The Effects of A Single Developmentally-Entrained Pulse of Testosterone in Female Neonatal Mice On Reproductive and Metabolic Functions in Adult Life

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Early postnatal exposures to sex steroids have been well-recognized to modulate predisposition to diseases of adulthood. There is a complex interplay between timing, duration and dose of endocrine exposures through environmental or dietary sources that may alter the sensitivity of target tissues to the exogenous stimuli. In this study, we determined the metabolic and reproductive programming effects of a single developmentally-entrained pulse of testosterone given to female mice in early postnatal period. CD-1 female mice pups were injected with either 5μg of testosterone enanthate (TE group) or vehicle (CON group) within 24 hours after birth and followed to adult age. 66% of testosterone-treated mice exhibited irregular cycling, anovulatory phenotype, and significantly higher ovarian weights than vehicle-treated mice. Longitudinal NMR measurements revealed that TE group had greater body weight, whole body lean, and fat mass than the control group. Adipose tissue cellularity analysis in TE group revealed a trend toward higher size and number than their litter-mate controls. The brown adipose tissue (BAT) of NT mice exhibited white fat infiltration with down-regulation of several markers including UCP-1, CIDEA, BMP-7 as well as BAT differentiation-related transcription regulators. Testosterone-injected mice were also more insulin resistant than control mice. These reproductive and metabolic reprogramming effects were not observed in animals exposed to TE at 3 and 6 weeks of age. Collectively, these data suggest that sustained reproductive and metabolic alterations may result in female mice from a transient exposure to testosterone during a narrow postnatal developmental window.

Pre and perinatal nutrition and hormonal perturbations have been well recognized as important modulators of adult heath and disease states. Seminal experiments by Phoenix et al (1) established the framework for the organizational and activational effects of hormones dichotomized by the duration of the resulting effects of exposure. The "organizational" effects were defined to be long-last-

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Abbreviations: ACAC, acetyl-CoA carboxylase α; ACACB, acetyl-CoA carboxylase β,ACC1, acetyl-coenzyme A carboxylase 1; ACS1, acetyl-coenzyme A synthetase 1; ACLY, ATP citrate lyase; AR, androgen receptor; ATGL, adipose triglyceride lipase; BAT, brown adipose tissue; BMP-7, bone morphogenetic protein 7; C/EBPα, CCAAT-enhancer-binding protein alpha; C/EBPβ, CCAAT-enhancer-binding protein beta; CIDEA, cell death-inducing DNA fragmentation factor, alpha subunit-like effector A; DHT, dihydrotestosterone; E2, 17β-estradiol; ERα, estrogen receptor alpha; ERβ, estrogen receptor beta; EVLOVL6, Fatty Acid Elongase 6; FASN, fatty acid synthase; FBPase, Fructose 2,6-bisphosphate; FoxO, Forkhead box O; FSH, follicle-stimulating hormone; G6Pase, Glucose 6-phosphatase; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GnRH, gonadotropin-releasing hormone;HSL, hormone sensitive lipase; IGF1R,insulin-like growth factor 1 receptor; IPGTT, intraperitoneal glucose tolerance test; IPITT, intraperitoneal insulin tolerance test; IRS, insulin receptor substrate; LH, luteinizing hormone; LPL, lipoprotein lipase; LXRα, liver X receptor alpha; NMR, nuclear magnetic resonance; PCOS, polycystic ovary syndrome; PGC-1α, peroxisome proliferator-activated receptor gamma coactivator 1 alpha; PEPCK, phosphoenolpyruvate carboxykinase; PPARγ, peroxisome proliferator-activated receptor gamma; PRDM16, PR domain containing 16; qPCR, quantitative PCR; SCD1, stearoyl-CoA desaturase 1;

ing effects while the "activational" effects of sex steroids on mating and nonmating behaviors modifications were limited largely to the duration of sex steroid exposure. Additional studies in songbirds and rodents established that organizational effects of androgens on neural circuits usually occur during the periods when masculinizing components are developing or maintained while the feminizing programming is prevented (2, 3). An important determinant of the neural organizational effects of steroids is the timing of sex steroid exposure. Male rats castrated at birth exhibit remarkable lifelong alterations in mating behaviors even when they receive testosterone replacement in adult life. Similarly, prenatal antiandrogen administration prevents masculinization of mating behaviors in adult males (4, 5). Consistent with the dramatic reprogramming in males associated with prenatal antiandrogen administration, prenatally and neonatally androgenized females show mounting behavior and altered sexual preferences (6, 7). These data suggest the existence of critical developmental periods in which discrete developmental processes may be susceptible to lifelong alterations as a result of perturbations in sex steroid exposure.

Exposure to endocrine modulators in the environment has long been recognized as a potential health risk. The origin of exposure may be diverse ranging from livestock excretion of steroidal supplements to aquatic life ingesting contaminants from surface water. Stavreva et al have shown androgenic activity in almost 35% of water samples collected across 14 states with in US (8). A recent study highlighted even more intriguing aspect this vexing problem where Qu et al demonstrated that metabolites of Trenbolone acetate, a common growth promoter used by for beef cattle production in the U.S., can undergo a photohydration effectively regenerating the parent steroid (9). This study is important in establishing that the exposure to steroidal endocrine modulators does not need to be extended over long duration or occur in supraphysiological doses; even transient exposures during critical developmental windows can induce profound alterations in adult reproductive and or metabolic reprogramming.

At the population levels, since the genetic composition is relatively slow to change, the significant role of rapid alterations in nutritional/hormonal exposures is being increasingly recognized. The Barker hypothesis, also known as the developmental origins of health and disease (10, 11), recognizes the importance of the environmental milieu during development to adult health. Although this hypothesis was originally based on nutritional deprivation in utero, its perspective has been extended to developmental exposure to sex steroid hormones and reproductive disruption in females in adult. It has been speculated that the increased prevalence of reproductive disorders in women may reflect the interplay between genetic and environmental factors (12–14).

Androgens have been shown to play important roles in reproductive function in females by acting as substrates for estradiol (E2) production, and by modulating ovarian function activationally and by organizational reprogramming. Studies in sheep (15–17), monkeys (18–20) and rodents (21) have shown that prenatal androgen exposure in utero may alter ovarian function in adult. Prenatal and neonatal androgen exposure to rodents results in anovulation (22, 23). These studies suggest that androgen exposure in early life may reprogram endocrine adaptations that permanently alter reproductive function.

Prenatal and neonatal factors may also influence the susceptibility to metabolic responses in adult life. While the precise mechanisms of steroid action during the development of metabolically active organs including liver and adipose tissue is not clear, prenatal androgenization has been reported to influence growth, adiposity and insulin sensitivity in female rodents, monkeys and sheep (24–29). However, the testosterone doses used in these studies were relatively large and sustained over varying durations of time.

