Possible mechanism for acceleration of meiotic progression of bovine follicular oocytes by growth factors *in vitro*

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The mechanism for the accelerating effects of epidermal growth factor (EGF) and insulin-like growth factor I (IGF-I) on the meiotic cell cycle of bovine oocytes cultured *in vitro* was investigated. Cumulus–oocyte complexes (COCs) were obtained from small (≤ 3 mm in diameter), medium (4–6 mm in diameter) or large (7–10 mm in diameter) ovarian follicles and cultured with or without a combination of EGF and IGF-I (growth factors). Growth factors significantly increased the frequency of first polar body extrusion of oocytes derived from small follicles at 16 h of culture (PB16 oocytes; with growth factors: 75%; without growth factors: 55%), but did not increase the frequency in oocytes from medium or large follicles. COCs from small follicles were cultured with individual growth factors and sampled for kinase activity. The frequencies of polar body extrusion in EGF only (67%) and EGF + IGF-I

(75%) treatment groups were significantly higher than those in the control (no growth factor) group (49%), but not significantly higher than in the IGF-I only group (63%). The H1 kinase activity at 6–8 h of culture in each group increased significantly from the baseline value at 0 h of culture, and the H1 kinase activities in the EGF only, IGF-I only and EGF + IGF-I treatment groups were significantly higher than those in the control group at 8 h of culture. MAP kinase activity was significantly higher than the baseline value and significantly higher than that in the control group at 6 h of culture in the EGF treatment group only. In conclusion, EGF and IGF-I act on COCs from small follicles to accelerate the meiotic cell cycle of the oocytes. This accelerating effect may be related to increased H1 and MAP kinase activities during the early stages of maturation.

Introduction

During ovarian follicular development, several growth factors and cytokines act as intra-ovarian regulators *in vivo* (Greenwald and Roy, 1994), and at the follicle the action of gonadotrophins is modulated by locally produced paracrine and autocrine growth factors (Bevers *et al.*, 1997). Sakaguchi *et al.* (2000) reported that the progression of meiosis in bovine oocytes enclosed with cumulus cells was accelerated by exposure of the oocytes to growth factors (epidermal growth factor (EGF) and insulin-like growth factor I (IGF-I)) in serum-free maturation medium, but that their subsequent developmental competence was not improved. Treatment with EGF during maturation culture decreased the time required for germinal vesicle breakdown (GVBD) of denuded oocytes in mice (Das *et al.*, 1991) and EGF has a maturation-promoting effect on denuded bovine oocytes (Lonergan *et al.*, 1996). However, it has also been reported that growth factors do not enhance or accelerate the progress of meiosis of denuded bovine oocytes *in vitro* (Lorenzo *et al.*, 1994; Sakaguchi *et al.*, 2000). Therefore, it appears likely that in

bovine oocytes under the experimental conditions used by Sakaguchi *et al.* (2000), the enclosing cumulus cells mediate the effects of growth factors on the progress of maturation.

Bovine oocytes recovered from follicles other than preovulatory follicles have yet to undergo certain pre-maturation events (Barnes *et al.*, 1993; Hyttel *et al.*, 1993). There have been many reports showing that oocytes derived from small follicles have low maturational and developmental competence *in vitro* (Tan and Lu, 1990; Pavlok *et al.*, 1992; Barnes *et al.*, 1993; Lonergan *et al.*, 1994; Kubota and Yang, 1998). In addition, the concentrations of growth factors in follicular fluid (Hsu *et al.*, 1987; Westergaard *et al.*, 1990; Spicer and Geisert, 1992) and of their receptors in ovarian granulosa cells (Spicer *et al.*, 1994) are dependent on follicular size. Elucidation of the mechanism by which growth factors stimulate the resumption of meiosis will lead to better understanding of the control of meiotic maturation in mammalian oocytes.

