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Comparison of bioactivities, binding properties and intra-follicular levels of bovine follistatins

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Running title: follistatin isoforms and bovine follicle development

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Abstract

Five isoforms of follistatin (FST) (M*r* 31, 33, 35, 37, 41kDa) were purified from bovine follicular fluid (bFF). Comparison of their activin- and heparan sulphate proteoglycan (HSP)-binding properties and bio-potencies in neutralization of activin-A action *in vitro* revealed that all five isoforms bound activin-A, but with different affinities. Only the 31kDa isoform (FST-288) bound to HSP. FST-288 also showed the greatest biopotency with 35 and 41kDa isoforms being least potent. To determine whether bovine follicle development is associated with changing intrafollicular FST and activin profiles, we analyzed bFF from dominant (DF) and subordinate (SF) follicles collected at strategic times during a synchronized estrous cycle. Total FST, activin-A and activin-AB were measured by immunoassay while individual FST isoforms were quantified by immunoblotting. Follicle diameter was positively correlated with estrogen:progesterone ratio (r=0.56) in bFF but negatively correlated with activin-A (r=-0.34), activin-AB (r=-0.80) and 'total' FST (r=-0.70) levels. Follicle diameter was positively correlated with abundance of the 41 kDa isoform (r=0.59) but negatively correlated with abundance of 33 and 31 kDa isoforms (r=-0.56, -0.41). Both follicle status (DF vs SF) and cycle stage affected total FST, activin-A, activin-B levels while follicle status, but not cycle stage, affected abundance of 41, 37, 33 and 31kDa FST isoforms. Collectively, these findings indicate that intrafollicular FST isoforms that differ in their ability to bind and neutralise activins and associate with cell-surface proteoglycans, show divergent changes during follicle development. Enhanced FST production may have an important negative role, either directly or via inhibition of the positive effects of activins, on follicle growth and function during follicular waves.

Introduction

Since the discovery and characterization of inhibins and activins over 25 years ago, numerous studies have investigated the involvement of these structurally-related yet reciprocally-acting proteins in the regulation of ovarian function (reviews:(Woodruff & Mather 1995, Knight & Glister 2001, Findlay *et al.* 2002, Phillips 2005, Knight *et al.* 2012). Regulatory roles impacting on follicle development are manifest both at the anterior pituitary (i.e. modulation of FSH secretion) and intraovarian level, with activin exerting local autocrine/paracrine

stimulatory effects that are opposed by inhibin and/or the activin-binding protein, follistatin (FST). For instance, in vitro studies have implicated activin as an important promoter of follicle growth/survival by virtue of its ability to upregulate cell proliferation, FSH receptor expression and enhance basal and FSH-induced oestrogen production by granulosa cells whilst simultaneously inhibiting progesterone production. In addition, activin suppresses LH-induced androgen production by theca cells. The above actions can be reversed by inhibin and/or FST. Inhibin antagonises activin signalling by competitive binding to the type 2 activin receptor on the cell surface while follistatin (FST) inhibits activin signalling by acting as a high affinity activin-binding protein (de Winter *et al.* 1996, Schneyer *et al.* 1997, Sugino *et al.* 1997, Harrison *et al.* 2006).

FST is a cysteine-rich, monomeric glycoprotein, encoded by a single gene that is highly conserved amongst vertebrates(Schneyer *et al.* 1997, Sugino *et al.* 1997, Hedger & de Kretser 2013). FST was first isolated from ovarian follicular fluid based on its activity to suppress FSH secretion by pituitary cells (Robertson *et al.* 1987). Multiple isoforms of FST protein exist, with up to six different isoforms identified in ovarian follicular fluid (Sugino *et al.* 1997, Glister *et al.* 2006). These are generated by alternate mRNA splicing to yield two core proteins (FST-288 and FST-315). FST-315 can undergo posttranslational cleavage of the carboxy-terminal sequence to yield FST-303. These three ‘core’ proteins can also be glycosylated to produce the six isoforms identified in porcine (Sugino *et al.* 1993, Sugino *et al.* 1997) and bovine (Glister *et al.* 2006) follicular fluid. It has previously been reported that FST can bind to heparan-sulphate proteoglycans (HSP) on the surface of granulosa and other cells with the shorter (FST-288) forms binding with much higher affinity than the longer (FST-315) forms, implying different roles in modulating activin function (Nakamura *et al.* 1991, Nakamura *et al.* 1997). Moreover, binding of FST-activin complexes to cell surface proteoglycans facilitates endocytosis and their subsequent lysosomal degradation (Hashimoto *et al.* 1997). In addition to their recognised roles in reproductive tissues, activins and FST are important regulators of haematopoiesis, immunoregulation, inflammation, wound healing and fibrosis (review: (Hedger & de Kretser 2013)).

With regard to previous studies on the ovarian inhibin-activin-FST system in various species, an important caveat is that a large majority has been carried out using just one FST form (recombinant human (rh)FST-288) and one activin form (rh activin-A), both of which have been widely available to the research community primarily from commercial sources. In

addition, very little information is available on the relative abundance of individual isoforms of FST and activin in different biological compartments including ovarian follicular fluid. With this in mind, the primary objective of the present study was to purify native FST isoforms from pooled bovine follicular fluid allowing a direct comparison of their activin- and heparan sulphate-binding properties as well as their bio-potencies in reversing the inhibitory effect of activin-A on thecal androgen production *in vitro*. In addition, we used quantitative immuno-analytical techniques (ELISA, western blotting) to test the hypothesis that post-emergence antral follicle growth in estrous cycle-synchronised cattle is associated with changing intrafollicular levels of different isoforms of FST and activin, reflecting differential production and roles of these proteins in follicle selection and dominance in this monovulatory species.

Materials and Methods

Purification of FST isoforms from pooled bFF

Pooled bovine follicular fluid (bFF; protein content 65mg/ml) was collected by needle aspiration of non-cystic follicles visible on the surface of cattle ovaries obtained from the slaughterhouse. bFF was stored at -80^{o}C in the presence of a protease inhibitor (0.005% w/v phenylmethylsulphonylfluoride, PMSF). After thawing, bFF was centrifuged (20,000g, 30min, 4^{o}C) and the supernatant (200ml) applied to an anti-FST immunoaffinity column. This was prepared by coupling 10mg of a 1:1 mixture of two anti-FST monoclonal antibodies (MAb; clones FST 1/1 and 8/1; gift from Prof NP Groome) to 5ml of n-hydroxysuccinamide-activated sepharose beads (GE Healthcare Ltd), according to the manufacturer's instructions. bFF (200ml; 13g protein) was cycled through the anti-FST immunoaffinity column at a flow rate of 0.5 ml/min, overnight at 4^{o}C. After washing the column with 100ml PBS the bound fraction (containing multiple FST isoforms as well as activins bound to FST) was eluted using 3 column volumes (15 ml) of 8M urea. Urea solution was deionised by passing it through a mixed bed ion exchange resin (Monobed, Amberlite) immediately before use. After immunoaffinity chromatography, subsequent steps used to isolate the FST isoforms were based upon the method described by Sugino et al. (Sugino *et al.* 1993) with some modifications. CHAPS (0.05% w/v final) and TRIS-HCl pH 7.8 pre-set crystals (20mM final) were added to the fraction eluted from the immunoaffinity column and it was applied to a fast

protein liquid chromatography (FPLC) Mono P anion exchange column (5 x 100 mm; Pharmacia Ltd), pre-equilibrated with 20mM Tris-HCl pH 7.8 and 0.05% w/v CHAPS. Four distinct peaks (A, B, C and D) were eluted with a linear gradient from 0 to 1M NaCl in 20mM Tris/HCl (pH 7.8) at a flow rate of 1ml/min over 40 minutes, with 0.5 min fractions collected. Pooled fractions comprising each peak, plus the non-retained (NR) fraction, were further purified by reversed phase HPLC using a Cosmosil C8 (4.6 x 150mm) column. The column was developed with a 5min linear gradient of 10-30% acetonitrile in 0.1% trifluoroacetic acid (TFA), followed by a 10min gradient of 30-40% at a flow rate of 1ml/min with fraction collected every 0.25 min. Highly enriched FST-containing fractions were designated as RP-HPLC peaks #1, #2, #3, #4 and #5.Throughout the purification procedure chromatographic eluates were monitored for UV absorbance at 280nm. Fractions were also analysed for FST immunoreactivity using a two-site ELISA (see below). Estimates of total protein concentrations and final yields were based on the assumption that a 1mg/ml solution of protein has a 280nm absorbance value of 1.0.

