Regulation of chromatin and chromosome morphology by histone H3 modifications in pig oocytes

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Abstract

Oocyte growth, maturation, and activation are complex processes that include transcription, heterochromatin formation, chromosome condensation and decondensation, two consecutive chromosome separations, and genomic imprinting. The objective of this study was to investigate changes in histone H3 modifications in relation to chromatin/chromosome morphology in pig oocytes during their growth, maturation, and activation. During the growth phase, histone H3 was acetylated at lysines 9, 14, and 18 (K9, K14, and K18), and became methylated at K9 when the follicles developed to the antral stage (oocyte diameter, 90 μm). When the fully grown oocytes (diameter, 120 μm) started their maturation, histone H3 became phosphorylated at serine 28 (S28) and then at S10, and deacetylated at K9, K14, and K18 as the chromosomes condensed. After the electroactivation of mature oocytes, histone H3 was reacetylated and dephosphorylated concomitant with the decondensation of the chromosomes. Histone H3 kinase activity increased over a similar time course to that of the phosphorylation of histone H3-S28 during oocyte maturation, and this activity decreased as histone H3-S10 and H3-S28 began to be dephosphorylated after the activation of the mature oocytes. These results suggest that the chromatin morphology of pig oocytes is regulated by the acetylation/deacetylation and the phosphorylation/dephosphorylation of histone H3, and the phosphorylation of histone H3 is the key event in meiotic chromosome condensation in oocytes. The inhibition of histone deacetylase with trichostatin A (TSA) inhibited the deacetylation and phosphorylation of histone H3, and chromosome condensation. Therefore, the deacetylation of histone H3 is thought to be required for its phosphorylation in meiosis. Although histone H3 acetylation and phosphorylation were reversible, the histone methylation that was established during the oocyte growth phase was stable throughout the course of oocyte maturation and activation.

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Introduction

During the maturation of pig oocytes, histone H3 is phosphorylated at serine 10 (S10) in condensed chromosomes and its phosphorylation correlates with histone H3 kinase activity (Bui *et al.* 2004). Recently, it was reported that histone H3 is deacetylated at lysines 9 and 14 (K9 and K14) during the maturation of mouse and pig oocytes (Akiyama *et al.* 2004, Endo *et al.* 2005). In somatic cells, histone modifications directly affect chromatin structure, and histone H3 modification sites are clustered within the first 30 amino acids. Histone H3 is acetylated at K9, K14, K18, and K23, phosphorylated at S10 and S28, and methylated at K4 and K9 (Cheung *et al.* 2000a).

The volume of the oocyte increases markedly during the growth phase. This increase during the early growth phase correlates with increased transcriptional activity (Bachvarova 1985). The nuclear morphology of growing oocytes is characterized by decondensed chromatin, with a vacuolated nucleolus. As the oocytes approach their fully grown size and acquire meiotic competence, the chromatin changes from the decondensed to the condensed state (Hirao *et al.* 1995), with a concomitant change in the morphology of the nucleoli from the transcriptionally active to the transcriptionally inactive state (Motlik *et al.* 1984). Histone acetylation is important in both chromatin remodeling and transcription in somatic cells (Struhl 1998). Although chromatin

changes during the growth phase in mammalian oocytes, there has been no evidence of the involvement of histone acetylation in oocyte growth. It has been suggested that there is a direct link between histone methylation and DNA methylation (Tamaru & Selker 2001). Recently, Hiura *et al.* (2006) showed that DNA methylation in the growing oocytes of adult ovaries took place due to the size of oocyte in mice. However, no evidence has yet been found regarding the histone methylation during the growth phase in mammalian oocytes. During mitosis in mammalian somatic cells, histone H3 is phosphorylated not only at S10, but also at S28 (Goto *et al.* 2002, Sugiyama *et al.* 2002). Furthermore, histone H3 phosphorylation at S28 occurs independently of its phosphorylation at S10 following the stimulation of the Ras-MAP kinase (MAPK) pathway (Dunn & Davie 2005). When the histone H3-S28 modification occurs in mammalian oocytes and how it is involved in chromosome condensation are unclear.

After maturation, the oocytes are arrested at metaphase of the second meiosis. Upon fertilization, the oocytes resume meiosis and complete the second meiotic division. The second polar body is extruded, the female chromosomes and sperm chromatin decondense, and two pronuclei are formed. We showed the histone H3 modifications in injected somatic cell nuclei in mature pig oocytes in a previous paper. In injected somatic nuclei, histone H3-S10 became phosphorylated in parallel with the deacetylation of histone H3 at K9 and K14 and, after the electroactivation of the oocytes, histone H3 was reacylated in concert with S10 dephosphorylation (Bui *et al.* 2006). However, in the normal somatic cell cycle, there is a positive correlation between the phosphorylation of histone H3-S10 and the acetylation of histone H3-K14, which promotes the delocalization of heterochromatin protein 1 (HP1) from the chromatin at G2 phase (Mateescu *et al.* 2004). How these histone modifications are related to changes in chromatin/chromosome morphology during meiosis in mammalian oocytes is not completely understood.

