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Androgen stimulates growth of mouse preantral follicles in vitro: interaction with follicle stimulating hormone and with growth factors of the TGFβ superfamily

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Androgens are essential for the normal function of mature antral follicles but also have a role in the early stages of follicle development. Polycystic ovary syndrome (PCOS), the commonest cause of anovulatory infertility, is characterized by androgen excess and aberrant follicle development that includes accelerated early follicle growth. We have examined the effects of testosterone and dihydrotestosterone (DHT) on development of isolated mouse preantral follicles in culture with the specific aims of investigating interaction with FSH, the steroidogenic pathway and with growth factors of the TGFβ superfamily that are known to have a role in early follicle development.

Both testosterone and DHT stimulated follicle growth and augmented FSH-induced growth and increased the incidence of antrum formation among the granulosa cell layers of these preantral follicles after 72h in culture. Effects of both androgens were reversed by the androgen receptor antagonist flutamide. FSH receptor (*Fshr*) expression was increased in response to both testosterone and DHT, as was that of *Star,* whereas *Cyp11a1* was downregulated. The key androgen-induced changes in the TGFβ signaling pathway were downregulation of *Amh, Bmp15* and their receptors. Inhibition of *Alk6 (Bmpr1b)*, a putative partner for *Amhr2* and *Bmpr2*, by dorsomorphin, resulted in augmentation of androgen-stimulated growth and modification of androgen-induced gene expression.

Our findings point to varied effects of androgen on preantral follicle growth and function, including interaction with FSH-activated growth and steroidogenesis and, importantly, implicate the intra-follicular TGFβ system as a key mediator of androgen action. These findings provide insight into abnormal early follicle development in PCOS.

Introduction

It has long been recognized that androgens have a physiological role in normal ovarian function. Androgens provide an obligatory substrate for estradiol production by maturing antral follicles (and may enhance either basal or FSH-induce steroidogenesis by isolated granulosa cells [1-4]) but there is also evidence that androgens may be necessary for the earliest stages of ovarian follicle development. Mice lacking a functional androgen receptor have impaired fertility (reduced litter size and/or reduced reproductive lifespan) but also show impaired growth and

enhanced atresia of preantral follicles [5-7]. Both aromatizable and non-aromatizable androgens have been shown to stimulate growth of isolated mouse preantral follicles [1] and effect activation of follicle development in fragments of bovine ovarian cortex [8,9].

Exposure to excess androgen, however, is associated with ovarian dysfunction. In experimental animals, androgens inhibit proliferation [10] and increase apoptosis [11] in granulosa cells from mature rat follicles. Importantly, ovarian dysfunction is a major feature of women with polycystic ovary syndrome (PCOS), a very common endocrine disorder in which ovarian hyperandrogenism is the key biochemical feature [12,13]. Infrequent or absent ovulation is characteristic and this is associated with arrest of antral follicles during the final stages of maturation [14]. However, anovulation in PCOS is also associated with aberrant development of preantral follicles, the key features of which are increased activation of follicle growth from the primordial stage [15], enhanced granulosa cell proliferation in small preantral follicles [16] coupled with apparent "stockpiling" of follicles at the primary stage [17]. Thus, ovarian dysfunction in PCOS would seem to have its origins at the earliest stages of follicle development at a point when, under physiological conditions, gonadotropin action is not obligatory and local growth factors are likely to play an important role. Furthermore, the abnormalities observed in preantral follicle development are characterized by enhanced rather than impaired activation and growth and there is evidence that androgens may play a part.

The prenatally androgenized sheep is a well-established large animal model of PCOS. Lambs born to ewes that have been exposed during pregnancy to large doses of exogenous testosterone or dihydrotestosterone (DHT) have both reproductive and metabolic abnormalities that are reminiscent of PCOS [18,19]. Critically, ovarian dysfunction includes not only evidence of disrupted neuroendocrine control of the ovulation cycle but also abnormal preantral follicle development. In particular, the pattern of early follicle development in the ovaries of the PA sheep mirrors that observed in ovarian tissue from women with PCOS; the proportion of growing follicles is increased and the primordial follicle population reciprocally diminished in ovary cortex from PA compared with control animals [20,21].

We have previously used isolated mouse preantral follicles in culture to examine the direct effects, on early developing follicles, of growth factors on follicle growth, granulosa cell proliferation and gene expression [22,23]. Here we have applied this methodology to the investigation of the effects of androgens with the specific aims of investigating the interaction of androgens with FSH and with growth factors of the TGFβ superfamily. These growth factors have a key role in ovarian follicular function and our previous studies of isolated mouse preantral follicles have provided evidence for the involvement of both inhibitory and stimulatory TGFβ molecules (and their endogenous inhibitors and binding proteins) in growth and function of small preantral follicles [22,23].

Material and Methods

Tissue collection, follicle isolation and culture

Whole ovaries were collected from C57BL/6 female mice aged 15-16 and 28 days post partum (dpp, Harlan). Mice were housed in accordance with the Animals (Scientific Procedures) Act of 1986 and associated Codes of Practice. Ovaries were removed, and those from 28 day-old mice were fixed in 10% neutral buffered formalin (Sigma). Preantral follicles were mechanically isolated from day 15-16 mice using acupuncture needles, as previously described [22,23] and placed in Liebovitz L15 medium (Life Technologies Ltd, Paisley, UK) supplemented with 1% (wt/vol) bovine

serum albumin (BSA; Sigma Aldrich Company Ltd, Dorset,UK). Individual follicles were then transferred into a single well (one follicle per well) in a 96 well plate containing 100µl Minimal Essential Medium alpha (MEM-α, Life Technologies) supplemented with 0.1%BSA, 75µg/ml penicillin (Sigma), 100µg/ml streptomycin sulphate (Sigma) and a cocktail of 5µg/ml Insulin, 5µg/ml transferrin and 5ng/ml sodium selenite (Sigma). Isolated follicles from each ovary were distributed randomly (by picking up from a slightly out-of-focus drop of culture medium) and evenly between treatments in a single 96 well plate. Up to 6 ovaries (plates) were cultured per experiment. Follicles were incubated in a humidified incubator in 5% CO_2 at 37°C for up to 72 hours.

Analysis of follicle development
Follicles were photographed daily and follicle area or diameter was measured using ImageJ 1.45s (https://imagej.nih.gov/ij/) at each time point. At the end of culture period, follicles were either snap frozen for expression studies or fixed in formalin for immunofluorescence studies.

During the course of this study images and growth data on 1307 follicles were acquired. These images, taken at 24h intervals, as well as measurements, were imported into a custom-made database (FileMaker Pro 11.v2; http://www.filemaker.com). This allowed automatic calculation of cumulative follicle growth in terms of relative size compared to the start of culture (time 0) for each follicle, as well as follicle diameter. The database also facilitated easy inspection of follicles during development, annotation of morphological features and the implementation of strict inclusion and exclusion criteria. Follicle images were scored by one observer (KH), in a database layout that lacked information about treatment. Follicles with a central spherical oocyte and intact layer of GCs were included in the analysis (Figure 1A). Follicles were excluded from analysis if, at the start of culture, their oocyte was misshapen (Figure 1B, G) or extruded from the follicle, if the follicle was atretic (darkened GCs; Figure 1E, F), or if the basal lamina was damaged with the oocyte being subsequently extruded from the follicle during culture (Figure 1C). Follicles were also excluded from analysis if they died during culture, with oocyte extrusion or the onset of atresia (Figure 1D).

The database also expedited search and export of data for follicles cultured under the same conditions for several further investigations, providing an extended dataset for analysis of developmental and morphological features such as antrum formation, or for comparing development of follicles of differing sizes in various treatments.

Effect of androgens on preantral follicle development
Isolated follicles were cultured in either control medium, or with androgen (either 10nM DHT or 10nM testosterone; Sigma) in the presence and absence of 20µM flutamide (a competitive AR inhibitor; Sigma). DHT, testosterone and flutamide were dissolved in ethanol and the final concentration of ethanol in each treatment and in control wells was 0.1%.

