

FULL LENGTH ARTICLE

SRSFs mediate the function of AR in the ovarian granulosa cells of patients with PCOS

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Abstract Ovarian hyperandrogenism is one of the characteristics of polycystic ovary syndrome (PCOS) and androgen receptor (AR) in ovarian granulosa cells (GCs) functions as an important element to the accumulation of androgens. This study verified the existence of alternative splicing variant of AR (AR-SVs) in the GCs of PCOS patients and found that the function of AR decreased significantly in the presence of AR-SVs. And compared to the normal individuals, the expression of Serine/arginine-rich splicing factor 2 (SRSF2) was higher and the expression of SRSF3 was lower in the GCs of patients with AR-SVs. More importantly, we found that the expression of SRSF2 was inhibited and that the expression of AR was decreased after the successful upregulation of miRNA-183, and testosterone (T) concentrations in the culture medium were increased. The results also showed that the expression of SRSF3 decreased when miRNA-124 was successfully upregulated, while the expression of AR significantly increased; however, the function of AR was also inhibited when T concentration in the culture medium was increased. This study has proved that SRSFs are regulated by corresponding miRNAs, and the altered expression of SRSFs interfered the alternative splicing process of AR and ultimately decreased the function of AR, leading to the accumulation of androgens in the ovary.

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Introduction

Polycystic ovary syndrome (PCOS) has an incidence of 4–18% among adolescent and reproductive women,¹ but its etiology is unknown. As a characteristic manifestation of PCOS, hyperandrogenism affects fertility in women.² Many scholars believe that the generation of PCOS-associated hyperandrogenism is closely related to the function of AR in GCs.^{3,4} Disorders in AR function can cause the accumulation of androgen in follicles.^{5,6} As a transcription factor, AR regulates the expression of various aromatase.⁷ Additionally, the expression of AR is crucial for the process of transformation from androgen to estrogen.⁸ However, high expression levels of AR have been detected in patients with PCOS, but the factor that promotes the elevated expression of AR is unknown, and this elevated AR expression may also be the result of a synergistic effect of multiple factors.⁹ Previously, Wang F et al found an alternative splicing variant of AR (AR-SV) in the GCs of PCOS patients with high androgen levels and identified two types of AR-SVs¹⁰: one type has a 69-bp insertion between exons 2 and 3, and the other type results from the loss of exon 3. The authors also proved that the production of AR-SV influenced the biological effect of AR. Therefore, an in-depth study of the generation and regulation mechanism of AR-SVs is merited to clarify the occurrence of hyperandrogenism.

Serine/arginine splicing factors (SRSFs) belong to a group of serine/arginine rich protein family that is directly involved in the alternative splicing process and play an important role in regulating the production of splicing variants. Studies have shown that SRSF1 (SF2/ASF) is likely to be affected by the extension rate of RNA polymerase II and results in the insertion or loss of exons.¹¹ In a study of prostate cancer, it was found that altered levels of SRSFs had a significant impact on the regulation of AR-SV production.¹² Skrdlant et al found that the mutation of SRSF2 (SC35) in myelodysplastic syndrome leads to the emergence of the splicing variant 5C of CDC25C during cell division.¹³ SRSF1 and SRSF2 can promote the binding of the small nuclear ribonucleoprotein (snRNP) U1 to the 5'-splice site and the snRNP U2 to the 3'-splice site, and these proteins also play an important role in the identification of the initial splicing site in the initial splicing body and mature splicing body.¹⁴ Changes in the expression level of SRSFs lead to changes in the expression of corresponding target genes. For example, the upregulation of SRSF1 increases the expression level of Vpr RNA, while the upregulation of SRSF2 increases the expression of Tat RNA, thereby inhibiting the transcriptional activity of genes.¹⁵ Different SRSFs can compete with one another to select their splicing sites and produce different splicing effects^{16–18}; for example, SRSF1 and SRSF2 compete to regulate the splicing of α -tropomyosin.¹⁹ SRSF3 plays a role as a splicing silencer, and direct evidence shows that SRSF3 is related to 83 genetic diseases, resulting in 67 disease-related gene mutations.²⁰ In recent years, some scholars have found that SRSF10 is involved in the regulation of five basic types of splicing events, among which the regulation of "cassette" exon access or removal events is predominant. SRSF3 and SRSF10 have also been indicated to directly participate in the regulation of alternative splicing of genes, and SRSF3 was

shown to have a tendency to promote exon retention, while SRSF10 was shown to have a tendency to promote exon shearing.^{21,22} Considering the interaction between SRSFs, we selected four SRSFs, namely, SRSF1, SRSF2, SRSF3 and SRSF10, for investigation in this study.

Posttranscriptional regulation of microRNAs (miRNAs) greatly influences ovarian function in women.²³ The stable expression of miRNA target genes plays an important role in the regulation of reproductive endocrine functions by transcriptional inhibition or degradation, especially in the process of oocyte maturation and follicle formation.²⁴ The expression of miRNAs in GCs may be directly involved in the regulation of follicle formation and the synthesis of steroid hormones.²⁵ MiRNA-183 was found to be downregulated in dihydrotestosterone (DHT)-induced PCOS in rats, and the downregulation of miRNA-183 was suggested to be related to the increase in androgens.²⁶ In another study, 80 miRNA precursors were transfected into ovarian GCs in primary culture. MiRNA-7 promoted a significant increase in the proliferation marker PCNA. Similarly, miRNA-29c and miRNA-183 dually promoted the accumulation of the pro-apoptotic marker Bax.^{27,28} In another study, the expression of miRNA-29c promoted cell division and proliferation.²⁹ Until now, studies have identified many miRNAs that are related to hyperandrogenism, but the target and regulatory mechanism of miRNAs remain unclear. In this study, six miRNAs, namely, miRNA-7, miRNA-10a, miRNA-505, miRNA-183, miRNA-124 and miRNA-29c, were screened through the prediction of target genes. The related target genes of miRNA-7, miRNA-10a and miRNA-505 were SRSF1, the related target gene of miRNA-183 was SRSF2, the related target gene of miRNA-124 was SRSF3, and the related gene of miRNA-29c was SRSF10.

The purpose of this study was to verify the importance of AR in the formation of hyperandrogenism, search for possible regulatory factors for the production of AR-SVs and promote a deeper understanding of the pathogenesis of hyperandrogenism in PCOS. The study of SRSFs and related miRNAs is conducive to finding possible means to regulate, reduce or inhibit the production of AR-SVs and to restore the conversion from androgens to estrogen and block the accumulation of androgens. This study also aimed to provide a new marker for the diagnosis of PCOS-related hyperandrogenism and open up new avenues for the treatment of PCOS-related hyperandrogenism.

Materials and methods

Patients

This study was approved by the Institutional Ethics Committee of the Second Affiliated Hospital of Chongqing Medical University and Chongqing Health Center for Women and Children, and written informed consent was obtained from all patients. The follicular fluid collected in this experiment did not contain the oocyte-cumulus-corona cell complex. The inclusion and exclusion criteria were strictly followed. A total of 71 follicular fluid samples were collected from patients with PCOS and women without PCOS undergoing *in vitro* fertilization (IVF) treatment at

the Center for Reproductive Medicine from Jun 2017 to Jan 2018.

After screening according to the exclusion criteria, 36 patients with PCOS and 35 control individuals were finally included in the present study. The inclusion and exclusion criteria were strictly set up, and the inclusion criteria of the control group (con) were as follows: (1) 25–45 female with regularly cycling menstrual period; (2) non-ovarian factor infertility (male or tubal factor infertility); (3) normal ovarian morphology by ultrasound; (4) the sex hormones at normal levels at the third day of the menstrual cycle; (5) the concentration of AMH is normal (0.24–11.78 ng/ml); (6) adopting long protocol controlled ovarian hyperstimulation (Triptorelin Acetate combined with Recombinant Human Follicle-stimulating Hormone). Each patient with PCOS met diagnostic criteria for PCOS based on the revised 2003 Rotterdam ESHRE/ASRM consensus criteria and excluded the related disorders. The inclusion criteria was also included: (1) 25–45 female; (2) adopting long protocol controlled ovarian hyperstimulation (Triptorelin Acetate combined with Recombinant Human Follicle-stimulating Hormone). Patients with a previous history of ovarian cancer, unexplained infertility, using immunomodulators and cytotoxic drugs within 6 months, or tubal obstruction or pelvic adhesions caused by endometriosis were excluded. The form contained basic information and hormone level of each participant was designed and filled out on the day before egg retrieval.

Follicular fluid collection and isolation of GCs

Follicular fluid containing primary GCs was centrifuged (2000 r at 4 °C for 10 min). Ten milliliters of supernatant was reserved in a sterile enzyme-free centrifuge tube. After labeling the name and the collection date on the tubes, the tubes were stored at -80 °C. The pelleted cells were resuspended in 1 ml PBS, and 2 ml 50% Percoll was added to purify the cells. After 25 min of centrifugation at 2500 r, the interphase was extracted and centrifuged (1500 r, 10 min, at room temperature). The sediment was regarded as the granulosa cell mass.

RNA isolation and RT-PCR analysis

Total RNA (including miRNA) was extracted using a total RNA Extraction kit (QIAGEN, Hilden, Germany). Mixtures with reverse transcriptase (including reverse transcriptase primers) and RT-PCR detection reagents (including primer probes) (QIAGEN, Hilden, Germany) were designed specific to the six target miRNAs. All operations were strictly performed according to the instruction manual. AR and SRSFs were detected using reverse transcriptase and SYBR Premix Ex Taq II (Tli RNaseH Plus). Both kits were purchased from Takara company (Japan). PCR was performed in a Bio-Rad PCR detector (California, USA).