In this study, we examined the correlation between the morphological changes in chromatin/chromosomes and histone H3 phosphorylation, acetylation, and methylation in pig oocytes throughout their growth, maturation, and activation phases, and measured the activities of Cdc2 and histone H3 kinases during these processes. We also examined the effect of histone acetylation on histone phosphorylation, using a histone deacetylase inhibitor.

Materials and Methods

Collection of pig oocytes

Pig ovaries were obtained from prepubertal gilts at a local slaughterhouse. The ovaries were washed once with 0.2% cetyltrimethylammonium bromide and twice with Dulbecco's PBS (PBS) containing 0.1% polyvinyl alcohol (PBS-PVA; Sigma Chemical Co.). Growing oocytes were

collected from follicles of various diameters in pig ovaries. Follicles 4–6 mm in diameter were dissected from ovaries using the technique described by Moor & Trounson (1977). After being opened in 25 mM HEPES-buffered TCM-199 (Earle's salt; Nissui Pharmaceutical Co. Ltd, Tokyo, Japan) containing 0.1% PVA (HEPES-199), oocyte–cumulus complexes with a piece of attached parietal granulosa tissue (oocyte–cumulus–granulosa cell complexes; OCGCs) were isolated from the follicles. To collect small follicles 0.1–3 mm in diameter, cortical slices (0.5–3 mm thick) were cut from the ovarian surface using a surgical blade. Under a microscope, preantral and antral follicles were dissected from the cortices, and the tissues surrounding the follicles were torn off. Oocytes–granulosa cell complexes were collected in HEPES-199. The oocytes from different-sized follicles were denuded of cumulus or granulosa cells with a small-bore pipette. The diameters of the oocytes (excluding the zona pellucida) were measured with an ocular micrometer (Olympus, Tokyo, Japan) attached to an inverted microscope, and the oocytes were then used for immunostaining and kinase assays.

Oocyte maturation and activation

OCGCs collected from follicles 4–6 mm were cultured for maturation. The maturation medium was bicarbonate-buffered TCM-199 supplemented with 10% heat-treated fetal calf serum (Biocell Inc., Carson, CA, USA), 0.1 mg/ml sodium pyruvate, 0.08 mg/ml kanamycin sulfate, 2.2 mg/ml sodium bicarbonate, and 0.1 IU/ml human menopausal gonadotropin (Pergonal; Teikoku Zoki, Tokyo, Japan) and two everted theca shells with gentle agitation in an atmosphere of 5% CO₂ in humidified air at 38.5 °C. The OCGCs were cultured for various times to obtain oocytes at different meiotic stages.

After maturation culture for 42 h, some oocytes were activated using a previously described protocol (Nguyen *et al.* 2003). Briefly, oocytes were washed thrice in solution composed of 0.3 mM mannitol, 0.1 mM MgSO₄, 0.05 mM CaCl₂, and 0.01% PVA. Oocytes were then transferred to 100 µl of field solution between two parallel stainless electrodes in a chamber (FTC-03; Shimadzu Co. Ltd, Kyoto, Japan). A single direct-current pulse of 1500 V/cm for 100 µs was supplied from an Electro Cell Manipulator (ECM 2000; BTX Inc., San Diego, CA, USA). Electro-activated oocytes were washed thrice in HEPES-199. They were then cultured for 2, 4, or 6 h in bicarbonate-buffered TCM-199 supplemented with 10% heat-treated fetal calf serum, 0.1 mg/ml sodium pyruvate, 0.08 mg/ml kanamycin sulfate, and 2.2 mg/ml sodium bicarbonate. After culture, the oocytes were used for immunostaining and kinase assays.

To examine the effects of histone deacetylase (HDAC) inhibition, OCGCs were cultured in maturation medium supplemented with 100 nM trichostatin A (TSA; Sigma Chemical Co.) for 27 h. The oocytes were then used for immunostaining experiments.

