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Distribution and Expression of Phosphorylated Histone H3 During Porcine Oocyte Maturation

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ABSTRACT Phosphorylation modification of core histones is correlated well with diverse chromatin-based cell activities. However, its distribution pattern and primary roles during mammalian oocyte meiosis are still in dispute. In this study, by performing immunofluorescence and Western blotting, spatial distribution and temporal expression of phosphorylated serine 10 or 28 on histone H3 during porcine oocyte meiotic maturation were examined and distinct subcellular distribution patterns between them were presented. Low expression of phosphorylated H3/ser10 was detected in germinal vesicle. Importantly, following gradual dephosphorylation from germinal vesicle (GV) to late germinal vesicle (L-GV) stage, a transient phosphorylation at the periphery of condensed chromatin was re-established at early germinal vesicle breakdown (E-GVBD) stage, and then the dramatically increased signals covered whole chromosomes from pre-metaphase I (Pre-MI) to metaphase II (MII). Similarly, hypophosphorylation of serine 28 on histone H3 was also monitored from GV to E-GVBD, indicating dephosphorylation of histone H3 maybe involved in the regulation of meiotic resumption. Moreover, the rim staining on the chromosomes and high levels of H3/ser28 phosphorylation were observed in Pre-MI, MI, and MII stage oocytes. Based on above results, such stage-dependent dynamics of phosphorylation of H3/ser 10 and 28 may play specific roles during mammalian oocyte maturation. *Mol. Reprod. Dev. 75: 143–149, 2008.* © 2007 Wiley-Liss, Inc.

Key Words: porcine oocyte; meiosis; histone; phosphorylation; chromatin condensation

INTRODUCTION

In eukaryotic cells, DNA binds to alkaline histones to constitute nucleosome. The core histones are often subject to phosphorylation modification on specific residues during cell division (Bradbury, 1992; Koshland and Strunnikov, 1996). Extensive studies reveal the phosphorylation of H2A and H4 on serine 1, H2B on serine 14/32, and H3 on serine 10/28 and threonine 3/11 (Preuss et al., 2003; Polioudaki et al., 2004; Sarmento et al., 2004). It has been widely documented that the phosphorylation at serines 10 and 28 is determined by Aurora kinases and phosphatase PP1, which are highly conserved events from yeast to vertebrates (Francisco et al., 1994; Murnion et al., 2001; Sugiyama et al., 2002).

By far, most studies concentrate on the phosphorylation of serine 10 on histone H3 (phH3/ser10). Mitotic phosphorylation of H3/ser10 commences in late G2 in pericentromeric heterochromatin of each chromosome and has spread throughout all chromosomes by metaphase. Dephosphorylation begins in anaphase and ceases immediately prior to detectable chromosome decondensation in telophase (Hendzel et al., 1997). Moreover, the similar phosphorylation patterns of H3/ser10 during meiosis were shown in some organisms (Wei et al., 1998; Kaszas and Cande, 2000).

Mounting evidence suggests that phosphorylation of histone H3 is linked with chromatin condensation (Nowak and Corces, 2004), chromosome segregation (Wei et al., 1998), and transcriptional activation (Sassone-Corsi et al., 1999). However, other studies revealed that histone H3 phosphorylation is not correlated with chromosome condensation either in mitosis (Hsu et al., 2000) or in meiosis (Cobb et al., 1999; Schmitt et al., 2002), but couples with the maintenance of sister chromatid cohesion in maize (Kaszas and Cande, 2000).

Notably, identified later and less studied than serine 10, the phosphorylation of serine 28 on H3 (phH3/ser28) shows a pattern similar to but different from that of serine 10 (Goto et al., 2002). It starts to be evident only in prophase (Goto et al., 2002). Nevertheless, the detailed role of phH3/ser28 still remains to be fully elucidated.

