Developmental Programming: Impact of Prenatal Testosterone Excess on Steroidal Machinery and Cell Differentiation Markers in Visceral Adipocytes of Female Sheep

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Abstract

Prenatal testosterone (T)-treated female sheep manifest reduced adipocyte size and peripheral insulin resistance. The small adipocyte phenotype may reflect defects in adipogenesis and its steroidal machinery. To test whether prenatal T treatment from gestational days 30 to 90 alters the visceral adipose tissue (VAT) steroidal machinery and reduces adipocyte differentiation, we examined expression of the steroidogenic enzymes, steroid receptors, and adipocyte differentiation markers at fetal day 90 and postnatal ages 10 and 21 months. Because gestational T treatment increases fetal T and maternal insulin, the contributions of these were assessed by androgen receptor antagonist or insulin sensitizer cotreatment, either separately (at fetal day 90 and 21 months of age time points) or together (10 months of age). The effects on adipogenesis were assessed in the VAT-derived mesenchymal stem cells (AT-MSCs) from pre- and postpubertal time points to evaluate the effects of pubertal steroidal changes on adipogenesis. Our results show that VAT manifests potentially a predominant estrogenic intracrine milieu (increased aromatase and estrogen receptor α) and reduced differentiation markers at fetal day 90 and postnatal 21 months of age. These changes appear to involve both androgenic and metabolic pathways. Preliminary findings suggest that prenatal T treatment reduces adipogenesis, decreases expression of differentiation, and increases expression of commitment markers at both pre- and postpubertal time points. Together, these findings suggest that (1) increased commitment of AT-MSCs to adipocyte lineage and decreased differentiation to adipocytes may underlie the small adipocyte phenotype of prenatal T-treated females and (2) excess T-induced changes in steroidal machinery in the VAT likely participate in the programming/maintenance of this defect.

Keywords

steroids, estrogen, testosterone, adipocytes, androgen antagonist, insulin sensitizer

Introduction

Metabolic diseases such as obesity, type 2 diabetes mellitus, and polycystic ovary syndrome (PCOS) are usually associated with systemic insulin resistance (IR) and adipose tissue dysfunction.^{1,2} The relationship between adipose tissue dysfunction and IR is so strong that either buildup of adipose tissue as in obesity or deficiency as in lipodystrophy is accompanied by IR.³ Various developmental insults stemming from disease states, under nutrition, obesity, stress, and exposures to environmental pollutants have been implicated in the development of IR and adipose tissue dysfunctions in the offspring.^{4,5} Large numbers of studies with animal models have provided evidence that developmental exposures to either native steroids or environmental endocrine disrupting chemicals result in IR and adipose tissue dysfunction.⁵⁻⁸ For instance, prenatal testosterone (T) excess leads to IR, dyslipidemia, and adipose tissue

dysfunctions in monkeys,^{9,10} sheep,^{11,12} and mice^{13,14} with the nature of dysfunction varying depending on the species, time of exposure, and developmental time points studied. In sheep, the model used in this study, effects of prenatal T excess are manifested as reduced adipocyte size in the absence of an increase in visceral adiposity,^{11,15,16} implying that the effects are at the level of adipocyte differentiation.

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Adipocytes, one of the major cell components of adipose tissue, are derived from adipose stem cells which are mesenchymal stem cell (MSCs) that are present in various tissues including adipose tissue and bone marrow, among others.^{17,18} Adipogenesis from MSCs involve a 2-step process.¹⁹ The first step, commitment of MSC into adipocyte lineage leading to the formation of preadipocytes,^{20,21} involves increased expression and activation of bone morphogenetic protein 4-associated zinc finger protein 423 (ZNF423; also known as ZFP423).²¹ The second step, the differentiation of the preadipocytes to become lipid-laden adipocytes, involves several transcriptional factors including peroxisome proliferator-activated receptor- γ (PPARG- γ) and CCAAT-enhancer-binding proteins^{22,23} and is regulated by hormones such as insulin-like growth factor 1 and steroid hormones.²⁴ Developmental insults have been found to increase AT-MSC proliferation, preadipocyte differentiation, and hypertrophy of adipocytes by affecting either or both steps.²⁵⁻²⁷

In addition to being involved in developmental programming of adipocyte differentiation, steroid hormones can exert direct effects on adipocytes with both estrogen (E) and T, influencing adipocyte proliferation and/or differentiation.²⁸ Adipose tissue predominantly expresses estrogen receptor 1 (ESR1), which mediates direct actions of E in reducing lipogenesis and promoting lipolysis²⁹ and reducing adipocyte size,³⁰ all of which could decrease adipocyte storage capacity. Additionally, the extent of E effects on adipogenesis seems to vary depending on the species, sex, and the fat depot, although in general it promotes adipogenesis.²⁹ In contrast, T inhibits adipogenesis and reduces lipolysis.^{29,31,32} Because gestational T treatment increases fetal T and E levels,³³ induces hyperandrogenism,³⁴ and elevates E in adult females³⁵ and adipose tissue produces steroids³⁶ intracrine changes in steroid hormones also have the potential to modulate adipogenesis throughout life. Using the prenatal T-treated sheep as the model, the aims of this study were to assess the effects of prenatal T excess on (1) the local steroidogenic enzymes and steroid receptors in the visceral adipose tissue (VAT), (2) developmental changes in the expression of markers of adipocyte differentiation in VAT, and (3) the capacity of AT-MSCs in VAT to differentiate into preadipocytes/adipocytes during pre- and postpubertal life. In addition, because gestational T exposure not only increased fetal T but also maternal insulin levels,³³ the contribution of androgenic and/or insulin-dependent pathways in programming the prenatal T-induced defects in adipocyte dysfunctions were also investigated.

Methods

Animal Breeding and Maintenance

All animal procedures used in these studies were approved by the Animal Care and Use Committee of the University of Michigan and are consistent with the National Institutes of Health Guide for Use and Care of Animals. All studies were conducted at the University of Michigan Sheep Research Facility (Ann Arbor, Michigan; 42° 18' N) using multiparous

Suffolk sheep. Details regarding animal maintenance, breeding, and lambing have been described previously.³⁷ In brief, ewes were group fed daily with 0.5 kg shelled corn and 1.0 to 1.5 kg alfalfa hay/ewe and were mated to raddled Suffolk rams. Once mated, pregnant females were randomly assigned to different treatment groups. After weaning at ~8 weeks of age, female lambs were maintained outdoors and fed a pelleted diet (Shur-Gain; Nutreco Canada Inc, Guelph, Canada) comprised of 3.6 MCal/kg digestible energy and 18% crude protein.

