

Localization of centromere proteins and their association with chromosomes and microtubules during meiotic maturation in pig oocytes

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Centromere proteins (CENPs) are required for the attachment of microtubules to chromosomes. However, their structure and mechanism of action are not well understood, especially in mammalian meiosis. The present study was conducted to examine (i) whether a human nuclear centromere autoantibody can be used to localize the CENPs in pig oocytes and (ii) the dynamics of CENPs and their association with microtubules and chromosomes during meiosis in pigs. Oocytes at various stages were double-labelled for CENPs, chromosomes or microtubules and examined by confocal fluorescence microscopy. Quantification of tubulin and CENPs in the oocytes was determined by immunoblotting. CENPs were detected in all oocytes from germinal vesicle (GV) to metaphase II (MII)

stages. The changes in the location were associated with chromosome movement and spindle formation. Tubulin was detected in the oocytes from GV to MII stages and no differences in content were observed. Two major CENPs at 80 kDa (CENP-B) and 50 kDa (CENP-D) were also found in the oocytes by the autoantibody and its content was significantly lower in the oocytes at GV stage compared with oocytes at other stages. These results indicate that the autoantibody used in this study can be used to detect CENPs in the kinetochores, and the proteins are expressed in pig oocytes at all stages during meiosis. As the localization of CENPs is associated with spindle formation and chromosome movement, CENPs may participate in cell cycle changes during meiosis in mammals.

Introduction

The kinetochore is a macromolecular structure that is associated with the centromeres of chromosomes and is responsible for establishing and maintaining the connection with the microtubules of the mitotic and meiotic spindles. Kinetochores consist mainly of proteins (Brenner *et al.*, 1981). So far, two types of centromere protein (CENP) have been found in the kinetochores. One type of CENP represents constitutive protein, such as CENP-A, CENP-B, CENP-C and CENP-D. These CENPs are present at the centromeres throughout the cell cycle and can be detected by anticentromere antibody (ACA), in autoimmune sera from patients with calcinosis, Raynaud's phenomenon, oesophageal dysmotility, sclerodactyly and telangiectasia (CREST) (Moroi *et al.*, 1980, 1981; Brenner *et al.*, 1981; Warburton *et al.*, 1997; Hudson *et al.*, 1998; Craig *et al.*, 1999). The other type of CENP represents transient proteins, such as CENP-E, CENP-F, MAD1, MAD2, bud1 and bud2. These proteins

can be detected in specific cell cycle stages, mainly in metaphase by specific antibodies (Craig *et al.*, 1999).

CREST has been used for study of CENPs and kinetochore structures for many years, mainly in mitosis. It would appear that different CENPs have different functions; most are associated with chromosome segregation, spindle formation and connection with chromosomes. It is believed that most CENPs are located in the kinetochores but some are located in the cytoplasm (Craig *et al.*, 1999). Irrespective of their locations, CENPs participate, in different ways, in searching and capturing the plus ends of microtubules after nuclear envelope breakdown, then attach the chromosomes to the spindle. After capturing the microtubules, these proteins regulate chromosome movement, find the defect in spindle structure and chromosome congregation on the spindle plate and release the signals to delay onset of anaphase until all the chromosomes have properly aligned on the spindle equatorial plate (Maney *et al.*, 1999; Hoffman *et al.*, 2001). Dysfunctional kinetochores or depletion of some CENPs may cause premature anaphase and then induce unequal distribution of sister chromatids during cell division, yield aneuploidy, and consequently result in tumour or severe congenital syndromes (Pennisi, 1998; Rieder and Salmon, 1998; Cimini *et al.*, 2001).