Dramatic changes of chromatin structure and function occur during oocyte maturation, which raises questions about particular histone phosphorylation

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patterns may exist in oocyte meiosis and play specific roles. Recently, two studies about the histone H3 phosphorylation in porcine oocyte were conducted in succession (Bui et al., 2004; Jelinkova and Kubelka, 2006). Interestingly, they presented totally different conclusions. Bui's data suggested that phosphorylation of H3/ser10 is involved in chromosome condensation, but the other group reported that neither Aurora B activity nor histone H3 phosphorylation is essential for chromatin condensation during meiotic maturation. Further, the distribution and expression of phosphorylated histone H3 at different developmental stages, especially around meiotic resumption, were not thoroughly investigated in mammalian oocyte. Obviously, there are not enough data for researchers to understand the regulatory mechanisms underlying porcine oocyte maturation. In this study, we examined the spatial distribution and temporal expression of phosphorylated serines 10 and 28 on histone H3 in meiosis in detail. Results showed that dephosphorylation of both serines 10 and 28 on histone H3 uniformly occurred around GVBD. With oocytes proceed to pre-metaphase I (Pre-MI) stage, the phosphorylation level of both H3/ser10 and 28 promptly increased, however, the differential distribution patterns between them were present.

MATERIALS AND METHODS

Chemicals

All chemicals used in this study were purchased from Sigma Chemical Company (St. Louis, MO) except for those specifically mentioned.

Collection and Culture of Porcine Oocytes

Cumulus-oocyte complexes were aspirated from antral follicles (3–8 mm in diameter) of ovaries collected from pre-pubertal gilts at a local slaughterhouse. Oocytes possessing compact cumulus and evenly granulated ooplasm were cultured with maturation medium TCM-199 (Gibco, Grand Island, NY) supplemented with 75 μg/ml potassium penicillin G, 50 μg/ml streptomycin sulfate, 0.57 mM cysteine, 0.5 μg/ml FSH, 0.5 μg/ml LH, and 10 ng/ml EGF. Each group of 30 oocytes was cultured in a 100 μl drop of maturation medium for up to 44 hr at 38.8°C in an atmosphere of 5% CO_2.

Immunofluorescence Microscopy

After removing the zona pellucida in acidified M2 (pH2.5), oocytes were fixed with 4% paraformaldehyde in PBS (pH7.4) for 30 min at room temperature. Samples were permeabilized with 1% Triton X-100 overnight at 37°C, and then incubated at 4°C overnight with mouse monoclonal anti-phH3/ser10 antibody (1:200, Upstate Biotechnology, Lake Placid, NY) or with rabbit polyclonal anti-phH3/ser28 antibody (1:200, Upstate Biotechnology). FITC (fluorescein isothiocyanate) conjugated goat anti-mouse or goat anti-rabbit (both 1:100, Santa Cruz Biotechnology, Santa Cruz, CA) IgG were used as secondary antibodies. Nuclear of oocytes was stained with 10 μg/ml propidium iodide (PI) for 5 min. Finally, cells were observed using a Confocal Laser-Scanning Microscope (Zeiss LSM 510 META, Jena, Germany), equipped with a 63 × Plan-Apochromat oil-immersion objective lens (1.5 NA). The detailed procedures followed the method described previously (Wang et al., 2006a).

Western Blot Analysis

Western blot analysis was conducted according to the procedures described by Fan et al. (2003) with minor modifications. Proteins extracted from 200 oocytes were separated by 15% SDS–PAGE and electrophoretically transferred onto nitrocellulose membrane. Then the membrane was incubated with mouse monoclonal anti-phH3/ser10 or rabbit polyclonal anti-phH3/ser28 antibodies overnight at 4°C. After three washes in TBST and incubation with horseradish peroxidase linked goat anti-mouse or goat anti-rabbit (1:1,000, Santa Cruz Biotechnology) IgG antibodies, the labeled proteins were detected by the ECL system. Equal protein loading was confirmed by detection of α-tubulin.

Data Analysis

The relative expression levels of phosphorylated histone H3/ser10 and 28 were evaluated by volume scan of band using Quantity One software (Bio-Rad Laboratories, Alfred Nobel Drive Hercules, CA), and analyzed by Student's *t*-test. Differences at $P < 0.05$ were considered significant.

