Interaction of bovine granulosa and theca cells in a novel serum-free co-culture system

C. Allegrucci¹, M. G. Hunter¹, R. Webb² and M. R. Luck^{1*}

¹Division of Animal Physiology, and ²Division of Agricultural Sciences, School of Biosciences, University of Nottingham, Sutton Bonington, Loughborough LE12 5RD, UK

The objective of this study was to develop a defined culture system in which bovine follicular and granulosa cells are grown in close contact with each other and with the extracellular matrix (ECM) component laminin. Granulosa and theca cells from follicles 4–6 mm in diameter were cultured on either side of laminin-coated BioCoat™ cell culture inserts in a serum-free medium containing 10 ng insulin ml⁻¹ at plating densities of 10⁵ and 3 × 10⁵ cells per membrane side. The cells adopted a clumped arrangement, maintained steroidogenic activity for at least 7 days and demonstrated paracrine communication by increased steroidogenesis and enhanced cell survival compared with cells in mono-culture. Co-cultured theca cells secreted significantly more androstenedione compared with cells in mono-culture. Granulosa cell viability was doubled by

co-culture with theca cells. Co-cultures at both cell plating densities were responsive to treatment with physiological combinations of either FSH, LH and LR3 insulin-like growth factor I (IGF-I) (treatment A) or FSH, LR3 IGF-I and androstenedione (treatment B). Significantly more androstenedione was secreted in the presence of treatment A compared with controls. In contrast, oestradiol secretion was increased only by treatment B. Progesterone secretion was unaffected by treatment and did not increase during culture. Co-cultures at the higher plating density demonstrated higher theca cell survival and better maintenance of the follicular cell phenotype. In conclusion, this novel co-culture system provides a unique model for the study of paracrine communication between ovarian somatic cells and cell–ECM interactions during follicle growth.

Introduction

Follicle development is a dynamic process controlled by a complex of extraovarian signals, including gonadotrophins, and locally produced ovarian hormones and growth factors.

During follicle growth, marked proliferation and differentiation of the follicular somatic cells takes place. These events are finely regulated by locally acting factors with both autocrine and paracrine actions. However, little is known about how granulosa and theca cells communicate on either side of the follicular basal lamina, particularly during the crucial expansion phases of follicle development (Webb *et al.*, 1999). The basal lamina creates a permeable barrier between the two follicular cell compartments and the presence of this extracellular matrix (ECM) boundary influences the interactions of follicular somatic cells (Luck, 1994; Rodgers *et al.*, 2000). Communication between granulosa and theca cells is exerted at the hormonal level. Indeed, co-ordination of cellular activity is required for hormone biosynthesis under gonadotrophic control (Webb *et al.*, 1999). Theca cells synthesize androgens, which are

the substrate for aromatization and act as paracrine hormones to control steroidogenesis induced by FSH in granulosa cells (Gore-Langton and Armstrong, 1994; Webb *et al.*, 1999). Oestradiol is also known to stimulate thecal production of androstenedione and decrease progesterone synthesis (Roberts and Skinner, 1990). Intraovarian regulators, such as cytokines and growth factors, mediate the effects of gonadotrophins in regulating granulosa–theca cell interactions. Communication between bovine granulosa and theca cells is mediated in part by a range of factors, including kit ligand, keratinocyte growth factor (KGF), hepatocyte growth factor (HGF), transforming growth factor β (TGF- β), epidermal growth factors (EGF and TGF- α), fibroblast growth factor, insulin-like growth factors I and II (IGF-I and -II), activin, inhibin and follistatin (Monniaux *et al.*, 1997; Webb *et al.*, 1999; Knight and Glister, 2001; Nilsson and Skinner, 2001). However, increasing evidence indicates that the interaction between granulosa and theca cells depends not only on paracrine communication, but also on their contact with the basal lamina (Luck, 1994; Rodgers *et al.*, 2000; Rodgers and Irving-Rodgers, 2002). Basement membrane and associated ECM components have different functions in many tissues, including control of cell anchorage, migration, division, differentiation and death (Adams and Watt, 1993;

*Correspondence

Email: martin.luck@nottingham.ac.uk

Streuli, 1999). The ECM can also interact with growth factors in a variety of ways to regulate cell behaviour. The ECM not only provides binding sites for growth factors, but also participates in the mechanism of reciprocal regulation, which is responsible for gene expression of ECM proteins and their receptors, and of expression of growth factors and their receptors (Adams and Watt, 1993; Streuli, 1999). Recent evidence supports the involvement of ECM components in regulating granulosa cell survival, proliferation and steroidogenesis in the ovarian follicles of several species (Aharoni *et al.*, 1997; Huet *et al.*, 2001; Le Bellego *et al.*, 2002). A continuous remodelling of the basal lamina occurs during follicle development and its composition is altered during this process (Luck, 1994; Rodgers *et al.*, 2000).

