# Inhibin removes the inhibitory effects of activin on steroid enzyme expression and androgen production by normal ovarian thecal cells

**J M Young** and **A S McNeilly**

MRC Human Reproductive Sciences Unit, The Queen's Medical Research Institute, 47 Little France Crescent, Edinburgh EH16 4TJ, UK

(Correspondence should be addressed to J M Young, who is now at Monash Institute of Medical Research, Centre for Reproduction and Development, 27–31 Wright Street, PO Box 5418, Clayton, VIC 3168, Australia; Email: julia.m.young@monash.edu)

## Abstract

Activin and inhibin are important local modulators of theca cell steroidogenesis in the ovary. Using a serum-free primary theca cell culture system, this study investigated the effects of inhibin on theca cell androgen production and expression of steroidogenic enzymes. Androstenedione secretion from theca cells cultured in media containing activin, inhibin and follistatin was assessed by RIA over 144 h. Activin (1–100 ng/ml) suppressed androstenedione production. Inhibin (1–100 ng/ml) blocked the suppressive effects of added activin, but increased androstenedione production when added alone, suggesting it was blocking endogenous activin produced by theca cells. Addition of SB-431542 (activin receptor inhibitor) and follistatin (500 ng/ml) increased androstenedione production, supporting this concept. Infection of theca cells with adenoviruses expressing inhibitory Smad6 or 7 increased androstenedione secretion, confirming that the suppressive effects of activin required activation of the Smad2/3 pathway. Activin decreased the expression levels of steroidogenic acute regulatory protein (STAR), whereas STAR expression was increased by inhibin and SB-431542, alone and in combination. *CYP11A* was unaffected. The expression of *CYP17* encoding 17$\alpha$-hydroxylase was unaffected by activin but increased by inhibin and SB-431542, and when added in combination the effect was further enhanced. The expression of 3$\beta$-hydroxysteroid dehydrogenase (*3$\beta$-HSD*) was significantly decreased by activin, while inhibin alone and in combination with SB-431542 both potently increased the expression of *3$\beta$-HSD*. In conclusion, activin suppressed theca cell androstenedione production by decreasing the expression of *STAR* and *3$\beta$-HSD*. Inhibin and other blockers of activin action reversed this effect, supporting the concept that endogenous thecal activin modulates androgen production in theca cells.

*Journal of Molecular Endocrinology* (2012) **48,** 49–60

## Introduction

Female fertility relies on a tightly controlled balance of hormonal signals and cellular interactions that result in the successful production of a mature oocyte for fertilization. Of these hormones, estradiol is a vital component required for oocyte maturation (Tesarik & Mendoza 1995). It is synthesized in granulosa cells from androgen precursors produced by theca cells that surround developing antral follicles, and is produced in increasing quantities by the growing follicle as development reaches the preovulatory stage (Young & McNeilly 2010). Before the expression of aromatase in granulosa cells, follicles are exposed to varying levels of androgens derived from thecal cells largely under the control of LH.

*In vitro*, testosterone inhibits meiotic maturation of the oocyte and subsequent embryo development (Laufer *et al.* 1984, Anderiesz & Trounson 1995). This negative effect of androgens would potentially be neutralized *in vivo* by expression of aromatase in the cumulus granulosa cells surrounding the oocyte in the preovulatory follicle, thus reducing exposure to these potentially damaging effects of excess testosterone and androgens.

In rodents, androgens not only can induce follicular atresia (Hillier & Ross 1979, Daniel & Armstrong 1986, Billig *et al.* 1993) but also have been shown to promote follicular development (Murray *et al.* 1998) and upregulate FSH-receptor expression in granulosa cells in culture (Tetsuka & Hillier 1997). In primates, intermittent exposure to androgens in normal female monkeys resulted in accelerated early stages of ovarian follicle development with reduced granulosa cell atresia and increased thecal cell mass, and enhanced granulosa cell FSH-receptor expression (Vendola *et al.* 1998, Weil *et al.* 1999). In contrast, chronic exposure led to intermittent or absent menstrual cycles (Billiar *et al.* 1985, Faiman *et al.* 1988). Thus, excess androgen production at an inappropriate time could directly affect the rates of follicle activation and lead to premature loss of primordial follicles.

In polycystic ovary syndrome (PCOS), follicle development is stalled, and a major characteristic is


DOI: 10.1530/JME-11-0134
0952–5041/12/048–049 
Online version via http://www.endocrinology-journals.org

the presence of excess androgens that are produced by an intrinsic ability of thecal cells to produce increased levels of androgens (Gilling-Smith *et al.* 1994, 1997, Nelson *et al.* 1999, 2001, Nelson-Degrave *et al.* 2005). It has been suggested that androgen-induced expression of insulin-like growth factor 1 (IGF1) and its receptor in early growing follicles may predispose thecal cells to produce more androgens (Vendola *et al.* 1999), while MEK/ERK phosphorylation has been shown to be decreased in thecal cells from patients with PCOS compared with normal subjects (Nelson-Degrave *et al.* 2005). This excess androgen itself has been implicated in the failure of follicles to continue to grow in PCOS.