Experiment Design

Study 1: Developmental impact of gestational T excess and the androgen receptor antagonist/insulin sensitizer interventions on adipose tissue steroidal machinery and markers of adipocyte cell differentiation. To determine the effects of gestational T excess and the relative contribution of androgen and insulin in modulating the steroidal machinery and markers of adipocyte differentiation, VAT from female offspring born to control (C); prenatal T treated (T); prenatal T + androgen receptor (AR) antagonist, flutamide treated (TF); and prenatal T + insulin sensitizer, rosiglitazone treated (TR) were studied at gestational day (GD) 90 and after birth at 21 months of age (Figure 1, top). Treatments for the prenatal T group consisted of twice-weekly intramuscular administration of 100 mg T propionate (1.2 mg/kg; Sigma-Aldrich, St. Louis, Missouri) suspended in 2-mL cottonseed oil from day 30 to day 90 of gestation. For the TF group, in addition to T, flutamide (15 mg/kg; Sigma-Aldrich)-filled capsule (Profill Capsule Filling System; Torpac, Fairfield, New Jersey) was administered daily, orally. This dose was chosen as it blocks the phenotypic virilization effects of both exogenous and endogenous androgens in males and prenatal T-treated female sheep.³⁸ For TR group, in addition to T, rosiglitazone (~0.11 mg/kg; GlaxoSmithKline, Research Triangle Park, North Carolina) tablets were administered orally on a daily basis. This dose has been previously shown to normalize the insulin to glucose ratio in the maternal circulation³³ and restore insulin sensitivity³⁹ in prenatal T-treated sheep. The number of female fetuses studied at GD90 was n = 6 for each of the groups. Year 2 sample sizes were C = 6, T = 5, TF = 9, and TR = 5. The effects of these interventions on estradiol positive feedback, puberty, insulin sensitivity, and adipocyte size distribution in this cohort of animals have been published,^{16,40-42} thus providing a framework for comparing adipocyte changes with adult phenotypic outcomes.

Study 2: Developmental impact of AR antagonist and insulin sensitizer dual intervention on steroidal machinery and adipocyte cell differentiation markers. This study examined if negation of androgen action and restoration of maternal insulin sensitivity in concert would prevent prenatal T-induced disruptions in local steroidal machinery and adipocyte differentiation (Figure 1). Pregnant sheep were randomly assigned to 1 of 3 groups: C (n = 8), T (n = 10), and TFR; n = 11, T + flutamide + rosiglitazone). The dosages of T, flutamide, and rosiglitazone as well as duration and mode of treatment are the

Figure 1. Top panel: Schematic showing the time line of prenatal treatments and the time point of VAT harvest for assessing the effects of prenatal T-treatment and androgen receptor antagonist and insulin sensitizer interventions on the VAT steroidal machinery and adipocyte differentiation markers (study 1). Bottom panel: Schematic showing the time line of prenatal treatments and the time points of VAT harvest for assessing the effects of dual intervention on steroidal machinery and adipocyte differentiation markers (study 2) and adipogenesis (study 3). VAT indicates visceral adipose tissue.

same as described in study 1. At 10 months of age (postpubertal), VAT (Figure 1) was collected following euthanasia with barbiturate overdose (Fatal Plus; Vortech Pharmaceuticals, Dearborn, Michigan) from C ($n = 7$), T ($n = 3$; only 3 female lambs were born with majority being males), and TFR ($n = 7$) female offspring. A portion of VAT was snap frozen for gene expression analysis (this study) and the remainder used for the adipogenesis study (study 3).

Study 3: Developmental impact of prenatal T excess and AR antagonist and insulin sensitizer dual intervention on adipogenesis of AT-MSC. To determine the impact of prenatal T on adipogenesis and the effects of dual intervention (AR antagonist +insulin sensitizer) in negating the detrimental effects of prenatal T on adipogenesis, VAT was collected from C ($n = 7$), T ($n = 3$), and TFR ($n = 7$) females at 5 months of age during a laparotomy and at 10 months of age following euthanasia of the animals (same set of animals as in study 2). The AT-MSCs were isolated from VAT using well-established procedures.⁴³ In brief, adipose tissues were minced and digested in 0.1% collagenase at 37°C for 1

hour in a shaker incubator. At the end of digestion, the mixture was centrifuged at 200g for 3 minutes at room temperature to remove the debris and adipocytes. The supernatant containing the stromal vascular fraction (SVF) was centrifuged at 800g for 5 minutes at 4°C. The resulting cell pellet was washed twice in 10 mL Dulbecco's Modified Eagle Medium containing 10% fetal bovine serum, 1X of antibiotic and antimycotic (Invitrogen, Carlsbad, California), plated onto cell culture dish and incubated at 37°C with 5% carbon dioxide (CO₂) in a cell culture incubator. The AT-MSCs were enriched by their capacity to adhere to culture dish plastic and cultured for 2 passages before storing in liquid nitrogen. All assays were run with cells at passage 2. Since 1 of 3 T animals did not yield sufficient VAT for AT-MSC isolation at 5 months, only 2 T animals were used in this study.

Differentiation of AT-MSCs into Preadipocytes/Adipocytes

Differentiation was induced by adipocyte differentiation medium (ZenBio, Research Triangle Park, North Carolina)

Table 1. Sequence of the Primers Used.

Gene ID	Forward Primer	Reverse Primer	Accession Number
STAR	AGCGCATGGAGGCTATGG	AATCTTCTGCAGGACCTTGATCTC	NM_174189.2
HSD3- β	CATCATCCACACTGCCTCTATCA	ACAGAAGCTGGGTACCTTTCACA	NM_001135932
CYP17	AGACATATTCCTGCGCTGA	GCAGCTTTGAATCCTGCTCT	XM_012102863.2
CYP19	CTGGCCTGGTGCGCATGGT	TGCGCCGCATGAGGGTCAAC	NM_001123000
ESR1	TCGTCTCGTTCCGTATGATG	GACAGAAATGTGTACACCCAGAAT	AY033393.1
ESR2	GCCGACAAGGAAGTGGTACAC	CCACGAAGCCCGGAATCT	NM_001009737.1
AR	GCCCATCTTTCTGAATGTCC	CAAACACCATAAGCCCCATC	XM_001253942
PPARG	CCTTACCACCGTTGACTTCT	GATACAGGCTCCACTTTGATTGC	HQ589347.1
LPL	AGGACACTTGCCACCTCATTC	TTGGAGTCCGGTTCCTCTTGTA	DQ997818
FABP4	AAACTGGGATGGGAAATCAACC	TGCTCTCTCGTAAACTCTGGTAGC	NM_00114667.1
CD36	GTACAGATGCAGCCTCATTTCC	TGGACCTGCAAATATCAGAGGA	X91503
ZNF423	CTGGCGGTGAAGCACAGCACGAG	GCAGGTGCTTCTCCAGCAGATGA	NM_001101893.1
SCD1	TTCCTGATCATGCCAACACCATG	GTCGGATAAATCTAGCGTAGCA	GU947654
SREBP	CTGCTGACCGACATAGAAGACAT	GTAGGGCGGGTCAAACAGG	HM443643.1
FASN	CCCAGCTCAACGAAACCA	GACGAGGTCAACACCCTTCC	AF479289.1
RPL19	CCTTGGCTCGCCGGA	CATGTGGCGGTCAATCTCTTA	XM_012186026.1

