Induction and maintenance of oestradiol and immunoreactive inhibin production with FSH by ovine granulosa cells cultured in serum-free media

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A serum-free ovine granulosa cell culture system is described that allows the induction of FSH-responsive oestradiol production by undifferentiated cells from small (< 3.5 mm) follicles ($P < 0.001$) and the maintenance of oestradiol production by differentiated cells from large (≥ 3.5 mm) follicles. Physiological doses of FSH stimulated ($P < 0.01$) proliferation of cultured granulosa cells from both small and large follicles. The synthesis of immunoreactive inhibin and progesterone by granulosa cells from small and large follicles increased ($P < 0.01$) with time of culture, and was not dependent on FSH. Inhibin secretion expressed on a per cell basis was not FSH responsive. Insulin and insulin-like growth factor I (IGF-I), in the presence of FSH, stimulated ($P < 0.001$) cell proliferation and oestradiol and inhibin production by granulosa cells from small and large follicles. There was a significant ($P < 0.001$) interaction between insulin and IGF-I in the stimulation of granulosa cell proliferation and differentiation. Both epidermal growth factor (EGF) and transforming growth factor α (TGF-α) in the presence of FSH stimulated cellular proliferation ($P < 0.001$) in a dose-responsive manner and concomitantly inhibited ($P < 0.001$) oestradiol and inhibin secretion. The development of this granulosa cell culture system will make it possible to study, *in vitro*, the cascade of events that controls granulosa cell differentiation and ultimately follicle selection in sheep.

Introduction

In sheep, as ovarian follicles grow from the gonadotrophin-dependent phase (2–3 mm diameter: Scaramuzzi *et al.*, 1993) to preovulatory size (4–6 mm), the mitotic activity of the granulosa cells declines (Turnbull *et al.*, 1977) and follicles destined to ovulate attain the ability to synthesize large amounts of both oestradiol (Scaramuzzi and Baird, 1977; McNatty *et al.*, 1985; McNeilly *et al.*, 1991) and inhibin (Tsonis *et al.*, 1988; Campbell *et al.*, 1990, 1991a). Both oestradiol and inhibin are almost exclusive products of granulosa cells (Engelhardt *et al.*, 1993; Hinshelwood *et al.*, 1993) and attainment of the ability to synthesize these two hormones is a key indicator of differentiated granulosa cell function. Experiments *in vivo* have shown that FSH alone can stimulate the growth of large ovarian follicles (Picton *et al.*, 1990) and a marked increase in the ovarian secretion of both oestradiol and inhibin (Campbell *et al.*, 1993a). Evidence has accumulated indicating that many of these actions of FSH are modulated in the ovary by a number of local factors, including many of the peptide growth factors

(Adashi *et al.*, 1985; May *et al.*, 1988; Scaramuzzi and Campbell, 1990). As much of this evidence has been derived from *in vitro* studies with rodents, their significance to follicle development in large domestic ruminants is not known. However, it has been shown that epidermal growth factor (EGF; Murray *et al.*, 1992), transforming growth factor α (TGF-α; Campbell *et al.*, 1994), insulin-like growth factor I (IGF-I; Campbell *et al.*, 1993b) and inhibin (Campbell *et al.*, 1992) can all modulate sheep ovarian follicle development and steroid secretion *in vivo*. These results provide strong confirmatory evidence that these factors may modulate follicle development in large domestic ruminants. Up until now it has not been possible to extend these findings *in vivo* to study the molecular basis of these effects *in vitro*, as the establishment of a physiologically relevant cell culture system for ovarian granulosa cells from large domestic ruminants has proved difficult. While there are numerous reports of granulosa culture systems for both sheep (Campbell, 1989; Webb and McBride, 1990; Monniaux and Pisselet, 1992) and cattle (Skinner and Osteen, 1988; Luck *et al.*, 1990; Langhout *et al.*, 1991; Spicer *et al.*, 1993; Gong *et al.*, 1994), many of the systems reported have the major deficiency that the granulosa cells luteinize spontaneously resulting in a rapid fall in oestradiol and inhibin secretion and a marked increase in progesterone secretion (Skinner and Osteen, 1988; Campbell, 1989; Luck *et al.*, 1990; Webb and McBride, 1990; Langhout *et al.*, 1991; Meidan *et al.*, 1992; Spicer *et al.*,

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1993; Gong *et al.*, 1994). Furthermore, the proliferative and differentiative responses of cultured granulosa cells to FSH are either lost (Monniaux and Pisselet, 1992) or can only be induced using supra-physiological hormone concentrations (Wrathall and Knight, 1993). Indeed, many of the reports of hormone production by cultured sheep and cattle follicular cells include only data for progesterone secretion (Monniaux and Pisselet, 1992). Alternatively, results are presented for the main follicular hormones without reference to the initial amount of hormone secretion (Wrathall and Knight, 1993; Gong *et al.*, 1994) or from relatively short-term cultures in which cells undergoing early luteinization retain the ability to synthesize some of these hormones (Skinner and Osteen, 1988; Gong *et al.*, 1994). Results from such studies can be misleading because the hormonal 'responses' to treatment may just reflect a change in the rate of luteinization. It is self-evident that to be able to make physiologically relevant inferences from cultured cells, it is essential to have a culture system in which the cells secrete the same hormones and exhibit the same hormonal responses as do cells *in vivo*.

This study reports the development of a serum-free culture system for granulosa cells that, without the use of attachment factors, allowed the induction of FSH-responsive oestradiol production by undifferentiated granulosa cells from small follicles and the maintenance of oestradiol production by differentiated cells from large follicles. With this culture system, cells from both follicle types are mitotically responsive to FSH. Using this culture system, the proliferative and steroidogenic effects of insulin, IGF-I, EGF and TGF-α on cells from small and large follicles were studied.

