Developmental programming: prenatal androgen excess disrupts ovarian steroid receptor balance

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Abstract

Steroid hormones play an important role in reproduction and the receptors through which they signal change in a developmental time, follicle stage, and cell-specific manner. Disruption in steroid receptor expression affects follicle formation and differentiation. In this study, using prenatal testosterone (T) and dihydrotestosterone (DHT)-treated female sheep as model systems, we tested the hypothesis that prenatal androgen excess disrupts the developmental ontogeny of ovarian steroid receptor protein expression. Pregnant Suffolk ewes were injected twice weekly with T propionate or DHT propionate (a non-aromatizable androgen) in cottonseed oil from days 30 to 90 of gestation. Changes in ovarian estrogen receptors (ER; ESR1, ESR2), androgen receptor (AR) and progesterone receptor (PGR) proteins were determined at fetal (days 90 and 140), postpubertal (10 months), and adult (21 months; only prenatal T-treated sheep studied) ages by immunohistochemistry. Prenatal T and DHT treatment induced selective increase in AR but not ER or PGR expression in the stroma and granulosa cells of fetal days 90 and 140 ovaries. An increase in ESR1 and decrease in ESR2 immunostaining coupled with increased AR expression were evident in granulosa cells of antral follicles of 10- and 21-month-old prenatal T but not DHT-treated females (analyzed only at 10 months). These findings provide evidence that an early increase in ovarian AR is the first step in the altered ovarian developmental trajectory of prenatal T-treated females, and manifestations of postnatal ovarian dysfunction are likely facilitated via altered equilibrium of antral follicular granulosa cell ER/AR protein expression.

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Introduction

Steroids play a key role in the growth, differentiation, and function of female reproductive tissues (Drummond et al. 2002, Yeh et al. 2002, Simpson et al. 2005, Padmanabhan et al. 2006). As such, inappropriate activation of the reproductive system by exposure to excess steroid hormones is a major concern, especially in the female. At risk is the female fetus whose mother has been exposed to exogenous steroids for a variety of reasons: failed contraception and continued exposure to contraceptive steroids, use of anabolic steroids, or inadvertent exposure to environmental compounds with estrogenic or androgenic activity (Bahrke et al. 1998, Crews & McLachlan 2006, Crain et al. 2008, Jellesen et al. 2008, Woodruff & Walker 2008). Evidence exists in support of excess native or environmental steroid exposure. For instance, serum testosterone (T) levels in 40% of female fetuses at mid-gestation were found to be in the male range (Beck-Peccoz et al. 1991). Amniotic fluid T levels were found to be also elevated in diabetic pregnancies (Barbieri et al. 1986). Furthermore, female stillbirth offspring of diabetic mothers have hirsutism, ovarian theca-lutein cysts, and thecal cell hyperplasia (Driscoll et al. 1960), indicative of excess androgen exposure. Evidence exists in support of biologically significant levels of unconjugated bisphenol-A, an environmental estrogen mimic, in maternal and fetal serum (Schonfelder et al. 2002, Padmanabhan et al. 2008). Experimentally, exposure of sheep or monkey fetuses to excess T during gestation culminates in a metabolic and reproductive phenotype (Abbott et al. 2006, Padmanabhan et al. 2006, Dumesic et al. 2007) similar to that of women with polycystic ovary syndrome (PCOS; Dunaif 1997, Rosenfield 1997, Franks et al. 2006), the most common cause of anovulatory infertility in women. Extensive investigations have been undertaken in sheep comparing prenatal T (signals through androgen receptor (AR) or estrogen receptor (ER), due to aromatization to estrogen), and dihydrotestosterone (DHT; non-aromatizable androgen; signals mainly through AR) to delineate the roles of androgen and estrogen in disrupting neuroendocrine feedback systems (Wood & Foster 1998, Robinson et al. 2002), and more
recently ovarian follicular recruitment (Steckler et al. 2005, Smith et al. 2009) and persistence (Manikkam et al. 2006, Steckler et al. 2007). These studies have shown that androgens program the disruptive effects of prenatal T excess on estradiol ($E_2$) negative but not positive feedback at the neuroendocrine level and follicular recruitment but not persistence at the ovarian level.

