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Dynamics and regulation of lysine-acetylation during one-cell stage mouse embryos

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ABSTRACT

Previous studies show that treatment of zygotes with trichostatin A (TSA), a histone deacetylase inhibitor (HDACi), impacts the subsequent development to a blastocyst as well as full-term development. To reveal the dynamics of protein acetylation, with and without TSA treatment during one-cell stage, we examined oocytes and zygotes by immunofluorescence and Western Blot analyses using anti-acetylated lysine and acetylated α-tubulin antibodies. In unfertilized oocytes, lysine acetylation level was extremely low over all but faintly detected in the spindle. Once oocyte activation occurs, a dramatic increase of lysine acetylation signal was observed mostly in the pronuclei and a fiber-like structure, the so called midbody, suggesting activation coupled up-regulation of lysine acetylation presumably in histones and α-tubulin. TSA treatment resulted in significantly more hyperacetylation not only in the midbody structure and pronuclei but also in the whole cytoplasm. Consistently, Western Blot analysis revealed that acetylation of proteins about 53 kDa and 11 kDa in size, corresponding to α-tubulin and histone H4 sizes respectively, were increased mainly after oocyte activation and exclusively enhanced by TSA treatment in zygotes. To confirm this behavior of acetylated nonhistone proteins, acetylated α-tubulin was examined and found to be faintly detected in the spindle of MII oocytes but later in whole in the cell of zygotes including the midbody, which was enhanced by TSA treatment. To elucidate the mechanism underlying up-regulation of lysine acetylation following oocyte activation, we assayed the HDAC activity, and found significant reduction of HDAC activity from MII to zygotic stages. Taken together, our data indicate that HDACs play an important role in maintaining low acetylated status in a MII oocyte. However, once an oocyte has been activated, histone and nonhistone proteins including α-tubulin are hyperacetylated partly due to a reduction of HDAC activity. TSA treatment of zygotes enhances their acetylation, which could affect subsequent embryonic development.

1. Introduction

Lysine acetylation is one type of reversible posttranslational protein modification and plays important roles in regulating protein function including gene expression for a wide range of cellular processes [1]. Lysine residues are acetylated by histone acetyltransferases (HATs) and deacetylated by histone deacetylases (HDACs), also called lysine deacetylases (KDACs). The modification change of core histone tails by HATs and HDACs contribute to the regulation of gene expression and works as epigenetic memory [2]. In addition to histones, other substrates for these enzymes include nonhistone proteins which are divided into two groups, nuclear and nonnuclear proteins represented by p53 and α-tubulin, respectively [1]. Treatment of cells with HDAC inhibitors (HDACi) such as trichostatin A (TSA) resulted in hyperacetylation of various proteins, suggesting a dynamic equilibrium of lysine acetylation *in vivo*.

In preimplantation embryos, it was suggested that the regulation of lysine acetylation through HDAC activity plays a pivotal role in the subsequent embryonic development rates [3]. Actually, treatment of the fertilized embryos with TSA leads to significant reduction of blastocyst rates [4]. In contrast, the treatment of parthenogenetic or round spermatid-injected embryos increased those rates [4]. More strikingly, treatment of the somatic cloned embryos with HDACi including TSA resulted in a more efficient *in vitro* development to the blastocyst stage from 2- to 5-fold [5]. Thus, the effects, harmful or not, of TSA treatment on embryonic development depend on their nuclear derivations [4]. In general, these effects of HDACi on embryonic development are supposed