Past studies have clearly established that reproductive/behavioral reprogramming of neural circuitry is sensitive to testosterone exposure in the developmental window. However, it is not known if the reproductive and metabolic tissues are direct targets of these organizational events. Accordingly, the aim of this study was to evaluate the programming effects of a single, developmentally-entrained pulse of testosterone (T) given to female mice in early postnatal period on the metabolic and ovarian tissue reproductive functions in adulthood. Specifically, we administered a single dose of testosterone to female pups after birth, which in preliminary experiments raised circulating testosterone concentrations to levels observed during the natural testosterone surge in male mice. The timing of testosterone injection during the immediate postnatal period after birth was intended to coincide with the timing of the postnatal testosterone surge in the male mice. The female mice that received either testosterone (TE) or vehicle (CON) during the immediate postnatal period were followed until 32 weeks of age and their hormone levels, estrous cycles, ovarian histology, body mass and composition, fat distribution, adipose tissue cellularity and gene expression, and insulin sensitivity were char-

SREBP1c, sterol regulatory element-binding protein 1c;T, testosterone; TE, testosterone enanthate; TGFβ1R, tumor growth factor beta 1 receptor; WAT, white adipose tissue

acterized. To test the hypothesis that the reproductive and metabolic phenotype was associated with testosterone exposure during the immediate postnatal period but not with testosterone exposure during other time windows (eg, time of weaning (~postnatal day 20) or time of sexual maturation (~ week 6)), we injected a single dose of testosterone to separate groups of female mice at 3 and 6 weeks of age and followed them into adulthood.

Materials and Methods

Animal Care and Diet Treatments

The study protocols were approved by the Boston University Institutional Animal Care and Use Committee. The pups delivered from pregnant CD-1 mothers (Charles River, Wilmington, MA) were injected subcutaneously within 24-hours after birth with either 5 μg of testosterone enanthate (TE) in 10μl oil or 10μl oil and their feet were tattooed to be segregated into appropriate groups. They were rubbed with the cage bedding, mixed, and redistributed randomly to mothers to minimize confounding due to the litter effect. After weaning on postnatal day 21, the mice were fed laboratory chow (Harlan Teklad global 18% protein rodent diet, #2018) ad libitum. Mice were sacrificed at age 32–33week for tissue analysis after 4hours fasting (7am-11am), and sacrifice was done within 2 hours after the morning fasting. At sacrifice, blood samples were collected by cardiac puncture after mice were anesthetized. Ovaries, inguinal subcutaneous, parametrial, mesenteric, perirenal fat pads and interscapular brown adipose tissue (BAT) were isolated, weighed and fixed in z-fix solution. Liver and kidney were isolated, weighed and fixed in 10% buffered formalin solution. In addition, isolated tissues were snap frozen in liquid nitrogen and stored at -80C until further processing. In independent experiments, pups were sacrificed at 3, 6 and 24 hours after TE or vehicle injection, blood samples were pooled from two to four female pups from the same litter due to the limited volume available from each pup for T measurements. Additionally, 3- and 6-week old female mice received a single injection of TE (5 μg/g BW) in 100μl oil or 100μl oil and were fed laboratory chow (Harlan Teklad global 18% protein rodent diet, #2018) ad libitum. Body weight, body lean mass and fat mass were determined every two weeks.

Hormone measurements

Plasma total T was measured using liquid chromatography tandem mass spectrometry (30). Mouse 17β-estradiol (E2), luteinizing hormone (LH) and follicle-stimulating hormone (FSH) were determined by ELISA (Calbiotech, La Jolla, CA), two-site sandwich immunoassay (31, 32) and RIA (33), respectively, at the University of Virginia Center for Research in Reproduction Ligand Assay and Analysis Core (Charlottesville, VA).

Oestrous cycle analysis and ovarian morphology

Oestrous cycle was determined by daily cytological examinations of vaginal smears obtained between 10 and 11 AM for 5 to 10 consecutive days before the sacrifice. The four stages of the cycle, oestrus (E), metoestrus (M), di-oestrus (D) and pro-oestrus (P) were recognized by the presence, absence or proportional numbers of epithelial cells, cornified cells and leukocytes. Occasionally mucous was seen, especially in acyclic females. Ovaries were collected from control (CON) and T-injected female mice (TE) at 32–33 weeks in oestrus stage (next day to pro-oestrus stage), and fixed in z-fix solution for morphological analysis. The specimens were processed for paraffin embedding. Sections were used for hematoxylin and eosin staining and for histological analysis with standard light microscopy. The number of corpora lutea was counted from 4–5 slides of each mouse, and the incidence of epithelial inclusion cysts and cystic dilated bursa were recorded.

Body composition

Whole body fat mass, lean body mass, free water and total body water were measured by nuclear magnetic resonance (NMR) using the EchoMRI-100™ QMR systems every two weeks. This noninvasive measurement does not need anesthesia or sedation.

Gene expression from ovary, white adipose tissue (WAT), BAT and liver

Total RNA was extracted from ovaries, adipose tissues and liver using miRNeasy Mini Kit according to the manufacturer's protocol (QIAGEN). First-strand cDNA was synthesized from the total RNA with SuperScript III-First Strand System (Invitrogen) per the manufacturer's protocol. Expression level of genes was quantified by 7900HT Fast Real-Time PCR System (Life Technologies) using SYBR Green PCR Reagents. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and 18S-ribosomal RNA (18S-rRNA) were used for normalization. The sequences for the quantitative PCR (qPCR) primer set are shown in Supplementary Table 1.

Adipose tissue cellularity

At euthanasia, the inguinal subcutaneous, parametrial, perirenal and mesenteric fat depots were dissected and weighed. Adipocytes were isolated from inguinal subcutaneous and parametrial adipose depots (n = 4 to 5 from each group) by collagenase digestion (1 mg/ml, Worthington Biochemical) in Krebs bicarbonate buffer, pH 7.4, containing 4% albumin (Millipore), 100 nM phenylisopropyl adenosine and 5 mM glucose (KRB-A) (34). After 1 hour of collagenase digestion in a shaking water bath (60 cycles/min, 37°C), isolated adipocytes were filtered through a 250-μm nylon mesh and washed three times with KRB-A buffer. The diameters of at least 300 isolated adipocytes were calculated using photomicroscopic method (35). The weighted average adipocyte weights of cells with a diameter over 20 micron were calculated (36) and total fat cell number was calculated assuming that the fat pads were 75% for inguinal and 85% for parametrial (34) .

Intraperitoneal glucose tolerance test (IPGTT) and intraperitoneal insulin tolerance test (IPITT)

At age 30–32 weeks of age, the mice were fasted for 6 hours (from 7 am to 1 pm) before IPGTT and IPITT. Blood glucose levels were measured in samples obtained from the tail vein using a Contour blood glucose monitoring system and test strips (BAYER). After fasting measurements, mice were injected intraperitoneally with glucose (2.0g/kg BW as a 20% glucose solu-

tion) or insulin (1.25 mU/kg BW), and tail vein samples were obtained at 15, 30, 45, 60, 90 and 120 minutes after the injection.