Considering that the genomic effects of steroids are mediated via intracellular receptors (Chan & O'Malley 1976, Tenbaum & Banahmad 1997, Beato & Klug 2002), and steroids have the potential to up- or down-regulate their own receptors (Chadha et al. 1994, Tetsuka & Hillier 1996, Drummond et al. 1999), the disruptive effects of prenatal T excess at the ovarian level may be mediated via altered developmental expression of ovarian steroid receptors. Changes in receptor expression would result in altered steroid signaling culminating in changes in follicle formation and differentiation (Rosenfeld et al. 2001, Drummond et al. 2002, Walters et al. 2008). During fetal ontogeny in sheep, expression of AR, ESR1, ESR2, and progesterone receptor (PGR) undergo progressive follicular type-, cell-, and time-specific changes (Juengel et al. 2006). In this study, using prenatal T- and DHT-treated female sheep as model systems, we tested the hypothesis that prenatal androgen excess disrupts the normal developmental progression of ovarian steroid receptor expression.

**Results**

**Antibody specificity**

Results from western blot analyses of ovarian and uterine (positive control) homogenate (left) and immunohistochemical recognition of the four steroid receptors in ovarian sections (middle) are summarized in Fig. 1. The negative controls (right) demonstrate the specificity of the antibody. Western blot analysis only found positive bands of appropriate sizes for each of the receptors studied (ESR1, ESR2, AR, and PGR). The ESR1 and ESR2 antibodies detected a single band at 66 and 55 kDa respectively. PGR (isoform B) was detected as a single band at 116 kDa while two bands were observed for AR around 110 kDa. In the absence of the primary antibodies, no specific bands were detected (not shown). Specific nuclear staining was detected for all four nuclear receptors studied in the ovaries (Fig. 1). Faint cytoplasmic staining was also evident and this was subtracted as background in the image analysis.

**Ovarian ESR1, ESR2, AR, and PGR localization**

In control animals, patterns of AR, ESR1, ESR2, and PGR immunostaining in the various ovarian compartments and follicular classes followed what has been reported earlier (Juengel et al. 2006). Representative patterns of AR immunostaining in primordial, primary, and pre-antral follicles from fetal day-140 ovaries and AR, ESR1, and ESR2 in antral follicles of 10-month old control, and prenatal T-treated females are shown in Fig. 2. In general, changes in steroid receptor expression induced by prenatal steroid treatment were evident only at the level of AR at all three fetal time points studied. In 10- and 22-month-old animals, prenatal steroid-induced changes were evident at the level of AR, ESR1, and ESR2. No changes in PGR were evident at any of the developmental time points studied. Prenatal steroid-induced changes in steroid receptor expression in the various ovarian compartments and follicular classes are discussed below within each developmental time point.
Fetal

Relative expression of ESR1, ESR2, AR, and PGR-B proteins in the ovaries of fetal days 90 and 140 are summarized in Fig. 3A. For reference, the previously published distributions of primordial and growing follicles in the contralateral ovary (Smith et al. 2009) are summarized in Fig. 3B. ESR1 and ESR2 proteins were expressed predominantly in granulosa of primordial, primary, and preantral (day 140 only) follicles and stroma at both fetal ages with very little expression in the oocytes. Expression levels of these two receptor proteins in prenatal T- and DHT-treated females in all ovarian compartments did not differ (P>0.05) from those of controls at these two ages. AR protein was expressed in the granulosa, theca, and stromal compartments (Fig. 2). In fetal D90 ovaries, expression was the highest in the stroma (P<0.05; Fig. 3A). Comparing across treatments, prenatal T and DHT excess increased (P<0.05) AR expression in granulosal, thecal, and stromal compartments (Fig. 3A). PGR-B was expressed in granulosa, theca, and stroma with expression being the highest in theca (P<0.05). Prenatal T and DHT did not alter expression pattern of PGR-B. Only a few antral follicles were present at fetal day 140 and hence were not quantified.

