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Myostatin is expressed in bovine ovarian follicles and modulates granulosal and thecal steroidogenesis

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Abstract

Myostatin plays a negative role in skeletal muscle growth regulation but its potential role in the ovary has received little attention. Here, we first examined relative expression of myostatin (MSTN), myostatin receptors (ACVR1B, ACVR2B and TGFBR1) and binding protein, follistatin (FST), in granulosa (GC) and theca (TC) cells of developing bovine follicles. Secondly, using primary GC and TC cultures, we investigated whether myostatin affects steroidogenesis and cell number. Thirdly, effects of gonadotropins and other intrafollicular factors on MSTN expression in GC and TC were examined. MSTN, ACVR1B, TGFBR1, ACVR2B and FST mRNA was detected in both GC and TC at all follicle stages. Immunohistochemistry confirmed follicular expression of myostatin protein. Interestingly, MSTN mRNA expression was lowest in GC of large estrogen-active follicles while GC FST expression was maximal at this stage. In GC, myostatin increased basal CYP19A1 expression and estradiol secretion whilst decreasing basal and FSH-induced HSD3B1 expression and progesterone secretion and increasing cell number. Myostatin also reduced IGF-induced progesterone secretion. FSH and dihydrotestosterone had no effect on granulosal MSTN expression whilst insulin-like growth factor and tumour necrosis factor-alpha suppressed MSTN level. In TC, myostatin suppressed basal and LH-stimulated androgen secretion in a follistatin-reversible manner and increased cell number, without affecting progesterone secretion. LH reduced thecal MSTN expression whilst BMP6 had no effect. Collectively, results indicate that, in addition to being potentially responsive to muscle-derived myostatin from the circulation, myostatin may have an intra-ovarian autocrine/paracrine role to modulate thecal and granulosal steroidogenesis and cell proliferation/survival.

Introduction

Ovarian follicle development is dependent on the actions and interactions of systemic and intra-ovarian regulatory signals. Whilst pituitary gonadotrophins (FSH, LH) are the key endocrine signals driving follicle development, a complex array of locally-produced growth factors also contribute to the modulation of follicular somatic cell proliferation and differentiation, 'initial' and 'cyclic' follicle recruitment, steroidogenesis, dominant follicle selection and ovulation (Campbell *et al.* 2003, Webb *et al.* 2003). Prominent amongst these are various members of the transforming growth factor-β (TGF-β) superfamily including growth and differentiation factor-9 (GDF9), anti-mullerian hormone (AMH), inhibins, activins and several bone morphogenetic proteins (BMP) including BMP2, BMP4, BMP6 and BMP7 (Shimasaki *et al.* 2004, Knight & Glister 2006). In the present study we examined the potential involvement of another TGF-β superfamily member, myostatin (also known as GDF8) in regulating ovarian follicle function.

Myostatin is well-recognised for its negative autocrine/paracrine role in skeletal muscle development (Otto & Patel 2010, Schiaffino *et al.* 2013). Myostatin-null mice show a pronounced increase in muscle mass due to muscle fibre hyperplasia and hypertrophy (McPherron *et al.* 1997). Naturally occurring inactivating mutations in the myostatin gene are also evident in several species including bovine (Kambadur *et al.* 1997), ovine (Clop *et al.* 2006), canine (Mosher *et al.* 2007) and human (Schuelke *et al.* 2004) and these also display a phenotype of substantially increased muscle mass. Conversely, upregulation of myostatin is associated with pathological conditions characterised by muscle wasting, notably sarcopenia and cachexia arising from late-stage cancer, chronic kidney failure and congestive heart failure (Elkina *et al.* 2011, Elliott *et al.* 2012).

Apart from skeletal muscle, myostatin has also been implicated in the regulation of cardiomyocyte and adipocyte function (review: (Elliott *et al.* 2012)), Moreover, investigations into the expression and potential functional role(s) of myostatin in reproductive organs including the human ovary have recently been reported (Chang *et al.* 2015, Fang *et al.* 2015, Chang *et al.* 2016a, Chang *et al.* 2016b).

Myostatin signals through the activin receptor type 2B (ACTR2B), forming a signalling complex with ACVR1B (ALK4) and/or TGFBR1(ALK5) that activates an intracellular Smad 2/3-dependent signal transduction pathway. Myostatin receptor activation can also signal in a Smad-independent manner via activation of MAPK and inhibition of Akt pathways (Rebbapragada *et al.* 2003). Binding of myostatin to its signalling receptors is modulated by follistatin (Amthor *et al.* 2004). Follistatin was initially identified as a secreted activin-binding protein but has since been shown to bind several other TGF-β ligands including BMP-2,-4,-6 and -7 (Fainsod *et al.* 1997, Iemura *et al.* 1998, Glister *et al.* 2004). Follistatin-null mice show decreased muscle mass (Matzuk *et al.* 1995) likely arising from diminished antagonism of myostatin signalling. Conversely, transgenic overexpression of follistatin promotes a hypermuscular phenotype resembling that of myostatin-null mice (Lee & McPherron 2001).

Global microarray studies of the bovine ovary revealed that myostatin mRNA is expressed in follicular granulosa (Skinner *et al.* 2008, Glister *et al.* 2014, Hatzirodos *et al.* 2014b) and theca cells (Glister *et al.* 2013, Hatzirodos *et al.* 2014a) although studies to confirm expression and explore the potential functional role(s) of myostatin in the bovine ovary have not been reported. Myostatin mRNA expression has also been documented in human reproductive tissues including ovary (Chang *et al.* 2015), myometrium (Islam *et al.* 2014) and trophoblast (Peiris *et al.* 2014) and recent evidence

from studies on luteinized granulosa cells supports various functional roles. For instance, treatment of human granulosa-lutein cells with myostatin down-regulated expression of steroidogenic acute regulatory protein (STAR) and reduced progesterone secretion, whilst increasing cytochrome P450 aromatase (CYP19A1) expression, FSHR expression and estradiol secretion (Chang *et al.* 2015, Fang *et al.* 2015, Chang *et al.* 2016a). An anti-proliferative effect of myostatin on human granulosa-lutein cells was also reported (Chang *et al.* 2016b). To our knowledge, there have been no reports on effects of myostatin on non-luteinized granulosa cells, nor on theca cells from any species.

Given the paucity of information on the ovarian expression and possible intraovarian role(s) of myostatin, particularly in relation to actions on non-luteinized follicular cells, the aims of the present study were to: (1) examine mRNA expression profiles for myostatin, its signalling receptors and binding protein (follistatin; FST) in granulosa (GC) and theca (TC) cells across different stages of bovine antral follicle development; (2) use non-luteinized bovine GC and TC culture models to investigate whether myostatin affects steroid production; (3) determine whether the effect of myostatin can be attenuated by follistatin; (4) investigate whether thecal and granulosal expression of myostatin mRNA is modulated by gonadotropins and several intrafollicular factors implicated in the regulation of follicular steroidogenesis.

Materials and Methods

Relative expression of myostatin, follistatin and myostatin receptor mRNAs in developing bovine antral follicles.

Relative mRNA expression for myostatin (MSTN), myostatin receptors (ACVR2B, ACVR1B and TGFBR1) and follistatin (FST) in theca and granulosa layers from bovine antral follicles was determined using RT-qPCR. Ovaries from randomly cycling cattle were obtained from an abattoir (Anglo Beef Processors, Guildford, UK) and selected for follicle dissection as described previously (Glister et al 2001; 2004; 2010). Briefly, antral follicles of diameter 3-18mm were dissected out and sorted by size into small (3-6mm; n = 30), medium (7-10mm; n = 43) and large (11-18mm; n = 37) categories. For each follicle GC and TC layers were retrieved for RNA extraction and follicular fluid recovered for steroid hormone analysis. Follicles in the large (11-18mm) category were subdivided retrospectively into large estrogen-active (LEA; E:P ratio >1) and large estrogen-inactive (LEI; E:P ratio <1) categories according to their intrafollicular ratio of estrogen to progesterone (E:P ratio). Isolated GC and TC were homogenised in 0.5ml of Tri reagent (Sigma UK Ltd, Poole) and stored at -80^{0}C for subsequent RNA purification. The number of GC and TC RNA extracts recruited to the study (n = 82 GC samples; n = 87 TC samples; see fig. 1 for n-values for individual follicle categories) was lower than the number of extracts processed because samples indicating >5% GC/TC cross contamination were rejected during an initial quality control screen. This involved a RT-qPCR-based comparison of relative transcript abundance of four GC/TC-specific 'marker' transcripts (FSHR and CYP19A1 for GC, CYP17A1 and INSL3 for TC) each normalized to β-actin transcript abundance (data not shown).

