

Activin promotes follicular integrity and oogenesis in cultured pre-antral bovine follicles

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ABSTRACT: The aim of this study was to determine the individual and combined effect of activin and follicle stimulating hormone (FSH) on somatic and germ cell development in cultured pre-antral follicles. Pre-antral bovine follicles (mean diameter 157 ± 3 , range 132–199 μm) were cultured for 8 days in serum-free medium in the presence of either 100 ng/ml of recombinant human activin A (rhAct A), 100 ng/ml rhAct A combined with a high (100 ng/ml) or low (50 ng/ml) concentration of recombinant FSH (rFSH) or 50 ng/ml rFSH alone. Intrafollicular connexin 43 expression and actin-based cell adhesion were assessed on Day 2 and 4 of culture. Steroidogenesis was evaluated after Day 4 and 8. Follicles exposed to 100 ng/ml activin maintained expression of connexin 43 at the follicular periphery. In the presence of activin, with or without 100 ng/ml or 50 ng/ml FSH, follicles were steroidogenic undergoing significant growth ($P < 0.01$), granulosa cell proliferation ($P < 0.01$) and antral cavity formation ($P < 0.05$) compared with cultured controls. Maximum oocyte growth occurred in the presence of 100 ng/ml activin alone with a significant percentage of these oocytes maintaining normal morphology over controls ($P < 0.05$). These results are consistent with a role for activin in maintaining oocyte granulosa cell interactions due to increased peripheral granulosa cell adhesion to the basement membrane and retention of adhesion at the surface of the zona pellucida. Thus, the polarized expression of cell contact interactions promoted by activin supports ongoing folliculogenesis.

Key words: pre-antral / bovine / *in vitro* / activin / FSH

Introduction

Pre-antral follicles are a potential source of fertilizable gametes. The ability to sustain pre-antral follicle growth *in vitro* while supporting acquisition of oocyte competence is of great interest both as a means of supplying oocytes for assisted reproductive technologies and of furthering the understanding of somatic cell/oocyte interactions in species undergoing prolonged follicular growth. Systems have been successfully defined by rodent studies with the birth of pups from *in vitro* matured oocytes derived from murine cumulus–oocyte complexes (Eppig and Schroeder, 1989), primordial follicles (Eppig and O'Brien, 1996) and cultured primary follicles (Spears *et al.*, 1994). These successes have yet to be repeated using incompetent oocytes from domestic species or humans which undergo a protracted developmental period *in vivo*. Development of systems supporting prolonged culture has been hindered by oocyte degeneration associated with the disruption of the contact between the oocyte and its companion somatic cells. It is essential therefore, to better understand how culture conditions impact on the maintenance of appropriate cell interactions during follicle development.

The pre-antral phase of follicle development is typified by growth and mRNA synthesis in the oocyte. Important follicular pre-antral events include the expression of growth and differentiation factor 9 (GDF 9) and junctional connexin (Cx) proteins. GDF 9 is an oocyte specific member of the transforming growth factor- β (TGF- β) superfamily associated with oocyte growth (Elvin *et al.*, 2000) and follicle differentiation (Webb *et al.*, 1999). Connexins form gap junctions which directly unite the oocyte to its surrounding granulosa cells as well as linking together neighbouring granulosa cells within the follicle (Carabatsos *et al.*, 2000). Several different connexin proteins have been localized in a number of mammalian species including cattle, with differential expression associated with follicle development and survival (Grazul-Bilska *et al.*, 1998; Johnson *et al.*, 1999; Wright *et al.*, 2001). Another TGF- β superfamily member activin, known to be involved in follicle activation (Findlay, 1993) and pre-antral development (Knight and Glistler, 2001; Ethier and Findlay 2001; Findlay *et al.*, 2002), is expressed by granulosa cells and oocytes. Activin is comprised of two beta subunits, A or B and exists as a homo or heterodimer, therefore three isoforms of activin exist; A, AB and B with activin A the predominant isoform. Systemically, activin promotes the release of follicle stimulating hormone (FSH)

from the anterior pituitary (Katayama *et al.*, 1990) although its intraovarian effects include promotion of aromatase activity, antral cavity formation and granulosa cell proliferation (Findlay, 1993; Mizunuma *et al.*, 1999; Zhao *et al.*, 2001). Activin has been localized in the oocytes and granulosa cells of rodent (Zhao *et al.*, 2001), porcine (van den Hurk and van de Pavert, 2001), caprine (Silva *et al.*, 2006) and bovine follicles (Hulshof *et al.*, 1997), in the granulosa cells of human follicles (Rabinovici *et al.*, 1992; Roberts *et al.*, 1993) and in the thecal layers of porcine (van den Hurk and van de Pavert, 2001) and human (Roberts *et al.*, 1993). Activin stimulated follicle growth *in vitro* has been demonstrated in pre-antral ovine (Thomas *et al.*, 2003) and caprine follicles (Silva *et al.*, 2006) and we have recently shown that activin enhances growth and survival of human pre-antral follicles *in vitro* (Telfer *et al.*, 2008). In rodent species, however, the effect of activin *in vitro* is less clear with studies demonstrating both promotion (Xiao *et al.*, 1992; Zhao *et al.*, 2001) and inhibition (Mizunuma *et al.*, 1999) of pre-antral follicle growth.

It has long been established that follicle growth becomes critically dependent on FSH at the emerging antral stage (Oktay *et al.*, 1998). Similarly it is accepted that activation of follicle growth and initial development can occur in the absence of FSH (Peters *et al.*, 1973) despite the presence of FSH receptors on the granulosa cells of early pre-antral follicles (O'Saughnessy *et al.*, 1996). The sensitivity therefore, of pre-antral follicular cells to FSH is unclear. FSH can promote connexin protein expression in cultured rat granulosa cells (Yogo *et al.*, 2002) as well as augmenting the action of activin (Xiao *et al.*, 1990; Findlay, 1993) and simulating FSH receptor expression in granulosa cells (Xiao *et al.*, 1992) indicating that a relationship between the follicular actions of FSH and activin exists.

We hypothesized that activin could promote oocyte and granulosa cell development in cultured pre-antral bovine follicles whilst maintaining oocyte/somatic cell association and that this effect could be enhanced by the addition of FSH. We further hypothesized that by increasing the concentration of FSH in the presence of activin, *in vitro* oocyte growth could be enhanced without disruption of the intercellular association between the oocyte and surrounding somatic cells.

To test this hypothesis, pre-antral bovine follicles were cultured in a concentration of activin, known to promote ovine (Thomas *et al.*, 2003) and human (Telfer *et al.*, 2008) follicle development. Follicles were cultured in activin alone, FSH alone or in activin combined with FSH at two different concentrations. The effects of these culture conditions on follicle and oocyte growth, maintenance of oocyte morphology and oocyte somatic cell association were evaluated.

