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## Enhanced Thecal Androgen Production Is Prenatally Programmed in an Ovine Model of Polycystic Ovary Syndrome

Kirsten Hogg, Julia M. Young, Elizabeth M. Oliver, Carlos J. Souza, Alan S. McNeilly, and W. Colin Duncan

Medical Research Council, Centre for Reproductive Health, The University of Edinburgh, The Queen's Medical Research Institute, Edinburgh EH16 4SA, United Kingdom

One of the hallmarks of polycystic ovary syndrome (PCOS) is increased ovarian androgen secretion that contributes to the ovarian, hormonal, and metabolic features of this condition. Thecal cells from women with PCOS have an enhanced capacity for androgen synthesis. To investigate whether this propensity is a potential cause, rather than a consequence, of PCOS, we used an ovine prenatal androgenization model of PCOS and assessed ewes at 11 months of age. Pregnant Scottish Greyface ewes were administered 100 mg testosterone propionate (TP) or vehicle control twice weekly from d 62 to 102 of gestation, and female offspring (TP = 9, control = 5) were studied. Prenatal TP exposure did not alter ovarian morphology or cyclicity, or plasma androgen, estrogen, and gonadotropin concentrations, at this stage. However, follicle function was reprogrammed *in vivo* with increased proportions of estrogenic follicles ( $P < 0.05$ ) in the TP-exposed cohort. Furthermore, *in vitro* thecal cells of follicles ( $>4$  mm) secreted more LH-stimulated androstenedione after prenatal androgenization ( $P < 0.05$ ), associated with increased basal expression of thecal *StAR* ( $P < 0.01$ ), *CYP11A* ( $P < 0.05$ ), *HSD3B1* ( $P < 0.01$ ), *CYP17* ( $P < 0.05$ ), and *LHR* ( $P < 0.05$ ). This provides the first evidence of increased thecal androgenic capacity in the absence of a PCOS phenotype, suggesting a thecal defect induced during fetal life. (*Endocrinology* 153: 450–461, 2012)

Polycystic ovary syndrome (PCOS) is the most common endocrine disorder in young women (1). Increased ovarian androgen production is a PCOS trait that is manifest clinically and contributes to the metabolic dysfunction and ovarian phenotype (1–3). According to the 2003 Rotterdam consensus, the criteria for the diagnosis of PCOS include the presence of hyperandrogenemia, a polycystic ovary morphology on ultrasound, and chronic anovulation (4). Polycystic ovaries are characterized by a polyfollicular phenotype, in which there is increased recruitment to, or persistence of, small antral-stage follicles (5) and there is an associated increased secretion of LH that contributes to enhanced ovarian androgen production (3).

The etiology of PCOS is not understood and although there are features of heritability (6), a robust genotype has not been identified (7). There is increasing evidence that

the adult PCOS phenotype is programmed in fetal life (8, 9). Exposure of a female fetus to increased androgen concentrations *in utero* results in ovarian, hormonal, and metabolic characteristics of PCOS in the adult (8, 10). Since features of PCOS occur in congenital virilizing disorders (11), it is likely that this experimental paradigm can inform us about the molecular mechanisms involved in the development of PCOS in women. This PCOS-like model has been developed in a number of species including the sheep (10, 12).

Evidence suggests that theca cells from women with PCOS function differently. Thecal cell androstenedione is increased in women with PCOS (3), which is augmented by increased expression of thecal LH receptor (*LHR*) (13) and intrinsic alterations in thecal steroidogenic gene expression (13, 14). Global enhancement of the steroido-

genic pathway is found in thecal cells of PCOS women, with up-regulation of *StAR* (13), *CYP11A*, and *CYP17* (13, 14) and increased enzyme activity of 17 $\alpha$ -hydroxylase, 3 $\beta$ -hydroxysteroid dehydrogenase (HSD), and 17 $\beta$ -HSD (14). Because hyperandrogenemia is a key etiological agent for many of the clinical features of PCOS, this is a key finding. However, these observations are present in thecal cells from women already manifesting the ovarian, hormonal, and metabolic features of PCOS. It is therefore not known whether enhanced thecal androgen production is a cause or consequence of PCOS. Thecal cells are recruited from stromal tissue surrounding follicles (15), which express androgen receptors and thus could be primed in fetal life during ovarian development. Alternatively, enhanced thecal androgen production could be manifest only in adult life as a result of the associated alterations in the metabolic and gonadotropin environment in women with PCOS.

In an ovine model of PCOS, maternal exposure to testosterone propionate (TP) from d 30 to d 90 of a 147 d gestation results in ovarian, hormonal, and metabolic features of PCOS (16–19), and ovarian cyclicity is absent by the second breeding season (20). There is also masculinization of the external genitalia (21, 22). This is avoided when TP exposure is commenced at d 60, although the PCOS-like features are more subtle (23, 24). We hypothesized that young adult ewes exposed to TP during mid-gestation would be predisposed to PCOS-like features that are not yet clinically manifest. This would allow assessment of the thecal cell with a reduced number of confounding factors.

The objective of this study was to characterize ewes at 11 months of age whose mothers had been treated with TP from d 62 to d 102 of gestation and compare these with control animals. We assessed the following: 1) ovarian morphology, the number of different classes of follicles, and follicular dynamics; 2) systemic hormone profiles and ovarian function; 3) thecal cell androgen production from individual follicles *in vivo* and *in vitro* in the presence or absence of exogenous LH; and 4) basal thecal cell expression of genes important for steroidogenesis and growth and development.

The results show that thecal cells from follicles matched in size and developmental stage in TP fetal-exposed ewes with normal endocrine and metabolic profiles have increased androgen synthesis, indicating that enhanced thecal androgen production is preprogrammed in fetal life.

## Materials and Methods

### Reagents

All reagents and chemicals were obtained from Sigma-Aldrich (Poole, UK) unless otherwise stated.

### Animal treatment and collection of tissue

All experiments were conducted under a UK Home Office project license after local ethical committee approval. Scottish Greyface ewes and Texel rams were mated and pregnancies were evenly and randomly divided between control and treatment groups. Pregnant ewes were administered 100 mg TP [1 ml im; AMS Biotechnology (Europe) Ltd., Abingdon, UK], in vegetable oil, twice weekly from d 62 to d 102 of a 147 d gestation. Controls (C; 1 ml im) were treated with vehicle only. The resulting females (TP = 9, C = 5) were killed at the end of the first breeding season (March) at 11 months of age. Before the animals were killed, estrous cycles were synchronized by the administration of 125 mg prostaglandin estrumate (im; Schering Plough Animal Health, Welwyn Garden City, UK) twice, 11 d apart, and animals were killed approximately 24 h after the second prostaglandin injection to ensure they were in the follicular phase. Both ovaries were immediately collected and weighed. One ovary was processed for histological assessment, whereas the other was immediately dissected for tissue culture and gene expression studies.