In mammalian oocytes, meiotic maturation under *in vitro* conditions is affected by many intrinsic and extrinsic factors. As the maturation/M-phase promoting factor (MPF) has been identified as a key regulator of both meiotic and mitotic cell cycles (Masui and Markert, 1971; Lohka *et al.*,

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1988), the role of MPF in driving the meiotic resumption of bovine oocyte maturation has been investigated (Campbell *et al.*, 1993; Wu *et al.*, 1997). MPF activity can be determined by measuring its kinase activity using exogenous histone H1 as a substrate (Nurse, 1990). In addition, other kinases are activated before or concurrently with MPF during initiation of meiosis in mammalian oocytes (Sirard *et al.*, 1998). One important group of these kinases is the mitogen-activated protein (MAP) kinases (Sun *et al.*, 1999), which are also called extracellular regulated protein kinases (ERKs) because they are regulated by a wide variety of extracellular signals (Mordret, 1993). Motlik *et al.* (1998) reported that, in cattle oocytes, p42-ERK2 and p44-ERK1 are phosphorylated at GVBD and remain phosphorylated until metaphase II. MAP kinase activity can also be determined by measuring its activity using exogenous myelin basic protein (MBP) as a substrate (Kalab *et al.*, 1996; Motlik *et al.*, 1998).

The aim of the present study was to investigate the mechanism of acceleration triggered by growth factors of meiotic progression of bovine follicular oocytes. Firstly, meiotic acceleration of oocytes in response to stimulation by growth factors (EGF and IGF-I) was observed using bovine cumulus-oocyte complexes (COCs) from follicles of different diameters. The results of this initial experiment indicated that only COCs from small follicles responded to the stimulation. Hence, the acceleration effects of individual growth factors on COCs derived from small follicles were examined and the effects of these growth factors on the kinetics of the cell cycle-regulating kinase activities of oocytes during early maturation were investigated.

Materials and Methods

Collection of follicular oocytes

Bovine ovaries (mainly from Holstein cows) were obtained from an abattoir and transported to the laboratory in physiological saline (0.9% (w/v) NaCl) in an insulated container within 3–6 h of recovery. The temperature of the container upon arrival was approximately 30°C. COCs were aspirated from peripheral antral follicles, which were grouped into three categories (small: ≤ 3 mm in diameter; medium: 4–6 mm in diameter; large: 7–10 mm in diameter), using a suction pump under low vacuum with an 18-gauge needle. Only evenly granulated oocytes with a compact cumulus investment were used. The COCs were washed four times with a Hepes-buffered Tyrode's medium supplemented with 0.2 mmol pyruvate l^{-1} and 0.3% (w/v) polyvinylpyrrolidone (TALP; Bavister *et al.*, 1983). All of the media used in this study were supplemented with 25 μg gentamycin sulphate ml^{-1} . The processing time from aspiration to culture was < 2 h. All chemicals were purchased from Sigma Chemical Company (St Louis, MO), unless stated otherwise.

In vitro maturation (IVM)

The basic medium for IVM was tissue culture medium 199 with Earle's salts (TCM199; Van Water Rogers,

Chicago, IL) supplemented with 0.2 mmol sodium pyruvate l^{-1} . Bovine COCs (7–10) were cultured in a 50 μl droplet of medium, with supplements as described below, under 10 ml paraffin oil in 60 mm Petri dishes at 39°C under 5% CO_2 in air with saturated humidity. The growth factors used in this study were recombinant human EGF and recombinant human IGF-I, both of which were used at a concentration of 10 ng ml^{-1} . Growth factors were reconstituted in TCM199 containing 1 mg bovine serum albumin fraction V (BSA) ml^{-1} , and were stored at -20°C until required. The final concentration of BSA in the maturation droplet of each treatment was $< 0.004\%$ (w/v).

Timing of polar body extrusion of oocytes from follicles of different sizes

In the first experiment, COCs were cultured with or without growth factors (EGF and IGF-I). For observation of completion of meiosis I, the oocytes were treated with hyaluronidase (2 mg ml^{-1} in TALP) for a few minutes and stripped free of cumulus cells by repeated pipetting through a small bore pipette. Examination for polar body extrusion was performed at 16 h after the onset of maturation. All denuded oocytes were examined for the presence of a polar body under a stereomicroscope (Dominko and First, 1997). Oocytes that extruded the first polar body within 16 h (PB16 oocytes) after the onset of maturation were mounted on a glass slide and fixed overnight in acetic acid-ethanol (1:3) to determine the cell cycle stage. After staining with 1% (w/v) orcein in 40% (w/v) acetic acid, the chromatin configuration of the oocytes that extruded the first polar body within 16 h was visualized under phase-contrast microscopy. The remaining oocytes were allowed to mature for a further 8 h and examined again for the presence of a polar body (PB24 oocytes).