Materials and Methods

Isolation and selection

Bovine ovaries from freshly abattoir-slaughtered heifers were transported at 33–38°C in HEPES buffered M199 media (Invitrogen Ltd, Paisley, UK) supplemented with amphotericin B (2.5 µg/ml; Invitrogen Ltd, Paisley, UK), pyruvic acid (25 µg/ml), penicillin G (75 µg/ml) and streptomycin (50 µg/ml; all Sigma Chemicals, Poole, UK). 10 to 40 ovaries were collected per abattoir visit. Under laminar flow conditions the ovaries were rinsed in 70% alcohol and fine strips of cortex removed using a scalpel; no more than two strips of tissue were removed from any one ovary to ensure no bias was introduced in sampling. The strips were then placed in dissection medium [Leibovitz medium supplemented with sodium pyruvate (2 mM),

glutamine (2 mM; both Invitrogen Ltd., Paisley, UK), bovine serum albumin (BSA; Fraction V, 3 mg/ml), penicillin G (75 µg/ml) and streptomycin (50 µg/ml; all Sigma Chemicals, Poole, UK)]. Follicles ($157 \pm 3 \mu\text{m}$) were dissected using 25 gauge needles; those with an intact basement membrane and no antral cavity were selected for culture.

Follicle culture

Eleven replicate experiments were established. Follicles were cultured individually in 96 well V-bottomed culture plates (Corning Costar Europe, Badhoevedorp, The Netherlands) in 150 µl of culture medium. For the control group bovine pre-antral follicles ($n = 41$) were incubated in McCoy's 5a medium with bicarbonate supplemented with HEPES (20 mM), glutamine (3 mM; both Invitrogen Ltd., Paisley, UK), BSA (Fraction V 0.1%), penicillin G (0.1 mg/ml), streptomycin (0.1 mg/ml), transferrin (2.5 µg/ml), selenium (4 ng/ml), insulin (10 ng/ml) and ascorbic acid (50 µg/ml), all Sigma Chemicals, Poole, UK.

Four treatment groups were established; 100 ng/ml recombinant human activin A (rhAct A; R&D Systems, Abingdon, UK; $n = 39$), 50 ng/ml recombinant FSH (rFSH; Sigma Chemicals Poole, Dorset, UK; $n = 37$), 100 ng/ml rhAct A with 50 ng/ml rFSH ($n = 43$) and 100 ng/ml rhAct A with 100 ng/ml rFSH ($n = 45$). A higher concentration of FSH alone (100 ng/ml) was also tested. In this treatment group follicle growth and oocyte integrity were severely compromised with few follicles surviving the culture period. To determine whether activin could ameliorate the effects of a high FSH concentration 100 ng/ml rhAct A combined with 100 ng/ml FSH was tested. On setting up each experiment, a number of follicles were randomly selected from the isolated follicle pool and fixed on the day of dissection (Day 0; $n = 19$).

Follicles were randomly assigned to treatment groups and incubated for a maximum of 8 days at 37°C in humidified air with 5% CO₂. Every fourth day half the culture medium was removed and replaced, concomitant with measuring of follicle diameters. Upon microscopic inspection on Days 4 and 8, any degenerate follicles were removed from culture and excluded from the study; the prevalence of follicle deterioration was not significantly different between treatment groups. No more than three follicles were removed from any treatment group over the total culture period.

Assessment of histology

On completion of culture follicles were fixed overnight Bouins solution and dehydrated in an eosin/alcohol solution in increasing concentrations (70, 90 and 100%). Thereafter follicles were immersed in cedar wood oil (BDH Laboratory Supplies, Poole, UK) for 24 h then placed in toluene (Fisher Scientific UK Ltd, Loughborough, UK) for 30 min to remove all traces of the oil. Follicles were embedded in paraffin wax at 60°C for 4 h, with hourly renewal of the wax to ensure complete clearance of all toluene. Six micrometre thick samples were sectioned, mounted on gelatin coated slides and left to dry overnight prior to staining with haematoxylin and eosin. Using a light microscope with a crossed micrometre, sections of follicles containing the nucleolus were assessed and the following parameters evaluated; follicle and oocyte diameter, antral cavity formation and oocyte integrity. Antral cavity formation was determined as the presence of one or more discreet fluid filled cavities within the granulosa cell population and was determined by inspecting all serial sections of fixed follicles. To distinguish antral cavity formation from incipient cavity formation i.e. the accumulation of follicular fluid between cells observed prior to emergence of a cavity, areas within the granulosa devoid of cells were measured. The diameter of a cavity was calculated by measuring the space at its widest and narrowest point and calculating a mean diameter value; areas with a diameter of $\geq 50 \mu\text{m}$ were considered to be established antral cavities. Spaces occurring between the basal lamina and the outermost layers of granulosa cells were occasionally observed within sections, however, these were considered to

be histological artefacts and not regarded as antral cavities. Oocyte integrity was assessed on the following morphological criteria; general shape, presence of an intact oolemma, evenly distributed ooplasm and visualization of both germinal vesicle and nucleolus. Tissue processing was performed independently of assessment of histology; treatment groups were unknown to examiner, and results were verified by an independent party.

Detection of Ki67 immunoreactivity in cultured follicles

To evaluate granulosa cell proliferation, six uncultured controls and 33 follicles cultured for 8 days were probed for detection of Ki67 immunoreactivity (control $n = 7$; 100 ng/ml rhAct A $n = 7$; 50 ng/ml rFSH $n = 6$, 100 ng/ml rhAct A and 50 ng/ml rFSH $n = 7$; 100 ng/ml rhAct A and 100 ng/ml rFSH $n = 6$). Follicles were fixed in 4% paraformaldehyde, dehydrated and processed for embedding in paraffin wax as described for histological assessment. Six micrometre sections were mounted on to positively charged slides, immersed in xylene and rehydrated through graded alcohol. Antigen retrieval was performed by microwaving for a total of 10 min (5 min on 850 W, 5 min on 600 W) in 0.01 M sodium citrate buffer followed by 20 min standing at room temperature. Following 2×5 min washes in phosphate buffered saline (PBS) 0.01 M, sections were incubated in 1.5% hydrogen peroxide for 10 min to block endogenous peroxidase then washed again in PBS as before. To reduce non-specific staining 1.5% horse serum (Vectastain Elite Kit, PK-6102, Vector Laboratories, CA, USA) was applied to the sections which were then probed with the Ki67 primary antibody, as per manufacturer's instructions (Ki67 mouse monoclonal antibody, Novocastra Laboratories Ltd, Newcastle Upon Tyne, UK) for 90 min at room temperature. Thereafter the sections were washed and probed with the secondary anti-mouse antibody labelled with horseradish peroxidase for 30 min (ABC-Elite Mouse IgG, Vectastain Elite Kit, PK-6102, Vectastain ABC Kit, Vector, Peterborough, UK). 3,3'-Diaminobenzidine was used to detect peroxidase and sections were counterstained with haematoxylin prior to dehydration with alcohol and xylene and mounted with coverslips. Non-specific antibody was detected by replacing the primary antibody with normal horse serum (1.5%). Quantitative determination of Ki67 staining was made on one section of each follicle probed with Ki67 antibody; the section containing the oocyte germinal vesicle and nucleolus. Under light microscopy the total number of granulosa cells in the section containing the germinal vesicle/nucleolus were counted for six or seven follicles per treatment. A total number of cells was calculated for each treatment by summing the six or seven sections counted. The number of cells counted per treatment was; uncultured controls 429, cultured control 843, activin alone 1437, FSH alone 890, activin and low concentration FSH 1196 and activin and high concentration FSH 1008. The proportion of proliferating cells was expressed as a proportion of the total of number of granulosa cells counted per treatment.