### Histology and stereological assessment of ovarian sections

One ovary from each animal was fixed in Bouins solution for 24 h and transferred to 70% ethanol before embedding in paraffin wax. The ovaries were serial sectioned (5  $\mu$ m), and every 20th section was stained with hematoxylin and eosin (H&E). All primordial, primary, preantral, and antral follicles were counted in at least eight sections from every ovary. Follicles were categorized into developmental stage according to standard classifications (25, 26). Counting was performed blindly and the average number of follicles per section for each developmental stage classification was calculated for each animal and these values used for statistical analysis.

### Immunohistochemistry and immunohistochemical assessment

Tissue sections (5  $\mu$ m) were processed for immunohistochemistry as previously described (27). Primary antibodies used were Ki67 (1:200; mouse monoclonal, NCL-Ki67-MMI; Novocastra Laboratories, Newcastle, UK) and cleaved caspase-3 (1:100; rabbit polyclonal, Asp175 no. 9961; Cell Signaling Technology Inc., Danvers, MA). The same method for follicle counting was used to count the number of antral follicles that were immunopositive for Ki67 and cleaved caspase-3. The average number of immunopositive and immunonegative follicles per section was calculated for each animal, and this value used in the proportional analysis.

### Collection of follicular fluid and thecal cell culture

One ovary from each animal was placed in a dish containing PBS and cut in half. Individual antral follicles were carefully dissected using scissors and fine forceps under a dissecting microscope and cleaned as described previously (28). The diameter (millimeters) of each individual follicle was recorded. Each follicle was transferred to an individual well containing 1 ml PBS in a 24-well plate, the follicle was cut in half to release the follicular fluid, and the granulosa cells were scraped out. The thecal shell was removed and the PBS containing follicular fluid and granulosa cells was separated by centrifugation at 400  $\times$  g for 10 min at room temperature. The fluid was collected and stored at  $-20$  C.

The thecal shell was peeled from the inside of the follicle shell and cut into two similar-size pieces. Half was immediately placed in 250  $\mu$ l RNAlater (Applied Biosystems, Carlsbad, CA) and stored at  $-20$  C, whereas the other half was cultured in 24-well plates, initially in 1 ml medium 199 for 1 h, and then transferred to media containing 100 ng/ml LH (ovine LH R-23; National Institute of Diabetes and Digestive and Kidney Diseases, Bethesda, MD) and cultured for 2 h at 37 C and 5% CO<sub>2</sub>. This concentration has been shown to maximally stimulate theca cell function (29). After the culture period, media was stored at  $-20$  C.

### Follicular and media hormonal measurements

Well-established and reported specific in-house assays were used to measure sex steroids by direct assay of serum extracts (30). Assay specificity was indicated by low cross-reactivity of known steroids that are structurally related. A RIA for androstenedione was performed in the follicular fluid and the culture media collected from the thecal cell cultures as described previously (29, 30). Briefly, a rabbit antiandrostenedione (1:320,000; 07-109216; MP Biomedicals, Illkirch, France) and a <sup>125</sup>I-radio-labeled androstenedione tracer (MP Biochemicals) were used with a donkey antirabbit secondary antibody (1:60; T022; Diagnostics Scotland, Edinburgh, UK) in normal rabbit serum (1:600; MP Biomedicals). The intra- and interassay coefficients of variation (CV) were 4 and 7.5%, respectively, and assay sensitivity was 10 pg/ml. For androstenedione measurements in media, concentrations were expressed as picograms per milliliter and in follicular fluid as nanograms per follicle after conversion based on initial dilution and follicle diameter.

An in-house ELISA was used to measure estradiol in ovine follicular fluid. Ninety-six microwell plates were precoated with donkey antish-eep IgG (1:200; 713-005-147; Jackson ImmunoResearch, Newmarket, UK) and stored overnight at 4 C in a humidity chamber. On d 2, coating buffer was discarded and the plate washed thoroughly with wash buffer (deionized water + 0.1% Tween 20). Estradiol antibody (in-house; ASMR32) was added to each well and incubated for 3 h at room temperature. The liquid was discarded and the plate washed repeatedly. The dilution of follicular fluid ranged from 1:2 to 1:100. In-house estradiol tracer conjugated to horse radish peroxidase was incubated with the sample for 2 h at room temperature, followed by washing steps. Substrate consisting of 0.1 M citrate phosphate buffer (pH 5.0), 0.03% (vol/vol) hydrogen peroxide and 0.1% (wt/vol) *o*-phenylenediamine dihydrochloride was added to each well. The plate was incubated for 30 min at room temperature in the dark and the reaction stopped with the addition of concentrated sulfuric acid diluted 1:10 in deionized water, and spectrophotometry was performed at 495 nm. The intra- and interassay CV were 6.7 and 10%, respectively, and assay sensitivity was 30 pg/ml. Estradiol values less than (<)30 ng/follicle were regarded as low and those follicles were classed as nonestrogenic. Estrogenic follicles were determined by values of, or greater than ( $\geq$ )30 ng/follicle.

### Follicular phase hormonal measurements

Progesterone was measured by RIA weekly over a 5-wk period from approximately 9 months of age, during the first breeding season, to determine ovulation frequency. The primary antibody used was rabbit anti-11 $\alpha$ -hydroxyprogesterone (1:6000; PROG-no. 51C; Pantex, Santa Monica, CA). The intra- and interassay CV were 8 and 10%, respectively, and assay sensitivity

was 400 pg/ml. Progesterone values greater than (>)1 ng/ml were considered representative of ovine luteal concentrations and therefore indicative of an ovulation (31, 32).

Thereafter estrous cycles were synchronized as described above, and serial blood sampling was conducted for a 6-h period commencing at 1000 h, on d 2 of the estrous cycle, and LH concentration was measured at 10-min intervals using an in-house LH RIA, as previously described (28, 33), using reagents supplied by the National Institute of Diabetes and Digestive and Kidney Diseases (ovine LH R-23) and a specific rabbit anti-ovine LH antiserum ASMR29 (28). The average value over all time points (37 in total) was calculated to give an overall representation of LH concentration. Peaks were defined if the LH concentration was >1 ng/ml and were approximately one third greater or more of that of the two preceding LH values, in line with previously published studies in the sheep (34). FSH concentration was similarly measured by in-house RIA (35) in samples collected at the beginning and end of serial sampling to give a representative FSH profile. Corresponding LH values at these time points (1000 and 1600 h) were used to calculate the LH to FSH ratio. The intra- and interassay CV for LH and FSH assays were <10% and assay sensitivity was 200 pg/ml and 1 ng/ml, respectively.