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Accurate segregation of chromosomes occurs in the highly ordered spindle. Any mistake in chromosome segregation results in aneuploidy and is associated with some diseases in humans. Mistakes in the chromosome distribution of oocytes or spermatozoa also result in aneuploid embryo formation, which causes early embryo death, spontaneous abortion and genetic diseases. Although the structure and function of kinetochores and associated CENPs have been widely studied in mitotic cells, little is known about them during meiosis, especially in females (Heald *et al.*, 1997; Polanski *et al.*, 1998; Brunet *et al.*, 1999). These studies indicate that kinetochores in meiotic oocytes behave differently from mitotic cells. However, information on mammalian meiotic kinetochores and associated proteins is limited and it is still not known whether there are species-specific differences. In the present study, pig oocytes were used as an experimental model for mammals to examine whether (i) CENPs in kinetochores of pig oocytes can be detected by CREST antibody and (ii) the dynamic location of CENPs and their association with chromosomes and microtubules can be found during meiosis.

Materials and Methods

In vitro maturation of oocytes

The collection and culture of oocytes were based on the procedures reported by Wang *et al.* (2000). Briefly, ovaries were collected from prepubertal gilts at a local abattoir and transported to the laboratory in 0.9% NaCl containing 75 µg penicillin G ml⁻¹ and 50 µg streptomycin sulphate ml⁻¹ maintained at 37°C. Oocytes were aspirated from medium-sized follicles (3–6 mm in diameter) with a 20-gauge needle fixed to a 10 ml disposable syringe. Cumulus–oocyte complexes surrounded by a compact cumulus mass with evenly granulated cytoplasm were selected and washed three times with maturation medium: TCM-199 supplemented with 0.57 mmol cysteine l⁻¹ (Sigma Chemical Co., St Louis, MO), 10 ng epidermal growth factor ml⁻¹ (Sigma), 10 iu equine chorionic gonadotrophin ml⁻¹ (Sigma), 10 iu hCG ml⁻¹ (Sigma) and 0.1% polyvinyl alcohol. A group of 50 oocytes was cultured in a 500 µl drop of maturation medium at 39°C in an atmosphere of 5% CO₂ in air and saturated humidity.

Oocytes were freed of cumulus cells at 0, 12, 24, 36 and 44 h after culture by treatment in the maturation medium containing 0.02% (w/v) hyaluronidase (Sigma) and repeated pipetting. Cumulus-free oocytes were used in the following experiments.

Assessment of nuclear maturation

At various time points of culture, the denuded oocytes were mounted on slides and fixed in 25% (v/v) acetic acid in ethanol for 48–72 h at room temperature (25°C),

stained with 1% (w/v) orcein in 45% (v/v) acetic acid and examined under a phase-contrast microscope at a magnification of ×400. The nuclear stages were classified as germinal vesicle (GV), pro-metaphase I (pro-MI), metaphase I (MI), anaphase I (AI), telophase I (TI) and metaphase II (MII) (Wang *et al.*, 1994).

Labelling of oocytes for examination of CENPs, chromosomes and microtubules

Cumulus-free oocytes at various stages were fixed with 4% formaldehyde in PHEM buffer (60 mmol Pipes l⁻¹, 25 mmol Hepes l⁻¹, 10 mmol EGTA l⁻¹, 4 mmol MgSO₄ l⁻¹, pH 7.0) for 20 min at room temperature. The fixed oocytes were rinsed three times for a total of 15 min in PBS and then treated for 10 min in 1% Triton-X100 in PHEM. After they were briefly washed in PBS, oocytes were blocked in 20% boiled normal goat serum (BNGS) in PHEM for 1 h at room temperature. Oocytes were double labelled for CENPs and chromosomes; microtubules and chromosomes; or CENPs and microtubules according to the following protocols.

Fixed oocytes were incubated in CREST (Cortex Biochem, San Leandro, CA) in 1:500 in PHEM containing 5% BNGS overnight at 4°C for labelling of CENPs and chromosomes. After they were washed four times in PBS with 0.05% Tween-20, the oocytes were incubated in fluorescein isothiocyanate (FITC)-conjugated goat anti-human IgG (Jackson ImmunoResearch, West Grove, PA) 1:200 in PHEM with 5% BNGS for 45 min at room temperature. The oocytes were then washed and stained with 10 µg propidium iodide ml⁻¹ in PBS–Tween for 2 min for chromosome examination before they were mounted on slides.

Fixed oocytes were incubated in FITC-conjugated monoclonal anti-α-tubulin (Sigma) 1:50 in PHEM with 5% BNGS for 1 h at room temperature for labelling of microtubules and chromosomes. The oocytes were then washed and stained with propidium iodide for chromosome examination using the methods described above.

