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ORIGINAL RESEARCH

Increased miR-6875-5p inhibits plasmacytoid dendritic cell differentiation via the STAT3/E2-2 pathway in recurrent spontaneous abortion

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ABSTRACT: Recurrent spontaneous abortion (RSA) is a common complication of early pregnancy. Dendritic cells (DCs) are thought to confer fetal–maternal immunotolerance and play a crucial role in ensuring a successful pregnancy. A decrease of plasmacytoid dendritic cells (pDCs) was found to be involved in RSA, but the underlying mechanisms of decreased pDC in RSA remain unclear. MicroRNAs (miRNAs) play critical roles in RSA as well as the development, differentiation and functional regulation of pDCs; however, the regulatory effect of miRNAs on pDC in RSA has not been fully investigated. Here we demonstrated that both the proportion of pDC and signal transducer and activator of transcription (STAT3)/transcription factor 4 (Tcf4/E2-2) expression decreased in the peripheral blood mononuclear cells and decidua of patients with RSA compared to those with normal pregnancy (NP), and there was a significantly positive correlation between pDC and *STAT3* mRNA. MiRNA microarray assay and quantitative reverse transcription PCR results showed that miR-6875-5p expression was markedly increased in women with RSA and negatively correlated with mRNA expression level of *STAT3*. Up-regulated miR-6875-5p could sensitively discriminate patients with RSA from NP subjects. Overexpression of miR-6875-5p significantly down-regulated the mRNA expression of *STAT3* and *E2-2* as well as the protein and phosphorylation level of STAT3, while miR-6875-5p knockdown showed opposite results. Dual luciferase reporter verified that miR-6875-5p regulated STAT3 expression by directly binding to its 3'untranslated region. Overall, our results suggested that increased miR-6875-5p is involved in RSA by decreasing the differentiation of pDCs via inhibition of the STAT3/E2-2 signaling pathway. miR-6875-5p may be explored as a promising diagnostic marker and therapeutic target for RSA.

Key words: recurrent spontaneous abortion / miR-6875-5p / STAT3 / plasmacytoid dendritic cells / post-transcriptional regulation

Introduction

Recurrent spontaneous abortion (RSA), defined as two or more consecutive miscarriages before 20 weeks of gestation (Kuon *et al.*, 2015), occurs in about 10% of all pregnancies (Branch *et al.*, 2010). Except for the known pathogenic factors, such as genetic, anatomical, infectious, hormonal, metabolic factors and disorders of auto- and alloimmune origin, the cause of approximately 50% of RSA cases remains unexplained (Imam *et al.*, 2011). Immunological factors have been postulated to play a role in the etiology of RSA, as the fetus is considered a semi homograft to the mother (Zhao *et al.*, 2010). Dendritic cells (DCs), the most specialized and potent among antigen-

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presenting cells, have the unique ability to induce immune responses and specific immune tolerance (Chorny et al., 2006; Galati et al., 2016). Studies had demonstrated that DCs exist in human decidua and are closely adjacent to a large number of clusters of T lymphocytes, which play an important role in the maintenance of pregnancy (Kammerer et al., 2008), and any disturbance DC distribution, maturation state and function may affect the pregnancy outcome, including disturbed pregnancy (Ahmadabad et al., 2016). The balance between myeloid DC (mDCs) and plasmacytoid DC (pDCs) is important for maintaining Th1/Th2 balance, which is significant for successful pregnancy, while an imbalance of mDCs/pDCs during pregnancy is associated with spontaneous abortion or RSA (Huang et al., 2016). Studies demonstrated that the level of mDCs was significantly increased while the fraction of pDCs was reduced in patients with spontaneous abortions (Huang et al., 2016). However, the factors and underlying mechanisms for triggering this change in RSA remain unclear.

Signal transduction and transcriptional activator protein (STAT) 3 is a prototypical member of the STAT family (Lin et al., 2010; Rizzuti et al., 2015). Studies have reported that the STAT3 signaling pathway plays a key role in the development, differentiation and functional regulation of DCs, especially pDCs (Cohen et al., 2008; Li et al., 2012). Activated STAT3 translocate into the nucleus, facilitating the transcription of transcription factor 4 (Tcf4/E2-2), a pDC-specific transcription regulator, thus, promoting pDC-specific or functional gene expression (Cisse et al., 2008; Ghosh et al., 2010). Although STAT3 and transcription factor E2-2 play an important role in the development, differentiation and functional regulation of pDCs, whether the decrease of pDCs in RSA patients is related to the STAT3/E2-2 pathway remains unclear, and the upstream regulation mechanism of STAT3 has not been clarified.

MicroRNAs (miRNAs), a class of conservative, endogenous small non-coding RNAs of 21–24 nucleotides in length, regulate gene expression by binding to the 3'-untranslated region (UTR) of their target mRNAs (Iwakawa and Tomari, 2015; Xu et al., 2015). miRNAs are reported to play critical regulatory roles in a wide range of biologic and pathologic processes including cell differentiation, proliferation, apoptosis, angiogenesis and inflammation (Rupaimoole et al., 2016; Zhou et al., 2018). An increasing number of studies have demonstrated that miRNAs are expressed abundantly in the human blood, decidua and villi, and that dysregulation of miRNAs is associated with RSA pathogenesis (Wang et al., 2016; Zhu et al., 2016; Zhou et al., 2018). For example, enhanced expression of miR-184 and miR-365 in decidual tissues promotes apoptosis of trophoblast cells and induces RSA by targeting zinc finger matrin-type 3 (WIG1) and serum/glucocorticoid regulated kinase 1 (SGK1), respectively (Zhao et al., 2017; Zhang et al., 2019). miR-133a is involved in the pathogenesis of RSA by restraining HLA-G expression (Wang et al., 2012). Moreover, miR-520 enhances trophoblast cell apoptosis by poly (ADP-ribose) polymerase 1 (PARP1) targeting and facilitated RSA (Dong et al., 2017). In addition, studies have demonstrated that miRNAs, such as miR-21, miR-146a, and miR-155, play an essential role in the development, differentiation and functional regulation of pDCs (Zhou et al., 2010; Karrich et al., 2013; Liu et al., 2017). However, the regulatory effect of miRNAs on pDCs in RSA has not been fully investigated.