The primer sequence for RT-PCR: AR-Forward: 5'-GTGGAAGCTGCAAGGTCTTC-3'; AR-Reverse: 5'-GGAGCTTG GTGAGCTGGTAG-3'; SRSF1-Forward: 5'-ATCTCATGAGGGA-GAAACTGCC-3'; SRSF1-Reverse: 5'-GCGAGATCTGCTATGAC GGG-3'; SRSF2-Forward: 5'-CCITCGTTCGCTTTCACGAC-3'; SRSF2-Reverse: 5'-GAGATCGAGAACGAGTGCGG-3'; SRSF3-

Forward: 5'-CACTATGTGGCTGCCGTGTA-3'; SRSF3-Reverse: 5'-ATCGGGACGGCTTGTGATTT-3'; SRSF10-Forward: 5'-AGT GGATTTGTGGACGGCAG-3'; SRSF10-Reverse: 5'-GAATGGCT TCTGCTACGCCG-3'; GAPDH- Forward 5'-GGATTTGGTCG-TATTGGCG-3'; GAPDH- Reverse 5'-CTTCCCGTTCTCAGCC TTGA-3'.

Sequencing

The primer design included the exons 2–4; and thus the primers were designed in exon 1 and exon 4. Primer5.0 was used as the primer design software, and primer specificity was tested in NCBI. The amplification products were sent to China Biological Engineering Company for sequencing after verifying successful amplification using standard OCR.

PremiRNA transfection

The human ovarian granular cell line COV434 (Sigma, Germany) was used to construct six miRNA precursor plasmids (Magen, Shanghai, China) after searching the miRBase Accession query number. Entranster™-H4000 (Entranster, Beijing, China) was used as the experimental reagent. The cells were transfected according to the operation manual, and the cell state was observed at 6 h, 24 h and 48 h after transfection. Transfection efficiency was determined by inverted fluorescence microscopy (Olympus, Japan), and cell groups with more than 80% transfection efficiency were chosen as the experimental subjects.

ELISA

The androstenedione (A) ELISA kit (KA1898), testosterone (T) (human) ELISA Kit (KA0236), estrone ELISA Kit (KA1908) and estradiol (E2) (human) ELISA Kit (KA0234) were all from Abnova. The assay was carried out according to the instructions of different kits. The dilution factor for the follicular fluid was 50 for A, 50 for T, 50 for E and 200 for E2. The culture supernatant of transfected cells was not diluted.

Western blotting analysis

Cells were lysed using RIPA Lysis Buffer (Thermo Scientific™, New York, USA). The full set of electrophoretic instruments was from Bio-Rad (USA). Anti-AR antibody (1:1000, ab133273, Abcam, Cambridge, UK), anti-β-tubulin antibody (1:5000, ab15568) and goat polyclonal secondary antibody to rabbit IgG (HRP-conjugated) (1:5000, ab6721) were used.

Statistical analysis

The clinical parameters are expressed as the means ± SD. Data analyses of all the experiments were performed using SPSS (version 13.0; SPSS Inc). Statistical comparisons between the control (CON) group and the PCOS group were performed with a nonparametric Mann-Whitney U test. The normal distribution of hormone level in the follicular fluid was examined, and an upper limit of 95% confidence

interval was obtained. The PCOS group was divided into two new groups: $T < \text{upper limit}$ was used to determine the low expression group (PCOS-L), and $T > \text{upper limit}$ was used to determine the high expression group (PCOS-H). Univariate analysis of the correlation between the expression of each miRNA and AR was determined by nonparametric Spearman's test. All results were considered statistically significant at $P < 0.05$.

Results

Parameter analysis of clinical samples

A total of 71 samples were included in this study according to the inclusion and exclusion criteria: 35 patients with tubal or male factor infertility were included in the CON group, while 36 patients who met the Rotterdam criteria were included in the PCOS group. There were no significant differences in age or dominant follicle number between the two groups, and the sizes of the included follicles were all greater than or equal to 15 mm. However, body mass index (BMI) and anti-Müllerian hormone (AMH) levels were significantly higher in the PCOS group than in the CON group ($P < 0.05$). The levels of serum luteinizing hormone (LH) and follicle-stimulating hormone (FSH) were changed in the PCOS group on the third day of menstrual cycles. Additionally, the concentration of LH in the PCOS group was higher ($P < 0.05$), the concentration of FSH was lower ($P < 0.05$), and the ratio of LH/FSH was obviously increased ($P < 0.05$) relative to the control (Table 1). The proportion of overweight and obesity patients in PCOS group was higher. The decline of ovarian function and the changes of gonadotropin in these patients indicated that the hypothalamic-pituitary-ovarian axis was changed.

Hormone status of the follicular fluid in the control and PCOS groups

The concentration of androgens (T and A) and estrogens (E and E2) in follicular fluid was determined by ELISA. The concentrations of T and A in follicular fluid were higher in the PCOS group than in the CON group ($P < 0.05$), while the

ratios of E/A and E2/T were lower in the PCOS group than in the CON group ($P < 0.05$) (Fig. 1). We further verified the normal distribution of T and A in the CON group (Fig. 2) and calculated the upper limit of the corresponding 95% confidence intervals. We determined 25.99 ng/ml as the upper limit of A and 28.04 ng/ml as the upper limit of T. Patients with higher A or T levels in follicular fluid were included in the PCOS-H group, and patients with lower A or T levels were included in the PCOS-L group. According to the pie chart analysis, 30 patients with higher levels of T or A accounted for 83.33% of patients with PCOS. Among them, 5 patients had elevated T levels alone, and only one patient had elevated A levels alone (Table 2), accounting for 16.67% and 3.33%, respectively, of the PCOS-H group (Fig. 3). All patients with higher androgens were included in the PCOS-H group. The proportion of patients with elevated androgen levels was higher, and androgen levels in follicular micro-environment were possibly elevated in serologically negative patients.

Expression of AR mRNA and AR-SV in human ovarian GCs

AR mRNA expression in the ovarian GCs of patients in the PCOS-H group was significantly higher than that of individuals in the CON group and the PCOS-L group ($P < 0.05$) (Fig. 4). All 71 cases were sequenced; although AR-SVs were not found in the CON group, they were found in 9 PCOS cases (including P1, P2, P10, P16, P17, P20, P24, P28 and P33), and the expression rate of AR-SVs in patients with PCOS was 27.27%. Additionally, the size difference of the two fragments is approximately 70 bp (Fig. 5). The expression of AR in PCOS-H patients were increased correspondingly and the AR-SV was present in ovarian granulosa cells of patients with hyperandrogen.

Relationship between AR-SV and hormone levels and AR mRNA expression levels

We split the subjects into two groups according to the type of AR expression. Subjects in the AR-FL group only expressed full-length AR mRNA, while subjects in the AR-SV group also expressed splice variants of AR mRNA. Furthermore, the concentrations of androgens T and A in the follicular fluid were higher in both the AR-SV and the AR-FL groups than in the CON group ($P < 0.05$), but the concentrations of T and A were higher in patients in the AR-SV group than in patients in the AR-FL group ($P < 0.05$). Consistent with these results, the E2/T and E/A ratios in the AR-SV and AR-FL groups were lower than those in the CON group ($P < 0.05$), and both ratios were significantly lower in the AR-SV group than in the AR-FL group ($P < 0.05$) (Fig. 6).

In addition, AR mRNA expression was significantly higher in the AR-SV group than in the CON group ($P < 0.05$), but there was no significant difference compared with the AR-FL group ($P > 0.05$) (Fig. 7). The existence of AR-SV was probably the reason for the high level of AR but the weakened function of AR.

Table 1 Clinical Parameters of con and PCOS group.

	con	PCOS
Age, years	29.00 ± 3.62	28.67 ± 3.39
BMI, kg/m ²	21.68 ± 2.60	24.06 ± 2.31*
AMH in serum, ng/L	3.99 ± 1.01	5.89 ± 2.12*
Antral follicle count	14.19 ± 3.61	14.56 ± 2.34
Day 3 LH in serum	2.36 ± 3.19	3.75 ± 2.10*
Day 3 FSH in serum	2.66 ± 1.25	1.96 ± 1.90*
Day 3 LH/FSH in serum	0.84 ± 0.98	1.89 ± 0.56*

BMI, body mass index; AMH, anti-Müllerian hormone; Antral follicle count, the size of the follicle > 15 mm; FSH, follicle stimulating hormone; LH, luteinizing hormone; Day 3, the third day of the menstrual cycle; Data are presented as mean ± SD. * $P < 0.05$ versus control group.