Theca and granulosa cells need to be cultured under serum-free conditions, in close proximity to each other and to ECM components to reproduce the *in vivo* environment of the follicle. Serum-free conditions are a crucial requirement because, although the addition of serum provides a complex mixture of hormones, growth and adhesion factors required for cell proliferation, it is impossible to control or know the identity of all the constituents of serum that affect cell function and differentiation. In addition, use of serum can apparently result in the luteinization of granulosa and theca cells (Gong *et al.*, 1994; Wrathall and Knight, 1995), as characterized by a decrease in oestradiol and androstenedione production and by a rapid and sustained increase in progesterone synthesis. Improved serum-free culture methods have been developed for granulosa (Campbell *et al.*, 1996; Gutierrez *et al.*, 1997; Picton *et al.*, 1999) and theca cells (Campbell *et al.*, 1998; Shores *et al.*, 2000), but these models use granulosa and theca cells in separate cultures or in mixed cultures without separation by an ECM-coated membrane. In a co-culture system, bovine granulosa and theca cells have been placed on opposite sides of a collagen type I membrane (Yada *et al.*, 1999; Tajima *et al.*, 2002). This system involved preliminary culture with serum and exposed the cells to high concentrations of insulin.

The aim of the present study was to develop an entirely serum-free co-culture of follicular somatic cells, which would resemble, as closely as possible, the *in vivo* conditions of follicle development, particularly with respect to ECM exposure and hormonal support. Although collagen type I participates in the maintenance of normal hormone secretion by granulosa cells, laminin is known to sustain follicle development by enhancing cell survival and proliferation (Huet *et al.*, 2001). Therefore, in the present study cultured granulosa and theca cells were cultured on opposite sides of a laminin-coated, porous membrane. This arrangement provides adjacent but separated cellular compartments with a contiguous fluid phase, which can be used to investigate cellular interactions. The use of defined media has allowed us to study the effects of physiological doses of IGF-I,

insulin and gonadotrophins and establish conditions under which co-cultured cells maintain their follicular phenotype.

Materials and Methods

Materials

Dulbecco's modified Eagle's medium and nutrient mixture F-12 Ham (DMEM-F12), and penicillin-streptomycin-glutamine were from Gibco-Invitrogen Corporation (Paisley, Renfrewshire). Falcon BD BioCoat™ cell culture inserts, mouse laminin (from Engelbreth-Holm-Swarm mouse tumour) were from BD Biosciences (Cowley, Oxford). Ovine FSH (NIDDK-oFSH-19-SIAPP, bioactivity 94 SI units mg⁻¹) was generously donated by National Institute of Diabetes and Digestive and Kidney Diseases (Torrance, CA). Bovine LH (USDA-bLH-B6-AFP11743B, bioactivity 2.3 SI units mg⁻¹) was generously donated by US Department of Agriculture (Beltsville, MD). ELISA kits for progesterone analysis were purchased from Ridgeway Science Ltd (Alvington, Gloucestershire). All other chemicals and reagents were purchased from Sigma-Aldrich Company Ltd (Poole, Dorset).

Isolation of theca and granulosa cells

Bovine ovaries, obtained from an abattoir at random stages of the oestrous cycle, were transported at ambient temperature in isotonic solution. After several washes in isotonic solution, selected ovaries were rinsed briefly in 70% (v/v) ethanol and then transferred into collection medium (DMEM-F12 medium containing 100.0 U penicillin ml⁻¹, 100.0 µg streptomycin ml⁻¹, 2.5 µg amphotericin B ml⁻¹). The stage of the oestrous cycle was determined morphologically, as described by Ireland *et al.* (1980). Healthy developing follicles were assessed according to Metcalf (1982) for a vascularized pink theca externa and amber follicular fluid without debris. Medium-sized follicles (4–6 mm in diameter) were selected and follicular fluid was aspirated using a syringe with a 19-gauge needle. Follicles were opened by making a small incision on the surface and the interior was washed with a few drops of collection medium to remove residual follicular fluid and cell debris. Mural granulosa cells were removed by gentle scraping of the follicle wall with a plastic inoculation loop and collected by aspiration. The theca cell layers were peeled away from the follicle and observed under a dissection microscope to check for the complete removal of granulosa cells. Chopped theca layers were digested by incubation with 1 mg collagenase ml⁻¹, 1 mg hyaluronidase ml⁻¹ and 1 mg dispase ml⁻¹ in PBS (Mg²⁺-Ca²⁺ free) for 1 h at 37°C (Campbell *et al.*, 1998). After 20 min of digestion, 100 µg DNase I ml⁻¹ was added to the digestion mixture. Dispersed granulosa and theca cells