Effect of initial follicle size on responsiveness to DHT
Upon completion of all the culture experiments, the database was mined for follicles cultured under control conditions and in the presence of DHT alone. Follicles were grouped in 5 size 'bins' according to initial diameter at 0h; <100µm, 100-109.99 µm, 110-119.99 µm, 120-129.99 µm, ≥130 µm in diameter. The overall growth of follicles in the presence and absence of DHT was compared using linear regression (Prism 6 for Mac OS X, version 6.0a (www.graphpad.com).

Effect of DHT on AR expression

Mechanically isolated follicles cultured for 24 or 72h in the presence or absence of 10 nM DHT were formalin-fixed, set in 2% (w/v) low melting point agarose (Sigma), before being dehydrated through an alcohol series, embedded in wax and serially sectioned (5µm) onto Superfrost charged slides (VWR, Lutterworth, UK). Immunofluoresence detection of AR was carried out as described below, and one central section of each follicle selected for analysis. Confocal images of individual follicles were imported into ImageJ, and split into RGB channels. Blue DAPI labeled nuclei were thresholded (same threshold value for all follicles) to produce a binary image (white DAPI positive, black DAPI negative) and define the nuclei. Nuclei were outlined using the 'Analyze Particles' command. In the green channel, AR labeling was thresholded (with the same threshold maintained for all follicles), the nuclear outlines were overlaid, and the area of labeling exceeding the threshold measured within the nuclear outlines, and for the GC layer as a whole. The proportion of nuclear area or GC area that exceeded the threshold (ie the proportion of the nuclei, or GC layer, that was AR positive) was compared for control and DHT-treated follicles at 24 and 72h.

Antrum formation

The database was further used for investigating antrum formation in the presence and absence of DHT, and under other treatment conditions. An antrum was clearly visible as a translucent area with a clear margin in the granulosa cells. Images were inspected and annotated for appearance of an early antrum by 72h of culture.

Effect of androgen and FSH on preantral follicle growth

Follicles were treated with control medium (including 0.1% ethanol vehicle), or with 10nm DHT and/or 10ng/ml FSH (recombinant human follicle stimulating hormone (rhFSH) (National Hormone and Peptide Program, National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK), Dr A.F. Parlow, Torrance, CA, USA). Follicles were maintained in culture for 24, 48 or 72 hours before being frozen for gene expression studies as previously described. Images of each individually cultured follicle were taken at 0, 24, 48 and 72 h, as appropriate.

Effect of inhibiting Alk6 on DHT action

Dorsomorphin (DSM, Sigma) was used as an inhibitor of the type I TGFβ receptors ALK2, ALK3 and ALK6 [24]. Follicles were cultured with vehicle alone, 10nM DHT alone, DHT and 1µM DSM [23], and DSM alone for 72 h. Follicle growth was assessed as described above. At the end of culture, follicles were snap frozen in liquid nitrogen for gene expression studies, as described below.

Expression studies

At the end of each appropriate culture period, approximately 5 follicles per treatment group were pooled into one tube and snap frozen in liquid nitrogen. Samples were then processed for RNA extraction using RNeasy microcolumns as per manufacturer's instructions (Qiagen, Manchester, UK). The resulting RNA was concentrated using vacuum centrifugation to provide a final volume of approximately 5µl and converted to cDNA using SuperScript III reverse transcriptase and random hexamers, according to manufacturer's instructions (Life Technologies). cDNA products were used for quantitative PCR as previously described [23]. Briefly, primers were designed using Primer3 plus v2.3.6 software (Table 1). cDNA or water (control) was added to a reaction mix that included 500nM of appropriate primers, ROX, KAPA SYBR FAST (KAPA Biosystems Limited, London, UK) and nuclease free water. Each sample or control mix was plated in duplicate and amplification was

carried out using an Applied Biosystems 7900HT Fast instrument. A melt curve analysis was carried out on each sample. *Atp5b* (Primer Design, Southampton UK) was determined to be an appropriate internal reference gene and therefore used in this series of studies. Expression levels were normalized to the internal reference gene and calculated as fold changes relative to the untreated control group, using the 2-delta-delta-cycle threshold $(2\text{-}\Delta\Delta\text{ct method})$ [25].

Immunofluorescence studies

Whole formalin-fixed, paraffin embedded ovaries were serially sectioned (5µm) to examine AR protein expression. For examination of the effect of treatment on AR protein expression, cell proliferation and apoptosis, formalin-fixed cultured follicles were set in 2% (w/v) low melting point agarose (Sigma), before being dehydrated through an alcohol series, embedded in wax and serially sectioned (5µm) onto Superfrost charged slides (VWR, Lutterworth, UK). Ovary and follicle sections were dewaxed and rehydrated prior to boiling in citrate buffer (10mM citric acid, pH6.0), and blocking of nonspecific binding with in 20% normal goat serum (NGS, Sigma). After this, with no washing step, sections were incubated in rabbit anti-mouse androgen receptor antibody NR-20 (sc-816, 1:200 dilution, Santa Cruz, Dallas, Texas), rabbit anti-mouse D3B5 Ki67 (1:200; New England Biolabs, Hitchin, UK) or rabbit anti-mouse cleaved caspase-3 (1:200; 9664, Cell Signaling) diluted in 2% NGS. The latter were subsequently exposed a 1:9 ratio of TUNEL enzyme:label (Roche) for 60 min at 37°C after PBS washes. The negative control for AR immunofluorescence was rabbit IgG (Sigma i8140) at the same concentration as that used for the primary antibody (0.5 µg/ml). The primary antibodies were incubated overnight at 4°C and then detected using an appropriate goat anti-rabbit Alexafluor secondary antibody (1:500; Life Technologies). All unstained nuclei were then visualized by incubation with DAPI (1:1000; Life Technologies) before sections were mounted under coverslips using Prolong Gold mounting medium containing DAPI (Life Technologies). Sections were visualized (x63 objective) and captured using a Leica inverted SP5 confocal laser-scanning microscope (Leica Microsystems).

Ki67-positive, caspase-positive, TUNEL positive and unlabeled DAPI stained cells or nuclei, were counted using the ImageJ plugin 'cell counter.jar' (https://imagej.nih.gov/ij/plugins/cell-counter.html) within ImageJ (1.45s). DAPI-labelled (blue), Ki67-positive (green), TUNEL-positive (green) nuclei, or caspase-positive (red) cells were counted in a single mid-section of the follicle, and the proportion of Ki67-positive, caspase-positive or TUNEL-positive nuclei/cells calculated.

Statistics

The relative area of follicles in different treatments (area at time_x/area at time_0, where $t_x = 24, 48$ or 72) was compared at each timepoint using one-way ANOVA with the appropriate post-hoc test for multiple comparisons (Prism 6). Gene expression values were $\log_2$ transformed and difference between treatments analysed using t-test. In all cases, a probability value (*P*) less than 0.05 was considered statistically significant.

Results

Follicle Culture

In this study, a total of 1307 follicles from 45 ovaries were cultured in various conditions. On average, 29 ± 0.68 (mean and SEM; range 22-40) follicles per ovary were cultured, and evenly distributed between treatments. Overall, less than 15% of follicles placed in culture were found, upon closer high power inspection, to be either

atretic or damaged at the outset (Figure 1), and were therefore excluded from further analysis. A very small number of follicles (12) died during culture; 4 controls, 3 in cultured in DHT alone and 5 in DHT+FSH.

Effect of androgens on preantral follicle growth

Dihydrotestosterone at doses of between 1 and 100 nM resulted in an increase in follicle growth compared with vehicle alone at 24, 48 and 72h in culture (Figure 2A). Although the largest response was achieved with a dose of 100 nM, there was little difference between the effects of the different doses. A similar dose-response range was found for the effects of testosterone on follicle growth (data not shown). In all subsequent studies a dose of 10 nM DHT or testosterone (approximating to physiological serum concentrations of testosterone) was therefore used.