Determination of histone H1 kinase and MAP kinase activities

In the second experiment, COCs from small follicles were assigned to four different maturation treatments: (i) no growth factors; (ii) EGF only; (iii) IGF-I only; and (iv) both EGF and IGF-I. In each treatment group, oocytes for measurement of kinase activities were sampled randomly at 0, 4, 6 and 8 h after initiation of maturation culture. At each time point, five COCs were stripped free of cumulus cells by repeated pipetting, and denuded oocytes were snap frozen in liquid nitrogen and stored at -70°C until use. The remaining COCs were examined for polar body extrusion, as described above. Five PB16 oocytes in each treatment group were also sampled for kinase assay and the remaining PB16 oocytes were fixed and stained for determination of cell cycle stage, as described above.

Kinase assay was performed according to Dominko and First (1996). The reaction mixture contained 5 μl sample, 1 μl histone H1 (1.0 mg ml^{-1}), 1 μl myelin basic protein (2.0 mg ml^{-1} ; MBP), 10 μCi $\gamma\text{-}^{32}\text{P}\text{-ATP}$ (> 3000 Ci mmol l^{-1} ; Amersham, Arlington Heights, IL) and 5 μl kinase buffer

Table 1. Polar body extrusion of bovine oocytes derived from follicles of different diameters cultured with or without growth factors

Polar body extrusion	Growth factors	Follicular size (diameter)*		
		Small (≤ 3 mm)	Medium (4–6 mm)	Large (7–10 mm)
PB16	–	108 (55 $\pm$ 5)	105 (63 $\pm$ 6)	40 (62 $\pm$ 5)
	+	147 (75 $\pm$ 2) ^a	99 (71 $\pm$ 7)	43 (61 $\pm$ 8)
PB24	–	59 (32 $\pm$ 3)	43 (26 $\pm$ 5)	16 (22 $\pm$ 5)
	+	32 (17 $\pm$ 2) ^a	35 (24 $\pm$ 6)	22 (30 $\pm$ 9)
No polar body	–	23 (13 $\pm$ 3)	16 (11 $\pm$ 3)	11 (16 $\pm$ 4)
	+	15 (8 $\pm$ 0)	8 (6 $\pm$ 2)	7 (9 $\pm$ 1)
Total	–	190	164	67
	+	194	142	72

*Values are number of oocytes (mean $\pm$ SE of five replicate experiments).

PB16 and PB24: oocytes that have extruded the first polar body by 16 or 24 h after the initiation of culture, respectively.

No polar body: oocytes that have not extruded their first polar body by 24 h after initiation of culture.

Growth factors: cultured with (+) or without (–) epidermal growth factor (EGF) and insulin-like growth factor I (IGF-I) in the maturation medium with 0.3% (w/v) polyvinylpyrrolidone.

^aSignificantly different from corresponding value without growth factors in culture medium ($P < 0.05$).

(80 mmol β -glycerophosphate l^{-1} , 20 mmol ethylene glycol-bis (β -aminoethyl ether), N,N,N',N' -tetraacetic acid l^{-1} , 15 mmol MgCl_2 l^{-1} and 1 mmol dithiothreitol l^{-1}) with inhibitors (1 mmol sodium ortho-vanadate l^{-1} , 10 μg aprotinin ml^{-1} and 10 μg pepstatin A ml^{-1}). The mixture was incubated for 30 min at room temperature and the reaction was stopped by the addition of 10 μl of $2 \times$ concentrated electrophoresis sample buffer containing 100 mmol dithiothreitol l^{-1} . The reactions were subjected to electrophoresis on 12.5% (w/v) SDS polyacrylamide gels, and phosphorylated histone and MBP bands were visualized by autoradiography. The intensity of the bands was analysed by imaging densitometry (Model GS-700; Bio-Rad, Hercules, CA).