Fig. 1. Method of achieving co-culture of bovine theca (outer surface) and granulosa (inner surface) cells on the laminin-coated membrane of the cell culture insert. Theca inoculation on the inverted insert took place in a deep container allowing at least 5 mm depth of medium above the membrane surface. Attachment of theca cells was allowed to proceed overnight before reversion of the insert and transfer to a culture plate well for granulosa inoculation.

were centrifuged separately at 800 *g* for 10 min and the pellets resuspended in 100 μ l of red blood cell lysis buffer (8.3 mg NH_4Cl ml^{-1} in 10.0 mmol Tris-HCl l^{-1} buffer) and incubated for 1 min. Isotonicity was restored by addition of 10 ml of collection medium and cells were then washed twice in collection medium by centrifugation at 800 *g* for 10 min. Granulosa and theca cells were resuspended in culture medium (DMEM-F12 medium containing 100.0 U penicillin ml^{-1} , 100.0 μ g streptomycin ml^{-1} , 2.0 mmol glutamine l^{-1} , 10.0 ng insulin ml^{-1} , 2.5 μ g apotransferrin ml^{-1} , 4.0 ng sodium selenite ml^{-1} and 1.0 mg BSA ml^{-1}) and counted using a haemocytometer. Cell viability, estimated by Trypan blue dye exclusion, was 20–30% and 80–90% for granulosa and theca cells, respectively.

Coating of cell culture inserts with laminin

Both sides of the Falcon BD BioCoatTM control cell culture inserts (24-well plates, 3 μ m pores) were coated with 5 μ g laminin cm^2 by soaking the inserts in a 24-well plate (solution DMEM-F12 medium). After 1 h of incubation at room temperature, the coating was allowed to air dry. Inserts were stored for up to 2 weeks at 4°C and then hydrated in culture medium for 30 min at 37°C before use.

Granulosa and theca cell co-culture

An inoculation chamber was created by positioning an Eppendorf 1.5 ml tube, previously opened by removing the cap and the rounded part of the tip, on the top of an upturned cell culture insert (Fig. 1). Viable theca cells (10^5 in 500 μ l of culture medium) were inoculated in the

chamber thus formed and allowed to attach overnight to the outer (convex) surface of the insert membrane. The insert was immersed in medium to a depth of about 5 mm above the level of the membrane during this time. The next day, the chamber was removed and the reverted insert fitted into a 24-well BD Falcon cell culture inserts companion plate, equilibrated with 500 μ l of culture medium. Freshly prepared granulosa cells (10^5 viable cells in 500 μ l of culture medium) were inoculated to the inside (concave) surface of the insert to obtain the co-culture arrangement (Fig. 1). The opposite arrangement, in which granulosa cells were first allowed to attach to the outer surface and then theca cells inoculated to the inner surface, was tested during optimization and was found to result in lower attachment rates. Granulosa and theca mono-cultures, used for comparison with co-cultures, were created using the procedures described above but omitting one of the types of cell. The effect of plating density on cellular activity was tested by comparing cultures with inoculations of 10^5 and 3×10^5 viable theca and granulosa cells. Cultures were maintained for 7 days at 37°C, 5% CO_2 and 95% humidity. Sixty-five per cent of the total medium in each well (650 μ l of 1 ml) was changed every day. Spent media were stored at –20°C until used for hormone measurement.

Co-culture treatments

The effects of FSH (0.3 ng ml^{-1}), LH (0.1 ng ml^{-1}), LR3 IGF-I (1 ng ml^{-1}) and androstenedione (10^{-7} mol l^{-1}) were tested on co-cultures (plating densities of 10^5 and 3×10^5 cells) in the following combinations: (i) LR3 IGF-I, FSH and LH (treatment A) and (ii) LR3 IGF-I, FSH and androstenedione (treatment B). Treatments were

added from the start of culture and at each change of medium.

Hormone measurement

Concentrations of oestradiol and androstenedione in unextracted culture medium from mono-cultures and co-cultures were measured by radioimmunoassay (Cutierrez *et al.*, 1997; Campbell *et al.*, 1998). Oestradiol assay sensitivity was 0.6 pg ml^{-1} and the inter- and intra-assay coefficients of variation were 9.4 and 7.5%, respectively. Androstenedione assay sensitivity was 8 pg ml^{-1} and the inter- and intra-assay coefficients of variation were 11.1 and 6.9%, respectively. Progesterone concentrations were determined by ELISA that was validated for culture medium (Picton *et al.*, 1999). Assay sensitivity was 0.5 ng ml^{-1} and the inter- and intra-assay coefficients of variation were 13.7 and 8.8%, respectively.

