Bovine follicle development is associated with divergent changes in activin-A, inhibin-A and follistatin and the relative abundance of different follistatin isoforms in follicular fluid

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Abstract

The aim was to determine whether follicle growth in cattle is accompanied by changes in levels of inhibin-A (inh-A), activin-A (act-A) and different Mr isoforms of follistatin (FS) in bovine follicular fluid (bFF), reflecting differential roles of these proteins during folliculogenesis. Follicles ($n=146$) from 2–20 mm diameter were dissected from ovaries of ~40 cattle. Immunoassays were used to measure total FS, act-A, inh-A, oestradiol (E) and progesterone (P) levels; immunoblotting was used to quantify the relative abundance of different FS isoforms. Follicle growth from 2–6 mm was associated with a 6-fold increase in inh-A and 30-fold increase in act-A; FS remained uniformly high from 2–10 mm. From 6–2 mm, inh-A remained high while act-A and FS fell 3-fold and 2-fold, respectively. Act-A/FS ratio increased 20-fold from 2–6 mm before falling slightly through to 20 mm. Act-A/inh-A ratio increased 6-fold from 2–6 mm before falling 2-fold from 6 to 17–20 mm. These findings imply a marked increase in relative activin ‘tone’ around the stage at which dominant follicle selection occurs. When larger follicles (13–20 mm) were subdivided according to E/P ratio, those with high (>5) E/P ratio had lower (2-fold; $P<0.001$) levels of inh-A and act-A in comparison to follicles with low (<5) E/P ratio, but there were

no significant differences in FS, act-A/inh-A ratio or act-A/FS ratio. Thus follicle size, but not oestrogenic status, has a major influence on the intrafollicular balance between act-A and its opposing factors, inh-A and FS. Six FS isoforms were detected in bFF (apparent Mr: 65, 41, 37, 35, 33 and 31 kDa) averaging 6, 13, 24, 26, 13 and 17% respectively of total FS. During growth from 2–20 mm the proportion of total FS represented by 65, 41 and 37 kDa isoforms increased ~2-fold while the proportion represented by the 33 and 31 kDa isoforms decreased by 3-fold and 1.6-fold, respectively. Treatment of bovine granulosa cells *in vitro* with FSH and IGF alone or in combination increased total FS secretion up to 12-fold but did not affect the relative abundance of the five different FS isoforms detected. While the functional significance of the intriguing shift in FS isoform abundance in bFF during follicle development remains to be established, we have shown that a marked increase in intrafollicular activin ‘tone’ accompanies bovine follicle growth from 3–6 mm, corresponding to the stage at which the FSH-dependent follicle selection mechanism operates in this species.

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Introduction

The mechanisms that control the cyclic recruitment, selection and dominance of ovarian follicles are not fully understood but there is compelling evidence that intra-ovarian factors play a key role in modulating the sensitivity of follicular cells to gonadotropins and other systemic factors. Of the numerous locally produced factors implicated in this process insulin-like growth factors (Adashi 1993, Fortune *et al.* 2004) and various members of the transforming growth factor beta (TGF β) superfamily including inhibins, activins and bone morphogenetic

proteins (BMP) (Ying 1988, Knight & Glister 2001, 2003, Welt *et al.* 2002, Shimasaki *et al.* 2004) feature prominently. With respect to TGF β superfamily members, *in vitro* studies have shown that activins and BMPs can up-regulate follicle-stimulating hormone (FSH)-receptor expression and enhance basal and/or FSH-induced oestrogen (E) production by granulosa cells whilst inhibiting progesterone (P) production and functional luteinization (Hasegawa *et al.* 1988, Xiao *et al.* 1992, Findlay 1993, Findlay & Drummond 1999). Conversely, activins (Hsueh *et al.* 1987, Hillier 1991, Wrathall & Knight 1995) and BMPs (Glister *et al.* 2005) suppress basal and/or