Statistical analysis

The observations on polar body extrusion and cell cycle stage were recorded as percentages and transformed by arcsine transformation to normalize the variance before analysis. The transformed data and densitometric value of the kinase activities were analysed by ANOVA using the GLM procedure (SAS, 1989). The transformed means of frequency of polar body extrusion and cell cycle stage in oocytes derived from follicles of specific sizes were compared by non-paired t test to evaluate the accelerating effects of EGF and IGF-I. The mean values of kinase activity as indicated by densitometric value at each time point were compared with the mean values at 0 h (before onset of maturation culture) by paired t test. All other means were compared by Fisher's least significant difference test. $P < 0.05$ was considered significant.

Results

Effects of follicular size on response to growth factors

A total of 829 COCs (139 from large follicles, 306 from medium follicles and 384 from small follicles) was examined in five replicates. Two-way ANOVA (follicular size and supplementation with growth factors) indicated that there were no main effects or interaction effects on the timing of polar body extrusion. However, for oocytes derived from small follicles, the frequency of PB16 oocytes in the growth factor (+) group was significantly higher than that in the growth factor (–) group, and the frequency of PB24 oocytes in the growth factor (+) group was significantly lower than that in the growth factor (–) group (Table 1). Observation of the chromatin configuration of PB16 oocytes indicated that 31–46% were in metaphase II, 52–62% were in anaphase I or telophase I of the meiotic cell cycle, and $< 8\%$ were in other stages or had degenerated (Fig. 1). There were no significant differences in the frequencies of cell cycle stages of PB16 oocytes collected from follicles of different sizes matured with or without growth factors.

Response of oocytes derived from small follicles to individual growth factors

COCs ($n = 1118$) from small follicles were prepared for treatment with individual growth factors and sampled for kinase assay. The timing of polar body extrusion of 818 oocytes in response to individual growth factors was examined in five replicate experiments. The frequencies of

Fig. 1. Cell cycle stage of bovine oocytes that have an extruded polar body within 16 h of the start of culture (PB16). ■: Metaphase II; ▨: anaphase I or telophase I oocytes; □: unmaturation oocytes. These oocytes with cumulus cells were derived from large (7–10 mm), medium (4–6 mm) or small (≤ 3 mm) follicles and treated with (+) or without (–) growth factors (GF; epidermal growth factor and insulin-like growth factor I) during maturation culture. Data presented are means of five replicate experiments. No significant differences were detected.

polar body extrusion of oocytes from small follicles treated with individual growth factors are shown (Table 2). EGF supplementation (EGF only and EGF + IGF-I) increased the frequencies of PB16 oocytes significantly and decreased the frequencies of PB24 oocytes compared with the control frequencies. IGF-I treatment also caused an increased frequency of PB16 oocytes and a decreased frequency of PB24 oocytes, although the differences from the control treatment were not significant. The frequency of oocytes without a polar body at 24 h in the EGF + IGF-I group was significantly lower than in the control and EGF only groups. Observation of the chromatin configuration of oocytes indicated that there were no significant differences in the frequencies of cell cycle stages of PB16 oocytes collected from small follicles and cultured with individual growth factors (Fig. 2).

Kinase activities in oocytes derived from small follicles and treated with growth factors

Determination of kinase activity was performed three times (total 300 oocytes). H1 kinase activity in each group of oocytes was increased significantly at 6–8 h of culture compared with the value at 0 h of culture, and the values in the EGF only, IGF-I only and EGF + IGF-I treatment groups at 8 h of culture were significantly higher than that in the control group (Fig. 3a). In the PB16 oocytes, the H1 kinase activity in each group of oocytes was decreased.

MAP kinase activity of oocytes in the EGF only group at 6 h of culture was significantly higher than activity at 0 h of culture. MAP kinase activity was also significantly higher in the EGF only group than in the control and EGF + IGF-I

Fig. 2. Cell cycle stage of bovine oocytes that have an extruded polar body within 16 h of the start of culture (PB16). ■: Metaphase II; ▨: anaphase I or telophase I oocytes; □: unmaturation oocytes. These oocytes with cumulus cells were derived from small (≤ 3 mm in diameter) follicles and treated with or without EGF and IGF-I during maturation culture. Data presented are means of five replicate experiments. No significant differences were detected.

groups at 6 h of culture (Fig. 3b). A significant increase in MAP kinase activity compared with at 0 h of culture was observed at 8 h of culture in the control, IGF-I only and EGF + IGF-I groups, at which point the value in the EGF only group was significantly higher than that in the control group. In the PB16 oocytes, MAP kinase activity remained high in each of the treatment groups without significant differences among the groups.