Detection of estradiol in culture media by enzyme immunoassay

The effect of the activin and FSH on estradiol secretion was determined by analysis of culture media removed on Day 4 and Day 8. Media from a total of 40 follicles was analysed; eight follicles per treatment. Concentrations of estradiol in reserved media were determined against pre-measured standard dilutions using Estradiol ELISA enzyme immunoassay kit (DRG Instruments, GmbH, Germany). Reserved media was diluted with freshly prepared culture medium (1 in 10), placed in microtiter wells coated with polyclonal (rabbit) antibody raised against the estradiol antigenic site, mixed with estradiol-horseradish peroxidase conjugate and incubated for 120 min. After incubation, the unbound conjugate was washed off and a substrate

solution of tetramethylbenzidine was added to allow colour development. The reaction was stopped after 25 min using dilute sulphuric acid, the wells washed and the absorbance of each well at 450 ± 10 nm measured using a microtiter plate reader. The absorbencies were converted to estradiol concentrations using Sigma Plot Version 9, with four parametric logistic functions (Systat Software Inc.) with an estradiol sensitivity of 25 pg/ml. Specificity of assay; 17β -estradiol 100%, oestriol 0.055% and oestrone 0.2%. The inter- and intra-coefficients of variation were 6.81 and 7.25%, respectively.

Confocal analysis of cell interactions during follicle culture

The impact of culture conditions on intra-follicular cell communication was examined by visualization of connexin 43, a gap junction protein found in homologous and heterologous cell junctions and crucial to normal folliculogenesis and oocyte development *in vitro* (Yogo et al., 2002; Gittens et al., 2005; Li et al., 2007). A total of 50 follicles were processed for confocal imaging and 39 randomly selected for comparison (see below). The size of these follicles was representative of each treatment group, i.e. FSH-only and control cultured follicles were smaller in diameter than activin exposed follicles. Antral cavity formation could not be verified by light microscopy after 2 or 4 days *in vitro*, and incipient cavities cannot be discounted. Comparisons were made between uncultured fixed follicles ($n = 4$) and follicles cultured for 2 ($n = 17$) or 4 ($n = 18$) days in the presence and absence of rhAct A, rFSH alone or rhAct A in combination with 50 ng/ml rFSH. Stromal overgrowth impeded imaging of follicles cultured for longer periods and obscured imaging of some processed follicles reducing the number of results obtained. As a result no statistical analysis was applied to the confocal images; each image represents a group containing either four or five follicles. Images of DNA (Hoechst), connexin 43 immunofluorescence and actin specific (phalloidin) stains were used to assay the distribution of adhesions, gap junctions and nuclear position from complete Z-stack image sets.

Following culture, follicles were fixed in 2% paraformaldehyde supplemented with 1 mg/ml polyvinyl alcohol, 40 μ M phenylarsine oxide and 100 μ M sodium orthovanadate at room temperature for 5 min. Following initial fixation follicles were subsequently fixed in microtubule stabilising buffer (100 mM PIPES, 5 mM $MgCl_2$, 2.5 mM EGTA, 2% formaldehyde, 0.1% Triton-X-100, 1 mM taxol, 10 U/ml apoprotinin and 50% deuterium oxide) for 30 min at 35°C and stored in wash solution (Barrett and Albertini, 2007) until ready for processing. Samples were incubated in an anti-connexin 43 (1:100; Santa Cruz, CA, USA) antibody for at least 24 h at 37°C with gentle agitation; antibodies were diluted in wash solution. Samples were then washed three times in ample wash solution for a total of 45 min at 37°C with gentle agitation. Secondary detection was carried out using an Alexa 488 Goat anti-rabbit IgG (1:800; Molecular Probes, CA, USA) in combination with 1 μ g/ml of Hoechst 33258 (Molecular Probes) and samples were again incubated at 37°C with gentle agitation for at least 24 h. Thereafter the samples were incubated for an additional 2 h in the presence of Rhodamine-phalloidin (1:100, Molecular Probes) to allow the detection of actin, a filamentous protein associated with gap junction stability (Albertini et al., 2001) followed by three rinses in wash solution for no less than a total of 45 min at 37°C with gentle agitation. Samples were mounted in medium containing 50% glycerol/PBS containing 25 mg/ml sodium azide and 1 μ g/ml of Hoechst 33258, to allow identification of intra-follicular cell position by localization of cell nuclei, using wax cushions to avoid compression of samples. Samples were analysed on a Zeiss LSM 5 Pascal confocal microscope using a 40x C-Apochromat objective (na 1.2), KrArg 405 and HeNe 543 lasers to allow the collection of a complete z-series of red, green and blue channels progressing through the entire oocyte of each follicle. Line scans were performed using Metamorph 7.5

with lines selected to run from basement membrane to basement membrane through the germinal vesicle at a 45° orientation and all channels were represented as individual histograms.

Statistical analyses

Follicle and oocyte diameters from both freshly isolated and cultured follicles were compared between replicate cultures and between treatments using ANOVA and where indicated two-sample *t*-tests were performed. Estradiol secretion and the number of granulosa cells staining positive for Ki67 were calculated and compared between experimental groups using one-way ANOVA; where indicated two-sample *t*-tests were applied to compare between specific groups. To compensate for multiple *t*-test comparisons, the Bonferroni Adjustment was applied. The proportions of intact oocytes and antral cavity occurrence were assessed using χ^2 analysis and Fisher's Exact Test.

Results

Follicle growth

Follicles were cultured for a maximum of 8 days in control medium ($n = 41$) or in medium containing 100 ng/ml rhAct A ($n = 39$),

50 ng/ml rFSH ($n = 37$), 100 ng/ml rhAct A and either 50 ($n = 43$) or 100 ng/ml rFSH ($n = 45$). Significant growth was observed between Day 0 and Day 8 in follicles cultured in control medium ($P < 0.01$) or in the presence of activin alone, FSH alone or activin with either concentration of FSH ($P < 0.01$). On Day 4 and Day 8 the diameters of follicles cultured in rhAct A alone or rhAct A with high dose rFSH were significantly greater than those cultured in control medium ($P < 0.01$) as illustrated in Fig. 1(i).

