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Activin B is produced early in antral follicular development and suppresses thecal androgen production

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Abstract

Little is known about the role of activin B during folliculogenesis. This study investigated the expression levels of activin/inhibin subunits (βA, βB, and α), steroid enzyme, and gonadotrophin receptors in theca (TC) and granulosa cells (GC) by QPCR and activin A and B and inhibin A protein levels in follicular fluid (FF) of developing sheep follicles during estrus and anestrus. The effect of activin B on androgen production from primary TC cultures in vitro was also assessed. During folliculogenesis, in anestrus and estrus, FF activin B concentrations and thecal and GC activin βB mRNA levels decreased as follicle diameter increased from 1–3 to >6 mm regardless of estrogenic status. Estrogenic preovulatory follicles had reduced concentrations of FF activins B and A, and TC and GCs expressed higher levels of activin βA mRNA at 3–4 mm, and TCs more inhibin α mRNA at >4 mm stages of development compared with nonestrogenic follicles. Activin B decreased androstenedione production from primary TCs in vitro, an effect blocked by inhibin A. Furthermore, the theca of large estrogenic follicles expressed high levels of inhibin α and activin βA mRNA suggesting local thecal derived inhibin A production. This would inhibit the negative effects of thecal activins B and A ensuring maximum androgen production for enhanced estradiol production by the preovulatory follicle(s).

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Introduction

Activin and inhibin are members of the TGFβ superfamily that consists of over 40 ligands including the bone morphogenetic proteins (BMPs), myostatin, and growth and differentiation factors (GDFs; Knight & Glister 2006). Activin and inhibin were originally identified from ovarian follicular fluid (FF) and have since been implicated in many ovarian processes necessary for normal and successful follicle development.

Structurally, activins are disulfide-linked dimers, where activin A and activin B are homodimers of activin βA and βB subunits respectively. Inhibin is composed of an α subunit heterodimerized with either a βA (inhibin A) or a βB (inhibin B) subunit (Knight et al. 2011). Activins signal through the SMAD2/3 pathway by interacting with one of the two activin type II receptors (ACVR2A (actRIIA) or ACVR2B (actRIIB)), which heterodimerize with the type I activin receptor ACVR1B (actRIB). The activin type IB receptor (also known as ACVR1B (ALK4)) is commonly regarded as the receptor that activins use, but activins B and activin AB (βA/βB heterodimer) might also signal through the type IC activin receptors (ACVR1C (ALK7)) (Bernard et al. 2006). The type I receptor initiates a signaling cascade by activating a phosphorylation cascade through SMAD2/3 that complexes with SMAD4 and translocates to the cell nucleus to modulate gene transcription. The effects of activin are antagonized by inhibin, which acts by blocking activin binding to its receptors and by follistatin, which forms complexes with activin extracellularly preventing activin availability (Knight et al. 2011).

Inhibins and activins are made by cells of the developing follicle and accumulate in high concentrations in the antral FF. Previous studies have shown that
granulosa cells (GCs) are the major source of follicular inhibin, which acts in a feedback mechanism to the pituitary to modulate gonadotrophin production (Padmanabhan & McNeilly 2001, McNeilly et al. 2003). Mouse models were used to investigate the importance of activin in development and fertility. Mice lacking the activin βA subunit are neonatal lethal (Matzuk et al. 1995) while activin βB null mice survived to adulthood (Vassalli et al. 1994). Activin B-deficient male mice exhibited normal fertility; however, females displayed profound inability to reproduce and their offspring are perinatal lethal. A GC-specific conditional activin βA knockout mouse was created and was sub-fertile (Pangas et al. 2007), and the same observation was found when mature βA was replaced with mature βB (Brown et al. 2000). These findings suggest that both activins A and B are important for regulating ovarian function and that activin B is necessary for female fertility. However, limited research has been carried out to determine the presence and function of activin B in somatic follicular cells due to a lack of specific assays for measuring activin B. While it was thought that activin B functions simply as an alternative to activin A, at least in thecal cells (TCs) from cattle activin B appears to have no activity while activin A will suppress androgen production (Knight et al. 2011). In sheep while the activin βB subunit mRNA and protein is expressed in both the testis and the ovary, no inhibin B is produced, and inhibin A is the only form produced and secreted by the testes and ovaries (McNeilly et al. 2002). These findings are interesting given that activin B is present during follicle development, and inhibin B functions during the follicular phase in human folliculogenesis but does not appear to be necessary for sheep fertility (McNeilly et al. 2002).

We have now used a new specific ELISA for activin B (Ludlow et al. 2009) to investigate the role of activin B in folliculogenesis. Using sheep, we determined the potential role of activin B in modulating thecal androgen production in vitro and pattern of production of activin B in vivo when follicular development continues in the face of low LH pulse frequencies in anestrus (McNeilly et al. 1982), when LH pulse frequency is highest in the late follicular phase of the normal cycle (Wallace et al. 1988), and after an induced preovulatory LH surge at a time when estradiol (E2) and androgen production is suppressed before ovulation (Baird et al. 1981). In these studies, the concentration of FF E2 was also measured and used to determine follicle dominance to compare levels of gene expression and protein accumulation in follicles in different states of health. Previous studies have shown that in follicles >2.5 mm in diameter, the size beyond which follicles are gonadotrophin dependent in sheep (McNeilly et al. 1986, 1987, McNeilly & Fraser 1987), FF levels of E2 directly reflect the state of health or atresia of follicles (Carson et al. 1981). High levels of E2 reflect healthy preovulatory follicles expressing high levels of LH receptors in both GCs and TCs (Webb & England 1982), and this is correlated with high output of E2 when the individual follicles are incubated in vitro (Webb & England 1982, McNeilly & Fraser 1987, McNeilly et al. 1987). In contrast, low levels of E2 both in FF and in in vitro culture are associated with follicles in varying states of atresia producing high levels of androgens (Carson et al. 1981, Webb & England 1982).