Steroid hormone measurements were also conducted. Androstenedione was measured by RIA as described above and treated in the same way as the culture media samples. Testosterone was measured by RIA, for which the primary antibody used was rabbit antitestosterone (1:600,000; R3S07-259; AMS Biotechnology, Oxfordshire, UK). The intra- and interassay CV were 4 and 8%, respectively, and assay sensitivity was 15 pg/ml. Peripheral estradiol concentrations were measured using the MAIA estradiol ELISA kit (37001; Inverness Medical UK, Cheshire, UK) as per the manufacturer's instructions, in which the intra- and interassay CV were 6 and 9%, respectively, and assay sensitivity was 0.3 pg/ml.

### Quantitative real-time PCR (qRT-PCR)

mRNA was extracted from the thecal cells stored in RNAlater (Applied Biosystems) using the RNeasy microkit (QIAGEN Ltd., Crawley, UK) with RNeasy MiniElute spin columns (QIAGEN), according to the manufacturer's instructions, and stored at  $-80$  C. Good-quality mRNA was obtained from individual follicles that were 3 mm or greater in diameter, and thecal gene expression was analyzed. RNA concentration and purity was measured using a NanoDrop 1000 spectrophotometer (Fisher Scientific Ltd., Loughborough, UK), and cDNA was synthesized from 100 ng RNA using the SuperScript VILO cDNA synthesis kit (Invitrogen, Paisley, UK) and stored at  $-20$  C.

Primers were designed for qRT-PCR (Table 1) using Primer3 Input version 0.4 software (Primer 3, Rozen S. and H. J. Skaltsky; <http://frodo.wi.mit.edu/primer3/>) and products validated by DNA sequencing. SYBR Green qRT-PCR was carried out as previously described (36), using reagents from Applied Biosystems. Gene expression was analyzed relative to internal house-keeping gene *GAPDH* and was quantified using the  $\Delta$ CT method.

### Statistical analyses

Values that were  $P < 0.05$  were considered statistically significant. For the analysis of follicle number, hormonal assessment and qRT-PCR gene expression experiments, in which the

**TABLE 1.** List of gene names and corresponding accession numbers for which forward and reverse primers were designed and PCR amplicon size

Gene (accession no.)	Forward sequence	Reverse sequence	Product size (bp)
<i>StAR</i> (NM_001009243)	GCATCC TCAAGACCAGGAG	CTTGACACTGGGGTTCCACT	194
<i>CYP11A</i> (NM_001093789.1)	CAACGTCCCTCCAGAAC TGT	CAGGAGGCAGTAGAGGATGC	172
<i>HSD3B1</i> (NM_001135932)	GGAGACAT TCTGGATGAGCAG	TCTATGGTGTCTGGTGTGGA	200
<i>CYP17</i> (NM_001009483)	AGACATAT TCCCTGCGCTGA	GCAGCTTTGAATCCTGCTCT	215
<i>LHR</i> (NM_214449.1)	TCCGAAAGCTTCCAGATGTT	GAAATCAGCGTTGTCCCAT	199
<i>AR</i> (XM_001253942)	GCCCATCT TTTCTGAATGTCC	CAAACACCATAAGCCCCATC	233
<i>ERβ</i> (NM_001009737.1)	GAGGCC TCCATGATGATGTC	GGTCTGGAGCAAAGATGAGC	208
<i>INHA</i> (NM_174094)	GAGCCCGAGGACCAAGATGTCTCC	CCTCAGCCTTCCAGCATCTGGC	91
<i>INHBA</i> (NM_174363)	GAAGAGACCCGATGTCACCCAGC	TGTCGTCTCTATCTCCACGTACCCG	113
<i>INHBB</i> (NM_176852)	ATGGCC TGGCCTGCTCCCG	CTTCAGGTAGAGCCACAGGCTGGC	101
<i>INSR</i> (XM_002688832.1)	CACCATCACTCAGGGGAAAC	CAGGAGGTCTCGGAAGTCAG	247
<i>IGF1R</i> (XM_002696504.1)	CCAAAACCGAAGCTGAGAAG	TCCGGGTCTGTGATGTTGTA	199
<i>GAPDH</i> (NM_001034034)	GGCGTGAACCACGAGAAGTATAA	AAGCAGGGATGATGTTCTGG	229

differences between the mean values from C and TP cohorts were compared, a Student's unpaired *t* test assuming equal variances was used. In the instances in which data were not normally distributed, a Mann-Whitney nonparametric test was alternatively used. An ANOVA followed by *post hoc* analysis (Bonferroni's multiple comparison test) was used to detect alterations in androstenedione secretion over increasing follicle size. A paired *t* test was performed to assess the effect of thecal cell LH response within treatment groups. Values are presented as mean  $\pm$  SEM. Proportional assessment of follicle growth and atresia dynamics and estrogenic status were subjected to a  $\chi^2$  test. These values are presented as a percentage.

## Results

### The effect of prenatal androgenization on ovarian morphology in adolescent sheep

There was no difference in the average ovarian weight between control animals ( $0.83 \pm 0.08$  g) and those exposed to increased prenatal androgens ( $1.09 \pm 0.09$  g;  $P > 0.05$ ), and overall body weights of animals at the time the animals were killed were not different (C,  $45.2 \pm 3.7$  vs. TP,  $46.8 \pm 2.2$  kg). Histological examination of H&E-stained ovarian sections did not reveal clear organizational differences between ovaries from control or TP-exposed offspring (Fig. 1A). Stereological assessment of follicle number and stage of development showed no differences in the numbers of primordial (Fig. 1B), primary, preantral, or antral follicles (Fig. 1C) as a result of prenatal androgenization. Whole ovarian follicular dissection also showed no differences in the numbers of antral follicles ( $\geq 2$  mm) per ovary between control and prenatally TP-exposed ewes (Fig. 1D). Immunohistochemical analysis of antral follicle cell proliferation and cell death was conducted using markers for the activated cell cycle (Ki67) and cell apoptosis (cleaved caspase-3). Prenatal androg-

