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Research Article

Functional Study of the Effect of miR-647 on the Prognosis of Polycystic Ovarian Syndrome (PCOS)

Hanieh Motahari Rad[1,3*], Fatemeh Akbarian[2], Zohreh Hashemian[4,5], Seyed Javad Mowla[1], Mojtaba Rezazadeh Valojerdi[3,6]

[1]Department of Molecular Genetics, Tarbiat Modares University, Tehran, Iran; [2]Department of Biological Sciences, Ale Taha University, Tehran, Iran; [3]Department of Embryology, Academic Center for Education, Culture and Research, Tehran, Iran; [4]Department of Human and Animal Cell Bank, Iranian Biological Resource Center, Tehran, Iran; [5]Department of Genetics, Royan Institute for Reproductive Biomedicine, Tehran, Iran; [6]Department of Anatomy, Tarbiat Modares University, Tehran, Iran

ABSTRACT

Purpose: Polycystic Ovarian Syndrome (PCOS) is a metabolic syndrome that is regulated by several genes and miRs. In a previous study, we showed that miR-647 was significantly downregulated in PCOS patients compared with normal controls. Furthermore, miR-647 exhibited a negative correlation with CYP11A1 in both follicular fluid and granulosa cells. The purpose of this study was to gain a preliminary understanding of the role of miR-647 in granulosa cells and its impact on the prognosis of PCOS patients.

Methods: A miR-647 Precursor (premiR) and a Sponge (miR-sp) were designed for the overexpression and knockdown of miR-647. Wild-type and mutant 3'-UTRs of CYP11A1 were also prepared as specific binding sites for miR-647. On the basis of bioinformatics studies, the low level of miR-647 in HEK-293T cells makes it suitable for testing the effectiveness of the premiR construct. A dual luciferase reporter assay was conducted to assess the function of miR-sp. The effect of miR-sp was investigated in GCN-01 (granulosa cells) cells by determining the expression of related genes *via* qRT–PCR. ELISA was used to quantify steroid hormones in the conditioned media of miR-sp-GCN-01.

Results: The significant reduction in miR-647, along with the significant increase in AR and CYP family genes in miR-sp-GCN-01, revealed a molecular pattern that closely resembled androgenic PCOS. The results of the luciferase reporter assay confirmed the specificity of miR-sp. Moreover, the levels of the hormones estradiol and progesterone significantly decreased in miR-sp-GCN-01.

Conclusion: Our data demonstrate that PCOS conditions can be simulated at the cellular level by downregulating miR-647, making it a potential in vitro model for PCOS.

Keywords: Polycystic ovarian syndrome; miR-647; Granulosa cells; miRNA sponge; *In vitro* model

INTRODUCTION

Polycystic Ovarian Syndrome (PCOS) is one of the most common diseases among women and affects the function of the ovaries due to excessive production of the androgen hormone. Approximately 2.2% to 26.7% of women of reproductive age worldwide suffer from PCOS, with approximately 70% of them being unaware of their condition. Various symptoms, including high levels of androgen hormones, disruption of the menstrual cycle and polycystic ovaries, can cause different phenotypes in patients. The main complications associated with these phenotypes include type 2 diabetes, gestational diabetes, blood clots, cardiovascular problems, mental disorders and uterine cancer. PCOS is caused by various factors, such as the

Correspondence to: Hanieh Motahari Rad, Department of Molecular Genetics, Tarbiat Modares University, Tehran, Iran; Email: Haniemotahary@gmail.com

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physiological conditions of the body, nutrition, lifestyle and genetics [1].

Among the most important genetic factors affecting the development and pathophysiology of PCOS are small noncoding RNAs called miRNAs (miRs). These factors contribute to the progression of this disease by regulating the functions of various cellular pathways, such as steroidogenesis, metabolic pathways, proliferation, focal adhesion and folliculogenesis. Abnormal expression of miRs is associated with many human diseases. Therefore, cellular and extracellular miRs have been widely reported as potential biomarkers for various diseases. In our previous study, we demonstrated for the first time that the expression of four circulating miRs, namely, miR-212-3p, miR-490-5p, miR-647 and miR-4643, significantly changes with different phenotypes of PCOS. Therefore, we suggest these miRs as potential molecular biomarkers for noninvasive and accurate identification of these subtypes, specifically indicating the dysfunction of ovarian steroidogenesis and follicular maturation arrest.

