Oocyte development in bovine primordial follicles is promoted by activin and FSH within a two-step serum-free culture system

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Abstract

Quiescent follicles of large mammals initiate growth within cultured pieces of ovarian cortex. Systems capable of sustaining *in vitro* development from this early stage until oocyte maturation would allow investigation of mechanisms regulating oocyte development in its entirety. The aims of this study were 1) to determine whether bovine follicles initiated to grow *in vitro* could be isolated from the cortical environment, and could undergo further development and 2) to evaluate the effect of activin and FSH on the development of secondary follicles derived from primordial follicles. Fragments of bovine ovarian cortex were cultured in serum-free medium for 6 days; thereafter, secondary follicles were isolated for further culture. After a maximum total of 21 days *in vitro*, follicles were either processed for histological assessment or opened to release the oocyte–cumulus complexes for inspection by light microscopy. Compared with control, significant follicle and oocyte growth were observed in activin-exposed follicles, with or without FSH, with some oocyte diameters measuring over 100 microns following a total *in vitro* period of 15 days. Significant oestradiol secretion was observed in follicles cultured in activin alone after a total of 9 days *in vitro* compared with other treatment groups; however, this effect was not sustained. In summary, this study demonstrates the promotion of primordial bovine follicle development within a two-step serum-free culture system with oocyte diameters > 100 μm achieved over 15 days *in vitro*. Further development of this system is needed to support complete oocyte growth and thereafter *in vitro* maturation.

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Introduction

Current understanding of the mechanisms underpinning oocyte development in immature follicles is incomplete. The ability to support the development of primordial bovine follicles through to acquisition of oocyte competency *in vitro* would not only advance the understanding of germ cell development in non-rodent species, but could also provide mature oocytes for assisted reproductive technologies in species understood to require a prolonged follicular growth period.

The mammalian follicle reserve mainly comprises primordial follicles (Gosden & Telfer 1987), which also represent the population of follicles least affected by apoptotic sequelae (Telfer 1998, Telfer *et al*. 1998), making them promising candidates for *in vitro* growth and subsequent oocyte harvesting. Individual culture of primordial follicles is technically demanding (Abir *et al*. 1997, Oktay *et al*. 1997); however, initiation of growth and early development *in vitro* have been achieved in human (Hovatta *et al*. 1997, 1999, Wright *et al*. 1999, Telfer *et al*. 2008) and large animal (Wandji *et al*. 1996, 1997, Braw-Tal & Yossefi 1997, Yang & Fortune 2006, 2007) follicles within small pieces of ovarian cortex. Previous studies using primate and bovine

models have demonstrated a reduction in the primordial population and a corresponding increase in the growing follicle population within gonadotrophin-free cultures of ovarian cortex with activated follicles reaching the secondary stage of development (Wandji *et al*. 1996, 1997, Braw-Tal & Yossefi 1997, Gigli *et al*. 2006, Yang & Fortune 2006, 2007). *In vitro* growth of follicles within cortical pieces can be successfully supported for extended periods of time (Hovatta *et al*. 1997, Louhio *et al*. 2000, Zhang *et al*. 2004, Segino *et al*. 2005, Fabbri *et al*. 2007); however, it is recognised that to achieve advanced development, follicles require to be released from the presumed inhibitory stromal environment (Abir *et al*. 1997, Telfer *et al*. 2008).

Multi-step systems have already been designed to support *in vitro* follicle development in mice with the birth of pups from *in vitro* matured oocytes derived from cumulus–oocyte complexes (Eppig & Schroeder 1989), and primordial (Eppig & O'Brien 1996) and primary follicles (Spears *et al*. 1994); however, to date, it has not been possible to repeat this success in large mammals using oocytes from such immature follicles. A major obstacle to progress in large species is oocyte degeneration associated with loss of oocyte and somatic

cell contact particularly over a prolonged culture period; therefore, it is imperative that an understanding of the impact of culture conditions on the maintenance of intercellular associations is realised.