Materials and Methods

Materials

The following were purchased from the Sigma Chemical Co. Ltd, Poole, Dorset: McCoy's 5a media with sodium bicarbonate, Penstrep containing 10 000 iu penicillin and 10 mg streptomycin ml^{-1}, BSA (tissue culture grade), transferrin, selenium, insulin (bovine), TGF-α, neutral red, trypsin, androstenedione and Trypan blue. The following were purchased from GIBCO BRL, Life Technologies Ltd, Paisley, Renfrewshire: Medium 199 (10 times concentrate), Hepes (1 mol l^{-1}), amphotericin (250 µg ml^{-1}), L-glutamine (200 mmol l^{-1}), Dulbecco's PBS without calcium or magnesium (DPBS^{-}), Dulbecco's PBS (DPBS^{+}). Human recombinant Long R3 IGF-I (LR3-IGF-I, media grade) was purchased from Gropep Pty Ltd (Adelaide, SA). Epidermal growth factor was purchased from Toyoba Co. Ltd (Osaka). The other chemicals used were Analar grade and were obtained from general laboratory suppliers. Ovine FSH (NIDDK-oFSH-S16; bioactivity 20 iu mg^{-1}) and LH (NIDDK-oLH-S26; bioactivity 2.3 iu mg^{-1}) were generously donated by the National Institute of Arthritis, Diabetes and Digestive and Kidney Diseases (Torrance, CA).

Sterile culture plates, bottles and dishes were obtained from Nunclon (Life Technologies, Paisley); sterile plastic syringes were obtained from Terumo Corp. (Terumo Europe, Leuven); sterile plastic universal containers and centrifuge tubes and Petri dishes were obtained from Greiner Labortechnik Ltd (Cam, Dursley, Glos) or Bibby Sterilin Ltd (Stone, Staffs).

Isolation of granulosa cells

Ovaries were collected directly from animals at the local abattoir and placed immediately in collection medium (Medium 199 containing 20 mmol Hepes l^{-1}, 100 kiu penicillin l^{-1}, 0.1 µg streptomycin l^{-1} and 1 mg amphotericin (Fungizone) l^{-1}) at 37°C. Follicle dissection was carried out in two stages under sterile conditions. Within 2–3 h, approximately 150–200 small (2–3.5 mm diameter) and 20–30 large (> 3.5 mm diameter) follicles were roughly dissected from the ovaries, and follicles with atretic morphology were discarded. When the desired number of follicles had been obtained, extraneous stromal tissue was removed under a dissecting microscope using watchmakers' forceps. Granulosa cells were isolated from small follicles by hemisecting the follicles in 5 ml DPBS^{-} at 37°C and the follicle halves flushed repeatedly up and down the barrel of a 1 ml syringe in a 25 ml Universal container. The thecal shells were then allowed to settle and the resulting granulosa cell suspension was collected. After centrifugation at 800 *g* for 10 min, the cell pellets were resuspended in culture medium (McCoy's 5a containing bicarbonate, 20 mmol Hepes l^{-1}, 100 kiu penicillin l^{-1}, 0.1 µg streptomycin l^{-1}, 3 mmol L-glutamine l^{-1}, 0.1% BSA (w/v), 10^{-7} mol androstenedione l^{-1}, 2.5 mg transferrin l^{-1}, 4 µg selenium l^{-1}). After a further wash, number of cells and viability was estimated using Trypan blue exclusion. Granulosa cells were isolated from large follicles by aspirating antral fluid using a 1 ml syringe fitted with a 23 gauge needle; the follicles were hemisected in 5 ml of DPBS^{-} at 37°C and the granulosa cells were gently scraped from the theca with a 10 µl inoculation loop. The resulting granulosa cell suspension was isolated by centrifugation as described for small follicles.

Granulosa cell cultures

Viable cells (50 000–100 000) from either small or large follicles were seeded in a volume of 50 µl into pre-prepared and equilibrated 96-well plates containing 200 µl of medium with various concentrations and combinations of either insulin (1–5000 ng ml^{-1}), LR3-IGF-I (1–100 ng ml^{-1}), FSH (0.1–20 ng ml^{-1}), LH (0.01–100 ng ml^{-1}), EGF (0.005–50 ng ml^{-1}) or TGF-α (0.005–50 ng ml^{-1}) replicated in quadruplicate. Cells were cultured in a humidified atmosphere with 3.75% carbon dioxide in air at 37°C. Cells were cultured for a total of 6 days and the medium was changed at intervals of 48 h. Disturbance of the cells was minimized by gently removing and replacing only 175 µl of media at each change. The spent medium was stored at − 20°C before assay.

Estimation of number of granulosa cells

The number of viable cells per well after 6 days of culture was estimated using the neutral red method (Borenfreund and Puerner, 1984). Briefly, at the end of the culture period spent medium was replaced with 200 µl of fresh culture medium containing 10 µg neutral red, and the plates were incubated for

3 h at 37°C. After incubation, the neutral red solution was removed and replaced with 200 μl of a solution containing 4% (w/v) formaldehyde and 1% (w/v) calcium chloride (formol–calcium) for 2–3 min. This solution was then removed and 200 μl of a solution containing 50% (v/v) ethanol and 1% (v/v) glacial acetic acid (acetic acid–ethanol) added. Absorbance of this solution was then determined at 540 nm using a multi-well plate reader. Absorbance was related directly to number of cells by culturing a large number of excess granulosa cells under similar conditions and incubating them with neutral red and isolating them after trypsinization (0.2% (w/v) trypsin and 0.08% (w/v) EDTA in DPBS⁻ for 20 min). They were then counted using a haemocytometer. These cells were then centrifuged at 800 g for 10 min and the cell pellet treated with formol–calcium and acetic acid–ethanol. The resulting solution was then serially diluted in acetic acid–ethanol and 200 μl aliquots in quadruplicate were placed in a blank 96-well plate and the absorbance at 540 nm was determined. The relationship between number of cells and absorbance was linear ($r > 0.99$) and the number of cells per well in the culture plates was estimated from the resulting linear regression equation. The limit of detection of the assay was 2×10^3 cells per well and the coefficients of variation were less than 5%.