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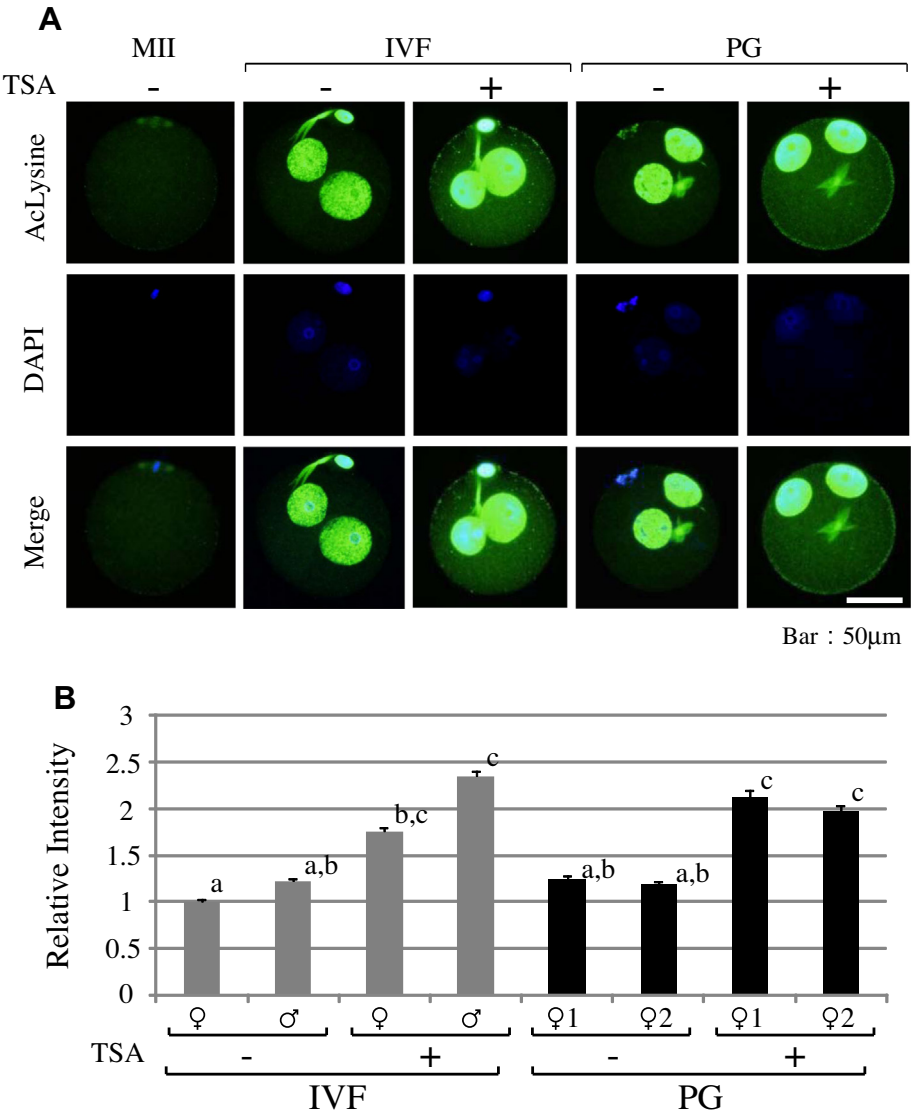

Fig. 1. Dynamics of lysine acetylation in MII oocytes, IVF and PG embryos with and without TSA treatment. (A) Embryos collected 10 h after oocyte activation or IVF were immunostained with anti-acetylated lysine antibody. Acetylated lysine (AcLysine) is shown in green. The DNA was counterstained with DAPI. Scale bar = 50 um (B) Quantification of acetylation in pronuclei in both IVF and PG embryos. In a PG embryo, the pronucleus which showed higher intensity is ♀1. Each value is shown after normalization by the mean value of these intensities female pronuclei in IVF embryos. These data are presented as the mean ±SEM. Values with different superscripts are significantly different at $P < 0.05$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

to be due to the status of histone acetylation. However, the specific targets of HDACi remain unclear and little is known about details of the impact on nonhistone proteins by HDACi treatment other than histones.

Based on previous cell culture experiments, HDACi treatment of embryos is expected to result in hyperacetylation of a variety of proteins including histone and nonhistone proteins. In this study, we focus on the dynamics of lysine acetylation at one-cell stage and its regulation, and also the impact of TSA treatment on lysine acetylation and α-tubulin as a nonhistone protein.

2. Materials and Methods

2.1. Animals and collection of oocytes-cumulus complexes

B6D2F1 (C57BL/6 X DBA/2) mice were obtained at 7–8 weeks of age from SLC (Hamamatsu, Japan). All procedures involving animal conformed to the Kinki University Guidelines for the Care and Use of Laboratory Animals. To superovulate, B6D2F1 mice were injected with 7 IU pregnant mare serum gonadotropin (PMSG) and 7 IU human chorionic gonadotropin (hCG), which were given 48 h apart. Fourteen to sixteen hours after hCG injection, oocyte-cumulus complexes (OCCs) were collected from the oviducts.