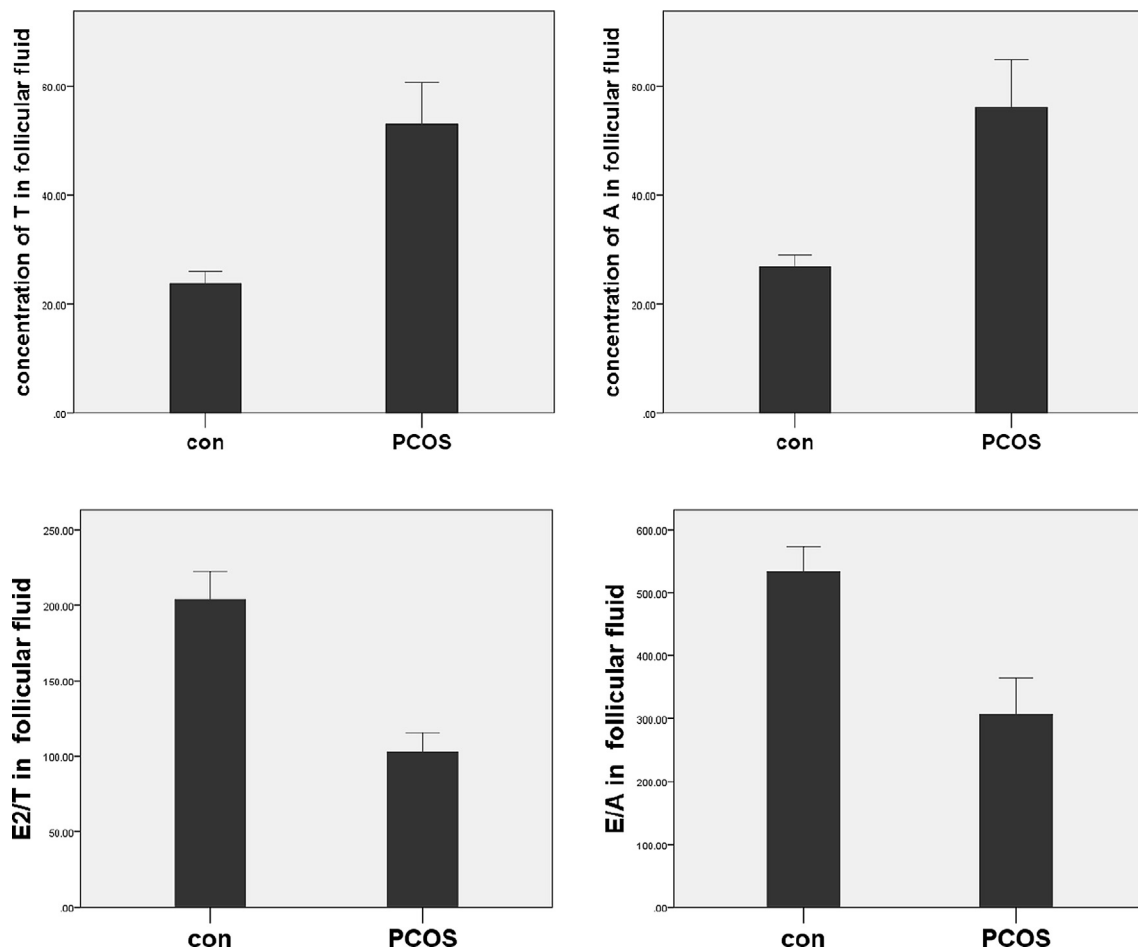


Figure 1 The concentration of T and A in the follicular fluid of con and PCOS group, and the ratio of E2/T to E/A.

Expression of six miRNAs and four SRSFs in the PCOS-H group and the AR-SV group

The expression levels of miRNA-7, miRNA-10a, miRNA-505 and miRNA-124 in the PCOS-H group were all higher than those in the CON group and the PCOS-L group ($P < 0.05$), but there was no significant difference between the PCOS-L group and the CON group. MiRNA-183 and miRNA-29c expression in the PCOS-H group was significantly lower than that in the CON group and the PCOS-L group ($P < 0.05$ and $P < 0.05$, respectively) (Fig. 8).

The expression levels of miRNA-7 and miRNA-505 were higher in the AR-SV group than in the AR-FL group ($P < 0.05$). Additionally, the expression levels of miRNA-10a and miRNA-124 in the AR-SV group were significantly higher than those in the CON group and the AR-FL group ($P < 0.05$). Furthermore, the expression of miRNA-183 in the AR-SV group was lower than that in the CON group and the AR-FL group ($P < 0.05$). However, the expression of miRNA-29c in the AR-FL and AR-SV groups was lower than that in the CON group (Fig. 9).

The expression level of SRSF2 mRNA in the ovarian GCs in the PCOS-H group was higher than that in the CON group and the PCOS-L group ($P < 0.05$), while the expression level of SRSF3 mRNA was lower in the PCOS-H group than in the

CON group and the PCOS-L group ($P < 0.05$). The expression levels of SRSF1 mRNA and SRSF10 mRNA in both the PCOS-L group and the PCOS-H group displayed no significant difference compared with those in the CON group ($P > 0.05$) (Fig. 10).

The expression of SRSF2 mRNA in the AR-SV group was significantly higher than that in the CON group and the AR-FL group ($P < 0.05$). In addition, SRSF3 mRNA expression was significantly lower in the AR-SV and AR-FL groups than in the CON group ($P < 0.05$). Nevertheless, there was no difference in the expression of SRSF1 mRNA in the AR-FL group or the AR-SV group compared with that in the CON group ($P > 0.05$). The expression of SRSF10 mRNA in the AR-SV group was higher than that in the CON group and the AR-FL group ($P < 0.05$) (Fig. 11). Six miRNAs were closely related to hyperandrogen of PCOS, and the change expression of SRSF2 and SRSF3 mediated the formation of AR-SV and the function of AR.

Expression of SRSFs in cells after the upregulation of miRNAs

We transfected the COV434 cells using six miRNA precursor plasmids. After the successful upregulation of six miRNAs in the COV434 cells (Figs. 12–14), the expression of four SRSFs

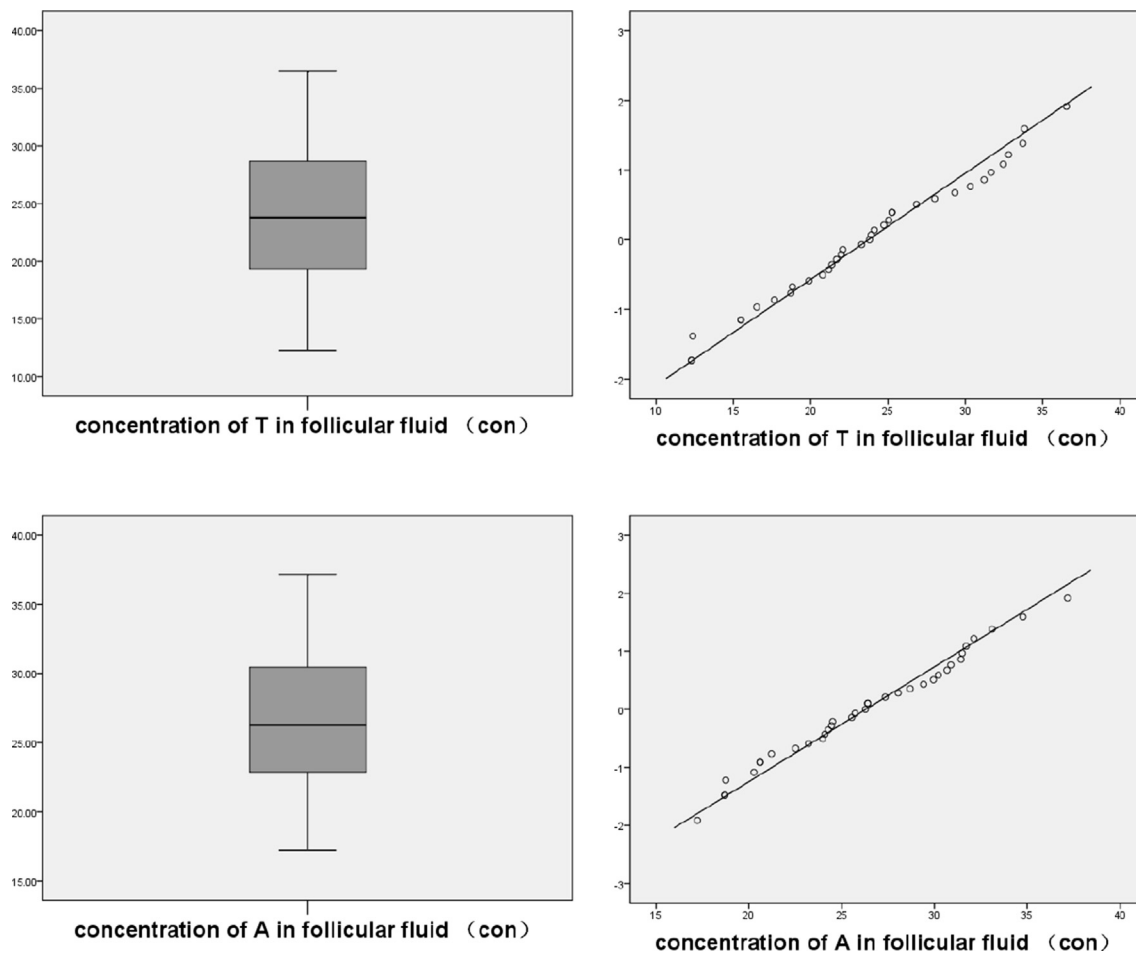


Figure 2 The normal distribution of T and A concentrations in the follicular fluid of control group.

Table 2 Ratio of higher androgen in PCOS patients.

	PCOS-L		PCOS-H		
			PCOS-T	PCOS-A	PCOS-A&T
Number of patient	6	5	1	24	

PCOS-L, No androgen raise; PCOS-T, only the concentration of T raise; PCOS-A, only the concentration of A raise; PCOS-A&T, both the concentration of A and T raise.

was significantly decreased. MiRNA had inhibitory effect on the corresponding SRSFs.