Flutamide inhibited DHT-induced follicle growth in a dose-dependent manner (Figure 2B) but as the effect was significant only at a flutamide concentration of 20μM, this dose was used in further studies

The effects of DHT (a non-aromatisable androgen) and testosterone (an aromatisable androgen) were compared. Both androgens significantly increased follicle growth over 72 hours in culture (Figure 2C). At all time points DHT and testosterone did not significantly differ in their stimulatory effect on follicle growth. The addition of flutamide to androgen treated follicles inhibited the effect of both androgens and brought follicle growth back in line with controls.

We were interested in whether the responsiveness of preantral follicles to DHT changed with follicle size. Taking an overview of all the data that we collated in the database during these studies, we explored how DHT affected the growth of follicles of different sizes. Isolated follicles placed in culture ranged in size from 75 µm to 150 µm in diameter. Intriguingly, while smaller follicles grew significantly more in the presence of DHT, there was no significant increase in the growth of larger follicles (>130µm) with DHT. This suggests that these follicles have become less androgen responsive (Figure 2D).

Expression of AR in whole mouse ovary and in cultured follicles

Immunofluorescence for AR showed expression in follicles from the primordial stage onwards in day 17 ovary (Figure 3A). In primordial and transitional follicles AR appeared to be expressed in the ooplasm more strongly than at later stages, and even in very early follicles, AR was clearly present in GCs (Figure 3C).

Immunofluorescence labeling of AR in follicles cultured in the presence and absence of DHT for 24 and 72h was quantified in the GC layer, as well as specifically in the nuclei (Figure 3 D-M). DHT treatment resulted in significantly increased expression of AR in the GCs following 24h (Figure 3N), and 72h (Figure 3P) of culture. Furthermore, translocation of AR to the nucleus was minimal in control follicles (Figure 3H), but was significantly increased in follicles exposed to DHT at both time points (Figure 3M, O, Q). Strong labeling of theca cell nuclei for AR was also observed following DHT treatment (Figure 3L, M, white arrows).

DHT and antrum formation

Examination of 219 follicles cultured under control conditions and 216 follicles cultured with DHT alone that were identified from the database showed that significantly ($P<0.0001$) more follicles cultured in DHT developed an antrum during 72h of culture (Figure 4A-G). We expected that this would be due to the increased follicle growth observed in the presence of DHT, and that antra would be seen in the largest follicles. Surprisingly, we found that there was no significant difference in diameter between follicles with and without an antrum in the presence of DHT

(Figure 4H), suggesting that this developmental change is not due to accelerated growth but to another, as yet unknown, mechanism.

The stimulatory action of DHT on follicle growth was accompanied by increased protein expression of the proliferation marker Ki67 in granulosa cells of preantral follicles following both 24 and 72h of culture (Figure 5 A-D). Follicles were assessed after 24 and 72h of culture for evidence of apoptosis using dual labelling for TUNEL (Figure 5 E, F) and active caspase -3 (Figure G, H). There was no significant difference between control and DHT-treated follicles in the incidence of these apoptotic markers. Generally, apoptosis was observed in a small proportion of granulosa cells at 24h (Figure 5 I, J) and 72h (Figure 5 M, N), with the exception of a few follicles showing more widespread TUNEL labeling (Figure 5 K, L).

Interaction of DHT with FSH
FSH (10 ng/mL) significantly increased the proportionate growth of cultured preantral follicles compared to control. When DHT was added to the cultures, the combination of DHT and FSH increased follicle growth in comparison to either individual treatment alone after 24 and, particularly, after 48 and 72 hours in culture (Figure 6A). Interestingly, we observed that, after 72 hours in culture with the combined treatment of DHT and FSH (but not with the individual treatments alone), the basal lamina surrounding the granulosa cell layers in some follicles was disrupted, allowing extrusion some granulosa cells vertically as well as horizontally, and thereby possibly decreasing the precision of follicle measurement.

Overall, the rate of growth of follicles in the presence of both DHT and FSH was significantly greater than that in either treatment alone. Furthermore, follicles in treatments alone or in combination grew at a significantly faster rate than control follicles (Figure 6B). The dose of FSH used here was the lowest dose that elicited a maximal response in a previous study [26]. We were interested to explore whether the presence of DHT would allow a lower dose of FSH to stimulate the same maximal response. This was not the case; lower doses of FSH resulted in decreased growth (Fig 6C).

Effects of androgens and FSH on gene expression

FSH receptor, steroid synthesis and action
FSH receptor (*Fshr*) expression was significantly increased in follicles cultured in DHT compared to vehicle-treated controls after 24h in culture whereas FSH treatment reduced expression of *Fshr*. This effect was partially reversed during co-treatment with DHT (Figure 6D). DHT alone had a small and insignificant effect on expression of *Star* but further enhanced FSH-stimulated *Star* expression.

We examined the time course of gene expression of androgen receptor (*Ar*), *Fshr*, *Star, Cyp11a1, Cyp17* and *Cyp19* during 72h of culture in presence of DHT (Figure 7). Androgen receptor expression was consistently suppressed by DHT whereas that of *Fshr* was increased at each time point. Expression of *Star* was significantly upregulated after 72h in culture whereas that of *Cyp11a1* was significantly reduced after 48h in culture and further reduced at 72h (Figure 7). *Cyp19* expression was undetectable, as was that of *Cyp17*.

Effect of DHT and testosterone on TGFβ ligands and receptors
In this series of studies we examined the effects of either DHT or testosterone on gene expression after 24h in culture (Figure 8). Expression of *Ar* was reduced and that of *Fshr* increased by DHT, as shown previously; similar changes were seen in response to testosterone.

We observed a significant interaction of androgens with ligands and receptors of the TGFβ superfamily in these studies (Figure 8). Exposure to androgens during culture resulted in reduction of expression of *Amh* and *Bmp15* by both DHT and testosterone. A similar trend was observed for *Gdf9* but the change was not significant in response to DHT. Testosterone, but not DHT, reduced expression of *Amhr2* and both androgens reduced *Bmpr2* expression. Expression of the type 1 receptors *Alk3, Alk4 and Alk5* were similarly reduced by both androgens but, again, only the actions of testosterone were significant. However both DHT and testosterone stimulated expression of *Alk6*. All androgen-induced changes in gene expression were negated by co-incubation with flutamide (data not shown).

To explore the functional significance of androgen-induced changes in gene expression of TGFβ ligands and their receptors, we examined the effects of the Alk2/3/6 inhibitor dorsomorphin (DSM) [24] (Fig 9A) on follicle growth and gene expression in the presence and absence of DHT. As shown in our previous study [23], DSM alone stimulated follicle growth at 24h (P<0.05) but here we show that the response to DHT was significantly enhanced by the addition of DSM (Figure 9B). DSM did not, however, influence DHT-dependent antrum formation.

DSM alone resulted in reduction in expression of *Amh* after 72h in culture but with no significant effect on *Gdf9* (Figure 9C). However, DSM further enhanced the inhibitory effect of DHT on *Amh* without influencing DHT-induced *Gdf9* expression. Neither DSM alone nor DSM with DHT had an effect on expression of *Bmp15*. DSM alone had no effect on *Amhr2* but led to increased expression of *Alk5* and, as anticipated, reduction in *Alk6* (Fig 9E). The addition of DHT resulted in greater production of *Amhr2* than either DSM or DHT alone and reversed the effects of DSM on *Alk5* and *Alk6* (Figure 9E).

There was no significant effect of DSM alone on *Ar* or *Fshr* and neither did DSM affect the response to DHT (Figure 9D). However, DSM alone enhanced expression of *Star* and further increased DHT stimulated expression (Figure 9F). Interestingly DSM alone led to an increase in *Cyp11a1* and reversed the inhibitory response to DHT (Figure 9F).