In the present study, we aimed to investigate the regulatory effect of miRNAs on pDC differentiation in patients with RSA. The results showed that both the proportion of pDCs and the expression of *STAT3/E2-2* decreased. A miRNA microarray assay and quantitative reverse transcription PCR (qRT-PCR) results indicated that miR-6875-5p expression increased significantly in RSA patients and there was a negative correlation between miR-6875-5p and STAT3 expression. Therefore, we hypothesized that increased miR-6875-5p might be involved in RSA by inhibiting pDC differentiation via suppression of STAT3. As expected, our results showed that over-expression of miR-6875-5p significantly down-regulated the expression of STAT3 by directly binding to its 3'UTR, then inhibited E2-2 expression; miR-6875-5p knock down showed opposite results. Ultimately, our findings suggested that miR-6875-5p regulates the STAT3 signaling pathway and interferes with the differentiation of pDCs. It provides a potential target and pathway for clinical treatment of RSA.

Materials and methods

Patients and samples

Between September 2019 and December 2020, 30 patients with RSA admitted to Shandong Province Maternal and Child Health Care Hospital and 30 women with a normal pregnancy (NP) that had no previous spontaneous abortion were included in this study. All the enrolled RSA patients had experienced two or more spontaneous abortions ranging from 6 to 10 weeks of gestation. All the RSA patients in our study underwent induced abortion rather than medical abortion. HCG level in blood was measured every other day to monitor threatened or inevitable abortion. If blood HCG level is obviously lower than normal, ultrasound would be performed. RSA was confirmed if the ultrasound showed disappearance of the fetal heartbeat, HCG level decrease and visualization of the abortus. Then, the patients were recommended to undergo an induced abortion within 24 h and the decidua tissue was collected immediately after the operation. In these patients, we excluded individuals with infections, endocrine or metabolic disorders, anatomic abnormalities, autoimmune diseases, and paternal or maternal chromosomal abnormalities. Detailed information about the study participants is summarized in Table I.

Specimen collection and preservation

Venous blood samples (drawn into EDTA blood collection tubes) from RSA patients were obtained within 24 h after onset of the symptoms, and the blood samples from NP subjects were collected before elective termination of early pregnancy. Decidual tissues were immediately separated through negative-pressure aspiration by physicians of Affiliated Hospital of Shandong University of Traditional Chinese Medicine and were carefully dissected free of myometrial tissue or attached placenta and visible blood clots. In addition, decidua tissue was confirmed microscopically by a pathologist. The cells of peripheral blood and decidua tissues were prepared within 2 h after the collection of samples. Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll density-gradient centrifugation. The protocol/IRB was approved by the Research Ethics Committee of Affiliated Hospital of Shandong University of Traditional Chinese Medicine and Research Ethics Committee of Shandong Provincial Hospital Affiliated to Shandong First Medical University. The IRB number is

Table I Clinical characteristics of subjects in the study.

Subject	RSA (mean ± SD, n = 30)	Control (mean ± SD, n = 30)	*P*
Age (years)	**30.23 ± 3.12**	**29.47 ± 2.91**	0.33
Number of miscarriages	2.87 ± 0.68	0	<0.0001
Gestation age (weeks)	**8.06 ± 0.69**	**7.75 ± 0.64**	0.07

Statistical significance was calculated using unpaired Student's *t*-test. The data are expressed as the mean± SD.

SDFMU20190605. Specimens were obtained after receiving informed written consent from each participant.

Flow cytometry detection

PBMCs and decidual cells were suspended in PBS and washed twice with 1×PBS. The anti-human CD11c (561355), anti-human CD123 (554529) conjugated antibodies were obtained from BD Biosciences (Franklin Lakes, NJ, USA) and incubated with cells for 30 min at 4 °C in the dark. Flow cytometry was performed using cell quest software flow cytometer (BD Biosciences) and results were analyzed using FlowJo 7.6 software (Treestar, Woodburn, OR, USA). Trypan blue test was performed before flow cytometry and it was found that the rate of survival cells was more than 80%.

RNA isolation and qRT-PCR

Total RNA was extracted using TRIzol reagent. RNA was reverse transcribed with the miRNA 1st Strand cDNA Synthesis Kit (Vazyme, Nanjing, China) for miRNA or the PrimeScript RT reagent Kit (Toyobo, Osaka, Japan) for mRNA. qRT-PCR using SYBR Green (Invitrogen, Waltham, MA, USA) was performed on an Applied Biosystems 7500 instrument. For miRNA and mRNA analysis, the primer sequences are shown in Table II. For each sample, the amplification reaction was performed in triplicate. Relative RNA quantification was performed via the comparative $2^{-\Delta\Delta Ct}$ method.

Microarray analysis

RNA samples from NP (n = 3) and RSA patients (n = 3) were sent to Biotech Corporation (Shanghai, China) for analysis by human miRNA microarray assay. Feature Extraction software (version10.7.1.1, Agilent Technologies, San Diego, CA, USA) was used to analyze array images to obtain raw data. Gene spring software (version 13.1, Agilent Technologies) was employed to finish the basic analysis with the raw data. The raw data was normalized with the quantile algorithm. Differentially expressed miRNAs were then identified through fold change as well as a *P* value calculated using two-tailed Student's *t*-test. To select the differentially expressed genes, we used threshold values > 2 and< -2-fold change and P value < 0.05.

Cell culture and transfection

The human monocyte cell line 293T was obtained from the Cell Resource Center (Shanghai Institutes for Biological Sciences). 293T cells were maintained in DMEM containing 10% FBS (Bioind, Kibbuiz, Israel) and 1% penicillin/streptomycin at 37°C and 5% CO_2. miR-6875-5p mimics/mimics negative control (NC) or miR-6875-5p inhibitor/inhibitor NC (INC) (GenePharma, Shanghai, China) were transfected into 293T cells at a final oligonucleotide concentration of 100 nM with Lipofectamine 2000 (Invitrogen, Waltham, MA, USA). The oligodeoxy nucleotide sequences used in this study are shown in Table III. Instantly, the cells were incubated at 37°C in a 5% CO_2 atmosphere for 24 h and then used for subsequent experiments.

Western blot analysis

Total protein was extracted by RIPA lysis buffer (Thermo Scientific, Waltham, MA, USA) containing protease and phosphatase inhibitors (Thermo Scientific) and quantified using the BCA Protein Assay Kit (Thermo Scientific). Protein was separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) then transferred onto a nitrocellulose membrane. Membrane was blocked with 5% skim milk and incubated overnight with primary antibodies at 4°C. Antibodies against STAT3 (1:1000, 9139, Cell Signaling Technology, Danvers, MA, USA), p-STAT3 (1:1000, 9131S, Cell Signaling Technology), and GAPDH (1:10000, ab181603, Abcam, Cambridge, MA, USA) were

Table II List of PCR primers used in the study.