Abbreviations: AR, androgen receptor; ESR1, estrogen receptor alpha; ESR2, estrogen receptor- β 2; FABP4, fatty acid-binding protein 4; FASN, fatty acid synthase; HSD3- β , 3- β hydroxysteroid dehydrogenase; LPL, lipoprotein lipase; PPARG, peroxisome proliferator-activated receptor; SCD1, stearoyl-coenzyme A desaturase 1; SREBP, sterol regulatory element-binding protein; STAR, steroidogenic acute regulatory protein.

according to the manufacturer's protocol. In brief, SVF cells were thawed and grown in preadipocyte medium (ZenBio) at 37°C with 5% CO₂ for 2 days. Cells were then plated onto 6-well plates in the density of 4×10^5 cells/well. The differentiation medium was added once the cells became confluent and incubated for 7 days. Cells were then cultured in adipocyte maintenance medium (ZenBio) for up to 14 days. Cells were collected at different time points for messenger RNA (mRNA) analysis or processed at the end of 14 days for oil red O staining.

Oil Red O Staining

At 14 days of differentiation, newly formed adipocytes attached to the culture plates were fixed with 4% formaldehyde in phosphate buffered saline, stained with 0.3% oil red O (Sigma-Aldrich) for 20 minutes, washed 2 $\times$ with isopropanol, and the stained cells visualized using a Nikon inverted microscope (DM IRE2; Nikon Instruments, Melville, New York).

RNA Extraction and Reverse Transcription Polymerase Chain Reaction (RT-PCR)

Total RNA was isolated from the VAT using Trizol (Invitrogen) or the AT-MSCs subjected to adipogenesis at day 0, 3, 7, and 14 of differentiation using an RNeasy kit (Qiagen, Germantown, Maryland) according to manufacturer's protocols. First strand complementary DNA (cDNA) was synthesized using SuperScript VILO cDNA Synthesis Kit (Invitrogen), and SYBR-Green qPCR was performed using SYBR Green Master Mix (BioRad, Hercules, California). Expression of each gene was normalized to the expression of housekeeping gene, ribosomal protein L19 (RPL19), and relative quantification of each gene was calculated according to the $\Delta\Delta C_t$ method. Amplification of specific transcripts was confirmed

by melting curve profiles at the end of each PCR. Primer sequences for each gene are given in Table 1.

Immunoblotting

For determining protein expression, VAT samples were homogenized in radioimmunoprecipitation assay buffer (Pierce RIPA Buffer; Thermo Scientific, Rockford, Illinois) containing protease inhibitors (Complete Mini; Roche Diagnostics, Indianapolis, Indiana) and phosphatase inhibitors (PhosSTOP; Roche Diagnostics) as described previously.⁴⁰ Briefly, tissue homogenates were centrifuged at 10 000g for 15 minutes at 4°C, the supernatant containing whole-tissue protein extract was collected, and protein concentrations determined using the detergent compatible protein assay kit (BioRad) as per manufacturer's recommendations. Equal amounts of protein (~40 μ g) were resolved on Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), transferred onto a nitrocellulose membrane, blocked in 5% milk, incubated with primary antibody overnight at 4°C, washed and probed with horse radish peroxidase-tagged secondary antibody raised against the species in which primary antibody was generated. Proteins were detected by electrochemiluminescent reaction using ProteinSimple FluorChem E system (San Jose, California). The levels of each protein, as well as the corresponding loading control (glyceraldehyde 3-phosphate dehydrogenase [GAPDH]), were determined in the same membrane after stripping and reblotting. Band densities were determined using the ImageJ software (NIH, Bethesda, Maryland). The antibodies used are aromatase ([CYP19] PA1-21398; Thermo Fisher Scientific, Waltham, Massachusetts), 17 α -hydroxylase/17,20-lyase ([CYP17] kindly provided by Dr Walter L Miller, University of California, San Francisco), ESR1 (MS-354-P1-A; NeoMarkers, Fremont, California), estrogen receptor 2 (ESR2; kindly provided

Figure 2. Effect of prenatal T excess on steroidogenic genes and steroid receptor mRNA expression in VAT from gestational day 90. Gene expression was quantified by RT-PCR and presented as mean $\pm$ SEM of fold changes of treated versus control samples. Differing alphabets above the histogram indicate significant changes by ANOVA and the numerical superscripts are Cohen's *d* values comparing (1) C versus T (within the circle) and (2) TF and TR versus T (within the square box). Cohen's *d* value ≥ 0.2 is considered small effect, ≥ 0.5 is considered medium effect, and ≥ 0.8 is considered large effect. ANOVA indicates analysis of variance; C, controls; mRNA, messenger RNA; T, prenatal T treated; TF, flutamide treated; TFR, prenatal T-treated animals cotreated with flutamide and rosiglitazone; TR, rosiglitazone treated; SEM, standard error of the mean; VAT, visceral adipose tissue.

by Dr Benita S Katzenellenbogen), AR (SC-816; Santa Cruz Biotechnology), and GAPDH (14C10; Cell Signaling Technologies, Danvers, Massachusetts).

Statistical Analysis

All data from studies 1 and 2 were analyzed using analysis of variance (GraphPad Prism 6 software) with Tukey post hoc analysis; data were presented as mean $\pm$ standard error of the mean, and the threshold for significance was set as $P \leq .05$. Additionally, Cohen's effect size analysis^{44,45} was also used to determine magnitude of differences between C and prenatal T-treated groups and the intervention versus prenatal T-treated groups. The computed Cohen *d* value ≥ 0.2 is considered as small, ≥ 0.5 as medium, and ≥ 0.8 as large magnitude effect.^{44,45} Since only data from 2 animals from the prenatal T-treated group were available for AT-MSC adipogenesis assay and assessment of adipocyte differentiation markers in study 3, these data were not subject to statistical analysis.

