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Developmental programming: prenatal testosterone excess disrupts antimullerian hormone expression in preantral and antral follicles

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Abstract

Objective—To investigate the impact of prenatal testosterone excess on the expression of key ovarian regulators implicated in follicular recruitment and persistence using a large animal model of polycystic ovarian syndrome.

Design—Interventional, animal model study.

Setting—Academic research unit.

Animals—25 female fetuses, 14 prepubertal female, and 24 adult female Suffolk sheep.

Interventions—Prenatal testosterone treatment.

Main outcome measures—Immunohistochemical determination of expression of antimullerian hormone (AMH), kit-ligand, and growth differentiation factor 9 (GDF9) in fetal, prepubertal, and adult ovarian tissues.

Results—Prenatal testosterone treatment reduced the AMH protein expression in granulosa cells of preantral follicles and increased its expression in antral follicles compared to age-matched adult controls. These differences were not evident in prepubertal animals. Protein expression of GDF9 and kit-ligand was not altered at any of the developmental time points studied.

Conclusion—Prenatal testosterone exposure is associated with changes in AMH expression in preantral and antral follicles in adult ovaries, similar to findings in PCOS women. These findings indicate that abnormal folliculogenesis in PCOS may be at least in part mediated by changes in AMH expression.

Keywords

PCOS; ovary; folliculogenesis; follicular recruitment; follicular persistence

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Introduction

Studies with animal models have found that exposure to excess testosterone (T) during development has a negative impact on reproduction and metabolism (1–3). In sheep, prenatal T excess (gestational day [D] 30–90) coinciding with early follicular differentiation (4), disrupts postnatal ovarian morphology and function (3, 5–6) culminating in an ovarian phenotype similar to that found in women with polycystic ovary syndrome (PCOS) (7–8). However, to what extent gestational T excess is a contributor to PCOS is controversial (9–10).

The ovarian disruptions induced by prenatal T excess include multifollicular morphology (5, 6), increased follicular recruitment (5, 11), and follicular persistence (12–13); traits also reported in women with PCOS (14). Little is known about the underlying mechanisms by which these ovarian disruptions are programmed to occur during adult life. Ovarian differentiation is a complex process with members of the transforming growth factor (TGF)- β family, among others, playing a role in mediating primordial germ cell migration, follicular recruitment, and selection (15). In the context of ovarian programming by prenatal T excess, it is of interest that T promotes early follicular differentiation (16), facilitates activation of primordial to primary follicles (17), and regulates oocyte-specific factors (18). Therefore, steroidal imbalance during critical stages of ovarian development may disrupt timing and / or level of expression of follicular growth and differentiation factors that are key to progression of normal ovarian differentiation. As such, delineation of the sequence of changes from the timing of the insult during fetal life to development of the pathology during later life of key regulatory factors would not only help identify key mediators, but also point to time points during reproductive life to target interventions.

The focus of this paper is on three key mediators of early follicular differentiation namely, antimüllerian hormone (AMH), kit-ligand (KL), and growth and differentiation factor-9 (GDF9). AMH, a member of the TGF- β superfamily and a granulosa cell product, has been implicated in repression of follicular recruitment as evidenced from enhanced recruitment in AMH null mice (19) and cultured human ovarian cortical biopses (20). KL, also a granulosa cell factor, is involved in granulosa cell proliferation, theca cell differentiation, and formation of the antral cavity (21). Mutant Kit mice manifest arrested early follicular development at the primary follicle stage (22). GDF9, an oocyte-specific marker, also plays a role in follicular recruitment as evidenced by the follicular arrest at the primary stage in the GDF9 knockout mice (23). Because T has been shown to affect follicular differentiation and all three factors are likely targets of T programming (16–18), early disruptions in any of these mediators may contribute to the increased follicular recruitment / persistence seen in prenatal T-treated females.

In this study, in view of the critical role AMH, KL, and GDF9 play in follicular growth and differentiation, using sheep as a model system, we tested the hypothesis that prenatal T excess disrupts the developmental expression of these key regulators in a manner consistent with enhanced follicular recruitment and follicular persistence (5, 11–13).