In spite of these studies, the modulation of androgen secretion by thecal cells from small antral stages as follicles develop into a preovulatory follicle during normal reproductive cycles is still unclear (Young & McNeilly 2010). Previous studies have shown that both BMPs (Glister *et al.* 2005, Campbell *et al.* 2006) and activin (Hillier *et al.* 1991*a*) can reduce androgen secretion from primary cultures *in vitro*. Furthermore, it was shown that inhibin alone could 'stimulate' androgen production and also neutralize the effects of added activin by human theca cells (Hillier *et al.* 1991*b*). However, there is no evidence that inhibin has a receptor coupled to any second messenger signaling pathway, but instead binds to betaglycan and the type 2 activin receptor to block the effects of activins (Lewis *et al.* 2000). Given that this is the case, the real effects of inhibin would then be to block the intrinsic inhibitory effects of activins that presumably are being produced by the thecal cells themselves. In this study, we have sought to clarify the role of inhibin on thecal cell androgen production by normal thecal cells maintained in primary cultures in conditions that prevent luteinization, thus mimicking the situation during normal follicle development. Using adenoviral vectors, follistatin and a specific chemical activin receptor blocker (SB-431542), we have shown that inhibin acts only as an activin inhibitor, rather than 'stimulating' androgen production. Instead inhibin removes the activin-induced brake on androgen production.

## Materials and methods

### Primary theca cell culture

Ovaries were obtained from sheep throughout the year and transferred to the laboratory in Medium 199 containing 20 mmol/l HEPES, 100 kIU/l penicillin, 0·1 ng/ml streptomycin and 1 mg/l amphotericin (Fungizone; all supplied by Sigma–Aldrich). Small follicles (<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 granulosa cells from the thecal cells as described previously (Campbell *et al.* 1996). The thecal cells 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 µg/l streptomycin, 3 mmol/l L-glutamine, 0·1% BSA (w/vol), 2·5 mg/l transferrin, 4 µg/l selenium, 10 ng/ml bovine insulin and 10 ng/ml LR3 IGF1). Ovine LH (code #AFP 8614B-NHPP-NIDDK supplied by Dr A Parlow, NHPP, Harbor-UCLA, Torrance, CA, USA) was also added to all culture media at 0·1 ng/ml ovine LH, unless otherwise stated. The cell pellets were then resuspended in culture media, and after a further wash, the number and viability of the cells were 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/well. Various concentrations and combinations of ligand and chemical treatments were added to the media in quadruplicate, and the exact details are given in the 'Results' section. Activin A (code #338-AC) and follistatin (code #669FO/CF) were obtained from R & D Systems (Abingdon, Oxon, UK), inhibin A was from NIBSC (code #91/624, Hertfordshire, UK) and SB-431542 was supplied by Sigma–Aldrich. Cells were cultured under standard culture conditions consisting of a humidified atmosphere with 5% $CO_2$ at 37 °C. For hormone analysis, cells were cultured for up to 6 days and the 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.* 1996).

### Adenovirus transfection

Recombinant adenoviruses were constructed from pcDNA3–Smad6, and pcDNA3–Smad7 expression plasmids obtained from Dr P Ten Dijke (The Netherlands Cancer Institute, Amsterdam, The Netherlands), as described previously (Fujii *et al.* 1999, Nakao *et al.* 1999), and were a generous gift from Dr Aristidis Moustakas (Ludwig Institute for Cancer Research, Uppsala, Sweden). Thecal cells were isolated and dissociated enzymatically as previously described and were plated in culture media under standard culture conditions at 75 000 cells/well and incubated overnight to allow cell attachment. The following day, all media were removed, infection of recombinant

adenoviruses was performed at concentrations of 10, 50 and 100 plaque-forming units (pfu)/cell overnight, and then the following day, the cells were resuspended in 200 μl culture media ± appropriate ligands/treatments. The culture media were then changed every 48 h up to 144 h, and stored at −20 °C for RIA.

### Steroid assays

Concentrations of androstenedione were determined from non-extracted cell culture media by a previously described RIA method (Campbell *et al.* 1998). The sensitivity of the androstenedione assay was ∼5 pg/ml, and the inter- and intra-assay variation was <15%.

### Quantitative RT-PCR

Thecal cell cultures for RNA collection were established as described above, but were plated at ∼750 000 cells/well in six-well plates and left to attach overnight. Treatments were added in fresh media the following day and left to incubate under standard culture conditions. After 24 h, another dose of ligand was added to the same media, and then again at 48 h. After the final dose was added at the 48 h time point, cells were incubated for a further 1 h, then media removed and stored at −20 °C for RIA, and cells lysed and RNA extracted using the Qiagen RNeasy Micro RNA extraction kit. RNA concentration and purity ($A_{260}/A_{280}$ ratio) were measured using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific). 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 listed in Table 1. The primers were pre-validated using conventional PCR and the product was sequenced to confirm authenticity. Quantitative RT-PCR (qRT-PCR) was carried out using Power SYBR Green (Applied Biosystems), whereby the primer efficiency was determined by generating standard curves. A 10 μl final reaction volume was prepared using 1 μl synthesized cDNA, 2× Power SYBR 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 and 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 to *GAPDH* was quantified by the $\Delta\Delta C_t$ method. Data were presented as average ± S.E.M. and the statistical analysis was performed using the Student's *t*-test. *P* values <0·05 were regarded as significant, and levels of significance were indicated on graphs for each gene analyzed.

