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Alteration in follistatin gene expression detected in prenatally androgenized rats

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Abstract

Impaired ovarian follicle development, the hallmark of polycystic ovarian syndrome (PCOS), is believed to be due to the changes in expression of related genes such as follistatin (FST).

Expression of FST gene and methylation level of its promoter in theca cells from adult female rats, prenatally exposed to androgen excess, during different phases of the estrus cycle was determined and compared with controls.

Eight pregnant Wistar rats (experimental group) were treated by subcutaneous injection of 5 mg free testosterone on day 20 of pregnancy, while controls ($n = 8$) received 500 ml solvent. Based on observed vaginal smear, adult female offspring of mothers were divided into three groups. Levels of serum steroidogenic sexual hormones and gonadotropins, expression and promoter methylation of the FST gene were measured using ELISA, cyber-green real-time PCR and bisulfite sequence PCR (BSP), respectively.

Compared to controls, the relative expression of FST gene in the treated group decreased overall by 0.85 fold; despite significant changes in different phases, but no significant differences in methylation of FST promoter.

Our results reveal that manifestation of PCOS-like phenotype following prenatal exposure to excess androgen is associated with irregularity in expression of the FST gene during the estrus cycle.

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Declaration of interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

Keywords

Follistatin; gene expression; methylation; PCOS; prenatally androgenized

Introduction

Polycystic ovary syndrome (PCOS), originally described by Stein and Leventhal in 1935, is the most prevalent gynecological endocrinopathy of reproductive age [1]. Oligo-ovulation or anovulation and hyperandrogenism, main criteria of the syndrome [2], are frequently associated with presence of insulin resistance, obesity, diabetes mellitus type 2 and metabolic syndrome [3]. The main pathophysiologic base of the syndrome has not yet been established. While several studies show it has a tendency to run in families [4,5], despite suggesting the existence of susceptibility genes, the specific gene(s) that play a determinant role in PCOS pathogenesis have not been identified [6], probably due to the multifactorial nature of this syndrome, i.e. a combination of genetic and environmental factors [7].

Prenatal exposure to excess androgen, an environmental factor, may induce PCOS and its related metabolic and reproductive derangements during pubertal development [8,9].

Many candidate genes have been examined for a possible role in development of this syndrome [10,11], of which follistatin (FST) gene is one considered because of its involvement in folliculogenesis. Follistatin known as the follicle-stimulating hormone (FSH) suppressing protein, due to its ability to prevent binding activin-binding protein, cooperates with inhibin to suppress the secretion of FSH from the pituitary [12]; it is one of the key mediators of follicular differentiation [13] in different stages of folliculogenesis; depending on the species [14]; and plays a role in reproductive processes by regulation of FSH secretion, development and action of rodent gonads, androgen production and pregnancy [15].

In this study, we examined whether excess of prenatal androgen alters the expression of this gene involved in folliculogenesis pathway that may play a role in development of PCOS.

Materials and methods

Animals and care

Animal experiments were handled in accordance with the standard ethical principles of laboratory animal care approved by the local ethics committee of the Research Institute for Endocrine Sciences with approval number RIES; 320 EC 90.09.07.

Sixteen female adult Wistar rats (age 85–95 days and body weight 170–180 g) were paired with adult males overnight in a polypropylene cage overnight under standard animal housing conditions (12 h–12 h light–dark cycles and controlled temperature of 22 ± 3 °C, relative humidity 45–55%). Observing the vaginal plug indicated initiation of pregnancy, pregnant rats were randomly divided into two groups, the experimental and controls (n = 8, each).

Hormonal treatment

On the 20th day of pregnancy, 5 mg of free testosterone (T1500; Sigma, Steinheim, Germany) dissolved in a 500 ml cocktail containing sesame oil (S3547; Sigma, Steinheim, Germany) and benzyl benzoate (B6630; Sigma, Steinheim, Germany) at a ratio of 4:1, was injected subcutaneously to pregnant rats, experimental group ($n = 8$), while controls ($n = 8$) received 500 ml solvent [16].