Statistics

All statistical analyses were performed using SAS 9.3 software (SAS Institute, Cary NC) and Prism software (version 4.0 and 6.0; GraphPad Software Inc.). Unpaired *t* test were performed to compare two groups if the data were normally distributed; Mann-Whitney-Wilcoxon test was used for data that were not normally distributed. Fisher's exact test was used for categorical data. Marginal models with repeated measurements (body weight, lean body mass and body fat mass), containing terms for baseline covariate, treatment, time effects and interaction between treatment and time were tested. If the interaction was found to be nonsignificant it was dropped from the model and overall treatment effect was tested. Statistical significance was set at 0.05 alpha level.

Results

Hormone levels (Table 1 and Figure 1)

To characterize the duration of testosterone elevation after the TE injection, circulating levels of T were measured 3, 6, and 24 hours after the injection. Blood collected from two to four female pups from the same litter was pooled due to the limited volume available from each pup. Circulating testosterone level was significantly higher in neonatal mice 3 and 6 hours after TE injection than in age-matched control pups injected with the vehicle. By 24 hours, the mean circulating testosterone levels in pups injected with TE had decreased to 52.8ng/dl. This rapid rise and subsequent decline in circulating testosterone levels in pups injected with TE is qualitatively similar to the natural endogenous testosterone surge observed in neonatal male mice during the first 24 hours after birth (Figure 1) (37). The circulating testosterone levels measured at 4, 12 and 32 weeks of postnatal life did not differ significantly between mice that had received TE or vehicle injection within 24 hours after birth.

To determine whether neonatal testosterone injection affected other reproductive hormone levels in adult life, circulating E2, LH and FSH levels were measured at 32–33 weeks of age, when the mice were in the oestrus phase. Overall, circulating E2, LH, and FSH levels did not differ significantly between the two groups (Table 1).

Cyclicity and Ovarian morphology (Table 2 and Figure 2)

Vaginal smears were monitored daily for 5–10 consecutive days to characterize the oestrous cycle. 66% (6 out of 9) of female mice that had received neonatal testosterone injection were acyclic (4 were in prolonged di-oestrus and 2 in extended oestrus) (Table 2). These perturbations were sensitive to the timing of testosterone exposure; the mice injected with TE at 3-, and 6-weeks of age had normal estrous cycles.

The mean ovarian weight at 32–33 weeks of age was higher in mice injected with testosterone in the neonatal period than in the control mice (160.2 ± 24.1 vs. 60.4 ± 6.6 mg, $P < .001$) compared to controls (Table 2). The histologic sections of the ovaries revealed an anovulatory phenotype; five of the nine (55%) testosterone-injected mice lacked a corpus luteum (Table 2). No significant difference was observed in the number of ovarian follicles between the testosterone-injected mice and their vehicle-injected control littermates. Although epithelial inclusion cysts, which are commonly seen in old female mice (38), were observed in both testosterone-injected and vehicle-treated mice, the testosterone-injected mice had a higher frequency of epithelial inclusion cysts (55%) than control mice (Table 2). The presence of ovarian cysts and cystic bursa likely contributed to the increased ovarian weight in NT mice.

Neonatal testosterone exposure had no effect on the steady state expression levels of sex hormone receptors and IGF-1R and TGFβ1R, which play an important role in follicular development (Supplemental Figure 1).

Body weight,d body composition and adiposity (Figure 3-5)

At the age of weaning (3 weeks of age), there were no significant differences in body weight between NT mice

Table 1. Blood reproductive hormone concentrations in control (CON) and T-treated (TE) mice at 32–33 weeks old

	CON	TE	P value[a]
Testosterone (ng/dl)	3.0 (0.0; 15.9)	5.2 (1.9; 10.2)	0.667
17β-Estradiol (pg/ml)	19.6 (11.2; 22.9)	18.0 (12.7; 28.1)	0.815
LH (ng/ml)	0.259 (0.178; 0.568)	0.862 (0.324; 1.425)	0.114
FSH (ng/ml)	4.966 (3.091; 5.338)	4.050 (2.912; 4.520)	0.228

Plasma samples were collected at the end of the study (32–33 weeks old). Values are Median (25% Percentile; 75% Percentile). [a]P values are from Mann Whitney test.

Table 2. Ovarian morphology in early postnatal testosterone-exposed (TE) and control (CON) female mice at 32–33 weeks old

	CON (n = 9)	NT (n = 9)	P value[b]
Ovarian weight (mg)[a]	60.44 ± 6.64	160.20 ± 24.11***	
Number of mice with			
Acycling	2 (22%)	6 (66%)	0.153
Ovary without corpus luteum	0 (0%)	5 (55%)	0.029
Epithelial inclusion cyst	3 (33%)	5 (55%)	0.637

[a]Values are mean ± SE. *** $P < 0.001$ *vs.* CON (unpaired *t* test). [b]P-values are from Fisher's exact test for a contingency table .

and CON mice. However, TE mice gained significantly more weight than CON mice after weaning (Figure 3A). As a result, TE mice had consistently higher body weight throughout the study period, compared with CON mice and there was a significant overall treatment effect ($P <$ .05) over the duration of the study. Body composition, ascertained using NMR scanning, also demonstrated higher lean body mass in TE mice (Figure 3B, $P <$ .01). Whole body fat mass was not statistically different in TE mice compared to CON mice over the duration of study (Figure 3C, P = .235), although, there was a consistent trend toward higher weight each depot in TE mice (Supplemental Table 3). However, the fat distribution calculated as depot weight as % dissected fat, was not changed by neonatal testosterone administration. Because testosterone is reported to regulate adipogenesis and adiposity we further examined the cellularity of inguinal subcutaneous and parametrial adipose tissue. No significant difference in the mean size of adipocyte or cell number of either depot was detectable with the number of samples examined (n = 4 to 5 from each group), so the increase in depot weight is likely to be accounted for by the trend toward higher size and number (Supplemental Table 4). In contrast, mice injected with testosterone at 3-, and 6-weeks of age did not display a significant difference in the body weight or body composition when compared with their vehicle-injected littermate controls (data not shown), again confirming the specificity of the timing of androgen exposure in altering body composition.

Since there were trends toward higher fat mass in the TE group (Figure 3C and Supplemental Table 3), we determined the expression levels of key genes in lipogenic and lipolytic pathways in adipose tissues. LPL, which hydrolyzes extracellular TG in lipoprotein for TG accumulation in adipocytes, showed 2.8-fold elevation in the inguinal subcutaneous adipose tissue in the TE mice relative to CON mice (Figure 4B, $P <$ .001, n = 9 for each group). This between-group difference in LPL expression was not observed in the parametrial adipose tissue (Figure 4C). This difference in LPL expression might contribute to the trend in increase fat mass. Adipocyte-derived resistin is known to be increased in obese rodents and strongly related to insulin resistance (39). The TE mice showed increased resistin expression in the inguinal subcutaneous adipose tissue compared with CON (Figure 4B, 1.8-fold elevation, $P <$ .01, n = 9 for each group). Neither the expression of AR nor ERα in specific adipose tissue depots was altered by neonatal testosterone exposure (Supplemental Figure 2).