**Table 1** Forward and reverse primer sequences for quantitative RT-PCR and respective amplicon size

| Gene (accession) | Nucleotide sequence (5′–3′) | Product size (bp) |
|---|---|---|
| *GAPDH* (NM_001034034) | | 229 |
| Forward | GGCGTGAACCACGAGAAGTATAA | |
| Reverse | AAGCAGGGATGATGTTCTGG | |
| *STAR* (NM_001009243) | | 194 |
| Forward | GCATCCTCAAAGACCAGGAG | |
| Reverse | CTTGACACTGGGGTTCCACT | |
| *CYP17* (NM_001009483) | | 215 |
| Forward | AGACATATTCCCTGCGCTGA | |
| Reverse | GCAGCTTTGAATCCTGCTCT | |
| *3β-HSD* (NM_001135932) | | 200 |
| Forward | GGAGACATTCTGGATGAGCAG | |
| Reverse | TCTATGGTGCTGGTGTGGA | |
| *CYP11A* (NM_001093789) | | 172 |
| Forward | CAGGAGGCAGTAGAGGATGC | |
| Reverse | CAACGTCCCTCCAGAACTGT | |
| *Id1* (NM_001097568) | | 151 |
| Forward | TCTGGGATCTGGAGTTGGAG | |
| Reverse | ATACGATCGTCCGCTGGAA | |

### Statistical analysis

For each cell culture experiment, cells from at least four sheep were pooled and treatments carried out in quadruplicate. The significance of treatment effects was determined by ANOVA, and individual comparisons between treatments were made using Student's *t*-test. All hormone data were expressed as picograms of hormone per microliter of cell culture media.

## Results

In this study, activin receptors (types IA, IB, IIA and IIB), Smad2/3, and the putative inhibin co-receptor betaglycan (transforming growth factor (TGF) βRIII) were identified in ovine theca and granulosa cells during folliculogenesis (data not shown).

### Activin suppressed androgen secretion

The effects of activin A at 1, 10 and 100 ng/ml on thecal androgen production are shown in Fig. 1A. Activin A was a potent inhibitor of androstenedione production from ovine thecal cells *in vitro* and was highly effective at the lowest dose tested (1 ng/ml). Viability evaluated by Neutral Red assay on completion of culturing at 144 h

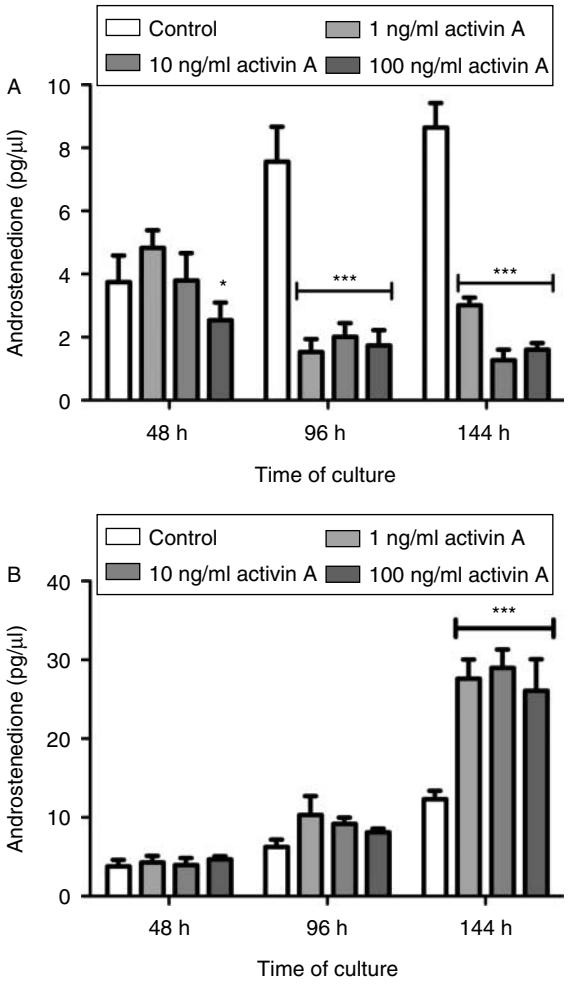


**Figure 1** Androstenedione production in theca cell cultures (A) in the presence of 0–100 ng/ml activin A and (B) effects of inhibin A on theca androstenedione production. Cell culture media were replaced every 48 h and androstenedione secreted into spent media was evaluated by RIA. Results show average ± S.E.M. Data provided are representative of at least four experiments, of which each experiment contained a separate pool of cells ($n=4$–5 sheep per pool), *$P<0{\cdot}05$ and ***$P<0{\cdot}001$ compared with the control group.

was not affected by any dose of activin (data not shown). Analyses also showed that theca cells maintained androgen production during the last 48 h culture period, indicating good health and steroidogenic capabilities. Furthermore, progesterone output measure by RIA (data not shown) remained at initial control levels throughout the culture period, indicating a lack of luteinization in the culture conditions as expected (Campbell *et al.* 1998).

### Inhibin increased androgen production

Inhibin increased ($P<0{\cdot}001$) androstenedione production from theca cells cultured *in vitro* (Fig. 1B). Results show a potent response to inhibin, which was highly effective at the lowest dose evaluated (1 ng/ml). Inhibin added alone is likely to act by blocking the suppressive effects of endogenously produced activin, and possibly also acts on BMPs that may be produced by theca cells *in vitro* (Wiater & Vale 2003).

### Inhibin blocked the suppressive effects of activin on theca steroidogenesis

Inhibin blocked the suppressive effects of added activin on theca androstenedione production at 144 h but not at 96 h (Fig. 2). The effects of inhibin became more significant over time, and on completion of the culture, inhibin antagonized ($P<0{\cdot}001$) activin suppression.

