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Research

Paracrine effects of oocyte secreted factors and stem cell factor on porcine granulosa and theca cells *in vitro*

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Abstract

Oocyte control of granulosa and theca cell function may be mediated by several growth factors via a local feedback loop(s) between these cell types. This study examined both the role of oocyte-secreted factors on granulosa and thecal cells, cultured independently and in co-culture, and the effect of stem cell factor (SCF); a granulosa cell derived peptide that appears to have multiple roles in follicle development. Granulosa and theca cells were isolated from 2–6 mm healthy follicles of mature porcine ovaries and cultured under serum-free conditions, supplemented with: 100 ng/ml LR3 IGF-1, 10 ng/ml insulin, 100 ng/ml testosterone, 0–10 ng/ml SCF, 1 ng/ml FSH (granulosa), 0.01 ng/ml LH (theca) or 1 ng/ml FSH and 0.01 ng/ml LH (co-culture) and with/without oocyte conditioned medium (OCM) or 5 oocytes. Cells were cultured in 96 well plates for 144 h, after which viable cell numbers were determined. Medium was replaced every 48 h and spent medium analysed for steroids.

Oocyte secreted factors were shown to stimulate both granulosa cell proliferation ($P < 0.001$) and oestradiol production ($P < 0.001$) by granulosa cells throughout culture. In contrast, oocyte secreted factors suppressed granulosa cell progesterone production after both 48 and 144 hours ($P < 0.001$). Thecal cell numbers were increased by oocyte secreted factors ($P = 0.02$), together with a suppression in progesterone and androstenedione synthesis after 48 hours ($P < 0.001$) and after 144 hours ($P = 0.02$), respectively. Oocyte secreted factors also increased viable cell numbers ($P < 0.001$) in co-cultures together with suppression of progesterone ($P < 0.001$) and oestradiol ($P < 0.001$). In granulosa cell only cultures, SCF increased progesterone production in a dose dependent manner ($P < 0.001$), whereas progesterone synthesis by theca cells was reduced in a dose dependent manner ($P = 0.002$). Co-cultured cells demonstrated an increase in progesterone production with increasing SCF dose ($P < 0.001$) and an increase in oestradiol synthesis at the highest dose of SCF (100 ng/ml). In summary, these findings demonstrate the presence of a co-ordinated paracrine interaction between somatic cells and germ cells, whereby oocyte derived signals interact locally to mediate granulosa and theca cell function. SCF has a role in modulating this local interaction. In conclusion, the oocyte is an effective modulator of granulosa-theca interactions, one role being the inhibition of luteinization.

Introduction

Within the ovarian follicle in mammals, oocyte growth and differentiation depends upon an intimate association between the somatic follicular cells and the developing germ cell [1]. Oocyte-granulosa cell communication is bi-directional and essential for both oocyte and follicular somatic cell function and development [2]. Studies have demonstrated that various aspects of follicular development also depend upon and/or are influenced by the presence of the oocyte [3–6]. For example, increased numbers of ovulations in sheep heterozygous for the $FecX^{I}$ gene are linked to an oocyte-derived gene, bone morphogenetic protein 15 (BMP-15) [7]. BMP-15 is an oocyte specific growth factor expressed in rat [8] and mouse [9] oocytes throughout folliculogenesis. Otsuka et al [8] showed that BMP-15 is responsible for FSH-independent granulosa cell proliferation *in vitro*. BMP-15 is also closely related to growth differentiation factor-9 (GDF-9) and these two factors have similar expression patterns in the ovary [9]. Follicular development is arrested at the primary stage in mice carrying an induced null mutation at the GDF-9 locus [10], a gene that is only expressed in oocytes [11]. It is likely that oocyte secreted factors provide a signalling mechanism to regulate the developmental fate of individual follicles. Although paracrine factors secreted by theca or granulosa cells, in addition to FSH, could regulate the development of individual follicles, the oocyte may play a dominant role in controlling follicle development [12].

Much work on oocyte-secreted factors has focussed on murine systems. Oocytes are capable of modulating steroid synthesis by murine cumulus cells *in vitro* [13], particularly by inhibiting progesterone production. The oocyte also affects granulosa cell proliferation and morphology [2,14,15]. However, little information is available on the effect the oocyte has on theca cell growth and function. It has been documented that theca cells affect oocyte growth and maturation in cattle [16,17] and that there is a theca-derived factor(s) which inhibits germinal vesicle breakdown [17]. Since oocyte granulosa cell communication is bi-directional [2] and there is bi-directional communication between the granulosa and theca cell compartments of the follicle [18], this raises the possibility that there is bi-directional communication between the oocyte and theca cells.

Stem cell factor (SCF) is one possible locally produced factor, encoded by the Steel (*Sl*) gene, and is thought to have many roles in follicular development [19]. SCF is essential for the colonisation of the gonad by germ cells and the continued survival and growth of germ cells in the ovine foetus [20]. SCF has also been implicated in the recruitment and early progression of primordial follicle development [21]. SCF mRNA is localised to granulosa cells at all stages of follicle growth in foetal and adult ovine ovaries [22]. Joyce et al [23] observed that SCF mRNA expression in mouse follicles was controlled by the oocyte depending on the stage of its growth and development. The nature of such control factors remains to be elucidated, but highlights the role of SCF during follicle growth *in vivo*. It has also been shown that SCF and its interaction with its receptor, *c-kit*, have a role in antrum formation, steroidogenesis and oocyte quality [24].

Previous studies on the effect of the oocyte on somatic ovarian cell function have used a variety of culture conditions. For example, cultures have been supplemented with serum [13,25], that causes the cells to luteinize. Culture vessels have been coated with attachment factors [26], which can also result in luteinization; and different populations of granulosa cells (cumulus versus mural cells) have been incubated. Primary serum-free culture systems have been developed for ovine [27], bovine [28] and porcine [29] granulosa cells and also for porcine theca cells [30]. The advantage of these serum free culture systems is that they maintain the follicular phenotype *in vitro*, in that spontaneous luteinization is avoided and the granulosa cells are able to maintain the activity of the aromatase enzyme complex [27–29].

Based upon the observation that follicular development is the result of a complex progression of cellular interactions, the aims of this work were to test the hypothesis that a porcine oocyte secreted factor(s) modulate granulosa and theca cell growth and function and to determine if any effects elicited by an oocyte secreted factor(s) are modified by SCF. These studies investigated the possibility that the communication between theca and granulosa cells is a key factor modulating the growth and function of these cells. We report here an investigation of porcine oocyte secreted factors using a physiologically relevant long-term, serum free culture system.

Materials and methods

Collection of oocyte conditioned medium (OCM)

All reagents were purchased from Sigma Chemical Co. Ltd., Poole, Dorset unless otherwise stated. Porcine ovaries from mature animals were collected from a local slaughterhouse. Only ovaries containing follicles >2 mm in diameter and of translucent appearance, not atretic [31], were selected. The ovaries were stored in dissection medium: M199 (Gibco RBL, Life Technologies) supplemented with 100 IU/ml penicillin and 0.1 mg/ml streptomycin, 0.1% BSA and 20 mM Hepes at 37°C until required for oocyte recovery.

Oocytes were aspirated from follicles between 2–6 mm in diameter. Oocytes possessing a compact (2–3 cell layers thick), intact cumulus mass and a dark ooplasm were selected and atretic oocytes or oocytes with an expanded

cumulus mass were rejected. The cumulus mass was mechanically removed from the oocytes by vortexing or repeated pipetting. The oocytes were washed by centrifugation and re-suspended in culture medium: McCoy's 5a medium with sodium bicarbonate supplemented with 20 mM Hepes, 100 IU/ml penicillin, 0.1 mg/ml streptomycin, 3 mM L-glutamine (Gibco BRL, Life Technologies), 4 ng/ml selenium, 2.5 μg/ml transferrin and 0.1% BSA (tissue culture grade). Oocyte conditioned medium was collected by culturing 1 oocyte per 1 μl medium in a humidified atmosphere with 5% CO_2 in air at 37°C. Oocyte conditioned medium (OCM) was collected after 24 and 48 hours of culture. Viability status of the oocytes was also assessed after 24 and 48 hours in culture using neutral red dye [33].