The objectives of this study were to measure the concentrations of activin B in FF and compare with those for activin A, inhibin A, and the estrogenic status of each individual follicle and correlate these measurements with the expression patterns of activin subunits (βA, βB), inhibin α, gonadotrophin receptors, and components of the steroidogenic pathway in TCs and GCs.

Results

Changes in activin B during folliculogenesis

The mean concentrations of activin B, A, and inhibin A, in the FF of follicles collected from sheep during anestrus, and the follicular phase (preovulatory and post-LH surge) are shown in Fig. 1. The concentrations of E2 were measured in the antral fluid of each follicle to allow segregation of follicles based on estrogenic status and are graphed into high and low estrogenic groups reflecting the dominant or subordinate status of each follicle respectively.

The results showed that levels of E2 were significantly lower in the antral fluid of follicles during anestrus compared with follicles during the follicular phase of the estrous cycle (P<0.05) (data not shown). There was also a significant decrease in the concentration of E2 following the LH surge in follicles 1–3, 3–4, and >6 mm in diameter (P<0.001). The greatest concentration of E2 was found in preovulatory follicles >6 mm in size (9449±1700 ng/ml).

Antral activin B concentration decreased as follicle size increased

Measurements showed that activin B concentration decreased (P<0.001) as follicle size increased during the three reproductive phases tested regardless of the estrogenic content of follicles (Fig. 1). At the small antral stage (1–3 mm in diameter), the average activin B concentration was 877±47 ng/ml, from 3–4 mm was 359±18 ng/ml, 173±17 ng/ml from 4–6 mm, and at >6 mm follicular activin B concentrations decreased to 72±6 ng/ml.

Significant differences were observed between the levels of activin B and the phase of the reproductive cycle within each follicle size group assessed. Small follicles (1–3 mm) from ovaries of sheep during anestrus had significantly less antral activin B than follicles during the follicular phase of the estrous cycle (P<0.01).
The concentration of activin B increased after the LH surge by ~30% from those concentrations observed in follicles just before the LH surge (P<0.05). In follicles measuring 3–4 and 4–6 mm in diameter, activin B levels were significantly lower during anestrus (P<0.001), but no significant differences were found between stages of the follicular phase (prior and post-LH surge). No differences in FF activin B concentrations were observed between cycling and anestrus follicles >6 mm in diameter, nor were any differences found preceding and following the preovulatory LH surge in follicles of this size.

When follicles were segregated based on the estrogenic status, the results showed that levels of FF activin B in estrogenic (high E₂) preovulatory follicles (>6 mm) during the follicular phase were lower than non-estrogenic (low E₂) follicles, measuring 71±9 and 106±14 ng/ml respectively (P<0.05; Fig. 1B). The concentration was also reduced after the LH surge in follicles >6 mm in diameter (Fig. 1C). During the anestrus phase, estrogenic follicles 3–4 mm in diameter had lower FF activin B levels than nonestrogenic follicles of the same size (P<0.001; Fig. 1A). No other differences between estrogenic and nonestrogenic follicle activin B concentrations were observed.

**Activin A and inhibin A levels follow a similar pattern**

The levels of activin A and inhibin A were measured from the same follicles used to map activin B concentrations. Data for all phases were averaged within size groups, and results showed that the concentration of activin A increased from 474±34 ng/ml in small antral follicles (1–3 mm) to 822±104 ng/ml in 3–4 mm, 1338±160 ng/ml in large antral follicles (4–6 mm), after which the levels of activin A in the FF decreased to 734±54 ng/ml (P<0.001) as follicles approached preovulatory size (>6 mm).

The concentration of antral inhibin A followed a similar pattern to that of activin A. The average concentration of FF inhibin in follicles, regardless of cyclic stage, increased from 526±37 ng/ml in 1–3 mm follicles to 849±86 ng/ml in 3–4 mm, 827±110 ng/ml in 4–6 mm, and then significantly decreased to 591±40 ng/ml in follicles >6 mm in diameter (P<0.05). Moreover, differences were found between inhibin A concentrations in 1–3 mm follicles during the three phases of the cycle assessed. The concentration of inhibin A was significantly higher (P<0.001) measuring 830±99 ng/ml after the LH surge and only 476±45 and 366±52 ng/ml before the LH surge and during anestrus respectively.

Estrogenic follicles >6 mm in diameter had 602±56 ng/ml and nonestrogenic follicles had 990±130 ng/ml FF activin A (P<0.01), and a reduced level of FF activin A was also found after the LH surge in estrogenic follicles >6 mm in diameter (P<0.05). However, no other differences in activin A concentration were found between estrogenic and nonestrogenic follicles at other stages of development (Fig. 1).