### Activin receptor inhibitor stimulated steroidogenesis

The small molecule inhibitor SB-431542 is a potent and specific inhibitor of activin receptors type IB (ALK4), TGFβRI (ALK5) and activin receptor type IC (ALK7) (Inman *et al.* 2002). In this experiment, the effects of this chemical activin receptor inhibitor were compared with those of inhibin. The manufacturer's instructions indicate that 10 µM SB-431542 is required for complete ablation of Smad2 phosphorylation by activin receptors IB, IC and TGFβRI. A dose response was therefore established from 0·1 to 10 µM, and the total androstenedione produced over time in culture is shown in Fig. 3. Results showed that SB-431542 increased androstenedione secretion at 0·1–2 µM, but had no effect from 5 to 10 µM (data not shown). Cell viability assessed on completion of the 144 h culture period showed that at concentrations

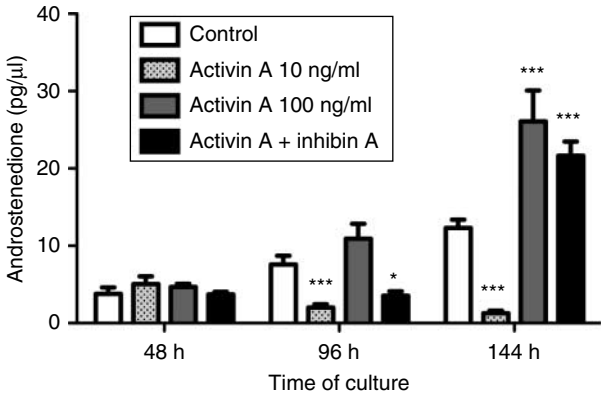


**Figure 2** Androstenedione production from theca cells in the presence of activin (10 ng/ml), inhibin (100 ng/ml) and both factors added together. Results give data of the average ± S.E.M. of four replicate wells of pooled theca cells from four sheep, androstenedione was measured by RIA. *$P<0{\cdot}5$ and ***$P<0{\cdot}001$ compared with the control group.

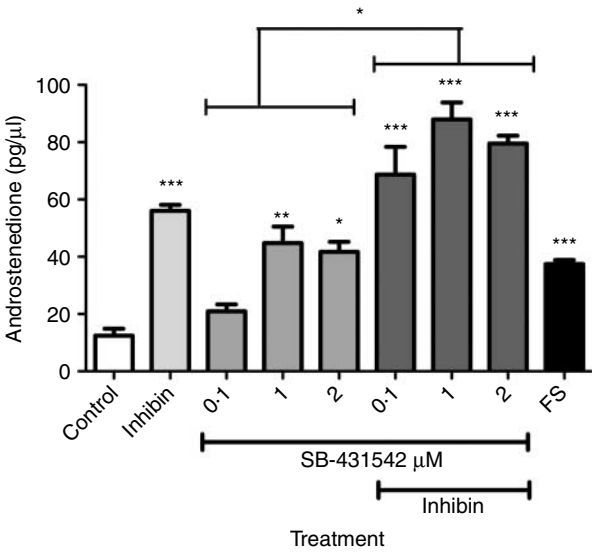


**Figure 3** Androstenedione secretion from theca cells in the presence of inhibin (10 ng/ml), SB-431542 (0·1–2 μM), or both SB-431542 and inhibin (10 ng/ml), and follistatin (FS, 500 ng/ml) over an accumulative period of 144 h. Data presented are the average±S.E.M. of four replicate wells, all from the same pool of cells ($n=4$ sheep pooled). *$P<0·05$, **$P<0·01$ and ***$P<0·001$ compared with the control group. The statistical significance of SB-431542 alone and together with inhibin is also shown above each treatment group as *$P<0·05$.

of ≥5 μM SB-431542, cell viability decreased indicating that SB-431542 had a detrimental effect on these cells (data not shown).

SB-431542 was added at doses ranging from 0·1 to 2 μM to maintain cell viability. Inhibin was used in combination with SB-431542 to evaluate whether the level of androgen secreted from cells was equal in cells treated with either inhibitor. If inhibin was merely causing androgen production by blocking the suppressive effects of activin, then the expected result would be equivalent to that observed when the chemical activin receptor inhibitor was present. Interestingly, in this experiment, the level of androstenedione secretion was greater in cell cultures treated with both inhibin and SB-431542 (Fig. 3).

### Follistatin inhibited activin suppression

Follistatin is a known antagonist of TGFβ superfamily members, including activin and BMPs, and was originally identified from the follicular fluid of ovarian follicles (Ueno *et al.* 1987). Follistatin (500 ng/ml) was added to cultures and androstenedione production measured (Fig. 3). The results show that the addition of follistatin increased androgen accumulation over 144 h in culture ($P<0·001$).