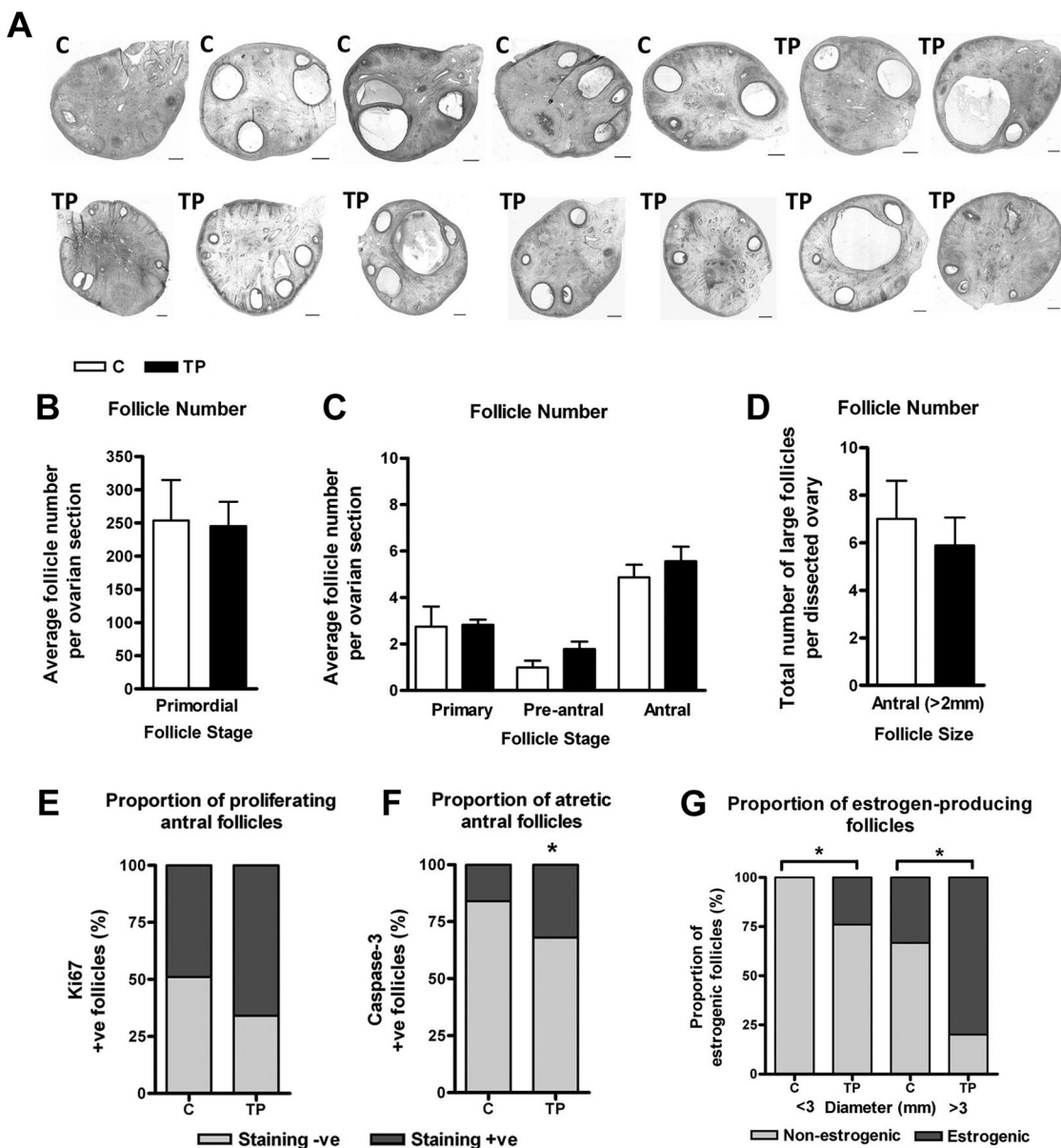
enization was not associated with increased proliferating follicles (Fig. 1E); however, it did result in a small increase in follicle cell death ( $P < 0.05$ ; Fig. 1F). Follicle fluid estradiol concentrations were also measured as an indication of follicle health. Assessment of the control cohort showed maturational differences in estradiol synthesis in follicles  $< 3$  mm and  $\geq 3$  mm diameter sizes ( $5.7 \pm 1.7$  vs.  $27.0 \pm 11.4$  ng/follicle, respectively;  $P = 0.053$ ). Ovaries from the prenatally androgenized cohort contained 25% more  $< 3$  mm estrogenic follicles ( $P < 0.05$ ) and 50% more  $\geq 3$  mm estrogenic follicles ( $P < 0.05$ ) than controls (Fig. 1G). These results indicate a clear shift in the proportion of healthy and potentially persisting follicles in the prenatally androgenized cohort in comparison with controls.

### The effect of prenatal androgenization on the hypothalamic pituitary ovarian axis

Representative profiles from the 6-h serial sampling of plasma LH concentrations are shown in Fig. 2A. Prenatal androgenization had no effect on pulse frequency (Fig. 2B), average LH concentrations (Fig. 2C), FSH concentrations (Fig. 2D), and therefore the LH to FSH ratio (C,  $0.69 \pm 0.14$  vs. TP,  $0.65 \pm 0.17$ ). In adolescent ewes, midgestation TP exposure did not alter ovarian cyclicity as assessed by progesterone measurement (Fig. 2E). In addition, there was no difference in systemic estradiol (Fig. 2F), androstenedione (Fig. 2G), or testosterone (Fig. 2H) concentrations in the control and prenatally TP-exposed cohorts. Therefore, in young adult ewes, midgestation androgenization did not appear to effect peripheral endocrine parameters by the first breeding season.