MiR-647 regulates the expression of numerous genes involved in various cellular processes, including cell proliferation, apoptosis and differentiation. Studies have shown that miR-647 plays a crucial role in the pathogenesis of several human cancers, including gastric cancer, hepatocellular carcinoma, non-small cell lung cancer and prostate cancer. miR-647 functions as a tumor suppressor by regulating the expression of key genes involved in cancer development and progression [2].

In this study, miR-647 and its target gene, CYP11A1, were selected for further cell studies on the basis of differential gene expression and correlation analysis conducted in our previous study. In that study, the expression level of miR-647 was significantly decreased in patients with PCOS. Furthermore, miR-647 exhibited a negative correlation with CYP11A1 in both follicular fluid and granulosa cells. By downregulating the expression level of miR-647 in granulosa cells, we are attempting to create an *in vitro* cell model of PCOS. Thus, we will investigate the molecular role of this miRNA in the progression of this disease and establish a laboratory model of PCOS by conducting molecular studies and hormonal tests in cells before and after transfection.

MATERIALS AND METHODS

Design and synthesis of constructs

miR-647 precursor (premiR-647) construct: The miR-647 precursor was amplified *via* the following forward and reverse primers: (1) 5'-TGTGGGTTGGGAGGCTCCTG-3' and (2) 5'-AGGGCAGAAAAGAGGCGTGACA-3'. The two oligonucleotides were annealed to create 687-nucleotide double-stranded DNA fragments with XhoI and EcoRI overhangs. To synthesize premir-647, PCR was performed on human DNA. The PCR cycles were repeated 40 times, with denaturation at 95° C, annealing at 60°C for 30 seconds and extension at 72°C for 30 seconds. The purified product was subsequently double-digested with both XhoI and EcoRI enzymes using Tango Buffer 2X (Thermo Scientific, USA) for 7 hours at 37°C. The insert and vector were then ligated *via* T4 DNA ligase (Vivantis Technologies, Malaysia). PremiR-647 was then cloned and inserted into the pEGFP-N1 plasmid (Addgene, USA) by performing a double digest with the aforementioned restriction sites (Supplementary Figure 1) and transformed into DH5α. To obtain a positive colony containing the plasmid and premiR-647, a colony PCR was performed on several colonies *via* Universal Primers (Sinaclon, Iran). Then, plasmid DNA was extracted *via* the GeneAll ExfectionTM Plasmid Kit (GeneAll biotechnology, South Korea) and the accuracy of the cloning procedures was confirmed *via* Sanger DNA sequencing (Macrogene, South Korea) [3].

miR-647 sponge (miR-647-sp) construct: The miRNA sponge sequence was constructed using three sequences that have complete complementarity to mature miR-647. These sequences were connected by "spacer" sequences. The miRNAsong tool (Barta T, Peskova L, Hampl A). miRNAsong: A web-based tool for the generation and testing of miRNA sponge constructs in silico. This technique was used to confirm the specific binding of the designed miRNA sponge sequence to the target miR-647 sequence. Since there is no array available for this sequence in humans, the target sequence was ordered into two separate probes: (1) 5'-CCGCTCGAGCGGGAAGGAAGTGAGTGCAGCCACCGC CCGAAGGAAGTGAGTGCAG-3', as the sense oligonucleotide and (2) 5'-GGAATTCCGTGGCTGCACTCACTTCCTTCGGGCGGTG GCTGCACTCACTTCCTTCGGGC-3', as the antisense oligonucleotide, with complementary 3' end sequences. Moreover, forward (5'-CCGCTCGAGCGGGAAGGAAGT-3') and reverse (5'-GGAATTCCGTGGCTGCACTCA-3') primers were designed to amplify the miR-647 sponge sequence, which includes restriction sites (EcoRI and XhoI) at their respective 5' ends.

To synthesize miR-647-sp, template DNA with complementary 3' ends, which allows the probes to hybridize after heat denaturation, was used for PCR to generate full-length products. The product was then purified from a 2% agarose gel after its size was confirmed and cloned and inserted into the mCherry plasmid. The plasmid was purified from a bacterial liquid culture and digested with XhoI and EcoRI enzymes in 2X Tango buffer for 7 hours at 37°C. The insert and vector were then ligated *via* T4 DNA ligase. The product was then cloned and inserted into the mCherry plasmid (Supplementary Figure 2) and transformed.