### Inhibitory Smads 6 and 7 increased androgen production

Smads 6 and 7 are intracellular signaling molecules that inhibit the signal transduction of BMP (Smad1/5/8), or BMP and activin (Smad2/3) signaling respectively. These inhibitory factors were transfected into theca cultures using adenoviruses to manipulate Smad signal transduction specifically. To establish the rate of infection required, the adenovirus construct was titred across concentrations from 10 to 100 pfu/cell seeded (Fig. 4A). Results indicated that 10 pfu/cell was sufficient to infect cells, and that at higher concentrations theca cell viability decreased. Overexpression of Smad6 or 7 by infecting cells at 10, 50 and 100 pfu/cell resulted in increased androstenedione production from theca cells in culture over two consecutive 48 h periods postinfection (Fig. 4A). It is not unexpected that the effects of Smad7 are more pronounced than those of Smad6 given that Smad7 inhibits both Smad1/5/8 and Smad2/3 (rather than just Smad1/5/8 for Smad6). BMPs have been previously shown to suppress androstenedione production in bovine and ovine theca cultures (Glister *et al.* 2005, Campbell *et al.* 2006), so blocking BMP signaling with Smad6 would remove this suppression which is presumably due to BMPs being produced endogenously by the thecal cells themselves. Adenovirus transfections were carried out at 10 pfu/cell thereafter.

As expected, when activin was added to cells overexpressing Smad6, the suppressive effects of activin were not markedly altered. However, when Smad7 was overexpressed, activin was unable to suppress androgen production ($P<0·001$; Fig. 4B). Inhibin effects were not affected by overexpression of either Smad6 or 7.

### Activin and inhibin modulate expression of genes required for steroidogenesis

Given that activin and inhibin modulate androstenedione production from ovine theca cells in culture, we assessed the effects of these factors on the expression levels of components of the steroidogenic pathway (Fig. 5). The expression of steroidogenic acute regulatory protein (*STAR*), which is responsible for transportation of cholesterol esters into the mitochondria for initiation of steroidogenesis, was decreased in the presence of added activin (50 ng/ml), and increased by SB-431542 alone (2 μM) and in combination with inhibin (100 ng/ml; $P<0·05$). Activin alone did not affect the expression of *CYP17* encoding 17α-hydroxylase (17αOH), whereas it was increased by inhibin ($P<0·05$) and SB-431542 ($P<0·001$), and when added in combination, the stimulatory effect was further enhanced ($P<0·001$). The expression level of 3β-hydroxysteroid dehydrogenase

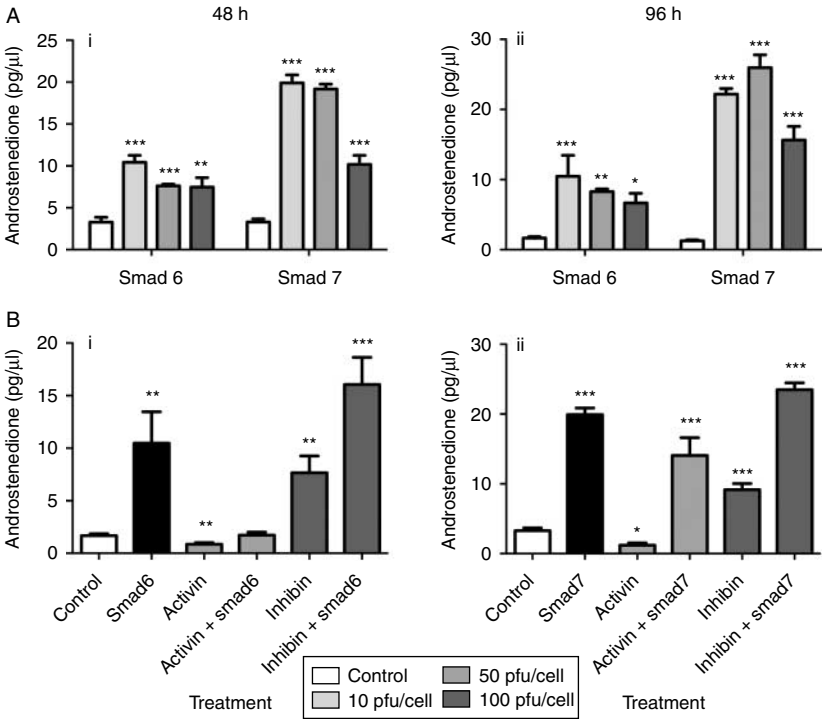


**Figure 4** (A) Androstenedione secretion from theca cells infected with either Smad6 or 7 for a 48 h period of culture. Cells were infected with 10, 50 and 100 pfu/cell overnight before addition of new media and the beginning of the first 48 h period. (B) Androstenedione production from theca cells infected with Smad6 or 7 in combination with activin (10 ng/ml) and inhibin (10 ng/ml). Cells were infected with 10 pfu adenovirus/cell overnight before addition of appropriate ligands. Data presented are the average ± S.E.M. of four replicate wells, all from the same pool of cells ($n=4$ sheep pooled). $^{*}P<0{\cdot}05$, $^{**}P<0{\cdot}01$ and $^{***}P<0{\cdot}001$ compared with the control group.

(*3β-HSD*) was significantly decreased ($P<0{\cdot}001$) by activin, while inhibin alone ($P<0{\cdot}01$) and in combination with SB-431542 ($P<0{\cdot}001$) both increased the expression of *3β-HSD*. There were no significant effects on the expression of cytochrome P450, family 11, subfamily A and polypeptide 1 (*CYP11A*), indicating that the effects of activin and inhibin are targeted primarily to the expression of *3β-HSD*.

### Expression of inhibitor of DNA-binding (*ID*) genes

*ID1* expression levels were significantly reduced in the presence of activin, inhibin and SB-431542 ($P<0{\cdot}05$), but the levels of *ID2* did not change significantly for any treatment group. *ID3* and *ID4* mRNA levels were below detection levels (data not shown).