lutinizing hormone (LH)-induced androgen production by theca cells *in vitro*. Inhibin, a physiological antagonist of activin, enhances thecal androgen production (Hsueh *et al.* 1987, Hillier 1991, Wrathall & Knight 1995). There is also evidence suggesting a positive role for granulosa cell-derived activin in enhancing oocyte developmental competence (Alak *et al.* 1998, Silva & Knight 1998). Each of the above actions of activin can effectively be neutralised by FS, a high affinity activin-binding protein initially identified in and isolated from ovarian follicular fluid (Ying 1988, Sugino *et al.* 1994). Inhibins, activins and FS are predominantly expressed by granulosa cells and are present in high concentrations ($\mu\text{g/ml}$ range) in bovine follicular fluid (bFF).

FS is a cysteine-rich, single chain glycoprotein encoded by a single gene. It exists as multiple size isoforms as a result of alternate mRNA splicing to generate two core proteins (FS-315 and FS-288) the larger of which is extended at the carboxy terminus. These core proteins undergo further post-translational modifications, including proteolytic cleavage and differential glycosylation to produce the six different isoforms that have been identified in ovarian follicular fluid of pigs and cows (Sugino *et al.* 1993, 1994, Welt *et al.* 2002). Although initially characterised as an activin-binding protein, FS also binds with lower affinity to several other TGF β superfamily members including inhibin (Shimonaka *et al.* 1991) and BMP-4, -6 and -7 (Otsuka *et al.* 2001, Amthor *et al.* 2002, Glister *et al.* 2004) but the extent to which this binding modulates the bioavailability of these ligands requires further investigation.

Evidence suggests that the relative activin 'tone' of a follicle (i.e. the ratio of activin to inhibin and/or FS) is an important determinant of its developmental fate (Hillier 1991, Hillier & Miro 1993) but there have been few direct studies to examine, at the protein level, the changing intrafollicular balance between activin and its opposing factors, inhibin and FS, during follicle development. Since granulosa cells can synthesise multiple FS isoforms with potentially distinct functional roles (Nakamura *et al.* 1991, Sugino *et al.* 1994, Schneyer *et al.* 2000, Welt *et al.* 2002), a further question that has yet to be addressed is whether the relative abundance of different FS isoforms in bFF vary during follicle development?

Therefore, the aims of the present study were: (1) to provide a detailed analysis of intrafollicular concentrations of inhibin, activin and FS proteins during bovine folliculogenesis; (2) to determine whether the relative abundance of individual FS isoforms varies during antral follicle growth in cattle and, if so, whether such changes are associated with other markers of follicle development including concentrations of E and P; and (3) to investigate whether *in vitro* exposure of isolated granulosa cells to FSH or IGF modifies the relative abundance of different FS isoforms secreted by the cells.

Materials and Methods

Collection of bFF samples

Ovaries from approximately 40 randomly cycling cattle (<18 month-old) were obtained from an abattoir over a period of several months. Upon arrival in the laboratory each batch of ovaries was placed on ice and antral follicles ($n=146$) ranging from 2–20 mm in diameter were removed using dissecting scissors and placed in ice-cold PBS until their diameters were recorded. To provide sufficient bFF sample volumes for analysis, follicles in the smallest (2 mm) size category were pooled (3–5 per pool) while follicles >3 mm were processed individually. Follicles were punctured with a needle and bFF recovered by centrifugation (13 000 g for 3 min) and transferred to a fresh tube to which 1% (v/v) protease inhibitor cocktail (Sigma UK Ltd) was added. bFF samples were stored at -20°C until analysed.