Immunofluorescence microscopy

Oocytes were fixed in PBS-PVA containing 4% paraformaldehyde and 0.2% Triton X-100 for 40 min. The fixed oocytes were washed twice in PBS-PVA for 15 min each and stored overnight in 1% BSA (BSA; International Reagents Corporation, Kobe, Japan) supplemented PBS-PVA (BSA-PBS-PVA) at 4 °C. The oocytes were blocked with 10% goat serum (DakoCytomation A/S, Glostrup, Denmark) in BSA-PBS-PVA for 45 min, and then incubated overnight with primary antibody at 4 °C. The primary antibodies used here were rabbit polyclonal anti-phospho-histone H3-S10 (Cell Signaling Technology Inc., MA, USA), rabbit polyclonal anti-phospho-histone H3-S28 (Upstate Cell Signaling Solutions, VA, USA), rabbit polyclonal anti-trimethyl-histone H3-K9 (Abcam, Cambridge, UK), rabbit polyclonal anti-acetyl-histone H3-K9 (Upstate), rabbit polyclonal anti-acetyl-histone H3-K14 (Upstate), and rabbit polyclonal anti-acetyl-histone H3-K18 antibodies (Upstate). To determine the integrity of the nuclear envelope and the meiotic stage, mouse monoclonal anti-lamin A/C (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) and mouse monoclonal anti- α -tubulin antibodies (Sigma Chemical Co.) were used. After the oocytes were washed thrice in BSA-PBS-PVA for 15 min each, they were incubated for 40 min at room temperature with conjugated secondary antibodies, Alexa-Fluor-488-labeled goat anti-rabbit IgG (Molecular Probes Inc., Eugene, OR, USA) or Alexa-Fluor-568-labeled goat anti-mouse IgG (Molecular Probes Inc.). After the oocytes were washed thrice in BSA-PBS-PVA for 15 min each, the DNA was stained with propidium iodide (400 μ g/ml; Sigma Chemical Co.) or 4,6-diamidino-2-phenylindole (DAPI) (2 μ g/ml; Molecular Probes Inc.). Then oocytes were washed and mounted on slides with Vectashield mounting medium (Vector Laboratories Inc., Burlingame, CA, USA), and observed with a confocal scanning laser microscope (Radiance 2100, Bio-Rad). For the negative control, all groups of oocyte were reacted with nonimmune rabbit serum instead of rabbit polyclonal primary antibody, or with mouse IgG instead of mouse MAB. The immunostaining data of all groups were compared with negative control to decide the localization of antigen.

Kinase assay

Each group of five oocytes was transferred into an Eppendorf tube with 1 μ l of PBS-PVA for the double kinase assay of Cdc2 and histone H3 kinases. To determine the meiotic stage of the oocytes, they were stained with 12 μ g/ml Hoechst 33342 (Polysciences Inc., Warrington, PA, USA) for 20 min and then observed under a fluorescence microscope. ice-cold extraction buffer (4 μ l) was then added, and the samples were frozen at -80 °C before the assay. The extraction buffer was composed of 80 mM β -glycerophosphate, 25 mM HEPES

(pH 7.2), 20 mM EGTA, 15 mM MgCl₂, 1 mM dithiothreitol (DTT), 1 mM 4-amidinophenylmethanesulfonyl fluoride hydrochloride (Sigma Chemical Co.), 0.1 mM Na₃VO₄, 1 μ g/ml leupeptin (Sigma Chemical Co.), and 1 μ g/ml aprotinin (Sigma Chemical Co.). After the oocytes were thawed, they were centrifuged at 10 000 $\times g$ for 2 min at 2 °C, added to 5 μ l of kinase buffer and 5 μ l of substrate solution, and incubated for 20 min at 37 °C. The kinase buffer was composed of 75 mM HEPES (pH 7.2), 75 mM β -glycerophosphate, 75 mM MgCl₂, 6 mM DTT, 10 mM EGTA, 60 μ M ATP, 15 μ M cAMP-dependent protein kinase inhibitor peptide (Sigma Chemical Co.), and 0.3 μ Ci/ μ l [γ -³²P]ATP (250 μ Ci/25 μ l; Amersham Pharmacia Biotech). The substrate solution was composed of 2.5 μ l of histone H1 (5 mg/ml, from calf thymus; Boehringer) and 2.5 μ l of histone H3 (1 mg/ml, from calf thymus; Boehringer). The reaction was terminated by the addition of 5 μ l of 4 $\times$ SDS sample buffer and boiling for 5 min. The samples were loaded onto an SDS-15% polyacrylamide gel to separate the labeled histones H1 and H3. After electrophoresis, the gels were dried and autoradiographed. The autoradiographs were scanned with an image analysis system with Image Master ID Elite software, version 3.00 (Amersham Pharmacia Biotech). The kinase activity of the oocytes at metaphase I or II was arbitrarily set to 100%, depending on the experiment, and the other bands were expressed relative to that as a mean percentage $\pm$ S.E.M.

Statistical analysis

Experiments of immunostaining were repeated thrice and at least a total of 40 immunostained oocytes were examined in each class with each antibody directed against modified histone H3. At least three replicates of each kinase assay were performed. Statistical differences in the activities of the kinases visualized on the autoradiographs were analyzed by one-way ANOVA (*F*-test) followed by Tukey's multiple range test. Other values were analyzed using the χ^2 -test. *P* values less than 0.05 were considered statistically significant. For quantitative analyses, the fluorescent images were subjected to densitometric analysis using Image-J software from the National Institutes of Health (<http://rsb.info.nih.gov/ij/>).