Determination of the number of viable cells

At the end of the culture period, the number of theca and granulosa cells was estimated by the cellular conversion of methylthiazole tetrazolium (MTT) (Mosmann, 1983). Theca and granulosa cells were detached from the insert membrane by treatment with 0.25% (w/v) trypsin-EDTA for 2 min at 37°C . After trypsin deactivation by the addition of fetal calf serum, cells were collected separately from the insert and centrifuged at 800 g for 10 min. The cells were then washed twice in DMEM-F12 medium (BSA- and phenol red-free) containing $10 \text{ mmol Hepes l}^{-1}$ and resuspended in $100 \mu\text{l}$ of the same medium. Cell suspensions were transferred to a 96-well plate and incubated with $10 \mu\text{l}$ of a MTT stock solution (5 mg ml^{-1}) for 4 h at 37°C . At the end of the incubation period, $100 \mu\text{l}$ of a MTT solubilization solution (consisting of 10% (v/v) Triton-X100, 0.1 N HCl in anhydrous isopropanol) was added to the wells to dissolve the formazan crystals. The resulting purple solution was measured using a spectrophotometer at 570 nm. The relationship between absorbance and the number of cells was determined by previously incubating known quantities of cells with MTT and creating a standard curve. The curve was linear over a range of 10^3 to 10^6 cells with a lower detection limit of 10^3 cells.

Statistical analyses

Data from time course experiments are presented as mean daily hormone concentration after correction for the residual amount of medium remaining in the wells during the medium change. Data are also expressed for the last day of culture as concentration of hormone per 20 000 viable cells measured at that time. The results presented in the present study are based on at least three independent cultures, each culture using wells and inserts in triplicate. The data were $(\log_{10} + 1)$ transformed to remove heterogeneity of variance before analysis.

Statistical analysis was performed by repeated measures of ANOVA using GenStat for Windows, 6th edition. Replicate cultures were treated as block structures; *P* values are shown for the overall analysis. The pooled variance was used to calculate the standard error of the difference (SED) between two means. Effects were considered significant at $P < 0.05$.

For the viable cell number determinations, data are presented as means $\pm$ SEM ($n \geq 3$). Statistical comparison of means was by ANOVA and, when indicated, by Tukey-Kramer *post hoc* test. The results were considered significantly different at $P < 0.05$.

Results

Granulosa and theca co-culture optimization

After coating, laminin formed a thin polymer (described by Yurchenco *et al.*, 1985, 1992) on both sides of the cell culture insert membrane, thus allowing both types of cell to interact with the ECM component. Theca cells showed better attachment compared with granulosa cells (approximately 80 and 10%, respectively; data not shown) after the overnight incubation and inversion of the insert. For this reason, theca cells were always seeded first during the co-culture procedures. Theca cell attachment was further improved by the physical removal of contaminating red blood cells using ammonium chloride treatment (data not shown).

Cell morphology and cell number

After overnight incubation, theca cells formed a network of well-attached cell clusters, which evolved into dense cell clumps by the end of the first day of culture. Some fibroblast-like cells with projections were also observed by the second day of culture, both at the base and between theca cell clumps. In contrast, granulosa cells started to form clumps by the second day of culture. After 4 days of culture, some elongated cells were visible at the base of the granulosa cell clumps and projections towards other clumps could be observed. No obvious difference in cell morphology was observed between mono- and co-cultures for either type of cell. Granulosa and theca cell morphology in co-culture, plated at a density of 10^5 cells per insert, is shown (Fig. 2a,b). The size and the shape of the clumps were dependent on the cell plating density; the clumps were bigger and more inter-connected at the higher cell plating density. Cells in co-culture were also distributed more evenly on the cell culture insert membrane at the higher cell plating density (Fig. 2c,d). No changes in cell morphology were observed with either treatment A (LR3 IGF-I, FSH and LH) or treatment B (LR3 IGF-I, FSH and androstenedione). After 7 days of culture, under basal conditions ($10 \text{ ng insulin ml}^{-1}$) at a plating density of 10^5 cells per insert, the number of theca and granulosa cells recovered from the mono-cultures was approximately 20% of

Fig. 2. Morphology of bovine granulosa and theca cells in co-culture. Cells were co-cultured for 7 days under basal conditions ($10 \text{ ng insulin ml}^{-1}$) at a density of either (a,b) 10^5 or (c,d) 3×10^5 of each type of cell per cell culture insert. Cells were observed using an inverted microscope fitted with a long distance objective. Representative images, corresponding to the same observation field but in different focal planes, are shown. (a,b) Co-culture density at 10^5 cells; (a) granulosa focal plane (granulosa cell clumps are indicated by arrows) and (b) theca focal plane (theca cell clumps are indicated by arrowheads). (c,d) Co-culture density at 3×10^5 cells; (c) granulosa focal plane (granulosa cell clumps are indicated by arrows) and (d) theca focal plane (theca cell clumps are indicated by arrowheads). Scale bars represent $100 \mu\text{m}$.