No significant differences in FF inhibin A content were found between the estrogenic and nonestrogenic follicles during the follicular phase of the estrous cycle (Fig. 1B). However, higher levels of FF inhibin were
observed after the LH surge in estrogenic follicles 1–3 mm in diameter (P < 0.05) and during anestrus in estrogenic follicles 1–3 and 3–4 mm in diameter (P < 0.01).

**Activin βB subunit expression during the reproductive cycle**

The follicles collected during anestrus, before and following the LH surge during the follicular phase of the estrous cycle, were divided into estrogenic and nonestrogenic based on the FF E2 concentrations.

The five follicles with the highest and five with the lowest levels of FF E2 within each size group (1–3, 3–4, 4–6, and >6 mm diameter) for each phase of the cycle (anestrus, preovulatory, and after the LH surge) were then selected for gene expression studies. Follicles were collected from n ≥ 3 sheep for each group analyzed. Each follicle was treated separately, and no pooling of mRNA or FF from individual follicles was carried out within this study.

The expression levels of mRNA encoding activin βA, βB, and inhibin α subunits were measured in TC and GC, and estrogenic follicles were found to have differential expression levels at specific stages of development.

During anestrus (Fig. 2), estrogenic follicles 1–3 mm in diameter expressed more mRNA encoding βA, βB, and α in TCs (P < 0.05) and more α in GCs (P < 0.001). Follicles from 3 to 4 mm in diameter produced more βA in TCs and more α and βA in GCs (P < 0.05). Follicles >4 mm in diameter were not found to have any differential levels between estrogenic and nonestrogenic follicles. There was a significant difference between the levels of βA mRNA expressed between theca cells in those follicles from 1 to 3 mm in diameter compared with follicles of 4–6 mm in diameter (P < 0.05).

During the preovulatory phase of estrus (Fig. 3), TC and GCs from 3 to 4 mm estrogenic follicles produced higher levels of βA mRNA than nonestrogenic follicles. TCs from follicles 4–6 and >6 mm in diameter produced higher levels of α subunit (P < 0.05), and TCs from follicles >6 mm also produced more βA mRNA (P < 0.01). No differences were observed in GC expression between estrogenic and nonestrogenic follicles in follicles >4 mm in diameter during the preovulatory phase. Significant differences were found between expression levels of the α subunit in TCs of follicles 1–3 mm compared with follicles >6 mm in diameter (P < 0.05) and between follicles 3–4 and >6 mm (P < 0.05).

After the LH surge (Fig. 4), TCs from estrogenic follicles 1–3 mm in diameter produced higher levels of mRNA encoding βA, βB, and α subunits (P < 0.05), and GCs produced more α mRNA (P < 0.001). TCs from estrogenic follicles 4–6 mm in diameter had reduced levels of βA subunit expression (P < 0.01). Significant differences were found between expression levels of βA subunit in TCs of follicles from size categories: 1–3 mm compared with 4–6 mm (P < 0.01), 3–4 mm compared with 4–6 mm (P < 0.01), and 4–6 mm compared with >6 mm (P < 0.01) follicles. Differences were also observed for βB expression: 1–3 mm compared with 4–6 mm (P < 0.001) and 4–6 mm compared with >6 mm (P < 0.01).

In our study, low levels of inhibin α protein were present in the theca layer with high levels present in GCs of preovulatory estrogenic follicles (Fig. 5C) identified by the expression of aromatase (Fig. 5A). In contrast, expression in the theca layer and GCs was either absent or at a low level in aromatase-negative follicles (Fig. 5C and D). Activin βA subunit was expressed in GCs and in the theca layer of both aromatase-positive and -negative follicles (Fig. 5E and F). Future studies will be required to determine the exact cell types that are expressing both...
TCs from preovulatory follicles (Fig. 7) of 3–4 mm in diameter expressed more mRNA encoding 3βHSD (P<0.05), and follicles >6 mm were found to have more StAR (P<0.01) and CYP17A1 (P<0.05) mRNA. No differences were found between estrogenic and nonestrogenic follicles during the preovulatory phase in levels of mRNA encoding LHR, FSHR, CYP19A1, and 3βHSD. Differences between the expression levels and size groups are displayed on the graphs in Fig. 7 (calculated grouped into sizes regardless of estrogenic status).

After the LH surge (Fig. 8), TCs from estrogenic follicles 4–6 mm in diameter contained more StAR and 3βHSD mRNA (P<0.05) and >6 mm estrogenic follicles had lower levels of mRNA encoding StAR and LHR (P<0.05). GCs from estrogenic follicles 1–3 mm in diameter expressed more CYP19A1 and 4–6 mm expressed less 3βHSD mRNA (P<0.05). Significant differences between expression levels of each follicle size group, regardless of estrogenic status, were calculated and are shown on the graphs in Fig. 8.

**Activin B suppresses androstenedione production**

Sheep TCs cultured in vitro in the presence of 1 ng/ml activin B produced 85% less androstenedione than control cells (Fig. 9). At concentrations of 10 and 100 ng/ml, activin B reduced androstenedione production by 93 and 99% compared with control levels (P<0.001). The effects of activin B were similar to levels observed for activin A-treated cells, which were also significantly suppressed in the presence of activin A (83% less than control levels at 1 ng/ml, P<0.001).

Inhibin A treatment at 1 ng/ml increased androstenedione production by 88% compared with the control level (P<0.001) and was also able to block the suppressive effects of activin B (79% increase) and activin A (70% increase) (P<0.01) at 100 ng/ml. The effects of activin and inhibin treatment did not alter cell viability as determined by neutral red assays (data not shown).

