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Changes in cyclin B localisation during pig oocyte *in vitro* maturation

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Summary

The localisation of cyclin B throughout *in vitro* maturation of pig oocytes was determined by indirect immunofluorescence using a monoclonal antibody specific for an epitope of the human cyclin B. Maturation of pig oocytes was induced by addition of Pergonal (2 UI/ml of FSH/LH) and β-oestradiol to the medium where isolated ovarian follicles were cultured for up to 72 h. Immature gametes with an intact germinal vesicle were observed during the first 30 h of culture. Only 10% were competent to reinitiate meiosis and showed germinal vesicle breakdown (GVBD) after 36 h. However, after 48–72 h, 60% of the oocytes accomplished their maturation and showed metaphase chromosomes. Immature oocytes showed cyclin B immunofluorescent staining in the cytoplasm, whereas mature oocytes showed the immunofluorescent label concentrated in the nucleus. Metaphase chromosomes showed an intense immunofluorescence. The migration of cyclin B to the nucleus and its association with metaphase chromosomes in pig oocytes able to progress through meiosis resembled the subcellular localisation of cyclin B and the distribution of maturation promoting factor (MPF) in mitotic dividing cells.

Keywords: Cyclin B, Meiosis, MPF, Oocyte maturation, Pig oocytes

Introduction

It has been shown that the structural and chemical interactions between the oocyte and its follicular environment plays a central role in maintaining female germ cells arrested at prophase I of meiosis (Lawrence *et al.*, 1978; Gilula *et al.*, 1978). In mammals, the preovulatory rise of luteinising hormone (LH) abolishes this blockage and triggers an irreversible commitment to maturation in some oocytes (Eppig, 1993). Competent oocytes reinitiate meiosis and progress from prophase I up to metaphase II driven by maturation promoting factor (MPF). MPF is a heterodimer constituted by a subunit with protein kinase activity, $p34^{cdc2}$, and a regulatory subunit, cyclin B. The serine/threonine protein kinase activity of MPF is regulated by several factors such as its subunit concentrations, its intracellular localisation and the phosphorylation – dephosphorylation on specific residues (reviewed by Norbury & Nurse, 1992; Masui, 1992; Taieb *et al.*, 1997). Therefore, variations in these parameters would modify the activity of MPF.

In some mammalian species such as pig, comparable levels of cyclin B and $p34^{cdc2}$ have been detected in immature and fully grown oocytes (Christmann *et al.*, 1994); therefore, the activity of MPF is not dependent on *de novo* synthesis of its subunits. However, the relevance of MPF dephosphorylation has clearly been shown in these oocytes. Inhibitors of protein phosphates completely blocked the rise in protein kinase activity of

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MPF (Kalous *et al.*, 1993) previously found during normal pig oocyte maturation (Naito & Toyoda, 1991; Mattioli *et al.*, 1991; Christmann *et al.*, 1994). Although the localisation of MPF is another factor involved in the control of its activity, it has not been determined whether the distribution of MPF changes during resumption of meiosis in pig oocytes.

Since it has been shown, in starfish oocytes, that as soon as cyclin B is synthesised it binds almost exclusively to p34 (Katsu *et al.*, 1993) and in this bound state migrates to the nucleus before the breakdown of the nuclear envelope (O. Okata *et al.*, 1993), the localisation of cyclin B can be used as tool to define the intracellular localisation of MPF.

In the present study the intracellular distribution of cyclin B during *in vitro* maturation of pig oocytes was determined using a monoclonal antibody specific for human cyclin B. It was observed that cyclin B is located in the cytoplasm of immature oocytes and, as meiosis is reinitiated, it becomes clearly associated with the nucleus and with metaphase chromosomes. These results suggest that MPF moves into the germinal vesicle in competent oocytes and becomes bound to chromosomes at metaphase.

Materials and methods

Oocyte maturation (Yoshida *et al.*, 1990; Betancourt *et al.*, 1993)

Porcine ovaries were collected from 6-month-old Yorkshire female pigs at a local slaughterhouse and were transported in less than 2 h to the laboratory in 0.157 M NaCl solution. Oocyte–cumulus complexes recovered from non-atretic follicles were rinsed twice with supplemented maturation medium adjusted to pH 7.4 and a final osmolarity of 280–290 mosmol/kg. Thirty to 40 oocytes showing a compact mass of surrounding cumulus cells were placed in 4-well plates (Nunc, Roskilde, Denmark) with 500 µl of the maturation medium. Oocytes were cultured for up to 72 h at 37 °C in a humidified atmosphere with 5% CO_2.

