

CYP19A1 Promoters Activity in Human Granulosa Cells: A Comparison between PCOS and Normal Subjects

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Abstract

Objective: Estrogen, a female hormone maintaining several critical functions in women's physiology, e.g., folliculogenesis and fertility, is predominantly produced by ovarian granulosa cells where aromatase enzyme converts androgen to estrogen. The principal enzyme responsible for this catalytic reaction is encoded by the *CYP19A1* gene, with a long regulatory region. Abnormalities in this process cause metabolic disorders in women, one of the most common of which is polycystic ovary syndrome (PCOS). The main purpose of this research was to determine the effect of the promoters on aromatase expression in cells with normal and PCOS characteristics.

Materials and Methods: In this experimental study, four promoters of the *CYP19A1* gene, including PII, I.3, I.4, and PII/ I.3 promoter fragments, were cloned upstream of the luciferase gene and transfected into normal and PCOS granulosa cells. Subsequently, the effect of follicle-stimulating hormone (FSH) on the activity of these regulatory regions was examined in the presence and absence of FSH. Western blotting was used to confirm aromatase expression in all groups. Data analysis was performed using ANOVA and paired sample t test, compared by post-hoc least significant difference (LSD) test.

Results: Luciferase results confirmed the intense activity of PII promoter in the presence of FSH. Moreover, the study demonstrated reduced activity of PII promoter in normal granulosa cells, possibly due to the regulatory region of I.3 next to PII.

Conclusion: FSH stimulates transcription of aromatase enzyme by affecting PII promoter, a process regulated by the inhibitory role of the I.3 region in PII activity in granulosa cells. Given the distinct role of these promoters in normal and PCOS granulosa cells, the importance of nuclear factors residing in these regions can be discerned.

Keywords: Aromatase, Granulosa, Luciferase, Polycystic Ovary Syndrome, Promoter

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Introduction

Women's reproductive system is complex machinery comprised of components such as hormones and ovarian factors working in perfect harmony. The normal reproduction process requires timely adequate secretion of hormones to regulate the input of a hormone from the central nervous system, the pituitary gland, and the ovary. Any disturbances in this process lead to reproductive system disorders and subsequent infertility. Hormones are the main actors in the process of folliculogenesis and ovulation, and imbalances in the secretion of hormones disturb the folliculogenesis process (1-3). Polycystic ovary syndrome (PCOS) is a common endocrinopathy disorder accompanied by an increase in androgen levels, disturbed follicular maturation process, and absent ovulation (4).

Generally, hormones are the main actors in folliculogenesis and ovulation. Estrogen, one of these major hormones, is essential for regulating hemostasis and pathological pathways, especially infertility (2).

Follicles are the primary source of local and circulatory estrogen in mammals. Synthesis of follicular estradiol depends on the effective reaction between pituitary gonadotropin hormone, follicle-stimulating hormone (FSH), luteinizing effective hormone (LH), cytokines, and growth hormones, among which FSH is the leading causative agent governing estrogen synthesis. Regulation of *CYP19A1* gene transcription is conducted via binding of FSH to the relevant receptor on granulosa cells and subsequent production of cAMP, NR5A1, and sequential signals. This gene encodes the aromatase enzyme, which plays a crucial role in the irreversible conversion of androstenedione to estrogen (5-7). In a PCOS woman; however, decreased level of the aromatase enzyme leads to the reduction of estrogens hormone, which finally caused the increase in androgen hormone. Production of androgens blocks the feedback between the ovaries and the pituitary gland. Therefore, the pituitary generates high levels of LH, which further produce more androgens from

Theca cells of the follicle. High levels of androgens inhibit follicular growth, and the egg cannot grow. Therefore, ovulation will not occur, and the premature oocyte becomes a small cyst with a thin wall instead of being released from the ovary. This leads to the production of androgens and prevention from follicular growth in the following months. In other words, acceleration of primary follicular growth is mainly due to an excessive increase in androgens, which results in excessive growth of small follicles (8).