Results

Chromatin morphology and histone H3 modifications during oocyte growth

The four typical patterns of chromatin, chromatin distributed over the nucleoplasm, filamentous chromatin (FC), stringy chromatin (SC), and germinal vesicle I (GVI) stages, were found in the oocytes from 0.1 to 0.2 mm secondary follicles, 0.5 to 1 mm early antral follicles, 2 to 3 mm middle antral follicles, and 4 to 6 mm late antral follicles respectively corresponding to oocyte diameters

Figure 1 Acetylation (Fig. 1-I) and methylation of histone H3 (Fig. 1-II) in growing pig oocytes. Oocytes were collected from secondary follicles (second follicle) and antral follicles. Oocytes from early antral follicles (diameter, 0.5–1 mm) had filamentous chromatin in the GV (FC), and oocytes from follicles of 2–3 mm diameter had stringy chromatin (SC). Oocytes from late antral follicles (diameter, 4–6 mm) were at germinal vesicle stage I (GVI). Oocytes were immunostained with anti-acetyl-histone H3-K14 (Ac-H3-K14) or anti-methyl-histone H3-K9 (Me-H3-K9), and Alexa-Fluor-488-labeled antibodies (green). The nuclear envelope was stained with anti-lamin A/C and Alexa-Fluor-568-labeled antibody (red), shown in the merged image. The DNA was counterstained with DAPI (blue). Scale bar=20 μ m.

Figure 2 Autoradiograph showing the changes in Cdc2 and histone H3 kinases activities in pig oocytes during the growth phase. Histone H1 (H1) and histone H3 (H3) were used as the substrates of Cdc2 and histone H3 kinases respectively. The oocyte stages are the filamentous chromatin stage (FC), the stringy chromatin stage (SC), germinal vesicle stage I (GVI), and metaphase I (MI).

of $60.0 \pm 3.2 \mu\text{m}$ ($n=65$), $89.7 \pm 2.5 \mu\text{m}$ ($n=70$), $115.3 \pm 3.0 \mu\text{m}$ ($n=69$), and $122.1 \pm 2.2 \mu\text{m}$ ($n=79$) respectively (Fig. 1).

No phosphorylation of histone H3 at S10 or S28 was detected in any growing or fully grown oocytes (data not shown). This is consistent with the results indicating that both Cdc2 and histone H3 kinases were inactive in those oocytes (Fig. 2). The acetylation of histone H3 at K9, K14, and K18 was detected in the chromatin of oocytes in the secondary follicles of 0.1–0.2 mm diameter (Fig. 2-I for H3-K14). The fluorescent signals for acetylation extended throughout the entire chromatin in growing oocytes and converged to the heterochromatin ring in fully grown oocytes.

No fluorescent signal for the form of histone H3 methylated at K9 was detected in the chromatin of oocytes from secondary follicles (Fig. 2-II). The methylation of H3-K9 appeared in the chromatin knobs in growing oocytes when the follicles increased from 0.5 to 1 mm in diameter. This methylation was observed at the

heterochromatin ring when oocytes reached the GVI stage with the final size.

Chromosome morphology and histone H3 modifications during oocyte maturation

In our culture system, the maturation stages of oocytes were classified as follows: the germinal vesicle I (GVI, 0 h), germinal vesicles II–IV (GVII–GVIV, 9–15 h), diakinesis (D, 18 h), metaphase I (MI, 27 h), anaphase I–telophase I (AI–TI, 31–33 h), and metaphase II (MII, 42 h; Fig. 3). In our previous study (Bui *et al.* 2004) and this study, we showed that the phosphorylation of histone H3-S10 is first detected in the clump of condensed chromosomes at the diakinesis stage after 18 h of culture (100%, 40/40 oocytes), and this phosphorylation is maintained thereafter during oocyte maturation. The fluorescent signal for phosphorylated histone H3-S28 was first seen in the chromatin around the nucleolus in oocytes at GVII stage after 9 h of culture (78%, 36/46 oocytes; Fig. 3B'). The phosphorylation of H3-S28 extended throughout the whole condensing chromosomes in oocytes at GVIII–IV stages after 15 h of culture (100%, 41/41 oocytes; Fig. 3C'). Thereafter, the phosphorylation of H3-S28 was maintained during oocyte maturation. Histone H3 kinase was activated after 9 h; its activity increased at 24 h and was highest in MI oocytes (Fig. 4). Cdc2 kinase became active at 24 h and reached maximum activity at 27 h.