In the following experiment, a two-step culture system was designed to promote the activation of follicle growth within fragments of ovarian cortex followed by isolation and individual culture of secondary follicles. The transforming growth factor-β superfamily member activin, previously associated with the maintenance of oocyte–somatic cell adhesion and a recognised promoter of preantral follicle development (Hulshof *et al.* 1997, Liu *et al.* 1998, Zhao *et al.* 2001, Thomas *et al.* 2003, Silva *et al.* 2006, Telfer *et al.* 2008), and FSH were chosen as culture medium supplements. Activation and early follicle growth occur in the absence of gonadotrophins (Peters *et al.* 1973, McNatty *et al.* 1990). Development beyond the early antral stage is critically dependent on the presence of FSH (Oktay *et al.* 1998); however, the precise effect of FSH on preantral follicle development is unclear. It has been demonstrated that FSH can augment the action of activin, which in turn simulates FSH receptor expression in cultured murine granulosa cells (Xiao *et al.* 1992), confirming a relationship between the follicular actions of FSH and activin. In this experiment, the effect of activin and activin in combination with FSH on the development of secondary bovine follicles grown *in vitro* was assessed with regard to follicle and oocyte growth, steroidogenesis and maintenance of normal oocyte morphology.

Results

Follicle growth and differentiation

To confirm the developmental stage of follicles in fresh tissue, a total of 12 fragments were randomly selected prior to culture, fixed in Bouin's solutions and processed as described for histological assessment. The mean distribution of follicles in freshly fixed tissue (day 0) was compared to that of ten fragments cultured for 6 days. Follicles were categorised by a modification of the system used by Wandji *et al.* (1996) as follows: 1) quiescent follicles: oocytes surrounded by a complete or incomplete single layer of cells, either all flattened (primordial) or a mixed layer of flattened and cuboidal cells (transitory), 2) primary follicles: follicles comprising an oocyte surrounded by a single complete layer of cuboidal granulosa cells, and 3) secondary follicles: multilaminar follicles with more than a complete single layer of granulosa cells. Follicle survival was high in cultured tissue fragments, with <10% follicle degeneration observed in any of the treatment groups. As shown in Fig. 1i, a significantly greater percentage (*P*<0.01) of growing follicles (primary) and a corresponding significant decrease in the percentage of quiescent follicles (*P*<0.01) were observed in cultured fragments compared with freshly fixed tissue, which contained predominantly quiescent follicles (Fig. 1ii). Secondary follicles (mean diameter 111±1.46 µm) were dissected from cultured cortical