Gene	Forward primer (5′–3′)	Reverse primer (5′–3′)
STAT3	GGGAAGAATCACGCCTTCTAC	ATCTGCTGCTTCTCCGTCAC
E2-2	CCATCTCTCTCAGCAGGCAC	CCCATGACCACCAGGCATAG
GAPDH	ACAACTTTGGTATCGTGGAAGG	GCCATCACGCCACAGTTTC
miR-6875-5p	ACTGCGTGAGGGACCCA	ACGCTCAGTTAATGCTAATCGTGATA
U6	ATTGGAACGATACAGAGAAGATT	GGAACGCTTCACGAATTTG

STAT3, signal transducer and activator of transcription 3; *E2-2*, transcription factor 4; *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase; miR-6875-5p, microRNA-6875-3p; U6, U6 small nuclear 1.

Table III miRNAs sequences.

miRNAs	Sequences (5′–3′)
NC	**Sense: UUCUCCGAACGUGUCACGUTT**
	Antisense: ACGUGACACGUUCGGAGAAGAATT
miR-6875-5p mimics	**Sense: UGAGGGACCCAGGACAGGAGA**
	Antisense: UCCUGUCCUGGGUCCCUCAUU
INC	CAGUACUUUUGUGUAGUACAA
miR-6875-5p inhibitor	UCUCCUGUCUGGGUCCCUCA

miRNA, microRNA; NC, mimics negative control; INC, inhibitor NC.

used. The immunoreactivity signals were visualized by an Immobile Western Chemiluminescent HRP Detection System (Millipore, Billerica, MA, USA) and quantified using Quantity One software 4.62 (Bio-Rad, Hercules, CA, USA).

Luciferase reporter assays

The wild-type (WT) and mutant (Mut1, Mut2) human *STAT3* mRNA 3'UTR luciferase reporter vectors were constructed by amplifying human WT, Mut1 or Mut2 *STAT3* mRNA 3'UTR and cloning into the pGL3-3M-Luc vector (Promega, Madison, WI, USA), respectively. WT, Mut1 or Mut2 luciferase reporter plasmid and miR-6875-5p mimics/NC or miR-6875-5p inhibitor/INC were co-transfected into the 293T cells using Lipofectamine 2000 reagent. After 24 h, the cells were collected for application in the Dual-Luciferase Reporter System (Promega, Madison, WI, USA) following the manufacturer's instructions.

Fluorescence in situ hybridization

A fluorescence *in situ* hybridization (FISH) assay was executed to observe the location and expression of miR-6875-5p in the decidual tissues of RSA patients and NP subjects. The decidual tissues were fixed with 4% paraformaldehyde at room temperature, embedded in paraffin, and slices prepared at 5 μm thickness. The paraffin slices were hybridized with specific Cy3-labeled miR-6875-5p probes at 37°C overnight. 4,6-diamidino-2-phenylindole (DAPI) was used for cell nucleus counterstain and the images were acquired with laser scanning confocal microscope (FV3000, Olympus, Tokyo, Japan). All procedures were conducted according to the manufacturer's protocol (Genepharma, Shanghai, China).

Statistical analysis

Unless otherwise stated, all experiments were performed at least three independent times. Statistical analysis was performed using SPSS16.0 statistical software (SPSS, Chicago, IL, USA). Values were represented as mean± SD. The 2-tailed Student's *t*-test was used to compare the data between any two groups through the normality and equal variance tests. If data for either normality or variance tests failed, the non-parametric Mann–Whitney *U* test was used. The diagnostic value was evaluated using the receiver operating characteristic (ROC) curve. *P* values< 0.05 were considered statistically significant.

Results

The population of pDCs is decreased in patients with RSA and positively correlated with STAT3

To explore the role of pDCs in RSA, the proportion of pDCs in the PBMC and decidua of both RSA and NP patients was investigated by flow cytometry. The data showed that, compared with NP group, the proportion of pDCs ($CD11c^{-}CD123^{+}$) decreased significantly in both the PBMC and decidua of RSA group (Fig. 1A and B). The mRNA expression of *STAT3* and *E2-2* was markedly down-regulated in the PBMC and decidua of RSA patients (Fig. 1C–F). Interestingly, the proportion of pDCs was positively related to *STAT3* expression (Fig. 1G and H). These results suggested that the proportion of pDCs was decreased in RSA patients and was positively related to the expression of STAT3.

miR-6875-5p increased in patients with RSA

To further explore the miRNAs potentially involved in RSA, a human miRNA microarray assay was performed in PBMC of RSA patients (n = 3) and NP women (n = 3). A total of 39 miRNAs were either up- or down-regulated by at least 1.5-fold (and *P* value< 0.05) in the RSA group, including 20 up-regulated miRNAs and 19 down-regulated miRNAs (Fig. 2A). The up-regulated miRNAs are shown in Fig. 2B. Of interest, in silico prediction of the miRNA-target gene interaction using TargetScan, miRDB database, and 20 up-regulated miRNAs revealed a high probability interaction between miR-6875-5p and STAT3 (Fig. 2C). qRT-PCR was performed to verify the expression of miR-6875-5p in samples of the microarray assay, PBMCs and decidua tissues of 15 patients with RSA and 15 women of NP, and the results revealed that miR-6875-5p expression was significantly increased in patients with RSA compared with NP subjects (Fig. 2D–F). The FISH assay identified that miR-6875-5p increased obviously in the decidua of RSA patients (Fig. 3A, red in middle panel). Furthermore, the expression of miR-6875-5p in PBMC and decidua were negatively correlated with that of *STAT3* (Fig. 3B and C). ROC analysis revealed that miR-6875-5p could sensitively discriminate RSA in PBMCs with an area under the curve (AUC) of 0.8867 (95% CI: 0.7336–0.9997) (Fig. 3D). These data indicated that up-regulated miR-6875-5p might suppress *STAT3* expression, and then inhibited pDC differentiation in RSA.