### The effect of prenatal androgenization on thecal androstenedione biosynthesis

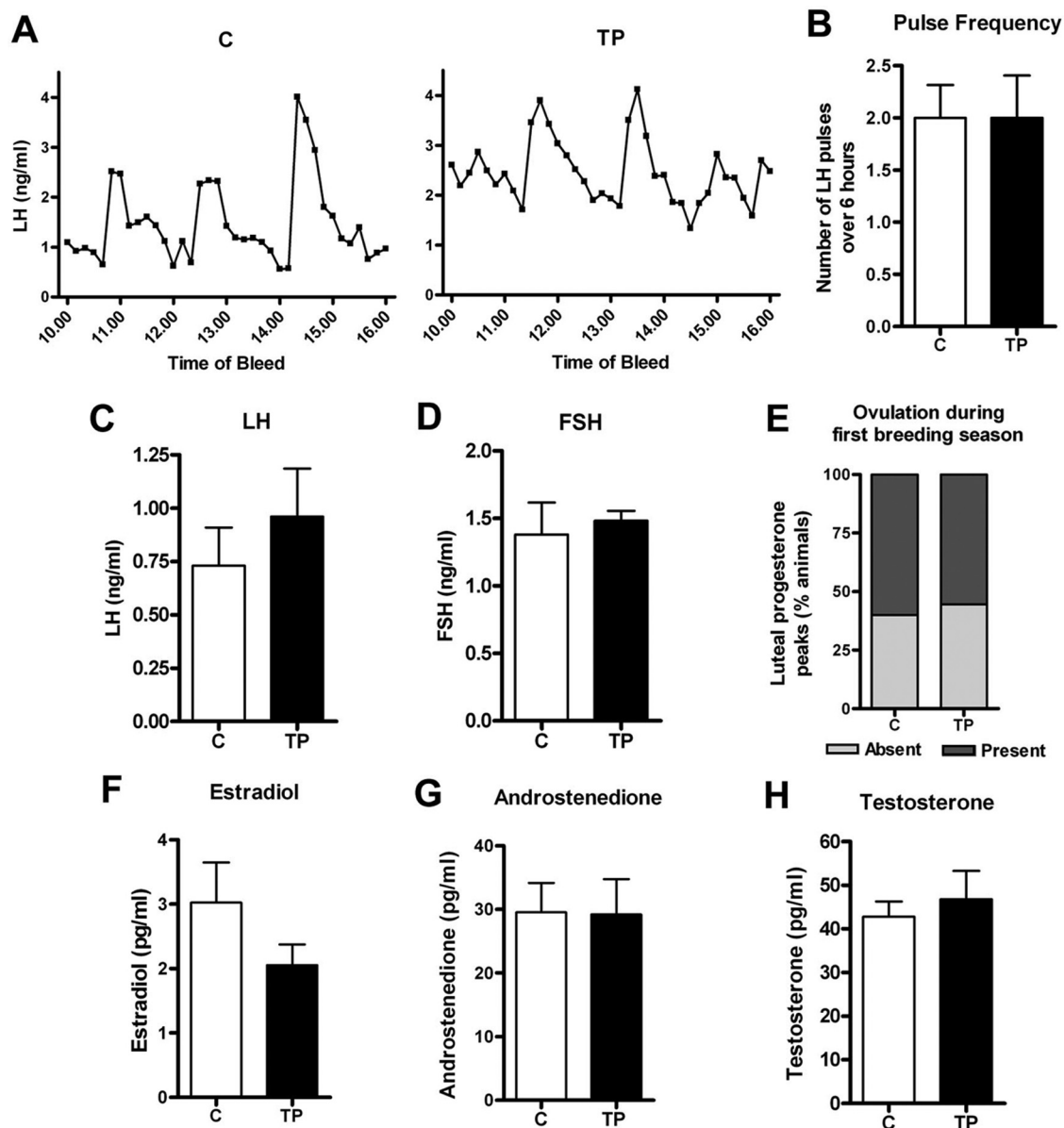
Androstenedione concentrations were measured *in vivo* in the follicular fluid of individual follicles. Analysis



**FIG. 1.** A, Representative tiled images of H&E-stained ovarian sections from prenatally C ( $n = 5$ ) and TP-exposed ( $n = 9$ ) adolescent ewes. Scale bars, 1 mm. Stereological quantification of primordial follicle number (B) and activated follicles (C), including primary, preantral, and antral follicles, was performed in the ovary processed for histology. D, Antral follicles were dissected from the opposite ovary and the number of follicles  $> 2$  mm was recorded. Data in B–D are presented as the mean  $\pm$  SEM and were analyzed using an unpaired  $t$  test. The proportion of proliferating (E) and atretic (F) antral follicles was quantified by reference to follicle immunostaining with Ki67 and cleaved caspase-3, respectively. G, The proportion of estrogenic follicles (follicular fluid estradiol  $> 30$  ng/follicle) from C and TP ovaries were determined. Data in E, F, and G are percentages and were analyzed using a  $\chi^2$  test where \*,  $P < 0.05$ .

was divided into follicle sizes of  $< 3$  mm, 3–4 mm, and  $> 4$  mm in diameter because there were maturational changes in androstenedione concentrations between these stages. Follicular fluid androstenedione concentrations decreased with increasing follicle size in control animals (overall ANOVA comparing  $< 3$  mm, 3–4 mm, and  $> 4$  mm sizes;  $P < 0.001$ ). In the TP-treated cohort, this finding was not observed due to a 5-fold increase ( $P = 0.055$ ) in follicular fluid androstenedione concentrations in follicles  $> 4$  mm compared with control follicles of this size (Fig. 3A).

*In vitro*, the small nonsignificant increase in basal and LH-stimulated thecal androstenedione secretion with follicle size in control animals was significant in the prenatally androgenized cohort (overall ANOVA  $P < 0.001$  and Bonferroni *post hoc* analysis for  $< 3$  mm vs. 3–4 mm groups,  $P < 0.01$ ; Fig. 3B). The thecal cells of follicles  $> 3$  mm responded to the addition of LH with either a trend or significant increase in androstenedione secretion in both the control and TP-treated groups (3–4 mm, C,  $P < 0.05$  and TP,  $P < 0.01$ , and  $> 4$  mm, TP,  $P < 0.05$ ; Fig. 3C). In



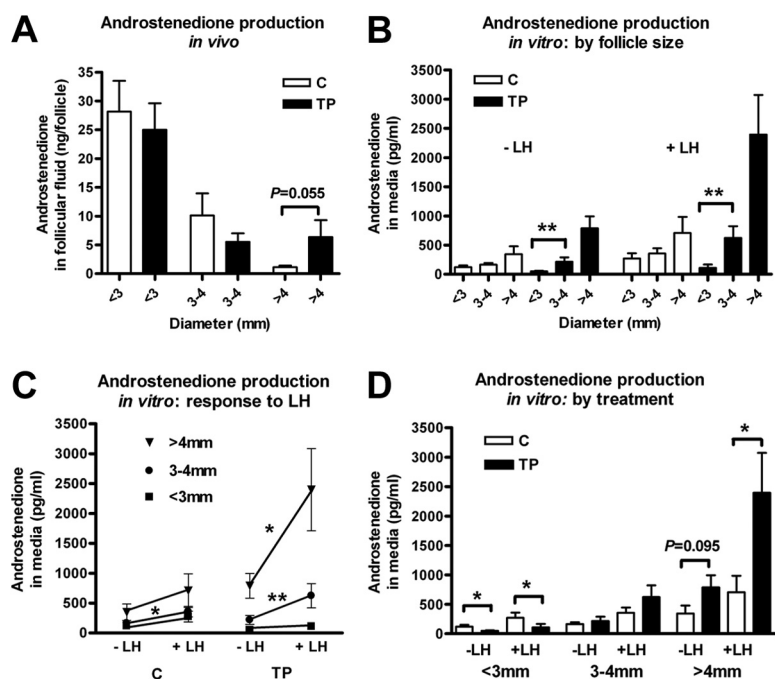
**FIG. 2.** A, Representative LH profiles from a C and TP individual in which LH concentrations are plotted as single values. B, The average number of LH pulses occurring over the sampling period in C and TP cohorts. Peripheral concentrations of LH (C) and FSH (D) were measured and treatment groups were analyzed using an unpaired *t* test. Values are mean  $\pm$  SEM. E, The proportion of animals that were ovulating by measurements of luteal progesterone over a 5-wk period from 9 months of age. Values are percentages and data were analyzed using a  $\chi^2$  test. Peripheral concentrations of estradiol (F), androstenedione (G), and testosterone (H) were measured and treatment groups were analyzed using an unpaired *t* test. Values are mean  $\pm$  SEM.

follicles  $>4$  mm, post-LH thecal androstenedione secretion was increased in the prenatal TP-exposed group ( $P < 0.05$ ; Fig. 3D). Indeed, androstenedione secretion from control LH-stimulated thecal cells from follicles  $>4$  mm was similar to the unstimulated thecal cell output from follicles  $>4$  mm in TP-exposed animals (Fig. 3D). Basal thecal androstenedione secretion also tended to be increased in follicles  $>4$  mm in the TP-treated group; however, this did not reach significance ( $P = 0.095$ ; Fig. 3D). Thecal androstenedione output was not altered in TP-exposed animals in 3–4 mm follicles; however, paradoxically,

there was decreased secretion in the follicles  $<3$  mm in both the absence ( $P < 0.05$ ) and presence ( $P < 0.05$ ) of LH (Fig. 3D); however, this finding was not mirrored *in vivo*. Nevertheless, clear enhancement of androgen secretion was observed in the largest antral follicles both *in vivo* and *in vitro* in the young, prenatally androgenized ewe.