Discussion

The results of the present study are: (i) a significant effect of growth factors on the timing of polar body extrusion of bovine oocytes was detected only when the oocytes were derived from small follicles; (ii) supplementation of the maturation medium with EGF accelerated the timing of polar body extrusion of oocytes derived from small follicles compared with that observed with IGF-I supplementation; and (iii) EGF and IGF-I increased H1 and MAP kinase activities of oocytes derived from small follicles during early maturation culture, and the increasing effect on MAP kinase activity was most pronounced in the EGF treatment group.

Arlotto *et al.* (1996) reported that as follicle diameter increases, the proportion of larger oocytes obtained from a specific follicular size class increases, and that larger oocytes extrude the first polar body slightly faster than do smaller oocytes under IVM culture supplemented with fetal calf serum and hormones. In the present study, under serum- and hormone-free conditions, the mean frequency of PB16 oocytes from small follicles was lower than that from medium or large follicles when growth factors were not present in the maturation medium, but the differences were not significant. Sakaguchi *et al.* (2000) demonstrated the

Table 2. Polar body extrusion of bovine oocytes derived from small (≤ 3 mm in diameter) follicles cultured with epidermal growth factor (EGF) or insulin-like growth factor I (IGF-I) or both

Polar body extrusion	Treatment*			
	Control	EGF only	IGF-I only	EGF + IGF-I
PB16	101 (49 $\pm$ 6) ^a	142 (67 $\pm$ 6) ^b	134 (63 $\pm$ 5) ^{ab}	138 (75 $\pm$ 4) ^b
PB24	85 (41 $\pm$ 5) ^a	46 (22 $\pm$ 6) ^b	65 (30 $\pm$ 6) ^{ab}	37 (21 $\pm$ 4) ^b
No polar body	21 (10 $\pm$ 1) ^a	24 (11 $\pm$ 1) ^a	17 (7 $\pm$ 2) ^{ab}	8 (4 $\pm$ 1) ^b
Total	207	212	216	183

*Values are number of oocytes (mean $\pm$ SE of five replicate experiments).

PB16 and PB24: oocytes that have extruded the first polar body by 16 or 24 h after the initiation of culture, respectively.

No polar body: oocytes that have not extruded their first polar body by 24 h after initiation of culture.

^{ab}Values in the same row with different superscripts are significantly different ($P < 0.05$).

accelerating effect of growth factors on the progress of meiosis in bovine oocytes as indicated by polar body extrusion using pooled oocytes from small and medium (1–6 mm in diameter) follicles. The results of the present study using oocytes from size-classified follicles demonstrate clearly that growth factors (EGF + IGF-I) accelerate the meiotic progress of oocytes enclosed by cumulus cells derived from small (≤ 3 mm in diameter) follicles only.

Lonergan *et al.* (1994) reported a clear relationship between follicle size and oocyte quality in terms of oocyte morphology after recovery and blastocyst yield after *in vitro* fertilization (IVF). Pavlok *et al.* (1992) demonstrated that oocytes from follicles 1–2 mm in diameter have a significantly lower competence to undergo IVM and IVF than do those from follicles 3–8 mm in diameter. Arlotto *et al.* (1996) also showed that oocytes from small (≤ 3 mm in diameter) follicles yielded fewer blastocysts than did those from larger (> 3 mm in diameter) follicles. Together with the results of the present study, which showed that only small follicle-derived oocytes are responsive to growth factors, these findings indicate that the lower developmental competence of oocytes from small follicles might be one explanation for the results of a previous study in which growth factors (EGF + IGF-I) in protein-free medium accelerated the progression of meiosis in oocytes, but did not improve blastocyst yield after IVF (Sakaguchi *et al.*, 2000).