strips (Fig. 1iii and iv), and grown for 6 days in control medium (*n*=31) or in medium supplemented with 100 ng/ml recombinant human activin A (rhAct A) alone (*n*=70) or 100 ng/ml rhAct A in combination with 50 ng/ml recombinant human FSH (rhFSH; *n*=57). Significant growth occurred in follicles supplemented with 100 ng/ml rhAct A in the presence (*P*<0.01) or absence (*P*<0.01) of 50 ng/ml rhFSH compared with control over a 12-day *in vitro* period. Activin-exposed follicles were cultured for a maximum of 21 days; due to poorer growth rates, control follicles were not cultured beyond 12 days. Follicle growth increased significantly at days 15 and 18 in the presence of 100 ng/ml rhAct A and 50 ng/ml rhFSH compared with activin alone (*P*<0.05); however, by day 21 of the
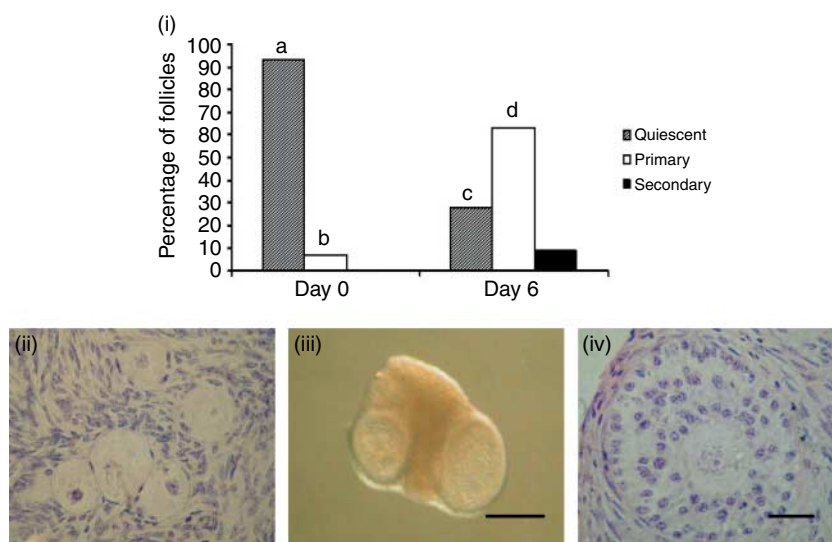
Figure 1 Effect of culture on the quiescent and growing follicle population in bovine cortical fragments. (i) a, b, c and d indicate differences between quiescent and primary follicle populations. A significant decrease (*P*<0.01) in the percentage of quiescent follicles was observed after 6 days in culture with a corresponding increase in the percentage of primary follicles (*P*<0.01). No secondary follicles were observed in freshly fixed (day 0) tissue which contained predominately quiescent follicles (ii). Secondary follicles were dissected from strips of bovine cortex following 6 days *in vitro* (iii), bar=100 µm and (iv), bar=25 µm.
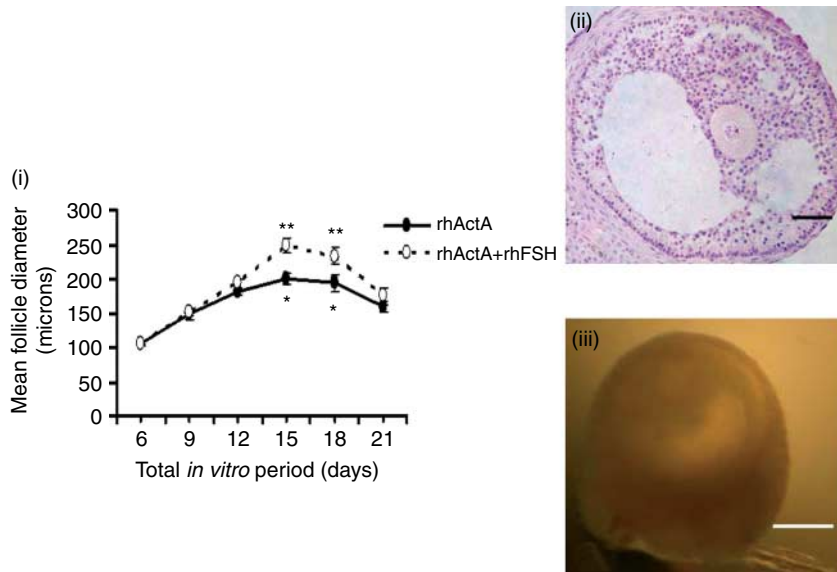
Figure 2 Effect of rhAct A and FSH on follicle development over 21 days *in vitro*. (i) Values are mean follicle diameters ±S.E.M. * and ** indicate significant differences betweens treatments. Mean follicle diameters were greater in the presence of 100 ng/ml rhAct A and 50 ng/ml FSH (open circles) on days 15 ($P<0.05$) and 18 ($P<0.05$) compared with 100 ng/ml rhAct A alone (closed circles). Follicles cultured in control medium for a total of 12 days were significantly smaller than those cultured in the presence of activin over the same time period (not shown). (ii) Antral cavity formation in a follicle cultured in the presence of 100 ng/ml rhAct A and 50 ng/ml FSH for 15 days; bar=50 μm. (iii) Formation of antra could occasionally be seen during the *in vitro* period; bar=100 μm.

in vitro period, mean follicle diameters in both groups had declined sharply (Fig. 2i). The presence of an antrum was determined on histological inspection (Fig. 2ii), though occasionally cavity formation could be seen by light microscopy during the *in vitro* period (Fig. 2iii). Significant antral formation was observed in follicles cultured in 100 ng/ml rhAct A alone ($P<0.05$) or 100 ng/ml rhAct A and 50 ng/ml rhFSH ($P<0.05$) compared with follicles cultured in control, with approximately a third of the follicles in both activin-exposed groups forming cavities.