2.2. Collection of sperms and in vitro fertilization

Sperms were collected from caudal epididymis for male B6D2F1 and cultured for 1 h in Human Tubal Fluid (HTF) medium containing 0.3% bovine serum albumin (BSA). After 1 h, collected OCCs were cultured with the sperms for 6 h in HTF medium containing 0.3% BSA. After 6 h, fertilized oocytes were transferred to KSOM AA medium and cultured for 4 h. These embryos were cultured with and without TSA treatment for a total of 10 h. The culture condition was at 37 °C in an atmosphere of 5% CO_2 in air.

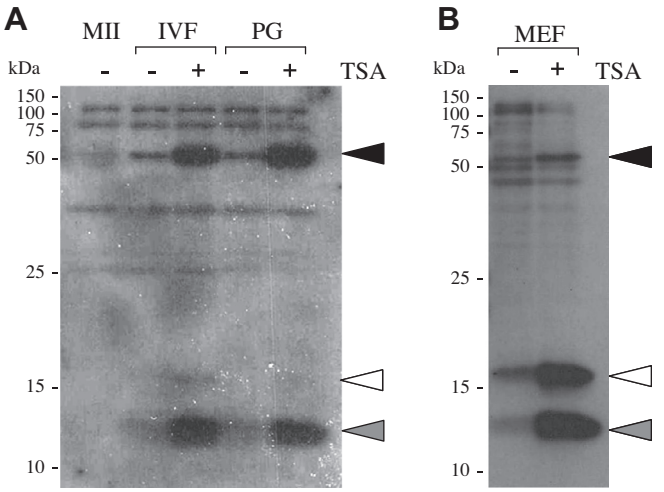

Fig. 2. Analysis of acetylated proteins with and without TSA treatment by immunoblotting. Acetylated proteins were detected by anti-acetylated lysine antibody. (A) In IVF and PG embryos, proteins of about 53 kDa (black arrowhead) and 11 kDa (gray arrowhead) were strongly acetylated 10 h after oocyte activation (PG) or *in vitro* fertilization (IVF), which were more acetylated in the presence of TSA. A 15 kDa band was faintly stained even in the presence of TSA (white arrowhead). (B) In MEFs, 53 kDa, 15 kDa and 11 kDa of proteins were also detected and more acetylated during TSA treatment. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

2.3. Parthenogenesis and TSA treatment

MII oocytes were collected from OCCs by using M2 medium containing 0.1% hyaluronidase. Collected MII oocytes were cultured in KSOM AA medium for 1–3 h before activation treatment. These oocytes were activated by 6 h of culture in KSOM AA medium supplemented with 10 mM $SrCl_2$, 5 mM ethyleneglycol bis (2-aminoethylether) tetraacetic acid (EGTA) and 5 μg/ml of cytochalasin B [6]. Activated embryos were cultured in KSOM AA for 4 h. The culture condition was at 37 °C in an atmosphere of 5% CO_2 in air.

These parthenogenetic or IVF embryos were cultured with and without 500 nM TSA treatment for a total of 10 h [4].

2.4. Immunofluorescence microscopy and quantitation of fluorescence intensity

Embryos were fixed by 0.4% paraformaldehyde. Fixed embryos were treated by phosphate-buffered saline (PBS) containing 0.2% BSA and 0.2% Triton-X 100 at 4 °C for overnight. Primary antibodies used were rabbit polyclonal anti-acetylated lysine (1:200 dilution; Cell Signaling Technology Inc., Beverly, MA, USA), mouse monoclonal anti-acetylated tubulin (1:10,000 dilution; Sigma–Aldrich, St. Louis, MO, USA) and rabbit polyclonal anti represented by α-tubulin (1:200 dilution; Cell Signaling Technology). The secondary antibodies were Alexa-Fluor-568-labeled goat anti-mouse IgG or Alexa-Fluor-488-labeled goat anti-rabbit IgG antibodies (1:400 dilution; Molecular Probes Inc., Eugene, OR, USA). The DNA was stained with 1 ug/mL 4,6-diamidino-2-phenylindole (DAPI; Wako, Ltd. Japan). Fluorescence images were quantitatively analyzed by using Image J software. Fluorescence intensities of pronuclei were measured manually outlining all pronuclei. The total intensity of pronuclei was calculated by multiplying the number of pronuclei pixels by average intensities and then subtracted the background value. For the cytoplasm, three different regions and total pixel size were measured. Total intensities were calculated by multiplying the average value of three regions by total pixels, and then subtracted the background value.