The enzyme aromatase is expressed periodically and specifically in the granulosa cells of the ovary and is essential for regulating folliculogenesis autocrine, endocrine control of reproduction in females, and coordination during gonadotropin secretion (7, 9). The wide regulatory area of the *CYP19A1* gene is about 93 kb and contains 11 discovered promoters. These promoters have been determined to regulate aromatase expression in various tissues in a tissue-specific manner (9-13). Various regulatory sequences of DNA, transcription factors, cytokines, and hormones bind to the aromatase promoter's regulatory regions in each cell type, resulting in the induction of aromatase expression in those cells (13). Among multiple promoters of *CYP19A1*, promoter II (PII) is actively involved in aromatase expression in granulosa cells (14-17). Besides, some studies indicated the presence of promoters I.3 and I.4 and promoter II for aromatase expression in ovarian cells (18, 19).

As mentioned, there are still ambiguities about the function of aromatase regulatory regions in granulosa cells and our aim was to clarify the most effective promoter of the *CYP19A1* gene in granulosa cells. In the present study, we examined the activity of PII, I.3, PII/I.3, and I.4 promoters in normal and PCOS granulosa cells via measurement of luciferase activity as a biosensor in the presence and absence of FSH. Moreover, we investigated aromatase expression in different groups of normal and PCOS granulosa cells.

Materials and Methods

Cell culture and morphological observation

Granulosa cells were obtained from samples of women with normal folliculogenesis and PCOS who had undergone assisted reproductive technology (ART) process. It

should be noted that the specimens were approved by the Embryology Department and Ethics Committee of Royan Institute (IR.ACECR.ROYAN.REC.1394.87). Primary culture and immortalization of normal granulosa cells (GCN) and PCOS granulosa cells (GCP) were performed in collaboration with the Iranian biological resource center and the Genetics Department of Royan Institute. GCN-01 and GCP-01 cell lines were banked at the Iranian biological resource center. GCP-01 cells were cultured in DMEM/Ham's F-12 medium supplemented with 17.5% fetal bovine serum (FBS, Gibco, US), 2.5% horse Serum, 4mM L-Glutamine (Gibco, US), 2 mM non-essential amino acids (Sigma, US), 100 IU/mL penicillin, 100 mg/mL streptomycin (Gibco, US), 100 ng/mL recombinant FSH (rFSH, Gonol-F, Merck, France), 100 ng/mL bFGF (Royan, Iran), 25 ng/mL epidermal growth factor (EGF, Royan, Iran), 0.5 µg/mL hydrocortisone (Merck, France), 50 µg/mL ascorbic acid (Sigma, US), 100 ng/mL cholera toxin (Sigma, US), and 1X insulin-transferrin-selenium (Gibco, US). In addition, GCN-01 cells were cultured in a DMEM/Ham's F-12 medium Supplemented as mentioned above but lacked horse Serum and rFSH (20).

GCP-01 and GCN01 were seeded in 6-well plates and treated with or without 100 ng/mL rFSH. Subsequently, the morphological features of GCP-01 and GCN-01 were examined under an optical microscope.

Promoter constructs

PII, I.3, PII/I.3, and I.4 promoters of CYP19 were amplified by polymerase chain reaction (PCR) from human genomic DNA in a reaction mixture of 120 µL containing 100 ng genomic DNA, Mastermix (Ampliqon, Denmark), and primers. Primers are listed in Table 1. After amplification, PCR products were purified by High Pure PCR Product Purification Kit (Roche). Next, fragments were digested using SacI and XhoI restriction enzymes (Fermentas) and subcloned into a pGL4-26 vector (Promega, Madison, WI, USA), which carries firefly luciferase as its reporter gene. In the first step, the presence of each promoter fragment in the vector was confirmed using colony PCR and double digestion by SacI and XhoI enzymes. In the end, the inserts were sequenced to ensure fidelity of the amplified sequences.

Table 1: Primers used for promoter constructs in the present study

Insert	Primer sequence (5'-3')	Annealing temperature (°C)	PCR product (bp)
I.3	F: ACTGGAGCTCTCAACGATGCCCAAG 3' R: ATCGCTCGAGAACAAGGAAGCCCAAG 3'	57	390
PII	F: ACTGGAGCTCCCTTGTGTTTACTTG 3' R: ATCGCTCGAGGACATAGTCTTCAGAG 3'	57	490
I.4	F: ACTGGAGCTCGAGAATGGGAATGTTG 3' R: ATCGCTCGAGTACCTGGTTTGATGG 3'	58	925
PII/I.3	F: ACTGGAGCTCTCAACGATGCCCAAG 3' R: ATCGCTCGAGGACATAGTCTTCAGAG 3'	57	835

F; Forward Primer (SacI restriction site: 5'-GAGCTC-3'), R; Reverser Primer (XhoI restriction site: 5'-CTCGAG-3'), and PCR; Polymerase chain reaction.