Granulosa cell culture

Mural granulosa cells were harvested from cattle ovaries obtained from an abattoir as described by Glister *et al.* (2001). Briefly, follicles of 4–6 mm diameter were dissected, aspirated, hemisected and the granulosa cell layer gently disrupted with the aid of a plastic inoculation loop. Cells were pelleted by centrifugation (800 g for 10 min) and subjected to an osmotic shock treatment to lyse any red blood cells present. Cells were pelleted, resuspended in a small volume of culture medium (McCoy's 5A modified medium supplemented with 1% (v/v) antibiotic-antimycotic solution, 10 ng/ml bovine insulin, 2 mM L-glutamine, 10 mM HEPES, 5 $\mu\text{g/ml}$ apotransferrin, 5 ng/ml sodium selenite, 0.1% BSA and 10^{-7}M androstenedione; all purchased from Sigma) and counted using a hemocytometer. Granulosa cells were seeded at a density of 0.5×10^6 viable cells/well in 12-well tissue culture plates (Nunc; Life Technologies Ltd) containing 1 ml pre-equilibrated culture medium with and without treatments (0.33 ng/ml FSH and 10 ng/ml LR3-IGF-1 alone and in combination; 12 replicate wells per treatment). These dose levels of FSH and LR3-IGF-1 were selected as being optimal on the basis of our previous dose-response studies on bovine granulosa cells (Glister *et al.* 2001, 2003). Plates were incubated at 38.5°C in 5% CO_2 and 95% air for 6 days. Conditioned medium was removed and replaced with fresh media (with and without treatments) every 2 days. For the final culture period (day 4–6) BSA was omitted from the culture medium to prevent protein overloading during the immunoblotting procedure. At the end of the 6-day culture period viable granulosa cell number was determined by uptake of neutral red dye (Campbell *et al.* 1996). For FS immunoblot analysis, conditioned media (day 4–6 of culture) pooled from 12 replicate wells per treatment were pooled

and centrifuged (10 min at 800 g) before desalting and concentrating to 0.1 ml using centrifugal ultrafiltration devices (10 000 MW cut-off; Amicon, Millipore, Bedford, MA, USA). Concentrations of inh-A, act-A, E and total FS were measured in aliquots of pooled conditioned media taken before the desalting/concentration procedure.

Hormone immunoassays

Concentrations of inh-A were determined using the two-site IRMA described by Knight and Muttukrishna (1994). Purified 32 kDa bovine inh-A (Knight *et al.* 1990) was used as a standard. The detection limit of the assay was 250 pg/ml with mean intra- and inter-assay coefficient of variations (CVs) of 5% and 10% respectively. Act-A levels were measured using a two-site ELISA (Knight *et al.* 1996). Human recombinant (hr) act-A (NIBSC, Potters Bar, Herts, UK) was used as a standard and the assay sensitivity was 100 pg/ml. Intra- and inter-assay CVs were 4% and 7% respectively. Total FS levels were determined using the ELISA described by Tannetta *et al.* (1998). Values are expressed in terms of hr-FS preparation provided by the National Hormone and Pituitary Programme (NHPP). Assay sensitivity was 100 pg/ml and intra- and inter-assay CVs were 7% and 9% respectively. Concentrations of P were determined by ELISA (Sauer *et al.* 1986). The detection limit was 10 pg/ml and intra- and inter-assay CVs were 7% and 10% respectively. Concentrations of E were determined by radioimmunoassay as described previously (Glister *et al.* 2001). The detection limit of the assay was 1.5 pg/ml and intra- and inter-assay CVs were 4% and 8% respectively.

SDS-PAGE/immunoblotting to resolve and quantify individual FS isoforms

Follicular fluid samples were diluted in non-reducing sample buffer (0.125 M Tris-HCl, pH 6.8, 10% (w/v) SDS, 30% (w/v) sucrose, 0.02% (w/v) bromophenol blue) and loaded at a concentration of 50 µg total protein/lane (follicles >3 mm) or 25 µg total protein/lane (follicles ≤ 3 mm). Each sample was analysed in duplicate on two separate gels. Desalted/concentrated conditioned media samples were diluted 50:50 with non-reducing sample buffer and 20 µl/lane loaded. All samples were heated to 95 °C for 5 min and then fractionated on 5% stacking/12.5% resolving SDS-PAGE gels (Mighty Small Tall system; Hoefer, San Francisco, CA, USA) run at 10 mA/gel. Proteins were then electrotransferred onto nitrocellulose membrane for 2 h at 10 V using a semi-dry transfer cell (Bio-Rad) and transfer buffer (48 mM Tris-base, 39 mM glycine, 1.3 mM SDS, 20% methanol). Gels were stained with Coomassie blue to check transfer efficiency. After blotting membranes were blocked overnight at 4 °C in PBS containing 5% Blotto and 1% BSA. Membranes