**Discussion**

This study provides novel information on the expression patterns and concentration profile of activin B in antral fluid of individual sheep follicles during folliculogenesis in anestrus and during the follicular phase of the estrous cycle before and after the preovulatory LH surge when follicles are exposed to radically different patterns of pulsatile LH secretion. Historically, activin B was thought to function similarly to activin A, albeit with less potency suggesting perhaps the use of different receptors for activin B (Corrigan et al. 1991, Mathews & Vale 1991, Nakamura et al. 1992). This study shows that the expression patterns of activin

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**Expression levels in components of steroidalogenic pathway**

During anestrus (Fig. 6), TCs from estrogenic follicles 1–3 to 4 mm in size produced less mRNA encoding StAR and more mRNA encoding CYP17A1 (CYP17) and 3βHSD (P<0.05). Estrogenic follicles 3–4 mm in size produced higher levels of CYP19A1 (CYP19) mRNA in GCs during anestrus (P<0.05, and higher expression of FSHR and 3βHSD was found in GCs of estrogenic follicles 3–4 mm in diameter (P<0.05). Significant differences between expression levels of different follicle sizes are shown on the graphs in Fig. 6.
The abundance of mRNA transcripts encoding activin/ inhibin subunits (βA, βB, and α) and steroidogenic pathway components (LHR, FSHR, 3βHSD, StAR, CYP17A1, and CYP19A1) were quantified in GC and/or TCs isolated from follicles ranging in diameter from 1–3 (small antral) to >6 mm (preovulatory). Follicles of these sizes were assessed due to the presence of antral fluid because these follicles contained sufficient GCs and TCs for individual follicle expression analyses and because this size range covers several important developmental stages. During these stages of development, follicles undergo the transition from gonadotrophin independence to becoming dependent (McNeilly et al. 1986), are selected for dominance, acquire LH receptors and necessary steroidogenic pathway components, produce higher levels of androgens and E2, and become dominant (estrogenic) in preparation for ovulation (Saramużżi et al. 1993, 2011, McNatty et al. 2007). The mechanism by which follicle dominance occurs in sheep is not well described (McNeilly et al. 1991, Saramużżi et al. 1993, 2011). Nevertheless, those follicles on a dominant growth track do have significantly higher levels of antral fluid E2 than those destined for atresia (Carson et al. 1981, Webb & England 1982). Therefore, in this study, antral E2 concentrations were measured and follicles segregated into dominant and subordinate follicle populations. While there is insufficient pulsatile LH support for high-level steroid production to occur during anestrus, follicles remain in a continuous cycle of growth and regression without actually reaching the point of ovulation. This study shows that there are differences in antral activin B and inhibin A protein concentrations between small (<4 mm) dominant follicles during anestrus, at a stage of development where follicle growth would normally be transitioning into gonadotrophin dependence during estrus. However, as follicles continue to grow in the gonadotrophin-dependent phase, the differences in activin and inhibin levels between dominant and subordinate follicles are lost. Aromatase expression in GCs from these follicles is also deficient, and no differences were found between estrogenic and non-estrogenic follicles in any genes assessed once follicles reached 4 mm in diameter. These results suggest that induction of aromatase and other steroidogenic enzymes in both TCs and GCs, even in large healthy estrogenic follicles, is dependent on the frequency of LH pulses that is limited in anestrus. This is supported by previous studies that showed that normal follicle steroid production is induced when LH pulses alone are replaced during anestrus (McNeilly et al. 1982, Wallace et al. 1986).

During the follicular phase of the estrous cycle, decreased concentrations of activins B and A were found in antral fluid from large dominant preovulatory follicles in comparison with subordinate size-matched follicles. At the preovulatory stage of
development, androgens are required in larger quantities as precursors for $E_2$ synthesis in neighboring GCs (Young & McNeilly 2010). This study confirms previous reports that activin A reduces androgen production (Hillier et al. 1991) and shows that activin B suppresses androgen production from TCs cultured in vitro, and it is not unexpected that follicles producing higher levels of antral $E_2$ also have lower levels of activins. At this preovulatory stage, inhibin is produced as an important feedback mechanism signaling to the pituitary gland to modulate FSH secretion. A significant increase in inhibin A levels was observed in antral fluid of estrogenic follicles 1–3 mm in diameter after the ovulatory LH surge. In sheep, only inhibin A is produced (McNeilly et al. 2002), and thus, the increased expression of inhibin $\alpha$ in both the TCs and the GCs of larger follicles that was observed would result in de novo synthesis of inhibin $\alpha$ subunit and heterodimerization with activin $\beta A$ to produce inhibin A rather than forming homodimers of $\beta A$. In this instance, inhibin A would function to reduce activin A production and also to increase androgen production by blocking the suppressive effects of residual activins A and B. It is possible that contamination of the TC preparations by GCs was responsible for the apparent expression of inhibin $\alpha$ in the TCs. In previous unpublished studies, we have shown minimal or absent expression of FSHR in the TCs prepared in exactly the same way as in this study, but we did not do this in this study. However, given the large difference in the threshold cycle ($C_T$) values for the QPCRs, in that the levels of inhibin $\alpha$ subunit mRNA were between three- and six-fold higher in the TCs than the GCs for the same follicle, this would suggest that the TC preparations would be contaminated with between three- and six-fold more GCs than TCs. This is not conceivable, but in future studies, $FSHR$ mRNA levels will need to be examined as complete evidence of the minimal or absent contamination of the TC preparations. In our study, low levels of inhibin $\alpha$ protein were present in the thecal layer as well as GCs of preovulatory estrogenic follicles. Future studies will be required to determine the exact cell types that are expressing each
subunit and therefore potentially contributing to the inhibin that controls androgen production.