All acetylations of histone H3 at K9, K14, and K18 were detected in oocytes at the GVI stage before maturation culture, as described above. The fluorescent signal for the acetylation of histone H3-K9 had disappeared completely at the diakinesis stage (72%, 31/43 oocytes). The signal for the acetylation of H3-K14 decreased and remained at a low level in the diakinesis stage, when the nuclear membrane start to break down (85%, 34/40 oocytes; Fig. 5-I C' for Ac-H3-K14). Thereafter, both H3-K14 (90%, 37/41 oocytes) and H3-K18 (100%, 45/45 oocytes) disappeared completely at MI. The fluorescent signal for acetylated histone H3-K9 appeared at a low level at TI (77%, 31/40

Figure 3 Immunofluorescent localization of phosphorylated histone H3-S28 (P-H3-S28) during the maturation of pig oocytes. PI stains the chromosome (red), and anti-phospho-histone H3-S28 and Alexa-Fluor-488-labeled antibodies stain P-H3-S28 (green) in the same oocyte. The meiotic stages are (A) germinal vesicle stage I (GVI), (B) germinal vesicle stage II (GVII), (C) germinal vesicle stage III (GVIII), (D) diakinesis (D), (E) metaphase I (MI), (F) anaphase I (AI), (G) telophase I (TI), and (H) metaphase II (MII). Numbers above the pictures represent the time in maturation culture. Scale bar = 10 μm .

Figure 4 Autoradiograph showing the changes in Cdc2 and histone H3 kinase activities during meiotic resumption in pig oocytes (Fig. 4-I). Histone H1 (H1) and histone H3 (H3) were used as the substrates of Cdc2 and histone H3 kinases respectively. The lower numbers represent the hours in maturation culture. The intensity of each phosphorylated histone H1 (Fig. 4-I) and histone H3 band (Fig. 4-II) was calculated by densitometric analysis. The activity of Cdc2 or histone H3 kinases after 27 h was set to 100%. The data are presented as mean $\pm$ S.E.M. Values with different superscripts are significantly different ($P < 0.05$). The experiments were conducted thrice with similar results.

oocytes). Similarly, the acetylation of H3-K14 appeared temporarily around AI and TI (93%, 39/42 oocytes; Fig. 5-I E' and F'). All these acetylation signals then disappeared completely when the oocytes reached MII (H3-K14 88%, 37/42 oocytes; Fig. 5-I G' and H3-K9 98%, 41/42 oocytes). However, the deacetylation of H3-K18 was maintained until MII (100%, 48/48 oocytes), without its reappearance at AI–TI.

The methylation of histone H3 at K9 was detected in oocytes at the GVI stage and was maintained throughout the meiotic maturation of the oocytes (Fig. 5-II).

Chromosome morphology and histone H3 modifications during oocyte activation

The typical meiotic stage observed in oocytes 2 h after electroactivation was AII–TII (95%, 38/40 oocytes; Fig. 6). After 4 h, most oocytes had reached TII (71%, 32/45 oocytes) and some had started to form a female pronucleus (FPN; 29%, 13/45 oocytes). The chromosomes had decondensed completely to form an FPN 6 h

after activation (98%, 47/48 oocytes). The activities of Cdc2 and histone H3 kinases were high in MII oocytes and started to decrease at the AII–TII stages. Both the kinases became inactive at the FPN stage, 6 h after activation (Fig. 7).

The phosphorylation levels of histone H3 at both S10 and S28 were high in oocytes at MII (Fig. 6-I A' and II A'; Table 1). The levels remained high at AII and started to decrease at TII (Fig. 6-I C' and II C'). The phosphorylation of histone H3-S10 completely disappeared at the FPN stage (Fig. 6-I D'). On the other hand, histone H3-S28 was still phosphorylated around the nucleoli of FPN stage (Fig. 6-II D').

The fluorescent signals for all the acetylated lysines in histone H3 were absent in MII oocytes before activation. The acetylation signal for H3-K14 reappeared first in AII oocytes 2 h after electroactivation. Then the signal for acetylated H3-K9 reappeared in oocytes at TII. Finally, the signal for acetylated H3-K18 reappeared in the FPN of activated oocytes (Table 1).

The methylation of histone H3-K9 in MII oocytes was maintained in oocytes at AII, TII, and the FPN stages (Table 1).