Discussion

The purpose of these studies was to examine the role of androgens in preantral follicle development to provide insight into the mechanism of aberrant early follicle development in human polycystic ovary syndrome (PCOS). Exposure to excess androgen has been postulated to underpin the probable developmental origin of PCOS and its adult manifestations of reproductive and metabolic dysfunction [14,27,28]. Using a mouse model of preantral follicle development that has been well validated in our laboratory, we have shown that exposure to both DHT and testosterone have a plethora of effects on both follicle growth and gene expression of key molecules implicated in follicle function. As shown in previous studies of cultured isolated follicles from mouse ovary [1,9], androgens stimulate follicle growth. This effect is mirrored in studies of prentral follicle development in the primate ovary following in vivo androgen treatment [29,30]. In addition to the increase of follicle diameter in response to androgens, our results show a significant increase in expression of the proliferation marker Ki67 whereas the expression of pro-apoptotic markers, TUNEL and caspase, was no different between treated and untreated follicles. Furthermore, there was a 10-fold increase of antrum formation, an index of accelerated growth, in the presence of DHT. Thus, although androgens have been shown to have pro-apoptotic effects in granulosa cells of large antral follicles [10,11], the predominant

action of testosterone and DHT in preantral follicles appears to be stimulation of granulosa cell proliferation and net follicle growth.

In sections of juvenile mouse ovary, we found that AR was expressed in the cytoplasm of oocytes, GCs and theca at all preantral stages, including primordial follicles. DHT treatment increased the overall expression of AR in cultured follicles, and, importantly, increased nuclear localization of AR, as would be expected in relation to androgen induced growth and differentiation. Increased expression of AR protein was interesting and paradoxical, as we have also demonstrated here that *Ar* <u>mRNA</u> is decreased by DHT treatment during culture.

With the exception of the specific experiment exploring the effect of DHT in the presence of FSH, all the follicles in this study were cultured without FSH. Use of the database allowed examination of the response of follicles of increasing size to DHT alone. Small follicles less than 130μm in diameter grew more in the presence of DHT. This is in contrast to our observations of the effects of FSH on preantral follicle growth in culture, in that responsiveness to added FSH was greatest in follicles larger than 130μm in diameter [26] leading us to conclude that follicles larger than 130μm in diameter had a greater requirement for FSH. It may be that the lack of response to DHT in larger follicles in the current study is due to the need for FSH to grow in vitro.

An important observation is the positive interaction with FSH in terms of both follicle growth and gene expression. Both FSH (as previously shown [26]) and DHT, independently, stimulated growth of preantral follicles in culture but FSH increased the responsiveness to DHT in a dose-dependent manner. After 72 hours in culture, the optimum dose of FSH induced a 30% increase in follicle growth above that of DHT alone. The amplifying effect of androgens on FSH-stimulated steroidogenesis was described more than 20 years ago by Wang and Greenwald [4] and recently Sen and colleagues have shown that DHT has both anti-apoptotic and pro-proliferative effects, the latter being mediated, at least in part, by increased FSH receptor expression [31]. Here we confirm the effects of DHT on FSH receptor gene expression and, in addition, show that testosterone has a similar effect. It is not clear whether the increase in the expression of *Fshr* is due to increased expression per cell or to the increased number of cells in follicles cultured in the presence of androgens (or indeed both). The action of both androgens was reversed by the androgen receptor antagonist flutamide, indicating that the effect of testosterone was not likely to be attributable to its possible conversion to estradiol. The most significant effect of androgens on the steroidogenic pathway was an increase in expression of *StAR*, which, of course, has a key role in making cholesterol available for steroid synthesis. Both DHT and FSH independently and interactively increased StAR expression. Conversely, expression of *Cyp11a1* was suppressed by exposure to androgens but these findings are in accord with observations in studies of the ovaries of day 90 fetal sheep whose mothers were treated with testosterone [18]

Very little has been reported to date on the interaction of androgens and the TGFβ family of growth factors. There are isolated reports that TGFβ1 inhibits androgen production by theca cells [32,33] but, as far as we are aware, there have been no systematic studies of the effects of androgens on TGFβ signaling. Our group has previously reported on expression and action of TGFβ growth factors in isolated mouse preantral follicles [22,23] and in the current study we examined the interaction of androgen with TGFβ ligands and their receptors. The most significant findings were inhibition by androgens of *Amh* and *Bmp15* expression. AMH has a well-described inhibitory action of preantral follicle growth [34] and BMP15 has a biphasic effect,

initially stimulating growth but then leading to reduced growth rate and apoptosis in mouse preantral follicles after 48h in culture [23]. In our studies, AMH expression was progressively reduced with time of exposure to androgens. Studies in sheep support this observation, AMH protein expression is lower in large preantral follicles in 10 and 21 month month old prenatally testosterone treated ewes, compared to untreated controls [35]. AMH and BMP15 activate receptor heterodimers that comprise the type 2 receptors AMHR2 and BMPR2 respectively and the type1 receptor Alk6 that is utilized by both ligands. Androgen-stimulated follicle growth is accompanied by reduced expression not only of *Amh* and *Bmp15* but also of *Amhr2* and *Bmpr2*. In addition, DSM, an inhibitor of Alk2/3/6 amplified DHT-stimulated follicle growth and further reduced expression of *Amh*. Taken together, these findings support the view that attenuation of the inhibitory effects of AMH and BMP15 play a role in the mechanism of androgen stimulation of preantral follicle development. There were also significant effects of DSM on the steroidogenic pathway in that, along with enhanced follicle growth, DSM amplified expression of key molecules involved early in the ovarian steroidogenic pathway, *Star* and *Cyp11a* (and modified the effects of DHT). This suggests a functional cross-talk between the TGFβ□system and the classic steroidogenic pathways. DSM has previously been described as an inhibitor of AMP kinase but although we cannot rule out the possibility that this mechanism may play a part in the observed effects on follicle growth, its high selectivity for the BMP signaling pathway [24] makes the latter a more plausible target.

   In addressing the issue of cross-talk between these pathways, one of the key findings in the current study was that, at 24h in culture, DHT treatment resulted in decreased expression of the oocyte specific gene *Bmp15* (as well as leading to decreased expression of *Amh* and *Amhr2*). These changes were accompanied by increased expression of *Star* and downregulation of *Cyp11a*. In this context, it is striking that direct treatment of human granulosa lutein cells and ovine GCs with rhBMP15 results in opposite effects; increased *AMH*, *AMHR2* and *CYP11A*, and decreased *STAR* [36,37,19]. This strongly suggests that BMP15 is a major regulator of both the rate limiting steps of steroidogenesis and of AMH and its receptor, and furthermore that DHT is having indirect actions on these genes via the consequent decreased levels of this oocyte derived growth factor. Indeed, the important role of the oocyte in stimulating AMH expression was first proposed in 2004 in elegant studies using co-cultures of isolated oocytes and preantral GCs from mice [38]. The presence of one or more oocytes in the immediate vicinity of GCs increased AMH expression more than 2-fold. Furthermore, studies in our laboratory comparing AMH expression in intact preantral follicles and follicles lacking an oocyte have shown that the absence of an oocyte results in almost complete loss of AMH expression after 24 hours of culture (Mora and Fenwick, unpublished data). Thus, the effect of DHT (or testosterone) on follicle growth appears to involve a complex interaction with both FSH and the intra-ovarian TGFβ system (Fig 10). These androgens have a direct stimulatory effect on growth and also increase FSH responsiveness by up-regulation of FSH receptor expression. In addition, DHT and testosterone reduce expression of AMH both directly and by inhibition of the oocyte-derived TGFβ growth factor, BMP15. BMP15 has been shown to stimulate AMH production by granulosa cells (and reduce expression of the growth-promoting GDF9 by the oocyte), hence reduction of BMP15 levels will contribute to reduced AMH levels. The net effect of androgens is therefore to contribute to granulosa cell proliferation by reversing the action of inhibitory TGFβ growth factors that are produced by the oocyte and surrounding granulosa cells. The current study has examined gene expression of these

key ovarian factors and it should, of course, be acknowledged that the changes observed do not necessarily imply similar effects on protein expression (as illustrated in this study with regard to AR gene and protein expression). Nevertheless, where we see evidence of functional changes (eg androgen-dependent amplification of both *Fshr* gene expression in parallel with increased follicle growth rate) the association between gene and protein expression can easily be implied.