Postpubertal

Mean changes in expression of the four receptor proteins in the various ovarian compartments of postpubertal females (10 months) are summarized in Fig. 4A. For reference, the previously published distributions of primordial and growing follicles in the same ovary used in this study (Smith et al. 2009) are summarized in Fig. 4B. As was the case with fetal ovaries, ESR1 and ESR2 immunostaining was predominant in granulosa of primordial, primary, and preantral follicles, with amounts in theca being lower than that in granulosa (P<0.05). Similar direction of changes was observed in the antral follicles with expression being high in granulosa, very low in oocytes, and intermediate in theca interna and externa (Fig. 2). Comparing across treatments, effects of prenatal T and DHT excess were unidirectional with both increasing ESR1 expression in granulosa of large preantral and antral follicles as well as in the stromal compartment (P<0.05; representative patterns in control and prenatal T-treated females in Fig. 2, right). ESR2 expression followed a completely different trajectory and was manifested in a tissue- and steroid-specific manner. Prenatal steroid excess induced changes in ESR2 expression only in antral follicles and the stromal compartment (P<0.05; representative patterns in control and prenatal T-treated females in Fig. 2, right). Prenatal T but not DHT excess reduced ESR2 expression in both granulosa and theca interna (P<0.05). By contrast, prenatal T and DHT excess increased ESR2 expression in the stroma (P<0.05). In theca externa, both treatments reduced the level of ESR2 immunostaining (P<0.05).
AR immunostaining followed a similar trend as in the fetuses with higher amounts found in granulosa and stroma \((P<0.05)\). In contrast to the fetal ovaries, prenatal T and DHT excess had no effect on AR protein expression in granulosa of primordial, primary, and small prenatal follicles. However, prenatal T excess increased granulosal expression of AR protein in large preantral and antral follicles while reducing expression in stromal cells \((P<0.05)\). Effects of prenatal DHT excess parallel that of prenatal T at the stromal level with both reducing AR immunostaining \((P<0.05)\). Changes in PGR-B for the most part were similar to that of fetal ovaries. Predominant PGR-B protein expression at this age was evident in thecal and stromal cells, with almost twice the amount as seen in granulosa cells \((P<0.05)\). Prenatal T and DHT treatment had no effect on PGR-B immunostaining in any ovarian compartment at this age \((P>0.05)\).

**Adult**

Changes in expression pattern of the four receptor proteins in the ovarian compartments of adult females are shown in Fig. 5. Note that the comparison is only between control and prenatal T as insufficient prenatal DHT females were born for including an adult group. Expression of ESR1 protein in follicular classes at this age paralleled that of fetal and 10-month-old animals. Prenatal T excess increased ESR1 immunostaining in granulosa and stroma \((P<0.05)\) as was the case with 10-month-old animals. Expression pattern of ESR2 protein also paralleled that of 10-month-old animals with prenatal T excess reducing expressions in granulosa and theca of antral follicles but increasing expression in stroma \((P<0.05)\). Both AR and PGR-B immunostaining also followed similar changes as in the 10-month-old females. While no effect of prenatal T excess was
Figure 4 (A) Relative expressions of ESR1, ESR2, AR, and PGR-B in the ovaries of 10-month-old control (open bars), prenatal T-treated (black bars) and prenatal DHT-treated (hatched bars) females. For each cellular compartment within each follicle type, values with different letters are significantly different ($P < 0.05$). Values represent mean ± S.E.M. of five control animals from five dams, six T-treated animals from six dams, and five DHT-treated animals from five dams. (B) Published summary of ovarian stereology results (Smith et al. 2009) from the same ovary used in this study, which document enhanced follicular depletion and recruitment in prenatally T- but not DHT-treated 10-month-old females for comparison. Asterisks indicate significant differences from controls.
seen in PGR-B, AR immunostaining was increased in granulosa cells of preantral and antral follicles but reduced in theca and stroma (P!0.05).

**Discussion**

Findings from this study provide evidence that prenatal T and DHT excess alters the ontogeny of ESR1, ESR2, and AR but not PGR-B protein expression in the ovary, in a developmental time, steroid, and follicular stage and cell-specific manner. Furthermore, the finding that the changes are manifested during fetal life exclusively at the level of AR but not ER/PGR indicates that resulting adult ovarian perturbations that the prenatal T-treated females manifest, namely disrupted ovarian morphology, follicular persistence, and depletion (Manikkam et al. 2006, Steckler et al. 2007, Smith et al. 2009), are reprogrammed via altered AR signaling. The significance of the changes in steroid receptor proteins observed during the different developmental time points is discussed below.

