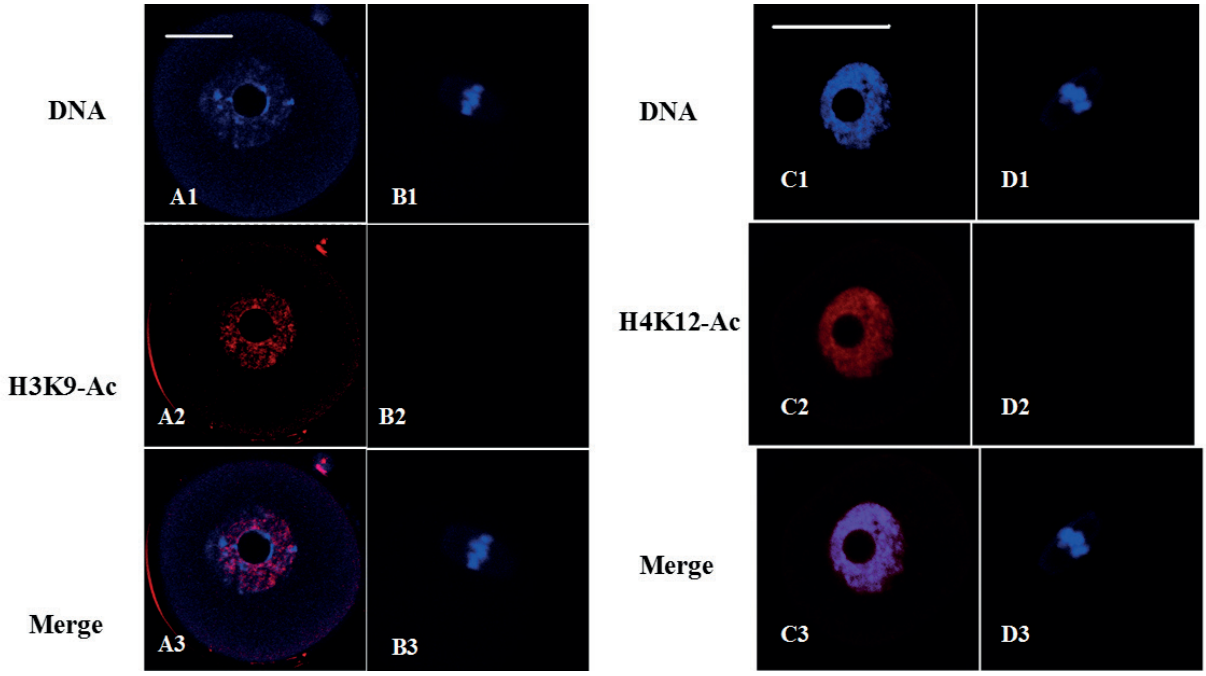

Figure 1. Changes in H3K9ac and H4K12ac in mouse oocytes at different maturation stages. Acetylated H3K9 and H4K12 were stained with red. The DNA was stained with blue (A1-A3, C1-C3): germinal vesicle (B1-B3, D1-D3): metaphase II matured *in vivo*. GV oocytes showed intense staining for H3K9ac and H4K12ac. MII oocytes matured *in vivo* had no staining for H3K9ac orH4K12ac. Bar=20 μm. Each experiment was repeated at least 3 times and similar results were obtained.

H4K12 (Figure C1-C3, D1-D3). Histone H3K9 and H4K12 acetylations disappeared during the meiotic maturation of mouse oocytes. HDAC1 can be detected in mouse oocytes. HDAC1 proteins localized mainly throughout the nucleoplasm in GV-intact oocyte and colocalized with chromosomes at metaphase II (MII) (Figure A1-A3, B1-B3).

Comparison of Histone Acetylation in Mouse oocytes Matured in vitro and in vivo

Western Blot analysis showed that acetylated H3K9 and H4K12 were absent in oocytes matured *in vivo*, while the elevated acetylation of H3K9 and H4K12 was detected in oocytes matured *in vitro* (Figure 3).

Effects of IVM on HDAC1 Expression in Mouse Oocytes

As shown in Figure 4, *in vivo* matured oocytes possessed significantly higher HDAC1 mRNA expression level than oocytes matured *in vitro* ($p<0.01$) (Figure 4). We also compared HDAC1 expression between these groups by Western blot.