RESULTS

Nuclear Maturation of Porcine Oocyte During In Vitro Culture

Nuclear status of oocytes at different time points was evaluated by orcein staining as described by Sun et al. (2001). As shown in Figure 1, at 0 hr of culture, about 96.3% oocytes were at the germinal vesicle (GV) stage. By 18 hr of culture, germinal vesicle breakdown (GVBD)

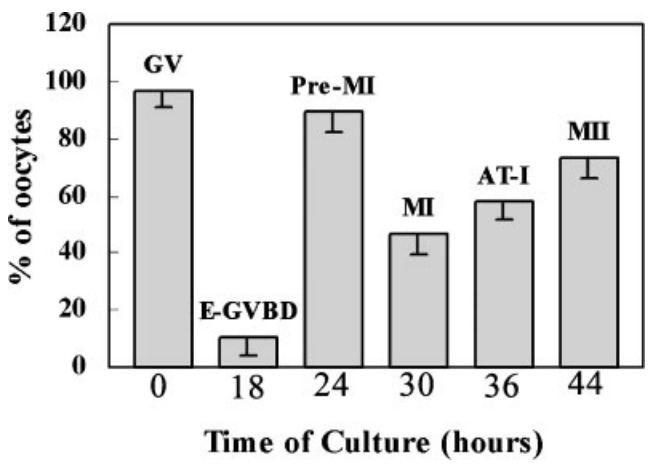

Fig. 1. Nuclear maturation time course of porcine oocyte during in vitro culture. Results are from three separate experiments. GV, germinal vesicle; E-GVBD, early-germinal vesicle breakdown; Pre-MI, pre-metaphase I; MI, metaphase I; AT-I, anaphase I and telophase I; MII, metaphase II. The graph shows the mean + SME of the results obtained in three independent experiments.

began to take place in a small proportion of oocytes (10.1%). After 24 hr of culture, about 89.2% of the oocytes proceeded to pre-metaphase of the first meiosis (Pre-MI). When the oocytes were examined at 30 and 44 hr, about 46.8% and 73.2% of the oocytes reached the metaphase I (MI) and II (MII) stages, respectively. It is worth noting that most oocytes (57.8%) were at the first meiotic anaphase/telophase (AT-I) after 36 hr of culture. These data are important for the design of following experiments and the analysis of Western blotting results.

Distribution and Expression of phH3/ser10 During Porcine Oocyte Maturation

Porcine oocytes at different developmental stages were immunolabeled with an antibody specific for phH3/ser10 and the results were shown in Figure 2. Apparent immunosignals were detected around the nucleolus and closely colocalized with chromatin in the GV (Fig. 2A). Importantly, with further condensation of chromatin, immunostaining of phH3/ser10 was barely observed on condensed chromatin in late GV (L-GV) oocytes (Fig. 2B; denoted by arrow) characterized by the intact nuclear membrane, clump-like chromatin and disappeared nucleolus (Motlik and Fulka, 1976). Surprisingly, just after GVBD (early GVBD—E-GVBD) with network-like chromatin, two different phosphorylation patterns of H3/ser10 were simultaneously detected: phH3/ser10 staining was absent in some oocytes (Fig. 2C), but rim staining around chromosomes in other oocytes (Fig. 2D; see insert). Intense signals tended to spread throughout the chromosome arms (Fig. 2E) at Pre-MI stage, and this distribution pattern was maintained through metaphase I, anaphase/telophase I to metaphase II (Fig. 2F–H).

Simultaneously, Western blotting was performed to examine the expression level of phH3/ser10 in porcine oocytes cultured for 0, 18, 24, 30, 36, and 44 hr (Fig. 3A,B). As a result, low expression of phH3/ser10 was detected at 0 hr and no distinct band was observed after 18 hr culture. With the meiotic resumption of oocytes, the expression level of phH3/ser10 dramatically increased from 24 to 44 hr. In general, immunoblotting results were consistent to that of immunofluorescent analysis.

Collectively, the stage-dependent dynamics of phH3/ser10 in porcine oocyte meiosis were presented, showing that gradual dephosphorylation from GV to L-GV followed by a transient phosphorylation at the periphery of chromosomes at E-GVBD stage, and then the dramatically increased signals covered whole chromosomes from Pre-MI to MII.

Distribution and Expression of phH3/ser28 During Porcine Oocyte Maturation

Similarly, subcellular distribution of phH3/ser28 at different stages was also determined by immunofluorescence as shown in Figure 4. Unexpectedly, the temporal-spatial distribution of phH3/ser28 was distinctly different from that of phH3/ser10. No apparent