Fixed oocytes were treated with a mixture of mouse anti-α-tubulin antibody (1:8000 in PHEM with 5% BNGS) (Sigma) and CREST (1:500 in PHEM with 5% BNGS) overnight at 4°C for labelling of microtubules and CENPs. After they were washed four times in PBST for 5 min each time, oocytes were incubated with a mixture of rhodamine red-conjugated goat anti-mouse IgG (Jackson ImmunoResearch) and FITC-conjugated goat anti-human IgG (both diluted 1:200 in PHEM with 5% BNGS) for 45 min at room temperature.

Confocal microscopy of stained oocytes

Oocytes were washed in PBS–Tween-20 after staining, then mounted on slides with antifade solution (0.5%

n-propyl gallate in 20 mmol Tris l⁻¹, pH 8.0, with 90% glycerol) and examined under a Leica confocal laser scanning microscope (TCS-4D) on the same day. Each experiment was repeated four times and at least 15 oocytes were examined each time.

Immunoblotting for quantities of tubulin and CENPs in the oocytes

Immunoblotting of tubulin was carried out according to the methods reported by Fan *et al.* (2002). Briefly, at 0, 12, 24, 36 and 44 h after culture, a total of 30 oocytes at each culture point was collected in SDS sample buffer and heated to 100°C for 4 min. Samples were frozen at -20°C until use after being cooled on ice and centrifuged at 12 000 *g* for 4 min. The proteins were separated by SDS-PAGE with a 4% stacking gel and a 10% separating gel for 2.5 h at 120 V and then transferred by electrophoresis on to nitrocellulose membrane for 2.5 h at 200 mA at 4°C. After it was washed three times in TBS (20 mmol Tris l⁻¹, 137 mmol NaCl l⁻¹, pH 7.4) for 30 min, the membrane was blocked for 2.5 h in TBST buffer (TBS with 0.1% Tween-20) containing 5% low-fat milk at room temperature. The membrane was then incubated in mouse anti- α tubulin (1:1000 in TBST with 0.5% low-fat milk) for 2 h at 37°C. After being washed three times in TBST, each time for 10 min, the membrane was treated in peroxidase-conjugated goat anti-mouse IgG (1:3000 in TBST with 0.25% low-fat milk) for 1 h at 37°C. The membrane was washed three times in TBST and processed using enhanced chemiluminescence.

Two hundred oocytes at various time points of culture were collected and boiled in SDS sample buffer for CENP analysis. Proteins were separated using the methods described for tubulin separation. After separation the membrane was incubated with CREST (1:4000 in TBST with 0.5% low-fat milk) overnight at 4°C. The washed membrane was then incubated with horseradish peroxidase-conjugated goat anti-human IgG (1:22 000 in TBST with 0.25% low-fat milk) (Jackson ImmunoResearch) for 1 h at 37°C. The membrane was washed three times in TBST and then the proteins were visualized using a chemiluminescence detection system. Immunoblot density was determined by the system of Personal Densitometer SI and Fragment Analysis software produced by Molecular Dynamics Inc. (Sunnyvale, CA).

Statistical analysis

Experiments were repeated four times except for immunoblotting, which was repeated three times. All percentage data were subjected to arc sine transformation before statistical analysis. Data were analysed by ANOVA.

Table 1. Maturation of pig oocytes cultured *in vitro*

Time of culture (h)	Number of oocytes examined	Number (%) of oocytes at stages			
		GV	Pre-MI-MI	AI-TI	MII
0	61	60 (98.4)	1 (1.6)	0 (0)	0 (0)
12	52	50 (96.2)	2 (3.8)	0 (0)	0 (0)
24	45	5 (11.1)	37 (82.2)	2 (4.4)	1 (2.2)
36	42	1 (2.4)	14 (33.4)	2 (4.8)	25 (59.5)
44	42	1 (2.4)	6 (14.3)	1 (2.4)	34 (81.0)

GV: germinal vesicle; pre-MI: pre-metaphase I; MI: metaphase I; AI: anaphase I; TI: telophase I; MII: metaphase II.