Maturation analysis

Oocytes were rinsed with saline solution, and cumulus removed by repeated pipetting with a fine-bore Pasteur pipette. Oocytes were fixed according to the air-drying fixation method of Tarkowski (1966). The cells were placed in a drop of 0.75% sodium citrate solution for 4 min, at room temperature, and fixed with one or two drops of fresh fixative (methanol:acetic acid, 3:1). The preparation was then dried by gentle blowing and stained with 2% acetic orcein for 5 min. Oocytes showing an intact germinal vesicle were considered as immature and those showing metaphase chromosomes were classified as mature oocytes.

Immunolocalisation of cyclin B

The intracellular localisation of cyclin B during *in vitro* oocyte maturation was determined using a commercial monoclonal antibody specific for human cyclin B by a modification of the methods previously described (Kung *et al.*, 1993; Sherwood *et al.*, 1993) for somatic cells. Cumulus-free oocytes were fixed at 4 °C with 2% (w/v) paraformaldehyde–phosphate-buffered saline (PBS), pH 7.3. After 2 h they were rinsed twice with PBS and permeabilised with 0.9% Triton X-100, 1 mM Hepes (pH 7.4), 150 mM NaCl, 4% fetal bovine serum for 10 min at room temperature. The cells were rinsed twice with PBS and incubated with 30 µl of anti-human cyclin B monoclonal antibody (Santa Cruz Biotechnology, CA; 1:30) for 2 h at 37 °C. They were rinsed with PBS and incubated with 30 µl of a fluorescein-conjugated goat anti-mouse IgG antibody (Sigma) diluted 1:500. After 30 min of incubation at 37 °C the oocytes were rinsed with PBS to eliminate the excess of antibody, mounted over a slide using glycerol:PBS (9:1) and observed under an epifluorescence Zeiss microscope (50 W mercury arc lamp) with fluorescein excitation at 490 nm. Fluorescence images were recorded on Kodak Ektachrome 100 ASA (Kodak, Mexico).

Electrophoresis and western blotting

Oocytes were released from 300 fresh pig ovaries using a domestic food processor. Thirty ovaries were processed at the same time with 200 ml of isolation buffer (PBS with 3 mM sodium azide). The oocytes were isolated using a series of nylon screens (500, 200, 150 and 120 µm), collected on an 80 µm screen and gently homogenised in a Potter-Elvehjem homogeniser with 1 ml of isolation buffer. The oocyte homogenate was centrifuged at 5600 *g* for 10 min. The supernatant was saved, aliquoted, and kept at –76 °C. Aliquots containing 40 µg of protein were separated by SDS-PAGE (Laemmli, 1970) using 12% polyacrylamide gels. Proteins were transferred to nitrocellulose paper according to Towbin *et al.* (1979). Nitrocellulose blot was incubated overnight with the anti-cyclin B antibody diluted 1:500. Bound antibody was detected using a goat anti-mouse IgG conjugated with peroxidase (Sigma), diluted 1:500, and the immune complex was revealed using a fresh solution of 0.05% (w/v) chloronaphthol and 200 µl of 30% hydrogen peroxide dissolved in 16% methanol-TBS (v/v).

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Results

In vitro maturation of pig oocytes

The maturation stage was analysed in at least 120 oocytes at 24, 30, 36, 42, 48 and 72 h of culture following the method previously described (Tarkowski, 1966). It was observed that up to 30 h most of the oocytes remained in the stage of intact germinal vesicle and were considered as immature. Germinal vesicle breakdown (GVBD) was observed in 25% of the oocytes after 36 h and in 42% between 42 and 48 h of culture. The highest percentage of maturation was reached after 72 h, when 60% of the oocytes showed metaphase chromosomes (Fig. 1).

Immunodetection of pig cyclin B

In order to define whether the monoclonal anti-human cyclin B antibody recognised the pig cyclin B, aliquots of 40 µg protein of the oocyte crude extract were separated in 12% polyacrylamide gels and transferred onto nitrocellulose paper. It was observed that only one 62 kDa band of the pig oocyte extract, migrating at the same level as the recombinant cyclin B, showed the ability to bind the anti-cyclin B antibody (Fig. 2), suggesting the presence of a common epitope in the two proteins. Therefore, this antibody was used to immunolocalise the pig cyclin B during oocyte maturation.