In pigs (Hsu *et al.*, 1987) and humans (Westergaard *et al.*, 1990), it has been reported that the concentration of EGF in small follicles is higher than that in medium and large follicles. In cattle it has been reported that specific EGF binding values are low in preantral follicles but show a 2.5- and 5.0-fold increase in both granulosa cells and the theca interna from antral I (0.16–0.50 mm in diameter) and antral II (0.6–3.0 mm in diameter) follicles, respectively (Wandji *et al.*, 1992). In contrast, the concentration of IGF-I (Spicer and Geisert, 1992) and its receptor (Spicer *et al.*, 1994) in small follicles is lower than in medium and large follicles in cattle. These results indicate that EGF plays a more important role during the early antral stages of follicular maturation than

does IGF-I. In the present study, a greater acceleration of the meiotic progress of bovine oocytes from small follicles was induced by EGF treatment than by IGF-I treatment compared with the control group. This difference in the ability of individual growth factors to accelerate the meiotic progress of oocytes derived from small follicles might be attributed to the stage-specific mechanisms of action of these growth factors in early follicular maturation.

Under conventional conditions for IVM culture, which is supplemented with fetal calf serum and hormones such as gonadotrophins and steroids, bovine oocytes with cumulus cells undergo GVBD at 6–9 h after the onset of maturation (Sirard *et al.*, 1989). It is widely accepted that MPF plays a pivotal role in driving meiotic resumption (Masui and Markert, 1971; Kishimoto *et al.*, 1984; Sorensen *et al.*, 1985; Nurse, 1990). Similar to other mammalian species, the kinetics of activation of MPF (assessed by H1 kinase activity) precede or occur simultaneously with GBVD in bovine oocytes (Tatemoto and Terada, 1996; Wu *et al.*, 1997), and MPF phosphorylates many of the proteins involved in nuclear membrane formation, chromatin condensation and microtubule reorganization (Verde *et al.*, 1992). In the present study, H1 kinase activity in oocytes derived from small follicles significantly increased from its baseline value at 6 h of maturation culture with or without growth factors. At 8 h of culture, the H1 kinase activity of oocytes in each treatment group continued to increase and the activity in oocytes treated with EGF or IGF-I or both was significantly higher than that of the control group. The good correlation between the rapid increases in H1 kinase activity caused by growth factors and the rapid meiotic progress of oocytes indicated by polar body extrusion indicates that acceleration of the meiotic progress of bovine oocytes by EGF and IGF-I might be related to an early activation of H1 kinase activity at about the time of GVBD.

In maturing bovine oocytes, MAP kinase, another essential kinase in the meiotic resumption of oocytes, is activated at the same time as MPF at about the time of GVBD (Fissore *et al.*, 1996; Motlik *et al.*, 1998). In rat

Fig. 3. (a) Histone H1 kinase and (b) MAP kinase activities in small follicle-derived oocytes treated with growth factors. Cumulus-oocyte complexes from small follicles (≤ 3 mm in diameter) were not treated with growth factors (control; □) or were treated with epidermal growth factor (EGF) only (▨), insulin-like growth factor (IGF-I) only (▤) or with EGF + IGF-I (■). In each treatment group, oocytes were selected at random for kinase assay at 0, 4, 6 and 8 h after initiation of maturation culture. Five PB16 (polar body extrusion at 16 h) oocytes in each treatment group were also sampled. Data are mean $\pm$ SE ($n = 3$ replicates). The densitometric values of kinase activities were analysed by ANOVA. The means of kinase activity indicated by the densitometric value at each time point were compared with the means at 0 h (before onset of maturation culture) by paired t test, and the means for the different treatments at the same time point were compared by Fisher's protected least significant difference test. *Significantly different from corresponding value at 0 h (before onset of maturation, $P < 0.05$). ^{ab}Values with different superscripts at the same time point are significantly different ($P < 0.05$).