Material and methods

Prenatal treatments

All procedures used were approved by the Institutional Animal Care and Use Committee of the University of Michigan and are consistent with National Research Council's Guide for the Care and Use of Laboratory Animals. The study was conducted at the University of Michigan Research Facility (Ann Arbor, MI; 42° 18'N). Details of prenatal T treatment as well as husbandry and nutrition of maternal sheep and newborn lambs have been published

previously (24). For generating prenatal T-treated females, pregnant sheep were injected (i.m.) twice weekly from days 30–90 of gestation with 100 mg T propionate (Sigma Chemical Co., St. Louis, MO) suspended in cottonseed oil. This dose of T produces adult male levels of T in maternal circulation and T levels in the female fetus that are in the range of that seen in male fetuses at D65 (25). Control animals did not receive vehicle since no differences in reproductive characteristics were found between vehicle-treated and non-treated controls in a previous study (26). Relative to fetal T concentrations achieved, it is of interest that forty percent of human female fetuses have been reported to have T in the male fetal range (27).

Ovaries were obtained from control and prenatal T-treated females at fetal D90 (6 fetuses from 6 dams per group), fetal D140 (6 control fetuses from 5 dams and 7 T-treated fetuses from 7 dams), prepubertal (5.5 mo of age; 8 control females from 8 dams and 6 T-treated females from 6 dams), postpubertal (10 mo of age; 5 control females from 5 dams and 6 T-treated females from 6 dams), and adult (21 mo of age; 5 control animals from 5 dams, 8 T-treated animals from 8 dams) age. Fetal ovaries were procured after the dams were euthanized by administration of a barbiturate overdose (Fatal Plus, Vortech Pharmaceuticals, Dearborn, MI, USA) and fetuses removed. Ovaries from adult ages (10- and 21-mo old) were collected 25 h after administration of a second prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$, 5 mg/ml Lutalyse; Pfizer Animal Health, New York, NY, USA) given 11 days apart. Details of euthanasia, ovarian collection, and tissue processing have been published (5, 28). Paraffin-embedded sections from one ovary from each animal were used in this study. Developmental changes in ovarian follicular distribution were determined by ovarian morphometry (5) and changes in protein expression of androgen, estrogen, and progesterone receptors (28) and members of the insulin signaling pathway (29) as determined by immunohistochemistry from the same set of animals have been previously published.

Immunohistochemistry

All sections were deparaffinized, and antigen retrieval was performed by heating in a pressure cooker (10 min). For AMH and KL immunohistochemistry, endogenous peroxidase activity was blocked with 3% H_2O_2 in methanol, and non-specific binding was blocked with 10% (v/v) normal goat and mouse serum, respectively. Sections were incubated at 4°C overnight with the following primary antibodies; AMH (polyclonal; sc-6886, Santa Cruz Biotechnologies, Santa Cruz, CA; dilution 1:100) and KL (monoclonal; sc-13126, Santa Cruz Biotechnologies, Santa Cruz, CA; dilution 1:200) or their respective negative control antisera, goat (sc-2043; Santa Cruz Biotechnologies, Santa Cruz, CA) or mouse antiserum (X0931; Dako North America, Inc. Carpinteria, CA).

The rest of the assay protocol was performed at room temperature. Sections assigned for AMH and KL detection were incubated with donkey anti-goat IgG-HRP (sc-2020; Santa Cruz Biotechnologies, Santa Cruz, CA) and horseradish peroxidase-labelled polymer (EnVision + DualLink System, Peroxidase, Dako North America, Inc. Carpinteria, CA) respectively, for 1 h at room temperature. AMH and KL detection was performed using chromogen 3,3'-diaminobenzidine (DAB; Dako North America, Inc. Carpinteria, CA) as per the manufacturer recommendation. Blocking of the endogenous peroxidase activity was confirmed by incubating some sections with DAB alone. The sections were then counterstained with hematoxylin-eosin, dehydrated, and mounted.