An intriguing effect of DHT on preantral follicle development was the precocious formation of an antrum, a feature that has also been reported in studies of prenatally androgenized sheep [39]. Initially it was thought to be due to the stimulatory effects of DHT on follicle growth, with the expectation that antra would be present in the largest follicles. However we found that there was no difference in the diameter of follicles with and without antra, suggesting that their appearance was due to effects of androgen on the expression of key genes, rather than achievement of a threshold diameter. Aquaporins facilitate water transport and are expressed in granulosa cells [40], and testosterone stimulated the swelling of porcine GCs in hypotonic culture medium, an effect that was reversed by flutamide, suggesting that aquaporins are sensitive to androgen [41]. A recent study by the same group confirmed that follicles from pigs that were exposed to flutamide in utero expressed less *Aqp5* mRNA and protein, demonstrating that androgen could potentially stimulate aquaporin expression leading to antrum formation [42].

Aberrant ovarian function associated with PCOS and hyperandrogenism has been well documented and it has been suggested that "programming" of ovarian function by androgens plays a key part [27]. This notion is supported by the results of studies of both rodent and large animal (sheep and monkey) models of PCOS that involve in vivo exposure to excess androgen [7,19,28,43]. Here, in an ex vivo model, we have examined the direct effects of androgens on follicle growth and gene expression and have uncovered pathways that are relevant to the abnormalities of follicle development that are characteristic of PCOS [14]. The enhancement of FSH activated follicle growth may well have a bearing not only on the accelerated growth of preantral follicles but also on the precocious responsiveness to LH that has been observed in small antral follicles in ovaries of women with PCOS [14,44]. Intriguingly, androgen action also implicates the participation of key members of the TGFβ☐superfamily and its receptors and there is little doubt that these growth factors have a fundamental role in activation and maintenance of early follicle development.

Acknowledgements

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None of the authors has any conflict of interest to declare

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Figure 1   Morphology of manually isolated follicles that were included in the analysis (84%; A), or excluded from analysis because of damage or atresia at the start of culture (<15%; B, C, E, F, G), or onset of atresia during culture (<1%; D). (A) Healthy follicle cultured under control conditions (72h) with a central oocyte (O) and even layer of granulosa cells (GCs). Follicle diameter shown in each image. The white dotted line (A''') indicates the basal lamina surrounding the GCs and shows the area that is measured. A nucleolus is arrowed (white arrow head). Note the lack of theca cells on the basal lamina. (B) Follicle with indistinct oocyte (white arrow) that becomes increasingly misshapen and shrunken with time. (C) Follicle with damaged basal lamina (black arrow), resulting in extrusion of GCs (white arrow). With time in culture the oocyte is extruded through the breach (black arrow). (D) Follicle that appears healthy at 0h but becomes atretic from 24h, with rounding up and progressive darkening of the GCs and loss of the basal lamina. (E, F) Atretic follicles at the start of culture (0h) with a detached basal lamina (black arrows) and darkened GCs. (G) Follicle with collapsing, indistinct oocyte (white arrow, 0h). (H) Sparse theca cells (black arrows) on basal lamina. Scale bars are 50 µm (A-G) and 25 µm (H).

Figure 2   Follicle growth in vitro in response to DHT and testosterone. (A) Response of individual follicles to increasing concentrations of DHT. The relative area of follicles in different treatments (area at time$_x$/area at time$_0$, where t$_x$ = 24, 48 or 72) was compared at each time point using one-way ANOVA with a Tukey's multiple comparisons test. *P<0.05, ***P<0.001, ****P<0.0001. Values are mean and 95%CI. Numbers in parentheses are numbers of follicles. (B) Response of follicles to DHT (10nM) in the presence of increasing doses of the AR inhibitor, flutamide. Values are mean and 95%CI. Statistical analysis as in (A). *P<0.05,

**P<0.01, ****P<00001. (C) Response of follicles to DHT (10nM) or testosterone (10nM) in the absence and presence of the flutamide (20µM). DHT- and T-stimulated follicle growth was reversed by flutamide. Values are mean and 95%CI. Statistical analysis as in (A). *P<0.05, **P<0.01, ***P<0.001, ****P<00001. (D) Effect of DHT on in vitro growth of follicles of different initial sizes. Values are means and SD, lines are regression slopes. Slopes were compared using linear regression. Follicles larger than 130µm in diameter at the start of culture did not grow more in the presence of DHT.

Figure 3   Androgen receptor (AR) is present in oocytes, GCs and theca cells of preantral follicles, and overall expression and nuclear localization of AR increases in the presence of DHT. (A) AR expression (green) in mouse prepubertal mouse ovary on day 17 postpartum. DAPI-labelled nuclei are blue. Scale bar = 50µm. White dotted lines indicate basal lamina surrounding follicles. GC = granulosa cell; T = theca; ose = ovarian surface epithelium; p = primordial; t = transitional; e1°= early primary; 1°+ = primary stage with second layer of GCs appearing; 2° = secondary stage. (B) Rabbit IgG control. Scale bar = 25 µm. (C) Strong labeling of ooplasm (white arrow) and GC cytoplasm (white arrowhead) in primordial (p) and transitional (t) follicles. Scale bar is 25µm. (D-M) Quantification of AR in formalin-fixed, paraffin-embedded sections of follicles cultured in the absence (D-H) and presence (I-M) of DHT. (D, I) Confocal image of follicle showing expression of AR (green) and DAPI labeled nuclei (blue). Scale bars are 25µm, white dotted box defines enlarged images (E-H, J-M). Images were imported into ImageJ, and split into RGB channels. Blue DAPI labeled nuclei (E, J) were thresholded (F, K; where white is DAPI positive and black DAPI negative), and nuclei were outlined using the 'Analyze Particles' command (F, K). In the green channel, AR labeling (G, L) was thresholded (with the same threshold maintained for all follicles), the nuclear outlines were overlaid, and the area of labeling exceeding the threshold measured within the nuclear outlines (H, M), and for the GC layer as a whole. Yellow arrows (H) indicate nuclei with little nuclear AR, and yellow arrow heads (M) indicate nuclei with strong nuclear AR. White arrowheads (L, M) show strong AR expression in theca cell nuclei. Values were plotted and compared between treatments using a Mann-Whitney test. Overall AR protein expression in the GC layer was significantly increased after 24h (N) and 72h (P) culture in the presence of DHT. Nuclear localization of AR was also significantly increased in the presence of DHT at 24h (O) and 72h (Q).

Figure 4   Follicles cultured in the presence of DHT had a 10-fold increased incidence of early antrum formation. (A) The number of follicles in the absence and presence of DHT that formed an antrum by 72h of culture. Numbers above the bars are the % of follicles that formed an antrum. P<0.0001; two-sided Fisher's Exact test. (B - F) Examples of preantral follicles with an antrum (arrowed, translucent area within the GCs), cultured in DHT. (G) Confocal image of DHT treated follicle exposed to DHT in vitro with oocyte (ooc) and antrum arrowed (H) Diameter of follicles at 48h (ie at the onset of possible antrum formation) that did, or did not, have an antrum at 72h. Four statistical comparisons were made (to compare diameter of follicles with and without antra within a treatment; and of follicles with, or without, antra between treatments), using one-way ANOVA with a Sidak's multiple comparison test. ***P<0.001, ns = not significant.

Figure 5 Follicles cultured in the presence of DHT had a greater proportion of Ki67-positive (green) nuclei than in control conditions after 24h (A), and 72h (B) hours of culture. Bars are means and 95% CI; treatments were compared using an unpaired t-test, **P<0.01. (C, D) Confocal images of Ki-67-positive nuclei (green); scale bars are 50µm. (E – H) There was no significant effect of treatment on TUNEL (green E,F) and caspase (red G,H) expression in control and DHT-treated follicles at 24 and 72h. Bars are medians; treatments were compared using a Mann-Whitney test. (I - G) Confocal images of TUNEL labeled (green) and active caspase-positive GCs (red) in follicles at 24h (I, J) and 72h (K-N) cultured in the absence (I,K,M) and presence (J,L,N) of DHT. Nuclei were counterstained with DAPI (blue). Scale bars are 25µm (I-N).