2.5. Immunoblotting

MII Oocytes or 10 h cultured embryos were collected after the zona pellucida was removed using Acid Tyrode's solution (ATS) and washed by PBS containing 0.1% polyvinylpyrrolidone (PVA). Oocyte or embryo lysates were resolved on polyacrylamide-SDS gel, and transferred to Hyperfilm ECL (GE healthcare Inc., Tokyo, Japan). These membranes were immunoblotted with specific antibodies. Primary antibodies used were rabbit polyclonal anti-acetylated lysine (1:1000 dilution), mouse monoclonal anti-acetylated tubulin (1:30,000 dilution) and rabbit polyclonal anti α-tubulin (1:200 dilution). Secondary antibodies were donkey polyclonal anti mouse-IgG-HRP (1:10,000 dilution) and donkey polyclonal anti rabbit-IgG-HRP (1:5000 dilution). Results were visualized using ECL Plus Western Blotting Detection Reagents (GE healthcare).

2.6. Measurement of HDAC activity

Oocytes and embryos were collected after removing zona pellucida using ATS and washed using 0.1% PBS–PVA. These were frozen in liquid nitrogen and preserved at −150 °C until they were used. These samples were lysed in a buffer containing 20 mM Tris–HCl (pH 8.0), 150 mM NaCl, 10% glycerol, and 0.5% NP-40 and incubated on ice for 15 min. Next, the substrate was added to these and incubated at 37 °C for 1 h. The substrates used were MCA-fused with a cortactin peptide (Ac-KGFGGk(Ac)-MCA). Then, 20 mg/ml Trypsin was added to these and incubated at 37 °C for 15 min. These samples were transferred to a 96-well black plate and measured by excitation 380 nm, emission 460 nm [7].

2.7. Statistically analysis

Data were analyzed by Scheffe tests for multiple mean comparisons using the statistical program SPSS version 12.0 (SPSS Inc., Chicago, IL, USA). All percentile data were subjected to arcsine transformation before statistical analysis.

3. Results

3.1. Dynamics of lysine acetylation during oocyte activation

To reveal the dynamics of lysine acetylation in oocytes, we performed immunostaining with anti-acetylated lysine antibody. Ovulated fresh oocytes were poorly stained in whole cell with weak staining in spindles including chromosomes, which should represent histone acetylation (Fig. 1A). However, 8 h after oocyte activation either via fertilization or parthenogenesis, acetylated lysine signal was increased specifically in pronuclei and midbody, where α- and β-tubulin are major components, in addition to the second polar body (Fig. 1A). Interestingly, male pronuclei tended to have a stronger acetylation signal yet not significantly (Figs. 1A and 1B). Thus, lysine acetylation was upregulated following oocyte activation. In the presence of TSA, acetylation was increased in pronuclei and cytoplasm as well as the second polar body (Figs. 1A and 1B). TSA treatment upregulated acetylation of not only nuclear but also nonnuclear proteins.

Immunoblotting with the same anti-acetylated lysine antibody revealed the molecular weights of proteins with acetylated lysine in one-cell stage embryos. Specifically proteins of about 53 kDa and 11 kDa were acetylated after oocyte activation, both of which were exclusively more acetylated after TSA treatment (Fig. 2A). Proteins of about 53 kDa and 11 kDa could be α-tubulin and histone H4 based on the above immunofluorescence results. In contrast, mouse embryonic fibroblast (MEF) also showed a highly