Transfection

GCN and GCP were grown in 12-well plates at 60–80% confluency, and transient transfection was carried out using the Lipofectamine 3000 kit (Invitrogen, US) according to the manufacturer's instructions. Cells were transfected with 1 mg of each pGL4-26/CYP19 promoter. The transfection experiments were performed in triplicate for each construct. After transfection, GCN and GCP were incubated in DMEM without FBS for 8 hours, and subsequently, the medium was replaced with complete media, with or without 100 ng/mL r-FSH (Gonal-f). After treatments, cells were incubated for 48 hours and observed for the firefly luciferase activities in the cell lysates.

Luciferase assay

Cells were washed with PBS and lysed in 30 μ L Cell Lysis Reagent (Promega, UK, Southampton, United Kingdom). Cell lysates were harvested, and spun for 15 minutes. The cell lysates (20 μ L) were added to 20 μ L of luciferin complex (Promega, UK, Southampton, United Kingdom). Luciferase bioluminescence measurements were performed at room temperature using a luminometer (Sirius tube Luminometer, Berthold Detection System, Germany). The activity was expressed as relative light units (RLU) emitted from total assays versus background activity. In addition, protein concentrations were measured by the Bradford method, and firefly luciferase activities were normalized against the total protein.

Western blot analysis

Total protein was extracted from all groups of transfected GCN and GCP. Protein concentration was determined using the Bradford assay. Here, 30 μ g of each sample protein was separated by SDS-PAGE and electroblotted onto Polyvinylidene fluoride (PVDF) membranes. The membranes were blocked using 5% non-fat skim milk in Tris-buffered saline containing Tween 20 and subsequently incubated overnight at 4°C with anti-aromatase (MCA2077S, Bio-Rad, US), anti- β actin (sc-47778, Santa Cruz, US). The membranes were then incubated for 60 minutes at room temperature with mouse secondary antibodies conjugated to horseradish peroxidase (STAR133, Bio-Rad, and DB9571, Kalazist, Iran). After washing with TBST for 15 minutes once and 5 minutes thrice, ECL reagent (BIO-RAD, Hercules, CA) was added, and the membranes were exposed to X-ray film (GE Healthcare, Cambridge, UK).

Statistical analysis

Statistical analyses were performed using the IBM SPSS statistic 21 software (IBM Corp., Armonk, NY). The ANOVA test determined significant differences between groups, while paired sample t test was used to compare the normal and PCOS subgroups. Also, significant differences between the subgroups were measured by the LSD test. A $P < 0.05$ was considered significant.

Results

Medium changes indicative of changes in cellular secretion after FSH treatment

Both GCN and GCP (normal and PCOS granulosa cells, respectively) were cultured in two groups for 6 hours at a density of 5×10^5 cells per well. One group of each normal or PCOS cells was treated with FSH. After 48 hours, without changing the medium, cellular morphology and alterations in the culture medium were precisely monitored by light microscopy. Steroids secretion by normal cells was significantly higher in the presence of FSH. These secretory changes were observed after treatment with FSH in the presence of secretory vacuoles in cells and lipid-like droplets suspended in cell culture medium. Also, secretion of the PCOS cells increased after FSH treatment, but this elevation was lower than that observed for normal cells (Fig. 1).

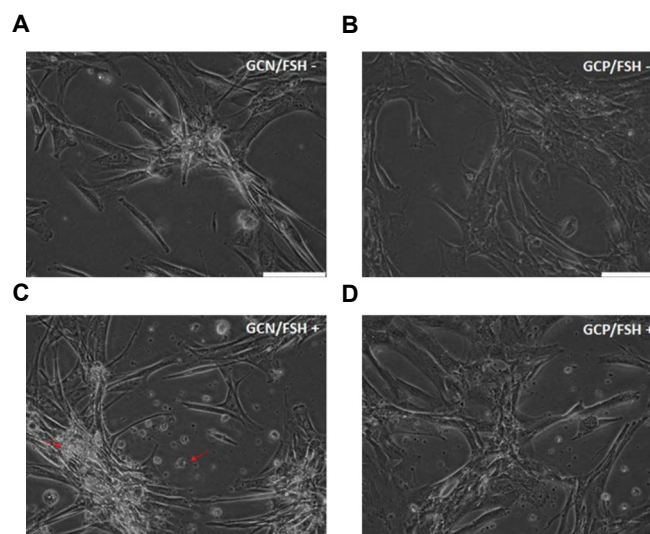


Fig. 1: FSH affects the morphology and culture medium of GCN and GCP. **A.** GCN was grown without FSH and **B.** With FSH. **C.** GCP was grown without FSH and **D.** With FSH (scale bar: 100 μ m). Red arrows indicate the lipid-like droplets. GCN; Normal granulosa cells, GCP; PCOS granulosa cells, PCOS; Polycystic ovary syndrome, and FSH; Follicle-stimulating hormone.