Granulosa and theca cell culture

Granulosa cells were collected and cultured as per Picton et al [29]. Briefly, follicles between 2–6 mm in diameter were dissected and each follicle was hemisected and granulosa cells were harvested from each follicle by scraping with an inoculation loop into Dulbecco's phosphate buffered saline (DPBS). The resultant cell suspension was washed by centrifugation in culture medium. Cell number and viability were determined using Trypan blue dye exclusion [32]. 1.5×10^5 viable cells in 50 μl aliquots were seeded into each well of a 96-well plate which contained 200 μl of culture medium (as before) supplemented with 10 ng/ml bovine insulin, 100 ng/ml testosterone, 100 ng/ml Long R3 IGF-1 (Groprep Pty Ltd., Adelaide, Australia) and 1 ng/ml porcine FSH (USDA-pFSH-I-2).

Following granulosa cell removal, the remaining follicle shells were rinsed in DPBS and the theca layer removed using forceps under a dissection microscope. The theca pieces were cut into small pieces and enzymatically dispersed using Hank's balanced salt solution supplemented with 20 mM Hepes, 0.5% w/v collagenase type II, 0.1% w/v hyaluronidase type I-S and 5% fetal calf serum in a shaking water bath at 37°C [30]. The digested cell suspension was washed by centrifugation and resuspended in culture medium: DMEM/Ham's F12 with Hepes and sodium bicarbonate supplemented with 3 mM L-glutamine, 4 ng/ml selenium, 2.5 μg/ml transferrin, 0.1% BSA, 100 IU/ml penicillin and 0.1 mg/ml streptomycin. Cell number and viability were determined by Trypan blue dye exclusion. 50×10^3 viable cells in 50 μl aliquots were seeded into each well of a 96-well plate which contained 200 μl of culture medium supplemented with 10 ng/ml bovine insulin, 100 ng/ml Long R3 IGF-1 and 0.01 ng/ml porcine LH (USDA-pLH-B-2). An optimal dose of LR3 IGF-1 (100 ng/ml) used in granulosa cell cultures was also found to be optimal for theca cell function [30], hence the utilization of control cultures with 100 ng/ml LR3 IGF-1. Shores et al [30] observed that a combination of LR3 IGF-1 (100 ng/ml), insulin (10 ng/ml) and LH (0.01 ng/ml) were required to maintain viable thecal cell numbers *in vitro*.

Granulosa and theca co-culture

Granulosa and theca cells were isolated and harvested as before. Cells were resuspended in culture medium: DMEM/ Ham's F12 with Hepes and sodium bicarbonate supplemented with 3 mM L-glutamine, 4 ng/ml selenium, 2.5 μg/ml transferrin, 0.1% BSA, 100 IU/ml penicillin and 0.1 mg/ml streptomycin. Viable cell number was determined using trypan blue dye exclusion. 50×10^3 viable cells per well (40×10^3 viable granulosa cells and 10×10^3 viable theca cells) were seeded in 50 μl aliquots into each well of a 96-well plate which contained 200 μl of culture medium supplemented with 10 ng/ml insulin, 1 ng/ml pFSH and 0.01 ng/ml pLH and 100 ng/ml LR3 IGF-1 [37,38]. Cells were cultured in a humidified atmosphere with 5% CO_2 in air at 37°C. After 48, 96 and 144 hours, 175 μl spent medium was removed and replaced with freshly equilibrated medium. Three independent cultures were carried out with 4 replicates per treatment in each culture.

In all cultures, after 144 hours, the number of viable granulosa and/or theca cells were assessed by the uptake of the vital dye, neutral red [33,27].

Experiment 1:*The effect of oocyte secreted factors on granulosa and theca cells*

OCM and granulosa cells were collected as before. 1.5×10^5 viable granulosa cells per well of a 96-well plate were cultured in 200 μl OCM supplemented with 10 ng/ml bovine insulin, 100 ng/ml testosterone, 1 ng/ml porcine FSH (USDA-pFSH-I-2) and with (100 ng/ml) or without LR3 IGF-1 (Gropep Pty Ltd., Adelaide). Control wells were also prepared containing basic McCoy's 5a medium supplemented with 10 ng/ml bovine insulin, 100 ng/ml testosterone, 1 ng/ml porcine FSH (USDA-pFSH-I-2) and 100 ng/ml LR3 IGF-1. Cells were also cultured with a series of OCM dilutions (1, 1:10, 1:100 and 1:1000) or 0, 5, 10 or 15 denuded oocytes per well in supplemented control medium as before.

50×10^3 viable theca cells per well of a 96 well pate were cultured in 200 μl OCM in supplemented control medium. Theca cells were also cultured in the presence of 0, 5, 10 or 15 denuded oocytes per well in supplemented control medium. Culture conditions, media changes and assessment of cell number were as before. Oocyte viability was also assessed by the uptake of neutral red dye.

Experiment 2:*The effect of oocyte secreted factors on granulosa and theca cells in co-culture*

Granulosa cells and theca cells were isolated and harvested as before. Cells were co-cultured as follows: 50 ×

10^3 viable cells per well (40×10^3 viable granulosa cells and 10×10^3 viable theca cells) were cultured 200 μl of OCM supplemented both with or without 100 ng/ml LR3 IGF-1 and supplements as before. Cells were also co-cultured in DMEM/ Ham's F12 (additives as before) supplemented with 5 oocytes per well. The cultures were terminated after 48 hours and viable cell numbers and oocyte viability were assessed.

Experiment 3:*Effect of stem cell factor on granulosa and theca cell function*

Granulosa and theca cells were cultured either individually or in co-culture as before with the addition of 0–100 ng/ml human stem cell factor (hSCF – Peprotech EC ltd., London, UK) with/without 5 oocytes per well. Supplements were as before. Culture conditions, media changes and assessment of viable cell numbers and oocyte viability were as before.

Cell viability

The average percentage viability (determined by trypan blue uptake) of granulosa cells and theca cells plated in all experiments were 45 (± 6.8) and 81 (± 2.4)%, respectively.

Oocyte viability (determined by the uptake of neutral red dye) was 90% and 87% after 24 and 48 hours of culture, respectively during the collection of OCM. Throughout all co-culture experiments, oocyte viability was in excess of 85%.

Assays

The concentrations of oestradiol, progesterone (granulosa, theca and co-culture experiments) and androstenedione (theca and co-culture experiments) were measured in unextracted culture medium by radioimmunoassay [34–39]. The sensitivities of the oestradiol (n = 6), progesterone (n = 6) and androstenedione (n = 8) assays were 1.0, 13.1 and 2.7 pg/tube, respectively. The intra- and inter-assay coefficients of variation were 10.1 and 15.4, 9.2 and 16.7, and 10.5 and 13.6% for oestradiol, progesterone and androstenedione, respectively.

Statistical analysis

Hormone data are expressed as hormone produced per 1000 viable cells per 48 hours after correction for any residual medium left in the wells after each medium change as described previously [27,29,30]. The data were log transformed ($\log_{10}(x + 1)$) to reduce heterogeneity of variance and subjected to analysis of variance (ANOVA) using Genstat 5 for windows version 4.1 [40]. Pooled variance was used to calculate the standard error of difference (SED) between means. Comparisons between each treatment were made using the Bonferroni t-test. Effects of treatments were considered significant at a probability level $P < 0.05$. The data are displayed as the mean value ± SED and the single error bar relates to the pooled data.

Results

Experiment 1

Granulosa cells cultured with OCM showed a significant increase in viable cell number ($P < 0.001$) which was amplified with the addition of LR3 IGF-1 (Figure 1A). In the presence of OCM, large interconnected cell clusters developed compared to controls. These cell clusters were viable as shown by neutral red assay (Figure 1B). OCM inhibited progesterone production both after 48 ($P < 0.001$) and 144 ($P < 0.001$) hours in culture (Figure 2A and 2B). A 100-fold dilution of OCM was still potent enough to inhibit progesterone production after 48 hours ($P < 0.001$). OCM in combination with LR3 IGF-1 also elicited a significant increase overall in oestradiol production after both 48 ($P = 0.03$) and 144 ($P < 0.001$) hours (Figure 2C and 2D). After 144 hours, this increase in oestradiol production was evident when OCM was diluted 1000-fold ($P = 0.041$). In keeping with these findings, oocyte co-culture also elicited both a reduction in progesterone production after 48 hours ($P < 0.001$) and 144 hours ($P < 0.001$) (Figure 3A and 3B), and an increase in oestradiol synthesis after 48 hours ($P < 0.001$) where cells were supplemented with 5 and 10 oocytes per well. Interestingly, after 96 hours in culture, the clusters of granulosa cells (which were expected to be largely mural cells and not cumulus cells) had restructured around the oocytes (Figure 3C and 3D).

