Maintenance of oestradiol production and expression of cytochrome P450 aromatase enzyme mRNA in long-term serum-free cultures of pig granulosa cells

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Studies were carried out to investigate the conditions required for maintenance of aromatase activity and expression in long-term cultures of pig granulosa cells. Cells from large (> 2 mm) and small (≤ 2 mm) follicles were cultured at 37°C with 5% CO_2 in McCoy's 5a medium supplemented with 0.1% (w/v) BSA, testosterone ($100\text{ }\mu\text{g l}^{-1}$), insulin ($10\text{ }\mu\text{g l}^{-1}$) and long R3 insulin-like growth factor I (IGF-I) ($100\text{ }\mu\text{g l}^{-1}$). Cells were cultured with five concentrations of USDA pFSH-I-2 (0 – $100\text{ }\mu\text{g l}^{-1}$) for 48, 96 or 144 h with or without fetal calf serum (FCS). The number of cells and oestradiol, progesterone and inhibin production were measured. In marked contrast to oestradiol production from cells cultured in plates precoated with FCS, $1\text{ }\mu\text{g FSH l}^{-1}$ was optimal for the maintenance of high oestradiol production by granulosa cells from large follicles after 144 h of serum-free culture. Culture with FCS promoted cell proliferation, reduced oestradiol production, and supported FSH-dependent ($P < 0.01$) increased progesterone and inhibin production indicating cellular luteinization. Northern blot analysis of total RNA from cells cultured with $1\text{ }\mu\text{g FSH l}^{-1}$ detected 2.5 and 1.8 kb transcripts encoding aromatase cytochrome P450 ($\text{P450}_{\text{arom}}$) and cholesterol side-chain cleavage cytochrome P450 (P450_{scc}), respectively. Transcript expression was hormone sensitive, irrespective of the presence of FCS. High concentrations of FSH ($100\text{ }\mu\text{g l}^{-1}$) stimulated expression of P450_{scc} but inhibited $\text{P450}_{\text{arom}}$ expression as the cells luteinized after 144 h of culture. This serum-free system, which maintains the aromatase enzyme complex, is fundamental if physiologically relevant observations are to be made of the mechanisms regulating follicle hierarchy development from long-term cultures of pig cells.

Introduction

It is now well established from experiments *in vivo* and *in vitro* that the ability to produce oestrogens and the establishment of a fully active aromatase enzyme system play a central role in the successful development of ovarian follicles (Hsueh *et al.*, 1984; Hutz, 1989; Tsang *et al.*, 1985). Insights into the biochemistry of steroid synthesis have indicated that several steps in the pathway leading to the production of ovarian oestrogen are catalysed by enzymes of the cytochrome P450 superfamily. Of these enzymes, 17α -hydroxylase cytochrome P450 ($\text{P450}_{17\alpha}$), P450_{scc} and $\text{P450}_{\text{arom}}$ have been localized within different cell types in the pig ovary (Conley *et al.*, 1994). In the follicular phase of the oestrous cycle, the production of oestrogen by preovulatory follicles in response to gonadotrophic stimulation results from the concerted action of both theca and granulosa cells

and the co-ordinated expression of this triad of enzymes (Guthrie *et al.*, 1994).

Although the primary role of gonadotrophins in the stimulation of ovarian function is established (Foxcroft and Hunter, 1985; Guthrie and Bolt, 1990; Conley *et al.*, 1994), the mechanisms regulating these processes are not fully understood. In recent years, considerable attention has focused on the establishment of model systems *in vitro* that reflect endocrine-dependent granulosa cell development *in vivo*. More specifically, cultured pig granulosa cells have been used to investigate temporal changes in the activity and gene expression of the steroidogenic enzymes, regulation of gonadotrophin receptor induction and function, and intercellular communication and cell growth. However, interpretation of the findings of studies *in vitro* are only meaningful if these gonadal cells maintain their *in vivo* biochemical characteristics during culture.

Historically, monolayers of pig granulosa cells cultured in the presence of serum have been used to investigate the

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effects of hormonal treatments on steroidogenesis (Erickson *et al.*, 1979; Ham and McKeehan, 1979; Ford and Lunstra, 1992). The addition of serum provides a complex mixture of hormones, nutrients, and growth and attachment factors that are necessary to support cell proliferation. However, interpretation of these studies has been complicated by the inhibitory effects of serum on follicular-type granulosa cell function. These effects are associated with increased cell proliferation (Erickson *et al.*, 1979; Orly *et al.*, 1980). Typically, in the presence of serum, granulosa cell monolayers rapidly lose the ability to secrete oestrogen, the key biochemical marker of follicular phenotype in these cultured cells (Henderson and Moon, 1979; Haney and Schomberg, 1981; May and Schomberg, 1984).