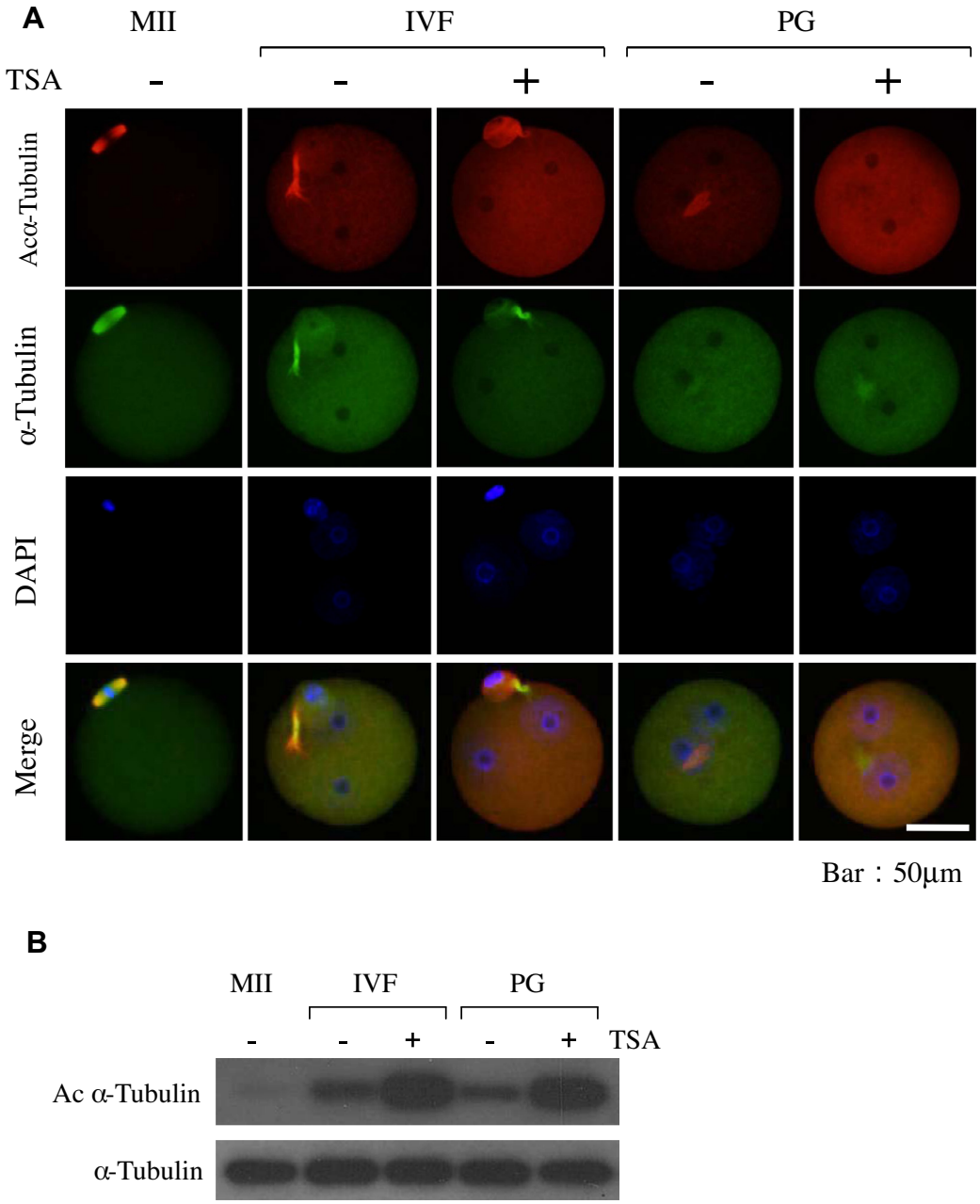

Fig. 3. Dynamics of α-tubulin acetylation in MII oocytes, IVF embryos and PG embryos with and without TSA treatment. (A) Embryos collected 10 h after oocyte activation (PG) or *in vitro* fertilization (IVF) as well as MII oocytes were immunostained with anti-acetylated α-tubulin and α-tubulin antibodies. Acetylated α-tubulin (Acα-Tubulin) and α-tubulin are shown in red and green, respectively. The DNA was counterstained with DAPI. Scale bar = 50 um (B) Western Blot analysis for quantification was performed by the same antibodies. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

acetylated 15 kDa band, corresponding to histone H3, in addition to 53 kDa and 11 kDa. Thus, zygotes and MEF as somatic cells also showed a similar but distinct staining pattern in terms of lysine acetylation and response to TSA (Figs. 2A and 2B). Taken together with the immunofluorescence data, it is suggested that lysine acetylation for both histone and nonhistone proteins is upregulated during one-cell stage in cell-cycle and HDAC dependent manner.