Female offspring of experimental (prenatally androgenized, PNA) and the control ($n = 23$, each) rats were kept with *ad libitum* food and water.

Sample collection

Blood samples were obtained from the abdominal aorta of the adult controls and prenatally androgenized female offspring (100–110 days of age) after deep anesthesia by intraperitoneal injection of pentobarbital sodium (P3761; 5 mg; Sigma, St. Louis, MO) dissolved in normal saline 0.9% [60 mg (kg body weight^{-1})]; sera were isolated and stored at −80 °C for subsequent measurement of hormone levels of testosterone (T), luteinizing hormone (LH) and FSH.

Simultaneously with blood sampling, theca cells were isolated, dispersed and stored following a standard protocol [17], after cutting both ovaries using sterile forceps, collected ovaries were placed in plates containing cold PBS (pH 7.4) on ice. Under a dissecting microscope, the adhering fat and surrounding tissues were removed; and follicles picked up one by one using a microforceps, were actively punctured with a 27 G needle and pushed to get rid of the fluid containing granulosa cells from the follicles; remaining cell layers of each follicle, composed of theca cells, were washed thrice with cold PBS to release remaining cells and tissues. Tissues obtained were transferred to RNA-DNase free tubes and, immediately, stored at −80 °C.

Determination of cyclicity, based on vaginal smears was performed before blood and tissue sampling to avoid neural stimulation and stress. Animals were then divided to four subgroups, i.e. proestrus, estrus, metestrus and diestrus.

Hormonal measurement

To measure hormonal levels, rat specific ELISA kits (CUSABIO, China) of T, LH and FSH, were used. Intra-assay coefficients of variations for all hormones were510%. The sensitivity of kits was <0.06 ng ml^{-1}, <0.15 mIU ml^{-1} and <0.45 mIU ml^{-1}, for T, LH and FSH, respectively.

RNA isolation and cDNA synthesis and real-time PCR

Using the Trizol protocol [18], total RNA was extracted from ovarian tissues. QuantiTec. Rev. Transcription kit (Qiagen, Germantown, MD) was used to synthesize cDNA. Quantity and purity of RNA and the cDNA were measured (NanoDrop 1000, Thermo-Scientific, Waltham, MA).

FST gene expression was quantitatively assessed by cyber-green real-time polymerase chain reaction (qRT-PCR); using appropriate primers: FST forward, 5′-CCAACTGCATCCCTTGTA-3′, FST reverse, 5′-CAGGTGATGTTGGAACAGT-3′, B-actin (housekeeping) forward, 5′-CCGTGAAAAGATGACCCAGATC-3′, B-actin reverse, 5′-CACAGCCTGGATGGCTACGT-3′. Reactions were performed in 15 μl volumes containing 7.5 μl cyber green-PCR Master Mix (Thermofisher Scientific, Waltham, MA), 0.6 ml (10 ng/μl) of each forward and reverse primers, 5.3 μl RNase-free water and 1 ml (100 ng/μl) of the total cDNA. PCR was performed for duplicate samples using the Rotor-Gene 6000 real-time PCR machine (Corbett Research, Sydney, Australia) in a 40-cycle program with initial denaturation (10 minutes at 95 °C), followed by a two-step amplification program (15 seconds at 95 °C, followed by 60 seconds at 60 °C and 30 seconds at 72 °C), per cycle.

The relative amount of mRNA in each sample was calculated based on its threshold cycle (Ct) compared to the Ct of the housekeeping gene. Expression of samples between the PNA and controls was compared by relative expression analysis using the $2^{-\Delta\Delta Ct}$.