**Fetal**

The selective increase in AR but not ER or PGR-B immunostaining in the stroma and granulosa of primordial and primary follicles of fetal day-90 ovary at the end of T treatment suggests that this is a key step in programming adult dysfunction. Administration of T propionate to prepubertal rats on day 5 of postnatal life also increased ovarian nuclear AR expression (Bukovsky et al. 2002). Developmentally, complete follicular differentiation (primordial to antral) is completed postnatally in rodents as opposed to sheep, in which it is completed in utero (Padmanabhan et al. 2007). The increased AR expression in fetal day-90 ovaries of prenatal T-treated sheep appears to be mediated by androgenic actions of T because the non-aromatizable androgen, DHT, also increased AR immunostaining in stromal as well as granulosa of primordial/primary follicles of day-90 fetuses.

Increase in AR immunostaining in stromal and granulosa compartments of fetal day-140 ovaries, coupled with enhanced follicular recruitment seen at this developmental time point in these very same animals (Smith et al. 2009), is consistent with androgens playing a role in early follicular differentiation (Hillier & Tetsuka 1997, Vendola et al. 1998, Walters et al. 2008). Androgens have been shown to recruit primordial follicles into the growing pool of developing follicles (Hillier & Tetsuka 1997, Vendola et al. 1998, McGee & Hsueh 2000). Recent studies using a well-characterized culture system have found that T stimulates primary to secondary follicle transition via an AR-dependent mechanism (Yang & Fortune 2006). Earlier findings that the AR knockout mice show accelerated follicle depletion at older ages (Shiina et al. 2006) and the testicular-feminized mice, which express a truncated non-functional AR, have a shortened reproductive lifespan (Young et al. 1989, Gaspar et al. 1991) indicate that AR might play a physiological role in determining the reproductive lifespan of the ovary. These findings are at odds with enhanced follicular depletion of the 10-month-old prenatal T-treated sheep manifest (Smith et al. 2009) in the face of increased AR during fetal life (this study). One possibility is that there is a threshold requirement of AR for the occurrence of normal ovarian

**Figure 5** Relative expressions of ESR1, ESR2, AR, and PGR-B in the ovaries of 21-month-old control (open bars) and prenatal T-treated (black bars) females. For each cellular compartment within each follicle type, values with different letters are significantly different (P<0.05). Values represent mean ± S.E.M. of five control animals from five dams, eight prenatal T-treated animals from eight dams. Note that stereology has not been undertaken with 21-month-old animals.
differentiation, amounts of AR above or below which would be detrimental to follicular survival.

Postpubertal and adult

Patterns of ESR1, ESR2, and AR immunostaining in ovarian compartments of adult controls were consistent for the most part with what has been described for sheep (Juengel et al. 2006). The altered equilibrium of ESR1 to ESR2 protein coupled with increased AR expression evident mainly in antral follicles of 10- and 21-month-old prenatal T-treated females might be a contributing factor in the development of follicular persistence that the prenatal T-treated females have been found to manifest in our earlier studies (Manikkam et al. 2006, Steckler et al. 2007). Antral follicles represent a stage in follicular development, at which maximal proliferation is occurring. Rodent studies have found that E2 synergizes with FSH in stimulating granulosal cell proliferation and steroidogenesis (Palter et al. 2001, Drummond 2006). The similarity in the direction of changes in ESR1 to ESR2 immunostaining in ovaries of 10- and 21-month-old animals is supportive of inherent reprogramming of the ovary.

Interestingly, ESR1 overexpressed mice are subfertile and show down-regulation of the Esr2 gene (Tomic et al. 2007). This observation raises the possibility that the decrease in ESR2 seen in granulosa of antral follicles of prenatal T-treated females may be secondary to ESR1 up-regulation. The increased numbers of antral follicles observed in ESR1 overexpressed mice (Tomic et al. 2007) parallel the increased number of antral follicles seen in the 10-month-old prenatal T-treated female sheep (same animals used in this study; Smith et al. 2009). These findings are consistent with a role for estrogen in promoting follicular growth via ESR1 receptor. Studies with ESR1 knockout mice that were anovulatory also indicated that ESR1 is not required for follicular recruitment or early differentiation but necessary for subsequent follicular growth (Dupont et al. 2000).