Results

Nuclear maturation of pig oocytes

At 0 and 12 h of culture most oocytes (98.4% and 96.2%, respectively) were at GV stage (Table 1). By 24 h, 88.9% of the oocytes underwent GV breakdown (GVBD), and most (82.2%) proceeded into pre-MI and MI. Only one oocyte (2.2%) reached MII. The proportions of oocytes at MII were increased to 59.5% and 81.0% when the oocytes were examined at 36 h and 44 h of culture, respectively. Some oocytes (2.4–4.8%) were at AI-TI stage at 24–44 h after culture.

Association of CENPs with chromosomes and microtubules in pig oocytes during meiosis

GV stage: CENPs were localized around the GV but quite randomly at the periphery of the chromatids and no obvious connection was observed between chromatids and CENPs. No microtubules were observed in the oocytes at the GV stage (Fig. 1).

Pre-MI stage: single chromosome can be observed and CENP staining was detected on both sides of chromosomes. The onset of connections between CENPs and microtubules, chromosomes and microtubules could be observed (Fig. 1).

MI stage: CENP staining was seen in two rows on two opposite sites of chromosomes that were aligned properly on the metaphase plate. A barrel-shaped spindle was formed and the CENPs were aligned in the spindle to form two lines perpendicular to the spindle axis; a complete connection between microtubules and CENPs was established (Fig. 1).

AI-TI stage: at AI stage, as the chromosomes started to separate, the CENPs were displaced to the polar regions of each chromosome mass, still retaining their orientation on the polar sides of the chromosomes. At TI stage, CENPs were dispersed disorderly over the two separated chromosome masses, but still on the polar side. The spindle was elongated in the longitudinal axis, and consequently two CENP lines were separated and translocated towards the opposite spindle poles. The