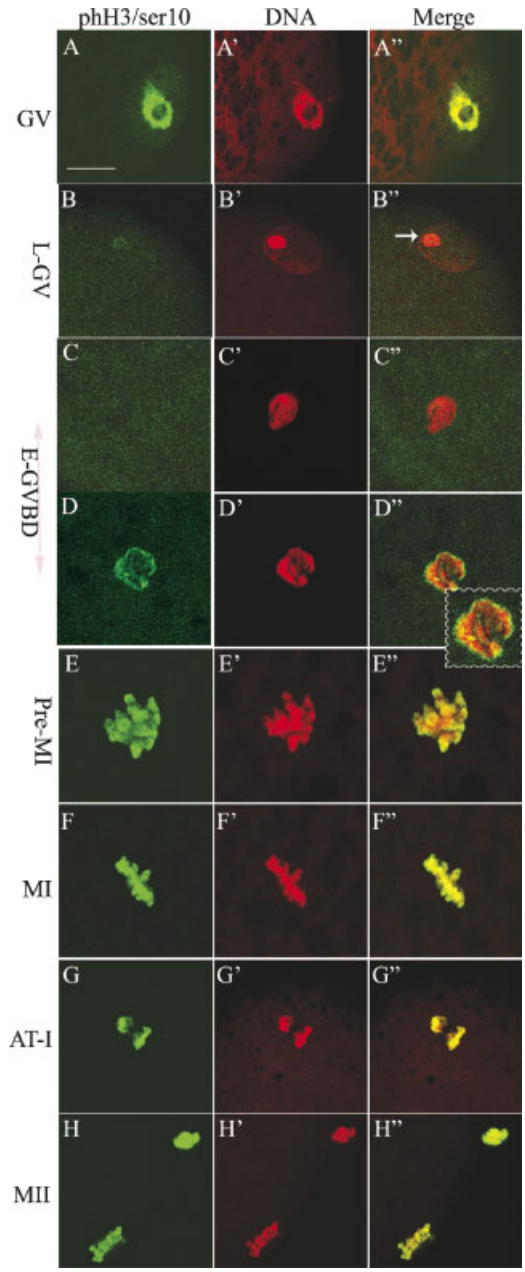

Fig. 2. Immunolocalization of phH3/ser10 at different stages of porcine oocyte. GV, noncultured oocytes at germinal vesicle stage (**A**,**A′**,**A″**); L-GV, oocytes at late germinal vesicle (**B**,**B′**,**B″**); E-GVBD, oocytes at early germinal vesicle breakdown (**C**,**C′**,**C″**; **D**,**D′**,**D″**); Pre-MI, oocytes at first pre-metaphase (**E**,**E′**,**E″**); MI, oocytes at first metaphase (**F**,**F′**,**F″**); AT-I, oocytes at first anaphase and telophase (**G**,**G′**,**G″**); MII, oocytes at second metaphase (**H**,**H′**,**H″**). Each sample is counterstained with PI to visualize DNA. Bar, 10 μm. [See color version online at www.interscience.wiley.com.]

signals of phH3/ser28 were detected in GV, L-GV, and E-GVBD stage oocytes (Fig. 4A–C). Importantly, in Pre-MI, MI, and MII oocytes, intense staining was clearly observed at the periphery of chromosomes (Fig. 4D,D″; E,E″; G,G″; see inserts), which strongly indicated that such a distribution pattern of phH3/ser28 may play specific roles in oocyte meiosis. Consistent with immunofluorescence analysis, the resulting Western blots