were then incubated overnight at 4 °C in PBS containing 5% Blotto, 1% BSA and a mixture of two monoclonal antibodies raised against recombinant human FS-288 (clone 1/1 and 8/1, 0.5 µg/ml). Membranes were washed three times in PBS/0.1% azide for 10 min each and then incubated with a ¹²⁵I-labelled goat-anti mouse secondary antibody (100 000 cpm./ml) at room temperature for 3 h. After washing, membranes were blotted dry using filter paper and wrapped in Saran wrap before being placed on a phosphor screen for 4 days. Western blots were imaged using a Phosphorimager (Molecular Dynamics) and analysed using Image J 1.32j. For quality control purposes, an aliquot of the same pooled bFF preparation was applied to one lane of each gel and carried through the FS immunoblotting procedure. The between gel CV for FS band intensity averaged 10.6%. In each sample the proportion of total FS represented by individual isoforms was calculated by expressing the band intensity for each isoform as a % of the total FS band intensity (i.e. sum of individual band intensities).

Statistical analysis

One-way and two-way ANOVA (with *post hoc* Fishers PLSD test where appropriate) of log-transformed data were used to evaluate between group differences in intrafollicular steroids, inh-A, act-A, total FS and the proportion of total FS represented by each of the six individual isoforms detected. Where indicated, Fisher's protected least significant difference (PLSD) test was used to make individual *post hoc* comparisons, providing ANOVA gave a significant F ratio. The same analyses were used to assess hormone secretion by cultured granulosa cells. Simple correlation analyses were also used to examine the relationship between some of the above variables. Unless stated otherwise, values are presented as arithmetic means ± S.E.M.

Results

Relationship between follicle size and E, inh-A, act-A and FS concentrations, act-A/inh-A ratio and act-A/FS ratio in bFF

As shown in Fig. 1a follicle growth from 2–6 mm was associated with a several hundred-fold increase in E concentration with the steepest increase between 11 and 20 mm. From 2–6 mm there was a progressive 6-fold increase in inh-A ($P < 0.0001$) and 30-fold increase in act-A ($P < 0.0001$) while FS remained at a uniformly high level from 2–10 mm (see Fig. 1 b, c and d). From 6–20 mm, inh-A remained high while concentrations of act-A and FS fell 3-fold ($P < 0.001$) and 2-fold ($P < 0.01$), respectively. Correspondingly, act-A/FS ratio (Fig. 1e) increased 20-fold ($P < 0.0001$) from 2–6 mm before falling

Figure 2 Representative immunoblot showing the presence of six different FS isoforms in five randomly selected bovine follicular fluid samples (lanes 1–5). The positions of the marker proteins used to calibrate the gel/blot are indicated (left) as are the apparent Mr-values of the FS bands identified (right).

slightly through to 20 mm (not significant). Act-A/inh-A ratio (Fig. 1f) increased 6-fold from 2–6 mm ($P<0.0001$) before falling 2-fold from 6 mm to 17–20 mm ($P<0.01$).

Relationship between follicle size and the relative abundance of different FS isoforms in bFF

A representative immunoblot showing the presence of six different bands of FS immunoreactivity in individual, randomly selected bFF samples is presented in Fig. 2. These bands had apparent Mr values of 65, 41, 37, 35, 33 and 31 kDa and, considering all follicles analysed, they represented 6, 13, 24, 26, 13 and 17% respectively of total FS immunoreactivity (i.e. sum of each individual immunoblot band intensity). Five of the six immunoreactive FS bands identified in bFF had apparent Mr values within the range corresponding to known molecular isoforms of FS. The weakest band with an apparent Mr of 65 kDa is too large to correspond to full length FS precursor or any known FS isoform and may represent dimerization of individual FS molecules. It was not possible to establish whether this 65 kDa band remained when gels were run under reducing conditions since this abolished binding of the FS antibodies.