Results

Effects of Gestational T Excess and AR Antagonist/Insulin Sensitizer Intervention on VAT Steroidal Machinery (Study 1)

Gestational day 90 fetuses. Effect size analysis revealed large effects of prenatal T treatment in increasing steroidogenic genes 3- β hydroxysteroid dehydrogenase (*HSD3B*) and

aromatase (*CYP19*) but not steroidogenic acute regulatory protein (*STAR*), and 17 α -hydroxylase/17,20-lyase (*CYP17*; Figure 2, left panels). Prenatal T treatment significantly increased steroid receptor *ESR1* and had large effects in increasing *ESR2* but not AR mRNA expression (Figure 2, right panels). Intervention with insulin sensitizer but not AR antagonist had large effects in preventing the increase in *HSD3B* and *CYP19*. The increase in *ESR1* was significantly reversed by cotreatment with insulin sensitizer while intervention with AR antagonist only had a partial effect. For *ESR2*, both interventions had large effects in preventing the increase induced by prenatal T treatment.

Twenty-one-month-old females. Prenatal T treatment significantly increased steroidogenic gene *CYP19* mRNA. Effect size analysis revealed small effects in increasing *HSD3B* and large effects toward increase for *STAR* and *CYP17* mRNA, respectively (Figure 3, left panels). Prenatal T treatment resulted in significant increase in steroid receptor *ESR2* mRNA. Effect size analysis showed medium and large effects of prenatal T treatment in increasing the expression of steroid receptors *ESR1* and AR mRNA, respectively (Figure 3, right panels). Intervention with AR antagonist or insulin sensitizer had no effect on prenatal T-induced changes in *HSD3B*, *CYP17*, and *ESR1* while both interventions were able to partially prevent the increase in *CYP19*, *ESR2*, and AR mRNA expression. For *STAR* gene while intervention with AR antagonist had no reversal insulin sensitizer had a partial effect in reversing the prenatal T-induced increase.

Figure 3. Effect of prenatal T excess on steroidogenic genes and steroid receptor mRNA expression in VAT from adult 21 months of age. Gene expression was quantified by RT-PCR and presented as mean $\pm$ SEM of fold changes of treated versus control samples. Differing alphabets above the histogram indicate significant changes by ANOVA and the numerical superscripts are Cohen's *d* values comparing (1) C versus T (within the circle) and (2) TF and TR versus T (within the square box). Cohen's *d* value ≥ 0.2 is considered small effect, ≥ 0.5 is considered medium effect, and ≥ 0.8 is considered large effect. ANOVA indicates analysis of variance; mRNA, messenger RNA; SEM, standard error of the mean; TF, flutamide treated; TR, rosiglitazone treated; VAT, visceral adipose tissue.

Consistent with the increased *CYP19* mRNA data, prenatal T excess increased *CYP19* protein level while having no effect on *CYP17* protein expression (Figure 4). As opposed to marginal, if any, effects on *ESR1* mRNA, prenatal T excess increased protein content of *ESR1* (Figure 4). No effect of prenatal T treatment on protein levels of *ESR2* was evident (Figure 4). Cohen's effect size analysis revealed large magnitude increase in *AR* protein content in VAT (Figure 4). Both *AR* antagonist and insulin sensitizer treatment partially prevented the increase in *CYP19* and *ESR1* protein while only *AR* antagonist reduced the prenatal T-induced increase in *AR* protein the VAT.

Effects of Prenatal T Excess and AR Antagonist/Insulin Sensitizer Intervention on Adipocyte Differentiation Markers (Study 2)

Gestational day 90 fetuses. Effect size analysis showed large effects of prenatal T treatment in reducing expression of lipoprotein lipase (*LPL*) and medium effects in reducing the *PPARG*, whereas there was a small effect toward increase in fatty acid-binding protein 4 (*FABP4*) gene expression (Figure 5, left panels). Intervention with either *AR* antagonist or insulin sensitizer had no effect in preventing the effects of prenatal T treatment on *PPARG* and *LPL* expression, whereas *AR* antagonist had medium effect in preventing the effect of prenatal T-induced changes in *FABP4*.

Twenty-one-month-old females. Effect size analysis found large effects of prenatal T in reducing *LPL* and *FABP4* and medium

effects in reducing *PPARG* mRNA expression (Figure 5, right panels). Cotreatment with *AR* antagonist or insulin sensitizer had large effects in preventing the prenatal T treatment-induced reduction in *PPARG* and *FABP4* while neither intervention prevented the effects of prenatal T on *LPL* expression.

Effects of Dual Intervention With AR Antagonist and Insulin Sensitizer on Adipose Tissue VAT Steroidal Machinery (Study 2)

At 10 months of age, prenatal T treatment had large, medium, and small effects in increasing *STAR*, *HSD3B*, and *CYP19* mRNA, respectively in the VAT (Figure 6, left panels). For steroid receptors, prenatal T treatment had medium effect in decreasing *ESR1* and large and medium effects in increasing *ESR2* and *AR* mRNA, respectively (Figure 6, middle panels). Dual intervention with *AR* antagonist and insulin sensitizer had large effects in reversing the increase in *STAR*, *ESR1*, and *ESR2* mRNA expression but had no effect in altering the prenatal T-induced changes in the remaining steroidogenic genes.

Effects of Dual Intervention With AR Antagonist and Insulin Sensitizer on Adipocyte Differentiation Markers (Study 2)

At 10 months of age, effect size analysis found large effects of prenatal T treatment in increasing *PPARG* expression and medium and large effects in decreasing for *LPL* and *FABP4*

Figure 4. Effect of prenatal T excess on steroidogenic enzymes and steroid receptor protein content in VAT from adult 21 months of age. Protein content for steroidogenic enzymes CYP19 and CYP17, and steroid receptors ESR1, ESR2, and AR was determined by immunoblotting. Results are mean $\pm$ SEM of ratio of densitometric values for the respective protein to GAPDH. Differing alphabets above the histogram indicate significant changes by ANOVA and the numerical superscripts are Cohen's *d* values comparing (1) C versus T (within the circle) and (2) TF and TR versus T (within the square box). ANOVA indicates analysis of variance; AR, androgen receptor; ESR1, estrogen receptor 1; ESR2, estrogen receptor 2; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; SEM, standard error of the mean; TF, flutamide treated; TR, rosiglitazone treated; VAT, visceral adipose tissue.

expression, respectively (Figure 6, right panels). Effect size analyses also revealed large effects of dual intervention with both AR antagonist and insulin sensitizer in preventing the prenatal T-induced increase in *PPARG* and decrease in *LPL* and *FABP4*.