According to the target scan online tool (McGeary SE, Lin KS, Shi CY, Pham T, Bisaria N, Kelley GM, Bartel DP). The biochemical basis of the efficacy of microRNA targeting. Science, Dec 5, 2019. The first 40 nucleotides in the 3' Untranslated Region (UTR) of the CYP11A1 gene provide a binding site for miR-647. To synthesize both wild-type and mutant 3' UTR fragments, two different forward primers and a shared reverse primer were designed (Table 1), with XhoI and NotI overhangs, respectively [4]. The fragments were subsequently produced *via* PCR, purified and digested with the abovementioned enzymes *via* NEB Buffer 3.1 (New England Biolabs, UK) for 7 hours at 37°C. The ligates that were inserted

were then cloned and inserted into the psiCHECK 2™ plasmid (Addgene, USA) and transformed into DH5α. Further steps followed the premiR-647 synthesis methodology (Supplementary Figure 3).

Table 1: The primer sequences of the designed constructs.

Construct	Primer sequences (F: forward; R: reverse)	Size of product (bp)
miR-647 Precursor	F: 5'-TGTGGGTTGGGAGGCTCCTG-3'	687
	R: 5'-AGGGCAGAAAAGAGGCGTGACA-3'	
miR-647 Sponge	F: 5'-CCGCTCGAGCGGGAAGGAAGT-3'	93
	R: 5'-GGAATTCCGTGGCTGCACTCA-3'	
Wild CYP11A1 3' UTR	F: 5'-CTGGCCCTTTAACCAGGAA-3'	243
	R: 5'-TGGTTCAGCTGTTTATTGTCTCC-3'	
Mutant CYP11A1 3' UTR	F: 5'-GGTCTCTGCATCTTCAGTCG-3'	152
	R: 5'-TGGTTCAGCTGTTTATTGTCTCC-3'	

Cell culture and transfection

On the basis of bioinformatics studies, the low expression level of miR-647 in HEK-293T cells was utilized to assess the effectiveness of the premiR-647 construct. Additionally, the GNC-01 cell line was used as a granulosa cell line to assess the quality of the miR-647-sp fragment and for other functional experiments [5].

HEK-293T and GNC-01 cells were obtained from the Iranian Biological Resource Center and maintained in DMEM (Gibco, USA) supplemented with 10% Fetal Bovine Serum (FBS, Gibco, USA) and 1% penicillin–streptomycin (Bio Basic, Canada) in a humidified atmosphere at 37 °C with 5% CO_2. Additionally, L-glutamine (4 mM), a nonessential amino acid (1x), bFGF (100 ng/ml), EGF (25 ng/ml), ascorbic acid (50 μg/ml), hydrocortisone (0.5 μg/ml), Insulin-Transferrin-Selenium (ITS 1x) and cholera toxin (0.75 μg/ml) were added to the GNC-01 culture medium.

The basal expression level of miR-647 was evaluated in both existing cell lines *via* RT–qPCR. Transient cell lines expressing premiR-647 and miR-64-sp, as well as their mock N1 plasmid, were generated *via* Lipofectamine 3000 (Invitrogen, USA) according to the manufacturer's instructions. HEK293T cells were plated in 24-well plates (7 × 104 cells per well) 24 hours prior to transfection. The cells were transfected with 500 ng of the pEGFP-N1 plasmid containing premiR-647. Furthermore, 3 × 104 GCN- 01 cells seeded in 24-well plates were transfected with 500 ng of mCherry plasmid containing miR-647-sp. Total RNA was extracted from transfected HEK-293T and GCN-01 cells for gene expression analysis at 24 h, 48 h and 72 h.

Luciferase reporter assay

HEK-293T cells were seeded in 48-well plates (35 × 10^3 cells per well) 24 h prior to transfection. Forty-eight hours after transfection (mock, premiR-647 and miR-647-sp), the cells were analyzed for luciferase activity *via* the Dual-Luciferase reporter assay kit following the manufacturer's instructions (Promega, UK). All transfections were performed in triplicate [6].