Assays

Concentrations of oestradiol (Webb *et al.*, 1985), inhibin p1–26α subunit (McNeilly *et al.*, 1989) and progesterone (Corrie *et al.*, 1981) in unextracted culture media were determined using radioimmunoassays. The sensitivities of the oestradiol, inhibin and progesterone assays were 1.4 pg ml⁻¹, 35 pg ml⁻¹ and 0.2 ng ml⁻¹, respectively. The intra- and interassay coefficients of variation for oestradiol, inhibin and progesterone were 10.3 and 8.7%, 6.0 and 8.0%, 8.3 and 5.3%, respectively.

Statistical analyses

Granulosa cells derived from small and large follicles were cultured and analysed separately on the basis of the observation that the diameter of a follicle is closely related to the stage of differentiation of its granulosa cells, and that the sheep antral follicle population can be classified into two physiologically distinct groups on the basis of size. Thus, sheep follicles of < 3.5 mm diameter contain antral fluid with low oestradiol and inhibin concentrations and mitotically active granulosa cells without LH receptors, whereas follicles of > 3.5 mm diameter contain antral fluid with high oestradiol and inhibin concentrations and mitotically inactive granulosa cells with abundant LH receptors (Turnbull *et al.*, 1977; Carson *et al.*, 1979; England *et al.*, 1981; Webb and England, 1982).

All hormone production data were expressed as pg of hormone produced per 1000 cells per 48 h, after correction for the residual amount of media left in the wells when the media was changed at 48 and 96 h. The number of cells used for this calculation was the number of cells after culture for 144 h and, with the exception of the time course data, only data from 96–144 h of culture are presented. As we have no direct estimates of viable number of cells after culture for 96 h, the

time course data have been expressed in terms of pg hormone ml⁻¹ of culture medium for each 48 h culture period, after correction for carry over. Each experiment was repeated at least three times and the significance of treatment effects was determined by analysis of variance on $\log_{10}$-transformed data, using replicate cultures as blocks. Individual comparisons between treatments were made using the Bonferroni *t* test.

## Results

Time course of hormone secretion

Granulosa cells from small follicles secreted little oestradiol during the first 48 h of culture (Fig. 1). In the absence of FSH, oestradiol production by these cells increased significantly ($P < 0.05$) with time, but in the presence of FSH, there was a marked sequential increase ($P < 0.001$) in oestradiol secretion after culture for 96 and 144 h. In contrast to oestradiol, both inhibin and progesterone secretion by cells from small follicles increased markedly and sequentially with time ($P < 0.001$), regardless of the presence or absence of FSH. Overall, FSH had a weak stimulatory effect on progesterone ($P = 0.03$) and inhibin ($P = 0.08$) production by granulosa cells from small follicles (Fig. 1).

Granulosa cells isolated from large follicles secreted 50–100 times more ($P < 0.001$) oestradiol during the first 48 h of culture than did cells from small follicles. In the absence of FSH, oestradiol secretion by these cells fell markedly with time ($P < 0.01$), but in the presence of FSH, the amount of oestradiol secretion was maintained (Fig. 1). In contrast to oestradiol, both inhibin and progesterone secretion by cells from large follicles increased sequentially with time ($P < 0.01$), regardless of the presence or absence of FSH. Overall, FSH had a stimulatory effect on inhibin ($P < 0.01$) and progesterone ($P < 0.01$) production by granulosa cells from large follicles.

Responses to FSH

Cultured sheep granulosa cells from both small and large follicles exhibited dose-dependent proliferative and oestrogenic responses to physiological doses of FSH (Fig. 2), but there were marked differences in the responsiveness of cells from small and large follicles to FSH. Cells from small follicles showed a maximal proliferative response at 0.5 ng FSH ml⁻¹ ($P < 0.01$) and a maximal oestrogenic response at 10 ng oFSH ml⁻¹ ($P < 0.001$). In contrast, FSH stimulated ($P < 0.01$) oestradiol production by the cells from large follicles in a dose-dependent manner, without affecting the number of cells, up to a dose of 1 ng ml⁻¹. Higher doses of FSH resulted in an increase ($P < 0.05$) in the number of cells, with a concomitant depression in oestradiol production.