Theca cell numbers were increased above controls (Figures 4A and 4B) when cultured with either OCM ($P = 0.02$) or oocytes ($P = 0.029$), although thecal cells did not aggregate around the oocytes as seen for the granulosa cell cultures (Figure 4C and 4D). As with granulosa cells, OCM inhibited progesterone synthesis by theca cells both after 48 ($P < 0.001$) and 144 hours ($P = 0.06$). However, oocyte co-culture elicited an increase in progesterone synthesis after 48 hours ($P = 0.02$). In addition, androstenedione synthesis was also inhibited by OCM after 48 hours ($P = 0.02$), and inhibited after 48 hours ($P = 0.01$) and 144 hours ($P = 0.03$) by the oocyte co-culture. In contrast to granulosa cells, oestradiol synthesis was unaffected by OCM, but suppressed by oocyte addition after 144 hours ($P < 0.001$).

Experiment 2

Granulosa and theca cells in co-culture showed a significant ($P < 0.001$) increase in viable cell number when cultured with either OCM or oocytes, but in the absence of LR3 IGF-1. Viable cell numbers were also significantly increased ($P < 0.05$), compared to controls in cultures supplemented with 5 oocytes per well. In keeping with the individual cell cultures (Experiment 1), both OCM and

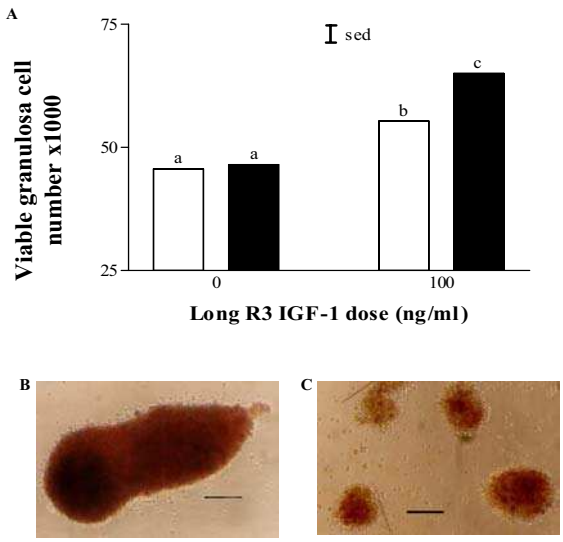

Figure 1
(A) Mean (± sed) number of viable granulosa cells after 144 hours in serum free culture in the presence (dark bars) and absence (light bars) of oocyte conditioned medium (OCM), 1 ng/ml pFSH and either an optimal dose of Long R3 IGF-1 (100 ng/ml) or no Long R3 IGF-1. Values are from 3 independent cultures, each treatment having 4 replicates. There was a significant effect of treatment ($P < 0.001$) on viable cell number. Bars with no common superscripts are significantly different ($P < 0.05$). (B) Granulosa cells cultured in the presence of OCM and (C) in the absence of OCM. Hormonal supplements are as before. Scale bar represents 100 μm.

oocyte co-culture suppressed progesterone synthesis in the presence of LR3 IGF-1 ($P < 0.001$). Both OCM and oocytes suppressed oestradiol synthesis by co-cultured cells ($P < 0.001$).

Experiment 3

Progesterone synthesis by granulosa cells was stimulated with SCF doses of 10 and 100 ng/ml after 48 hours ($P < 0.001$) (Figure 5A). However, oocyte co-culture reduced progesterone synthesis at all SCF doses ($P = 0.05$). In contrast, a 25% increase in oestradiol production from control values was observed with a concentration of 100 ng/ml SCF ($P < 0.05$). Oocyte co-culture also elicited an increase in oestradiol production with 0 and 10 ng/ml SCF compared to controls (cells cultured alone) (Figure 5B). After 144 hours in culture, a similar trend in oestradiol production was observed, but did not reach statistical significance ($P = 0.07$). Overall oestradiol concentrations were up to almost 7-fold higher after 144 hours in culture.

Viable theca cell numbers were significantly reduced by SCF ($P < 0.001$) and these were further reduced with oocyte co-culture ($P = 0.05$). In contrast to granulosa cells, SCF elicited an overall decrease in progesterone after 48 hours ($P = 0.002$), which was further reduced in oocyte cultures at 0 and 10 ng/ml SCF (Figure 6A). However, theca cells co-cultured with oocytes and supplemented with 100 ng/ml SCF significantly increased progesterone synthesis ($P = 0.05$). After 144 hours in culture, there was an overall significant ($P < 0.001$) effect of SCF dose and oocyte co-culture on androstenedione production (Figure 6B). Androstenedione production was significantly ($P < 0.05$) increased when theca cells were supplemented with 10 and 100 ng/ml SCF, both with and without oocyte co-culture, compared to controls. Theca cells co-cultured with oocytes secreted less androstenedione at SCF doses of 10 or 100 ng/ml, compared to oocyte free cultures and this difference was significant ($P < 0.05$) in the presence of 100 ng/ml SCF. Interestingly, this pattern of androstenedione synthesis was observed after 48 hours, but did not reach statistical significance ($P = 0.08$). In contrast to granulosa cells, thecal cells demonstrated a SCF dose dependent decrease in oestradiol production both with and without oocyte co-cultures after 48 ($P = 0.031$) and 144 ($P < 0.001$) hours (Figure 6C and 6D). Similar to granulosa cells, oocyte co-culture increased oestradiol synthesis in the absence of SCF after 48 hours in culture. However, after 144 hours in culture, this effect was reversed and oocyte co-culture in the absence of SCF decreased oestradiol production. Also, 10 ng/ml SCF increased oestradiol synthesis above that of controls ($P = 0.05$).

Granulosa and theca cells co-cultured both in the presence or absence of oocytes showed an overall increase in progesterone production after 48 hours in culture ($P < 0.001$). However, oocyte co-cultures at all SCF doses suppressed progesterone production compared to controls (Figure 7A). This pattern of progesterone secretion was repeated after 144 hours in culture ($P = 0.025$) (Figure 7B).

Oestradiol production was significantly inhibited ($P < 0.001$) after 48 hours when cells were co-cultured with oocytes at all SCF doses compared to controls. After 144 hours in culture, a similar pattern of oestradiol production was observed, but was not statistically significant.

Discussion

These data support the proposal that the porcine oocyte secretes a factor(s) that modulates both granulosa and thecal cell proliferation and steroidogenesis in physiologically relevant, previously validated, serum free culture systems [29,30]. These serum free systems maintain the aromatase complex, maintain thecal cell LH responsiveness and prevent full luteinization of cells in culture