3.2. Dynamic change of α-tubulin acetylation during oocyte activation

A 53 kDa protein was a target protein of lysine acetylation in oocyte and zygote. Next we analyzed the acetylation status of α-tubulin, a most likely candidate of the 53 kDa protein in terms of its size, localization like midbody and abundance, which was among the acetylated nonhistone proteins to be first discovered and was reported to be highly acetylated concomitantly with oocyte activation [8]. Consistent with the above immunofluorescent results, we found a low acetylation level of α-tubulin in spindle in MII oocyte but, a high acetylation level in midbody and cytoplasm in activated oocytes (Fig. 3A). Further, TSA treatment significantly increased these signals of acetylated α-tubulin. This increased acetylation in α-tubulin after oocyte activation and in the presence of TSA was also confirmed by immunoblotting (Fig. 3B), where results showed similar response to a 53 kDa protein detected by anti-acetylated lysine antibody (Fig. 2A). These analyses with anti-acetylated α-tubulin antibody demonstrated a

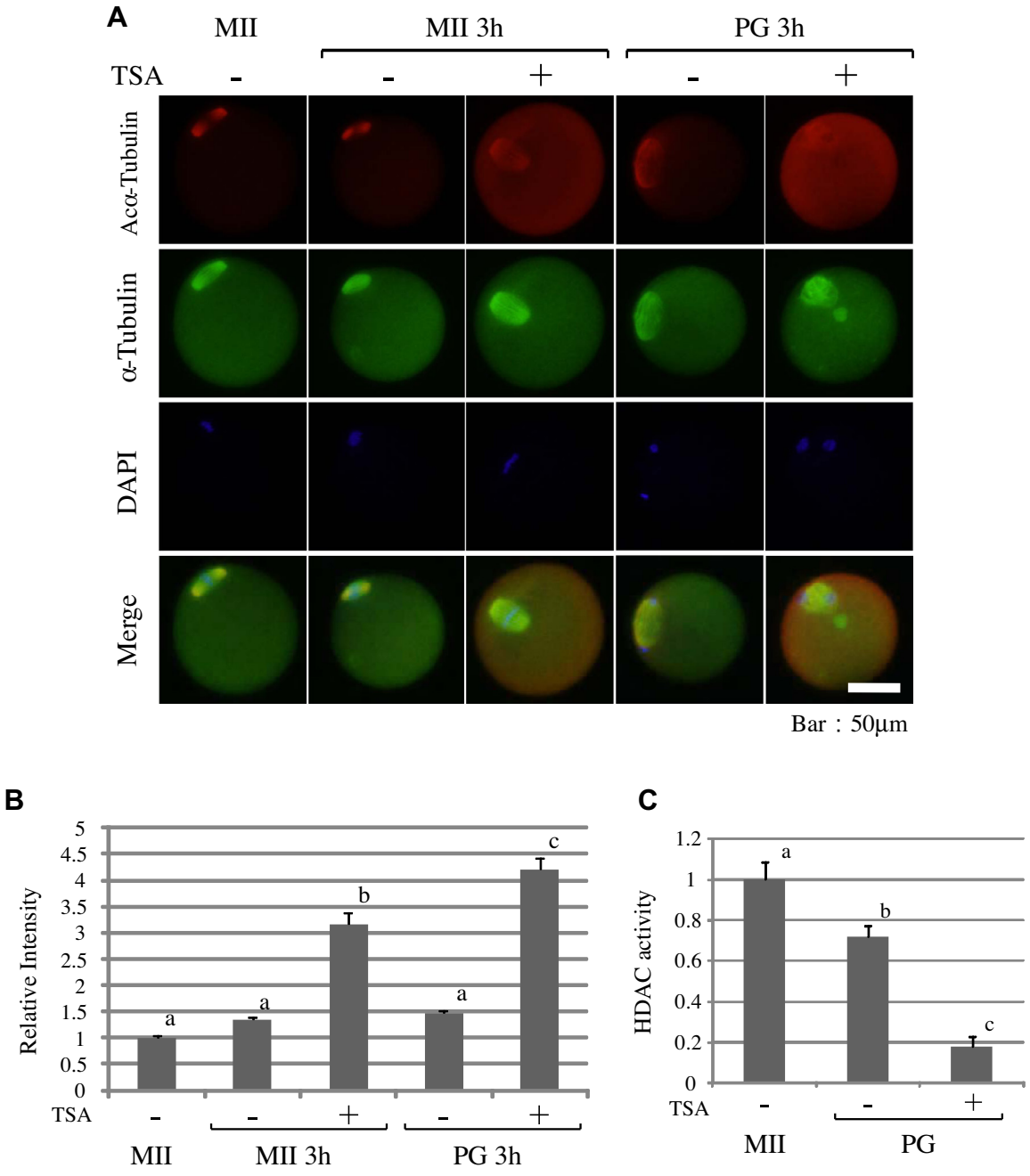

Fig. 4. Different acetylation statuses of α-tubulin between unactivated and activated oocytes under TSA. (A) Activated oocytes were collected 3 h after oocyte activation (PG 3 h) with or without TSA. Unactivated oocytes were also incubated for 3 h with or without TSA. They were immunostained with anti-acetylated α-tubulin and α-tubulin antibodies. Acetylated α-tubulin (Acα-Tubulin) and α-tubulin are shown in red and green. The DNA was stained with DAPI (blue). Scale bar = 50 um. (B) Acetylation signals of α-tubulin in the oocytes were measured. These data are presented as the mean ±SEM. Values with different superscripts are significantly different at $P < 0.05$. (C) Different HDAC activities between unactivated and activated oocytes. PG embryos were collected 10 h after activation. HDAC activities of MII and the activated oocytes were measured using the substrate, MCA-fused with cortactin peptide. Activated oocytes (PG) showed a significant reduction in the HDAC activity which further decreased under TSA. Values with different superscripts are significantly different at $P < 0.05$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

similar pattern to that of anti-acetylated lysine antibody. That is, that α-tubulin remains mostly unacetylated in oocyte but once activation occurs its acetylation level is increased and enhanced by TSA.

3.3. Insight into upregulation of α-tubulin acetylation during oocyte activation