In contrast to oestradiol, inhibin synthesis by cells from small follicles was not responsive to FSH when the results were expressed on a per cell basis (Fig. 2). Similarly, inhibin production by granulosa cells from large follicles was unresponsive to FSH up to 10 ng FSH ml⁻¹ and higher doses were inhibitory ($P < 0.05$).
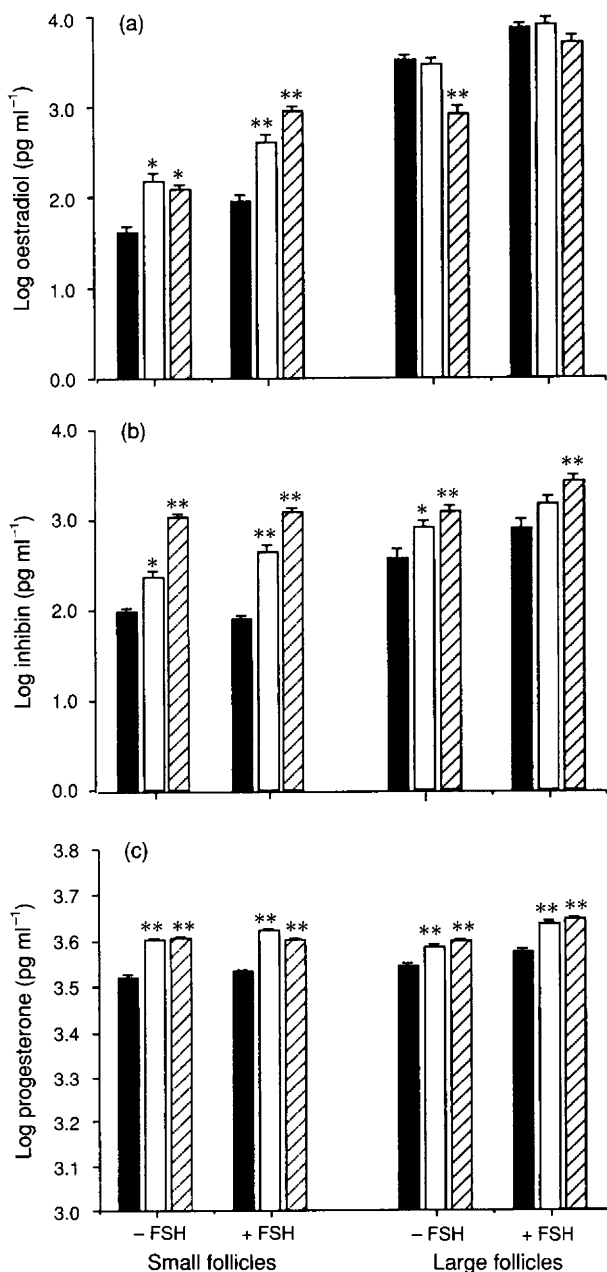
Fig. 1. The changes in the concentration of (a) oestradiol, (b) inhibin and (c) progesterone in media of cells from small (< 3.5 mm) and large ($\geq$ 3.5 mm) sheep ovarian follicles after culture for 0–48 h (■), 48–96 h (□) and 96–144 h (▨) in the presence or absence of 10 ng oFSH-S16 ml^{-1}. Cells were cultured under identical serum-free conditions in the presence of 10 ng insulin ml^{-1} and 10 ng IGF-I analogue, Long R3 IGF-I ml^{-1}. Values are least squares means $\pm$ SEM of three independent replicate cultures. *$P < 0.05$, **$P < 0.01$ compared with culture for 0–48 h within each time course (repeated samples ANOVA).

Interaction between insulin and IGF-I in the presence of FSH

Cell morphology. Both insulin and LR3 IGF-I had a marked effect on the gross morphology of cultured granulosa cells from small follicles. Despite being plated initially as a dispersed monolayer, by the end of the culture period the granulosa cells

had formed into dense interconnected clumps. These clumped cells retained the normal spheroidal appearance of granulosa cells, but seemed to be anchored to the culture plate by more elongated spindle-shaped cells, which also projected to adjacent clumps of cells. Although granulosa cells from large follicles tend to be more clumped when they are first plated than do cells from small follicles, high doses of insulin or IGF-I resulted in similar changes in the morphology of cultured granulosa cells from large follicles as those observed for cells from small follicles.

Cell proliferation. After culture for 6 days under serum-free conditions in the presence of FSH, but in the absence of either insulin or IGF, the number of granulosa cells was low (10% and 31% of the initial number of cells plated from large and small follicles, respectively; Fig. 3). For granulosa cells from small follicles cultured in the absence of LR3 IGF-I, insulin increased the number of cells in a dose-responsive manner ($P < 0.001$). There was a significant ($P < 0.001$) interaction between insulin and LR3 IGF-I in the stimulation of proliferation of granulosa cells from small follicles (Fig. 3), and the addition of increasing doses of LR3 IGF-I resulted in a flattening of the proliferative dose–response to insulin, so that at the highest dose (100 ng LR3 IGF-I ml^{-1}), cells were not responsive ($P = 0.9$) to insulin (Fig. 3). In contrast to granulosa cells from small follicles, the effects of both insulin and LR3 IGF-I on cells from large follicles were mainly restricted to maintaining the number of cells at the number plated, rather than stimulating proliferation (Fig. 3). For granulosa cells from large follicles, cultured in the absence of LR3 IGF-I, insulin increased the number of cells in a dose-responsive manner ($P < 0.001$), and the number of cells was maintained by a dose of 5 µg ml^{-1}. There was a significant ($P < 0.001$) interaction between insulin and LR3 IGF-I in the regulation of number of cells from large follicles (Fig. 3). Doses of 1 and 10 ng LR3 IGF-I ml^{-1} led to maintenance of the number of cells, while the highest dose of 100 ng LR3 IGF-I ml^{-1} stimulated ($P < 0.01$) the granulosa cells from large follicles to proliferate both in the absence and presence of low doses of insulin (Fig. 3). At the highest dose of LR3 IGF-I, high doses of insulin led to an inhibition of this effect.