Reverse transcription quantitative PCR (RT–qPCR)

The expression levels of miR-647, CYP11A1 and other related miRs that were previously examined in PCOS and control cases (miR-212-3p, miR-490-5p and miR-4643) were measured in treated and control cells. The methods used for RNA extraction, cDNA synthesis and RT-PCR are described previously. After analysis, the expression of this miR decreased the most after 24 h because of the sponge effect. Next, the expression levels of genes involved in steroidogenesis, folliculogenesis and focal adhesion were measured after 24 hours of treatment. The genes measured included Anti-Mullerian Hormone (AMH) Androgen Receptor (AR), the cytochrome P450 family (CYP17 and CYP19A1), Growth Differentiation Factor 9 (GDF9) and very-long-chain 3-oxoacyl-CoA reductase (HSD17B12).

Quantification of steroid hormones

To investigate the effects of the sponge fragment on the steroidogenesis pathway and the expression of genes examined in the previous step, the levels of steroid hormones, including estradiol, progesterone, testosterone and 17OH-progesterone, in the culture media of treated and control cells were investigated *via* ELISA [7].

Statistical analysis

The expression levels of the miRs and genes were normalized to the expression levels of miR-16-5p and GAPDH, respectively. All the data were analyzed at least 3 times *via* the ΔΔCT method with GraphPad prism 6 software. The statistically significant differences were tested *via* ordinary ANOVA. All histograms are

presented as the means ± SDs and differences were considered significant when P< 0.05.

RESULTS AND DISCUSSION

MiR-647 is expressed in the GCN-01 and HEK-293T cell lines

The expression level of miR-647 was evaluated in GCN-01 cells and HEK-293T cells via qRT–PCR. There was significant differential expression of miR-647 between these cell lines. MiR-647 was upregulated in GCN-01 cells (expression level=29.86; p<0.05), whereas it was downregulated in HEK-293T cells (expression level=0.00011; p<0.05), as expected on the basis of bioinformatics studies (Figure 1) [8].

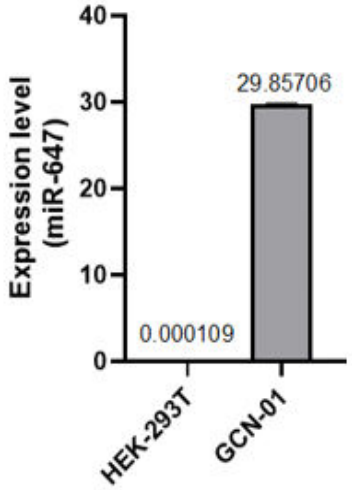

Figure 1: The expression level of miR-647 in two selected cell lines, GCN-01 and HEK-293T. MiR-647 is upregulated in GCN-01 cells (29.857), whereas this miRNA has almost no expression in the HEK-293T cell line (0.000).

Functional efficacy testing and optimization of miR-647-sp *in vitro*

After being seeded in 24-well plates, the cells were transfected with mock N1 and mCherry plasmids, as well as plasmids containing precursor and sponge fragments (Figure 2A). The expression level of miR-647 was analyzed 48 hours after transfecting HEK-293T cells with premiR-647, cells were transfected with both vectors containing the miR precursor and sponge fragment and a mock vector was used. The expression level of miR-647 in the cells containing premiR-647 increased approximately 132-fold, as depicted in Figure 2B. Additionally, the expression level of miR-647 in cells transfected with both vectors containing premiR-647 and miR-647-sp also significantly decreased. This decrease can be attributed to the specific function of the sponge fragment.

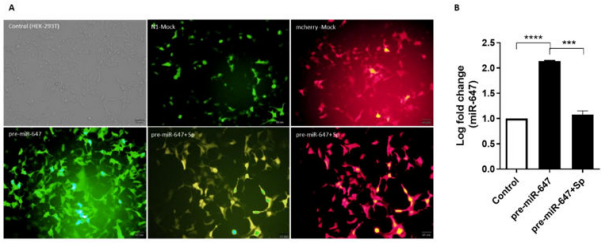

Figure 2: A) HEK-293T cells in the visible state (gray), cells containing the N1-mock plasmid and premiR-647 (green) and cells containing the mCherry-mock plasmid and miR-647-sp (red). B) The expression level of miR-647 was measured in HEK-293T cells transfected with premiR-647, cells transfected with vectors containing both premiR-647 and miR-647-sp and the control group. Statistical analysis revealed significant changes in miR-647 expression in both transfected groups compared with the control group (***P ≤ 0.001, ****P ≤ 0.0001).