oocytes, MAP kinase activation alone was insufficient to maintain progression through meiotic maturation and MAP kinase was also implicated in the regulation of microtubule organization (Zernicka-Goetz *et al.*, 1997). These authors also reported that the spindle did not form immediately after MPF activation, but rather after the appearance of MAP

kinase activity. In bovine oocytes, the rapid and maximum activation of MAP kinase induced by the product of the proto-oncogene *mos* results in accelerated resumption of meiosis and GVBD (Fissore *et al.*, 1996). The results of the present study indicate that the MAP kinase activity in oocytes treated with EGF only significantly increased from the baseline value at 6 h of maturation culture but did not in other treatment groups including the EGF + IGF-I group. From 6 h to 8 h of culture, treatment with EGF without IGF-I resulted in higher MAP kinase activity than did the other treatments. This activation effect of EGF on MAP kinase might also be involved with a higher acceleration effect of EGF on meiotic progress. However, the reason why EGF in combination with IGF-I did not have the same ability to increase MAP kinase activity as EGF only is unclear. Further studies are required to clarify the interaction between EGF and IGF-I during maturation of bovine oocytes, including the effects on the kinetics of other cell cycle-regulating kinases such as protein kinases A and C (Rose-Hellekant and Bavister, 1996; Sirard *et al.*, 1998).

The results from analysis of cell cycle stage of fixed oocytes in the present study showed that 55–73% of PB16 oocytes were at the anaphase I to telophase I stage. This finding, together with early activation of kinase activities in oocytes treated with growth factors, indicates that growth factors accelerate the early stage of oocyte maturation before the formation of the first polar body. Dominko and First (1997) demonstrated that a higher proportion of fertilized oocytes developed to the advanced preimplantation stage when insemination of PB16 oocytes was delayed for 8 h than when the oocytes were inseminated immediately after polar body extrusion. Although these workers did not analyse the cell cycle stage of polar body oocytes and in the present study the cell cycle progression of the PB16 oocyte after polar body extrusion was not examined, it is possible that delayed insemination of the PB16 oocytes allows the PB16 oocytes at the anaphase I or telophase I stage to form the metaphase II plate, as well as complete cytoplasmic maturation.

Sakaguchi *et al.* (2000) demonstrated that growth factors do not affect the timing of the polar body extrusion of denuded bovine oocytes in the serum-free culture system and that the acceleration effects of growth factors on the polar body extrusion of bovine oocytes are probably mediated by the enclosing cumulus cells. In rodents, EGF may promote oocyte maturation by disrupting oocyte communication with cumulus cells through the gap junctions (Dekel and Sherizly, 1985), and generation of a positive signal within cumulus cells in response to treatment with growth factors and hormones may act on the oocyte to stimulate GVBD (Downs *et al.*, 1988; Downs, 1989). Prochazka *et al.* (2000) reported that the disruption of communication or a positive signal might result in rapid increases in H1 and MAP kinase activities in pig oocytes at the germinal vesicle stage. Thus, it can be speculated that the acceleration effect of growth factors on the meiotic progress of bovine oocytes derived from small follicles is

exerted through disruption of the communication between cumulus cells and oocytes or by generating the positive signal to the oocyte.

Fair *et al.* (1995) reported that as follicle size increases to > 3 mm in bovine follicular oocytes, the growth in oocyte diameter reaches a plateau at about 120 µm and oocytes acquire full meiotic competence with transcriptional activity at a diameter of about 110 µm. These authors also demonstrated that 60% of oocytes derived from follicles 1–2 mm in diameter were < 110 µm in diameter. Therefore, some of the oocytes from small follicles (≤ 3 mm in diameter) might be at the final growing phase. The results of the present study indicate that growth factors may act on these oocytes at the final growing phase and accelerate their meiotic progress through enhancing cell cycle-related kinase activities. Examining the changes in diameter of oocytes from follicles of different sizes during maturation culture with or without growth factors will provide additional information to help to explain the effects of growth factors on maturation of bovine oocytes.

In conclusion, EGF and IGF-I act on COCs from small follicles and accelerate the progress of the meiotic cell cycle of oocytes, as indicated by early polar body extrusion. The accelerating ability of EGF may be greater than that of IGF-I, and these two growth factors have an additive promoting effect on the timing of the first polar body extrusion of oocytes derived from small follicles. The accelerating effect may be related to increased H1 and MAP kinase activities in oocytes mediated by cumulus cells. It is suggested that these possible mechanisms for the action of growth factors on the progress of bovine oocyte maturation must be taken into consideration when these factors are used for improvement of IVM systems.

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