DNA extraction and bisulphite treatment

DNA was extracted from 2 mm disks of theca layer samples, fixed on the FTA classic Whatman cards (Whatman International Ltd, Maidstone, UK), using QIAamp DNA Investigator Kit (Qiagen Ltd, Germantown, MD). DNA concentration was measured by spectrophotometry and subsequently adjusted to 40 ng/μl. Bisulphite treatment of 2 μg of each sample was undertaken using the EZ DNA Methylation-Lightening™ kit (Zymo Research, Irvine, CA), by adding 130 μl of Lightning Conversion Reagent to 20 μl of a DNA sample in a PCR tube by the following incubation steps: 98 °C for eight minutes and 54 °C for 60 minutes followed by several steps of adding binding and washing buffers to a Zymo-Spin™ IC Column and centrifuging at full speed (>10 000 × *g*) for 30 seconds after each step and finally, eluting the converted DNA in 10 μl of 0.1 × TE buffer.

Primer design

Using forward (5′-TAGAGGTGTTGGGGATTAATTTTAG-3′) and reverse (5′-AAAAACATCCACTTCAATCCTACAC-3′) primers, designed by Methprimer online software [19], according to these criteria: Island size > 100, GC Percent > 50.0, Obs/Exp40.6, the second CpG island of FST gene was amplified.

Amplification and purification

Hot-start PCR was carried out with HotStar Taq Master Mix Kit (Qiagen Ltd., Germantown, MD), using 10 μl bisulphite treated DNA, 12.5 μl Master Mix, 0.6 μl of each primers (final concentration: 10 μM) in 25 μl total volume using T100™ Thermal Cycler machine (Bio-Rad, CA, USA). A touch-down amplification was performed with an initial step of 95 °C for 15 min and 11 cycles of 94 °C for 1 min, 63–58 °C for 30 s (decreasing 0.5 °C/cycle), 72 °C for 1 min, followed by 30 cycles of 94 °C for 1 min, 58 °C for 30, 72 °C for 1 min and finally 72 °C for 8 min.

Confirmation of PCR product quality was established on 2% agarose gels with ethidium bromide staining. For purification of up to 20 μl PCR products, the QIAquick PCR Purification Kit (Qiagen Ltd., Germantown, MD) was used.

Direct sequencing methylation analysis, data analysis

Using power read DNA sequencing service, primers and purified PCR products were sent to Eurofins MWG Operon Eurofins Genomics Company (Gilroy, CA). The methylation index (MtI) at each gene promoter, and for each sample, was calculated as the average value of mC/(mC + C) for all examined CpGs in the gene.

Statistical analysis

The results are presented as mean ± standard error of the mean. GraphPad (La Jolla, CA) ver. 6 was used to perform statistical analysis of data. Non-normally distributed variables were analyzed with the Mann–Whitney U test. p values < 0.05 are considered significant.

Results

Hormone profile

Table 1 displays a comparison of hormone levels between the control and the PNA adult female offspring. Overall, in the estrous cycle, testosterone and LH levels, and the LH/FSH ratio were significantly increased in the PNA adult female offspring, compared with control rats ($p < 0.05$); however, there were no significant changes in some phases.

Relative gene expression

The observed expression rate of the FST gene in the PNA group was 0.85 times ($p = 0.058$) less than the controls (Graph 1), although there was significantly higher expression in the metestrus (2.61-fold, $p = 0.007$) phases. Expression of FST had significant reductions in the proestrus and estrus phases, compared to controls (Graph 2).

Promoter methylation analysis

In comparison with controls, there was no significant change in methylation levels of 17 CpG sites in the promoter of the FST gene, being 55 ± 11.39 and 52.88 ± 7.66% for the PNA group and the controls, respectively (Graph 1).

Discussion

To determine the possible effects of prenatal exposure to excess androgen on developing adult PCOS, we investigated the expression of FST gene, as a component of the folliculogenesis pathway. Results showed that prenatal androgen excess causes alteration in expression of FST gene in different phases of estrus cycle.

While increased levels of serum FST in PCOS patients introduced the FST gene as a candidate gene of PCOS [20,21], the results of genetic association studies have been controversial [11,22,23]. Therefore, FST is still a possible target, the effect of which needs to be investigated on the development of this syndrome.