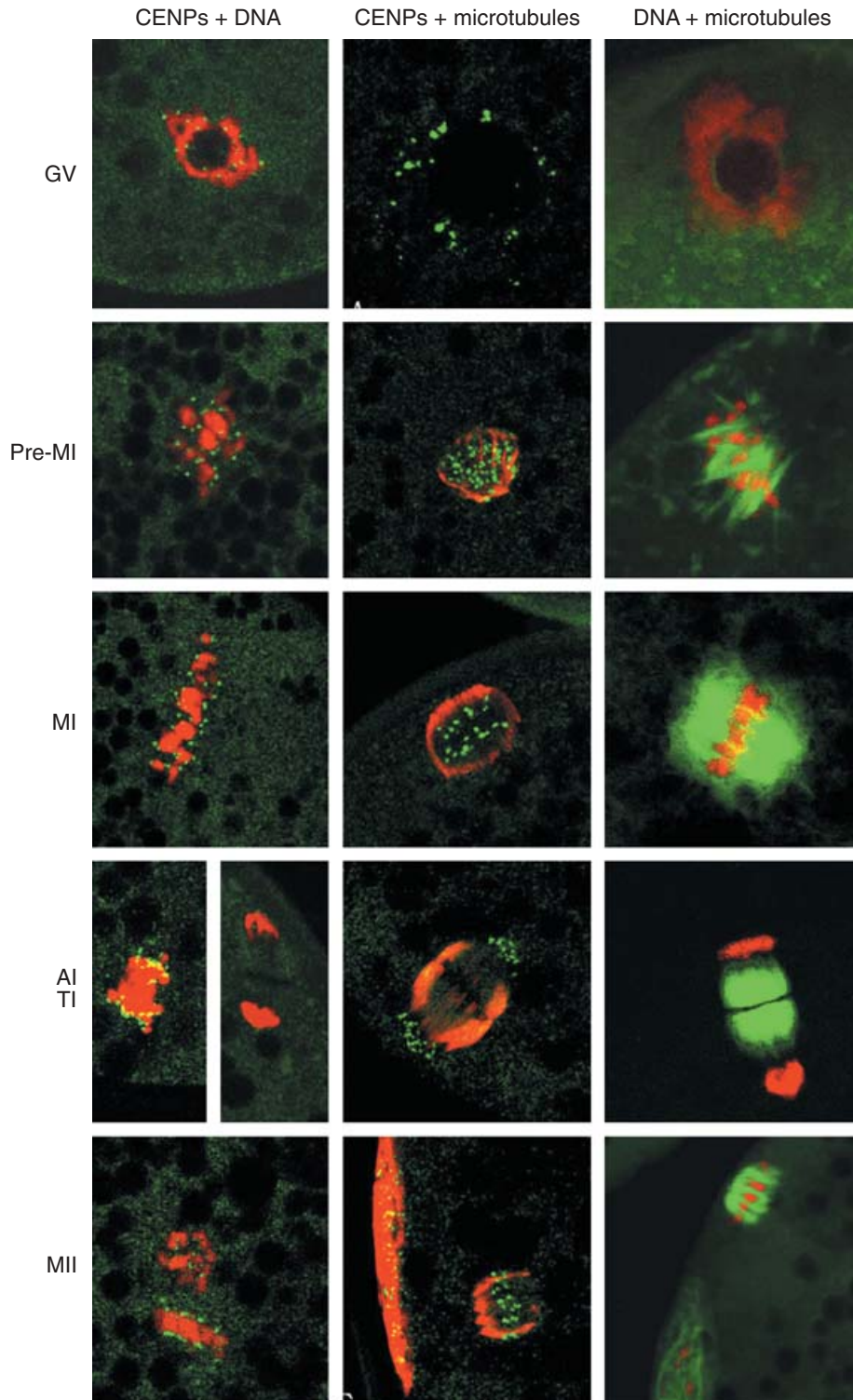


Fig. 1. Confocal micrographs of immunostaining of centromere proteins (CENPs), microtubules and DNA (nucleus) of pig oocytes during meiotic maturation. Oocytes at each stage were double-stained. Left column: CENPs (green) and DNA (red); middle column: CENPs (green) and microtubules (red); right column: DNA (red) and microtubules (green). GV: germinal vesicle; pre-MI: pre-metaphase I; MI: metaphase I; AI: anaphase I; TI: telophase I; MII: metaphase II.