Primary granulosa and theca cell culture models

Ovaries from randomly cycling cattle were collected from a local abattoir. As described previously (Glister *et al.* 2001, Glister *et al.* 2005) GC and TC were isolated from 4-6mm diameter follicles, plated out in either 96-well (75,000 cells/well; for steroid secretion experiments) or 24-well (250,000 cells/well; for RNA extraction experiments) plates and cultured for 7 days. To preserve a non-luteinized cellular phenotype (Gutierrez *et al.* 1997, Campbell *et al.* 1998, Glister *et al.* 2001, Glister *et al.* 2005, Sahmi *et al.* 2006) chemically-defined serum-free media was used throughout the culture period. This consisted of McCoy's 5A modified medium supplemented with 1% (v/v) antibiotic-antimycotic solution, 10 ng/ml bovine insulin, 2 mM L-glutamine, 10 mM Hepes, 5 μg/ml apotransferrin, 5 ng/ml sodium selenite, 0.1% BSA. In the case of GC cultures, media was also supplemented with 10^{-7} M androstenedione as aromatase substrate (all media and supplements were purchased from Sigma). Media were replenished and treatments added on days 2 and 4 (see below). Cultures were terminated on day 7 when conditioned media were retained for hormone assays and viable cell number was determined by neutral red uptake assay as described elsewhere (Glister *et al.* 2001)

Effects of myostatin on granulosal and thecal steroid secretion and viable cell number

Recombinant human myostatin (R&D Systems; 94% amino acid sequence homology with bovine myostatin) was added to wells to give final concentrations of 0.08, 0.4, 2, 10, 50 and 100ng/ml in the presence and absence of gonadotropin (FSH or LH). Highly purified ovine FSH (oFSH 19SIAPP) and LH (oLH-S-16) were provided by the NHPP (Torrance, CA, USA). In GC cultures, FSH was used at a final concentration of 0.3

ng/ml, shown previously to elicit optimal estradiol secretion (Glister *et al.* 2001, Glister *et al.* 2004). GC were also treated with myostatin (100ng/ml) in the presence and absence of LR3 IGF-1 analogue (Sigma;10 and 50 ng/ml) since IGF-1 is also a potent stimulator of estradiol secretion (Gutierrez *et al.* 1997, Glister *et al.* 2001). In the case of TC cultures, LH was used at a final concentration of 150 pg/ml, shown previously to elicit maximal androstenedione secretion (Glister *et al.* 2005). Control wells received an equivalent volume of culture medium as vehicle.

Can follistatin neutralize the effect of myostatin on thecal androstenedione secretion?

To examine whether follistatin can neutralize the suppressive effects of myostatin on thecal androgen secretion, TC were treated with myostatin (100ng/ml) in the presence/absence of recombinant human follistatin-288 (R&D systems; 96% amino acid sequence homology with bovine follistatin) at 0.25 and 1.25µg/ml. These concentrations were shown previously to reverse the effects of 50 ng/ml activin and BMP6 on bovine GC (Glister *et al.* 2004).

Effect of myostatin on granulosal expression of steroidogenic pathway components

To evaluate the effects of myostatin on expression of key transcripts involved in steroidogenesis (CYP11A1, HSD3B1, CYP19A1, FSHR) GC were cultured in 24-well plates (250,000 cells/well) and exposed to fixed concentrations of myostatin (100 ng/ml) in the presence and absence of an optimal concentration of FSH (300 pg/ml). At the end of culture, media were removed and cell lysates were prepared for total RNA extraction and RT-qPCR analysis.

Do gonadotropins and other factors modulate MSTN expression by cultured GC and TC?

GC (n> 4 independent batches of cells) plated out in 24-well plates were cultured in the presence/absence of FSH (300 pg/ml) and several other intrafollicular factors shown previously to modulate steroidogenesis at the concentrations used here, including LR3 IGF-1 analogue at 10 ng/ml (Glister *et al.* 2001), TNFα at 10 ng/ml (Glister *et al.* 2014) and DHT at 100nM (Wu *et al.* 2011, Hasegawa *et al.* 2017). RNA was harvested at the end of culture for evaluation of relative gene expression by RT-qPCR. TC (n=9 independent batches of cells) plated out in 96-well plates were treated with LH (150 pg/ml) in the presence/absence of BMP6 (10 ng/ml) shown previously to suppress thecal androgen production (Glister *et al.* 2005, Glister *et al.* 2013).

RNA isolation, cDNA synthesis and real-time PCR

Total RNA was isolated using Tri-reagent as described previously (Glister *et al.* 2010). cDNA was synthesized from 1µg of RNA using the AB High Capacity cDNA synthesis kit (Thermo Fisher Scientific; used according to manufacturers protocol) in a 20µl reaction primed with random hexamers. PCR primers (see table 1) were designed using Primer-BLAST' (http://www.ncbi.nlm.nih.gov/tools/primer-blast) with BLAST specificity checking against all known bovine (Bos taurus) transcripts to exclude potential amplification of off-target sequences. Primer pairs were also validated using agarose gel electrophoresis to demonstrate amplification of a single product of the predicted size. Melt curve analyses was included in each PCR assay to confirm the

amplification of a single product in each sample. cDNA template log-dilution curves were used to demonstrate satisfactory PCR efficiency and linearity. PCR assays were carried out in a volume of 14µl containing 5µl cDNA template, 1µl each forward and reverse primers (final concentration 0.36µM) and 7µl QuantiTect SYBR Green QPCR 2x Master Mix (Qiagen, Crawley, W. Sussex, UK). Samples were processed on a StepOne Plus thermal cycler (Applied Biosystems) with cycling conditions: 15min at 95^{o}C (one cycle only) followed by 15s at 95^{o}C and 1min at 60^{o}C for 40 cycles. The ΔΔCt method (Livak & Schmittgen 2001) was used to compare the relative abundance of each mRNA transcript. Ct values for each transcript in a given sample were first normalized to the corresponding ß-actin Ct value (i.e. ΔCt value). In the case of theca and granulosa tissue samples ΔCt values for each transcript in a given sample were then normalized to the mean ΔCt value for that transcript in all tissue samples. Resultant ΔΔCt values were converted to fold-differences using the formula: fold-difference = $2^{(-\Delta\Delta Ct)}$. In the case of cell culture experiments ΔCt values were normalized to the corresponding ΔCt value for vehicle-treated control cells. ΔΔCt values were then converted to fold-differences using the formula: fold-difference = $2^{(-\Delta\Delta Ct)}$.

Steroid hormone assays

Steroid concentrations were determined by competitive ELISA as described previously (Glister *et al.* 2010, Glister *et al.* 2013, Glister *et al.* 2014). The progesterone assay had a detection limit of 20pg/ml and intra- and inter-assay CVs were 8% and 10% respectively. The androstenedione ELISA had a detection limit of 30 pg/ml and intra- and inter-assay CVs were 7% and 10% respectively. The estradiol ELISA had a detection limit of 15 pg/ml and intra- and inter-assay CVs were 6% and 9% respectively.

226

227 ***Immunohistochemistry***

228 Bovine ovaries were dissected into segments and fixed in formalin for 48 hours, before
229 being dehydrated through an alcohol series, embedded in wax and sectioned (5μm) onto
230 Superfrost charged slides (VWR, Lutterworth, UK). Sections were dewaxed and
231 rehydrated prior to boiling in citrate buffer (10mM citric acid, pH6.0), blocking of
232 endogenous peroxidase (3% H_20_2 in methanol) and blocking of nonspecific binding
233 with 20% normal goat serum (NGS, Vector Laboratories Ltd, Peterborough, UK). After
234 this, sections were incubated overnight at 4°C in rabbit antibody against GDF8 (1:200;
235 sc-28910, Santa Cruz) diluted in 2% NGS. Control sections were incubated with normal
236 rabbit serum (1:200) diluted in 2% NGS. Primary antibody binding was detected using
237 biotinylated goat anti-rabbit diluted 1:250 in 2% NGS and Vector Elite ABC reagents
238 (Vector), prepared as per manufacturers instructions. Visualization of bound antibodies
239 was achieved using 3,3'–diaminobenzidine tetrahydrochloride (DAB; Vector), prior to
240 slides being counterstained with haematoxylin, dehydrated through an alcohol series
241 and mounted with coverslips using DPX mounting medium. Sections were imaged
242 using a Zeiss Axioscop 2 microscope and AxioCam digital camera.