Oocyte growth and cumulus complex morphology

Follicles grown *in vitro* were isolated and cultured for up to 21 days in the presence or absence of 100 ng/ml rhAct A, or in the presence of 100 ng/ml rhAct A in combination with 50 ng/ml rhFSH. As shown in Fig. 3i, significant oocyte growth occurred over 12 days in isolated secondary follicles cultured in the presence of 100 ng/ml rhAct A with or without 50 ng/ml rhFSH compared with oocytes within cortical fragments cultured for 6 days only ($P<0.01$) and isolated secondary follicles cultured in control medium ($P<0.05$). The normality of oocyte morphology was assessed by determining the general circularity of the oocyte and the integrity of the oolemma and zona pellucida, and the presence of a germinal vesicle and nucleolus in each section was evaluated as illustrated in Fig. 3ii and iii. Therefore, grossly spherical oocytes with an intact oolemma, distinguishable zona pellucida, a visible germinal vesicle and defined nucleolus were considered morphologically normal. Oocytes not meeting these criteria were deemed abnormal. After 12 days
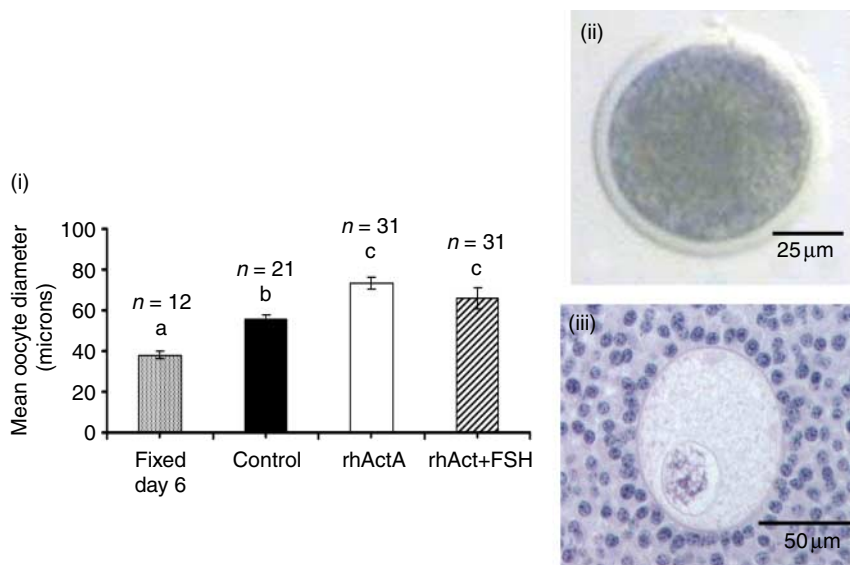
Figure 3 Effect of rhAct A and FSH on oocyte growth and morphology over 12 days *in vitro*. (i) Values are mean oocyte diameters ±S.E.M.; a, b and c indicate differences between treatments. Mean oocyte diameters were significantly greater in the presence of 100 ng/ml rhAct A with or without 50 ng/ml FSH compared with the diameters of oocytes cultured in control medium ($P<0.05$) and with those of oocytes of follicles cultured in strips for 6 days only ($P<0.01$). (ii) Morphologically normal oocyte grown *in vitro* in the presence of 100 ng/ml rhAct A alone for 12 days; bar=25 μm. (iii) Histological image of morphologically normal *in vitro* grown oocyte cultured in the presence of 100 ng/ml rhAct A and 50 ng/ml rhFSH for 12 days; bar=50 μm.
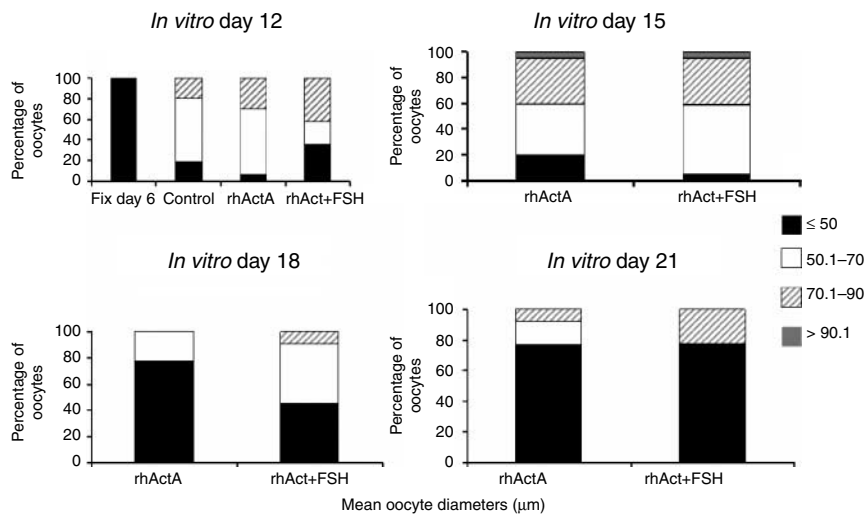
Figure 4 Distribution of oocytes by size at days 12, 15, 18 and 21. Maximum oocyte diameters were achieved after 15 days *in vitro* in the presence of 100 ng/ml rhAct A or 100 ng/ml rhAct A and 50 ng/ml rhFSH with some oocytes exceeding 100 μm in diameter. By day 21, the percentage of oocytes of diameter ≤50 μm had risen sharply in both groups indicating that culture conditions were suboptimal.