the initial plating number. The number of granulosa cells recovered from co-cultures was significantly higher than that from mono-cultures ($P < 0.001$). No significant difference in theca cell survival was observed between mono-cultures and co-cultures (Fig. 3a). Treatment of the co-culture with either combination of treatment hormones (treatment A or treatment B) under the same experimental conditions resulted in an increase in the number of viable theca cells in comparison with basal conditions (treatment A: $P < 0.01$; treatment B: $P < 0.001$). The treatments did not affect the number of viable granulosa cells in co-culture compared with the number observed under basal conditions (Fig. 3b). Theca cells in co-culture at 3×10^5 cell plating density survived better compared with those at the lower cell density in all culture conditions (basal conditions: $P < 0.001$; treatment A: $P < 0.01$; treatment B: $P < 0.05$).

The increased number of viable theca cells at 3×10^5 cell plating density was related to the higher number of cells in culture as no significant difference in the number of cells was observed when cultures were performed in basal conditions, or with either combination of treatment hormones (Fig. 3c). This plating density effect was not observed for granulosa cells. The number of viable granulosa cells in co-culture at 3×10^5 cells plating density was the same at 10^5 cells and was unaffected by treatments (Fig. 3b,c).

Hormone production under basal conditions

Mono-culture and co-culture at a plating density of 10^5 cells. Androstenedione concentrations were higher in co-culture than in mono-culture ($P < 0.05$) and varied significantly with time ($P < 0.001$; Fig. 4a).

Fig. 3. The number of viable bovine theca and granulosa cells at the end of the culture period. The number of cells was estimated by methylthiazole tetrazolium conversion by metabolically active cells. Values are means $\pm$ SEM ($n=6$ independent cultures). Statistical comparisons of the means were performed by one-way ANOVA followed by Tukey–Kramer *post hoc* test ($^{a,b}P < 0.05$). (a) Cell survival in mono-culture and co-culture in basal conditions at plating density of 10^5 cells; (b) cell survival in co-culture at plating density of 10^5 cells under basal conditions (BC), treatment A (TA) and treatment B (TB); and (c) cell survival in co-culture at plating density of 3×10^5 cells under basal conditions (BC), treatment A (TA) and treatment B (TB).

Fig. 4. Time course of secretion of androstenedione, oestradiol and progesterone under basal conditions ($10 \text{ ng insulin ml}^{-1}$) at cell plating density of either (a) 10^5 or (b) 3×10^5 bovine cells. (a) Secretion profiles of mono-cultures and co-cultures (◆: theca mono-culture; ●: granulosa mono-culture; ■: co-culture). (b) Secretion profiles of co-cultures at plating density of 3×10^5 cells (■) compared with those at plating density of 10^5 cells (□). Values are the means of eight separate cultures in triplicate. Data were log transformed to remove heterogeneity of variance before repeated measures of ANOVA. The pooled variance was used to calculate the SED between means. Differences between means greater than the SED are significant ($P < 0.05$).

Androstenedione concentrations declined markedly in theca mono-culture, whereas a more constant hormone production was observed in co-culture.

Oestradiol concentrations were low and not significantly different between mono-culture and co-culture (Fig. 4a). Co-cultured cells produced more progesterone compared with the respective mono-cultured cells ($P < 0.001$; Fig. 4a), but no difference between co-culture and mono-culture was observed in the pattern of secretion over time.

Co-culture at a plating density of 3×10^5 cells. Co-cultures with 3×10^5 cells under basal conditions produced threefold more androstenedione compared with co-cultures at the lower cell density ($P < 0.01$). However, no significant difference was observed in androstenedione production with time in culture (Fig. 4b).

Oestradiol concentrations under basal conditions were increased significantly ($P < 0.001$) compared with co-cultures at the lower cell plating density. In addition, a significantly distinct reduction in production was observed during the period of culture ($P < 0.001$; Fig. 4b). Co-cultures with 3×10^5 cells under basal conditions produced threefold more progesterone compared with co-cultures at the lower cell plating density ($P < 0.001$). There was also a significantly different secretion pattern ($P < 0.05$; Fig. 4b).

Effect of LR3 IGF-I, FSH and LH on hormone production on co-cultures

Androstenedione. Treatment A increased the concentration of androstenedione in co-cultures when cells were plated at a density of 10^5 cells (Fig. 5a).