243

244 ***Statistical analysis***

245 Steroid concentrations were log-transformed prior to statistical analysis to reduce
246 heterogeneity of variance. RT-qPCR data were analysed as ΔΔCt values (i.e. $\log_2$
247 values) before conversion to fold-difference values for graphical presentation of relative
248 transcript abundance. ACTB was used as the normalization control and showed uniform

expression level across experimental groups being compared. Results were evaluated using one- and/or two-way ANOVA and, where indicated, post-hoc pairwise comparisons were made using Fisher's protected least significant difference (PLSD) test. Results of cell culture experiments are based on a minimum of three replicate experiments using independent batches of cells (see figure legends for numbers of replicates)

Results

Relative expression of myostatin, follistatin and myostatin receptors in theca and granulosa layers

Myostatin

MSTN mRNA expression was found in both TC and GC of all antral follicles examined and overall expression level was higher in TC than GC (Figure 1A). Interestingly, while MSTN expression level in TC was uniform across antral follicle development, expression in GC fell ~15-fold to a nadir in large estrogen active (LEA) follicles. However, a higher expression level was maintained in GC of large estrogen inactive (LEI) follicle. (Fig. 1A). Immunohistochemistry confirmed myostatin protein expression in both TC and GC of antral follicles (Fig. 2). In addition myostatin immunoreactivity was evident in preantral follicles and in vascular smooth muscle cells. Both oocytes and granulosa cells of primordial, primary and secondary follicles exhibited positive immunostaining for myostatin (Fig 2)

Follistatin

FST mRNA expression was found in both TC and GC at all stages of follicle development examined with much higher expression levels in GC than TC (Fig. 1B). Interestingly, the expression of FST in GC sharply increased in LEA follicles but remained low in LEI follicles; this was opposite to what was observed for MSTN.

Myostatin receptors (ACVR2B, ACVR1B and TGFBR1)

ACVR1B, TGFBR1 and ACVR2B mRNA expression was found in both TC and GC at all stages of follicle development examined. The expression of ACVR2B and ACVR1B was generally higher in GC than TC while TGFBR1 expression levels were broadly similar in the two cell types. No notable changes in cell-specific patterns of expression of these receptors between each stages of follicle development were evident (Fig. 1C,D,E respectively).

Effect of myostatin on basal and FSH-induced steroid secretion by GC

Myostatin promoted a marked increase in basal estradiol secretion by cultured GC (~12-fold; $P<0.0001$; Fig. 3A) but did not modulate the >30-fold increase in estradiol secretion elicited by FSH. Myostatin suppressed both basal ($P<0.01$) and FSH-induced ($P<0.001$) progesterone secretion (Fig. 3B). In addition, myostatin promoted a modest though significant increase in cell number under basal conditions (~20% increase; $P<0.001$), but not under FSH-stimulated conditions (Fig. 3C).

Effects of myostatin on GC expression of steroidogenesis-related transcripts

293 The stimulatory action of myostatin on basal estradiol secretion was accompanied by a
294 ~10-fold increase in CYP19A1 expression level (P<0.05; Fig. 3D). Concomitantly, a
295 reduction in CYP11A1 and HSD3B1 expression level was observed (P<0. 05; Fig. 3EF)
296 that mirrored the myostatin-induced decrease in progesterone secretion. Myostatin did
297 not affect FSHR expression (data not shown).

298

299 ***Effect of myostatin on basal and IGF1-induced secretion of estradiol and***
300 ***progesterone by GC***

301 Fig.4 confirms the stimulatory effect of myostatin treatment (100 ng/ml) on basal
302 estradiol secretion by GC. However, myostatin did not modulate the stimulatory effect
303 of the LR3-IGF1 analogue on estradiol secretion or viable cell number. Myostatin
304 reduced both basal and IGF-induced progesterone secretion (P<0.05) but did not modify
305 the IGF-induced increase in viable cell number.

306

307 ***Effects of FSH, LR3 IGF-1, TNFα and DHT on expression of MSTN mRNA by***
308 ***cultured GC***

309 Fig. 5 shows that treatment of cultured GC with FSH elicited a ~50-fold upregulation of
310 CYP19A1 expression (p<0.05) and estradiol secretion but did not affect MSTN
311 expression. Treatment with IGF-1 analogue also promoted a marked increase in
312 CYP19A1 expression (~10-fold; P<0.05) and estradiol secretion that was accompanied
313 by a 60% reduction in MSTN expression (P<0.05). Treatment with TNFα had no effect
314 on basal CYP19A1 expression but abolished FSH-induced upregulation of CYP19A1

315 expression and estradiol secretion. TNFα suppressed MSTN expression by ~80%
316 (P<0.05) under both basal and FSH-stimulated conditions. Treatment with DHT did not
317 affect expression of either MSTN or CYP19A1.

318 ***Effects of myostatin on thecal steroid secretion and viable cell number***

319 Myostatin suppressed androstenedione secretion in a dose-dependent manner (P<0.001)
320 with an IC_{50} of ~10 ng/ml under LH-stimulated conditions (Fig. 6A). No effect of
321 myostatin on progesterone secretion was observed (Fig. 6B). Viable cell number was
322 increased (~25%; P<0.0001) by myostatin under both basal and LH-stimulated
323 conditions (Fig. 6C). LH increased both androstenedione and progesterone secretion but
324 did not affect viable cell number.

325 ***Can follistatin neutralize the effect of myostatin on androstenedione secretion?***

326 Treatment of cells with myostatin alone decreased androstenedione secretion by ~80%
327 (P<0.000; Fig. 7). Co-treatment with follistatin partially reversed this inhibitory action
328 (P<0.001). Treatment with follistatin alone tended to increase androstenedione secretion
329 but the effect was not statistically significant.
330

331 ***Effects of LH and BMP6 on MSTN mRNA expression by cultured TC***

332 Fig. 8 shows that treatment of cultured TC with LH elicited a 4-fold increase in
333 CYP17A1 expression and androstenedione secretion that was accompanied by a 40%
334 suppression of MSTN expression (p<0.05). Treatment with BMP6 profoundly
335 suppressed basal and LH-induced CYP17A1 expression and androstenedione secretion.
336 Whilst BMP6 alone did not affect MSTN expression, it reversed the suppressive effect
337 of LH on MSTN expression.

Discussion

In this study, we first provide novel information on the spatio-temporal pattern of mRNA expression of myostatin, its signalling receptors and the binding protein (FST), at different stages of bovine antral follicles development. Expression of mRNA for MSTN and its receptors was found in both GC and TC at all antral follicle stages examined, consistent with and extending previous evidence from global microarray studies (Skinner *et al.* 2008, Glister *et al.* 2013, Glister *et al.* 2014, Hatzirodos *et al.* 2014a, Hatzirodos *et al.* 2014b). Immunohistochemistry confirmed corresponding expression of myostatin protein in follicular granulosa and theca interna layers of antral follicles. Moreover, myostatin immunoreactivity was observed at earlier follicle stages than those we analysed for mRNA expression, with positive staining in both oocytes and GC of primordial, primary and secondary follicles and both GC and TC of late preantral and early antral follicles. The inverse mRNA expression pattern of MSTN and FST we observed in GC of large estrogen-active follicles is of interest since follistatin is known to bind to and inhibit myostatin signaling (Lee & McPherron 2001, Amthor *et al.* 2004), a finding confirmed in this study by its ability to attenuate the effect of myostatin on thecal androgen production. These results suggest, therefore, that GC-derived myostatin and follistatin interact to regulate ovarian follicle physiology. In particular, these observations suggest that autocrine/paracrine signalling by GC-derived myostatin is attenuated in large healthy follicles (i.e. low myostatin/high follistatin), such as those reaching the preovulatory stage of development. By contrast, at earlier antral follicle stages (i.e. high myostatin/low follistatin), myostatin signalling via a Smad 2/3

dependent pathway may contribute to the suppression of thecal androgen production whilst upregulating granulosal estradiol production and down-regulating progesterone production. Thus, myostatin appears to act to prevent/delay premature follicle maturation and luteinisation in a similar manner to that suggested previously for activins and BMPs (Findlay *et al.* 2002, Knight & Glister 2006), both of which can attenuate thecal androgen production, enhance granulosal estrogen output whilst suppressing granulosal progesterone output.

The present results from experiments on non-luteinized ovarian cell models clearly support the above with myostatin suppressing androgen secretion by theca cells. In the case of granulosa cells, myostatin enhanced basal CYP19A1 expression and estradiol secretion whilst suppressing CYP11A1 and HSD3B1 expression and secretion of progesterone. In addition, treatment of human granulosa-lutein cells with myostatin was recently reported to enhance FSH-induced upregulation of aromatase/estradiol production, while inhibiting LH-induced upregulation of StAR/progesterone production (Chang *et al.* 2016a). Moreover, the present study found that myostatin increased viable cell number in both TC and GC cultures suggesting a positive effect on cell proliferation and/or survival. This finding contrasts with a report that myostatin reduces proliferation of human granulosa-lutein cells, evidently by upregulating connective tissue growth factor expression (Chang *et al.* 2016b). The reason for this discrepancy is not known but may reflect the effect of luteinisation, or a species difference.