Another possibility to consider is that it is not the increased ESR1 but the relative expression of ESR1 to ESR2 that is more important, because the 10-month-old prenatal DHT-treated females, which failed to show the decrease in ESR2 (this study), did not show increased presence of antral follicles (Smith et al. 2009). Because ESR1 and ESR2 can form homodimers as well as heterodimers (Pettersson et al. 1997) and both ESR1 and ESR2 are expressed in the granulosa, theoretically they should be capable of forming ESR1:ESR1 and ESR2:ESR2 homodimers as well as ESR1:ESR2 heterodimers and alter signal transduction. ESR1 and ESR2 homodimers and heterodimers can have different affinities/specificities for estrogen or other potential estrogen-like ligands (Sun et al. 1999) and cause differential gene activation (McInerney et al. 1998, Pettersson et al. 2000). Earlier studies have found that ESR2, when present within a heterodimer, repressed ESR1 activity and sensitivity to E2 (Hall & McDonnell 1999). More recent studies have suggested that the main determinants of the transcriptional activities of ESR1 and ESR2 are not their binding ability but rather the individual concentration of the two receptors in target cells and the structure of the estrogen ligand (Gougelet et al. 2007, Bhavnani et al. 2008). As such, a given ligand could exert opposite activities depending on the type of ER expressed. Therefore, regulation of ovarian differentiation and subsequent function in adulthood by estrogen appears to be complex and involve intricate interactions/cell signaling pathways coordinated by different receptor–receptor interactions (Pettersson et al. 1997, McInerney et al. 1998, Hall & McDonnell 1999, Sun et al. 1999, Pettersson et al. 2000, Gougelet et al. 2007, Bhavnani et al. 2008). If so, induction of changes in the ESR1:ESR2 ratio such as that seen in prenatal T-treated females would perturb the effects of estrogen in regulating ovarian function.

The disruptive effects of altered ER signaling may be compounded further by altered AR signaling in antral follicles of 10- and 21-month-old females. Findings from this study coupled with earlier findings of multifollicular ovaries, reduced inhibin/activin b mRNA expression (West et al. 2001), enhanced follicular recruitment (Steckler et al. 2005, Smith et al. 2009 (same animals as used in this study)), and follicular persistence (Manikkam et al. 2006, Steckler et al. 2007), in prenatal T-treated sheep all point to disrupted intra-follicular androgen signaling. It is known that androgens promote atresia and/or arrest of follicles (Hillier & Tetsuka 1997, Vendola et al. 1998, McGee & Hsueh 2000). Furthermore, T antagonizes the anti-apoptotic effects of E2 in rat granulosal cells in early antral and preantral follicles (Billig et al. 1993). Our earlier findings of follicular persistence in prenatal T- but not DHT-treated females (Manikkam et al. 2006, Steckler et al. 2007) are therefore consistent with increased AR protein in granulosal cells of antral follicles of prenatal T- but not DHT-treated females. The fact that the direction of changes in AR expression was tissue/follicle stage specific and a function of the nature of prenatal steroid exposure emphasizes the specificity of this regulation and the importance of these findings to ovarian function.

The finding that AR expression was increased only in granulosal cells of antral follicles from adult animals is also consistent with reduced ESR2 expression seen in prenatal T-treated females. Earlier studies have found that AR expression in the granulosa of late antral follicles is repressed by the activation of ESR2 (Cheng et al. 2002). Repression of AR changes the follicular environment from androgen to estrogen dominance, a critical step for the survival of the follicle (Billig et al. 1993, Hillier & Tetsuka 1997, Britt & Findlay 2002, Drummond 2006, Yang & Fortune 2006). If AR expression remains high,
as is the case in prenatal T-treated females, antral follicles cannot achieve estrogen dominance and their fate is atresia or arrest. However, parallel studies performed using the same ovaries as used in this study (Smith et al. 2009) found no differences in the percentage of atretic antral follicles between control and prenatal T-treated females at 10 months of age. On the contrary, our earlier findings of follicular persistence in the prenatal T-treated females (Manikkam et al. 2006, Steckler et al. 2007) provide evidence in support of arrested follicular development. It remains to be determined whether this arrest is mediated via a shift in the balance of apoptotic to anti-apoptotic factors in these follicles. It is of interest that granulosa cells of antral follicles derived after gonadotropin stimulation from women with PCOS, whose reproductive attributes the prenatal T-females duplicate, also exhibit increased AR expression compared with controls (Catteau-Jonard et al. 2008) and flutamide, an anti-androgen, restores ovulatory function in anovulatory women with PCOS (De Leo et al. 1998).