Previously, we reported alterations following an investigation of the effects of prenatal androgenization on expression of three genes involved in steroidogenesis of thecal cells [24], and hypothesized that FST expression, as an important local regulator of steroidogenesis in human ovaries, could also be altered. FST exerts its effect via enhancement of estradiol production in addition to neutralizing the effect of activin on steroid production [25].

In rats, as a non-primate species, levels of FST increase between the proestrus and estrus phases and return to proestrus values in the diestrus phase [26]. Another study investigating localization of FST in the rat tissues, using immunohistochemistry, showed that intensity of staining changed with follicle development, because no immunoreactivity was observed in all follicles (primordial to primary); however, secondary to graafian follicles displayed moderate to strong staining and finally luteal cells of the corpus luteum became negative [27]; indicating the importance of increase of FST expression in the development of pre-ovulatory follicles, it however decreases after ovulation to form corpus luteum.

In PCOS status, due to the presence of anovulation, follicles are typically arrested at preantral and antral stages [28]; since the diestrus is a dominant phase in the PCOS rats [29], our observation of sharply higher expression of FST in metestrus and diestrus phases, corresponds with results of the previous studies, indicating higher serum FST in PCOS patients [20,21].

Our results of a marginally significant decrease of FST gene expression in PCOS rats, resulting from prenatal exposure to excess androgen may be due to follicular arrest, as a key feature of PCOS, indicating that FST has a role in development of pre-ovulatory follicles and its decreased expression leads to follicular arrest in PCOS conditions.

Methylation of promoters is a possible mechanism in controlling gene expression [30]. Here we also examined the methylation level of 17 CpG sites in promoter of FST and found no significant changes, although the promoter methylation levels of PNA slightly increased compared to controls, which is consistent with the results of decrease in expression of FST gene in the PNA group.

To the best of our knowledge, this is the first study using a new rat PCOS model to compare FSTgene expression in different phases of the estrus cycle and also promoter methylation analysis. Time and budget constraints limited us from examining serum levels of FST, more genes or more CpG sites of the FST gene. Hence serum level measurement of FST, inhibin and activin levels, plus studying the expression of other genes related to the folliculogenesis pathway such as activin, inhibin, FSH and its receptor, methylation level of other CpG sites of FST gene is needed. Investigating the mechanisms of epigenetics regulation could also be beneficial to ascertain new genetic aspects of PCOS, the role of epigenetic mechanisms of its development and the effects of prenatal exposures.

In conclusion, it seems PCOS development, resulting from prenatal excess androgen exposure, interrupts the physiological mechanism of the folliculogenesis axis leading to irregularities in FST expression during the estrus cycle.