### The effect of prenatal androgenization of thecal cell gene expression

In the theca cells of follicles  $\geq 3$  mm, there was an up-regulation in the expression of steroidogenic acute regu-



**FIG. 3.** Androstenedione concentrations were measured *in vivo* in follicular fluid (A) and *in vitro* in culture media before and after thecal stimulation with LH (B–D). *In vitro* data are grouped by follicle size (B) and are compared using an ANOVA followed by a Bonferroni pair-wise *post hoc* test (indicated by asterisks), and overall ANOVA outcomes for the effect of increasing follicle diameter on androstenedione production in the TP-exposed cohorts before (–) and after (+) LH were  $P < 0.001$ . Additionally, *in vitro* data are grouped by response to LH and analyzed using the paired *t* test (C) or by prenatal treatment (D) and are analyzed using the unpaired *t* test. Data are divided into follicle diameter classifications of sizes of <3 mm, 3–4 mm, and >4 mm. Values are mean  $\pm$  SEM and  $P < 0.05$  was considered significant. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ .

latory (*StAR*) gene in prenatally androgenized offspring ( $P < 0.01$ ; Fig. 4A). Gene expression was also assessed in the same cohorts with follicle groups split into non-estrogen-producing and healthy estrogen-producing follicles to ensure that gene expression changes were not a consequence of a higher number of estrogenic follicles in the TP cohort. A trend for increased *StAR* expression was present in nonestrogenic follicles ( $P = 0.11$ ) and was significantly up-regulated in healthy estrogenic follicles ( $P < 0.05$ ; Fig. 4A). There was also an up-regulation in thecal *CYP11A* gene expression in these follicles ( $P < 0.05$ ; Fig. 4B), which occurred predominantly in the nonestrogenic follicles ( $P < 0.05$ ; Fig. 4B). Prenatal androgens were also found to program thecal cell expression of *HSD3B1* ( $P < 0.01$ ), *CYP17* ( $P < 0.05$ ), and *LHR* ( $P < 0.05$ ), which were up-regulated in follicles  $\geq 3$  mm (Fig. 4, C–E). This trend remained present in nonestrogenic ( $P = 0.078$ ,  $P = 0.085$ , and  $P = 0.084$ , respectively) and estrogenic ( $P < 0.05$ ,  $P < 0.05$ , and  $P = 0.076$ ; Fig. 4, C–E, respectively) follicles.

Although it initially appeared that thecal cell androgen receptor (*AR*) gene expression was not affected by prenatal androgenization (Fig. 4F), group separation revealed that

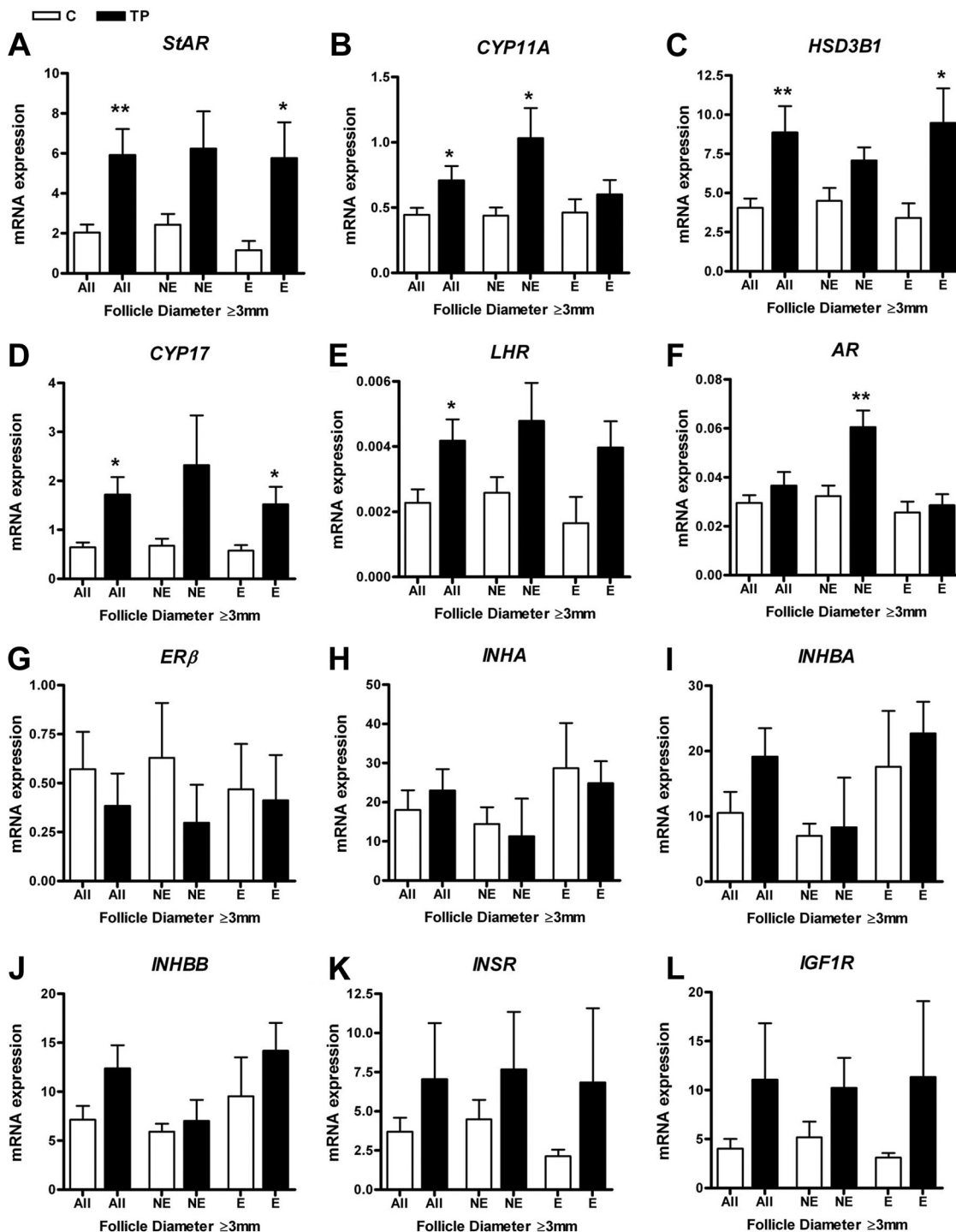
follicles that were not producing estrogens had up-regulated *AR* compared with controls, whereas estrogen-producing follicles did not (Fig. 4F). There was no effect of prenatal androgenization on thecal cell receptivity to estrogens (*ER $\beta$* ; Fig. 4G). Similarly, this treatment did not program changes in thecal *INHHA* (Fig. 4H), *INHBA* (Fig. 4I), or *INHBB* (Fig. 4J) expression. Finally, the thecal cell expression of receptors for insulin (*INSR*; Fig. 4K) or IGF-I (*IGF1*; Fig. 4L) were not significantly altered by prenatal androgenization. These gene expression findings demonstrate up-regulation of thecal cellular steroidogenic machinery in TP-exposed animals that is associated with augmented steroid production in these ovaries.