While changes in ER and AR likely play a role in the manifestation of ovarian disruptions in the adult prenatal T-treated females, the lack of changes in PGR-B indicate that this isoform of PGR is not involved. However, the finding that double PGR (PGR-A and PGR-B) knockout mice fail to ovulate (Lydon et al. 1995) provides evidence that other PGR isoforms may be involved. Consistent with this premise, cystic ovaries of rats manifested higher expression of PGR-A, lower expression of the PGR-C, and no change in PGR-B isoform (Salvetti et al. 2009). Changes in the expressions of other PGR forms were not evaluated in this study.

The fetal ovarian changes in AR and changes in AR/ER at 10 months of age are consistent with the effects of prenatal T and DHT treatments on follicular recruitment, depletion, and ovarian morphology reported in these very same animals (Smith et al. 2009). The increased fetal ovarian AR expression (this study) coupled with the increased follicular recruitment (Smith et al. 2009) in fetal day-140 prenatal T- and DHT-treated fetuses supports a role for androgen in early follicular activation. Similarly, increased follicular depletion and ovarian disruptions seen in the 10-month-old prenatal T-treated but not DHT-treated females (same animals used in this study; Smith et al. 2009) coupled with selective changes in the expression of ESR2 in antral follicles of 10-month-old prenatal T- but not DHT-treated females (this study) are also consistent with a role for estrogen. In distinguishing between androgenic versus estrogenic contribution, similarity of responses between prenatal T (aromatizable androgen) and prenatal DHT-treated (non-aromatizable androgen) females was used as an index to imply androgenic action. While evidence exists that DHT can be metabolized into 5α-androstane-3β,17β-diol (3β-diol) and has the ability to act via the ESR2 receptor (Handa et al. 2008), there is very little information available regarding the extent and impact of this conversion at the ovarian level. Nonetheless, selective changes in AR and ER expressions seen in this study help to distinguish which receptor signaling are involved and the developmental time point when this occurs.

Finally, it is unclear how the fetal changes in AR induced by prenatal T excess reprogram the ovary to culminate in adult ovarian dysfunction. A likely possibility involves epigenetic modifications of key ovarian regulatory genes. Earlier studies have found that sex steroids have the ability to influence the methylation state of DNA sequences and both ER and AR acetylation can be regulated by physiologic stimuli (Leader et al. 2006a, 2006b, Vottero et al. 2006, Xue et al. 2007). The findings from this study are consistent with the recent proposal of a two-step process (Tang et al. 2008) whereby early insult from in utero T treatment resets the course of ovarian development and the manifestation of adult ovarian phenotype requires subsequent exposure to ovarian steroids such as that occurring during puberty (Tang et al. 2008).

In summary, this comprehensive reproductive lifespan study relates the impact of prenatal exposure to excess sex steroids on the developmental trajectory of ovarian steroid receptors. The findings provide evidence that the first step in the altered trajectory of ovarian differentiation of prenatal T-treated females involves an early increase in ovarian AR and that manifestation of postnatal ovarian dysfunction is likely mediated via an increase in the ratio of ESR1/ESR2 receptor coupled with an increase in granulosal expression of AR resulting in increased AR signaling and consequent follicular persistence. The findings may be of translational significance to women with polycystic ovarian disease, the reproductive and metabolic phenotypes of whom the prenatal T-treated females manifest.

Materials and Methods

Breeding and prenatal treatment

All procedures used in this study were approved by the Institutional Animal Care and Use Committee of the University of Michigan and were consistent with the National Research Council’s Guide for the Care and Use of Laboratory Animals. Two to three-year-old Suffolk ewes were purchased locally and bred in a farm receiving oversight from University of Michigan, Department of Laboratory Animal Medicine. Day of mating was determined by visual confirmation of a paint mark left by an intact ram on the hindquarter of bred ewes. Beginning on day 30 of gestation and continuing until day 90 of gestation, pregnant ewes were injected i.m. twice weekly with 100 mg of T propionate (1.2 mg/kg; Sigma–Aldrich Corp.) or 100 mg DHT propionate (Steraloids, Inc., Newport, RI, USA) suspended in cottonseed oil (Sigma–Aldrich Corp). The dose and mode of treatment were chosen based on the large body of data available documenting postnatal reproductive disruptions.
Prenatal T treatment produces circulating concentrations of T in pregnant sheep and female fetuses in the range seen in intact adult males and male fetuses respectively (Wood et al. 1991). Details of prenatal treatments, husbandry, and nutrition of maternal sheep as well as newborn and growing lambs until 4 months of age have been published previously (Manikkam et al. 2004).