To evaluate the functional efficacy of our synthetic miR-647-sp, we measured the normalized firefly activity (*i.e.*, firefly luciferase activity/Renilla luciferase activity) in HEK-293T cells 24 hours after cotransfection. HEK-293T cells were transfected with premiR-647, miR-647-sp, wild-type/mutant UTRs and mock vectors (N1 and mCherry). Since the expression of miR-647 is very low in HEK-293T cells, we transfected this construct simultaneously with premiR-647 to assess the function of the sponge fragment. Subsequently, luciferase assays were performed. A significant alteration in normalized luciferase activity was observed, as shown in Figure 3A, indicating an abnormal negative relationship between miR-647 and its target gene. Furthermore, the lack of significant changes in expression among the samples in Figure 3B indicates that the sponge fragment has a reducing effect, preventing the decrease in luciferase activity [9].

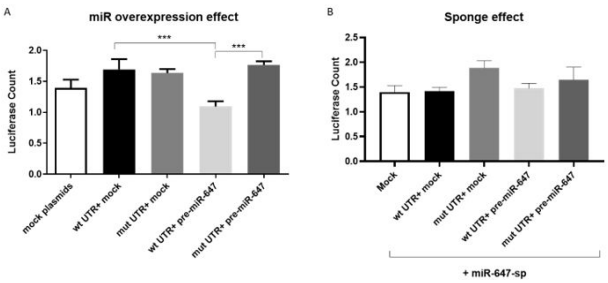

Figure 3: A) Luciferase activity counts in samples transfected with premiR-647 and wild-type/mutant UTRs. This was done to assess the quality of the premiR-647 construct. B: PremiR-647 was used with miR-647-sp and wild-type/mutant UTRs to assess the effectiveness of miR-647-sp (***P ≤ 0.001).

Altered miR-647 expression levels in GCN-01 cells transfected with miR-647-sp

To investigate the effects of sponge fragments, we assessed the expression levels of miR-647 and the *CYP11A1* gene in both the

treated and control cells. Our analysis revealed that the most significant result was the decrease in miR expression due to the sponge effect after 24 h (Figure 4). We also measured the expression levels of other miRNAs (miR-212-3p, miR-490-5p and miR-4643) and previously studied genes (*AMH, AR, CYP11A1, CYP17A1, CYP19A1, CYP21A2, GDF9, HSD17B12* and *INTGB4*) in treated cells for 24 hours (Figure 5).

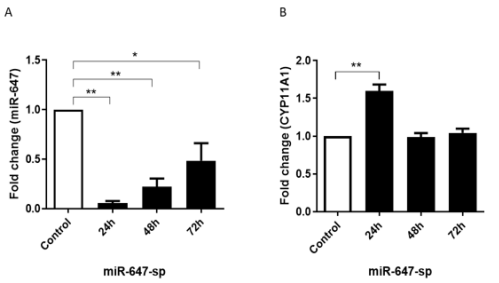

Figure 4: The fold changes in A) miR-647 and B) CYP11A1 (a specific target of miR-647) in granulosa GCN-01 cells transfected with miR-647-sp at three time points (24, 48 and 72 hours) (*P ≤ 0.05, **P ≤ 0.01).

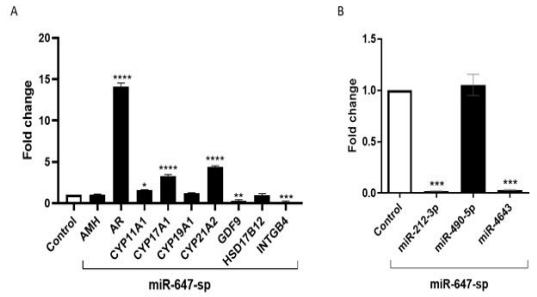

Figure 5: The fold changes in A) PCOS-related genes (*AMH, AR*, the CYP family, *GDF9, HSD17B12* and *INTGB4*) and B) miRs (miR-212-3p, miR-490-5p and miR-4643) in GCN-01 granulosa cells transfected with miR-647-sp after 24 hours (*P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, ****P ≤ 0.0001).