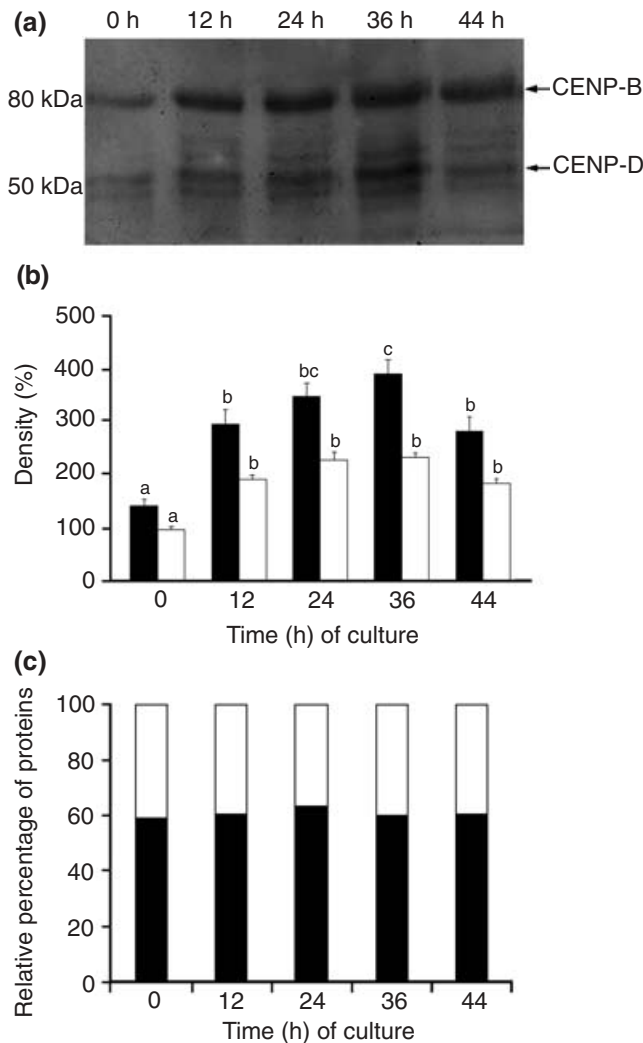


Fig. 2. Quantification of centromere proteins (CENPs) in pig oocytes during meiotic maturation. (a) Immunoblots of CENPs in oocytes cultured for 0, 12, 24, 36 and 44 h. Two-hundred oocytes at each culture time point were examined by immunoblotting. (b) Density analysis indicates that significantly ($^{abc}P < 0.01$ among the different culture times) low density was observed in the 80 kDa (CENP-B; ■) and 50 kDa (CENP-D; □) proteins in the oocytes at the germinal vesicle stage. (c) Relative amount of each CENP (CENP-B: ■; CENP-D: □), which was calculated by density of each protein to total density of two proteins.

CENPs were still aligned vertically to the spindle axis until the oocytes reached TI (Fig. 1).

MII stage: CENPs were rearranged and again aligned on the outside of the chromosome line and formed two lines that were similar to that in MI oocytes. CENPs were also detected in the first polar body but were not aligned in an orderly manner, indicating that CENPs were also released together with chromosomes from the oocytes during the polar body extrusion. The connection between CENPs and microtubules was re-established in

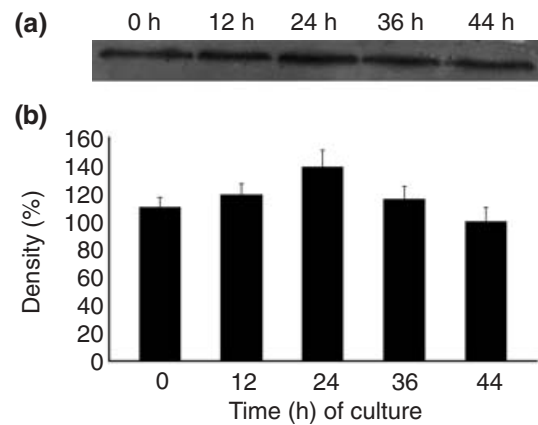


Fig. 3. Quantification of tubulin in pig oocytes during meiotic maturation. (a) Immunoblots of tubulin in the oocytes cultured for 0, 12, 24, 36 and 44 h. Thirty oocytes were examined and (b) no difference was observed in the quantification of tubulin among the oocytes cultured for 0–44 h.

the oocytes at MII stage and CENPs were re-aligned in two lines (Fig. 1).

Quantification of CENPs in pig oocytes during meiosis

Immunoblotting analysis revealed that some CENPs were present in pig oocytes: CENP-B, an 80 kDa protein, accounting for 59–62% of the total CENPs; and CENP-D, a 50 kDa protein, accounting for 38–41% of the total CENPs (Fig. 2). Density analysis indicated that the amount of CENP-B and CENP-D in the oocytes increased during oocyte maturation and was highest after 36 h of culture and then decreased in the oocytes cultured for 44 h. The density at GV stage (0 h of culture) was significantly ($P < 0.01$) lower than that in the oocytes after culture for 12–44 h (Fig. 2).

Quantification of tubulin in pig oocytes during maturation

Tubulin, a 24 kDa protein, was detected in the oocytes cultured for various times (Fig. 3). No significant difference was observed in the quantity of tubulin by western blotting.

Discussion

To the authors' knowledge, the present study is the first detailed examination of CENPs in the kinetochores and their relationship with microtubules and chromosomes during oocyte maturation in pigs. The present results indicate that at least two CENPs are present in pig oocytes from GV to MII stages and these CENPs can be detected by the anticentromere antibody CREST. From the molecular mass, they are CENP-B and CENP-D,

as observed in mitotic cells. The connection between CENPs and microtubules was initiated as the oocyte underwent GVBD, completed at MI and re-established after the oocyte reached MII. These results are consistent with those reported for mitosis; these CENPs were present in all cell cycles from GV to MII. The locations of these proteins were changed in a similar way to the chromosome segregation and spindle formation, indicating that they may participate in the control of chromosome alignment and segregation during oocyte meiotic maturation.