## Discussion

Midgestation androgenization from d 62 to d 102 of gestation does not lead to a polycystic ovarian morphology in young adult ewes. Other studies, albeit in the Poll Dorset breed of sheep, have shown that polycystic ovaries are present in 5-wk-old lambs as a consequence of earlier and prolonged exposure to TP from d 30 to d 90 (16). In PCOS, the stockpiling of primary follicles has been described, in which a greater number of growing follicles are observed through developmental stages (37). A reciprocal decrease in primordial follicle number and increase in primary and antral follicles is reported in women with PCOS (38). In the d 30–90 prenatally androgenized sheep, although there is no effect by fetal d 90 (39), there is a decrease in ovarian reserve observed in the fetal d 140 ovary (40) and also in the 8-month ewe, although not in the 20-month ewe (41). In the sheep PCOS model, follicular recruitment to growing follicles is enhanced in addition to increased follicular persistence (42, 43), which could contribute to an overall polycystic morphology. Studies have also revealed, through treatment of pregnant ewes with the nonaromatizable androgen, dihydrotestosterone, that the dysregulated recruitment of follicles is an androgenic effect, whereas enhanced follicular persistence is likely a consequence of estrogenic programming (39, 43).

In the current study, ovarian reserve is not affected at 11 months by prenatal TP exposure in the Scottish Greyface ewe, in agreement with previous findings that germ cell number is not altered in the d 90 fetal ovary in response to





**FIG. 4.** Thecal gene expression was measured *in vivo* by qRT-PCR using RNA extracted from individual follicles  $>3$  mm in diameter (all; C = 12, TP = 12). Data are further separated into follicles that were nonestrogenic (NE; C = 7, TP = 3) or estrogenic (E; C = 5, TP = 9). Genes assessed were steroidal acute regulatory protein (*StAR*) (A), *CYP11A* (B), *HSD3B1* (C), *CYP17* (D), *LHR* (E), *AR* (F), estrogen receptor (*ER $\beta$* ) (G), *INHA* (H), *INHBA* (I), *INHBB* (J), insulin receptor (*INSR*) (K), and IGF-I receptor (*IGF1R*) (L). Mean data  $\pm$  SEM are presented and an unpaired *t* test was performed where \*,  $P < 0.05$  and \*\*,  $P < 0.01$ .

maternal TP administration from d 60–90 (36). Midgestation androgenization also did not result in an increased number of primary, preantral or antral follicles at 11 months; however, the estrogenic status measured for individual follicles in the contralateral ovary revealed a sta-

tistically significant increase in the proportion of healthy estrogen producing follicles compared with the control cohort. This may be indicative of enhanced follicular persistence in these animals that is not yet reflected by follicle number. Differential findings between this study and oth-

ers may be a consequence of breed as well as alternative treatment periods, in which earlier exposure to androgens over the period of sexual differentiation leads to an overall more severe masculinized phenotype (21, 22). Since the androgenic programming effects of TP have been demonstrated to be important in the sheep for the programming of increased follicular recruitment (39, 43), it is possible that the shorter period of TP exposure in our study does not induce such changes. Similarly to ovarian morphology and follicle number, animals exposed to androgens from d 62 to d 102 did not exhibit peripheral hyperandrogenemia, had normal LH to FSH ratios as well as hypothalamic pituitary ovarian function, and were fundamentally not displaying the phenotypic criteria for diagnosis of PCOS.

We therefore hypothesized that, before marked alteration in ovarian morphology and the endocrine milieu, it would be possible to detect intrinsic ovarian alterations that may not be a consequence of the adult environment. An assessment of thecal cell steroidogenic function was subsequently carried out. Thecal cell secretion of androstenedione was determined *in vivo* by measurement in follicular fluid. In the largest antral follicles (>4 mm), there was a trend for increased androstenedione secretion by ovarian follicles of prenatally androgenized animals. Androstenedione concentrations in follicular fluid were still raised in this cohort, despite an increase in the number of follicles that were producing large amounts of estradiol. In fact, when follicles were compared by estrogenic status, the trend for increased androstenedione secretion was still present compared with controls, although sample size was too small to reach robust conclusions. It seems, however, that the rate of aromatization to estradiol by the granulosa cell is not responsible for the differences in the local concentration of androstenedione. These *in vivo* data represent a potential source of hormone dysregulation that could contribute to the development of a systemic hyperandrogenic phenotype.

In PCOS, LH concentration, pulse frequency, and peak amplitude are elevated (44). There is a positive association between LH and peripheral androgen levels (45), and LH stimulates excessive ovarian androgen production by PCOS thecal cells *in vitro* (3). In the sheep PCOS model, androgenization from fetal d 30 to d 90 leads to a perturbed gonadotropin milieu that is characterized by LH hypersecretion and LH pulses that are increased in amplitude and frequency (23, 46, 47). In the first breeding season, we did not observe disrupted LH concentrations or altered pulse dynamics during the estrus phase in sheep that were androgenized from d 62 to d 102; however, it should be noted that other midgestation models of androgenization in Suffolk sheep have reported subtle altera-

tions in LH frequency measured through more frequent blood sampling than this study. Nevertheless, ovulation was unaffected in these animals (23). Although the endocrine milieu of the animals in the current study appeared normal, we hypothesized that in the presence of an abnormal adult hormonal environment, the hyperandrogenic phenotype observed in thecal cells, would be augmented.