Functional analysis of the promoter regions in normal and PCOS cells

The mentioned promoters were cloned upstream of the luciferase reporter gene of the pGL4-26 plasmid. These were transiently transferred to the normal and PCOS granulosa cells in different groups. The four designed promoter constructs included PII, I.3, PII-I.3, and PI.4. Luciferase assays were performed on cultures maintained with or without FSH. The luciferase assay results after normalization against total protein showed that in both cells, the promoter PII had a significant role in luciferase expression as a 2.3-fold increase was observed in normal cells and a 2.2-fold increase in PCOS cells. However, the PII-I.3 promoter fragment has a different pattern in luciferase expression in normal and PCOS cells. A 1.7-fold increase compared to the control and a 0.3-fold

decrease compared to the promoter PII alone were observed in normal cells. In addition, a 0.5-fold decrease compared to the control and a 0.8-fold decrease compared to the promoter PII alone were detected in PCOS cells. Increased activity of the promoter I.3 in the normal cell was not significant. In contrast, the reduction in its activity in the PCOS group was significantly larger than that in the normal group and the control subgroup. Also, FSH removal in this subgroup did not show any significant changes in promoter activity. Promoter I.4 activity decreased in both normal and PCOS groups; however, this decrease reached a significant level only in the normal group. Promoter I.4 exhibited similar behavior in the presence or absence of FSH (Fig.2).

Evaluation of aromatase and β actin gene expression

Cell preparation protocol for protein extraction was similar

to that described for luciferase assay. Total proteins were extracted from all cell groups. Expression of β actin protein as internal control and aromatase were determined to assess changes in expression induced by vector transfection and FSH treatment using western blotting. Subsequently, the density of the western blot bands was evaluated by ImageJ software. The aromatase expression and luciferase assay were normalized against β actin expression in each group. After analysis of the results, it was found that transfection with the promoter fragments caused no significant change in protein expression. However, FSH treatment increased by 2.3-fold in normal cells and 2.1-fold in PCOS cells. It should be noted that comparing between before- and after-FSH-treatment values showed that aromatase expression in normal cells was 1.5 times more than in PCOS cells (Fig.3).