Effects of Prenatal T Excess and AR Antagonist–Insulin Sensitizer Dual Intervention on Adipogenesis of AT-MSc (Study 3)

Since only 2 T animals were available for this assessment, a statistical analysis was not possible. Differentiation of AT-MSc into adipocytes as evident from reduced oil red O staining (Figure 7) was lower relative to controls in both T animals at both pre- and postpubertal time points. Adipocyte differentiation of dual intervention group was similar to that of controls at both pre- and postpubertal time points suggestive of potential reversal of prenatal T-induced effects. The mRNA expression levels of adipocyte differentiation markers were consistent

with the observed changes in adipogenesis with reduced expression levels of *PPARG*, *FABP4*, *LPL*, and *CD36* (Figure 8) evident 7 days after induction of differentiation in the prenatal T-treated group at both pre- and postpubertal time points. This pattern of decreased expression was also evident 14 days after initiation of differentiation for *PPARG* and *LPL* genes at the prepubertal time point and for *LPL* and *FABP4* mRNA at the postpubertal time point. There was no indication of any changes in the expression of stearoyl-coenzyme A desaturase 1, sterol regulatory element-binding protein, and fatty acid synthase at both pre- and postpubertal time points (Supplemental Figure 1). Expression level of *PPARG*, *FABP4*, *LPL*, and *CD36* at both pre- and postpubertal time points in the dual intervention group were similar to control (except *CD36* 14 days after initiation of differentiation) suggestive of potential prevention of prenatal T-induced effects on adipogenesis.

To examine whether prenatal T excess has any effects on lineage commitment of AT-MSc into preadipocyte, the expression level of *ZNF423*, an adipocyte lineage commitment marker, was evaluated before (day 0) and after (day 3) induction of

Figure 5. Effect of prenatal T excess on adipocyte differentiation related genes in VAT from gestational day 90 fetuses and 21 months of age adult female sheep. Gene expression was quantified by RT-PCR and results were shown as mean $\pm$ SEM. Results represent fold changes treated versus control samples. Numerical superscripts are Cohen *d* values comparing (1) C versus T (within the circle) and (2) TF and TR versus T (within the square box). Cohen's *d* value ≥ 0.2 is considered small effect, ≥ 0.5 is considered medium effect, and ≥ 0.8 is considered large effect. SEM indicates standard error of the mean; TF, flutamide treated; TR, rosiglitazone treated; VAT, visceral adipose tissue.

differentiation. In contrast to the reduced expression of the markers of adipocyte differentiation, the expression of *ZNF423* in the prenatal T-treated animals appear to be higher before and after initiation at both pre- and postpubertal time points (Figure 9). Expression level of *ZNF423* in the dual intervention group was similar to controls in the postpubertal time point but not at the prepubertal time point (increased relative to controls).

Discussion

The findings of the present study provide evidence in support of altered adipogenesis with increased AT-MSC commitment to preadipocytes and decreased preadipocyte differentiation into adipocytes (preliminary findings) in the VAT of prenatal T-treated female sheep. These changes may contribute to the

formation of small adipocyte phenotype^{15,16} of the prenatal T-treated female sheep. Additionally, the changes detected in steroidogenic enzymes and steroid receptor expression in the VAT suggestive of an estrogenic intracrine steroidal milieu point to estrogenic contribution in programming and maintaining the reduced adipogenesis, cell differentiation, and adipocyte size phenotype. The significance of these findings and the involvement of androgenic and metabolic pathways in programming these defects are discussed below.

Impact of Prenatal T Treatment on VAT Steroidal Machinery

This is the first report of existence of multiple steroidogenic enzymes (*STAR*, *HSD3B*, *CYP17*, and *CYP19*) in the VAT depot of sheep, a precocial species—a finding that is consistent with other studies in altricial species and humans indicating adipose tissue depots expresses multiple steroidogenic enzymes.⁴⁶ The finding that prenatal T treatment increased the mRNA expression of *STAR*, *CYP17*, and *CYP19* and protein content of *CYP19* in the VAT at 21 months of age suggests that prenatal T treatment has reprogrammed the steroidogenic biosynthetic machinery resulting in altered steroidal machinery. The increased protein expression of *CYP19* coupled with *ESR1* indicates a predominant estrogenic action in the VAT from prenatal T-treated female sheep. Other studies have shown that increased E signaling in the adipose tissue reduces lipid uptake through inhibition of LPL and promotes lipid mobilization by stimulation of lipolysis.^{29,47} As such the increased estrogenic action in the VAT would be expected to reduce the lipid storage capability of the adipocytes and contribute to dyslipidemia and ectopic lipid accumulation. Consistent with this premise prenatal T-treated sheep not only have reduced adipocyte size^{15,16} with reduced LPL expression (this study) but also manifest dyslipidemia and ectopic lipid accumulation in liver and muscle.⁴⁸

Additionally, the finding that VAT from prenatal T-treated fetuses manifests increases in *CYP19*, *ESR1*, and *ESR2* mRNA in the absence of increase in *CYP17* and *AR* mRNA indicates that E likely played a role in altering the trajectory of adipocyte differentiation such that the intracrine adipocyte milieu is predominantly estrogenic. These data together with the observations that gestational T treatment increases fetal E levels⁴⁹ and prenatal T-induced reduction in adipocyte size was not reversed by cotreatment with AR antagonist¹⁶ provide further support that defects in adipocyte size maybe programmed by the estrogenic pathway.

Impact of Prenatal T Treatment on Expression of Adipocyte Cell Differentiation Markers

The reduced expression of adipocyte differentiation markers (*PPARG*, *LPL*, and *FABP4*) in the VAT at 21 months of age is consistent with our finding of reduced adipocyte size in prenatal T-treated adult female sheep.^{15,16} Evidence that adipose tissue-specific knockout of *PPARG* induces an insulin

Figure 6. Effect of prenatal T exposure on steroidogenic genes (left panels), steroid receptor (middle panels), and adipocyte differentiation markers (right panels) mRNA expression in VAT from 10 months of age female sheep. Gene expression was quantified by RT-PCR and presented as mean $\pm$ SEM of fold changes of treated versus control samples. Differing alphabets above the histogram indicate significant changes by ANOVA and the numerical superscripts are Cohen *d* values comparing (1) C versus T (within the circle) and (2) TFR versus T (within the square box). One T female was found to have a kidney abscess at the time of euthanasia ($\blacksquare$). Cohen's *d* value ≥ 0.2 is considered small effect, ≥ 0.5 is considered medium effect, and ≥ 0.8 is considered large effect. ANOVA indicates analysis of variance; mRNA, messenger RNA; SEM, standard error of the mean; TFR, prenatal T-treated animals cotreated with flutamide and rosiglitazone; VAT, visceral adipose tissue.

resistant state in the mouse,⁵⁰ raises the possibility that the reduced PPARG expression in the VAT of prenatal T-treated sheep may have contributed to their insulin resistant state.¹¹ Peroxisome proliferator-activated receptor gamma is a ligand-activated transcriptional factor that is predominantly expressed in adipose tissues⁵¹ with its expression regulated by dietary fatty acids and hormones such as insulin⁵² and E.⁵³ Studies in mice have found that administration of E results in reduced expression of PPARG and its target genes *LPL* and *FABP4*.⁵³ As such the increased estrogenic action in the VAT of prenatal T-treated sheep (this study) may have contributed to the reduced expression of adipocyte differentiation markers and subsequently the impaired differentiation of adipocytes.