Ovaries procured from control, prenatal T, and DHT-treated females on fetal day 90 (six control fetuses from six dams, six T-treated fetuses from six dams, and six DHT-treated fetuses from five dams), fetal day 140 (six control fetuses from five dams, seven T-treated from seven dams, and five DHT-treated from five dams), 10 months of age (five control animals from five dams, six T-treated animals from six dams, and five DHT-treated animals from five dams), and 21 months of age (five control animals from five dams, six T-treated animals from six dams, and eight T-treated animals from eight dams) were utilized in this study. There were insufficient DHT-treated females born to include a 21-month-old prenatal DHT-treated group.

For collection of ovaries from fetuses, dams were killed by administration of a barbiturate overdose (Fatal Plus, Vortech Pharmaceuticals, Dearborn, MI, USA) and fetuses removed. Biweekly progesterone monitoring found that all controls were cycling at both 10- and 21-month age. Barring one prenatal T-treated female, all prenatal T- and DHT-treated females were cycling at 10 months of age although the T animals manifested irregular cycles. At 21 months of age, three prenatal T-treated animals were anovulatory and the other five showed irregular cycles. To avoid influence of cycle stage in cycling females at the time of study (data not shown).

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Western blotting

Details of antibodies used are summarized in Table 1. To test specificity of the antibodies, sheep ovarian tissues were homogenized in a radio-immunoprecipitation assay lysis buffer consisting of 1% v/v IGEPAL CA630 (octylphenyl-polyethylene glycol), 0.5% w/v sodium deoxycholate, 0.1% w/v SDS, 1 mM EDTA, 50 mM sodium fluoride (all from Sigma–Aldrich Corp.), 0.1 M PBS, and a protease inhibitor cocktail (Complete Mini Protease Inhibitor Cocktail Tablets, Roche). Ovarian homogenates were centrifuged at 12 000 g for 20 min and supernatant stored frozen at −80°C. Forty micrograms of protein were separated by SDS-PAGE (10% resolving gel). Proteins were transferred to nitrocellulose membranes (Amersham), blocked for 1 h in 2% w/v non-fat milk in Tris-buffered saline containing 0.05% v/v Tween 20 (Sigma–Aldrich Corp.), and then incubated overnight at 4°C with specific primary antibodies (Table 1). Following washing, membranes were treated for 1 h with corresponding secondary peroxidase-conjugated antibody (Table 1). Immunopositive bands were visualized with a chemiluminescent detection kit (ECL, Amersham).

Immunochemistry

A streptavidin–biotin immunoperoxidase method as described previously (Salvetti et al. 2007) was used for immunohistochemical detection. After deparaffinizing and antigen retrieval in 10 mM sodium citrate solution (pH 6.0) by boiling in

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pressure cooker (ESR1 and PGR) or microwaving (AR and ESR2; Table 1), endogenous peroxidase activity in ovarian sections was quenched with 1% (v/v) H$_2$O$_2$ in methanol. To eliminate nonspecific binding, sections were incubated with 10% (v/v) normal goat serum for 20 min at room temperature before incubating with the primary antibodies for 18 h at 4°C and then with respective biotinylated secondary antibody for 30 min at room temperature. The visualization of antigens was achieved with streptavidin–peroxidase (BioGenex, San Ramon, CA, USA) using 3,3-diaminobenzidine (DAB; Dako, Carpinteria, CA, USA) as chromogen. Ovarian sections were counterstained with Mayer’s hematoxylin, dehydrated, and mounted. Quenching of residual endogenous peroxidase activity was confirmed by incubating some sections with DAB alone. The specificity of the secondary antibodies was tested with negative control sections processed as above except for replacement of primary antibodies with non-immune serum. As multiple series of histological processing were involved, serial sections of a non-experimental set of sheep ovaries were included with each series to allow normalization across series. Each immunohistochemical series included randomly selected slides with ovarian sections from different ages and treatments. Follicle classes were distinguished using the following criteria: primordial, single layer of flattened granulosa cells; primary, partial or one complete layer of cuboidal granulosa cells; small preantral, more than one to five layers granulosa cells; large preantral, more than five layers of cuboidal granulosa cells, and antral, those with an antral cavity.