As follicles developed from small antral to large preovulatory follicles, dynamic switches in activin and inhibin subunit gene expression occurred. Several studies have previously looked at expression of βA, βB, and α subunits in follicles of human ovaries, and although not in complete agreement, the evidence indicates that various degrees of expression for all three subunits were found in GCs and TCs (Yamoto et al. 1992, 1993, Roberts et al. 1993, Jaatinen et al. 1994, Roberts & Barth 1994). In adult monkey ovaries, the βB subunit was observed in small antral follicles before α or βA mRNA was detected (Schwall et al. 1990). Activin βA and βB subunit mRNA was detected in GCs and TCs from small porcine antral follicles (van den Hurk & Van de Pavert 2001). In sheep ovaries, no inhibin α had previously been detected in TCs but was detected in GCs of some healthy antral follicles at all stages of the estrous cycle (Tisdall et al. 1994). In antral follicles expressing βA, the α subunit and follistatin were also always detected; however, the converse was not true. In preantral and atretic sheep follicles, no α or βA was detected, but follistatin was present. Activin has been localized to oocytes and GCs in rodents, pigs, and cows (Hulshof et al. 1997, van den Hurk & Van de Pavert 2001, Zhao et al. 2001) and in human GCs and TCs (Roberts et al. 1993). The effects of activin on follicular development are controversial where some studies have reported a stimulatory effect (Thomas et al. 2003, Silva et al. 2006, Tellier et al. 2008) and others indicated an inhibitory effect (Mizunuma et al. 1999, Knight et al. 2011).

Expression levels of activin receptors, betaglycan, follistatin, intracellular smads, and presence of the many TGFβ signaling antagonists have implications on efficacy of activin signal transduction (Knight et al. 2011, Scaramuzzi et al. 2011). For example, expression of the inhibin receptor betaglycan is higher in TCs than in GCs, and expression increased as follicle growth progressed in antral bovine follicles (Glister et al. 2010). Betaglycan is also in TCs and GCs of human follicles and in rodents (Drummond et al. 2002, Liu et al. 2003). In sheep, betaglycan was expressed in the oocytes of primordial follicles and in GCs and TCs of preantral follicles (McNatty et al. 2007). The presence of betaglycan in these cells indicates that TCs and GCs are target cells of inhibin, as betaglycan functions by mediating functional antagonism of activin signaling (Lewis et al. 2000). In cows, the expression of the type I activin receptor in TCs fell in large estrogenic follicles (Glister et al. 2010), suggesting an additional control mechanism for reducing activin signaling.

The results of this study suggest that synthesis of activin and inhibin subunits occurred at specific developmental time points throughout folliculogenesis, subsequently a corresponding amount of protein accumulated in the antral fluid of growing follicles. Follicles destined for ovulation synthesized greater levels of mRNA encoding activin βA in TCs and GCs and inhibin α in TCs and had lower levels of activins A and B in the FF of >6 mm dominant follicles compared with subordinate follicles. We found that activins A and B suppressed androgen production in TCs, and inhibin blocks this suppressive effect. Therefore, in estrogenic follicles, higher production of activin βA and activin A at 3–4 mm in diameter suggests that androgen synthesis is being kept in check at this stage by activin A production. Once follicles reach gonadotrophin dependence and GCs of the selected preovulatory follicle(s) switch on aromatase (CYP19A1) expression to produce E2, inhibin α mRNA is synthesized producing inhibin A that functions to block the suppressive effects of activin on androgen production. The result would increase androgen synthesis,
which is required as a precursor for E<sub>2</sub> synthesis in neighboring GCs. However, it has previously been assumed that the increase in inhibin production was entirely produced by the GCs of the estrogenic follicle. In our study, it is clear that the TCs produce inhibin and thus a local TC-derived inhibin system is activated to enhance androgen production by blocking local activins A and B inhibition.

After ovulation, the secretion of E<sub>2</sub> and thecal androgens decreases dramatically (Baird et al. 1981). In this, the expression of inhibin α and activin BA subunits in estrogenic follicles is also dramatically reduced to be equivalent to the level of nonestrogenic follicles shortly after the LH surge signaling ovulation. The maintained levels of α inhibin and activin BA subunits producing inhibin A and the maintained levels of inhibin A in the FF of these follicles >3 mm diameter after the preovulatory LH surge is in accordance with the maintained secretion of inhibin by the ovary after the preovulatory surge in sheep (McNeilly et al. 1989, Campbell et al. 1990).