While the predominantly estrogenic action at fetal day 90 (this study) coupled with our earlier findings of increased fetal E levels⁴⁹ suggests that decreased adipogenesis is likely programmed by E, the higher PPARG expression and lack of changes in the expression of estrogenic genes (ESR1 and CYP19) in the VAT at the postpubertal age (10 months) likely reflect a compensatory

adaptation, a finding consistent with the insulin sensitive state at this time point in the prenatal T-treated sheep.¹⁶

Impact of Prenatal T Treatment on Adipogenesis From AT-MSCs

Consistent with the reduced expression of adipocyte differentiation markers in the VAT, our findings, albeit preliminary, suggest adipocyte formation is likely lower in the prenatal T-treated females. An impairment in adipocyte formation was also reported in subcutaneous AT-MSC from the prenatally T-treated macaque model.⁵⁴ Whether such changes also exist in the subcutaneous depot in the sheep and visceral depot in the macaque remains to be determined. Preliminary observation of absence of adipocyte differentiation at both pre- and postpubertal time points suggests that the pubertal hormonal changes may have no influence and the disruption is likely organized during the window of the prenatal T treatment. However, because gestational T exposure also alters VAT steroidal

Figure 7. Differentiation of VAT AT-MSC, isolated before and after puberty, into adipocytes visualized utilizing oil red O stain at 14 days following induction. C indicates controls; T, prenatal T treated; TFR, prenatal T-treated animals cotreated with flutamide and rosiglitazone; VAT, visceral adipose tissue.

Figure 8. Effect of prenatal T exposure on expression of adipocyte differentiation related genes before and after puberty. Gene expression of *PPARG*, *FABP4*, *LPL*, and *CD36* was quantified by RT-PCR at day 0, 3, 7, and 14 following induction of differentiation. Controls and TFR are shown as mean $\pm$ SEM, the fold changes in 2 samples from prenatal T-treated animals are marked in black dots. AT-MSC, VAT-derived mesenchymal stem cell; *FABP4* indicates fatty acid-binding protein 4; *LPL*, lipoprotein lipase; *PPARG*, peroxisome proliferator-activated receptor; SEM, standard error of the mean; VAT, visceral adipose tissue.

Figure 9. Effect of prenatal T exposure on expression of adipocyte lineage commitment marker *ZNF423* before and after puberty. Gene expression of *ZNF423* was quantified by RT-PCR before (day 0) and after (day 3) induction of differentiation. Controls and TFR were shown as mean $\pm$ SEM, the fold changes in 2 samples from prenatal T-treated animals were marked in black dots. TFR indicates prenatal T-treated animals cotreated with flutamide and rosiglitazone; SEM, standard error of the mean.

machinery, intracrine steroidal changes may be the mediary via which such organization is facilitated.

As opposed to reduced adipocyte formation from visceral AT-MSCs from prenatal T-treated sheep, our preliminary finding of the increase in adipocyte lineage marker *ZNF423* is suggestive of increase in commitment of VAT AT-MSC to preadipocytes, a finding that is similar to observations in the subcutaneous AT-MSCs of prenatally androgenized rhesus macaques.⁵⁴ The increase in commitment may contribute to the increased proportion of small adipocytes evidenced in prenatal T-treated sheep^{15,16} and monkeys,⁵⁴ a premise that needs to be investigated in a larger group of prenatal T-treated animals.

Impact of Steroidal and Metabolic Interventions on VAT Steroidal Machinery, Adipocyte Differentiation, and Adipogenesis

Visceral adipose tissue steroidal machinery. The finding that prenatal intervention with either AR antagonist or insulin

sensitizer was able to partially reverse the increase in VAT CYP19 at both mRNA and protein levels suggests that both androgenic and metabolic pathways are involved in setting the increased estrogenic action in the VAT. However, the reduced estrogenic action expected as a result of the reduction in CYP19 as well as ESR1 with both interventions is not consistent with reported absence of reversal in adipocyte size.¹⁶ One possibility is that the partial reduction achieved with CYP19 and ESR1 protein may have been insufficient or alternatively a reflection of the intracrine androgen/E balance as partial reversal of AR was evident with AR cotreatment. In the context of programming, the complete reversal of *AR* mRNA, partial of *ESR2* but not *ESR1*, with either AR antagonist or insulin sensitizer in fetal day 90 VAT indicates synergy between steroidal and metabolic pathways in inducing such programming defects.

Adipocyte differentiation and adipogenesis. As is the case with the VAT steroidal machinery, our findings that intervention with either AR antagonist or insulin sensitizer or dual interventions are able to reverse the expression of adipocyte differentiation genes *PPARG* and *FABP4* in 10- and 21-month old animals suggest that both androgenic and metabolic pathways are involved in prenatal T-induced programming of defects in adipocyte differentiation. However, the finding that neither interventions had any effect in reversing the prenatal T treatment-induced changes in adipocyte size¹¹ coupled with their inability to completely reverse the estrogenic action (this study), and the increase in fetal E levels,⁴⁹ supports involvement of E also in mediating adipocyte differentiation. Additional studies utilizing E receptor antagonist alone or in combination with AR antagonist and insulin sensitizer are required to narrow down the relative contribution of androgenic, estrogenic, and metabolic pathways in programming and maintaining adipocyte differentiation.

Translational Relevance and Conclusions

Although adipose tissue is recognized as an endocrine organ⁵⁵ and express various steroidogenic enzymes⁵⁶ and it is well known that steroids can influence adipose distribution and function, the role played by intracrine steroidal milieu in adipocyte differentiation and IR is unclear. Demonstration of altered steroidal machinery in the VAT of prenatal T-treated female sheep that manifest a PCOS reproductive and metabolic phenotype raises the possibility, such defects may underlie adipocyte dysfunction and insulin sensitivity defects seen in prenatal T-treated sheep. Association of increased distribution of small adipocytes in humans with insulin resistance coupled with decreased expression of adipocyte differentiation markers,⁵⁷ aspects also seen in prenatal T-treated female sheep, suggests that the prenatal T-treated sheep model may be of translational relevance in understanding human metabolic diseases including PCOS. Additionally, studies in prenatal T-treated sheep—a precocial model—is likely to be of benefit in determining the role played by

environmental agents with steroidogenic potential in programming defects in adipogenesis.