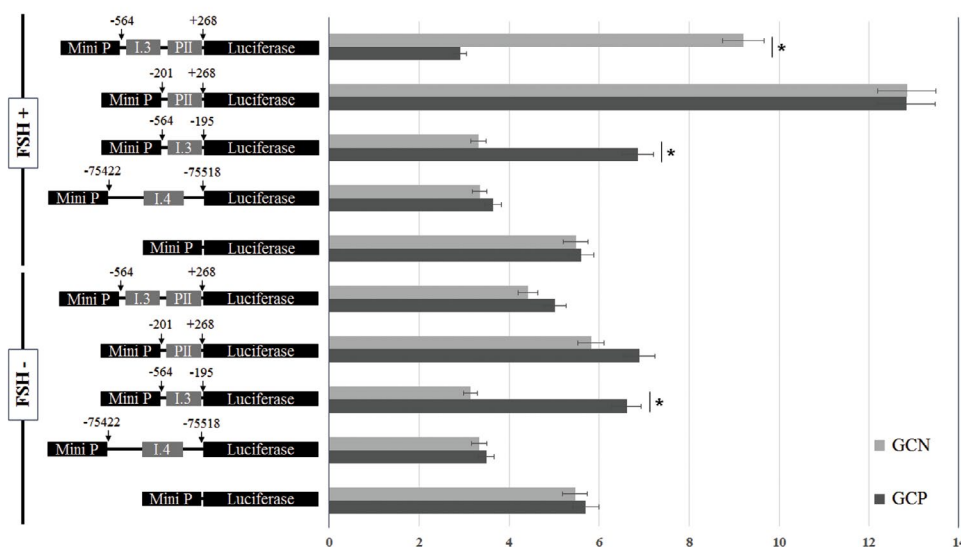


Fig.2: Luciferase assays of several CYP19 promoters constructs under FSH treatment. Relative luciferase activities (right) measured in GCN and GCP (normal and PCOS granulosa cells, respectively) transfected with one pGL4-26-CYP19 construct (PII/I.3, PII, I.3 and I.4; left) were treated with or without FSH. Transfection experiments were replicated three times for each construct. *: Significant differences in activity construct between GCN and GCP (paired sample t test, P<0.05), GCN; Normal granulosa cells, GCP; PCOS granulosa cells, PCOS; Polycystic ovary syndrome, and FSH; Follicle-stimulating hormone.

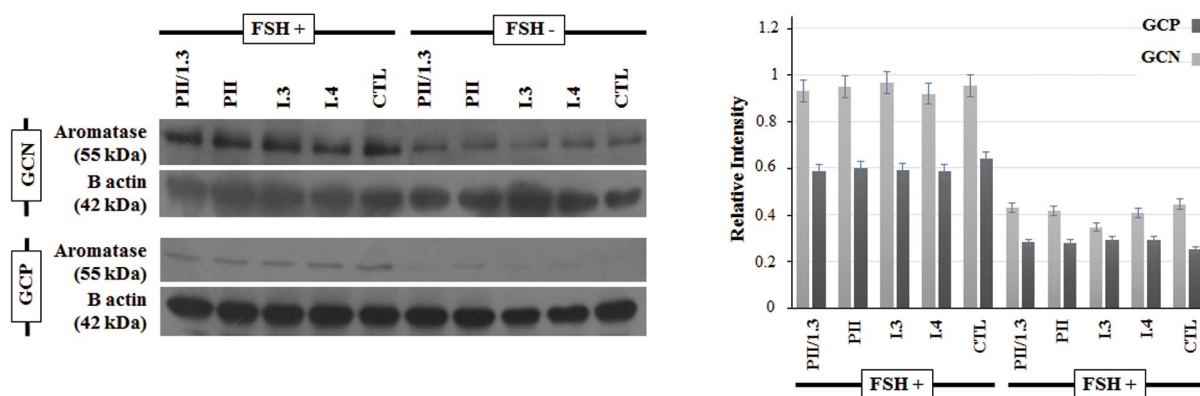


Fig.3: Western blot analysis of aromatase protein expression. **A.** Aromatase expression was investigated in the transfected and treated GCN and GCP. β -actin was used as a loading control. **B.** Histogram for aromatase expression based on western blot results. GCN; Normal granulosa cells, GCP; PCOS granulosa cells, PCOS; Polycystic ovary syndrome, and FSH; Follicle-stimulating hormone.

Discussion

In this study, we showed that in the presence of FSH, the PII promoter is the main promoter that influences ovarian aromatase gene expression; however, the PII promoter activity was affected by promoter region I.3. We also found that the effect of region I.3 on the PII promoter was not similar in the normal and PCOS cells.

According to the previous studies, we investigated 4 promoter regions, PII (-201/+268), PI.3 (-564/-195), PII/I.3 (-564/+268), and I.4 (-75422/-75518), among 11 promoters of the *CYP19A1* gene. Investigation of the role of conserved regulatory sequences in these promoter regions can help to discuss the results.

The PII promoter is coded as the closest promoter to the coding region. This promoter is naturally the main promoter of the ovary, which participates in aromatase expression in seminal vesicles, bladder, testis, and prostate tissues. It was also shown to induce unusual aromatase expression in breast cancer, endometriosis, hepatocellular carcinoma, adrenocortical tumors, and Sertoli and PI.3 to each other and to the coding region, these were considered the promoter region in some studies (21, 22). Therefore, it is considered a relatively strong promoter (8-12). The I.3 promoter is located approximately 200 bp upstream of the PII promoter and acts as a *CYP19A1* promoter in breast cancer, adipose tissue, seminal, and blood vessels (8, 9). Due to the proximity of the promoters PII and PI.3 to each other and to the coding region, these were considered the promoter region in some studies (21, 22).