Immunolocalisation of cyclin B

Based on the results described above, the anti-human cyclin B monoclonal antibody was used to determine the intracellular localisation of cyclin B during pig oocyte maturation as previously reported for starfish oocytes (Ookata *et al.*, 1993). It was observed that immature oocytes, with intact germinal vesicle, showed the immunofluorescence dispersed throughout the cytoplasm (Fig. 3*A*). Oocytes cultured for 36 h showed a bright immunofluorescence inside the germinal vesicle, with the cytoplasm signal absent (Fig. 3*B*). After 48 and 72 h, oocytes presented the immunofluorescent label associated with metaphase chromosomes (Fig. 3*C*). Control oocytes were incubated with secondary antibody alone and showed no fluorescent label (Fig. 3*D*).

Discussion

The results presented here support previous observations showing that pig oocytes required more than 24 h in culture to acquire the competence to reinitiate the meiotic division cycle *in vitro* (Naito & Toyoda, 1991;

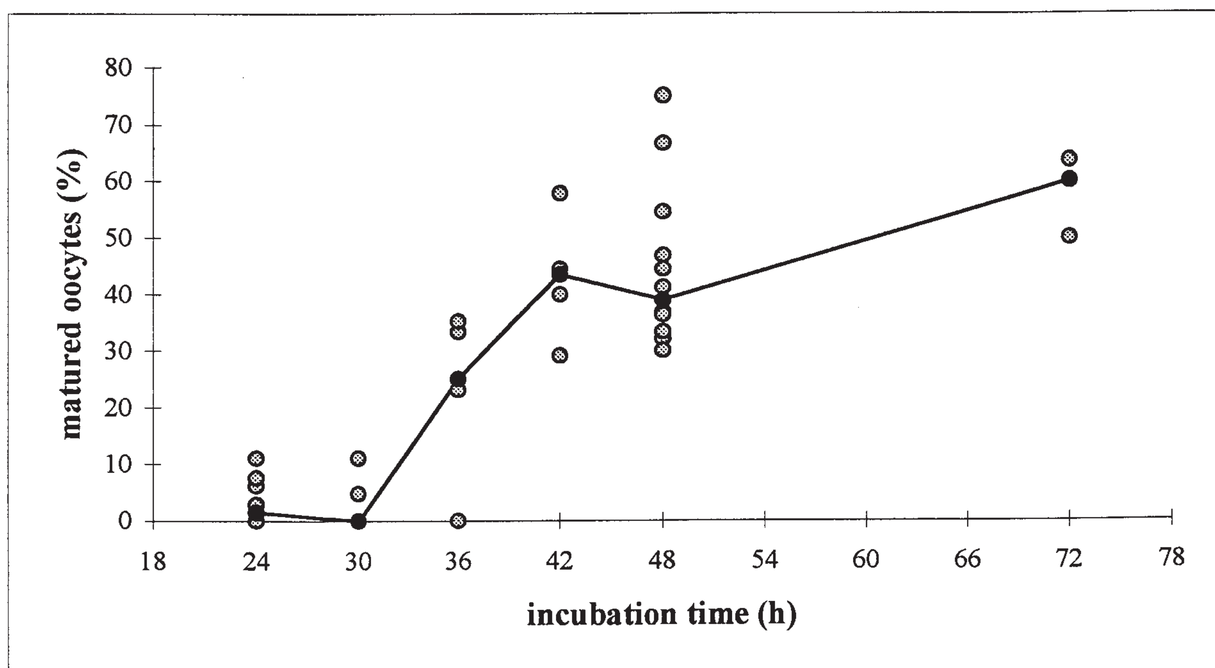

Figure 1 Kinetics of *in vitro* maturation of pig oocytes. Fully grown oocytes after 4, 12, 24, 36, 48 and 72 h of culture at 37 °C were liberated from cumulus cells by repeated pipetting through a fine-bore Pasteur pipette. Cumulus-free oocytes were fixed with methanol:acetic acid (3:1) and stained with acetic orcein. Batches of at least 10 oocytes were observed to determine the maturation stage reached, following the method previously described (Tarkowski, 1966). Open circles show the average of at least 20 oocytes per assay, and black circles the median values at each time.