As shown in Fig. 3, during follicle growth from 2–20 mm the proportion of total FS represented by the 65, 41 and 37 kDa isoforms increased 2-fold ($P<0.001$), the proportion represented by the 35 kDa isoform remained constant, while the proportion represented by

Figure 1 Relationship between follicle diameter and concentrations of (a) E (b) inh-A (c) act-A (d) FS and (e) act-A/FS ratio and (f) act-A/inh-A ratio in bovine follicular fluid. Values are means $\pm$ S.E.M. ($n=6-27$). For each panel ANOVA gave a $P<0.0001$ indicating a highly significant effect of follicle size.

the 33 and 31 kDa isoforms decreased by 3-fold and 1.6-fold, respectively ($P < 0.001$).

Classification of follicles according to E/P ratio

Considering all bFF samples, follicle diameter was positively correlated with E/P ratio ($r = 0.36$; $P < 0.0001$) and negatively correlated with FS concentration ($r = -0.55$; $P < 0.0001$) and act-A/inh-A ratio ($r = -0.25$; $P < 0.01$). To further explore the relationship between oestrogenic status and the intrafollicular balance of inhibin, activin and FS, follicles > 6 mm in diameter were arbitrarily assigned two size classes: medium (6–12 mm) and large (13–20 mm). Follicles in each size category were then arbitrarily categorised as having high oestrogenic status (E/P ratio > 5), or low oestrogenic status (E/P ratio < 5).

The resultant mean E/P ratios in the four different follicle categories are shown in Fig. 4a. With regard to inh-A concentration 2-way ANOVA revealed a highly significant effect of oestrogenic status ($P < 0.0001$) and a statistical interaction ($P < 0.005$) between size-class and oestrogenic status (Fig. 4d). Within the small follicle category inh-A levels were similar in high-oestrogenic follicles and low-oestrogenic follicles but in the large follicle category inh-A levels were 2-fold lower in highly oestrogenic follicles. As shown in Fig. 4b and c, mean concentrations of both act-A and FS in large follicles were 2–3-fold lower than in small follicles ($P < 0.0001$). Act-A was also affected by oestrogen status, being lower in highly oestrogenic follicles, but there were no differences with respect to FS. Likewise there was no effect of oestrogenic status on the relative abundance of the six individual isoforms of FS (data not shown). There were no significant effects of (nor statistical interaction between) follicle size class or oestrogenic status on intrafollicular act-A/FS ratio (Fig. 4e). However, act-A/inh-A ratio in large follicles was significantly lower than small follicles ($P < 0.001$) but there was no 'size-class' $\times$ 'oestrogenic status' interaction (Fig. 4f).

Granulosa cell cultures: effects of FSH and IGF on secretion of FS, act-A, inh-A and E

As shown in Fig. 5 treatment of isolated granulosa cells with FSH alone enhanced secretion of E and inh-A ($P < 0.05$) while IGF analogue alone enhanced secretion of FS, E, inh-A and act-A ($P < 0.05$). Co-treatment with FSH

Figure 3 Relationship between follicle diameter and the relative abundance of six different FS isoforms in bovine follicular fluid. The proportion of total FS represented by individual isoforms was calculated by expressing the band intensity for each isoform as a % of the total FS band intensity (i.e. sum of individual band intensities). The apparent Mr-values of the six isoforms were (a) 65 (b) 41 (c) 37 (d) 35 (e) 33 and (f) 31 kDa. Values are means $\pm$ S.E.M. ($n = 6$ –27) and P-values (ANOVA) are indicated.