Fig. 5. Time course of secretion of androstenedione, oestradiol and progesterone by co-cultures treated with treatment A and treatment B at cell plating density of either (a) 10^5 or (b) 3×10^5 bovine cells. (a) Secretion profiles of co-cultures at plating density of 10^5 cells treated with treatment A (○) and treatment B (▲) compared with basal conditions (■). (b) Secretion profiles of co-cultures at plating density of 3×10^5 cells treated with treatment A (○) and treatment B (▲) compared with basal conditions (■). Values are the means of four separate cultures in triplicate. Data were log transformed to remove heterogeneity of variance before repeated measures ANOVA. The pooled variance was used to calculate the SED between means. Differences between means greater than the SED are significant ($P < 0.05$).

Androstenedione concentrations were significantly higher ($P < 0.05$) compared with those measured under basal conditions, but hormone production over time did not change significantly. The increase in secretion on the last day of culture was almost twofold more than that seen under basal conditions (Table 1). Under treatment A, co-cultures with 3×10^5 cells secreted significantly ($P < 0.001$) more androstenedione compared with that produced by cells under basal conditions at the same cell plating density. The secretion profile changed significantly during culture ($P < 0.05$), as shown (Fig. 5b). The treatment resulted in an almost threefold increase in androstenedione concentrations, as measured on day 7 of culture (Table 1). Co-cultures with 3×10^5 cells produced significantly more androstenedione compared with co-cultures at the lower cell plating density in the

same experimental conditions ($P < 0.005$). However, no significant difference was observed in production over time.

Oestradiol. Treatment A, at a plating density of 10^5 cells, produced no significant difference in oestradiol concentrations when compared with co-cultures under basal conditions (Fig. 5a). Treatment B significantly increased oestradiol concentrations ($P < 0.001$). The pattern of secretion over the time of culture was significantly different ($P < 0.001$) and the increased hormone production was maintained throughout the culture period (Fig. 5a and Table 1). Co-cultures with 3×10^5 cells showed a significant increase in oestradiol concentrations in response to treatment A when compared with those under basal conditions ($P < 0.005$; Fig. 5b).

Table 1. Content of androstenedione (log pg per 2×10^4 cells per 24 h), oestradiol (log pg per 2×10^4 cells per 24 h) and progesterone (log ng per 2×10^4 cells per 24 h) in bovine co-cultures at day 7

Culture conditions	10^5 cells			3×10^5 cells		
	Androstenedione	Oestradiol	Progesterone	Androstenedione	Oestradiol	Progesterone
Basal	2.10 ^a (125.9)	0.40 ^a (2.5)	0.40 ^a (2.5)	1.87 ^a (74.1)	0.30 ^a (1.99)	0.60 ^a (4.0)
LR3 IGF-I, FSH, LH	2.45 ^b (281.8)	0.24 ^a (1.7)	0.24 ^a (1.7)	2.31 ^b (204.2)	0.44 ^a (2.75)	0.30 ^b (2.0)
LR3 IGF-I, FSH, androstenedione		0.89 ^b (7.8)	0.20 ^a (1.6)		0.76 ^b (5.75)	0.33 ^b (2.1)

Results are log of median steroid concentrations (absolute concentrations are given in parentheses).

The SED is 0.17 for androstenedione, 0.13 for oestradiol and 0.11 for progesterone. ^{ab}Values with different letters within the same column are significantly different ($P < 0.05$).

IGF-I: insulin-like growth factor I.

However, the secretion profiles over time were not significantly different. Treatment B markedly increased the concentration of oestradiol ($P < 0.001$) and a different pattern of secretion was observed over time ($P < 0.001$; Fig. 5b and Table 1). Both combinations of treatment hormones increased oestradiol production compared with co-cultures at the lower cell density under the same experimental conditions ($P < 0.001$) and a significantly distinct profile of production was observed during culture ($P < 0.001$).

Progesterone. Neither treatment affected progesterone concentrations when compared with cells cultured under basal conditions (Fig. 5 and Table 1). However, when progesterone concentrations were measured on the last day of culture and corrected for the number of viable cells present at that time, treatments had resulted in a significant decrease in secretion ($P < 0.005$; Table 1). Progesterone concentrations at the higher plating density, under either combination of treatment hormones, were significantly higher ($P < 0.01$) than those at the lower plating density under the same treatments and a significant difference in secretion over time was observed ($P < 0.005$; Fig. 5a).

Discussion

The present study is to our knowledge the first to describe a serum-free co-culture system for granulosa and theca cells supported on a membrane coated with an ECM component. In this system, cells cultured under basal conditions ($10 \text{ ng insulin ml}^{-1}$) retained the ability to secrete steroid hormones for at least 7 days. In addition, steroidogenesis was influenced by hormone treatment and plating density. When compared with monocultures, co-cultured cells showed evidence of paracrine communication through enhanced steroidogenesis and cell survival.