Detection of Ki67 antigen

Granulosa cell proliferation was confirmed by detection of the proliferating nuclear antigen Ki67 in either six or seven growing follicles from each treatment group. Significant granulosa cell proliferation was detected in FSH only cultured follicles compared with control ($P < 0.01$). The percentage of positively staining granulosa cells was significantly higher in follicles cultured in 100 ng/ml rhAct A ($P < 0.01$) and 100 ng/ml rhAct A with either 50 ($P < 0.01$) or 100 ng/ml rFSH ($P < 0.01$) compared with those follicles cultured in control or in 50 ng/ml rFSH alone as illustrated in Fig. 1(ii–v).

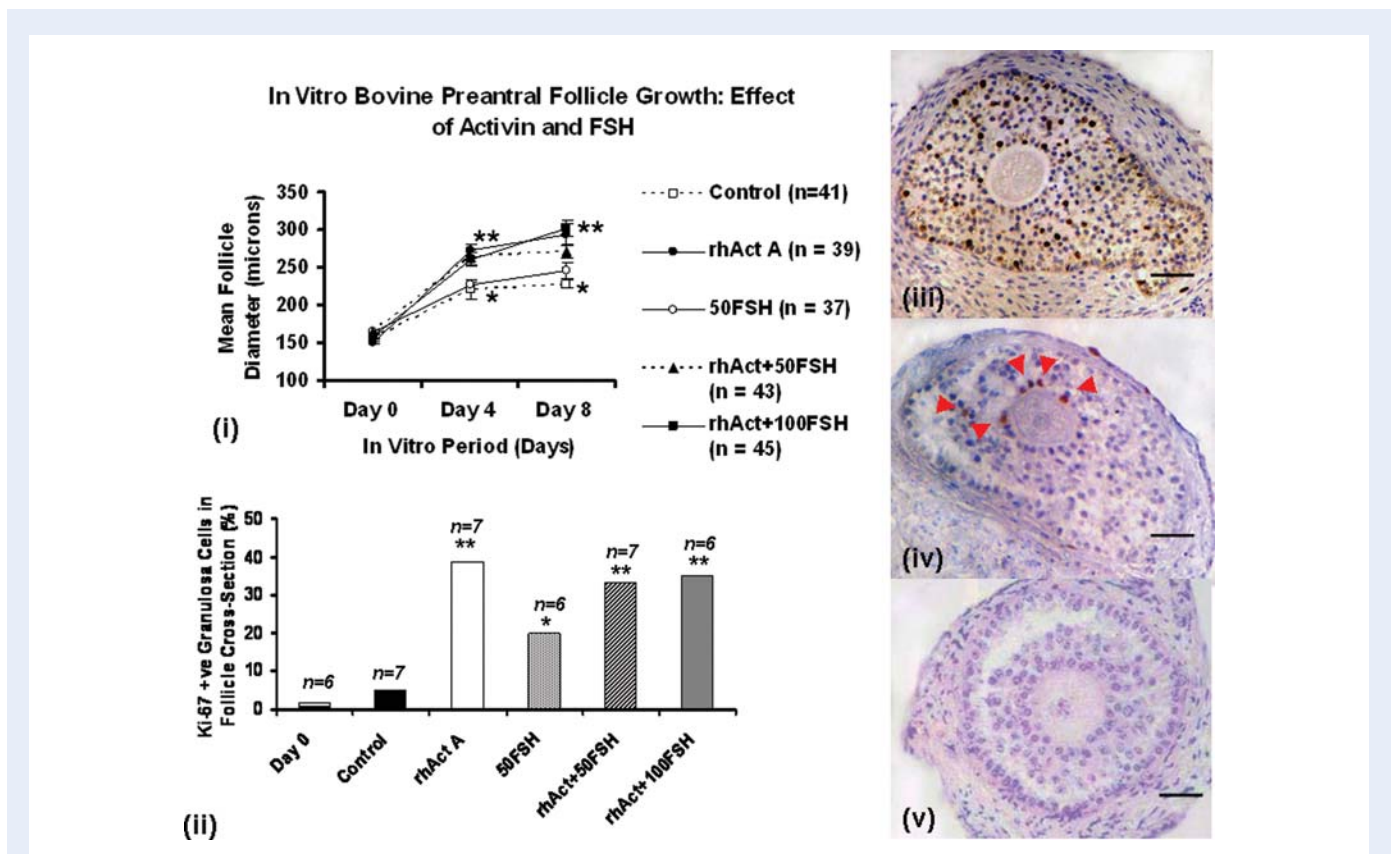


Figure 1 (i) *In vitro* follicle growth. After 8 days follicles cultured in activin alone ($P < 0.01$) or activin and high dose FSH ($P < 0.01$) were significantly larger than those cultured in control medium. Values are mean \pm SEM. Asterisks (* versus **) denote significant differences between treatments at the same time points (ii) Immunohistochemical detection of Ki67 positive cells. Values are Ki67 positive granulosa cells expressed as a percentage of the total number of granulosa cells per treatment. Asterisks (none versus * or **, and * versus **) denote significant differences between values ($P < 0.01$); n denotes number of follicles probed per treatment. (iii) Ki67 positive granulosa cells (brown) in an activin only exposed follicle. (iv) A small number of Ki67 positive cells indicated by (closed triangle) in a control cultured follicle. (v) Negative control; primary antibody omitted, follicle cultured in 50 ng/ml rFSH. Scale bar represents 50 microns.