In summary, the results from the study provide evidence that prenatal T treatment programs an increased estrogenic action in the sheep VAT, reduces expression of adipocyte differentiation genes, and provides preliminary evidence that it also decreases the adipogenic potential of the AT-MSCs. While the limited sample size in study 3 precludes from making substantial inferences on the effects of prenatal T treatment on adipogenesis, the findings from studies 1 and 2 in concert with our previous observations of reduced adipocyte size and IR phenotype¹⁵ provide a possible linkage for such defects contributing to the metabolic phenotype of the prenatal T-treated sheep. Additional studies with large sample size, determination of the local steroidal content in the VAT, and interventions utilizing E antagonists or aromatase inhibitors should confirm the defects in adipogenesis and help identify the role played by E in programming or maintaining the defects in adipocyte differentiation.

Authors' Note

Muraly Puttabyatappa and Chunxia Lu contributed equally to this work.

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Supplemental Material

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References

1. Kloting N, Bluher M. Adipocyte dysfunction, inflammation and metabolic syndrome. *Rev Endocr Metab Disord*. 2014;15(4):277-287.
2. Diamanti-Kandarakis E, Dunaif A. Insulin resistance and the polycystic ovary syndrome revisited: an update on mechanisms and implications. *Endocr Rev*. 2012;33(6):981-1030.
3. Grundy SM. Adipose tissue and metabolic syndrome: too much, too little or neither. *Eur J Clin Invest*. 2015;45(11):1209-1217.
4. Lukaszewski MA, Eberle D, Vieau D, Breton C. Nutritional manipulations in the perinatal period program adipose tissue in offspring. *Am J Physiol Endocrinol Metab*. 2013;305(10):E1195-E1207.
5. Janesick AS, Blumberg B. Obesogens: an emerging threat to public health. *Am J Obstet Gynecol*. 2016;214(5):559-565.
6. Casals-Casas C, Desvergne B. Endocrine disruptors: from endocrine to metabolic disruption. *Annu Rev Physiol*. 2011;73:135-162.
7. Padmanabhan V, Sarma HN, Savabieasfahani M, Steckler TL, Veiga-Lopez A. Developmental reprogramming of reproductive and metabolic dysfunction in sheep: native steroids vs. environmental steroid receptor modulators. *Int J Androl*. 2010;33(2):394-404.
8. Chevalier N, Fenichel P. Endocrine disruptors: new players in the pathophysiology of type 2 diabetes? *Diabetes Metab*. 2015;41(2):107-115.
9. Abbott DH, Dumesic DA, Eisner JR, Colman RJ, Kemnitz JW. Insights into the development of polycystic ovary syndrome (PCOS) from studies of prenatally androgenized female rhesus monkeys. *Trends Endocrinol Metab*. 1998;9(2):62-67.
10. Eisner JR, Dumesic DA, Kemnitz JW, Colman RJ, Abbott DH. Increased adiposity in female rhesus monkeys exposed to androgen excess during early gestation. *Obes Res*. 2003;11(2):279-286.
11. Cardoso RC, Puttabyatappa M, Padmanabhan V. Steroidogenic versus metabolic programming of reproductive neuroendocrine, ovarian and metabolic dysfunctions. *Neuroendocrinology*. 2015;102(3):226-237.
12. Padmanabhan V, Veiga-Lopez A. Sheep models of polycystic ovary syndrome phenotype. *Mol Cell Endocrinol*. 2013;373(1-2):8-20.
13. Roland AV, Nunemaker CS, Keller SR, Moenter SM. Prenatal androgen exposure programs metabolic dysfunction in female mice. *J Endocrinol*. 2010;207(2):213-223.
14. Maliqueo M, Benrick A, Stener-Victorin E. Rodent models of polycystic ovary syndrome: phenotypic presentation, pathophysiology, and the effects of different interventions. *Semin Reprod Med*. 2014;32(3):183-193.
15. Veiga-Lopez A, Moeller J, Patel D, et al. Developmental programming: impact of prenatal testosterone excess on insulin sensitivity, adiposity, and free fatty acid profile in postpubertal female sheep. *Endocrinology*. 2013;154(5):1731-1742.
16. Cardoso RC, Veiga-Lopez A, Moeller J, et al. Developmental programming: impact of gestational steroid and metabolic milieu on adiposity and insulin sensitivity in prenatal testosterone-treated female sheep. *Endocrinology*. 2016;157(2):522-535.
17. Ksiazek K. A comprehensive review on mesenchymal stem cell growth and senescence. *Rejuvenation Res*. 2009;12(2):105-116.
18. Augello A, Kurth TB, De Bari C. Mesenchymal stem cells: a perspective from in vitro cultures to in vivo migration and niches. *Eur Cell Mater*. 2010;20:121-133.
19. Ali AT, Hochfeld WE, Myburgh R, Pepper MS. Adipocyte and adipogenesis. *Eur J Cell Biol*. 2013;92(6-7):229-236.
20. Ren G, Chen X, Dong F, et al. Concise review: mesenchymal stem cells and translational medicine: emerging issues. *Stem Cells Transl Med*. 2012;1(1):51-58.
21. Cawthorn WP, Scheller EL, MacDougald OA. Adipose tissue stem cells meet preadipocyte commitment: going back to the future. *J Lipid Res*. 2012;53(2):227-246.