Hormone production. Despite a ten-fold difference in the amounts secreted, the effects of both insulin and IGF-I on the pattern of oestradiol and inhibin secretion by cells from small and large follicles were similar. There was a highly significant ($P < 0.001$) interaction between insulin and IGF-I in the stimulation of hormone production by granulosa cells from small and large follicles. Insulin alone stimulated oestradiol and inhibin secretion in a dose-responsive manner ($P < 0.001$), and the supraphysiological dose of 5 µg ml^{-1} resulted in maximal secretion. A dose of 1 ng LR3 IGF-I ml^{-1} in the absence of, or at low doses of insulin (1–10 ng ml^{-1}), stimulated ($P < 0.01$) oestradiol and inhibin production, but had little effect at higher doses of insulin. A dose of 10 ng LR3 IGF-I ml^{-1} led to a further stimulation ($P < 0.05$) of oestradiol and inhibin production in the absence, or at low doses, of insulin, but tended to be inhibitory at supraphysiological doses of insulin. In contrast, increasing the dose of LR3 IGF-I to 100 ng ml^{-1} resulted in a marked suppression ($P < 0.001$) of oestradiol and inhibin secretion in the absence of insulin, and cells exposed to this
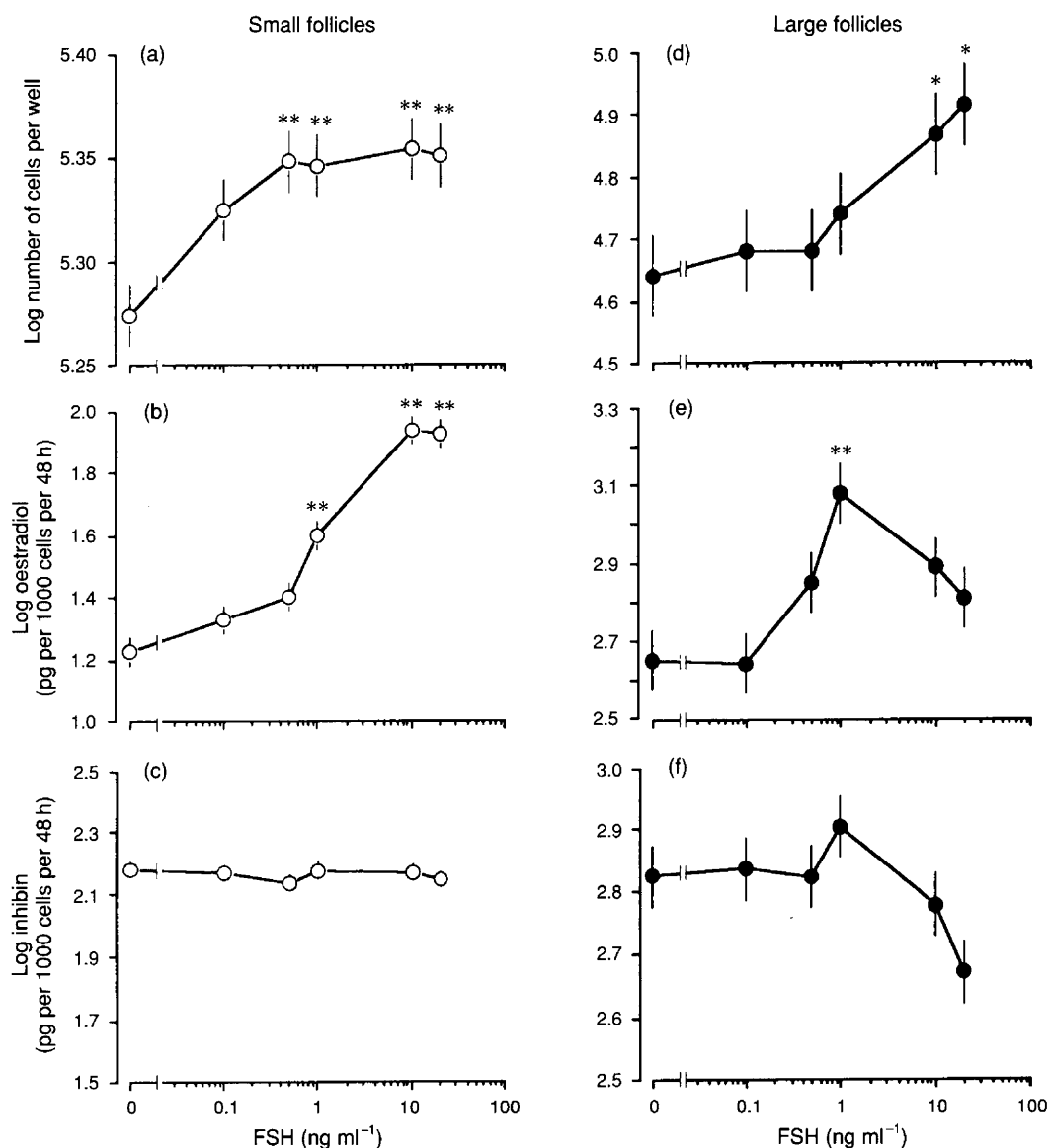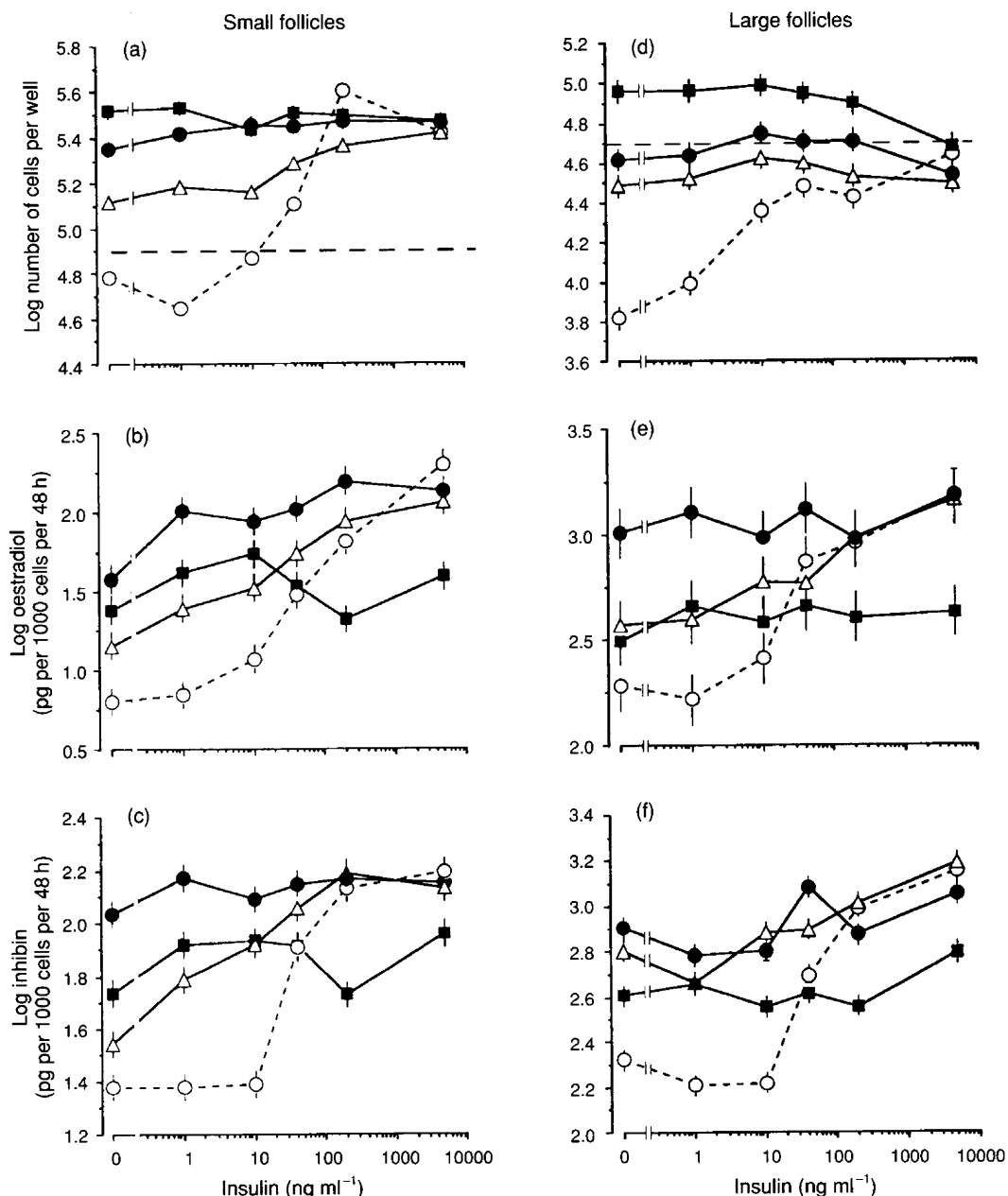
Fig. 2. The effect of oFSH-S16 on (a, d) number of cells, (b, e) oestradiol and (c, f) inhibin production by sheep granulosa cells isolated from small (< 3.5 mm; a–c) and large (≥ 3.5 mm; d–f) follicles after culture for 6 days under serum-free conditions in the presence of 10 ng insulin ml^{-1} and 10 ng IGF-I analogue, Long R3 IGF-I ml^{-1}. Note that the oestradiol and inhibin data have been corrected for the effect of number of cells. Values are least squares means ± SEM of four independent replicate cultures. *$P < 0.05$, **$P < 0.01$ compared with zero dose of FSH (ANOVA with Bonferroni test).