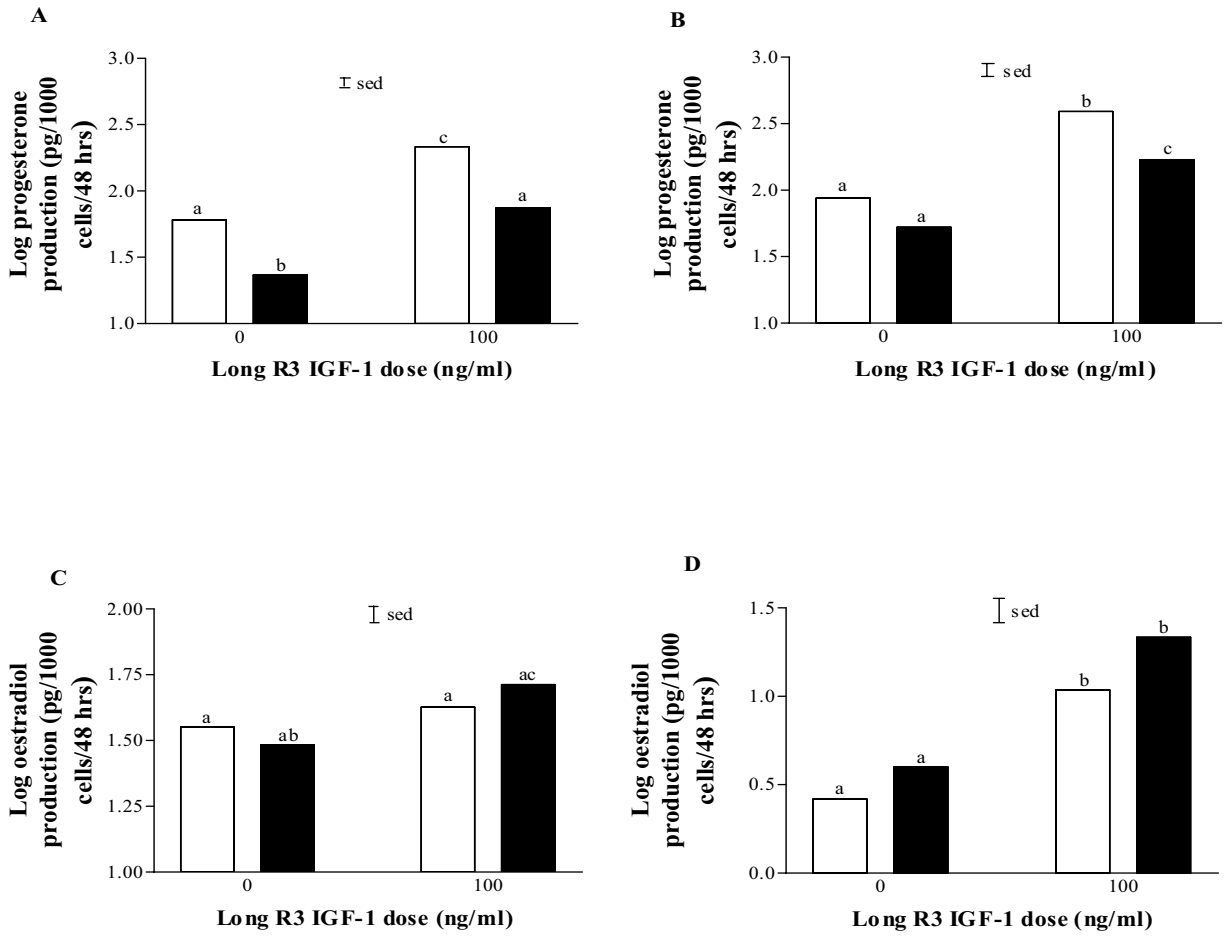

Figure 2
Mean (± sed) progesterone production by granulosa cells after (A) 48 hours and (B) 144 hours and mean (± sed) oestradiol production by granulosa cells after (C) 48 hours and (D) 144 hours in serum free culture in the presence (dark bars) and absence (light bars) of oocyte conditioned medium (OCM), 1 ng/ml pFSH and either an optimal dose of Long R3 IGF-1 (100 ng/ml) or no Long R3 IGF-1. Values are from 3 independent cultures, each treatment having 4 replicates. There was a significant effect of treatment ($P < 0.001$) on progesterone production at both time points. Bars with no common superscripts are significantly different ($P < 0.05$).

unlike previous systems, which used serum in culture and resulted in a luteinized phenotype rather than a follicular phenotype. The current results also demonstrate that this oocyte-secreted factor(s) is capable of stimulating cell proliferation together with suppression in progesterone production, and that there is an interaction with LR3 IGF-1 and SCF. In addition, we have shown for the first time to our knowledge, that the porcine oocyte secretes a factor(s) that can stimulate oestradiol synthesis by non-luteinized, FSH-responsive mural granulosa cells and suppress androstenedione synthesis by LH stimulated thecal cells. The study has also demonstrated that granulosa and theca interactions are mediated not only by somatic cell-derived factors, but also by oocyte-derived factors.

OCM increased viable granulosa and thecal cell number and altered the gross morphology of the cells since there was formation of large interconnected clusters

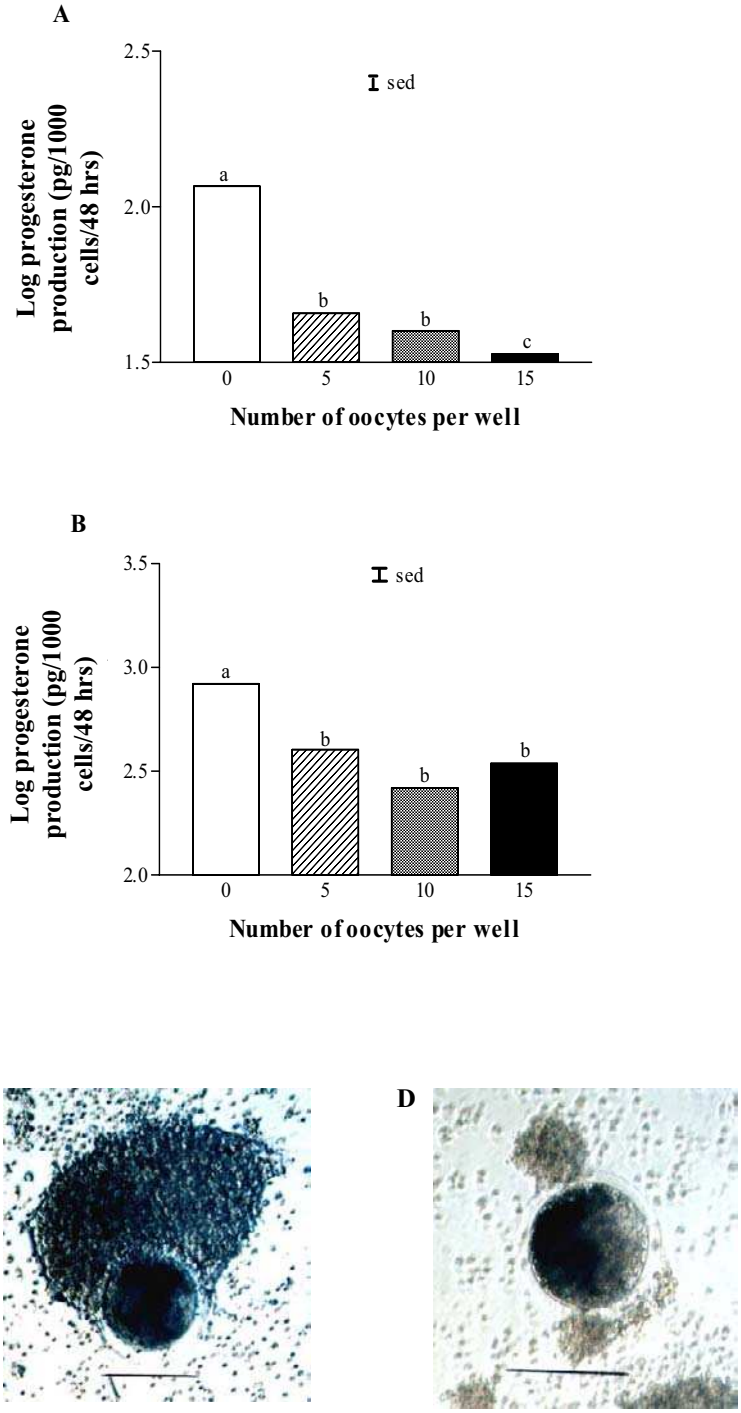

Figure 3
Mean (± sed) $\log_{10}$ progesterone production by granulosa cells after (A) 48 hours and (B) 144 hours in serum free culture supplemented with either 0, 5, 10 or 15 oocytes per well, 1 ng/ml pFSH and 100 ng/ml Long R3 IGF-1. Values are from 3 independent cultures, each treatment having 4 replicates. There was a significant effect of treatment ($P < 0.001$) on progesterone production at both time points. Bars with different superscripts are significantly different ($P < 0.05$). Morphological appearance of the organisation of granulosa cells around the oocytes when co-cultured under serum-free conditions: (C) granulosa cells supplemented with 10 oocytes per well and (D) granulosa cells supplemented with 5 oocytes. Scale bar represents 100 μm.