Expression of AR in cells and the hormone level in conditioned medium after the upregulation of miRNAs

The expression level of AR decreased ($P < 0.05$) after the upregulation of miRNA-183, and the expression level of AR was significantly increased in the miRNA-124 group ($P < 0.05$). However, there was no significant change in AR

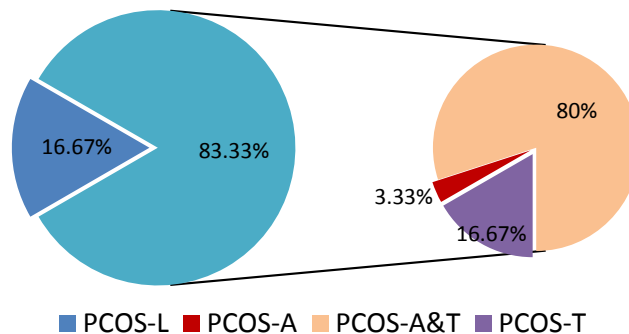


Figure 3 The ratio of high androgen group in PCOS. PCOS-L, No androgen raise; PCOS-A, only the concentration of A raise; PCOS-A&T, both the concentration of A and T raise; PCOS-T, only the concentration of T raise.

expression after the upregulation of miRNA-7, miRNA-10a, miRNA-505 or miRNA-29c ($P > 0.05$) (Figs. 15 and 16).

The concentration of T in the cell culture medium was differentially increased after the transfection of miRNA-7,

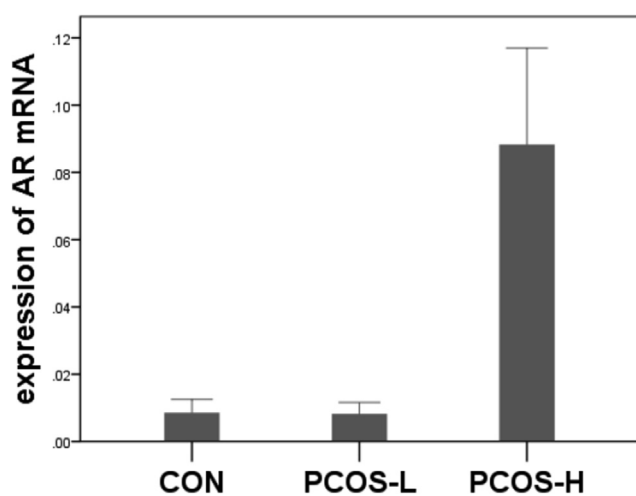


Figure 4 The expression of AR mRNA in CON, PCOS-L and PCOS-H.

miRNA-505, miRNA-183, miRNA-124 and miRNA-29c ($P < 0.05$) but was not significantly changed after the transfection of miRNA-10a ($P > 0.05$). The concentration of A in the cell culture medium showed no significant difference in any of the groups ($P > 0.05$). The E2/T ratio in the cell culture medium after the transfection of miRNA-7, miRNA-505, miRNA-183, miRNA-124 and miRNA-29c was lower than that after the transfection of blank control ($P < 0.05$), but there was no difference after the transfection of miRNA-10a ($P > 0.05$). Nevertheless, there was no change in the E/A ratio after the transfection of any of the six miRNAs (Fig. 17). The up-regulation of miRNA-183 and miRNA-124 regulated the expression of AR, reduced AR function, and eventually led to the accumulation of androgen.

Discussion

The DNA binding domain (DBD) can bind with the androgen response element (ARE) on the aromatase gene to activate the transcription of aromatase. In this study, we found that AR mRNA expression was significantly higher in the ovarian GCs of patients with higher androgens in follicular fluid than in those of control patients, suggesting that the increase in AR expression did not enhance the function of transforming androgens. Therefore, we believe that not only the expression level of AR but also the structure of AR were changed under conditions of high androgens. Two types of AR-SVs have been previously reported to be closely related to hyperandrogenism in PCOS, and these two AR-SVs are generated by insertion or deletions of corresponding sequences during the splicing of AR precursor mRNA.¹⁰ The AR-SVs significantly reduces the ability of AR to bind to ligands, which severely affects the function of AR, resulting in the inhibition of the transcriptional activity of AR.^{30,31} In this study, all 9 patients expressing AR-SVs belonged to the PCOS-H group, and these patients accounted for 30% of the PCOS-H group. Therefore, we believe that AR-SVs are

closely correlated with hyperandrogenism. Additionally, the level of follicular fluid androgen was higher in the patients of AR-SV group than that in the patients of AR-FL group, indicating that the formation of AR-SVs affects the function of AR and leads to the local accumulation of androgens. Alternatively, signals from excess androgen could have induced the formation of AR-SVs due to prolonged periods of high androgen status, further increasing the accumulation of androgens. Regardless of the causes of AR-SV generation, the production of AR-SVs caused functional changes in AR, which in turn caused the dysfunction in ovarian GCs.

The level of AR mRNA in patients with PCOS was significantly higher than that in control individuals, but there was no significant difference in AR mRNA expression between the AR-FL group and the AR-SV group. Thus, even though the expression level of AR mRNA in patients with PCOS had increased, AR function was inhibited due to the generation of AR-SVs rather than being promoted. Nevertheless, the expression of AR-SVs reduced the proportion of normal AR, and there may have been other underlying splicing variants in the AR-FL group or other factors affecting AR function. AR-SVs inhibit the expression of aromatase induced by AR. Due to the effect on the function of AR as a transcription factor, androgen metabolism is eventually disrupted. Unfortunately, we did not identify AR-SVs that lack exon 3 or other AR-SVs. This could have been due to technological limitations or a small percentage of the missing splicing variants. Furthermore, the sample size was limited, and no patients expressed other types of AR-SVs. Therefore, we will continue to collect samples in follow-up studies. By increasing the sample pool, we will be able to perform a better study on the expression of AR-SVs.

We also examined the expression status of four SRSFs and six miRNAs in ovarian GCs. Based on our data, changes in the expression levels of SRSF2 and SRSF3 were closely related to the formation of AR-SVs in patients with PCOS. Previous studies on SRSF2 and SRSF3 have demonstrated that both proteins are directly involved as splicing factors in the splicing regulation of the target gene.³² SRSF2 can directly bind with target genes. In addition, it influences the alternative splicing process of target genes by changing the extension rate of RNA polymerase II.³³ SRSF2 is involved in a variety of splicing regulation of target genes.^{34–36} SRSF3 can act as a splicing silencer to suppress the splicing of exons.²⁰ The synergistic competition between SRSF3 and other factors may lead to the loss of exons during splicing.²² Therefore, we believe that changes in the expression of SRSF2 and SRSF3 can affect the variable splicing process of AR and promote the formation of AR-SVs. This process changes the expression level of AR and leads to AR dysfunction. In addition, this process decreases the ability of ovarian GCs to transform androgens. Nonetheless, SRSF10 levels were slightly increased in the AR-SV group, indicating that SRSF10 may have some synergistic regulatory effect on the formation of AR-SVs.

MiRNAs change the expression level of genes by binding complementarily with the untranslated region of target genes³⁷ and alter the regulatory effect of target genes on downstream factors.^{38,39} By examining six miRNAs in the

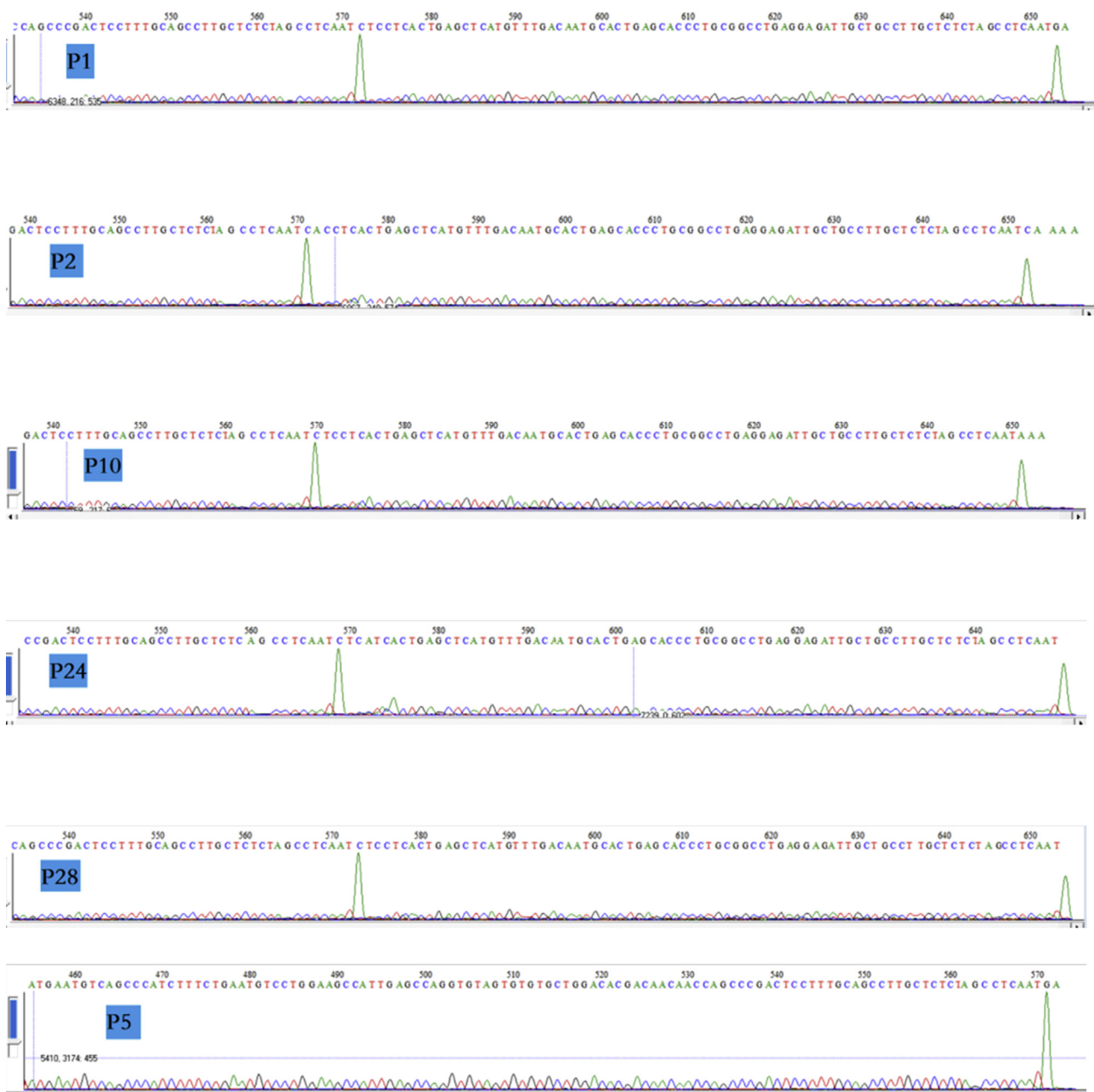


Figure 5 The peak figure of some samples such as five AR-SV expression samples (P1, P2, P10, P24 & P28) and one non-SR-SV expression sample P5. (P1, P2, P10, P24 & P28 have two terminational sharp peaks, there is 70 basic group between two peaks, by contrast P5 only has one terminational sharp peak).