To examine how the upregulation of lysine acetylation is coupled to oocyte activation, we compared lysine acetylation in 3 h cultured oocytes in the presence of TSA with and without oocyte activation. Although there was no significant difference in the acetylation level between unactivated and activated oocytes at the time point of 3 h, TSA treatment revealed a significant increase of acetylation in α-tubulin in activated oocytes (Figs. 4A and 4B), suggesting a mechanism of an oocyte activation-coupled upregulation of lysine acetylation.

To get further insight into the mechanism, we also assayed the HDAC activity during oocyte activation and found a significant reduction after oocyte activation (Fig. 4C). Thus, oocyte-activation

achieves upregulation of lysine acetylation apparently by reduction of HDAC activities.

4. Discussion

Lysine-acetylation, one type of post-translational modification, plays pivotal roles in various cellular functions. HDACi treatment of one cell stage embryos affects subsequent embryonic developmental rates [3–5], suggesting the significance of lysine acetylation status during this time period for developmental potentials. However, its dynamics and regulation in oocytes and embryos have been largely unknown. To reveal the impact of TSA treatment on the lysine acetylation status at one-cell stage, we have used indirect immunofluorescence to describe two issues for the first time: dynamics of pan-lysine acetylation and α-tubulin acetylation in one-cell stage embryos with or without HDACi and the oocyte activation-coupled regulation of lysine acetylation.

Histone acetylation status during one-cell stage has been well-studied to show that the acetylation level at a variety of lysine residues in the N-terminus of both histone H3 and H4 are increased during the one-cell stage after oocyte activation usually enhanced by HDACi such as TSA [9,10]. On the other hand, Schatten and colleagues previously described that α-tubulin acetylation is increased after oocyte activation [8]. We observed similar dynamics of lysine-acetylation in pronuclei as well as in cytoplasm after oocyte activation. Those dynamics of lysine-acetylation are shifted to more hyperacetylation by TSA. Further, our Western Blot data revealed that two proteins, 53 kDa and 11 kDa, were detected as the proteins acetylated after oocyte activation and hyperacetylated after TSA treatment in both IVF and parthenogenetic embryos. Thus, during one-cell stage, acetylation in, at least, histones and α-tubulin are dynamically increased, suggesting a common mechanism underlying dynamic regulation of lysine acetylation. It is also noted that embryos and somatic cells (MEF) apparently showed a striking difference of lysine acetylation with the additional size of 15 kDa, possibly for H3, suggesting that histone H4 may be more rich in acetylation than H3 in the zygote.