Deglycosylation of purified FST isoforms

A 1μg aliquot each of the purified FST isoforms was treated with a mixture of five different deglycosylation enzymes (E-DEGLY kit; Sigma) according to the manufacturer's instructions. Treated versus non-treated samples were run on SDS-PAGE and stained with silver nitrate according to the method of (Morrissey 1981).

SDS-PAGE and Western Blotting

Purified bovine FST fractions and bFF samples harvested from cycle-synchronized heifers (see below) were analysed by SDS-PAGE/Western blotting as described previously (Glister et al 2006). SDS-PAGE was performed under non-reducing conditions using 12.5% gels. Gels were either stained with Coomassie blue to detect total proteins or subjected to electrophoretic transfer to nitrocellulose membranes for immunodetection of individual FST isoforms using a 1:1 mixture of FST MAb (clone 1/1 and clone 8/1). ^{125}I-labelled horse anti-mouse IgG was used as secondary antibody to detect membrane-bound anti-FST MAb using a phosphor screen and Phosphorimager (Molecular Dynamics) and images were analysed using Image J

(v1.32J) software. The between-gel coefficient of variation for FST band intensity averaged 10.6%, based on estimates for a pooled bFF quality control sample included on each gel.

Bioassay for FST isoform activity

The biological potency of each purified bovine FST isoform, along with that of recombinant human (rh) FST-288 (R&D Systems, UK), was determined using an *in vitro* bovine theca cell bioassay based upon the ability of FST to block the suppressive effect of activin on androstenedione production by LH-treated theca cells. Bovine theca cells were isolated and cultured as described previously (Glister *et al.* 2005). The cells were cultured under serum-free conditions for 6 days with media changed and treatments, including LH (150pg/ml), added on day 2 and 4. Before addition to cells, FST and activin treatment combinations were diluted in culture medium and pre-incubated for 4h at room temperature to allow any FST-activin association to occur. A near-maximally effective concentration of activin-A (10ng/ml giving ~85% suppression of androstenedione secretion) was used and the molar ratio of FST to activin-A was adjusted to 0:1, 2:1, 4:1, 8:1 or 16:1. Molar ratio, rather than mass ratio, was used to account for the differences in molecular mass amongst the different FST isoforms. At the end of the culture period conditioned media were removed and stored at -20^{o}C until assayed for androstenedione; viable cell number was determined by neutral red dye uptake assay. Each experiment was repeated four times using independent batches of cells.

Biacore surface plasmon resonance experiments to determine binding affinities

To determine the binding affinity of purified FST isoforms for immobilised rh Activin-A and heparan sulphate proteoglycan (HSP), experiments were performed using the Biacore 3000 surface plasmon resonance (SPR) instrument (Biacore International, Stevenage, Herts). rh Activin-A (R&D Systems) and HSP (Sigma) were immobilised on the surface of separate CM5 sensor chips (~500 resonance units) according to the manufacturer's protocol (Biacore). BSA was immobilised on an adjacent lane of each chip (~500 resonance units) to act as independent controls for rh activin-A and HSP. Purified bovine FST isoforms and rhFST-288 were passed over both chips at a flow rate of 30µl/min. Hepes-buffered saline (10mM Hepes, 150mM NaCl, 3.4 mM EDTA, 0.005% Tween20, pH 7.4) was used for sample dilution and as

a running buffer. Each FST isoform was run over immobilised rhAct-A, HSP and BSA lanes at a range of concentrations (6.25, 12.5, 18.75, 25, 37.5, 50, 75, 100 nmol/l). For chip regeneration bound FST was eluted from the rh activin-A or HSP coated surface using 50mM NaOH and 1M NaCl. Binding curves were corrected by subtraction of the blank and evaluated using Biacore evaluation software. Each experiment was repeated at least 3 times and kinetic data, including KD, were calculated using the Biacore evaluation software.

Comparison of biological activities and FST binding properties of activins A, AB and B

The ability of rh activin-A, activin-AB and activin B to suppress androstenedione secretion by bovine theca cells was compared by treating cells for 4 days with 0, 2, 10 and 50 ng/ml of each protein. The binding affinities of rh activin-A, activin-AB and activin B for immobilized rhFST-288 were evaluated by Biacore SPR analysis. Bioassay and binding experiments were repeated three times.

Collection of ovarian samples from estrous cycle-synchronized cattle

The bovine follicular fluid (bFF) samples analysed for activin and FST content in the present study were collected as part of a previous investigation focusing on analysis of inhibin and inhibin subunits (Sunderland *et al.* 1996). A detailed description of the animals and experimental procedures used to obtain the bFF samples, as well as their serum hormone profiles and bFF concentrations of inhibins, estradiol and progesterone can be found in the aforementioned publication. All animal work was carried out in accordance with regulations set down by the UCD BioMedical Centre, Dublin, Ireland, and by the Cruelty to Animals Act (Ireland).

Briefly, estrous cycles of 28 crossbred beef heifers aged 15-18 months were synchronised using a 10-day progestagen implant regimen (Crestar: Intervet Ireland Ltd, Dublin) with PGF2α analogue (PG; Prosolvin: Intervet Ireland Ltd) administered two days prior to implant removal to initiate luteolysis. Follicular development was monitored by transrectal ultrasonography from the time of PG administration until approximately 12h before ovariectomy. Animals were allocated to one of five groups according to the time of

ovariectomy as follows: day 0 (follicular phase, pre-LH surge; n=5), day 1 (follicular phase, post-LH surge, pre-ovulation; n=6), day 3 (post-ovulation, selection phase; n=6), day 6 (post-ovulation, dominance phase; n=5) and day 12 (post ovulation, atretic phase; n=6).

Excised ovaries were placed in ice-cold PBS and the number of follicles ≥5mm and their diameters were recorded before bFF was aspirated from each follicle. The bFF samples were aliquotted and stored at -80˚C until analysis. For the present study follicles from each pair of ovaries were classified as the DF (largest or morphologically dominant follicle), SF1 (second largest or first subordinate follicle) or SF2p (bFF pooled from all remaining subordinate follicles ≥5mm). In addition, bFF concentrations of estradiol (E) and progesterone (P) were used to classify follicles as either estrogen active (EA; E/P ratio >1) or estrogen inactive (EI; E/P ratio < 1) (Ireland & Roche 1982).

Hormone immunoassays

Concentrations of androstenedione in theca cell-conditioned media were determined by radioimmunoassay (Glister *et al.* 2005). The detection limit of the assay was 50 pg/ml and the intra- and inter-assay CVs were 8 and 10% respectively. 'Total' (i.e free + activin-bound) FST levels in bFF samples and chromatographic fractions were determined by two-site ELISA (Tannetta *et al.* 1998). Values are expressed in terms of a rhFST preparation provided by Dr A Parlow (NHPP, USA). Assay sensitivity was 100pg/ml and intra- and inter-assay CVs were 7% and 9% respectively. 'Total' activin A concentrations in bFF were measured by 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 100pg/ml. Intra- and inter-assay CVs were 4% and 7% respectively. 'Total' activin-AB concentrations were measured using the two-site ELISA (Evans *et al.* 1997). The assay was calibrated using purified preparation of porcine activin-AB (gift from H Sugino) and had a sensitivity of 200pg/ml. Intra- and inter-plate CVs were 5% and 8% respectively. Activin-B concentrations were evaluated using a more recently developed 2-site ELISA (Ludlow *et al.* 2009). The assay was calibrated using hr Activin-B (R&D systems) and the sensitivity in this laboratory was about 100 pg/ml. Intra- and inter-plate CVs were 9% and 12% respectively.