dose of LR3 IGF-I were relatively unresponsive to insulin (Fig. 3). On the basis of the results of this study, a combined dose of 10 ng insulin ml^{-1} and 10 ng LR3 IGF-I ml^{-1} was chosen for subsequent studies, as these doses are in the physiological range and appear to give the best balance between cell proliferation or maintenance and stimulation of hormone production.

Effect of mitogenic growth factors

Both EGF and TGF-α, in the presence of FSH (10 ng ml^{-1}), stimulated dose-responsive increases in the proliferation of granulosa cells ($P < 0.001$) from both small and large follicles,

and a concomitant inhibition of oestradiol ($P < 0.001$) and inhibin ($P < 0.001$) synthesis (Fig. 4). The presence or absence of FSH had no significant effect on the stimulatory effects of EGF and TGF-α on cellular proliferation, but hormone production was low in the absence of this gonadotrophin (data not shown). Owing to the heterologous nature of the growth factors used, their respective biopotencies were not analysed, but both these growth factors stimulated maximal proliferative responses by granulosa cells from both size classes at doses of 500 pg ml^{-1}. However, granulosa cells from small follicles were more sensitive to these mitogenic growth factors; a dose of 10–20 pg ml^{-1} was required to stimulate 50% of the maximum increase in number of cells, compared with a dose of
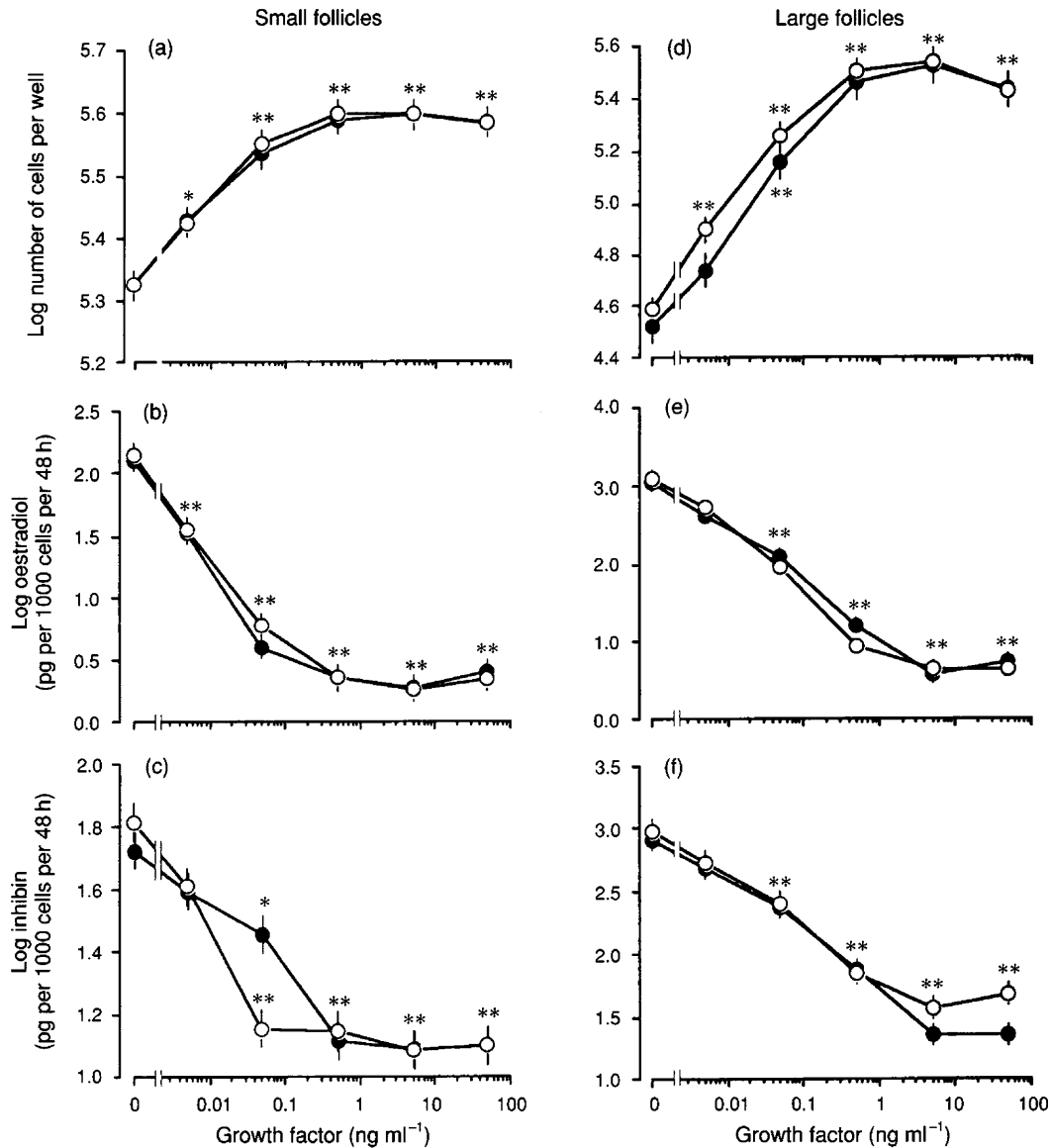
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Fig. 3. The effect of insulin on (a, d) number of cells, (b, e) oestradiol and (c, f) inhibin production by sheep granulosa cells isolated from small (< 3.5 mm; a–c) and large (≥ 3.5 mm; d–f) follicles after culture for 6 days under serum-free conditions in combination with 0 (○), 1 (△), 10 (●) or 100 (■) ng IGF-I analogue, Long R3 IGF-I ml⁻¹. All cultures were performed in the presence of 10 ng oFSH-S16 ml⁻¹. (– – –) indicates the number of cells plated at the start of culture. Note that the oestradiol and inhibin data have been corrected for the effect of number of cells. Values are least squares means ± SEM of four independent replicate cultures (ANOVA with Bonferroni). For clarity, significance asterisks have been omitted from this figure; the significance of main effects are described in the results.