An intrafollicular IGF system is firmly implicated in the autocrine/paracrine regulation of follicle development, steroidogenesis and dominant follicle selection (Campbell *et al.* 1995, Glister *et al.* 2001, Silva & Price 2002, Webb *et al.* 2003). Like FSH, IGF-1 can upregulate granulosal estradiol secretion; moreover, IGF-1 can augment follicular

386 responsiveness to FSH, providing a potential mechanism for selecting the dominant
387 follicle from the cyclically-recruited growing cohort (Campbell *et al.* 1995, Webb *et al.*
388 2003). It was therefore pertinent to investigate whether myostatin affected the GC
389 response to IGF-1 treatment. Although the results showed no effect on IGF-induced
390 estradiol production or cell number, myostatin increased basal estradiol production and
391 cell number whilst reducing basal and IGF-induced progesterone production. As such,
392 these observations further support the notion that myostatin has a role to delay
393 premature follicle maturation and luteinisation.

394 Whilst circulating or intrafollicular concentrations of myostatin in cattle have not been
395 reported to our knowledge, serum concentrations of 10-20 ng/ml in cynomolgus
396 monkey and human, ~24 ng/ml in rat and ~80 ng/ml in mouse have been documented
397 (Furihata *et al.* 2016, Hedayati *et al.* 2016, Palandra *et al.* 2016). A myostatin
398 concentration of ~3 ng/ml has been reported for human follicular fluid (Chen *et al.*
399 2012). Since myostatin suppressed thecal androgen production and granulosal
400 progesterone production in vitro with an IC50 value of ~10 ng/ml, it seems plausible
401 that levels reaching the well-vascularized theca interna from peripheral blood could be
402 sufficient to exert a regulatory action, regardless of the additional 'local' contribution
403 (perhaps considerable?) of TC and/or GC-derived myostatin. On the other hand, given
404 the greater diffusional barrier needed to reach the avascular granulosal layer, combined
405 with the somewhat higher myostatin concentration (~50 ng/ml) needed to upregulate
406 GC estradiol production, it is possible that GC are primarily responsive to locally
407 produced myostatin acting in an autocrine/paracrine manner. The establishment of a
408 bovine myostatin assay to allow comparison of endogenous concentrations in peripheral

409 blood and ovarian follicular fluid of cattle in different physiological states and in
410 follicles at different stages of development, would be useful in this regard.

411 As a first step towards investigating which endocrine and local paracrine and/or
412 autocrine signals regulate myostatin expression in bovine ovarian follicles, we found
413 that an LH-induced increase in thecal CYP17A1 expression and androstenedione
414 secretion was accompanied by reduced MSTN expression level, consistent with a
415 negative autocrine/paracrine action of myostatin on thecal androgen production, and
416 with in the findings of our myostatin dose-response study. Indeed, it is possible that the
417 stimulatory action of LH on thecal androgen production could be due, in part, to LH-
418 induced suppression of myostatin expression. The finding of a reduced MSTN mRNA
419 abundance in TC producing more androgen could reflect increased androgen receptor-
420 mediated signalling since raised androgen levels are also associated with decreased
421 MSTN expression in rat skeletal muscle tissue (Mendler *et al.* 2007). However, another
422 intraovarian growth factor, BMP6, shown here and elsewhere (Glister *et al.* 2005,
423 Glister *et al.* 2013) to greatly reduce thecal CYP17A1 expression and androstenedione
424 secretion, did not affect thecal MSTN expression, casting doubt on androgen having a
425 direct effect. Furthermore, treatment of cultured GC with the potent non-aromatisable
426 androgen DHT had no effect on MSTN expression, suggesting an absence of androgen
427 receptor-dependent regulation of granulosal MSTN expression. Consistent with
428 previous findings (Gutierrez *et al.* 1997, Glister *et al.* 2001) treatment of GC with FSH
429 and IGF analogue both promoted substantial increases in estradiol secretion but only
430 IGF analogue modulated MSTN expression, eliciting a ~60% reduction. This suggests
431 a possible interaction between IGF and myostatin signalling at the intrafollicular level
432 that warrants further investigation. In skeletal muscle IGF-1 is a prominent positive

433 regulator of muscle cell proliferation and differentiation whilst myostatin opposes this
434 action (Valdes *et al.* 2013). Despite this, IGF signalling upregulates myostatin
435 expression in skeletal muscle tissue models, suggesting an inhibitory auto-regulatory
436 loop (Yang *et al.* 2007, Kurokawa *et al.* 2009, Valdes *et al.* 2013).

437 The pro-inflammatory cytokine, TNFα, is also expressed at the intraovarian level and is
438 implicated in the regulation of follicle and luteal growth/regression and steroidogenesis
439 (Sheldon *et al.* 2014, Samir *et al.* 2017). Consistent with earlier findings (Glister *et al.*
440 2014) we showed that TNFα abolished FSH-induced upregulation of CYP19A1 and
441 estradiol secretion by GC. This was accompanied by a marked reduction in MSTN
442 expression reinforcing the view that myostatin has a positive role in granulosal estrogen
443 production. In skeletal muscle models, activation of the TNFα pathway suppresses
444 myogenesis but upregulates myostatin expression (Ono & Sakamoto 2017). Moreover,
445 IGF can reverse the TNF-α induced suppression of myogenesis (Zhao *et al.* 2015)
446 indicating interactions between positive (IGF1) and negative (myostatin, TNF-α)
447 regulators of myogenesis. Further studies are needed to decipher the regulatory signals
448 that contribute to the regulation of myostatin expression by ovarian follicular cells and
449 to place these in a physiological context.

450 With respect to myostatin-null mice, there are few, if any, references to their ovarian
451 phenotype and the potential impact of the mutation on gonadal function and fertility is
452 unknown to us. However, an in vivo study involving active immunization of female
453 mice against myostatin, showed that the number of developing ovarian follicles in their
454 female progeny was ~50% lower than that of control mice, with a similar diminution in
455 litter size (Liang *et al.* 2007). Double-muscled cattle with myostatin mutations,
456 reportedly show delayed puberty, reduced female fertility and a higher incidence of

457 dystocia and perinatal calf mortality/morbidity is associated with the large size of calves
458 (McPherron & Lee 1997). However, we are not aware of any studies examining whether
459 perturbations in ovarian follicle dynamics or steroidogenesis occur in double-muscled
460 cattle. Whilst information is currently lacking on the above, it is possible that the
461 physiological actions of myostatin in the ovary are functionally redundant owing to
462 compensatory effects of other TGF-β ligands (e.g activins) that can signal via the same,
463 or overlapping, receptors to elicit similar regulatory actions on theca and granulosa cells.

464 In summary, this study provides novel information on the expression of myostatin, its
465 signalling receptors and the binding protein, follistatin, in theca and granulosa cells of
466 developing bovine antral follicles. Myostatin expression in GC declined to a very low
467 level in large estrogen-active follicles in which expression of follistatin was maximal,
468 suggesting attenuation of GC-derived myostatin signalling at this stage. Since myostatin
469 suppressed thecal androgen production in a dose-dependent manner, an effect partially
470 rescued by follistatin, it is hypothesised that attenuation of myostatin signalling in large
471 antral follicles could facilitate thecal androgen production required as a substrate for
472 granulosal aromatase enzyme and estrogen synthesis. Paradoxically, however,
473 myostatin was found to promote CYP19A1 expression and estradiol production by
474 granulosa cells under 'basal' conditions whilst suppressing CYP11A1 and HSD3B1
475 expression and progesterone production (see Fig. 9). Taken together, this suggests a role
476 for myostatin in delaying follicle progression towards pre-ovulatory maturation and
477 luteinisation, in a manner similar to that suggested for granulosa-derived activin
478 (Findlay *et al.* 2002, Knight & Glister 2006). Further in-depth studies in other species,
479 including whole animal models, are required to confirm and extend these in vitro
480 observations based on bovine ovarian cell culture models. It is also speculated that

muscle-derived myostatin conveyed to the ovary via the systemic circulation may contribute to the regulation of follicle function. In a similar manner, testicular steroidogenesis and gametogenesis may be influenced by circulating and/or locally-produced myostatin although we are not aware of any studies, to date, examining this possibility.

Declaration of interests

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

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690

691 **Table 1** List of primers used for real-time PCR

692

693

694

695 **Figure Legends**

696 **Fig. 1.** Relative abundance of mRNA transcripts for (A) MSTN, (B) FST, (C)
697 ACVR1B, (D) TGFBR1 and (E) ACVR2B in theca and granulosa layers of small (3-
698 6mm), medium (7-10mm) and large (11-18mm) bovine antral follicles. Large follicles
699 are subdivided into estrogen active (E:P ratio >1) and estrogen-inactive (E:P ratio <1)
700 categories referred to as LEA and LEI follicles, respectively. Intrafollicular E:P ratios
701 for each follicle category are shown in panel F. Numbers in parenthesis in panel A are
702 n-values for each group. Values are mean ± SEM and summarized two-way ANOVA
703 results are shown. Within each cell type means without a common letter are
704 significantly different ($P<0.05$).