in vitro, no significant difference in oocyte morphology was observed between the treatment groups (control $n=31$, activin $n=20$, and activin and FSH $n=20$), with 55–65% of all the oocytes cultured appearing morphologically normal. Oocyte–cumulus complexes were released from follicles cultured in 100 ng/ml rhAct A with or without 50 ng/ml rhFSH on days 15 (activin $n=15$, and activin and FSH $n=15$), 18 (activin $n=10$, and activin and FSH $n=8$) and 21 (activin $n=5$, and activin and FSH $n=6$) of the total *in vitro* period, and the oocyte complexes were measured and assessed for the presence of adherent cumulus cells and an intact zona pellucida. Follicle degeneration increased steeply with time. At day 15, <5% of follicles cultured in the presence of activin alone and <7% of those cultured in activin combined with FSH were degenerate, whereas by day 18, this had risen to 32 and 39% respectively. By day 21, almost 50% of the oocytes from both treatment groups were degenerate. As shown in Fig. 4, maximum oocyte diameters (>90.1 μm) were achieved in follicles cultured for a total of 15 days in the presence of activin with or without FSH. A significantly greater proportion of oocytes had adherent cumulus cells when FSH was present in the culture medium ($P<0.05$; Fig. 5i) compared with oocytes cultured in activin alone at days 15 and 18; however, by day 21, nearly all the oocytes from both treatment groups were denuded on release (Fig. 5ii). Zona pellucidae were present and

intact in all oocytes released from follicles on days 15, 18 and 21; however, by day 21, oocytes from both treatments appeared grossly misshapen (Fig. 5iii).

Oestradiol secretion

Media from follicles cultured individually in control medium (day 9, $n=7$; day 12, $n=7$), activin alone (day 9, $n=9$; day 12, $n=9$) or activin with FSH (day 9, $n=7$; day 12, $n=7$) were analysed for oestradiol (OE_2) content by immunoenzyme assay. Significant OE_2 secretion was observed in follicles cultured in activin alone ($P<0.05$) compared with the other treatment groups; however, this effect was not sustained, and by day 12, no significant difference was detected between treatments (Table 1).

Discussion

If mechanisms regulating germ cell development in non-rodent mammals are to be fully understood and competent oocytes are to be routinely produced *in vitro*, systems supporting the growth of large mammalian follicles from the earliest stages are a prerequisite. Although major milestones in bovine follicle growth have been realised *in vitro*, i.e. primordial follicle activation (Wandji *et al*. 1996, Fortune *et al*. 1998, Gigli *et al*. 2006, Yang & Fortune 2006, 2007), preantral follicle growth (Wandji *et al*. 1996, Hulshof *et al*. 1997,
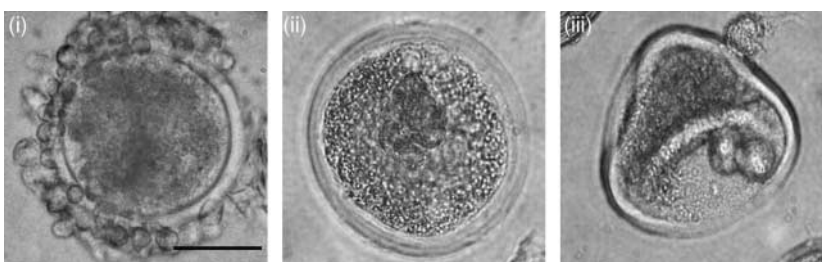
Figure 5 Oocyte morphology on aspiration after 15–21 days in culture. (i) Cumulus cells adhering to an oocyte released from a follicle cultured in the presence of activin and FSH for 15 days. (ii) Oocyte cultured in activin alone for 18 days; intact zona pellucida was visible but no adherent cumulus. (iii) Grossly misshapen oocyte released from a follicle cultured in 100 ng/ml rhAct A and 50 ng/ml rhFSH for 21 days.