## Discussion

There is contradictory evidence for a role of activin in follicle development, where some studies have shown stimulatory effects (Li *et al.* 1995, Smitz *et al.* 1998, Liu *et al.* 1999, Zhao *et al.* 2001, Thomas *et al.* 2003, McLaughlin *et al.* 2010), while others suggest activin inhibits development (Mizunuma *et al.* 1999, Ding *et al.* 2010), prevents luteinization (Myers *et al.* 2008), or has no effect at all (Fortune *et al.* 2000). Moreover, the concept of activin acting alone is far removed from the *in vivo* situation where many other factors are present that can antagonize and modulate the efficiency of activin signaling.

Studies on ovarian somatic cells have been conducted using media containing serum, which have been shown to induce luteinization of cells, thus changing biological functionality to principally the secretion of progesterone rather than androgens (Hillier *et al.* 1991*b*, Shukovski *et al.* 1993, Wrathall & Knight 1995). In this study, serum-free conditions were employed, and theca cells were cultured for a maximum of 144 h in the presence of low-dose LH to maintain androgen steroidogenic capacity. This study extends on previous findings in luteinized theca cells that activin A suppresses androgen production from thecal cells cultured *in vitro* without affecting cell viability (Hsueh *et al.* 1987, Hillier & Miro 1993, Wrathall & Knight 1995).

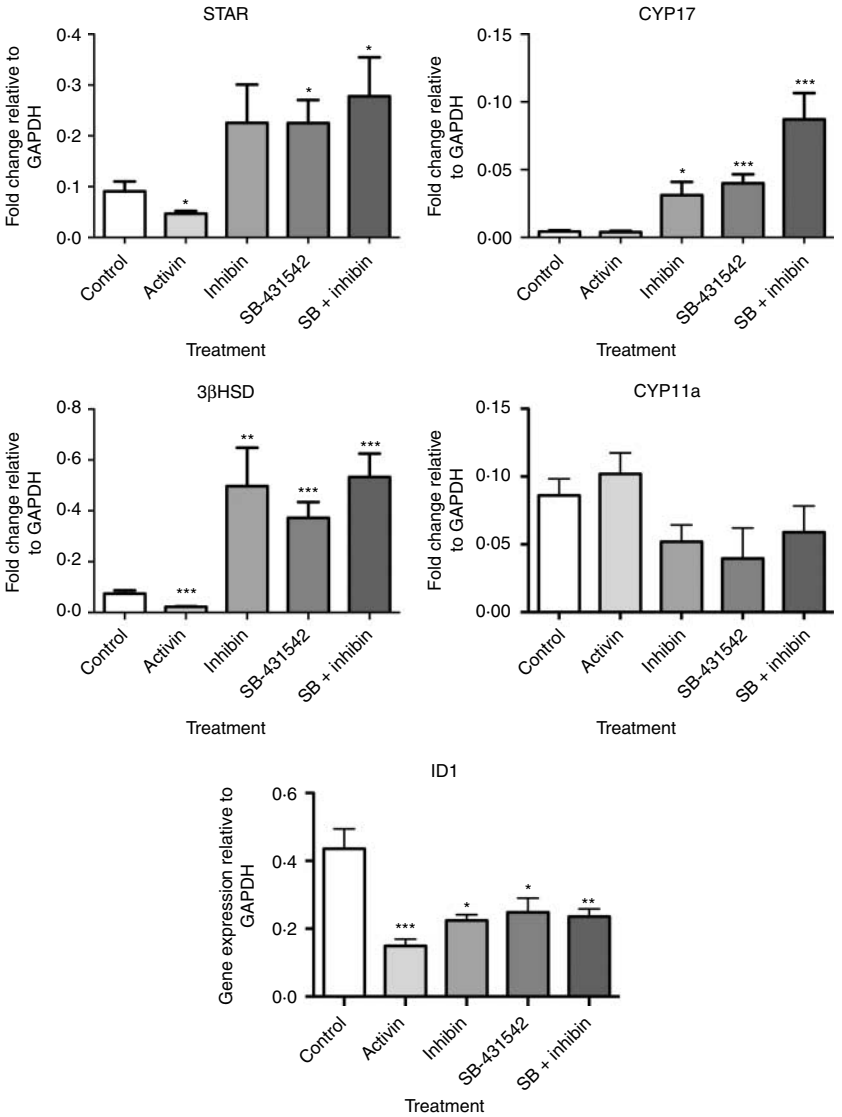


**Figure 5** The expression of steroidogenic acute regulatory protein (STAR), 17α-hydroxylase (CYP17), 3β-hydroxysteroid dehydrogenase (3β-HSD), cholesterol side-chain cleavage cytochrome P450 (CYP11A) and inhibitor of DNA binding 1 (ID1) was assessed in theca cells cultured alone, or in the presence of activin (50 ng/ml), inhibin (100 ng/ml) and/or SB-431542 (2 μM). Cells were cultured for a total of 64 h, whereby fresh ligand was added to each well at 24 and 48 h and then an hour before RNA isolation. Data are the average ±S.E.M. for four replicate experiments (each experiment from a pool of cells harvested from four to five sheep) containing four replicate wells per treatment. $^{*}P<0{\cdot}05$, $^{**}P<0{\cdot}01$ and $^{***}P<0{\cdot}001$ were assessed by Student's *t*-tests compared with the control group.