Figure 4 Bar graphs showing (a) oestradiol/progesterone ratio (E/P ratio) (b) FS concentration (c) act-A concentration (d) inh-A concentration (e) act-A/FS ratio and (f) act-A/inh-A ratio in follicles categorised arbitrarily according to size class (Medium=6–12 mm; Large=13–20 mm) and oestrogenic status (High=E/P ratio>5; Low=E/P ratio<5). Values are means $\pm$ S.E.M. and *P*-values from 2-way ANOVA are indicated.

and IGF analogue promoted an even greater response. Act-A/FS ratio and act-A/inhibin ratio was also raised by combined treatment with FSH and IGF. A representative FS immunoblot of granulosa cell-conditioned media samples is shown in Fig. 6. Five of the six different FS isoforms found in bFF were detected (65, 41, 37, 35, 31 kDa) and, considering all samples analysed, these represented 18, 30, 28, 16 and 8% of total FS immunoreactivity. However, the relative amount of each FS isoform increased in parallel in response to treatment with FSH and IGF analogue, alone and in combination (Fig. 7a). Consequently, when the data were expressed on a proportional basis (Fig. 7b) there was no difference between treatments in the proportion of total FS represented by each individual isoform.

Discussion

To investigate the involvement of the inhibin–activin–FS system in folliculogenesis we examined intrafollicular

concentrations of these proteins in antral follicles from 2–20 mm in diameter, a size range which spans the cyclic recruitment, selection, dominance and atretic phases of follicle development in cattle (Webb *et al.* 1999, Mihm *et al.* 2002). We also applied the same analyses to conditioned medium from isolated granulosa cells cultured with/without FSH and IGF. Dynamic changes in FF concentrations of inh-A, act-A and FS as well as act-A/inh-A ratio and act-A/FS ratio were detected. The most novel and striking finding was a sharp increase in intra-follicular activin ‘tone’ that accompanied follicle growth from 3–6 mm in diameter. Increased activin ‘tone’ was reflected by a 30-fold increase in act-A concentration that far outpaced the concomitant 6-fold increase in inh-A concentration; FS concentrations were uniformly high throughout follicle growth from 2–9 mm and greatly exceeded act-A concentrations until follicles grew beyond 6 mm. Since FS binds to act-A with a 2:1 stoichiometry to neutralise its bioactivity (Sugino *et al.* 1994), these data suggest that little or no free act-A would be available for

Figure 5 Effects of FSH and LR3-IGF, alone and in combination, on the secretion of (a) FSH (b) E (c) inh-A and (d) act-A by bovine granulosa cells *in vitro*. Panels (e) and (f) show act-A/FSH ratio and act-A/inh-A ratio in media samples. Values are means $\pm$ S.E.M. ($n=4$ independent cultures); bars with different letters are significantly different ($P < 0.05$).

interaction with activin receptors, until follicles reached 6 mm in diameter and it is notable that this is the size at which the first appreciable increase in intrafollicular E was detected, coincident with upregulation of granulosa cell expression of P450 aromatase (Webb *et al.* 1999). This also corresponds to the approximate size (4–5 mm) at which FSH support becomes obligatory for further follicle growth in cattle (Gong *et al.* 1996) and to the stage at which the follicle selection mechanism is considered to operate in this species (Webb *et al.* 1999, Mihm *et al.* 2002). Thus, our novel observational findings are consistent with evidence from functional *in vitro* studies that activin upregulates expression of FSH receptors, thereby sensitising follicles to pituitary FSH and promoting enhanced P450 aromatase expression and E production (Hasegawa *et al.* 1988, Xiao *et al.* 1992, Findlay 1993, Knight & Glister 2001). For methodological reasons,

we only analysed follicles from 2 mm in diameter and so the possibility that the intrafollicular balance between inhibin, activin and FS fluctuates in preantral and/or smaller antral-stage follicles should not be ruled out.