The kinetochore is highly conserved during evolution (Rieder, 1982; Rieder and Salmon, 1998) and it can be specifically discerned by CREST in cells during mitosis or meiosis (Moroi *et al.*, 1980, 1981; Brenner *et al.*, 1981; Schatten *et al.*, 1988; Simerly *et al.*, 1990). Similar to mouse oocytes (Schatten *et al.*, 1988), in the present study the alterations of CENPs (in kinetochores) were in agreement with the motion of the chromosome during pig oocyte maturation. It has been reported that micro-injection of anti-kinetochore or centromere antibodies to mouse oocytes interfered with chromosome congression at prometaphase I stage and caused unaligned chromosomes in the spindle when they entered into metaphase and resulted in abnormal meiosis (Simerly *et al.*, 1990). These results indicate that these CENPs are involved in the alignment and separation of chromosomes during meiosis.

The major function of kinetochores is to establish the connection between chromosomes and microtubules, thus participating in chromosome alignment at metaphase, and separation at anaphase. So far, two types of CENP have been found to be associated with kinetochore function. One type represents constitutive proteins, such as CENP-A, CENP-B, CENP-C and CENP-D. The other type represents transient proteins, such as CENP-E, CENP-F, MAD1, MAD2, bub1 and bub2 (Craig *et al.*, 1999). The constitutive CENPs are present at the centromeres throughout the cell cycle and can be detected by the anticentromere antibody CREST. The present study indicates that CENPs in the kinetochores of pig oocytes can also be detected by CREST and these proteins are expressed in pig oocytes at all stages from GV to MII. The present results also show that two major CENPs could be detected by CREST: CENP-B and CENP-D. It appears that these results are different from those observed in mouse oocytes (Schatten *et al.*, 1988) and mammalian mitotic cells (Yen *et al.*, 1991; Warburton *et al.*, 1997). CENP-A, a 17 kDa protein, and CENP-B were detected by CREST in mouse oocytes (Schatten *et al.*, 1988). However, CENP-A, CENP-B and CENP-C were detected by CREST in human mitotic cells (Warburton *et al.*, 1997) and CENP-A, CENP-B, CENP-C and CENP-D were detected in HeLa cells (Yen *et al.*, 1991). It is not known whether the differences observed among these studies are due to different species, different cells, different materials or other factors. Recently, most

studies have been conducted to examine the location of CENPs; their functions have not been widely examined. It has been reported that CENP-B null mice are viable (Perez-Castro *et al.*, 1998) and can undergo normal meiosis and mitosis (Hudson, *et al.*, 1998; Tomascik-Cheeseman *et al.*, 2002). However, injection of anti-CENP-B antibodies into human and mouse somatic cells resulted in disruption of centromere assembly during interphase and inhibited the function of kinetochores in mitosis (Bernat *et al.*, 1990, 1991; Simerly *et al.*, 1990). The effects of other constitutive CENPs are not clear. It is necessary to investigate the effects of individual proteins on chromosome segregation with more specific anti-CENP antibodies.

As kinetochore functions are largely dependent on the transient proteins, the localization and function of the transient proteins have been well studied, especially in mitosis, such as Mad1, Mad2, Bub1, Bub2 and BubR1 and motor proteins (CENP-E and cytoplasmic dynein). Most of these proteins are located in the kinetochores and the activity of these proteins (kinases) is related to the chromosome movement and anaphase onset during mitosis. Mad2 and CENP-E have been widely studied. It has been found that Mad2 senses unattached kinetochores and delays premature anaphase until all kinetochores are attached by microtubules (Hoffman *et al.*, 2001). Kallio *et al.* (2000) showed that Mad2 is present continuously on kinetochores during mouse female meiosis, even at MII, indicating different intracellular level and localization between mitosis and meiosis. The localization of CENP-E is also different between somatic cells and oocytes in pigs (Lee *et al.*, 2000), but it is implicated in the spindle checkpoint and in chromosome alignment in both mitosis and meiosis. It would appear that all CENPs in the kinetochores work together, but through different mechanisms, to ensure accurate chromosome segregation during both mitosis and meiosis (Craig *et al.*, 1999).