A more physiologically acceptable approach is the use of chemically defined serum-free culture systems which allow more detailed examination of factors regulating both replication and cytodifferentiation of ovarian cells. To date, different approaches have been used for the serum-free culture of granulosa cells, including totally serum-free systems (Barnes and Sato, 1980; Mondschein *et al.*, 1989), and those that use fibronectin (Ford and Lunstra, 1992), collagen (Lino *et al.*, 1985a,b) or precoating with FCS (Wrathall and Knight, 1993) to aid cellular attachment. In culture systems including serum, it is possible to measure oestradiol production in the first 48 h of culture after which, characteristically, aromatase activity declines exponentially as the cells luteinize (May and Schomberg, 1981; Stoklosowa *et al.*, 1982). This is also possible with systems in which attachment factors are used (Buck and Schomberg, 1987). The terminal differentiation of the cultured cells is indicated by a marked increase in progesterone secretion in response to stimulation by FSH. Improved serum-free culture methods have been reported for ovine and bovine granulosa cells (Campbell *et al.*, 1996; Gutierrez *et al.*, 1997). The approach of Campbell *et al.* (1996) offers a major advance over previous studies because, depending on the physiological status of the cultured cells, it is possible to induce and maintain oestradiol production in response to physiological concentrations of gonadotrophins in the absence of both attachment factors and serum. Development of a similar serum-free system for pig cells will allow detailed examination of factors regulating the replication and differentiation of ovarian cells in this economically important species.

The maintenance of normal follicular aromatase activity and oestradiol production is fundamental if long-term cell culture systems are to be used to extend physiological understanding of the cellular and molecular regulation of follicular function in pigs. Therefore, the objective of the present study was to develop a long-term serum-free culture system that sustains aromatase activity in pig granulosa cells cultured in the presence of physiological concentrations of FSH. The experiments aimed to identify and optimize the conditions required for the maintenance of preovulatory oestradiol production and expression of the P450_{arom} enzyme complex in granulosa cells cultured in the absence of serum or attachment factors. Oestradiol, progesterone and inhibin production were measured as steroidal and non-steroidal discriminants, respectively, of the differentiated status of the cultured cells.

Materials and Methods

Follicles were dissected from adult pig ovaries obtained from the local abattoir. Granulosa cells from the follicles were used to conduct a series of experiments to compare the morphology and steroidogenic properties of cells grown in defined serum-free medium, medium supplemented with FCS, and serum-free medium in plates precoated with FCS. All media were equilibrated in a humidified atmosphere with 5% CO₂ in air at 37°C before use. All tissue culture media and additives were obtained from Sigma Chemical Co. Ltd, Poole, unless otherwise stated.

Experiment 1

Granulosa cells were cultured according to Wrathall and Knight (1993). Pig ovaries were collected and washed in Dulbecco's modified Eagle's medium (DMEM) and Ham's F12 solution (Flow Laboratories, High Wycombe) containing, 100 kiu penicillin l⁻¹, 0.1 µg streptomycin l⁻¹ and 1 mg amphoterecin l⁻¹, at 37°C. Granulosa cells were recovered aseptically from follicles 4–6 in mm diameter by scraping the inner surface of the follicles with a sterile inoculating loop. The cells were washed twice by centrifugation at 800 g for 10 min in 1:1 (v/v) DMEM and Ham's F12 solution containing 100 kiu penicillin l⁻¹, 0.1 µg streptomycin l⁻¹, 3 mmol L-glutamine l⁻¹, 10 mg transferrin l⁻¹ and 5 µg selenium l⁻¹. The cells were then cultured in plates precoated with 10% (v/v) FCS in DMEM. For culture, the medium detailed above was further supplemented with 100 µg testosterone l⁻¹ and 0.3% (w/v) tissue culture grade BSA (for the first 24 h only). Cells were cultured with 0, 10 or 100 µg pig FSH l⁻¹ (USDA pFSH-I-2; biological potency 81.6 U mg⁻¹) as reported previously (May and Schomberg, 1981, 1984; Buck and Schomberg, 1987; Ford and Lunstra, 1992; Wrathall and Knight, 1993; Howard and Ford, 1994) in the presence of a range of concentrations of bovine insulin (5, 10, 50, 100 and 500 mg l⁻¹) as described by Lino *et al.* (1985a,b), Maruo *et al.* (1988) Mondschein *et al.* (1989), Hylka *et al.* (1989) and Wrathall and Knight (1993). Cells were inoculated at a concentration of 5 × 10⁵ viable cells ml⁻¹ (May and Schomberg, 1984; Hylka *et al.*, 1989; Howard and Ford, 1994) and were cultured in a humidified atmosphere with 5% CO₂ in air at 37°C. Fresh medium and treatments were added at 24 h intervals for 96 h. Spent medium was stored at -20°C until assayed for oestradiol. Treatments were in replicates of four and the culture was repeated twice.

Experiment 2

Pig ovaries were collected and washed in medium 199 containing 20 mmol Hepes l⁻¹, 100 kiu penicillin l⁻¹, 0.1 µg streptomycin l⁻¹ and 1 mg amphoterecin l⁻¹ and all follicles ≥ 1 mm in diameter were dissected under sterile conditions. Granulosa cells were harvested from large steroidogenically differentiated follicles > 2 mm in diameter and small undifferentiated follicles ≤ 2 mm in diameter according to Campbell *et al.* (1996). Briefly, after dissection, follicular fluid

was aspirated from follicles > 2 mm in diameter, the follicles were hemisected, and granulosa cells (LGCs) were collected into Dulbecco's PBS without calcium or magnesium (DPBS) by scraping the follicle shells with a sterile inoculation loop. The LGCs were harvested by centrifugation at 800 *g* for 10 min. The LGCs were then washed twice in DPBS and were resuspended in McCoy's 5a medium containing bicarbonate, 20 mmol Hepes l⁻¹, 100 kiu penicillin l⁻¹, 0.1 µg streptomycin l⁻¹, 50 mg gentamicin l⁻¹, 3 mmol L-glutamine l⁻¹, 0.1% (w/v) BSA, 100 µg testosterone l⁻¹, 2.5 mg transferrin l⁻¹, 4 µg selenium l⁻¹, 10 µg bovine insulin l⁻¹ and 100 µg long R3-IGF-I l⁻¹ (media grade; Gropep Pty Ltd, Adelaide). After hemisection, granulosa cells from small follicles (SGCs) were collected by repeated aspiration of the follicle shells through a sterile 1 ml syringe into 5 ml DPBS at 37°C. The follicle shells were discarded and the SGC suspension was harvested by centrifugation as described for LGCs.