In the ovary, a major factor modulating activin bioactivity is inhibin, which is produced by granulosa cells once follicles reach the preantral stage of development (Roberts *et al.* 1993). It has been unclear whether inhibin acts by simply blocking binding of activin and BMPs to their receptors or it can elicit its biological effects through an unidentified mechanism not involving antagonism of activin/BMP receptors since no signaling pathways have been identified with inhibin bioactivity (Bernard *et al.* 2001, 2002). Association of inhibin with the membrane-anchored proteoglycan, betaglycan (also known as TGFβRIII), has been shown to increase the affinity of inhibin binding to activin type II receptors (Lewis *et al.* 2000). However, it is unclear if inhibin can elicit any signaling through betaglycan in a manner similar to TGFβ2 that utilizes the small cytoplasmic domain of this receptor for downstream signal transduction (Blobe *et al.* 2001).

In bovine follicles, the abundance of betaglycan increased approximately fivefold as bovine follicles grew and decreased by 50% when follicles reached the final stages of development, indicating an important role during the stages of development (Glister *et al.* 2010). Betaglycan is also found in granulosa and thecal cells of sheep follicles (data not shown).

In this study, inhibin had a potent and robust stimulatory effect on androstenedione production when added to cell culture media alone. Furthermore, it blocked the suppressive effects of activin added to culture medium, confirming previous findings (Hillier *et al.* 1991*b*, Hillier & Miro 1993, Wrathall & Knight 1995, Campbell & Baird 2001). The observation that inhibin-treated cells produce considerably more androgen than cells with no inhibin added indicates that theca cells might produce activin and/or BMPs that act in an autocrine manner to maintain the level of androgen production at a basal level. The production of activin βA subunit was investigated in these cultures, and it was confirmed that theca cells produce activin βA (data not shown).

Follistatin is produced by granulosa cells and is abundant in follicles as soon as two layers of granulosa cells have developed, and is maintained at high levels of expression through all stages of follicle development up to ovulation (Shimasaki *et al.* 1989, Nakatani *et al.* 1991, Roberts *et al.* 1993, Braw-Tal *et al.* 1994, Tisdall *et al.* 1994, Izadyar *et al.* 1998, Sidis *et al.* 1998, Silva & Knight 1998). While follistatin was shown to have only a 10% affinity for BMPs as for activins (Glister *et al.* 2004), it was shown to reverse the suppressive effects of added activin A, TGFβ and BMP4/6/7 on thecal androgen production (Hsueh *et al.* 1987, Hillier & Miro 1993, Wrathall & Knight 1995, Cortvrindt *et al.* 1997, Glister *et al.* 2005). In our study, when added in excess, follistatin alone increased androgen production from theca cells, supporting the concept that theca cells produce activins/BMPs endogenously *in vitro*.

To further address this concept, we used the non-signaling chemical activin receptor inhibitor SB-431542 as a comparative measure against inhibin (Bak *et al.* 2009). Androstenedione production was enhanced from theca cultures to levels similar to those produced by inhibin-treated cells, and an additive effect was observed when inhibin and SB-431542 were added in combination. This may be due to a dynamic mechanism of inhibin action compared with the binding of the chemical compound, or the fact that inhibin also interferes with BMP signaling in addition to blocking activin signaling since SB-431542 does not appear to block BMPs effectively.

Intracellular inhibitory Smads were utilized to investigate signaling mechanisms. The overexpression of inhibitory Smads 6 and 7 alone (Fujii *et al.* 1999, Nakao *et al.* 1999, Kaivo-Oja *et al.* 2003) resulted in increased androstenedione production from theca cultures over time. Moreover, the inhibition of signaling by Smad7 was observed to have a more potent effect than that caused by Smad6. This result is logical given that Smad6 blocks BMP signaling through Smad1/5/8, whereas Smad7 blocks both Smad1/5/8 and Smad2/3 pathways. Of importance, these results show that by blocking activin and BMP signal transduction intracellularly, theca cells produce greater amounts of androstenedione than control cells over time.

As expected, since Smad6 affects the Smad1/5/8 pathway, it had no effect on the ability of activin A to suppress androstenedione production and equally did not block the stimulatory effects of inhibin. Activin signaling was blocked by the overexpression of Smad7 but not by Smad6. Inhibin had an additive effect on androstenedione production when Smad6 or 7 was overexpressed. Taken together, these studies indicate that inhibin acts similar to Smad signaling inhibitors Smad6 or 7 by blocking Smad signal transduction and increasing androgen production.

While activin and inhibin modulate theca cell steroidogenesis, it has not been clear how this is achieved. Gene expression analyses showed that activin suppressed expression levels of *STAR* and *3β-HSD*, whilst inhibin and SB-431542 induced expression of *CYP17* and *3β-HSD*. A previous study using a *CYP17* promoter–luciferase reporter system in the H295R cell line *in vitro* suggested that Smad3 directly inhibited *CYP17* promoter activity (Derebecka-Holysz *et al.* 2008), but this was not replicated in this study with activin-induced Smad2/3 in primary thecal cells. The most potent and significant effects of activin, inhibin and SB-431542 were found on the expression levels of *3β-HSD*, indicating an important role for these factors in modulating 3β-HSD abundance. 3β-HSD is required for synthesis of both progesterone and androgens in theca cells, and 17αOH is required for androgen production. If inhibin stimulates or removes an activin-induced inhibition of *3β-HSD* expression, then it would make sense that more progesterone would be produced compared with androgens since the substrate pregnenolone is being used to make progesterone, therefore limiting the substrate available to 17αOH for 17-hydroxypregnenolone synthesis. However, past research has not shown any effect of inhibin on progesterone secretion from theca cells cultured in media containing 5% FCS (Hillier *et al.* 1991*b*), which may be due to luteinization. Testosterone is synthesized from androstenedione in theca cells by the enzyme 17β-hydroxysteroid, which has not been assessed in these studies, as this enzyme is not thought to be regulated by activins/inhibins due to the lack of response in testosterone levels in these and other studies (Shukovski *et al.* 1993). Furthermore, in this study, activin, inhibin, SB-431542, Smad6 or 7 did not

have any effect on testosterone production (data not shown).