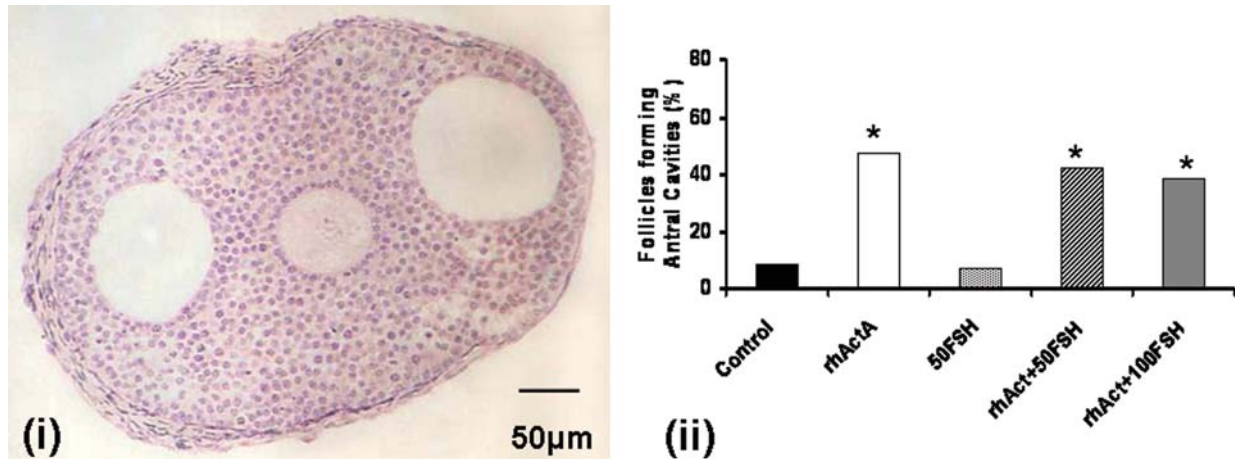


Figure 2 Formation of antral cavities *in vitro*. (i) Established cavitation (two cavities either side of the central oocyte) in a follicle cultured in the presence of 100 ng/ml rhAct A for 8 days. (ii) Antral cavity formation expressed as a percentage of the total number of follicles examined in each treatment group after 8 days *in vitro*. Asterisks denote significant differences between compared with control or 50 ng/ml rFSH ($P < 0.05$).

Antral cavity formation

The presence of an antrum was determined by light microscopy on histological evaluation of follicles fixed at Day 8 of the culture period as illustrated in Fig. 2(i). The percentage incidence of antral cavity formation is shown in Fig. 2(ii). Significant antral formation was observed in follicles cultured in 100 ng/ml rhAct A ($P < 0.05$), 100 ng/ml rhAct A and 50 ($P < 0.05$) or 100 ng/ml rFSH ($P < 0.05$) compared with follicles cultured in control medium or in 50 ng/ml rFSH alone.

Estradiol secretion

Assayed media indicated that all growing follicles were steroidogenically active after 4 days *in vitro* (Fig. 3). Mean estradiol secretion was significantly higher in all activin-exposed follicles at Day 4 compared with control ($P < 0.01$) and FSH alone ($P < 0.01$). The estradiol output of individual follicles varied between Days 4 and 8; with mean estradiol levels maintained in some treatments (control, FSH alone, activin with low dose FSH) and decreased in others (activin alone, activin with high dose FSH; $P < 0.01$).

Oocyte growth and integrity

Oocyte diameters increased in all cultured follicles over time compared with follicles fixed at Day 0; the largest oocytes achieved were cultured in activin alone, where a total increase of over 20 μm was observed, the final mean oocyte diameter in this group measuring 65 μm . Oocytes with a grossly spherical appearance, even ooplasm surrounded by an intact oolemma and a visible germinal vesicle and nucleolus were considered morphologically normal as illustrated in Fig. 4(i). In this study, oocytes lacking in any one of these determinants were deemed suboptimal (Fig. 4(ii)). The percentage of oocytes showing normal oocyte morphology was significantly greater following culture in activin alone ($P < 0.05$), activin and low dose FSH ($P < 0.05$) and FSH alone ($P < 0.05$) compared with control or activin and high FSH cultures (Fig. 4(iii)).

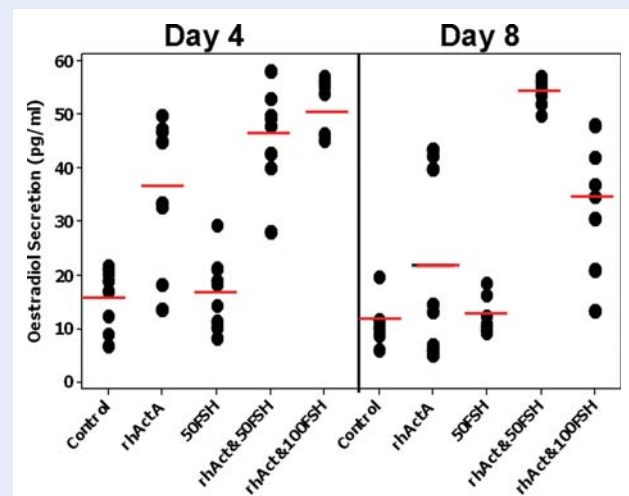


Figure 3 Effect of activin and FSH on *in vitro* steroidogenesis. 17β estradiol levels were measured in reserved culture media from eight individual growing follicles from each treatment. Continuous line denotes mean estradiol level. Levels of estradiol fluctuated in media from individual follicles during the culture period.

Cell-cell and cell-matrix interactions

Actin appeared to be evenly and thickly distributed at the periphery and the granulosa pellucida zona interface in uncultured controls (Fig. 5A). Sparse actin networking was observed in control follicles cultured for either 2 or 4 days *in vitro* ($n = 4$) with poor definition of the granulosa cell-zona pellucida interface. In the presence of FSH either alone ($n = 4$; Fig. 5C) or combined with activin ($n = 5$; Fig. 5E) actin distribution appeared reduced throughout the granulosa and at the granulosa-zona pellucida interface in comparison with activin alone suggesting decreased concentration of intercellular contact in these treatments. In contrast, over the same time period, actin distribution