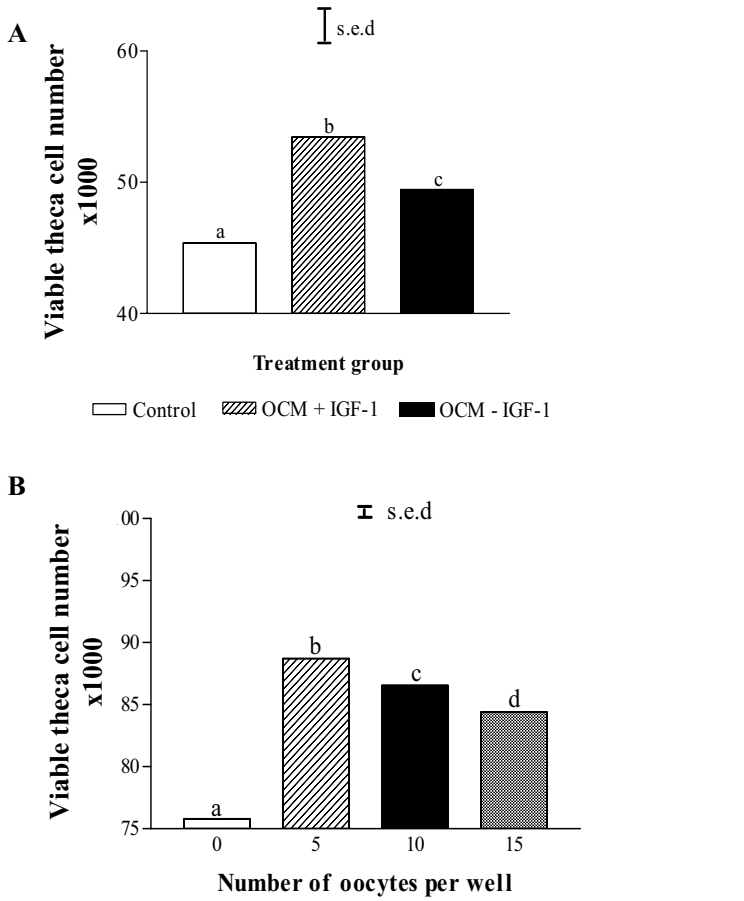

Figure 4
Mean (± sed) number of viable theca cells after 144 hours in serum free culture in (A) the presence of 100 ng/ml LR3 IGF-1, 0.01 ng/ml LH and 10 ng/ml insulin (control – light bars), supplemented with oocyte conditioned medium (OCM) (hatched bars) and OCM supplementation without LR3 IGF-1 (dark bars); (B) in the presence of either 0, 5, 10 or 15 oocytes per well supplemented with 100 ng/ml LR3 IGF-1, 0.01 ng/ml LH and 10 ng/ml insulin. Values are from 3 independent cultures, each treatment having 4 replicates. There was a significant ($P = 0.02$) effect of OCM on viable theca cell number. There was a significant effect of oocyte number per well on viable cell numbers ($P = 0.029$). Bars with different superscripts are significantly different ($P < 0.05$). Gross morphology of porcine theca cells co-cultured with oocytes. C and D represent theca cells supplemented with 5 oocytes after 144 hours in culture and (D) stained with neutral red. Scale bar represents 100 μm.

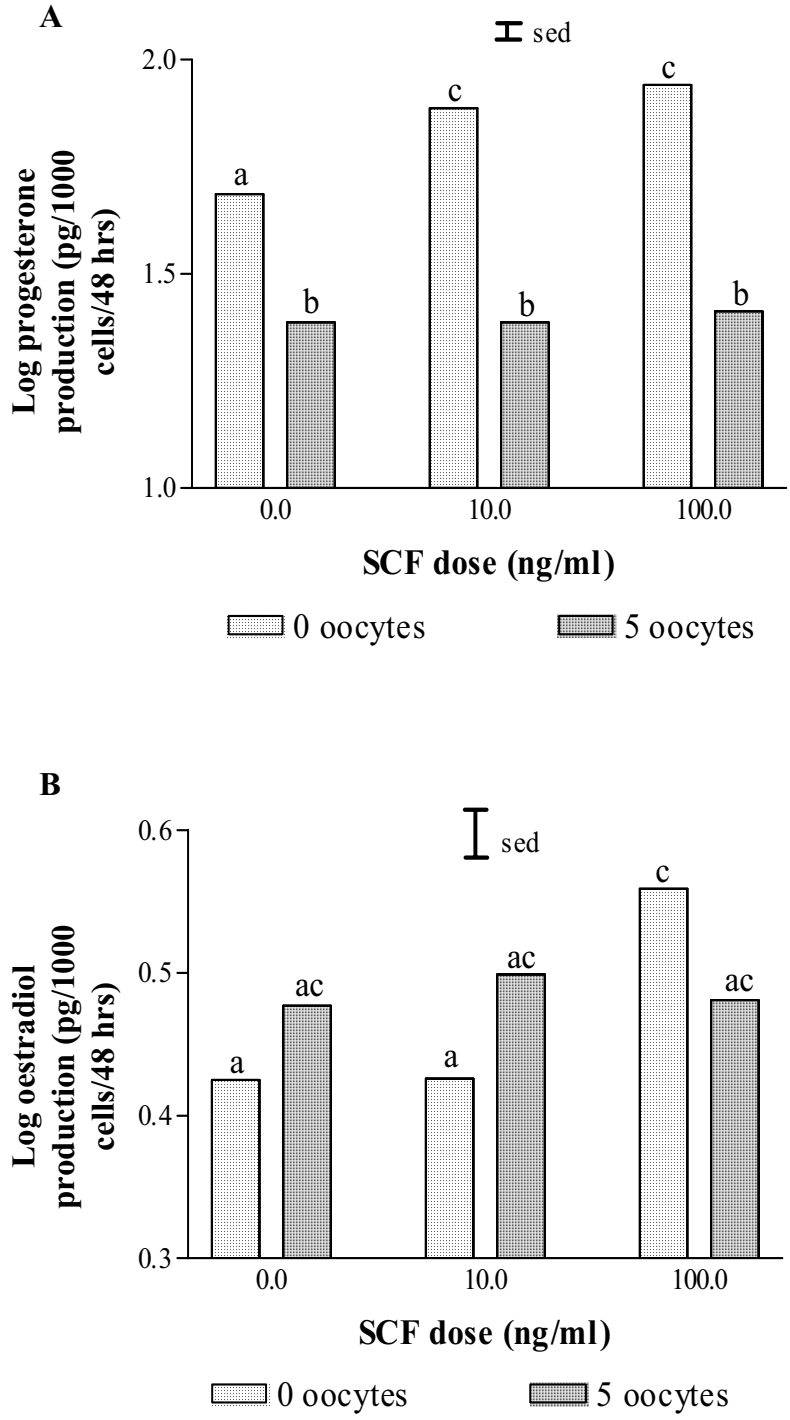

Figure 5
Mean ± sed $\log_{10}$ (A) progesterone and (B) log oestradiol production by granulosa cells after 48 hours in serum free culture supplemented with either 0, 10 or 100 ng/ml SCF ± 5 oocytes per well. Values are from 3 independent cultures, each having 4 replicates. There was a significant effect of treatment ($P < 0.001$) on progesterone production and significant effect of treatment ($P = 0.004$) on oestradiol production. Bars with different superscripts are significantly ($P < 0.05$) different.

Figure 6

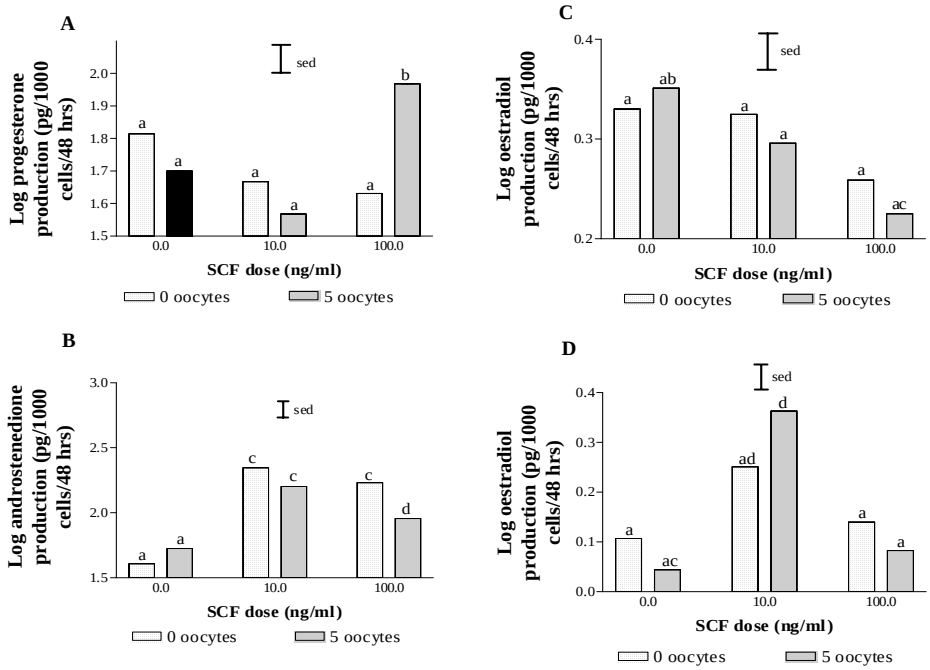

Figure 6
Mean (± sed) (A) $\log_{10}$ progesterone production by theca cells after 48 hours; (B) $\log_{10}$ androstenedione production by theca cells after 144 hours; (C) $\log_{10}$ oestradiol production by theca cells after 48 and (D) 144 hours in serum free culture supplemented with either 0, 10 or 100 ng/ml hSCF ± 5 oocytes per well. Values are from 3 independent cultures, each having 4 replicates. There was a significant effect of treatment in (A): P = 0.002, (B): P < 0.001, (C): P = 0.031 and (D): P < 0.001. Bars with different superscripts are significantly (P < 0.05) different.