Table 1 Oestradiol secretion in bovine follicles activated and grown *in vitro*.

	Mean oestradiol secretion (pg/ml)	
	Day 9	Day 12
Control	28.45	32.61
100 ng/ml rhAct	41.31*	35.28
100 ng/ml rhAct A + 50 ng/ml rFSH	30.78	33.33

*Denotes a significant difference between treatment groups at day 9 ($P<0.05$).

Katska & Ryńska 1998, Gutierrez *et al.* 2000, Saha *et al.* 2000, Thomas *et al.* 2001, 2007), oocyte–cumulus cell complex development (Ralph *et al.* 1995, Hirao *et al.* 2004), and oocyte maturation and fertilisation (Hirao *et al.* 2004), gaps still exist and a complete system capable of supporting the development of immature follicles through to oocyte competency is yet to be defined. It is not known exactly how long it takes for primordial follicles to reach the secondary stage *in vivo*; however, previous studies have observed a significant reduction in the primordial follicle population with a concomitant increase in the later stages of development in bovine cortical strips grown over 7 (Wandji *et al.* 1996, Gigli *et al.* 2006), 10 (Yang & Fortune 2006, 2007) and 20 days (Fortune *et al.* 1998) *in vitro*, with primordial activation being observed as early as the second day of culture (Fortune *et al.* 1998). In this study, the mean diameter of follicles dissected from cortical fragments cultured for 6 days was $111 \pm 1.46\,\mu m$, considerably larger than 40 μm, the limit for exclusion from the experiment and smaller than the minimum diameter required for antral formation (Webb *et al.* 1999), indicating that secondary follicles that were present entered the growth phase *in vitro* from the quiescent pool, and that subsequent antral cavity formation occurred *in vitro*. This was confirmed by histological analysis as no secondary or antral follicles were observed in freshly fixed cortical fragments.

In this study, activin with or without FSH promoted follicle growth up to 18 days *in vitro*, and the formation of antral cavities in cultured secondary follicles was observed after 12 days. This demonstrates a markedly enhanced rate of development, as it is estimated that *in vivo* bovine primordial follicles take several months to reach the antral stage (Lussier *et al.* 1987, Gosden *et al.* 1994). Previous studies have achieved antral cavity formation after 7 and 10 days *in vitro* (Gutierrez *et al.* 2000, Itoh *et al.* 2002); however, both studies cultured larger preantral follicles ($166 \pm 2.15\,\mu m$). Activin and activin with FSH promoted follicle growth with follicle diameters increasing from 111 up to 200 μm (activin alone) and 247 μm (with FSH) with concomitant increase in oocyte diameters from 39 μm to a maximum of 107 μm over 15 days *in vitro*. Normal oocyte morphology was maintained in the presence of activin

with or without FSH; however, their competency is yet to be tested. Hirao *et al.* (2004) successfully cultured, matured and fertilised bovine oocyte–cumulus complexes *in vitro* with a mean oocyte diameter of 95 μm on aspiration (Hirao *et al.* 2004). In this study, the largest oocytes were observed on day 15; moreover, in the presence of 100 ng/ml rhAct A and 50 ng/ml rhFSH, oocytes retained adherent cumulus cells until day 18, indicating that a close association between the germ and somatic cells was being maintained by culture conditions; however, extending the culture to 21 days resulted in oocyte degeneration. It is well established that maintenance of bidirectional communication between the oocyte and its surrounding somatic cells is vital to the production of a healthy oocyte (Albertini *et al.* 2001, Eppig 2001). Our results support the idea that to facilitate complete oocyte development *in vitro*, an additional culture step would need to be introduced to support the development of oocytes outwith the large antral follicle (Telfer & McLaughlin 2007).