No two follicles are in the same microenvironment of growth factors, contain the same numbers of GCs and TCs, or have the same blood supply at any one time (McNatty et al. 2007). These results show that there are differences in the levels of activin and inhibin subunit expression in TCs and GCs of follicles that are destined for ovulation in comparison to those that regress. In this study, we have assessed the expression levels of steroidogenic enzymes and pathway components in TCs and GCs. In general, all follicles 1–3 mm in diameter expressed mRNA encoding StAR, CYP17A1, and 3βHSD in TCs and CYP19A1 in GCs. As follicles progressed along the developmental pathway, CYP17A1 expression levels increased dramatically in TCs of 3–4 mm follicles. This change signifies the point at which androgen production begins and CYP17A1 is a rate-limiting enzyme required for this process (Young & McNeilly 2010). As expected, follicles during anestrus displayed reduced expression patterns of steroidogenic pathway components in comparison with the preovulatory follicular phase primarily due to the reduced LH pulse frequency in anestrus as normal steroid secretion can be induced by replacement of LH pulses alone (McNeilly et al. 1982, Wallace et al. 1986, McLeod & McNeilly 1987).

Before the LH surge, TCs of estrogenic follicles >6 mm in diameter produce more mRNA encoding CYP17A1 and StAR, and these levels dropped dramatically following the LH surge. This may be due to downregulation of LH receptors, and we have shown in this study that the LH surge also caused a reduced level of LHR expression in TCs of estrogenic follicles >6 mm in diameter compared with subordinate follicles. In GCs of the same follicles, levels of CYP19A1 expression were high before the LH surge but then radically reduced following the LH surge. The fact that estrogenic follicles require greater amounts of androgen production in order to produce more E<sub>2</sub> supports this data, which showed higher expression levels of steroidogenic pathway components in both TCs and GCs before the LH surge. The reduction in steroid enzyme levels in both the TCs and GCs explains the dramatic reduction in secretion of E<sub>2</sub> and androgens after the LH surge.

In summary, this is the first study showing that FF activin B protein levels are highest in small antral follicles and decrease as follicle size increased. Dominant preovulatory follicles had reduced amounts of FF activin B protein compared with subordinate follicles of the same size. In addition, the data showed the expression patterns of activin βB subunit in TCs and GCs of antral follicles varied according to the estrogenic status of the follicle as well as the size and stage of the estrous cycle and anestrus. Furthermore, this study suggests, for the first time, that in sheep follicles, the
TCs are able to synthesize all subunits required to produce activin B, A, and inhibin A in a controlled manner to autoregulate androgen production at the local TC level regardless of inhibin production by the GCs during follicular development.

Materials and Methods

Follicle collection

Ovaries were obtained from Scottish Blackface ewes in accordance with the UK Home Office Guideline and Project Licence approved by the local University of Edinburgh Ethical Review Process (ERP) and the Animals (Scientific Procedure) Act 1986. The ovaries from ten sheep were collected during the anestrus phase (July), and the estrous cycles in a further 20 sheep were synchronized by withdrawal of progestogen-impregnated sponges (60 mg medroxyprogesterone acetate per sponge; Intervet Laboratories Ltd, Cambridge, UK) in November. Luteolysis was induced on day 10 of the subsequent cycle using a synthetic PGF2α analog (PG) (100 μg i.m. Estrumate; Schering-Plough Animal Health, Welwyn Garden City, UK). Ovaries were collected 36 h after PG in the late follicular phase from ten sheep just before the LH surge and ten sheep 20 h after a preovulatory LH surge induced by the i.v. injection of GNRH agonist (500 μg Buserelin; Sanofi-Aventis, Guilford, UK; Crawford et al. 2006) just before ovulation. Follicles containing an antral cavity were dissected from the ovary and measured and then hemisected in individual wells. The GCs scraped and then rehydrated in decreasing concentrations of alcohol (100, 90, 75%). After a 5 min wash in water and another in 0.05 M lysis buffer (RNeasy Micro kit, Qiagen) and stored at K(−80°C). The FF/PBS solution washed out from the theca shell into 1 ml PBS and then centrifuged to separate the GCs from the FF. The FF/PBS solution from each individual follicle was stored at K(−80°C) for gene expression analyses.

Immunohistochemistry for inhibin α and βA subunit

Sections from archived ovaries from two sheep collected in a previous study in the late follicular phase (at the same time as in this study, 36 h after PG) (Gubbay et al. 2006) were used to determine whether inhibin α and activin βA subunits were present in the thecal layer of follicles. Follicles were classified as either estrogen positive and thus preovulatory or estrogen negative, on the basis of the presence or absence of P450 aromatase determined by immunohistochemistry.

Sections (5 μm thickness) were dewaxed in xylene and rehydrated in decreasing concentrations of alcohol (100, 90, and 75%). After a 5 min wash in water and another in 0.05 M...
TBS, sections were blocked in 3% H2O2 in methanol for 30 min to inhibit endogenous peroxidase activity. Following another wash in water (5 min) and TBS (2×5 min), combined avidin–biotin block was performed according to the manufacturer’s instructions (Vector Laboratories, Peterborough, UK). Nonspecific background was blocked for 30 min at room temperature with 10% normal goat serum (Diagnostics Scotland, Carluke, UK) diluted 1:5 in TBS containing 5% BSA. Slides were incubated with primary antibodies diluted in normal blocking serum at 4°C overnight in a humidity chamber. Aromatase was detected using a primary antibody raised in mouse at 1:50 dilution and activin (Ludlow et al. 2009)). Negative controls were performed by replacing the primary antibody with normal blocking serum at 4°C overnight in a humidity chamber. Aromatase was detected using a primary antibody raised in mouse at 1:50 dilution and activin βA by the E4 mouse antibody also diluted to 1:50. Inhibin α primary antibody (PPG/14/6) was biotinylated and used at 1:100 dilution (source of antibodies (Ludlow et al. 2009)). Negative controls were performed by replacing the primary antibody with normal blocking serum. After washing in TBS (2×5 min) to remove excess primary antibody slides were incubated with a biotinylated secondary antibody diluted 1:500 in normal serum for 30 min. Inhibin α slides did not require a biotinylated secondary antibody as the primary antibody was biotinylated. After another wash cycle, all slides were incubated in streptavidin HRP (Vector Laboratories) diluted 1:1000 in TBS. Secondary antibody as the primary antibody was biotinylated. After another wash cycle, all slides were incubated in streptavidin HRP (Vector Laboratories) diluted 1:1000 in TBS for 30 min. Bound antibody was visualized using 3,3′-diaminobenzidine tetrahydrochloride (Dako, Ely, Cambridge-shire, UK). Sections were counterstained with hematoxylin, dehydrated, mounted, and visualized by light microscopy.