Nevertheless, 1kb upstream of exon II, the three promoter regions PII, I.3, and I.6 are located (23, 24). So far, several conserved regulatory sequences have been identified in this area, indicating the critical role of this region in aromatase gene expression. The most important segments of these regions are the TATA box (-26 to -31), a putative forkhead element (-69 to -82), a conserved region that responds to GATA transcription factors (-165 to -179), and an uncharacterized putative SFRE (-184 to -193) (25-27). The cis-regulatory elements steroidogenic factor-1 (SF-1) and CRE-like sequence (CLS) are located upstream of the PII promoter. CLS responds to both cAMP as an activator and Jun proteins as inhibitors of *CYP11A1* gene expression (28). Other regulatory factors that can be mentioned as the C/EBP conservation sequence are located upstream of SF-1 and CLS (17, 29). There are two conserved sequences for NR5A1/NR5A2 and FOXL2 that bind to the forkhead box and act as a transcription factor. These elements are involved in granulosa cells proliferation, steroid hormones synthesis, and ovary apoptosis regulation. Increased levels of NR5A2, along with forskolin, induce the activity of *CYP19A1* and FOXL2 as aromatase inhibitors in the ovary (30). In granulosa cells, cis-elements located upstream of the PII promoter respond to several nuclear receptors, including NURR1 and NGFI-B, that suppress aromatase expression (29). A forkhead element in -516 responds to the mutant FOXL2 (C134W) in granulosa cell tumors, which in turn

induces the expression of aromatase in these cells (24). Previous studies investigated the role of some regulatory elements in this area in other cells, which may have a role in ovarian cells, however, they were neglected. For instance, upstream of -211, there are several repressor elements that can inhibit the activity of the PII promoter in MG-63 cells (31). In addition, in upstream of the PII promoter, there are a number of conserved sequences with a silencing role in breast cancer cells. These areas respond to Gata 4 and perhaps other factors but their role is not so far elucidated (27).

The promoter I.4 is located approximately 73 kb upstream of exon II and has been implicated in aromatase expression as the primary promoter in adipose tissue, skin, seminal vesicles, bladder, placenta, bone, and blood vessels (8, 9, 32). Several regulatory sequences in this region are involved in aromatase expression in different tissue cells. For example, glucocorticoid responsive factors in normal breast cells and ROR alpha regulatory factors in breast cancer cells are known as regulatory factors in this area (33-36). However, the effect of the promoter I.4 on the protein expression of the *CYP19A1* gene has not been established in ovarian cells; only in one case, exon I.4 in aromatase transcripts was reported in granulosa cells (18).

Considering the present work results and data reported by previous studies, it can be concluded that the PII promoter is stimulated in the presence of FSH, which leads to aromatase protein expression. Also, inhibitory factors in promoter I.3, which is located upstream of the PII promoter, control the PII promoter and prevent aromatase overexpression resulting in the reduction of the activity of the PII promoter. These results indicated that promoter I.3 acts as enhancer elements (27, 31). However, in the granulosa cells of PCOS individuals, the inhibitory effect of the promoter I.3 is sufficient to inhibit PII promoter activity. Furthermore, aromatase is not sufficiently expressed in these cells leading to PCOS symptoms such as increased androgen and decreased estrogen. Taken together, the data indicate the critical role of the promoter I.3 in the regulation of aromatase gene expression, which has received less attention so far.

This study is a preliminary step to discover how promoter regions are involved in aromatase expression in the normal and PCOS granulosa cells. Therefore, it is likely that nuclear elements interact with the PI.3 region and affect aromatase expression in the normal and PCOS granulosa cells. Further studies are needed to understand the mechanisms of transcriptional regulation in the PII region, in particular I.3, to recognize the nuclear factors that are involved.

In brief, we found that FSH has stimulatory effects on the PII promoter, and a functional relationship between the promoter region I.3 and the PII promoter exists in normal granulosa cells and PCOS. We found that the I.4 promoter was not involved in the expression of aromatase protein, neither in normal nor in PCOS granulosa cells.

These results could help discover the complexity of CYP19 expression in ovarian cells, especially in PCOS granulosa cells.

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Author's Contributions

Z.H., P.A., S.H., M.Sh.; Contributed to conception and design. Z.H.; Contributed to all experimental work, data and statistical analysis, and interpretation of data. P.A., S.H.; Were responsible for overall supervision. A.A.-Y.; Contributed to concept and design of molecular part. M.K., F.A.; Cooperated with some experimental tests. Z.H.; Drafted the manuscript, which was revised by P.A., A.A.-Y. All authors read and approved the final manuscript.

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