Figure 6 FSH further stimulates DHT-stimulated follicle growth after 48h of culture (A). Relative area of follicles in different treatments was compared at each time point using one-way ANOVA with a Tukey's multiple comparisons test. *P<0.05, **P<0.01, ***P<0.001, ****P<00001. Values are mean and 95%CI. Numbers in parentheses are numbers of follicles. (B) Growth trajectory over time of follicles cultured in the absence and presence of DHT and/or FSH. A combination of DHT and FSH stimulates a greater rate of growth than DHT alone (P<0.0001), FSH alone (P<0.0001) or control (P<0.0001). FSH or DHT alone stimulates a greater rate of growth than control (FSH, P<0.0001; DHT, P<0.002). The rate of growth in FSH alone is significantly greater than that in DHT alone (P<0.0004). Values are means and SEM, lines are regression slopes. Slopes were compared using linear regression. Numbers in parentheses are numbers of follicles. (C) Follicles cultured in the presence of DHT and increasing doses of FSH showed significant growth enhancement in the presence of 1ng/ml and 10 ng/ml FSH, but not in the lowest dose of FSH (0.1ng/ml). Statistical comparisons as in (A). (D) Change in expression of *Fshr* and *Star* in 4 samples of 5 follicles each cultured for 24h in the presence of DHT alone, FSH alone, or DHT and FSH in combination. Data have been $\log_2$ transformed and treatments were compared to control (0), and between treatments using an unpaired t-test. Fold change indicated in right-hand y axis. Asterisks by error bars indicate significant difference in expression from control, differences between treatments indicated by asterisks on horizontal lines, P values as in (A). Values are mean and SEM.

Figure 7 Time-course of hormone receptor and steroidogenic enzyme gene expression in isolated follicles during 72h of culture in the presence of DHT. Change in expression of *Ar*, *Fshr1*, *Star* and *Cyp11a1* in 4 - 6 samples of 5 follicles each cultured for 24, 48 and 72h in the presence of 10 nM DHT. Data have been $\log_2$ transformed and DHT treatment was compared to time matched control samples (0), and between time points using an unpaired t-test. Fold change is indicated in right-hand y axis. Asterisks by error bars indicate significant difference in expression from control, differences between time points are indicated by asterisks on horizontal lines, *P<0.05, **P<0.01, ***P<0.001, ****P<00001. Values are mean and SEM.

Figure 8 Effect of DHT (A) and Testosterone (B) on gene expression of hormone receptors, and members of the TGFβ superfamily (ligands and receptors). Isolated follicles were cultured in the presence and absence of DHT or Testosterone for 24h. Data have been $\log_2$ transformed and DHT or testosterone treatment was compared to control samples (0) using an unpaired t-test. Fold change is indicated on

right-hand y axis. Asterisks by error bars indicate significant difference in expression from control, *P<0.05, **P<0.01, ***P<0.001. Values are mean and SEM.

Figure 9   Effect of Dorsomorphin (DSM, an inhibitor of Alk 2, 3 and 6) on DHT-stimulated follicle growth in vitro, for 72h. (A) TGFβ receptor inhibition by DSM. (B) Isolated follicle growth in the presence and absence of DHT and/or DSM. Relative area of follicles in different treatments was compared at each time point using one-way ANOVA with a Tukey's multiple comparisons test; *P<0.05, **P<0.01, ***P<0.001, ****P<00001. Values are mean and 95%CI. Numbers in parentheses are numbers of follicles. (C-F) Effect of DHT alone, DSM alone and DHT in combination with DSM on gene expression following 72h of culture. Data have been $\log_2$ transformed and DHT, DSM or DHT+DSM treatment was compared to control samples (0) using an unpaired t-test. Fold change is indicated in right-hand y axis. Asterisks by error bars indicate significant difference in expression from control, differences between treatments indicated by asterisks on horizontal lines; *P<0.05, **P<0.01, ***P<0.001, ****P<00001. Values are mean and SEM. (C) TGFβ ligands. (D) Hormone receptors. (E) TGFβ receptors. (F) Steroidogenic enzymes.

Figure 10   Putative pathways of androgen action on preantral follicle growth. Testosterone or DHT act via the androgen receptor, causing a measurable increase in granulosa cell proliferation. This may be mediated directly, or indirectly by increased FSHR (stimulating GC proliferation) or decreased AMH (reducing AMH inhibition). AMH can be further reduced by androgen-induced reduction of oocyte-specific BMP, which normally stimulates AMH levels. Inset box summarizes androgen-induced inhibition of TGFβ ligands, and type I and II TGFβ receptors, with the exception of *Alk6*.

Table 1: Primers used for quantitative PCR assays.

Gene Symbol	Primer sequence (5'-3')	GenBank Accession	Product Size (bp)
Amh	Fwd: ggggcacacagaacctct	NM_007445.2	124
	Rev: gcaccttctctgcttggttg		
Bmp15	Fwd: gagaaccgcacgattggag	NM_009757.4	134
	Rev: agttcgtatgctacctggtttg		
Gdf9	Fwd: tcacctctacaataccgtccgg	NM_008110.2	139
	Rev: gagcaagtgttccatggcagtc		
Fshr	Fwd: acaactgtgcattcaacggaac	NM_013523.3	187
	Rev: gacctggccctcaacttctt		
Ar	Fwd: attcctggatgggactgatg	NM_013476.4	246
	Rev: gcccatccactggaataatg		
Star	Fwd: aagaacaacccttgagcacct	NM_011485.4	267
	Rev: ctccctgctggatgtaggac		
Cyp11a1	Fwd: ctgggcactttggagtcagt	NM_019779.3	185
	Rev: aggacgattcggtctttcttc		
Amhr2	Fwd: acagcatgaccatatcgttcg	NM_144547.2	122
	Rev: gagtcaagtagtggcataaggag		
Bmpr2	Fwd: actgggaggtgtttatgaggtg	NM_007561.4	150
	Rev: ggaacttgggtctctgcttct		
Alk3 (Bmpr1a)	Fwd: tgactttagcaccagaggatacc	NM_009758.4	177
	Rev: cagagccttcatacttcatacacc		
Alk4 (Acvr1b)	Fwd: caacatgaagcactttgactcc	NM_007395.3	177
	Rev: tcacatacaacctttcgcatct		
Alk5 (Tgfbr1)	Fwd: attgctccaaaccacagagtag	NM_009370.3	157
	Rev: caccaatagaacagcgtcgag		
Alk6 (Bmpr1b)	Fwd: aggaggatggagagagtacagc	NM_007560.4	166
	Rev: ccagaggtgacaacaggcatt		