Follicles supplemented with 100 ng/ml rhAct A alone secreted significant OE_2, which together with antral formation is indicative of follicle differentiation. Increased OE_2 peaked at day 9, and was not maintained throughout the culture period; however, the reduced steroidogenesis observed at day 12 did not have a deleterious effect on oocyte growth or morphology.

In summary, this study demonstrates the potential of secondary bovine follicles derived from primordial follicles activated *in vitro* to support continued oocyte development within isolated follicles. The presence of activin and FSH enhanced follicle and oocyte growth *in vitro*, with some follicles containing oocytes with diameters $>100\,\mu m$ after a 15-day culture period. These results provide another encouraging step towards the realisation of complete *in vitro* growth of large mammalian oocytes.

Materials and Methods

Tissue preparation and fragment culture

Bovine ovaries from freshly killed heifers were transported from the local abattoir at 33–38 °C in HEPES-buffered M199 media (Invitrogen Ltd) supplemented with amphotericin B (2.5 μg/ml; Invitrogen Ltd), pyruvic acid (25 μg/ml), penicillin G (75 μg/ml) and streptomycin (50 μg/ml; all chemicals obtained from Sigma Chemicals). Under laminar flow conditions, ovaries were rinsed in 70% alcohol, and fine cortical strips were removed using a scalpel. The strips were placed in a dissection medium (Leibovitz medium, Invitrogen Ltd) supplemented with sodium pyruvate (2 mM), glutamine (2 mM; both obtained from Invitrogen Ltd), BSA (Fraction V, 3 mg/ml), penicillin G (75 μg/ml) and streptomycin (50 μg/ml; Sigma Chemicals)). Under light microscopy, any damaged, haemorrhaged or excess stromal tissue was excised, allowing the pieces to flatten and enhancing microscopic visualisation. The flat tissue pieces were dissected into fragments of between 0.5 and

1 mm^2, and re-examined under light microscopy, and all visible follicles were measured. Any follicle of mean diameter >40 µm was excised from the tissue fragments to ensure that the presumptive population of follicles was unilaminar, i.e. primordial or transitory. Seven replicate cultures were established with 12–33 cortical fragments in each. The fragments were cultured individually in 24-well cell culture plates (Corning B.V. Life Sciences Europe, Amsterdam, The Netherlands) containing McCoy's 5a medium with bicarbonate supplemented with HEPES (20 mM; Invitrogen Ltd), glutamine (3 mM; Invitrogen Ltd), 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 obtained from Sigma Chemicals). Fragments were cultured for 6 days at 37 °C in humidified air with 5% CO_2, with half the media being removed and replaced every second day. A number of cortical fragments were randomly selected, and fixed on each day a culture was established (day 0) to allow comparison between cultured and *in vivo* follicular parameters.

Follicle isolation and culture

After 6 days, a number of cortical fragments were removed from each culture and fixed in Bouin's solution prior to secondary follicle dissection to allow comparison of follicle growth between day 0 and 6 (total $n=10$). The remaining cortical fragments ($n=233$) were transferred to pre-warmed Leibovitz dissection medium supplemented as described earlier, and preantral follicles (111 ± 1.46 µm) were mechanically dissected with 25-gauge needles, and those with an intact basement membrane and no antral cavity were selected for culture. A total of 170 follicles were dissected, 12 follicles were fixed immediately as day 6 follicle controls, and 158 follicles were randomly assigned to treatment groups. Selected follicles were individually placed in 96-well V-bottomed culture plates (Corning Costar Europe, Badhoevedorp, The Netherlands) in 150 µl of the culture medium. For the control group ($n=31$), follicles were cultured in McCoy's 5a medium supplemented as described earlier. For the treatment groups, follicles were cultured in McCoy's 5a medium as described before with either 100 ng/ml rhAct A (R&D Systems, Abingdon, UK; $n=70$) or 100 ng/ml rhAct A in combination with 50 ng/ml rhFSH (Sigma Chemicals; $n=57$). The follicles were cultured individually for a maximum of 15 days at 37 °C in humidified air with 5% CO_2, and on every third day, half the culture medium was removed and replaced concomitant with follicle diameter measurement using a dissecting microscope with a crossed micrometer. Upon microscopic inspection, any damaged or degenerate follicles were excluded from the study. The prevalence of follicle deterioration was not significantly different between the treatment groups.