The present finding that follicle growth from 9–20 mm was associated with a highly significant 2-fold reduction in 'total' FS concentration in FF, is inconsistent with previous immunohistochemical evidence in cattle, that granulosa cells of large, functionally dominant follicles contain more FS than granulosa cells of smaller or subordinate follicles (Singh & Adams 1998). Indeed, when we classified large (13–20 mm) follicles on the basis of E/P ratio, follicles with a high E/P ratio tended (not significant) to have lower FS contents than those with low E/P ratio; there was certainly no indication of a positive association between FS levels and oestrogenicity. In agreement with our data, Li *et al.* (1997) reported an inverse

Figure 6 Representative immunoblot showing the presence of five different FS isoforms in bovine granulosa cell-conditioned culture medium. Lanes 1–4 correspond to the following treatments: (1) control, (2) FSH alone, (3) IGF alone and (4) FSH+IGF. The positions of the marker proteins used to calibrate the gel/blot are indicated (*left*) as are the apparent Mr-values of the FS bands identified (*right*).

association between FS concentration in FF and follicle size in pigs. However, analysis of human follicular aspirates revealed no change according to follicle size or functional status (Erickson *et al.* 1995, Schneyer *et al.* 2000). Similarly, it was reported that granulosa cell expression of the two alternately spliced FS mRNA transcripts, corresponding to FS-315 and FS-288, did not vary during growth of human follicles from 6–23 mm (Fujiwara *et al.* 2001). The explanations for these apparent species differences are not known.

Our observation that antral follicle growth is associated not only with a decrease in total FS concentration in FF but also with a distinct shift in the relative abundance of different FS isoforms is novel and intriguing but, at this stage, we can only speculate as to the functional significance of this finding. While all FS isoforms are capable of binding activin with high affinity, the shorter isoforms, notably FS-288, also have a high affinity for sulphated proteoglycans on the cell surface, longer isoforms (e.g. FS-315) do not (Sugino *et al.* 1993, 1994). It has been suggested that the shorter membrane-anchored FS isoforms act to sequester activin on the cell surface, while the longer isoforms may bind activin in the circulation and extracellular fluid (Sugino *et al.* 1994, Schneyer *et al.* 1997, Wang *et al.* 2000). Since the relative abundance of the larger 41 and 37 kDa FS isoforms increased during follicle growth while the abundance of the smaller 33 and 31 kDa FS isoforms decreased, this could reflect changing granulosa cell expression of the two FS mRNA splice variants throughout follicle growth. However, a study of human granulosa cells revealed no change in expression (or ratio of expression) of FS-288 and FS-315 mRNA splice variants follicles with follicle size (Fujiwara *et al.* 2001).

An alternative possibility is that the extent of post-translational modification of the two FS core proteins (FS-288 and FS 315) might change as follicles grow. Thus granulosa cells might proteolytically cleave FS-315 into the truncated form (FS-303), or glycosylate FS-315, FS-303 or FS-288 to different extents according to the stage of follicle growth. Thirdly, the relative abundance of different FS isoforms in the FF compartment might be affected by changes in cellular binding, cellular uptake, rate of degradation or rate of exit of FS isoforms through the follicle wall. Thus, the observed decline in the relative abundance of the smaller FS isoforms (33 and 31 kDa) during follicle growth could reflect their selective depletion from FF as a result of enhanced binding to cell surface proteoglycans. Given the substantially (~1000-fold) lower concentration of FS in the peripheral circulation, it is most improbable that the FS isoform distribution pattern in FF is influenced by FS from extra-follicular sources.