Statistical analysis

Data from in vitro bioassays and Biacore experiments comparing different FST isoforms we isolated were evaluated using ANOVA and *post-hoc* Fisher's PLSD test. One-way and two-way ANOVA of log-transformed data were used to evaluate between-group differences in bFF levels of activin-A, activin-AB, 'total' FST and the proportion of 'total' FST represented by each of the six individual isoforms detected. Where indicated, *post hoc* Fishers PLSD test was subsequently used to make individual pair-wise comparisons. Linear correlation analyses were also used to examine the relationship between the above variables amongst the entire sample set. Unless stated otherwise, values are presented as arithmetic means ±SEM.

Results

Chromatographic purification of bovine ovarian FST isoforms

Fig. 1 (lane 1) shows the presence of six different bands of FST immunoreactivity in pooled bFF (starting material). These bands had apparent molecular weights (M*r*) ranging from 31-65kDa. When FST was purified by affinity chromatography on an immobilised FST-MAb column, the eluate contained five M*r* forms ranging from 31-41 kDa (fig.1, lane 2). When this material was applied to an anion exchange column (Mono P), five major regions of FST immunoreactivity (fig.1, lanes 3-7) were identified (using FST ELISA and Western blotting) corresponding to peaks A, B, C and D plus the non-retained fraction (NR) that passed straight through the column (fig. 2). Each of these five anion exchange fractions was further fractionated by reversed-phase HPLC to generate the final five highly purified FST isoforms (#1 - #5) shown in fig.1A lanes 8-12. The purity of each isoform was judged to be ≥74% based on densitometric analysis of FST immunoblots and ≥79% based on densitometric analysis of coomassie blue-stained gels (see Fig 1B and table 1). The purified FST preparations represent 8810-11475-fold purification factors and the final combined yield was 17.7% (206μg FST in total; 12-86μg of each isoform) from 200ml of bFF starting material (see table 2). The amount of each FST isoform purified was quantified using a combination of ELISA (calibrated using rh FST-288), UV absorbance (280nm) and densitometric quantification of western blots using a calibration curve generated by running known amounts of hrFST-288 alongside the purified forms (see table 3). Relative to the rh FST-288 standard

(100%), cross reactivities of purified bovine isoforms #1 - #5 in the ELISA were 93, 86, 80, 103 and 95%, respectively with each isoform yielding a parallel dilution curve.

Characterisation of purified FST isoforms

As it is known that potential Asn-linked glycosylation sites exist in FST proteins (Sugino et al, 1993) the purified proteins were treated with a mixture of deglycosylation enzymes, that included PNGase F which cleaves all asparagines-linked oligosaccharides, to ascertain whether the various forms were glycosylated variants of the core FST proteins. The multi-glycosylated protein fetuin, included in the deglycosylation kit, served as a positive control; upon deglycosylation it ran as a smaller forms on SDS-PAGE (fig. 3 lanes 1 and 2). When enzyme-treated FST isoforms were electrophoresed under non-reducing conditions, #1, #2 and #3 did not undergo a mobility shift, running at 31, 33 and 35 kDa respectively (fig.3 lanes). Upon deglycosylation #5 underwent a mobility shift from 41 to 35 kDa (fig. 3 lanes). Unfortunately, insufficient amounts of #4 (apparent Mr 37 kDa) were available for inclusion in the deglycosylation experiment. Based on the size at which they ran on SDS-PAGE, we deduce from these observations that #1 is a non-glycosylated variant of core protein 288, #2 is the non-glycosylated variant of core protein 303, #3 is the non-glycosylated variant of core protein 315, with #5 appearing to be the glycosylated variant of core protein 315.

Biological potencies of purified FST isoforms

The biological properties of the five purified bovine FST forms, along with hr FST-288, were compared using an in vitro theca cell bioassay. As shown in fig.4, activin-A suppressed LH-induced androstenedione secretion by ~85% ($p<0.0001$). Co-treatment of cells with FST dose-dependently reversed the suppressive effect of activin on LH-induced androstenedione secretion. Each FST isoform neutralised activin-A bioactivity to a greater or lesser degree with ED_{50} values ranging from 1.7 fold to 4.4 fold molar excess of FST over activin (ED_{50} data summarised in table 3). Bovine #1 (31kDa) had the same biopotency as hr FST-288 (31kDa) from a commercial source (R&D systems). Conversely, #3 and #5 (35- and 41kDa respectively) had the lowest biopotencies of all forms tested (39% and 53% relative to hr FST-288 at 100%). In the absence of activin treatment (fig. 4B), none of the FST forms tested

had any significant effect on thecal cell androstenedione production consistent with the lack of endogenous activin production by these cells.

Activin-binding properties of purified FST isoforms

To investigate whether the differences observed between FST isoforms in neutralising activin bioactivity reflected different binding affinities of these FST forms for activin, the dissociation constant (KD) of each form for immobilised activin-A was quantified using Biacore (table 4). The KD was significantly greater for each of the larger FST isoforms (33, 35, 37 and 41kDa) than for the smallest isoform #1 (31kDa) and hr FST-288, indicating lower affinity binding. The 35kDa and 41kDa isoforms formed the least stable complexes with activin-A, with KD values some 20-fold higher (~33nM) than for the 31kDa isoform (1.6nM).

Heparan sulphate-binding properties of purified FST isoforms

The differential ability of FST isoforms to bind to cell-surface heparin sulphate proteoglycans (HSP) has been implicated as a method whereby FST modulates the activity of activin in different extracellular compartments. We therefore used Biacore to examine the ability of our purified bovine FST forms to bind to immobilized HSP. As shown in table 4, isoform #1 (32kDa) and hr FST-288 of the same molecular weight both bound to HSP with similar KD values (~3nM). However, no quantifiable binding of FST isoforms #2, #3, #4 and #5 to immobilised HSP was observed.

Comparison of biological potencies and FST binding properties of activins A, AB and B

In terms of their ability to suppress androstenedione secretion in the theca cell bioassay (table 5) activin-A was about two-fold more potent that activin-AB whereas activin B did not elicit a response at the maximum concentration tested (50 ng/ml). Biacore analysis showed that activin-AB and activin-B bound to immobilised rh FST-288 with ~2-fold higher affinity than activin-A (table 5). There were insufficient amounts of purified bovine FST isoforms

available to carry out a systematic evaluation of their binding affinities for the three different rh activin isoforms.

Analysis of FST and activin isoforms in bFF samples from estrous cycle-synchronized heifers

As circulating gonadotrophin (LH, FSH) and steroid hormone (E2, P4) profiles and detailed information on ovarian ultrasonography and intrafollicular E2, P4 and inhibin concentrations in these heifers have been documented in a previous report (Sunderland *et al.* 1996) only new information pertaining to the analysis of FST and activin isoforms in bFF is presented in this paper (Fig. 5). Preliminary analysis of a subset of pooled bFF samples showed that activin B concentrations (214±25 ng/ml) were substantially (~20-fold) lower than activin-A (4333±379 ng/ml) and ~6-fold lower than activin-AB (1259±71 ng/ml) concentrations. Given the limited bFF sample volumes available it was not feasible to determine activin B concentrations in this sample set.

Overall, follicle diameter was positively correlated with E/P ratio ($r=0.56$; $P<0.0001$) and negatively correlated with activin-A ($r=-0.34$; $P=0.003$), activin-AB ($r=-0.51$; $P<0.0001$) and 'total' FST measured by ELISA ($r=-0.70$; $P<0.0001$). Using SDS-PAGE/Western blotting 5-6 different FST isoforms were detected in individual bFF samples (apparent Mr: 65, 41, 37, 35, 33 and 31 kDa) representing, on average, 6, 13, 24, 26, 13 and 17%, respectively of total FST (Fig. 6). Follicle diameter was positively correlated with % abundance of the 41kDa ($r=0.59$; $P<0.0001$) and 35kDa ($r=0.29$; $p=0.014$) FST isoform but negatively correlated with % abundance of the smaller 33kDa ($r=-0.56$; $p<0.0001$) and 31 kDa ($r=-0.41$; $p< 0.001$) FST isoforms. There was a particularly marked effect of follicle status on the % abundance of the 33kDa isoform that showed up to a 3-fold difference ($P<0.0001$) between DF and SF2p follicles.