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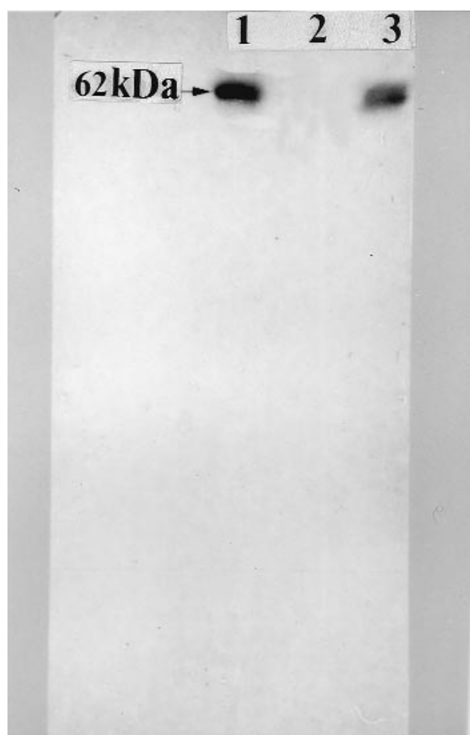

Figure 2 Western blot of pig oocyte cyclin B. A crude extract from oocytes obtained from 300 pig ovaries was made using a domestic food processor. Non-disrupted tissue was eliminated by filtration through nylon screens of different pore sizes. The oocyte homogenate was centrifuged at 5600 *g* for 10 min, and the supernatant was saved and used to determine the presence of cyclin B. Aliquots containing 40 μg of oocyte protein, 15 μg of recombinant cyclin B, and 40 μg of zona pellucida protein were separated on 12% polyacrylamide gels and transferred to nitrocellulose paper. Nitrocellulose paper was incubated with the monoclonal antibody specific for human cyclin B. Peroxidase-conjugated anti-mouse IgG antibody was used to detect anti-cyclin B antibody binding. Lane 1, recombinant cyclin B; lane 2, zona pellucida; lane 3, pig oocyte extract.

Christmann *et al.*, 1994). Since 30–40 oocytes per well were cultured and a high percentage of them were induced to reinitiate meiosis under the conditions employed, it was possible to determine both the kinetics of maturation and the immunolocalisation of cyclin B throughout this process using a monoclonal antibody able to recognise pig cyclin B.

The cytoplasmic immunolocalisation of cyclin B in immature pig oocytes unable to progress through meiosis was similar to the cyclin B distribution in somatic cells at interphase (Pines & Hunter, 1991). The association of cyclin B with metaphase chromosomes has also previously been observed in somatic cells during mitosis (Adlakha *et al.*, 1982; Pines & Hunter, 1991) as well as in starfish oocytes during reinitation of meiosis (Ookata *et al.*, 1993).

The concentration of cyclin B in the germinal vesicle before GVBD suggests that oocytes have acquired the competence to progress through meiosis. This interpretation is supported by previous work showing that nuclear envelope breakdown occurs after transport of cyclin B and MPF into the nucleus (Pines & Hunter, 1991; Ookata *et al.*, 1993).

The *in vitro* reinitation of meiosis and GVBD in pig oocytes has been shown to be coincident with an increase in the phosphorylation of histone by MPF. Immature oocytes showed a protein kinase activity 10 times lower than that of mature oocytes (Naito & Toyoda, 1991; Christmann *et al.*, 1994). Since the kinetics of pig oocyte maturation previously cited and those described here are very similar, a correlation can be made between maturation and the distribution of cyclin B as well as between protein kinase activity and the distribution of MPF. The high affinity of cyclin B for $p34^{cdc2}$ makes the assembly of MPF possible as soon as cyclin B is synthesised (Ookata *et al.*, 1993). Therefore, its intracellular distribution can be extrapolated to the MPF localisation. Furthermore, the use of anti-cyclin B antibodies has been proposed to be more specific for MPF detection than antibodies against $p34^{cdc2}$ because the latter could also detect other cell-cycle-dependent protein kinases (Ookata *et al.*, 1993).

Taking into account all these observations, the cytoplasmic localisation of cyclin B in immature oocytes and the nuclear concentration of cyclin B in competent oocytes could be indicative of the cytoplasmic and nuclear distribution of MPF in immature and competent oocytes, respectively. It can also be hypothesised that the inactive, phosphorylated form of MPF is localised in the cytoplasm of immature oocytes whereas the active, dephosphorylated type of MPF can be localised both in the cytoplasm of fully grown oocytes (Petr *et al.*, 1994) and within the nucleus of competent germ cells where it becomes bound to the chromosomes during metaphase. However, further work is required to prove this hypothesis as well as to define the substrates phosphorylated by MPF and their role in the nuclear and cytoplasmic events leading to GVBD and progression of meiosis up to metaphase in pig oocytes.