study, we found that the change in androgen concentrations in follicular fluid of patients with PCOS was accompanied by a change in miRNA expression in GCs. These six miRNAs were related to hyperandrogenism in PCOS, consistent with the results of other studies. We further analyzed the expression of these six miRNAs in PCOS patients with AR-SVs. Surprisingly, changes in the expression levels of miRNA-183 and miRNA-124 in the ovarian GCs of patients in the AR-SV group were found to be related to the

formation of AR-SVs. When androgen concentrations in the follicular fluid of patients with PCOS were increased, miRNA-183 expression was decreased, SRSF2 expression was increased, while miRNA-124 expression was increased, and SRSF3 expression was significantly decreased. Therefore, in our opinion, changes in miRNA expression may regulate the expression of the target SRSF genes and lead to the abnormal splicing regulation of SRSFs. This in turn can promote changes in AR expression and structure and block

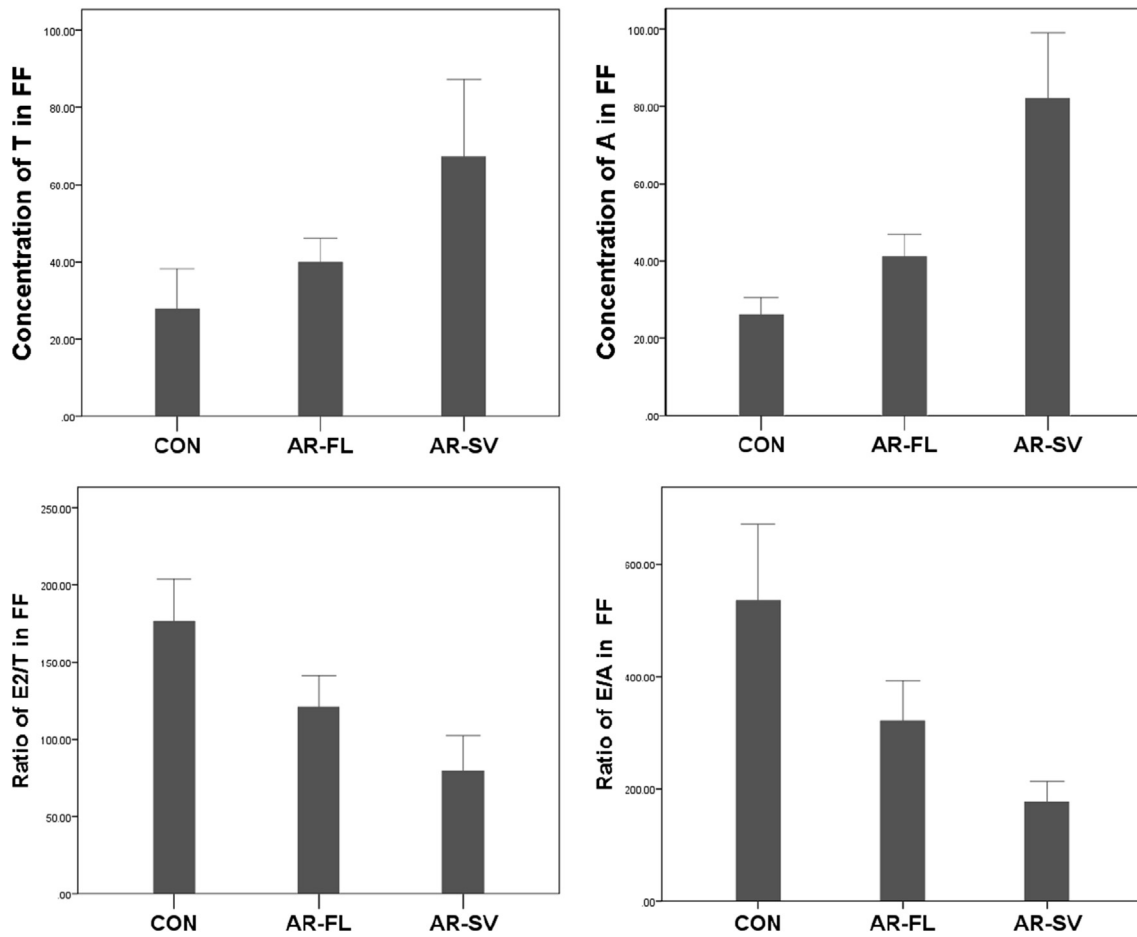


Figure 6 The concentration of T, A, E2/T and E/A in follicular fluid of AR-FL and AR-SV.

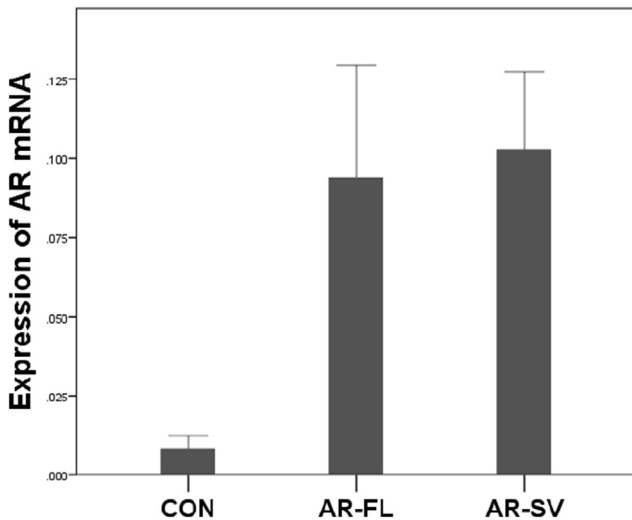


Figure 7 The expression of AR mRNA in CON, AR-FL and AR-SV.

the transformation of androgens in ovarian GCs, eventually leading to the dysfunction of ovarian GCs.

To further clarify the regulatory effect of miRNAs on their target genes, we successfully upregulated miRNAs in

cells and found that all four types of SRSFs decreased by varying degrees. The results indicated that the six miRNAs served as inhibitory factors on the corresponding SRSFs. In other words, the expression of SRSFs was reduced to different degrees, and miRNAs inhibited the expression of target genes. When miRNA-7, miRNA-505, miRNA-10a and miRNA-29c were upregulated, the expression of SRSF1 and SRSF10 decreased, but there was no significant change in the expression of AR mRNA or protein. Nevertheless, when miRNA-183 and miRNA-124 were upregulated, the expression levels of SRSF2 and SRSF3 were significantly decreased, along with changes in AR mRNA expression. Furthermore, when the other 5 miRNAs except miRNA-10a were upregulated in cells, T concentrations in the medium increased to different degrees. Because of the strong biological activity of T in the body, which is 5–10 times that of A, and the low concentration of A in the CON group, which is close to the low concentration standard, the concentration of A did not significantly change after the transient transfection of miRNAs. Taking the effect on estrogens into account, the change in the E2/T ratio was consistent with the increase in T concentration. The above results suggest that miRNA-183 may change the activity of SRSF2 as a splicing factor by inhibiting the expression of SRSF2 and AR. This disrupts AR function, leading to a reduction in androgen utilization and the