Two-way ANOVA revealed a significant effect of cycle day ($P<0.05$) and follicle status ($P<0.01$) (i.e. DF, SF1, SF2p) on follicle diameter, E/P ratio, activin-A, activin-AB and 'total' FST concentrations (Fig 5). Furthermore, FST concentration was much lower in DF than in SF1 or SF2p ($P<0.05$). During growth and selection of the 'first wave' follicles in the early luteal phase (i.e. days 3 to 6) 'total' FST, activin-A, activin-AB concentrations decreased in DF ($P<0.05$) but were maintained or increased in SF1 and SF2p. During subsequent atresia of DF

(i.e. days 6 to 12), 'total' FST and activin-AB remained lower in DF than in SF1 and SF2p (Fig. 5). Two-way ANOVA revealed a significant ($P<0.05$) effect of follicle status (i.e., DF, SF1, SF2p) on % abundance of the 41, 37, 35, 33 and 31kDa FST isoforms and an effect of cycle day on % abundance of the 65kDa FST isoform (Fig. 6).

In 'follicular phase' samples (Fig.5), follicle status significantly affected total FST and activin-AB concentrations, activin/FST ratio and E/P ratio. FST concentration was much lower in DF than in SF1 or SF2p. There was also a significant effect of cycle day (i.e. pre- versus post-LH surge) on activin-A, activin-AB and FST levels. Follicle status, but not cycle day, also affected the % abundance of the 41, 33 and 31kDa FST isoforms (Fig. 6).

Discussion

It is well established that FST can selectively attenuate signalling by activins and, to a lesser degree, other ligands belonging to the TGFβ superfamily (e.g. BMP2, BMP4, BMP7, BMP15, GDF9, myostatin/GDF8) by forming a stable inactive complex with the ligand in the extracellular compartment thus blocking binding to signalling receptors (Sugino *et al.* 1993, Nakamura *et al.* 1997, Phillips & de Kretser 1998, Chang 2008). However, the existence of multiple isoforms of FST in biological fluids such as ovarian FF and serum (Robertson 1992, Sugino *et al.* 1993, Nakamura *et al.* 1997, Schneyer *et al.* 1997, Glister *et al.* 2006) is indicative of a more complex scenario, the potential significance of which has yet to be fully resolved. FST isoforms differ in their ability to bind to proteoglycans on the cell surface and extracellular matrix with the shorter, carboxy-terminal truncated form (FST-288) showing a much higher affinity for HSP than the long isoform (FST-315). This reflects the ability of the carboxy-terminal domain of FST-315 to mask the HSP binding region found within the FS1 domain (Keutmann *et al.* 2004, Hedger & de Kretser 2013). Activin-binding activity involves the amino terminal domain, FS1 and FS2 domains (Keutmann *et al.* 2004, Chang 2008). Differences in the propensity of FST isoforms to bind to cell surface proteoglycans is indicative of differential roles in controlling activin distribution and bioavailability at local and/or distant target tissue levels.

In view of substantive evidence implicating the inhibin-activin-FST system in ovarian follicle development, in this study we devised an effective chromatographic scheme to purify five native isoforms of FST from bovine ovaries (overall FST yield 17.7%; ~10,000-fold purification factor) thus permitting a comparison of their biological activities and binding affinities for activin-A and HSP. We show that all five FST isoforms are capable of blocking the suppressive effect of activin on androgen secretion by bovine theca cells. The smallest isoform (bovine #1; 31kDa, deduced to be the non-glycosylated, carboxy-terminal truncated form, FST-288) represented about 17% of total FST in bFF and was the most bio-potent, being equipotent with rh FST-288 (31kDa) obtained from a commercial source. It also exhibited KD values for binding to activin-A and HSP that were very similar to those for rh FST-288. In contrast, two of the larger isoforms we isolated (35kDa, 41kDa) showed reduced biopotency (39 and 53% respectively) as well as significantly lower affinity for activin-A and undetectable binding to HSP. On the basis of their mobility on SDS-PAGE before and after enzymatic deglycosylation it was deduced that the 35kDa isoform (bovine #3) is non-glycosylated FST-315 while the 41kDa isoform (bovine #5) is its glycosylated counterpart. Respectively, these FST isoforms represented 26% and 13% of total FST in bFF. The other two isoforms we purified, bovine #2 (33kDa) and #4 (37kDa) had relative bio-potencies that were not significantly different from FST-288 (77% and 89%, respectively). The affinity of the 37kDa isoform for activin-A was ~3-fold less than that of FST-288 and neither the 33kDa or 37kDa isoform showed detectable binding to HSP. The 33kDa isoform was deduced to be a non glycosylated variant of FST-303 core protein since no mobility shift occurred upon deglycosylation. The 33kDa form represented on average 13% of the total FST in bFF although, as discussed later, it showed a substantial follicle status-dependent variation *in vivo* (6-21% of total FST). The chromatographic yield of the 37kDa FST isoform was low (only 12μg recovered) and there was insufficient material to further characterise it by deglycosylation mobility shift analysis. However, on the basis of previous characterisation of porcine FST isoforms (Sugino *et al.* 1993) it is considered likely to be a glycosylated form of FS-303.

The native bovine FST isoforms we isolated differed both in their ability to neutralize activin-A bioactivity in vitro and in their binding affinity for activin-A. This observation contrasts with an early report (Sugino *et al.* 1993) that different FST isoforms purified from porcine FF displayed very similar activin binding activities. However, activin binding was estimated using a polyethyleneglycol precipitation method with ^{125}I-labelled activin-A, as opposed to

direct binding of FST to unlabelled activin-A immobilized on a Biacore sensorchip, as used in our study. Another study employing the Biacore approach (Hashimoto *et al.* 2000) reported that hrFST-288 bound activin-A with an affinity an order of magnitude higher than hrFST-315, a finding in agreement with our comparison of native bovine FST isoforms.

To our knowledge this is the first report to compare both the bioactivities and FST binding properties of the three activin isoforms. In terms of their relative bioactivity it has been reported previously that activin B is much less potent that activin-A in various in vitro bioassays (Sugino *et al.* 1997, Schneyer *et al.* 2003), a finding in agreement with our study using a bovine theca cell bioassay. Using immobilized hrFST-288 we also found that the binding affinities for activin-AB and activin-B were significantly higher than for activin-A and suggest that this could contribute to the higher biopotency of activin-A. However, using a radioligand binding assay it was reported (Schneyer *et al.* 2003) that activin-B bound to hrFST-288 with a lower affinity than activin-A, in disagreement with our finding based on the Biacore technique. Regardless of this discrepancy, the present study shows that levels of activin B in bovine follicular fluid are much lower than activin-AB that, in turn, are lower than activin-A. Collectively, these observations suggest that activin B has a minimal physiological role(s) in bovine antral stage follicles.