Effect of histone deacetylation inhibition on chromosome condensation and histone H3 phosphorylation

Histone H3 acetylation decreased in parallel with the appearance of histone H3 phosphorylation during oocyte maturation. We examined the effect of HDAC inhibition by TSA on the deacetylation of histone H3-K14 and the phosphorylation of H3-S10 and H3-S28. TSA inhibited oocyte germinal vesicle breakdown (GVBD) and maintained histone H3-K14 acetylation (100%, 40/40 oocytes), when the control oocytes had reached MI with the deacetylation of H3-K14 after 27 h (98%, 41/42 oocytes; Fig. 8-I). Treatment with TSA induced changes in chromatin morphology to that of the decondensed stage. The perinucleolar heterochromatin rim showed a limited response to TSA, remaining in close apposition to the nucleolus (Fig. 8-II B and D). The histone H3 phosphorylation at S10 did not occur in the chromosome (95%, 39/41 oocytes) and phosphorylation at S28 just occurred around the nucleus (90%, 37/41 oocytes) during 27 h treatment. (Fig. 8-II B' and D'). This differed from the control oocytes, which reached MI with condensed chromosomes and high histone H3 phosphorylation at both S10 and S28 (Fig. 8-II A' and C').

In all of the experiment groups, the negative control oocytes did not show any fluorescent signals at any stage.

Discussion

In this study, the regulation of chromatin/chromosome morphology by histone H3 modifications throughout

Figure 5 Acetylation (Fig. 5-I) and methylation of histone H3 (Fig. 5-II) during the maturation of pig oocytes. Oocytes were immunostained with anti-acetyl-histone H3-K14 (Ac-H3-K14) or anti-methyl-histone H3-K9 (Me-H3-K9), and Alexa-Fluor-488-labeled antibodies (green). The nuclear envelope was stained with anti-lamin A/C and Alexa-Fluor-568-labeled antibodies (red, A''-C''), and microtubules were stained with anti- α -tubulin and Alexa-Fluor-568-labeled antibodies (red, D''-G''). The DNA was counterstained with DAPI (blue). The meiotic stages are (A) germinal vesicle stage I (GVI), (B) germinal vesicle stage III (GVIII), (C) diakinesis (D), (D) metaphase I (MI), (E) anaphase I (AI), (F) telophase I (TI), and (G) metaphase II (MII). Scale bar = 20 μ m.

oocyte growth, maturation, and activation was investigated. The chromatin of oocytes in the secondary follicles was distributed throughout the nucleoplasm and was in the decondensed state. As the oocyte grew, the chromatin became more heterogeneous, and heavily condensed in part to form a heterochromatin rim around the nucleolus (GVI stage). The acetylation of histone H3-K9, H3-K14, and H3-K18 was detected in chromatin spreading throughout the nucleoplasm of the growing pig oocytes in secondary follicles of 0.1–0.2 mm diameter (Fig. 9). The histone H3 acetylation status was maintained, while the chromatin configuration changed from decondensed to a perinucleolar heterochromatin sheath during the growth of the pig oocytes. In the growing pig oocytes, Cdc2 and histone H3 kinases were inactive, and there was no histone H3 phosphorylation at S10 or S28. This finding differs from the situation in somatic cells, where histone H3-S10 is phosphorylated in both interphase and mitosis (Cheung *et al.* 2000a), and the phosphorylation of H3-S10 has been observed during both transcriptional activation and chromosome condensation during mitosis (Cheung *et al.* 2000a). It has been suggested that growing oocytes have high transcriptional activity (Crozet 1983), although no phosphorylation of histone H3 was detected in oocytes during the growth phase. This result suggests that the phosphorylation of histone H3 is not involved in the transcriptional activity of growing oocytes and the phosphorylation of histone H3 differs between meiotic and mitotic interphases.

In fungi and plants, DNA methylation acts downstream from histone H3-K9 methylation (Tamaru & Selker 2001). In the present experiments, no methylation signal for histone H3-K9 was detected in pig oocytes from secondary follicles. The methylation of H3-K9 appeared in growing oocytes from early antral follicles, and it increased thereafter during the growth phase. This pattern of histone H3 methylation in our results appears to correspond to the pattern of DNA methylation in mouse growing oocytes, in which DNA methylation in differentially methylated region for imprinted genes is established with oocyte size-dependent manner (Hiura *et al.* 2006). The present experiments indicate that the acquisition of methylation at histone H3-K9 is probably related to oocyte diameter and the change from chromatin distributed throughout the nucleoplasm to chromatin with a filamentous morphology in early antral follicles.

Histone H3-S10 is phosphorylated in condensed chromosomes in pig oocytes during maturation, as shown in our previous paper (Bui *et al.* 2004). Furthermore, the phosphorylation of histone H3-S28 was detected earlier in condensing chromosomes in pig oocytes at the GVII–IV stage (Fig. 9). Since chromosome condensation begins at this point, the phosphorylation of H3-S28 might be one of the key events initiating meiotic chromosome condensation. The activity of histone H3 kinase increased in oocytes, corresponding to the phosphorylation of histone H3-S28. The activity of H3 kinase increased further around GVBD, concomitant