For GDF9 immunostaining, after antigen retrieval with a pressure cooker, avidin and biotin endogenous activity were blocked with 12.5 IU/ml avidin (A9275, Sigma Chemical Co., St. Louis, MO) and 0.1 mg/ml biotin (B4501, Sigma Chemical Co., St. Louis, MO). Sections were incubated with the GDF9 primary antibody overnight at 4°C (dilution 1:100). Details of generation of the ovine GDF9 antibody have been described previously (30). Sections

were incubated for 30 min at room temperature with a biotinylated secondary antibody (dilution: 1:500; rabbit anti-mouse IgG (E354, Dako North America, Inc. Carpinteria, CA) followed by an avidin-biotinylated horseradish peroxidase complex (K0355, Dako North America, Inc. Carpinteria, CA) for 30 min. Tyramide signal amplification biotin system was used for GDF9 (NEL700A001KT, Perkin Elmer, Boston, MA) which consisted of incubation with biotinyl-tyramide (dilution 1:50 for 10 min) followed by a second incubation with streptavidin- horseradish peroxidase complex (dilution 1:100 for 30 min). The large number of sections involved required multiple series of histological processing. Each series were blocked to include ovarian sections from all ages and treatments. For GDF9, sections from a non-experimental ovary with multiple oocytes in antral follicles were included with each run as a positive control.

Image analysis

To avoid overlap of measures in follicles, immunohistochemical quantification for each marker was performed in two ovarian sections, the first one-third into the ovary and the second two-thirds into the ovary. Follicle classes were distinguished using the following criteria: primordial (PRI), single layer of flattened granulosa cells; transitional (TRA), mixed layer of flattened and cuboidal cells; primary (PRY), partial or one complete layer of cuboidal granulosa cells; small preantral (SPF), two to five layers granulosa cells; large preantral (LPF), more than five layers of cuboidal granulosa cells; small antral (SAF), antrum formed and < 1 mm of diameter; and large antral (LAF), antrum formed and ≥ 1 mm of diameter.

For all developmental stages during both fetal and postnatal life, 40 primordial, 40 transitional, and 40 primary follicles per ovary were studied. Each ovary section was divided into 4 quadrants and five follicles from each quadrant were chosen sequentially clockwise to a total of 20 follicles per section. All preantral and antral follicles present in a given section were studied. Very few follicles above the primary stage were observed in the fetal D140 ovaries. Degree of expression was categorized in a 0–5 scale for all markers studied.

To relate the degree of expression from categorical scale, densities of AMH, KL, and GDF9 from individual follicles were quantified (100 follicles/marker) under brightfield illumination using a Leica DMR microscope, Diagnostic Instruments Spot RT camera, and Spot RT software v3.5.1. All images for each marker were captured using the same magnification and camera settings. The immunopositivity of granulosa cells (AMH and KL) and oocytes (GDF9) were measured using IMAGEJ software (National Institutes of Health, Bethesda, MD, USA) after background subtraction (50 pixels) with a fixed threshold for each marker. Mean density was calculated for three areas of each follicle (AMH and KL) and one area for the oocytes (GDF9). Mean density obtained for each follicle was then correlated to the categorical measure (0–5 scale) for each of the markers. Pearson correlation coefficients (R^2) between these measures were 0.933, 0.897, and 0.895 for AMH, KL, and GDF9, respectively (Figure 1), thus confirming the validity of the categorical method used.

Statistical analyses

The degree of expression of AMH and KL in the granulosa cell layer was analyzed using a linear mixed effect model controlling for correlation between follicles from the same female. Degree of expression for GDF9 (oocyte expression) was analyzed using ANOVA on repeated measures with multiple comparisons adjusted with either Tukey or Newman-Keul methods. When more than one fetus was studied per dam, the data were averaged before analyses. All analyses were carried out using SAS for Windows release 9.1.3 (SAS Institute, Cary, NC). All results are presented as least square means $\pm$ SEM. Significance was defined as $P < 0.05$.

Results

Representative immunostaining of AMH, KL, and GDF9 from 10-mo-old control and prenatal T-treated females are shown in Figure 2. Results from quantitative analysis of the expression of these proteins from immunohistochemical analyses are shown in Figure 3.