For both LGCs and SGCs, 1.5×10^5 viable cells per well were seeded into 96-well plates (Nunc, Life technologies Ltd, Paisley). Cells were cultured for 48, 96 or 144 h in a total volume of 250 µl with 0, 0.1, 1.0, 10.0 or 100.0 µg pig FSH l⁻¹ in the absence or presence of a step-down concentration of FCS (10% (v/v) FCS for the first 48 h and 2.5% (v/v) FCS thereafter, as used by Ford and Lunstra (1992)). The range of FSH concentrations used encompassed the basal (4.9–7.3 µg l⁻¹) and surge (28.5–34.4 µg l⁻¹) concentrations of FSH measured in the plasma of different breeds of pig during the periovulatory period (Hunter *et al.*, 1993). Only 175 µl culture medium per well was replenished at each change of media to minimize the disturbance of cells cultured in serum-free conditions. Spent medium was stored at –20°C until assay. Three repeat cultures were carried out with four replicates per treatment in each culture.

At the end of culture, the effect of treatments on the number of viable cells and cell morphology was assessed by measuring the uptake of the vital dye neutral red after a 3 h incubation period (Borenfreund and Puerner, 1984; Campbell *et al.*, 1996). Incorporation of the dye by viable cells was assessed by measuring absorbance at 540 nm using a multi-well plate reader. The number of cells was calculated by comparing the absorbance of treated cells with that of standard preparations of cultured cells. The limit of detection of the assay was 2000 viable cells per well.

Northern blot analysis

Additional LGCs were cultured at a concentration of 4.4×10^6 viable cells per well in six-well culture plates to enable measurement of expression of mRNA encoding the steroidogenic enzymes P450_{arom} and P450_{sc}. Three replicate wells were cultured for 48 or 144 h with 1 or 100 µg FSH l⁻¹ in the presence or absence of FCS. Each culture was carried out according to the method for Expt 2, and was repeated three times.

At the end of each culture, spent media was removed and the cells were snap-frozen in liquid nitrogen and stored at –70°C for subsequent RNA preparation. Total RNA was extracted from pooled cells for each treatment using phenol–chloroform (Chomczynski and Sacchi, 1987).

Electrophoretic separation was carried out on 25 µg aliquots of total RNA using denaturing agarose formaldehyde gels (Doody *et al.*, 1990). After electrophoresis, RNA was capillary blotted onto nylon membranes (Zetaprobe; Bio-Rad Laboratories Ltd, Hemel Hempstead) which were air dried and baked at 80°C in a vacuum oven for 2 h. Specific mRNA species were detected in separate sequential hybridization experiments carried out at 42°C with ³²P-labelled cDNA probes (Megaprime; Amersham International plc, Amersham) to bovine P450_{sc} (Lauber *et al.*, 1993) and human P450_{arom} (Means *et al.*, 1989). The specific activity of each probe was 1×10^6 c.p.m. ng⁻¹ cDNA. Blots were exposed to X-ray film (Kodak X-OMAT-AR-2; Sigma Chemical Co. Ltd, Poole) at –70°C. After exposure to each probe, blots were stripped according to the manufacturer's recommendations. Blots were stripped and reprobed with a ³²P-labelled oligonucleotide probe directed against 18S ribosomal RNA (Saunders *et al.*, 1993) to enable quantitation and to control RNA loading. Expression of each RNA species was quantified by electronic autoradiography (Instantimager; Packard Instrument company, Meriden). mRNA for each enzyme was expressed as arbitrary relative intensity units after correction for both background and 18S ribosomal RNA loading (Samaras *et al.*, 1992). Northern analyses were carried out on RNA from each of three separate culture experiments.

Assays

The concentrations of oestradiol (Expts 1 and 2) and inhibin p1-26α subunit (Expt 2) were assessed in unextracted culture medium using radioimmunoassays (Webb *et al.*, 1985; McNeilly *et al.*, 1989). Progesterone concentration (Expt 2) was measured using a commercially available ELISA kit (Ridgeway Science Ltd, Alvington). The sensitivities of the oestradiol, progesterone and inhibin assays were 1.4, 1.5 and 35.0 ng l⁻¹, respectively. The intra- and interassay coefficients of variation were 2.7 and 20.0%, 14.4 and 15.5%, and 6.5 and 11.8% for oestradiol, progesterone and inhibin, respectively. All of the assays used have been validated for the measurement of oestradiol, progesterone and inhibin in medium with and without serum.