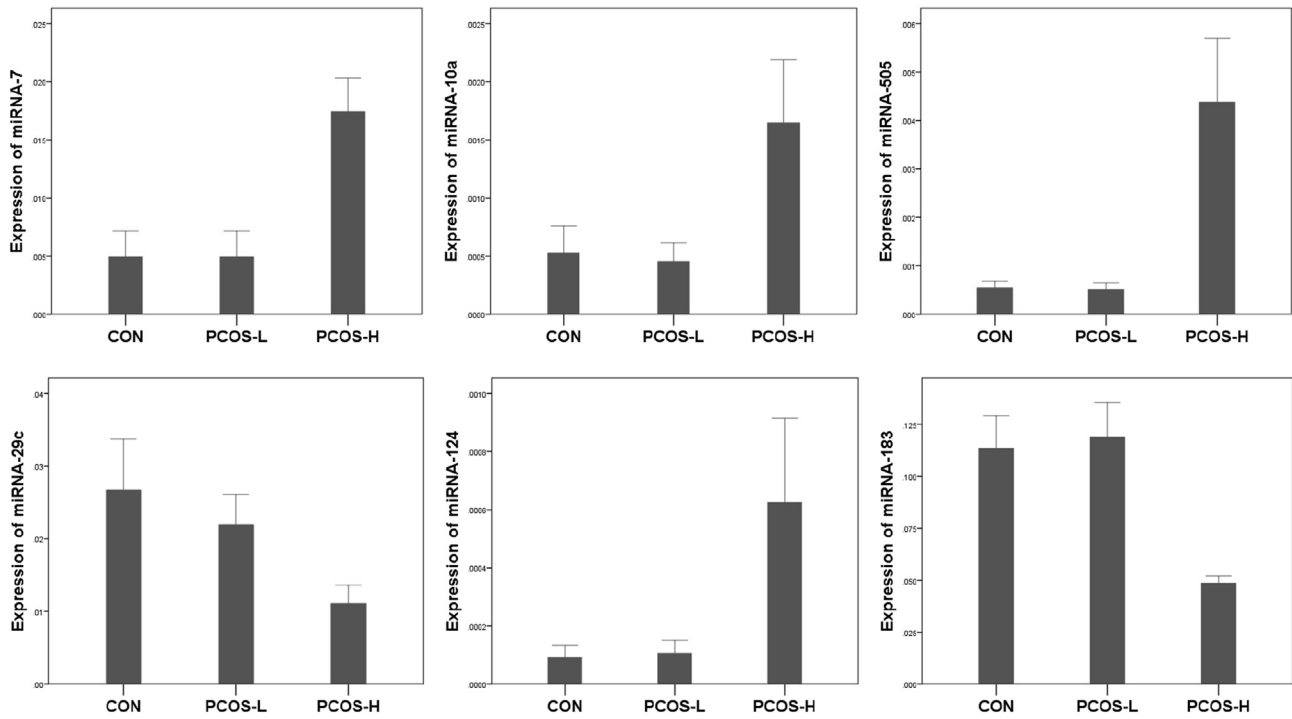


Figure 8 The expression of six miRNAs in CON, PCOS-L and PCOS-H groups.

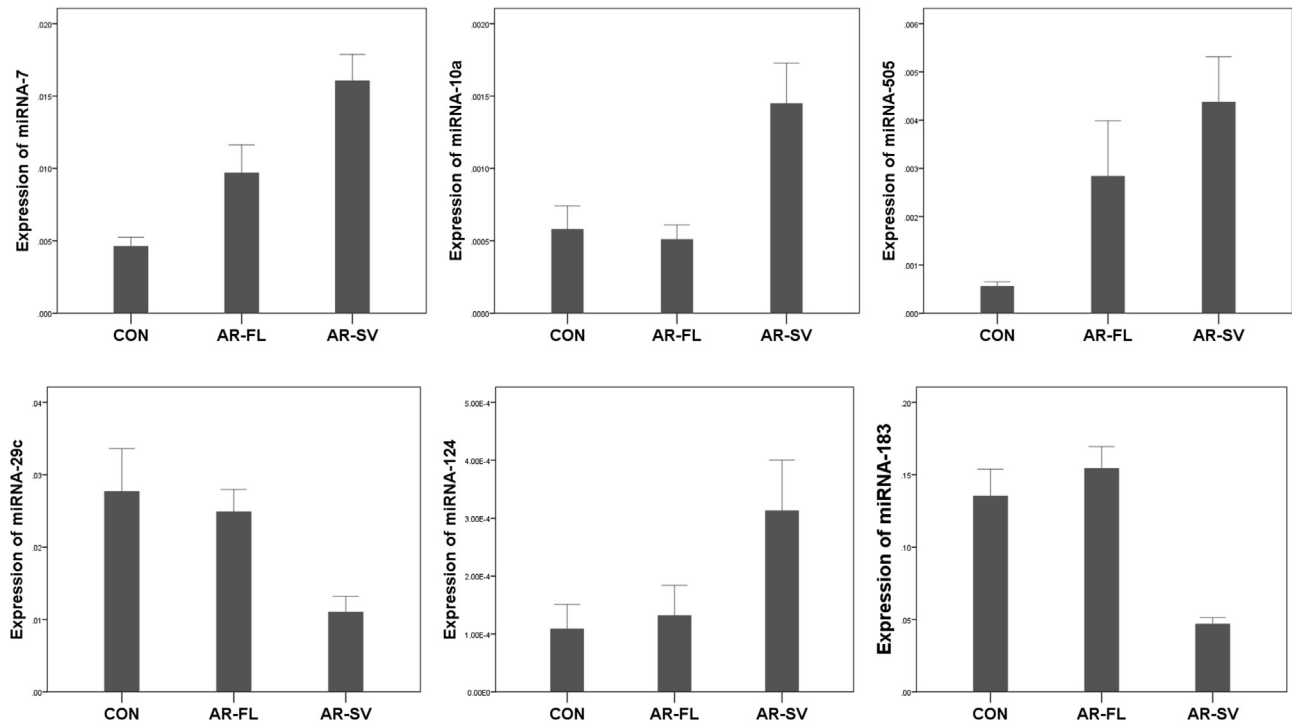


Figure 9 The expression of six miRNAs in CON, AR-FL and AR-SV groups.

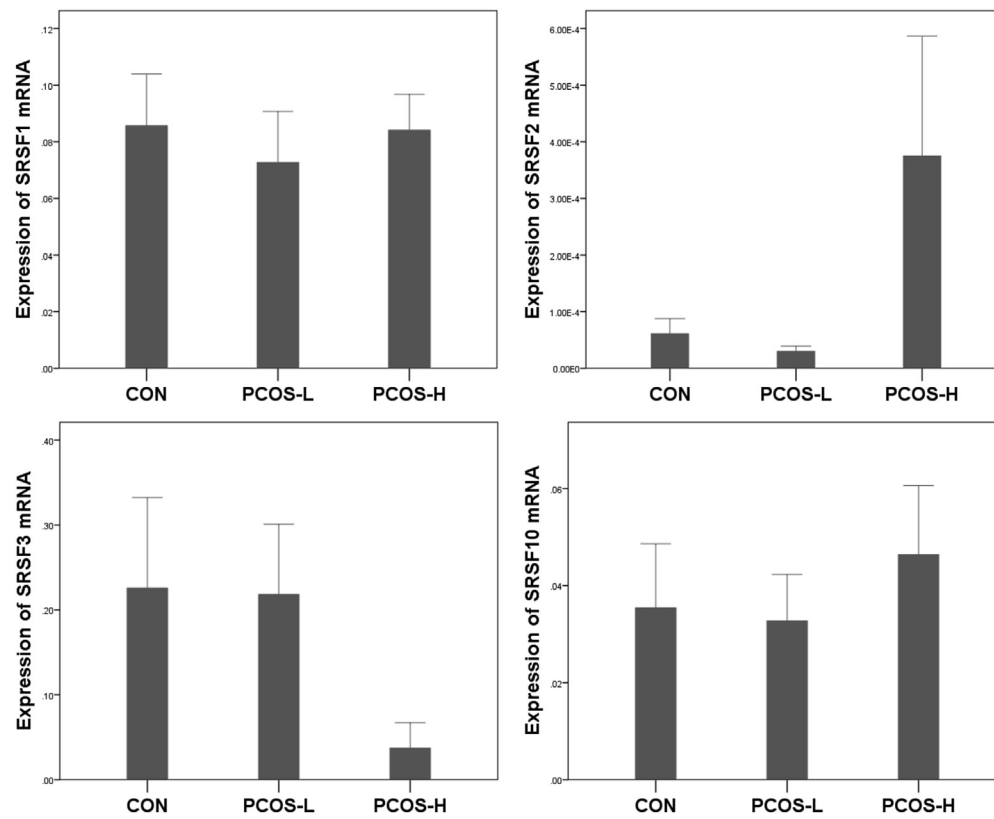


Figure 10 The expression of SRSFs mRNA in CON, PCOS-L and PCOS-H groups.

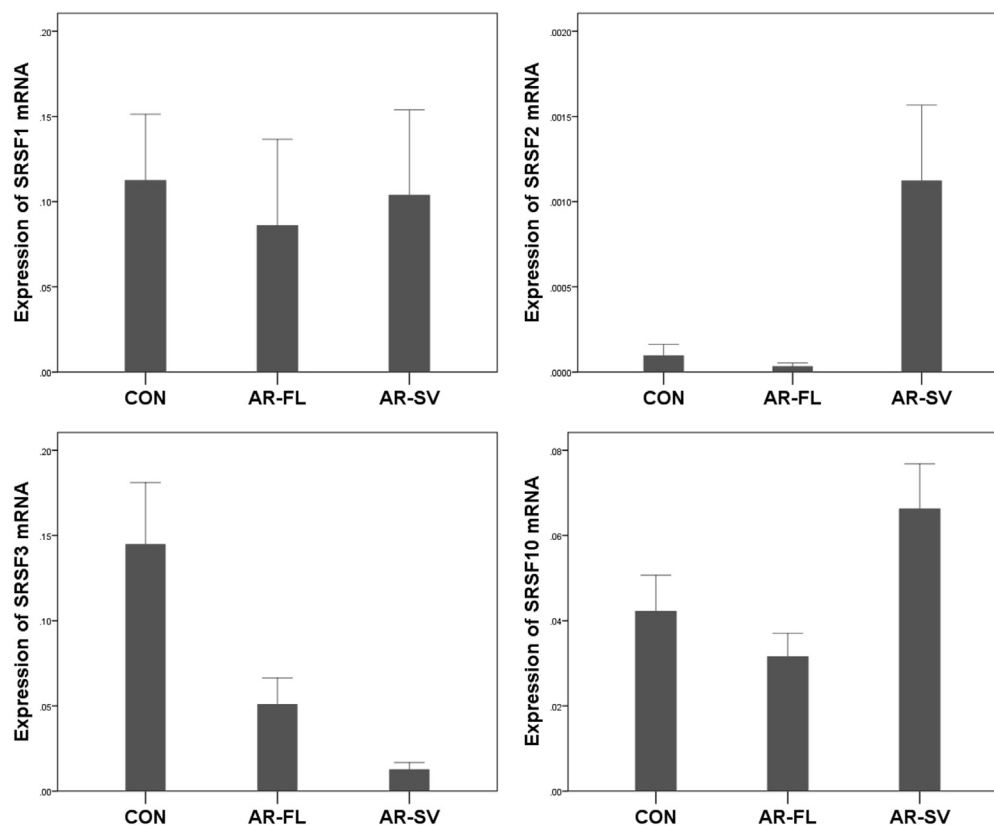


Figure 11 The expression of SRSFs mRNA in CON, AR-FL and AR-SV groups.