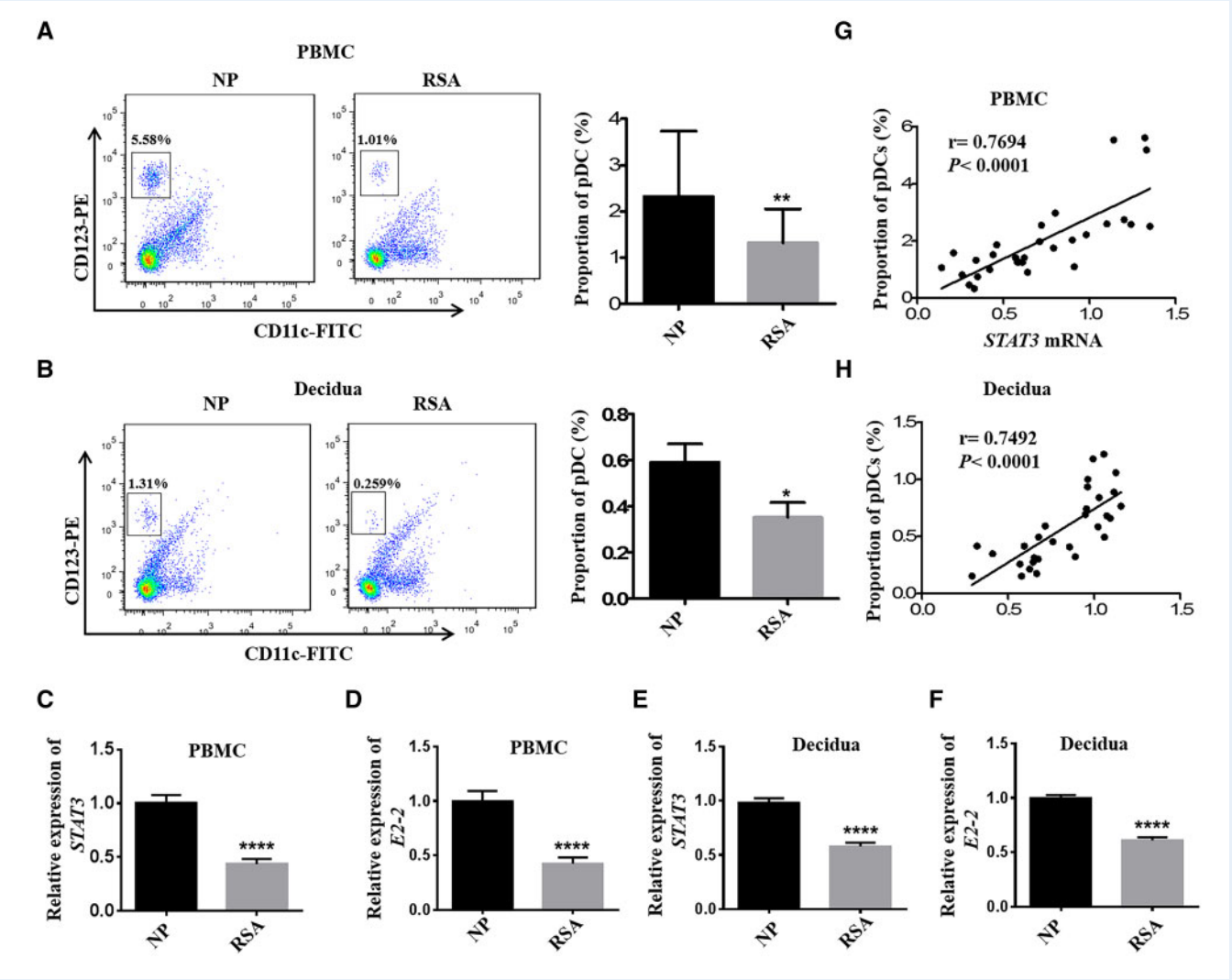

Figure 1 pDCs, STAT3, and E2-2 decreased in patients with recurrent spontaneous abortion. (A) The proportion of plasmacytoid dendritic cells (pDCs) ($CD11c^{-}CD123^{+}$) cells in peripheral blood mononuclear cells (PBMC) of women with a normal pregnancy (NP) (n = 30) and patients with recurrent spontaneous abortion (RSA) (n = 30) was analyzed by flow cytometry. **(B)** The proportion of pDCs ($CD11c^{-}CD123^{+}$) cells in decidua of NP (n= 30) and RSA patients (n = 30) was analyzed by flow cytometry. **(C** and **D)** The mRNA expression of signal transduction and transcriptional activator protein 3 (*STAT3*) and transcription of transcription factor 4 (*E2-2)* were quantified by qRT-PCR in PBMC of NP (n = 30) and RSA patients (n = 30). **(E** and **F)** The mRNA expression of *STAT3* and *E2-2* were quantified by qRT-PCR in decidua of NP (n = 30) and RSA patients (n = 30). **(G)** Correlation between *STAT3* and the proportion of $CD11c^{-}CD123^{+}$ in PBMC of NP subjects (n = 15) and RSA patients (n = 15). **(H)** Correlation between *STAT3* and the proportion of $CD11c^{-}CD123^{+}$ in decidua of NP subjects (n = 15) and RSA patients (n = 15). Statistical significance was calculated using unpaired Student's *t*-test. The data are expressed as the mean ± SD. $^{**}P< 0.01$, $^{****}P< 0.0001$.

miR-6875-5p inhibits the STAT3/E2-2 signaling pathway

To determine the effect of miR-6875-5p on STAT3 expression, we altered miR-6875-5p expression and determined the levels of STAT3, and the STAT3 downstream gene *E2-2*, in 293T cells. The data showed that miR-6875-5p mimics enhanced, while the inhibitor suppressed, miR-6875-5p expression effectively (Fig. 4A and B). Moreover, overexpression of miR-6875-5p reduced *STAT3* and *E2-2* mRNA expression efficiently, while down-regulated miR-6875-5p enhanced *STAT3* and *E2-2* mRNA level (Fig. 4C–F). Meanwhile, the protein levels of both total and phosphorylated STAT3 (p-STAT3) were markedly decreased in miR-6875-5p mimics group, while miR-6875-5p inhibitor transfection showed the opposite effect (Fig. 4G and H). In summary, the results suggested that miR-6875-5p has a negative regulatory effect on the expression of STAT3.