(experiment 1). Such clusters of cells and the presence of gap junctions have been observed previously in serum free conditions for ovine and bovine granulosa cell systems [27,28,41] and in porcine thecal cell cultures [30]. Interestingly, granulosa cells were restructured around oocytes, whereas theca cells did not appear to form connections with oocytes. Cell proliferation was also observed when granulosa and theca cells were co-cultured independently with 5 or 10 oocytes per well (experiment 1). This is in agreement with co-culture experiments in other species (rat and bovine: [14] where cell proliferation has also been observed.

The interactions between oocyte secreted factors and somatic cells were studied further by the use of granulosa-theca cell co-culture (experiment 2) and the cellular interactions were extended by the addition of SCF (experiment 3). Porcine oocyte secreted factor(s) modulated granulosa and theca cell function when the somatic cells were cultured together. OCM increased viable cell numbers above controls, both with and without LR3 IGF-1. However, addition of 5 oocytes per well increased granulosa and theca viable cell numbers in the absence of LR3 IGF-1. This provides evidence that co-culturing cells stimulates cellular interactions necessary for cell growth and differentiation without the requirement for LR3 IGF-1.

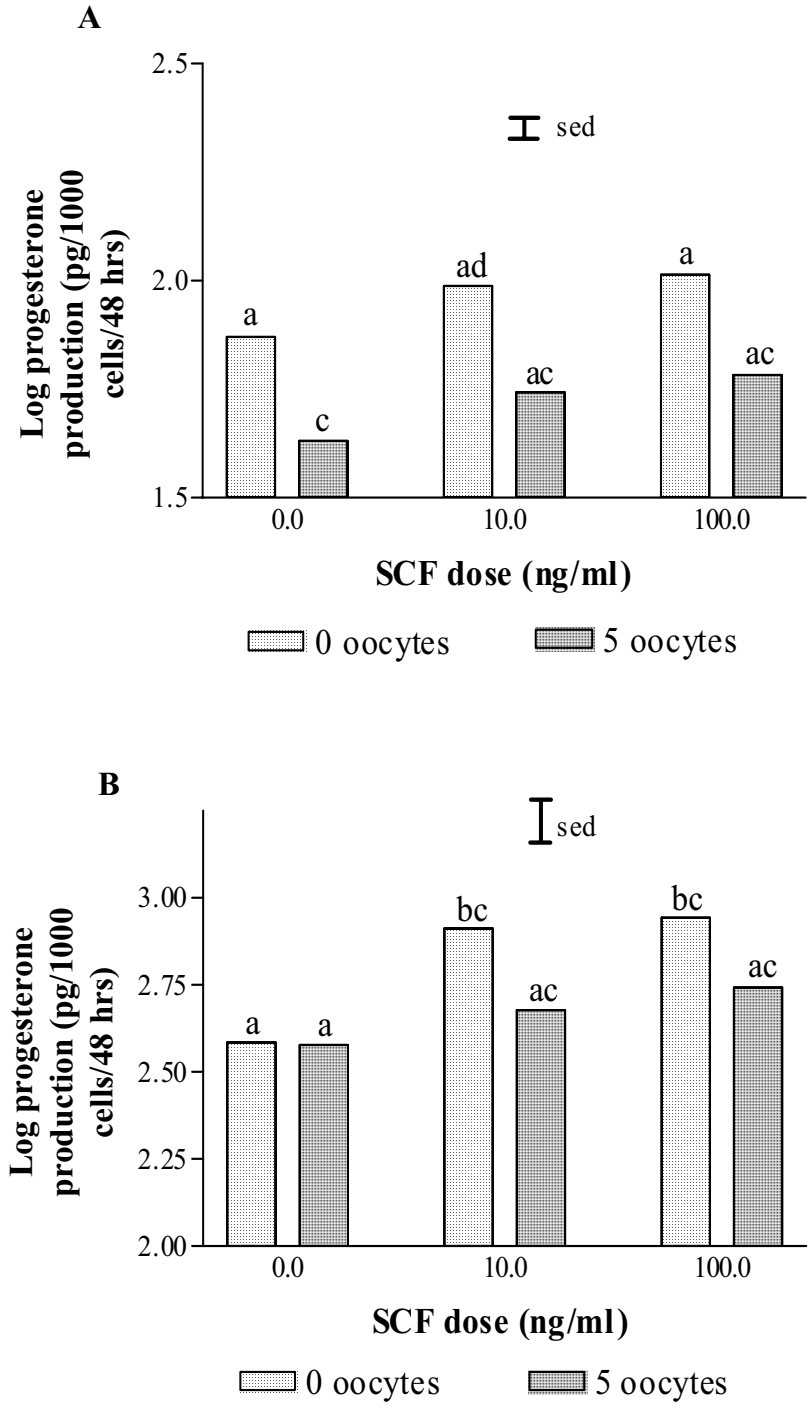

Figure 7
Mean (± sed) $\log_{10}$ progesterone production by co-cultured granulosa and theca cells after (A) 48 and (B) 144 hours in serum free culture supplemented with either 0, 10 or 100 ng/ml hSCF ± 5 oocytes per well. Values are from 3 independent cultures, each having 4 replicates. There was a significant effect of treatment in (A) ($P < 0.001$) and in (B) ($P = 0.025$). Bars with different superscripts are significantly ($P < 0.05$) different.

The results demonstrate that oocyte secreted factors are potent inhibitors of luteinization. OCM and oocyte co-culture consistently reduced progesterone production by granulosa cells compared to control cultures (experiment 1). This observation is in agreement with studies in mice and cattle [13,42]. Overall progesterone synthesis increased between 48 and 144 hours and this may indicate that granulosa cells become less responsive to OCM with time. These effects may be associated with i) changes in intracellular signalling pathways (such as c-*jun* or c-*myc* expression); ii) proteolysis of oocyte secreted proteins; iii) reduced production of secreted factors over the incubation period or iv) increased metabolism of any secreted factors by the granulosa cells *in vitro* and remain to be elucidated. In contrast, the opposite effect was observed with oestradiol production. Throughout culture, OCM and oocyte co-culture significantly stimulated oestradiol production. This effect was repeated even with a 1000-fold dilution of OCM (with LR3 IGF-1) and at 144 hours, granulosa cells were still responsive to OCM, which indicates that sensitivity to OCM may increase throughout culture. Oocyte viability after culture was in excess of 85%, which would support the belief that the presence of the oocyte would allow the continued secretion of oocyte factors such as BMP15 and/or GDF-9, which are multifunctional oocyte secreted proteins and hence candidates for the control of ovarian cell function [43,44,8]. Granulosa and theca cells, cultured with OCM, showed a 65% and 15% fall in progesterone levels respectively, compared to controls, after 48 hours. These oocyte-secreted factors appear to be more potent in granulosa cell function than theca cell function, raising the possibility of a cell specific action of oocyte secreted factor(s).

We report for the first time that oocyte secreted factor(s) enhanced oestradiol production. The effect of oocyte secreted factors on porcine granulosa cell steroidogenesis has been studied previously [25], suggesting that the oocyte suppresses oestradiol production. Coskun et al, [25] cultured oocytectomized complexes which contain cumulus granulosa cells whereas the granulosa cells cultured in the present study were isolated by scraping the wall of dissected follicles and were expected to be largely mural granulosa cells and hence more oestrogenic in nature.

Thecal cell progesterone production was also suppressed by OCM and oocyte co-culture (experiment 1). Androstenedione production was also inhibited when theca cells were supplemented with OCM, either with or without LR3 IGF-1. Reduced androstenedione synthesis was also observed when theca cells were co-cultured with 15 oocytes. However, these effects were only observed after 48 hours in culture. The finding that there was an oocyte-dependent decrease in androstenedione production indicates that precursor availability may also play a role in the inhibition of oestradiol synthesis.

OCM and oocyte co-culture suppressed progesterone production by co-cultured granulosa and theca cells after the addition of LR3 IGF-1 (experiment 2). It can be postulated that the oocyte-induced reduction in progesterone production is elicited by inhibiting 3β HSD activity and enzymes responsible for pregnenolone synthesis. Oocyte secreted factors may also inhibit androstenedione synthesis by the attenuation of side chain cleavage P450 enzyme. If this is the case, then precursors for androstenedione synthesis are also reduced, although this remains to be elucidated. It is also reasonable to hypothesize that oocyte secreted factors reduce theca cell oestradiol synthesis because of low levels of precursor availability. This is overcome in the granulosa cell cultures because of the supplementation with exogenous testosterone. In addition, the observation that OCM stimulated oestradiol production indicates that oocyte secreted factors potentiate aromatase P_{450} activity.