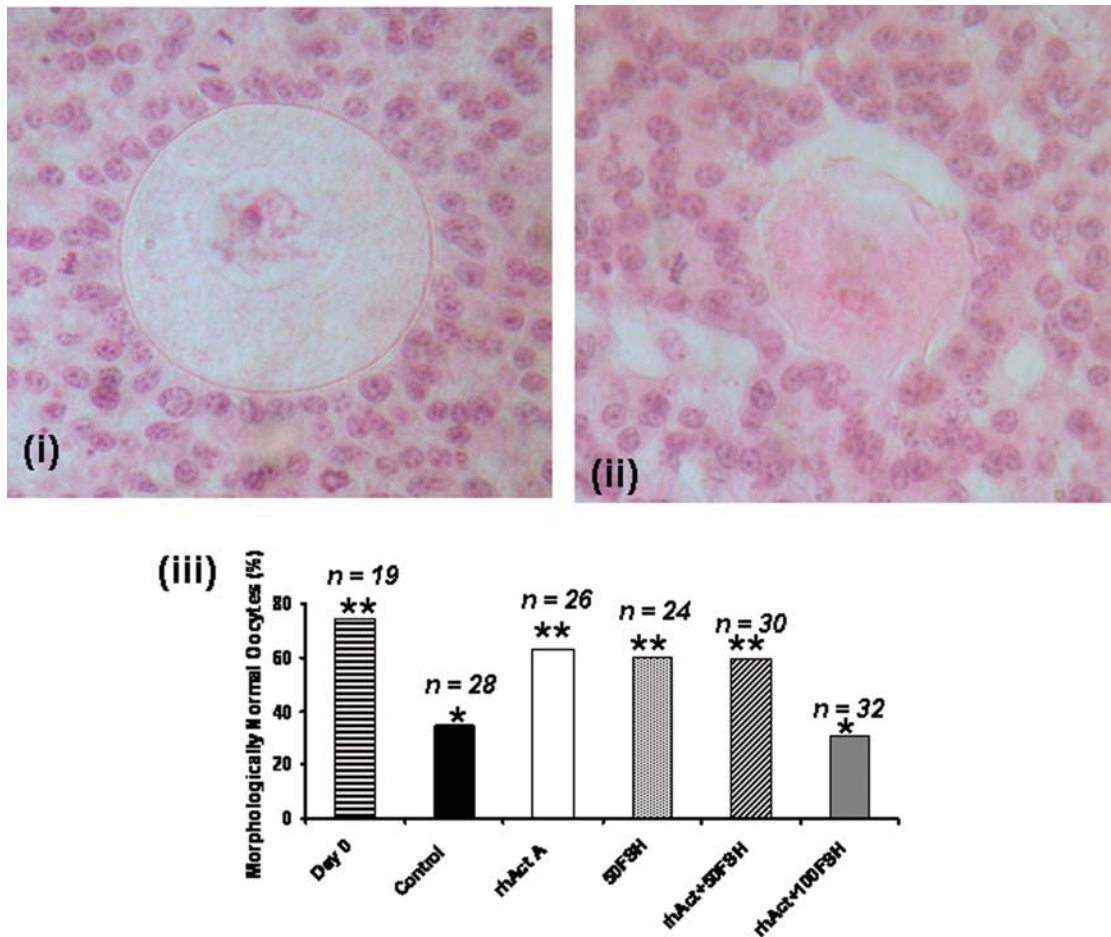


Figure 4 Oocyte integrity in cultured pre-antral bovine follicles. (i) Morphologically normal oocyte after 8 days *in vitro* (ii) Morphologically abnormal oocyte after 8 days *in vitro*. (iii) Effect of Activin and FSH on oocyte morphology. Values are healthy oocytes as expressed as a percentage of the total number of oocytes analysed per treatment group; asterisks denote significant differences between treatments. Normal oocyte morphology was significantly reduced in follicles cultured in control medium ($P < 0.05$) or 100 ng/ml rhAct A with 100 ng/ml rFSH ($P < 0.05$) compared with other groups.

in follicles cultured in 100 ng/ml rhAct A alone ($n = 5$) was distinct and extensive throughout the granulosa indicating the maintenance of intercellular junctions with dense bundles of actin projections located at the granulosa–zona pellucida interface (Fig. 5D).

A conspicuous loss of gap junctions was observed in follicles cultured for 4 days with no supplements ($n = 4$; Fig. 5I) compared with fresh follicles ($n = 4$; Fig. 5G). Notably, in the presence of activin some degree of connexin expression (Fig. 5M and O) and a distinct gradient of expression were visible from the peripheral mural cells to those surrounding the oocyte where expression was minimal. This pattern was less apparent in the presence of FSH alone where connexin expression was scant with patchy concentration in the mural granulosa (Fig. 5K).

The observation that activin sustains a high degree of somatic cell association with the zona pellucida suggests a role in the differential adhesion of somatic cells towards the oocyte. To test this idea, line scans of Day 0 controls and Day 4 cultured follicles were generated to monitor the density of gap junctions and adhesions between the basement membrane and the zona pellucida (Fig. 6). As shown in

Fig. 6 the major difference between Day 0 (Fig. 6A) and control cultured follicles is the disappearance of gap junctions (Fig. 6B). Actin density at the zona pellucida is diminished and nuclear positioning is more dispersed. In contrast, gap junction density is maintained in the presence of activin (Fig. 6C). Moreover high levels of actin-based adhesion to the zona are seen and the distribution of gap junctions has assumed a distinctly peripheral disposition.

Discussion

Systems supporting *in vitro* ruminant and human pre-antral follicle growth have been developed but routine production of fully grown oocytes from such systems has yet to be realized. Previous studies have identified that a combination of activin and FSH can promote development in cultured rodent pre-antral follicles (Xiao *et al.*, 1990, 1992; Li *et al.*, 1995; Ola *et al.*, 2008); however, the effect of this combination of supplements on *in vitro* germ cell development is unclear. This study demonstrates that activin promotes growth and differentiation of bovine pre-antral follicles although maintaining

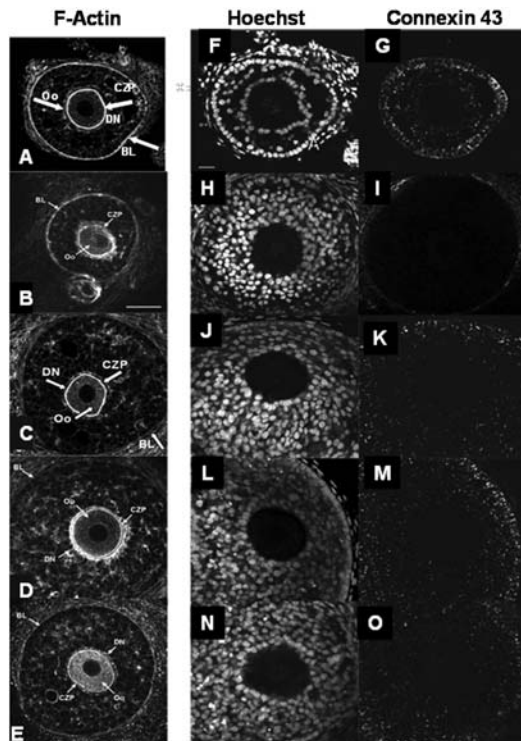


Figure 5 Confocal imaging of F-Actin, Hoechst and Cx43 distribution in bovine follicles. (A) Uncultured follicle: pronounced actin around the oocyte (Oo) in defined dense network (DN). Actin shown in granulosa–zona pellucida interface (CZP) and basal lamina (BL). (B–E) Confocal imaging of the effect of activin and FSH on F-actin distribution after 2 days *in vitro*. (B) Control: poorly defined granulosa–zona pellucida interface (CZP), very modest actin arrangement visible within the granulosa. (C) FSH-only: actin network (DN) visible around the oocyte; small areas of actin network visible within the granulosa. (D) Activin-only: defined DN of actin around the Oo, extensive network of actin throughout the granulosa cells extending to the basal lamina (BL). Prominent DN at the granulosa–zona pellucida interface; distinct projections extending from the granulosa cells. (E) Activin and low dose FSH: actin visible around the oocyte; moderate network visible within the granulosa. Scale bar represents 50 microns. (F, G) Uncultured fixed follicles (F) Hoechst: evenly spaced cells surrounding the oocyte and closely packed layer at the basement membrane; some unevenly distributed granulosa throughout the follicle; (G) Cx43: evident throughout the follicle with fewer plaques near the oocyte and greater density of lateral adhesion between cells at the basement membrane. (H, I) Control follicles (H) Hoechst: evenly distributed granulosa without definable patterning or close association with the oocyte or basement membrane; (I) Cx43: negligible staining in the granulosa. (J, K) 50 ng/ml rFSH-only (J) Hoechst: regular cellular packing throughout the follicle (K); Cx43: plaques most abundant between granulosa nearest the basement membrane; fewer, smaller plaques adjacent to the oocyte (L, M) 100 ng/ml rhAct A-only (L) Hoechst: well defined closely packed layer of granulosa at the basement membrane, dense granulosa throughout follicle and surrounding the oocyte (M) Cx43: small plaques diffuse throughout the follicle with a predominance toward the basement membrane. (N, O) 100 ng/ml rhActA and 50 ng/ml rFSH (N) Hoechst: dense granulosa throughout the follicle; well defined layer of granulosa cells surrounding the oocyte. (O) Cx43: plaques concentrated in outermost layers of granulosa with scant definition nearer the oocyte. Scale bar represents 20 μm .

normal oocyte morphology associated with a distinct pattern of intra-follicular cell adhesions with connexin 43 protein expression concentrated towards the follicle periphery and F-actin distribution density maximal at the cumulus zona interface.