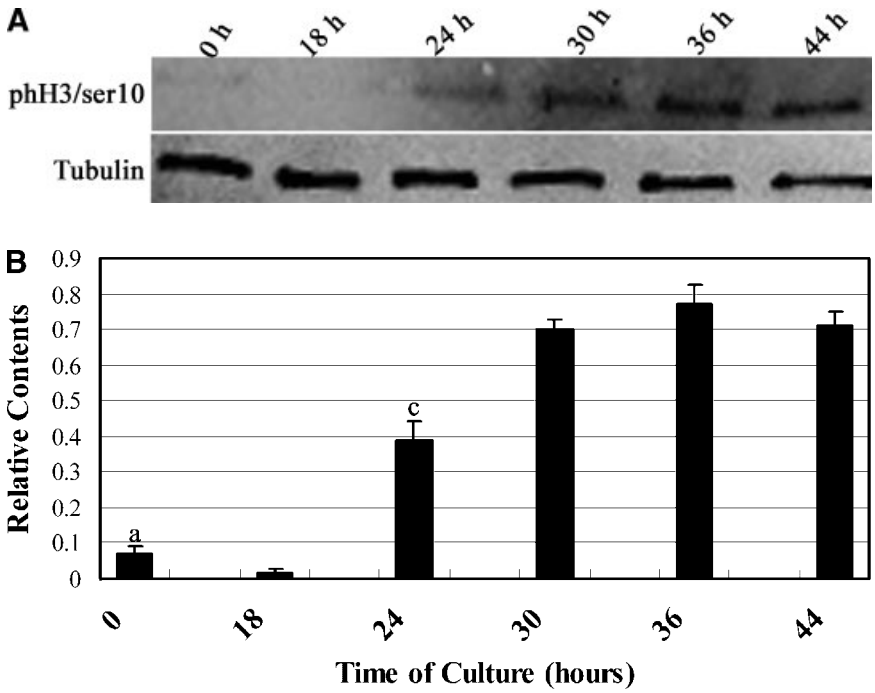

Fig. 3. Expression of phH3/ser10 during porcine oocyte maturation. **A**: Oocytes cultured for 0, 18, 24, 30, 36, or 44 hr were analyzed by immunoblotting. A control blot of α-tubulin shows equal protein loading between the various samples. **B**: Semi-quantitative analysis of Western blotting results. Different superscripts indicate statistical differences ($P < 0.05$).

revealed that phH3/ser28 levels markedly increased when oocytes were cultured for 24, 30, 36, and 44 hr compared to those cultured for 0 and 18 hr (Fig. 5A,B), implying that increasing levels of phH3/ser28 may associated with the further condensation of chromatin after GVBD.

Exceptionally, at anaphase/telophase I stage, phH3/ser28 colocalized with the chromatin (Fig. 4F) and its expression level significantly decreased compared with those at MI and MII stages (Fig. 5B). These results indirectly underscored that such a unique spatial distribution and temporal expression pattern of phH3/ser28 may tightly correlate with chromosome decondensation in AT-I oocytes.

Altogether, dephosphorylation of both serines 10 and 28 on histone H3 uniformly occurred around GVBD, which may be required for the orderly meiotic resumption. With oocytes proceed to the Pre-MI stage, the phosphorylation level of both H3/ser10 and 28 promptly increased, however, the differential distribution patterns between them were revealed.

DISCUSSION

It has been recognized that histone H3 phosphorylation is a conserved event in species ranging from *Tetrahymena* (Wei et al., 1998) to mammals (Hendzel et al., 1997; Cobb et al., 1999). Phosphorylation of histone H3 at serine 10 has been regarded as a marker for mitosis (Bradbury, 1992). Substantial reports suggest that phosphorylated histone H3 initiates in late G2 interphase cells, becomes maximal at metaphase, and then dephosphorylates at late anaphase and telophase (Prigent and Dimitrov, 2003).

In this study, by performing immunofluorescence and Western blotting, our results revealed that phosphorylation of serines 10 and 28 were temporally and spatially related to oocyte meiotic maturation.

We paid particular attention to the transition from GV to GVBD, during which dramatic changes in chromatin structure and function occur. As expected, some specific events were uncovered by this study. First, we observed H3/ser10 is phosphorylated at GV stage by immunostaining (Fig. 2A), which is consistent with our conclusion derived from mouse oocytes (Wang et al., 2006b), but contrary to that reported by Bui et al. (2004). The low expression level of phH3/ser10 (Fig. 3) as revealed by the present study may explain the undetected signal in Bui et al.'s research. Second, from late GV to E-GVBD, the staining uniformly diminished either on phH3/serine 10 or on phH3/ser28 (Figs. 2B and 4B). Most recently, our study on the acetylation changes during porcine oocyte maturation revealed that the deacetylation of all lysine residues on histones H3 and H4 occurred during GV to GVBD period. Moreover, by adding TSA (a general inhibitor of deacetylases) to the culture, the onset of GVBD was significantly delayed (Wang et al., 2006a). Based on these observations, we are greatly encouraged to speculate that histone dephosphorylation/deacetylation from GV to GVBD may