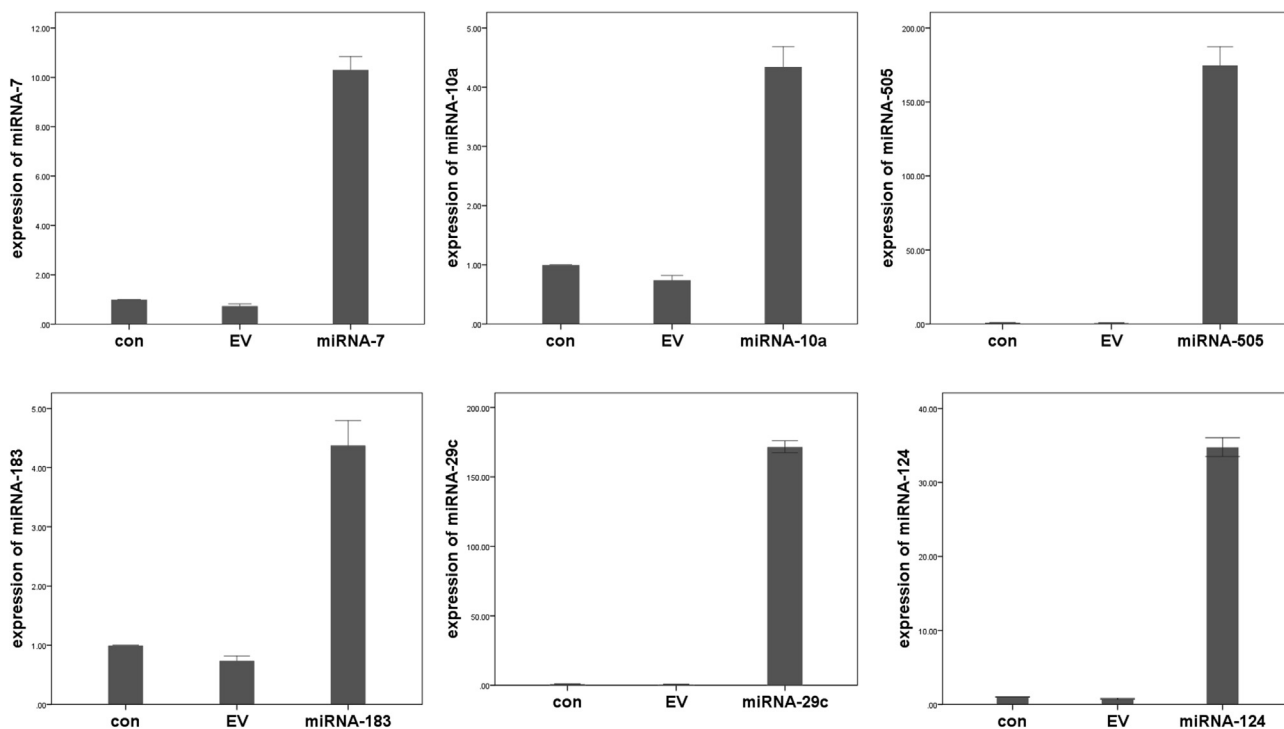


Figure 12 The expression of six miRNAs after the transfection.

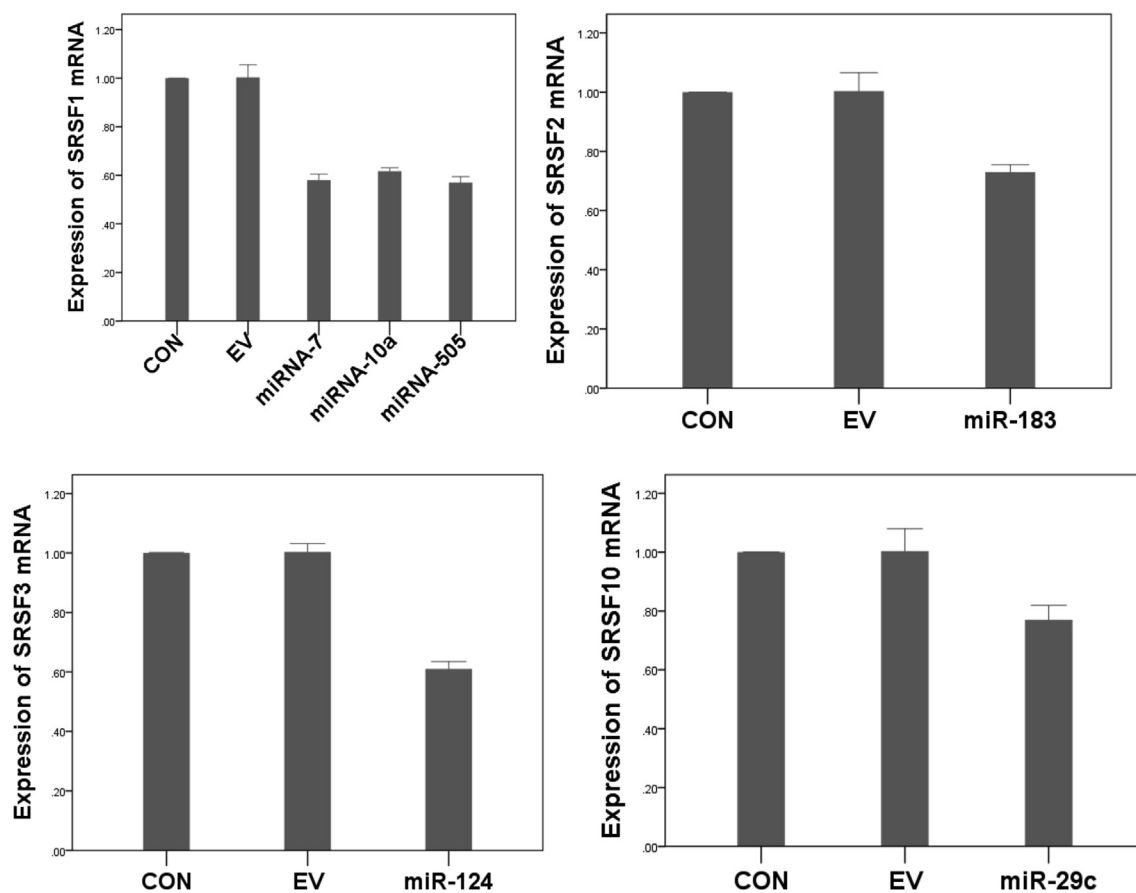


Figure 13 The expression of SRSFs mRNA after the transfection.

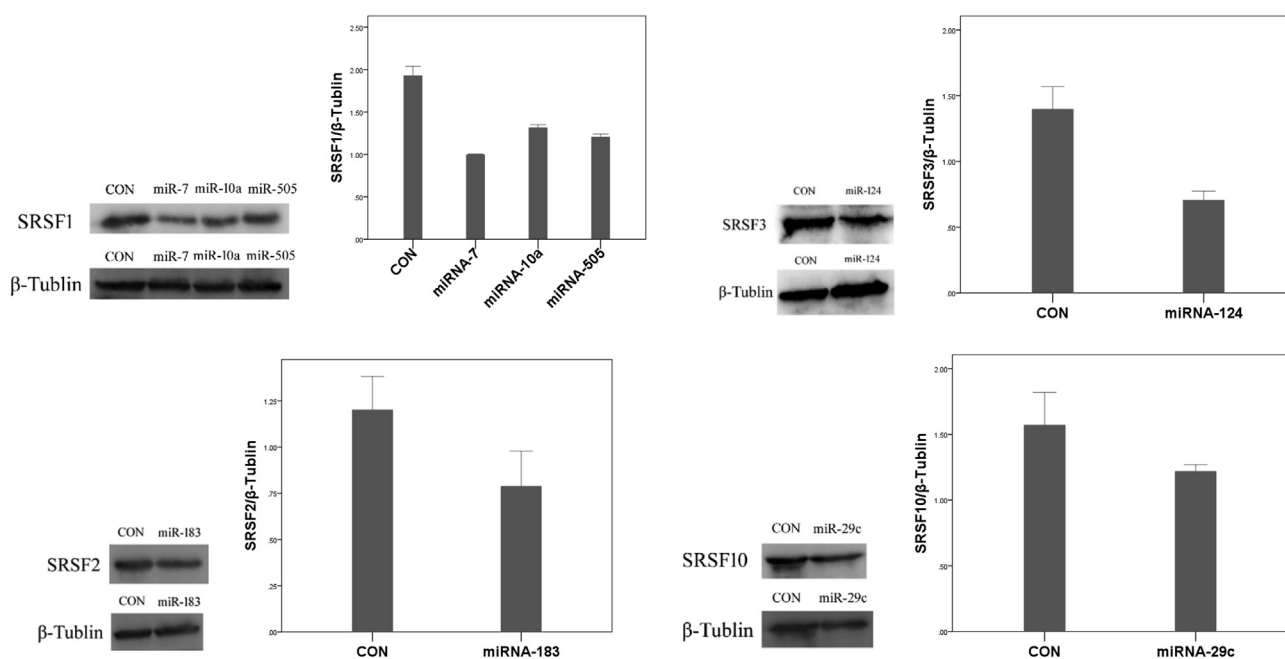


Figure 14 The expression of four SRSFs after the transfection of miRNA-29c.