Pre-antral gap junction protein expression is vital to normal folliculogenesis and oocyte maturation *in vivo*. Expression of connexin proteins is highly regulated with specific proteins associated with particular follicular compartments, stages of development and health status (Johnson et al., 1999; Vozzi et al., 2001; Wright et al., 2001; Gittens et al., 2005; Li et al., 2007). In bovine follicles the major connexin proteins are Cx43, 37, 32 and 26 with Cx43 the principal connexin forming gap junctions between somatic cells in healthy growing pre-antral follicles (Vozzi et al., 2001). In this study, follicles cultured in the presence of activin alone grew significantly with diameters increasing from a mean of 157 μm on isolation to a mean of >290 μm over 8 days *in vitro*. Significant Ki67 antibody localization in this group confirmed granulosa proliferation and confocal imaging showed Cx43 expression concentrated in the mural granulosa layers of these follicles. Da Silva-Buttkus et al. (2008) demonstrated that in mice, multi-laminar follicles are formed by the unidirectional proliferation of granulosa cells innermost towards the oocyte, confirmed by Ki67 localization (Da Silva-Buttkus et al., 2008). In this study, the notably peripheral distribution pattern of Cx43 protein seen in the presence of activin may be indicative of a similar spatial pattern of growth with extensive intercellular communication, evidenced by Cx43, required in the peripheral granulosa layers during rapid proliferation *in vitro*. Cx43 expression is negligible in the granulosa of atretic bovine follicles (Wiesen and Midgley, 1994). In this study, follicles cultured in control medium grew slowly and showed oocyte degeneration when compared with activin supplemented follicles. Confocal imaging of control cultured follicles showed no localization of Cx43 protein within the granulosa supporting the idea of activin as a promoter of Cx43 expression in proliferating granulosa *in vitro*. The impact of culture conditions on connexin protein expression and follicle survival could be explored further by localization of the oocyte specific connexin proteins 26 and 37 as well as Cx32, found in the granulosa of atretic, but not healthy, bovine follicles (Johnson et al., 1999). In the presence of FSH alone, follicle growth, antral cavity formation and steroidogenesis occurred but not significantly compared with control follicles. When activin was combined with both concentrations of FSH tested a significant increase was observed with respect to these parameters compared with culture in FSH alone. The effects observed in activin alone were not significantly affected by the addition of either concentration of FSH, moreover addition of activin could not ameliorate a significant loss of oocyte degeneration in the presence of a high concentration of FSH.

In the bovine ovary, antral cavities are usually established in follicles of 200 μm diameter (Moss et al., 1954; Lussier et al., 1987; McNatty et al., 2000). In the present study, the mean follicle diameter after 8 days *in vitro* was >220 and >240 μm in control and FSH, respectively, and >270 μm in all activin exposed follicles. Activin promoted significant antral cavity formation with over a third of all the activin exposed follicles developing cavities. The greatest percentage of cavity formation occurred in activin alone although interestingly, despite a mean follicle diameter in this group of over 290 μm , less than half (48%) formed cavities in contrast with the expected morphology of similar sized bovine follicles *in vivo*. In rodents kit-ligand, connexin 37