The serum-free medium and the combination of treatment hormones and concentrations used in the present study were based on those used in previous

serum-free culture systems, developed for bovine and ovine granulosa cells (Campbell *et al.*, 1996; Gutierrez *et al.*, 1997) and for ovine and pig theca cells (Campbell *et al.*, 1998; Picton *et al.*, 1999; Shores *et al.*, 2000). Cell culture inserts coated with laminin were chosen to support cells in culture. Laminin is one of the most abundant ECM components within the basal lamina (Zhao and Luck, 1995; Colognato and Yurchenco, 2000; Rodgers *et al.*, 2000) and is capable of preventing apoptosis and maintaining cell morphology to the same extent as intact ECM (Aharoni *et al.*, 1997). In particular, it has been reported that laminin improves survival of rat and sheep granulosa cells (Aharoni *et al.*, 1997; Huet *et al.*, 2001; Le Bellego *et al.*, 2002). However, rat and sheep granulosa cells cultured in serum-free conditions on a laminin substratum did not retain the gross morphology of cell clumps, described as the 'follicular phenotype' by several authors (Campbell *et al.*, 1996; Gutierrez *et al.*, 1997). The bovine cells in the system used in the present study rapidly attained the clumped morphology after being plated as a dispersion, which mimics the ovarian follicle environment in which only cells in contact with the ECM components of the basement membrane assume a 'flattened' morphology; the subsequent layers of cells, faraway from the basement membrane, assume a 'more rounded morphology' (Irving-Rodgers and Rodgers, 2000). Cells in the system used in the present study also retained endocrine features associated with the follicular phenotype and survived at rates comparable with those of cells in serum-free monoculture. ECM-induced granulosa cell differentiation is modulated by gonadotrophins and growth factors (Aharoni *et al.*, 1997; Huet *et al.*, 2001), so it is possible that a higher degree of differentiation is obtained when cultures are performed in the presence of these regulators. For this reason, the culture system used in the present study was characterized first under basal conditions (in the presence of $10 \text{ ng insulin ml}^{-1}$), and subsequently in the presence of physiological concentrations of gonadotrophins and growth factors.

Under basal conditions, comparison between monocultures and co-cultures was performed at a plating

density of 10^5 cells. This plating density was initially chosen in accordance with the optimal cell density reported in previous serum-free culture studies carried on an equivalent surface area (Gutierrez *et al.*, 1997; Campbell *et al.*, 1998; Shores *et al.*, 2000). Granulosa cells showed a twofold increase in viability when co-cultured with theca cells. This finding is consistent with reports that bovine theca cells secrete a range of factors (for example, TGF- α , KGF and HGF) that can sustain granulosa cells in culture (Skinner and Coffey, 1988; Parrott *et al.*, 1994). A similar positive effect of theca cells on granulosa cell viability has been reported by different authors in co-culture experiments in which cells were cultured in partial serum-free conditions (first cultured with serum and then in serum-free medium; Bendell *et al.*, 1988; Tajima *et al.*, 2002).

Co-operation between granulosa and theca cells was also apparent at the hormone level. The concentrations of androstenedione were higher in co-cultures compared with mono-cultures, indicating a positive effect of granulosa cells on thecal steroidogenesis. A similar feature has been reported in studies of bovine theca and follicle wall preparations cultured in serum-free conditions (Fortune, 1986) and by bovine theca and granulosa cells co-cultured in partial serum-free conditions (Wrathall and Knight, 1995; Yada *et al.*, 1999). The mechanisms responsible for this effect are still unknown, but it has been suggested that oestradiol and inhibin secreted by granulosa cells can induce an increased secretion of androstenedione by theca cells (Fortune, 1986; Roberts and Skinner, 1990; Wrathall and Knight, 1995; Webb *et al.*, 1999). This mechanism may be applicable to the system used in the present study as granulosa cells cultured in serum-free medium supplemented with androstenedione are capable of producing oestradiol and inhibin (Campbell *et al.*, 1996; Glister *et al.*, 2001). We did not observe any oestradiol accumulation when cells were cultured under basal conditions because exogenous androstenedione was not included in the medium and was therefore unavailable as an aromatase substrate. Although androstenedione concentrations were higher in co-cultures, oestradiol concentrations in co-cultures were not different from those measured in mono-cultures. This apparent discrepancy can be explained on the basis that androstenedione concentrations in co-cultures remained at concentrations to which the granulosa aromatase cytochrome P450 (P450 aromatase) is relatively insensitive; androstenedione affinity for P450 aromatase in the ovary is in the nanomolar range (Conley, 2001) and androstenedione concentrations in co-cultures are sub-nanomolar.