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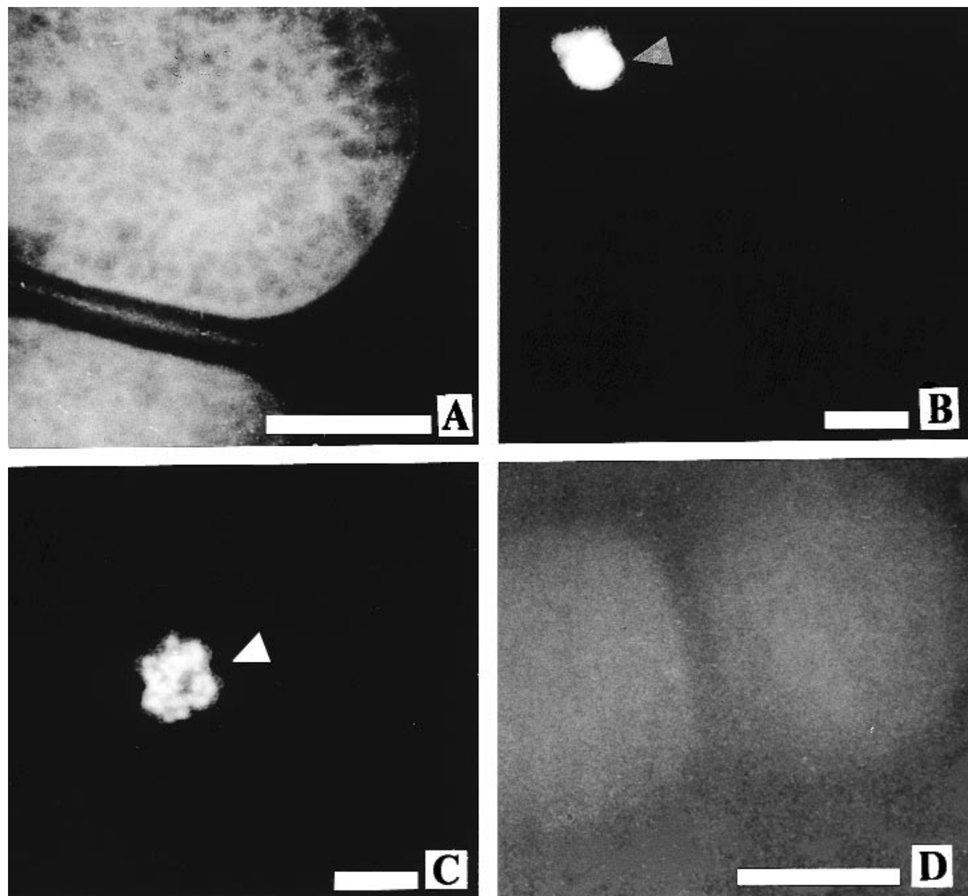

Figure 3 Immunolocalisation of cyclin B in pig oocytes during the reinitiation of meiosis. After 24 and 48 h in culture, cumulus-free pig oocytes were fixed with paraformaldehyde–PBS and oocytes permeabilised with 150 mM NaCl, 1 mM Hepes, pH 4, 10% HFS and 0.9% Triton X-100. The fixed oocytes were incubated sequentially with a monoclonal antibody specific for human cyclin B and a fluorescein-conjugated goat anti-mouse IgG. The immunofluorescence was observed using a Zeiss epifluorescence microscope. Images were recorded on Kodak Ektachrome 100 ASA film. (*A*) Immunolocalisation of cyclin B in two oocytes recently isolated from pig ovary (×400). (*B*) Immunolocalisation of cyclin B in the germinal vesicle (arrowhead) of immature oocytes after 24 h of culture (×1000). (*C*) Mature oocytes showing metaphase chromosomes (arrowhead) after 48 h of culture (×1000). (*D*) Negative control. Mature oocytes without incubation with the anti-human cyclin B antibody (×400). Scale bar in (*A*) and (*D*) represents 50 µm and in (*B*) and (*c*) represents 10 µm approximately.

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