In the control group, AMH immunostaining was restricted to the cytoplasm of granulosa cells of small preantral and larger follicles at D140. A small number of preantral follicles was present at fetal D140 precluding statistical analysis. In prepubertal animals, AMH was found in small preantral, large preantral, small antral, and large antral follicles. AMH expression was higher in the large preantral compared to small preantral follicles ($P < 0.05$) but similar between large preantral and small antral in prepubertal, 10- and 21-mo-old animals (Fig. 3, panel B). In prepubertal animals, AMH expression was lower in large antral compared to small antral stage (Fig. 3, panel B). AMH expression in small preantral, large preantral and small antral in 10- and 21-mo-old control females were follicle stage dependent and followed the same pattern as in prepubertal animals.

The increase in AMH expression between the small preantral and the large preantral stage and similar level of expression between large preantral and small antral stage in control females were also evident in prenatal T-treated females at all 3 ages, although AMH expression in the large preantral follicles of 10- and 21-mo-old prenatal T-treated females were lower compared to age matched controls ($P < 0.05$). The AMH decline seen in controls from small antral to large antral in the prepubertal and 10-mo-old control females was not evident in the prenatal T-treated groups. As such, AMH expression was higher in the large antral follicles of 10-mo-old prenatal T-treated females compared to controls ($P < 0.05$).

KL immunostaining was localized to the granulosa cell compartment at all follicle stages. Level of KL immunoexpression did not differ between follicle types or treatment groups in any of the fetal or postnatal ages studied (Fig. 3, panel B). GDF9 immunostaining was restricted to oocytes at all stages evaluated. Percentage of follicles from the primordial to the primary stage that expressed GDF9 at D90 and D140 are shown in Fig. 3, panel A. At both fetal ages not all oocytes were found to express GDF9. There was no treatment effect of prenatal T on the percentage of follicles expressing GDF9 or degree of GDF9 expression (Fig. 3, panel B) at any fetal age. In all postnatal ages (prepubertal, 10- and 21-mo-old), the expression of GDF9 was follicle stage dependent ($P < 0.01$, $P < 0.05$, and $P < 0.05$ for prepubertal, 10- and 21-mo-old, respectively). Degree of GDF9 expression increased with follicular stage until the large preantral stage in both control and T-treated females at all 3 ages studied ($P < 0.05$). No significant differences in GDF9 expression were found between treatment groups.

Discussion

Findings from this study provide evidence that prenatal T treatment leads to selective disruption in AMH protein expression during adult life in a follicle-specific manner, but not KL or GDF9. The relevance of these changes during the different developmental time points and its translational relevance is discussed below.

Developmental expression of AMH, KL, and GDF9

Restriction of AMH expression to small preantral and larger follicles at both fetal stages (D90 and D140) parallel what is seen in humans (31). Limited number of such follicles present at these fetal ages precluded assessment of treatment effects. Immunocytochemical studies with human ovaries have also found AMH positive cells only at 36 weeks of gestation in secondary follicles (31) as opposed to its detection as early as day 30 of

gestation in the Sertoli cells in the male (32). In contrast to AMH, expression of KL and GDF9 was evident as early as fetal D90; previous studies have detected expression of GDF9 by RT-PCR at day 56 of gestation (33–34) and by in situ hybridization at day 135 (35).

Restricted expression of AMH to granulosa cells in adult ovaries with highest expression in large preantral and small antral follicles declining in large antral follicles also confirms previous findings in rodents and cattle (36–37) and human (38). The developmental changes in AMH in control females support the concept that the direction of changes in AMH expression is consistent with cyclic follicular recruitment and follicular survival (19). A previous study conducted in sheep found the proportion of AMH-positive follicles to increase with increasing follicle size (39). Similar expression of KL across follicle stages at all ages studied in this study is also in agreement with previous findings in rodents and sheep (40–41), but to our knowledge there are no reports addressing expression of GDF9 across developmental time points.