Evidently, this study is also the first to compare the bioactivities of multiple native FST isoforms using an homologous (i.e. same species) ovarian bioassay. Many studies have used FST-dependant inhibition of FSH release from rat anterior pituitary cells as a heterologous bioassay for purified porcine or bovine FST preparations (Robertson *et al.* 1987, Sugino *et al.* 1993, Sugino *et al.* 1997) with the possibility that species differences could affect the relative responses observed. In fact, bovine isoform #1, evidently unglycosylated core protein FST-288, behaved identically to hrFS-288 in our bovine theca cell bioassay and had the highest biopotency of the five FST isoforms purified. Similarly, the activin binding affinity of isoform #1 was not significantly different to hrFS-288 and was also the highest of the five isoforms. Moreover, of the different FST preparations tested, only hrFST-288 and bovine isoform #1 had the ability to bind to heparan sulphate proteoglycan with both exhibiting a similar KD value of 3-4nM, close to the value reported by Sugino and colleagues (Sugino *et al.* 1997) for the binding of porcine FST-288 to granulosa cell membranes (KD 2nM). These observations support the notion that FST-288 is the most effective of all the isoforms at neutralising activin-A activity, at least in the present *in vitro* bioassay. This can arise by a

two-step mechanism: not only does it bind to activin-A with the greatest affinity, thereby blocking its interaction with activin signalling receptors, but it also has the greatest propensity to bind to cell surface associated heparan sulphate proteoglycans, thereby becoming concentrated on the cell surface to provide an even more effective 'barrier' to prevent activin association with its signalling receptors. In addition, it has been shown that activin: FST complexes bound to heparan sulphate proteoglycans on the cell surface are endocytosed leading to their subsequent lysosomal degradation (Hashimoto *et al.* 1997, Sugino *et al.* 1997). The KD value we observed for FST-288-activin-A binding was of the same order of magnitude (low nM range) as that for FST-288-HSP binding indicating stable complex formation in both cases. In further work it would be of interest to use the Biacore technique to examine the binding kinetics of ternary complex formation/dissociation (HSP-FST-activin) in an attempt to model likely interactions at or near the cell surface *in vivo*.

We deduce that purified bovine isoforms #3 and #5 are non-glycosylated and glycosylated variants of core protein FST-315, respectively. They showed the lowest activin biopotencies of the five isoforms purified, up to 60% less effective at neutralising activin in bioassay compared to isoform #1 (FST-288). Moreover, their affinity for activin-A was an order of magnitude less than for isoform #1/FST-288. Although this finding conflicts with a previous report that different isoforms of porcine FST show similar activin-binding affinities (Sugino *et al.* 1993) our finding is in agreement with a later study comparing recombinant human FST-288 and FST-315 activin-binding affinities (Hashimoto *et al.* 2000). The explanation for this discrepancy is not known but, consistent with earlier findings for porcine (Sugino *et al.* 1993, Sugino *et al.* 1997), the larger isoforms of bovine FST we isolated displayed much lower (non-quantifiable) binding to HSP.

Having examined the biological activities, activin-binding and HSP-binding properties of the different naturally occurring FST isoforms purified from bovine ovaries, we asked the question: do their relative intrafollicular expression levels vary during bovine follicle development *in vivo*? To address this we used a quantitative immunoblotting approach to analyse individual bFF samples harvested from the follicles of oestrous cycle-synchronized cattle that had been ovariectomized at five key stages of follicle 'wave' development (Sunderland *et al.* 1996). Additionally, we used a panel of specific 2-site immunoassays to quantify 'total' FST, activin-A and activin-AB concentrations in the same samples; attempts to quantify activin B in individual samples using a new immunoassay (Ludlow *et al.* 2009)

were unsuccessful due to the much lower concentration present and the insufficient sample volumes available. Concentrations of activin-A were around 3-fold higher than activin-AB and estimated to be at least 20-fold higher than activin-B levels, indicating a minimal physiological role for activin-B in the bovine antral follicle.

Although interpretation of the resultant dataset is complex owing to the number of different analytes, time-points and follicle status categories involved, the findings are broadly consistent with those of an earlier study involving analysis of size-ranked follicles from the ovaries of randomly cycling cattle obtained from an abattoir (Glister *et al.* 2006). In particular, total FST levels in bFF were inversely related to follicle diameter and E/P ratio in both studies. Likewise, the average proportion of total FST represented by each different isoform was very similar in both studies. FST-288 core protein represented about 17% of total FST while glycosylated/nonglycosylated forms of the carboxy-terminal extended core proteins FST-303 and FST-315 represented 13/24% and 26/13% respectively. The relatively low abundance of FST-288 present in bFF is still much higher than the estimate of 1% reported for porcine FF (Inouye *et al.* 1991) suggesting a pronounced species variation. A low abundance of FST-288 present in FF most likely reflects its high affinity for HSP that would result in its adhesion to the plasma membrane of granulosa cells lining the follicular antrum and cumulus cells surrounding the oocyte. By contrast, FST-303 and FST-315 would tend to remain in the liquid phase, likely diffusing further from their site of secretion (granulosa cells) to sequester activins and other TGFβ family ligands arising from other intra- or extra-ovarian sources, and perhaps acting as an 'activin reservoir'. Interestingly, while each FST isoform showed some variation in relation to follicle status and stage of cycle, the 33kDa isoform (deduced to be FST-303 core protein) showed the greatest difference in relative abundance between dominant and subordinate follicles, being up to 3-fold higher in the latter. Whilst the explanation and/or physiological significance of this intriguing observation remains obscure it is speculated that dominant follicles may show decreased expression of a specific protease that cleaves FST-315 into FST-303 (Welt *et al.* 2002). Consistent with this suggestion, the abundance of the 41kDa and 35kDa isoforms (glycosylated/nonglycosylated FST-315) tended to show the reverse pattern to FST-303, being highest in dominant follicles. The nature of the 65kDa band representing about 6% of immunoreactive FST detected by immunoblotting in bovine FF remains unknown since it was not one of the isoforms purified in the present study.

Regarding the physiological interpretation of these data, on days 0, 1 and 6 of the cycle (see Fig. 5) the "selected" healthy DF was clearly distinguished from atretic SFs that were smaller and had lower E/P ratio, but higher total FST (and 33kDa, 31kDa FST forms), activin-A and activin-AB. These findings imply that high FST and activin levels may hinder growth and E production of SFs, which is critical for onset of follicle dominance. This idea is supported by the change in FST and activin levels from Day 3 (before selection) to Day 6 (when DF is present) when levels of total FST (and 33 kDa and 37 kDa FSH isoforms) and activin-A and -AB decrease as the size and E/P ratio increase in the DF. Moreover, on Day 3 before onset of dominance, when DF and SF showed a similar size and E:P ratio, total FST (and most FST isoforms) and activin levels remained similar between follicle types. From Days 6 to 12 as the DF from Day 6 became atretic, as indicated by its greatly reduced E/P ratio, its diameter and total FST, activin-A and –AB levels remained unchanged. However, the relative abundance of several FST isoforms (31kDa, 37kDa) increased as the DF from Day 6 became atretic whilst other isoforms (35kDa, 41kDa, 65kDa) showed a corresponding decrease. The interpretation of this intriguing observation remains unknown but, overall, the above findings imply that other factors (like inhibin) may be critical for loss of non-ovulatory DF function.

Expression and secretion of FST by granulosa cells has been shown to be upregulated by FSH and IGF1 (Klein *et al.* 1991, Lindsell *et al.* 1994, Glister *et al.* 2001, Glister *et al.* 2003, Glister *et al.* 2006). However, the relative abundance of different FST isoforms secreted by cultured bovine granulosa cells was found to be very similar under basal, FSH- and IGF1-stimulated conditions (Glister *et al.* 2006) making it difficult to relate the present changes in intrafollicular FST isoform profiles to presumptive changes in FSH and/or IGF action occurring in vivo. Expression of FST by granulosa cells is also upregulated by activin (Michel *et al.* 1992, Fazzini *et al.* 2006) but whether activin affects the FST isoform distribution pattern is not known. Intrafollicular levels of activin-A, activin-AB and 'total' FST followed a similar pattern of change in the present study being lowest in dominant follicles at all cycle stages examined with the exception of day 3. For the most part, the mass ratio of 'total' activins (i.e. activin-A+AB) to FST levels was maintained around 1:1 at all follicle stages examined, consistent with FST-activin complex formation and at least partial neutralization of activin signalling in accordance with the 2:1 binding stochiometry (Shimonaka *et al.* 1991, Welt *et al.* 2002, Chang 2008). This apparent lack of saturation of activin's FST binding capacity implies that the bioavailability of other locally expressed TGFβ ligands capable of binding FST, such as BMP-2, -4, -7 and GDF9, may by modulated by intrafollicular FST. An

important caveat is that levels of activins and FST in bFF may provide little indication of their relative concentrations and interactions at or near the cell surface. The challenge remains to devise experimental approaches to address this issue.