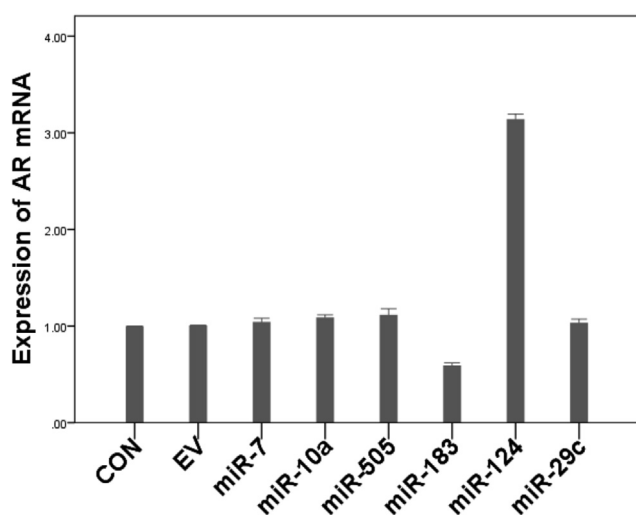


Figure 15 The expression of AR protein after up-regulated different miRNAs separately.

accumulation of androgens. However, the upregulation of miRNA-124 can also change the splicing regulation of SRSF3 by inhibiting the expression of SRSF3 and promoting the expression of the AR gene and protein. In this study, the upregulation of miRNA-124 also increased the androgen content in the culture medium, indicating that the increase in AR expression did not enhance the

function of AR but rather inhibited the transformation of androgens. Therefore, we speculated that miRNA-124 promotes the expression of AR-SVs and changes the structure of AR. Although the expression level of AR was increased, the activity of AR was not, and the accumulation of androgens was obvious. The upregulation of miRNA-7, miRNA-505 and miRNA-29c also increased the androgen content of the cell culture medium, but the expression level of AR was not altered. The reason may be that these miRNAs affect the transformation of androgen to estrogen. For example, the expression of aromatase may be directly affected.

Several researchers have attempted to use miRNAs to treat diseases through the principle of miRNA interference of corresponding target genes.^{40–42} These researchers combined miRNA-34a with docetaxel on a nanocarrier and injected them into mice with metastatic breast cancer, and this inhibited tumor growth and metastasis.⁴³ MiRNA-145 can increase the sensitivity of pancreatic cancer cells to the anticancer drug gemcitabine by inhibiting the expression of the target gene p70S6K1.⁴⁴ In this study, we found that miRNA-183 and miRNA-124 had a regulatory effect on the expression of AR and affected the function of ovarian cells, resulting in the local accumulation of androgens. Further research on the regulatory mechanism of miRNAs on AR and androgen transformation needs to be conducted to improve AR activity by changing the level of miRNAs, reducing local androgen status and promoting follicular development, which is important to restore fertility in patients with PCOS.

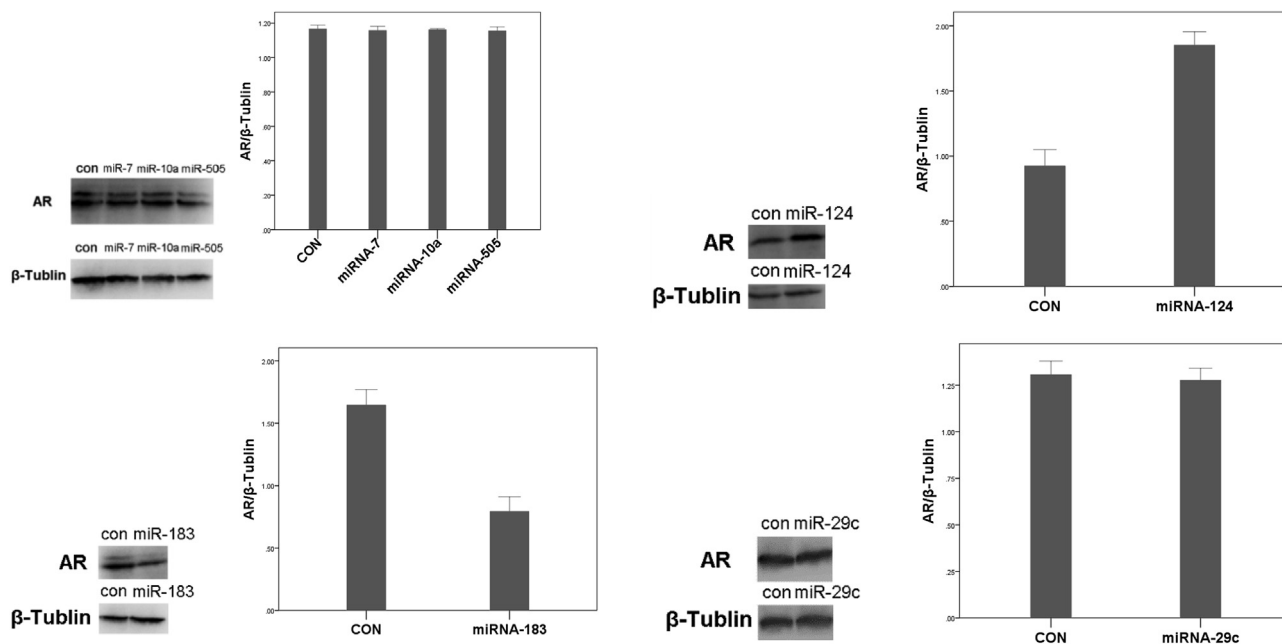


Figure 16 The expression of AR protein after up-regulated miRNA-29c.

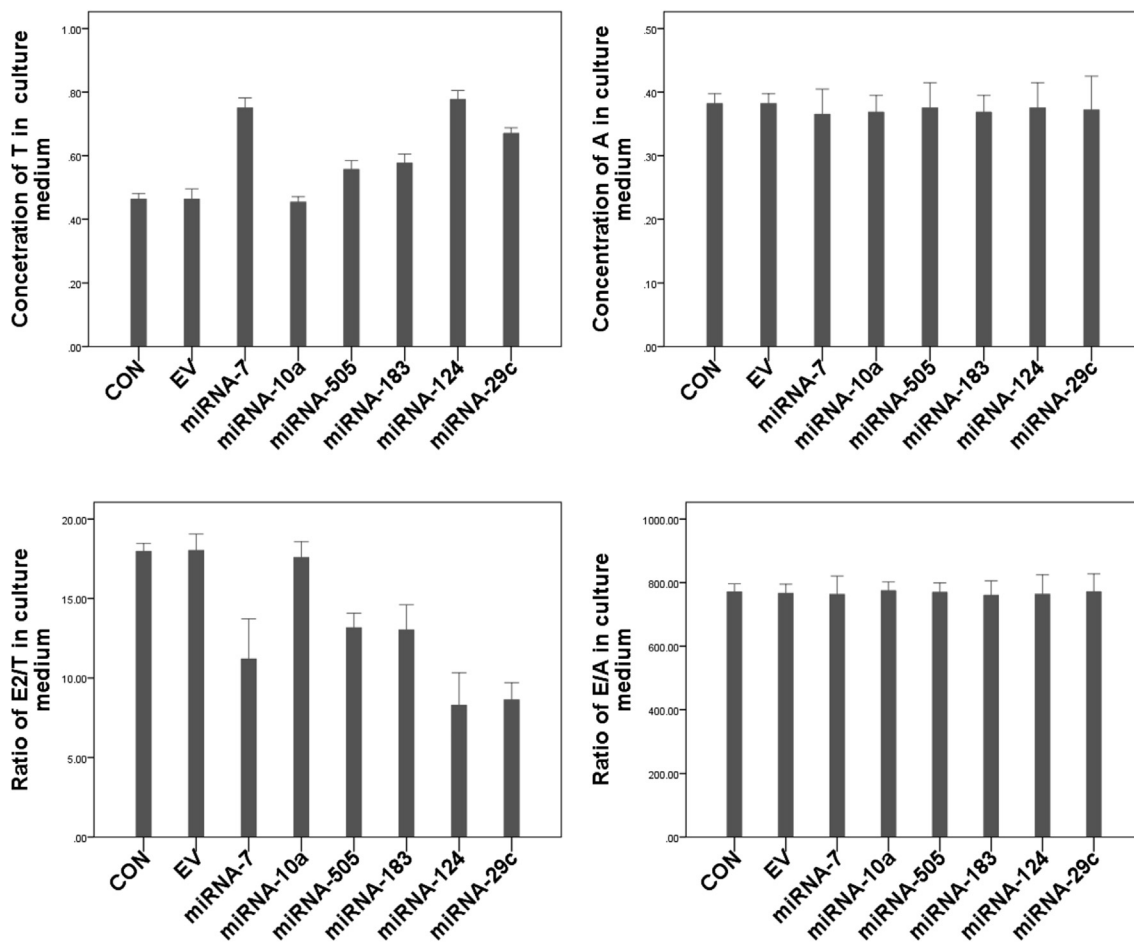


Figure 17 The ratio of E2/T and E/A in culture medium after up-regulated different miRNAs separately.

Conflict of Interests

The authors declare no conflicts of interest.

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