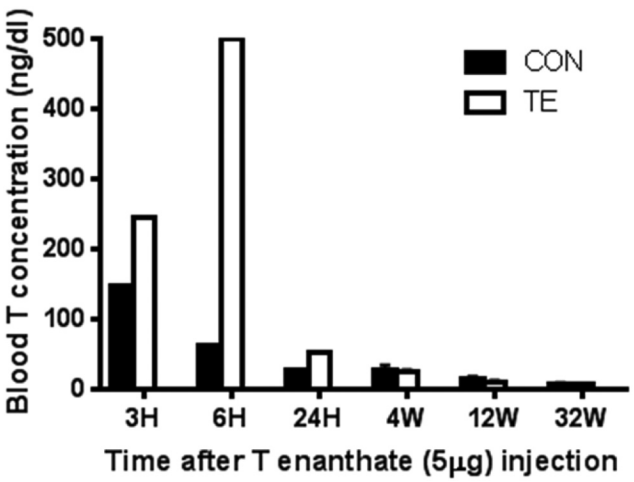

Figure 1. Plasma T concentration after TE injection. Plasma samples collected at 3H, 6H and 24H after testosterone enanthate injection were pooled and used for total testosterone measurements. Two to four female mice within the same litter were used for plasma pooling. Plasma samples were also collected at 4 (n = 6), 12 (n = 6) and 32 weeks of age (n = 9) after testosterone enanthate injection. Values at 4, 12 and 32 weeks are mean ± S.E. Mann-Whitney-Wilcoxon test was performed for between group comparisons of plasma testosterone levels at 4, 12 and 32 weeks.

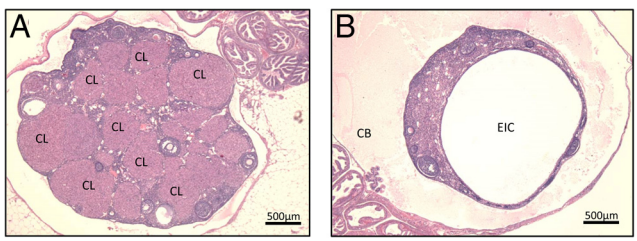

Figure 2. Ovarian morphology of control (CON) and testosterone-injected (TE) mice. A, Hematoxylin-eosin (HE)-stained ovarian section of a normal cycling mouse (CON) with healthy follicles and a corpus lutea. B, HE-stained ovarian section of an acyclic mouse with ovarian cyst and cystic bursa, but without a corpus luteum (TE). CL, Corpus luteum; EIC, Epithelial inclustion cyst; CB, Cystic bursa. Magnification, X4; Scale bar, 500 μm.

TE mice showed significantly higher weights of the interscapular brown adipose tissue (BAT) ($P < .01$, n = 9 for each group) and the BAT accumulated more lipid in multilocular cells (Figure 5A). During the fat tissue dissection at the time of euthanasia at 32–33 weeks, we noted white fat infiltration into brown fat (Figure 5B). We therefore examined a series of functional and differentiation transcripts to assess if the BAT differentiation was affected by neonatal testosterone exposure. UCP-1 is a member of the mitochondrial inner membrane proteins and is highly expressed in BAT. Real-time PCR showed a 28% reduction of transcript level of UCP-1 in TE mice compared to CON mice (Figure 5C, $P = .074$). CIDEA, a mitochondria-specific marker in BAT, was also reduced by 34% in TE mice relative to CON mice (Figure 5C, $P = .089$). mRNA expression levels of BMP-7, another BAT-specific marker, and BAT differentiation-related transcription factors (PPARγ, C/EBPβ and PRDM16) and transcriptional coactivator (PGC-1α), in TE mice were consistently reduced in comparison with the CON group but the differences between groups were not statistically significant (Figure 5D). The expression levels of AR, ERα and ERβ in BAT were numerically lower in the TE group but did not differ significantly between groups (Figure 5E); ERβ was undetectable in both groups as expected.

Glucose and insulin tolerance (Figure 6)

To investigate whether changes in body mass and composition observed in mice exposed to neonatal testosterone administration affected glucose tolerance and response to insulin, IPGTT and IPITT were performed at the age of 30 and 32 weeks, respectively. TE and control mice showed similar fasting glucose levels (~ 100 mg/dl). After exogenous glucose loading (2.0 g glucose/kg BW), blood glucose levels increased to a higher level in TE mice than in CON mice (206.8 ± 9.7 vs 172.8 ± 7.5 mg/dl), respectively at 15 minutes ($P < .05$). However, the blood glucose area-underthe-curve (AUC) did not differ between the TE and CON mice. After intraperitoneal administration of 1.25 mU insulin/kg BW, the TE mice displayed a significantly lesser reduction in blood glucose levels compared with their litter-mate controls. The blood glucose AUC was significantly greater in the TE mice compared with the CON mice, consistent with their higher level of insulin resistance. Two of 9 mice from the CON had blood glucose levels were < 25 mg/ dl at 45–60min after insulin administration; these values were recorded as 25 mg/ dl in the statistical analyses.

Discussion

We show here that transient exposure of female newborn mice to testosterone within 24-hours of birth is associated with substantial reproductive and metabolic alterations in adult life. The female mice injected with a single dose of testosterone during the neonatal period displayed higher frequency of oestrous cycle abnormalities, including acyclicity and the lack of corpora lutea, higher ovarian mass, and a greater frequency of epithelial inclusion cysts than those injected with the vehicle alone. These phenotypic effects were not associated with a concomitant alteration in circulating levels of testosterone and estradiol during adult life. Furthermore, in comparison to vehicle-injected controls, the mice exposed to testosterone transiently after birth had higher body weights, a higher trajectory of body weight gain, a body composition characterized by increased lean tissue mass, and a greater degree of insulin tolerance. Collectively, these data demonstrate that even transient androgen exposure during a relatively narrow developmental window can induce sustained effects on reproductive and metabolic functions throughout adult life.

These reproductive and metabolic features were observed in mice exposed to testosterone during the immediate postnatal period, and were not observed in mice

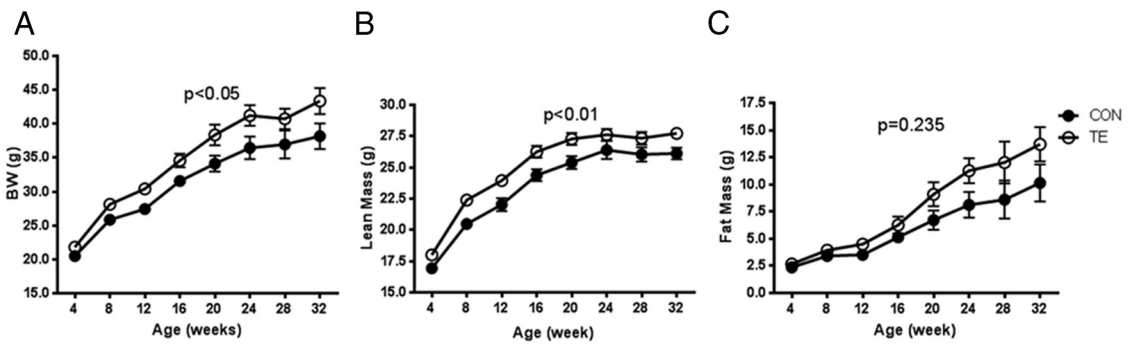

Figure 3. A, The trajectories of body weight of control (closed circle) and T-treated mice over the 32 weeks of postnatal life (open circle). B, Absolute lean body mass of control and T-treated mice during the postnatal life. C, Absolute body fat mass in control and T-treated mice. Data represent mean ± S.E. (n = 9). P-values are for overall treatment effect from marginal model.