Primary TC culture

Ovaries were obtained from sheep during the estrous cycle or in anestrous throughout the year and transferred to the laboratory in Medium199 containing 20 mmol/l HEPES, 100 kIU/l penicillin, 0.1 mg/ml streptomycin, and 1 mg/l amphotericin (Fungizone) (all supplied by Sigma–Aldrich). Small follicles (1–3.5 mm in diameter) were dissected from ovaries in Dulbecco’s PBS without calcium or magnesium, with particular attention given to removal of all extraneous stromal tissues surrounding the thecal layers. Follicles were then hemisected and washed vigorously using a 1 ml syringe, flushing repeatedly, to separate GCs from the theca shells as described previously (Campbell et al. 1998). The thecal shells were then dispersed in an enzyme mixture containing 10 ml PBS, 5 g/l collagenase, 1 g/l hyaluronidase, 1 g/l protease, and 0.001% donor calf serum (vol/vol), for ~ 10 min at 37°C with gentle agitation. The reaction was stopped by addition of 2 ml FCS, and cells were then washed by centrifugation at 800 g for 5 min and resuspended in culture media (DMEM-F12 with 100 kIU/l penicillin, 0.1 mg/ml streptomycin, 3 mmol/l l-glutamine, 0.1% BSA (w/vol), 2.5 mg/l transferrin, 4 mg/l sodium, 10 ng/ml bovine insulin, 0.1 ng/ml ovine LH (code# AF8 8614-BNHP-NIDDK supplied by Dr A Parlow, NHPP, Harbor-UCLA, Torrance, CA, USA) and 10 ng/ml LR3 IGF1). The cell pellets were then resuspended in culture media and after a further wash, the number and viability of cells was estimated using Trypan Blue exclusion. Cell viability was routinely more than 95%.

Cells were plated in 96-well plates at 75 000 cells in a total of 200 μl media per well. Actinin A (code# 338-AC) and activin B (code# 659-AB) were obtained from R&D Systems (Abingdon, Oxon, UK) and inhibin A from NIBSC (code # 91/624, Hertfroshire, UK). Cells were cultured under standard culture conditions consisting of a humidified atmosphere with 5% CO2 at 37°C. For hormone analysis, cells were cultured for up to 6 days and media changed every 48 h and stored at −20°C for analysis at a later stage. At the end of the culture period, the cell viability was determined by neutral red dye uptake, as described elsewhere (Campbell et al. 1998).

Immuoassays

RIA

Concentrations of androstenedione were determined from nonextracted cell culture media using previously described RIA methods (Campbell et al. 1998). The sensitivity of the androstenedione assay was ~ 5 pg/ml and inter- and intra-assay variation was < 15%. Each experiment was repeated at least three times with a separate pool of cells taken from different animals (n ≥ 4 animals in each pool of cells). The significance of treatment effects was determined using Student’s t-test. The data shown is representative of replicated experiments and is presented as a percentage of the control androstenedione production with significance levels indicated (**P<0.01 and ***P<0.001).

ELISA

FF concentrations of E2, actinin A, activin B, and inhibin A were measured using ELISA. Activin B concentrations were measured using the activin B ELISA that incorporates the use

Table 1 Primer sequences used for QPCR assays and amplicon sizes.