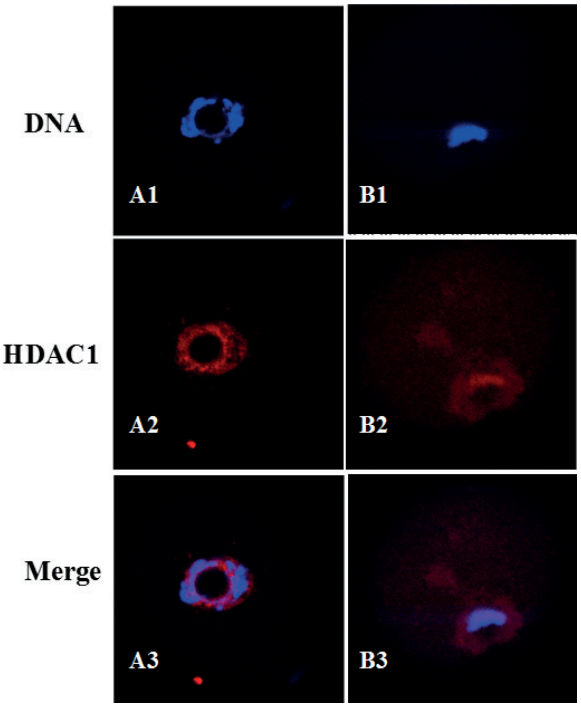

Figure 2. Localization of HDAC1 in mouse oocytes HDAC1 were stained with red. The DNA was stained with blue (A1-A3): germinal vesicle; B1-B3: metaphase II.

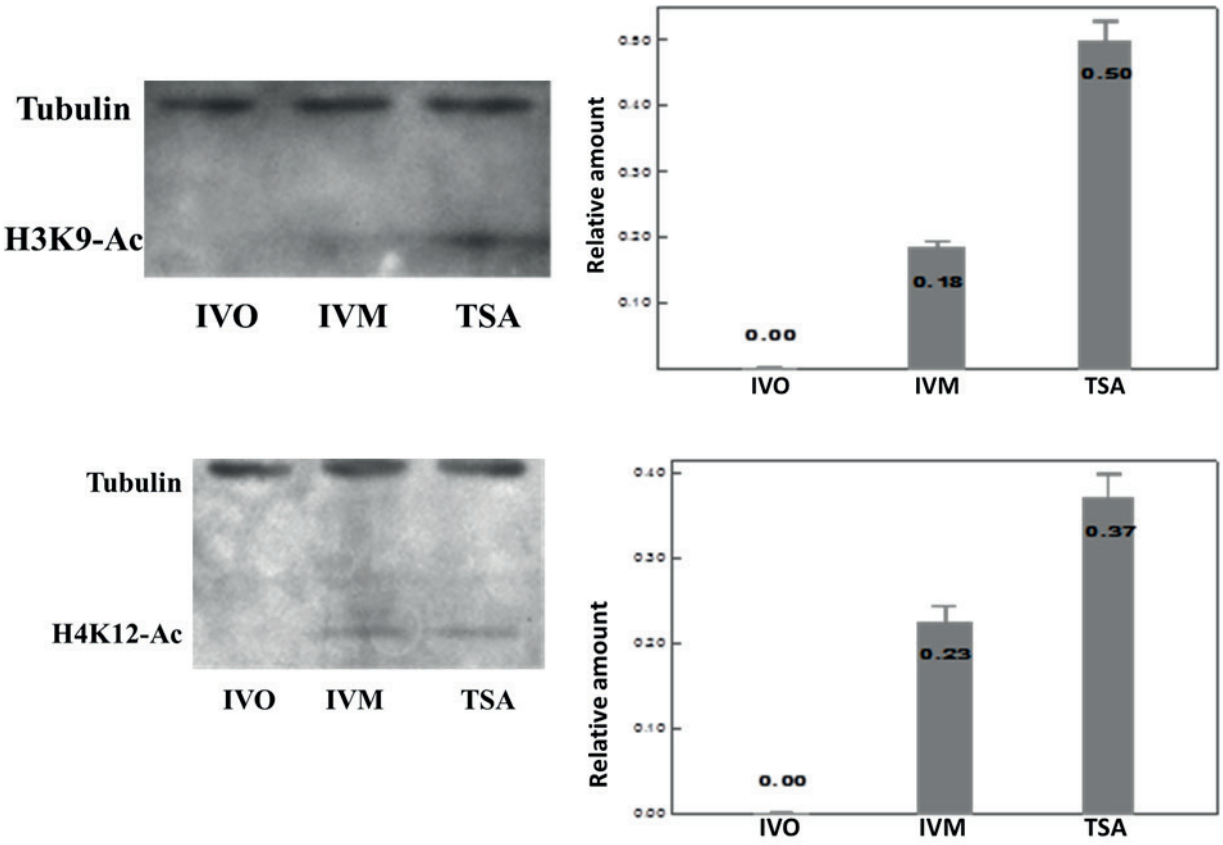

Figure 3. Histone acetylation of mouse oocytes during in vitro and *in vivo* maturation Protein levels of histone modification patterns (H3K9ac, H4K12ac) were detected via Western blot in oocytes matured *in vivo vs. in vitro*. Two hundred oocytes were loaded per lane. α-Tubulin served as a loading control. The data is represented as mean ±SD. Comparisons were made to the TSA-treated positive control (p<0.01).