Statistical analysis

All hormone data were expressed as hormone produced per 10000 viable cells per 48 h after correction for any residual medium left in the wells at each media change. The number of cells represents the number of viable cells at the end of each 48 h culture period. The data obtained at 96 h of culture were intermediary between that at 48 and 144 h, and were not included in further analysis. Univariate repeat measure analysis was carried out to determine the effect of time, follicle size, FSH concentration, FCS and the FSH–FCS interaction on cultured cells with replicate cultures used as the block structure. Hormone production data were analysed after logarithmic transformation ($\log_{10}(x + 1)$) of hormone produced per 48 h by 10^4 cells to correct for heterogeneity of

variance. The statistical significance of the difference between mean steroid production in the first and last 48 h of culture was assessed by ANOVA followed by Bonferroni's test. Similar analyses were carried out on northern blots from three repeat cultures to determine the effect of FSH on expression of mRNA encoding P450_{scc} and P450_{arom}. All data presented are least square means $\pm$ SEM.

Results

Experiment 1

Granulosa cells cultured in plates precoated with serum formed flattened epithelioid monolayers. There was no significant effect of FSH or insulin on cell proliferation or oestradiol secretion after 96 h of culture. Furthermore, none of the concentrations of insulin or FSH was able to maintain oestradiol production and a rapid decrease in oestradiol production was recorded for all combinations of FSH and insulin after the first 24 h of culture. This decline was significant ($P < 0.05$) after 72 h of culture (Fig. 1). After 96 h of

Fig. 1. Comparison of serum-free culture strategies for the maintenance of oestradiol production per cell by pig granulosa cells from large follicles. Cells cultured using the methodology of Wrathall and Knight (1993) in plates precoated with 10% (v/v) FCS with (□) 0 or (■) 10 µg USDA pFSH-I-2 l⁻¹ (method 1) were compared with cells cultured according to the method of Campbell *et al.* (1996) in plates with no precoating with (▨) 0 or (▩) 1 µg FSH l⁻¹ (method 2). Bars with different letters are significantly different ($P < 0.05$). The inset graph shows the rapid decline in oestradiol production in cells cultured according to method 1 in the presence of 10 µg FSH l⁻¹. Inset values are means $\pm$ SEM of two independent replicate cultures; * $P < 0.05$.

culture, oestradiol production was below the limit of detection of the assay even in the presence of high concentrations of FSH and insulin. Irrespective of the concentration of insulin, after 96 h, cells cultured in plates precoated with 10% (v/v) FCS in the presence of 0, 10 and 100 ng FSH ml⁻¹, produced only 0.4, 0.3 and 0.6%, respectively, of the initial oestradiol production. These data are in marked contrast to the oestradiol production maintained in LGCs cultured in the serum-free conditions in Expt 2 (Fig. 1).

Experiment 2

Cell morphology. Although the cells cultured under serum-free conditions were well dispersed at the initiation of culture, within 48 h, they had formed dense interconnected clumps of rounded cells, irrespective of the size of the follicles from which the cells were harvested. These cells were shown to be viable when incubated with neutral red dye (Fig. 2a). Elongated spindle-shaped cells projected between clumps and anchored the clumps to the plate. During extended culture, the clumps merged due to the outgrowth of granulosa cells along the spindle cells (Fig. 2b). In contrast, all granulosa cells cultured in the presence of serum formed flattened epithelioid monolayers (Fig. 2c). After 144 h, cells cultured in the presence of FCS were confluent.

Cell proliferation. After 48 h of culture with FCS, there was a significant five- to tenfold increase ($P < 0.01$) in the number of viable cells compared with that in the initial inoculum, irrespective of follicular size. In contrast, in the absence of serum, a 1.6–3-fold increase in the number of viable cells was recorded in the first 48 h of culture for both SGCs and LGCs. Although no further increase in the number of viable cells was detected after 48 h of culture, the significant effect of FCS on the number of cells was maintained after 144 h of culture ($P < 0.01$) and was dependent on FSH concentration. High concentrations (10 and 100 µg FSH l⁻¹) significantly inhibited the proliferation of both LGCs ($P < 0.05$) and SGCs ($P < 0.01$) cultured with FCS. Similar concentrations of FSH inhibited the number of viable cells after 144 h of culture under serum-free conditions. However, this effect was only significant for SGCs ($P < 0.01$). There was a significant interaction between FCS and FSH in determination of the number of viable cells (LGCs: $P < 0.05$; SGCs: $P < 0.01$), irrespective of the culture period. For LGCs, 1 µg FSH l⁻¹ was optimal for maintenance of the number of viable cells between 48 and 144 h of culture in the absence of serum (Fig. 3a,e). There was no significant difference in the effect of treatment on the number of cells between LGCs and SGCs after 48 h. The response of large and small cells to treatment was significantly different at 144 h of culture ($P < 0.01$).