22. Cristancho AG, Lazar MA. Forming functional fat: a growing understanding of adipocyte differentiation. *Nat Rev Mol Cell Biol.* 2011;12(11):722-734.
23. Farmer SR. Transcriptional control of adipocyte formation. *Cell Metab.* 2006;4(4):263-273.
24. Rosen ED, Spiegelman BM. Molecular regulation of adipogenesis. *Annu Rev Cell Dev Biol.* 2000;16:145-171.
25. Taylor PD, Poston L. Developmental programming of obesity in mammals. *Exp Physiol.* 2007;92(2):287-298.
26. Regnier SM, Sargis RM. Adipocytes under assault: environmental disruption of adipose physiology. *Biochim Biophys Acta.* 2014;1842(3):520-533.
27. Padmanabhan V, Cardoso RC, Puttabyatappa M. Developmental programming, a pathway to disease. *Endocrinology.* 2016;157(4):1328-1340.
28. Cooke PS, Naaz A. Role of estrogens in adipocyte development and function. *Exp Biol Med (Maywood).* 2004;229(11):1127-1135.
29. Newell-Fugate AE. The role of sex steroids in white adipose tissue adipocyte function. *Reproduction.* 2017;153(4):R133-R149.
30. D'Eon TM, Souza SC, Aronovitz M, Obin MS, Fried SK, Greenberg AS. Estrogen regulation of adiposity and fuel partitioning. Evidence of genomic and non-genomic regulation of lipogenic and oxidative pathways. *J Biol Chem.* 2005;280(43):35983-35991.
31. Gupta V, Bhasin S, Guo W, et al. Effects of dihydrotestosterone on differentiation and proliferation of human mesenchymal stem cells and preadipocytes. *Mol Cell Endocrinol.* 2008;296(1-2):32-40.
32. Dieudonne MN, Pecquery R, Leneuve MC, Giudicelli Y. Opposite effects of androgens and estrogens on adipogenesis in rat preadipocytes: evidence for sex and site-related specificities and possible involvement of insulin-like growth factor 1 receptor and peroxisome proliferator-activated receptor gamma2. *Endocrinology.* 2000;141(2):649-656.
33. Abi Salloum B, Veiga-Lopez A, Abbott DH, Burant CF, Padmanabhan V. Developmental programming: exposure to testosterone excess disrupts steroidal and metabolic environment in pregnant sheep. *Endocrinology.* 2015;156(6):2323-2337.
34. Comim FV, Nobrega JE Jr, Santos JT, et al. *Prenatally Androgenised Sheep Exhibit Changes in the Wool: Relevance for Polycystic Ovary Syndrome.* Endo 2017 99th Annual Meeting of the Endocrine Society; Orlando, FL; 2017.
35. Veiga-Lopez A, Astapova OI, Aizenberg EF, Lee JS, Padmanabhan V. Developmental programming: contribution of prenatal androgen and estrogen to estradiol feedback systems and periovulatory hormonal dynamics in sheep. *Biol Reprod.* 2009;80(4):718-725.
36. Li J, Papadopoulos V, Vihma V. Steroid biosynthesis in adipose tissue. *Steroids.* 2015;103:89-104.
37. Manikkam M, Crespi EJ, Doop DD, et al. Fetal programming: prenatal testosterone excess leads to fetal growth retardation and postnatal catch-up growth in sheep. *Endocrinology.* 2004;145(2):790-798.
38. Jackson LM, Timmer KM, Foster DL. Sexual differentiation of the external genitalia and the timing of puberty in the presence of an antiandrogen in sheep. *Endocrinology.* 2008;149(8):4200-4208.
39. Veiga-Lopez A, Lee JS, Padmanabhan V. Developmental programming: insulin sensitizer treatment improves reproductive function in prenatal testosterone-treated female sheep. *Endocrinology.* 2010;151(8):4007-4017.
40. Lu C, Cardoso RC, Puttabyatappa M, Padmanabhan V. Developmental programming: prenatal testosterone excess and insulin signaling disruptions in female sheep. *Biol Reprod.* 2016;94(5):113.
41. Padmanabhan V, Veiga-Lopez A, Herkimer C, et al. Developmental programming: prenatal and postnatal androgen antagonist and insulin sensitizer interventions prevent advancement of puberty and improve LH surge dynamics in prenatal testosterone-treated sheep. *Endocrinology.* 2015;156(7):2678-2692.
42. Abi Salloum B, Herkimer C, Lee JS, Veiga-Lopez A, Padmanabhan V. Developmental programming: prenatal and postnatal contribution of androgens and insulin in the reprogramming of estradiol positive feedback disruptions in prenatal testosterone-treated sheep. *Endocrinology.* 2012;153(6):2813-2822.
43. Chazenbalk G, Singh P, Irge D, Shah A, Abbott DH, Dumesic DA. Androgens inhibit adipogenesis during human adipose stem cell commitment to preadipocyte formation. *Steroids.* 2013;78(9):920-926.
44. Cohen J. A power primer. *Psychol Bull.* 1992;112(1):155-159.
45. Nakagawa S, Cuthill IC. Effect size, confidence interval and statistical significance: a practical guide for biologists. *Biol Rev Camb Philos Soc.* 2007;82(4):591-605.
46. Meseguer A, Puche C, Cabero A. Sex steroid biosynthesis in white adipose tissue. *Horm Metab Res.* 2002;34(11-12):731-736.
47. Tchernof A, Calles-Escandon J, Sites CK, Poehlman ET. Menopause, central body fatness, and insulin resistance: effects of hormone-replacement therapy. *Coron Artery Dis.* 1998;9(8):503-511.
48. Puttabyatappa M, Andriessen V, Mesquitta M, Zeng L, Pennathur S, Padmanabhan V. Developmental programming: impact of gestational steroid and metabolic milieu on mediators of insulin sensitivity in prenatal testosterone-treated female sheep. *Endocrinology.* 2017;158(9):2783-2798.
49. Veiga-Lopez A, Steckler TL, Abbott DH, et al. Developmental programming: impact of excess prenatal testosterone on intrauterine fetal endocrine milieu and growth in sheep. *Biol Reprod.* 2011;84(1):87-96.
50. He W, Barak Y, Hevener A, et al. Adipose-specific peroxisome proliferator-activated receptor gamma knockout causes insulin resistance in fat and liver but not in muscle. *Proc Natl Acad Sci U S A.* 2003;100(26):15712-15717.
51. Vidal-Puig AJ, Considine RV, Jimenez-Linan M, et al. Peroxisome proliferator-activated receptor gene expression in human tissues. Effects of obesity, weight loss, and regulation by insulin and glucocorticoids. *J Clin Invest.* 1997;99(10):2416-2422.

52. Houseknecht KL, Cole BM, Steele PJ. Peroxisome proliferator-activated receptor gamma (PPARgamma) and its ligands: a review. *Domest Anim Endocrinol*. 2002;22(1):1-23.
53. Jeong S, Yoon M. 17beta-Estradiol inhibition of PPARgamma-induced adipogenesis and adipocyte-specific gene expression. *Acta Pharmacol Sin*. 2011;32(2):230-238.
54. Keller E, Chazenbalk GD, Aguilera P, et al. Impaired preadipocyte differentiation into adipocytes in subcutaneous abdominal adipose of PCOS-like female rhesus monkeys. *Endocrinology*. 2014;155(7):2696-2703.
55. Kershaw EE, Flier JS. Adipose tissue as an endocrine organ. *J Clin Endocrinol Metab*. 2004;89(6):2548-2556.
56. MacKenzie SM, Huda SS, Sattar N, Fraser R, Connell JM, Davies E. Depot-specific steroidogenic 695 gene transcription in human adipose tissue. *Clin Endocrinol (Oxf)*. 2008;69(6):848-854.
57. McLaughlin T, Sherman A, Tsao P, et al. Enhanced proportion of small adipose cells in insulin-resistant vs insulin-sensitive obese individuals implicates impaired adipogenesis. *Diabetologia*. 2007;50(8):1707-1715.