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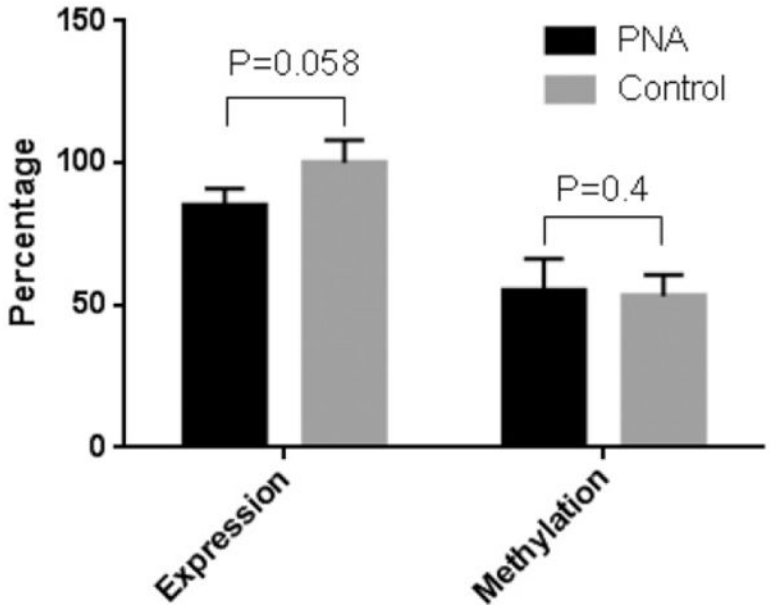
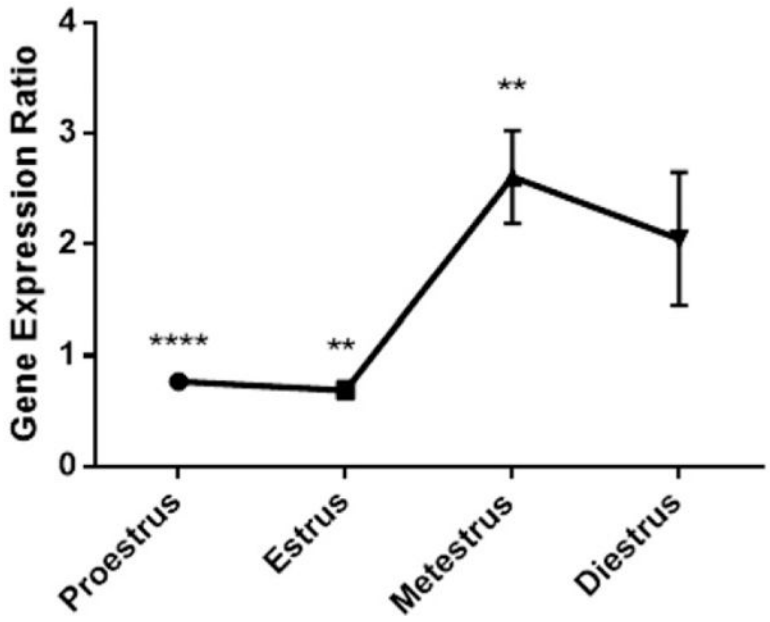


Graph 1.

Expression ratio and the promoter methylation percentage of FST gene in the PNA and control group. Data are presented as mean ± SEM.



Graph 2.

FST gene in different phases of estrus cycle. Data are presented as mean ± SEM, ** p values < 0.01, **** p values < 0.0001.

Table 1

Testosterone and gonadotropins levels in the studied groups.

Phase	Group	Testosterone ng/ml	LH ng/ml	FSH ng/ml	LH/FSH ng/ml
Proestrus	PNA ($n = 6$)	2.46 ± 0.16 *	1.54 ± 0.26	41.55 ± 2.8 †	0.036 ± 0.005 *
	Control ($n = 6$)	1.47 ± 0.55	1.43 ± 0.19	68.54 ± 8.2	0.023 ± 0.0035
Estrus	PNA ($n = 5$)	2.82 ± 0.33 *	1.77 ± 0.23	66.75 ± 6.5	0.027 ± 0.004
	Control ($n = 6$)	1.56 ± 0.32	1.11 ± 0.28	59.38 ± 9.03	0.024 ± 0.007
Metestrus	PNA (0 = 6)	2.73 ± 0.44	2.28 ± 0.75	42.41 ±7.4 †	0.056 ± 0.016 *
	Control ($n = 5$)	2.44 ± 0.3	0.98 ± 0.13	75.58 ± 5.5	0.013 ± 0.001
Diestrus	PNA ($n = 6$)	4.05 ± 1.09	2.0 ± 0.3 *	51.14 ± 8.4	0.045 ± 0.009 *
	Control ($n = 6$)	2.29 ± 0.2	1.03 ± 0.15	52.12 ± 10.5	0.021 ± 0.003
Total	PNA ($n = 23$)	3.02 ± 0.32 *	1.90 ± 0.22 ‡	50.04 ± 3.69 *	0.041 ± 0.05 ‡
	Control ($n = 23$)	2.00 ± 0.14	1.17 ± 0.10	63.96 ± 6.00	0.021 ± 0.00

Data are presented as mean ± SEM.

* p values < 0.05.

† p values < 0.01.

‡ p values < 0.001.