Hormone production. The results demonstrate that it is possible to maintain high oestradiol production between 48 and 144 h in cultures of LGCs in the absence of FCS. Comparison of the mean data at each time point identified 1 µg FSH l⁻¹ as the concentration required to maintain the highest oestradiol production after 144 h of culture in the

Fig. 2. Comparison of the morphology of pig granulosa cells from follicles > 2 mm in diameter after (a) 48 h culture in the absence of fetal calf serum (FCS), (b) 144 h culture in the absence of FCS, and (c) 144 h culture in the presence of FCS. Cells were stained with the vital dye neutral red to indicate viability. Cells in serum-free culture were characterized by the formation of discrete clumps within 48h of culture (a), which became interconnected by spindle-shaped cells after 144 h of culture (b). In contrast, in the presence of serum, cells formed flattened epitheloid monolayers (c). For comparison, (d) shows monolayers of pig luteal cells cultured for 144 h in the presence of FCS. Scale bars represent 5 µm.

absence of serum (Fig. 3b,f). At this FSH concentration, there was no difference in oestradiol production between 48 and 144 h of culture ($P = 0.412$). After 144 h of culture with 1 µg FSH l⁻¹, oestradiol production (LGCs: 51.00 ng per 10 000 cells in 48 h; SGCs: 22.55 ng per 10 000 cells in 48 h) was 3.4- and 4.6-fold higher, respectively ($P < 0.05$), than steroid production in cells cultured with FCS (LGCs: 14.84 ng per 10 000 cells in 48 h; SGCs: 4.90 ng per 10 000 viable cells in 48 h).

At both 48 and 144 h, cells from large follicles produced more oestradiol *in vitro* than cells from small follicles ($P < 0.01$; Fig. 4), irrespective of the presence of serum. Owing to the variability of the data, FSH had no significant effect on oestradiol production by either cell type at either time point and there was no significant interaction between FSH and FCS on oestradiol production by LGCs. In contrast, a significant interaction between FSH and FCS was recorded for SGCs at both 48 and 144 h of culture ($P < 0.01$).

There was a significant effect of FSH ($P < 0.01$) and FCS ($P < 0.05$), and a significant interaction between FSH and FCS on progesterone production (LGCs: $P < 0.01$; SGCs: $P < 0.05$) for both cell types and time points studied in Expt 2. Overall, FSH stimulated a dose-related increase ($P < 0.01$) in progesterone production by LGCs cultured with and without serum at both 48 and 144 h of culture. Furthermore, after 144 h of culture with FCS, LGCs were less sensitive

($P < 0.05$) to the stimulatory effects of FSH than cells cultured without serum, as evidenced by a shift in the dose-response curve to the right (Fig. 4a,b). A similar dose-dependent effect of FSH on progesterone production after 144 h of culture was observed for SGCs cultured with FCS (Fig. 4c,d). At 1 µg FSH l⁻¹ there was an increase in the amount of progesterone produced ($P < 0.01$; Fig. 3c) by LGCs between 48 and 144 h of culture in the absence of serum. A similar increase in progesterone production was observed for SGCs, irrespective of the presence of serum ($P < 0.01$). There was no significant difference in overall progesterone production by cells from large or small follicles at either 48 ($P = 0.197$) or 144 h ($P = 0.342$) of culture, irrespective of the presence of serum.

There was a significant effect of FSH ($P < 0.01$) on inhibin production by both LGCs and SGCs at each time point of culture in Expt 2. Furthermore, inhibin production was significantly higher in all cells cultured with FCS ($P < 0.01$; Fig. 4), irrespective of the source of the cells. In addition, there was a significant interaction between FSH and FCS ($P < 0.01$) for LGCs after 144 h of culture. After 144 h of culture in the presence of serum, there was a dose-dependent increase ($P < 0.01$) in inhibin production over control values in response to concentrations of ≥ 1 µg FSH l⁻¹. Similar responses ($P < 0.01$) were recorded for SGCs cultured with FCS (Fig. 3h). In the absence of serum, inhibin production decreased between 48

Fig. 3. Comparison of the effect of 1 µg USDA pFSH-I-2 I⁻¹ on (a,b) the number of cells, and (c,d) oestradiol, (e,f) progesterone and (g,h) inhibin production by pig granulosa cells isolated from large (> 2 mm; a,c,e,g) and small (≤ 2 mm; b,d,f,h) follicles and cultured for 0–48 and 96–144 h in the (■) presence or (□) absence of fetal calf serum. Note that the hormone production data have been corrected for the effect of the number of cells. The values are the least square means ± SEM of three independent replicate cultures. Bars with different letters are significantly different as indicated within each category (ANOVA with Bonferroni's test).

and 144 h in both LGCs and SGCs cultured with 1 µg FSH I⁻¹ (Fig. 3d,h). This effect was significant ($P < 0.05$) for LGCs. In the first 48 h of culture, overall inhibin production was higher

in cells from large follicles ($P < 0.01$) than in those from small follicles. However, this difference was not apparent after 144 h of culture.

Fig. 4. The effect of USDA pFSH-I-2 on (□,■) oestradiol, (○,●) progesterone and (△,▲) inhibin production per cell by pig granulosa cells isolated from (a,b) large (> 2 mm) and (c,d) small (≤ 2 mm) follicles and cultured for 144 h in the absence (open symbols) or presence (solid symbols) of fetal calf serum (FCS). The values are the least square means $\pm$ SEM of three independent replicate cultures. Comparison of the effects of FCS on hormone production with similar concentrations of FSH, within follicle class size are shown: * $P < 0.05$; ** $P < 0.01$ (ANOVA with Bonferroni's test).