Despite many similarities, the results also highlight the differences between cell function under the influence of either OCM or oocyte co-culture. For example, as observed with granulosa cells, only 5 oocytes per well were sufficient to effect an increase in viable cell numbers, whereas several thousands of oocytes were required to condition medium before any such effect was observed. This implies that oocyte secretions are extremely potent and are of a labile nature. Other workers have reported the labile nature of oocyte secretions [2]. *In vivo*, a single oocyte is bathed in approximately 10–250 μl of follicular fluid [45] in medium-sized follicles (2–6 mm) which emphasises how potent oocyte secreted factors must be in their physiological control of granulosa cell development and function.

SCF has many roles in follicle development [19] and is a granulosa cell derived peptide [46] which is expressed at high levels in mural granulosa cells in murine preovulatory follicles [47]. The addition of SCF to granulosa cell cultures in the present study (experiment 3) elicited an increase in progesterone production after 48 hours in culture. Morphological appearance of the granulosa cells however, did not suggest luteinization. SCF increased oestradiol after 48 hours in culture and similarly oocyte co-culture also increased oestradiol concentrations above controls (Experiment 3). These effects are in agreement with Experiment 1 and also correspond with the finding that human follicular fluid concentrations of SCF are positively correlated to oestradiol concentrations [48].

The receptor for SCF, c-kit, has been isolated to bovine the theca cells [46]. However, the results demonstrate that

SCF acted in an autocrine manner in granulosa cells, which suggests that there is either c-kit present on granulosa cells or that SCF is capable of eliciting a biological response through receptors similar to c-kit or through c-kit receptor sub-types. A decrease in cell number was observed when theca cells were supplemented with SCF. This is in contrast to Parrott et al, [46] who reported an increase in theca cell number and thereby highlights differences in the action of SCF between mono- and poly-ovulatory species. Very little data is available on SCF levels in ovarian tissue, however, Tanikawa et al, [48] reported a range of 0.116 – 3.26 ng/ml SCF in human follicular fluid, which is comparable to the lower concentrations of SCF used in the dose response study (Experiment 3).

Progesterone production by co-cultured granulosa and theca cells showed an increase with SCF, both with and without oocytes, throughout culture. However, progesterone suppression was observed with oocyte co-culture. This was identical to the pattern observed with granulosa cells alone. This suggests that granulosa cells are the major source of progesterone in this co-culture system and that SCF may be responsible for cellular differentiation, including the stimulation of 3β-HSD activity.

Theca cells became more sensitive to SCF with time in culture in terms of increased androstenedione production at both doses of SCF. This is in agreement with studies in cattle demonstrating that SCF stimulates androgen production by theca cells [46].

Oestradiol production also followed an interesting trend. In contrast, after 48 hours, SCF reduced oestradiol production, both in the presence and absence of oocytes. However, after 144 hours, theca cells, co-cultured with oocytes and 10 ng/ml SCF, stimulated oestradiol concentrations – supporting an inverse relationship between cellular differentiation and steroidogenesis. 100 ng/ml SCF inhibited oestradiol production by co-cultured cells compared with lower doses. A similar effect was observed when either granulosa or theca cells were cultured alone (with 100 ng/ml SCF). Oestradiol production was also inhibited in theca and oocyte co-cultures, compared to controls, at all doses of SCF. However, these observations are in contrast to granulosa and oocyte co-culture, where 10 ng/ml SCF stimulated oestradiol production. If porcine theca cells are the main source of SCF, these results support a role of theca cells in modulating granulosa cell steroidogenesis.

The results demonstrate that oocyte derived factors may inhibit 3β-HSD activity but stimulate the aromatase enzyme complex. Huang et al, [49] showed that SCF, in combination with IGF-1, increased the expression of StAR, LH receptor, $P450_{scc}$, CYP17 and 3β-HSD in theca cell cultures which supports a modulatory role for SCF. The findings of the present study indicate the presence of a co-ordinated interaction between somatic and germ cells whereby oocyte derived signals are mediated between the granulosa and theca cells and that granulosa cells secrete factors that promote theca cell differentiation. The present study also supports the role of feedback signals from the granulosa cells to the theca cells. A positive feedback loop has been identified between theca and granulosa cells that is mediated by keratinocyte growth factor (KGF), hepatocyte growth factor (HGF) and SCF [50].

In conclusion, this work has demonstrated that the oocyte is a potent modulator of ovarian cell proliferation and an inhibitor of luteinization. It has also shown that SCF can dramatically affect ovarian cell function by modulating the interaction between granulosa and theca cells. This supports the hypothesis that local granulosa-theca cell interactions play an important role in regulating cellular function within ovarian follicles and that oocyte secreted factors have a key role in this feedback loop. Although the identification of oocyte secreted factor(s) have not yet been elucidated, we have recently demonstrated the presence of a functional BMP system in the porcine ovary whereby BMP receptors were localised particularly in the oocytes and granulosa cells, along with BMP expression in the oocyte [51]. Further investigations will reveal the functional significance and mechanisms of action of BMPs and other oocyte factors on follicular cell function.

Authors' contributions

VB carried out all experiments and drafted the manuscript. MM provided technical assistance. RW and MGH co-ordinated the study and gave advice during manuscript preparation. All authors read and approved the manuscript.

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References

1. Downs S: **The influence of glucose, cumulus cells, and metabolic coupling on ATP levels and meiotic control in the isolated mouse oocyte.** *Developmental Biology* 1995, **167:**502-512.
2. Eppig JJ, Wigglesworth K, Pendola F and Hirao Y: **Murine oocytes suppress expression of luteinizing hormone receptor messenger ribonucleic acid by granulosa cells.** *Biology of Reproduction* 1997, **56:**976-984.
3. Vanderhyden BC: **Oocyte-secreted factors regulate granulosa cell steroidogenesis.** *Zygote* 1996, **4:**317-321.
4. Erickson GF and Shimasaki S: **The physiology of folliculogenesis: the role of novel growth factors.** *Fertility and Sterility* 2001, **76(5):**943-949.
5. Matzuk MM, Burns KH, Viveiros MM and Eppig JJ: **Intercellular communication in the mammalian ovary: oocytes carry the conversation.** *Science* 2002, **296:**2178-2180.
6. Eppig JJ, Wigglesworth K and Pendola FL: **The mammalian oocyte orchestrates the rate of ovarian follicular development.** *Proceedings of the National Academy of Science* 2002, **99(5):**2890-2894.