705

706 **Fig. 2** Immunohistochemical staining of bovine ovary sections showing myostatin
707 immunoreactivity (brown) in oocyte and granulosa cells of primordial (pF) and primary
708 (PrF) follicles (A), secondary (SF) follicles (B,C) and in thecal (T) and granulosal (G)
709 layers of antral follicles (AF) (D,E). Myostatin immunoreactivity was also evident in
710 vascular smooth muscle cells (bv) (E). No staining was observed in control sections
711 treated with normal rabbit serum instead of primary antibody (F).

712

713 **Fig. 3** Effect of myostatin on basal and FSH-induced secretion of (A) estradiol and (B)
714 progesterone by bovine granulosa cells, and on (C) viable cell number; Panels (D-F)
715 show the effect of myostatin ± FSH on expression of CYP19A1, CYP11A1 and
716 HSD3B1 mRNA, respectively. Values are means ± sem (n = 5 independent cultures).

717 Results of 2-way ANOVA are summarized; *P<0.01, **P<0.01 ***P<0.001 compared
718 to respective control with zero myostatin (panels A, B, C). In panels D-F means without
719 a common letter are significantly different (P<0.05).
720
721
722 **Fig. 4** Effect of myostatin on basal and LR3 IGF-1-induced secretion of (A) estradiol
723 and (B) progesterone by bovine granulosa cells and on (C) viable cell number. Values
724 are means ± SEM (n = 3 independent cultures). Means without a common letter are
725 significantly different (P<0.05).
726

727 **Fig. 5** Effect of different treatments known to modulate GC steroidogenesis on
728 granulosal expression of (A) MSTN and (B) CYP19A1 and on (C) secretion of estradiol.
729 Values are means ± SEM (n = 4 independent cultures); Means without a common letter
730 are significantly different (p<0.05).

731

732 **Fig. 6** The effects of myostatin on basal and LH-induced secretion of (A)
733 androstenedione and (B) progesterone by bovine theca cells. Panel (C) shows effects on
734 viable cell number. Values are mean ± SEM (n=12 independent cultures); Two-way
735 ANOVA p-values are shown

736

737 **Fig. 7** Ability of follistatin to antagonize myostatin-induced suppression of thecal
738 androstenedione secretion. Values are means ± SEM (n=6 independent cultures)

739

740 **Fig. 8** Effect of LH and BMP6 on thecal expression of (A) MSTN and (B)
741 CYP17A1and on (C) secretion of androstenedione. Values are means ± SEM (n = 8
742 independent cultures); means without a common letter are significantly different
743 (p<0.05).

744

745 **Fig. 9** Schematic diagram illustrating potential involvement of systemic and/or locally
746 produced myostatin in the modulation of thecal and granulosal steroidogenesis.

Target	Accession number	Forward primer 5' to 3'	Reverse primer 5' to 3'	Amplicon size (bp)
LHCGR	NM_174381.1	ATTGCCTCAGTCGATGCCCAGACC	AAAAAGCCAGCCGCGCTGC	92
STAR	NM_174189	TTTTTTCCTGGGTCCTGACAGCGTC	ACAACCTGATCCTTGGGTTCTGCACC	103
CYP11A1	NM_176644	CAGTGTCCCTCTGCTCAACGTCC	TTATTGAAAATTGTGTCCCATGCGG	99
HSD3B1	NM_174343.2	GCCACCTAGTGACTCTTTCCAACAGCG	TGGTTTTCTGCTTGGCTTCCTCCC	111
FSHR	NM_174061.1	GCCAGCCTCACCTACCCCAGC	AATTGGATGAAGGTCAGAGGTTTGCC	75
CYP17A1	NM_174304	GACAAAGGCACAGACGTTGTGGTCA	TGATCTGCAAGACGAGACTGGCATG	301
CYP19A1	NM_174365	TCTGTCCCCACTGAATCCTCCTGG	GGGTTTCATGGTGCTGTGTGGC	102
MSTN	NM_001001525.2	GTTCGATGTCCAGAGAGATGCCAGC	ACTTGCGTTAGAAGATCAGACTCCGTGG	114
ACTB	NM_173979.3	ATCACCATCGGCAATGAGCGGTTC	CGGATGTCGACGTCACACTTCATGA	128