STAT3 is a direct target gene of miR-6875-5p

To validate the post-transcriptional suppressive effect of miR-6875-5p on *STAT3*, we located the miR-6875-5p binding sites on *STAT3* 3'UTR through TargetScan, and found that there are two potential

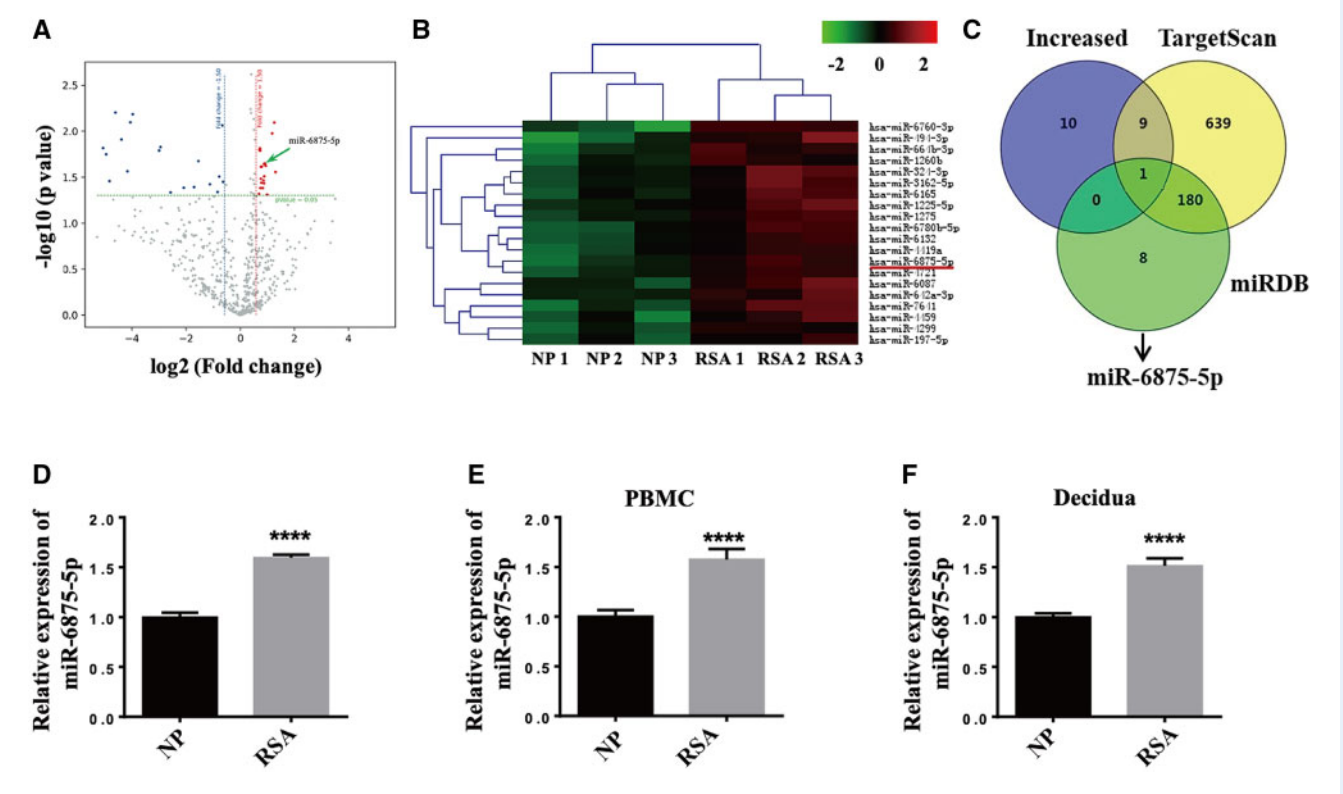

Figure 2 The expression profile of global miRNAs in PBMC of women with RSA and NP. (A) Volcano plot of differentially expressed miRNAs in PBMC of NP and RSA patients, the red point in the plot represents the significant up-regulated, and the blue point represents the significant down-regulated miRNAs. **(B)** Heat map of 20 up-regulated miRNAs (fold change >1.5, $P < 0.05$). **(C)** Schematic representation showing the predicted potential miRNA that might target STAT3 screened by using increased miRNA in microarray assay, TargetScan and miDB prediction. **(D)** The expression of miRNA-6875-5p was analyzed using qRT-PCR in microarray assay sample (n = 3). **(E)** The expression level of miRNA-6875-5p in PBMC of NP (n = 15) and RSA (n = 15) patients. (**F**) The expression level of miRNA-6875-5p in decidua of NP (n = 15) and RSA (n = 15) patients. Statistical significance was calculated using unpaired Student's *t*-test. The data are expressed as the mean± SD. ****$P<$ 0.0001.

complementary miR-6875-5p binding sites for *STAT3* (Fig. 5A). To specify which site is true for miR-6875-5p binding, the WT, Mut1 and Mut2 3'UTR for STAT3 were cloned into a pGL3-3M-Luc vector, and co-transfected with miR-6875-5p mimics/NC or miR-6875-5p inhibitor/INC into 293 T cells for a dual-luciferase reporter assay (Fig. 5A). The results showed that miR-6875-5p mimics can significantly reduce luciferase reporter activity of WT and Mut1 3'UTR plasmid for *STAT3*, without affecting that of Mut2 3'UTR plasmid (Fig. 5B–D). Meanwhile, miR-6875-5p inhibitor markedly increased the luciferase reporter activity of WT and Mut1 3'UTR plasmid, but had no effect on the luciferase reporter activity of Mut2 3'UTR plasmid (Fig. 5E–G). These results suggested that *STAT3* is a direct target of the miR-6875-5p, which bind to *STAT3* 3'UTR at the 1412–1419 bp site.

Discussion

Human pregnancy is considered to be a unique immunological paradigm. During pregnancy, the immune system is faced with the challenge. It requires maternal tolerance to avoid fetal rejection, meanwhile allowing effective immunity to protect both mother and fetus from pathogens infection (Mor *et al.*, 2017). Failure to promote immune tolerance to paternal antigens may result in pregnancy loss (Sasaki *et al.*, 2004). DCs participate in the important immune regulation process at different stages of pregnancy (Zarnani *et al.*, 2007; Blois *et al.*, 2008; Dauven *et al.*, 2016). PDCs, as an important member of the DCs, play essential roles in establishing immune tolerance (Manches *et al.*, 2008). On the one hand, pDCs can induce Treg generation or inhibit T cell activation by secreting indoleamine 2, 3-dioxygenase, expressing inducible costimulatory- ligand or autocrine transforming growth factor-β (Munn *et al.*, 2004; Ito *et al.*, 2007; Uto *et al.*, 2018). On the other hand, activated pDCs can promote Th2 cell differentiation through OX40L-dependent mechanism (Gilliet and Liu, 2002; Ito *et al.*, 2004). Besides, a study found that the percentage of conventional DCs (cDCs) was significantly reduced concurrent with an increase in pDCs during NP, while disrupting the balance between cDCs and pDCs will induce abortion (Fang *et al.*, 2016). Moreover, Huang et al. found that the proportion of pDCs decreased in patients with RSA, which differed from Ehrentraut's results showing no changes of pDCs in peripheral blood of miscarriage patients compared with NP women (Huang *et al.*, 2016; Ehrentraut *et al.*, 2019). In the present study, increased miR-6875-5p expression was identified in patients,