7. Galloway SM, Gregan SM, Wilson T, McNatty KP, Juengel JL, Ritvos O and Davis GH: **Bmp15 mutations and ovarian function.** *Molecular and Cellular Endocrinology* 2002, **191:**15-18.
8. Otsuka F, Yao Z, Lee T, Yamamoto S, Erickson GF and Shimasaki S: **Bone morphogenetic protein-15. Identification of target cells and biological functions.** *Journal of Biological Chemistry* 2000, **275(50):**39523-39528.
9. Dube JL, Wang P, Elvin J, Lyons KM, Celeste AJ and Matzuk MM: **The bone morphogenetic protein 15 gene is X-linked and expressed in oocytes.** *Molecular Endocrinology* 1998, **12:**1809-1817.
10. Dong J, Albertini DF, Nishimori K, Kumar TR, Lu N and Matzuk MM: **Growth differentiating factor 9 is required during early ovarian folliculogenesis.** *Nature* 1996, **38:**531-535.
11. McGrath SA, Esquela AF and Lee SJ: **Oocyte specific expression of growth/ differentiation factor-9.** *Molecular Endocrinology* 1995, **9:**131-136.
12. Hsueh AJW, McGee EA, Hayashi M and Hsu SY: **Hormonal regulation of early follicle development in the rat ovary.** *Molecular and Cellular Endocrinology* 2000, **163:**95-100.
13. Vanderhyden BC, Cohen JN and Morley P: **Mouse oocytes regulate granulosa cell steroidogenesis.** *Endocrinology* 1993, **133(1):**423-426.
14. Lanuza GM, Fischman ML and Baranao JL: **Growth promoting activity of oocytes on granulosa cells is decreased upon meiotic maturation.** *Developmental Biology* 1998, **197:**129-139.
15. Gilchrist RB, Ritter LJ and Armstrong DT: **Mouse oocyte mitogenic activity is developmentally co-ordinated throughout folliculogenesis and meiotic maturation.** *Developmental Biology* 2001, **240(1):**289-293.
16. Kotsuji F, Kubo M and Tominaga T: **Effect of interactions between granulosa and thecal cells on meiotic arrest in bovine oocytes.** *Journal of Reproduction and Fertility* 1994, **100(1):**151-156.
17. Richard FJ and Sirard M: **Effects of follicular cells on oocyte maturation. II: Theca cell inhibition of bovine oocyte maturation in vitro.** *Biology of Reproduction* 1996, **54:**22-28.
18. Yada H, Hosokawa K, Tajima K, Hasegawa Y and Kotsuji F: **Role of ovarian theca and granulosa cell interaction in hormone production and cell growth during the bovine follicular maturation process.** *Biology of Reproduction* 1999, **61:**1480-1486.
19. Yoshida H, Takakura N, Kataoka H, Kunisada T, Okamura H and Nishikawa S: **Step-wise requirement of c-kit tyrosine kinase in mouse ovarian follicle development.** *Developmental Biology* 1997, **184:**122-137.
20. Gentry PC, Smith GW, Anthony RV, Zhang Z, Long DK and Smith MF: **Characterisation of ovine stem cell factor messenger ribonucleic acid and protein in the corpus luteum throughout the luteal phase.** *Biology of Reproduction* 1996, **54:**970-979.
21. Parrott JA and Skinner MK: **Thecal cell granulosa cell interactions involve a positive feedback loop among keratinocyte growth factor, hepatocyte growth factor, and kit ligand during ovarian follicular development.** *Endocrinology* 1998, **139(5):**2240-2245.
22. Tisdall DJ, Fidler AE, Smith P, Quirke LD, Stent VC, Heath DA and McNatty KP: **Stem cell factor and c-kit gene expression and protein localisation in the sheep ovary during fetal development.** *Journal of Reproduction and Fertility* 1999, **116:**277-291.
23. Joyce IM, Pendola FL, Wigglesworth K and Eppig JJ: **Oocyte regulation of kit ligand expression in mouse ovarian follicles.** *Developmental Biology* 1999, **214:**342-353.
24. Reynaud K, Cortvrindt R, Smitz J and Driancourt M: **Effects of kit ligand and anti-kit antibody on growth of cultured mouse preantral follicles.** *Molecular Reproduction and Development* 2000, **56:**483-494.
25. Coskun S, Uzumcu M, Lin YC, Friedman CI and Alak BM: **Regulation of cumulus cell steroidogenesis by the porcine oocyte and preliminary characterisation of oocyte-produced factor(s).** *Biology of Reproduction* 1995, **53:**670-675.
26. Eppig JJ, Pendola FL and Wigglesworth K: **Mouse oocytes suppress cAMP-induced expression of LH receptor mRNA by granulosa cells in vitro.** *Molecular Reproduction and Development* 1998, **49:**327-332.
27. Campbell BK, Scaramuzzi RJ and Webb R: **Induction and maintenance of oestradiol an immunoreactive inhibin production with FSH by ovine granulosa cells cultured in serum-free media.** *Journal of Reproduction and Fertility* 1996, **106:**7-16.
28. Gutierrez CG, Campbell BK and Webb R: **Development of a long-term bovine granulosa cell culture system: induction and maintenance of estradiol production, response to follicle-stimulating hormone, and morphological characteristics.** *Biology of Reproduction* 1997, **56:**608-616.
29. Picton HM, Campbell BK and Hunter MG: **Maintenance of oestradiol production and expression of cytochrome P450 aromatase enzyme mRNA in long-term serum-free culture of pig granulosa cells.** *Journal of Reproduction and Fertility* 1999, **115:**67-77.
30. Shores EM, Picton HM and Hunter MG: **Differential regulation of pig theca cell steroidogenesis by LH, insulin-like growth factor I and granulosa in serum free culture.** *Journal of Reproduction and Fertility* 2000, **118(2):**211-219.
31. Hay MF, Cran DG and Moor RM: **Structural changes occurring during atresia in sheep ovarian follicles.** *Cell and Tissue Research* 1976, **169:**515-529.
32. Tennant JR: **Evaluation of the trypan blue technique for determination of cell viability.** *Transplantation* 1964, **2(6):**685-694.
33. Borenfreund E and Puerner JA: **A simple quantitative procedure using monolayer cultures for cytotoxicity assays (HTD/NR-90).** *Journal of Tissue Culture* 1984, **9:**7-9.
34. Webb R, Baxter G, McBride D, Nordblum GD and Shaw MPK: **The measurement of testosterone and oestradiol-β using iodinated tracers and incorporating an affinity chromatography extraction procedure.** *Journal of Steroid Biochemistry* 1985, **23(6A):**1043-1051.
35. Gong G: **The role of growth hormone, insulin-like growth factor-I and insulin in the control of ovarian follicular growth and development in the heifer.** *PhD thesis. University of Edinburgh* 1992.
36. Corrie JET, Hunter WM and MacPherson JS: **A strategy for radioimmunoassay of plasma progesterone with use of a homologous site ^{125}I labelled radioligand.** *Clinical Chemistry* 1981, **27(4):**594-599.
37. Law AS, Baxter G, Logue DN, O'Shea T and Webb R: **Evidence for the action of bovine follicular fluid factors other than inhibin in suppressing follicular development and delaying oestrus in heifers.** *Journal of Reproduction and Fertility* 1992, **96(2):**603-616.
38. Thompson S, Wallace AM and Cook B: **A ^{125}I-radioimmunoassay for measuring androstenedione in serum and in blood spot samples from neonates.** *Clinical Chemistry* 1989, **35:**1706-1712.
39. Campbell BK, Mann GE, McNeilly AS and Baird DT: **The pattern of ovarian inhibin, estradiol and androstenedione secretion during the estrous cycle of the ewe.** *Endocrinology* 1990, **127(1):**227-235.
40. Lawes Agricultural Trust: **Genstat 5 version 4.1.** *IACR, Rothamstead, UK* Third1997.
41. Gutierrez CG, Glazyrin AL, Robertson GW, Campbell BK, Gong JG, Bramley TA and Webb R: **Ultra-structural characteristics of bovine granulosa cells associated with maintenance of oestradiol production in vitro.** *Molecular and Cellular Endocrinology* 1997, **134:**51-58.
42. Glister C, Groome NP and Knight PG: **Oocyte-mediated suppression of follicle-stimulating hormone and insulin-like growth factor induced secretion of steroids and inhibin-related proteins by bovine granulosa cells in vitro: possible role of transforming growth factor α.** *Biology of Reproduction* 2003, **68:**758-765.
43. Yamamoto N, Christenson LK, McAllister JM and Strauss JF: **Growth differentiation factor-9 inhibits 3'5'-adenosine monophosphate-stimulated steroidogenesis in human granulosa and theca cells.** *Journal of Clinical Endocrinology and Metabolism* 2002, **87(6):**2849-2856.
44. Vitt UA, Hayashi M, Klein C and Hsueh AJW: **Growth differentiation factor-9 stimulates proliferation but suppresses the follicle-stimulating hormone induced differentiation of cultured granulosa cells from small antral and preovulatory rat follicles.** *Biology of Reproduction* 2000, **62:**370-377.
45. Foxcroft GR and Hunter MG: **Basic physiology of follicular maturation in the pig.** *Journal of Reproduction and Fertility* 1985, **Suppl 33:**1-19.
46. Parrott JA and Skinner MK: **Direct actions of kit-ligand on theca cell growth and differentiation during follicle development.** *Endocrinology* 1997, **138(9):**3819-3827.

47. Motro B and Bernstein A: **Dynamic changes in ovarian c-kit and steel expression during the estrous reproductive cycle.** *Developmental Dynamics* 1993, **197:**69-79.
48. Tanikawa M, Haraha T, Masayuki I, Enatsu A, Iwabe T and Terakawa N: **Presence of stem cell factor in follicular fluid and its expression in the human ovary.** *Fertility and Sterility* 2000, **73(6):**1259-1260.
49. Huang CTF, Weitsman SR, Dykes BN and Magoffin DA: **Stem cell factor and insulin-like growth factor-I stimulate luteinizing hormone independent differentiation of rat ovarian theca cells.** *Biology of Reproduction* 2001, **64:**451-456.
50. Parrott JA and Skinner MK: **Theca cell-granulosa cell interactions that induce primordial follicle development and promote folliculogenesis.** *Biology of Reproduction* 1997, **56(Suppl 1):**125.
51. Quinn R, Shuttleworth G and Hunter MG: **Localisation of bone morphogenetic protein (BMP)-15 and BMP receptors in the porcine ovary.** *Biology of Reproduction* 2002, **66(Suppl 1):**110-111.