Progesterone concentrations in co-cultures were significantly higher compared with those in mono-cultures. The increased progesterone production in co-cultures was a synergistic effect of the presence of the two types of cell, rather than an additive one. Despite the higher concentrations of progesterone, as

compared with those of androstenedione and oestradiol, several features indicate that cells in the present co-culture system did not undergo luteinization. First, progesterone secretion by luteinizing cells typically increases extremely rapidly over the duration of culture (Luck *et al.*, 1990); concentrations in the cultures used in the present study remained constant. Second, oestradiol and androstenedione synthesis continued throughout the culture period and remained responsive to low doses of FSH, LH and the IGF-I analogue, LR3-IGF-I. Secretion of oestradiol by luteinizing cells declines rapidly to minimal or undetectable levels within the first few days of culture (Luck *et al.*, 1990). The low oestradiol concentrations observed in the cultures of the present study may be the result of culture on a laminin-coated membrane. It has been shown that laminin suppresses oestradiol, but increases progesterone secretion in granulosa cell culture in serum-free conditions (Huet *et al.*, 2001). Nevertheless, addition of exogenous androstenedione significantly stimulated oestradiol production during all periods of culture, demonstrating that granulosa cells maintained their P450 aromatase activity.

Treatment of co-cultures with either combination of treatment hormones produced not only a positive effect on hormone secretion, but also significantly affected theca cell survival. An almost twofold increase in the number of theca cells was measured at the end of culture compared with cells cultured under basal conditions. In contrast, no effect was observed on granulosa cell viability. These results indicate an anti-apoptotic effect of treatments on theca cells. The proliferative effect of LR3-IGF-I on granulosa and theca cells has been shown in previous experiments conducted under serum-free conditions. In particular, it has been reported that LR3-IGF-I, at the same concentration used in our study, stimulated proliferation of ovine theca cells in the presence of low doses of LH (Campbell *et al.*, 1998), but not of bovine granulosa cells in the presence of low doses of FSH (Gutierrez *et al.*, 1997). The positive effect exerted by treatments on theca cell viability in co-culture was not observed when cells were plated at higher density; theca cells cultured under basal conditions survived better at the higher plating density, with no further increase in number of cells even in the presence of treatments. Previous studies demonstrated that plating density had a detrimental effect on thecal hormone secretion (Campbell *et al.*, 1998; Shores *et al.*, 2000). In the system used in the present study, the culture surface area is approximately the same as that of a well in the conventional 96-well plates used in previous culture studies. It was proposed that the absence of any detrimental effect of increasing the number of cells may be the result of having the cell culture inserts fitted in a 24-well plate and incubated in 1 ml of medium (four times larger volume than in previous studies; Campbell *et al.*, 1998; Shores *et al.*, 2000), thereby avoiding the negative effects of metabolic by-product accumulation.

Co-culture at the higher plating density under basal conditions produced significantly more androstenedione, oestradiol and progesterone compared with that at the lower plating density. Furthermore, secretion of androstenedione and oestradiol was stimulated by treatment with both combinations of treatment hormones. The addition of exogenous androstenedione increased oestradiol secretion indicating an active P450 aromatase in these culture conditions. Interestingly, progesterone concentrations, measured on the last day of culture and corrected for the number of viable cells in culture, were decreased compared with production by cells in basal conditions. These results indicate that cells are more able to retain their follicular phenotype when cultured at a higher plating density. Thus, it was concluded that plating density has a marked effect in this co-culture system, increasing both cell viability and hormone production.

A previous *in vitro* model described granulosa and theca cell interactions in a co-culture system on a collagen membrane (Kotsuji *et al.*, 1990; Yada *et al.*, 1999). In that system, serum was used for the initial 48 h of culture to allow cell attachment and then super-physiological doses of insulin were used for the rest of the culture period (Kotsuji *et al.*, 1990; Yada *et al.*, 1999; Tajima *et al.*, 2002). These differences in design between the two systems preclude a direct comparison of results, although the earlier studies also found that theca cells maintained granulosa viability by preventing apoptosis (Tajima *et al.*, 2002) and that granulosa cells increased androstenedione production by theca cells (Yada *et al.*, 1999).

In conclusion, a serum-free co-culture system has been developed for bovine granulosa and theca cells in which paracrine communication across a laminin-coated membrane promotes steroidogenesis and cell survival. Co-cultured cells respond to physiological concentrations of gonadotrophins and an IGF-I analogue and maintain a follicular phenotype, especially when plated at a higher density. Taken together, these characteristics indicate that the co-culture system provides a model for the study of cell-cell and cell-ECM interactions and may be useful for investigating tissue remodelling during follicle growth.

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