Impact of prenatal T excess on AMH, KL, and GDF9

During normal follicular differentiation, AMH expression increases from the primary to the antral stage and then declines as the selected follicle progresses towards the preovulatory stage (36, 38). The reduced AMH expression in the large preantral stage and the lack of decrease in AMH expression in large antral follicles might, at least in part, account for the disrupted follicular differentiation in prenatal T-treated females, namely follicular recruitment (5, 11) and follicular persistence (12–13).

Due to the limited number of positively stained follicles for AMH during fetal ages, it was not possible to assess prenatal T treatment effects. A previous study in the Poll Dorset sheep reported that the proportion of non-growing and early transitional follicles (<50% cuboidal granulosa cells) positive for AMH was lower in T fetuses (fetal age not specified) than in controls (6.5 vs. 4.7%, $P=0.05$) (39). Since AMH is postulated to have an inhibitory role on primordial follicular recruitment (19, 20) it is likely that the reduced number of transitional follicles expressing AMH (39) may underlie the increased follicular recruitment seen in prenatally T-treated sheep (5, 11). Although T can stimulate expression of KL *in vitro* (42) and KL has a major role in the establishment of the ovarian germ cell population (43), prenatal T-treatment did not alter expression of KL in this study implicating involvement of other regulators.

AMH, the only regulator where changes have been observed, has been implicated in establishment of a FSH threshold for follicular recruitment; the AMHKO mice have increased number of growing follicles despite lower FSH levels (19), supportive of AMH playing a role in reducing the threshold for FSH requirement. The reduction in AMH expression in large preantral follicles in the prenatal T-treated group, a stage when AMH levels are high in control ovaries (38), may therefore help overcome AMH inhibition of FSH sensitivity. This in turn would increase the number of follicles being recruited thus contributing to the multifollicular morphology of prenatal T-treated females (5–6). Interestingly, aberrant FSH sensitivity is postulated to be the underlying cause for the abnormal follicle development in PCOS women (44). Because T downregulates AMH expression in cultured bovine follicles (45), it is possible that changes in AR expression may account for reduced expression of AMH in preantral follicles. Increase in AR is evident in preantral follicles of prenatal T-treated D140 fetuses, when AMH suppression is beginning to be evident (28). Although changes in AMH expression have been reported to influence expression of KL (46), a corresponding change in KL was not evident in the current study. Paradoxically, the effect of prenatal T treatment on AMH expression was evident postpubertally but not prepubertally, although increased follicular recruitment is evident prepubertally (47). Conceivably, defects in AMH expression evident postpubertally in

prenatal T females may be a function of reproductive maturity and associated hormonal changes. Paradoxically, the loss of the prenatal T-treatment effect on AMH overexpression in large antral follicles in year 2 relative to year 1 is not accompanied by an improvement in ovulatory frequency (12), indicative of involvement of other players. Interestingly, in humans, onset of PCOS symptoms coincides with puberty (48). Higher expression of AMH in large antral follicles, as opposed to reduced expression in large preantral follicles is suggestive of abnormal or incomplete maturation of preovulatory follicles in prenatal T-treated females. In humans, AMH expression is lost during the preovulatory follicle development (38). We hypothesize that the lack of reduction of AMH expression in large antral follicles in prenatal T females may prevent these follicles from proceeding to the preovulatory state, thus contributing to follicular persistence seen in these females (12–13).

In relating the findings from this animal model to PCOS, it is important to address when during folliculogenesis follicle growth gets arrested. Although there are several reports addressing abnormal growth at early stages of follicular development (14, 49), follicular persistence has not been systematically evaluated in women with PCOS. One time transvaginal or transabdominal ultrasonography can not discern whether the higher number of follicular count is due to follicular persistence or the result of a higher follicular turnover as hypothesized earlier (14). Presence of >12 antral follicles (2–9 mm) is used as cut off in the humans is ~20 mm (51) as opposed to ~8 mm in Suffolk sheep (52), follicles appear to arrest at small antral follicle stage in women with PCOS (~7–8 mm), as opposed the larger size of arrested follicles in prenatal T-treated females.