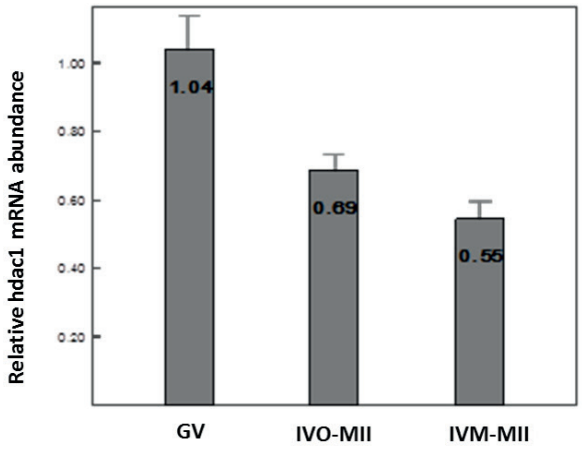

Figure 4. Relative HDAC1 mRNA levels of oocytes matured *in vivo* and *in vitro*. The experiment was conducted 3 times. The data is represented as mean ±SD (p<0.01).

When cultured *in vitro*, the significant decrease of HDAC1 protein level was observed compared with oocytes matured *in vivo* (p<0.01) (Figure 5).

Discussion

As observed in the present study, it could be inferred that there is a further need for the detailed exploration of mechanisms behind oocyte meiosis. However, it could be hypothesized that deregulation of specific HDACs responsible for variable patterns of H3K9 as well as H4k12 might be determined by IVM conditions. Moreover, epigenetic processes are of immense importance as they are able to reveal epigenetic risks and safety aspects of ART. HDAC1 played a role in the ATP-dependent chromatin remodeling, participated in the downregulation of a variety of DNA-binding transcription factors[1-3]. The significantly decreased HDAC1 mRNA and protein levels in MII oocytes from IVM strongly implied that IVM procedure downregulated the expression of HDAC1 during meiosis. During the growth of the mammalian follicular oocyte, the oocyte actively transcribed

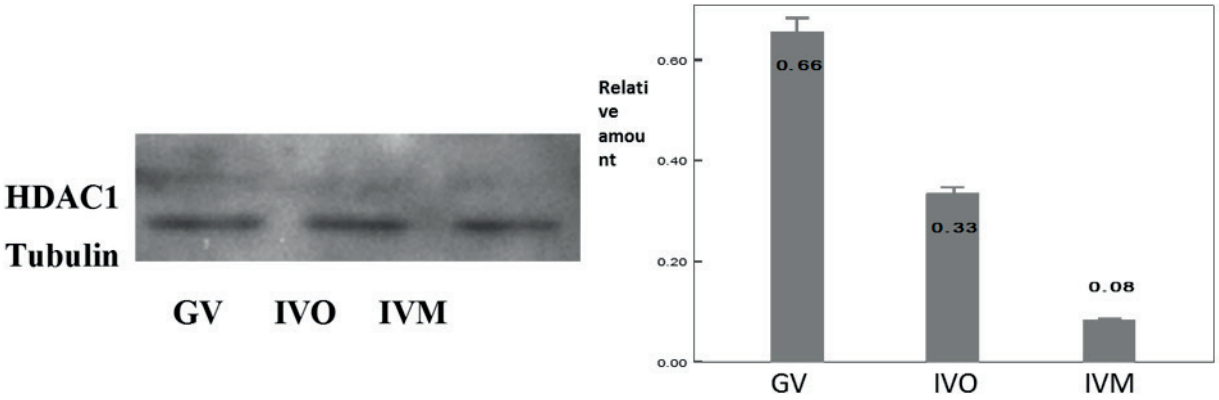

Figure 5. Western blot analysis of HDAC1 expression. HDAC1 protein expression was detected via Western blot in oocytes matured *in vivo vs. in vitro*. Two hundred oocytes were loaded per lane. α-Tubulin served as a loading control. The data is represented as mean ±SD.

and produced stable RNA to support early embryonic cleavage. Since there is no transcriptional activity during the final stage of oocyte maturation[4,5], the pattern of histone acetylation would not be associated with gene expression. It is more likely that reprogramming of histone acetylation may be required for genome-wide chromatin remodeling. The defects of HDAC1 expression in MII oocytes suggested that the down-regulation of HDAC1 gene resulted from IVM occurred at the early stage of oocyte maturation.

It has been revealed that IVM oocytes failed to acquire full remodeling competence because of the disturbance of acetylation[6]. HDAC1 may be the most responsible HDAC due to its close association with chromatin. The reduced level of HDAC1 expression in oocytes matured *in vitro* suggested that IVM affected the HDAC1 gene expression, then protein syntheses, and might interrupt its activity in the later stage[7].

The level of acetyl-histone H3 & H4 is an index for the evaluation of global histone acetylation in chromatin. Histone acetylation is involved in gene expression regulation and genome reprogramming in oocytes and embryos[8,9]. IVM could affect the expression of HDAC1 in MII oocytes leading to some changes on histone acetylations, which might be one reason for the low birth rate after IVM. Furthermore, a recent report has suggested the possibility of IVM to be safe in newborn but its detrimental effects were not certain[10].

Conclusions

We observed that IVM procedures significantly affects histone acetylation as well as HDAC1 levels but the exploration of precise mechanism requires further investigation in future studies.

Acknowledgments

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Conflict of interest

The authors declare no conflicts of interest.

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