Northern blot analysis

P450_{arom} mRNA was detected in total RNA extracted from LGCs after 48 and 144 h of culture with 1 $\mu\text{g FSH l}^{-1}$ in the presence or absence of FCS (Expt 2) (Figs 5 and 6). A single P450_{arom} mRNA transcript of approximately 2.5 kb was detected in all RNA extracted from LGCs cultured with 1 $\mu\text{g FSH l}^{-1}$, but not in RNA extracted from fresh pig luteal tissue. Increasing the concentration of FSH in the culture medium from 1 to 100 $\mu\text{g FSH l}^{-1}$ resulted in the loss of P450_{arom} expression in both LGCs and SGCs, irrespective of the inclusion of serum in the culture medium. Analysis of individual northern blots from each of three independent cultures (Fig. 6a) indicated that FSH had a significant effect on P450_{arom} expression ($P < 0.01$). However, there was no significant effect of FCS or interaction between FSH and FCS on P450_{arom} expression in these cultured cells.

P450_{ssc} mRNA was detected as a 1.8 kb transcript in all cultured granulosa cells and in luteal tissue. The intensity of the hybridization signals indicated that the amount of P450_{ssc} mRNA was consistently higher in freshly extracted luteal tissue (20.1 ± 8.2 , $n = 3$) than in cultured cells. A transient decrease in P450_{ssc} gene expression was observed in LGCs cultured for 144 h in the absence of serum and with either 1 or 100 $\mu\text{g FSH l}^{-1}$ (Fig. 6b) compared with expression after 48 h of culture. In contrast, in the presence of FCS, there was a progressive increase in P450_{ssc} gene expression with

increasing time in culture and with increasing FSH concentration. Owing to the variability of data, the interaction between FSH and FCS approached significance ($P = 0.06$). The effects of both FSH and FCS on P450_{ssc} gene expression were not significant. Expression of P450_{ssc} mRNA was 1.5–7-fold higher than P450_{arom} mRNA in cells cultured with 1 $\mu\text{g FSH l}^{-1}$, irrespective of the presence of serum. In contrast, P450_{ssc} mRNA gene expression was 90–234-fold higher than P450_{arom} expression in cells cultured for 144 h with 100 $\mu\text{g FSH l}^{-1}$.

Discussion

These data demonstrate that it is possible to maintain high aromatase activity and oestradiol production in long-term serum-free cultures of pig granulosa cells that maintain their follicular phenotype *in vitro*. The amount of oestradiol produced by LGCs after 144 h of culture in Expt 2 is within the range of aromatase activity and oestradiol production in short-term incubations of an equivalent number of granulosa cells isolated from preovulatory pig follicles (Grant *et al.*, 1989; Hunter *et al.*, 1994). This is in marked contrast to the exponential decline in oestradiol production after 24 h using the more conventional culture methods of Expt 1 (Wrathall and Knight, 1993). After 144 h of culture, the maintenance of oestradiol production was associated with FSH-stimulated

Fig. 5. A representative northern blot showing regulation of mRNA expression for aromatase cytochrome P450 (aromatase) and cholesterol side-chain cleavage cytochrome P450 (SCC) in pig granulosa cells isolated from large (> 2 mm) follicles and cultured for 48 or 144 h with 1 or 100 $\mu\text{g USDA pFSH-I-2 l}^{-1}$ in the presence or absence of fetal calf serum (FCS). Total RNA was isolated and used for northern analysis by sequential probing for the two transcripts. Ribosomal bands are indicated on the left and specific hybridizing bands are indicated on the right. 18S ribosomal RNA loading of the same blot is shown in the bottom panel.

increases in progesterone synthesis and maintenance of inhibin production. These data confirm the suitability of this species-specific serum-free culture system for use with ovarian cells from polyovular species such as pigs, as well as ruminant species (Campbell *et al.*, 1996; Gutierrez *et al.*, 1997). Furthermore, the measurements of hormone production by the cultured cells in the present study are supported by the detection of P450_{arom} and P450_{sc} gene expression in LGCs after 144 h of culture, irrespective of the inclusion of serum in the culture medium. This extends the observations made in sheep (Campbell *et al.*, 1996) and cattle (Gutierrez *et al.*, 1997).

The data presented for the size and nature of the transcripts for P450_{arom} and P450_{sc} in Expt 2 conform to those described for pig follicles (Guthrie and Bolt, 1990; Tilley *et al.*, 1992; Conley *et al.*, 1994). Furthermore, the decline in P450_{arom} gene expression between 48 and 144 h of culture indicates that the transcripts were fully functional, as the decrease in expression correlated with the decrease in oestradiol production in cells cultured with 100 $\mu\text{g FSH l}^{-1}$. Pharmacological concentrations of FSH ($\geq 100 \mu\text{g l}^{-1}$) have been widely used to stimulate differentiated functions in cultured pig cells, such as LH-hCG receptor induction and

Fig. 6. Comparison of the relative signal intensity of mRNA for (a) aromatase cytochrome P450 and (b) cholesterol side-chain cleavage cytochrome P450 from large follicle granulosa cells cultured for 48 or 144 h with 1 or 100 $\mu\text{g USDA pFSH-I-2 l}^{-1}$ in the (□) presence or (■) absence of fetal calf serum (FCS). The data are expressed in relative intensity units as a percentage of the corresponding expression of 18S ribosomal RNA; values are least square means $\pm$ SEM for three northern blots from three independent replicate cultures.

progesterone secretion (May and Schomberg 1981; Stoklosowa *et al.*, 1982; Buck and Schomberg, 1987). The results of the present study indicate that such concentrations induce luteinization and downregulate both the activity and expression of P450_{arom} in cultured cells. In the present study, P450_{arom} mRNA was not detected in cells cultured with 100 $\mu\text{g FSH l}^{-1}$. This evidence supports *in vivo* studies demonstrating that, after exposure to the preovulatory gonadotrophin surge, luteinization of the granulosa and theca cells and the formation of the corpus luteum is characterized by the downregulation of P450_{17 α '} and P450_{arom} and a concomitant increase in P450_{sc} activity and mRNA expression (Conley *et al.*, 1995).