The compromised AMH expression in antral follicles may also be secondary to altered steroid receptor balance, since large antral follicles of prenatal T females manifest increased expression of AR and ESR1, and a decrease in ESR2 (28). In support, estrogens (53) and androgens (45) have been found to regulate follicular AMH expression in vitro.

Genetic studies in PCOS subjects, whose ovarian phenotype the prenatal T animals recapitulate, have suggested a role for both GDF9 and AMH, although AMH contribution to the abnormal ovarian morphology is debated (54–55). Variants in GDF9 gene in PCOS women are found to be associated with hirsutism scores, potentially as a result of increased theca cell growth in the ovary leading to elevated androgens (54). Because prenatal T excess leads to a formation of a less developed and disorganized theca layer in antral follicles (5), and KL and GDF9 play a role in theca cell formation (56) and differentiation (57), respectively, a reduction in KL or GDF9 expression in theca cells of antral follicles of adult females was expected. Failure of such changes to occur suggests that underdeveloped and disorganized theca cells in prenatal T females do not involve changes in KL or GDF9, but are likely associated with changes in extracellular matrix proteins (58) such as seen in PCOS, where a decrease in fibrillin-3 expression has been reported (59).

This study demonstrates, for the first time, that prenatal T treatment programs changes in AMH expression in preantral and antral follicles in adult ovaries. Findings from this study are in agreement with findings in PCOS women, where AMH expression in granulosa cells is elevated (60). They are also consistent with the hypothesis that abnormal folliculogenesis in PCOS may be mediated, at least in part, by AMH and that reduced AMH expression may itself be a function of excess prenatal T exposure. Whether such changes are mediated via androgenic or estrogenic actions of T (because T can be aromatized to estrogen) warrants further research.

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Capsule

Prenatal testosterone leads to changes in antimullerian hormone protein expression that likely account for the increased follicular recruitment and persistence found in this animal model of polycystic ovary syndrome.

Figure 1. Correlation between the degree of expression from categorical scale and mean density after quantification from individual follicles is shown for AMH (*top*), KL (*middle*), and GDF9 (*bottom*). Pearson correlation coefficient is shown as R^2 .

Figure 2.

Top panel: Representative negative and positive control images from AMH (*left*), KL (*middle*), and GDF9 (*right*) immunostaining in large preantral follicles from 10-mo-old ovaries. Bottom panel: Representative images of AMH (*left columns*), KL (*middle columns*), and GDF9 (*right columns*) immunostaining in primordial, primary, small preantral, large preantral, small antral, and large antral follicles in 10-mo-old control and prenatal T-treated females. Bar = 50 μm.

Figure 3. Panel A: Percentage of follicles that expressed GDF9 at fetal ages D90 and D140. Panel B: Impact of prenatal T on expression of AMH, KL, and GDF9 in ovaries of prepubertal (*top panels*; 5.5-mo-old), postpubertal (*middle panels*; 10-mo-old) and adult (*bottom panels*; 21-mo P-old) sheep (control: *open bars*; prenatal T-treated: *closed bars*) sheep. Data expressed as mean $\pm$ SEM. Significant differences between treatment groups within follicle class are indicated by asterisks (* $P < 0.05$). Significant differences between follicle class within marker and age are indicated by different letters ($a \neq b$; $P < 0.05$).

Figure 4.

Schematic summarizing direction of changes in AMH expression across follicular stages and the impact of prenatal T excess. During normal follicular differentiation (*left side*), AMH levels increase from primordial to the preantral stage and is implicated in suppression of follicular recruitment. AMH expression is reduced in large antral follicles thus contributing to increased follicular FSH sensitivity. Prenatal T (*right side*) reduces expression of AMH in preantral follicles thus contributing to increased follicular recruitment. Lack of AMH suppression in large antral follicles of prenatal T-treated animals may reduce FSH sensitivity and thus contributing towards arrested follicular growth and persistence. Abbreviations: AMH: antimüllerian hormone, FSH: follicle stimulating hormone, Gr: granulosa cells, Oc: oocyte, T: testosterone, and Th: theca cells.