Quantification of CENPs indicates that both CENP-B and CENP-D in pig oocytes are significantly increased after oocytes undergo GVBD and reach the highest values until oocytes reach MII. These results may indicate that the CENPs are synthesized not only in fully grown oocytes, but also in oocytes undergoing meiotic maturation. The increased quantity may also indicate that CENPs are associated with chromosome segregation during meiosis. Although previous studies have examined CENPs in some mammalian oocytes (Schatten *et al.*, 1988; Simerly *et al.*, 1990; Lee *et al.*, 2000) by fluorescence microscopy or immunoblotting, quantification of these proteins during the cell cycle has not been reported. Further studies are necessary to address whether species-specific differences are present.

The spindle is also an essential cellular structure for guaranteeing the accurate separation of two sets of chromatids during cell division. According to the 'search and capture' model of spindle formation (Vernos and

Karsenti, 1995, 2001; Brunet *et al.*, 1999), there are two major events during the mitotic spindle assembly. First, the two centrosomes, which nucleate microtubules, migrate to opposite sides of the nucleus, thus spindle bipolarity can be established before nuclear envelope breakdown. Second, after nuclear envelope breakdown, kinetochores randomly capture and stabilize the dynamic microtubules from centrosomes and form kinetochore fibres. Then chromosomes move to the equatorial plane of the spindle when both sister kinetochores are connected to the opposite poles by kinetochore fibres. This random and continuous process ends when all chromosomes align on the metaphase plate (Pennisi, 1998; Rieder and Salmon, 1998). However, in mammalian oocytes, as observed in pig oocytes in the present study, the bipolarity of spindle is not predefined during early maturation. Tubulin begins to polymerize into microtubules in all directions around the condensed chromatin after GVBD. As oocytes proceed to MI stage, microtubules gradually organize into a bipolar spindle. A similar pathway of spindle formation has been observed during oocyte maturation in *Drosophila*, *Xenopus* and mice (Vernos and Karsenti, 1995; Heald *et al.*, 1997; Brunet *et al.*, 1999). Again, these results indicate that organization of the spindle formation is different between mitosis and meiosis.

Although no microtubules were detected in the oocytes at GV stage, immunoblotting indicates that tubulin is sufficient in the oocytes at the GV stage and the quantity of tubulin is the same in the oocytes at the GV stage compared with other stages. These results are consistent with those reported in other animals (Wu *et al.*, 1997; Josefsberg *et al.*, 2000), indicating that tubulin is present in the cytoplasm of immature, maturing and mature oocytes. Wang *et al.* (2000) found that β -actin was also present in pig oocytes at the same amount from GV stage to MII. These results indicate that major cytoskeleton proteins (both actin and tubulin) in pig oocytes are synthesized during oocyte growth but not in the fully grown oocytes, which is consistent with the studies reported in mouse oocytes (Wassarman, 1983). The component(s) that initiates the polymerization of microtubules is still unclear. Some signals may be given to tubulin only after oocytes start GV breakdown. When microtubule polymerization starts, kinetochores also start to catch the plus ends of microtubules. It has been reported that the interactions between kinetochores and microtubules in mouse oocytes are not established until about 8 h after GVBD (Brunet *et al.*, 1999), just before the final alignment of chromosomes in the spindle by checking cytoplasmic linker protein 170 (CLP-170), a 170 kDa transient protein of kinetochore (Dujardin *et al.*, 1998). This finding may indicate that CLP-170 remains on kinetochores until meiosis is complete (Brunet *et al.*, 1999). However, the exact mechanisms for initiation of microtubule polymerization and spindle formation are still unknown.

In summary, kinetochores are important structures in oocytes that control the chromosome alignment and connect microtubules and chromosomes during chromosome movement, thus participating in accurate completion of meiosis. The method used in the present study is valid for labelling CENPs in the kinetochores in pig oocytes. Further experiments are necessary to examine the functions of these proteins associated with spindle formation and chromosome alignment.

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