Figure 6 Histone H3 dephosphorylation at S10 (Fig. 6-I) and S28 (Fig. 6-II) in electro-activated MII pig oocytes. Oocytes were cultured in the maturation medium for 42 h, and were then electroactivated. Oocytes were examined after 2, 4, or 6 h by immunostaining with anti-phospho-histone H3-S10 or H3-S28 primary antibody, and Alexa-Fluor-488-labeled secondary antibody for phosphorylated histone H3 (green), and anti- α -tubulin and Alexa-Fluor-568-labeled antibodies for microtubules (red). The DNA was counterstained with DAPI (blue). Meiotic stages are (A) metaphase II (MII), (B) anaphase II (AII), (C) telophase II (TII), and (D) female pronucleus stage (FPN). 1st pb, First polar body; 2nd pb, second polar body. Dotted circles in A'–D' indicate oocyte chromosomes. Scale bar = 20 μ m.

with the increase in Cdc2 kinase activity. It has been suggested that, in *Aspergillus nidulans*, NIMA kinase plays a role in the mitotic phosphorylation of histone H3, and NIMA and Cdc2 kinases cooperate to promote the activity of each (De Souza *et al.* 2000). Aurora kinase is also a mitotic histone H3 (S10) kinase (Crosio *et al.* 2002), and it is activated depending on the activation of Cdc2 kinase by indirect pathways, such as the inhibition of protein phosphatase 1 in mammalian cells (Marumoto *et al.* 2002). These findings and our present results suggest that Cdc2 kinase may promote histone H3 kinase activity to phosphorylate histone H3 in the condensed chromosome.

In the present experiments, the methylation of histone H3-K9 was maintained during oocyte maturation, perhaps to preserve maternal genomic imprinting. On the other hand, the acetylation of histone H3-K9, H3-K14, and H3-K18 disappeared completely around GVBD (Fig. 9). The deacetylated status of histone H3-K18 was maintained during oocyte maturation, although histone H3-K9 and H3-K14 were temporarily reacetylated around the MI/II transition. Since there is no transcriptional activity during oocyte maturation (Bachvarova 1985), the change in histone acetylation would not be associated with gene expression. Cdc2 kinase activity fell during

Figure 7 Autoradiograph showing the changes in Cdc2 and histone H3 kinase activities in pig oocytes after electroactivation. Histone H1 (H1) and histone H3 (H3) were used as the substrates of Cdc2 and histone H3 kinases respectively (Fig. 7-II). The intensity of each phosphorylated histone H1 (Fig. 4-I) and histone H3 band (Fig. 4-III) was calculated by densitometric analysis. The activity of Cdc2 or histone H3 kinases in MII oocytes was set to 100%. The data are presented as mean ± s.e.m. Values with different superscripts are significantly different (*P* < 0.05). The experiments were conducted thrice with similar results.

the MI/MII transition in pig oocytes, as shown previously (Bui *et al.* 2004). This oscillation pattern of Cdc2 kinase activity coincided with the temporal acetylation of histone H3-K9 and H3-K14. Thus, it is

likely that histone H3 acetylation at these residues is dependent on Cdc2 kinase activity.

The ‘histone code’ hypothesis predicts that specific combinations of histone modifications provide regulatory information through changes in the chromatin structure (Strahl & Allis 2000, Jenuwein & Allis 2001). According to this hypothesis, the modification of one residue in a histone may affect the type and frequency of the modifications at other sites. In pig oocytes after 9 h of culture, histone H3 began to be phosphorylated at S28, while histone H3 acetylation at K9, K14, and K18 was maintained. Thereafter, histone H3 started to be deacetylated, and was completely deacetylated concomitant with the increase in phosphorylation at S10 (Fig. 9). This suggests that the deacetylation of histone H3 and the phosphorylation of H3-S10 possibly affect on each other. This corresponds to studies of budding yeasts and nematodes, in which a decrease in the acetylation of histone H3-K9 was observed when the H3-S10 phosphorylation level increased (Hsu *et al.* 2000). Furthermore, in TSA-treated pig oocytes, histone H3 acetylation at K14 remained concomitant with the inhibition of phosphorylation at S10 in the chromosome. However, it has been suggested that the phosphorylation of histone H3-S10 in somatic cells correlates with the acetylation of histone H3-K14 during prophase (Mateescu *et al.* 2004) and facilitates the acetylation of K14 (Cheung *et al.* 2000b). These observations suggest that the modification of histone H3 in somatic cells is different from its modification in oocytes. Our experiment with TSA treatment suggests that the deacetylation of oocyte histone H3 is required for the phosphorylation of histone H3-S10, although TSA is thought to regulate cell-cycle molecules necessary for the G2–M transition (Noh & Lee 2003). On the other hand, the phosphorylation of histone H3 at S28 occurred around the nucleoli in TSA-treated pig oocytes. It has been shown in immunolocalization studies of mouse somatic cells that the phosphorylation events at histones H3-S10 and H3-S28 occur independently in distinct chromatin regions (Dunn & Davie 2005). In pig oocytes, the phosphorylation events at H3-S10 and H3-S28 are thought to be also regulated by different kinases and/or phosphatases. It is interesting to note that the S28 residue is further from K9,

Table 1 Histone H3 modifications in pig oocytes after activation.