60–100 pg ml⁻¹ for granulosa cells from large follicles. In contrast, granulosa cells from both size classes were equally sensitive to the inhibitory effects of these growth factors on hormone production, with the effective 50% dose being about 5 pg ml⁻¹. Exposure of granulosa cells from both small and large follicles to either EGF or TGF-α resulted in a marked change in the morphology of the cultured cells. Instead of the rather irregular clumping observed with insulin or LR3 IGF-I,

the proliferative growth factors stimulated the formation of discrete, condensed spherical balls of cells.

Discussion

This is the first time that an ovine granulosa cell culture system that allows the induction of FSH-responsive oestradiol

Fig. 4. The effect of epidermal growth factor (EGF) (○) and transforming growth factor (TGF-α) (●) on (a, d) number of cells, (b, e) oestradiol and (c, f) inhibin production by sheep granulosa cells isolated from small (< 3.5 mm; a–c) and large (≥ 3.5 mm; d–f) follicles after culture for 6 days under serum-free conditions in the presence of 10 ng insulin ml^{-1}, 10 ng IGF-I analogue, Long R3 IGF-I ml^{-1} and 10 ng oFSH-S16 ml^{-1}. Note that the oestradiol and inhibin data have been corrected for the effect of number of cells. Values are least squares means ± SEM of four independent replicate cultures. *$P < 0.05$, **$P < 0.01$ compared with zero dose of growth factor (ANOVA with Bonferroni). Where only one significance asterisk is present at each dose of growth factor, this refers to either EGF or TGF-α.

production by undifferentiated cells from small follicles and the maintenance of oestradiol production by differentiated cells from large follicles has been described. In this culture system, physiological doses of FSH can stimulate cellular proliferation, which is apparently a unique finding, as positive proliferative responses of sheep (Monniaux and Pisselet, 1992) or cattle (Gong *et al.*, 1993) granulosa cells to FSH *in vitro* have not been reported previously. In addition, the proliferative and oestrogenic responses of the granulosa cells depend not only on the amount of FSH stimulation but also on the stage of differentiation of the granulosa cells. Thus, low doses of FSH can stimulate undifferentiated granulosa cells from small follicles to

proliferate, whereas higher, but still physiological, doses of FSH can induce the synthesis of oestradiol. Conversely, differentiated granulosa cells from large follicles exposed to low doses of FSH are refractory to the proliferative effects of FSH but sensitive to the oestrogenic effects, whereas higher doses of FSH can stimulate proliferation while concomitantly depressing oestradiol production. These responses to FSH *in vitro* parallel closely the changes observed in granulosa cells *in vivo* during FSH-induced follicle development (McNatty *et al.*, 1985; Picton *et al.*, 1990; Campbell *et al.*, 1993a) and suggest that this granulosa cell culture system is an appropriate physiological model.

Inhibin, a second major hormone produced by non-luteinized granulosa cells (Luck *et al.*, 1990; Bramley *et al.*, 1992), is also secreted in increasing amounts by granulosa cells from both small and large follicles maintained in serum-free culture. In contrast to oestradiol, inhibin production was not responsive to FSH when the results were expressed on a per cell basis. This observation supports *in vivo* findings that ovarian inhibin secretion is not acutely responsive to FSH (Campbell *et al.*, 1991b) and suggests that the increase in ovarian inhibin secretion observed after FSH infusion (Tsonis *et al.*, 1988; Campbell *et al.*, 1993a) mainly reflects an increase in the number of granulosa cells. However, it is clear from both the present experiment and from the findings of Campbell *et al.* (1991a) that granulosa cells from large follicles secrete more inhibin on a per cell basis than do granulosa cells from small follicles. This finding indicates that this increase in inhibin production is a non-FSH-dependent result of granulosa cell differentiation.