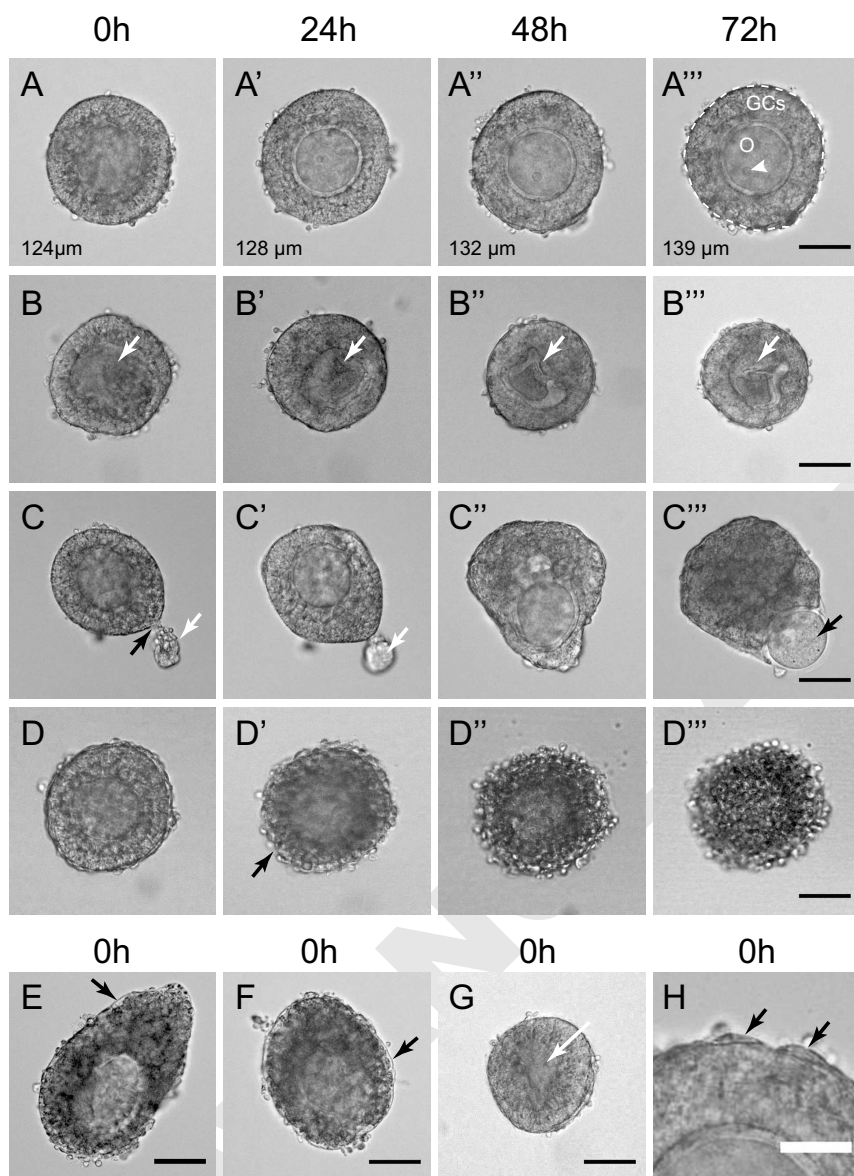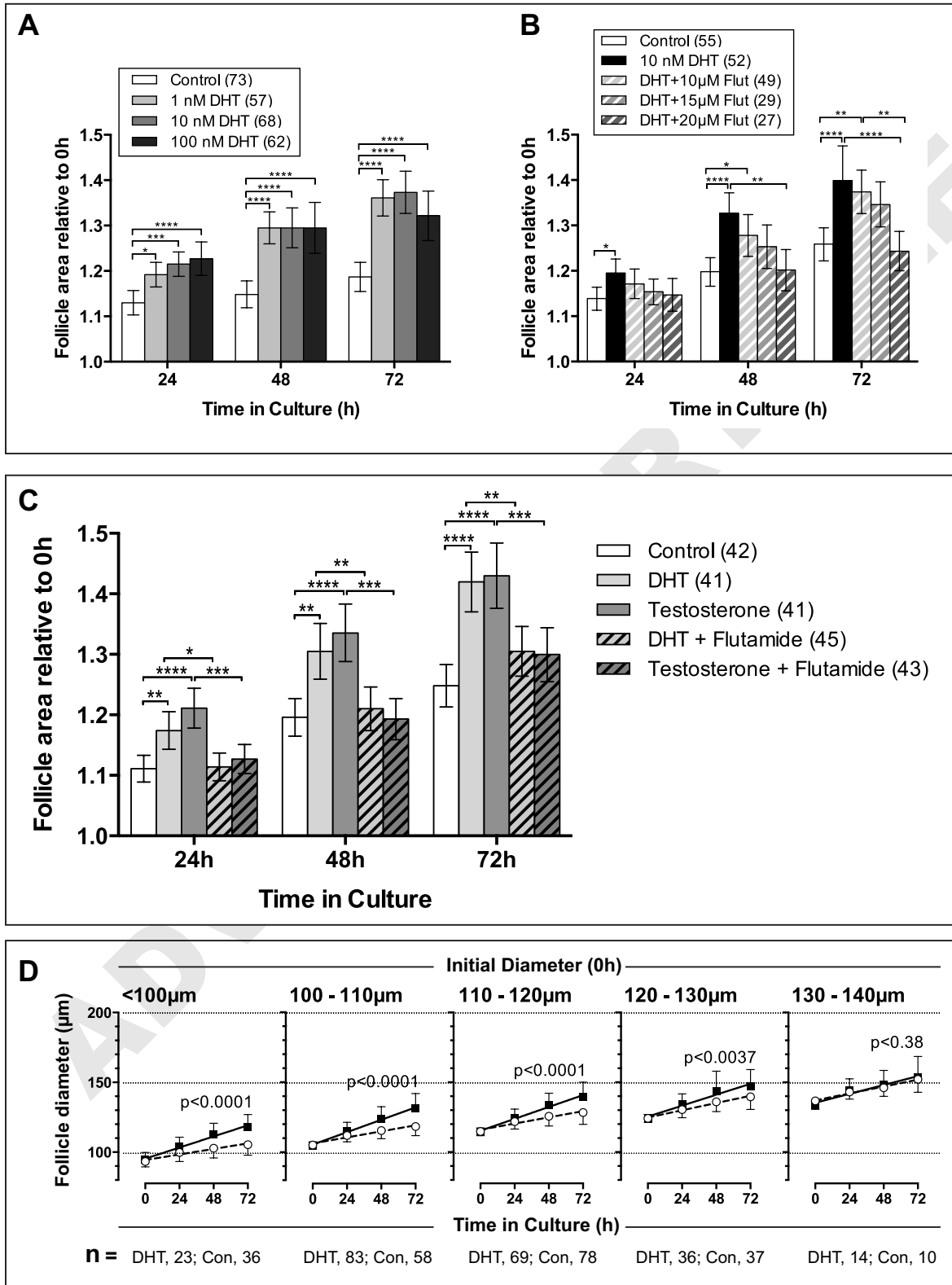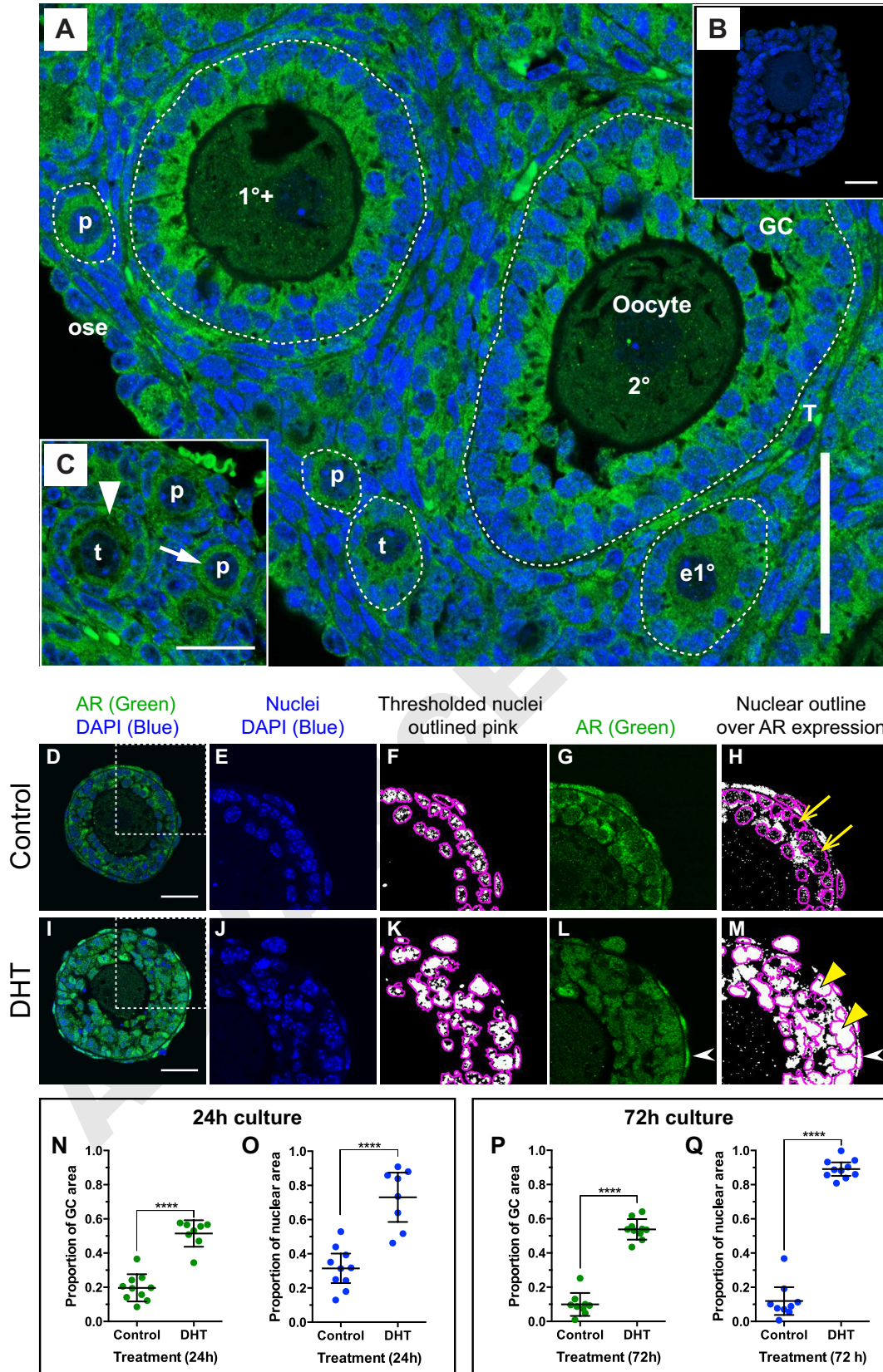| Peptide/Protein Target	Antigen Sequence	Name of Antibody	Manufacturer, Catalog No., RRID No.	Species Raised in Monoclonal or Polyclonal	Dilution Used	RRID
Androgen receptor	Epitope mapping at N-terminus	AR (N-20)	Santa Cruz Biotechnology, sc-816	Rabbit, polyclonal	1 in 200	AB_1563391
Ki67	Recombinant protein specific to the amino terminus of Ki-67 protein.	Ki-67 (D3B5) (Mouse Preferred; IHC Formulated)	Cell Signaling Technology, 12202	Rabbit, monoclonal	1 in 200	AB_2620142
Cleaved caspase-3	Large fragment (17/19 kDa) resulting from cleavage adjacent to Asp175	Cleaved caspase-3 (Asp175) (5A1E)	Cell Signaling Technology, 9664	Rabbit, monoclonal	1 in 200	AB_2070042