Expression of the inhibitor of DNA-binding protein genes (*ID1–4*) was assessed and the results agreed with previous studies on granulosa cells from sheep follicles (Hogg *et al.* 2010). Levels of mRNA encoding *ID1* were suppressed in the presence of activin A, but no changes in *ID2* expression were observed while *ID3* and *ID4* were undetectable. In our previous study (Hogg *et al.* 2010), activin decreased *ID3* expression in granulosa cells; moreover, this study also provided evidence that in atretic follicles, *ID3* and *ID4* expression was partial or absent. Since primary theca cells were cultured for a short period of time and cells remained healthy and viable, changes in *ID2* were not expected. In other experiments, BMPs increased *ID1–4* expression in theca cultures (data not shown). BMPs are functional in the sheep follicle as evidenced by the presence of p-Smad1/5/8 in theca and granulosa cells (Hogg *et al.* 2010) and from previous *in vitro* studies on bovine thecal cells (Glister *et al.* 2010) and also our current results using Smads 6 and 7, both of which inhibit BMP signaling. Thus, it may be that in our culture system, any endogenous BMPs that are being produced are insufficient to maintain *ID1–4* expression or are counteracted by the endogenous activin being produced by the theca cultures themselves. The failure of inhibin and the activin receptor blocker SB-431542 to maintain or increase *ID2* requires further investigation.

In humans, inhibin B is the predominant form of inhibin present during the follicular phase of the cycle, and inhibin A in the luteal phase (Groome *et al.* 1996). However, inhibin B is not found in sheep ovaries. So, these studies were carried out using only inhibin A (McNeilly *et al.* 2002). In patients diagnosed with PCOS, studies have shown that circulating inhibin B levels were higher while activin A was lower in the follicular phase (Anderson *et al.* 1998, Lockwood *et al.* 1998, Norman *et al.* 2001, Shen *et al.* 2005). Inhibin A levels were reported to be lower in the follicular fluid of PCOS follicles compared with control follicles, and there were no differences in levels of activin A and inhibin B levels (Magoffin & Jakimiuk 1998). Activin has been previously shown to increase theca and granulosa cell proliferation (Miro & Hillier 1996, Duleba *et al.* 2001). *In vitro* studies using primary cultures of theca cells from PCOS ovaries produced more androgens than normal controls, had higher levels of steroid intermediates produced during steroidogenesis and had higher levels of *STAR, LHR, CYP17 and CYP11A* expression than in size-matched control follicles (Gilling-Smith *et al.* 1994, Nelson *et al.* 1999, Wickenheisser *et al.* 2000, Jakimiuk *et al.* 2001, Nelson *et al.* 2001). Ovaries from PCOS women have also been shown to have 1·5-fold higher expression of betaglycan than controls that would enhance inhibin binding and ability to block activin action, whereas no difference in activin receptor (types IA, IB, IIA and IIB) expression was detected (Zhu *et al.* 2010). These alterations in inhibin and activin concentrations coupled with increased thecal layers may allow increased androgen production in PCOS patients, caused not by an active excess secretion, but by a reduction in the local inhibition of androgen production by the reduced exposure to activins.

In conclusion, our present studies have confirmed that the biological effects of activin on theca cell androgen production are inhibitory, whereas inhibin acts in an opposing manner by causing an increase in the production of androgens. Furthermore, these effects of activin are modulated through the Smad signaling pathway that results in decreased levels of *STAR* and *3β-HSD* gene expression. The studies also show that inhibin appears to act almost exclusively by blocking the inhibitory action of activins, a concept supported by the similar responses of increased androgen production when activin action was blocked by the activin receptor blocker SB-431542, the activin-binding protein follistatin and the Smad2/3 signaling pathway by Smad7. All the results also support the novel concept that theca cells themselves produce activins, and potentially BMPs which autoregulate androgen production locally. As follicles develop and acquire aromatase, they require more androgen precursor, and this is induced by increased production of inhibin by the granulosa cells, which then act to negate the suppressive effects of thecal, and granulosa cell activins, and BMPs. Therefore, inhibin does not stimulate androgen production, but acts to remove the effects of the inhibitor, activin, on suppressing CYP17 and 3β-HSD, thus allowing increased androgen production.

## 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.

## Funding

This study was funded by an MRC grant to A S M (G00007.01).

## Acknowledgements

We acknowledge Kirsten Hogg for her gift of primers for quantitative RT-PCR assays. We also extend thanks to Joan Docherty and staff at the Marshall Building for help with all the animal work.

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Received in final form 18 October 2011
Accepted 14 November 2011
Made available online as an Accepted Preprint 14 November 2011