which received TE injection at 3 or 6 weeks of age, indicating the critical importance of the timing of testosterone exposure in inducing the reproductive and metabolic phenotype. Rodents exposed to androgen in utero during late pregnancy have shown inconsistent changes in adult reproductive function. For instance, in one study, prenatal androgen exposure had no effect on adult reproductive function in Wistar rats, although there was a reduction in the primordial follicle pool (22); exposure to higher testosterone doses for 4 days during late gestation induced hyperandrogenism, ovarian cysts and anovulatory cycles in Sprague Dawley rats (21). Recently, Tyndall et al (22) reported that sustained testosterone administration from postnatal day 1 to postnatal day 24 induced anovulation, while testosterone administration during a later postnatal period (days 15 to 24) did not affect ovarian phenotype in Wistar rats. These findings are in agreement with our data that the early postnatal window is critical for inducing the reproductive and metabolic phenotype that we observed in testosterone-injected mice.

Several regimens of prenatal as well as postnatal sex steroid administration, including serial prenatal injections or long-term postnatal injections have been shown in well-conducted studies to induce the development of cystic ovaries, acyclicity and immature follicular development in sheep, monkey and rodents (15–22). The testosterone dose used in the present study - 5 μg TE - is among the lowest doses of testosterone used in published reports. More importantly, this dose of TE was effective in transiently raising circulating testosterone concentrations in female pups into the range observed naturally in male newborn mice during this period. The peak testosterone levels were observed 6 hours after the injection and testosterone levels decreased to near normal levels within 24 hours after the injection, confirming that the testosterone exposure was transient and mimicked the natural testosterone surge in male mice.

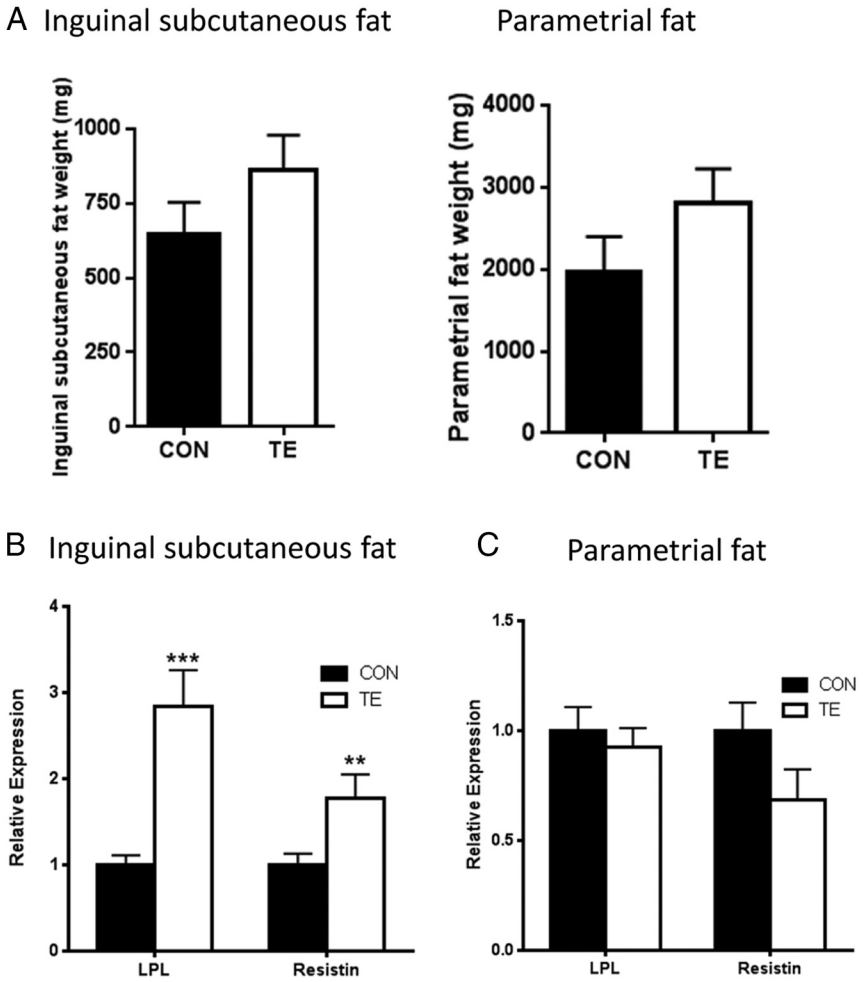

Figure 4. A, Adipose tissues weights of postnatal control and T-treated mice at 32–33 weeks old. Data represent mean ± S.E. (n = 9) (B) Expression of LPL and Resistin in early postnatal testosterone-exposed (TE, n = 9) and control (CON, n = 9) female mice aged 32–33wk in inguinal subcutaneous adipose tissue. C, Expression of LPL and Resistin in early postnatal testosterone-exposed (TE, n = 9) and control (CON, n = 9) female mice aged 32–33wk in parametrial adipose tissue. Values are relative expression ($2(-\Delta\Delta C_T)$), ΔC_T values (C_T of target gene – C_T of housekeeping gene) were used for unpaired *t* test. **,$P < .01$ vs. CON; *** $P < .001$.

Although some of the phenotypic features of testosterone-injected mice – acyclicity and anovulation, and increased adiposity and insulin resistance – are similar to those observed in women with polycystic ovary syndrome, extrapolation of findings from rodent models to human disease warrants substantial caution. Circulating testosterone levels in adult life did not differ between testosterone and placebo-injected mice. Also the reproductive developmental windows in rodents and humans differ substantially. However, these data are consistent with the notion that even brief durations of exposures to environmental endocrine disruptors with androgenic or antiandrogenic activity during critical developmental windows could potentially exert long term effects on reproductive and metabolic function.

The testosterone-injected mice had higher body weight and greater lean body mass over the study period. While sum of the major fat depots and individual fat depots dis-

played clear trend towards increased tissue weights, the difference didn't reach statistical significance. The strongest trends toward higher adipose tissue weights were observed in two intra-abdominal depots, mesenteric and the perirenal ($P < .1$ Supplemental Table 3). Within the sample size (n = 9 per group), NT did not significantly affect body fat distribution as calculated as the mass of each depot as a percentage of the total dissected tissue (Supplemental Table 3), nor the size or number of adipocytes in the inguinal or parametrial depots (Supplemental Table 4). However, as strongest trends were in the mesenteric depot, further studies should include this depot, the only true visceral depot in the mouse. These observations suggest that neonatal programming effect by testosterone tend to increase overall adiposity, but selective effect to increase the mass of specific depots is not evident. This is similar to phenotypic changes observed in the ovariectomized C57/BL6 mice (40), but contrasts with data in women with high androgen levels in PCOS who are often characterized by accumulation of visceral fat (41). Taken together, our results highlight the need for better powered studies to further dissect the depot-specific influence of neonatal exposure to sex steroids.