<table>
<thead>
<tr>
<th>Gene (accession)</th>
<th>Forward primer (5′−3′)</th>
<th>Reverse primer (5′−3′)</th>
<th>Insert size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH (NM_001034034)</td>
<td>GCGCTGAAACACGCGAAGTATAA</td>
<td>AAGCAGGGATGTATCTCGG</td>
<td>229</td>
</tr>
<tr>
<td>S100A (NM_001098243)</td>
<td>GCATCCTCAAGACGACGAG</td>
<td>CTTGACACTGGGTTCCACT</td>
<td>194</td>
</tr>
<tr>
<td>CYP17A1 (NM_001009483)</td>
<td>AGCATATCTGCTGGATCAG</td>
<td>GCACGTGTTGACTCTCTCT</td>
<td>215</td>
</tr>
<tr>
<td>38HSD (NM_001135392)</td>
<td>GGAGACATTCTGGATGAGCAG</td>
<td>TCTATGGTGCTGGTGTGGA</td>
<td>200</td>
</tr>
<tr>
<td>CYP19A1 (NM_001123300)</td>
<td>AATCAGGCTCTGGAAAGC</td>
<td>ACGTCCACATGCGCAAGTC</td>
<td>152</td>
</tr>
<tr>
<td>Activin BA (NM_174363.1)</td>
<td>GGAGAAGCCCGATGGCCACCCAG</td>
<td>TGTGCGTGTCTTACCCGAGCTTGCC</td>
<td>113</td>
</tr>
<tr>
<td>Activin BB (NM_176852.1)</td>
<td>ATGGCCTGCTGGCTCTTC</td>
<td>CCTTGAACCTAGGCAAGCCTTGG</td>
<td>101</td>
</tr>
<tr>
<td>Inhibin a (NM_174094.3)</td>
<td>GAGGCCCGAGGACAGATGCTTC</td>
<td>CCTCCGCTCTCCAGCATGTCGG</td>
<td>91</td>
</tr>
<tr>
<td>LH (NM_211449)</td>
<td>GCCAGTGACAGTACGTGGT</td>
<td>CTATCAGGATGGTTCCAT</td>
<td>199</td>
</tr>
</tbody>
</table>

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of MAB 46A/F as both the capture and the detection antibody (Ludlow et al. 2009). This assay also uses a SDS and heat pretreatment step to remove any binding proteins that may interfere. The FF of each individual follicle was diluted into PBS when extracted from follicles, and this diluted sample was added directly to the assay. The assay had a lower detection limit of 19 pg/ml.

Inhibin A concentrations in FF were measured using a plate modification of a standard in-house inhibin A ELISA as described previously with a sensitivity of 2 pg/ml and an intra- and interplate coefficients of variation <10% (McNeilly et al. 2002). Activin A concentrations were measured using a two-site ELISA kit that measured total activin A (Knight et al. 1996; Oxford Bio-Innovation, Oxfordshire, UK), following the manufacturer's instructions. This assay had a sensitivity <78 pg/ml and inter- and intraplate coefficients of variation <10%.

An in-house ELISA was used to measure E2 in ovine FF. Briefly, 96 microwell plates were precoated with donkey antisseep IgG (1:200; 713-005-147; Jackson ImmunoResearch, Suffolk, UK). Plates were sealed and stored overnight at 4°C in a humidity chamber. On day 2, coating buffer was discarded and the plate washed thoroughly with wash buffer (deionized water +0.1% Tween-20). E2 antibody (In-house ASMR32; 1:300,000, diluted in PGBS) was added to each well and incubated for 3 h at room temperature. The liquid was discarded and the plate washed repeatedly. The dilution of FF ranged from 1:2 to 1:100. The appropriate controls discarded and the plate washed repeatedly. The dilution was calculated by accounting for the initial dilution of the FF contained in each follicle was calculated.

The GCs and TCs from all follicles isolated were sorted according to the content of E2 measured from the FF collected. The five follicles with the highest and five with the lowest levels of E2 within each size group (1–3, 3–4, 4–6, and >6 mm diameter) for each phase of the cycle (anestrus, before LH surge, and after LH surge) were then selected for gene expression studies. Each follicle was treated separately, and no pooling of RNA or FF from individual follicles was carried out within this study.

The GCs from each individual follicle were lysed using passive lysis buffer (PLB; Qiagen RNeasy Micro RNA extraction kit), and each individual theca shell was transferred from RNAlater into PLB and lysed in a tissue lyser (Qiagen). RNA was extracted using the RNeasy Micro RNA extraction Kit, and RNA concentration and purity (A260/A280 ratio) were measured using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Loughborough, Leicestershire, UK). RNA was stored at −80°C until cDNA was synthesized from 200 ng total RNA per reaction using SUPerscript VILO cDNA synthesis kit (Invitrogen) in a 20 μl reaction.

Primer sets for expression analysis were designed to amplify short regions of the target genes crossing an intron/exon boundary and are described in Table 1. The primers were prevalidated using conventional PCR and the product was sequenced to confirm authenticity. Quantitative RT-PCR was carried out using PowerSYBR Green (Applied Biosystems, Paisley, Renfrewshire, UK), whereby primer efficiency was determined by generating standard curves. A 10 μl final reaction volume was prepared using 1 μl synthesized cDNA, 2 μl PowerSYBR Green PCR Master Mix, 5 μM primer pairs, and nuclease-free water. The qRT-PCR cycling program consisted of a denaturing step (95°C for 10 min), annealing, and extension step (95°C for 15 s, 60°C for 1 min) repeated 40 times, and a dissociation step (95°C, 60°C, and 95°C for 15 s each), using a real-time thermal cycler from Applied Biosystems (ABI-7500). Each sample was measured in duplicate and negative controls included a reaction using cDNA prepared leaving out reverse transcriptase and a reaction substituting cDNA with nuclease-free water.

The relative expression level of each target gene was normalized to that of the housekeeping gene GAPDH for each individual sample/follicle and was quantified using the ΔΔCt method.

### Statistical analysis

Data are presented as average ± S.E.M. and the statistical analysis was performed using GraphPad Prism version 4.0 (GraphPad Software, Inc., San Diego, CA, USA) using an unpaired Student's t-test to compare the means of two groups or ANOVA for more than two groups. P values <0.05 were regarded as significant, and levels of significance are indicated on graphs for each gene analyzed (*P<0.05, **P<0.01, and ***P<0.001).

### Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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