The denoted 53 kDa protein was likely α-tubulin based on the molecular weight, abundance and immunofluorescence patterns including a strong signal on the midbody. α-Tubulin was among the first acetylated nonhistone proteins to be discovered [11]. Acetylated α-tubulin in microtubules has been already described in detail during early development [8]. The immunofluorescence study by anti-acetylated lysine antibody illustrated a quite similar pattern to that of anti-acetylated α-tubulin antibody. These data strongly suggest that α-tubulin is one of the major substrates for increased acetylation after oocyte activation. On the other hand, we clearly showed that in the presence of TSA, α-tubulin acetylation level significantly increased without oocyte activation by TSA treatment within 3 h, suggesting an equilibrium state maintained a lower level of acetylation with high HDAC activity in ovulated oocytes. Three hours after oocyte activation, α-tubulin acetylation level increased more than that without oocyte activation, which may be due to a concomitant change of accessibility or higher activity of HAT. Thus, our data show that extremely low acetylation level of proteins in unactivated MII oocytes are maintained by a high activity of HDACs. Consistently, MII oocytes express abundant HDACs including HDAC 1–4, which decrease after the two-cell stage except HDAC1 [12].

We previously reported that the treatment of one-cell stage embryos with TSA 20 h after fertilization significantly reduces the blastocyst rate and the rate of offspring born with some abnormalities, suggesting that HDAC activity in one-cell stage embryos is essential for normal development [4]. In contrast, the treatment of PG embryos with TSA 20 h after oocyte activation showed no reduction but rather an increase of blastocyst rates. Further, continuous inhibition of HDAC for 10 h or even 20 h following oocyte activation improves the development of cloned embryos [5,13]. Thus, these results suggest that, during the one cell stage, optimization of HDAC activity for nuclei with different epigenetic statuses should be required for improving the subsequent developmental rates. Our current study shows that an oocyte dynamically changed the acetylation status in a whole cell and that TSA induced hyperacetylation not only in pronuclei but also in cytoplasm including nonhistone proteins such as α-tubulin. This research may provide new insight into the mechanism underlying how TSA treatment affects subsequent development. TSA treatment may improve development of the cloned embryos by increasing acetylation in not only histone but also nonhistone proteins such as α-tubulin. Further analysis is required for future studies to examine this possibility.

To date, the mechanism underlying the dynamics of lysine acetylation remain unknown. Since the lysine residues are acetylated by HATs and deacetylated by HDACs, the steady state of lysine acetylation is determined by the balance of these enzyme activities and accessibilities to substrates. Actually, our data indicated that after 3 h culture under TSA to inhibit HDACs oocytes showed a significant increase of acetylation of α-tubulin, suggesting that HDACs play an important role in keeping lysine acetylation levels lower in α-tubulin. Further, oocyte activation with TSA increased acetylation of α-tubulin within 3 h when compared to unactivated oocytes, indicating that the balance of lysine acetylation is shifting to a higher acetylation state probably by increasing HAT activities or/and more access of HATs to α-tubulin. We also found a significant reduction of HDAC activities after oocyte activation. Taken together, one-stage embryos exhibit dynamic reorganization of the lysine acetylation state triggered by oocyte activation probably through altered regulation of HAT and HDAC activities.

Thus, it seems that a general increase of the cellular acetylation level is triggered by oocyte activation both in histones and nonhistone proteins, which implies that HAT or/and HDAC activity is regulated by oocyte activation. Actually, we found that HDAC activity became significantly lower after oocyte activation in this study, indicating a reduction of HDAC activities contributes to the increased acetylation after oocyte activation.

Our findings here provide an insight into the dynamics of lysine acetylation and its regulation in pronuclei and cytoplasm during the one cell stage with and without HDACi. Future studies should be focused on the identification of acetylated proteins in oocytes to affect embryonic development as well as the oocyte activation-coupled regulation mechanism of HAT and HDAC activities in oocytes.

Acknowledgments

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