Triglyceride accumulation within the adipose tissue occurs primarily via LPL hydrolysis of lipoprotein triglyceride (42). The TE mice exhibited higher mRNA levels of LPL in inguinal subcutaneous fat depot, which would be predicted to lead to an increase in adipocyte size as the animals aged. Overall, LPL expression pattern in inguinal subcutaneous fat in these mice are consistent with their morphologic features as seen in a previous report (43). Adipocyte-derived resistin is known to be strongly related to insulin resistance (39). The TE mice showed increased resistin expression in the inguinal subcutaneous adipose tissue compared with CON (Figure 4C), consistent with the observed insulin resistance. The mechanisms by which neonatal androgen exposure leads to systemic insulin resistance in adult mice need further investigation.

The BAT in TE mice exhibited higher mass with increased white fat infiltration. The transcriptional analysis of BAT-specific markers UCP-1, CIDEA and growth factor BMP-7 and other BAT differentiation-related genes is also consistent with the phenotypic observation (Figure 5 C-D). We recognize that the steady state gene expression profiles may be quite different than during the transient exposure. However, even a slight shift in expression over the cumulative lifespan can have significant effect on body composition. Accordingly, expression data obtained at a single time point may not necessarily reflect the dynamic changes during an animal's life or during different metabolic states.

The imprinting effects of neonatal testosterone exposure on mating and nonmating behaviors in rodents, song birds, and in other animal models have been well characterized, and generally felt to be due to the epigenetic effects of testosterone. Further research is needed to determine whether the reproductive and metabolic phenotype in adult mice life after a transient testosterone exposure is

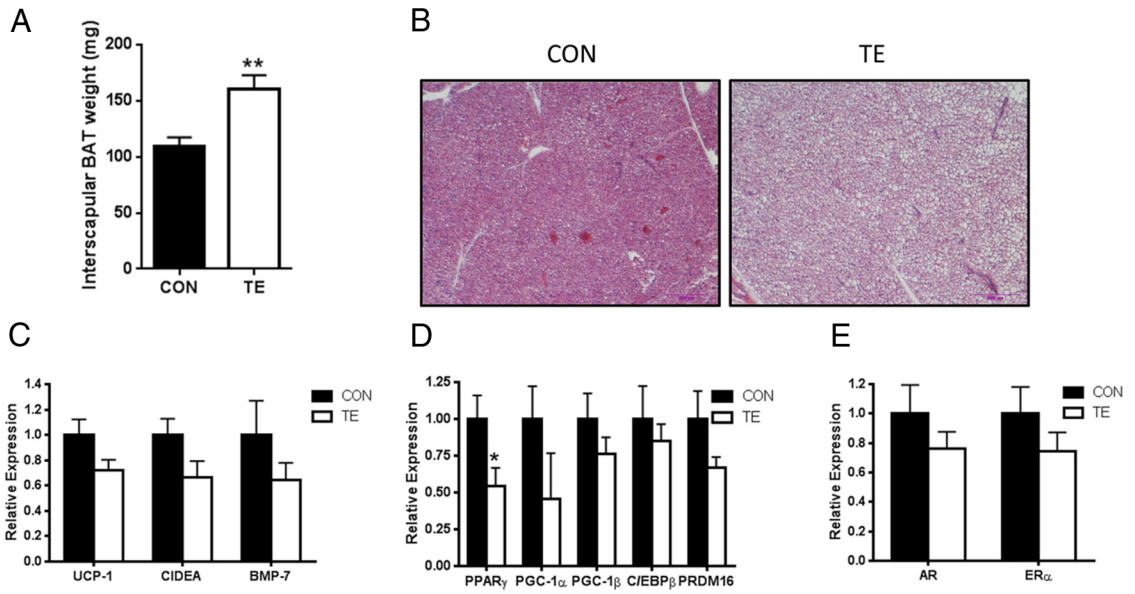

Figure 5. A, Interscapular brown adipose tissue (BAT) weights of control (CON) and T-treated (TE) mice at 32–33 weeks old. Data represent mean ± S.E. (n = 9). **,$P < .01$ vs. CON (unpaired t test). B, Hematoxylin-eosin (HE)-stained section of the interscapular BAT in CON and TE mice. Magnification, X10, Scale bar, 200 μm. Figure 5. C, -(E) Brown adipose tissue-specific mRNA expression. C, Expression of UCP-1, CIDEA and BMP-7 mRNA in early postnatal testosterone-exposed (TE, N = 9) and control (CON, n = 9) female mice aged 32–33wk were measured by quantitative RT-PCR. D, Gene expression of BAT differentiation-related transcription factors. E, Gene expression of nuclear receptors. Values are relative expression ($2(-\Delta\Delta C_T)$), ΔC_T values (C_T of target gene – C_T of housekeeping gene) were used for unpaired *t* test. *,$P < .05$ vs. CON.

due to epigenetic effects related to alterations in DNA methylation, histone modifications, or microRNAs. It is also not clear whether these reproductive and metabolic effects are due to shared mechanistic pathways induced by testosterone or to different pathways. We do not know whether these are AR-mediated effects or whether these effects require testosterone's conversion to estradiol and mediation of ER-mediated mechanisms, as has been reported for some imprinting effects of testosterone on rodent behaviors and on adult ovary (23, 44). The TE mice did not reveal clear differences in the expression pattern of AR, ERα, or ERbeta. While in this study we focused on the transcriptional markers of signaling mechanisms associated with neonatal testosterone exposure, several chromatin modifying enzymes have been shown to interact with and modulate action of AR and ER mediated pathways. It is likely that additional epigenetic pathways may be involved in the regulation of the observed metabolic and reproductive reprogramming.

In summary, we have shown that a single injection of low, physiologically relevant dose of testosterone to neonatal female mice induces reproductive and metabolic alterations that are sustained throughout adult life. The findings from this study provide further support for the hypothesis that early postnatal period in mice may be a critical developmental window of susceptibility to environmental endocrine disruptors. Because the reprogramming that occurs during early life may go unrecognized until adulthood, but may contribute to metabolic and reproductive alterations, further studies should establish the mechanisms and transcriptional or epigenetic markers of such preprogramming by androgens.