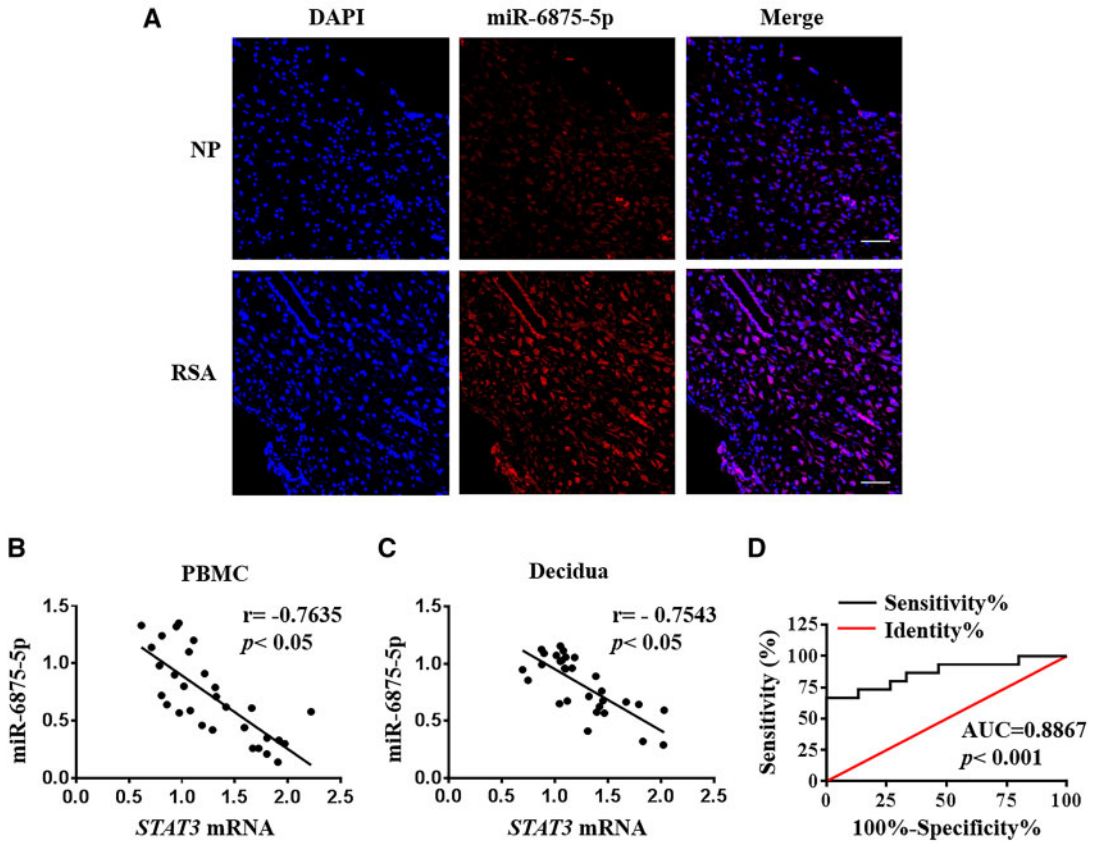

Figure 3 miR-6875-5p was up-regulated in patients with RSA and negatively correlated with STAT3. (A) FISH was performed to observe the location and expression level of miR-6875-5p in decidual tissues (a representative experiment, from three independent experiments, Scale bar, 50μm, 200×). **(B)** Correlation between STAT3 mRNA and miR-6875-5p in PBMC of NP (n = 15) and RSA (n = 15) patients. **(C)** Correlation between *STAT3* mRNA and miR-6875-5p in decidua of NP (n = 15) and RSA (n = 15) patients. **(D)** Diagnostic value of miR-6875-5p for RSA was assessed by ROC curve (n = 15).

with RSA and we demonstrated that increased miR-6875-5p could inhibit STAT3 expression, which in turn restricted the differentiation of pDCs.

Increasing evidence has shown that the differentiation of DCs is controlled by a few transcription factors (Cisse et al., 2008; Donninelli *et al.*, 2018). Among these transcription factors, STAT3 has been discovered to play an important role in modulating both the development and the activation of pDCs (Laouar *et al.*, 2003; Sun *et al.*, 2017). STAT3 was necessary for proliferation of bone marrow progenitors to Flt3L stimulation, and pDC numbers reduced in STAT3-deficient bone marrow cultures (Esashi *et al.*, 2008). Li *et al.* (2012) reported that STAT3 regulates pDC development by stimulating Flt3L-responsive expression of the pDC regulator Tcf4 (E2-2). Inhibition of STAT3 activation completely abrogates IL-27-induced upregulation of the co-regulatory molecule B7 Homolog-1 expression in liver pDCs (Matta *et al.*, 2012). However, the role of STAT3 in the down-regulation of pDCs in RSA and the underlying mechanisms are still largely unknown. In our study, the mRNA level of *STAT3* and *E2-2* were markedly down-regulated in PBMC and decidua of RSA patients. Interestingly, the proportion of pDCs was positively related to *STAT3* expression. These results suggested that the reduced pDCs in RSA may be mediated by the down-regulated STAT3.miRNAs are small non-coding RNAs that regulate gene expression by binding to the 3'UTR of their target genes, thereby decreasing gene expression (Iwakawa and Tomari, 2015). Recently, increasing evidence has demonstrated that miRNAs are expressed differently in patients with RSA, and dysregulation of miRNAs such as miR-365, miR-520 and miR-184 has been associated with RSA pathogenesis (Zhao *et al.*, 2017; Zhang *et al.*, 2019). miRNAs also play an important role in the differentiation and functional regulation of pDCs (Zhou *et al.*, 2010; Wu *et al.*, 2015). miR-21 is crucial for modulation of pDC activation (Liu *et al.*, 2017). miR-23b is capable of inducing tolerogenic DC activity and Treg responses *in vitro* through inhibition of the Notch1 and NF-kB signaling pathways (Zheng *et al.*, 2012). However, the regulatory effect of miRNAs on pDC in RSA has not been fully investigated. In the present study, our miRNA microarray analysis showed that a total of 39 miRNAs were either up- or down- regulated by at least 1.5-fold (with *P* value< 0.05) in the PBMC of RSA patients, indicating that miRNAs play important roles in RSA. We used both TargetScan and miRDB datasets to predict candidate miRNAs that may target STAT3 and