In culture, androstenedione secretion was significantly increased with increasing follicle size in follicles from TP-exposed animals before and even more so after treatment with LH, in comparison with controls. In addition, the thecal cell response to LH was much greater for prenatally androgenized animals, particularly in the follicles >4 mm. In parallel with the *in vivo* data, androstenedione secretion was nonsignificantly increased in the TP group, compared with controls, under basal culture conditions. However, upon LH treatment of thecal cells, the increase in androstenedione concentration in culture media was more exaggerated and was 3-fold > in controls. These findings demonstrate that in the prenatally androgenized ewe, not only do follicular thecal cells have an increased propensity to secrete androgens *in vivo*, but also an altered hormonal environment simulated in this study by the addition of exogenous LH could contribute substantially to an overall ovarian hyperandrogenic phenotype. These data are in agreement with the findings of excessive basal and LH-stimulated androgen production by human PCOS thecal cells (3). The reduction in androstenedione secretion from follicles <3 mm in diameter is also of interest. It is difficult to reconcile but, combined with the subtle morphological changes seen by immunohistochemistry, may suggest further evidence for aberrant follicular function in this model.

To investigate the intrinsic properties that might warrant increased ovine thecal androgen production, *in vivo* gene expression analyses were conducted in cells collected from the noncultured portion of the thecal shell. Studies assessing the factors that might lead to increased steroid production in human PCOS thecal cells identified alterations in gene expression related to the steroidogenic biosynthesis pathway, including the up-regulation of *StAR* (13), *CYP11A*, and *CYP17* (13, 14) as well as increased enzymatic activity of 17 $\alpha$ -OH, 3 $\beta$ -HSD, and 17 $\beta$ -HSD (14). Assessment of our prenatally androgenized ovine model also identified the up-regulation of many genes involved in steroid biosynthesis including *StAR*, *CYP11A*, *HSD3B1* and *CYP17*. Furthermore, thecal *LHR* gene expression was increased in these animals, a finding that has also been reported in human thecal cells from PCOS patients (13). Granulosa cells were also collected at this time; however, yields from individual follicles were found to be too low to obtain satisfactory RNA concentrations and

purities. Study of the FSH receptor (*FSHR*) in granulosa cells of individual follicles and the above steroidogenic genes as well as *CYP19* (aromatase) would have been of interest and may have provided explanation for the increased proportions of estrogenic follicles present in the androgenized cohort. Nevertheless, these data provide further evidence for functional ovarian programming by prenatal androgens in the sheep, and like in human studies of PCOS thecal cells, there may be an increased drive of cholesterol through the steroidogenic pathway and an enhanced sensitivity to LH due to an increase in thecal *LHR*, present in this ovine model. Given that the thecal cell effects of prenatal androgenization include global up-regulation of steroidogenic genes as well as hypersecretion of androgens both *in vivo* and *in vitro*, it is perhaps surprising that there is no measurable alteration in peripheral androgen concentrations. We suspect that the capacity for aromatization can maintain the normal circulating androgen concentrations at this stage.

Since the activins and inhibins have opposite effects on thecal androstenedione production, suppressing and promoting secretion, respectively (48–50), we hypothesized that local signaling by these factors could contribute to the ovarian phenotype. Prenatal androgenization did not, however, alter the expression of the inhibin subunits in the thecal cells of these animals. Unfortunately, due to the inability to investigate inhibin subunit expression in the granulosa cells, which are the predominant site of ovarian activin and inhibin production, assessment was limited to the thecal cells. Other investigators have studied the expression of the activin and inhibin subunits in the d 30–90 prenatally androgenized 5-wk-old lamb and, through *in situ* hybridization, have found that *INHBB* mRNA was in fact down-regulated in whole follicles (16).

The stimulatory effect of insulin and IGF-I on ovarian androgen production has been documented (51–54), and therefore, we investigated whether receptor gene expression for these ligands, *INSR* or *IGF1R*, were affected by prenatal androgen exposure. These genes were not altered by increased exposure to prenatal androgens. It is, however, interesting to note that in this same ovine model of midgestation androgenization, there is a slightly exaggerated insulin response to glucose load, although basal insulin concentration and glucose clearance are not changed at this stage. Also, there is increased hepatic *IGF1* mRNA expression in these young adult sheep (55). These findings might indicate early physiological perturbations in the environment that, as they develop, could influence adult ovarian function. Although we show that altered theca steroidogenesis is the predominant abnormality in these young sheep, it remains impossible to fully temporarily

separate theca cell alterations and minor metabolic changes.

It is clear that there are intrinsic ovarian abnormalities that are programmed by prenatal exposure to androgens that are not associated with an abnormal PCO-like morphology or an altered steroid or gonadotropin environment. This suggests the presence of a pre-PCOS phenotype that is observable in this ovine model of PCOS, in which midgestation androgens program more subtle reproductive anomalies than is reported in animals exposed to androgens for a prolonged gestational period as well as in different breeds. We have previously shown in this model that just 10 d of androgen exposure, commencing at d 60, is sufficient to alter protein expression in the d 70 fetal ovary (36). The AR is strongly expressed in the interstitial somatic streams of the d 70 fetal ovary, and therefore, androgens can directly act on the ovary during this time. Prenatal TP treatment results in a trend for increased AR in the d 70 ovary and presents a mechanism for the programming of ovarian somatic cells that may include pre-granulosa and/or thecal progenitor cells (36). Furthermore, this treatment programs an up-regulation of regulatory proteins involved in proliferation and differentiation known as the inhibitors of differentiation and also down-regulates steroidogenic gene expression in the midgestation ovary (36). Interestingly, contemporary testosterone exposure *in utero* reduced the expression of steroidogenic enzymes and LH receptor in the d 90 fetal ovary (36), whereas previous prenatal exposure increased theca steroidogenic capacity as young adults. The effects in the fetus may be secondary to the suppressed gonadotropins (Hogg, K. and W.C. Duncan, unpublished data), which are normal in the adult, or another indicator of early disordered ovarian steroidogenic capacity. These changes in the fetal ovary as a consequence of exposure to androgens may be linked to the intrinsic alterations observed in the adult ovary.

Further studies will be required to dissect the longitudinal changes to ovarian function as a consequence of these early changes. However, this study has for the first time characterized thecal cell androgen production and steroidogenic gene expression in a prenatal androgenization model. Midgestation androgenization programs enhanced thecal cell androgen production in the adult sheep ovary, which is likely driven by the universal up-regulation of genes involved in steroid biosynthesis. Further augmentation of these pathways may occur through ovarian exposure to an altered adult environment including elevated LH and insulin levels, which are common features of PCOS.

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Address all correspondence and requests for reprints to: Kirsten Hogg, Medical Research Council, Centre for Reproductive Health, The Queen's Medical Research Institute, 47 Little France Crescent, Edinburgh EH16 4SA, United Kingdom. E-mail: k.hogg@sms.ed.ac.uk.

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