**Image analysis**

Two sections, the first one-third into the ovary and the second two-thirds into the ovary, were used for immunohistochemical quantification. Considering the follicular sizes (McNatty et al. 1999) and the distance between the two sections (~200 μm in fetal and >10 mm in adult), there is little likelihood of follicular overlap. All growing follicles in both sections were analyzed (ranged between 8 and 15 in follicular classes). For primordial follicles, the slides were scanned left to right from the top and the first 20 primordial follicles that were distinct and showed no overlap with neighboring follicles were utilized. Only healthy follicles without atretic signs (apoptotic/pycnotic nucleus and loss of cell adhesion in granulosa) were evaluated. Image analysis was performed using Image Pro-Plus 3.0 system (Media Cybernetics, Silver Spring, MD, USA). Images were digitized at 40 magnification using an Olympus C5060 digital camera mounted on a conventional light microscope (Olympus BH-2; Olympus Co., Tokyo, Japan). Details of image analysis have been described earlier (Shan et al. 1997, Wang et al. 1999, 2000, Zhu et al. 2000, Salvetti et al. 2007). The nuclei were visualized and identified using AutoPro macro language, an automated sequence operation created to measure the optical density (OD). The images of immunostained slides were converted to an 8 bit gray scale, with the background staining of the histological slides set to zero and the most intense staining set at two. This calibration was carried out using specific tools of the software that determine, through a histogram analysis of the images, the intensity values corresponding to background (represented mainly by cytoplasmic and antral staining) and the most intense staining (in nucleus) independently for each receptor and considering the respective positive and negative controls in each assay. These values were verified and normalized with the controls carried across various runs and the same region (verified by image comparison) was used for calibration. The OD was measured as a mean gray intensity of each pixel divided by the total number of pixels measured. The OD was calculated separately for each follicular compartment (oocyte, granulosa, theca interna, and theca externa) and stroma.

For primordial, primary, and small preantral follicles, all granulosa cells within each follicle were analyzed. For large preantral and antral follicles, 100 cells/compartment/follicle were quantified. To avoid subjectivity and differences in location of cells relative to antrum and theca, vertical rows of all cells spanning between the theca and antrum were quantified until 100 cells were counted (Fig. 1). For each ovary, 200 stromal cells were analyzed, in the centre of the ovary. Sections were analyzed with the observer blinded to treatment.

The major strength of the imaging approach used in this study is visualization of *in situ* localization of proteins within cells of interest. In the past decade, computerized image analysis systems have been developed to obtain objective and accurate quantification of nuclear markers (Lejeune et al. 2008). This approach has been successfully applied by other investigators to quantify steroid receptors in different tissues and validated for diagnostic, prognostic, and therapeutic purposes (Shan et al. 1997, Wang et al. 1999, 2000, Zhu et al. 2000, Salvetti et al. 2007, Lejeune et al. 2008). Since the immunostaining and image analyses are optimized for each protein, quantitative comparisons across proteins are not possible.

**Statistical analyses**

The OD of all cells within each follicular compartment (granulosa, theca, and oocyte) within a follicle class was first averaged and then a group mean across follicles was derived for each follicle type within an animal. When more than one fetus was studied per dam, the data were averaged before analyses. For 21-month-old females, because comparison of data from cycling and anovulatory prenatal T-treated animals revealed no differences in steroid receptor expression, data from cycling and anovulatory animals were treated as one group for analysis. A statistical software package (SPSS 11.0 for Windows; SPSS Inc., Chicago, IL, USA) was used for performing the statistical tests. When more than two groups were involved (control, T-treated, DHT-treated; all ages except 21 months), data were compared by analyses of variance, followed by Duncan’s multiple range tests. For comparing results between two groups (control versus T-treated, 21 months), Student’s *t*-test was utilized. A *P*<0.05 value was considered significant. Results are expressed as mean ± S.E.M.

**Declaration of interest**

The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.
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Received 24 November 2008
First decision 16 January 2009
Accepted 3 March 2009