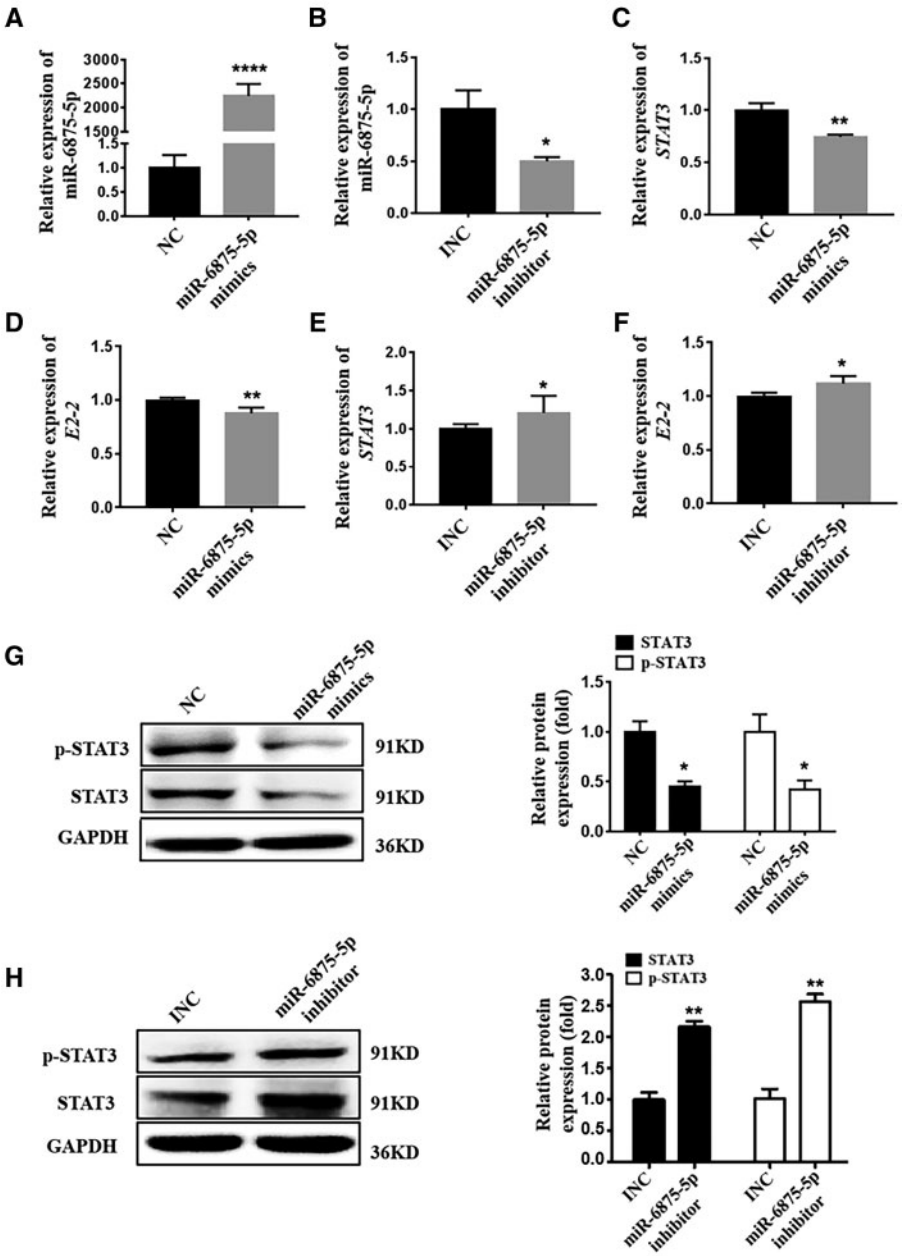

Figure 4 miR-6875-5p inhibited the STAT3/E2-2 signaling pathway. (A and **B)** The level of miRNA-6875-5p in 293T cells transfected with mimics negative control (NC)/miRNA-6875-5p mimics or inhibitor NC (INC)/miRNA-6875-5p inhibitor (data were pooled from three independent experiments, with n = 3 per group). **(C–F)** *STAT3*, *E2-2* mRNA expression were detected in 293T cells transfected with NC/miRNA-6875-5p mimics or INC/miRNA-6875-5p inhibitor by qRT-PCR (data were pooled from three independent experiments, with n = 3 per group). (**G** and **H**) See Supplementary information for the uncropped western blots. The protein levels of STAT3 and p-STAT3 in 293T cells transfected with NC/miRNA-6875-5p mimics or INC/miRNA-6875-5p inhibitor were measured by western blot (a representative blot, from three independent experiments). Statistical significance was calculated using unpaired Student's *t*-test. The data are expressed as the mean ± SD. **P*< 0.05, ***P*< 0.01, *****P*< 0.0001.

there were 829 and 189 miRNAs which may target STAT3 in the two datasets, respectively. The candidate miRNAs, described above, were compared with the 20 up-regulated miRNAs from the microarray, and only miR-6875-5p was identified. Therefore, we hypothesized that miR-6875-5p might be play an important role in RSA by regulating pDCs differentiation.

miR-6875-5p is located on human chromosome 7q22.1. It has been reported that miR-6875-5p measured from serum can be used to