In conclusion we have shown that activin-A and –B as well as naturally occurring isoforms of bovine ovarian FST that differ in their biological activity, binding affinities for activin and cell surface proteoglycans, display follicle status-dependent differences in their intrafollicular abundance during the bovine oestrous cycle. Given the prominent autocrine/paracrine regulatory roles that activins and other TGFβ superfamily members are thought to engage in throughout folliculogenesis, these findings underscore the inherent complexity and multifactorial nature of this system. Further research is needed to delineate the biological significance of multiple FST isoforms as extracellular modulators of signalling by activins and, likely, other TGFβ family members secreted by the oocyte, granulosa and/or theca cells.

Declaration of Interest

The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work

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Table 1. Estimates of the molecular weight and relative purity of the five follistatin isoforms isolated from bovine follicular fluid

Purified Follistatin isoform	Apparent molecular weight (kDa)	Total Yield (based on A_{280}nm) (μg)	Purity (Western blot) (%)	Purity (Coomassie -stained gel) (%)	Purity (Follistatin ELISA) (%)
Bovine #1	31	93	83	92	93
Bovine #2	33	53	75	82	85
Bovine #3	35	55	85	79	79
Bovine #4	37	12	74	88	~100
Bovine #5	41	19	75	82	95

Table 2. Purification table for bovine follistatin isoforms

Fraction	**Total Protein[a] (mg)**	**Follistatin[b] (μg)**	**Purification (-fold)**	**Recovery (%)**
bFF (start material)	13,000	1,164	1	100
Immunoaffinity eluate	3.28	733	2,497	63
Anion exchange				
Non-retained #	nd	127.4	nd	10.95
Peak A	nd	24.3	nd	2.08
Peak B	nd	110.9	nd	9.53
Peak C	nd	25.4	nd	2.18
Peak D	nd	115.9	nd	9.97
Total	**nd**	**403.9**	**nd**	**34.7**
RP-HPLC				
Bovine #1	0.093	86.6	10,400	7.44
Bovine #2	0.053	45.3	9,553	3.89
Bovine #3	0.055	43.4	8,810	3.73
Bovine #4	0.012	12.3	11,475	1.06
Bovine #5	0.019	18.0	10,721	1.55
Total (#1 - #5)	**0.232**	**205.6**	**10,191**	**17.7**

[a] based on absorbance at 280nm; [b]based on follistatin ELISA; nd, not determined

Table 3. Comparison of biological potencies of the five isoforms of follistatin isolated from bovine follicular fluid with that of human recombinant follistatin 288

Follistatin isoform	Molecular weight (kDa)	Mean IC_{50} in bioassay (FST:Activin ratio)	Relative biopotency (%)
hrFST-288	31	1.7	100
Bovine #1	31	1.7	100
Bovine #2	33	2.2	77
Bovine #3	35	4.4	39
Bovine #4	37	1.9	89
Bovine #5	41	3.2	53

Table 4. Comparison of activin-A-binding and heparin sulphate proteoglycan (HSP)-binding properties of five isoforms of follistatin isolated from bovine follicular fluid

Follistatin isoform	Molecular Weight (kDa)	KD (Activin-A) (nM)	KD (HSP) (nM)
hrFST-288	31	1.8 ± 0.61^{ab}	3.93 ± 0.57^{a}
Bovine #1	31	1.6 ± 0.16^{a}	2.94 ± 1.36^{a}
Bovine #2	33	2.9 ± 0.30^{ab}	nd
Bovine #3	35	33.2 ± 13.9^{c}	nd
Bovine #4	37	4.4 ± 1.48^{b}	nd
Bovine #5	41	34.3 ± 4.14^{c}	nd
p-value (ANOVA)		*<0.001*	*0.24*

nd: not determined as binding too low to quantify

Table 5. Comparison of follistatin•-binding properties and biological potencies (bovine theca cell bioassay) of three recombinant human activin isoforms

Activin isoform	KD (FST) (nM)	Bioassay IC_{50} (ng/ml)
Activin-A	0.36 ± 0.02^{a}	7.3 ± 2.7^{a}
Activin-AB	0.15 ± 0.01^{b}	14.7 ± 7.8^{a}
Activin-B	0.14 ± 0.01^{b}	$>50^{b}$ (NR)
p-value (ANOVA)	<0.01	<0.01

•recombinant human FST-288

NR, no detectable response at 50ng/ml

Figure Legends

Fig. 1 SDS-PAGE/western blotting analysis of chromatographic fractions generated during purification of FST isoforms from bovine follicular fluid (bFF). Samples were electrophoresed under non-reducing conditions using 12.5% acrylamide gels. In **A** proteins were transferred to nitrocellulose membrane and immunostained with FST antibody; in **B** the gel was directly stained for total protein using coomassie blue.

Fig. 2 Elution profile (absorbance at 280nm) for the anion exchange FPLC step after applying the highly-enriched fraction from the FST immunoaffinity column.

Fig. 3 SDS/PAGE analysis of four purified bovine FST isoforms before (-) and after (+) enzymatic deglycosylation. Samples were electrophoresed under non-reducing conditions using 12.5% acrylamide gels and total protein was detected by silver-staining. Lanes 1-2 show the behaviour of the control protein (Fetuin) supplied with the deglycosylation kit.

Fig. 4 Panel **A:** Comparison of the bio-potencies of the five purified isoforms of bovine FST and rh FST-288 in reversing the activin-A-induced suppression of androstenedione secretion by bovine theca cells *in vitro*. Panel **B** shows that none of the FST preparations affected 'basal' androstenedione secretion by cells cultured in the absence of activin-A.

Fig. 5 Changes in follicle diameter and E/P ratio, activin-A, activin-AB, combined activin-A/-AB and total FST concentrations in follicular fluid obtained from cattle (n=28) ovariectomized at five time-points during a synchronized estrous cycle. The horizontal bar beneath the x-axis distinguishes the follicular phase (FP) from the luteal phase (LP). Values are means ±SEM and results of 2-way ANOVA are indicated on each panel.

Fig. 6 Changes in the relative abundance (%) of six different M*r* isoforms of FST in follicular fluid obtained from cattle (n=28) ovariectomized at five time-points during a synchronized estrous cycle. The horizontal bar beneath the x-axis distinguishes the follicular phase (FP) from the luteal phase (LP). Values are means ±SEM and results of 2-way ANOVA are indicated on each panel.