While the physiological significance of these multiple FS isoforms remains to be investigated, it is generally assumed that all serve to diminish the association of activins, and perhaps other TGF β superfamily ligands, with their cognate receptors. However, it is possible that in some circumstances FS might serve to enhance presentation of activin and/or other ligands to cell surface receptors (Schneyer *et al.* 1997), this might be expected when the binding affinity with FS is relatively low, as is the case with BMPs (Iemura *et al.* 1998, Amthor *et al.* 2002, Glister *et al.* 2004). There is also evidence that binding of activin to FS anchored to the cell surface promotes internalization and subsequent lysosomal degradation of activin within the cell (Hashimoto *et al.* 1997). The presence of a large molar excess of FS over act-A in small antral follicles suggests that FS would be available to bind and potentially modulate the bioavailability of ligands other than act-A, at least in follicles <5 mm in diameter. In follicles growing beyond 6 mm, act-A increased to a level that would effectively saturate the available FS; given the very high affinity of activin for FS, we predict that this would displace other less avidly-bound FS ligands (e.g. BMPs) in FF, perhaps enhancing their interaction with signalling receptors on the cell surface.

We carried out *in vitro* experiments on isolated granulosa cells to test the hypothesis that treatment with known FS secretagogues (FSH, IGF) would modify the relative abundance of different FS isoforms, secreted by the cells and thus help explain, at least in part, the shift in the FS isoform distribution pattern observed during follicle development. In confirmation of our previous findings (Glister *et al.* 2001, 2003 2004) there was a clear-cut enhancement of inh-A, act-A and total FS secretion in response to treatment with FSH and IGF. Combined treatment with FSH and IGF also increased act-A/FS ratio and act-A/inh-A ratio, as was observed during follicle growth from 4–6 mm. However, the relative

Figure 7 Effects of FSH and LR3-IGF, alone and in combination, on the secretion of five different FS isoforms by bovine granulosa cells in serum-free culture. Panel (a) shows that the relative amount of each isoform secreted increased in response to the different treatments while panel (b) shows that the % of total immunoreactive FS (i.e. sum of individual band intensities) represented by each isoform was not affected by the different treatments. Values are means $\pm$ S.E.M. ($n=4$ independent experiments) and P -values from 2-way ANOVA are presented.

abundance of the five different FS isoforms detected in cell-conditioned medium was remarkably constant across treatments, leading us to reject this hypothesis. Since FSH and IGF treatment promoted a concomitant increase in E secretion by the granulosa cells (up to 30-fold), this *in vitro* observation actually corroborates our *ex vivo* analysis of bFF samples in which we found no relationship between oestrogenic status and follicle isoform distribution pattern in bFF. Rather, follicle size was the key factor affecting the relative abundance of the different FS isoforms in bFF.

The gradual decline in act-A concentration and act-A/inh-A ratio we observed in bFF during follicle growth from 7–20 mm supports the proposal of Hillier & Miro (1993) that the relative inhibin ‘tone’ increases as follicles approach pre-ovulatory status. In human follicles, this shift in inhibin ‘tone’ is mainly due to an increase in inh-B rather than inh-A (Magoffin & Jakimiuk 1998). In bovine

follicles, however, concentrations of inh-B are very much lower than inh-A concentrations, questioning the physiological relevance of inh-B in cattle (Knight & Glister 2003). Despite the clear increase in inh-A during follicle growth from 2–10 mm, classification of large (12–20 mm) follicles on the basis of E/P ratio showed an inverse association between E/P ratio and inh-A concentration. This observation accords with previous studies in cattle (Guilbault *et al.* 1993, Sunderland *et al.* 1996) and further supports *in vitro* evidence that inhibin may have an inhibitory effect on E secretion by bovine granulosa cells (Jimenez-Krassel *et al.* 2003).

To extend the present study we are currently analysing bFF samples harvested from the ovaries of cycle-synchronised cattle subjected to daily ovarian ultrasound monitoring. We have also isolated the five different FS isoforms (31–41 kDa) from pooled bFF and are

assessing their biological potencies using a bovine theca cell-based bioassay. It is anticipated that this additional information will shed further light on the intriguing finding of a follicle size-dependent shift in intrafollicular FS concentration and isoform distribution pattern. In conclusion, a marked increase in intrafollicular activin 'tone' accompanies bovine follicle growth from 3–6 mm in diameter, and this corresponds to the stage at which the FSH-dependent follicle selection mechanism is considered to operate in this species.

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