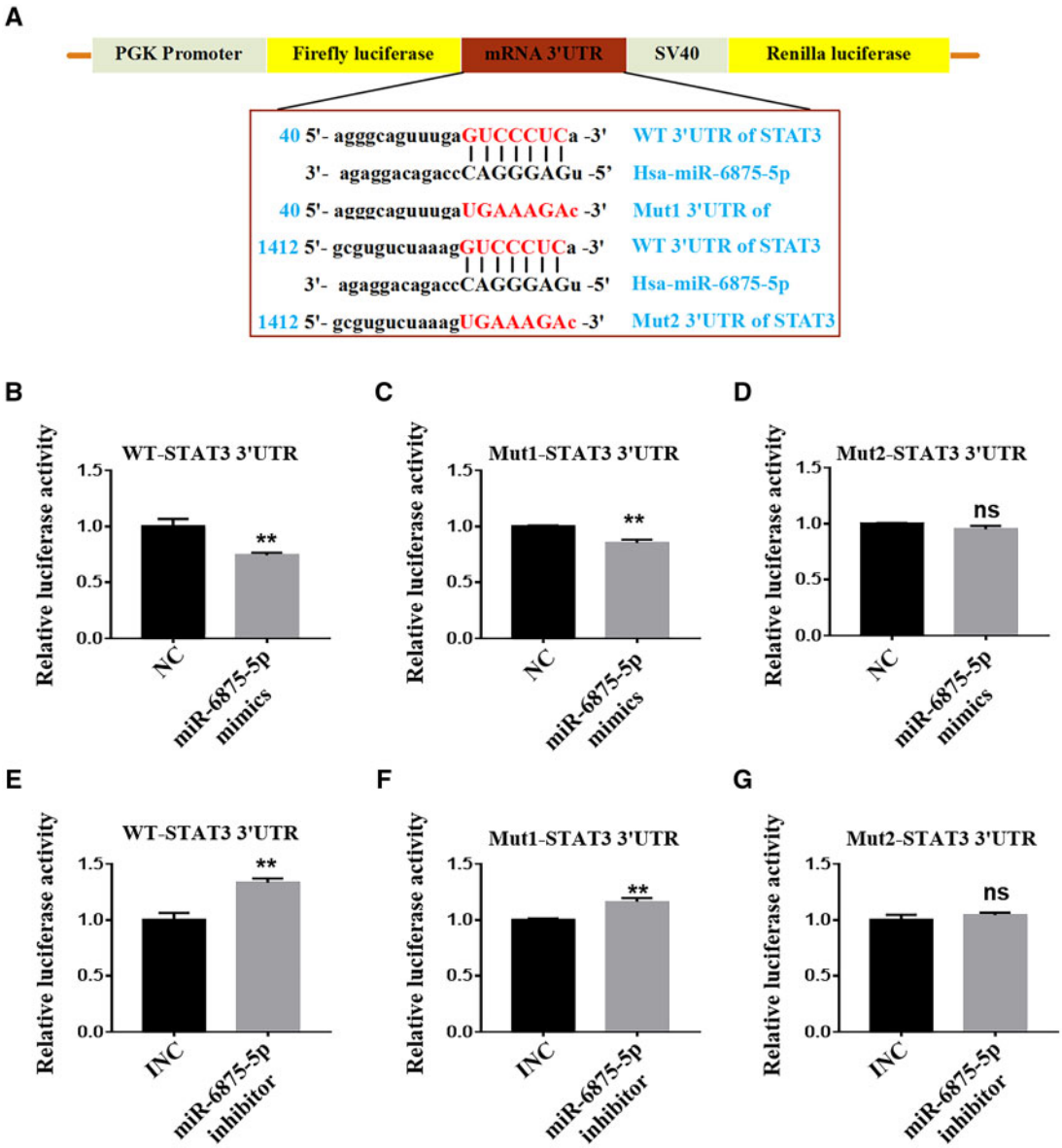

Figure 5 STAT3 is a direct target of miR-6875-5p. (A) Schematic representation of miRNA-6875-5p putative binding sequence in the 3'untranslated region (3'UTR) of *STAT3*, luciferase activities of wild-type (WT) and mutant (Mut1, Mut2) constructs. **(B–D)** The luciferase activity was determined by co-transfecting the vectors (*STAT3* 3'UTR-WT, Mut1, Mut2) combined with NC, miRNA-6875-5p mimics into 293T cells (data were pooled from three independent experiments, with n = 3 per group). **(E** and **G)** The effect of miR-6875-5p inhibitor on luciferase activity of *STAT3* 3'-UTR-WT, Mut1, Mut2 vectors (data were pooled from three independent experiments, with n = 3 per group). Statistical significance was calculated using unpaired Student's *t*-test. The data are expressed as the mean± SD. $^{**}P < 0.01$.

detect breast cancer in the early stages (Shimomura *et al.*, 2016). Metastatic colorectal cancer patients with high expression of miR-6875 and miR-6826 have been reported to have a poor response to immunotherapy and poor prognosis (Kijima *et al.*, 2017). miR-6875-3p can promote the proliferation, invasion and metastasis of hepatocellular carcinoma via the BTG2/FAK/AKT pathway (Xie *et al.*, 2019). To date, the biological functions and potential underlying molecular mechanisms of miR-6875-5p in RSA or pregnancy-related diseases still remain unexplored. In this study, the expression of miR-6875-5p was significantly higher in both the PBMC and decidua of patients with RSA and this was negatively correlated with *STAT3*. Overexpression of miR-6875-5p can significantly decrease STAT3/E2-2 expression by directly binding to *STAT3* 3'UTR. These findings confirmed that increased miR-6875-5p down-regulates the differentiation of pDCs via inhibiting the STAT3/E2-2 signaling pathway.

In conclusion, our study reveals for the first time that up-regulation of miR-6875-5p is associated with decreased pDCs through inhibiting the STAT3/E2-2 pathway in RSA. The finding reveals the potential

pathogenesis of RSA from the perspective of epigenetic immune regulation and indicates that miR-6875-5p might be a potential diagnostic marker and promising therapeutic target for RSA. Nevertheless, it should be noted that the underlying mechanisms of miRNA action in RSA are diverse, and might involve various target genes: miRNAs may have regulatory effects on other cells in decidual tissue, such as decidual cells, trophoblast, T-cells, and macrophages. Further studies are needed to fully understand the role of miR-6875-5p in RSA.

Supplementary data

Supplementary data are available at *Molecular Human Reproduction* online.

Data availability

The raw data supporting the conclusions of this manuscript will be made available by the corresponding author, without undue reservation. The datasets presented in this study can be found in NCBI using the accession GSE178619.

Authors' roles

X.L. conceived and designed the study. X.-X.Z., X.-Q.Y., and G.-Z.H. conducted most of the experiments. R.W. and Q.G. contributed to the technical support. Z.Z., L.Z., C.C., X.-X.F. and K.X. analyzed the data. X.-X.Z. and X.L. drafted the manuscript.

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Conflict of interest

All authors declare no conflicts of interest.

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