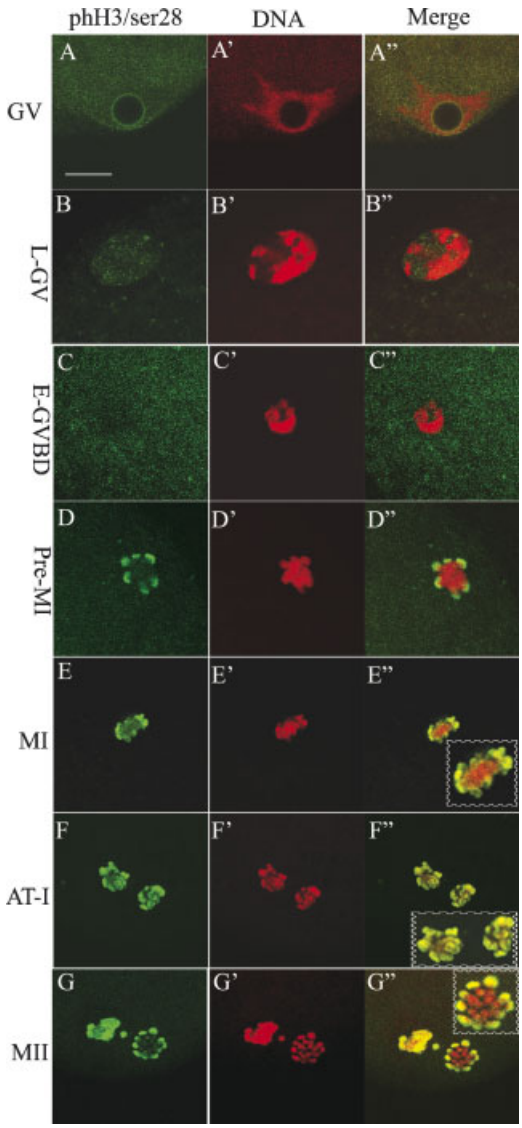

Fig. 4. Immunolocalization of phH3/ser28 at different stages of porcine oocyte. GV, noncultured oocytes at germinal vesicle stage (**A**,**A′**,**A″**); L-GV, oocytes at late germinal vesicle (**B**,**B′**,**B″**); E-GVBD, oocytes at early germinal vesicle breakdown (**C**,**C′**,**C″**); Pre-MI, oocytes at first pre-metaphase (**D**,**D′**,**D″**); MI, oocytes at first metaphase (**E**,**E′**,**E″**); AT-I, oocytes at first anaphase and telophase (**F**,**F′**,**F″**); MII, oocytes at second metaphase (**G**,**G′**,**G″**). Each sample is counterstained with PI to visualize DNA. Bar, 20 μm. [See color version online at www.interscience.wiley.com.]

participate in the regulation of meiotic resumption in porcine oocytes.

Importantly, our results for the first time showed that phosphorylation of H3/ser10 re-established at the periphery of the chromosome just after GVBD (Fig. 2D″; see insert), then spread throughout the whole chromosomes at Pre-MI stage (Fig. 2E), and such a high level of expression maintained until MII stage (Figs. 2F–H and 3A,B). In contrast, the presence of phH3/ser28 rim around meiotic chromosomes (60× magnification) persisted from Pre-MI to MII except for the AT-I stage, which was different from the report that both phH3/ser10 and 28 showed similar colocalization with chromatin in porcine oocyte meiosis (Jelinkova and Kubelka, 2006). At a large extent, such discrepancies perhaps were caused by their using the low magnification of lens (40×) equipped on confocal microscope. Being supportive, we also observed rim-fluorescence distribution of phH3/ser28 during mouse oocyte meiosis (Wang et al., 2006b). Such a unique phosphorylation pattern of H3/ser28 may play specific roles during mammalian oocyte maturation. It is worth noting that two different phosphorylation patterns of H3/ser10 were simultaneously observed in E-GVBD stage (Fig. 2C,D). As explained in other cell types (Prigent and Dimitrov, 2003), this phenomenon may indicate that, accompanying with the completion of dephosphorylation, rephosphorylation of H3/ser10 also starts from pericentromeric heterochromatin in porcine oocytes after meiotic resumption. Interestingly, we could not detect the similar phosphorylation dynamics on H3/ser28 in the corresponding stage.

In addition, in mitotic cells, H3 phosphorylation was gradually reduced or even vanished at late anaphase and early telophase (Prigent and Dimitrov, 2003). However, all results derived from mammalian oocytes in previous (Bui et al., 2004; Jelinkova and Kubelka, 2006) and present studies (Figs. 2G and 4F) revealed that histone H3 phosphorylation persists into late telophase I. This remarkable difference may play a role in the establishment of differential segregation mechanisms between homologous chromosomes in meiosis I and sister chromatids in mitotic cells (Wang et al., 2006b).