Oocyte stage	Histone H3 modifications					
	Me-H3-K9	Ac-H3-K9	Ac-H3-K14	Ac-H3-K18	P-H3-S10	P-H3-S28
MI	+	–	–	–	+	+
AII	+	–	±	–	+	+
TII	+	±	±	–	±	±
FPN	+	+	+	+	–	+/–

The fluorescence intensity observed for each sample is denoted as described in Materials and Methods. The fluorescence of oocyte chromosomes at MI stage was used as the control value and set to 100%. The fluorescence intensity observed for each sample is expressed relative to this value as follows: > 60%, (+) high signal; 20–60%, (±) low signal; < 20%, (–) no signal; and (+/–) phosphorylated histone H3 around the nucleoli. Meiotic stages are: metaphase II (MI), anaphase II (AII), telophase II (TII), and female pronucleus stage (FPN).

Figure 8 Effects of trichostatin A (TSA) on the chromatin configuration, and the acetylation (Fig. 8-I) and phosphorylation of histone H3 (Fig. 8-II) in pig oocytes. Oocytes were cultured with (+) or without (-) 100 nM TSA for 27 h, and immunostained with anti-acetyl-histone H3-K14 (Ac-H3-K14), anti-phospho-histone H3-S10 (P-H3-S10), or anti-phospho-histone H3-S28 (P-H3-S28) antibody, and Alexa-Fluor-488-labeled antibody (green). The nuclear envelope was stained with anti-lamin A/C and Alexa-Fluor-568-labeled antibodies (red). The DNA was counterstained with DAPI (blue). Scale bar=20 μ m.

K14, and K18 than is the S10 residue. Therefore, the phosphorylation of S28 is possibly not influenced by the acetylation of other residues.

After the electroactivation of pig oocytes, the phosphorylation of histone H3 at S10 and S28 decreased at AII, and become dephosphorylated at TII. The activities of histone H3 and Cdc2 kinases decreased during the AII–TII stage. Together with the chromosome condensation observed during oocyte maturation, these results suggest that the phosphorylation/dephosphorylation of histone H3 at S10 and S28 is regulated by histone H3 kinase. The reacylation of histone H3 at K9 and K14 was observed in parallel with the drop in phosphorylation at H3-S10 and H3-S28 at the AII–TII stage. This result supports the inference drawn from the results of

oocyte maturation, that histone phosphorylation coincides with the acetylation status of the neighboring lysine. Histone acetylation has been thought to be associated with DNA replication, as well as with the regulation of transcription (Vogelauer *et al.* 2002). The reappearance of acetylation toward the pronucleus stage seems to be preparatory to DNA replication and transcription activity in pig embryos, and the chromosomes were completely decondensed at the time of pronucleus formation.

The methylation of histone H3-K9 remained high during oocyte maturation and activation, regardless of the acetylation of lysine residues or the phosphorylation of serine residues, in pig oocytes (Fig. 9). It has been suggested that histone methylation and DNA

Figure 9 Schematic depiction of histone modifications during the growth, maturation, and activation of pig oocytes.

methylation are stable and potentially stabilize epigenetic modifications through critical developmental transitions (Kubicek & Jenuwein 2004). This observation is similar to that of a study in the mouse, in which methylated H3-K9 was detected in chromosomes in MII oocytes and in the female PN of one-cell embryos (Liu *et al.* 2004). This suggests that chromatin modifications take place specifically in the maternal or paternal genomes of the mouse zygote (Morgan *et al.* 2005) and global histone demethylation occurs in the paternal pronucleus (Arney *et al.* 2002). The hypomethylation of histone H3-K9 in the male pronucleus implies that oocyte cytoplasmic histone H3 is demethylated at K9 and/or histone methylase activity in the oocyte is low in fertilized oocytes. The mechanisms responsible for protecting the maternal pronucleus from histone demethylation are not clear at present. However, dimethylation of H3-K9 in the maternal genome has recently been suggested to play a role in preventing histone demethylation in the female PN during the first cell cycle (Santos *et al.* 2005).

In conclusion, the findings of this study suggest that chromosome condensation/decondensation in pig oocytes is regulated by the acetylation/deacetylation and phosphorylation/dephosphorylation of histone H3. It is also clear that histone H3-K9 methylation is

established in parallel with oocyte growth. Although histone acetylation and phosphorylation were reversible, the stable methylation of H3-K9 was maintained throughout the course of oocyte maturation and activation.

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