A reduction in P450_{sc} gene expression per microgram 18S

ribosomal RNA was also observed in LGCs cultured with $1 \mu\text{g FSH l}^{-1}$ for 144 h under serum-free conditions. This observation was not due to inequality in RNA loading. However, this result may correspond to the transient reduction of both P450_{ssc} mRNA in pig follicles (Guthrie *et al.*, 1994) and of the progesterone content of pig follicular fluid (Eiler and Nalbandov, 1977; Ainsworth *et al.*, 1980) that is consequential to the preovulatory LH surge. This transient decline would be followed by a significant increase in both the activity and expression of the enzyme with the establishment of a fully functional corpus luteum.

Fully differentiated granulosa cells from large follicles produced significantly more oestradiol than granulosa cells from small undifferentiated follicles. These findings support previous observations for pig granulosa cells harvested from different sized follicles (Maruo *et al.*, 1988; Grant *et al.*, 1989) and for incubation of whole pig follicles (Picton and Hunter, 1995). These results demonstrate that *in vitro*, granulosa cell differentiated status and steroid production changes with the developmental status of the follicle from which the cells were initially collected. Furthermore, contrary to the results reported for small sheep follicles (Campbell *et al.*, 1996), the culture conditions used were not sufficient to induce further aromatase activity and expression in cells from small follicles.

In Expt 2, a chemically defined serum-free culture system was developed to support the maintenance of follicular-type biochemical functions in pig granulosa cells. This represents a significant advance over previously published systems (evaluated in Expt 1) for the culture of pig ovarian cells in the presence of FCS (May and Schomberg, 1981, 1984; Maruo *et al.*, 1988), in plates precoated with serum (Wrathall and Knight, 1993) or attachment factors (Lino *et al.*, 1985a,b; Ford and Lunstra, 1992) and in serum-free medium (Mondschein *et al.*, 1989), in which it was not possible to maintain measurable oestradiol production after 48 h of culture. In the majority of these studies, the success of the culture system was judged by the ability of the cells to maintain their attachment to the culture vessel and by the establishment of epithelioid monolayers that are morphologically similar to those obtained when cells are cultured with serum (May and Schomberg, 1981). May and Schomberg (1981) reported that pig granulosa cells do not plate well or survive in moderate to long-term cultures in the absence of serum. In addition, it has been reported that pig granulosa cells terminally differentiate in medium containing 10% FCS in response to the combined action of $10\text{--}100 \mu\text{g FSH l}^{-1}$ and $1 \text{ mg insulin l}^{-1}$ (May and Schomberg, 1981; Buck and Schomberg, 1987), as assessed by increased progesterone production and the concomitant loss of the cells capacity to synthesize oestradiol after 48 h in culture.

The combination of insulin and long-R3 IGF-I used in the present experiments favoured the maintenance of follicular type functions in pig granulosa cells, rather than stimulation of cell proliferation with the attendant loss of aromatase activity and oestrogen production. It is likely that the facilitative effects of insulin and IGF-I act both at the cell membrane, as pig granulosa cells have binding sites for insulin and IGF-I (Otani *et al.*, 1985; Maruo *et al.*, 1988), and intracellularly. They maintain the cells in culture by

enhancing metabolism, as well as inducing, sustaining and amplifying the actions of FSH on differentiated cell function (Maruo *et al.*, 1988). The concentrations of insulin and long R3 IGF-I and the plating density of the cells used in Expt 2 were based on data published for ovine cells (Campbell *et al.*, 1996) after further optimization for use with pig granulosa cells (H. M. Picton, unpublished observations). Long-R3 IGF-I (Howard and Ford, 1994) and insulin (May and Schomberg, 1981) are critical for aspects of granulosa cell development *in vitro*. The concentrations of long-R3 IGF-I used in the present study fell within the range of physiological IGF-I concentrations in pig follicular fluid and serum. Hammond *et al.* (1985) reported that intrafollicular concentrations of IGF-I and insulin were 63 ± 17 and $0.6 \pm 0.07 \mu\text{g l}^{-1}$, respectively, in small follicles and 104 ± 12 and $0.45 \pm 0.04 \mu\text{g l}^{-1}$, respectively, in large follicles. These data, in conjunction with the results for ovine (Campbell *et al.*, 1996) and bovine cells (Gutierrez *et al.*, 1997), indicate that there are species specific differences in the culture requirements for the induction and maintenance of oestrogen production by granulosa cells harvested from follicles of a similar differentiated status from each species. These critical differences include the ratio of the two metabolic hormones insulin and IGF-I and the optimal plating density of the cells. Furthermore, the differences in the FSH concentrations required to stimulate oestradiol production after 144 h of culture among these species cannot be totally attributed to differences in the biological potency of the purified FSH preparations used in each of the studies.