Steroid hormone levels in granulosa GCN-01 cells transfected with sponge-containing vectors

To validate and supplement the RNA-level gene expression data, steroid hormone levels were measured as indicators of enzyme function. The levels of estrogen, progesterone, testosterone, estradiol and DHEA were measured in the culture medium of the cells. Interestingly, the levels of estradiol and progesterone hormones were significantly lower in the miR-647-sp-transfected GCN-01 cells than in the mock-transfected cells (Figures 6 and 7) [10].

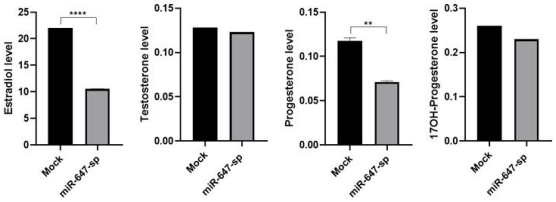

Figure 6: The hormonal changes (estradiol, testosterone, progesterone and 17-OH-progesterone) in the culture medium of GCN-01 cells treated with miR-647-sp after 24 hours compared with control GCN-01 mock-transfected cells (**P ≤ 0.01, ****P ≤ 0.0001).

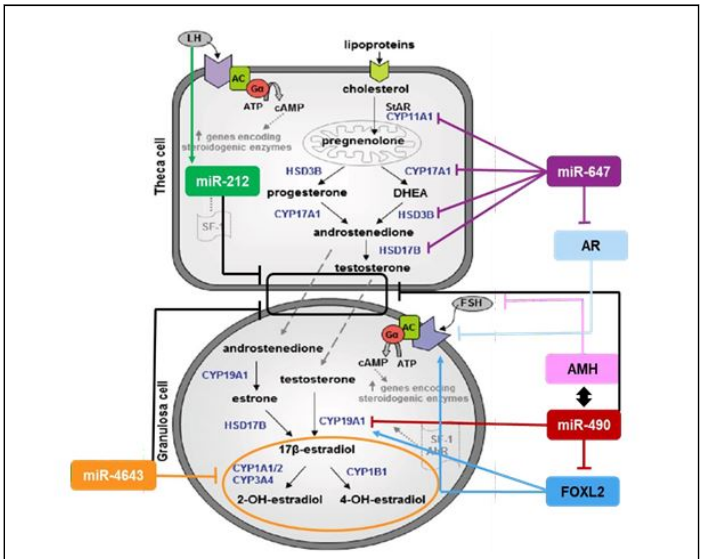

Figure 7: Role of the studied miRs (miR-212, miR-490, miR-647 and miR-4643) in steroid hormone biosynthesis pathways in theca and granulosa cells of the ovary.

CONCLUSION

PCOS is an intrinsic hormonal disorder that affects the biosynthesis of steroid hormones, folliculogenesis and oocyte maturation. These pathways are controlled by genetic factors, such as miRs. miRs are important posttranscriptional factors that play a key role in regulating signaling pathways in the ovary. These molecules regulate the expression of their targets by binding to the 3' UTRs of mRNAs. Thus, identifying miRNAs, along with their relevant physiological and therapeutic roles, is essential. miRNAs located in follicular fluid are considered noninvasive biomarkers for diagnosing and predicting the prognosis of ovarian disease. Our previous study suggested important roles for miR-212-3p, miR-490-5p, miR-647 and miR-4643 in steroidogenesis, metabolic pathways, oocyte maturation, proliferation, cell contact and focal adhesion. This was determined by predicting their potential target genes and examining their expression levels in PCOS. Our results revealed a significant negative correlation between miR-647 and CYP11A1 in both the follicular fluid and cumulus cells of PCOS patients. Therefore, in this study, we investigated the molecular role of miR-647 in the progression of this disease

through functional assays in cell lines before and after transfection. Additionally, we developed an *in vitro* model of PCOS.

Dysregulation of miR-647 is a common feature of many types of cancer. Xiangyang et al. reported that the overexpression of miR-647 in hepatocellular carcinoma inhibits the PIPRF gene, thereby regulating tumor cell proliferation, migration and invasion. In gastric cancer, miR-647 has been found to regulate cell proliferation and metastasis in tumors by targeting *ANK2, FAK, MMP2, MMP12, CD44* and *SNAIL1*. Notably, several studies have evaluated different miRs involved in various important ovarian pathways as candidate markers for PCOS. However, this is the first study to evaluate the role of miR-647 in granulosa cell lines.