Table 1: List of primers used for quantitative RT-PCR

Fig 1

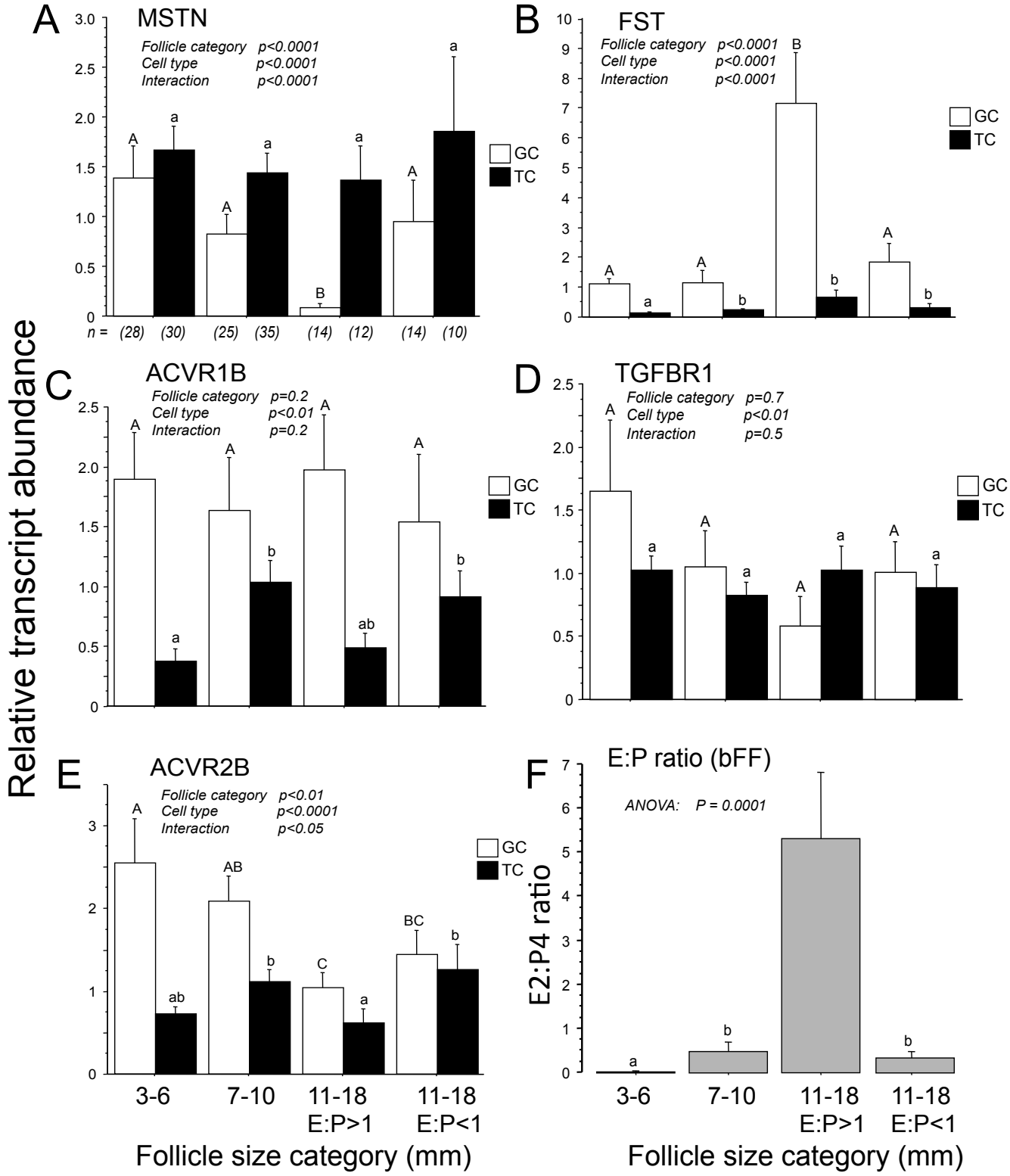

Fig. 2

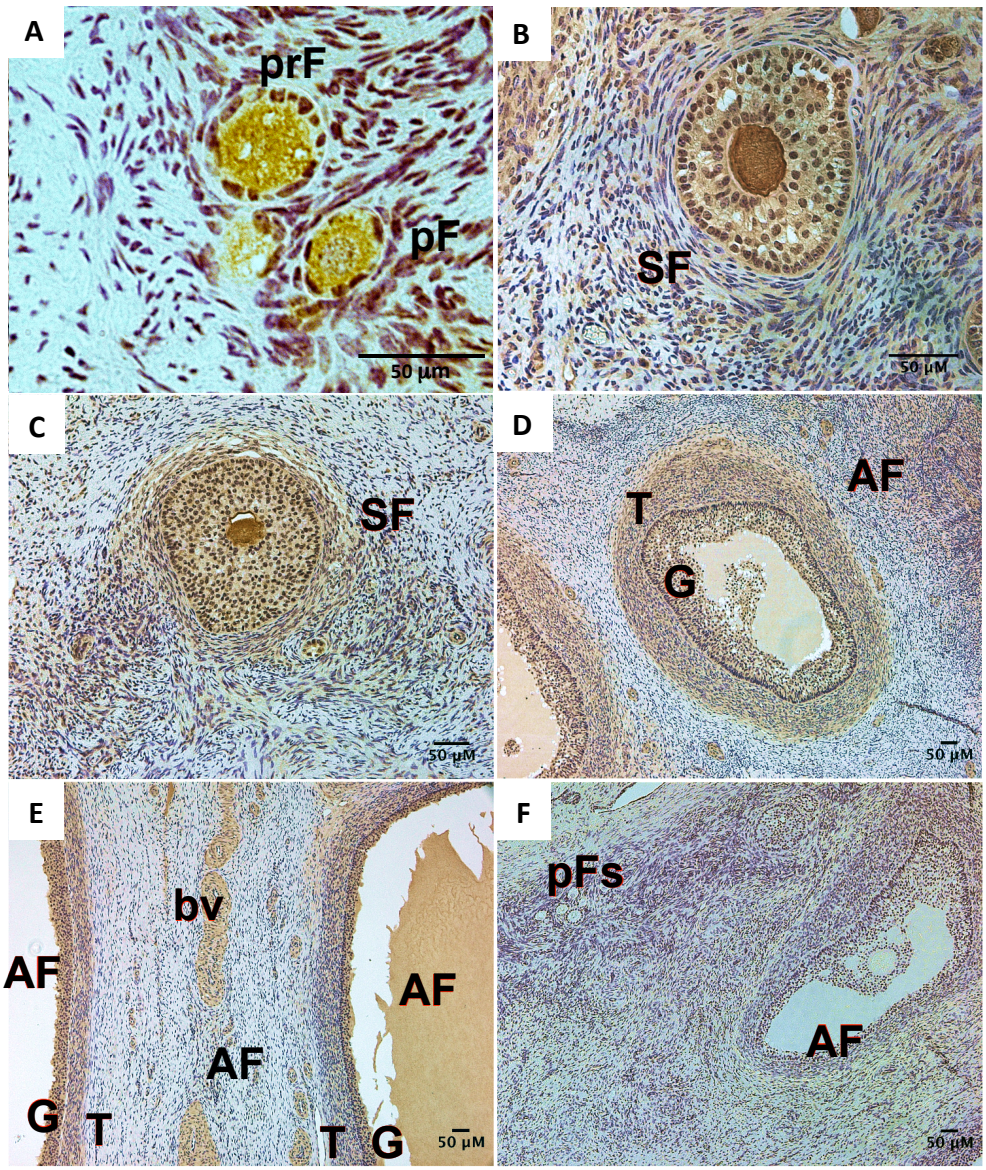