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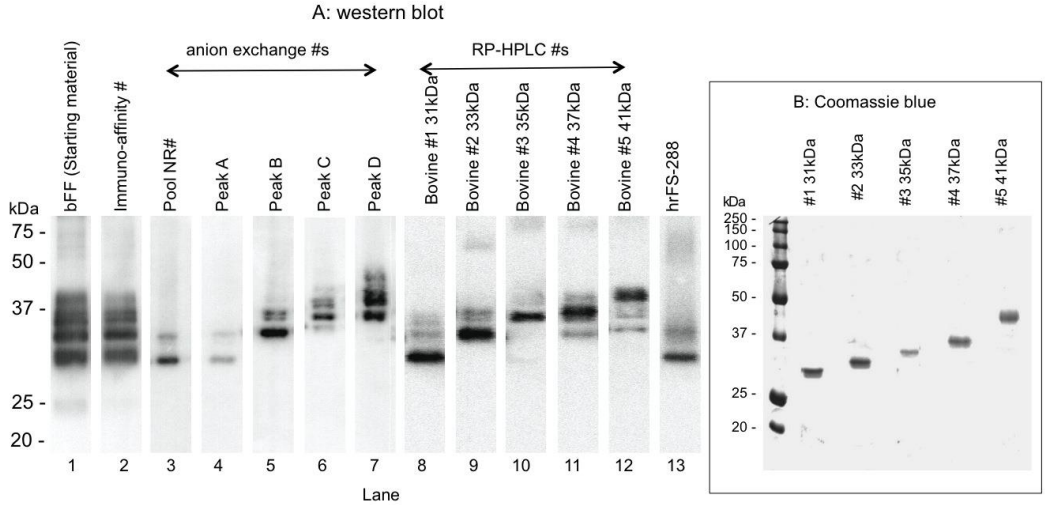
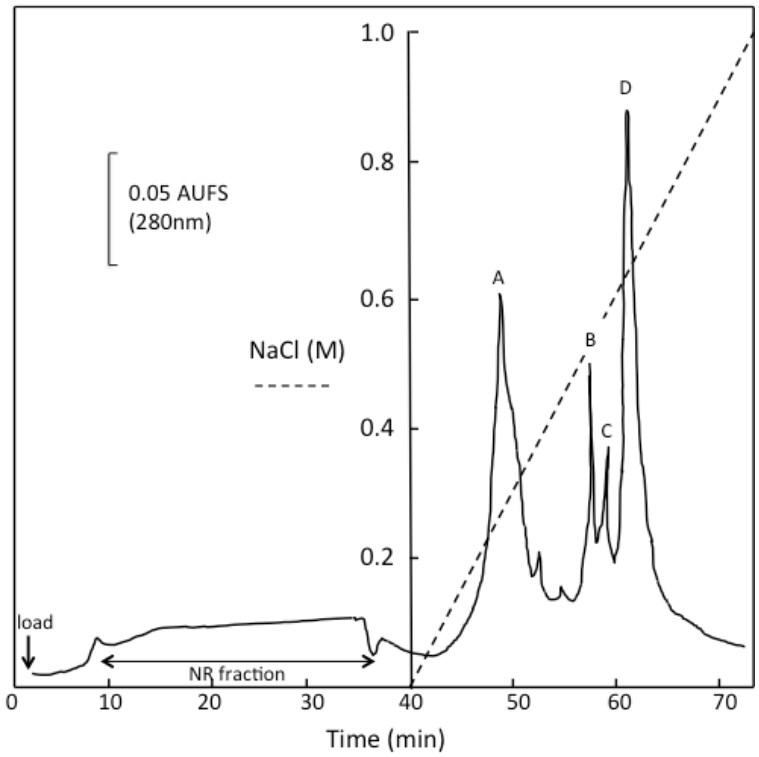
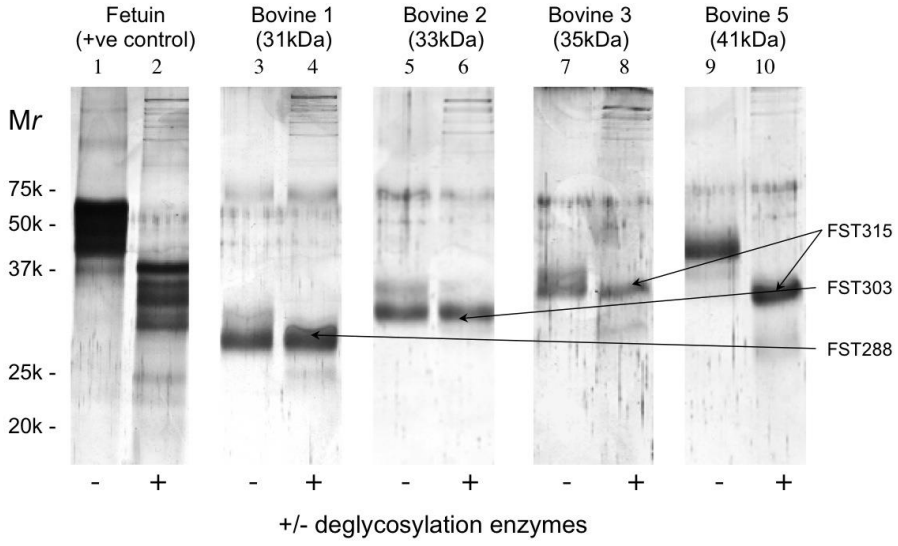
Fig. 1