Figure 1

	0h	24h	48h	72h
A	124μm	A' 128 μm	A'' 132 μm	A''' GCs O 139 μm
B		B'	B''	B'''
C		C'	C''	C'''
D		D'	D''	D'''
	0h	0h	0h	0h
E		F	G	H

Figure 2

Figure 3

A 1°+   p   ose   GC   Oocyte   2°   T   p   t   e1°

B

C p   t   p

AR (Green) DAPI (Blue)   Nuclei DAPI (Blue)   Thresholded nuclei outlined pink   AR (Green)   Nuclear outline over AR expression

Control   **D**   **E**   **F**   **G**   **H**

DHT   **I**   **J**   **K**   **L**   **M**

N 24h culture — Proportion of GC area — Treatment (24h) — Control, DHT — ****

O Proportion of nuclear area — Treatment (24h) — Control, DHT — ****

P 72h culture — Proportion of GC area — Treatment (72h) — Control, DHT — ****

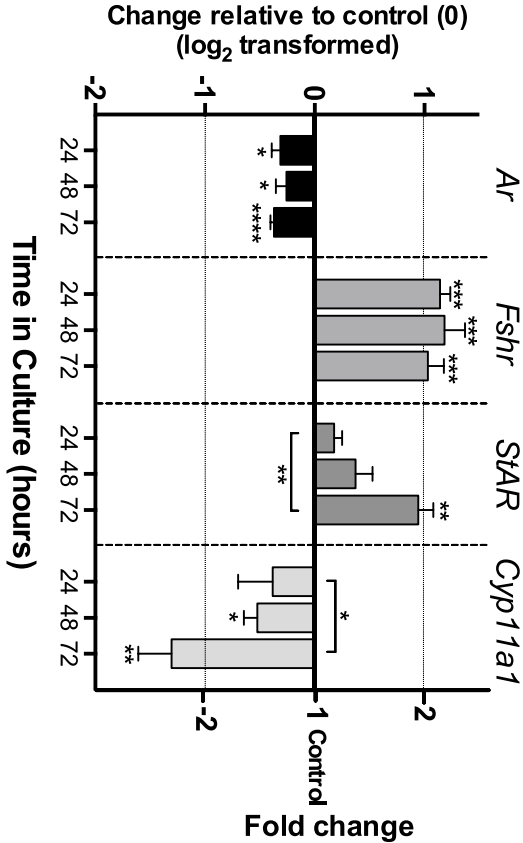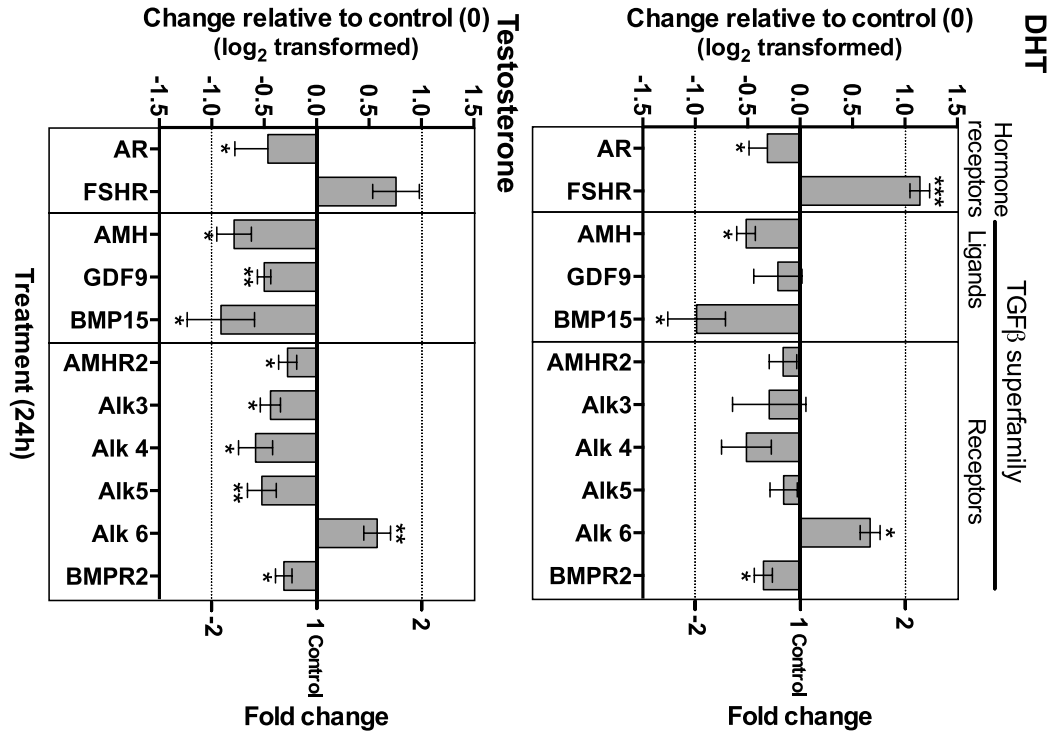**Q** Proportion of nuclear area — Treatment (72 h) — Control, DHT — ****

Figure 4

Figure 5

Figure 6

Figure 7

Figure 8

Figure 9

Figure 10