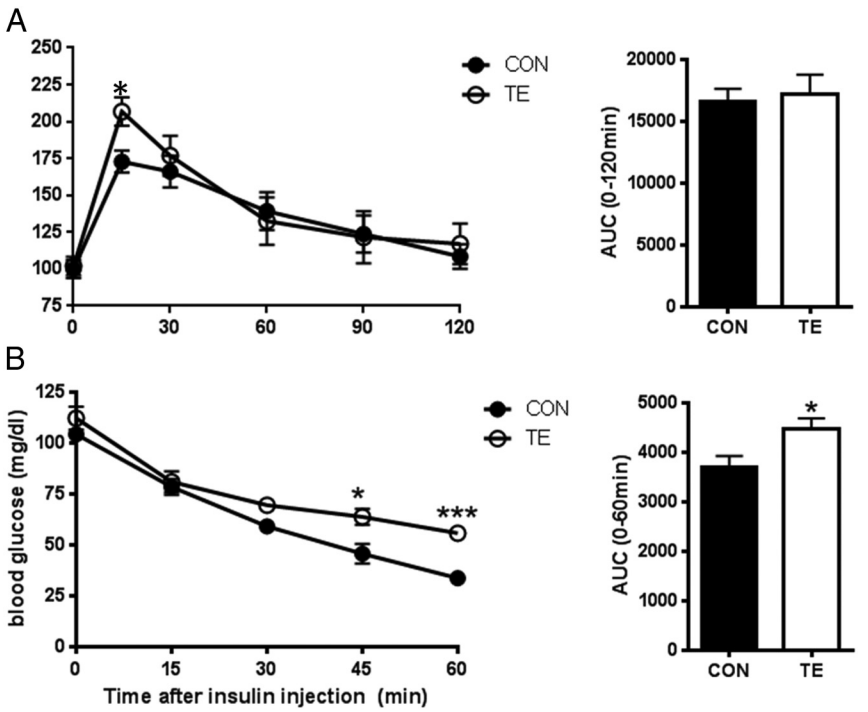

Figure 6. Intraperitoneal glucose tolerance test (IPGTT) and intraperitoneal insulin tolerance test (IPITT) of control and testosterone-treated mice. The IPGTT and IPITT were performed at 30 and 32 weeks old, respectively. A, IPGTT in control (CON, closed circles) and testosterone-treated (TE, open circles) mice and the corresponding 0- to 120-minute area under the curve (AUC). B, IPITT in control (CON, closed circles) and testosterone-treated (TE, open circles) mice, and the corresponding 0- to 60-minute AUC. Data represent mean ± SEM (n = 9). Log-transformed data were used in the analysis of AUC for *t* test; *,$P < .05$; ***, $P < .001$. Two of 9 mice from the control had blood glucose levels < 25 mg/dl at 45–60min. Their blood glucose levels were recorded as 25 mg/dl at 60min for statistical analysis.

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References

1. **Phoenix CH, Goy RW, Gerall AA, Young WC.** Organizing action of prenatally administered testosterone propionate on the tissues mediating mating behavior in the female guinea pig. *Endocrinology*. 1959; 65:369–382.
2. **Nottebohm F, Arnold AP.** Sexual dimorphism in vocal control areas of the songbird brain. *Science*. 1976;194:211–213.
3. **Pohl-Apel G.** The correlation between the degree of brain masculinization and song quality in estradiol treated female zebra finches. *Brain research*. 1985;336:381–383.
4. **Thornton JE, Irving S, Goy RW.** Effects of prenatal antiandrogen treatment on masculinization and defeminization of guinea pigs. *Physiol Behav*. 1991;50:471–475.
5. **Goh DW, Middlesworth W, Farmer PJ,**

Hutson JM. Prenatal androgen blockade with flutamide inhibits masculinization of the genitofemoral nerve and testicular descent. *Journal of pediatric surgery*. 1994;29:836–838.