Low but measurable oestradiol production and aromatase mRNA expression were maintained in LGCs cultured in the presence of serum in Expt 2. This was in contrast to the rapid decline in oestradiol production measured in Expt 1. These data highlight the requirement of optimized concentrations and combinations of FSH, insulin, long-R3 IGF-I and cell plating density for the maintenance of oestradiol production and the retention of follicular-type characteristics *in vitro* (as seen in Expt 2). Once these components of the culture system have been optimized (Campbell *et al.*, 1996; Gutierrez *et al.*, 1997), the expected inhibitory effects of serum on oestradiol production can be neutralized. This observation is further supported by the 87% loss in oestradiol production per LGC within 24 h when a suboptimal number of cells were cultured in Expt 1 in the presence of high concentrations of insulin in plates precoated with FCS.

Reports on inhibin production by cultured pig cells are limited, but suggest that inhibin production from mature and immature granulosa cells can be stimulated by FSH (Michel *et al.*, 1989). In contrast to the present results, Michel *et al.* (1989) reported that the ability of granulosa cells to produce inhibin decreased during extended culture. In addition, low inhibin secretion has been reported during the luteal phase of the pig oestrous cycle (Hasegawa *et al.*, 1988) and for cultured pig luteal cells (Michel *et al.*, 1989). No difference in inhibin production was detected in Expt 2 between cells from large and small follicles, but hormone production was lower in the absence of serum. In the presence of serum, inhibin production increased throughout culture but the cells became less sensitive to the stimulatory effects of FSH as evidenced by a shift in the FSH

dose-response curve. It is possible that the discrepancy between this report and published data is the result of different assay methods. The inhibin assay used in the present study measures total immunoreactive inhibin, including inhibin A and B and non-bioactive isoforms of the free α -subunit. Alternatively, the discrepancy may result from differences in the ability of the culture systems to maintain differentiated functions in granulosa cells.

In Expt 2, in the absence of serum and irrespective of follicle diameter, cells in culture formed distinct clumps of viable rounded cells that closely resembled the morphology of granulosa cells in pig follicles *in vivo* (Chang *et al.*, 1977). The clumps formed within 48 h of culture and were morphologically distinct from the epithelioid monolayers formed in the presence of serum. Clumped cells have been observed in both bovine and ovine granulosa cells cultured under similar serum-free conditions (Campbell *et al.*, 1996; Gutierrez, *et al.*, 1997). Therefore, it is highly unlikely that the clumped morphology is indicative of stress and partial cell lysis in cultured pig cells. Indeed, marked differences in cell morphology have been observed in rat and human granulosa cells cultured with or without serum or extracellular matrix proteins (Furman *et al.*, 1986; Amsterdam and Rotmensch, 1987). In the present study, all cells were inoculated as dispersed suspensions and rapid aggregation, as observed by time-lapse video photography (H. M. Picton, unpublished observations), occurred within 6 h of the start of culture, but only in cells cultured in the absence of serum. In Expt 2, the presence of serum prevented clumping and prompted the cells to attach to the plastic, flatten and proliferate, with the establishment of epithelioid monolayers.

The morphological differences in the cultured cells in the presence or absence of FCS and the associated changes in their actin cytoskeleton (Ben-Ze'ev and Amsterdam, 1988) may have a role in the transduction of endocrine and paracrine steroidogenic signals in these cells, so affecting their ability to produce significant amounts of oestradiol in response to FSH *in vitro*. Ford and Lunstra (1992) reported that pig mural granulosa cells formed tightly bound aggregates that retained higher oestradiol production *in vitro* than cells aspirated with follicular fluid. In the present study, the cell populations were harvested by rigorous repeat pipetting (SGCs) and scraping of the follicle wall (LGCs), and would include a high proportion of mural cells. This could account, in part, for their ability to produce oestradiol after extended culture. In addition, the physiological morphology of the clumped cells, together with their ability to secrete compounds such as fibronectin *in vitro* as determined by western blotting (H. M. Picton, unpublished observations), may have prompted development of an extensive system of gap junctions between the cells in response to stimulation by FSH and insulin (Amsterdam *et al.*, 1988). Ultrastructural characterization of multilayer cultures of human granulosa cells grown on collagen revealed that these rounded cells have abundant mitochondria, lipid droplets and numerous gap junctions (Ben-Rafael *et al.*, 1988). While the formation of a network of gap junctions may be responsible for the maintenance of oestradiol production by the cultured cells in the present study, the oestradiol produced may further enhance cell-cell

communication by increasing intercellular processes and gap junctions, and FSH-stimulated progesterone production (Hutz, 1989). The presence of two distinct cell types (rounded clumped cells and spindle-shaped cells) have been reported in serum-free cultures of ovine (Campbell *et al.*, 1996) and bovine granulosa cells (Gutierrez, *et al.*, 1997). The significance of different morphologies in the serum-free cultured cells has yet to be fully evaluated.

In conclusion, these results demonstrate the development of a long-term serum-free culture system for pig cells that supports the maintenance of normal differentiated granulosa cell function *in vitro*. Both the activity and expression of the steroidogenic enzymes P450_{arom} and P450_{scc}, which are responsible for the production of oestradiol and progesterone by these cultured cells, were regulated in an FSH-dependent manner. Exploitation of this physiologically relevant culture system for the extended culture of pig cells will facilitate evaluation of the possible paracrine and autocrine pathways involved in regulating the development of follicular hierarchy in this polyovular species.

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