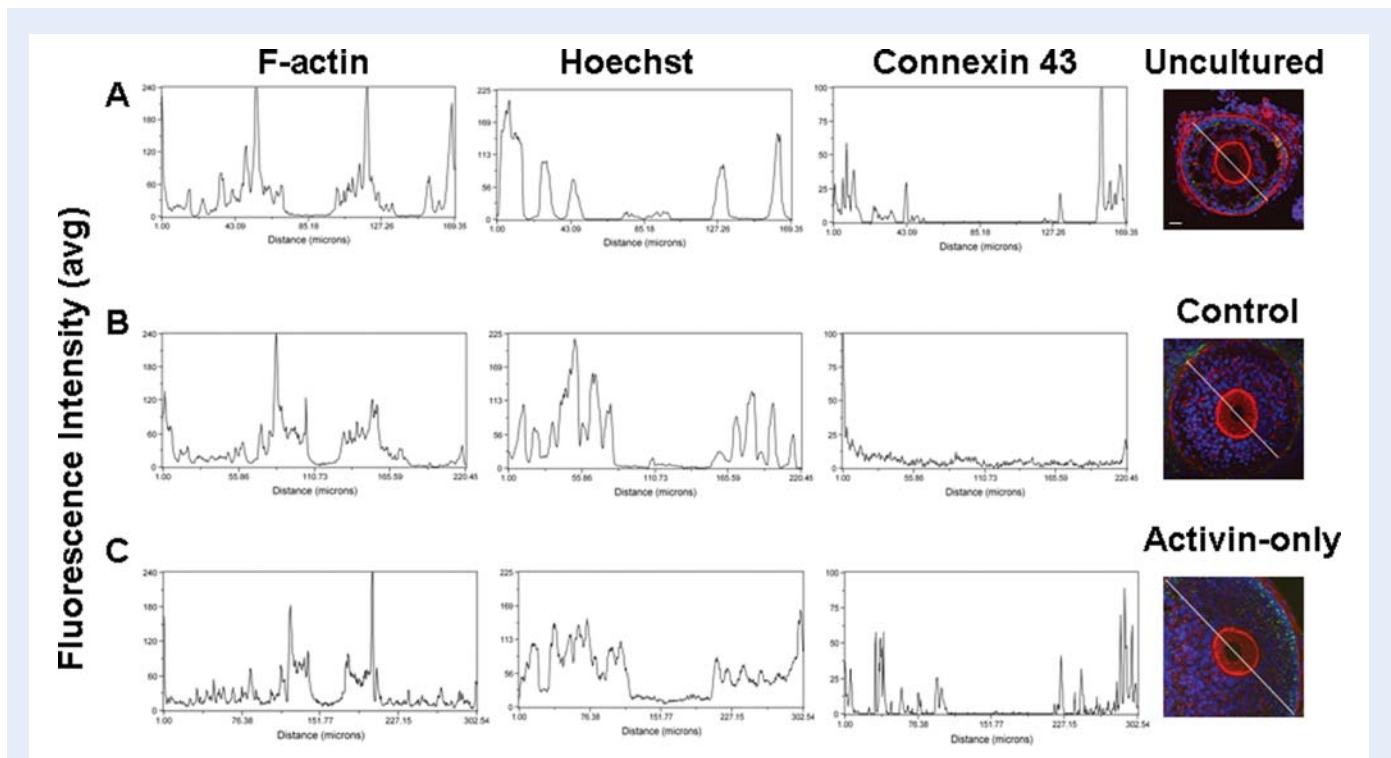


Figure 6 F-actin, Hoechst and connexin 43 distribution patterns in uncultured follicles and follicles cultured for 4 days. Scans performed by defining a line from basement membrane to basement membrane passing through the germinal vesicle. Each colour channel is drawn on the same y-axis scale. **(A)** Uncultured: F-actin: primarily at the oocyte cortex and zona pellucida; Hoechst: few granulosa cells occurring in distinct areas (layers); Cx43: plaques occur in a clear gradient with high density large plaques at the basement membrane subsiding toward the oocyte. **(B)** Control: F-actin: localization heaviest at the oocyte cortex; Hoechst: a greater density of granulosa cells within the follicle than observed in uncultured control; Cx43: uniformity of plaque density between the basement membrane and the oocyte. **(C)** 100 ng/ml rhAct A-only: F-actin: cortical and cytoplasmic patterning throughout with greatest density around the oocyte; Hoechst: tightly packed granulosa cells throughout the follicle; Cx43: polarized distribution with the highest density of plaques at the basement membrane and subsiding toward the oocyte. Scale bar represents 20 μm .

and GDF-9 have been identified as essential for antral cavity formation (Yoshida *et al.*, 1997; Simon *et al.*, 1997; Carabatsos *et al.*, 1998). Moreover, in the presence of FSH, activin promotes cavitation in rat cumulus–oocyte complexes (Li *et al.*, 1995) therefore investigation of whether activin promotes the expression of these proteins in cultured bovine follicles is required.

In vivo production of estrogen occurs in the granulosa cells of antral follicles where it exerts a mitogenic effect. Steroidogenesis occurs in cultured pre-antral ovine follicles in the presence of activin (Thomas *et al.*, 2003) and in pre-antral bovine follicles in the presence of FSH (Wandji *et al.*, 1996). In this study, steroidogenesis was supported from Day 4 onwards in all treatment groups. FSH alone did not increase mean estrogen secretion above the basal rate in agreement with Itoh *et al.* (2002); however, activin alone and activin combined with either concentration of FSH investigated induced significant steroidogenesis by Day 4 of the culture period ($P < 0.01$). Media tested on Day 8 may contain residual estradiol therefore it is not possible to ascertain the proportion of *de novo* steroidogenesis between Days 4 and 8 in this system. The mean level of estrogen secretion was maintained in all treatments between Days 4 and 8 except in activin alone and activin with high dose FSH where the mean level decreased as illustrated in Fig. 3. Mechanical dissection may impact on the thecal cell endowment of each follicle causing variability of

available androgens *in vitro* resulting in fluctuating estrogen synthesis in individual follicles, however, perturbation of the P450 aromatase enzyme system cannot be discounted. Histological analysis demonstrated that oocyte integrity had significantly decreased by Day 8 in the presence of activin and high concentration FSH, and this is a possible contributory factor to the reduction in steroidogenesis observed in this group. Extension of the culture period to allow further oocyte growth with subsequent *in vitro* maturation would allow for a more thorough assessment of reduced steroidogenic capability and its impact on oocyte competency.

Oocyte growth was previously demonstrated in bovine follicles cultured in 50 ng/ml FSH (Itoh *et al.*, 2002). In this study, the effect of this concentration as well as 100 ng/ml rFSH were evaluated in the presence of 100 ng/ml rhAct A to determine if (i) activin and FSH could act synergistically to promote growth and (ii) by increasing the concentration of FSH an enhanced rate of oocyte and somatic cell development could be achieved. After 8 days *in vitro* oocytes cultured in the presence of activin alone achieved a mean diameter equivalent to that observed in a 13-day study of pre-antral bovine follicles cultured in with 50 ng/ml FSH (Itoh *et al.*, 2002). Preservation of normal oocyte morphology *in vitro* correlated positively with the presence of activin alone, FSH alone and activin combined with low dose FSH whereas on comparison, a significantly lower percentage of

morphological healthy oocytes were observed in control ($P < 0.05$) or activin with high dose FSH ($P < 0.05$). Interestingly, in the latter group, oocyte deterioration was not deleterious to granulosa proliferation in agreement with Wandji et al. (1996).

The ability of the oocyte and its surrounding somatic cells to directly exchange molecules through gap junctions is facilitated by transzonal projections (TZPs) stabilized by actin-rich filamentous membrane extensions, found near the zona pellucida (Motta et al., 1994). A positive correlation between oocyte growth and maintenance of an intra-follicular F-actin TZP network has been demonstrated in cultured human pre-antral follicles (Xu et al., 2009). Confocal analysis of somatic and germ cell contacts and germinal vesicle chromatin was performed on a number of cultured and freshly isolated follicles. Although all cultured follicles fostered some degree of granulosa cell adhesion to the zona pellucida, in the presence of activin alone the appearance of focal adhesions by granulosa at the zona surface was more extensive and regularly distributed than other treatment groups. TZPs are dynamic regulators of paracrine signalling with fewer projections observed post-ovulation than during the earlier stages of follicle development (Albertini et al., 2001; Vozi et al., 2001). It is suggested that the FSH dosages utilized in this study whereas initially stimulating growth and steroidogenesis were sufficiently high to trigger a dose dependent premature retraction of TZPs over time. This disruption of somatic and germ cell signalling ultimately results in oocyte degeneration as irrespective of media supplementation, small incompetent oocytes are unable to survive *in vitro* without somatic cell contact. This implies that to maintain optimal co-ordinated follicular growth *in vitro*, a reduced basal dose of FSH (1–10 ng/ml) should be considered (Campbell et al., 2004).

In conclusion this study demonstrates that activin is associated with somatic cell proliferation, antral cavity formation and maintenance of normal oocyte morphology of bovine pre-antral follicles *in vitro*. Confocal imaging indicates that in the presence of activin the relative densities of actin and connexin 43 are not uniformly distributed throughout the follicle rather that they are differentially arranged with differing gradients of intercellular contact between follicular cell types established. These results indicate that activin maintains oocyte integrity by effecting a polarization of adhesion events towards the oocyte and gap junctions towards the periphery which is, to the best of our knowledge, the first time this distribution pattern has been reported.

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