A growing number of studies revealed that histone H3 phosphorylation is correlated with the chromatin condensation in several cell types (Hendzel et al., 1997; Van Hooser et al., 1998; Wei et al., 1998). Nevertheless, other genetic and cellular data meanwhile suggested that phosphorylation of histone H3 was not correlated with

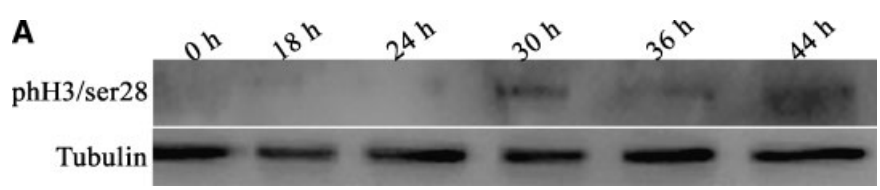

Fig. 5. Expression of phH3/ser28 during porcine oocyte maturation. **A**: Oocytes cultured for 0, 18, 24, 30, 36, or 44 hr were analyzed by immunoblotting. A control blot of α-tubulin shows equal protein loading between the various samples. **B**: Semi-quantitative analysis of Western blotting results. Different superscripts indicate statistical differences ($P < 0.05$).

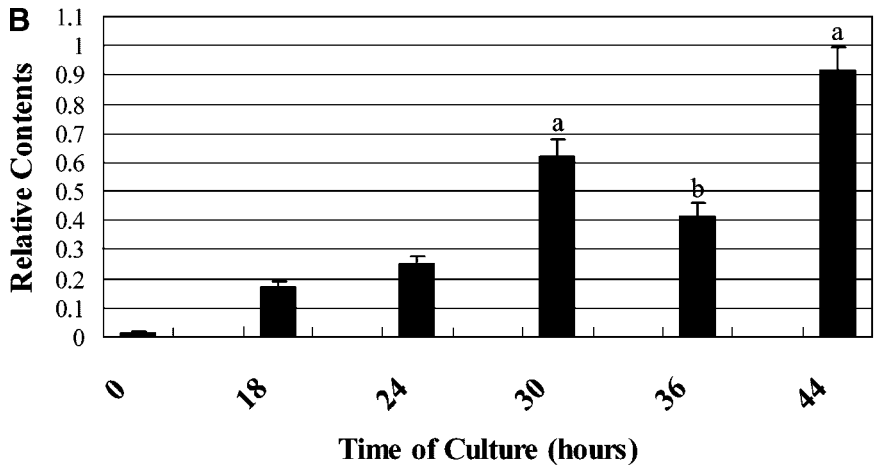

Fig. 5. (*Continued*)

chromosome condensation (Hsu et al., 2000; Kaszas and Cande, 2000). Concerning the relationship between histone H3 phosphorylation and chromatin condensation in mammalian oocyte, different studies presented the totally contrary conclusions (Bui et al., 2004; Jelinkova and Kubelka, 2006). In the present study, the expression level of both phH3/ser10 and 28 significantly increased from Pre-MI (Figs. 2E, 3, 4D, and 5), when chromatin condensation dramatically takes place. This result indicates that phosphorylation of H3/ser10 may be coupled with chromosome condensation. However, the phosphorylation level of H3/ser10 was reduced from GV to GVBD (Figs. 2B, 3, 4B, and 5), although during which the chromatin gathering are also present. Based on these observations, we conclude that phosphorylation of H3/ser10 maybe correlated with chromatin condensation in some stages but not in all processes. In contrast, phH3/ser28 is more likely a critical factor associated with chromatin condensation (Goto et al., 2002). It has been proposed that the phosphorylation of histone amino-terminal tails might reduce their affinity for DNA and facilitate the movement of nucleosomes and access of chromatin to condensation factors and thus promote further chromosome condensation (Koshland and Strunnikov, 1996).

CONCLUSIONS

Differential phosphorylation patterns of H3/ser10 and 28 in porcine oocyte meiosis were presented. Gradual dephosphorylation of histone H3 uniformly occurred around GVBD, which perhaps participated in the regulation of meiotic resumption. With oocytes proceed to the Pre-MI stage, the phosphorylation level of both H3/ser10 and 28 promptly increased, which maybe correlated with chromosome condensation after GVBD.

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