As with inhibin, progesterone secretion by cultured granulosa cells from both size classes increased markedly with time, independently of FSH-stimulation. It is unclear whether this increase in progesterone secretion is simply a reflection of the increase in the steroidogenic potential of the cells as they differentiate or whether it indicates that a proportion of the cells have luteinized. As neither oestradiol nor inhibin are secreted by the ovine corpus luteum (Rodgers *et al.*, 1989), the fact that the cells in this culture system secrete both oestradiol and inhibin indicates that we have at least partially overcome the problem of spontaneous luteinization of granulosa cells in culture. However, it is difficult to determine how much progesterone granulosa cells secrete *in vivo*, as the theca uses granulosa-derived progesterone as a substrate for androgen synthesis (Dorrington *et al.*, 1987) and the concentration of progesterone in follicular fluid may therefore not indicate the amount of production. McNatty *et al.* (1984) reported that progesterone comprises 80% of the steroid secreted by bovine granulosa cells during short term incubations, and this estimate agrees favourably with the results of the present study that indicate that granulosa cells secrete about ten times more progesterone than oestradiol during the initial 48 h of culture. Certainly the amount of progesterone secreted by granulosa cells from large follicles stimulated by IGF-I and FSH reported in this experiment is 100 times less than the amount of progesterone secretion by similarly stimulated luteinized sheep granulosa cells (Monniaux and Pisselet, 1992). In addition, in cattle it has been observed that expression of mRNA encoding for cytochrome P_{450} side chain cleavage is higher in differentiated granulosa cells from the dominant follicle than in undifferentiated cells from small follicles (Xu *et al.*, 1995). It is therefore likely that the increase in progesterone secretion by cultured granulosa cells with time mainly reflects an increase in the steroidogenic potential of the cells as they differentiate.

We have hypothesized that the physiological basis of follicle selection is the differential expression of factors that modulate the action of gonadotrophins on follicular cells at key points during the process of follicle development (Campbell *et al.*, 1995). We have used the ovarian autotransplant model to show that intraovarian infusion of both EGF (Murray *et al.*, 1992) and TGF-α (Campbell *et al.*, 1994) *in vivo* leads to the acute inhibition of ovarian hormone secretion that is associated with

induction of atresia in the large follicle population (Radford *et al.*, 1987; Campbell *et al.*, 1994). Alternatively, intraovarian infusion of LR3 IGF-I results in an acute and prolonged stimulation of ovarian steroid secretion (Campbell *et al.*, 1993b), indicating that IGF-I can act *in vivo* to enhance hormone production by gonadotrophin-dependent follicles. In the present experiment, we used the granulosa cell model system to confirm and extend the results of these studies *in vivo*. Both EGF and TGF-α stimulated marked increases in proliferation of granulosa cells from both small and large follicles and a concomitant inhibition of oestradiol and inhibin synthesis. In contrast to the proliferative growth factors, both insulin and IGF-I can stimulate granulosa cell proliferation in conjunction with increasing oestradiol and inhibin synthesis (on a per cell basis). While the effects of insulin at supraphysiological doses are probably mediated via the type 1 IGF receptor (Adashi *et al.*, 1985), we have shown that both IGF-I and insulin interact at physiological concentrations to influence both cellular proliferation and hormone production, suggesting that both these metabolic hormones act through their own receptors to stimulate granulosa cell differentiation. The similarity of the responses to the putative intraovarian factors examined *in vivo* and *in vitro* provides further evidence that physiologically relevant inferences can be drawn from this granulosa cell culture system. Furthermore, these observations support the hypothesis that the stimulatory action of gonadotrophins can be modulated in the ovary by locally produced factors. The development, for the first time, of a physiological granulosa cell culture system for sheep provides us with a valuable model to further elucidate the mechanism of follicle selection in this species.

The culture system for sheep granulosa cells described in this paper has three key features. The first is the use of a totally serum-free and attachment factor-free culture system. During initial attempts to establish a sheep culture system, it was found that, although cell viability was high when cultures were performed in the presence of serum or with donor calf serum, vitronectin or fibronectin treated plates, oestradiol production by cells cultured under these conditions was low or was not responsive to FSH (Campbell, 1989). The second key feature of this culture system is the inclusion of insulin or IGF-I, as cell viability and hormone production is low in serum-free culture in their absence. As in many serum-free culture systems, insulin alone at supraphysiological concentrations was used initially in this system (Campbell and Scaramuzzi, 1991) and while many of the effects of insulin at these doses are probably mediated via the type 1 IGF receptor (Adashi *et al.*, 1985), the results of this study show that both IGF-I and insulin interact at physiological concentrations to influence both cellular proliferation and hormone production. The LR3 IGF-I analogue used in these studies is ten times more potent than is native IGF-I (Howard and Ford, 1994; B. K. Campbell, R. J. Scaramuzzi and R. Webb, unpublished), presumably owing to its decreased affinity for IGF binding proteins. The final key feature of this culture system is the plating density used. Preliminary studies showed that the induction of oestradiol production by cells from small follicles and the maintenance of oestradiol production by cells from large follicles is critically dependent on cell density, as at low density (< 20 000 cells per well), cell survival is extremely poor, whereas at high density (> 100 000

cells per well), plating efficiencies are high but oestradiol production is low (B. K. Campbell, unpublished). It is possible that this effect of cell density is mediated through the promotion of the clumping of the granulosa cells that is essential for differentiation of the cells through cell-to-cell contact. These three principles have been successfully applied in the development of improved granulosa cell culture systems for both cattle (Guttierez *et al.*, 1994) and pigs (Picton *et al.*, 1994).

In conclusion, this paper reports the development of a physiological culture system for sheep granulosa cells that allows proliferative and differentiative responses to physiological concentrations of FSH *in vitro*. Using this model system, we have demonstrated that these stimulatory actions of FSH can be enhanced by physiological concentrations of insulin and IGF-I or attenuated by TGF-α and EGF. This cell culture model therefore provides a physiologically relevant model to study the cascade of events that control granulosa cell differentiation and ultimately follicle selection in sheep.

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