Assessment of histology

A total of 12 fragments of fresh cortical tissue and ten fragments of tissue cultured for 6 days were fixed for 24 h in Bouin's solution and processed for histological inspection as described below. The developmental stage of the follicles that were present in the tissue was assessed by light microscopy, and the proportions of quiescent and growing follicles were compared between the groups. After a total of 12 days *in vitro* (6 days within cortical fragments and 6 days in individual follicle culture), 20 follicles each from both activin-supplemented treatment groups and all control-cultured follicles ($n=30$) were fixed in Bouin's solution for 24 h, and were then dehydrated in an eosin/alcohol solution in increasing concentrations (70, 90 and 100%). Following dehydration, the follicles were immersed in cedarwood oil (BDH Laboratory Supplies, Poole, UK) for 24 h and were then placed in toluene (Fisher Scientific UK Ltd, Loughborough, UK) for 30 min to remove all traces of the oil. The follicles were then embedded in paraffin wax at 60 °C for 4 h, with the wax being renewed every hour to ensure complete clearance of all the toluene. Six-micrometre-thick samples were sectioned, mounted on gelatine-coated slides and left to dry overnight prior to staining with haematoxylin and eosin. Using a light microscope with a crossed micrometer, sections of follicles containing the nucleolus were assessed, and the following parameters were evaluated: follicle and oocyte diameter, antral cavity formation and oocyte integrity. In total, 1–2 follicles from each activin-treated group cultured beyond a total of 12 days were fixed and processed as described above; the remaining follicles from these groups cultured for more than 12 days were assessed as detailed below.

Assessment of oocyte–cumulus complexes

A number of follicles cultured in 100 ng/ml rhAct A with ($n=35$) or without ($n=45$) 50 ng/ml rhFSH remained *in vitro* for a maximum total period of 21 days. Following 15, 18 and 21 days *in vitro*, a number of follicles from each treatment group were removed to preheated Leibovitz dissection media supplemented as described earlier, and were gently opened using 25-gauge needles. Using a light microscope with a crossed micrometer, the released oocyte complexes were inspected, and oocyte diameter, zona pellucida integrity, cumulus cell adherence and oocyte morphology were evaluated.

Measurement of OE$_2$ by enzyme immunoassay

To evaluate follicular steroidogenesis *in vitro*, concentrations of OE$_2$ in reserved culture media were measured against standard dilutions using OE$_2$ ELISA kit (DRG Instruments, GmbH, Marburg, Germany). Media removed from follicles cultured in control ($n=7$) or in the presence of 100 ng/ml rhAct A with ($n=7$) or without ($n=9$) 50 ng/ml rhFSH on days 9 and 12 of the total *in vitro* period were analysed. As per the immunoassay kit instructions (DRG Instruments), reserved media were diluted with freshly prepared culture medium (1 in 10), placed in microtiter wells coated with polyclonal (rabbit) antibody raised against the OE$_2$ antigenic site, mixed with OE$_2$–HRP conjugate and incubated for 120 min. After incubation, the unbound conjugate was washed off, and a substrate solution of tetramethylbenzidine was added to allow development of colour. After 15 min, the reaction was stopped using dilute sulphuric acid, the wells were washed, and the absorbance of

each well at 450 ± 10 nm was measured using a microtiter plate reader. The absorbencies were converted to OE_2 concentrations using Sigma Plot Version 9, with four parametric logistic functions (Systat Software Inc., Hounslow, UK) and OE_2 sensitivity of 25 pg/ml. Specificity of the assay was as follows: OE_2 100%, oestriol 0.055% and oestrone 0.2%. The inter- and intra-coefficients of variation were 6.81 and 7.25% respectively.

Statistical analyses

Inter- and intra-treatment differences were compared by measuring mean follicle diameters, mean oocyte diameters and OE_2 production on every third day of culture using a one-way ANOVA with subsequent t-tests. The proportions of quiescent and primary follicles and normal oocyte morphology were compared using χ^2 analysis. The proportions of antral cavity and zona pellucida formation and cumulus cell adherence were compared using Fisher's exact test.

Declaration of interest

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