Fig. 3

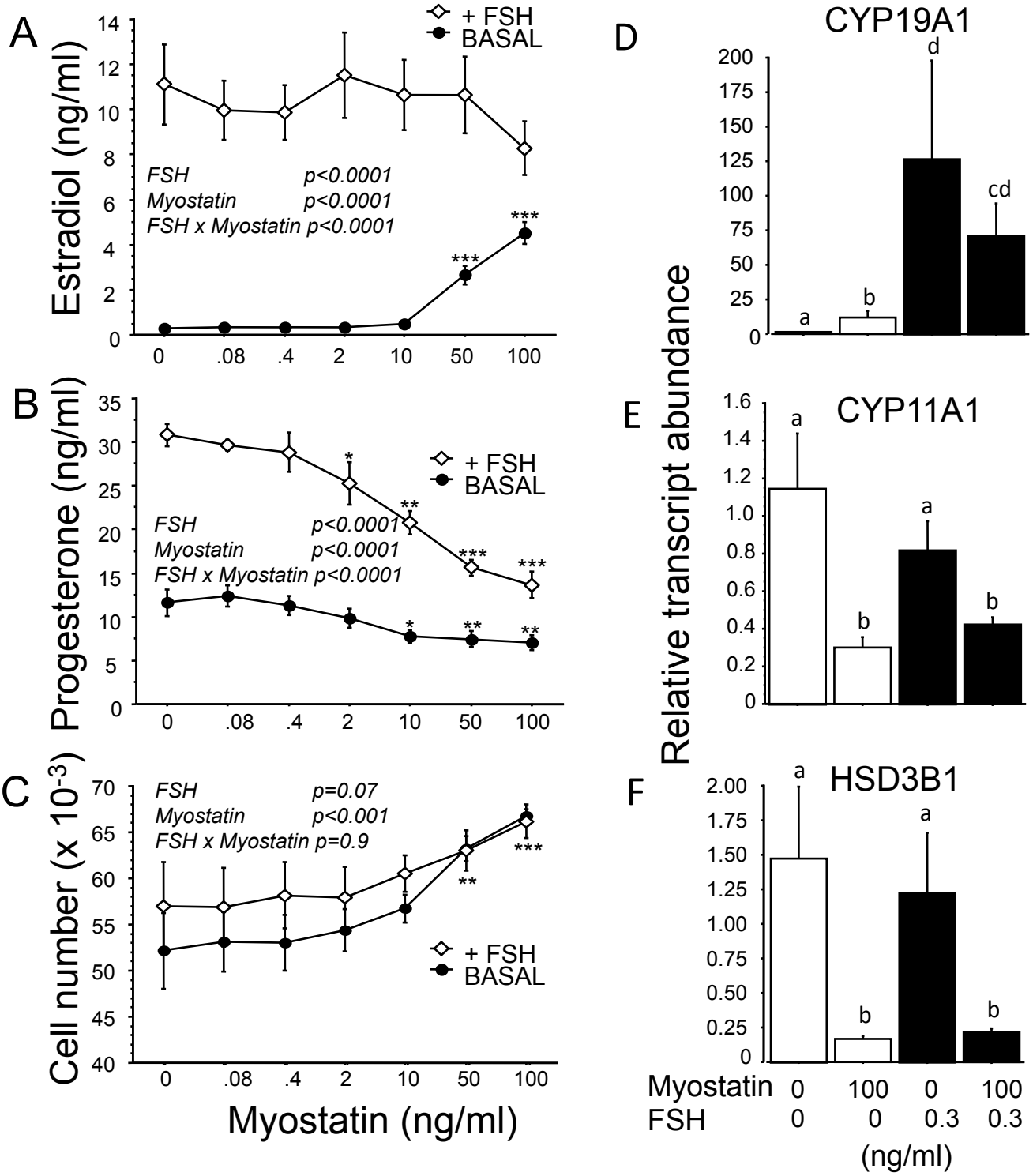

Fig. 4

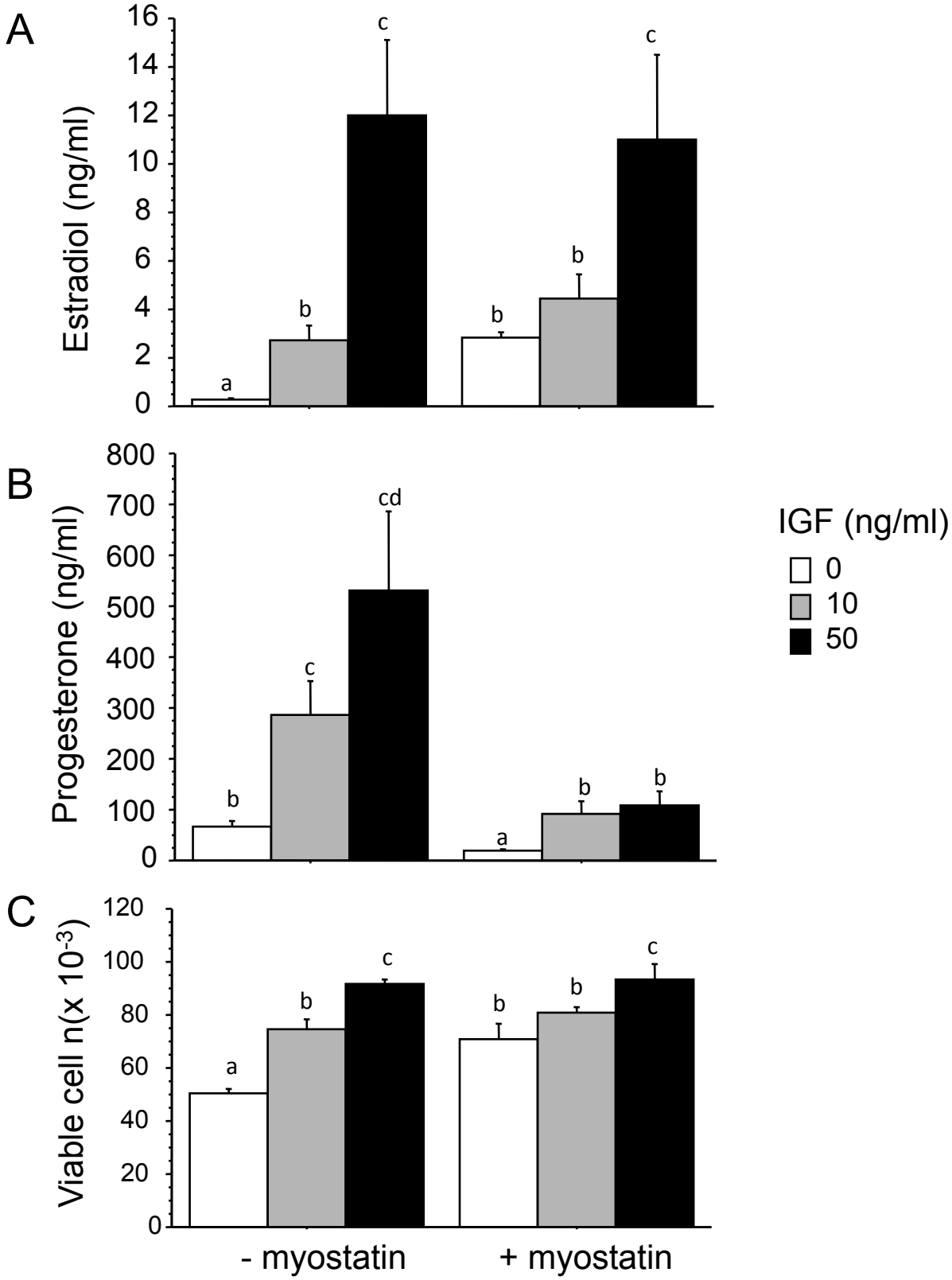

Fig 5

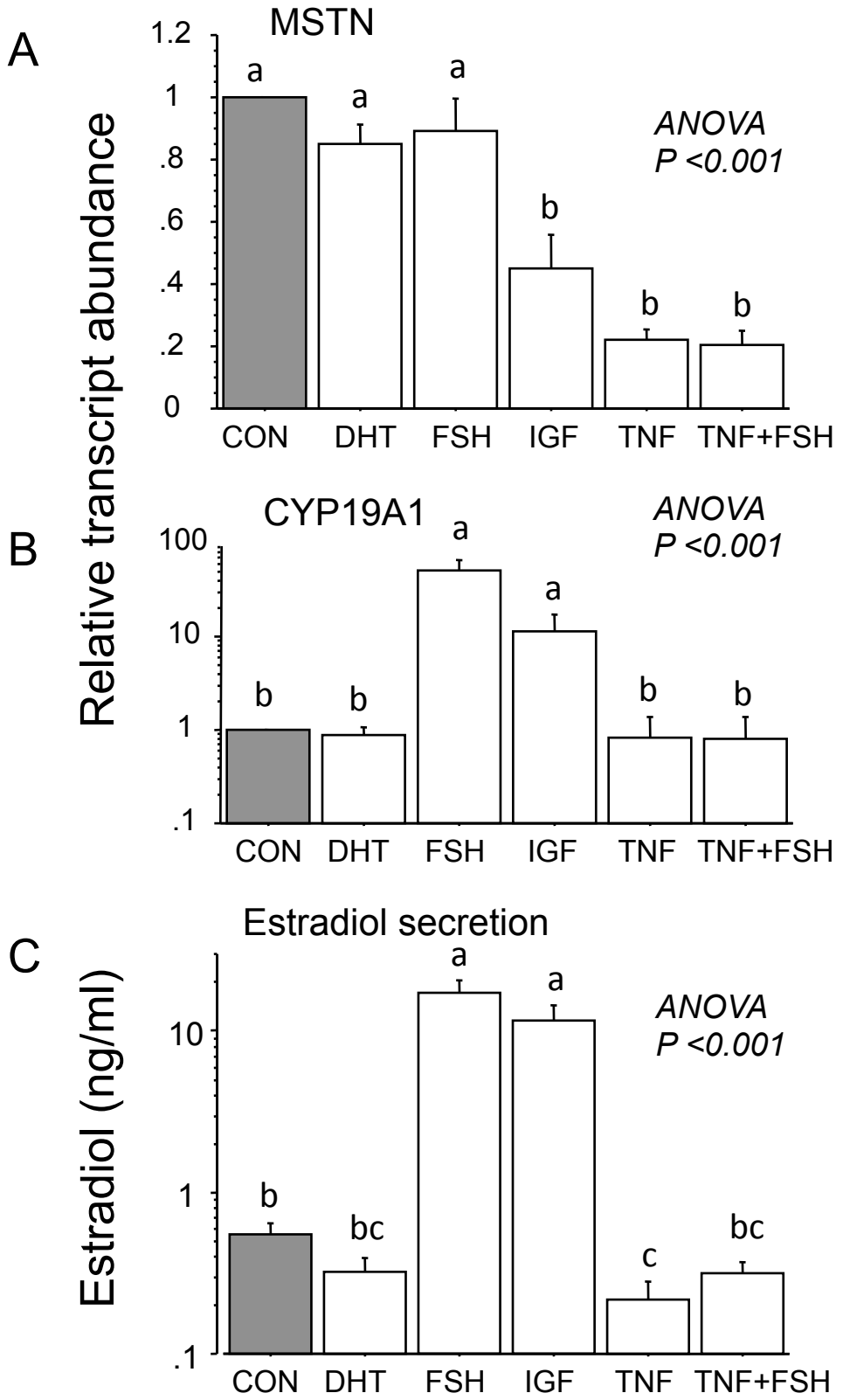