6. **Goldfoot DA, Van Der Werff Ten Bosch JJ.** Mounting behavior of female guinea pigs after prenatal and adult administration of the propionates of testosterone, dihydrotestosterone, and androstanediol. *Hormones and behavior*. 1975;6:139–148.
7. **Feder HH, Goy RW.** Effects of neonatal estrogen treatment of female guinea pigs on mounting behavior in adulthood. *Hormones and behavior*. 1983;17:284–291.
8. **Stavreva DA, George AA, Klausmeyer P, Varticovski L, Sack D, Voss TC, Schiltz RL, Blazer VS, Iwanowicz LR, Hager GL.** Prevalent glucocorticoid and androgen activity in US water sources. *Scientific reports*. 2012;2:937.
9. **Qu S, Kolodziej EP, Long SA, Gloer JB, Patterson EV, Baltrusaitis J, Jones GD, Benchetler PV, Cole EA, Kimbrough KC, Tarnoff MD, Cwiertny DM.** Product-to-parent reversion of trenbolone: unrecognized risks for endocrine disruption. *Science*. 2013;342:347–351.
10. **Barker DJ, Gluckman PD, Godfrey KM, Harding JE, Owens JA, Robinson JS.** Fetal nutrition and cardiovascular disease in adult life. *Lancet*. 1993;341:938–941.
11. **Paneth N, Susser M.** Early origin of coronary heart disease (the "Barker hypothesis"). *BMJ*. 1995;310:411–412.
12. **Zama AM, Uzumcu M.** Epigenetic effects of endocrine-disrupting chemicals on female reproduction: an ovarian perspective. *Frontiers in neuroendocrinology*. 2010;31:420–439.
13. **Crain DA, Janssen SJ, Edwards TM, Heindel J, Ho SM, Hunt P, Iguchi T, Juul A, McLachlan JA, Schwartz J, Skakkebaek N, Soto AM, Swan S, Walker C, Woodruff TK, Woodruff TJ, Giudice LC, Guillette LJ, Jr.** Female reproductive disorders: the roles of endocrine-disrupting compounds and developmental timing. *Fertility and sterility*. 2008;90:911–940.
14. **Diamanti-Kandarakis E, Bourguignon JP, Giudice LC, Hauser R, Prins GS, Soto AM, Zoeller RT, Gore AC.** Endocrine-disrupting chemicals: an Endocrine Society scientific statement. *Endocrine reviews*. 2009;30:293–342.
15. **Steckler TL, Roberts EK, Doop DD, Lee TM, Padmanabhan V.** Developmental programming in sheep: administration of testosterone during 60–90 days of pregnancy reduces breeding success and pregnancy outcome. *Theriogenology*. 2007;67:459–467.
16. **Steckler T, Manikkam M, Inskeep EK, Padmanabhan V.** Developmental programming: follicular persistence in prenatal testosterone-treated sheep is not programmed by androgenic actions of testosterone. *Endocrinology*. 2007;148:3532–3540.
17. **Hogg K, McNeilly AS, Duncan WC.** Prenatal androgen exposure leads to alterations in gene and protein expression in the ovine fetal ovary. *Endocrinology*. 2011;152:2048–2059.
18. **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;9:62–67.
19. **Zhou R, Bruns CM, Bird IM, Kemnitz JW, Goodfriend TL, Dumesic DA, Abbott DH.** Pioglitazone improves insulin action and normalizes menstrual cycles in a majority of prenatally androgenized female rhesus monkeys. *Reprod Toxicol*. 2007;23:438–448.
20. **Abbott DH, Tarantal AF, Dumesic DA.** Fetal, infant, adolescent and adult phenotypes of polycystic ovary syndrome in prenatally androgenized female rhesus monkeys. *American journal of primatology*. 2009;71:776–784.
21. **Amalfi S, Velez LM, Heber MF, Vighi S, Ferreira SR, Orozco AV, Pignataro O, Motta AB.** Prenatal hyperandrogenization induces metabolic and endocrine alterations which depend on the levels of testosterone exposure. *PloS one*. 2012;7:e37658.
22. **Tyndall V, Broyde M, Sharpe R, Welsh M, Drake AJ, McNeilly AS.** Effect of androgen treatment during foetal and/or neonatal life on ovarian function in prepubertal and adult rats. *Reproduction*. 2012;143:21–33.
23. **Alexanderson C, Eriksson E, Stener-Victorin E, Lystig T, Gabrielsson B, Lonn M, Holmang A.** Postnatal testosterone exposure results in insulin resistance, enlarged mesenteric adipocytes, and an atherogenic lipid profile in adult female rats: comparisons with estradiol and dihydrotestosterone. *Endocrinology*. 2007;148:5369–5376.
24. **Eisner JR, Dumesic DA, Kemnitz JW, Abbott DH.** Timing of prenatal androgen excess determines differential impairment in insulin secretion and action in adult female rhesus monkeys. *J Clin Endocrinol Metab*. 2000;85:1206–1210.
25. **Eisner JR, Dumesic DA, Kemnitz JW, Colman RJ, Abbott DH.** Increased adiposity in female rhesus monkeys exposed to androgen excess during early gestation. *Obesity research*. 2003;11:279–286.
26. **Recabarren SE, Padmanabhan V, Codner E, Lobos A, Duran C, Vidal M, Foster DL, Sir-Petermann T.** Postnatal developmental consequences of altered insulin sensitivity in female sheep treated prenatally with testosterone. *Am J Physiol Endocrinol Metab*. 2005;289:E801–806.
27. **Gill JW, Hosking BJ.** Acute prenatal androgen treatment increases birth weights and growth rates in lambs. *J Anim Sci*. 1995;73:2600–2608.
28. **Hansen LR, Drackley JK, Berger LL, Grum DE.** Prenatal androgenization of lambs: I. Alterations of growth, carcass characteristics, and metabolites in blood. *J Anim Sci*. 1995;73:1694–1700.
29. **King AJ, Olivier NB, Mohankumar PS, Lee JS, Padmanabhan V, Fink GD.** Hypertension caused by prenatal testosterone excess in female sheep. *Am J Physiol Endocrinol Metab*. 2007;292:E1837–1841.
30. **Sir-Petermann T, Codner E, Perez V, Echiburu B, Maliqueo M, Ladron de Guevara A, Preisler J, Crisosto N, Sanchez F, Cassorla F, Bhasin S.** Metabolic and reproductive features before and during puberty in daughters of women with polycystic ovary syndrome. *J Clin Endocrinol Metab*. 2009;94:1923–1930.
31. **Fallest PC, Trader GL, Darrow JM, Shupnik MA.** Regulation of rat luteinizing hormone beta gene expression in transgenic mice by steroids and a gonadotropin-releasing hormone antagonist. *Biology of reproduction*. 1995;53:103–109.
32. **Haavisto AM, Pettersson K, Bergendahl M, Perheentupa A, Roser JF, Huhtaniemi I.** A supersensitive immunofluorometric assay for rat luteinizing hormone. *Endocrinology*. 1993;132:1687–1691.
33. **Gay VL, Midgley AR, Jr., Niswender GD.** Patterns of gonadotrophin secretion associated with ovulation. *Federation proceedings*. 1970;29:1880–1887.
34. **Shaughnessy S, Smith ER, Kodukula S, Storch J, Fried SK.** Adipocyte metabolism in adipocyte fatty acid binding protein knockout mice (aP2-/-) after short-term high-fat feeding: functional compensation by the keratinocyte [correction of keritinocyte] fatty acid binding protein. *Diabetes*. 2000;49:904–911.
35. **Lavau M, Susini C, Knittle J, Blanchet-Hirst S, Greenwood MR.** A reliable photomicrographic method to determining fat cell size and number: application to dietary obesity. *Proceedings of the Society for Experimental Biology and Medicine Society for Experimental Biology and Medicine*. 1977;156:251–256.
36. **Kursawe R, Eszlinger M, Narayan D, Liu T, Bazuine M, Cali AM, D'Adamo E, Shaw M, Pierpont B, Shulman GI, Cushman SW, Sherman A, Caprio S.** Cellularity and adipogenic profile of the abdominal subcutaneous adipose tissue from obese adolescents: association with insulin resistance and hepatic steatosis. *Diabetes*. 2010;59:2288–2296.
37. **Motelica-Heino I, Castanier M, Corbier P, Edwards DA, Roffi J.** Testosterone levels in plasma and testes of neonatal mice. *J Steroid Biochem*. 1988;31:283–286.
38. **Fleming JS, McQuillan HJ, Millier MJ, Beaugie CR, Livingstone V.** E-cadherin expression and bromodeoxyuridine incorporation during development of ovarian inclusion cysts in age-matched breeder

and incessantly ovulated CD-1 mice. *Reproductive biology and endocrinology : RB&E*. 2007;5:14.

39. **Park HK, Ahima RS.** Resistin in rodents and humans. *Diabetes, metabolism journal*. 2013;37:404–414.
40. **Grove KL, Fried SK, Greenberg AS, Xiao XQ, Clegg DJ.** A microarray analysis of sexual dimorphism of adipose tissues in high-fat-diet-induced obese mice. *International journal of obesity*. 2010;34:989–1000.
41. **Escobar-Morreale HF, San Millan JL.** Abdominal adiposity and the polycystic ovary syndrome. *Trends Endocrinol Metab*. 2007;18:266–272.
42. **Garcia-Arcos I, Hiyama Y, Drosatos K, Bharadwaj KG, Hu Y, Son NH, O'Byrne SM, Chang CL, Deckelbaum RJ, Takahashi M, Westerterp M, Obunike JC, Jiang H, Yagyu H, Blaner WS, Goldberg IJ.** Adipose-specific lipoprotein lipase deficiency more profoundly affects brown than white fat biology. *The Journal of biological chemistry*. 2013;288:14046–14058.
43. **Palou M, Priego T, Sanchez J, Rodriguez AM, Palou A, Pico C.** Gene expression patterns in visceral and subcutaneous adipose depots in rats are linked to their morphologic features. *Cellular physiology and biochemistry : international journal of experimental cellular physiology, biochemistry, and pharmacology*. 2009;24:547–556.
44. **Alexanderson C, Stener-Victorin E, Kullberg J, Nilsson S, Levin M, Cajander S, Lonn L, Lonn M, Holmang A.** A single early postnatal estradiol injection affects morphology and gene expression of the ovary and parametrial adipose tissue in adult female rats. *J Steroid Biochem Mol Biol*. 2010;122:82–90.