MiR-647 potentially modifies the processes of steroidogenesis by targeting key factors involved in cholesterol catabolic pathways, including AR, CYP1A1/2, CYP11A1/B2, CYP17A1 and HSD17B12. The decrease in the expression of miR-647 in the follicular fluid of individuals with PCOS and the significant increase in the expression of these genes in the cumulus cells of the androgenic PCOS group may indicate excessive activation of the aforementioned pathways and an increase in androgenic products in the follicular fluid of PCOS patients.

According to our investigation, the normalized luciferase activity in HEK-293T cells revealed a significant negative relationship between miR-647 and its target gene. As miR-647 is overexpressed in granulosa cells, we initially reduced its expression in GCN-01 cell lines by using designed miRNA sponges. Our analysis revealed that the most significant result was the decrease in miR-647 expression due to the sponge effect. Consistent with our results, Du and colleagues reported that the androgen receptor can reduce the activities related to this gonadotropin by inhibiting the FSH receptor, leading to PCOS. Furthermore, the expression of miR-647 is significantly downregulated in the follicular fluid of patients with PCOS, which supports its involvement in follicular development and function. Thus, the function of this gene in the HEK-293T and GCN-01 cell lines was confirmed through a dual luciferase assay, which validated the previous data.

The results of examining the expression of the *AMH, AR, CYP11A1, CYP17A1, CYP19A1, CYP21A2, GDF9, HSD17B12* and *INTGB4* genes in miR-647-sp-transfected GCN-01 cells revealed a somewhat similar pattern to the androgenic PCOS that we reported in another paper. The significant increase in the expression of the *CYP21A2, CYP17, CYP11A1* and *AR* genes in the treated cells indicates an increase in the production of androgens and their subsequent accumulation in cells, mimicking PCOS conditions.

Furthermore, our results suggest that the significant decrease in the expression of *GDF9* and *INTGB4* in the treated cells was due to the progression of PCOS in these cells. The GDF9 protein plays a significant role in the expansion of cumulus cells. Luteinizing hormone activates this protein from inactive proteoglycans through proteases, also affecting its specific receptors. Other studies have also demonstrated a decrease in the expression of the *GDF9* gene in patients with PCOS which fully supports the *in vitro* data. In addition to gene expression, examining the changes in other miRNAs (miR-490, miR-212 and miR-4643) and their relationship with miR-647 also provides further confirmation of the transition of cells from a normal state to PCOS.

In addition to molecular investigations at the RNA level, hormonal analysis of the GCN-01 cell culture medium revealed a decrease in the levels of progesterone and estradiol, which are downstream products of aromatase CYP19A1, similar to the conditions observed in PCOS. PCOS, as an endocrine disorder, can affect the levels of various hormones in the body, such as estrogen, progesterone, testosterone, estradiol and DHEA. The secretion of estrogen and progesterone differs between PCOS patients and normal individuals. According to a study reported in 2021, progesterone levels decrease in patients with PCOS due to persistent ovulation and impaired luteal development. However, estrogen levels are reportedly increased in patients with PCOS and altered expression of estrogen receptors in granulosa cells may play an essential role in ovulation dysfunction. As the target gene for miR-647, CYP11A is the side-chain cleavage enzyme that catalyzes the conversion of cholesterol to pregnenolone. This enzyme is responsible for the first and rate-limiting step in the biosynthesis of all steroid hormones. Thus, our results were consistent, demonstrating changes in estrogen and progesterone levels following transfection with the miR-647 sponge.

In conclusion, the results of the present study demonstrate the significant role of miR-647 in the regulation and progression of PCOS. Furthermore, gaining more insight into the molecular mechanisms of action for this miRNA is essential for the development of new diagnostic and therapeutic approaches.

AUTHOR CONTRIBUTIONS

Conceptualization, M.R.V. and S.J.M.; formal analysis, H.M.R.; investigation, H.M.R.; resources, Z.H.; writing original draft preparation, F.A.; writing review and editing, H.M.R. and M.R.V.; supervision, M.R.V. and S.J.M.; all authors have read and agreed to the published version of the manuscript.

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DATA AVAILABILITY STATEMENT

All the data generated or analyzed during this study are included in this published article.

CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

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