Fig. 6

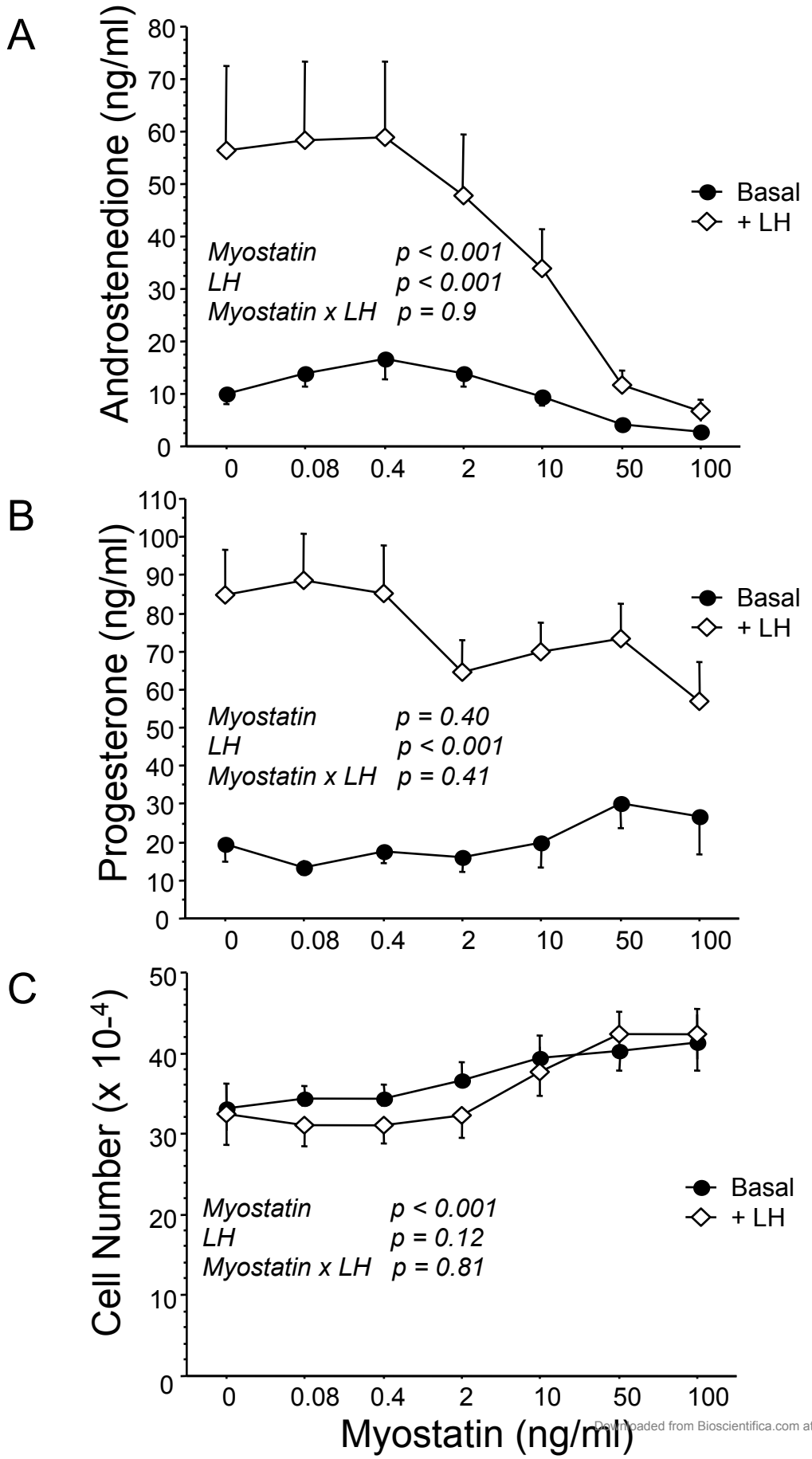

Fig. 7

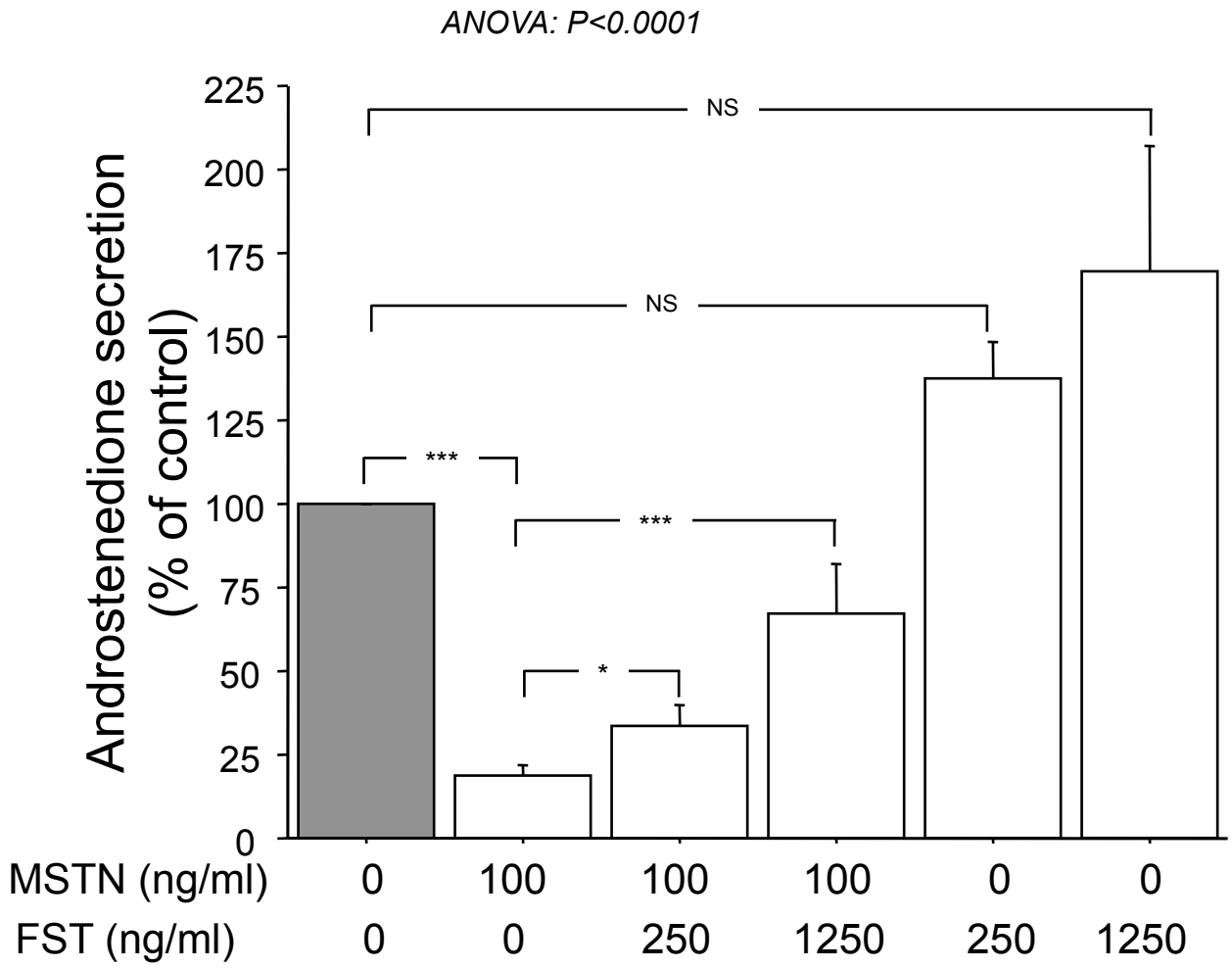

Fig. 8

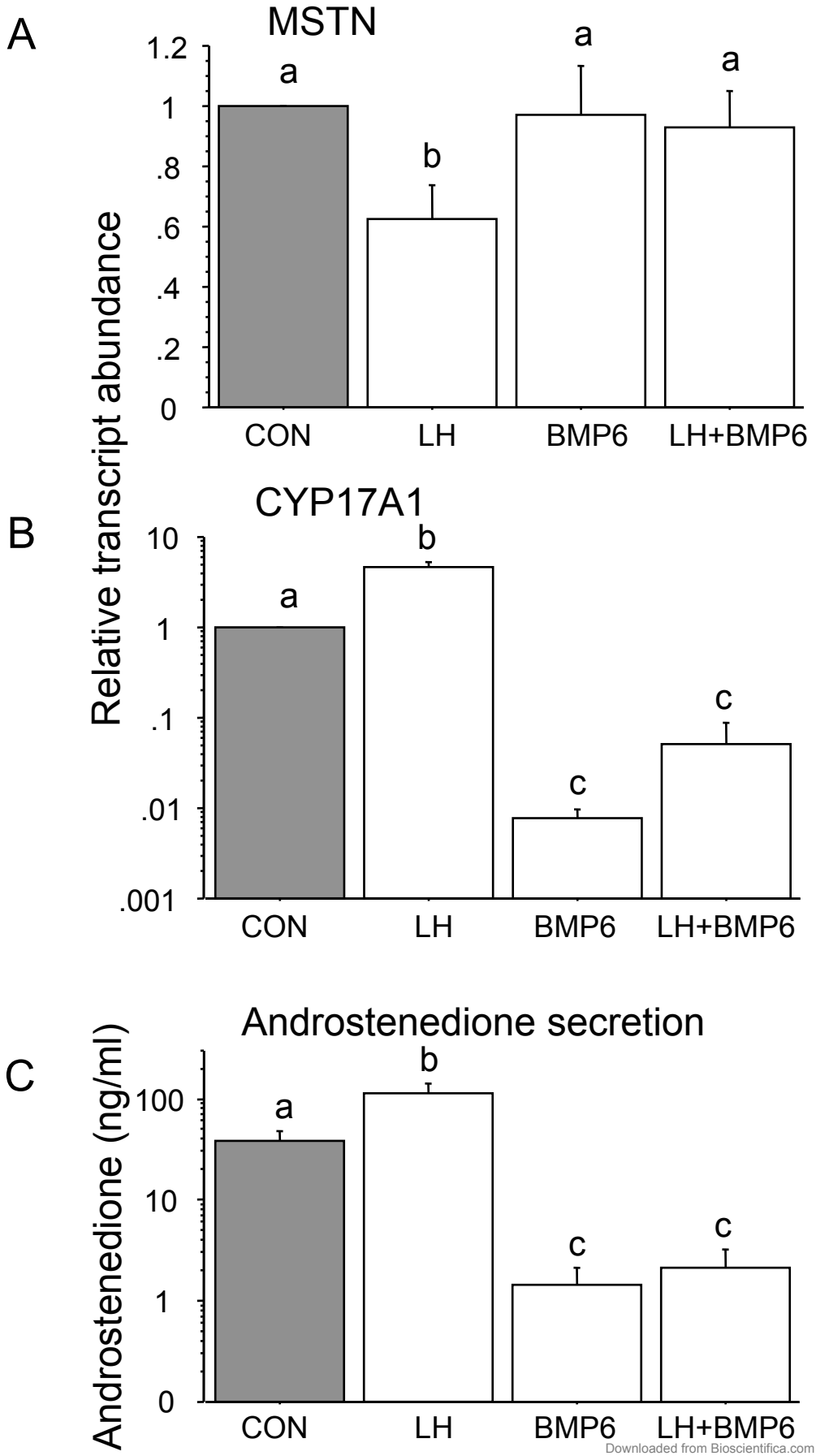

Fig. 9