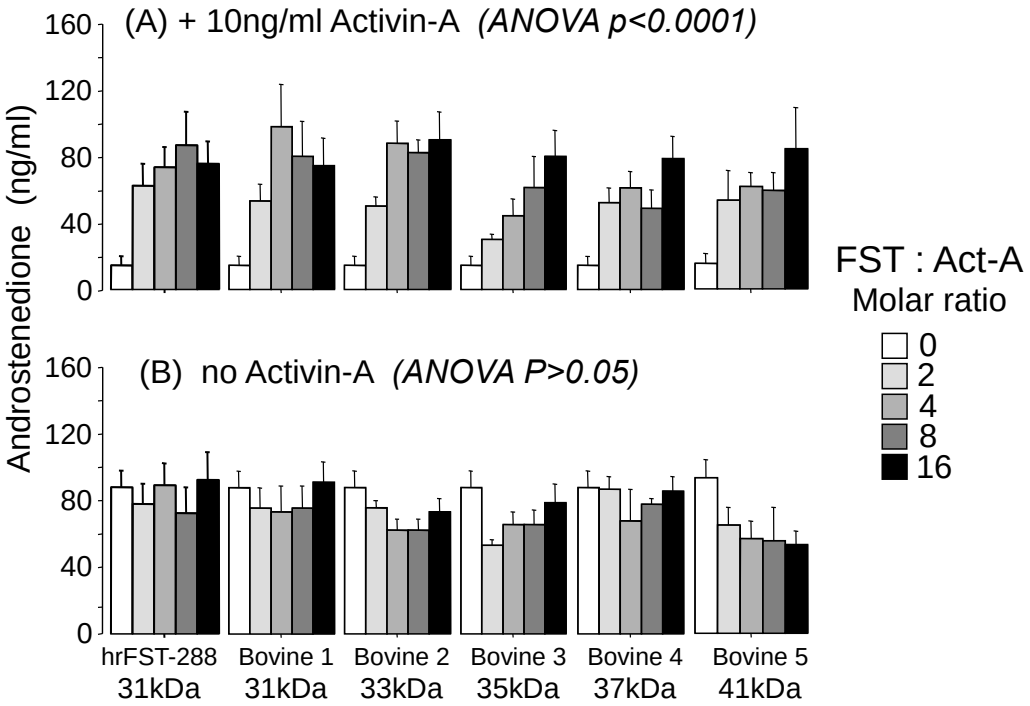
837
838
839

Fig. 3



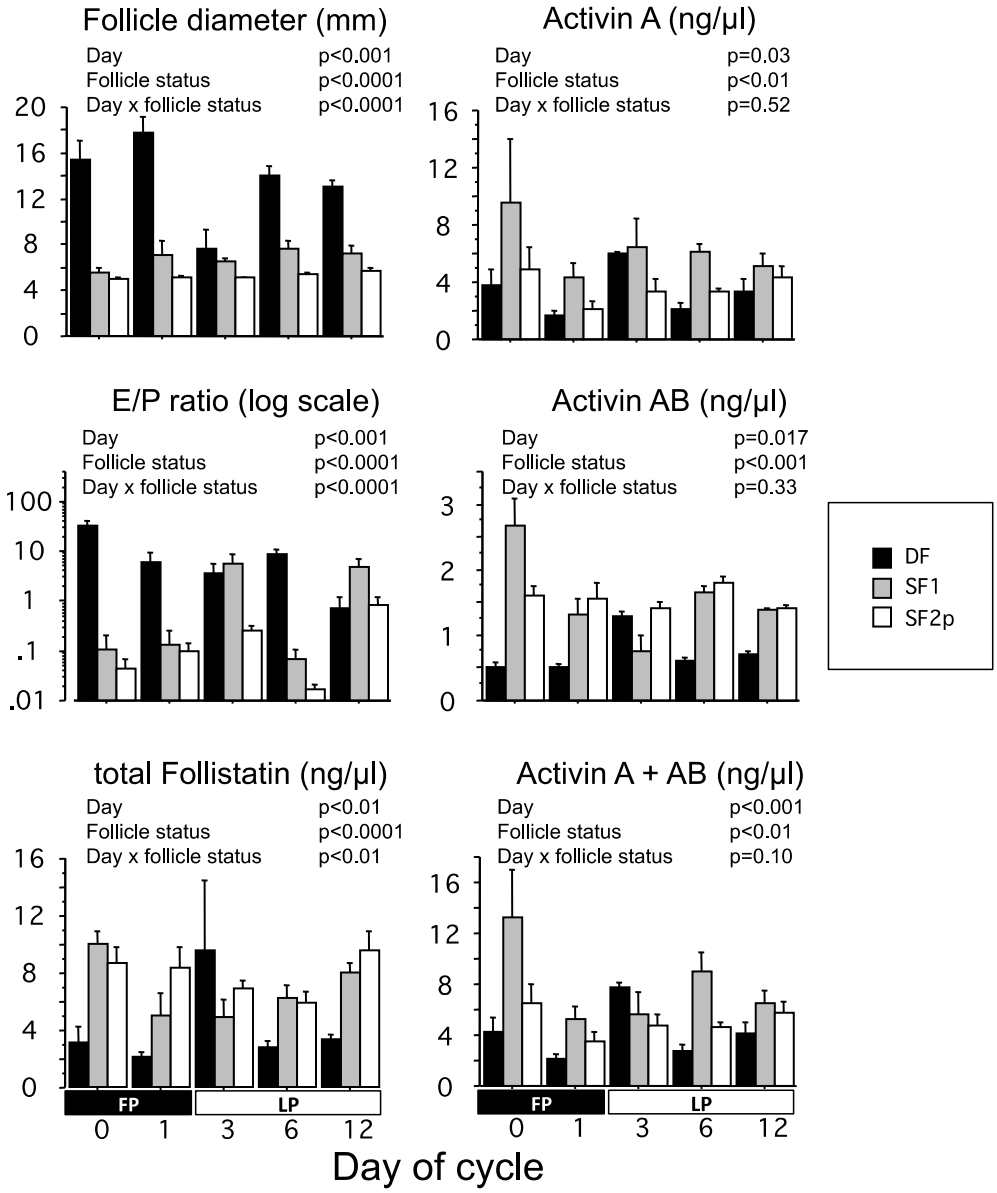
840
841
842

Fig. 4


(A) + 10ng/ml Activin-A (ANOVA p<0.0001)
(B) no Activin-A (ANOVA P>0.05)
Androstenedione (ng/ml)
160
120
80
40
0
FST : Act-A
Molar ratio
0
2
4
8
16
hrFST-288
31kDa
Bovine 1
31kDa
Bovine 2
33kDa
Bovine 3
35kDa
Bovine 4
37kDa
Bovine 5
41kDa

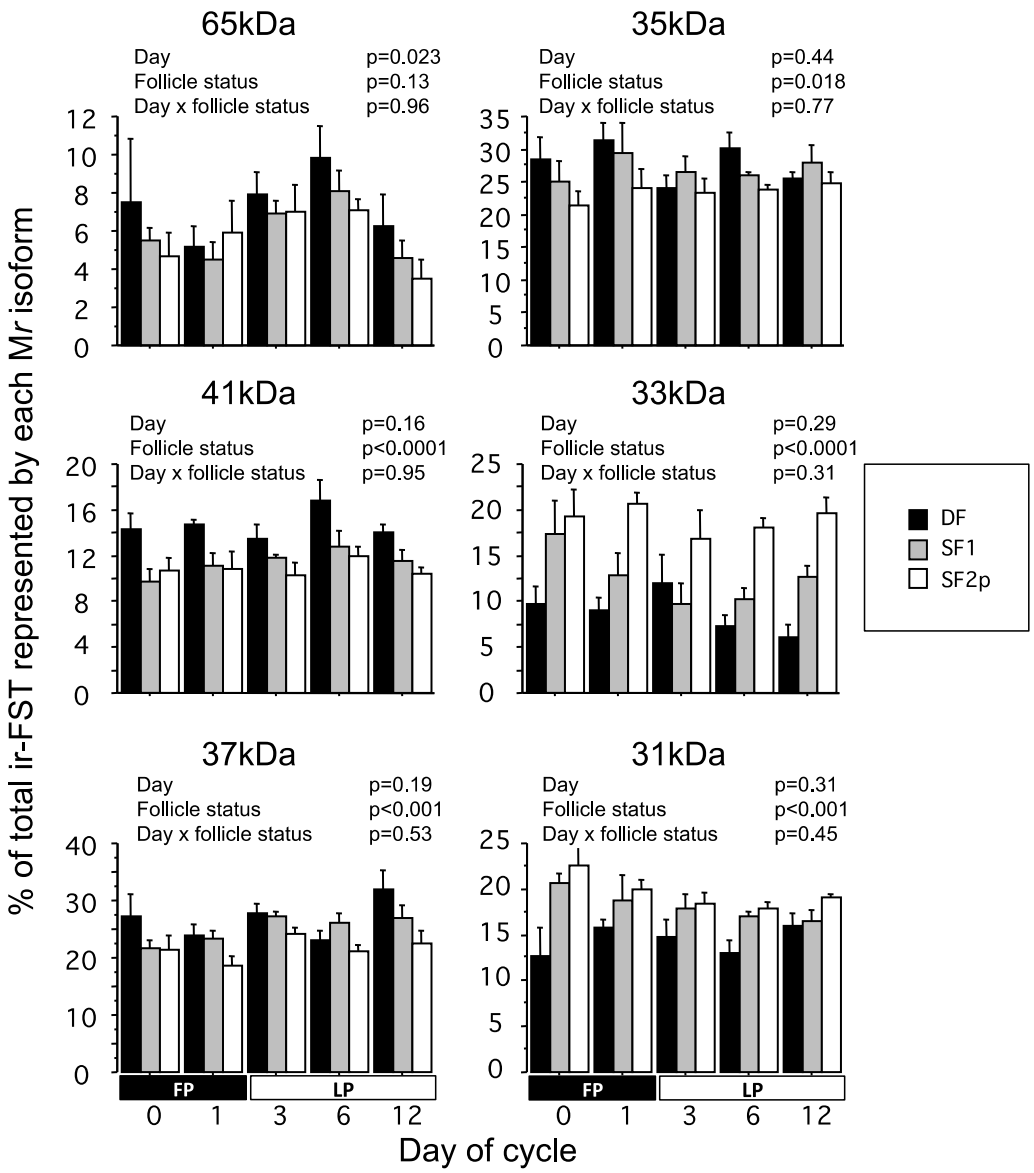
843

Fig. 5


Follicle diameter (mm)
Day p<0.001
Follicle status p<0.0001
Day x follicle status p<0.0001
Activin A (ng/µl)
Day p=0.03
Follicle status p<0.01
Day x follicle status p=0.52
E/P ratio (log scale)
Day p<0.001
Follicle status p<0.0001
Day x follicle status p<0.0001
Activin AB (ng/µl)
Day p=0.017
Follicle status p<0.001
Day x follicle status p=0.33
DF
SF1
SF2p
total Follistatin (ng/µl)
Day p<0.01
Follicle status p<0.0001
Day x follicle status p<0.01
Activin A + AB (ng/µl)
Day p<0.001
Follicle status p<0.01
Day x follicle status p=0.10
FP
LP
0 1 3 6 12
Day of cycle

844
845

Fig. 6


65kDa
Day p=0.023
Follicle status p=0.13
Day x follicle status p=0.96
35kDa
Day p=0.44
Follicle status p=0.018
Day x follicle status p=0.77
41kDa
Day p=0.16
Follicle status p<0.0001
Day x follicle status p=0.95
33kDa
Day p=0.29
Follicle status p<0.0001
Day x follicle status p=0.31
37kDa
Day p=0.19
Follicle status p<0.001
Day x follicle status p=0.53
31kDa
Day p=0.31
Follicle status p<0.001
Day x follicle status p=0.45
DF
SF1
SF2p
FP
LP
0 1 3 6 12
Day of cycle
% of total ir-FST represented by each Mr isoform

846
847